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The role of soluble FMS-like tyrosine kinase (sFLT1) and FAS associated proteins in pregnancies complicated by preeclampsia and intrauterine growth restriction (IUGR).

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THE ROLE OF SOLUBLE FMS-LIKE TYROSINE KINASE (sFlt1) AND FAS ASSOCIATED PROTEINS IN PREGNANCIES COMPLICATED BY PREECLAMPSIA AND INTRAUTERINE GROWTH RESTRICTION (IUGR)

By

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ABSTRACT

Current thinking suggests that preeclampsia is associated with activation of the maternal vascular endothelium in response to factors released from the placenta due to placental hypoxia. Failure of physiological modification of spiral arteries due to impaired trophoblast invasion results in a several-fold increase in the risk of developing pre-eclampsia and/or (IUGR). The defect behind impaired trophoblast invasion is not fully explained and the aetiological factor(s) linked with the development of pre-eclampsia, compared to normotensive IUGR, is not known. In this thesis, I examined placental and serum levels of fms-like tyrosine kinase 1 (sFlt1) and placental growth factor (PlGF), as mediators of angiogenesis, and Fas and FasL, as mediators of apoptosis, in three groups; preeclampsia, normotensive IUGR and controls who had abnormal mid-trimester uterine artery Doppler.

Uterine artery Doppler flows were examined in 553 women at 24 weeks. 97 of them had abnormal uterine artery Doppler flow and were enrolled in this study. 86 women were followed up; among them eight women developed preeclampsia and seven developed normotensive IUGR. Umbilical artery Doppler examination 24 hours before delivery in both groups, showed significantly lowered resistance indices in the preeclampsia compared to the normotensive IUGR group.

I examined placental and serum levels of fms-like tyrosine kinase 1 (Flt1) and Placental Growth Factor (PlGF) in three groups. Soluble Flt1 acts as an antagonist for both Vascular Endothelial Growth Factor (VEGF) and PlGF. Placental Flt1 and serum sFlt1 were higher and serum PlGF was lower in the preeclampsia group compared to the other two groups. This could be responsible for the systemic manifestations of

preeclampsia. This dysregulation in serum sFlt1 and PlGF was found as early as 24 weeks in pregnancies with abnormal uterine artery Doppler examination. The normotensive IUGR group had significantly elevated serum sFlt1 compared to controls. This could be due to an element of placental hypoxia in the IUGR group.

To investigate the in-vivo effect of sFlt1 on impaired placental angiogenesis and trophoblast invasion, I examined the correlations between uterine artery Doppler resistance indices and serum sFlt1 and PlGF at 24 weeks. Significant correlations were found between these markers and uterine artery Doppler pulsatility index (PI) and resistance index (RI) on both the placental and non-placental sides at 24 weeks.

Fas and Fas ligand (FasL) are membrane proteins that mediate cellular apoptosis, and recently were related to cellular growth and migration. Using western blotting and immunohistochemistry, placental expression of Fas (western blotting) and (FasL) (immunohistochemistry) was assessed in the three study groups. No differences in placental Fas or Fas ligand were found between the groups. In addition, serum levels of Fas and FasL were measured at 24 weeks and within 24 hours of delivery in the same groups. Serum Fas was not different between the three study groups at 24 weeks and within 24 hours of delivery. Serum FasL was below the kit's detection threshold in the samples studied.

In conclusion, placental Flt1 and its soluble form sFlt1 seem to play an important role in the pathophysiology of preeclampsia. In addition, sFlt1 correlated positively with the severity of impaired trophoblast invasion and could play a central role in blocking placental angiogenesis in these pregnancies. This needs further evaluation. Fas and

FasL do not seem to have a role in impaired placentation and development of preeclampsia and IUGR.

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Converting data rigorously into a thesis suitable for submission is always a big challenge. Professor Philip Baker, Dr Louise Kenny, Dr Anthony Smith, Dr Sarah Vause and Mr. Sean Hughes provided inspired suggestions and encouragement throughout the course of writing up this thesis.

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dearly like to list them all, but rules of confidentiality mean that the names of those to whom I owe the most must remain forever outside the public domain. The least I would say is that their altruism was humbling.

INTRODUCTION

1.1 Definition of Preeclampsia

Preeclampsia is a serious disorder of human pregnancy, which because of a lack of complete understanding of aetiology and pathophysiology, is defined by the presenting symptoms and signs. In this thesis, the definition of preeclampsia made by other authors has been taken to imply significant hypertension and proteinurea, although different thresholds have been used in many of the studies cited. Patients recruited into the present study have preeclampsia as defined according to the guidelines of the International Society for the Study of Hypertension in Pregnancy (ISSHP). This defines preeclampsia as hypertension of at least 140/90 (diastolic blood pressure recorded at Korotkoff 5) on two separate occasions at least four hours apart and in the presence of at least 300mg protein in a twenty-four hour collection of urine, arising de novo after the 20th week of gestation in a previously normotensive woman and resolving completely by the 6th post partum week (Davey *et al.*, 1988).

1.2 Incidence of Preeclampsia

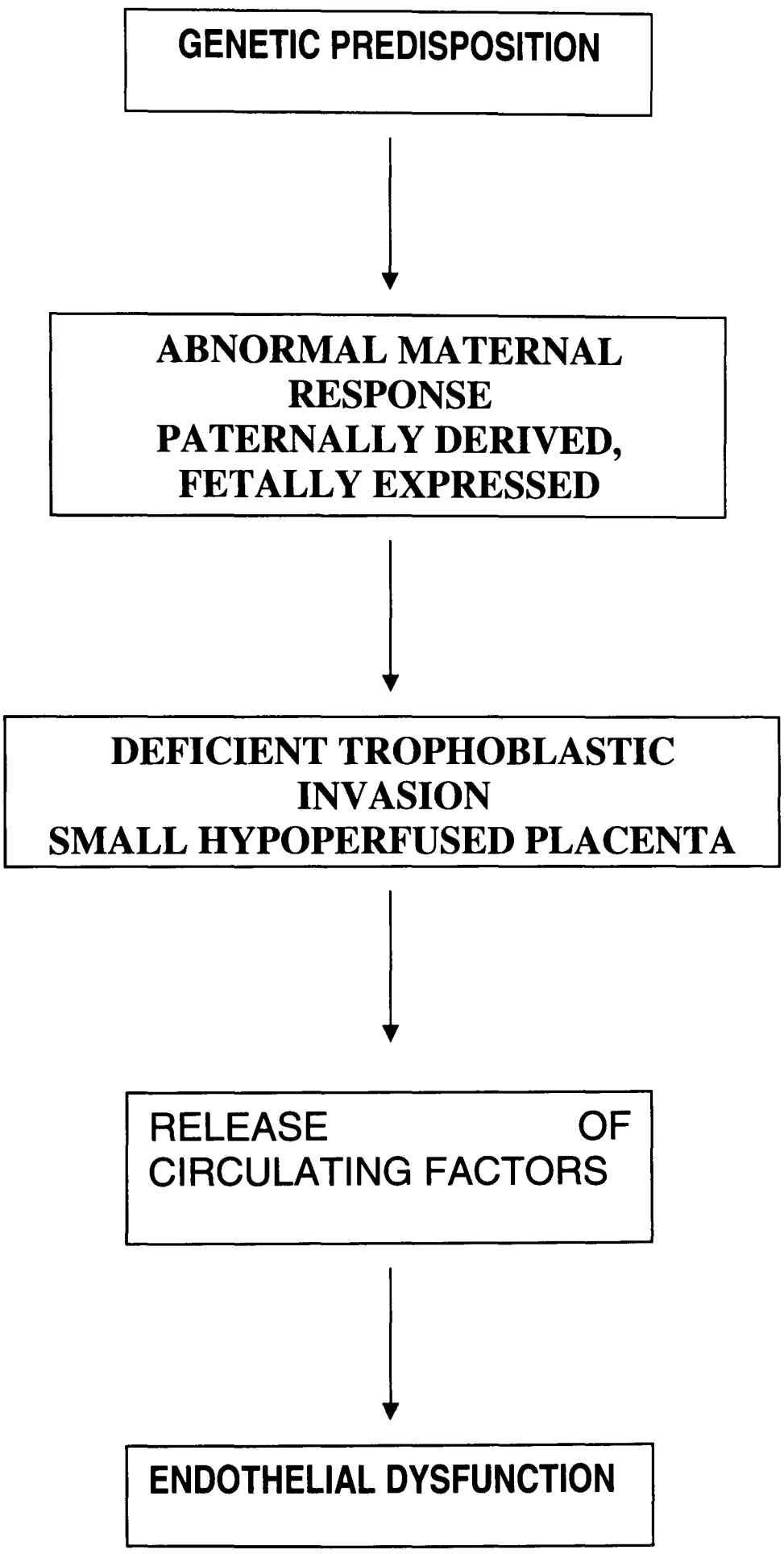
Preeclampsia complicates 2-5% of pregnancies in the United Kingdom. It is a leading cause of maternal mortality and is responsible for considerable perinatal mortality and morbidity (HMSO). Furthermore, preeclampsia carries health care implications in adult life. Infants born of pregnancies complicated by preeclampsia are at an increased risk of hypertension, heart disease and diabetes (Barker, 1992). Therapy of preeclampsia is now, as it has been for the last 100 years, delivery of the fetus and placenta. Consequently, part of the increased perinatal mortality is due to iatrogenic prematurity. It is estimated that 15% of pre-term births are secondary to delivery for preeclampsia (Meis *et al.*, 1995). An appropriate therapeutic strategy based upon the

underlying cause of the disease is clearly required to reduce the impact of this condition. However, the precise aetiology of preeclampsia is poorly defined.

1.3 Aetiology of Preeclampsia

The precise aetiology of preeclampsia, which has been termed the 'disease of theories' (Zwiefel, 1916), has eluded physicians since the time of Hippocrates. At the turn of the last century, the presence of a circulating toxin of fetal origin was postulated as the cause of eclampsia, and hence the disease became known as 'toxaemia of pregnancy'. More than a century later, the toxaemic theory remains the favoured hypothesis although the focus is now on the placenta rather than the fetus. It is now widely accepted that preeclampsia is associated with abnormal placental implantation, which is believed to result in relative placental ischaemia. Furthermore, as yet unidentified maternal or fetal genes may confer susceptibility. The syndrome of preeclampsia, which normally presents in the third trimester, possibly results from an immunologically based maternal response to the faulty placental implantation occurring earlier in pregnancy. This response, which can involve almost every major system of the body, reflects the involvement of the maternal vascular endothelium, now widely regarded as the target cell of the disease process. These observations have led to the development of an aetiological model of preeclampsia (see Figure 1.1).

Figure 1.1 The proposed pathogenesis of preeclampsia.



1.3.1 The Genetics of Preeclampsia

A familial factor has been recognised in the pathogenesis of preeclampsia for many years. Epidemiological studies have demonstrated a 3-4-fold increase in the incidence of preeclampsia in first-degree relatives of affected women (Chesley *et al.*, 1986; Arngrimsson *et al.*, 1990). Family genetic studies have previously been reported to support models of maternal inheritance due to a recessive gene with a population frequency of 0.16 to 0.31 (Chesley *et al.*, 1986; Arngrimsson *et al.*, 1990), or a dominant gene with a population frequency of 0.14 and 48% penetrance (Arngrimsson *et al.*, 1990). However, data on the incidence of preeclampsia in identical twin sisters, although limited, indicates that discordance is more common than concordance, implicating factors other than the maternal genotype (Thornton *et al.*, 1999).

One possible factor is the contribution of the fetal genotype. Epidemiological data have been reported, consistent with fetal recessive inheritance (Cooper *et al.*, 1979) or, maternal-fetal sharing of a recessive gene (Liston *et al.*, 1991). Genetic studies of preeclampsia are beset by difficulties. Extended pedigrees are relatively uncommon. In part this is due to the fact that males cannot be tested for susceptibility and that females can only be tested if they become pregnant. Furthermore, preeclampsia is predominantly a disease of first pregnancy and therefore the interaction between maternal and fetal genotypes can only be tested once. Finally, the definition and diagnosis of preeclampsia, in even the recent past, has been variable and imprecise. Hence, retrospective analysis of hospital records, when they are available, is often frustrating and unreliable. There are presently several studies in progress that are attempting to address the complex issues surrounding genetic studies in preeclampsia.

One such study, the Genetics of Preeclampsia Collaborative Study (GOPEC) is a multi-centre UK project. The aim is to utilise transmission disequilibrium testing (TDT), in order to examine distortions in the transmission of marker alleles from heterozygous (informative) parents to affected individuals. In the first instance, the GOPEC study will investigate various candidate genes, which have been implicated by previous studies.

The genetic inheritance of preeclampsia is undoubtedly complex. However it is highly likely that the advent of complete disclosure of the human genome and rapidly evolving genotyping technology will make genome wide screening by TDT a realistic possibility within the next few years.

1.3.2 Immunological Aspects

The underlying reason for the failure of trophoblast invasion in preeclampsia is unknown. It has been suggested that abnormal placentation reflects an abnormal maternal immunological response to fetal antigens derived from the father. The increased prevalence of preeclampsia in multiple pregnancies, molar pregnancies and those associated with increased placental mass suggests that fetal antigen load and trophoblast volume have a pathological role in this disorder (Taylor, 1997).

There is extensive epidemiological evidence to implicate immunological factors in the aetiology of preeclampsia. These data suggest that prior exposure to paternal antigen is protective against preeclampsia (Taylor, 1997). Preeclampsia is largely a disease of the first pregnancies (Misra *et al.*, 1997). It is proposed that the normal fetal maternal transfusion that occurs during pregnancy and particularly during delivery exposes the mother to products of fetal (and hence paternal) genome, protecting her in subsequent

pregnancies. In keeping with this concept, the protective effect of first pregnancy is partially lost if a women has a child with a new father (Trupin *et al.*, 1996).

Li and Wi (2000) conducted a cohort study based on 140,147 women with two consecutive births during 1989-1991 identified through linking of annual California birth certificate data. Among women without preeclampsia in the first birth, changing partners resulted in a 30% increase in the risk of preeclampsia in the subsequent pregnancy compared with those who did not change partners. However, among women with preeclampsia in the first birth, changing partners resulted in a 30% reduction in the risk of preeclampsia in the subsequent pregnancy. These findings demonstrate that preeclampsia is a disease of primipaternity rather than primigravidity and are consistent with the hypothesis that normal pregnancy reflects a state of tolerance to the foreign paternally derived antigens of the fetus, whereas in women with preeclampsia this immunological tolerance is impaired.

In line with this hypothesis, several studies have demonstrated that the incidence of preeclampsia may be related to the duration of prior exposure to paternal antigens in sperm. During a protracted sexual relationship women develop an immune response against paternal antigens expressed on spermatozoa or in seminal fluid (Robillard *et al.*, 1996), which is possibly impaired in women using barrier methods of contraception (Klonoff-Cohen *et al.*, 1989), and enhanced by oral exposure (Koelman *et al.*, 2000). As would be predicted, women inseminated with sperm that are not from their husband have an increased risk of preeclampsia (Smith *et al.*, 1997). Furthermore, women conceiving with donated embryos have an even greater risk of

developing preeclampsia presumably because the entire fetal genome is allogenic in these pregnancies (Salha *et al.*, 1999).

1.3.3 The Role of the Placenta

The pivotal role of the placenta in the pathogenesis of preeclampsia has been established by several clinical observations. Firstly, It has long been recognised that removal of the placenta leads to resolution of the disease and indeed this remains the mainstay of current clinical management. Secondly, preeclampsia can occur in the absence of a fetus. Indeed hydatiform moles are associated with an increased incidence of preeclampsia (Scott *et al.*, 1976). Finally, the incidence of preeclampsia is increased in pregnancies associated with hyperplacentosis such as multiple pregnancy and diabetes (Szulman *et al.*, 1982; Coonrod *et al.*, 1995; Sibai *et al.*, 2000).

Thirty years ago, morphological examination of placental bed biopsies from women with preeclampsia demonstrated shallow invasion of trophoblasts and failure of vascular remodelling (Brosens *et al.*, 1972). Applications of contemporary bioscientific approaches have provided detailed insight into deficient mechanisms in this process.

Within the placenta in early gestation, trophoblasts differentiate and give rise to sub-populations of cell. One subpopulation, the extravillous trophoblast, invades the uterine wall (interstitial invasion) and its blood vessels (endovascular invasion). During normal pregnancy, the trophoblasts destined to be endovascular, adopt a more endothelial cell-like phenotype and invade the uterine spiral arteries progressing back

as far as the myometrial segments. Immuohistochemical studies of placental bed biopsies suggest that trophoblast cells and endothelial cells transiently coexist on walls of partially modified spiral arteries (Pijnenborg *et al.*, 1983; Zhou *et al.*, 1997b). Trophoblast cells migrate along the luminal surfaces of vessels, invading them and partially replacing the endothelial cells and most of the musculoelastic tissue in the vessel walls (Pijnenborg *et al.*, 1980; Enders *et al.*, 1991; Blankenship *et al.*, 1993; Meekins *et al.*, 1997). The vessels are subsequently restructured such that they have little smooth muscle and become larger in diameter. This creates a high flow, low resistance circulation that maximises maternal blood flow to the placental villi at the maternal-fetal interface. There is contrasting evidence as to whether trophoblasts themselves are important in arterial remodelling. Although it has been suggested that some changes in the decidual vessels occur independently, as part of the maternal response to pregnancy, there is also strong evidence that invasive interstitial trophoblasts prepare the decidual spiral arteries for endovascular trophoblast migration (Pijnenborg *et al.*, 1983; Blankenship *et al.*, 1997). The invasive trophoblast may play an important role in inducing further changes either by interactions or factors produced by the interstitial trophoblast or by direct cellular interactions of the endovascular trophoblast with the cells of the vessel that they subsequently replace.

The pathogenesis of preeclampsia and intrauterine growth restriction is associated with trophoblasts failing to adopt an endothelial cell-like phenotype and endovascular invasion failing to proceed beyond the superficial portions of the spiral arteries in early pregnancy (Lim *et al.*, 1997; Meekins *et al.*, 1994; Zhou *et al.*, 1997a). The cause of this impaired placentation is not fully understood but may in part be due to poor invasive properties of the trophoblastic cells or changes in the maternal decidual

tissues, which regulate trophoblast behaviour, perhaps mediated via multifunctional cytokine pathways. The cytotrophoblastic expression of adhesion molecules, which influence invasion, is altered in women with preeclampsia (Pijnenborg *et al.*, 1996). In vitro studies have shown lower attachment of trophoblasts from pre-eclamptic placentas on fibronectin and vitronectin compared to normotensive controls, which may reflect differences in expression of matrix receptors (Pijnenborg *et al.*, 1996). Maternal factors leading to inhibition of trophoblast invasion include reduced expression of the histocompatibility antigen HLA-G (Colbern *et al.*, 1994), local inflammatory cell behaviour (Butterworth *et al.*, 1991) and cytokine regulation of integrin expression (Vinatier *et al.*, 1995).

The end result of altered differentiation is inadequate trophoblast invasion. The incomplete remodelling of the maternal vasculature results in inadequate uteroplacental perfusion, particularly later in pregnancy. The subsequent hypoxia/ischaemia is presumed to trigger the release of a circulating factor(s) into the maternal circulation.

1.3.4 The Link Between Reduced Placental Perfusion and Maternal Systemic Disease

The link between deficient trophoblastic invasion early in pregnancy and the widespread endothelial dysfunction manifesting much later has eluded researchers for many years. The observation that terminating the pregnancy, and more specifically, that delivery of the placenta results in resolution of the disease suggest that the placenta is the focus of production of the putative factor(s) that influences the endothelial cell. This hypothesis, and the nature of the circulating factor(s), has been

investigated in a wide variety of both in vivo and ex vivo studies. One of the first studies to investigate the potential of circulating factors was reported over a decade ago and is widely credited with reviving the concept of toxaemia (Rodgers *et al.*, 1988). This much cited study demonstrated that serum from women with preeclampsia was cytotoxic to cultured human umbilical vein endothelial cells (HUVECS) when compared with control sera from normal pregnant women. The cytotoxic effect of serum of preeclamptic women was reduced after 24 to 48 hours post partum. This rapid disappearance of cytotoxic activity suggested that the putative factor(s) had a short half-life and provided evidence that it was related to the products of conception. Interestingly, the cytotoxic effect persisted when a combination of antenatal sera from normal pregnant women and women with preeclampsia was utilised, providing further evidence of an active circulating factor as opposed to a relative deficiency of a protective factor. This landmark study has subsequently been extended by other investigators in a wide variety of cultured cell types. It now appears that the circulating factor(s) induce an alteration in endothelial function (Tsukimori *et al.*, 1992; Roberts *et al.*, 1992) rather than gross morphological injury and cell death as was initially suggested (Rodgers *et al.*, 1988). This contention is supported by the observation that when seeded in the presence of sera from women with preeclampsia, endothelial cells proliferate, attach and spread in a manner similar to those seeded in sera from normal pregnant women. Moreover, endothelial cells continue to grow well during incubation with serum from women with preeclampsia, and any metabolic changes can be reversed by replacing the serum from women with preeclampsia with standard culture medium (Lorentzen *et al.*, 1991).

Several investigators have used the technique of small vessel myography to study endothelial function in isolated vessels. Using an isometric technique, Ashworth *et al.* (1998) observed that incubation of vessels isolated from normal pregnant women with plasma from women with preeclampsia induced an alteration in endothelium-dependent responses, such that the response to bradykinin was markedly impaired. Under these conditions vessels from normal pregnant women mimicked the behaviour of vessels isolated from women with preeclampsia (Ashworth *et al.*, 1998). Further investigation of this phenomenon has revealed that the effect was independent of the parity of the patient from whom the vessel was isolated, but was specific to vessels from pregnant women (Hayman *et al.*, 2001).

In summary, there is an abundance of evidence to implicate the presence of a placentally derived circulating factor that targets the endothelial cell in preeclampsia and induces widespread alterations in function. Evidence suggests that there are a number of characteristics any putative factor must possess. These include the ability to pass freely into the maternal circulation and to produce functional alterations including an increase in cellular permeability and possibly cell turnover, an alteration in prostacyclin and nitric oxide production and subsequent functional change in the response to endothelium-dependent vasodilators. Although definitive identification of one single factor has not been made, a number of factors have been advanced as potential candidates for this role.

1.3.4.1 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis (1, 3), and promotes endothelial cell proliferation and migration (Clark *et al.*, 1998).

VEGF was shown to be essential for embryonic development in mice (Ferrarea *et al.*, 1996; Carmeliet *et al.*, 1996) and its action cannot be compensated for by other closely related factors (Joukov *et al.*, 1996; Olofsson *et al.*, 1996; Counetal *et al.*, 1990). The receptors for VEGF-the *fms*-like tyrosine kinase, Flt-1 (Shibuya *et al.*, 1990), and the kinase domain receptor, KDR are present in endothelial cells present in many tissues (Peters *et al.*, 1993; Millauer *et al.*, 1993; Olander *et al.*, 1991). Flt-1 but not KDR, on the other hand is highly expressed by trophoblast cells (Charnock-Jones *et al.*, 1994; Clark *et al.*, 1996).

Angiogenesis is a complex process necessitating the interaction of numerous cell types that leads to the coordinated development of a complex three-dimensional vascular structure. Many factors are involved in angiogeneis, and it is the balance of stimulators such as VEGF and inhibitors that determines the net result (O'Reilly *et al.*, 1994; Hanahan & Folkman, 1996). In the placenta, there is profound angiogeneis as high-capacity transport develops between the maternal and fetal circulation (Clark *et al.*, 1996) (see above). In human placentation, while there is profound angiogenesis within the placental villi, there is little angiogenesis in maternal tissue. This suggests that there are locally acting factors that regulate vascular growth.

VEGF has been implicated to act as an endothelial survival factor by promoting scaffold formation and cell attachment in microvascular endothelial cells (Ahmed *et al.*, 2000; Watanabe & Dvorak, 1997). Withdrawal of VEGF from xenografted c6 gliomas resulted in blood vessel regression and endothelial cell death (Benjamin & Keshet, 1997). As VEGF declines throughout gestation, these data suggest a possible mechanism for the regression of the capillary nets during stem villous formation (see

above) (Ahmed *et al.,* 2000). Five genes for VEGF have so far been identified and these are designated as VEGF-A (Leung *et al.,* 1989), -B (Paavonen *et al.,* 1996), -C (Joukov *et al.*, 1996), -D (Yamada *et al.*, 1997) and the virally encoded -E (Lyttle *et al.*, 1994). There are three receptors for VEGF-A; VEGFR-1, VEGFR-2 and VEGFR-3. VEGF-A is highly expressed in the placenta and previous studies showed strong expression of its antagonist, Flt-1, in both villous and extravillous trophoblast (EVT) cells (Charnock-Jones *et al.,* 1994; Clark *et al.,* 1996). Expression of VEGF-A in different placental cell types has been shown to increase under hypoxic conditions (Wheeler *et al.*, 1995).

In preeclampsia, there is circumstantial evidence that antagonism of VEGF may have a role in hypertension and proteinurea. VEGF is a well-known promoter of angiogenesis; it also induces nitric oxide and vasodilatory prostacyclins in endothelial cells, suggesting a role in decreasing vascular tone and blood pressure (Morbidelli *et al.,* 1996; He *et al.,* 1999). VEGF has been implicated in glomerular healing, and anti-VEGF compounds have been found to increase apoptosis, impair glomerular capillary repair, and increase proteinurea in a rat model of mesangioproliferative nephritis (Ostendorf *et al.,* 1999). Furthermore, exogenous VEGF was found to accelerate renal recovery in rat models of glomerulonephritis and experimental thrombotic microangiopathy (Masuda *et al.,* 2001; Kim *et al.,* 2000). More recently, exogenous VEGF was shown to ameliorate post-cyclosporin-mediated hypertension, endothelial dysfunction, and nephropathy (Kang *et al.,* 2001). In recent antiangiogenic clinical trials, VEGF signalling inhibitors have resulted in hypertension and proteinurea (Maynard *et al.,* 2003). The study by Clark *et al* (1998) showed that the placenta secretes a soluble form of VEGF antagonist, namely sFlt-1, which naturally regulates

the potent angiogenic action of VEGF. sFlt-1 is believed to play a role in regulating placental and decidual angiogenesis.

Collectively these data suggest that VEGF is important not only in blood vessel regulation but also in maintaining the glomerular filtration barrier. A recent study describing new knockout mice, reduction of VEGF production by podocytes alone led to massive proteinurea and glomerular endotheliosis (Eremina *et al.*, 2002). Additionally, VEGF-neutralising antibodies in clinical cancer trials have resulted in proteinurea (Yang *et al.*, 2000). These reports support the hypothesis that VEGF deficiency in the glomerulus may produce proteinurea.

The role of VEGF in preeclampsia has received substantial attention. Several authors have reported increased systemic VEGF levels in women with preeclampsia (Baker *et al.*, 1995; Bosio *et al.*, 2001; Hunter *et al.*, 2000; Sharkey *et al.*, 1996). Other authors have reported reduced levels (Lyall *et al.*, 1997; Reuvekamp *et al.*, 1999; Livingston *et al.*, 2000). In reviewing the methodology of these studies carefully, all the studies reporting decreased levels used a commercially available ELISA kit (R&D Systems), which measures *free* (unbound) VEGF as previously shown by others (Hornig *et al.*, 2000; Banks *et al.*, 1998; Anthony *et al.*, 1997). On the other hand, all studies reporting increased VEGF in preeclampsia used either radioimmunoassay or a non-R&D ELISA system, measuring *total* (bound and unbound) VEGF (Baker *et al.*, 1995; Bosio *et al.*, 2001; Hunter *et al.*, 2000; Sharkey *et al.*, 1996). Under many circumstances, these two entities would be intererchangeable. However, in pregnancy, circulating sFlt1 is present at very high levels (the mean sFlt1 level in normal-term pregnancy was 0.5+/- 0.2 ng/ml), compared with the nonpregnant state, in which sFlt1

levels are relatively low (the mean sFlt1 level in healthy female volunteers was 0.15 +/- 0.04 ng/ml (Maynard *et al.,* 2003). Therefore, in normal pregnancy, and especially in preeclampsia where circulating levels of sFlt1 are extremely high, most VEGF is bound to circulating sFlt1 (Jelkmann *et al.,* 2001). Free VEGF levels, which more accurately reflect effective, circulating VEGF, will thus be substantially lower than total VEGF levels. Seen in this light, the previously confusing and contradictory literature on VEGF supports the hypothesis that preeclampsia is characterised by normal to high total VEGF levels.(perhaps induced by placental hypoxia) but low free VEGF and PlGF levels, owing to a great excess of sFlt1.

1.3.4.2 Placental Growth Factor (PlGF)

Placental growth factor is a secreted dimeric glycoprotein that shares significant homology with VEGF, hence its role in angiogenesis. Like VEGF, PlGF is a potent angiogenic growth factor capable of inducing proliferation, migration, and activation of endothelial cells (Ziche *et al.,* 1997). Unlike the case of VEGF, abundant expression of PlGF is restricted to the placenta (Maglione *et al.,* 1991; Maglione *et al.,* 1993), with the primary site of synthesis being the trophoblast (Khaliq *et al.,* 1996; Vourela *et al.,* 1997; Shore *et al.,* 1997). Also in contrast VEGF, low oxygen tension results in downward regulation of PlGF expression in primary cultures of human trophoblasts (Shore *et al.,* 1997).

The high basal expression of PlGF in trophoblasts and decreased expression during hypoxia in vitro suggest that PlGF may be altered in preeclampsia. Cross-sectional studies in normal pregnancy show that PlGF levels rise during the second trimester and peak at the early third trimester before falling to low levels by delivery.

Decreasing PlGF levels near term may be the result of acute hypoxia, because labour was associated with significantly reduced serum levels of PlGF.

In healthy pregnant women, the serum levels of PlGF increased by approximately four times from the late first trimester to the late second trimester (Torry *et al.,* 1998). This increase may reflect growth of the placenta, which is concomitant with the need to recruit and maintain an adequate placental circulation. Low levels of PlGF during the first trimester may reflect the paucity of maternal blood flow into the intervillous spaces at this time (Jaffe *et al.,* 1997). Serum PlGF levels declined steadily from peak levels at approximately 28 to 30 weeks' gestation to term but remained slightly higher than levels found during the first trimester.

PlGF mRNA expression has been found exclusively in villous trophoblasts, whereas protein localization was also noted in the fetal stem vessels, implying that trophoblast-derived PlGF may act in a paracrine fashion to influence placental blood vessels (Vourela *et al.,* 1997). A study by Shore et al showed that hypoxia severely inhibited PlGF mRNA expression in normal trophoblasts (Shore *et al.,* 1997). Torry *et al.* (1998) showed that serum PlGF levels were significantly lower in preeclampsia compared with gestational-age matched controls, and that although PlGF levels decreased gradually with gestational age in healthy pregnancies, the levels in preeclampsia showed no significant changes with gestational age, as they were almost one fifth the normal range. Importantly, the low levels of PlGF noted in patients with preeclampsia were not due to a decrease in placental size, and most probably represent trophoblast response to hypoxia. There was significant drop of the level of PlGF at term, and during labour due to the hypoxic effect on the placenta by the

increasing myometrial tone in the third trimester and uterine contractions in labour. In cases of preeclampsia, however, there was no correlation between mode of delivery and the level of PlGF in the serum or cord blood, suggesting that low expression of PlGF in preeclampsia preclude additional perturbations in placental perfusion from having a significant effect (Torry *et al.,* 1998).

1.3.4.3 Placental soluble fms-like tyrosine kinase 1 (sFlt1)

Placental soluble fms-like tyrosine kinase 1 (sFlt1), a splice variant of the VEGF receptor (Flt1) lacking the transmembrane and cytoplasm domains, acts as a potent VEGF and PlGF antagonist (Kendall *et al.,* 1996; Shibuya *et al.,* 2001). It is produced by a number of tissues, including the placenta (He *et al.,* 1999). Its physiologic role is unclear. Recently, both placental sFlt1 expression and sFlt1 levels in the amniotic fluid have been noted to be elevated in preeclampsia (Zhou *et al.,* 2002; Vuorela *et al.,* 2000).

A recent study by Maynard *et al.* (2003) demonstrated excess serum sFlt1 levels in preeclampsia. In their study, excess sFlt1 production most likely originated in the placenta as its mRNA was upregulated in the placentas and the serum levels fell within 48 hours after delivery. They also demonstrated that administration of sFlt1 to pregnant rats induces hypertension, proteinurea and glomerular endotheliosis, the classic lesions of preeclampsia. The systemic effects of sFlt1 do not require the presence of pregnancy or the placenta, as hypertension and glomerular changes occurred in both non-pregnant and pregnant rats when infused with sFlt1. This suggests a direct effect of sFlt1 on the maternal endothelium. Their work suggested that sFlt1 acts through its antagonism of both VEGF and PlGF, since the sole VEGF

antagonist, sFlk1, did not produce the preeclampsia phenotype in pregnant rats. The preeclampsia phenotype that developed in rats was associated with decreased circulating levels of free VEGF and PlGF, resulting in endothelial dysfunction in vitro and was rescued by exogenous VEGF and PlGF. This suggests that, although the primary trigger for abnormal placental development and excess sFlt1 production in preeclampsia remains speculative, excess sFlt1 alone may be sufficient to produce generalised endothelial dysfunction and some of the clinical phenotype noted in preeclampsia. Interestingly, the pathologic effects of sFlt1 were dose dependent in the animal model; rats treated with low-dose sFlt1, with plasma levels similar to those seen in preeclamptic women, generally showed milder renal pathology as compared with rats treated with higher dose of sFlt1. A possible explanation is that sFlt1 may be one of several factors elaborated by the placenta that influence the severity of preeclampsia. It is also possible that the recombinant adenoviral-linked sFlt1, which was used to induce preeclampsia in these animals, has a less potent in vivo activity than endogenous sFlt1 present in human serum. Alternatively more prolonged, sustained levels of sFlt1 are required to produce severe disease. Furthermore, none of the treated animals in this study, developed thrombocytopenia, which is present in humans with the condition of haemolysis, elevated liver function tests, and low platelets (HELLP) syndrome, a variant of preeclampsia. In view of the above and because patients with mild preeclampsia had only slightly elevated sFlt1 levels, they concluded that sFlt1 may be causative in most but not all cases of preeclampsia in humans and that additional factors might be involved in HELLP syndrome.

The above study, however, did not distinguish whether sFlt1 production by the placenta is a primary or secondary event. Hypoxia has been shown to increase sFlt1

production by placental cytotrophoblasts (Hornig *et al.*, 2000). If placental hypoxia is an early event in preeclampsia, sFlt1 release may occur as a secondary phenomenon. It has been proposed that placental angiogenesis is defective in preeclampsia, as evidenced by failure of the cytotrophoblasts to convert from an epithelial to an endothelial phenotype (referred to as pseudovasculogenesis) and invade maternal spiral arteries (Zhou *et al.*, 1997). It seems plausible that angiogenic molecules such as VEGF, PlGF, and sFlt1 may be important regulators of early placental development and pseudovasculogenesis. In fact, it has recently been shown that exogenous sFlt1 inhibits placental cytotrophoblast invasion in vitro (Zhou *et al.*, 2002). Thus excess placental sFlt1, in addition to its direct effect on the maternal endothelium in the third trimester, may also play a more primary role in deranged placental development in preeclampsia. In the study by Maynard *et al.* (2003), the sFlt1 treated-rats did not develop the placental pathological changes typical of preeclampsia, such as infarcts and shallow spiral invasion. However, this may reflect the fact that sFlt1 protein was administered in the early second trimester, after spiral artery invasion had already been established.

In vitro studies on the chorioallantoic membrane of the chicken have demonstrated that VEGF binding to both Flt-1 and KDR mediates branching angiogenesis, while PlGF binding to Flt-1 alone results in non-branching angiogenesis (see above) (Wilting *et al.*, 1996; Kurz *et al.*, 1998). In the human placenta; VEGF, PlGF and the two receptors are differentially expressed throughout gestation: VEGF and KDR are most intense during early gestation and decline as pregnancy advances (Vuckovic *et al.*, 1996) while PlGF and Flt-1 increase towards term (Clark *et al.*, 1996). Correlations of these growth factors effects and their expression patterns throughout

gestation with the development of the villous angioarchitecture (Kaufmann *et al.*, 1985) suggest that VEGF and KDR are involved in the first two trimesters of pregnancy in the establishment of the richly branched capillary beds of the mesenchymal and immature intermediate villi, while PlGF and Flt-1 are more likely to be involved in the formation of the long, poorly branched, terminal capillary loops in the last trimester (Ahmed *et al.*, 2000).

1.3.4.4 Neurokinin B

Neurokinin B is a neuropeptide and one of three known mammalian tachykinins; the others being substance P and neurokinin A. The tachykinins are normally restricted to the nervous tissue and exert their effects peripherally by release from nerve endings and activation of the neurokinin receptors, NK1, NK2 and NK3. Neurokinin B preferentially binds to the NK3 receptor, activation of which has been demonstrated to induce hypertension by contraction of the rat portal vein and mesenteric vasculature (D'Orleans-Juste *et al.*, 1991) and increase canine heart rate (Thompson *et al.*, 1998).

Neurokinin B was previously thought to be restricted to the brain. However, Page *et al.* (2000) have recently reported that the syncytiotrophoblast of the human placenta expresses neurokinin B mRNA. Moreover they found that plasma levels of the peptide were significantly elevated in women with preeclampsia, as compared with normal pregnant women. They speculated that, in response to placental ischaemia consequent upon defective trophoblastic invasion, placental production of neurokinin B increases, in order to increase blood pressure and correct the hypoperfusion of the feto-placental unit. The subsequent stimulation of the neurokinin B receptors is hypothesised to cause constriction of the mesenteric vascular bed and the portal veins, leading to an

increase in blood pressure, damage to the liver and kidneys and the symptoms of abdominal pain. Reduction in blood flow to the liver is speculated to lead to an accumulation of undetoxified metabolites, such as lipid peroxides, which may contribute to endothelial cell damage and dysfunction. The authors also suggest that in severe cases of preeclampsia, concentrations of neurokinin B may be sufficient to stimulate peripheral NK 1 receptors on platelets and neutrophils and hence contribute to the other symptoms of preeclampsia associated with activation of these cells. Finally, the authors suggest that increased secretion of neurokinin B may pre-date the development of clinical signs and symptoms of preeclampsia and therefore increased levels of neurokinin B in early pregnancy may identify pregnancies destined to develop preeclampsia. The neurokinin B hypothesis is attractive, particularly as there are several potent and commercially available selective antagonists for NK receptors, allowing for the implementation of a much-needed pharmacological intervention early in the disease process. However, the findings of this small study await confirmation. In particular, the localisation of NK receptors in the human vasculature and the effect of neurokinin B on human vascular tone await elucidation. Quantitative analysis of the placental production of neurokinin B in normal and compromised human pregnancy and circulating maternal plasma levels in all three trimesters of pregnancy await clarification in a longitudinal study. Undoubtedly this area will be the focus of intense research in the immediate future.

1.3.4.5 Syncytiotrophoblast microvillous membranes (STBM)

STBM have been proposed to be the factor linking the defective placentation to the endothelial dysfunction (Smarason *et al.*, 1993). Morphological evaluation of placentas from women with preeclampsia shows abnormally shaped syncytiotrophoblast microvilli and areas of focal necrosis, associated with a reduced number of microvilli. These changes are similar to those seen in placental villi, cultured under hypoxic conditions. In vitro experiments have demonstrated that STBM can interfere with endothelial cell growth in cultured endothelial cells by suppressing proliferation and disrupting the cell monolayer (Smarason *et al.*, 1993). Furthermore, perfusion of isolated subcutaneous arteries with a high concentration of STBM abolishes acetylcholine-induced vasodilatation (Cockell *et al.*, 1997).

STBM prepared from placentas of normal women and women with preeclampsia had similar effects on endothelial cells, indicating the effect of STBM in preeclampsia to be quantitative rather than qualitative (Smarason *et al.*, 1993). Knight *et al.* (1998) studied the presence of STBM in pregnant women and reported the concentrations of STBM to be significantly increased in the maternal circulation of women with preeclampsia, with concentrations in the uterine vein exceeding that in peripheral venous circulation, indicating the placental origin of the STBM. The mechanism by which STBM exerts effects on endothelial cells is unclear. However recent evidence has emerged linking STBM with oxidative stress. It has been reported that incubation of cultured endothelial cells with STBM produced a substance that activates peripheral leukocytes and primes peripheral monocytes to give greater responses after activation (von Dadelszen *et al.*, 1999).

1.3.4.6 The role of oxidative stress

More recently, evidence has emerged to support a role for oxidative stress in linking reduced placental perfusion with systemic maternal pathophysiology (Hubel, 1999). Oxidative stress is a pathological state, implicated in the aetiology of many disorders including atherosclerosis, in which pro-oxidants dominate over antioxidants. The resultant increase in the formation of reactive oxygen species can damage cell membranes, proteins and DNA. Epidemiological studies have revealed that many of the risk factors associated with the development of preeclampsia such as obesity, black race, lipid abnormalities, insulin resistance and raised serum homocysteine are also associated with the risk of developing atherosclerosis in later life. These observations have led to the emerging hypothesis that suggests reduced placental perfusion generates oxidative stress and leads to widespread endothelial dysfunction in preeclampsia. The hypothesis proposes that reduced placental perfusion results in the generation of reactive oxygen species (Hubel *et al.*, 1997; Roberts *et al.*, 1999). Placental blood flow is known to be reduced by posture, activity and uterine contractions and to rapidly return to normal with the termination of these stimuli. In normal pregnancy it is suggested that adaptive mechanisms prevent this from resulting in hypoperfusion-reperfusion oxidative stress. Evidence for this concept is the presence of stable metabolites generated by oxidative stress in the placentas of women with preeclampsia (Wang *et al.*, 1992). Furthermore, enzyme activities and mRNA expression of the placental antioxidant enzymes superoxide dismutase and glutathione peroxidase have been found to be significantly decreased in placentae from women with pregnancies complicated by preeclampsia as compared to those from normal pregnant women (Wang *et al.*, 1996). This is thought to lead to an abnormal increase in placental production of lipid peroxides in preeclampsia. In addition, markers of

lipid peroxidation, including malondialdehyde (Hubel *et al.*, 1996) and 8-epiprostaglandin-F2α (Barden *et al.*, 1996) are increased in the plasma of women with preeclampsia. Furthermore, it has been reported that both the water-soluble antioxidant, ascorbic acid and the lipid-soluble antioxidants alpha-tocopherol and beta-carotene levels are decreased in the plasma of women with preeclampsia compared with normal pregnant women, suggesting that antioxidant nutrients may be utilized to a greater extent to counteract free radical-mediated cell disturbances, resulting in a reduction in antioxidant plasma levels in this disease (Mikhail *et al.*, 1994). The increase in lipid peroxide formation by the placenta in preeclampsia may account for many of the pathological changes seen in this disease. With half-lives extending up to minutes, lipid peroxides formed at a primary site may accumulate in lipoproteins and be transferred throughout the circulation. Elevated lipid peroxide levels in plasma inhibit the enzyme prostaglandin synthase, with a consequent fall in the production of prostacylin. However, platelet thromboxane A2 synthesis is unaffected by such compounds. This could potentially lead to an alteration in the ratio of prostacylin to thromboxane production, comparable to that observed in preeclampsia (Wang *et al.*, 1991). Lipid peroxides have also been noted to induce smooth muscle contractions in a variety of isolated arterial preparations. Furthermore, elevation of circulating levels of lipid peroxide products induced by deprivation of vitamin E in rats produced an increased pressor responsiveness to angiotensin II and a decreased isolated mesenteric artery relaxation to acetylcholine (Hubel *et al.*, 1989). Recently, the results of a prospective randomised placebo controlled trial suggested that supplementation with vitamins C and E in women at increased risk of preeclampsia was associated with a significant decrease in plasma markers of vascular endothelial cell activation and placental insufficiency (Chappell *et al.*, 1999). This

study also demonstrated a significant reduction in the occurrence of preeclampsia in the treated group. Confirmation of the clinical usefulness of this therapy by a larger trial is awaited, but the findings do support the involvement of oxidative stress in the genesis of endothelial dysfunction in preeclampsia.

1.4 Endothelial dysfunction in preeclampsia

The vascular endothelium is no longer thought to be simply an inert lining of the vasculature but is now recognised as a highly specialised, metabolically interactive interface between blood and the underlying tissues. Furchgott and Zawadzki (1980) first demonstrated the obligatory role of the endothelium in mediating acetylcholine-induced relaxation in isolated arterial strips or rings. In addition to modulating vascular tone, the endothelium is now known to play a role in maintaining thromboresistance, as well as participating in the inflammatory response.

As our understanding of the role of the endothelium has evolved, a concept has formed that the endothelial cell is the target of the disease process in preeclampsia. This hypothesis is attractive as the ubiquitous nature and diverse functions of the vascular endothelium could account for the complex multisystem nature of the clinical manifestations of the disease process.

The concept of endothelial cell involvement in preeclampsia is by no means novel. A textbook in use over 70 years ago stated that;

"One of the few points to emerge clearly from the study of the pathology of eclampsia is that the toxin acts as an endothelial poison, particularly affecting the walls of capillary vessels" (Johnstone, 1939).

However the significance of this hypothesis was largely ignored for over sixty years, until a series of publications from Roberts and co-workers revived this concept (Roberts *et al.*, 1993; Roberts *et al.*, 1989; Rodgers *et al.*, 1988). With an increased awareness of the physiological significance of vascular endothelial cell function, the seemingly unrelated signs of hypertension, prtoeinurea, oedema, and hypercoagulability converged to provide clinical evidence of a unifying pathophysiological mechanism: systemic, maternal endothelial cell dysfunction.

There is extensive evidence for endothelial involvement in preeclampsia. The best-characterised morphological abnormality of this syndrome, once assumed to be pathognomonic of the condition, involves endothelial cells (Spargo *et al.*, 1959). This distinctive renal lesion, glomerular endotheliosis, is found in up to 80% of women with preeclampsia and consists of glomerular capillary cells engorged with intracellular inclusions. The absence of this lesion in any other form of hypertension and that fact that it disappears completely after delivery, suggest that it is unlikely to be secondary to hypertension or hypoperfusion. Campbell and Campbell (1983) described direct evidence of a loss of endothelial cell integrity with a resulting increase in vascular permeability when they found that an albumin-bound dye, Evan's blue, disappeared from the intravascular compartment of women with preeclampsia at an increased rate compared with normal pregnant women.

There is a substantial body of supporting evidence from studies of circulating endothelial cell markers in preeclampsia. Levels of fibronectin, factor VIII antigen, von Willebrand factor, tissue plasminogen activator and plasminogen activator inhibitor-1 have been found to be elevated in the circulation of women with

preeclampsia (Roberts *et al.*, 1989; Friedman *et al.*, 1995). Although these substances are synthesised by several different cell types, the only common source of production is the vascular endothelium. Several studies have reported increased levels of circulating endothelin-1 in preeclampsia (Taylor *et al.*, 1990; Mastrogiannis *et al.*, 1991) that may reflect an increased synthesis by activated endothelial cells (MacCumber *et al.*, 1989; Yoshimoto *et al.*, 1990). It is recognised that an intact endothelium is a vital component of the coagulation system (Roberts *et al.*, 1993). Widespread deposition of fibrin, associated with diffuse vascular damage suggesting activation of the coagulation system is a recognised pathological feature of preeclampsia. However, unless preeclampsia is complicated by disseminated intravascular coagulation, routine coagulation tests are normal. More sensitive indicators of coagulation abnormalities have been reported in a high proportion of women with preeclampsia including an altered ratio of factor VIII related antigen to coagulation activity (Redman *et al.*, 1977), a reduction in the platelet count (Redman *et al.*, 1978) and an increase in the levels of plasma β-thromboglobulin (Ayhan *et al.*, 1990). Significantly, these changes antedate the onset of clinical manifestations of the disease by several weeks.

One of the most striking and consistent pathophysiological abnormalities of women with preeclampsia is an increased sensitivity to pressor agents such as angiotensin II (Gant *et al.*, 1973; Gant *et al.*, 1987) and noradrenaline (Magness *et al.*, 1994). Gant *et al.* (1973) performed a longitudinal study throughout pregnancy and reported that whereas normal pregnancy was associated with a relative refractoriness to angiotensin II, women destined to develop preeclampsia exhibited increased sensitivity to infused angiotensin II from as early as 18 weeks. This finding has been confirmed by an in

vitro study by Aalkjaer *et al.* (1985) who used the technique of wire myography to investigate the functional characteristics of isolated omental resistance vessels from women with preeclampsia, normal pregnant women and non-pregnant women. The vessels from the women with preeclampsia had an increased responsiveness to angiotensin II compared with the vessels from the normotensive pregnant women whereas the angiotensin II responsiveness of the vessels from the women with preeclampsia and from the non-pregnant women were similar. However, no difference in responsiveness to norepinephrine was found between any of the groups. Pascoal *et al.* (1998) evaluated the responses of omental arteries from normal pregnant women and women with preeclampsia to either KCl or vasopressin. They reported that maximal contraction due to either agent was significantly augmented in vessels from women with preeclampsia.

The suppressed response to pressor agents in normal pregnancy and the increased sensitivity observed in preeclampsia is yet to be fully elucidated. Data from animal studies supports a role for the sympathetic nervous system in modulating vascular tone in pregnancy. Parent *et al.* (1990) compared the sensitivity of pregnant and non-pregnant rat mesenteric arteries to noradrenaline and phenylephrine. They reported a significant decrease in sensitivity to these pressor agents in vessels isolated from pregnant animals compared to those isolated from non-pregnant animals. They further demonstrated that removal of the endothelium had no significant influence on this alteration leading to the suggestion that the changes observed in pregnancy were neurologically mediated. Davidge *et al.* (1992) also examined rat mesenteric arteries from pregnant and non-pregnant animals and reported that arteries from pregnant animals were less sensitive to phenylephrine than arteries from non-pregnant animals.

Furthermore, cyclo-oxygenase inhibition had no effect on the vasoconstrictor response in arteries from either group. Inhibition of nitric oxide and endothelial cell removal had a similar twofold increase in phenylephrine sensitivity in arteries from both the pregnant and non-pregnant rats suggesting that the decreased sensitivity to phenylephrine during pregnancy was not modulated acutely by nitric oxide or by prostaglandin products of the cyclo-oxygenase pathway.

Nelson *et al.* (1998) examined responses to electrical field stimulation (EFS) of perivascular nerves in human uterine arteries removed from pregnant and non-pregnant patients undergoing hysterectomy. EFS induced-constrictions were significantly lower in uterine arteries from pregnant than from non-pregnant patients. When arterial rings were precontracted, the response to EFS was biphasic, consisting of an initial constriction followed by a postconstriction relaxation. The EFS-induced relaxation was endothelium independent and was greater in uterine arteries from pregnant than from non-pregnant patients. The relaxation was enhanced by guanethidine and superoxide dismutase, inhibited by nitric oxide synthase inhibitors, blocked by tetrodotoxin, and unaffected by atropine, propranolol, or indomethacin. The results demonstrated that human uterine arteries respond to EFS with contraction and relaxation and that these responses may be mediated, respectively, by norepinephrine and, in part, by nitric oxide released from periarterial nerves.

McCarthy *et al.* (1994); Knock *et al.* (1996) and Ogoghoi *et al.* (1996) studied the effects of preeclampsia on endothelium-dependent relaxation of subcutaneous resistance arteries. McCarthy *et al.* used acetylcholine; Knock *et al.* used bradykinin and Oguogho *et al.* used acetylcholine and histamine to investigate endothelium-

dependent responses in isometrically mounted arteries isolated from normal pregnant women and women with preeclampsia. In all of these studies an impairment of endothelium-dependent relaxation in arteries from women with preeclampsia was observed. No difference to relaxation to sodium nitroprusside was observed between the groups in any of the studies indicating that differences in vascular smooth muscle behavior did not underlie the difference in vasorelaxation.

Pascoal *et al.* (1998) reported a similar impairment to acetylcholine in isometrically mounted omental arteries from women with preeclampsia when compared to vessels isolated from normal pregnant women. Ashworth *et al.* (1997) compared endothelium-dependent responses in isometrically mounted small myometrial arteries isolated from normal pregnant women and women with preeclampsia. They reported significantly impaired responses to bradykinin in vessels from women with preeclampsia as compared with responses in vessels from normal pregnant women. There are at least three endothelium-derived agents responsible for mediating endothelium-dependent relaxation in resistance arteries, nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF). Among these, the one most extensively studied is nitric oxide.

Nitric oxide in normal pregnancy and in preeclampsia

Nitric oxide is synthesised from L-arginine in a reaction catalysed by the enzyme nitric oxide synthase (NOS). The production of nitric oxide requires molecular oxygen, and the presence of at least four cofactors, with the concomitant production of citrulline. NOS is present in at least three isoforms: of these the endothelial (eNOS) and neuronal (nNOS) isoforms are constitutive. These isoforms of NOS are activated

by an influx of calcium into cells. The third isoform, inducible NOS (iNOS) is different from the constitutive forms in that it is functionally independent of calcium. iNOS is expressed in a wide variety of cells, including macrophages, neutrophils and mast cells and is produced in response to immunological stimuli (Michel *et al.*, 1993). The role of nitric oxide in activated immune cells is to inflict lethal oxidative injury on pathogens.

Mammalian adaptation to normal pregnancy involves profound alterations in the cardiovascular system, resulting in a decrease in total peripheral resistance. This is a reflection of widespread maternal vasodilatation. Data from animal studies suggests that this is in part mediated by increased biosynthesis of nitric oxide in pregnancy (Weiner *et al.*, 1994; Yang *et al.*, 1996). However, caution should be employed when extrapolating the results of animal studies to the human state, as it is recognised that there is intra- and inter-species variation in the contribution of nitric oxide to vasorelaxation. Indeed, the balance of evidence supporting a role for increased nitric oxide generation in human pregnancy is far less convincing. Many studies investigating nitric oxide generation in human pregnancy rely on the measurement of nitrite and nitrate, two stable end products of nitric oxide metabolism. Increased serum concentrations of nitrite in the plasma of normotensive pregnant women compared to non-pregnant women have been reported (Seligman *et al.*, 1994). In contrast however, other investigators have failed to observe any difference in plasma concentration or urinary excretion of nitrate or in exhaled nitric oxide in normal pregnancy (Cameron *et al.*, 1993; Brown *et al.*, 1995; Morris *et al.*, 1995). There are similarly conflicting results regarding nitric oxide production in pregnancies complicated by preeclampsia with some studies reporting increased levels (Nobunaga

et al., 1996; Smarason *et al.*, 1997; Lyall *et al.*, 1995), others finding decreased levels (Seligman *et al.*, 1994) and some reporting no difference (Cameron *et al.*, 1993).

The conflicting results of these studies may be explained in part by differences in the methodology. However, studies of this nature are intrinsically difficult as it is recognised that dietary intake of nitrate is known to affect plasma levels and urinary excretion. The dietary intake of the patients in the studies cited was not controlled since measurements were often made in women presenting with severe preeclampsia. Furthermore, urinary excretion of any substance is dependent upon the glomerular filtration rate, which is commonly reduced in preeclampsia. Although some investigators attempt to control for this by analysing urinary nitrate/nitrate against creatinine clearance, this has been done on random spot collections instead of the more accurate 24-hour urine collection. To address these problems, large cohorts of women undergoing strict dietary control need to be studied with longitudinal analysis of 24-hour urine collections. Conrad *et al.* (1999) performed such a study and they demonstrated that plasma levels and urinary excretion of nitrate and nitrite does not increase in normal pregnancy, but that the urinary excretion of these metabolites is modestly reduced in preeclampsia.

One final caveat that applies to all studies of nitric oxide biosynthesis is that it is possible that the local production of nitric oxide increases or decreases by small but biologically significant amounts and only in certain vascular beds. Such changes would therefore be obscured by analysis of whole body synthesis.

There is now increasing evidence that nitric oxide synthesis may be regulated via the production of endogenous inhibitors of nitric oxide synthesis. Holden *et al.*, 1998

measured the circulating concentration of one of these inhibitors, asymmetric dimethylarginine (ADMA), throughout normal pregnancy, in preeclampsia and in normal non-pregnant women. They reported that the early fall in blood pressure in normal pregnancy was accompanied by a significant fall in the plasma ADMA concentration and as pregnancy progresses the concentration increases again. Furthermore, when pregnancy was complicated by preeclampsia, concentrations of ADMA were found to be significantly higher than in the non-pregnant or normal pregnant state. The role of ADMA in nitric oxide regulation in both normal pregnancy and preeclampsia requires clarification. It is possible that enhanced production of ADMA may be of pathophysiological importance in preeclampsia and lead to decreased synthesis of nitric oxide. Alternatively, increased circulating concentrations of ADMA may represent a feedback response to enhanced nitric oxide production in preeclampsia.

Normal pregnancy is associated with profound changes in the cardiovascular system. In particular, normal pregnancy is characterised by an attenuated response to vasoactive agonists, such as angiotensin II, the net effect being vasodilatation. The technique of myography has allowed the isolation and study of small resistance arteries from various maternal vascular beds. The study of these small calibre arteries is particularly pertinent as vessels of this size contribute most to precapillary resistance. Myography studies provide evidence that the vascular changes of normal pregnancy are not entirely mediated via nitric oxide.

McCarthy *et al.* (1994) used a small vessel wire myograph to examine the endothelium-dependent relaxation of subcutaneous arteries in normal pregnant and

non-pregnant women. They found no significant difference in the degree of acetylcholine-mediated relaxation between vessels from the two groups. This would argue against a universal increase in receptor mediated nitric oxide production in the human myometrial vasculature in pregnancy. However, they also reported that the inhibition of both cyclooxygenase and NOS only modestly attenuated the relaxation to acetylcholine in vessels from normal pregnant and non-pregnant women, suggesting the presence of an additional vasoactive mediator such as endothelium derived hyperpolarizing factor (EDHF).

Pascoal and Umans (1996) studied small arteries from the omentum of women with preeclampsia and normal pregnant women. They demonstrated that the vessels from normal pregnant women completely relaxed to acetylcholine and bradykinin in an endothelium-dependent but nitric oxide-independent manner, again suggesting the presence of EDHF. They also found that the vessels from women with preeclampsia exhibited impaired responses to acetylcholine compared with vessels from normal pregnant women.

Ashworth *et al.* (1997) studied endothelium-dependent relaxation in the resistance arteries of the myometrial vascular bed. They also reported a marked reduction in endothelium-dependent relaxation in vessels from women with preeclampsia and a significant nitric oxide-independent component to bradykinin induced relaxation in the vessels of normal pregnant women.

In summary, endothelium-dependent, nitric oxide independent vasorelaxation has been described by a number of groups in resistance arteries from a wide variety of

vascular beds in normal pregnancy, suggesting that the role of nitric oxide in normal pregnancy may be limited. Furthermore, impaired endothelium-dependent relaxation has been observed in vessels from women with preeclampsia.

1.5 Role of trophoblast apoptosis in preeclampsia

1.5.1 Trophoblasts and immune tolerance

Different mechanisms, either alone or in combination, play a role in conferring maternal immune tolerance against the semi-allogeinc fetal allograft. The proliferation, invasion, and differentiation of trophoblast cells during implantation is a tightly controlled process of intracellular signalling that is mediated by cytokines, growth factors, and hormones (Norwitz *et al.,* 2001; Wegmann & Guilbert, 1992; Mellor & Munn, 2000; McMaster *et al.,* 1994). An extensive array of cytokines is produced at the trophoblast-maternal interface that contributes to the well being of the fetoplacental unit. These cytokines help to regulate the maternal immune response and so maintain a successful pregnancy (Athnassakis *et al.,* 2000). Trophoblasts themselves possess certain properties that could promote tolerance. These include a lack of MHC antigen expression (Bulmer *et al.,* 1992), the presence of immunosuppressive substances such as transforming growth factor-B (Kauma *et al.,* 1990), and the expression of CD59, an inhibitor of compliment cascade (Tedesco *et al.,* 1993). In addition, Fas and FasL interaction seem important in conferring immune privilege to the invading trophoblasts (see below).

Normal pregnancy is characterised by a lack of maternal cell-mediated immune response against the implanting embryo. Instead, humoral immunity predominates during pregnancy (Wegmann & Guilbert, 1992; Wegmann *et al.,* 1993). This switch, from cytolytic to humoral immunity, is the result of maternal changes in the cytokine profile (Wegmann *et al.,* 1993). The balance between T helper (Th)-1, or proinflammatory cytokines and Th-2, or anti-inflammatory cytokines is thought to be crucial for determining the success or failure of pregnancy. It is currently believed

that production of proinflammatory cytokines such as interleukin 2 (IL-2), tumour necrosis factor (TNF), and interferon is suppressed in normal pregnancy, whereas production of Th-2 cytokines, such as IL-4, -6, and -10, is enhanced (Mellor & Munn, 2000; Roth & Fisher, 1999).

Placental and decidual tissues isolated from normal pregnancy, however, have been shown to produce both Th-1 and Th-2 cytokines (Tranchot-Diallo *et al.,* 1997; Bennett *et al.,* 1998). The type of cytokines thought to be potentially harmful to pregnancy in humans as well as in mice are those classified as pro-inflammatory cytokines. For example excess production of TNF and gamma interferon is involved in preterm delivery (Casey *et al.,* 1989; Casey *et al.,* 1990). Besides influencing the maternal immune response, many of these cytokines have been shown to affect trophoblast physiology, specifically the proliferation and apoptosis of trophoblast cells (Hunt, 1989; Yai *et al.,* 1994).

1.5.2 Definition of apoptosis

Apoptosis was first described as a morphologic pattern of cell death characterised by cell shrinkage, membrane blebbing, and chromatin condensation culminating in cell fragmentation. The terminal deoxynucleotidyl transferase deoxy-UTP-nick end labelling (TUNEL) method may identify apoptosis before the characteristic morphologic changes become apparent (Ishihara *et al.,* 2002). Although TUNEL labelling is correlated with apoptosis, it has been suggested that this method might also detect fragmented DNA in necrotic cells within a tissue. Therefore, study of cellular ultrastructural changes in apoptosis, combined with TUNEL labelling permit a reliable differentiation between apoptosis and cell necrosis (Gold *et al.,* 1994).

1.5.3 Death receptors

Integral to many forms of apoptosis is a family of at least 14 different cyteine proteases called caspases. Caspases are synthesised as inactive precursors called procaspases. Cleavage and activation of procaspases can occur following a variety of stimuli including DNA damage and death receptor activation (Joza *et al.,* 2002; Herr & Debatin, 2001). Death receptors belong to a superfamily of receptors involved in proliferation, differentiation and apoptosis called the Tumour Necrosis Factor (TNF) superfamily (Krammer, 1999). Death receptors are type I integral receptors with a conserved extracellular domain containing two to four cytoseine rich pseudo-repeats, a single transmembrane region and a conserved intracellular death domain about 80 amino acids in length that binds to adaptor proteins and initiates apoptosis (Golstein, 1997; Griffith & Lynch, 1998; Schulze-Osthoff *et al.,* 1998; Idriss *et al.,* 2000). Each receptor can bind with one or more than one type of ligand expressed on adjacent cells. Binding of ligand to receptor induces receptor trimerisation and clustering on the plasma membrane is required to initiate apoptosis in cells. Among the different death receptors, Fas (CD95/Apo1) and Fas ligand (FasL) will be discussed here.

Fas receptor

Fas mediated apoptosis can be regulated by a variety of signalling pathways in cells and is required for normal cell function (6). One of the principal roles of Fas receptor is regulating the immune response and this is the most clearly characterised function of Fas receptor. However, Fas receptor is expressed on most tissues and also plays an important role in regulating the function of many tissues (Curtin & Cotter, 2003).

FasL Receptor

In contrast to Fas receptor, expression of FasL (CD95L), a type II transmembrane molecule belonging to the tumour necrosis factor receptor superfamily, is limited to certain leukocytes and tissues with immune privilege (Griffith *et al.,* 1995). Fas-FasL interactions leading to clonal deletion of antigen presenting cells have been implicated in the establishment of graft tolerance (Kabelitz, 1998), and provide one explanation for the immune privilege status of certain tissues, including the placenta, cornea and testis (Griffith *et al.,* 1995). Fas-mediated apoptosis regulates other cells involved in adaptive and is a principal mechanism by which cytotoxic T lymphocytes (CTL) induce apoptosis in cells expressing foreign antigens (Medema *et al.,* 1997). Progression and metastasis of tumours is associated with resistance to Fas-receptor mediated apoptosis (French & Tschopp, 2002). In addition, upregulation of Fas ligand often occurs in tumour cells following chemotherapy and may play a key role in immune evasion by eliminating infiltrating lymphocytes (Strand *et al.,* 1996; Pinkoski & Green, 2000).

Fas-FasL interaction

Activation of Fas receptor by the Fas ligand initiates a caspase cascade culminating in apoptosis in sensitive cells. Effective formation of a protease complex called Death inducing signalling complex (DISC) is required in Fas-mediated apoptosis (Curtin & Cotter, 2003). Immediately following Fas receptor ligation with Fas ligand, microaggregates of Fas receptor form on the cell surface independent of caspase activity (Algeciras-Schimnich *et al.,* 2002). Trimerisation of Fas receptor is the minimal event required for effective DISC formation (Schneider *et al.,* 1998). Activation of caspase 8 occurs following DISC formation and directly regulates the

formation of large Fas receptor aggregates on the plasma membrane of cells and increased DISC activity. These large clusters of Fas receptor are endocytosed and recycled (Algeciras-Schimnich *et al.,* 2002). Caspase 8 activation appears to be the principal initiator during apoptosis (Curtin & Cotter, 2003).

Although Fas is viewed as the prototypical death receptor in susceptible cells, Fas may mediate apoptosis-independent processes such as proliferation, angiogenesis, fibrosis, and inflammation (Hohlbaum *et al.,* 2002). The most striking example of apoptosis-independent Fas function is derived from transgenic mice and pancreatic islet cell transplantation studies in which beta cells were genetically engineered to express Fas ligand in an effort to confer immune privilege through apoptotic deletion of invading, Fas-expressing T-cells (Chervonsky *et al.,* 1997; Allison *et al.,* 1997; Kang *et al.,* 1997). These studies surprisingly revealed extensive neutrophil infiltration and islet cell destruction, rather than preserved pancreatic morphology.

In non-immune tissues, the expression of Fas and FasL correlates with the presence of tissue-specific micro-environment factors and cytokines, which are known to locally modulate immune reactions (Chen *et al.,* 1998). Therefore, changes in Fas and FasL expression reflect not only a shift in the cytokine profile (Garcia-Velasco *et al.,* 1998) but also alterations in the hormonal environment of these tissues (Song *et al.,* 2000).

1.5.4 Apoptosis and trophoblast function

The role of apoptosis in the regulation of trophoblast function is not well defined as yet. Apoptosis, assessed by the TUNEL method was noted in the nuclei of both cytotrophoblasts and syncytiotrophoblasts, being most abundant in very early 4- to 5-

weeks placentas, less abundant in early and midterm placentas, and least abundant in term placentas (Ishihara *et al.,* 2000). It was found that Bcl-2 protein, an apoptosis-inhibiting gene product, was expressed in the cytoplasm of syncytiotrophoblasts, being least abundant in very early 4-5 weeks placentas, less abundant in early and midterm placentas, and most abundant in term placentas. It is possible that high levels of Bcl-2 protein expression in syncytiotrophoblasts in normal term placentas may prevent or slow the death of terminally differentiated trophoblasts that progressively accumulate during pregnancy (Ishihara *et al.,* 2000). Recently, it has been suggested that Bcl-2 blocks Fas-Fas ligand mediated apoptosis, suggesting that Bcl-2 protein and Fas antigen may play important roles in regulating apoptosis of human placental trophoblast (Ishihara *et al.,* 2000).

Despite expressing both Fas and FasL, villous trophoblasts undergo apoptosis in limited numbers during normal pregnancy. However trophoblasts exhibit increased apoptosis in pregnancies complicated by fetal growth restriction and preeclampsia (Ishihara *et al.,* 2002; Allaire *et al.,* 2000). The majority of the apoptotic nuclei in these studies were found in the syncytiotrophoblasts of abnormal placentas. DiFederico et al, however, reported that apoptosis of the invasive cytotrophoblasts in severe preeclampsia was more widespread compared with normal uncomplicated placentas (DiFederico *et al.,* 1999). More recently, Levy et al showed increased apoptosis in trophoblast layer of villi from pregnancies complicated by fetal growth restriction associated with preeclampsia, which was associated with up-regulation of p53, but not members of the Bcl-2 family of proteins (Levy *et al.,* 2002). In a previous study, the same group showed that hypoxia enhances apoptosis in cultures of human trophoblasts, which is associated with increased expression of the proapoptotic

proteins p53 and Bax and with a lower expression of the antiapoptotic protein Bcl-2 (Levy *et al.,* 2000). These findings implicate the mitochondrial pathway in enhanced apoptosis in cultured trophoblasts exposed to low oxygen tension.

1.5.5 Placental Fas and Fas ligand (FasL)

Characterisation of the Fas/FasL pathway has evolved dramatically since its original description as the mechanism controlling the turnover of peripheral activated mature T cells (Nagata *et al.,* 1994). It has become evident that the Fas/FasL system plays a major role in continuos tissue haemostasis (Song *et al.,* 2000). Furthermore, understanding the Fas/FasL interactions has changed the paradigm regarding the establishment and maintenance of immune privilege. Once believed to depend on physical barriers and anatomical isolation, immune privilege is now viewed as an active process by which infiltrating, activated lymphoid cells are eliminated by the Fas/FasL apoptotic pathway (Bechmann *et al.,* 1999).

Mor *et al.* (1998) suggested that placental expression of FasL serves as the key component for establishing immune privilege at the maternal-fetal interface. Contrary to the old concept of a physical barrier, the trophoblast is now believed to function as a dynamic obstacle, averting activated and potentially detrimental T cells. Once they recognise placental alloantigens, activated T cells express Fas, which interacts with FasL on placental trophoblast cells. Consequently, the activated T cells undergo Fas-mediated apoptosis.

Aschkenazi *et al.* (2002) showed that susceptibility to Fas does not necessarily correlate with its trophoblast cell expression, and that cellular inhibitors must exist in

the Fas-mediated signalling pathway to prevent apoptosis. In their study, for example, IL-10 treatment increased trophoblast Fas expression, but unexpectedly increased trophoblast resistance to apoptosis, suggesting a mechanism of action other than regulation of Fas expression. This could be through activation of FLIP, an intracellular component of the Fas apoptotic pathway, as IL-10 treatment increased both the expression and activation of FLIP in trophoblast cells.

Mice with *gld* mutation are deficient in FasL and have smaller litters with decreased viability (Hunt *et al.,* 1997). Studies in these mice suggest that the Fas-FasL pathway contributes to preservation of the fetal allograft by preventing the trafficking of activated leukocytes between the mother and the fetus during normal gestation, observations also supported in humans (Uckan *et al.,* 1997; Jiang & Vacchio, 1998; Kauma *et al.,* 1999). These studies suggest that interactions between FasL-positive placental trophoblasts and activated maternal lymphocytes that express Fas contribute to the induction of tolerance through a clonal deletion mechanism (Guller & LaChapelle, 1999). Conversely, alternative mechanisms may be contributory to establishing maternal-fetal tolerance during pregnancy as *gld* mice can reproduce (Hunt *et al.,* 1997), and clonotypic maternal T cells that remain after clonal deletion are unresponsive to fetal antigen challenge (12-9). This leads us to think that the role of Fas and FasL in trophoblast physiology is presumably more complex than originally anticipated. Aschkenazi et al (Jiang *et al.,* 1998) showed that despite the expression of Fas and FasL by first-trimester trophoblast, it is particularly resistant to Fas-mediated apoptosis. They found that susceptibility to Fas does not necessarily correlate with its trophoblast cell expression and that cellular inhibitors such as Interleukin 10 exist in the Fas-induced signalling pathway to prevent apoptosis.

1.5.6 Fas-FasL system and preeclampsia

As establishment of normal maternal-fetal tolerance appears to involve components of the Fas-FasL pathway (Hunt *et al.,* 1997; Uckan *et al.,* 1997; Jiang *et al.,* 1998), which also may mediate rejection of transplanted tissues (Griffith *et al.,* 1995; Kabelitz, 1998). Thus, it is reasonable to speculate that aberrant function of the Fas-FasL pathway might also be involved in a maternal rejection of the fetal allograft (Kuntz *et al.,* 2002). The contribution of this pathway to the pathogenesis of preeclampsia is further suggested by associated maternal and neonatal complications, including hepatic inflammation, endothelial injury and neutrophil activation, which can be mediated by the Fas-FasL pathway (Kuntz *et al.,* 2002).

Allaire et al showed that placentas from preeclamptic pregnancies have increased apoptosis compared to placentas from normal pregnancies. Using immunohistochemistry, they demonstrated that trophoblastic FasL expression was reduced in preeclamptic placentas compared to normal controls, while Fas expression was enhanced in these placentas (Allaire *et al.,* 2000). They proposed that the combination of reduced FasL expression and enhanced Fas could account for the increased placental apoptosis in cases of preeclampsia. On the other hand, increased Fas expression could be attributed to placental neutrophil stimulation. High levels of neutrophil activation have been described in patients with preeclampsia (Clark *et al.,* 1998; Greer *et al.,* 1991). It is possible that a dense neutrophil infiltration may alter the surrounding environment at the maternal-fetal interface, thereby promoting the up-regulation of Fas-expression in non-immune cells such as trophoblast and vascular endothelium and allowing FasL-induced inflammation and apoptosis (Varani *et al.,* 1992). Another study, which evaluated placental Fas and FasL expression in

preeclampsia using immunohistochemistry, showed enhanced *FasL* expression in the trophoblasts from preeclamptic placentas, while there was no difference in Fas expression between the two groups.

It seems, however, that the role of Fas/FasL in trophoblast apoptosis is far from clear. A study by Payne et al demonstrated that Fas expressed by human placental cytotrophoblasts does not mediate apoptosis (Payne *et al.,* 1999). A possible explanation for this could be the low levels of trophoblast Fas expression constitutively (Balkundi *et al.,* 2003). Recent evidence suggests that cells are more sensitive to Fas mediated apoptosis when levels of Fas expression increase (Xiao *et al.,* 2002). A recent study of the regulation of Fas/FasL in human trophoblasts in cases of chorioamnionitis showed that Fas on trophoblasts is biologically active and by specifically blocking FasL receptors, a reduction in TNF-a and IFN-induced apoptosis was noticed (Balkundi *et al.,* 2003). In summary, the role of Fas/FasL surface proteins in the trophoblasts is poorly defined and abnormalities of expression of these proteins in cases of abnormal placentation are yet to be defined. Its thought that critical events during normal pregnancy, such as immunoprotection are regulated by cytokines produced locally at the mother-fetus interface (Norwitz *et al.,* 2001), and the locally produced cytokines may exert their effect via the Fas/FasL pathway (Aschkenazi *et al.,* 2002).

Serum soluble Fas and FasL in preeclampsia

Soluble Fas, an alternatively spliced product of the Fas gene (see above), protects cells from apoptosis by antagonizing the binding between membrane form of the Fas and the Fas ligand (Cheng *et al.,* 1994). Elevated serum soluble Fas levels are found

in patients with autoimmune disease, malignancy, hepatitis, congestive heart failure and infection (Knipping *et al.,* 1995; Shen *et al.,* 1996; Lio *et al.,* 1998; Okuyama *et al.,* 1997). A study by Hsu *et al.* (2001) showed increased serum Fas but not FasL levels in preeclampsia compared to normal gestational age matched controls. The source for the elevation of serum levels of soluble Fas in-patients with preeclampsia was not clear in that study. The activated trophoblast, maternal immune cells, or abnormal shedding from the injured vascular endothelium are possible sources of the soluble Fas antigen in cases of preeclampsia.

A study by Harirah *et al.* (2001) showed elevated serum soluble Fas levels in the syndrome of Haemolysis, Elevated Liver Enzymes and Low Platelets (HELLP). Serum FasL, however, was not significantly raised in this condition, indicating a possible role of Fas but not FasL in the pathogenesis of this disease. Again, several explanations for the increased Fas in HELLP syndrome were proposed; this included release of Fas from injured hepatocytes, activation of vascular endothelium, or abnormal release of Fas by trophoblasts. In vitro studies have documented upregulation of Fas expression by endothelial cells when stimulated with tumour necrosis factor (TNF) (Sata *et al.,* 2000). In addition, elevated serum Fas could act as anti-apoptotic factor in the circulation, which results in delayed maternal neutrophil apoptosis. This in turn could result in abnormal systemic inflammatory response including endothelial dysfunction and multiple organ affection characteristic of preeclampsia (Von Dadelszen *et al.,* 1999).

The study by Kuntz *et al.* (2001) challenged the idea of elevated serum soluble Fas in preeclampsia and alternatively demonstrated higher soluble FasL levels in paired

maternal and cord blood sera of preeclamptic pregnancies compared to normal controls. In contrast, soluble Fas levels were similar between the groups. In this study, the source of these proteins was unclear, with the trophoblast being a likely candidate. A second source of the elevated levels of sFasL might be membrane associated FasL released from the surface of activated neutrophils (Liles *et al.,* 1996). This possibility is supported both by the lower neutrophil surface expression of FasL in preeclamptic gestations, and by the observation that preeclampsia is associated with neutrophil activation (Redman *et al.,* 1999).

Elevated serum FasL in preeclampsia in the study by Kuntz *et al.* (2001) could contribute to the clinical presentation of the disease. Serum FasL and the membrane-bound form associated with microvesicles are bioactive (Martinez-Lorenzo *et al.,* 1996; Martinez-Lorenzo *et al.,* 1999), and can mediate a variety of pathologic conditions including cytopenias, hepatic inflammation, autoimmune disorders, and rejection syndromes (Liu *et al.,* 2000; Pinkoski *et al.,* 2000). So far, however, the exact effect of elevated serum soluble Fas or FasL in preeclampsia is far from clear.

1.6 Intra-Uterine Growth Restriction (IUGR)

1.6.1 Aetiology of IUGR

One of the biggest difficulties in approaching the problem of intrauterine fetal growth restriction (IUGR) or the small for gestational age fetus (SGA) fetus is the diverse aetiology associated with these conditions (Table 1). Being small does not in itself mean that the fetus is growth restricted, although for convenience it is often assumed to be so. As birth weight is a simple, universal standard, paediatric studies tend to be on patients who are SGA at birth, so in perinatology, being SGA in utero is usually the starting point for the investigation of the fetus suspected of IUGR.

Table 1.1 A list of some of the common causes of being SGA at birth

Fetal
Constitutional
Chromosomal anomaly
Genetic Dwarfing syndromes
Infection

Uteroplacental
Impaired placental invasion (e.g. preeclampsia)
Drugs (e.g. smoking, cocaine, etc).
Placental tumours

Maternal
Renal disease
Cardiovascular disease
Systemic lupus erythematosus
Sickle cell disease
Undernutrition

The use of ultrasound has enabled us to study the physiology of normal pregnancy, as well as diagnosing complications related to structural and chromosomal anomalies. One of the most important advances made with ultrasound has been the ability to accurately date a pregnancy, especially in the first half. Indeed, apart from ultrasound

measurement before 18 weeks (still not available to many around the world), we have advanced little from the often uncertain dating by Naegele's rule (Berg & Bracken, 1992).

The failure of a fetus to reach its expected growth potential, or to exceed it, can be the result of any of a number of complications, acting alone or in combination. Although there is some overlap in the following groups, for convenience we divide the causes of growth failure into maternal, uteroplacental and intrinsic fetal causes. Animal and biochemical studies primarily attempt to understand the effects of maternal/uteroplacental supply on growth, and to understand the hormonal and biochemical changes in the animal fetus that accompany such deprivation. Whatever the cause, IUGR has far reaching implications for the developing fetus. In addition to the known increase in perinatal morbidity and mortality, epidimiological studies have found a correlation between low birth weight, perinatal and neonatal mortality and subsequent death from cardiovascular disease up to 70 years later (Barker, 1992). This information suggests that we should approach the problem of IUGR on three fronts: pre-pregnancy education about diet/drug use, etc., identification of patients at risk early in pregnancy, and with ultrasound the early detection and optimum management of the compromised fetus.

In an analysis of a specific group of patients (Abrams & Newman, 1991) found that maternal smoking, ethnic minority status and low maternal ($<10^{th}$ centile) booking weight were the most significant risk factors for the delivery of a small for gestational age fetus. Under nutrition is probably the most common causes of SGA fetuses in the

world. In addition, substance abuse such as tobacco, marijuana and alcohol is a well-known cause of SGA fetuses.

Fetal structural and/or chromosomal abnormality is recognised as a cause of IUGR. Symmetrical IUGR tends to start earlier and require delivery earlier than asymmetric IUGR. A ten-year paediatric study (Mili *et al.,* 1991) found that the overall incidence of major birth defects was 3.6%; the incidence in babies with a birth weight < 1,500 gms was 16.2% and with a birth weight >1500<2000 gms was 13.2%. There is a strong relationship between low birth weight and cardiac malformations. IUGR can also result from intrauterine infection, with rubella being the classical example (Merril & Weiner, 1992). The introduction of cordocentesis has allowed in utero assessment of infection through analysis of the fetal immune response.

1.6.2 Diagnosis of the Small for Gestational Age (SGA) fetus

Clinical suspicion

Clinical suspicion from abdominal palpation, reduced weight gain or a previous SGA baby led to a suspicion of IUGR (less than 10^{th} centile) in 50% of cases and action in only one third of those suspected (Rosenberg et al 1982a). Symphysial-fundal height (SFH) can be measured easily and predicted low birth weight with a sensitivity and specificity of 86% and 90%, respectively in a study that excluded more than half of the women entered into it (Belizan *et al.,* 1978).

In a study of high-risk pregnancies with certain menstrual dates and early ultrasound examinations, sensitivity ranged from 79% to 86% and specificity from 79% to 92% depending on the type of growth curves used (Cnattingius *et al.,* 1984). When the use

of SFH curves was examined in actual practice with different observers the values fell to 56% and 85%, respectively (Rosenberg *et al.*, 1982b). Recently, the idea of customised growth charts was introduced. This means that maternal height; weight and race are to be taken into consideration when measuring the SFH, as normograms for SFH measurements can be affected by the above factors.

Ultrasound and biophysical assessment

The idea that ultrasound can be used to determine fetal size and fetal growth is not new (Campbell & Dewhurst, 1971), but because of the inherent errors in the use of ultrasound, and our failure to always identify the pregnancies most suitable for surveillance, much work continues to be published on this topic (Skorvon *et al.*, 1991). There are many difficulties in adopting in-utero growth models from other sources because of the differing attributes of a population, the sample size and the formulae used among others. However, a study of the accuracy of 20 published formulas for predicting birth weight using estimated fetal weight by ultrasound found no differences between the different formulae (Medchill *et al.*, 1991).

Villar & Belizan (1982) have described three different types of IUGR depending on the time of onset of placental insufficiency. The three growth patterns defined were 1. Chronic or symmetric IUGR; 2. Subacute or asymmetric IUGR; and 3. Acute IUGR. Each is thought to have different aetiologies and prognostic significance. Serial ultrasound assessment can identify reduced growth velocity, in which serial fetal biometry measurements cross the centiles in the growth charts. These fetuses are more likely to suffer from IUGR rather being constitutionally small for age. Serial ultrasound, however, cannot always differentiate between the varying types of IUGR.

In practice, if an insult occurs before 24 weeks gestation, it is more likely to present as symmetric IUGR, whereas if the insult is in the third trimester, the fetus is more likely to demonstrate an asymmetric IUGR pattern.

Manning *et al.* (1980) described the fetal biophysical score (BPS). The score contained five variables: fetal breathing movement, fetal movement, fetal tone, quantitative amniotic fluid volume (qAFV) determination, and cardiotocographic fetal heart rate testing over a 30 minute observation interval. The BPS may be regarded as an acute index of fetal well being, in view of the known association between fetal oxygenation and the presence or absence of these biophysical activities (Vintzileos *et al.,* 1987). The association between abnormal biophysical activity (low BPS) and rising morbidity and mortality is well established (Manning *et al.,* 1985, Vintzileos *et al.,* 1987). Gross perinatal mortality ranges from 1.5 per 1000 for a BPS of 8 or 10, rising to 17.5 per 1000 for a BPS of 6, and 500 per 1000 for a BPS of zero. In practice, the score can be misleading, as the various parameters are not weighted correctly for the importance of their presence or absence.

The two parameters consistently associated with a poor outcome are reduced amniotic fluid volume (AFV), clinically known as oligohydramnios, and an abnormal fetal heart rate pattern. There are three causes of oligohydramnios, 1. Premature rupture of the membranes (PROM), 2. Renal ageneis, and 3. Placenta insufficiency. In the absence of PROM and renal disorders, oligohydramnios is the best biophysical predictor of a poor perinatal outcome (Manning *et al.,* 1981). Oligohydramnios is often a late sign: while useful in confirming a perilous situation for the fetus, it is less useful in the early stages of identifying IUGR.

The normal fetus has active-inactive cycles of 20-50 minutes, with the cycle length generally increasing with gestational age. Normal FHR analysis (Wheeler & Murrils, 1978), reflects this normal activity, and is an established test of fetal well being. When intervention is based on abnormal FHR analysis, perinatal mortality in a high risk group can be reduced to <3 per 1000. However, there are a number of problems with false positive and false negative results, which limit the use of FHR analysis as a lone indicator of fetal well-being. Maternal obesity, drugs, prolonged inactivity cycle, excessive activity are but some of the problems that can lead to false positive results.

The real danger with the use of FHR testing in isolation is the risk of false negative. A meta-analysis of all randomised trials using intervention based on FHR findings reported that the perinatal mortality in the group with a normal FHR tracing was three times that of the abnormal FHR group. This presumably reflects the fact that an abnormal FHR trace was acted upon, while a conservative approach was used in the group with normal FHR testing. This would suggest that some fetuses with a normal FHR trace were compromised, but not to the point of cerebral/cardiac ischaemia. In this group of fetuses, a normal pattern was falsely reassuring and occasionally fatal. While the assessment of growth and biophysical scoring, in particular AFV and FHR testing, are useful tests of fetal well being, there was a need for further understanding of the pathophysiological response of the fetus to placental insufficiency. In this context, Doppler examination of the fetoplacental circulation was developed to critically examine this response (see below).

1.6.3 Long-term consequences of IUGR

If an organ or fetus receives an insult at a critical period of development, the fetus is capable of compromising by reprogramming it's potential for development (Lucas, 1991). This reprogramming appears to be permanent, implying that the true potential of the IUGR fetus can never be realised (Law *et al.,* 1991). This work is supported by Hinchcliffe *et al.* (1991), who found the number of nephrons in kidneys of asymmetrically growth retarded fetuses were considerably lower at birth and in the first year of life, compared with kidneys from fetuses and infants of normal weight. There is also evidence that babies born small for gestational age are physically and developmentally behind their appropriate for gestational age (AGA) counterparts (Pryor, 1992). In a long term follow up of patients with cerebral palsy (CP) of unknown aetiology, the risk of a small (short and thin) baby developing cerebral palsy is very much greater than appropriately grown fetus. The risk of CP is even greater if the fetus is symmetrically small (small, thin and small head), excluding fetuses with chromosomal anomalies (Blair & Stanley, 1992). All this evidence points to the primary need for prevention of growth retardation or the early recognition of at risk groups so that potential intervention can be utilised at an early stage of the disease.

1.6.4 Trophoblast apoptosis in IUGR

Smith *et al.* (1997) showed that the incidence of apoptosis is significantly higher in placentas from pregnancies with intrauterine growth restriction compared to controls. This was supported by Ishihara *et al.* (2002), but showed that increased apoptosis in these placentas is mostly localised to the syncytitrophoblast and not the cytotrophoblast layer of the placenta. This was also true of placentas in cases of severe preeclampsis at term. This, however, was challenged by a study of the

morphology of placenta from normal and IUGR pregnancies (Battistelli *et al.*, 2004). This study showed that, although villi in IUGR placentas appeared longer, thinner and less vascularized compared to control placentas, they showed no difference in typical apoptotic features compared to normal placentas.

The aetiology of increased apoptosis in IUGR placentas is not entirely clear. Hypoxia due to impaired spiral artery remodeling could be implicated. Crocker *et al.* (2003) showed, in an in-vitro model, that both syncytiotrophoblast and cytotrophoblast from IUGR pregnancies have increased susceptibility to hypoxia-induced apoptosis compared to control placentas. The reason for this was not clear. On the other hand, Kilani *et al* (2003) showed that only extreme hypoxia results in increased trophoblast apoptosis in *normal* trophoblast.

Other causes of increased trophoblast apoptosis in IUGR include enhanced p53 expression (Levy *et al.*, 2002) or reduced Bcl-2 expression (Ishihara *et al.*, 2002). Bcl-2 protein, an apoptosis inhibiting-gene product, is expressed in the cytoplasm of syncytiotrophoblasts and is least abndant in very early 4-5 weeks placentas, less abundant in early and midterm placentas, and most abundant in term placentas. Apoptosis, on the other hand, was most abundant in very early 4-5 weeks placentas, less abundant in early and mid-term placentas, and least abundant in term placentas (Ishihara *et al.*, 2000). This possibly indicates a possible role of Bcl-2 protein in preventing apoptosis. In addition, Bcl-2 protein blocks Fas-Fas ligand-mediated apoptosis, suggesting that both Bcl-2 protein and Fas antigen may play important roles in regulating apoptosis of human trophoblasts. Immunohistochemichal studeis of placental Bcl-2 expression in normal term placentas compared to placentas from

severe preeclampsia or IUGR at term showed that Bcl-2 protein was abundantly immunolocalised in the syncytiotrophoblasts in normal term placentas, but least aundant in term placentas in term placentas complicated by severe preeclampsia or IUGR (Ishihara *et al.*, 2002).

Recent in-vitro study of the role of Fas/FasL in spiral artery remodelling suggested that apoptotic changes in the endothelial layer of spiral arteries cultured with trophoblast in the lumen were detected after 20 hours and were possibly mediated by Fas/FasL interaction (Ashton *et al.*, 2005). Allaire *et al.* (2000) showed that placental FasL is downregulated AND Fas is upregulated in cases of preeclampsia. One third of these cases had small for gestational age pregnancies (defined as birth weight $<5^{th}$ centile for gestational age). Strictly speaking, this does not mean that all these pregnancies had IUGR. The in-vivo role of placental Fas-FasL interaction in spiral artey remodelling, placental invasion and the clinical syndrome of IUGR remain to be studied.

1.7 Placental vasculogenesis

1.7.1 Stages of placental angiogenesis

Maternal blood flow into the intervillous space increases 20-fold during pregnancy due to vasomotor changes of the distal intramyometrial portions of the uteroplacental arteries, and to transformation and dilatation of the decidual segments (Ahmed *et al.*, 2000). The latter changes are due to invasion of the vessels by trophoblast cells that replace the maternal endothelium and media of the spiral arteries (Benirschke & Kaufmann, 1995). Therefore, in contrast with other placental exchange systems, the human placenta does not have an endothelium-lined maternal microvascular system connecting arteries and veins (Ahmed *et al.*, 2000). In addition, the core of the placental villi is supplied by an endothelium lined fetoplacental system, whose development is similar to that of the systemic vascular beds. The only major exception to this is the lack of innervation by the autonomic nervous system (Reily & Russell, 1977).

Vascularization of the placental villi starts at day 21 post-conception (Demir *et al.*, 1989) and is the result of local de novo formation of capillaries rather than protrusion of embryonic vessels into the placenta. The villous trees at this stage are made up of solid trophoblastic or primary villi and secondary villi, which are characterised by a loose mesenchyme (Benirschke & Kaufmann, 1995). There appears to be a paracrine role in the initiation of vasculogenesis and mediating trophoblast invasion as mesenchymal derived macrophages, maternal decidua and decidual macrophages express angiogenic growth factors.

1.7.1.1. Branching angiogenesis

From the stage of primary and secondary villi until the end of first trimester, the villous vasculature increases in number rather than types of vessels (Ahmed *et al.*, 2000). The number of fetal blood vessels containing capillaries increases, and sprouting and branching angiogenesis from the existing capillaries gives rise to a primitive capillary network surrounded by an incomplete layer of pericytes (Benirschke & Kaufmann, 1995). The villous trees and their vascular bed continuously expand from the peripheral ends of the newly differentiated villous stems, giving rise to a new capillary network which in turn differentiates into stem villi (Castelluci *et al.*, 1990).

1.7.1.2 Non-branching angiogenesis

From 26 weeks of gestation until term the villous vascular growth undergoes a change from branching to non-branching angiogenesis due to the formation of a new villous type, the mature intermediate villi that specialise in gas exchange. These form at the tips of the villous tree and are long (>1000 um) and slender (80-120 um diameter) containing one or two long, poorly branched, capillary loops. These arise as a result of a decrease in trophoblast proliferation and an increase in endothelial proliferation along the entire length of the capillary giving a final capillary loop exceeding 4000 um in length (Kaufmann *et al.*, 1985). As these capillaries grow in excess of the villi, they coil and bulge through the trophoblastic surface forming the terminal villi. Each of these is supplied by one or two capillary coils that are covered by a very thin trophoblastic lamella or the vasculosyncytial membrane (Benirschke & Kaufmann, 1995). These specialised structures are the main site of diffusional gas exchange between the maternal and fetal circulations. As gestation increases these terminal

capillaries focally dilate to form large sinusoids, this dilatation counterbalances the effects of the long poorly branched capillaries on total fetoplacental vascular impedance (Kingdom & Kaufmann, 1988). Increasing fetal blood pressure also aids this dilatation and fetoplacental blood flow rises throughout gestation to 40 percent of fetal cardiac output by term (Hendriks *et al.*, 1989).

Placentas from the pathological condition of IUGR display a failure of elongation, branching and dilatation of these capillary loops, and failure of terminal villi formation (Krebs *et al.*, 1996; Macara *et al.*, 1996). Consequently, fetoplacental blood flow is severely impaired and transplacental gas exchange is poor, placing the fetus at risk of hypoxia and acidosis.

1.7.2 Placental morphology in IUGR and preeclampsia

The underlying pathology of increased vascular resistance in IUGR must reside either in the stem arteries (increased vasomotor tone) or in the vascular arrangement of the capillaries within gas-exchanging villi (Kingdom *et al.*, 1997). Stereologic two-dimensional studies of placental structure from cases of IUGR with absent or reversed end-diastolic flow (AREDF) in umbilical artery Doppler have indicated a reduction in the elaboration of the peripheral gas-exchanging parts of the villous tree in early-onset IUGR (Hitschold, 1998; Jackson *et al.*, 1995). Immunohistochemistry and transmission electron microscopy of the placental villous structure in these cases showed that the terminal villi were characterised by reduced numbers of proliferating cytotrophoblast, with excess numbers of overlying syncytiotrophoblast nuclei (Kingdom & Kaufmann, 1999). Since syncytial fusion is thought to focally retard apoptosis in the syncytiotrophoblast (Huppertz *et al.*, 1998), these findings were

interpreted as representing accelerated trophoblast ageing (Kingdom & Kaufmann, 1999). Villous trophoblasts in these cases is characterised by reduced numbers of non-branching capillaries (ie predominance of non-branching angiogenesis, see below). The combination of arrested trophoblast turnover and failure to evoke an adaptive angiogenic response in these placentas suggest that these villi have been exposed to a higher than normal oxygen tension- a situation termed placental hyperoxia (Macara *et al.,* 1996).

Fractional extraction of oxygen from the intervillous space is greatly reduced with ARED in the umbilical arteries, such that uteroplacental venous blood has a 30% higher oxygen content than under normal circumstances (Pandi *et al.,* 1992). Severe early-onset IUGR with AREDF is very uncommon in unselected pregnancies (Mason *et al.,* 1993). The pathology of AREDF and thus the concept of placental hyperoxia is thus the exception to the rule that the placenta adapts to reduced uteroplacental blood flow (Kingdom & Kaufmann, 1999). In these cases, hypoxia exists at the level of the fetoplacental circulation rather than the intervillous space. This is in contrast to placentas in cases of preeclampsia with preserved end diastolic flow (EDF) in the umbilical artery, which show villous trophoblast vascular structure characterised by predominance of branching angiogenesis. In these cases, hypoxia exists at the level of the intervillous space, while the fetoplacental circulation will have significantly higher oxygen saturation compared to cases of IUGR with AREDF.

1.7.4 Oxygen regulation of vascular growth factors

Successful placentation involves the development of low-impedance uteroplacental circulation following trophoblast invasion and transformation of the maternal

intramyometrial portion of the spiral arterioles. An oxygen tension gradient exists between the maternal decidua and the villous placenta during embryonic development. Hypoxia stimulates cytotrophoblast proliferation and inhibits trophoblast invasion (Genbacev *et al.*, 1996). At around 12 weeks' gestation, the intervillous space switches from relative hypoxia to an enhanced oxygen state. This striking rise in oxygen may be the trigger for the trophoblast to change from its proliferative state within "hypoxic" cell columns to an invasive extravillous trophoblast that is responsible for trophoblast invasion. In preeclampsia and IUGR, the uterine blood vessels do not undergo adequate vascular development transformation, so the rate of delivery of oxygenated blood to the fetus falls "see below". This "uteroplacental insufficiency" has led to the notion of placental hypoxia in these two disorders.

Ultrastructural studies in normal and severe IUGR placentas have challenged the idea of placental hypoxia. The terminal villi of IUGR placents showed reduced cytotrophoblast proliferation, stromal fibrosis, and poor villous angiogenesis as indicated by straight and unbranched capillaries (Krebs *et al.*, 1996; Macara *et al.*, 1996). There was also congestion of fetal capillaries by erythrocytes, which may further limit oxygen transfer from the intervillous space to the fetus. These findings were interpreted as evidence of placental hyperoxia, which refers to the oxygen tension in the intervillous space being closer to the maternal arterial oxygen level in severe IUGR than is the case in normal pregnancy at the same gestation. Since hypoxia promotes angiogenesis, the relatively high oxygen levels in the intervillous space in contact with IUGR placental villi will limit angiogenesis. Similar changes were noticed in placental hyperoxia induced in a primate experimental model (Panigel

& Myers, 1972). The observation that oxygen content in uteroplacental venous blood in IUGR was significantly higher than in control pregnancies supports the idea of poor fetal oxygen extraction in IUGR (Pardi *et al.*, 1992; Burton *et al.*, 1996).

The usual response to uteroplacental ischaemia, or to pregnancy at high altitudes (4-6) is for the villous tree to undergo increased capillarization, resulting in increased fractional extraction of oxygen to meet the demand of the growing fetus. It seems that this adaptive mechanism fails badly in severe IUGR. The molecular regulation of villous development and placental angiogenesis must involve the interaction of the cellular components of the placenta (trophoblast, stromal cells, and endothelium) and the local oxygen gradients established both within each villous and between the cellular compartments (Ahmed & Kilby, 1997). The human placenta is richly endowed with angiogenic growth factors and their receptors, including VEGF, PlGF and Flt1. A study by Ahmed (1997) showed that hypoxia upregulates VEGF and downregulates PlGF. Recent studies show that the intensity of immunostaining for VEGF and PlGF in severe IUGR placenta was no different from that in normal placenta (Ahmed, 1997; Lyall *et al.*, 1997), supporting the notion that the placenta was not hypoxic at delivery. These findings were confirmed by western blotting which confirmed the lack of change in VEGF levels in severe IUGR (Ahmed, 1997). In preeclampsia, however, levels of PlGF were decreased and those of VEGF increased, which may indicate that that in preeclampsia, the intervillous space and anchoring villi (source of invading extravillous trophoblast) remain hypoxic. Kingdom and Kaufmann identified three types of hypoxia relating to the uteroplacental unit; "preplacental hypoxia" as in pregnancy at high altitude and maternal anaemia, "uteroplacental hypoxia" as in preeclampsia, and "postplacental

hypoxia", as in severe IUGR with absent end-diastolic flow velocity in the umbilical artery (Macara *et al.,* 1996), which indicates elevated fetoplacental vascular resistance (Morrow *et al.,* 1989).

1.8 Doppler Ultrasound

Christian Doppler (1843) suggested that the frequency of light and sound could change with movement. Experimental proof was provided by Buys Ballot in 1845 (Jonkman, 1980). Musicians with perfect pitch compared the trumpet note played by a stationary horn player and one on a moving train, playing before and after passing a station. The musicians scored the notes in eighths of a note and the results compared well with those expected on theoretical grounds.

Doppler ultrasound is now a widely used technique for the investigation of blood flow. As equipment has developed, so has the range of applications and the confidence in the information obtained. Competent use of Doppler ultrasound techniques requires an understanding of three key components: haemodynamics within vessels, the capabilities and limitations of Doppler ultrasound, and the different parameters, which contribute to the Doppler sonogram.

1.8.1 The Doppler principle applied to ultrasound

In medical imaging, the term Doppler ultrasound encompasses continuos wave Doppler (CW), pulsed wave Doppler (PW) and colour Doppler imaging (CDI). Processing for each differs considerably but the underlying principles are similar. When ultrasound is reflected from a moving subject, in this case red blood cells, the movement of the target causes a frequency shift in the reflected signal and the ultrasound receiver detects this frequency shift. The frequency shift, known as the Doppler shift frequency or Doppler frequency, can be related to the flow velocity within a vessel. The Doppler frequency is substantially altered by three factors:

1. The magnitude of the Doppler frequency increases as blood flow velocity increases.
2. The Doppler frequency increases as the transmitted ultrasound frequency increases. This means that higher frequency probes are more sensitive to low velocities. Unfortunately, attenuation of ultrasound in tissue is frequency-dependent so that high frequency probes (5 MHz and above) may not provide adequate penetration when examining deep vessels. Conversely, the use of low frequency ultrasound in deep abdominal vessels may lead to low flow velocities not being detected.
3. The Doppler frequency increases as the Doppler ultrasound beam becomes more aligned to flow direction (Doppler angle becomes smaller). This is of utmost importance in the use of Doppler ultrasound. All Doppler ultrasound equipment employ filters to cut out the high amplitude, low frequency Doppler signals resulting from tissue movement, for instance due to vessel wall motion. This filter frequency limits the minimum flow velocities that can be measured, the actual value being dependent on the transmitted frequency and beam/flow angle.

1.8.2 Continuous Wave Doppler Ultrasound

Continuous wave (CW) ultrasound is the simplest type of Doppler ultrasound. Because CW systems transmit and receive ultrasound continuously, Doppler shifts are obtained from all vessels in the path of the ultrasound beam (until ultrasound becomes sufficiently attenuated due to depth). Relatively inexpensive Doppler ultrasound systems are available which employ CW probes to give Doppler output without the addition of B-mode images. One disadvantage of a Doppler only system is that the

beam/flow angle may not be known, preventing velocity measurements being derived from the Doppler frequency shifts.

1.8.3 Pulsed Wave Doppler Ultrasound

A limitation of CW systems is that they are not range-specific, which can make it difficult to isolate the Doppler signals from a particular vessel. Pulsed wave (PW) systems transmit short pulses of ultrasound, the echoes of which are received after a short interval of time. PW systems allow measurement of the depth (or range) at which blood flow is observed (by measurement of the time taken for the pulse to be transmitted to and reflected back from the blood cells). Additionally, the size of the sample volume (or range gate) can be changed by altering the transmitted pulse length and the time over which the signal is received. In this way, the region of examination of blood flow can be altered to suit specific clinical requirements.

1.8.4 Colour Doppler Imaging (CDI) and Spectral Doppler

Colour Doppler imaging (CDI) - also referred to as Doppler flow imaging (CFI) or colour Doppler ultrasound (CDU) - produces a colour coded map of Doppler frequency shifts superimposed onto a B-mode ultrasound image. Although CDI uses PW ultrasound, its processing differs from the used to provide the Doppler sonogram. The latter employs several long pulses to enable a full spectral analysis of the region of interest to be obtained over a small interval of time. This is repeated to generate the sonogram in real time. Since CDI may have to produce several thousand colour points of flow information for each frame superimposed on the B-mode image, analysis is simpler. CDI uses fewer, shorter pulses along each colour scan line of the image to give a mean frequency shift and a variance at each small area of measurement. This

frequency shift is converted to a colour pixel. The scanner then repeats this for several lines to build up the colour image, which is then superimposed onto the B-mode image (Figure 2.1).

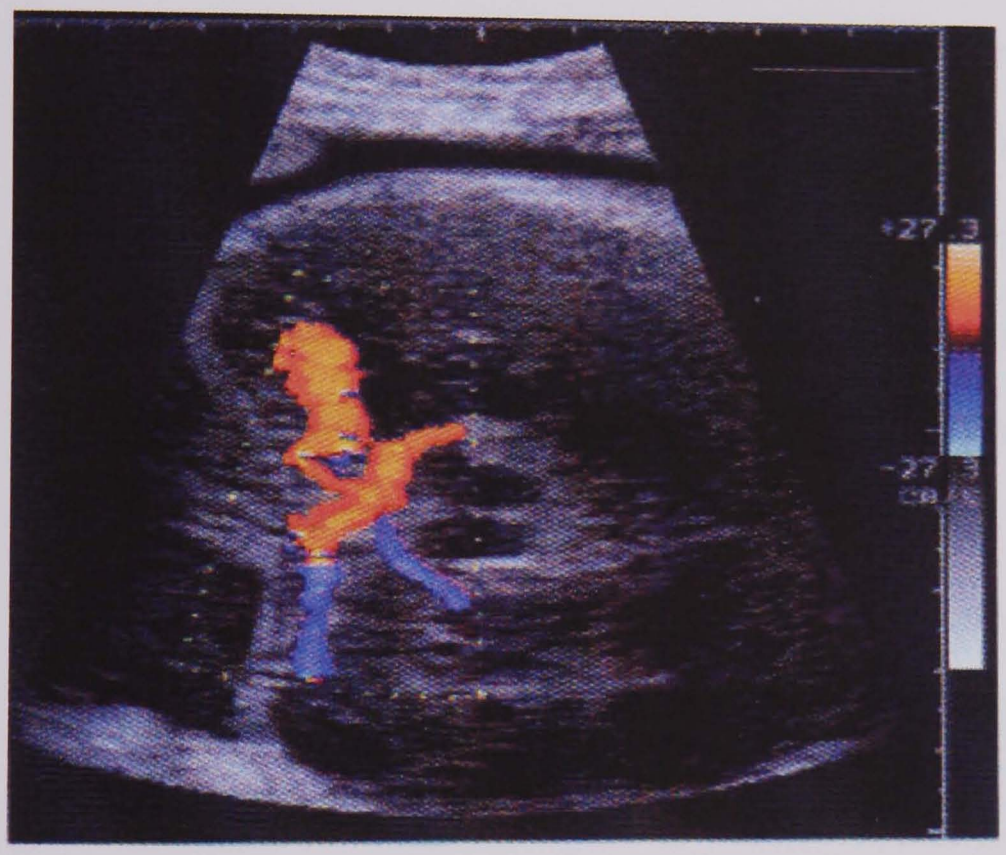
The transducer elements are switched rapidly between B-mode and CDI to give an impression of a combined simultaneous image. The pulses used for CDI are typically 3-4 times longer than those for the B-mode image with a corresponding loss of axial resolution. Assignment of colour to frequency shifts is usually based on direction (for example red for Doppler shifts towards the ultrasound beam and blue for shifts away from it) and magnitude (different colour hues or lighter saturations for higher frequency shifts) (Kremkau,1992). The colour Doppler image is dependent on general Doppler factors, particularly the need for a good beam/flow angle. Because the beams in a linear array transducer are unidirectional, curved vessels can have different colour hues and possibly colour reversal throughout the area of investigation.

Increased sensitivity to low velocity flow can be achieved by decreasing the pulse repetition rate (some manufacturers call this altering the scale). Ultimately, sensitivity to low velocity flow is limited by the filter frequency used to eliminate noise from tissue motion.

The use of CDI can decrease the available frame rate, because the same transducer elements are used for both B-mode and CDI. For each frame, B-mode imaging requires only one pulse per scan line, CDI typically requires 5-10. Broadly, the frame rate increases with increased pulse repetition frequency, but decreases as the number of colour lines increases (e.g. due to a large area of colour flow examination) and

decreases as the number of pulses per line is increased (to improve the quality of colour signal). The loss of frame rate can be of particular importance in obstetric scanning where the fast fetal heart rate results in rapid changes in instantaneous flow.

Figure 1.2 Colour Doppler image of the Circle of Willis and the Middle Cerebral artery superimposed on the B-mode image.



1.8.5 Blood flow measurement using Doppler ultrasound

Velocity measurement

Theoretically, once the beam/flow angle is known, velocities can be calculated from the Doppler spectrum as shown in the Doppler equation. However, errors in the measured velocity may still occur (Evans *et al.,* 1989; Gill, 1985). Errors can arise in the measurement of the ultrasound beam/flow velocity angle; use of high angles (>60) may give rise to errors, because the velocity vector may not be in the direction of the vessel axis. In addition, small changes in the angle will result in comparatively large

changes in the velocity measurement. Other causes of errors include faults in the formation of the Doppler spectrum due to causes such as use of filters removing low velocity components and the non-uniform insonation of the vessel lumen. Furthermore, errors can arise in the calculation packages which the manufacturers provide for analysis of the Doppler spectrum

Flow waveform analysis

Qualitative analysis of the flow waveform shape and spectrum has proved to be a useful technique in the investigation of many vascular beds. It has the advantage that derived indices are independent of the beam/flow angle. The flow waveform shape arises from the action of a pulsatile pressure waveform acting on a distal impedance. Changes in flow waveform shape have been used to investigate both proximal disease (e.g in the adult peripheral arterial circulation) and distal changes (in fetal circulations). If waveform analysis is to be used to observe changes in one component of the proximal or distal vasculature, consideration must be made to ensure that other components are not affecting the waveform significantly.

Resistance Indices

It is beyond the scope of this introduction to detail all the various indices which have been used to describe the shape of slow waveforms. There are variable indices used to describe Doppler waveforms in a quantitative way, usually as a guide to some kind of classification. The following indices are described briefly because they are commonly used in many vascular beds and because the calculations necessary to produce them are available on many commercial ultrasound scanners. They are all based on the maximum Doppler shift waveform. The heights referred to are of the outline of the

waveform, which can be in units of frequency or velocity; all indices are non-dimensional.

Pourcelot's resistance index

Pourcelot's Resistance Index (RI) (1974), also known as Resistance Index is defined as;

(Peak Systolic Height - Minimum Diastolic Height) over (Peak Systolic Height)

RI=(S-D)/S

As diastolic flow falls, the value of RI increases, if there is no diastolic flow, RI=1. RI values greater than are possible if there is negative diastolic flow.

Systolic /diastolic ration (A/B ratio)

The systolic diastolic (S/D) ratio is calculated by dividing the peak systolic height by the minimum diastolic height. As diastolic flow falls, S/D increases; when there is no diastolic flow, the S/D ratio is infinity.

Pulsatility Index (PI)

The pulsatility index (PI) (Gosling, 1975) is defined as,

PI=(Peak Systolic Height-Minimum Diastolic Height)/Mean Waveform Height.

PI=(S-D)/M

Because of the need to measure the mean height of the waveform, this takes slightly longer to calculate than RI or the S/D ratio. It does, however, give a broader range of values, for instance describing a range of waveform shapes when there is no end diastolic flow. In addition to these indices, the flow waveform may be described or

categorised by the presence or absence of a particular feature. Generally, a low pulsatility waveform is indicative of low distal resistance and high pulsatility waveform occurs in high resistance vascular beds. Care should be taken when trying to interpret indices as an absolute measurement of either upstream or downstream factors. For example, alterations in heart rate can alter the flow waveform shape in vessel and cause significant changes in the value of indices.

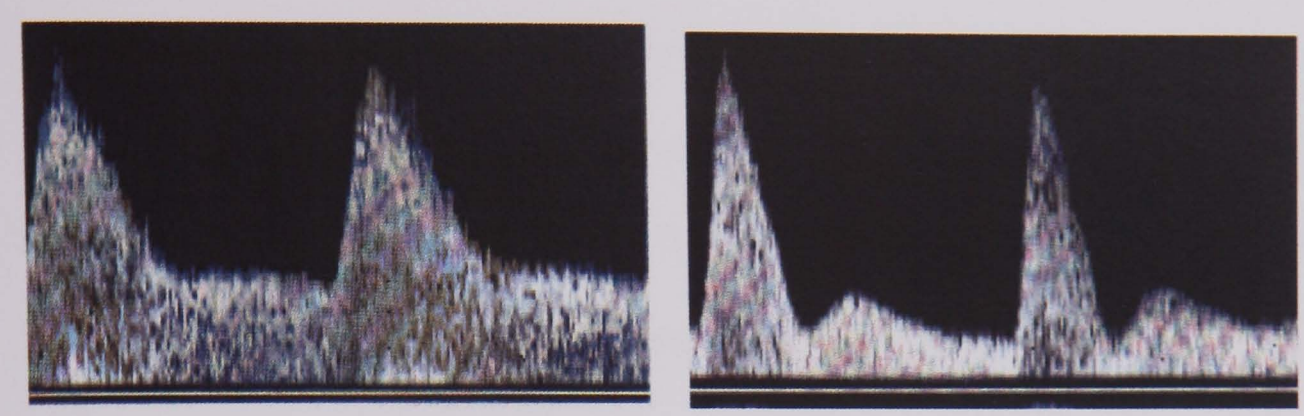
Peak velocity and Time Averaged Velocity

The angle of insonation is required to calculate velocity measurements. With PW/CDI systems it is possible to estimate the angle of insonation. The peak velocity is the maximum systolic velocity, and time averaged mean velocity (TAV) is calculated by averaging velocities throughout the cardiac cycle.

Qualitative analysis

The flow velocity waveform can be analysed by qualitative assessment. In particular the presence or absence of early diastolic notching (Fig 1.3) and the presence or absence of end-diastolic frequencies have been used in the evaluation of the uterine and umbilical arteries waveforms, respectively. Although this approach lacks the attraction of being able to place a numerical value on a particular waveform, it has proven useful in analysis of waveform resistance (see below).

Figure 1.3 High (A) and low (B) resistance waveforms obtained from the uterine artery.

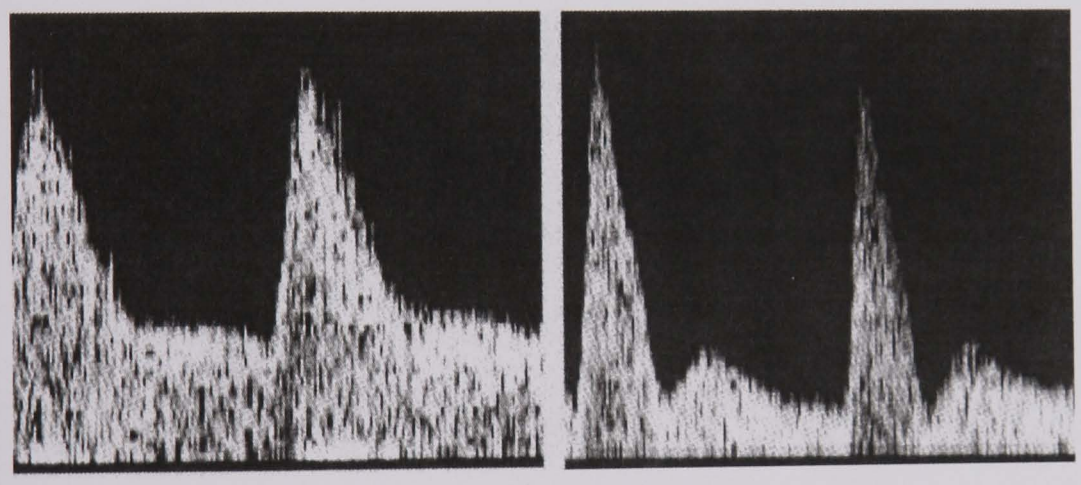


(A) (B)

1.9 Screening for placental insufficiency by uterine artery Doppler

Impaired trophoblastic invasion of the maternal spiral arteries is associated with increased risk for subsequent development of intrauterine growth restriction, preeclampsia and placental abruption. A series of screening studies involving assessment of impedance to flow in the uterine arteries have examined the potential value of Doppler in identifying pregnancies at risk of the complications of impaired placentation (Fig 1.3 and 1.4).

Figure 1.4 Qualitative assessment of uterine artery Doppler flow velocity waveforms. Good end diastolic flow (A), and early diastolic notching (B).



(A) (B)

1.9.1 Studies in selected populations

Arduini *et al.* (1987) examined 60 women who had essential hypertension, renal disease or a previous pregnancy complicated by pregnancy induced hypertension. They measured impedance to the flow in arcuate arteries at 18-20 weeks of gestation and defined a resistance index (RI) (see above) more than 0.57 as abnormal. The test had a sensitivity of 64% for the development of pregnancy-induced hypertension (PIH) (Table 1).

In a similar study, Jacobson *et al.* (1990) examined 91 women who had chronic hypertension, history of preeclampsia or fetal loss and a variety of other medical conditions. They measured impedance to flow in the arcuate arteries at 24 weeks of gestation and defined an abnormal result as RI more than 0.57. The sensitivity of the test for PIH was 44% (Table 1). Similar study by Zimmerman *et al.* (1997) examined 172 women and defined an abnormal result as RI more than 0.68, found a sensitivity of increased impedance in the prediction of preeclampsia of 56%. (Table 1).

Table 1.2 Screening for pregnancy-induced hypertension in high-risk pregnancies be measurement of impedance to flow in the arcuate or uterine arteries.*

	Arduini et al, 1987	*Jacobson et al, 1990*	*Zimmerman et al 1997*
Gestation at Doppler	18-20 weeks	24 weeks	21-24 weeks
Patients	60	91	172
Prevalence of preeclampsia	37%	29%	18%
Sensitivity	64%	44%	56%
Specificity	94%	73%	83%
Positive predictive value	70%	33%	435
Negative predictive value	80%	81%	89%

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1.9.2 Studies in unselected populations

Several studies in unselected populations have examined the value of Doppler assessment of the uteroplacental circulation in the prediction of preeclampsia and/or IUGR. The early studies were limited by the use of continuous wave Doppler, which is a blind investigation. Recent studies, however, have used colour Doppler ultrasound to assess flow in the uterine artery at the point where it crosses the external iliac artery, which is a more reproducible examination. Discrepant results between the studies may be the consequence of differences in Doppler technique for sampling and the definition of abnormal flow velocity waveform, differences in the populations examined (the prevalence of preeclampsia varied from as low as 2% to as high as 24%), the gestational age at which women were studied, and different criteria for the diagnosis of preeclampsia and IUGR.

One stage screening

Bewley *et al.* (1991) calculated the average resistance index from the left and right uterine and arcuate arteries in 925 pregnancies at 16-24 weeks gestation. When the RI was greater than the 95th centile, there was a 10-fold increase in risk for a severe adverse outcome, defined by fetal death, placental abruption, IUGR or preeclampsia. However, the sensitivity of the test for preeclampsia or IUGR was only 24% and 19% respectively with a specificity of of about 95% for both.

Bower *et al.* (1993) examined the uterine arteries in 2058 pregnancies at 18-22 weeks. An abnormal result, defined by a resistance index above the 95th centile or the presence of an early diastolic notch in either of the two uterine arteries, was found in 16% of the pregnancies. The sensitivity of the test was 75% for preeclampsia and 40%

for IUGR, and the specificity was 86% for both. This study highlighted the fact that abnormal Doppler results provide a better prediction of the more severe types of pregnancy complications. Thus, the sensitivity for mild preeclampsia was only 29%, but for moderate/severe disease the sensitivity was 82%. Similarly, the sensitivity for birth weight below the 10^{th} centile was 38% and, for birth weight below the 5^{th} centile, it was 46%.

Two-stage screening

Steel *et al.* (1990) examined the uterine arteries in 1014 nulliparous women by continuous wave Doppler at 18 weeks of gestation and, in those with increased impedance (RI>0.58), the Doppler studies were repeated at 24 weeks. A screen-positive result (increased impedance at 24 weeks) was found in 12% of cases, and the sensitivity of the test for preeclampsia was 63% and for IUGR was 43% ($<5^{th}$ centile).

Bower *et al.* (1993) examined the uterine arteries in 2437 unselected pregnancies by CW Doppler at 20 weeks gestation. In those with increased impedance to flow (RI>95^{th} centile or early diastolic notch in either of the two uterine arteries), the Doppler studies were repeated by colour Doppler at 24 weeks. Persistently increased impedance was observed in 5.4% of the patients (compared to 16% at 20 weeks). It was reported that increased impedance provides good prediction of preeclampsia (but not of non-proteinuric hypertension). Furthermore, in terms of low birth weight, abnormal waveforms provide better prediction of severe (below the 3^{rd} centile) rather than mild (below the 10^{th} centile) IUGR.

Harrington *et al.* (1996) examined the uterine arteries in 1233 unselected women by CW Doppler at 20 weeks gestation. In those with increased impedance (RI>95th centile or early diastolic notch in either of the uterine arteries), the Doppler studies were repeated by colour Doppler at 24 weeks. Persistently increased impedance was observed in 8.9% of the patients. The sensitivity of the test for preeclampsia was 77% and for IUGR 32%. Bilateral notching at 24 weeks was observed in 3.9% of patients; the sensitivity for preeclampsia was 55%, and for IUGR 22%. The respective sensitivities for those complications leading to delivery before 35 weeks were 81% and 58%. Tables 2,3 and 4 summarise the main characteristics and results of the different studies used to assess the value of uterine artery Doppler in the prediction of preeclampsia and IUGR.

Table 1.3 Characteristics of uteroplacental Doppler-screening studies in unselected populations*.

Author	*N*	*Doppler*	*Vessel*	*Abnormal result*	*Gestational age (weeks)*
One-stage					
Campbell et al 1986	126	PW	Arcuate	RI>0.58	16-18
Hanretty et al 1989	291	CW	Arcuate	RI>0.52	26-30
Bewley et al 1991	925	CW	Uterine and arcuate	Mean RI>95TH centile	16-24
Bower et al 1993	2058	CW	Uterine	RI>95TH centile or notch	18-22
Valensise et al 1993	272	Colour	Uterine	Mean RI>0.58	22
North et al 1994	457	Colour	Uterine*	RI>0.57	19-24
Chan et al 1995	334	CW	Uterine	RI>90th centile and bilateral notches	20
Irion et al 1998	1159	Colour	Uterine*	Mean RI>0.57	26
Kurdi et al 1998	946	Colour	Uterine	RI>0.55 and bilateral notches	19-21
Two-stage					
Steel et al 1990	1014	CW/CW	Uterine	RI>0.58	18 & 24
Bower et al 1993	2437	CW/Colour	Uterine	RI>95th centile or notches	20 & 24
Harrington et a 1996	1233	CW/Colour	Uterine	RI>95th centile or notches	20 & 24
Frusca et al 1997	419	CW/Colour	Uterine	Mean RI>0.58	20 & 24

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+Only the uterine artery on the placental side was studied.
PW, Pulsed wave, CW continuos wave

Table 1.4 Results of the uteroplacental Doppler screening studies on unselected populations for the prediction of preeclampsia*.

Author	Outcome measure	Prevalence (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
One-stage screening						
Campbell et al 1986	PET 1	11.9	67	64	20	93
Hanretty et al 1989	PET 2	24.1	7	94	26	76
Bewley et al 1991	PET 3	4.6	24	95	20	96
Bower et al 1993	PET 4	2.5	75	86	12	99
Valensise et al 1993	PET 4	3.3	89	93	31	99
North et al 1994	PET 4	3.3	27	90	8	97
Chan et al 1995	PET 5	6.9	22	97	36	94
Irion et al 1998	PET 4	4.0	26	88	7	
Kurdi et al 1998	PET 3	2.2	62	89	11	99
Two-stage screening						
Steel et al 1990	PET 4	1.9	63	89	10	99
Bower et al 1993	PET 4	1.8	78	95	22	99
Harrington et al 1996	PET 6	3.6	77	94	31	99
Frusca et al 1997	PET 4	1.9	50	92	11	99

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Definitions of hypertensive disease (PET) used by different studies

PET 1 Blood pressure rise (systolic > 30 mmHg or diastolic > 15 mmHg) with proteinurea or generalised oedema

PET 2 Blood pressure > 140/90 mmHg, requiring further investigation or treatment

PET 3 Blood pressure > 140/90 mmHg and proteinurea (>150 mg/24 h)

PET 4 Blood pressure > 140/90 mmHg and proteinurea (>300 mg/24 h)

PET 5 Blood pressure > 140/90 mmhg and proteinurea (>300 mg/24 h) or non-proteinuric hypertension before 37 weeks.

PET 6 Blood pressure rise (systolic > 30 mmHg and diastolic > 25mmHg) with proteinurea (> 500 mg/24h). PPV Positive Predictive Value, NPV Negative Predictive Value

Table 1.5 Results of uteroplacental Doppler screening studies for the prediction of IUGR in unselected population

Author	Outcome measure	Prevalence (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
One-stage screening						
Campbell et al 1986	IUGR<10 th Centile	14.3	67	65	24	92
Hanretty et al 1989	IUGR<5th Centile	5.5	6	93	5	95
Bewley et al 1991	IUGR<5th Centile	5.7	19	95	19	95
Bower et al 1993	IUGR<5th Centile	5.2	46	86	15	97
Valensise et al 1993	IUGR<10 th Centile	7.7	67	95	54	97
North et al 1994	IUGR<10 th Centile	6.6	47	91	27	96
Irion et al 1998	IUGR<5th Centile	11	29	89	25	
Kurdi et al 1998	IUGR<5th Centile	6.0	37	89	22	95
Two-stage screening						
Steel et al 1990	IUGR<5th Centile	7.2	37	90	23	95
Bower et al 1993	IUGR<5th Centile	4.9	30	95	23	96
Harrington et al 1996	IUGR<10 th Centile	10.6	32	94	38	92
Frusca et al 1997	IUGR<10 th Centile	7.2	43	94	36	96

* Reproduced with permission from Nicolaides, Rizzo and Hecher (*eds.*) Placental and fetal Doppler. Parthenon Publishing; 2000, 94.
PPV Positive Predictive Value
NPV Negative Predictive Value

One -stage screening at 23 weeks

Albaiges *et al.* (2000) used colour Doppler to examine the uterine arteries in 1757 singleton pregnancies attending for routine ultrasound examination at 23 weeks. Increased impedance was observed in 7.3% of patients, including 5.1% with mean PI of more than 1.45 and 4.4% with bilateral notches. Increased pulsatility index identified 35.3% of women who later developed preeclampsia and 80% with preeclampsia requiring delivery before 34 weeks; the respective value for bilateral notches were 32.3% and 80%. These findings suggest that a one-stage colour Doppler-screening program at 23 weeks identifies most women who subsequently develop the serious complication of impaired placentation associated with delivery before 34 weeks. The screening results are similar if the high-risk group is defined either as those with increased PI or those with bilateral notches.

More recently, Papageorghio *et al.* (2001) in a multicentre study assessed the use of transvaginal uterine artery Doppler at 23 weeks gestation as screening method for the development of preeclampsia and IUGR. They examined 8335 women with singleton pregnancies and the presence of an early diastolic notch in the waveform was noted, and the mean PI of the two arteries was calculated. Screening characteristics in the prediction of preeclampsia and the delivery of a low-birth weight infant were calculated. The sensitivity for increased PI above the 95^{th} centile for preeclampsia with fetal growth restriction was 69%, for preeclampsia without fetal growth restriction was 24%, for fetal growth restriction without preeclampsia was 13%, for preeclampsia irrespective of fetal growth restriction was 41% and for fetal growth restriction irrespective of preeclampsia was 16%. The sensitivity for fetal growth restriction when defined as birth weight less than 5^{th} rather than 10^{th} centile was

higher (19% vs 16%). The sensitivity for both preeclampsia and fetal growth restriction was inversely related to the gestational age at delivery; when delivery occurred before 32 weeks, the sensitivity for all cases of preeclampsia with fetal growth restriction, preeclampsia without fetal growth restriction and fetal growth restriction without preeclampsia increased to 93%, 80% and 56% respectively. The sensitivity of bilateral notches in predicting preeclampsia and/or fetal growth restriction was similar to that of increased PI but the screen positive rate with notches (9.3%) was much higher than that with increased PI (5.1%). They concluded that a one-stage colour Doppler screening program at 23 weeks identifies most women who subsequently develop severe preeclampsia and/or fetal growth restriction.

Comprehensive analysis of uterine artery Doppler flow velocity waveforms for the prediction of preeclampsia

Aquilina *et al.* (2000) evaluated the performance of different velocimetric indices of uterine artery flow velocity waveforms (FVW's) at 20 weeks gestation, alone or in combination with qualitative analysis, and established the optimum screening method for the prediction of preeclampsia. They studied both uterine arteries at 20 weeks gestation in a total of 614 primiparous women, and created receiver operator curves (ROC) curves for the A/B ratio, RI and systolic/early diastolic (A/C) ratio for placental and non-placental uterine arteries, individually or in combination with the presence of unilateral or bilateral notches.

The highest sensitivity (88%) and specificity (83%) was obtained using bilateral notches/mean RI > or = 0.55 (50^{th} centile) and unilateral notch/mean RI > or = 0.65 (80^{th} centile). Placental velocimetric indices performed better than mean indices but

the differences in sensitivity at a set false-positive rate were not statistically significant.

Uterine artery Doppler screening in multiparous women

Harrington *et al.* (2004) recently studied the value of second-trimester uterine artery Doppler in the prediction of complications resulting from uteroplacental insufficiency in low- and high-risk multiparous women. 628 multiparous women were examined at 20 weeks. 458 had no known risk factors and 170 had clinically identifiable high-risk factors at booking. These were history of preeclampsia, small for gestational age (SGA) birth weight (<5TH centile), placental abruption, stillbirth or early neonatal death. The screening efficacy of uterine artery Doppler for adverse perinatal outcome in low-risk multiparous women was poor and does not justify routine screening. In high-risk multiparous women, mid-trimester abnormal uterine artery Doppler identifies the vast majority of women who develop complications secondary to uteroplacental insufficiency (sensitivity 81.4%, specificity of 89.0%). Normal uterine artery Doppler studies in these women confers a risk of adverse outcome similar to that of women with an uncomplicated obstetric history.

A study of the effect of parity on second trimester uterine artery Doppler flow velocity and waveforms showed that parity has a significant effect on the resistance index and the prevalence of early diastolic notching in the uterine artery flow waveforms. These findings suggest that some permanent modification may persist in the maternal vessels after a successful pregnancy, altering their impedance in subsequent pregnancies. For the prediction of severe early onset preeclampsia, uterine

artery Doppler indices showed a trend towards being better predictors of disease in nulliparous compared with parous women (Prefumo *et al.*, 2004).

1.10 Haemodynamic response of the growth-restricted fetus to hypoxia

1.10.1 The fetus in normal pregnancy

Fetoplacental circulation

The umbilical cord contains two arteries and a vein that branch into chorionic vessels that pass through the chorionic plate and are continous with the cotyledonary arteries and veins. These branch into villous arteries, veins and capillaries of the fetal cotyledons and are situated in the stroma and in the terminal villi (Boyd & Hamilton, 1970). During pregnancy, there is a progressive change in the type of villi that predominate. In the first trimester, they are largely stem villi, in the second, intermediate, and in the third, terminal villi budding off from the intermediate villi (Kaufmann *et al.,* 1979). These small terminal villi have a large surface area to volume ratio for efficient gas and nutrient exchange. The placenta has an initial reparative response to ischaemia. Reduced maternal blood supply leads to a proliferation of cytotrophoblastic cells and a thickening of the basement membrane (Fox, 1983).

Fetal Circulation

The combined output of the fetal heart at 30 weeks is approximately 550 mls/minute (Reed, 1987). About 400 mls/minute passes through the thoracic aorta (Griffin *et al.,* 1985), and the remainder supplies the head and neck. About 50% of aortic flow is in turn directed immediately back to the placenta via the umbilical arteries representing approximately 40% of the fetal cardiac output. The well-oxygenated venous return from the placenta passes in the umbilical vein. In the liver, umbilical venous blood flow is divided, with half the blood passing into the ductus venosus, and the rest draining into the portal system and hepatic veins.

Fetal oxygenation

Oxygenation is the process of transporting molecular oxygen from air to the tissues of the body. In the fetus, this involves, first, oxygen transfer across the placental, second, reversible binding of oxygen to fetal haemoglobin and fetal blood flow, and third, oxygen consumption for growth and metabolism.

In normal fetuses, the blood oxygen tension is much lower than the maternal, and it has been suggested that this is due either to incomplete venous equilibration of uterine and umbilical circulation and/or high placental oxygen consumption (Soothill *et al.*, 1986(b); Nicolaides *et al.*, 1989). Studies in a variety of animals have also demonstrated that the umbilical venous blood *pO2* is less than half the maternal arterial *pO2* and this has led to the concept of "Mount Everest in utero". However, the high affinity of fetal haemoglobin for oxygen, together with high fetal cardiac output in relation to oxygen demand, compensates for the low fetal *pO2* (Battaglia & Meschia, 1986).

Fetal Hypoxia

Small-for-gestational age fetuses may be constitutionally small, with no increased perinatal death or morbidity, or they may be growth-restricted due to many causes (see above). One of which is reduced placental perfusion (i.e. uteroplacental insufficiency).

Cross sectional studies in pregnancies with growth-restricted fetus have shown that increased impedance to flow in the uterine and umbilical arteries is associated with fetal hypoxaemia and acidaemia (Soothill *et al.*, 1986(a); Nicolaides *et al.*, 1988).

These data support the findings from histoplathological studies that, in some pregnancies with small-for-gestational age fetuses, there are:

Failure of the normal development of maternal placental arteries into low-resistance vessels and therefore reduced oxygen and nutrient supply to the intervillous space (Brosens *et al.*, 1972).

Reduction in the number of placental terminal capillaries and small muscular arteries in the tertiary stem villi and therefore impaired maternal-fetal transfer (Giles *et al.*, 1985) (see above).

Animal studies have demonstrated that, in fetal hypoxaemia, there is redistribution in blood flow, with increased blood supply to the brain, heart and adrenals and a simultaneous reduction in the perfusion of gut and kidneys (Peeters *et al.*, 1979). Doppler ultrasound has enabled the non-invasive assessment of the fetal haemodynamic response to hypoxia.

1.10.2 Doppler assessment of fetal circulation in IUGR

Arterial circulation

Pathological studies have demonstrated that increased impedance in the umbilical arteries becomes evident only when at least 60% of the placental vascular bed is obliterated (Giles *et al.*, 1985). In pregnancies with reversed or absent end-diastolic frequencies (EDF) in the umbilical artery, compared to those with normal flow, mean placental weight is reduced and cross-sectional diameter of terminal villi is smaller (Karsdorp *et al.*, 1996). In pregnancies with fetal growth restriction, those with absent EDF, compared to those with normal Doppler, have more fetal stem vessels with medial hyperplasia and luminal obliteration, and those with reversed EDF have even

more poorly vascularised terminal villi, villous stromal haemorrhages, "haemorrhagic endovasculitis" and abnormal thin-walled fetal stem vessels (Salafia *et al.*, 1997).

Clinical studies of umbilical arterial flow velocity waveform in IUGR have reported progressive increase in impedance to flow until absence and, in extreme cases, reversal of EDF (Figure 1.5). The latter represents the extreme end of the spectrum and this finding is associated with a high perinatal mortality, as well as an increased incidence of lethal fetal structural and chromosomal defects (Todros *et al.*, 1999; Trudinger *et al.*, 1985).

In terms of monitoring IUGR pregnancies, abnormal waveforms in the umbilical artery are an early sign of fetal impairment. Bekedam *et al.* (1990) followed up IUGR fetuses longitudinally and reported that abnormalities in the umbilical artery preceded the occurrence of cardiotocographic signs of fetal hypoxaemia in more than 90% of cases. The median time interval between absence of EDF and the onset of decelerations was 12 days (range 0-49 days).

Fetal arterial blood flow redistribution

In fetal hypoxaemia, there is an increase in the blood supply to the brain, myocardium and the fetal adrenal glands and reduction in the perfusion of the kidneys, gastrointestinal tract and lower extremities (Figures 1.6 & 1.7) (Harrington *et al.*, 1999). Although knowledge of the factors governing circulatory readjustments and their mechanism of action is incomplete, it appears that partial pressures of oxygen and carbon dioxide play a role, through their action on chemoreceptors. This mechanism allows preferential delivery of nutrients and oxygen to vital organs,

thereby compensating for diminished placental resources. However, compensation through cerebral vasodilatation is limited and a plateau corresponding to a nadir of PI in cerebral vessels is reached at least 2 weeks before the development of the fetus is jeopardised (Arduini *et al.*, 1992). Vyas *et al.* (1990) reported that in severe IUGR, and concomitant to severe oxygen deficit, there was a sudden rise in middle cerebral artery PI; they suggested that vascular dilatation may be suppressed by the development of cerebral oedema, or a consequence of alterations in flow due to reduced cardiac contractility and a fall in absolute cardiac output. Consequently, arterial vessels are unsuitable for longitudinal monitoring of the growth-restricted fetus. Venous velocity waveforms give more information regarding fetal well-being or compromise.

Fetal arterial Doppler studies are useful in the differential diagnosis of normal small for gestational age (SGA) fetuses from growth-restricted ones. In the hypoxaemic group, due to impaired placental perfusion, the PI in the umbilical artery is increased, in the fetal middle cerebral artery, the PI is decreased; consequently, the ratio in PI between the umbilical artery and middle cerebral artery (UA/MCA) is increased (Hecher *et al.*, 1992).

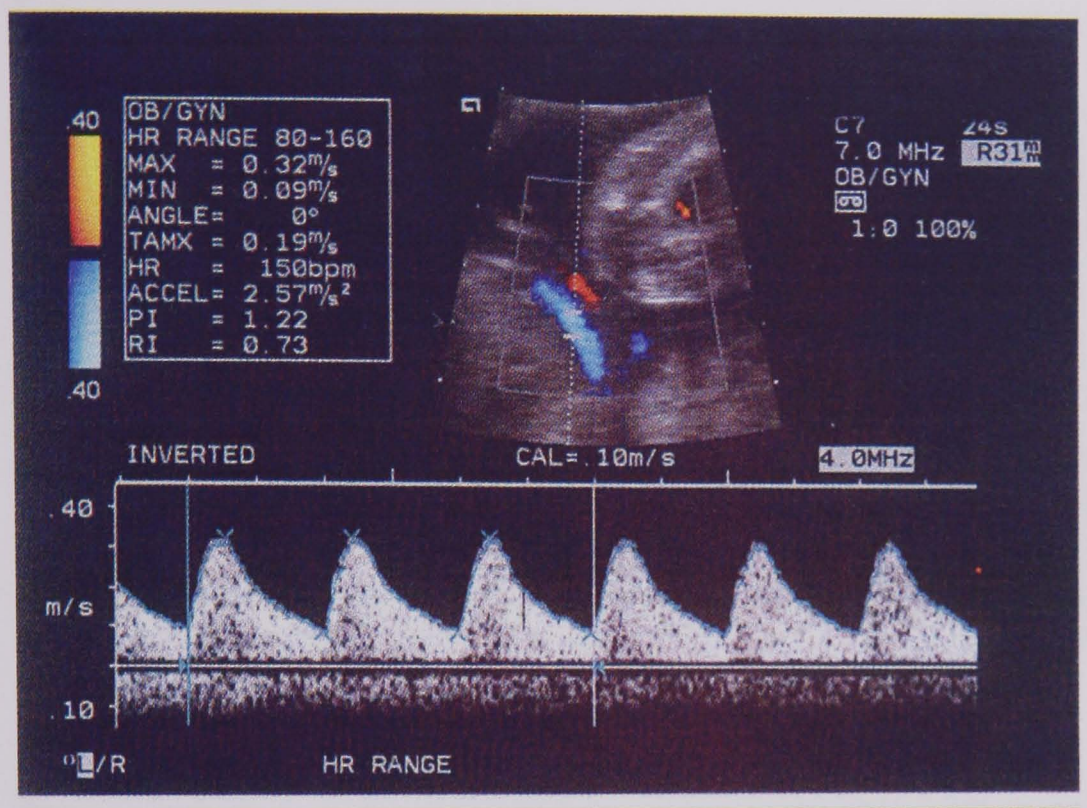
Fetal venous Doppler

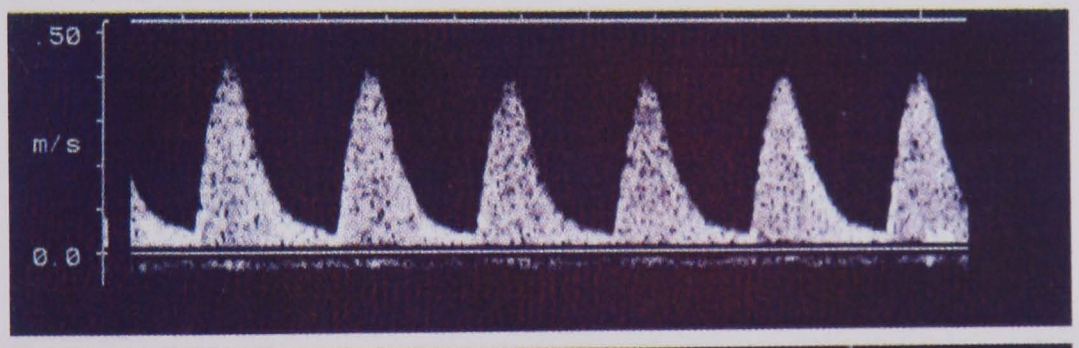
Animal studies have shown that, in severe hypoxaemia, there is redistribution in the umbilical venous blood towards the ductus venosus at the expense of hepatic blood flow contributing to the fetal cardiac output is increased (Reuss & Rudolph, 1980). Under unfavourable conditions, the ductus venosus seems to ensure blood flow directly to the fetal heart and, in extreme conditions, umbilical blood may pass

exclusively through the ductus venosus. During hypoxaemia and increased cardiac afterload, studies in fetal sheep showed an increase in peak systolic forward flow, and during atrial contraction retrograde flow occurs. In contrast, reductions in afterload are associated with an increase in peak diastolic forward flow, indicating that fetal systemic vascular resistance has a major influence on venous return and filling patterns of the right heart (Reuss *et al.,* 1983). This means that increased placental resistance and peripheral vasoconstriction, as seen in fetal arterial redistribution, cause an increase in right ventricular afterload, and thus ventricular end-diastolic pressure increases. This may result in highly pulsatile venous blood flow waveforms and umbilical venous pulsations due to transmission of atrial pressure waves through the ductus venosus (Kiserud *et al.,* 1998). The development of these pulsations is close to the onset of abnormal fetal heart rate patterns and is frequently associated with acidaemia and fetal endocrine changes (Rizzo *et al.,* 1995). In the ductus venosus on the other hand, Colour Doppler examination in the severely hypoxaemic IUGR fetus shows abnormal waveforms with absent or reversal of flow during atrial contraction and markedly increased pulsatility (Hecher & Hackeloer, 1997) (Figure 1.8).

Fetal venous Doppler studies are useful in monitoring the IUGR redistributing fetus. Normal venous flow suggests continuing fetal compensation, whereas abnormal flow indicates the breakdown of haemodynamic compensatory mechanisms. In a study of 41 fetuses displaying arterial blood flow redistribution, Hecher & Hackeloer (1997) showed that, while there were no differences in arterial PI values between fetuses with normal and abnormal biophysical profile, venous pulsatility was significantly increased in compromised fetuses compared to the non-compromised group.

Figure 1.5 Flow velocity waveforms from the umbilical artery in a growth-restricted fetus demonstrating progressive deterioration from normal waveform (top) to low but positive diastolic flow, absent and finally reversed end-diastolic flow (bottom).





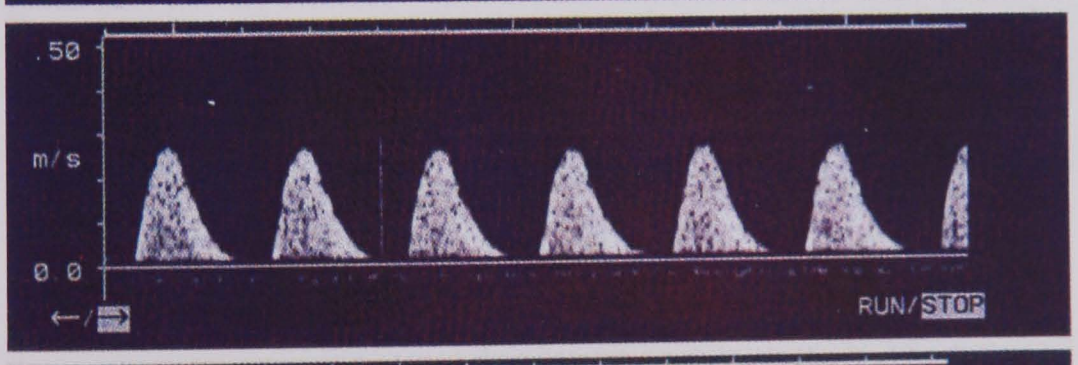


Figure 1.6 Colour Doppler examination of the circle of Willis (left). Flow velocity waveforms from the middle cerebral artery in a normal fetus with low diastolic velocities (right, top) and in a growth-restricted fetus with high diastolic velocities (right, bottom).

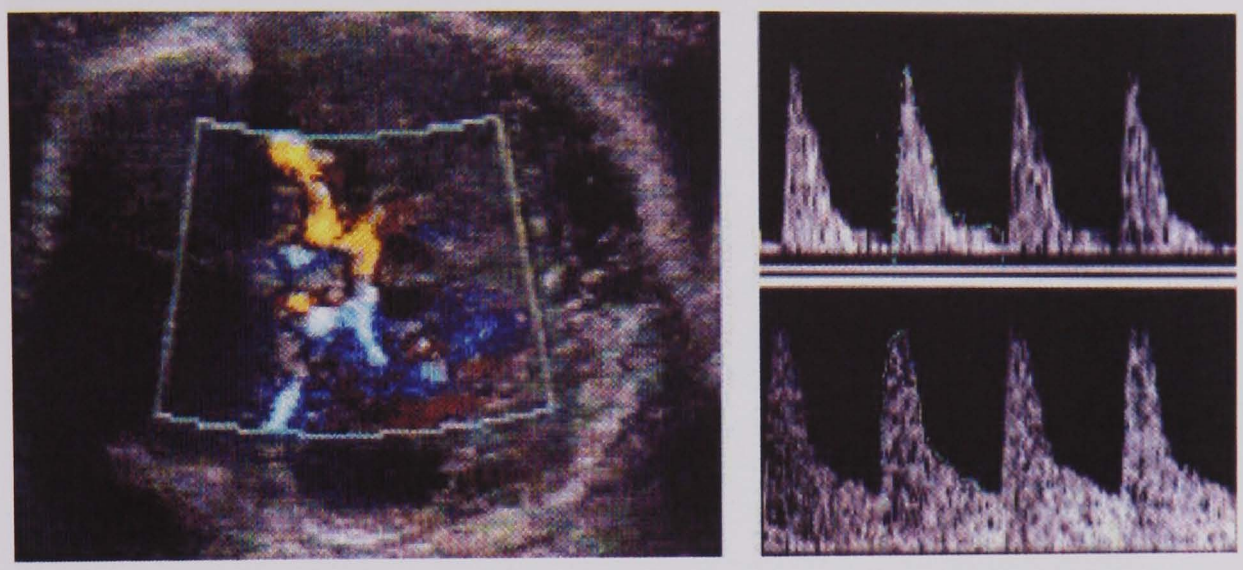


Figure 1.7 Colour Doppler examination of the descending aorta (left) with normal flow velocity waveforms showing positive flow velocities during diastole (right, top) and in a growth-restricted fetus with reversed end-diastolic velocities (right, bottom).

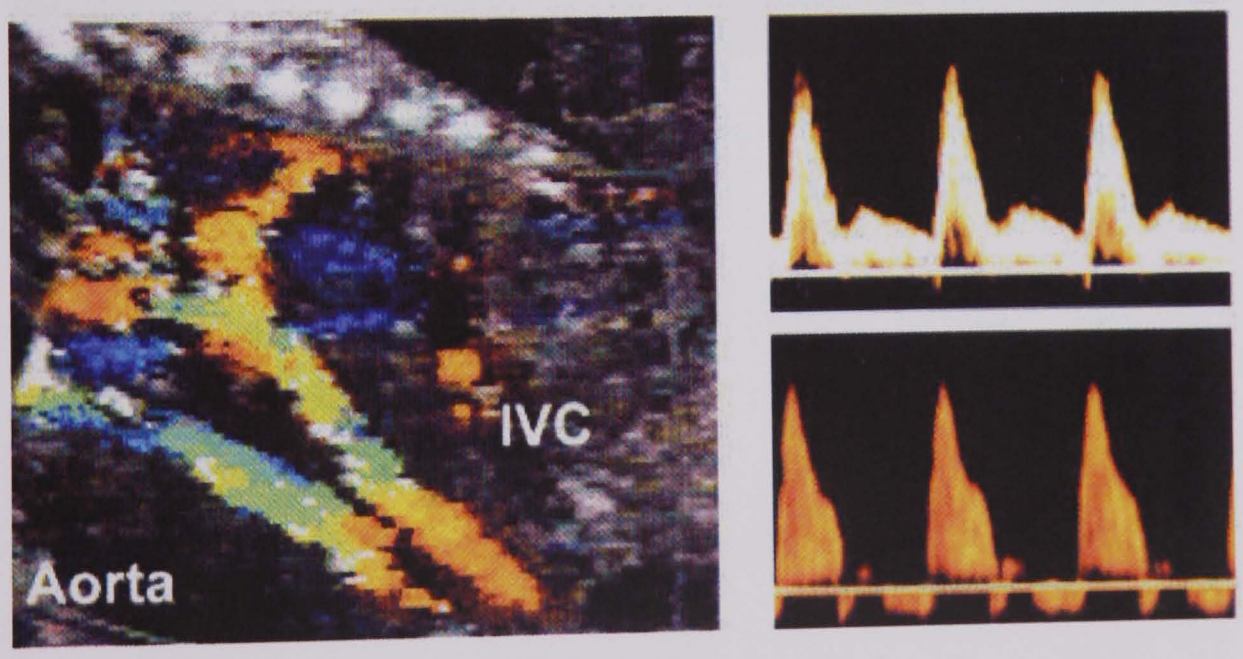
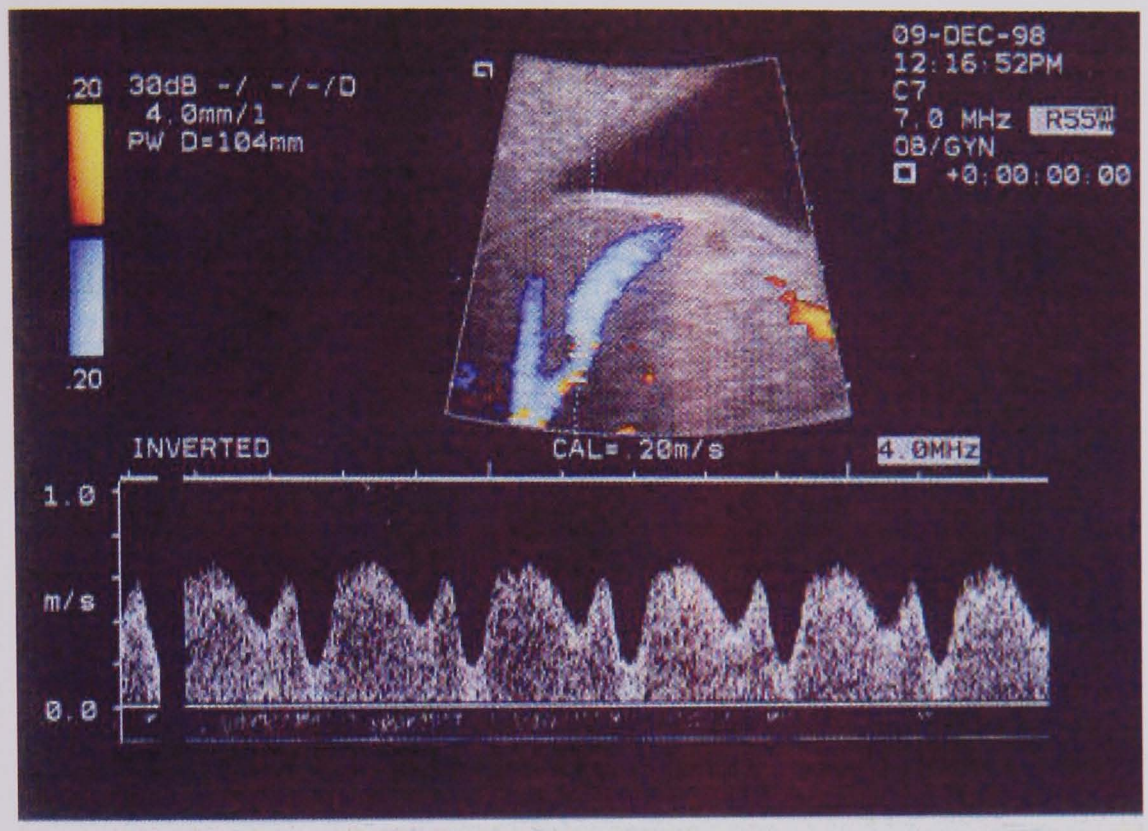
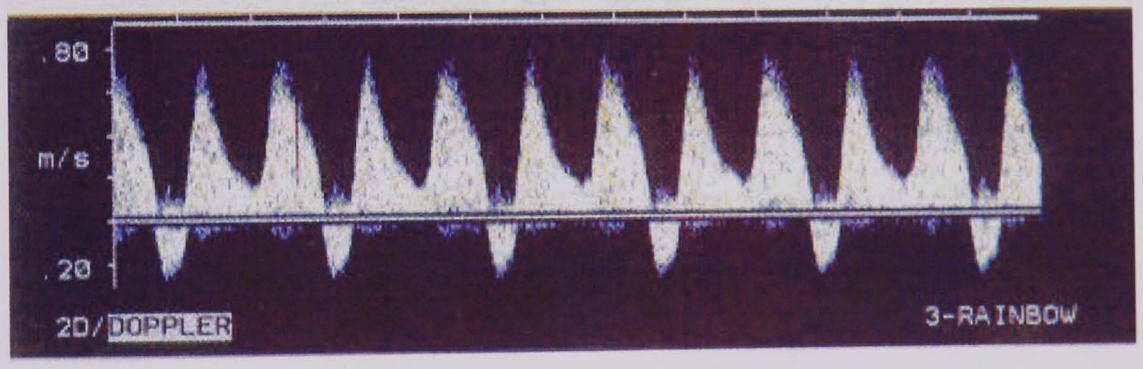


Figure 1.8 Colour Doppler examination of the ductus venosus with normal flow velocity waveforms (top). Abnormal waveform with reversal of flow during atrial contraction and markedly increased pulsatility in a growth restricted fetus (bottom).





1.10.3 Timing of delivery

In the management of the very preterm (before 33 weeks of gestation) IUGR fetus, there is still uncertainty as to whether iatrogenic delivery should be undertaken before the development of signs of severe hypoxaemia, with a consequent risk of prematurity-related neonatal complications, or whether delivery should be delayed, but the risks of prolonged exposure to hypoxia and malnutrition imposed by placental insufficiency. Fetuses with severe early-onset growth restriction during the late second or early third trimester are capable of tolerating chronic hypoxaemia without damage for much longer than a well-nourished late third-trimester fetus with a high-energy consumption.

In the growth-restricted hypoxaemic fetus, redistribution of well-oxygenated blood to the vital organs, such as the brain, heart and adrenals, represents a compensatory mechanism to prevent fetal damage. When the reserve capacities of the circulatory redistribution reach their limits, fetal deterioration may occur rapidly. In clinical practice, it is necessary to carry out serial Doppler investigations to estimate the duration of fetal blood flow redistribution. The onset of abnormal venous Doppler results indicates deterioration in the fetal condition and iatrogenic delivery should be considered. During the current study, we followed the above general guidance in timing the delivery of the preterm fetuses with IUGR with or without preeclampsia.

1.11 Summary and aims of current study

In discussing preeclampsia in 1939, Johnstone concluded that "no completely satisfactory explanation of the disease or source of the toxin can as yet be offered" and this conclusion is no further advanced today (Johnstone 1939). It is difficult to construct a unifying hypothesis. However, the primary event in preeclampsia appears to be abnormal trophoblastic invasion early in pregnancy. Maternal genotype and fetal phenotype may confer susceptibility and as a result the maternal immunological response to fetal antigen is abnormal. The resulting hypoperfused placenta secretes a factor into the maternal circulation, which targets the vascular endothelium and gives rise to the clinical sequelae of the disease. The nature of this factor(s) remains unclear and the effect on endothelial function at cellular level has not been fully elucidated. The balance between VEGF and its inhibitors play a fundamental role in maintaining vascular endothelial integrity. Disturbance in this balance, such as overexpression of sFlt1 may tip the balance towards generalised endothelial dysfunction such as that seen in preeclampsia. Recent evidence suggests that placental expression of molecules such as Flt1, PlGF, Fas and FasL is altered in preeclampsia. However, the exact role of these molecules in cases of abnormal placentation is far from clear.

Therefore the aims of the work carried out in this thesis are:

1. Compare mid-trimester serum levels of sFlt, PlGF, Fas and FasL in pregnancies with abnormal mid-trimester uterine artery Doppler that subsequently develop preeclampsia, normotensive IUGR and normal outcome.
2. Establish the correlation between the resistance indices of the uterine artery Doppler and the above serum markers at 24 weeks.

3. Compare serum levels of these markers in preeclampsia, normotensive IUGR and controls.
4. Assess placental expression of Flt1, PlGF, Fas and FasL in cases of preeclampsia, normotensive IUGR and control pregnancies.

MATERIALS AND METHODS

2.1 Ethical approval

Ethical approval was obtained from East London and the City Research Ethics Committee and the Homerton Hospital local research committee prior to commencement of the study. Written informed consent was obtained from all women participating in the study by the investigator and written patient information sheets were given to them. The patients' General Practitioner (GP) was informed of the patients' participation in the study.

2.2 Patient Selection

Uterine artery Doppler examination

553 healthy pregnant women were examined using uterine artery Doppler at 24 weeks. Among these, there were 395 primiparous and 158 multiparous women. Gestational age was determined by means of ultrasound examination at 10-12 weeks. All primigravid women and multiparous women with previous pregnancy complication (see table) attending for routine anomaly scans at 20 weeks were offered uterine artery Doppler examination at 23-24 weeks.

Table 2.1 Risk factors in multiparous women who were offered uterine artery Doppler examination

- History of hypertension in pregnancy,
- History of delivery of small baby,
- History of placental abruption,
- History of stillbirth,
- History of previous neonatal death.

Among women who underwent uterine artery Doppler flow examination, 97 women had abnormal result defined as above. 63 women were primiparous and 34 were multiparous. Eleven women did not attend for follow up (dropped out). The remaining 86 women were enrolled in the study after obtaining appropriate consent and giving them information leaflets. Only women with complete outcome information were enrolled in this study. Seven women developed intrauterine growth restriction, defined as fetal biometry measures less than the 5^{th} centile for gestational age and associated abnormal umbilical artery Doppler or serial fetal growth measurements crossing the growth centiles with evidence of redistribution on middle cerebral artery Doppler examination. Eight women developed preeclampsia (<36 weeks to match the IUGR group) defined according to the ISSHP. For each woman with preeclampsia,

two normotensive controls with delivery at term of a normally grown infant were selected.

Serial growth scans and Doppler

Women enrolled in the study were followed up on a fortnightly basis (and more frequently if abnormality was detected) until delivery. Uterine artery Doppler flow examination and serial fetal biometry growth scans including head circumference (HC), abdominal circumference (AC) and femur length (FL) were performed in each visit and plotted on fetal growth charts produced by Snijders and Nicolaides (1994). Maternal blood pressure (BP) and urine check for protein was done in each visit. If there was evidence of proteinurea on urine dipstick examination, then 24-hour urine collection for protein was performed.

Fetal Doppler examination was performed if there is suspicion of fetal growth restriction. This included umbilical artery, middle cerebral artery and fetal aorta. Ductus venosus (DV) Doppler was performed in cases where there is evidence of IUGR and fetal arterial redistribution with absent end-diastolic flow (EDF) in umbilical artery Doppler examination (see below).

2.3 Techniques of Doppler examination

All Doppler studies were done by the investigator using ATL 5000 ultrasound machine with a 3.5-MHz probe colour flow mapping, and a 100-Hz high-pass filter. The quality of the velocity waveforms was maximized by using the smallest possible angle of insonation (< 30 degrees). At least four consecutive waveforms with clear sharp outline were obtained in each Doppler measurement. The RI and PI were calculated using built-in calibration in the machine. 553 women were examined using

uterine artery Doppler at 24 weeks. They were 395 primiparous and 158 multiparous women.

Uterine arteries

Uterine artery Doppler examination was performed with the transabdominal approach, by placing the transducer in the relevant iliac fossa. After that, the course of the uterine artery was followed from the lateral pelvic wall, across the external iliac artery. Pulsed Doppler was then applied 1-cm medial to the cross over point and with a Doppler angle<30 degrees to obtain accurate flow velocity waveforms. In addition to PI and RI, the presence or absence of a notch was noted in each of the waveforms in both uterine arteries. A notch was considered to be present when there was a clearly defined upturn of the flow velocity waveform at the beginning of diastole, which was present in all waveforms. Each uterine artery was sampled twice and the same operator performed all measurements. Abnormal uterine artery Doppler was defined as flow velocity waveforms with bilateral notches and mean RI > 0.55, or unilateral notch and mean RI > 0.65 (Aquilina et al 2000).

Umbilical artery

The umbilical artery waveform was measured from a free-floating loop of cord during fetal quiescence. Generally signal was obtained from both the umbilical artery and vein, following the above methodology. Three types of flow waveforms were identified:

1. Normal PI and RI and normal end diastolic flow (EDF).
2. High PI and RI (>90th centile for GA) with positive EDF.
3. Absent or reversed EDF. All these cases had high PI and RI.

Middle cerebral artery (MCA)

A cross section through the fetal head at the level used for biparietal diameter measurement was obtained. The transducer was then moved caudally and the circle of Wellis was identified. The course of the MCA was identified using colour flow mapping. The course of the vessel allows optimum angles of insonation for obtaining accurate flow velocity waveforms. Pulsed Doppler was used and PI and RI were measured using, built in calibrations. The evidence of arterial redistribution was noted and abnormal MCA Doppler was defined as PI and/or RI less than the 5^{th} centile for gestational age.

Fetal aorta

Doppler flow waveforms from fetal aorta were obtained using the same principles as above. Because of the position of the aorta in fetal body, Doppler measurements necessitated angling of the ultrasound probe to maintain the insonation angle less than 30 degrees. Three types of flow waveforms were identified:

1. Normal PI and RI and normal end diastolic flow (EDF).
2. High PI and RI (>90^{th} centile for GA) with positive EDF.
3. Absent or reversed EDF (AREDF). All these cases had invariably high PI and RI.

Ductus venosus (DV) Doppler

The course of the ductus venosus as it courses through the liver between the umbilical vein and the right atrium was identified using colour flow mapping. Both cross sectional and longitudinal views of the DV were obtained and pulsed Doppler was

obtained when the fetus was quiescent. Presence, absence or reversal of "a wave" was noticed in the flow waveforms. DV Doppler was only performed in cases of IUGR with evidence of fetal compromise on arterial Doppler examination.

Doppler measurements from MCA, fetal aorta and ductus venosus were obtained to assess fetal well-being and timing of delivery.

2.4 Blood collection

Blood was collected from the 86 women with abnormal mid-trimester uterine artery Doppler examination during the same visit (24 weeks) after obtaining appropriate consent and serum was stored at -80C for further assay (see below). Blood was collected again from these women within 24 hours of delivery and serum was stored at -80C for further assay. Placental samples were obtained at the time of delivery and stored at -80C for further analysis (see below). Serum and placental samples were obtained from normotensive gestational age matched controls with appropriately grown fetuses delivered by Caesarean Section using similar protocols and stored at -80C for further analysis.

5 ml of blood was collected in a sterile vacuum tube and allowed to stay in room temperature for 30 minutes to allow clot to form. Serum was separated by centrifugation at 1500 g/minute for 10 minutes at 4C and kept in aliquots at -80C until further assay. Assays were performed for Fas, Fas Ligand, sFlt1 and PlGF in the samples collected. Specimens were randomly ordered for analysis, and the clinical outcome of pregnancy was not known whilst performing the assay.

2.5 Placental sample collection

3-5 cotyledons from the maternal side of the placenta were collected from women enrolled in the study. Placental samples were exclusively collected from women who underwent Caesarean delivery (both cases and controls) to avoid the potential effect labour could have on placental expression of the studied antigens. The samples were collected immediately after delivery and washed with normal saline three times, then snap frozen in liquid nitrogen. After that, samples were immediately stored at -80C for further analysis. Immunohistochmeistry was performed for Fas and FasL. Western blotting was performed for Fas, Fas Ligand, Flt1 and PlGF.

2.6 Enzyme Linked Immune Sorbent Assay (ELISA)

2.6.1 Soluble Fas ELISA

Principle

We used the sFas ELISA kit (Medical and Biological Laboratories MBL, Nagoya, Japan), Code no. 5251, to measure sFas protein by sandwich ELISA. The assay uses Fas antibodies against two different epitopes. One of the antibodies is polyclonal and recognizes intracellular domain (No. 305-319), and another one is momoclomal antibody and recognizes extracellular domain (No. 110-120 a.a.).

In the wells coated with anti-Fas polyclonal antibody, samples to be measured or standards are incubated. After washing, a peroxidase conjugated anti-Fas monoclonal antibody is added in the microwell and incubated. After another washing, the peroxidase substrate is mixed with the chromagen and allowed to incubate for an additional period of time. An acid solution is then added to each well to terminate the

enzyme reaction and to stabilize the developing colour. The optical density (O.D.) of each well is then measured at 450 nm using a microplate reader. The concentration of sFas is calibrated from a dose response curve based on reference standards.

2.6.2 Soluble FasL ELISA

Principle

We used the sFasL ELISA kit (Medical and Biological Laboratories MBL, Nagoya, Japan), Code no. 5255, to measure sFasL protein by sandwich ELISA. The assay uses FasL antibodies against two different epitopes. In the wells coated with anti-Fas ligand monoclonal antibody, 4H9, samples to be measured or standards are incubated. After washing, a peroxidase conjugated anti-Fas Ligand monoclonal antibody, 4A5, is added in the microwell and incubated. After another washing, the peroxidase substrate is mixed with the chromagen and allowed to incubate for an additional period of time. An acid solution is then added to each well to terminate the enzyme reaction and to stabilize the developing colour. The optical density (O.D.) of each well is then measured at 450 nm using a microplate reader. The concentration of sFas Ligand is calibrated from a dose response curve based on reference standards.

2.6.3 Soluble sFlt1 ELISA

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF R1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any VEGF R1 present. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF R1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added

to the wells and color develops in proportion to the amount of VEGF R1 bound in the initial step. The color development is stopped and the intensity of the color is measured.

2.6.4 Soluble PlGF ELISA

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for PlGF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any PlGF present. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for PlGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of PlGF bound in the initial step. The color development is stopped and the intensity of the color is measured.

2.7 WESTERN BLOTTING

2.7.1 Homogenisation of placental samples

Into a 1.5ml microfuge tube, 2.3mg dithiothreitol and 4.6mg β-glycerophosphate were weighed out. 1.5ml Homogenisation Buffer (H.B.) were pipetted into the microfuge tube, followed by 75µl protease inhibitors (one aliquot). The solution was subsequently vortexed.

100µl of H.B. were applied to clean *Duall* glass tissue grinders. Approximately 10-15 mg of tissue, stored in the –80C freezer, were homogenised in the tissue grinders, on ice. The homogenate was pipetted into a labelled 1.5ml microfuge tube, and any excess tissue, in the grinder, was ground up in a further 50µl. Once complete, approximately 50µl of H.B. were pipetted down the sides of the grinder, to remove any protein adhered to the glass. The

homogenate was removed and pooled with the homogenate from the initial grind. The process took 15-20 minutes per sample.

Each homogenate was centrifuged at 13,000 x g (11,700 rpm in a *Herous Biofuge pico)* for five minutes. The supernatants were decanted into fresh 1.5ml microfuge tubes, and the pellets were either discarded or re-suspended in Homogenisation Buffer (H.B.).

The protein concentration of each sample was estimated by performing a Lowry assay. The following standard curve was set up, using 1.5ml microfuge tubes. Standards were duplicated, whilst unknowns were repeated a further four times.

1.5 mg/ml BSA(µl)	dH2O (µl)	H.B. (µl.)	Sample (µl.)	[Protein] (µg/µl)
0	27	3	0	0.00
2	25	3	0	1.0
5	22	3	0	2.5
10	17	3	0	5.0
15	12	3	0	7.5
20	7	3	0	10.0
0	27	3	3	Unknown

Bio-Rad Dc Protein Assay reagents A, B and S were subsequently added, in the following quantiles, to each microfuge tube. (20µl. of reagent S were added to each ml of reagent A):

1. 150µl of reagents A/S per tube+vortexed.
2. 1200µl. of reagent B per tube + vortexed immedaitely.

The solutions were transferred to cuvettes, and the absorbance read after 5 minutes, using a *Jenway Genova* spectrophotometer, set at a wavelength absorbance of 750nm. The protein concentration of each sample was subsequently determined using the *Graph Pad Prism 3.0* computer software. Following the addition of x2 Laemmli Sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125M Tris-HCl, pH 6.8) in a 1:1 ratio, the samples were heated at 100C for 10 minutes and stored at -20C

2.7.2 SDS-Electrophoresis

Preparation for Electrophoresis & Western Blotting

1. Before casting the electrophoresis gel all the apparatus were cleaned with *Purite Select* deiodinised water (dH2O) and dried using paper towel.

   600ml of electrode / running buffer were prepared by combining 120 ml of 5X electrode / running buffer (see figure 2.1) with 480 ml of deiodinised water.

Figure 2.1 Preparation of 5X electrode / running buffer, pH 8.3

Tris base	9g	(15g/l)
Glycine	43.2g	(72g/l)
SDS	3g	(5g/l)

The running buffer was stored at 4C (i.e in the fridge).

2 Blotting buffer was prepared (3.03g Tris-base, 14.4g glycine, 200ml methanol per litre, pH 8.3) in advance and stored at 4C (i.e. in the fridge). The pH should be -8.3. Neither acid nor base was added to adjust the pH.

3 The Bio-Ice cooling unit, full of deionised water, was placed in a -20C freezer overnight.

Assembling the Glass Plate sandwiches

SDS-Electrophoresis was performed through the use of the *Bio-Rad Mini-Protean II* electrophoresis system. These instructions were adapted from the Mini-protean II Electrophoresis Cell instruction manual.

1. The gel sandwich was assembled on a clean surface. The longer glass plate was laid on the surface first. Two spacers (1.5mm thick) were placed on each of the shortest sides of the glass plate. The shorter glass plate was then placed on top of the spacers, in alignment with the bottom of the longer glass plate and spacers.
2. The four screws on the clamp assembly were loosened, to allow the insertion of the glass plate gel sandwich. The clamp assembly was then stood upright, with the screws facing away from the operator. The glass plates were slid gently alongside the acrylic pressure plate of the clamp assembly, on the side opposing the four screws.
3. The clamp assembly was placed into the alignment slot of casting stand, so that the clamp screws faced away from the operator. The bottom of the two glass plates and spacers were then allowed to align along the base of the alignment slot. Next, the alignment card was slide between the glass plates and spacers to ensure a parallel alignment of spacers. Finally the two pairs of clamp screws were gently tightened.
4. Following the removal of the alignment card, the alignment of the bottoms of the glass plate, spacers and clamp assemly was checked by eye. All three

surfaces were therefore flush. The complete sandwich clamp assembly was transferred to the casting slot on the opposite side of the casting stand from the alignment slot. The bottom of the clamp assembly/glass plates formed a seal with the grey silicon gaskets at the base of the casting slots.

5. In order to cast a second gel, steps 1-4 were repeated, but the complete sandwich clamp assembly was transferred to the casting slot on the same side of the alignment slot.

Casting the Discontinous Polyacrylamide Gels

A discontinous polyacrylamide ge system was used which consisted of a seperating gel (lower gel) and stackingf gel (upper gel). Discontinous gel systems allow better resolution of proteins than continous polyacrylamide gels, which employ only one continous gel, due to differing acidities of the stacking gel (pH 6.8) and seperating gel (pH 8.8). This sudden change in pH causes the proteins of similar molecular masses to 'bunch together', therefore aiding resolution.

1. A 1.5 mm comb was placed in between the two glass plates of each glass plate sandwich. A marker pen was then used to draw a line 0.5 mm below the teeth of the comb. The comb was subsequently removed.
2. A 10% or 7.5% acrylamide separating gel solution was prepared by combining the components listed in figure 2.2.

Figure 2.2 Preparation of 10% and (7.5%) Separating Gel Solutions

Deiodinized water	8.1 ml	(9.7 ml)
1.5 M Tris-HCl, pH 8.8	5.0 ml	(5.0 ml)
10% (w/v) SDS stock	200 ul	(200 ul)

(stored at room temperature)		
Acrylamide/Bis (30% stock)	6.6 ml	(5.0 ml)
Vortexed		
10% ammonium persulfate (APS)	100 ul	(100 ul)
(i.e. 100mg/ml, fresh daily)		
TEMED	10 ul	(10 ul)
Total volume	20 ml	(20 ml)
Vortexed		

3. Upon the addition of APS and TEMED the solution sets. To cast the separating gel, the separating gel solution was poured between the two glass plates of the gel sandwiches, down the side of one of the spacers, using a disposable Pasteur pipette. The solution was poured up to the drawn lines. This procedure was carried out slowly to prevent mixing the solution with air bubbles.
4. The solution was immediately overlaid with a layer of deionised water. The separating gel solution was left to set for 30 minutes.
5. A 4% stacking gel solution was prepared by combining the components listed in Figure 2.3.

Figure 2.3 Preparation of Stacking Gel Solution

Deionised water	6.1 ml
0.5 M tris-HCl, pH 6.8	2.5 ml
10% (w/v) SDS	100 ul
Acrylamide/bis (30% stock)	1.33 ml
Vortexed	

10% ammonium persulfate (APS)	50 ul
TEMED	10 ul
Total volume	10 ml

Vortexed

6. The layers of deionised water, overlaying the separating gels, were removed using absorbent paper towel. Upon the addition of APS and TEMED the solution sets. Thus the solution was immediately poured between the glass plates, down the side of a spacer, using Pasteur pipette.
7. A comb (1.5 mm thick) was placed into the top of each stacking gel. The stacking gel solutions were then left to set for 20 minutes. The hot block was set at 40c, in preparation for the Kaleidoscope markers.
8. The comb was slowly removed upwards. The areas where the teeth of the comb had penetrated the surface of the stacking gel solution later served as sample loading wells.
9. The loading wells were rinsed with deionised water. The gels were then ready to clip onto the inner cooling core.

Assembling the upper buffer chamber

1. Initially, the cleanliness of the grey U-shaped inner cooling core gaskets was inspected, to prevent leakage.
2. The clamp assemblies/gel sandwiches were then released from the casting stand.
3. With the inner cooling core lying flat on a clean surface, a clamp assembly/gel sandwich was clipped into position onto the inner cooling core. This was

achieved by sliding the clamp assembly wedges under the locator slots on the top of the inner cooling core, with the assembly screws facing outward and the glass plates facing the cooling core, until the base of the clamp assembly clipped into the cooling core latch.

4. The inner cooling core was turned over and another clamp assembly/gel sandwich was clipped onto this side of the inner cooling core.

Loading the samples

1. The inner cooling core, complete with attached clamp assemblies, was inserted into the *Mini-Protean II* cell. The upper buffer was then filled with electrode/running buffer. The remainder of the electrode / running buffer was poured into the lower buffer chamber, until at least 1 cm of the gel bases were covered in buffer. **N.B. It was important to ensure that the upper buffer chamber was full before loading the samples. If the upper buffer chamber leaked, the chamber was only re-filled once the samples had ran into the gel, i.e. to avoid displacement of the samples from the wells.** The hot block was set to 100C and the samples were boiled for 10 minutes.
2. Set volumes of the homogenised myometrial samples (containing known amounts of total protein) were loaded into each sample loading well, at the top of the stacking gel, using a 2-20 ul *HTL Lab Mate* pipette and long, fine pipette tips. The volumes of samples to be loaded, for a given total amount of protein, was determined by the following equation:

Volume loaded (ul) = Amount (ug) / Concentration (ug/ul)

A solution of standard marker proteins, of known molecular masses, was then applied to one of the sample loading wells. A few drops of bromphenol blue were also added to the upper buffer chamber.

3. The lid was placed on top of the *Mini-Protean II* cell, ensuring that the colours of the plugs on the lid matched the colours of the jacks on the inner cooling core.
4. The electrical leads, leading from the lid, were attached to a *Bio-Rad Power Pac 300.*
5. In order to run the samples into and through the gels, a constant current was applied across the gels. The current applied was 30mA/gel (i.e. 60 mA for two gels), which resulted in a voltage of approximately 200V, therefore the voltage was set to 300V. Once the samples had ran into the Separating gel, the currant was increased to 40mA/gel (i.e. 80 mA for two gels). The voltage will decrease at this step.

Removing the gel

1. At the end of the electrophoresis run, the power supply was turned off and the electrical cables were disconnected from the power pack.
2. After the removal of the *Mini-Protean II* cell lid, the inner cooling core was removed from the cell and the lower and upper buffer chambers were decanted.
3. The inner cooling core was laid horizontally and the clamp assemblies detached by pushing the cooling core latch downwards and up on the clamp assembly.

4. The clamp assembly screws were loosened and the glass plate gel sandwiches were slid out from the clamp assembly.
5. To access each gel, one of the spacers was removed and the upper plate was gently prised off using a thumb. The gel stuck to one of the glass plates.
6. The stacking gel was removed with a scalpel blade. The bottom left hand corner of the gel was removed to orientate the gel.
7. The gel was detached from the glass plate by gentle agitation with a thumb, either immersed under blotting transfer buffer or in dry conditions. If the protein in the gel was to be Western blotted, then the gel was allowed to equilibrate for 30 minutes, submerged in blotting buffer, as SDS-gels often shrink in blotting buffer (see Section 4.2).

Gel Staining

Occasionally it is desirable to visualise the total protein profile, separated in gels during electrophoresis. This may be to check protein separation, or it can be conducted after Western blotting (see Section 4), to determine the extent of protein transfer from a particular gel. One such stain is Coomassie Blue/ Brilliant Blue:

1. Gels were immersed separately in Brilliant Blue R (0.25% (w/v) brilliant blue R, 40% (v/v) methanol, 7% (v/v) acetic acid) overnight, with gentle agitation, in suitable plastic containers.
2. The Brilliant Blue R stain was decanted. To increase the contrast between stained proteins and gel, i.e. to remove background, the gel was de-stained in a solution consisting of 40% methanol and 10% acetic acid, for 1 to 3 hours, with several changes of the de-stain solution.

2.7.3 Western blotting

Western blotting was performed through the use of the *Bio-Rad Mini Trans-Blot Electrophoresis Transfer Cell.* All the equipment, including the *Mini Trans-Blot Cell,* gel cassettes and fibre pads were initially rinsed in deionised water.

Preparation for blotting

1. PVDF membranes were used, onto which the separated proteins were blotted from the gels. PVDF membranes, and filter papers, were cut to the same dimensions as fibre pads. The PVDF membrane was 'activated' by immersion in methanol, which allows the proteins to bind to the membrane. The PVDF membranes were only handled whilst wearing latex gloves and using forceps.
2. Acrylamide gels shrink in blotting buffer, therefore the gels and membranes were soaked in blotting buffer (in a plastic tray) for 30 minutes to allow them to equilibrate.
3. The Bio-Ice cooling unit was inserted into the buffer chamber, a few minutes before the blotting procedure began. A magnetic flea was also placed in the centre, at the bottom, of the chamber. The blotting chamber was then placed on top of a magnetic stirrer.

Assembly of the Gel Holder Cassette

1. The fibre pads were submerged in blotting buffer, and any air bubbles were removed by compressing the pads, e.g. with a 5ml pipette tip.
2. The gel holder cassette was opened by sliding and lifting the latch. Once the gel holder cassette was immersed in blotting buffer, a bubble-free buffer

saturated fibre pad was laid in the centre of the open cassette, followed by a piece of filter paper.

3. A piece of methanol-activated PVDF membrane was then placed on top of the filter paper, followed by the gel, another piece of filter paper and the other saturated fibre pad. Whilst inserting the different layers, care was taken to exclude any possible trapped air bubbles in the blotting sandwich.
4. The cassette was then closed over and fastened. The cassette was held firmly during closure, thereby minimising movement, and subsequent miss-alignment, of the different sandwich layers.
5. The buffer tank was half filled with blotting buffer. An electrode module was then placed vertically into the buffer tank, followed by the insertion of the blotting sandwich into the immersed electrode module. The electrode module is colour coded to distinguish the anode from the cathode. The sandwich was inserted with the membrane facing the red (+) anode portion of the electrode module, whilst the gel faced the black (-) cathode. Thus the negatively charged proteins moved out of the gel, towards the anode, and bound to the PVDF membrane during the blotting procedure.
6. The blotting tank was completely filled with blotting buffer. The lid was then attached to the top of the tank, so that the red anode pin inserted into the red jack on the lid, and the black cathode pin was sheathed by the black jack on the lid.
7. The magnetic stirrer was switched on. The red electrical wire was then plugged into the red socket, and the black electrical wire was plugged into the black socket, in the *Bio-Rad power pac 300.*

8. The power pack was set to 100 volts, at constant voltage, which resulted in a current of 200-330 mA. The transfer proceeded for 2 hours. N.B. The ice was changed every 15 minutes after 1 hour of transfer.

2.7.4 Protein fixing and staining

1. Before fixing, a 10x stock solution of Tris Buffered Saline (TBS) (18.2 g/l Tris base, 87 g/l NaCl, made up to 800 ml, pH to 8.0 and then made up to a litre) was prepared. 500 ml of 1x tween-20 TBS (Tw-TBS) solution were prepared, incorporating 50 ml of 10 x TBS, 0.1 % (0.5 ml) Tween-20 and 1.0% (5g) powdered milk. N.B. The tip of the pipette was trimmed before pipetting the Tween-20.
2. Following the completion of the transfer procedure, the PVDF membranes were trimmed to the exact size of the gel. The gels were retained for immediate staining in a Coomassie Blue R solution.
3. The trimmed PVDF membranes were incubated in glutaraldehyde solution (100 ul of a 25% gluteraldehyde solution in 100ml of deionised water) for 10 minutes. Gluteraldehyde helps to cross-link the bound proteins to the PVDF membrane, therefore minimising protein loss during the stripping procedure (see below).
4. The gluteraldehyde solution was decanted and any excess gluteraldehyde was removed by washing in deionised water for 15 minutes, with frequent solvent changes.
5. To visualise the loading and banding patterns of bound proteins, the PVDF membranes were stained in Ponceau S solution (0.1% Ponceau S (w/v) in 55 acetic acid (v/v)) for 20 seconds. The protein standards of known molecular mass

were marked onto each membrane, using a ballpoint pen. The PVDF membranee were de-stained in Tw-TBS (with several changes).

2.7.5 Blocking the membrane

During Western Blotting, a primary antibody that binds to a specific epitope on the surface of the protein is used to visualise the protein of interest. However, the antibody can also non-specifically bind directly to the PVDF membrane, thereby contributing to the background and masking the signal relating to the protein of interest. Thus the membranes were first incubated in a blocking solution of Tw-TBS incorporating 5% milk powder (i.e. 0.8 g Marvel in 20ml Te-TBS), for at least 30 minutes on a gyro-rocker at 30 rpm, which coats the membrane with proteins such as casein and albumin. It is much less likely that the antibody will bind to the coated or 'blocked' membrane, compared to the non-coated membrane, therefore minimising the background.

2.7.6 Exposure of the membrane to primary and secondary antibodies

1. Commercially available primary antibody solutions, which bind to specific proteins of interest, were diluted in 10-20 ml Tw-TBS, according to the supplier's guidelines.
2. The PVDF membranes were incubated in primary antibody solution, in a large weighing boat, either overnight at 4C, or for 1 hour at room temperature, with gentle agitation on a gyro-rocker (25 rpm).

3. After exposure to the primary antibody, the solution was decanted and frozen for re-use (labelled property, including each date that it was used). The membranes were transferred to a plastic dish, rinsed in Tw-TBS and washed in Tw-TBS for 35 minutes with 6 changes of solution and a gyro-rocker speed of 40 rpm.
4. The PVDF membranes were then incubated in a commercially available secondary antibody, diluted in 10-20 ml Tw-TBS, in a large weighing boat, for 1 hour at room temperature. Secondary antibodies bind to the constant region of the primary antibody, and are usually conjugated to a horseradish peroxidase (HRP) enzyme, which is utilised during the developing step of the procedure. Different types of secondary antibodies are available, which are raised against the constant region of antibodies from one particular animal. Thus, if the primary antibody was raised in a rabbit, the appropriate secondary antibody, which would bind to the primary antibody, should have been raised against rabbit antisera.
5. After exposure to the secondary antibody, the solution was decanted. The membranes were transferred to a plastic dish, rinsed in Tw-TBS, and washed in Tw-TBS for 35 minutes with 6 changes of solution. The water bath was also switched on, to allow it to heat to 80C.

2.7.7 Developing-visualising the protein bands

1. *Kodak GBX developer* and *fixer* solutions were prepared by diluting 103 ml of each stock solution to make 473 ml. Each solution could then be stored in dark containers, or poured directly into plastic trays for immediate use.
2. The membranes were transferred to a weighing boat and incubated 10-20ml of *Pierce Super Signal West Pico Chemiluminescent Substrate* for 5

minutes. The substrate is supplied as two solutions, which are active when combined in a 1:1 ratio. The HRP enzyme converts the substrate into a chemilumiescent product, which can be visualised using *Kodak X-OMAT AR Scientific Imaging Film.*

3. Excess substrate was removed from the membranes by sliding the membranes along the edge of a plastic container. The membranes were then placed face up inside an Amersham Pharmacia Biotech Hypercassette, under a clear plastic sheet.
4. In the dark room, sheets of the imaging film were exposed to the membranes for various durations of time, e.g. 30 seconds, 1 minute, 5 minutes by directly placing the film on top of the plastic covered membranes and closing the hypercassette lid.
5. Immediately following each membrane / film exposure period, each imaging film was placed into the developer solution, using forceps, until dark bands could be easily visualised. N.B. The films were moved around a little to prevent stripes from developing on the films.
6. Each film was immediately rinsed in deionised water and fixed in fixer solution for about 60 seconds. The films were hung up to drip dry.
7. Once dry, the markers on the membrane were drawn into each film.
8. The density of each band was determined using a densitometry package, such as *Bio-Rad Molecular Analyst* Software package.

2.7.8 Stripping of the membrane

To compare the abundance of one protein compared with another, or with a phosphorylated form of the same protein, the membranes can be incubated for a second time in a different primary antibody. Before the second primary antibody

incubation, the first primary antibody must first be stripped from the membrane. This can be achieved using the following procedure.

1. The PVDF membranes were transferred to a plastic dish, containing Tw-TBS, and washed for 5 minutes.
2. A stock of strip buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.8) was prepared.
3. 20 ml of strip buffer were used per membrane. Immediately prior to use, 140 ul of β-marcaptoethenol (β-ME) were added per 20 ml of strip buffer, in a fume hood.
4. The Tw-TBS solution was decanted, and the strip buffer, with β-ME, was poured over each membrane. The plastic containers were sealed and placed incubated in a water bath, set at 80C, for 45 minutes.
5. The membranes were rinsed three times in deionised water and equilibrated in Tw-TBS for 15 minutes, on a gyro-rocker at 35 rpm, with frequent changes of Tw-TBS solution. The antibody incubations were then followed as outlined above. The primary antibody used was anti-actin at 1/1000 concentration, and the secondary was anti-mouse at 1/2000 concentration.

2.7.9 Naphthol Blue Black membrane staining

Once the Western blotting procedures was complete, the proteins bound to the PVDF membranes were visualised to verify the sample loadings, using the Naphthol Blue Black staining system.

1. A Naphthol Blue Black solution (0.1% (w/v) aphthol Blue Black, 10% methanol, 2% acetic acid) was prepared.

2. The membranes were incubated for at least 30 minutes, in a plastic dish containing the staining solution, on a gyro-rocker set at around 30 rpm.

3. The staining solution was decanted and retaind for re-use.

4. The membranes were washed in deionised water for 3-5 minutes on a gyro-rocker set at 30 rpm.

5. To increase the contrast between the protein and the membarne, the membranes were incubated in a de-stain solution (20% (v/v) methanol, 7% acetic acid) for 5-10 minutes, or until the stained protein bands could be visualised clearly, on a gyro-rocker set at 30 rpm. The de-stain removes the stain that is not bound to the protein.

6. The membranes were removed from the de-stain solution and allowed to dry.

2.8 Immunohistochemistry

Three-micron sections of paraffin-embedded placental tissue were deparaffinized in xylene. The avidin-biotin complex method was used for staining using the Vector Elite Kit (PK-6200). A positive and negative tissue control was included in each batch of staining. A placenta from an uncomplicated pregnancy delivered at term was used for this purpose. The negative control was processed identically to the samples, but without the addition of the primary antibody. The positive control is treated in the same way as the samples, in the presence of the primary antibody. These controls allow the specificity of the staining to be monitored so conditions can be altered if necessary and allows for any batch-to-batch differences to be accounted for.

All slides were counter-stained with Mayers Haemalum to facilitate the recognition of tissue structure and hence make it possible to identify the cells or area of interest, i.e the villous trophoblast. Finally, slides were dehydrated in alcohol, cleaned in xylene and mounted in Canada Balsam (VWR international, UK).

An initial optimization study was performed on primary antibodies to achieve optimal specific staining with minimal background staining (DAKO Corporation, 2001). The placental control tissue was used for optimization.

2.9 Statistical analysis

Statistical analysis was used using Microsoft Statistical Package for Social Sciences (SPSS)-10, Chicago, IL, USA. When comparing means, data was considered non-parametric and Mann-Whitney u test was used. Correlations between different variables were performed using Spearman correlation coefficient. A p value < 0.05 was considered significant.

RESULTS

CHAPTER 1

Perinatal outcome and umbilical artery Doppler flow in pregnancies with abnormal 24-week uterine artery Doppler

3.1 Introduction

Uterine artery Doppler examination is used as a screening tool for pregnancy complications related to uteroplacental insufficiency (Harrington et al, 2004). This technique, however, suffers from significant limitations including variable sensitivity and positive predictive value (see chapter 1). In addition, its value as screening tool for uteroplacental complications in multiparous women is debatable (Harrington et al, 2004, Prefumo et al, 2004). Despite these drawbacks, uterine artery Doppler examination remains the one screening modality for preeclampsia and IUGR that has been most-critically evaluated.

In this study, uterine artery Doppler examination at 23-24 weeks was used to screen for the development of preeclampsia and IUGR. These pregnancies were followed until delivery. I compared perinatal outcome in pregnancies that developed preeclampsia before 36 weeks, with pregnancies that developed normotensive IUGR. I also compared umbilical and uterine artery Doppler flow and resistance indices within 24 hours of delivery between the two groups. Umbilical artery Doppler flow reflects placental angiogenic response, and differences in umbilical artery Doppler flow between the two groups might be due to differences in the quality of angiogenesis within placental villi (Ahmed and Kilby, 1997). In addition, I compared uterine artery Doppler flow between the two groups to assess the effect of impaired trophoblastic invasion on the development of the syndrome of preeclampsia (ie are there any differences in uterine artery flow between the two groups (preeclampsia and IUGR) at the time of disease?)

3.2 Methods

In the uterine artery Doppler screening study at 24 weeks, 86 women attended follow up for fortnightly growth scans and uterine artery Doppler flow examination, along with BP checks and urine analysis for protein. Fetal Doppler examination was performed in cases where there was suspicion of growth restriction on ultrasound examination. Using pulsed Doppler, flow velocity waveforms were obtained and resistance indices (RI and PI) were measured in the umbilical artery, middle cerebral artery and fetal aorta (see chapter 2). Doppler of the DV was performed if the IUGR was associated with evidence of fetal compromise by arterial Doppler examination. Doppler examination was then performed three times per week up to the time of delivery. Timing of delivery was decided on clinical grounds, as well as cardiotocographic examination, in conjunction with Doppler ultrasound examination results.

Preeclampsia was defined according to the definition by the ISSHP (see chapter one). IUGR was defined as fetal weight less than the 5^{th} centile for gestational age along with abnormal umbilical artery Doppler examination (see below) or fetal biometry measurements crossing the fetal growth centiles over at least two fetal biometry examinations 10-14 days apart. This excluded any constitutionally (normal) SGA fetuses from the study. Abnormal umbilical artery Doppler was defined as absent or reversed EDF and/or resistance indices (PI and RI) > 95^{th} centile for gestational age (Harrington et al, 1999). Abnormal uterine artery Doppler was defined as persistence of unilateral/bilateral notches with PI and RI > 95^{th} centile for gestational age. Statistics were performed with Microsoft Statistical Package for Social Sciences

(SPSS)-10, Chicago, IL, USA. Data were considered non-parametric and Mann-Whitney *U* test was used for comparisons.

3.3 Results

In the study group, 15 pregnancies delivered before 36 weeks due to pregnancy complications related to uteroplacental insufficiency. Eight women developed preeclampsia as defined by the ISSHP (see chapter 1). The gestational age of the preeclampsia group at delivery ranged between 28-36 weeks (median 32 weeks). Birth weight ranged between 720g-2680g (median 1360g). There were two intrauterine fetal deaths in this group. One was associated with severe IUGR and AREDF in umbilical artery Doppler flow at 28 weeks. The other fetal death was a complication of placental abruption at 30 weeks. The fetus was appropriately grown with a birth weight of 1250g (10^{th} centile). There were no neonatal deaths in this group. Antenatal umbilical artery (UA) Doppler examination 24 hours before delivery revealed positive EDF in all cases except one that had AREDF at 28 weeks and intrauterine fetal death.

Seven other pregnancies in the study group were complicated by normotensive IUGR ($<5^{th}$ centile), which required delivery before 36 weeks for fetal compromise. Four pregnancies had AREDF on umbilical artery Doppler examination 24 hours before delivery. None of these pregnancies had preeclampsia. The gestational age at delivery was not significantly different from the preeclampsia group (range 27-35, median 34 weeks) ($p=0.712$). Birth weight ranged from 500-1728g (median 953g), which was also not statistically different from the preeclampsia group ($p=0.674$). Among this group, there was one fetal death in utero due to severe uteroplacental insufficiency and one neonatal death.

When comparing uterine artery resistance indices between the preeclampsia and the normotensive SGA groups, no significant differences in RI and PI were found at 24 weeks (p=0.92 for PI and 0.71 for RI). Furthermore, uterine artery Doppler examination within 24 hours of delivery in both groups showed persistent bilateral notches and PI and RI > 95th centile in all cases. Again, there were no significant differences in uterine artery resistance indices between the two groups at that time (p=0.61 for PI and 0.52 for RI).

When comparing umbilical artery Doppler flow within 24 hours of delivery in both groups; PI was significantly lower in preeclamptic pregnancies compared to normotensive IUGR [median 1.22, range (0.94-1.40) vs 1.39, (1.25-4.68)]; (p=0.01). Umbilical artery RI was also significantly less in the preeclampsia group compared to IUGR, [median 0.68, range (0.63-0.81) vs 0.83 (0.69-1.00)]; (p=0.007).

Table 3.1 illustrates the demographic characteristics of both groups, table 3.2 illustrates the perinatal outcome and table 3.3 summarizes the uterine and umbilical artery Doppler findings in both groups

Table 3.1 Demographic characteristics of the preeclampsia and the IUGR groups

	Preeclampsia (n=8)	IUGR (n=7)
Race		
Afrocarribean	4	3
White	3	2
Asian	1	2
Maternal age		
Median (range)	27 (19-35)	25 (16-34)
Parity		
Primiparous	7	5
Multiparous	1	2

Table 3.2 Perinatal outcomes in preeclamptic and IUGR pregnancies

	Preeclampsia (n=8)	IUGR (n=7)	P value (Mann-Whitney u test)
GA at delivery* Median (range)	32 (28-36)	34 (27-35) (p=0.712)	0.712
Birth weight Median (range)	1360 (720-2680)g	953 (500-1728)g (p=0.674)	0.674
Fetal death in utero	2	1	
Neonatal death	0	2	

*GA Gestational age

Table 3.3 Uterine and umbilical artery Doppler findings in preeclamptic and IUGR pregnancies

	Preeclampsia (n=8)	IUGR (n=7)	P value (Mann-Whitney U test)
Uterine artery Doppler (24 weeks) Bilateral notches PI RI	 Yes 1.60 (1.22-2.26) 0.73 (0.64-0.84)	 Yes 1.49 (1.19-1.87) 0.72 (0.65-0.78)	 0.35 0.77
Uterine artery Doppler (within 24hr of delivery) Bilateral notches PI RI	 Yes 1..05 (0.98-1.78) 0.67 (0.60-0.81)	 Yes 1.02 (0.96-1.57) 0.66 (0.59-0.80)	 0.61 0.52
Umbilical artery Doppler (within 24hr of delivery) PI	1.22 (0.94-1.4)	1.39 (1.25-4.68)	0.01
Umbilical artery Doppler (within 24hr of delivery) RI	0.68 (0.63-0.81)	0.83 (0.69-1.00)	0.007
Umbilical artery AREDF*	1	4	

*AREDF Absent/reversed EDF

3.4 Discussion

In our study, we used uterine artery Doppler flow examination at 24 weeks to screen for the development of pregnancy complications related to uteroplacental insufficiency, namely preeclampsia and IUGR. The exact pathophysiology underlying the difference in outcome between the two groups (development of preeclampsia compared to normotensive IUGR) is not clear. We compared perinatal outcome, birth weight, umbilical artery Doppler flow and uterine artery Doppler resistance indices in the two groups in search for differences that could point to the underlying mechanism behind this apparent difference. We found no differences in the gestational age at delivery, birth weight or perinatal mortality between the two groups. There were also no significant differences in uterine artery resistance and pulsatility indices between the two groups at 24 weeks and within 24 hours of delivery. This excludes differences in placental invasion between the two groups as a cause for the difference in outcome. Umbilical artery Doppler flow, however, was significantly more favourable in the preeclampsia group, with significantly lower PI and RI and only one case with AREDF in the Doppler flow. This might reflect different placental response in the two groups at the micro-vascular level. Despite the significant difference in umbilical artery Doppler flow examination between the two groups, this was not reflected in differences in perinatal outcome.

In this study only pregnancies delivered before 36 weeks were included to minimize the effect gestational age would have on umbilical artery Doppler examination. All normotensive IUGR pregnancies were delivered before 36 weeks; so only preeclamptic pregnancies that delivered before 36 weeks were included. This could explain the low positive predictive value (PPV) of uterine artery Doppler examination

in predicting preeclampsia in my study (10%), compared to other studies that reported PPV of up to 36% (Chan *et al.*, 1995). Other factors that contribute to this difference is using different cut off points for uterine artery Doppler flow resistance indices and the use of different definitions of preeclampsia.

Villous vascularization during the first and second trimesters is largely determined by sprouting and branching angiogenesis, which leads to the formation of immature villous trees, whose vascular core is characterized by continuously growing networks of fetal capillaries and their supply vessels (Benirschke & Kaufmann, 1995). At the end of the second trimester, there is a switch to non-branching angiogenesis, which forms long, poorly branched terminal capillary loops. As the third trimester progresses, these loops form the capillary network of the mature intermediate and terminal villi responsible for nutrient and gas exchange between mother and fetus (Kaufmann *et al.*, 1988). The net effect of these angiogenic phases is an exponential increase in the volume of fetal capillaries within the placenta, representing about one-quarter of the feto-placental blood volume by term (Luckhardt *et al.*, 1996). This process coincides with the known increase in end-diastolic flow velocities (and a fall in the pulsatility index) in the umbilical artery, suggesting that these capillaries are an important determinant of fetoplacental vascular impedance in the normal placenta (Hendricks *et al.*, 1989).

Todros *et al.* (1999) showed that the characteristics of umbilical artery Doppler flow velocity waveforms in IUGR fetuses indicate angiogenesis within placental stem and gas-exchanging villi. They showed that pregnancies complicated by IUGR in which positive EDF was documented in the umbilical artery before delivery had an adaptive

response in the villous placenta, characterized by enhanced branching angiogenesis. This results in greater numbers of highly branched terminal villi (Kingdom & Kaufmann, 1997) and compensates, at least in part, for the underlying impairment of uteroplacental blood flow indicated by abnormal uterine artery Doppler findings. By contrast, they showed that the pattern of villous vascularization in cases of IUGR complicated by absent or reversed EDF suggested that this adaptive process failed to occur (Macara *et al.,* 1996; Krebs *et al.,* 1996), which might be due to an apparent lack of placental villous regulation of vascular growth through VEGF and its receptors (Todros *et al.,* 1999). They suggested that hypovascularization of stem villi or reduced peripheral villous branching in placentas from fetuses with IUGR and AREDF could result from abnormal differentiation of capillary networks in immature intermediate villi during early gestation, rather than some obliterative process in the third trimester. If the former concept is true, the placental origin of IUGR might begin much earlier in gestation than previously thought.

Roberts & Cooper (2001) suggested that the systemic syndrome of preeclampsia is a result of two-stage process. The first starts at the level of the placenta with placental hypoxia being the main factor involved. The second stage involves the release of cytokines from the placenta to the systemic circulation that triggers the systemic manifestations of preeclampsia. According to this theory, placental hypoxia (due to a variety of conditions) seems to be a key factor in the development of preeclampsia. In this study, I demonstrated that umbilical artery Doppler waveforms from preeclamptic pregnancies are different from the normotensive IUGR ones, with significantly lower resistance indices and more favorable blood flow in the preeclamptic group. This difference in umbilical artery flow could affect the level of placental oxygen in these

pregnancies, and in turn affect the maternal systemic response to placental-derived factors in the hypoxic group. In other words, pregnancies where umbilical artery blood flow is preserved with lower PI and RI tend to have hypoxic placentas (Kingdom & Kaufmann, 1997). This might explain the difference in outcome (development of preeclampsia) in these two groups.

It is possible that women in preeclampsia were delivered on clinical grounds for maternal reasons before serious deterioration in fetal condition and umbilical artery Doppler flow. Hence, the lower umbilical artery Doppler flow resistance indices and more favourable flow pattern (one case with AREDF) in the preeclampsia group, compared to four in the IUGR group. Larger studies are needed to verify the differences between the two groups. In the next chapter, we assess differences in placental angiogenic factors both in the serum and placenta in the two groups compared to controls.

CHAPTER 2

Serum and placental Flt1 and PlGF in high-risk women with abnormal 24-week uterine artery Doppler that develop preeclampsia and normotensive IUGR.

4.1 Introduction

Preeclampsia is associated with defective uteroplacental vascularization, along with impaired angiogenesis and vascular transformation of the uteroplacental unit. Vascular Endothelial Growth Factor-A (VEGF-A) is one of the most important growth factors for endothelium, as it induces angiogenesis and endothelial cell proliferation (see chapter 1). Placental Growth Factor (PlGF) is a member of the VEGF family closely related to VEGF-A. The two main receptors for VEGF-A and PlGF, namely VEGFR-1 (Flt-1) and VEGFR-2 (KDR), are essential for the development of embryonic vasculature (Fong *et al.,* 1995). Flt-1 is expressed by trophoblast cells and thought to play a physiological function during pregnancy (Charnock-Jones *et al.,* 1994). A soluble form of VEGFR-1 (sFlt-1) can be detected in peripheral blood. Soluble Flt-1 has a strong antagonistic activity and neutralizes the effects mediated by VEGF and PlGF effects (Kendall & Thomas, 1993; Hornig *et al.,* 2000; Yamaguchi *et al.,* 2002). Both villous and extravillous trophoblastic cells produce sFlt-1 (Clark *et al.,* 1998). Recent evidence suggests that placental dysregulation of sFlt1 and PlGF could be implicated in the pathogenesis of preeclampsia through disordered angiogenesis in the trophoblast and hence abnormal trophoblast invasion (Maynard *et al.,* 2003; Ahmad & Ahmed, 2004)

In the last study, I demonstrated significant differences in umbilical artery blood flow within 24 hours of delivery between preeclamptic and normotensive IUGR pregnancies in women with abnormal uterine artery Doppler. This could be due to differences in placental angiogenesis between the two groups. In this chapter, placental Flt1 and PlGF and their soluble serum forms are assessed in cases of abnormal uterine artery Doppler that develop preeclampsia and normotensive IUGR.

4.2 Methods

553 women were examined using uterine artery Doppler at 24 weeks. Among these, there were 395 primiparous and 158 multiparous women. All primigravid women and multiparous women with previous pregnancy complication (see materials and methods) attending for routine anomaly scan at 20 weeks were offered uterine artery Doppler examination at 24 weeks. Among women who underwent uterine artery Doppler flow examination, 97 women had an abnormal result (see materials and methods). 63 women were primiparous and 34 were multiparous. Eleven women did not attend for follow up (dropped out). The remaining 86 (56 primiparous and 30 multiparous) women were enrolled in the study after obtaining appropriate consent and giving them information leaflets. Blood was collected from the 86 women with abnormal 24-week uterine artery Doppler examination during the same visit, after obtaining appropriate consent; serum was stored at -80C for further assay (see below).

Women enrolled in the study were scanned at fortnightly intervals for growth, uterine artery Doppler and fetal Doppler examination (if there is evidence of slowing of fetal growth). These pregnancies were followed for the development of preeclampsia and IUGR. Eight women developed preeclampsia at gestations less than 36 weeks, seven women developed normotensive IUGR; four of these had evidence of AREDF in umbilical artery Doppler examination, compared to one in the preeclampsia group. For details of perinatal outcome, see chapter one.

Blood was collected from these women within 24 hours of delivery when umbilical and uterine artery Doppler examination was performed. Serum was stored at -80C (see materials and methods) for further assay. The control group comprised 16 women

from the study group with normal pregnancy outcome that delivered at term. Blood was collected from these pregnancies at 24 weeks (at the time of uterine artery Doppler examination) and at gestations compared to the time of delivery of the abnormal outcome group (27-36 weeks). Serum sFlt1 and PlGF were measured in the three groups at 24 weeks and the time of the disease by *Enzyme Linked Immune Sorbent Assay (ELISA)*

Serum sFlt-1 ELISA

Reagent preparation

All reagents were brought to room temperature before use.

Wash Buffer - 20 mL of Wash Buffer Concentrate were diluted into distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B were mixed together in equal volumes within 15 minutes of use and protected from light. 200 uL of the resultant mixture is required per well.

VEGF R1 Standard - The VEGF R1 Standard was reconstituted with 1.0 mL of distilled water. This reconstitution produced a stock solution of 20 ng/mL. The standard were mixed to ensure complete reconstitution and allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

900 uL of Calibrator Diluent RD6Z were pipetted into the 2000 pg/mL tube. 500 uL of Calibrator Diluent RD6Z were pipetted into the remaining tubes. The stock solution was used to produce a dilution series (seven dilutions were made). Each tube was thoroughly mixed before the next transfer. The 2000 pg/mL standard serves as the high standard. Calibrator Diluent RD6Z serves as the zero standard (0 pg/mL).

Assay procedure

All reagents and samples were brought to room temperature before use. All samples, controls and standards were assayed in duplicate.

1. All reagents, working standards and samples were prepared as directed in the previous sections.

2. 100 uL of Assay Diluent RD1-71 were added to each well.

3. 100 uL of Standard, control or sample* were added per well and covered with the adhesive strip provided. The wells were then incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 rpm 50 rpm.

4. Each well was aspirated and washed, repeating the process three times for a total of four washes using 400ul of Wash Buffer.

5. 200 uL of VEGF R1 Conjugate were added to each well and covered with a new adhesive strip. The wells were then incubated for 2 hours at room temperature on the shaker.

6. The aspiration/wash was repeated as in step 4.

8. 200 uL of Substrate Solution were added to each well and incubated for 30 minutes at room temperature on the bench top and protected from light.

9. 50 uL of Stop Solution were added to each well. If color change did not appear uniform, the plate was gently tapped to ensure thorough mixing.

10. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm.

Serum PlGF ELISA

Reagent preparation

All reagents were brought to room temperature before use.

Wash Buffer - 20 mL of Wash Buffer Concentrate were diluted into distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B were mixed together in equal volumes within 15 minutes of use and protected from light. 200 uL of the resultant mixture is required per well.

PlGF Standard - The PlGF Standard was reconstituted with 1.0 mL of Calibrator Diluent RD6-11. This reconstitution produced a stock solution of 1000 pg/mL. The standard were mixed to ensure complete reconstitution and allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

500 uL of Calibrator Diluent RD6-11 were pipetted into each tube. The stock solution was used to produce a dilution series (seven dilutions were made). Each tube was thoroughly mixed before the next transfer. The 1000 pg/mL standard serves as the high standard. Calibrator Diluent RD6-11 serves as the zero standard (0 pg/mL).

Assay procedure

All reagents and samples were brought to room temperature before use. All samples controls and standards were assayed in duplicate.

1. All reagents, working standards and samples were prepared as directed in the previous sections.

2. 100 uL of Assay Diluent RD1-22 were added to each well.

3. 100 uL of Standard, control or sample* were added per well and covered with the adhesive strip provided. The wells were then incubated for 2 hours at room temperature.

4. Each well was aspirated and washed, repeating the process three times for a total of four washes using 400ul of Wash Buffer.

5. 200 uL of PlGF Conjugate were added to each well and covered with a new adhesive strip. The wells were then incubated for 2 hours at room temperatur.

6. The aspiration/wash was repeated as in step 4.

8. 200 uL of Substrate Solution were added to each well and incubated for 30 minutes at room temperature on the bench top and protected from light.

9. 50 uL of Stop Solution were added to each well. If color change did not appear uniform, the plate was gently tapped to ensure thorough mixing.

10. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm.

Western blotting

Western blotting was used to study placental expression of Flt1 and PlGF using the principles described in chapter two. Four placental samples from pregnancies complicated by preeclampsia (<36 weeks) with preserved EDF in umbilical artery Doppler, four samples from normotensive IUGR with AREDF in umbilical artery Doppler and four gestational age matched controls (28-32 weeks) were studied. All disease samples had abnormal midtrimester uterine artery Doppler at 24 weeks and at the time of delivery while the controls had normally growing fetus with normal umbilical and uterine artery Doppler. All cases and controls were delivered by Caesarean Section.

Supernatants of homogenized placental samples were thawed in ice, then heated for 10 minutes at 90 degrees. Kleidoscope and marker samples were thawed in ice and heated for one minutes at 40C and 90C respectively. All samples were centrifuged at 13G for 40 seconds, then 50 micrograms of each sample was loaded to the wells in the

prepared gel. Using running buffer, separating electrophoresis was started at 60 Amperes for 10 minutes then at 80 Amperes for 40 minutes, after which the stacking gel was removed. The gels were subsequently incubated for 30 minutes in transfer buffer. Protein was transferred from the gel to blotting membrane with blot buffer and two hours were allowed for protein transfer at 100V under cooling conditions using ice. Membranes were then incubated overnight in blocking solution in a cold room.

Membranes were then incubated in primary antibody solution (Flt1 and PlGF, R&D systems, Abingdon, UK) at a concentration of 1:500 ug/L for two hours in the shaker. Extensive washing was then performed for 30 minutes followed by secondary antibody incubation (anti mouse IgG, heavy and light chain specific peroxidase conjugate, Calbiochem, USA) at a concentration of 1/2000 for one hour. Membranes were then extensively washed for 30 minutes, after which they were incubated for five minutes in ECL solution (equal volumes of Supersignal West Pico stable peroxide solution and Supersignal West Pico Luminol enhancer solution (Piercenet, Rockford, IL, USA). Films were then developed in a dark room. Protein stripping was then performed and actin expression in the three groups was assessed according to the technique in chapter two. The intensity of staining for the studied protein and actin was assessed using *Bio-Rad Molecular Analyst* Software package densometer. The ratio between the studied protein and actin expression was assessed. Differences in protein expression ratios between the three groups were assessed using Mann-Whitney *U* test.

4.3 Results

Serial evaluation of serum sFlt1 and PlGF

Serum sFlt1 levels were significantly higher at 24 weeks in women who developed preeclampsia (n=8), compared to the normal outcome group (n=16). The median of sFlt1 in the preeclampsia group was 2848 (range: 1022-5243) pg/ml compared to 1145 (range: 297-2139) pg/ml in the normal group; (p=0.001). Furthermore, the median for PlGF was significantly lower in the preeclampsia group at 24 weeks, compared to those with a normal outcome [188 (range: 50-315) versus 532 (range: 203 -1129) pg/ml; p=0.001].

Serum sFlt1 levels were also elevated at 24 weeks in pregnancies with abnormal uterine artery Doppler, which subsequently developed normotensive IUGR (n=7). The median (range) for the IUGR group was 2846 (934-3624) pg/mL compared to controls with a normal outcome (n=16); [median 1145 (range: 297-2139) pg/ml]; p=0.003. On the other hand, serum PlGF levels were significantly lower in the IUGR group [median 292., range (50-820) pg/mL] compared to the control group (see above); p=0.019. At 24 weeks, there were no significant differences in serum sFlt1 (p=0.345) and PlGF (p=0.372) levels between women who subsequently developed preeclampsia and those who developed normotensive IUGR.

The ratio between sFlt1 and PlGF (sFlt1/PlGF) was also assessed in the three groups. Women who subsequently developed preeclampsia or normotensive IUGR had significantly higher sFlt1/PlGF ratios compared to normal controls. There were no significant differences between the preeclampsia and IUGR groups at 24 weeks. Using sFlt1/PlGF ratio did not add a significant advantage over the use of sFlt1 alone

in predicting preeclampsia or IUGR. Table 4.1-4.3 summarizes the differences between serum level of sFlt1 and PlGF at 24 weeks in women who subsequently developed preeclampsia, normotensive IUGR and controls. Figures 4.1-4.3 illustrate the differences in PlGF and sFlt1 and sFlt1/PlGF ratio between the three groups at 24 weeks.

When following serum sFlt1 and PlGF levels longitudinally, there were no significant changes in serum sFlt1 and PlGF in the control group with advancing gestation. There was, however, a several fold increase in sFlt1 levels in cases of preeclampsia (n=8) at the time of the disease (in the 24 hours before delivery) [median 41,425 (range: 5000-300,000) pg/ml] compared to gestational age matched controls (n=16) [median 1295 (range: 293-5657) pg/ml)]; ($p<0.001$). Similar to the control group, PlGF concentrations did not change significantly with gestation in women with preeclampsia. Serum PlGF, however, was significantly lower in the preeclampsia group at the time of the disease (in the 24 hours before delivery) [median 287.5 (range: 50-425) pg/mL] compared with gestational age matched controls [median 867 (range: 114-6014) pg/mL] $p=0.02$.

In the IUGR group, there were no significant changes in sFlt1 levels [median 3500 (range 2255-5243) pg/mL] ($p=0.63$) and PlGF levels [median 200 (range 50-337) pg/mL] ($p=0.37$) at the time of disease (in the 24 hours before delivery) compared to levels at 24 weeks. When comparing serum sFlt1 levels in IUGR pregnancies to preeclamptic and control pregnancies at the time of the disease, serum sFlt1 was significantly elevated in IUGR compared to controls ($p=0.006$), and significantly lower than that in preeclamptic pregnancies ($p=0.002$). In addition, there were

significant changes in PlGF in the IUGR group compared to gestational age matched controls. PlGF was significantly lower in IUGR compared to controls (p=0.005), but was not different from preeclamptic pregnancies (p=0.118). When comparing sFlt1/PlGF ratio in the three groups, preeclamptic pregnancies had significantly higher ratios compared to IUGR (p=0.021) and control pregnancies (p<0.001). The ratios in IUGR pregnancies were also significantly higher than controls (p=0.001).

Tables 4.4-4.6 illustrate sFlt1 and PlGF levels in preeclamptic, normotensive IUGR and control pregnancies at the time of the disease (within 24 hours of delivery in the preeclampsia and IUGR groups). Figures 4.4 and 4.5 illustrate the differences in sFlt1 between the three groups. Figure 4.6 illustrates differences in PlGF between the three groups. Figures 4.7 and 4.8 illustrate differences in sFlt1/PlGF ratios between the three groups at the time of the disease.

Table 4.1 Differences in sFlt1 and PlGF at 24 weeks in women with abnormal uterine artery Doppler who subsequently developed preeclampsia and normal controls.

	Preeclampsia (n=8) Median (range)	Controls (n=16) Median (range)	p
sFlt1	3168 (1022-5243)	1145 (297-2139)	0.001
PlGF	188 (50-315)	532 (203-1129)	0.001
SFlt1/PlGF ratio	18.57 (5.50-60.04)	1.467 (0.88-9.58)	0.001

Table 4.2 Differences in sFlt1 and PlGF at 24 weeks in women with abnormal uterine artery Doppler who subsequently developed preeclampsia and normotensive IUGR.

	Preeclampsia (n=8) Median (range)	IUGR (n=7) Median (range)	P
sFlt1	3168 (1022-5243)	2846 (934-3624)	0.345
PlGF	180 (50-315)	292 (50-820)	0.372
sFlt1/PlGF	18.57 (5.50-60.04)	11.15 (3.49-93.40)	0.563

Table 4.3 Differences in sFlt1 and PlGF at 24 weeks in women with abnormal uterine artery Doppler who subsequently developed normotensive IUGR and control pregnancies.

	IUGR (n=7) Median (range)	Controls (n=16) Median (range)	P
sFlt1	2846 (934-3624)	1145 (297-2139)	0.003
PlGF	292 (50-820)	532 (203-1129)	0.019
sFlt1/PlGF	11.15 (3.49-93.40)	1.467 (0.88-9.58)	0.002

Figure 4.1 Box and plot comparison between serum sFlt1 at 24 weeks in women with abnormal uterine artery Doppler who subsequently developed normotensive IUGR (n=7), preeclampsia (n=8), and normal outcome (n=16). The preeclampsia and IUGR groups have significantly higher sFlt1 levels at 24 weeks compared to the normal outcome (control) group. Boxes represent interquartile range, where the line represents the median. Whiskers at top and bottom represent the highest and lowest values.

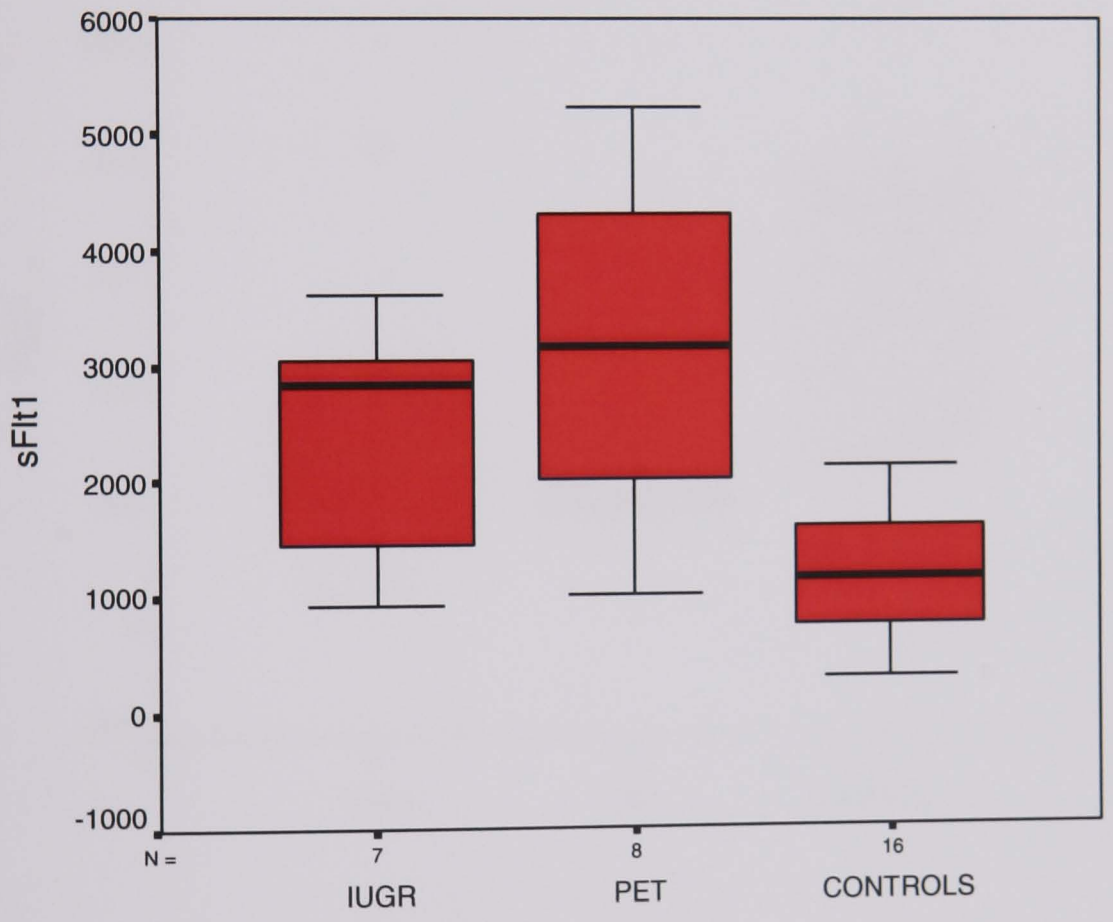


Figure 4.2 Box and plot comparison between serum PlGF at 24 weeks in women with abnormal uterine artery Doppler who subsequently developed normotensive IUGR (n=7), preeclampsia (n=8), and normal outcome (n=16). The preeclampsia and IUGR groups have significantly lower PlGF levels at 24 weeks compared to the normal outcome (control) group. Boxes represent interquartile range, where the line represents the median. Whiskers at top and bottom represent the highest and lowest values.

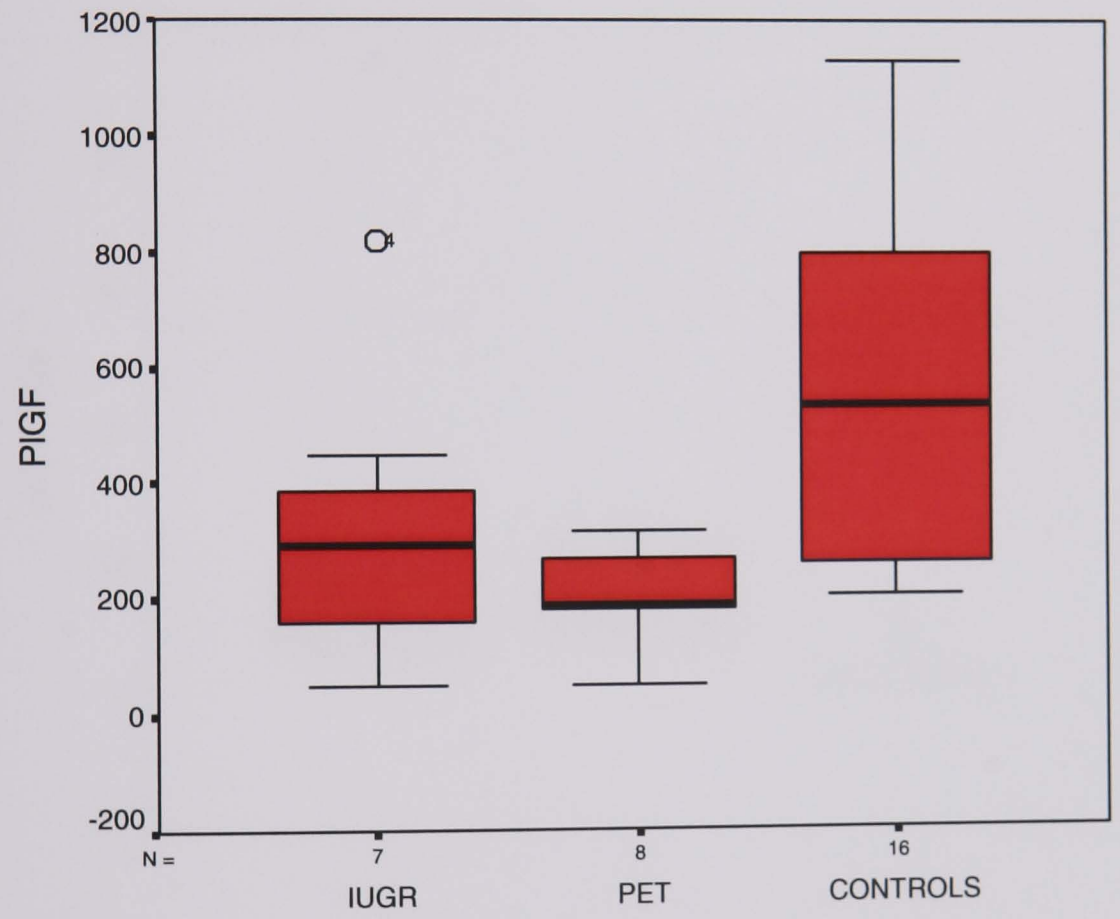


Figure 4.3 Box and plot comparison between serum sFlt1/PlGF ratio at 24 weeks in women with abnormal uterine artery Doppler who subsequently developed normotensive IUGR (n=7), preeclampsia (n=8), and normal outcome (n=16). The preeclampsia and IUGR groups have significantly higher sFlt1/PlGF ratios at 24 weeks compared to the normal outcome (control) group. Boxes represent interquartile range, where the line represents the median. Whiskers at top and bottom represent the highest and lowest values.

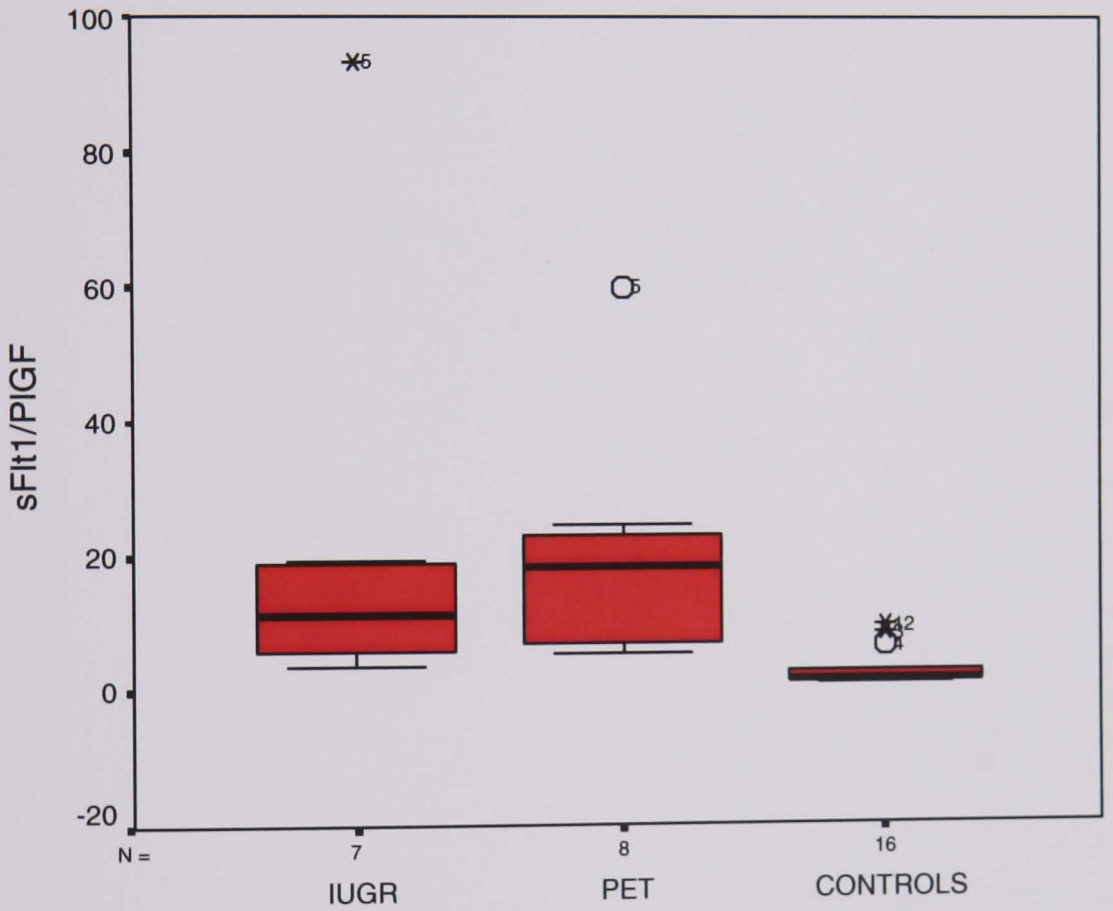


Table 4.4 Differences in sFlt1 and PlGF within 24 hours of delivery in women with abnormal uterine artery Doppler who developed preeclampsia and gestational age matched controls.

	Preeclampsia (n=8) Median (range)	Controls (n=16) Median (range)	p
sFlt1	41,425 (5,000-300,000)	1295 (293-5,657)	<0.001
PlGF	287.5 (50-425)	867 (114-6,014)	0.02
sFlt1/PlGF ratio	138.1 (72.66-1127.8)	1.344 (0.05-14.02)	<0.001

Table 4.5 Differences in sFlt1 and PlGF within 24 hours of delivery in women with abnormal uterine artery Doppler who subsequently developed preeclampsia and normotensive IUGR.

	Preeclampsia (n=8) Median (range)	IUGR (n=7) Median (range)	P
sFlt1	41,425 (5,000-300,000)	3500 (2255-5243)	0.002
PlGF	287.5 (50-425)	200 (50-337)	0.118
sFlt1/PlGF	138.1 (72.66-1127.8)	11.15 (3.49-93.40)	0.021

Table 4.6 Differences in sFlt1 and PlGF within 24 hours of delivery in women with abnormal artery Doppler who developed normotensive IUGR and gestational age matched control pregnancies.

	IUGR (n=7) Median (range)	Controls (n=16) Median (range)	P
sFlt1	3500 (2255-5243)	1295 (293-5,657)	0.006
PlGF	200 (50-337)	867 (114-6,014)	0.005
sFlt1/PlGF	11.15 (3.49-93.40)	1.344 (0.05-14.02)	0.001

Figure 4.4 Box and plot comparison between serum sFlt1 at the time of the disease in women with abnormal uterine artery Doppler who developed normotensive IUGR (n=7), preeclampsia (n=8), and normal outcome (n=16). The preeclampsia and IUGR groups have significantly higher sFlt1 levels compared to the normal outcome (control) group. Boxes represent interquartile range, where the line represents the median. Lines at top and bottom represent the highest and lowest values.

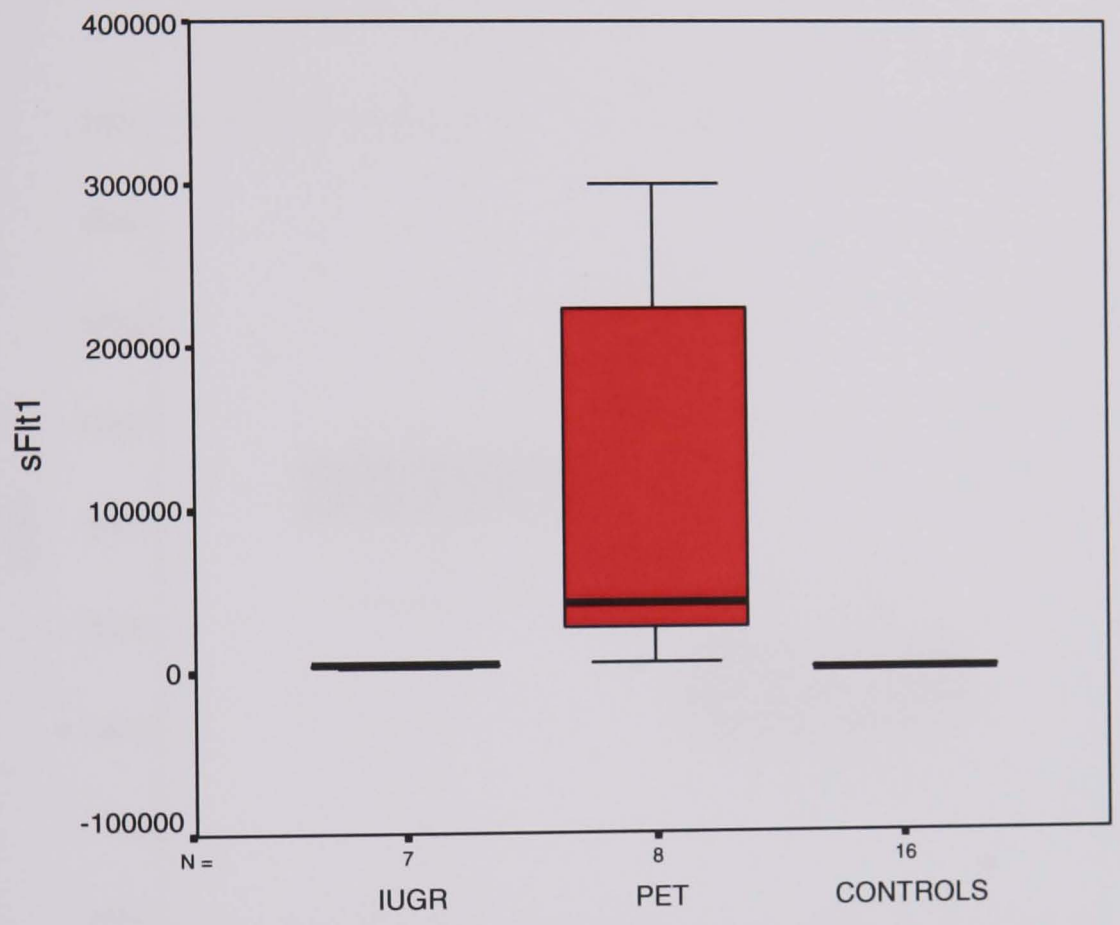


Figure 4.5 Box and plot comparison between serum sFlt1 at the time of the disease in women with abnormal uterine artery Doppler who developed normotensive IUGR (n=7), and gestational age matched controls (n=16). The IUGR group have significantly higher sFlt1 levels compared to the normal outcome group. Boxes represent interquartile range, where the line represents the median. Lines at top and bottom represent the highest and lowest values.

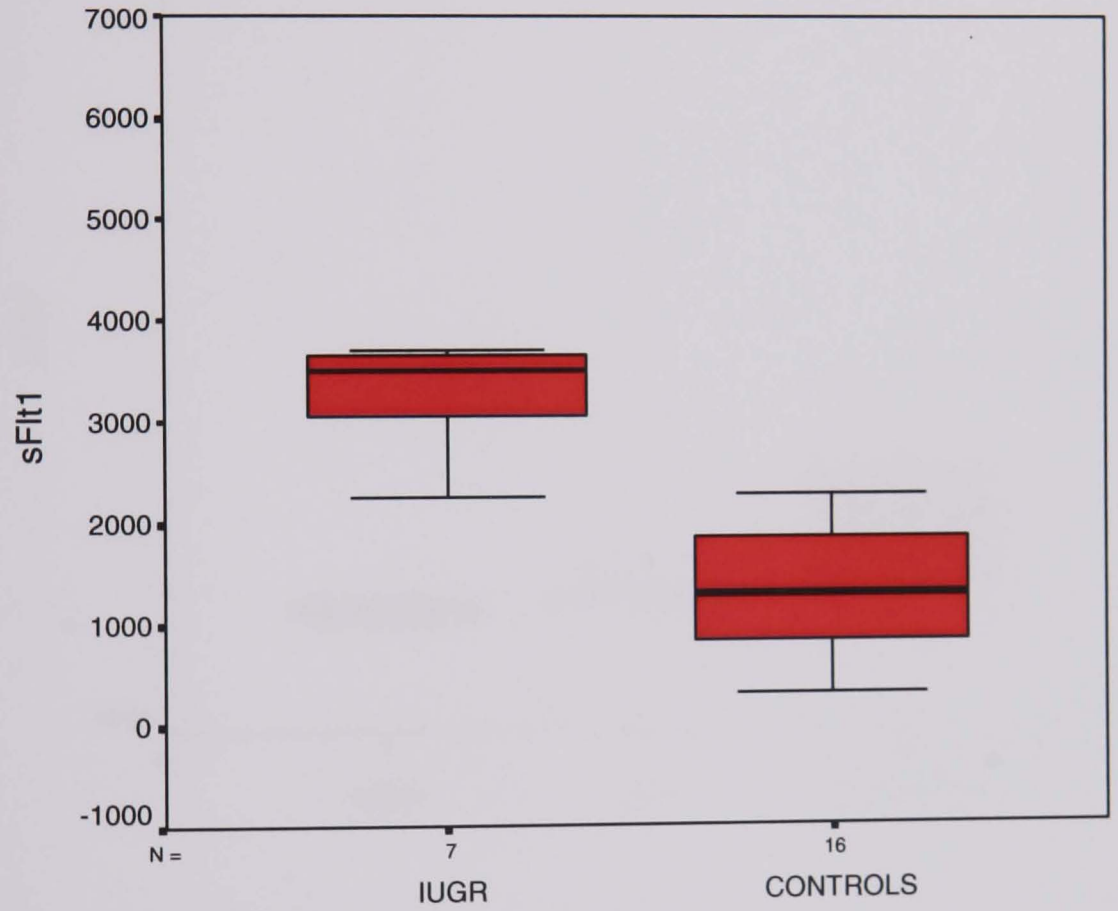


Figure 4.6 Box and plot comparison between serum PlGF at the time of the disease in women with abnormal uterine artery Doppler who developed normotensive IUGR (n=7), preeclampsia (n=8), and normal outcome (n=16). The preeclampsia and IUGR groups have significantly lower PlGF levels compared to the normal outcome (control) group. Boxes represent interquartile range, where the line represents the median. Lines at top and bottom represent the highest and lowest values.

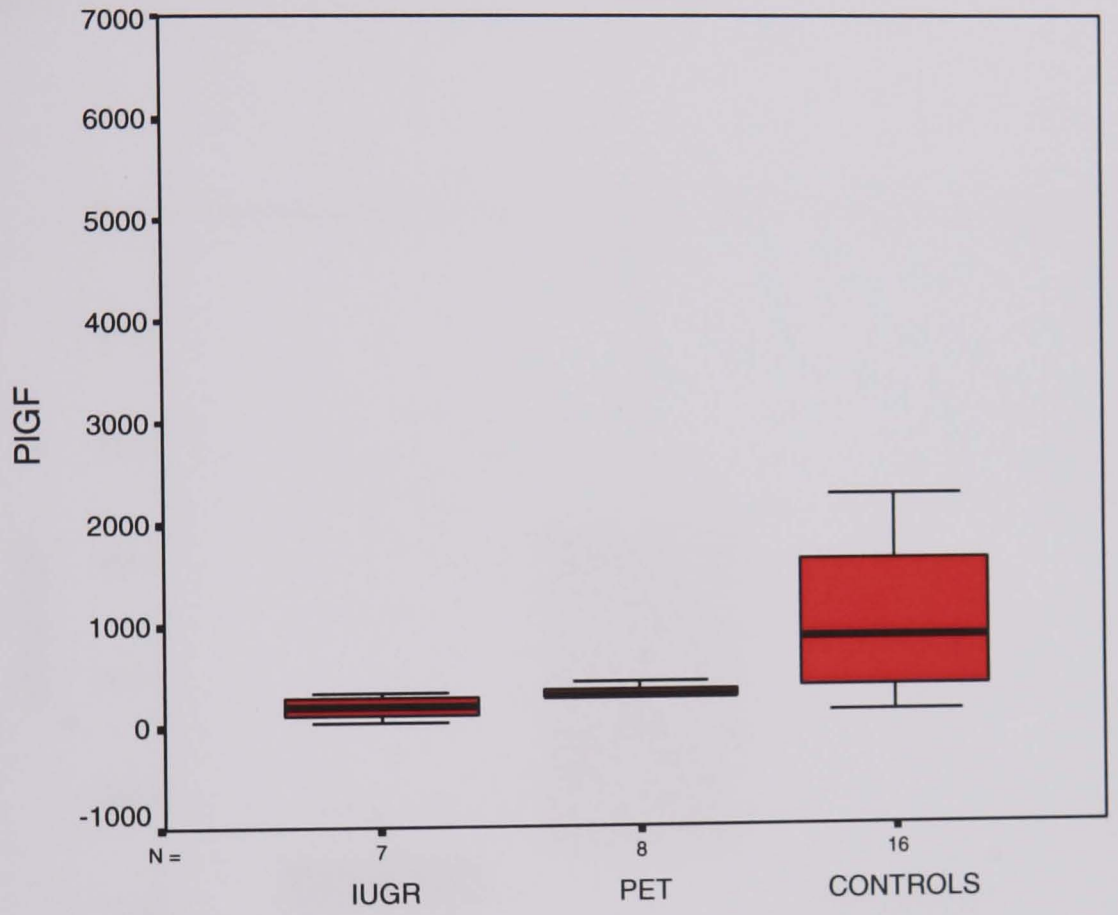


Figure 4.7 Box and plot comparison between serum sFlt1/PlGF ratios at the time of the disease in women with abnormal uterine artery Doppler who developed normotensive IUGR (n=7), preeclampsia (n=8), and normal outcome (n=16). The preeclampsia and IUGR groups have significantly higher sFlt1/PlGF ratios compared to the normal outcome (control) group. Boxes represent interquartile range, where the line represents the median. Lines at top and bottom represent the highest and lowest values.

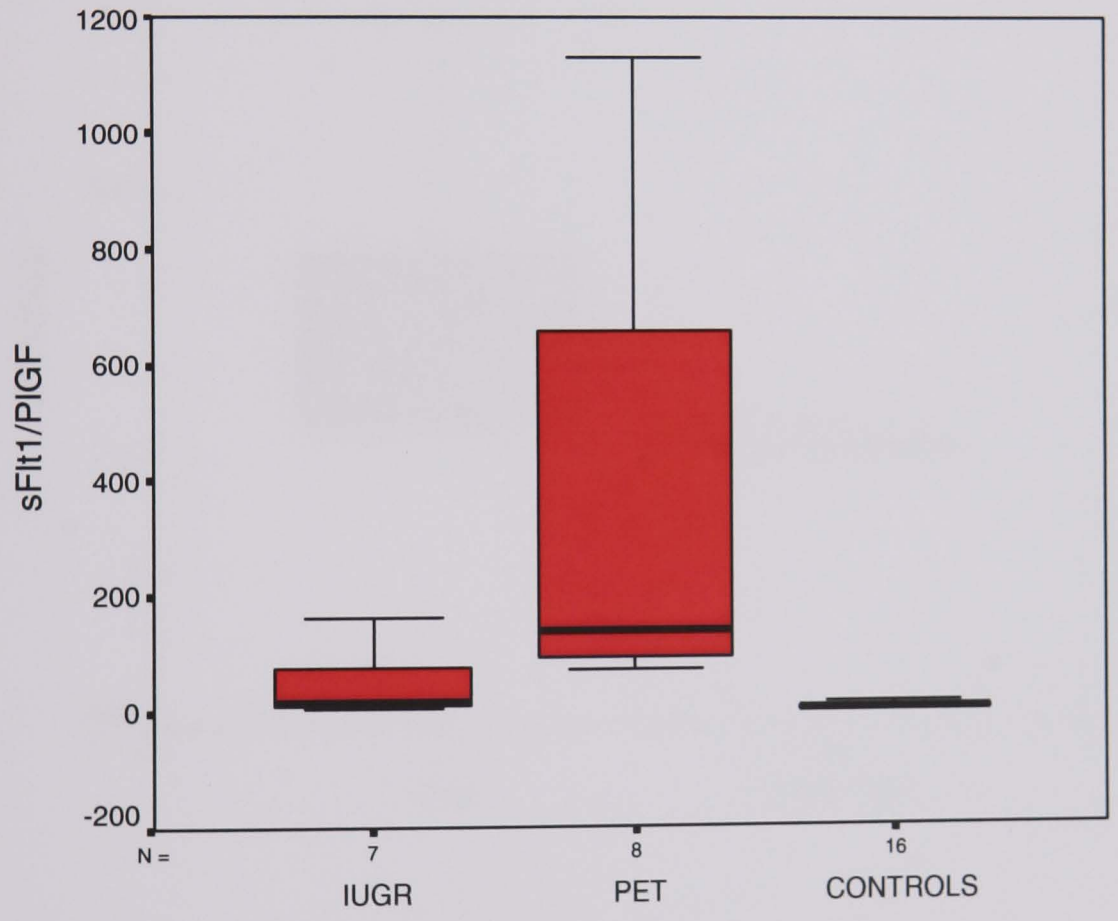
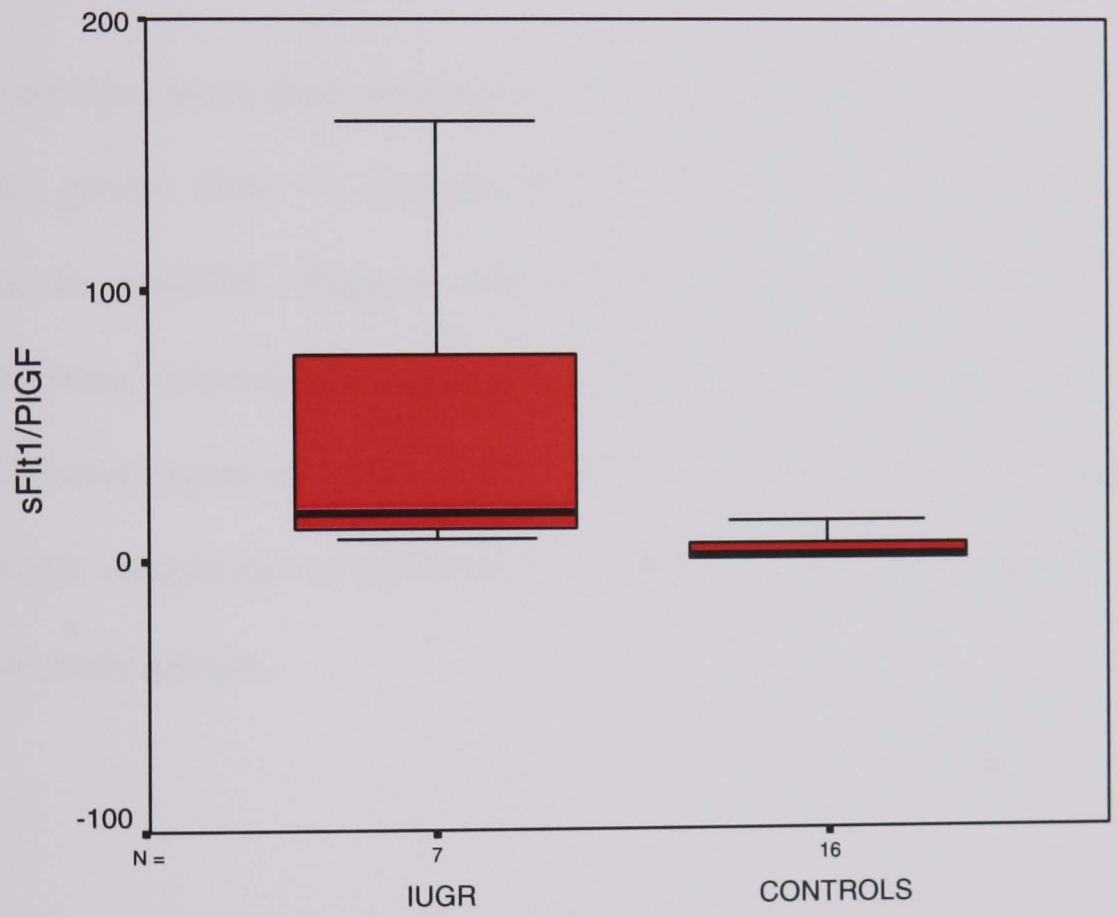


Figure 4.8 Box and plot comparison between serum sFlt1/PlGF at the time of the disease in women with abnormal uterine artery Doppler who developed normotensive IUGR (n=7), and normal outcome (n=16). The IUGR groups have significantly higher sFlt1 levels compared to the normal outcome (control) group. Boxes represent interquartile range, where the line represents the median. Lines at top and bottom represent the highest and lowest values.



Placental expression of sFlt1 and PlGF

Placental expression of PlGF and Flt1 in the three groups was detected using western blotting at the corresponding molecular weight of 50 kDa and 180 KDa, respectively. There was a significant increase in placental expression of Flt1 in preeclampsia compared to IUGR placentas (p=0.021) and controls (p=0.043). There were no differences in placental expression of Flt1 between IUGR and control placentas (p=0.773). Figure 4.9 illustrates placental Flt1 expression in the study groups.

On the other hand, there were no differences in placental PlGF expression in the three study groups (PET vs controls; p=0.773), (PET vs IUGR; p=0.149), (IUGR vs controls; p=0.386). Figures 4.10 and 4.11 illustrate trophoblast PlGF and actin expression, respectively in the study groups. Table 4.7 summarises Flt1/actin and PlGF/actin expression ratios in the studied placentas. Figures 4.12 and 4.13 are a box and plot comparison of placental Flt1 and PlGF expression, respectively between the three study groups.

Figure 4.9 Placental expression of Flt1 in pregnancies complicated by normotensive IUGR (I) (right four lanes), preeclampsia (P) (middle four lanes) and gestational age matched controls (C) (left four lanes).

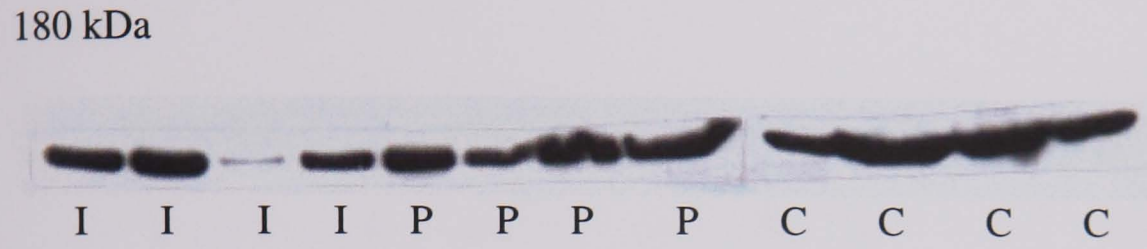


Figure 4.10 Placental expression of PlGF in IUGR (I) preeclampsia (P) and controls (C) pregnancies. No differences were found in placental PlGF expression was found between the three groups.

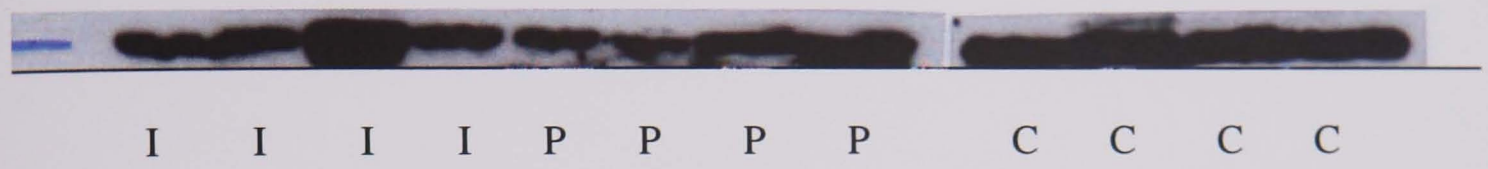


Figure 4.11 Actin expression in the groups studied; I: IUGR pregnancies, P: preeclampsia, C: control pregnancies.

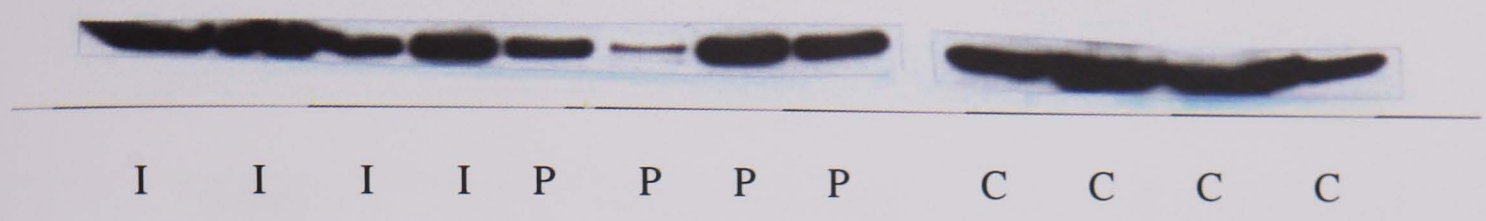


Table 4.7 Flt1/Actin and PlGF/Actin ratios in western blotting from placentas in the three study groups.

	Flt1 Median (Range)	PlGF Median (Range)
IUGR	0.752 (0.347-0.889)	1.266 (0.826-5.893)
PET	1.498 (0.904-28.618)	2.669 (1.780-20.783)
CONTROLS	0.83 (0.642-0.953)	2.365 (1.427-4.34)

Figure 4.12 Box and plot comparison between placental Flt1 expression in normotensive IUGR (n=4), preeclampsia (PET) (n=4) and controls (n=4). Preeclamptic placentas have significantly higher Flt1 expression compared to the IUGR (p=0.021) and control groups (p=0.043). No differences were found between the IUGR and control groups (p=0.773). Boxes represent interquartile range, where the line represents the median. Lines at top and bottom represent the highest and lowest values.

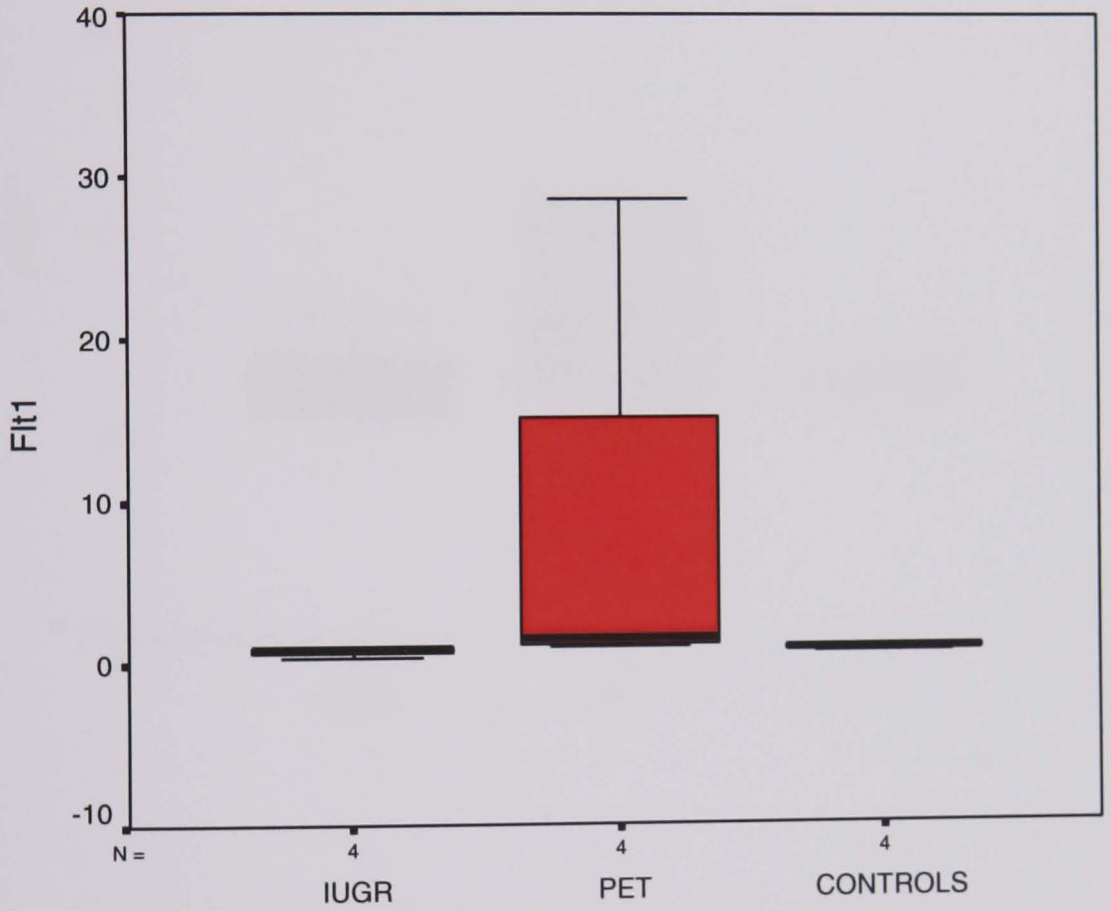
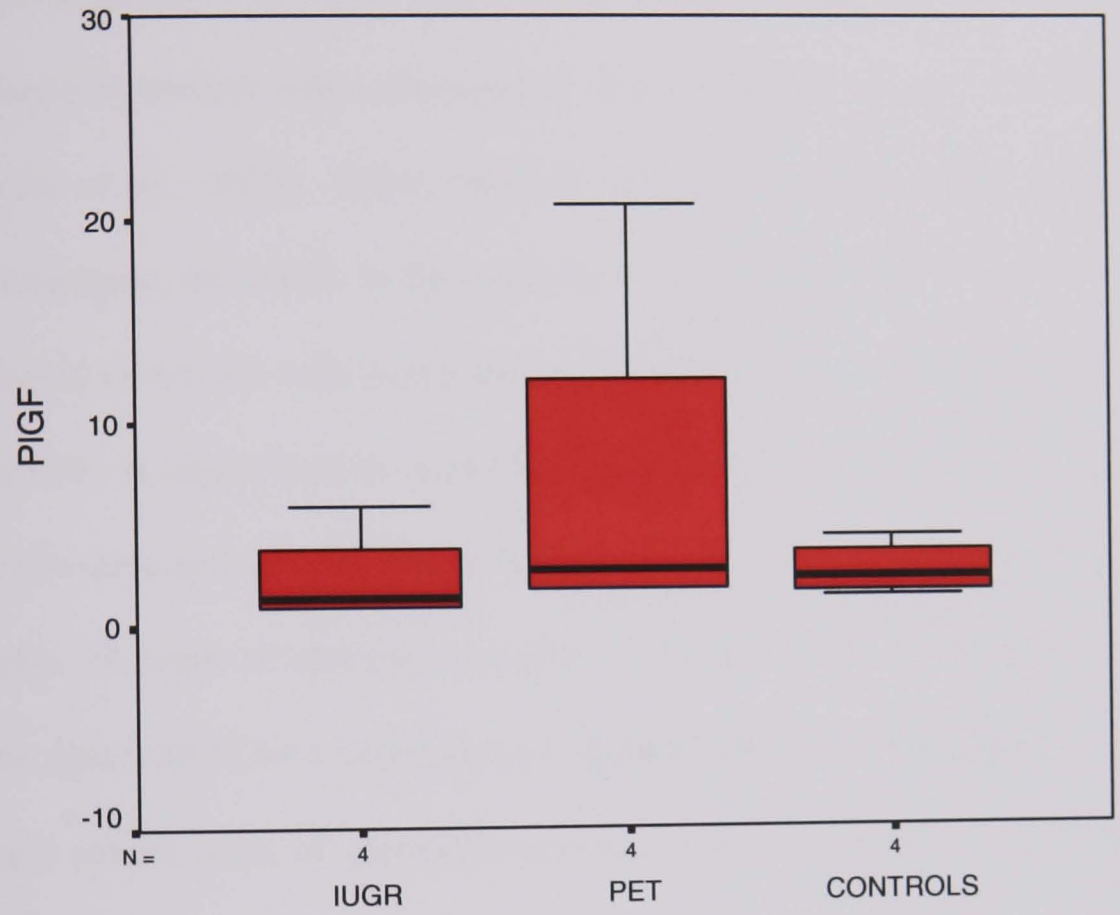


Figure 4.13 Box and plot comparison between placental PlGF expression in normotensive IUGR (n=4), preeclampsia (PET) (n=4) and controls (n=4). No differences were found in placental PlGF expression between the three groups. Boxes represent interquartile range, where the line represents the median. Lines at top and bottom represent the highest and lowest values.



4.4 Discussion

My observation of raised sFlt1 serum concentrations in established pre-eclampsia confirms and extend the recent work of Maynard *et al* (2003) and Koga *et al* (2003). I report here that concentrations of sFlt1 increased in the second trimester, before evidence of clinical disease and that these are accompanied by a fall in PlGF concentrations. The latter observation confirms studies that showed PlGF to be reduced in women who subsequently develop pre-eclampsia (Chappell *et al.*, 2002a; Taylor *et al.*, 2003). These markers of the angiogenic pathway may, alone or in combination, be useful in the prediction of the disease. In this study, sFlt1 was also elevated in women with normotensive IUGR from as early as 24 weeks. This suggests that sFlt1 is implicated in impaired trophoblast invasion in these pregnancies (as they all had abnormal uterine artery Doppler at 24 weeks). Serum sFlt1 was also elevated within 24 hours of delivery in cases of IUGR despite the absence of preeclampsia. This again could be a reflection of impaired placental angiogenesis and invasion, or might reflect state of placental hypoxia, which is not as severe as that in cases of preeclampsia.

Recently Thadhani, *et al* (2004) have reported no difference between sFlt1 levels in first trimester samples (10.6 week's gestation) of low risk women destined to develop pre-eclampsia. The low risk controls (10.6 week's gestation) showed very similar concentrations to those of our 'high risk' women with a normal outcome, studied at 24 weeks. The discrepancy between these studies is most likely to arise from the different gestational ages of study, since the placental function only becomes obviously abnormal in the second trimester. This is supported by a previous study in which placental markers in the maternal serum (e.g. PAI-2, leptin and PlGF)

demonstrated abnormal concentrations only from 20 week's gestation onwards (Chappell *et al.*, 2002a). Additionally, the women we have studied here were all known to have abnormalities of the uteroplacental circulation, and may therefore represent a group with particularly severe placental pathology.

The members of the VEGF family contribute to growth, proliferation and survival of endothelial cells and are therefore hypothesized to play a substantive role in placental development and growth (Autiero *et al.*, 2003). New developments in our understanding of interrelationships between these molecules have shown the importance of dynamic intra- and intermolecular cross talk. As an antagonist of VEGF-A and PlGF, it has been postulated that sFlt1 may act as a 'decoy'-receptor, through interference with the signals normally leading to the activation of VEGF-R2, thereby down regulating the angiogenic activity of VEGF-A and PlGF function. In addition, sFlt1 is able to act in a dominant negative manner to directly inhibit both VEGF-R1 and VEGF-R2 signaling (Autiero, *et al* 2003). PlGF plays a distinct role in regulating endothelial cell survival and therefore excess sFlt1 may inhibit this directly (Autiero *et al.*, 2003).

Maynard *et al.* (2003) hypothesized that in preeclampsia, excess sFlt1 causes widespread endothelial dysfunction by interfering with the normal physiological effects of VEGF and/or PlGF. They demonstrated that both preeclamptic serum and normal serum with added sFlt1 had profound anti-angiogenic effects in vitro and resulted in impaired tube formation in the HUVEC (Human Umbilical Vein Endothelial Cells) model, compared to angiogenesis noted when serum from normal pregnant women was used. In that study, circulating sFlt1 at levels observed in

preeclampsia blocked the dose-dependent increase in vasodilatation induced by VEGF or PlGF, which suggests that sFlt1 in preeclamptic patients might oppose physiological vasorelaxation, thus contributing to hypertension. Furthermore, exogenous sFlt1 administered to pregnant rats resulted in hypertension and proteinurea with renal lesions similar to the ones seen in preeclampsia, mainly glomerular enlargement with occlusion of the capillary loops by swelling and hypertrophy of endocapillary cells (glomerular endotheliosis), and focal foot-process effacement in podocytes. These changes were also observed in non-pregnant rats injected with sFlt1, which suggests that the systemic effects of sFlt1 do not require the presence of pregnancy or the placenta to develop.

In pre-eclampsia the process of decidual spiral arteriole transformation is often incomplete and may lead to poor perfusion and episodic hypoxia, which are potent stimuli for expression of placental VEGF-A and its receptors, whilst having a suppressive effect on PlGF. This could result in increased binding of VEGF-A and PlGF to abundant sFlt1 and a reduction of free VEGF-A and PlGF. Indeed, such a profile was observed in the present study and in the reports of Maynard *et al.* (2003) and Koga *et al.* (2003). All these data are consistent with PlGF having important effects on maternal endothelial cells during pregnancy and when this is inhibited the maternal syndrome of pre-eclampsia ensues. Together, this could result in loss of the vasodilatory action of free VEGF-A at the level of the maternal endothelium (Autiero *et al.*, 2003).

A study by Mckeeman *et al.* (2004) showed that serum sFlt1 concentration in preeclampsia was elevated compared to normal controls throughout gestation (starting

at 12 weeks). This, however, reached statistical significance at 30 weeks and at pre-delivery. Another study by Levine *et al.* (2004) demonstrated that serum sFlt1 concentration started to increase at 21 to 24 weeks gestation, in women who later developed preeclampsia. In this study, serum sFlt1 concentration increased steeply at 29 to 32 weeks. In these two studies (Mckeeman *et al.*, 2004; Levine *et al.*, 2004) serum sFlt1 concentration in normal controls remained constant until 33 to 36 weeks gestation, when a constant rise started till delivery. In my study, there were no differences in serum sFlt1 and PlGF with advancing gestational age in the control group. This could be attributed to that both controls and disease samples were collected before 36 weeks (ie. before the expected rise in serum sFlt1).

In this study, I have demonstrated that there is significant dysregulation of serum levels of sFlt1 and PlGF at 24 weeks, in pregnancies with abnormal uterine artery Doppler who developed severe early onset preeclampsia and normotensive IUGR pregnancies, when compared to normal controls. Significant differences in serum levels of sFlt1 between the preeclampsia and IUGR groups were also found at the time of the disease, despite similar changes in uterine artery Doppler resistance indices at 24 weeks and at the time of disease in these two groups. The control group in this study comprised women with abnormal uterine artery Doppler with normal pregnancy outcome. There might be an argument that this group is considered a "high-risk" group and strictly speaking, not normal controls. On the other hand, uterine artery Doppler, used as a screening method in my study, is known to have low positive predictive value for abnormal pregnancy outcome. This suggests that more than half of pregnancies considered high-risk using this screening method have normal pregnancy outcome, without evidence of placental dysfunction. Larger study

comparing serum sFlt1 and PlGF in pregnancies with abnormal uterine artery Doppler that develop preeclampsia and/or IUGR with normal uterine artery Doppler controls is needed. Elevated level of serum sFlt1 in normotensive IUGR compared to controls suggests that there is an element of placental hypoxia in IUGR cases compared to controls. The IUGR group studied comprised of seven cases, four of which had AREDF in umbilical artery Doppler. The rest had positive EDF, a factor contributing to placental hypoxia in face of reduced uterine artery blood flow due to failed modification of spiral arteries.

The source of elevated sFlt1 in preeclampsia is not clear. Trophoblast is a potential source as both villous and extra villous trophoblastic cells produce both sFlt1 and PlGF (Tsatsaris *et al.*, 2003). Studies of sFlt1 in preeclampsia showed significant drop 24 hour after delivery. In the study by McKeeman *et al.* (2004), serum sFlt1 concentrations at 37 weeks in preeclampsia were not significantly higher than controls. There was, however, a significant increase at the time of disease in the preeclampsia group, which often was not far from 37 weeks. This casts some doubt over the role of the placenta as the only source of sFlt1, as a significant elevation in serum sFlt1 in the preeclampsia group compared to controls is to be expected at that advanced gestation, if the placenta was the major source of sFlt1. Other potential sources include the liver and kidneys; both are affected in preeclampsia (He *et al.*, 1999) and could be contributing to the significant increase in sFlt1 in preeclampsia at the time of delivery.

My observations in this study may have important implications for the prediction and thereby prevention of pre-eclampsia. It could be argued that as this study was

undertaken using second trimester samples that there would be limited value for potential therapeutic intervention. However, in a previous study of vitamin C and E supplementation in the second trimester of high-risk pregnancies, a significant reduction in the incidence of pre-eclampsia was observed compared to placebo (Chappell *et al.*, 1999). In that study, vitamin supplementation was associated with improvement in serum markers of placental function (Chappell *et al.*, 2002b). Clinical trials of antioxidant prophylaxis currently underway will provide an opportunity to validate the use of sFlt1 and PlGF in prediction of pre-eclampsia and to assess whether antioxidants reduce sFlt1. The current study also has implications for the development of a novel prophylactic strategy. Theoretically placental angiogenesis and maternal endothelial function could be restored by the administration of PlGF and/or VEGF, or by blocking the production or action of sFlt1 leaving VEGF-A and PlGF available for binding to membrane bound VEGF receptors.

The use of sFlt1 measurement alone or in combination with PlGF levels as a predictive indicator of pre-eclampsia needs to be confirmed in a larger prospective study of high-risk women, and in women at lower risk. However, these novel data further implicate angiogenic factors and their endogenous regulators in the etiology of pre-eclampsia and offer new hope for predictive and preventative strategies.

In my study, I also demonstrated significant elevation in placental expression of Flt1 in preeclampsia compared to normotensive IUGR and controls at the time of delivery. All placentas studied were obtained at the time of pre-labour elective Caesarean Section, which avoids the confounding effect labour could have on placental expression of the studied proteins. My findings supports and extends the findings by

Maynard, *et al* (2003) that showed enhanced expression of Flt1 (using reverse transcriptase-polymerase chain reaction (RT-PCR)) in preeclamptic placentas compared to *term* controls. Another study of sFlt1 expression in preeclampsia by Tsatsaris *et al.* (2003) showed increased expression of sFlt1 mRNA in preeclamptic placentas compared with uncomplicated pregnancies. This increase was also found in placentas from pregnancies complicated by normotensive intrauterine growth restriction (IUGR). The authors attributed placental over expression of Flt1 and sFlt1 in preeclampsia to the hypoxic state of the placenta. In their study, Flt1 and sFlt1 were also over expressed in normotensive IUGR placentas. These findings made the authors challenge the concept of placental hyperoxia in growth-restricted placentas with AREDF in umbilical artery Doppler introduced by Kingdom and Kaufmann (1997). In Tsatsaris *et al.* (2003) study, however, only 20% of their patient's population had AREDF in umbilical artery Doppler. In my study, all placentas studied in the IUGR group had AREDF in umbilical artery Doppler, whilst all preeclamptic placentas had positive EDF in umbilical artery Doppler flow.

Placental hypoxia seems the major factor triggering the expression of Flt1 in trophoblast. Flt1 is known to be upregulated by hypoxia, mediated through Hypoxia Inducible Factor (HIF-1α) binding to a hypoxia response element in the Flt1 gene promoter (Gerber *et al.*, 1997). In addition, hypoxia stimulates alternate splicing of the Flt1 gene and the release of serum sFlt1 (Kendall & Thomas, 1993). This explains the significant increase in placental Flt1 and serum sFlt1 in cases of preeclampsia in my study. The placenta in this condition is known to be hypoxic (all cases had preserved EDF in umbilical artery Doppler). On the other hand, Placental expression of Flt1 in cases of IUGR with AREDF in umbilical artery Doppler was not different

from gestational-age matched controls. This suggests the absence of placental hypoxia in these cases of IUGR compared to preeclampsia.

I found no differences in PlGF expression between preeclamptic, IUGR and control placentas. This supports the findings of the study by Tsatsaris *et al.* (2003), which showed that placental PlGF mRNA expression was not different between preeclamptic and normotensive IUGR pregnancies. In my study, western blotting was used instead of RT-PCR to assess Flt1 and PlGF expression in placental trophoblast. Western blotting detects protein expression at only one point in the tissue studied and does not necessarily reflect overall changes in synthesis and expression of that protein. In addition, western blotting is more valid in demonstrating the positive difference in protein expression between tissues rather than proving the absence of differences. Another draw back of this study is that the control group included only gestational-age-matched pregnancies, which had other complications such as preterm rupture of the membranes and infection. This could potentially influence Flt1 and PlGF expression in these placentas.

A study by Ahmad & Ahmed (2004) of the effect of placental sFlt1 on placental angiogenesis showed that levels of sFlt1 released from placental villous explants were significantly higher in preeclamptic and fetal growth-restricted placentas compared with normal term placentas. This, however, was not associated with increase in expression of the full-length Flt1. This suggests that sFlt1 release from preeclamptic placenta is triggered without enhanced expression of the parent protein (Flt1) itself. My findings do not support this, as I found a significant increase in placental Flt1 expression in preeclamptic placentas compared to both IUGR and controls.

Ahmad & Ahmed (2004) also suggested that elevated placental sFlt1 is implicated in impaired placental angiogenesis and trophoblast invasion in pregnancies that develop preeclampsia and IUGR. My findings of elevated sFlt1 in pregnancies with abnormal mid-trimester uterine artery Doppler that develop preeclampsia and IUGR suggests that sFlt1 could be a factor involved in impaired trophoblast invasion in these pregnancies that persist till the time of delivery. Cases of preeclampsia, however, have a several fold increase in serum sFlt1 just before delivery compared to normotensive IUGR. It seems that placental hypoxia at this stage is the main trigger responsible for the release of sFlt1 in preeclampsia and could be responsible for the widespread endothelial dysfunction seen in this condition.

CHAPTER 3

The relation between 24-week uterine artery Doppler resistance and serum soluble fms-like tyrosine kinase 1 (sFlt1) and placental growth factor (PlGF)

5.1 Introduction

Development of the feto-placental-uterine unit requires the invasion of the uterus by extravillous trophoblast cells, remodelling of the uterine spiral arterioles and development of a vast capillary network within the villous core of the placenta. Appropriate placental angiogenesis is critical for the successful transfer of oxygen and nutrients by the placenta from the maternal circulation into the fetus. Insufficient adaptation of the decidual and intramyometrial portions of the spiral arterioles in preeclampsia results in reduced utero-placental blood flow, leading to local placental hypoxia (Frusca *et al.,* 1989; Lunell *et al.,* 1982). Placental oxygen tension is known to affect placental production of VEGF-A, PlGF and sFlt1.

Evidence suggests that major reductions in umbilical artery blood flow (represented by absent/reversed EDF and or raised resistance indices) is associated with a pattern of villous maldevelopment in the placenta in which the peripheral villi contain slender, unbranched and uncoiled capillary loops (Kingdom *et al.*, 1997). In chapter one, I reported that pregnancies complicated by preterm preeclampsia had lower umbilical artery Doppler resistance indices before delivery, when compared to normotensive IUGR pregnancies. This indicates distinct placental response in these pregnancies with impaired placental invasion and abnormal uterine artery Doppler flow. Todros *et al.* (1999) suggested that pregnancies complicated by preeclampsia (with and without fetal growth restriction) with positive end diastolic frequencies (EDF) in umbilical artery Doppler before delivery had an adaptive response in the villous placenta, characterized by branching angiogenesis, which compensates for the underlying impairment of uteroplacental blood flow. By contrast, this pattern of villous vascularization was lacking in the fetal growth restriction group complicated by absent or reversed EDF in umbilical artery Doppler. This indicates that umbilical

artery Doppler flow could indirectly reflect the pattern of placental villous development, and consequently oxygen level within the placenta (placentas with preserved EDF in umbilical artery Doppler are more hypoxic compared to placentas with AREDF in umbilical artery Doppler (Kingdom *et al.*, 1997)).

In the last study (chapter 2), placental expression of Flt1 was upregulated in preeclampsia, possibly related to placental hypoxia. This also resulted in increased serum sFlt1 production. The increase in serum sFlt1 was, however, seen in cases of both preeclampsia and normotensive IUGR, despite significantly higher serum levels in cases of preeclampsia. This could indicate a more pronounced level of placental hypoxia in preeclampsia, compared to IUGR. The increase in serum sFlt1 in cases of preeclampsia and IUGR was noticed from as early as 24 weeks, in pregnancies with abnormal uterine artery Doppler. Placental sFlt1, through blocking of VEGF-A and PlGF, could be implicated in defective angiogenesis and trophoblast invasion in these placentas, indirectly seen as abnormal uterine artery Doppler.

The aim of this study was: 1. To investigate the correlation between uterine artery resistance indices (pulsatility index (PI) and resistance index (RI)) at 24 weeks and serum levels of PlGF and sFlt1; 2. To examine the effect of placental hypoxia within 24 hours of delivery (reflected by umbilical artery PI and RI) on corresponding serum levels of sFlt1, in pregnancies complicated by preeclampsia and normotensive IUGR.

5.2 Methods

Women enrolled in this study were the same study group with abnormal uterine artery Doppler, that were followed up and enrolled in the previous study (see chapter 2). Among the 31 women with abnormal uterine artery Doppler at 24 weeks gestation, eight women developed preeclampsia before 36 weeks gestation, seven developed normotensive IUGR and sixteen were enrolled as controls. Abnormal uterine artery Doppler was defined as bilateral notches with a mean RI>0.55, or unilateral notch with a mean RI>0.65. Uterine artery Doppler velocity flow waveforms were obtained via the transabdominal approach, using the technique described in *Materials and Methods*, and the position of the placenta was noticed. Resistance and Pulsatility index (RI and PI) in both placental and non-placental side were recorded, along with the mean RI and PI, using built in software in the ultrasound machine (ATL-5000).

Blood was collected from women with abnormal uterine artery Doppler at the time of the examination and serum was stored at -80C, using the technique described in chapter 2. Serum assay for sFlt1 and PlGF, in women with abnormal uterine artery Doppler, was performed using sandwich ELISA technique as described in *Materials and Methods*. Data was considered non-parametric and correlation coefficients between uterine artery resistance indices [PI, RI (placental and non-placental side), and mean PI and RI] and corresponding serum levels of sFlt1 and PlGF were then calculated using Spearman correlation coefficient.

To investigate the effect of umbilical artery blood flow on serum levels of sFlt1 and PlGF, women with abnormal uterine artery Doppler that developed preeclampsia (n=8) and normotensive IUGR (n=7) had umbilical artery Doppler examination and

blood collected in the same setting within 24 hours of delivery. Serum was stored at –80C for further assay of sFlt1 and PlGF.

5.3 Results

Correlation between uterine artery resistance indices and serum sFlt1 and PlGF:

Table 5.1 illustrates the clinical outcome and serum levels of sFlt1 and PlGF at 24 weeks in the study and control groups and the corresponding uterine artery Doppler resistance indices. Using the Spearman correlation coefficient, significant positive correlation was found between sFlt1 levels and uterine artery PI and RI, in both the placental and the non-placental sides and the mean value for RI and PI. Table 5.2 summarizes the correlation coefficients between uterine artery resistance indices at 24 weeks and sFlt1 levels. Figures 5.1-5.6 illustrate the correlations between uterine artery resistance indices at 24 weeks and sFlt1 levels (n=31).

On the other hand, PlGF was negatively correlated with all uterine artery resistance indices at 24 weeks. Table 5.3 illustrates the correlation coefficients between PlGF and uterine artery resistance indices (RI, PI (placental and non-placental sides) and mean RI and PI) and the corresponding p value. Figures 5.7-5.12 illustrate the correlation between PlGF and uterine artery resistance indices at 24 weeks.

Table 5.1 Serum sFlt1, PlGF and uterine artery resistance indices at 24 weeks.

No	Outcome	sFlt1	PlGF	RI (P)	RI (NP)	PI (P)	PI (NP)	Mean RI	Mean PI
1	IUGR	1516	292	0.63	0.66	1.11	1.26	0.65	1.19
2	IUGR	3255	306	0.61	0.76	1.11	1.74	0.69	1.43
3	IUGR	2846	168	0.70	0.73	1.39	1.50	0.72	1.45
4	IUGR	2860	449	0.68	0.79	1.26	1.87	0.74	1.44
5	IUGR	1393	20	0.74	0.82	1.70	2.04	0.78	1.87
6	IUGR	3858	820	0.58	0.78	0.98	1.70	0.68	1.34
7	IUGR	3624	325	0.71	0.79	1.41	1.70	0.75	1.56
8	PET	1022	186	0.72	0.73	1.59	1.61	0.73	1.60
9	PET	1472	252	0.59	0.69	1.04	1.40	0.64	1.22
10	PET	3334	178	0.72	0.77	1.65	1.85	0.73	1.75
11	PET	2606	315	0.65	0.71	1.25	1.54	0.68	1.40
12	PET	3002	10	0.57	0.76	0.85	1.70	0.67	1.28
13	PET	4693	191	0.83	0.85	2.25	2.27	0.84	2.26
14	PET	5243	284	0.74	0.77	1.52	1.67	0.76	1.60
15	PET	3943	180	0.79	0.84	1.92	2.15	0.82	2.04
16	Normal	783	573	0.57	0.58	0.97	0.98	0.58	0.98
17	Normal	1250	937	0.51	0.70	0.77	1.25	0.60	1.01
18	Normal	1682	255	0.59	0.65	1.03	1.16	0.62	1.10
19	Normal	1784	266	0.57	0.75	0.94	1.57	0.66	1.26
20	Normal	798	730	0.60	0.64	1.06	1.22	0.62	1.14
21	Normal	928	350	0.40	0.80	1.05	2.03	0.60	1.54
22	Normal	1482	500	0.65	0.71	1.17	1.40	0.68	1.29
23	Normal	1376	493	0.56	0.71	0.95	1.50	0.64	1.23
24	Normal	1679	1129	0.40	0.69	0.53	1.37	0.55	0.95
25	Normal	757	764	0.66	0.66	1.24	1.26	0.66	1.25
26	Normal	704	564	0.61	0.75	1.07	1.69	0.68	1.38

No	Outcome	sFlt1	PlGF	RI (P)	RI (NP)	PI (P)	PI (NP)	Mean RI	Mean PI
27	Normal	2139	203	0.52	0.78	0.79	1.82	0.65	1.31
28	Normal	1519	1025	0.61	0.76	1.11	1.24	0.69	0.97
29	Normal	386	250	0.71	0.73	0.98	1.10	0.72	0.91
30	Normal	735	833	0.60	0.65	1.02	1.07	0.63	0.85
31	Normal	359	255	0.55	0.62	0.95	1.15	0.58	0.87

IUGR:Intrauterine Growth Restriction, PET Preeclampsia

P: Placental Side, NP: Non-placental side

Table 5.2 Correlations between uterine artery resistance indices at 24 weeks and serum sFlt1. Using Spearman correlation coefficient, significant positive correlation was found between uterine artery resistance indices and serum sFlt1 at 24 weeks.

	Spearman Correlation Coefficient	**p value**
RI (placental side)	0.362	**0.045**
RI (non-placental side)	0.632	**<0.001**
Mean RI	0.575	**0.001**
PI (placental side)	0.391	**0.030**
PI (non-placental side)	0.720	**<0.001**
Mean PI	0.620	**<0.001**

Table 5.3 Correlations between uterine artery resistance indices at 24 weeks and serum PlGF. Using Spearman correlation coefficient, significant negative correlation was found between uterine artery resistance indices and serum PlGF at 24 weeks.

	Spearman correlation coefficient	**p value**
RI (placental side)	-0.437	**0.014**
RI (non-placental side)	-0.382	**0.034**
Mean RI	-0.485	**0.006**
PI (placental side)	-0.372	**0.040**
PI (non-placental side)	-0.479	**0.006**
Mean PI	-0.526	**0.002**

Figure 5.1 Correlation between uterine artery Doppler resistance indices (RI) of the placental side at 24 weeks and the corresponding serum sFlt1. Using Spearman correlation coefficient, significant positive correlation exists between the two groups (r=0.362, p=0.045)

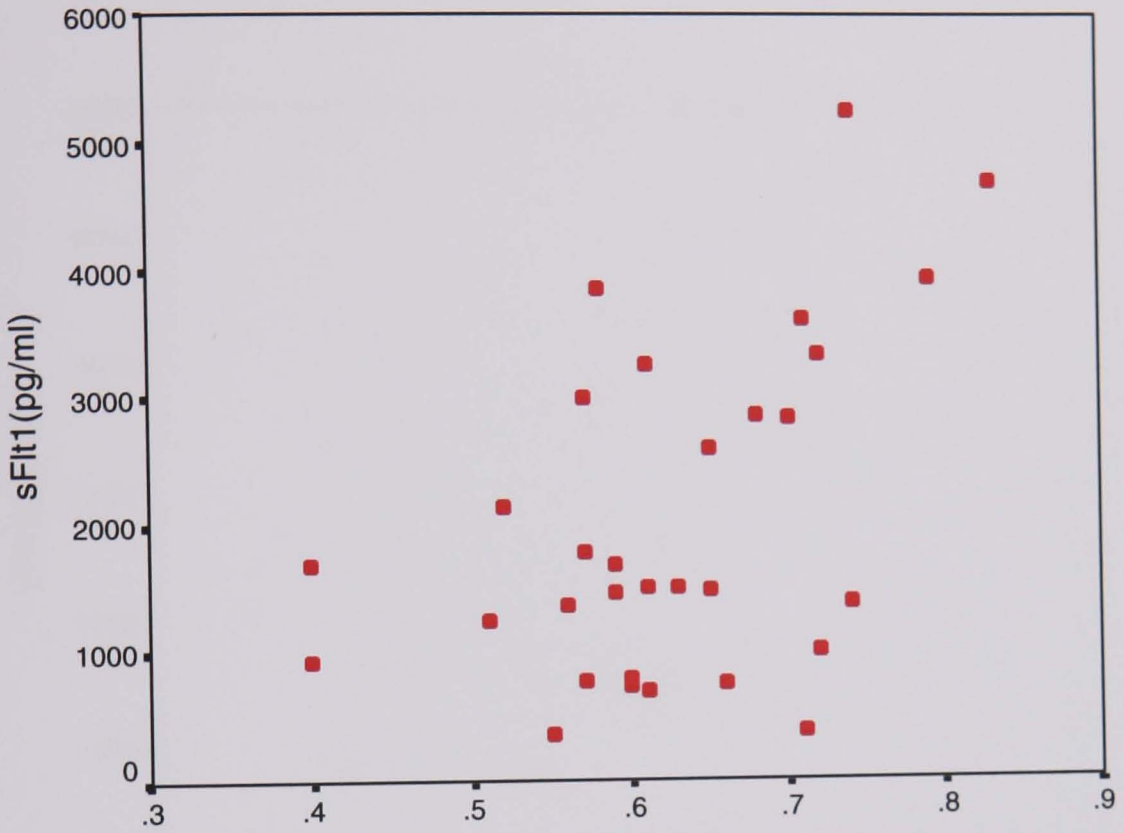


Figure 5.2 Correlation between uterine artery Doppler resistance indices (RI) in the non-placental side at 24 weeks and the corresponding serum sFlt1. Using Spearman correlation coefficient, significant positive correlation exists between the two groups ($r=0.632$, $p<0.001$)

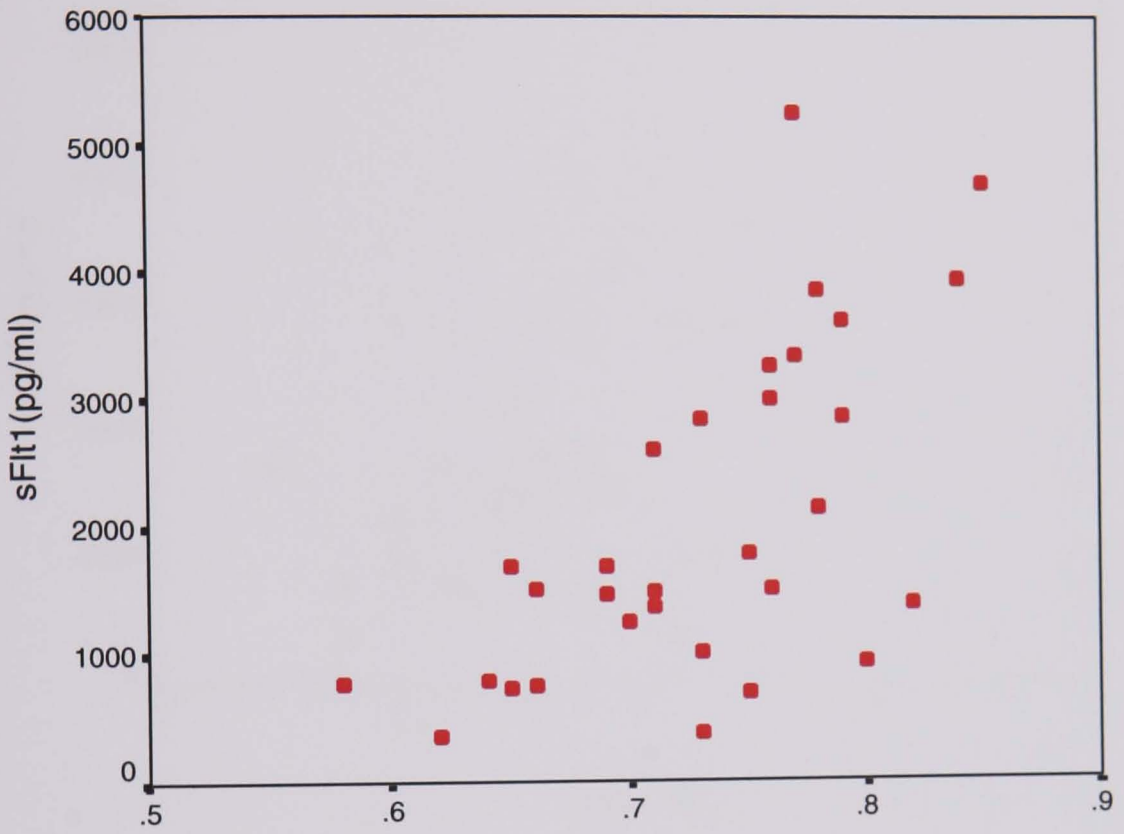


Figure 5.3 Correlation between uterine artery mean resistance indices (mean RI) at 24 weeks and the corresponding serum sFlt1. Using Spearman correlation coefficient, significant positive correlation exists between the two groups (r=0.575, p=0.001).

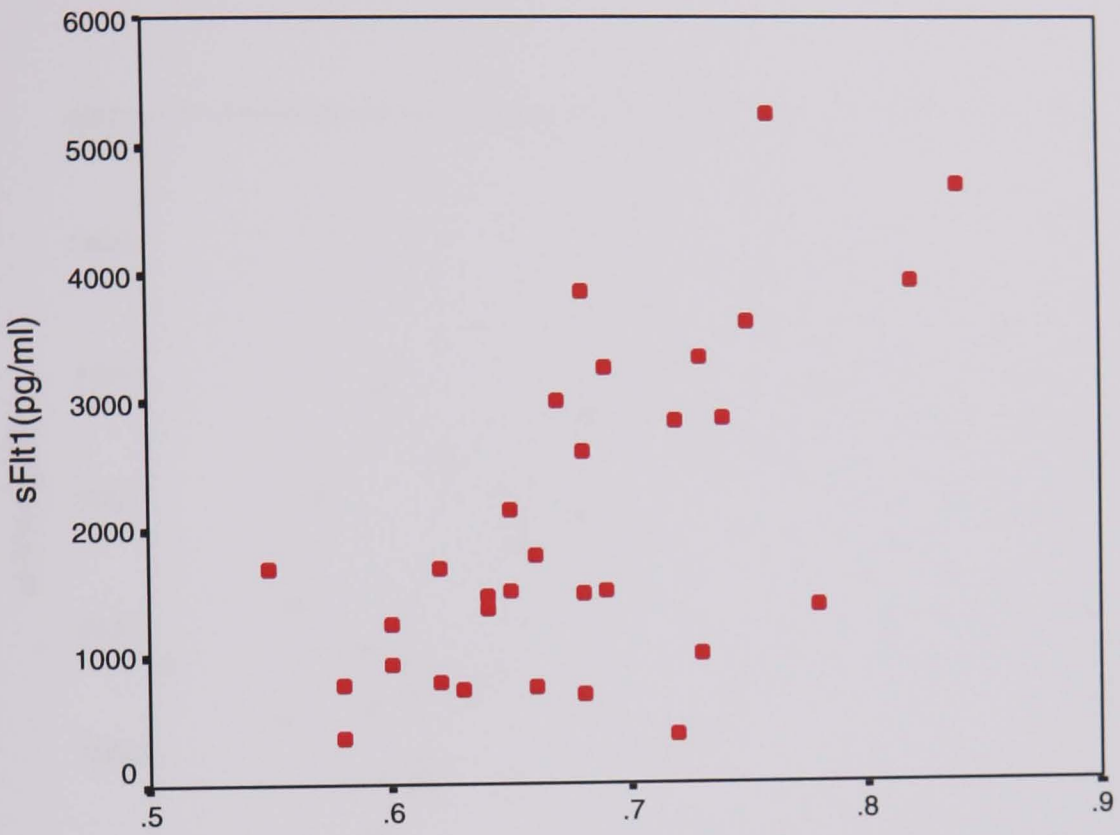


Figure 5.4 Correlation between uterine artery Doppler pulsatility indices (PI) of the placental side at 24 weeks and the corresponding serum sFlt1. Using Spearman correlation coefficient, significant positive correlation exists between the two groups (r=0.391, p=0.03)

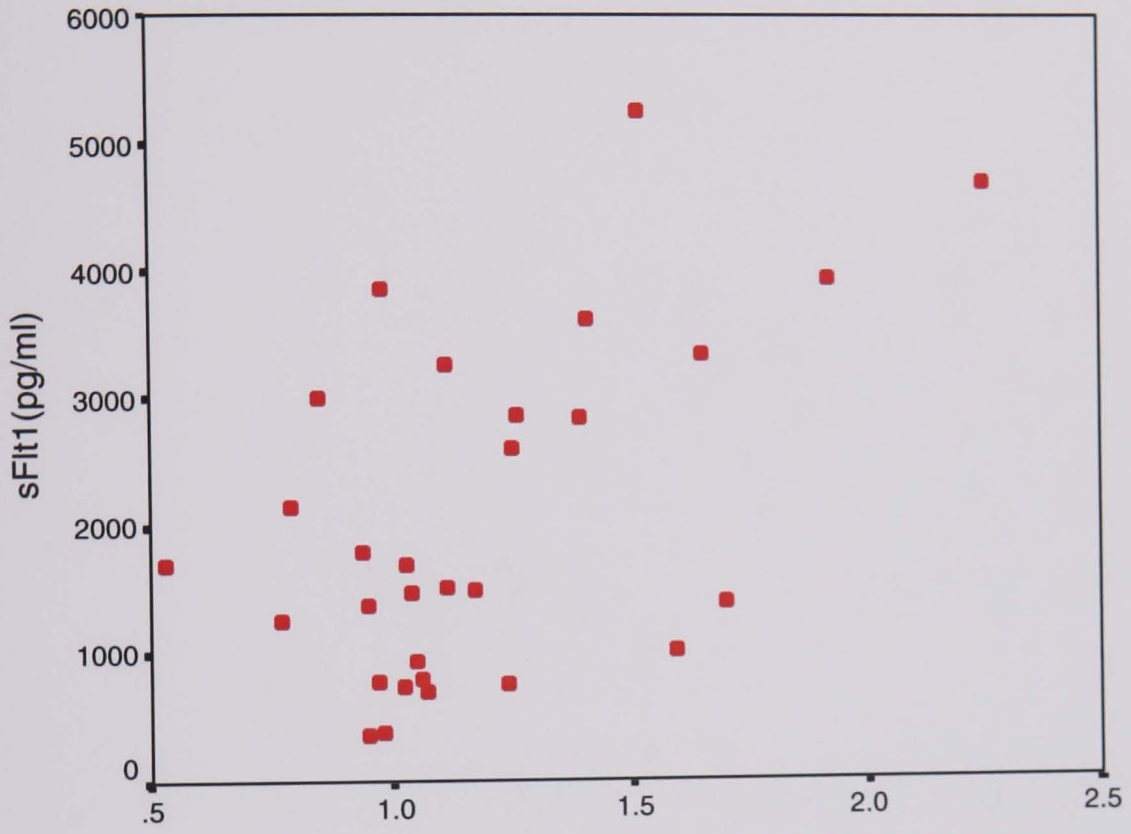


Figure 5.5 Correlation between uterine artery Doppler pulsatility indices (PI) of the non-placental side at 24 weeks and the corresponding serum sFlt1. Using Spearman correlation coefficient, significant positive correlation exists between the two groups ($r=0.720$, $p<0.001$)

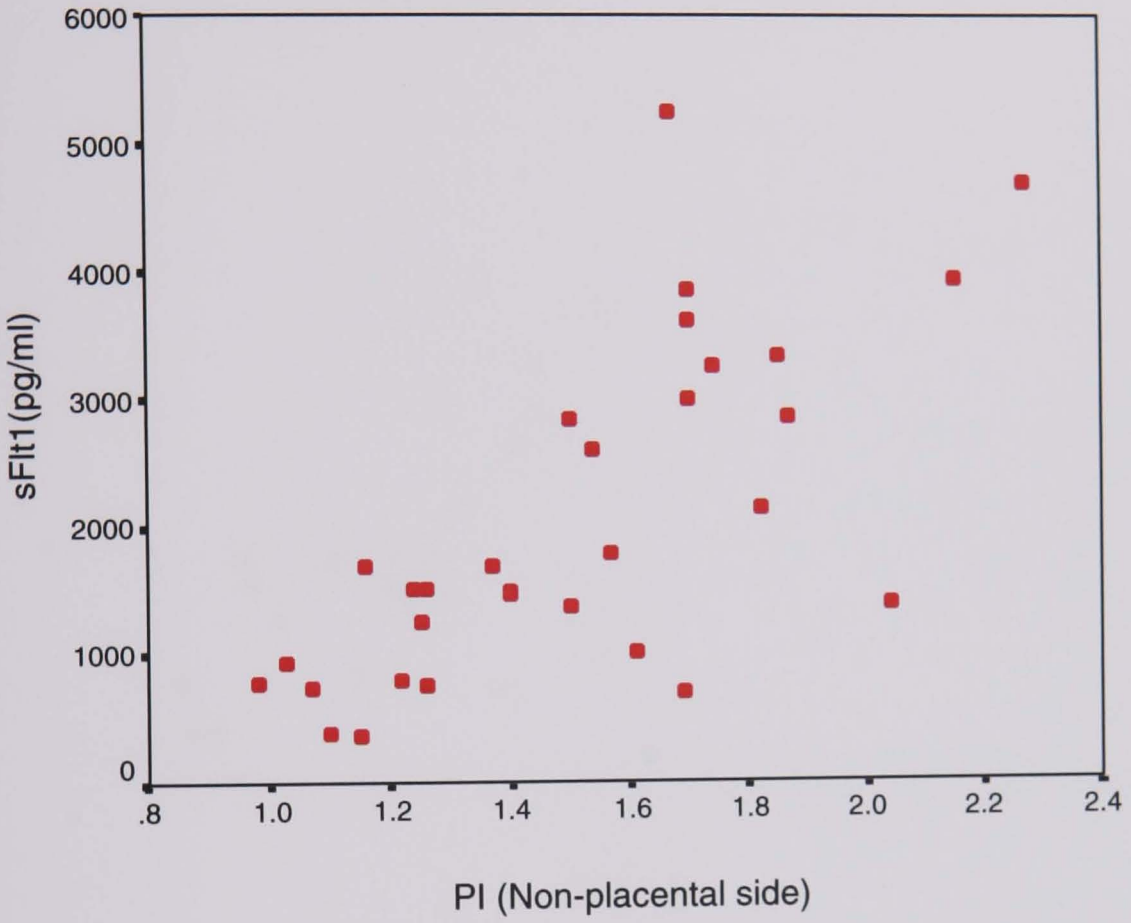


Figure 5.6 Correlation between uterine artery Doppler mean pulsatility indices (mean PI) at 24 weeks and the corresponding serum sFlt1. Using Spearman correlation coefficient, significant positive correlation exists between the two groups (r=0.620, p<0.001)

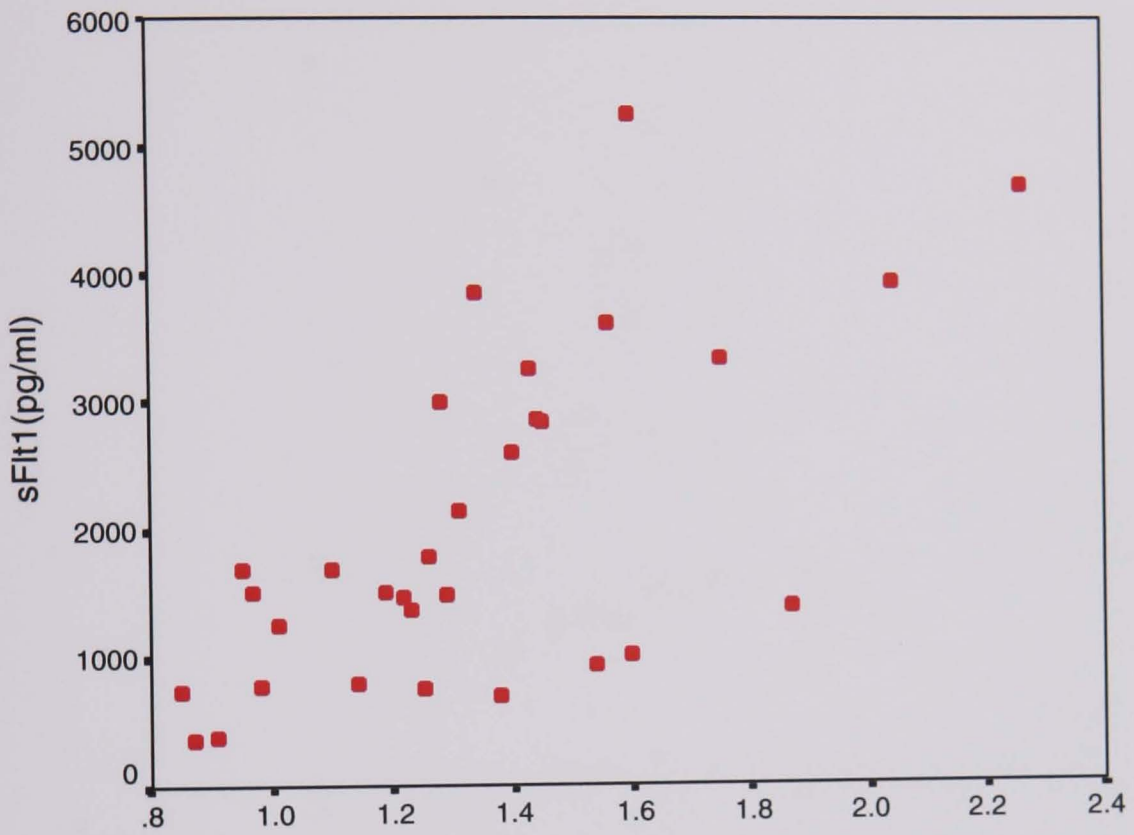


Figure 5.7 Correlation between uterine artery Doppler resistance indices (RI) of the placental side at 24 weeks and the corresponding serum PlGF. Using Spearman correlation coefficient, significant negative correlation exists between the two groups (r=-0.437, p=0.014)

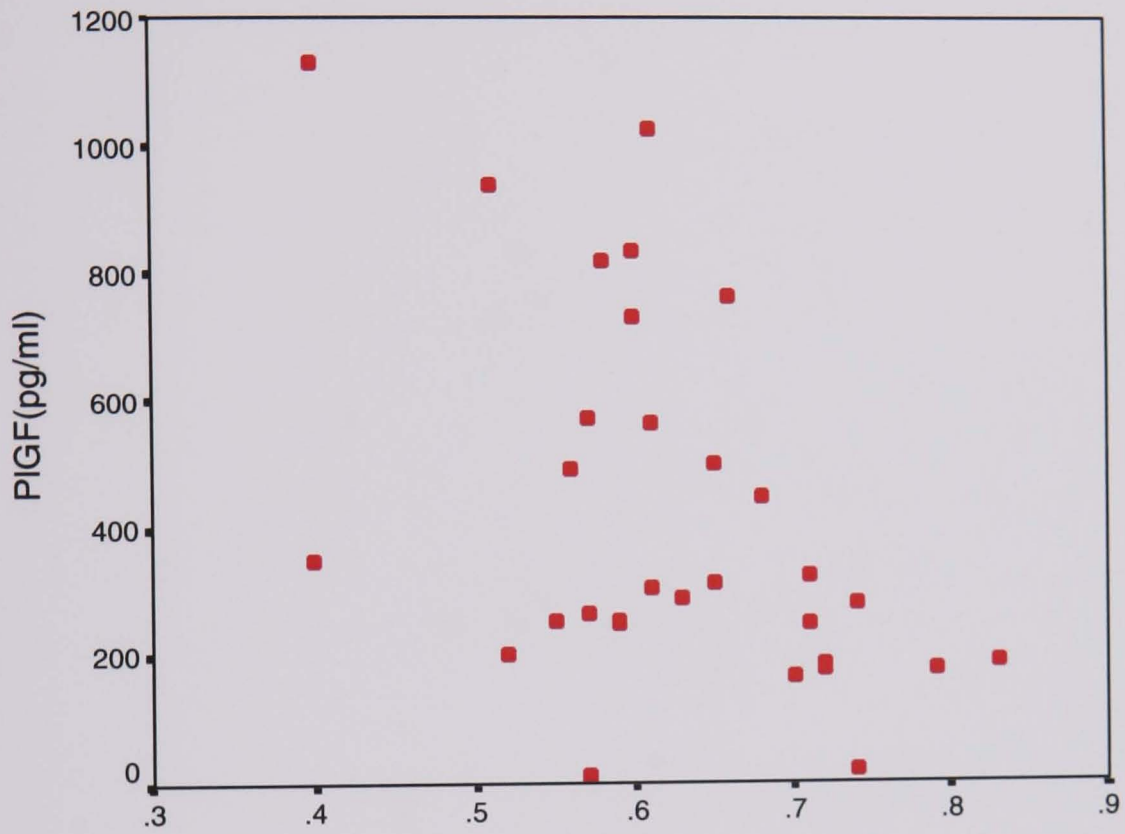


Figure 5.8 Correlation between uterine artery Doppler resistance indices (RI) in the non-placental side at 24 weeks and the corresponding serum PlGF. Using Spearman correlation coefficient, significant negative correlation exists between the two groups (r=-0.382, p=0.034).

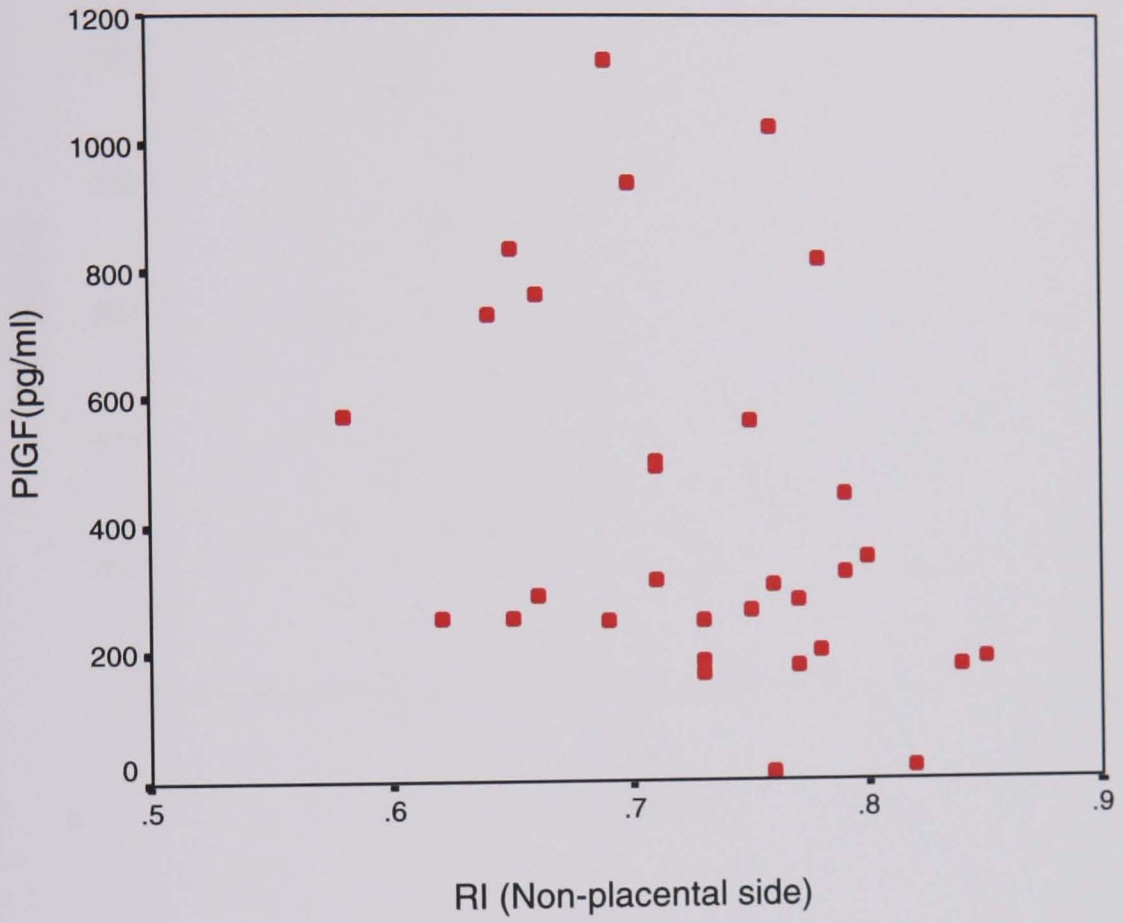


Figure 5.9 Correlation between uterine artery mean resistance indices (mean RI) at 24 weeks and the corresponding serum PlGF. Using Spearman correlation coefficient, significant negativecorrelation exists between the two groups (r=-0.485, p=0.006)

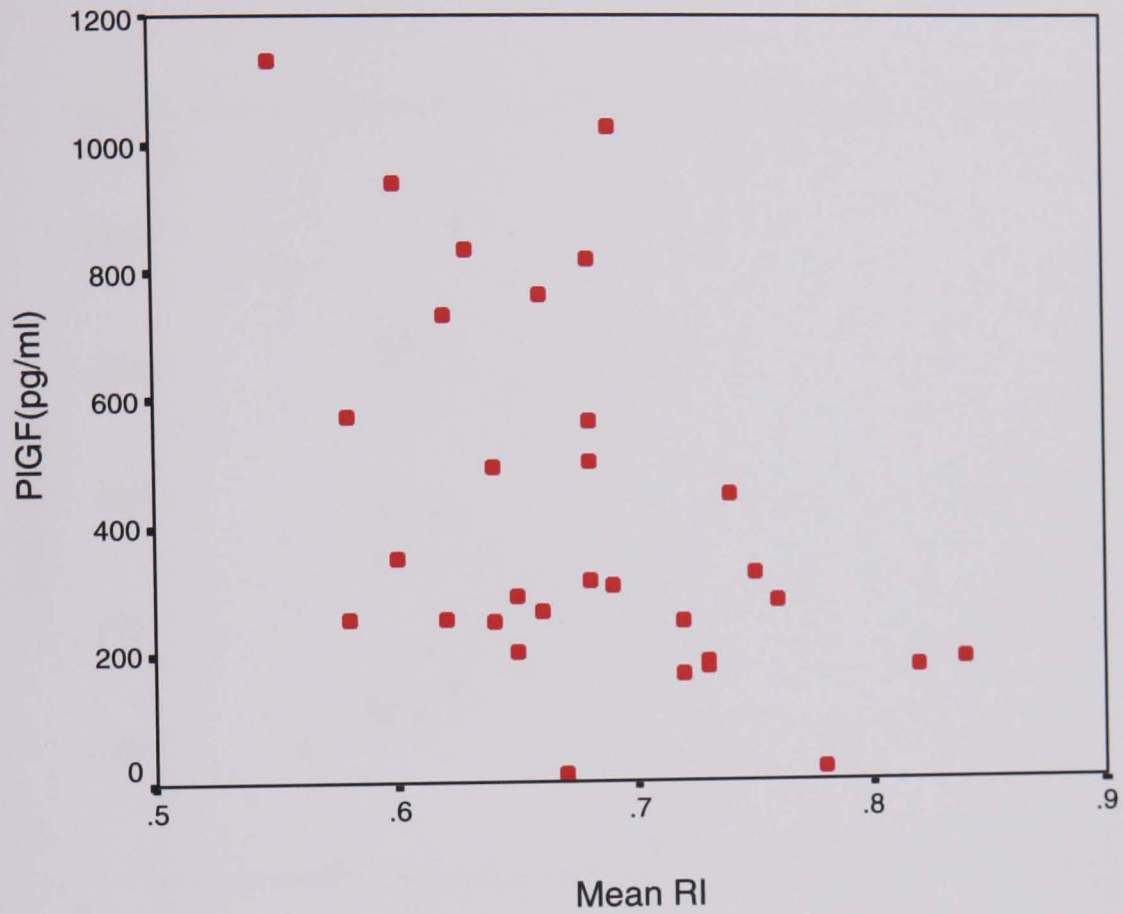


Figure 5.10 Correlation between uterine artery Doppler pulsatility indices (PI) of the placental side at 24 weeks and the corresponding serum PlGF. Using Spearman correlation coefficient, significant negative correlation exists between the two groups (r=-0.372, p=0.04).

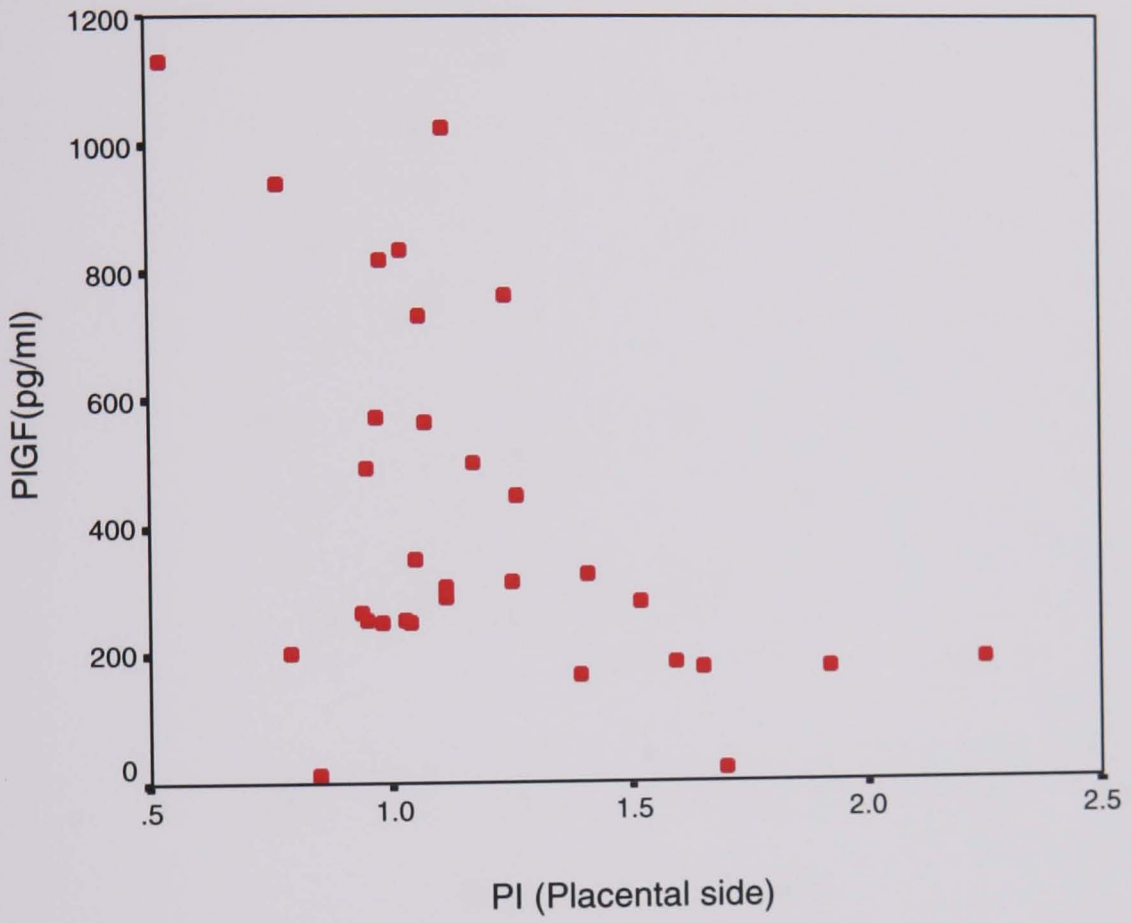


Figure 5.11 Correlation between uterine artery Doppler pulsatility index (PI) of the non-placental side at 24 weeks and the corresponding serum PlGF. Using Spearman correlation coefficient, significant negative correlation exists between the two groups (r=-0.479, p=0.006).

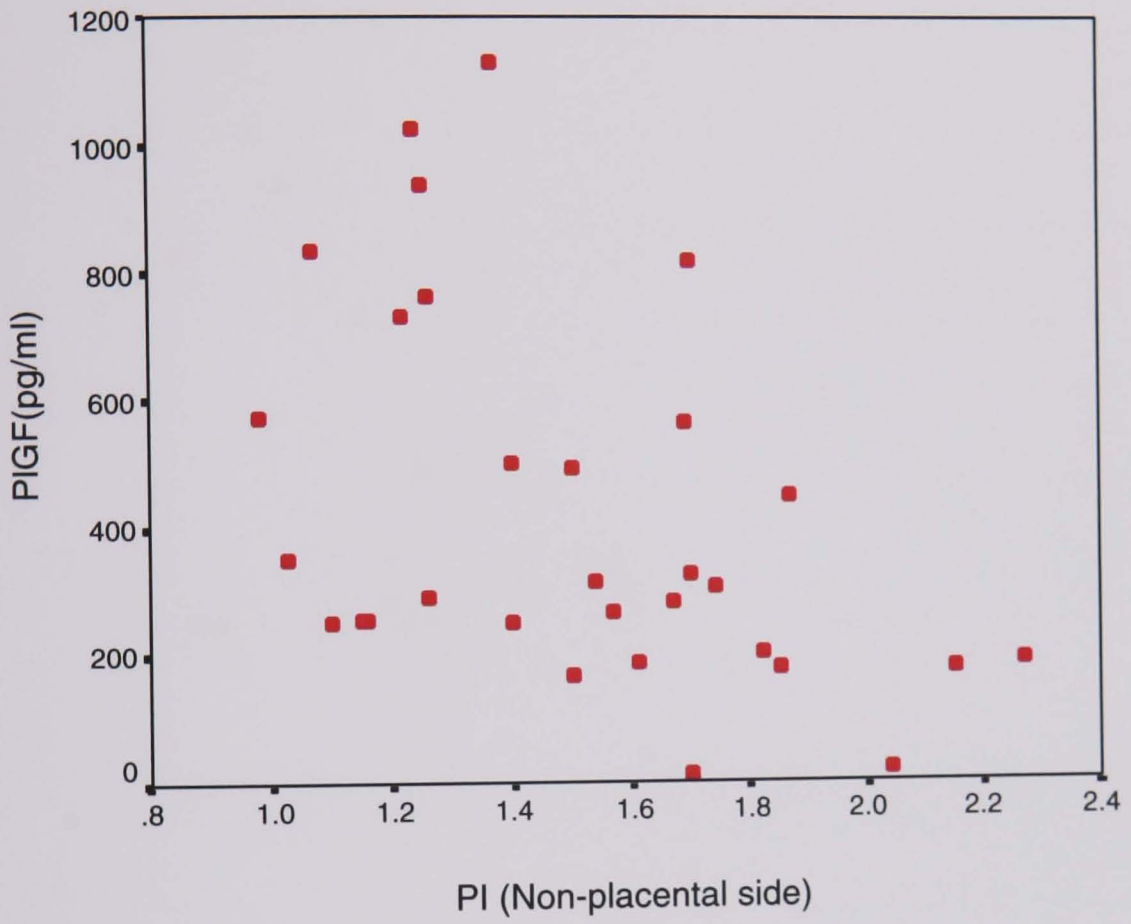
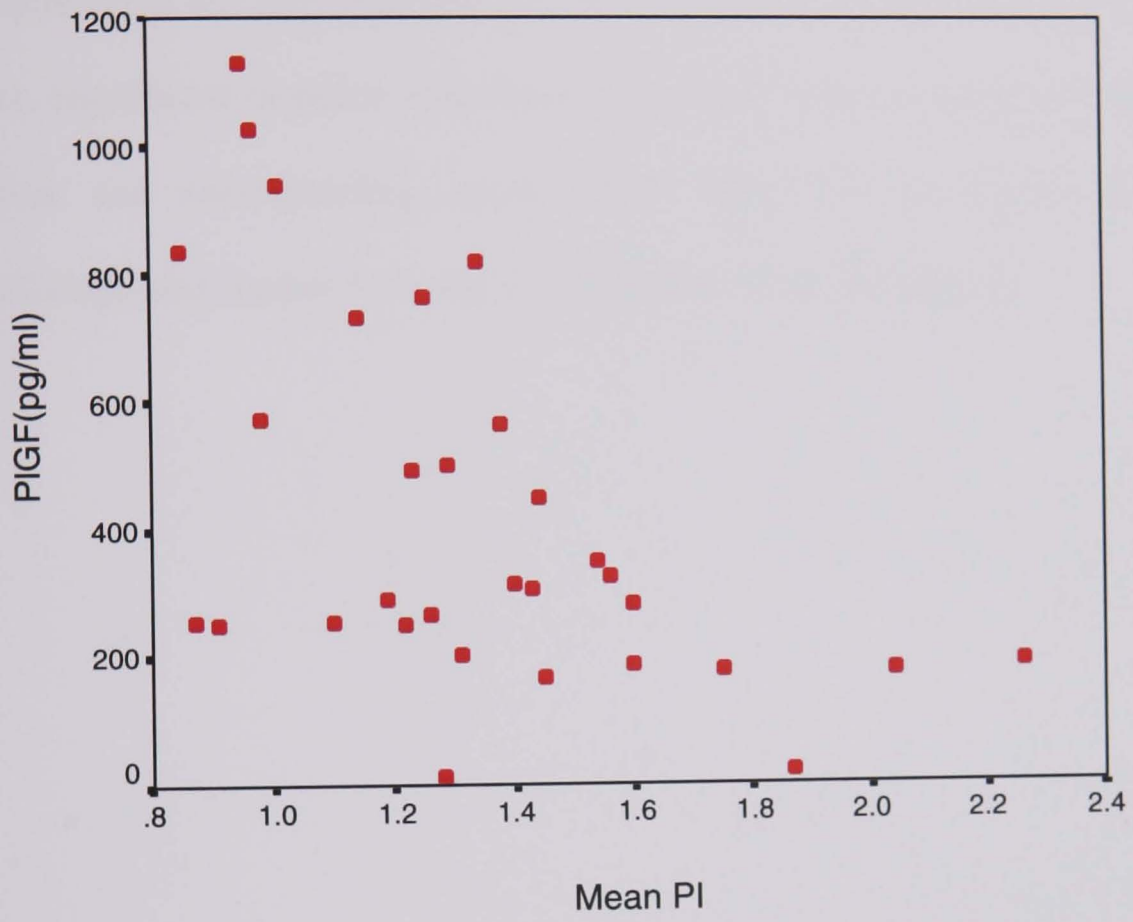


Figure 5.12 Correlation between uterine artery Doppler mean pulsatility indices (mean PI) at 24 weeks and the corresponding serum PlGF. Using Spearman correlation coefficient, significant negative correlation exists between the two groups (r=-0.526, p=0.002).



Correlation between umbilical artery resistance and serum sFlt1 within 24 hours of delivery

To investigate the effect of placental blood flow on serum levels of sFlt1, I used the Spearman correlation coefficient to correlate umbilical artery resistance indices (RI and PI) within 24 hours of delivery in cases of abnormal pregnancy outcome (preeclampsia and normotensive IUGR) (n=15) and the corresponding serum levels of sFlt1. Significant negative correlation was found between umbilical artery resistance indices and corresponding serum sFlt1. Table 5.4 summarises the correlation coefficients and figures 5.13 and 5.14 illustrate these correlations.

Table 5.4 Spearman correlation coefficients between umbilical artery resistance indices and corresponding serum sFlt1 level within 24 hours of delivery in cases of preeclampsia and normotensive IUGR (n=15).

	Spearman Correlation Coefficient	**p value**
Umbilical artery RI	-0.665	**0.007**
Umbilical artery PI	-0.674	**0.006**

Figure 5.13 Correlation between umbilical artery Doppler resistance indices (RI) and the corresponding serum sFlt1 within 24 hr of delivery in preeclampsia and IUGR. Using Spearman correlation coefficient, significant negative correlation exists between the two groups (r=-0.665, p=0.007).

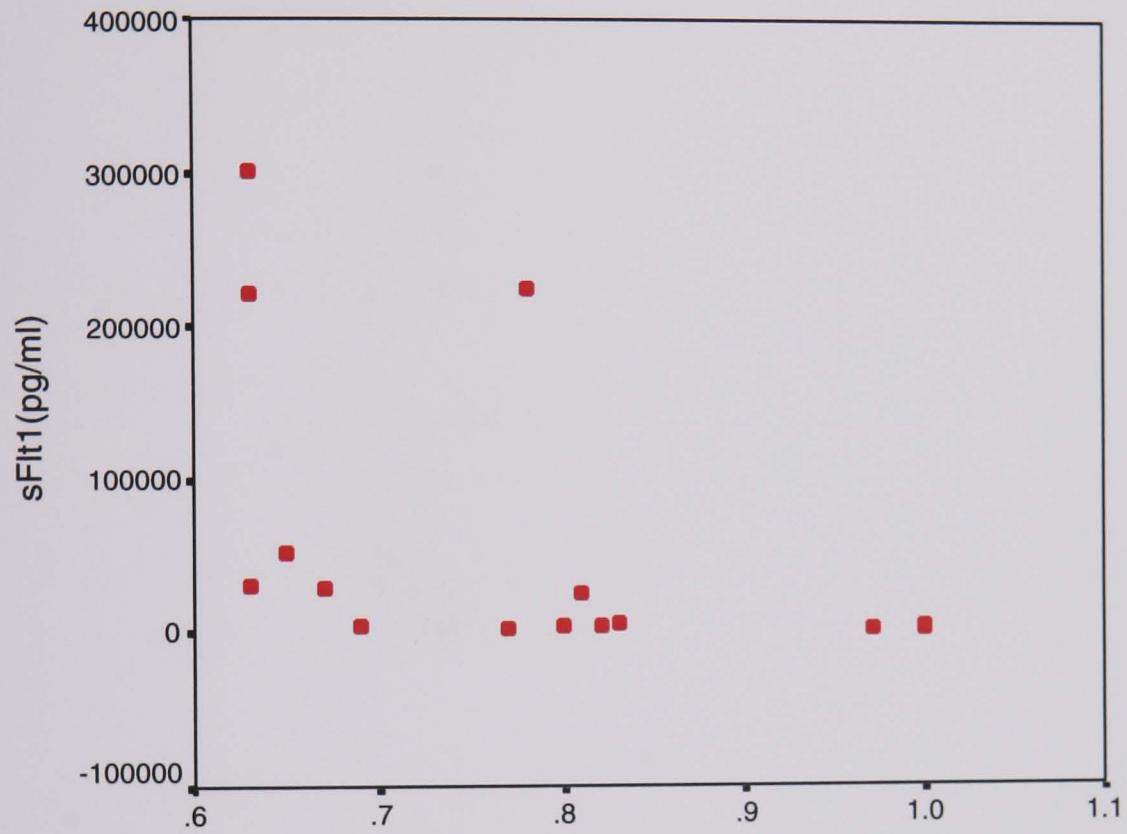
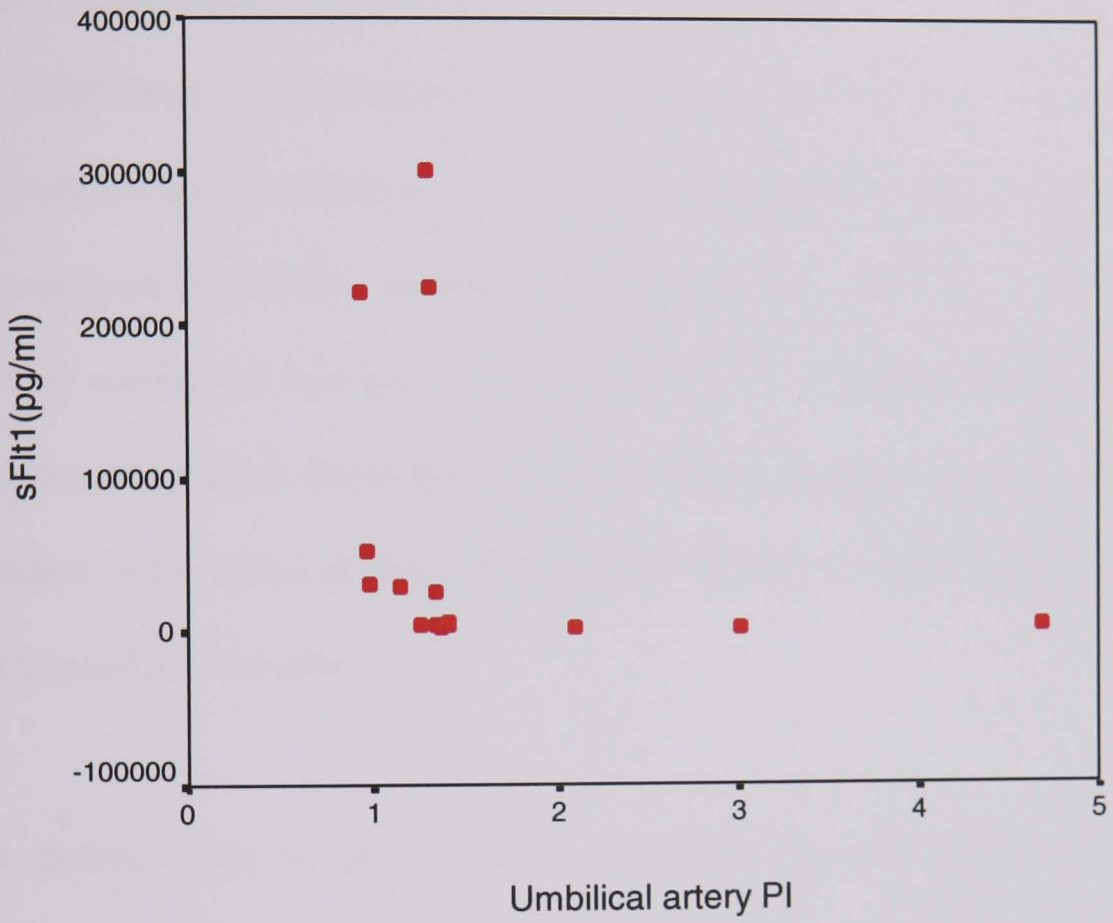


Figure 5.14 Correlation between umbilical artery Doppler pulsatility indices (PI) and the corresponding serum sFlt1 within 24 hours of delivery in cases of preeclampsia and IUGR. Using Spearman correlation coefficient, significant negative correlation exists between the two groups (r=-0.674, p=0.006).



Discussion

In this study, the in-vivo effect of varying degrees of impaired placentation and angiogenesis at 24 weeks on serum levels of sFlt1 and PlGF was assessed. This study demonstrates positive correlations between uterine artery Doppler resistance indices at 24 weeks and serum sFlt1 levels. In addition, negative correlations were found between these indices and serum PlGF. I demonstrated in chapter 2 that serum sFlt1 and PlGF levels were abnormal in pregnancies with abnormal uterine artery Doppler at 24 weeks. Serum values were abnormal in pregnancies that developed preeclampsia or normotensive IUGR, without major differences between the two groups. The positive correlation between serum sFlt1 and uterine artery resistance indices and the negative correlation between PlGF and those resistance indices provides further evidence of the effect of placental sFlt1 and PlGF on impaired placental angiogenesis and trophoblast invasion.

The pathogenesis of preeclampsia is thought to act at three levels: defective placentation, placental ischaemia, and endothelial cell dysfunction (Chung *et al.*, 2004). Vascular endothelial growth factor is a critical regulator for vascular growth and function. VEGF actions are mediated via binding to its receptors, which include VEGFR-1 (Flt1) and VEGFR-2 (Flk1/KDR). VEGFR-2 is the major signal transducer of VEGF in endothelial cells and mediates most known VEGF bioactivities. In contrast, Flt1 has been implicated in the inhibition of VEGF-dependent endothelial function (Giles *et al.*, 2000; Zeng *et al.*, 2001).

Recently Ahmad and Ahmed (2004) showed that elevated sFlt1 in patients with preeclampsia has anti-angiogenic effects during placental development. They showed

that conditioned media from preeclamptic placentas attenuated endothelial cell migration and in vitro tube formation, two key markers of angiogenesis. This suggests that raised levels of sFlt1 in those placentas may explain the poorly developed feto-placental vasculature associated with this disorder. In normal pregnancy, the rapid growth of the placenta and the associated vascularization occurs from the second trimester on wards. In preeclampsia, during this period, circulating levels of sFlt1 are elevated. The study by Ahmad and Ahmed (2004) showed that in preeclampsia there is loss of >70% of PlGF activity in the placenta. This strongly supports the premise that the VEGF/PlGF axis is dysregulated in this disorder. They showed that removal of sFlt1 by immunoprecipitation from preeclamptic controlled medium significantly restored angiogenesis. This further suggests that the elevated sFlt1 in preeclampsia is likely to be responsible for the poorly developed feto-placental vasculature associated with this disorder.

My study is the first to assess the in vivo relation between uterine artery resistance indices and serum sFlt1 and PlGF in mid trimester. My findings support and extend the study by Ahmad and Ahmed (2004), that suggested that sFlt1 is a major factor responsible for impaired placental angiogenesis and development in midtrimester in complicated pregnancies. The increase in serum sFlt1 was associated with a concomitant drop in free PlGF, which suggests bindings of PlGF by its antagonist sFlt1.

A study of placental bed expression of Flt1 in preeclampsia and intrauterine growth restriction compared to normal controls showed significant reduction in Flt1 mRNA expression in preeclamptic placental beds compared to controls. Flt1 expression was

also reduced in placental beds from IUGR pregnancies but this did not reach statistical significance (Tsatsaris *et al.,* 2003). In this study, it was not possible to determine whether this modification was a cause or a consequence of the pathologic status of pregnancy. The authors suggested that low levels of Flt1 in placental beds might explain the defective uterine vascularization associated with early-onset preeclampsia. In my study, the association between uterine artery resistance indices and serum levels of sFlt1 and PlGF indicates a role for these markers, both at placental and serum level, in modulating trophoblast invasion of the spiral arteries and the physiological changes in uterine artery resistance seen in normal pregnancies. On the other hand, upregulation of sFlt1 expression at 24 weeks in cases of abnormal uterine artery Doppler could represent a response to placental hypoxia due to defective trophoblast invasion. Hypoxia has been shown to increase sFlt1 production by placental cytotrophoblasts (Hornig *et al.,* 2000). This provides evidence that placental hypoxia is an early event in these pregnancies.

Disordered placentation is responsible for a wide range of pregnancy complications, ranging from miscarriage through second trimester fetal death and the classic third trimester complications of preeclampsia, intrauterine growth restriction and placental abruption. Placentation begins with implantation of the blastocyst beneath the uterine epithelium and differentiation into embryonic and extraembryonic tissues (Cross, 1998). A maternal uteroplacental circulation is gradually established in tandem with a fetoplacental circulation. Close co-ordination of the development of these circulations is essential to meet the rising fetal demands for oxygen. Any disturbance of the balance between these circulations will alter intra-placental oxygen content. An increasing body of evidence supports the concept that villous development depends

upon local oxygen conditions, and in turn that disturbed placental oxygenation is a feature of many pathological conditions (Kingdom and Kaufmann, 1999) including preeclampsia.

The data on Flt1 expression in normal and preeclamptic placentas is controversial. I demonstrated in the last chapter that there is upregulation of Flt1 expression in preeclamptic placentas, compared to placentas from IUGR and control pregnancies at the time of delivery. This might be a result of a lowered placental oxygen level within the placenta in preeclampsia with abnormal uterine artery Doppler. Placentas from pregnancies with AREDF in umbilical artery Doppler are known to have impaired branching angiogenesis, characteristic of normal third trimester placentas. The cause of this is not entirely clear but the reduced number and maldevelopment of peripheral villi result in a marked impairment of oxygen extraction from the intervillous space (Krebs *et al.,* 1996). This results in a raised placental oxygen level that potentially offsets impaired oxygenation, resulting from impaired trophoblast invasion. In contrast, placentas from preeclamptic pregnancies with positive end-diastolic frequencies have a normal pattern of stem artery development, increased capillary angiogenesis and development of terminal villi, as signs of an adaptive mechanism (Todros *et al.,* 1999). This causes local placental hypoxia and possibly upregulation of Flt1 and its soluble form sFlt1 (Hornig *et al.,* 2000).

In this study, I have demonstrated that there is an inverse correlation between umbilical artery resistance indices and serum sFlt1 levels within 24 hours of delivery. This provides further evidence of the role of placental hypoxia in preeclampsia. Preeclamptic pregnancies had significantly lower umbilical artery Doppler resistance

indices that resulted in increased placental blood flow from the fetal side and consequently placental hypoxia in the face of diminished uteroplacental blood supply. Hypoxia is known to up regulate placental sFlt1 production, which could be responsible for the widespread endothelial dysfunction seen in the disease (Maynard *et al.,* 2003). In contrast, normotensive IUGR pregnancies with raised umbilical artery resistance indices and lower umbilical artery blood flow had significantly lower serum sFlt1 levels, possibly due to relatively higher oxygen level within the placenta. This is due to lowered placental blood flow from the fetal side rather than higher uteroplacental blood flow.

In summary, this study reports that there is significant dysregulation of expression of sFlt1 and PlGF in pregnancies with abnormal uterine artery Doppler resistance indices from as early as 24 weeks. This dysregulation correlated with the severity of uteroplacental dysfunction. Abnormalities in PlGF and sFlt1 expression could be a contributing factor to impaired placental angiogenesis and trophoblast invasion in these pregnancies. On the other hand, this dysregulation could represent a response of the placenta to hypoxia caused by impaired spiral artery invasion by trophoblast. Further research in this area is needed to accurately describe the role of these molecules in pregnancies with abnormal uterine artery Doppler. These findings provide potential therapeutic approaches for the prevention and treatment of preeclampsia and suggest that pharmacological intervention to inhibit sFlt1 may be worthy of investigation.

CHAPTER 4

Serum and placental Fas and Fas ligand in high-risk women with abnormal 24-week uterine artery Doppler

6.1 Introduction

Apoptosis, or controlled cell death, is an important feature of placental cell management in normal pregnancy (Smith et al., 1997). There is evidence that placentas of pregnancies complicated by pre-eclampsia or IUGR show enhanced apoptosis when compared with placentas of normal pregnancies (DiFederico et al., 1999; Allaire et al., 2000; Ishihara et al., 2002). Furthermore, physiological changes in the spiral arteries are absent in pregnancies complicated by preterm pre-eclampsia and intrauterine growth restriction, which, in turn, causes placental hypoxia (Harrington et al., 1997). It is not clear whether increased placental apoptosis in these pregnancies is the cause of impaired placental invasion or is a direct result of placental hypoxia. The cause of impaired trophoblast invasion in pregnancies with abnormal mid-trimester uterine artery Doppler that subsequently develop preeclampsia and/or intrauterine growth restriction (IUGR) is far from clear. I showed in chapters 2 and 3 important dysregulation of the VEGF system and its receptors in these pregnancies. This affects placental angiogenesis and possibly trophoblast growth and invasion, but does not provide explanation of the increased apoptosis in these placentas.

The placenta has always been viewed as an immune-privileged site. The factors behind this immune privilege are not entirely clear yet. Breaking of this immune privilege was viewed responsible for inadequate placental function and hence development of conditions such as preeclampsia and intrauterine growth restriction (Allaire *et al.,* 2000). The Fas-Fas ligand system is believed to affect the death of inflammatory cells in immune-privileged sites such as the cornea of the eye and the testis. Many tissues express Fas membrane protein, whereas Fas ligand is expressed

only by circulating lymphocytes and in cells in immune-privileged sites. At those privileged sites, Fas-expressing peripheral T cells bind to Fas ligand in tissues expressing it. Fas-Fas ligand interaction delivers a signal to induce apoptosis in peripheral lymphocytes (Griffith *et al.,* 1995). Prior research has suggested that Fas ligand is normally expressed in human trophoblast throughout pregnancy and induces apoptosis in circulating activated T cells (Bamberger *et al.,* 1997). The expression of Fas ligand by the placenta might affect survival of the fetal allograft through the induction of apoptosis of circulating maternal leukocytes, allowing cytotrophobalsts to invade into the myometrium while escaping immune recognition. Alterations in the balance of mutual induction of apoptosis might affect diseases associated with abnormal placentation (eg pre-eclampsia, IUGR, placenta acreta/percreta, choriocarcinoma) (Allaire *et al.,* 2000). Recent studies have shown that membrane Fas activation may transduce dual apoptosis and non-apoptosis pathways. The non-apoptotic functions of Fas include proliferation, fibrosis, inflammation and angiogenesis (Hohlbaum *et al.,* 2002).

This study evaluates serum and placental levels of Fas and FasL in pregnancies with abnormal 24 week uterine artery Doppler that develop pregnancy complications due to uteroplacental insufficiency, namely pre-eclampsia and IUGR pregnancies, compared to normal controls.

6.3 Methods

Serum Fas and FasL assay in pregnancies with abnormal mid-trimester uterine artery Doppler.

In the uterine artery Doppler screening study at 23-24 weeks, 86 women attended follow up for fortnightly growth scans and uterine artery Doppler flow examination, along with BP checks and urine analysis for protein. Fetal Doppler examination was performed in cases where there was suspicion of growth restriction on ultrasound examination. Using pulsed Doppler, flow velocity waveforms were obtained and resistance indices (RI and PI) were measured in the umbilical artery, middle cerebral artery and fetal aorta (see chapter 2). Doppler of the DV was performed if the IUGR was associated with evidence of fetal compromise by arterial Doppler examination. Doppler examination was then performed three times per week up to the time of delivery. Timing of delivery was decided on clinical grounds, as well as cardiotocographic examination, in conjunction with Doppler ultrasound examination results.

Preeclampsia was defined according to the definition by the ISSHP (see chapter one). IUGR was defined as fetal weight less than the 5^{th} centile for gestational age along with abnormal umbilical artery Doppler examination (see below) or fetal biometry measurements crossing the fetal growth centiles over at least two fetal biometry examinations 10-14 days apart. This excluded any constitutionally (normal) SGA fetuses from the study. Abnormal umbilical artery Doppler was defined as absent or reversed EDF and/or resistance indices (PI and RI) > 95^{th} centile for gestational age (Harrington et al, 1999). Abnormal uterine artery Doppler was defined as persistence of unilateral/bilateral notches with PI and RI > 95^{th} centile for gestational age.

Statistics were performed with Microsoft Statistical Package for Social Sciences (SPSS)-10, Chicago, IL, USA. Data were considered non-parametric and Mann-Whitney *U* test was used for comparisons.

In this cohort, 15 pregnancies delivered before 36 weeks due to pregnancy complications related to uteroplacental insufficiency. Eight women developed preeclampsia. The gestational age of the preeclampsia group at delivery ranged between 28-36 weeks (median 32 weeks). Birth weight ranged between 720g-2680g (median 1360g). There were two intrauterine fetal deaths in this group. One was associated with severe IUGR and AREDF in umbilical artery Doppler flow at 28 weeks. The other fetal death complicated placental abruption at 30 weeks. The fetus was normally grown with a birth weight of 1250g (10^{th} centile). There were no neonatal deaths in this group. Antenatal umbilical artery (UA) Doppler examination 24 hours before delivery revealed positive EDF in all cases except one that had AREDF at 28 weeks and intrauterine fetal death.

Seven other pregnancies in the study group were complicated by normotensive IUGR ($<5^{th}$ centile) that needed delivery before 36 weeks due to fetal compromise. Four pregnancies had AREDF in umbilical artery Doppler examination 24 hours before delivery. None of these pregnancies had preeclampsia. The gestational age at delivery was not different from the preeclampsia group (range 27-35, median 34 weeks) ($p=0.712$). Birth weight ranged from 500-1728g (median 953g), which was not different from the preeclampsia group ($p=0.674$). Among this group, there was one fetal death in utero due to severe uteroplacental insufficiency and one neonatal death.

Blood was collected from pregnancies enrolled in the study at 24 weeks (the time of uterine artery Doppler examination) and within 24 hours of delivery. Serum was stored at –80C for further assay. Serum Fas and FasL was measured in the preeclampsia, IUGR and gestational age matched control (n=16) groups both at 24 weeks and at the time of disease using *Enzyme Linked Immune Sorbent Assay (ELISA)*

Enzyme Linked Immune Sorbent Assay (ELISA)

1.Serum Fas ELISA

Procedure

- Preparation of Reagents

1. Wash solution

   100 ml of Wash concentrate was diluted with 900 ml of distilled water.

2. Conjugate solution

   Peroxidase conjugated anti-Fas monoclonal antibody 1:101 was diluted with conjugate diluent by adding 10ul of the Peroxidase conjugated anti-Fas monoclonal antibody to 1000ul of the conjugate diluent.

3. Standards

   a) sFas calibrator was reconstituted with 100ul of distilled water.

   b) The reconstituted calibrator 1:100 was diluted with Assay diluent accurately to make the first standard by adding 10ul of the reconstituted Calibrator to 990ul of Assay diluent.

   c) The first standard was serially diluted with Assay diluent to make 7 standards. Assay diluent was used for 0 ng/ml standard.

- Preparation of samples

1. Serum was used for sFas ELISA assay after thawing in room temperature for 60 minutes.
2. Dilution: each sample was diluted 1:5 with Assay diluent by adding 50ul of sample to 200ul sample diluent.

- Sample incubation

  Duplicate assays were used.

  1. 150ul of prepared samples and standards were added to 96-well polyvinyl plate as the same order of assay run. After that, 100ul of each sample were transferred to the antibody-coated microwell simultaneously using multichannel pipette. Pipetting was completed as quickly as possible
  2. The plate was incubated for 60 minutes at room temperature (22C) with shaking at 200 rpm.

- Washing

  The well contents were discarded and the wells were washed with wash solution and the contents were discarded. The wells were washed four times using wash bottles. Washing buffer was used at room temperature (23C).

- Conjugate incubation

  1. Conjugate solution was poured into the vessel. After removing wash solution that remained completely, 100ul of conjugate solution were pipetted to each well with multichannel pipette.
  2. The conjugate solution in the wells was incubated for 60 minutes at room temperature with shaking at 200 rpm.

- Washing

The well contents were discarded and the wells were washed with wash solution and the contents were discarded. The wells were washed four times using wash bottles. Washing buffer was used at room temperature (23C).

- Substrate incubation

  1. Substrate reagent was pored into the vessel by adding 100ul of prepared Substrate reagent to each well. Substrate reagent was used at room temperature and the vessel used was different from the one used for pouring Conjugate solution.
  2. The substrate was incubated at room temperature for 30 minutes with shaking at 200 rpm.

- Stopping reaction

  Stop solution was poured into the vessel. 100ul of stop solution was pipetted to each well with multichannel pipette.

- Reading

  The absorbance of each well was read at 450 nm. Reading was done promptly after stopping reaction.

- Calculation

  The mean absorbance value of each standard was calculated, and was plotted and was plotted to construct a standard curve (Absorbance on the vertical axis, concentration (ng/ml) on the horizontal axis. The concentration of samples was calculated by multiplying the value read from the standard curve by dilution factor (X 5).

Serum FasL ELISA

Procedure

- Preparation of Reagents

1. Wash solution

   100 ml of Wash concentrate was diluted with 900 ml of distilled water.

2. Conjugate solution

   Peroxidase conjugated anti-Fas Ligand monoclonal antibody 1:101 was diluted with conjugate diluent by adding 10ul of the Peroxidase conjugated anti-Fas monoclonal antibody to 1000ul of the conjugate diluent.

3. Standards

   d) sFas Ligand calibrator was reconstituted with 100ul of distilled water (250ng/ml).

   e) The reconstituted calibrator (250ng/ml) 1:50 was diluted with Assay diluent accurately to make the first standard by adding 20ul of the reconstituted Calibrator to 980ul of Assay diluent.

   f) The first standard was serially diluted with Assay diluent to make 6 standards. Assay diluent was used for 0 ng/ml standard.

4. Substrate solution

   Just prior to colour development, equal volumes of substrate A and substrate B. The solution was used as soon as possible using disposable new pipette and vessel, as the substrate solution is easy to oxidized by metal ion.

- Preparation of samples

  1. Serum was used for sFas ELISA assay after thawing in room temperature for 60 minutes.
  2. Dilution: each sample was diluted 1:5 with Assay diluent by adding 50ul of sample to 200ul sample diluent.
  3. Dilution

Each sample was diluted 1:2 with assay diluent by adding 100ul of sample to 100ul Assay diluent.

- Sample incubation

Duplicate assays were used.

1. 150ul of prepared samples and standards were added to 96-well polyvinyl plate as the same order of assay run. After that, 100ul of each sample were transferred to the antibody-coated microwell simultaneously using multichannel pipette. Pipetting was completed as quickly as possible
2. The plate was incubated for 60 minutes at room temperature.
3. Washing

The well contents were discarded and the wells were washed with wash solution and the contents were discarded. The wells were washed four times using wash bottles. Washing buffer was used at room temperature (23C).

- Conjugate incubation

3. Conjugate solution was poured into the vessel. After removing wash solution that remained completely, 100ul of conjugate solution were pipetted to each well with multichannel pipette.
4. The conjugate solution in the wells was incubated for 60 minutes at room temperature.

- Washing

The well contents were discarded and the wells were washed with wash solution and the contents were discarded. The wells were washed four times using wash bottles. Washing buffer was used at room temperature.

- Substrate incubation

4. Substrate reagent was pored into the vessel by adding 100ul of prepared Substrate reagent to each well. Substrate reagent was used at room temperature and the vessel used was different from the one used for pouring Conjugate solution.
5. The substrate was incubated at room temperature for 30 minutes.

- Stopping reaction

  Stop solution was poured into the vessel. 100ul of stop solution were pipetted to each well with multichannel pipette.

- Reading

  The absorbance of each well was read at 450 nm. Reading was done promptly after stopping reaction.

- Calculation

  The mean absorbance value of each standard was calculated, and was plotted and was plotted to construct a standard curve (Absorbance on the vertical axis, concentration (ng/ml) on the horizontal axis. The concentration of samples was calculated by multiplying the value read from the standard curve by dilution factor (X 2).

Placental expression of Fas and FasL in preeclamptic, normotensive IUGR and control pregnancies.

Western blotting

Western blotting was used to study placental expression of Fas and FasL using the principles described in chapter two. Four placental samples from pregnancies complicated by preeclampsia with preserved EDF in umbilical artery Doppler, four samples from normotensive IUGR with AREDF in umbilical artery Doppler and four

gestational age matched controls were studied. The gestational age of the placentas studied (disease and control groups) ranged from 28-32 weeks. All disease samples had abnormal midtrimester uterine artery Doppler at 24 weeks.

In summary, supernatants of homogenized placental samples were thawed in ice, and then heated for 10 minutes at 90 degrees. Kleidoscope and marker samples were thawed in ice and heated for one minutes at 40C and 90C respectively. All samples were centrifuged at 13G for 40 seconds, and then 50 micrograms of each sample was loaded to the wells in the prepared gel. Using running buffer, separating electrophoresis was started at 60 Amperes for 10 minutes then at 80 Amperes for 40 minutes, after which the stacking gel was removed. The gels were subsequently incubated for 30 minutes in transfer buffer. Protein was transferred from the gel to blotting membrane with blot buffer and two hours were allowed for protein transfer at 100V under cooling conditions using ice. Membranes were then incubated overnight in blocking solution in a cold room.

Membranes were then incubated in primary antibody solution (Fas and FasL, R&D systems, Abingdon, UK) at a concentration of 1:500 ug/L for two hours in the shaker. Extensive washing was then performed for 30 minutes followed by secondary antibody incubation (anti mouse IgG (for Fas) and anti goat IgG (for FasL), heavy and light chain specific peroxidase conjugate, Calbiochem, USA) at a concentration of 1/2000 for one hour. Membranes were then extensively washed for 30 minutes, after which they were incubated for five minutes in ECL solution (equal volumes of Supersignal West Pico stable peroxide solution and Supersignal West Pico Luminol enhancer solution (Piercenet, Rockford, IL, USA). Films were then developed in a

dark room and membrane stripping was then performed to assess actin expression according to the technique in chapter 2. The intensity of staining for the studied protein and actin was then assessed using *Bio-Rad Molecular Analyst* Software package densometer. Fas/actin ratio was used to assess level of Fas protein expression in the three study groups.

Immunohistochemistry

Three-micron sections of paraffin-embedded placental tissue were deparaffinized in xylene. The avidin-biotin complex method was used for staining using the Vector Elite Kit (PK-6200). The primary antibodies used were polyclonal rabbit Fas L (N-20) antibodies (Santa-Cruz Biotechnology, Santa Cruz, California). A positive and negative tissue control was included in each batch of staining. A placenta from an uncomplicated pregnancy delivered at term was used for this purpose. The negative control was processed identically to the samples, but without the addition of the primary antibody. The positive control is treated in the same way as the samples, in the presence of the primary antibody. These controls allow the specificity of the staining to be monitored so conditions can be altered if necessary and allows for any batch-to-batch differences to be accounted for.

All slides were counter-stained with Mayers Haemalum to facilitate the recognition of tissue structure and hence make it possible to identify the cells or area of interest, i.e the villous trophoblast. Finally, slides were dehydrated in alcohol, cleaned in xylene and mounted in Canada Balsam (VWR international, UK).

An initial optimization study was performed on Fas L antibodies to achieve optimal specific staining with minimal background staining (DAKO Corporation, 2001). The placental control tissue was used for optimization, and a primary antibody dilution of 1:80 was used for Fas L. Two observers who were blinded to clinical conditions examined the specimens using the intensity of staining of the trophoblast. The differences in Fas ligand expression were assessed in the trophoblast from the three groups.

6.3 Results

Serum Fas and FasL levels at 24 weeks in women with abnormal uterine artery Doppler

FasL level was below the kits detection level in all cases at 24 weeks. There were no differences in serum Fas level at 24 weeks in women with abnormal uterine artery Doppler that subsequently developed preeclampsia, normotensive IUGR or normal outcome.

Serum Fas and FasL levels at the time of the disease in women with abnormal uterine artery Doppler

FasL levels were below the kits detection level in all cases at the time of the disease. When following serum Fas longitudinally in the three study groups, no significant changes in Fas level with gestational age were found. In addition, there were no significant differences in serum Fas between pregnancies that developed preeclampsia, normotensive IUGR (within 24 hr of delivery) and normal outcome. Tables 6.1-6.3 summarize serum Fas values in the three study groups at 24 weeks and at the time of the disease. Figures 6.1 and 6.2 show a box and plot presentation of serum Fas levels in the three study groups at 24 weeks and at the time of the disease, respectively.

Table 6.1 Serum Fas values at 24 weeks and at the time of the disease in women who developed preeclampsia (n=8), compared to control pregnancies (n=16). No significant differences were found between the two groups at 24 weeks and at the time of the disease.

	Preeclampsia Median (range) (ng/ml)	Control Median (range) (ng/ml)	P value[+]
24 weeks	12.23 (3.05-16.05)	11.56 (4.93-23.12)	0.976
Disease	12.71 (2.66-22.59)	9.15 (1.92-23.12)	0.372

[+] Mann Whitney *U* Test

Table 6.2 Serum Fas values at 24 weeks and at the time of disease in women who developed normotensive IUGR (n=7), compared to control pregnancies (n=16). No significant differences were found between the two groups at 24 weeks and at the time of the disease.

	IUGR Median (range) (ng/ml)	Control Median (range) (ng/ml)	P value[+]
24 weeks	7.47 (1.67-9.86)	11.56 (4.93-23.12)	0.061
Disease	7.71 (4.34-12.20)	9.15 (1.92-23.12)	0.423

[+] Mann Whitney Test

Table 6.3 Serum Fas values at 24 weeks and at the time of disease in women who developed preeclampsia (n=8), compared to normotensive IUGR pregnancies (n=7). No significant differences were found between the two groups at 24 weeks and at the time of the disease.

	Preeclampsia Median (range) (ng/ml)	IUGR Median (range) (ng/ml)	P value[+]
24 weeks	12.23 (3.05-16.05)	7.47 (1.67-9.86)	0.165
Disease	12.71 (2.66-22.59)	7.71 (4.34-12.20)	0.132

[+] Mann Whitney Test

Figure 6.1 Box and plot diagram showing serum Fas levels at 24 weeks in women who subsequently developed preeclampsia (n=8), normotensive IUGR (n=7) and normal outcome (n=16). No significant differences were found in the three groups at 24 weeks. Boxes represent interquartile range, where the line represents the median. Whiskers at top and bottom represent the highest and lowest values.

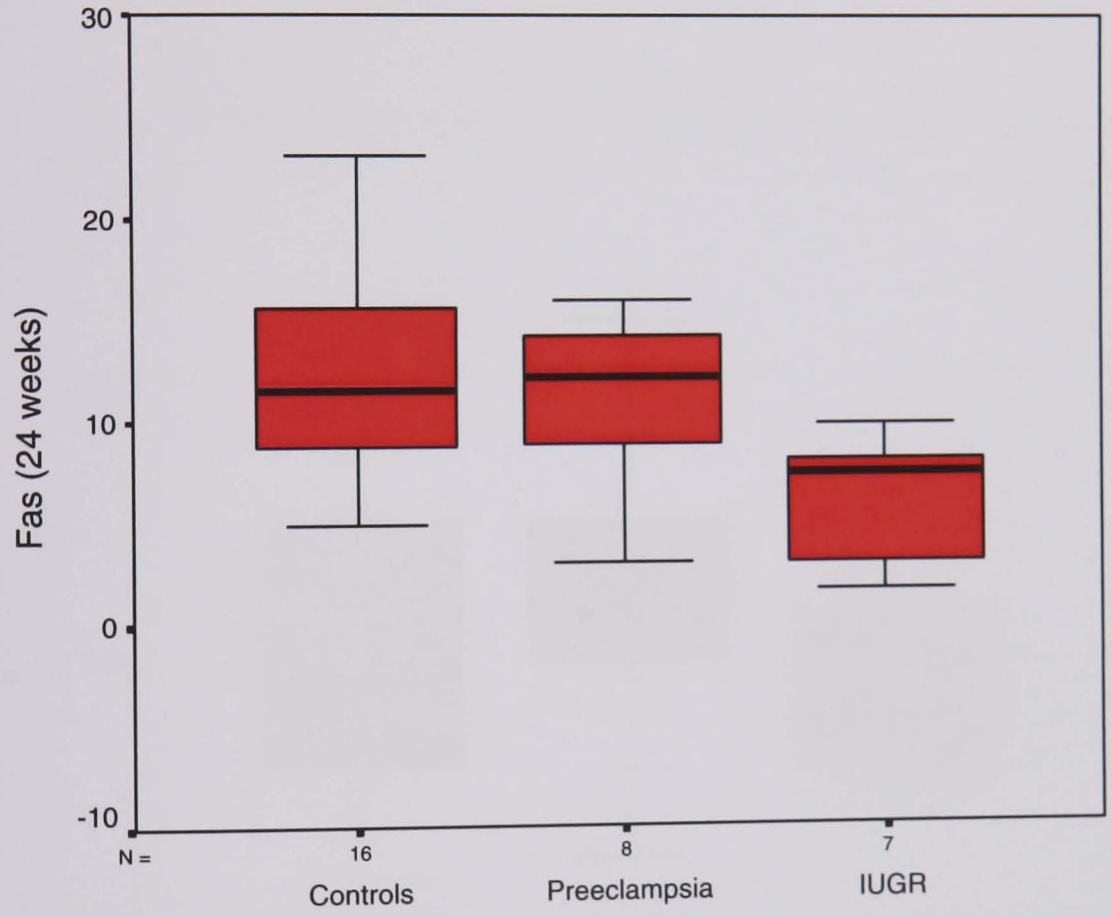
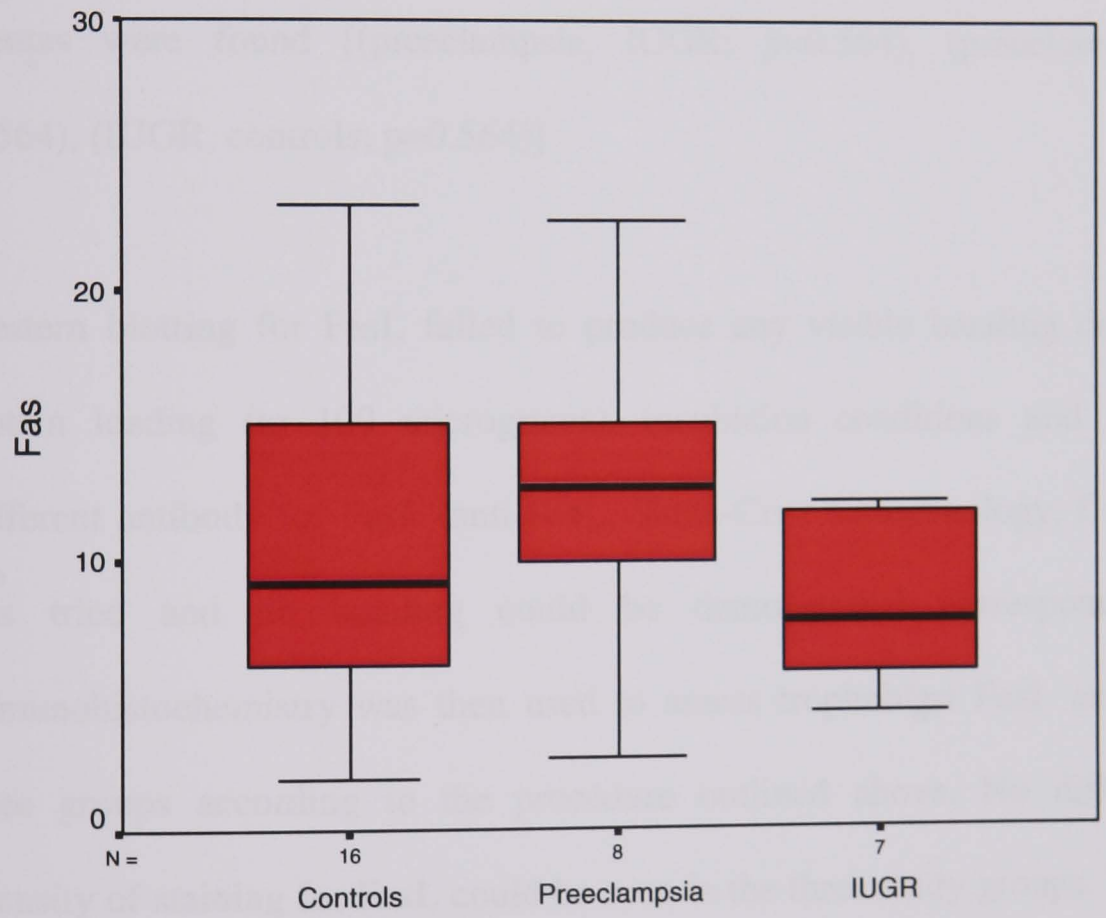


Figure 6.2 Box and plot diagram showing serum Fas levels at the time of the disease in women with abnormal uterine artery Doppler who developed preeclampsia (n=8), normotensive IUGR (n=7) and normal outcome (n=16). No significant differences were found in the three groups at the time of the disease. Boxes represent interquartile range, where the line represents the median. Whiskers at top and bottom represent the highest and lowest values.



Placental expression of Fas and FasL in women with preeclampsia, normotensive IUGR and gestational-age matched controls.

Western blotting for Fas demonstrated clear bands at a molecular weight of 45kDa corresponding to the Fas protein. Figures 6.3 and 6.4 illustrate placental expression of Fas and actin in the three study groups, respectively. Table 6.4 shows densometer values for Fas/actin ratio in the placentas studied from the three groups. No differences in placental expression of Fas between preeclamptic, normotensive IUGR and control placentas were found [(preeclampsia, IUGR; p=0.564), (preeclampsia, controls; p=0.564), (IUGR, controls; p=0.564)].

Western blotting for FasL failed to produce any visible banding despite changing protein loading (to 100 micrograms), incubation conditions and reaction times. Different antibody for FasL (anti-FasL, Santa-Cruz biotechnology, California, USA) was tried and no banding could be demonstrated corresponding to FasL. Immunohistochemistry was then used to assess trophoblast FasL expression in the three groups according to the procedure outlined above. No differences in the intensity of staining for FasL could be seen in the three study groups. Figures 6.5 and 6.7 demonstrate placental expression of FasL in the three groups using immunohistochemistry.

Figure 6.3 Western blotting for trophoblast expression of Fas in placentas from normotensive IUGR (I), preeclamsia (P) and control (C) pregnancies with abnormal uterine artery Doppler. No significant differences in placental expression of these proteins were found between the three study groups.

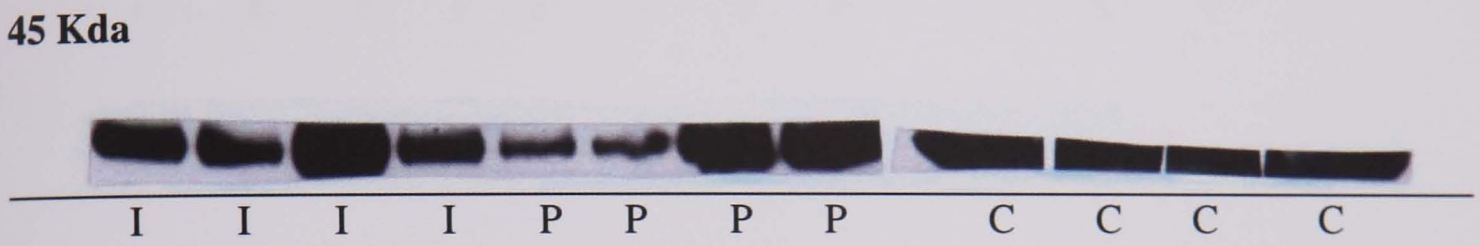


Figure 6.4 Actin expression in the groups studied; I: IUGR pregnancies, P: preeclampsia, C: control pregnancies.

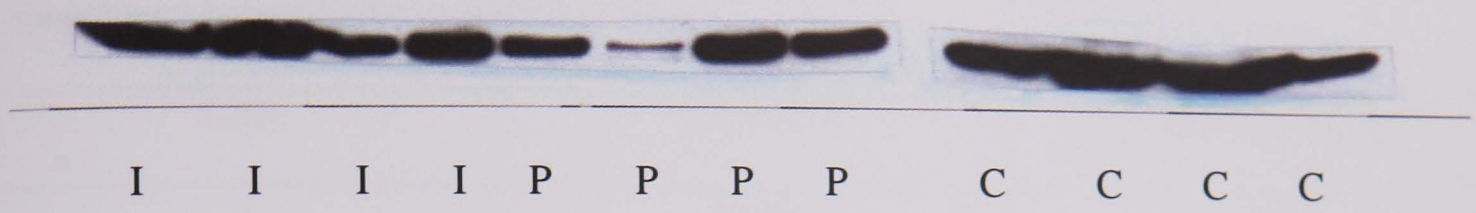


Table 6.4 Fas/actin ratio in placentas from the study groups (normotensive IUGR, preeclampsia, and controls). No significant differences in placental Fas expression were found between the groups (see text).

Case	Condition	Fas/Actin ration
1	IUGR	1.333
2	IUGR	1.077
3	IUGR	5.532
4	IUGR	1.354
5	PET	0.609
6	PET	14.038
7	PET	1.943
8	PET	2.741
9	Control	3.969
10	Control	0.88
11	Control	0.80
12	Control	1.462

Figure 6.5 Box and plot presentation of placental Fas expression. Western blotting showed no significant differences in placental Fas expression between IUGR, preeclampsia and control placentas.

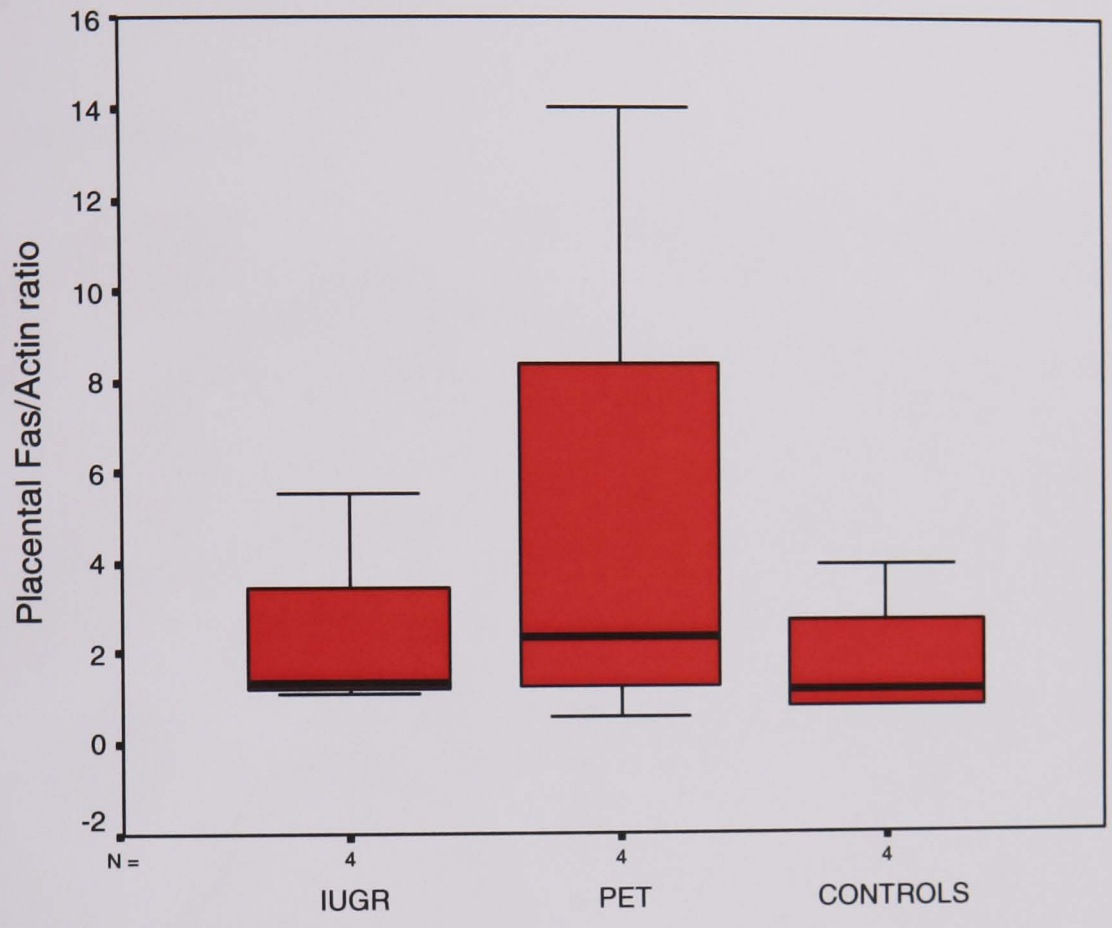
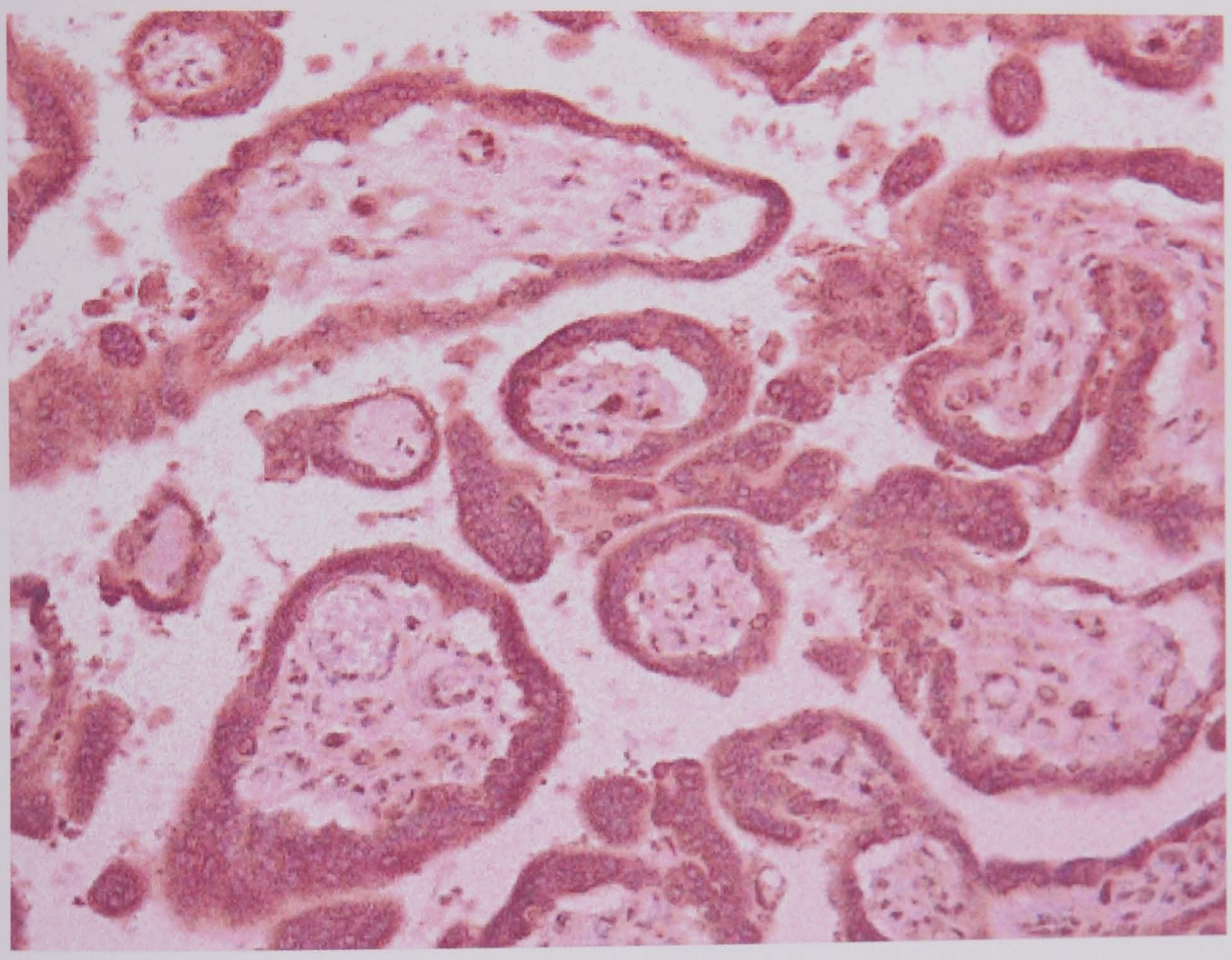
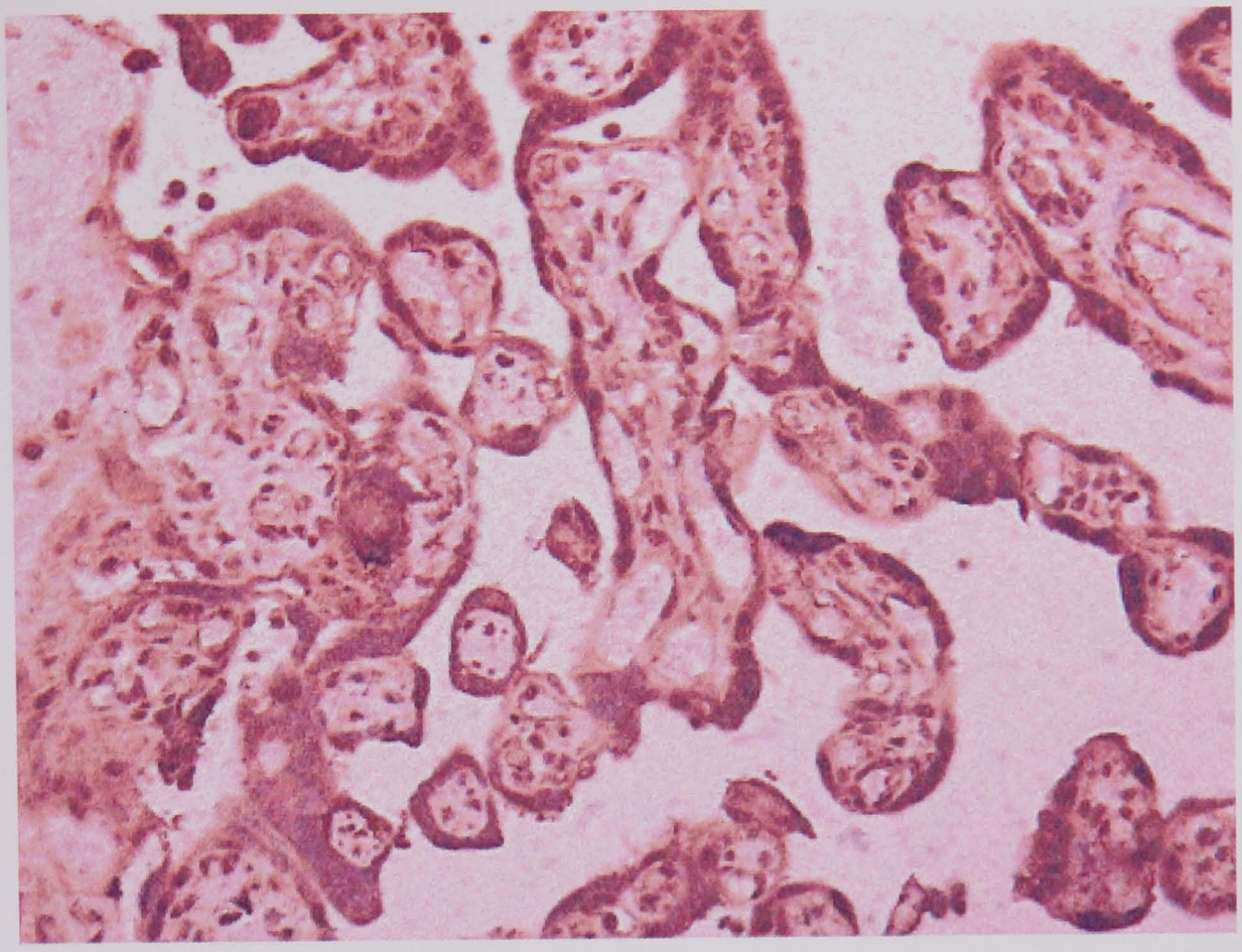


Figure 6.6 Representative samples of immunohistochemistry for trophoblast FasL expression in placentas from preeclampsia (A), normotensive IUGR (B), and normal controls (C). FasL is a membrane surface protein. No significant differences in staining intensity of trophoblast cell membranes were found between the three groups.

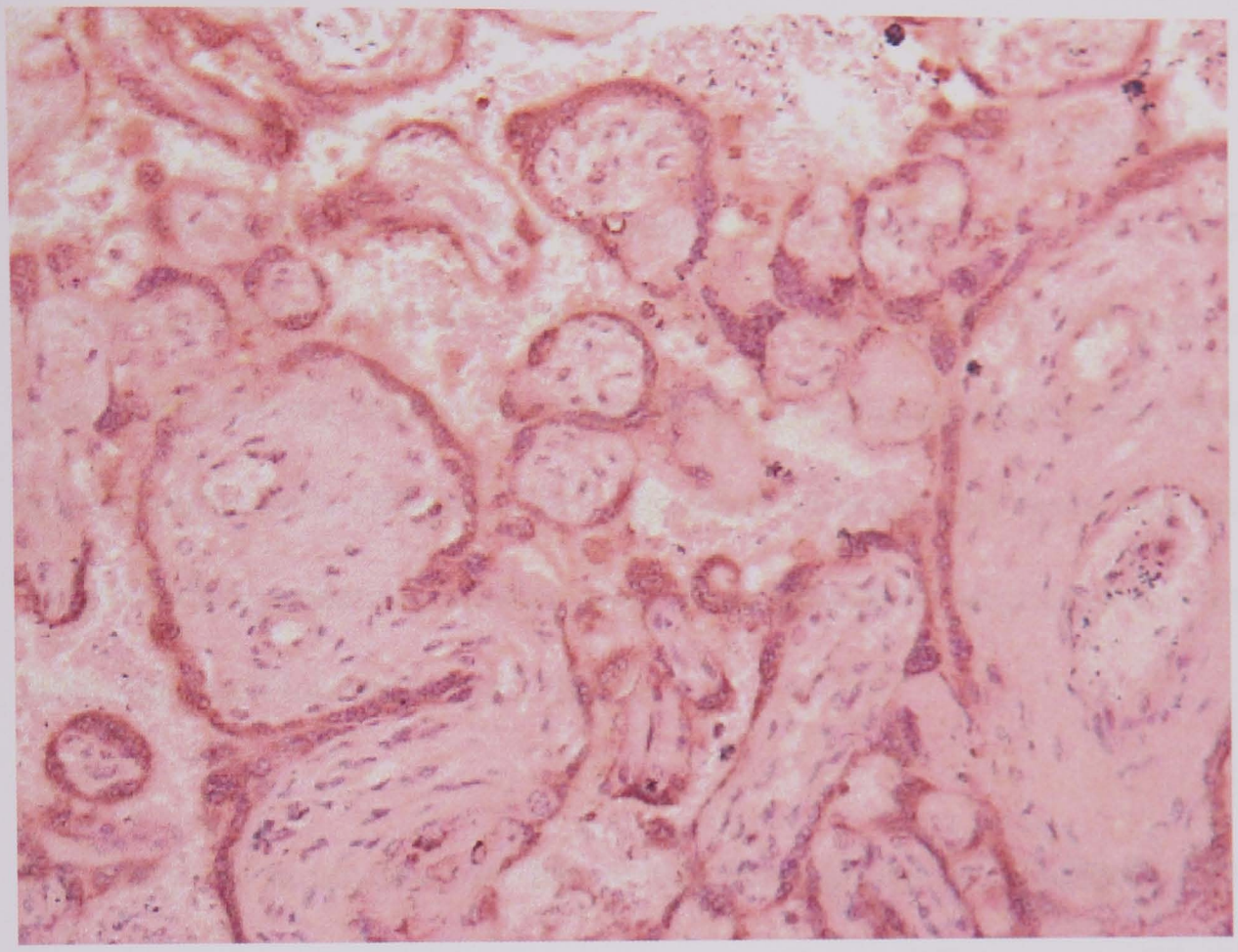
(A) Preeclampsia:



(B) Normotensive IUGR with AREDF in umbilical artery Doppler



(C) Control



6.4 Discussion

Fas (CD95, APO-1) is a ubiquitously expressed member of the tumour necrosis factor receptor superfamily, which mediates diverse cellular responses, including proliferation, inflammation, angiogenesis, and apoptosis (Jarad *et al.*, 2002). Among receptor-mediated apoptosis pathways, signals transduced by Fas stimulation have been so extensively characterized that Fas is viewed as the prototypical death receptor in susceptible cells (Nagata, 1997). However, in some cell types, Fas may mediate apoptosis-independent processes such as proliferation, angiogenesis, fibrosis, and inflammation (Hohlbaum *et al.*, 2002).

My study on serum level of Fas in the three groups (preeclampsia, normotensive IUGR and controls) did not show any significant differences at 24 weeks (with abnormal uterine artery Doppler) or at the time of the disease. I used kits for measuring Fas and FasL produced by MBL international (Nagoya, Japan). A study of serum soluble Fas levels in preeclampsia by Hsu *et al* (2001) showed that pregnant women with preeclampsia had significantly higher serum soluble Fas levels than normotensive controls. Another study by the same group of serum soluble Fas levels in the syndrome of hemolysis, elevated liver enzymes and low platelets (HELLP) showed elevated serum soluble Fas but not FasL in HELLP pregnancies compared to gestational age matched controls (Harirah *et al.*, 2001). These findings were later challenged by Kuntz *et al* (2001), who showed that serum soluble Fas levels were similar in both preeclamptic and control pregnancies. Serum soluble FasL, on the other hand, was significantly elevated in preeclamptic gestations, both in serum and umbilical cord blood. The differences in the findings of these groups could be attributed to using different manufacturing kits. The first group used ELISA kits for

the detection of Fas and FasL by Oncogene Research Products (Cambridge, MA, USA), while the second group used kits manufactured by MBL international (Nagoya, Japan). In summary, the findings in my study do not support a role for Fas in the pathogenesis of preeclampsia or normotensive IUGR.

In this study, I evaluated trophoblast Fas and FasL expression in pregnancies complicated by pre-eclampsia and normotensive IUGR with abnormal uterine artery Doppler, compared with control pregnancies. No differences were found in Fas and FasL expression in placentas from *preterm* pre-eclampsia and IUGR compared to gestational-age matched controls. This supports and extends previous findings by Ishihara *et al* (2002) regarding placental Fas expression in preeclampsia.

The immune factors responsible for maintenance of the fetal and placental semi-allogenic graft appear to lie at the uterus-placenta interface. Expression of FasL and complement regulatory proteins, along with failure to express MHC class I and II molecules in the placenta were thought to be crucial factors for the maintenance of pregnancy (Weetman, 1999). This is the first study to evaluate Fas and Fas ligand expression in placentas from pregnancies complicated by preeclampsia and IUGR with abnormal uterine artery Doppler. All pregnancies in the study groups delivered at a preterm gestational age (<36 weeks). This, in comparison to the study by Ishihara *et al* (2002) of the placental expression of Fas antigen in severe pre-eclampsia or IUGR at *term,* which showed no difference in Fas expression in placentas from normal pregnancies and those complicated by pre-eclampsia and IUGR. They, however, confirmed the earlier finding of increased apoptosis in syncytiotrophoblast of these

placentas. Reduced expression of Bcl-2, a survival factor, in the diseased trophoblast was found, which could probably contribute to enhance apoptosis in these placentas.

Mor *et al* (1998) illustrated that placental expression of Fas/Fas ligand is vital for the survival of invading trophoblast and seems to be involved in regulation of placental growth in first trimester normal pregnancies. In their study, Fas and FasL expression was confirmed by immunohistochemistry and double immunoflouresence in villous trophoblast cells from normal first trimester placentas and seems to be important in maintaining homeostasis during the process of normal trophoblast growth and invasion (Runic *et al.,* 1996). Acshkenazi *et al* (2002) showed that Fas expression in early pregnancy trophoblast is affected by cytokines secreted by T helper and T suppresser lymphocytes. This balance affects Fas expression, activation, and in turn may affect the ability of invading trophoblast to survive. A recent study of the function of Fas membrane proteins in renal tubular cells demonstrated a relation between membrane Fas induction and β integrin expression and function in the absence of apoptosis. It was found that β integrin induction is an additional pathway that is regulated by constitutively expressed Fas. β Integrins play an important role in the endometrial phenotype change that occurs during the secretory phase and the first stage of implantation, and has a clear role in trophoblast invasion during the early stages of pregnancy (Merviel *et al.,* 2001). In an in-vitro model of extra villous trophoblast invasion of uterine spiral arteries, Ashton *et al.* (2005) showed that endothelial apoptosis, induced by extravillous trophoblast, is a possible mechanism by which the endothelium in spiral arteries is removed, and consequently vascular remodeling may occur. Apoptosis was blocked by caspase inhibition and FasL blocking antibody, providing evidence that Fas/FasL interactions have an important

role in trophoblast-induced endothelial apoptosis. This study used extravillous trophoblast to induce apoptosis in spiral artery endothelium. My study, on the other hand, assessed placental trophoblast expression of Fas and FasL. This could be a factor causing the difference between these two studies.

The aetiology of increased apoptosis in human trophoblast in preeclampsia and IUGR is not entirely clear. The study by Allaire *et al* (2000) showed reduced Fas ligand and enhanced Fas expression in trophoblast from pregnancies complicated by preeclampsia. They concluded that reduced Fas ligand expression in the placental trophoblast could account for the increased apoptosis given the role of Fas ligand in evading immune detection of the placenta by maternal cytotoxic T lymphocytes and Natural Killer (NK) cells. Koenig and Chegini (2000), on the other hand, found increased Fas ligand expression in the syncytiotrophoblast from pregnancies complicated by pre-eclampsia. Both studies, however, relied solely on immunohistochemistry for the assessment of Fas/FasL expression. Immunohistochemistry has limitations in evaluating tissue protein expression, attributed to the technique-related variability and section-dependent staining intensity. Other factors that could contribute to apoptosis in human trophoblasts is hypoxia. Levy *et al* (2000) demonstrated that hypoxia up-regulated p53 and Bax expression in cultured trophoblasts; both enhance apoptosis at a cellular level through the mitochondrial pathway. Hypoxia also reduced the expression of Bcl-2, a cellular survival factor that protects against apoptosis (Levy *et al.*, 2000). These findings implicate the mitochondrial pathway in enhanced apoptosis in cultured trophoblasts exposed to low oxygen tension. A recent study by Levy *et al* (2002) showed a two-

fold increase in p53 expression in placental villi from placentas of fetuses with IUGR compared with placentas from normal pregnancies.

Studies on the role of Fas ligand as the enforcer of immune privilege does not fit the established paradigm of this role. When Allison *et al* (1997) used transgenic mice expressing FasL on their islet β cells for transplantation, they found that rather than being the solution to the transplantation immunologist's rejection problem, expression of FasL caused a more rapid rejection of islet cells accompanied by a "granulocyte infiltration". Kang *et al* (1998) used an entirely different approach to test the same question, using adenoviruses to confer FasL expression on islet cells. They also found accelerated rejection accompanied by "massive neutrophilic infiltrates".

In summary, although the Fas/FasL system seems important for early pregnancy implantation and trophoblast survival, the findings in this study do not support a role for Fas or Fas ligand in abnormal placentation in pregnancies complicated by preterm pre-eclampsia and IUGR. Further research is needed to the etiology of impaired trophoblast invasion and reduced trophoblast mass available to regulate maternal-fetal exchange in these conditions.

GENERAL DISCUSSION

The work carried out in this thesis characterises abnormalities within the serum and the placenta of women with abnormal midtrimester uterine artery Doppler, that develop preeclampsia, compared to normotensive IUGR and controls. This provides an insight into the mechanisms and consequences of abnormal placentation.

Preeclampsia has been sometimes termed the "disease of theories," as several models for its pathogenesis have been proposed. But, as of today, no satisfactory unifying hypothesis has emerged (Roberts & Cooper, 2001). For the fetus to develop normally, it must receive sufficient oxygen and nutrients. These are supplied via the maternal spiral arteries in the uterus. During normal pregnancy, cytotrophoblasts convert from an epithelial to an endothelial phenotype (a process termed pseudo-vasculogenesis) and invade maternal spiral arteries. By the end of the first trimester, this vascular remodelling increases the bulk flow and the supply of nutrients and oxygen to the fetus (Goldman-Wohl & Yagel, 2002; Zhou *et al.,* 2002). In preeclampsia, pseudo-vasculogenesis is defective, and the resultant placental ischaemia has been proposed as a trigger for the release of unknown-placenta derived factors. The latter would induce systemic endothelial dysfunction and therefore contribute to the renal, cardiovascular, and neurological manifestations of preeclampsia. Despite intensive efforts, the precise nature of the placenta-derived factors has remained enigmatic for years.

The research in this thesis looked at differences in the serum and placenta between preeclamptic and normotensive IUGR pregnancies with abnormal uterine artery Doppler. Strict criteria were employed and pregnancies included had regular growth scans, uterine and umbilical artery Doppler examination and clinical assessment for

preeclampsia. Strict definitions were used for inclusion in the IUGR group, so as to avoid including normal but "small for gestational age" pregnancies. This, in a way, limited the number of patients enrolled in the study, as only patients who were followed from 24 weeks were eligible. I also compared pregnancies that were matched for gestational age, to limit the potential confounding effect of gestational age on sFlt1 and PlGF. Pregnancies with abnormal mid-trimester uterine artery that developed preeclampsia after 36 weeks were not included as the upper gestational-age in the normotensive IUGR group was 35 weeks. This explains the relatively low positive predictive value of uterine artery Doppler in predicting abnormal pregnancy outcome in my study, compared to other studies.

Uterine artery Doppler examination at 24 weeks showed no difference in uterine artery resistance indices between pregnancies that developed preeclampsia compared to normotensive IUGR. This provided evidence that shallow trophoblast invasion was the basic pathology behind both conditions, but there was no difference in the degree of impairment of trophoblast invasion between the two groups, which could explain the difference in clinical outcome. Uterine artery Doppler resistance was measured in the 24 hour period prior to delivery, in pregnancies enrolled in the study, expecting a difference in trophoblast invasion between the two groups (preeclampsia and IUGR), which could account for the difference in clinical outcome. Again, no differences could be found between the two groups in uterine artery Doppler resistance before delivery. As uterine artery resistance indirectly reflects depth of trophoblast invasion, my findings rule out differences in trophoblast invasion as the underlying cause for the difference in clinical outcome between preeclampsia and normotensive IUGR. The unexpected finding in my study, however, was the significant differences in

umbilical artery resistance between the two groups in the 24-hour period prior to delivery. This reflects different angiogenic responses within the placenta in the two groups and indirectly a reflection of the placental oxygen level, which could be contributing to the systemic manifestations of preeclampsia.

Recent work by Maynard *et al* (2003) and Ahmad & Ahmed (2004) report the novel insight that circulating levels of two angiogenic growth factors, soluble fms-like tyrosine kinase 1 (sFlt1) receptor and Placental Growth Factor (PlGF), may play a more important role than previously believed. Their study revealed that in pregnant women with preeclampsia, the placenta produces elevated levels of sFlt1, which captures free VEGF and PlGF. As a result, the normal vasculature in the kidney, brain, lungs, and other organs is deprived of essential survival and maintenance signals and becomes dysfunctional. In this thesis, I report a novel insight into differences in serum sFlt1 and PlGF between preeclamptic and normotensive IUGR pregnancies with abnormal uterine artery Doppler, compared with controls that had a normal pregnancy outcome. It appears that sFlt1 plays a central role in the systemic manifestations of preeclampsia through antagonism of VEGF. Whereas dynamic surges of high VEGF levels mediate angiogenesis in the embryo and in the adult during disease, continuous low levels of VEGF are required for endothelial cells to survive prolonged periods and function properly (Luttun & Carmeliet, 2003). Thus, when sFlt1 plasma levels rise, they may reduce the circulating VEGF and PlGF levels below a critical threshold required for maintenance of the established vasculature in the adult. The resultant endothelial dysfunction may disrupt the blood brain barrier and cause intracranial hypertension, oedema in the liver, and affect glomerular function.

The finding of significant differences in umbilical artery Doppler between the preeclampsia and normotensive IUGR groups provides further evidence that placental hypoxia could be the centre piece in the development of preeclampsia. Absent end diastolic flow in umbilical artery Doppler reflects predominance of non-branching angiogenesis within the placenta, a factor that results in higher placental oxygen levels. That this situation could arise in the face of uteroplacental ischaemia is superficially hard to understand until one appreciates that the catabolic severely-IUGR fetus (with reduced activity) is surviving with minimal oxygen consumption (Pardi *et al.,* 1993). Fractional extraction of oxygen from the intervillous space is greatly reduced with AREDF in the umbilical arteries, such that uteroplacental venous blood has 30% higher oxygen content than under normal circumstances (Pardi *et al.,* 1992). Early-onset IUGR and abnormal umbilical artery Doppler is very uncommon in unselected pregnancies (Mason *at al.,* 1993), while between 5-10% of such pregnancies are often conveniently assigned as IUGR based on birth weight centiles (the correct term for the latter is small-for-gestational age). The pathology of AREDF in umbilical artery Doppler, and thus the phenomenon of placental hyperoxia, is thus the exception to the rule that the placenta adapts to reduced uteroplacental blood flow (Kingdom *et al.,* 1998).

My findings of increased placental Flt1 and serum sFlt1 in preeclampsia supports the notion of appropriate placental adaptation to reduced uteroplacental placental blood flow in these pregnancies. This, in turn, resulted in placental hypoxia and upregulated *sFlt1* expression and consequently the syndrome of preeclampsia. This sequence of events does not start in cases of normotensive IUGR (especially with AREDF in umbilical artery) due to much lower degree of placental hypoxia. My data, however,

demonstrated that serum sFlt1, in the 24 hours before delivery, in cases of normotensive IUGR, was significantly higher than controls. This could be due to a degree of placental hypoxia in these cases, albeit not as severe as that in cases of preeclampsia. This degree of placental hypoxia is to be expected since three out of the seven cases of normotensive IUGR had preserved end-diastolic flow in umbilical artery Doppler. This, in the face of impaired uteroplacental blood flow, would result in a degree of placental hypoxia that upregulates serum sFlt1. In addition, the level of sFlt1 needed to trigger widespread endothelial dysfunction could be different between different pregnancies, depending on the baseline degree of endothelial susceptibility in the maternal vasculature. My findings of inverse correlations between serum sFlt1 and umbilical artery resistance indices at the time of delivery in affected pregnancies, provides further evidence of the apparent effect of umbilical artery blood flow on placental hypoxia and consequently serum sFlt1.

The cause of impaired trophoblast invasion and spiral artery remodelling in preeclampsia and IUGR is not clear. Abnormal uterine artery Doppler provides indirect in-vivo evidence of impaired trophoblast invasion. Ahmad & Ahmed (2004), showed that sFlt1 inhibits in-vitro placental angiogenesis and endothelial cell migration. In this thesis, I provided further evidence of the role of sFlt1 on impaired trophoblast invasion and placental angiogenesis, as positive correlations were found between uterine artery resistance indices at 24 weeks and the corresponding serum sFlt1. This sheds unprecedented light on the pathogenesis of impaired vascular remodeling in pregnancies with abnormal uterine artery Doppler that develop preeclampsia and IUGR.

There are a few limitations to the research in this thesis. First, serum values for sFlt1 and PlGF were used to reflect placental expression of these markers in the mid-trimester. Whether this association between placental and serum expression of these markers remains valid needs further evaluation. Furthermore, placental expression of Flt1 and PlGF was assessed in villous, but not the extra villous trophoblast. This makes it difficult to draw conclusions on the role of placental Flt1 and PlGF in trophoblast invasion and spiral artery remodeling, a function of extra-villous cytotrophoblasts. However, it could be reliably concluded that differences in placental expression of Flt1 between preeclampsia and IUGR do exist, and most likely a result of differences in placental hypoxia between the two groups.

Increased trophoblast apoptosis occurs in placentas from pregnancies complicated by IUGR and preeclampsia. The potential effect of apoptosis on impaired trophoblast invasion and spiral artery remodelling is not clear. I studied trophoblast expression of Fas and FasL in placentas from pregnancies with abnormal uterine artery Doppler. No differences in Fas and FasL expression were found between the groups studied. Furthermore, levels of serum soluble Fas were not different between the preeclampsia, normotensive IUGR and control groups at 24 weeks and at the time of the disease. This is in contrast to previous reports that showed conflicting evidence about serum Fas levels in preeclampsia. My findings do not support a role for the Fas/FasL system in the pathogenesis or the clinical manifestations of preeclampsia or IUGR. In my study, however, placental expression of Fas and FasL was evaluated in villous rather than extravillous trophoblast. Therefore, no conclusion could be made about the role of these surface proteins in impaired placentation. Recent evidence suggests that Fas/FasL interaction is involved in endothelial apoptosis induced by extra-villous

trophoblast during spiral artery remodeling in vitro (Ashton *et al.*, 2005). The in-vivo role of Fas and FasL in impaired placentation needs to be investigated in a study of expression of these surface markers in extra-villous trophoblasts in cases of abnormal uterine artery Doppler that develop preeclampsia and IUGR.

In summary, the clinical syndrome of preeclampsia and IUGR is the concluding episode of a story of failed physiological modification of the uterine circulation by the developing placenta. The work described in this thesis demonstrates that:

1. Serum sFlt1 correlates positively with uterine artery resistance indices at 24 weeks and provides further evidence of the role sFlt1 in failed pseudo-vasculogenesis and maternal spiral artery remodeling in preeclampsia and IUGR.
2. Placental Flt1 and serum sFlt1 is upregulated in preeclampsia compared to normotensive IUGR and controls. This is due to placental hypoxia in preeclampsia and, through capturing free VEGF and PlGF; serum sFlt1 contributes to the widespread endothelial dysfunction in this condition.
3. Placental and serum Fas is not changed in pregnancies with preeclampsia or IUGR, and consequently the role of this protein in the pathogenesis of these conditions is doubtful.

REFERENCES

Aalkjaer, C., Danielsen, H., Johannesen, P., Pedersen, E.B., Rasmussen, A. & Mulvany, M.J. (1985). Abnormal vascular function and morphology in pre-eclampsia: a study of isolated resistance vessels. *Clin Sci (Colch)*, **69,** 477-82.

Abrams, B., Newman, V. (1991). Small-for-gestational-age birth: maternal predictors and comparison with risk factors of spontaneous preterm delivery in the same cohort. *Am J Obstet Gynecol,* **164,** 785-90.

Ahmad, S., Ahmed, A. (2004). Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia. *Circ Res,* **95,** 884-91.

Ahmed, A. (1997). Heparin-binding angiogenic growth factors in pregnancy. *Trophoblast Res,* **10,** 215-58.

Ahmed, A., Dunk, C., Ahmad, S., Khaliq, A. (2000). Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF) and soluble Flt-1 by oxygen--a review. *Placenta,* **21 Suppl,** A:S16-24.

Ahmed, A., Kilby, M. (1997). Hypoxia or hyperoxia in placental insufficiency? *Lancet,* **350,** 826-7.

Albaiges, G., Missfelder-Lobos, H., Lees, C., Parra, M., Nicolaides, K.H. (2000). One-stage screening for pregnancy complications by color Doppler assessment of the uterine arteries at 23 weeks' gestation. *Obstet Gynecol,* **96,** 559-64.

Algeciras-Schimnich, A., Shen, L., Barnhart, B.C., Murmann, A.E., Burkhardt, J.K., Peter, M.E. (2002). Molecular ordering of the initial signaling events of CD95. *Mol Cell Biol*, **22,** 207-20.

Allaire, A.D., Ballenger, K.A., Wells, S.R., McMahon, M.J., Lessey, B.A. (2000). Placental apoptosis in preeclampsia. *Obstet Gynecol*, **96,** 271-6.

Allison, J., Georgiou, H.M., Strasser, A., Vaux, D.L. (1997). Transgenic expression of CD95 ligand on islet beta cells induces a granulocytic infiltration but does not confer immune privilege upon islet allografts. *Proc Natl Acad Sci U S A*, **94,** 3943-7.

Anthony, F.W., Evans, P.W., Wheeler, T., Wood, P.J. (1997). Variation in detection of VEGF in maternal serum by immunoassay and the possible influence of binding proteins. *Ann Clin Biochem*, **34,** 276-80.

Aquilina, J., Barnett, A., Thompson, O., Harrington, K. (2000). Comprehensive analysis of uterine artery flow velocity waveforms for the prediction of pre-eclampsia. *Ultrasound Obstet Gynecol*, **16,** 163-70.

Arduini, D., Rizzo, G., Romanini, C. (1992). Changes of pulsatility index from fetal vessels preceding the onset of late decelerations in growth-retarded fetuses. *Obstet Gynecol*, **79,** 605-10.

Arduini, D., Rizzo, G., Romanini, C., Mancuso, S. (1987). Utero-placental blood flow velocity waveforms as predictors of pregnancy-induced hypertension. *Eur J Obstet Gynecol Reprod Biol*, **26,** 335-41.

Arngrimsson, R., Bjornsson, S., Geirsson, R.T., Bjornsson, H., Walker, J.J. & Snaedal, G. (1990). Genetic and familial predisposition to eclampsia and pre-eclampsia in a defined population. *Br J Obstet Gynaecol*, **97,** 762-9.

Aschkenazi, S., Straszewski, S., Verwer, K.M., Foellmer, H., Rutherford, T., Mor, G. (2002). Differential regulation and function of the Fas/Fas ligand system in human trophoblast cells. *Biol Reprod*, **66,** 1853-61.

Ashton, S.V., Whitley, G.S., Dash, P.R., Wareing, M., Crocker, I.P., Baker, P.N., Cartwright, J,E. (2005). Uterine spiral artery remodeling involves endothelial apoptosis induced by extravillous trophoblasts through Fas/FasL interactions. *Arterioscler Thromb Vasc Biol*, **25,** 102-8.

Ashworth, J.R. (1998) The role of the vascular endothelium in pregnancy, pre-eclampsia and intrauterine growth restriction. M.D. Thesis, School of Human Development, University of Nottingham.

Ashworth, J.R., Warren, A.Y., Baker, P.N. & Johnson, I.R. (1997). Loss of endothelium-dependent relaxation in myometrial resistance arteries in pre-eclampsia. *Br J Obstet Gynaecol*, **104,** 1152-8.

Athanassakis, I., Papadimitriou, L., Koumantakis, E., Vassiliadis, S. (2000). Th1- and Th2-type lymphokine-assisted induction and release of chemokine receptors from primary human trophoblast cells. *Hum Immunol*, **61,** 651-7.

Autiero, M., Waltenberger, J., Communi, D., Kranz, A., Moons, L., Lambrechts, D., Kroll, J., Plaisance, S., De Mol, M., Bono, F., Kliche, S., Fellbrich, G., Ballmer-Hofer, K., Maglione, D., Mayr-Beyrle, U., Dewerchin, M., Dombrowski, S., Stanimirovic, D., Van Hummelen, P., Dehio, C., Hicklin, D.J., Persico, G., Herbert, J.M., Communi, D., Shibuya, M., Collen, D., Conway, E.M., Carmeliet, P. (2003). Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med*, **9,** 936-43.

Ayhan, A., Akkok, E., Urman, B., Yarali, H., Dundar, S. & Kirazli, S. (1990). Beta-thromboglobulin and platelet factor 4 levels in pregnancy and preeclampsia. *Gynecol Obstet Invest,* **30**, 12-4.

Baker, P.N., Krasnow, J., Roberts, J.M., Yeo, K.T. (1995). Elevated serum levels of vascular endothelial growth factor in patients with preeclampsia. *Obstet Gynecol,* **86,** 815-21.

Balkundi, D.R., Ziegler, J.A., Watchko, J.F., Craven, C., Trucco, M. (2003). Regulation of FasL/Fas in human trophoblasts: possible implications for chorioamnionitis. *Biol Reprod,* **69,** 718-24.

Bamberger, A.M., Schulte, H.M., Thuneke, I., Erdmann, I., Bamberger, C.M., ASA, S.L. (1997). Expression of the apoptosis-inducing Fas ligand (FasL) in human first and third trimester placenta and choriocarcinoma cells. *J Clin Endocrinol Metab*, **82,** 3173-5.

Banks, R.E., Forbes, M.A., Searles, J., Pappin, D., Canas, B., Rahman, D., Kaufmann, S., Walters, C.E., Jackson, A., Eves, P., Linton, G., Keen, J., Walker, J.J., Selby, P.J. (1998). Evidence for the existence of a novel pregnancy-associated soluble variant of the vascular endothelial growth factor receptor, Flt-1. *Mol Hum Reprod*, **4,** 377-86.

Barden, A., Beilin, L.J., Ritchie, J., Croft, K.D., Walters, B.N. & Michael, C.A. (1996). Plasma and urinary 8-iso-prostane as an indicator of lipid peroxidation in pre-eclampsia and normal pregnancy. *Clin Sci (Colch)*, **91,** 711-8.

Barker, D.J.P. (1992). Fetal growth and adult disease. *Br J Obstet Gynecol,* **99,** 275-6.

Battaglia, F.C., Meschia, G. (1986). *An Introduction to Fetal Physiology*. 154-67. London: Academic Press.

Battistelli, M., Burattini, S., Pomini, F., Scavo, M., Caruso, A., Falcieri, E. (2004). Ultrastructural study on human placenta from intrauterine growth retardation cases. *Microsc Res Tech*, **65,** 150-8.

Bechmann, I., Mor, G., Nilsen, J., Eliza, M., Nitsch, R., Naftolin, F. (1999). FasL (CD95L, Apo1L) is expressed in the normal rat and human brain: evidence for the existence of an immunological brain barrier. *Glia*, **27,** 62-74.

Bekedam, D.J., Visser, G.H., van der Zee, A.G., Snijders, R.J., Poelmann-Weesjes, G. (1990). Abnormal velocity waveforms of the umbilical artery in growth retarded fetuses: relationship to antepartum late heart rate decelerations and outcome. *Early Hum Dev*, **24,** 79-89.

Belizan, J.M., Villar, J., Nardin, J.C., Malamud, J., De Vicurna, L.S. (1978). Diagnosis of intrauterine growth retardation by a simple clinical method: measurement of uterine height. *Am J Obstet Gynecol,* **131,** 643-6.

Benirschke,K., Kaufmann, P. eds (1995). *Pathology of human placenta,* London: Springer-Verlag.

Benjamin, L.E., Keshet, E. (1997). Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. *Proc Natl Acad Sci U S A,* **94,** 8761-6.

Bennett, W.A., Lagoo-Deenadayalan, S., Stopple, J.A., Barber, W.H., Hale, E., Brackin, M.N., Cowan, B.D. (1998). Cytokine expression by first-trimester human chorionic villi.*Am J Reprod Immunol,* **40,** 309-18.

Berg, A.T. and Bracken, M.B. (1992). Measuring gestational age: an uncertain proposition. *Br J Obstet Gynecol,* **99,** 280-2.

Bewley, S., Cooper, D., Campbell, S. (1991) Doppler investigation of uteroplacental blood flow resistance in the second trimester: a screening study for pre-eclampsia and intrauterine growth retardation. *Br J Obstet Gynaecol,* **98,** 871-9.

Blair, E., Stanley, F. (1992). Intrauterine growth and spastic cerebral palsy II. The association with morphology at birth. *Early Hum Dev*, **28,** 91-103.

Blankenship, T.N. & Enders, A.C. (1997). Expression of platelet-endothelial cell adhesion molecule-1 (PECAM) by macaque trophoblast cells during invasion of the spiral arteries. *Anat Rec*, **247,** 413-9.

Blankenship, T.N., Enders, A.C. & King, B.F. (1993). Trophoblastic invasion and modification of uterine veins during placental development in macaques. *Cell Tissue Res*, **274,** 135-44.

Bosio, P.M., Wheeler, T., Anthony, F., Conroy, R., O'herlihy, C., McKenna, P. (2001). Maternal plasma vascular endothelial growth factor concentrations in normal and hypertensive pregnancies and their relationship to peripheral vascular resistance. *Am J Obstet Gynecol*, **184,** 146-52.

Bower, S., Bewley, S., Campbell, S. (1993). Improved prediction of preeclampsia by two-stage screening of uterine arteries using the early diastolic notch and color Doppler imaging. *Obstet Gynecol*, **82,** 78-83.

Bower, S., Schuchter, K., Campbell, S. (1993). Doppler ultrasound screening as part of routine antenatal scanning: prediction of pre-eclampsia and intrauterine growth retardation. *Br J Obstet Gynaecol*, **100,** 989-94.

Boyd, J.D., Hamilton, W.J. (1970). The human placenta. Cambridge, 207-74. Heffer & Sons.

Brosens, I., Robertson, W.B., Dixon, H.G. (1972). The role of the spiral arteries in the pathogenesis of pre-eclampsia. *Obstet Gynecol Annu,* **1,** 177-91.

Brown, M., Tibben, E., Zammit, V., Cario, G. & Calton, M. (1995). Nitric oxide secretion in normal and hypertensive pregnancies. *Hypertension in Pregnancy*, **14,** 85-92.

Brown, M., Tibben, E., Zammit, V., Cario, G. & Calton, M. (1995). Nitric oxide secretion in normal and hypertensive pregnancies. *Hypertension in Pregnancy*, **14,** 85-92.

Bulmer, J.N. (1992). Immune aspects of pathology of the placental bed contributing to pregnancy pathology. *Baillieres Clin Obstet Gynaecol*, **6,** 461-88.

Burton, G.J., Reshetnikova, O.S., Milovanov, A.P., Teleshova, O.V. (1996). Stereological evaluation of vascular adaptations in human placental villi to differing forms of hypoxic stress. *Placenta,* **17,** 49-55.

Cameron, I., van Papendorp, C., Palmer, R., Smith, S. & Moncada, S. (1993). Relationship between nitric oxide synthesis and increase in systolic blood pressure. *Hypertension in Pregnancy*, **12,** 85-92.

Campbell, D.M. & Campbell, A.J. (1983). Evans Blue disappearance rate in normal and pre-eclamptic pregnancy. *Clin Exp Hypertens B*, **2,** 163-9.

Campbell, S., Dewhurst, C.J. (1971). Diagnosis of the small-for-dates fetus by serial ultrasonic cephalometry. *Lancet,* **ii,** 1002-6.

Campbell, S., Pearce, J.M., Hackett, G., Cohen-Overbeek, T., Hernandez, C. (1986). Qualitative assessment of uteroplacental blood flow: early screening test for high-risk pregnancies. *Obstet Gynecol*, **68,** 649-53.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., Nagy, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*, **380,** 435-9.

Casey, M.L., Cox, S.M., Beutler, B., Milewich, L., MacDonald, P.C. (1989). Cachectin/tumor necrosis factor-alpha formation in human decidua. Potential role of cytokines in infection-induced preterm labor. *J Clin Invest*, **83,** 430-6.

Casey, M.L., Cox, S.M., Word, R.A., MacDonald, P.C. (1990). Cytokines and infection-induced preterm labour. *Reprod Fertil Dev,* **2,** 499-509.

Castellucci, M., Scheper, M., Scheffen, I., Celona, A., Kaufmann, P. (1990). The development of the human placental villous tree. *Anat Embryol (Berl)*, **181,** 117-28.

Chan, F.Y., Pun, T.C., Lam, C., Khoo, J., Lee, C.P., Lam, Y.H. (1995). Pregnancy screening by uterine artery Doppler velocimetry--which criterion performs best? *Obstet Gynecol*, **85,** 596-602.

Chappell, L. & Bewley, S. (1998). Pre-eclamptic toxaemia: the role of uterine artery Doppler. *Br J Obstet Gynaecol*, **105,** 379-82.

Chappell, L.C., Seed, P.T., Briley, A., Kelly, F.J., Hunt, B.J., Charnock-Jones, D.S., Mallet, A.I., Poston, L. (2002 (a)). A longitudinal study of biochemical variables in women at risk of preeclampsia. *Am J Obstet Gynecol*, **187,** 127-36.

Chappell, L.C., Seed, P.T., Kelly, F.J., Briley, A., Hunt, B.J., Charnock-Jones, D.S., Mallet, A., Poston, L. (2002 (b)). Vitamin C and E supplementation in women at risk of preeclampsia is associated with changes in indices of oxidative stress and placental function. *Am J Obstet Gynecol*, **187,** 777-84.

Charnock-Jones, D.S., Sharkey, A.M., Boocock, C.A., Ahmed, A., Plevin, R., Ferrara, N., Smith, S.K. (1994). Vascular endothelial growth factor receptor localization and activation in human trophoblast and choriocarcinoma cells. *Biol Reprod*, **51,** 524-30.

Chen, J., Sun, Y., Nabel, G. (1998) Regulation of the proinflammatory effects of Fas ligand (CD95L). *Science,* **282,** 1714-1717.

Cheng, J., Zhou, T., Liu, C., Shapiro, J.P., Brauer, M.J., Kiefer, M.C., Barr, P.J., Mountz, J.D. (1994). Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. *Science*, **263,** 1759-62.

Chervonsky, A.V., Wang, Y., Wong, F.S., Visintin, I., Flavell, R.A., Janeway, C.A. Jr., Matis, L.A. (1997). The role of Fas in autoimmune diabetes. *Cell*, **89,** 17-24.

Chesley, L.C. & Cooper, D.W. (1986). Genetics of hypertension in pregnancy: possible single gene control of pre-eclampsia and eclampsia in the descendants of eclamptic women. *Br J Obstet Gynaecol*, **93,** 898-908.

Chung, J.Y., Song, Y., Wang, Y., Magness, R.R., Zheng, J. (2004). Differential expression of vascular endothelial growth factor (VEGF), endocrine gland derived-VEGF, and VEGF receptors in human placentas from normal and preeclamptic pregnancies. *J Clin Endocrinol Metab*, **89,** 2484-90.

Clark, D.E., Smith, S.K., He, Y., Day, K.A., Licence, D.R., Corps, A.N., Lammoglia, R., Charnock-Jones, D.S. (1998). A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternal circulation. *Biol Reprod.*, **59**,1540-8.

Clark, D.E., Smith, S.K., Sharkey, A.M., Charnock-Jones, D.S. (1996). Localization of VEGF and expression of its receptors flt and KDR in human placenta throughout pregnancy. *Hum Reprod*, **11,** 1090-8.

Clark, P., Boswell, F., Greer, I.A. (1998). The neutrophil and preeclampsia. *Semin Reprod Endocrinol,* **16,** 57-64.

Cnattingius, S., Axelsson, O., Lindmark, G. (1984). Symphysis-fundus measurements and intrauterine growth retardation. *Acta Obstet Gynecol Scand*, **63**, 335-40.

Cockell, A.P., Learmont, J.G., Smarason, A.K., Redman, C.W., Sargent, I.L. & Poston, L. (1997). Human placental syncytiotrophoblast microvillous membranes impair maternal vascular endothelial function. *Br J Obstet Gynaecol*, **104,** 235-40.

Colbern, G.T., Chiang, M.H. & Main, E.K. (1994). Expression of the nonclassic histocompatibility antigen HLA-G by preeclamptic placenta. *Am J Obstet Gynecol*, **170,** 1244-50.

Conn, G., Bayne, M.L., Soderman, D.D., Kwok, P.W., Sullivan, K.A., Palisi, T.M., Hope, D.A., Thomas, K.A. (1990). Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor. *Proc Natl Acad Sci U S A*, **87,** 2628-32.

Connolly, D.T., Heuvelman, D.M., Nelson, R., Olander, J.V., Eppley, B.L., Delfino, J.J., Siegel, N.R., Leimgruber, R.M., Feder, J. (1989). Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest.*, **84,**1470-8.

Conrad, K., Kerchner, L., Smith, R. & Mosher, M. (1999). Plasma levels and 24 hour urinary excretion on NOx and cGMP in normal pregnancy and preeclamspia: cross sectional and serial studies in women on a NOx-reduced diet. *Journal of the Society for Gynaecological Investigation*, **6,** 286.

Coonrod, D.V., Hickok, D.E., Zhu, K., Easterling, T.R. & Daling, J.R. (1995). Risk factors for preeclampsia in twin pregnancies: a population-based cohort study. *Obstet Gynecol*, **85,** 645-50.

Cooper, D.W. & Liston, W.A. (1979). Genetic control of severe pre-eclampsia. *J Med Genet*, **16,** 409-16.

Crocker, I.P., Cooper, S., Ong, S.C., Baker, P.N. (2003). Differences in apoptotic susceptibility of cytotrophoblasts and syncytiotrophoblasts in normal pregnancy to those complicated with preeclampsia and intrauterine growth restriction. *Am J Pathol,* **162,** 637-43.

Cross, J.C. (1998). Formation of the placenta and extraembryonic membranes. *Ann N Y Acad Sci*, **857,** 23-32.

Curtin, J., Cotter, T. (2003). Live and let die: regulatory mechanisms in Fas-mediated apoptosis. *Cell Signal,* **15,** 983-992.

Davey, D.A. & MacGillivray, I. (1988). The classification and definition of the hypertensive disorders of pregnancy. *Am J Obstet Gynecol*, **158,** 892-8.

Davidge, S.T. & McLaughlin, M.K. (1992). Endogenous modulation of the blunted adrenergic response in resistance-sized mesenteric arteries from the pregnant rat. *Am J Obstet Gynecol*, **167,** 1691-8.

Davidge, S.T., Signorella, A.P., Hubel, C.A., Lykins, D.L. & Roberts, J.M. (1996). Distinct factors in plasma of preeclamptic women increase endothelial nitric oxide or prostacyclin. *Hypertension*, **28,** 758-64.

Demir, R., Kaufmann, P., Castellucci, M., Erbengi, T., Kotowski, A. (1989). Fetal vasculogenesis and angiogenesis in human placental villi. *Acta Anat (Basel),* **136,** 190-203.

DiFederico, E., Genbacev, O., Fisher, S.J. (1999). Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall. *Am J Pathol,* **155,** 293-301.

Doppler J.C. (1843). Uber das farbige Licht der Dopplersterne und einiger andere Hestirne des Himmels. Abhandlungen d. Konigl. *Bohmischen Gesellschaft der Wissenschafter Sers,* **2,** 465-82.

D'Orleans-Juste, P., Claing, A., Telemaque, S., Warner, T.D. & Regoli, D. (1991). Neurokinins produce selective venoconstriction via NK-3 receptors in the rat mesenteric vascular bed. *Eur J Pharmacol*, **204,** 329-34.

Enders, A.C. & King, B.F. (1991). Early stages of trophoblastic invasion of the maternal vascular system during implantation in the macaque and baboon. *Am J Anat*, **192,** 329-46.

Eremina, V., Wong, M.A., Cui, S., Schwartz, L., Quaggin, S.E. (2002). The role of VEGF-A in glomerular angiogenesis. *J Am. Soc. Nephrol*, **13,** 100A. (Abstract).

Evans, D.H., McDicken, W.N. Skidmore, R., Woodcock, J.P. (eds.). 1989. Doppler ultrasound: Physics, Instrumentation and Clinical Applications. Wiley, Chichester.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L.., Hillan, K.J., Moore, M.W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature, **380**,439-42.

Fong, G.H., Rossant, J., Gertsenstein, M., Breitman, M.L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*, **376,** 66–70.

Fox, H. (1983). Placental pathology. In: Studd ed. *Progress in Obstetrics and Gynaecology.* **3,** 114-9. London, Melbourne, New York. Churchill Livingstone.

French, L.E., Tschopp, J. (2002). Defective death receptor signaling as a cause of tumor immune escape. *Semin Cancer Biol*, **12,** 51-5.

Friedman, S.A., Schiff, E., Emeis, J.J., Dekker, G.A. & Sibai, B.M. (1995). Biochemical corroboration of endothelial involvement in severe preeclampsia. *Am J Obstet Gynecol*, **172,** 202-3.

Frusca, T., Morassi, L., Pecorelli, S., Grigolato, P., Gastaldi, A. (1989). Histological features of uteroplacental vessels in normal and hypertensive patients in relation to birthweight. *Br J Obstet Gynaecol*, **96,** 835-9.

Frusca, T., Soregaroli, M., Valcamonico, A., Guandalini, F., Danti, L. (1997). Doppler velocimetry of the uterine arteries in nulliparous women. *Early Hum Dev*, **48,** 177-85.

Furchgott, R.F. & Zawadzki, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288,** 373-6.

Gant, N.F., Daley, G.L., Chand, S., Whalley, P.J. & MacDonald, P.C. (1973). A study of angiotensin II pressor response throughout primigravid pregnancy. *J Clin Invest*, **52,** 2682-9.

Gant, N.F., Whalley, P.J., Everett, R.B., Worley, R.J. & MacDonald, P.C. (1987). Control of vascular reactivity in pregnancy. *Am J Kidney Dis*, **9,** 303-7.

Garcia-Velasco, J., Arici, A., Naftolin, F., Zreick, T., Mor, G. (1998). Macrophage-derived growth factors regulate FasL expression in endometrial stromal cells. *Mol Hum Reprod,* **5,** 642-650.

Genbacev, O., Joslin, R., Damsky, C.H., Polliotti, B.M., Fisher, S.J. (1996). Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia. *J Clin Invest*, **97,** 540-50.

Gerber HP, Condorelli F, Park J, Ferrara N. (1997). Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *J Biol Chem*, 272, 23659-67.

Giles, W.B., Trudinger, B.J., Cook, C.M. (1985). Fetal umbilical artery flow velocity-time waveforms in twin pregnancies. *Br J Obstet Gynaecol*, **92,** 490-7.

Gill, R.W., Kossoff, G., Warren, P.S., Garrett, W.J. (1984). Umbilical venous flow in normal and complicated pregnancy. *Ultrasound Med Biol,* **10,** 349-362.

Gille, H., Kowalski, J., Yu, L., Chen, H., Pisabarro, M.T., Davis-Smyth, T., Ferrara, N. (2000). A repressor sequence in the juxtamembrane domain of Flt-1 (VEGFR-1) constitutively inhibits vascular endothelial growth factor-dependent phosphatidylinositol 3'-kinase activation and endothelial cell migration. *EMBO J,* **19,** 4064-73.

Gold, R., Schmied, M., Giegerich, G., Breitschopf, H., Hartung, H.P., Toyka, K.Y. (1994). Differentiation between cellular apoptosi and necrosis by the combined use of in situ tailing and nick translation techniques. *Lab Invest,* **71,** 219-25.

Goldman-Wohl, D., Yagel, S. (2002). Regulation of trophoblast invasion: from normal implantation to pre-eclampsia. *Mol Cell Endocrinol*, **187,** 233-8.

Golstein P. (1997). Cell death: TRAIL and its receptors. *Curr Biol*, **7,** R750-3.

Greer, I.A., Dawes, J., Johnston, T.A., Calder, A.A. (1991). Neutrophil activation is confined to the maternal circulation in pregnancy-induced hypertension. *Obstet Gynecol*, **78,** 28-32.

Griffin, D.R., Teague, M.J., Tallet, P., Willson, K., Bilardo, C., Massini, L., Campbell, S. (1985). A combined ultrasonic linear array scanner and pulsed Doppler velocimeter for the estimation of blood flow in the foetus and adult abdomen--II: Clinical evaluation. *Ultrasound Med Biol*, **11,** 37-41.

Griffith, T.S., Brunner, T., Fletcher, S.M., Green D.R., Ferguson T.A. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science,* **270,** 1189-1192.

Griffith, T.S., Lynch, D.H. (1998). TRAIL: a molecule with multiple receptors and control mechanisms. *Curr Opin Immunol,* **10,** 559-63.

Guller, S., LaChapelle, L. (1999). The role of placental Fas ligand in maintaining immune privilege at maternal-fetal interfaces. *Semin Reprod Endocrinol*, **17,** 39-44.

Hanahan, D., Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*, **86,** 353-64.

Hanretty, K.P., Primrose, M.H., Neilson, J.P., Whittle, M.J. (1989). Pregnancy screening by Doppler uteroplacental and umbilical artery waveforms. *Br J Obstet Gynaecol*, **96,** 1163-7.

Harirah, H., Donia, S.E., Hsu, C.D. (2001). Serum soluble Fas in the syndrome of hemolysis, elevated liver enzymes, and low platelets. *Obstet Gynecol*, **98,** 295-8.

Harrington, K., Cooper, D., Lees, C., Hecher, K., Campbell, S. (1996). Doppler ultrasound of the uterine arteries: the importance of bilateral notching in the prediction of pre-eclampsia, placental abruption or delivery of a small-for-gestational-age baby. *Ultrasound Obstet Gynecol*, **7,** 182-8.

Harrington, K., Fayyad, A., Thakur, V., Aquilina, J. (2004). The value of uterine artery Doppler in the prediction of uteroplacental complications in multiparous women. *Ultrasound Obstet Gynecol,* **23,** 50-5.

Harrington, K., Goldfrad, C., Carpenter, R.G., Campbell, S. (1997). Transvaginal uterine and umbilical artery Doppler examination of 12-16 weeks and the subsequent development of pre-eclampsia and intrauterine growth retardation. *Ultrasound Obstet Gynecol*, **9,** 94-100.

Harrington, K., Thompson, M.O., Carpenter, R.G., Nguyen, M., Campbell, S. (1999). In third trimester fetuses the ratio in pulsatility index between the descending thoracic aorta and the middle cerebral artery may be more useful. Doppler fetal circulation in pregnancies complicated by pre-eclampsia or delivery of a small for gestational age baby. 2. Longitudinal analysis. *Br J Obstet Gynaecol,* **106,** 453-66.

Harrington, K., Thompson, M.O., Carpenter, R.G., Nguyen, M., Campbell, S. (1999). Doppler fetal circulation in pregnancies complicated by pre-eclampsia or delivery of a small for gestational age baby: 2. Longitudinal analysis. *Br J Obstet Gynaecol*, **106,** 453-66.

Hayman, R., Warren, A., Johnson, I. & Baker, P. (2001). Inducible change in the behavior of resistance arteries from circulating factor in preeclampsia: an effect specific to myometrial vessels from pregnant women. *Am J Obstet Gynecol*, **184,** 420-6.

He, H., Venema, V.J., Gu, X., Venema, R.C., Marrero, M.B., Caldwell, R.B. (1999). Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through flk-1/KDR activation of c-Src. *J Biol Chem*, **274,** 25130-5.

He, Y., Smith, S.K., Day, K.A., Clark, D.E., Licence, D.R., Charnock-Jones, D.S. (1999). Alternative splicing of vascular endothelial growth factor (VEGF)-R1

(FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Mol Endocrinol*, **13,** 537-45.

Hecher, K., Hackeloer, B.J. (1997). Cardiotocogram compared to Doppler investigation of the fetal circulation in the premature growth-retarded fetus: longitudinal observations. *Ultrasound Obstet Gynecol,* **9,** 152-61.

Hecher, K., Spernol, R., Stettner, H., Szalay, S. (1992). Potential for diagnosing imminent risk to appropriate- and small-for-gestational-age fetuses by Doppler sonographic examination of umbilical and cerebral arterial blood flow. *Ultrasound Obstet Gynecol,* **2,** 266-71.

Hendricks, S., Sorensen, T.K., Wang, K.Y. Bushnell, J.M., Seguin, E.M., Zingheim, R.W. (1989). Doppler umbilical artery waveforms indices-normal values from fourteen to forty two weeks. *Am J Obstet Gynecol,* **161,** 761-5.

Herr, I., Debatin, K.M. (2001). Cellular stress response and apoptosis in cancer therapy. *Blood*, **98,** 2603-14.

Hinchliffe, S.A., Lynch, M.R., Sargent, P.H., Howard, C.V., Van Velzen, D. (1992). The effect of intrauterine growth retardation on the development of renal nephrons. *Br J Obstet Gynaecol*, **99,** 296-301.

Hitschold, T.P. (1998). Doppler flow velocity waveforms of the umbilical arteries correlate with intravillous blood volume. *Am J Obstet Gynecol*, **179,** 540-3.

Hohlbaum, A.M., Saff, R.R., Marshak-Rothstein, A. (2002). Fas-ligand—iron fist or Achilles' heel? *Clin Immunol,* **103,** 1-6.

Holden, D.P., Fickling, S.A., Whitley, G.S. & Nussey, S.S. (1998). Plasma concentrations of asymmetric dimethylarginine, a natural inhibitor of nitric oxide synthase, in normal pregnancy and preeclampsia. *Am J Obstet Gynecol*, **178,** 551-6.

Hornig, C., Barleon, B., Ahmad, S., Vuorela, P., Ahmed, A., Weich, H.A. (2000). Release and complex formation of soluble VEGFR-1 from endothelial cells and biological fluids. *Lab Invest* , **80,** 443–454.

Hsu, C.D., Harirah, H., Basherra, H., Mor, G. (2001). Serum soluble Fas levels in preeclampsia. *Obstet Gynecol*, **97,** 530-2.

Hubel, C.A. (1999). Oxidative stress in the pathogenesis of preeclampsia. *Proc Soc Exp Biol Med*, **222,** 222-35.

Hubel, C.A., Griggs, K.C. & McLaughlin, M.K. (1989). Lipid peroxidation and altered vascular function in vitamin E-deficient rats. *Am J Physiol*, **256,** H1539-45.

Hubel, C.A., Kagan, V.E., Kisin, E.R., McLaughlin, M.K. & Roberts, J.M. (1997). Increased ascorbate radical formation and ascorbate depletion in plasma from women with preeclampsia: implications for oxidative stress. *Free Radic Biol Med*, **23,** 597-609.

Hubel, C.A., McLaughlin, M.K., Evans, R.W., Hauth, B.A., Sims, C.J. & Roberts, J.M. (1996). Fasting serum triglycerides, free fatty acids and malondialdehyde are increased in preeclampsia, are positively correlated, and decrease within 48 hours post partum. *Am J Obstet Gynecol*, **174**, 975-82

Hunt, J.S. (1989). Cytokine networks in the uteroplacental unit: macrophages as pivotal regulatory cells. *J Reprod Immunol*, **16,** 1-17.

Hunt, J.S., Vassmer, D., Ferguson, T.A., Miller, L. (1997). Fas ligand is positioned in mouse uterus and placenta to prevent trafficking of activated leukocytes between the mother and the conceptus. *J Immunol*, **158,** 4122-8.

Hunter, A., Aitkenhead, M., Caldwell, C., McCracken, G., Wilson, D., McClure, N. (2000). Serum levels of vascular endothelial growth factor in preeclamptic and normotensive pregnancy. *Hypertension*, **36,** 965-9.

Huppertz, B., Frank, H.G., Kingdom, J.C., Reister, F., Kaufmann, P. (1998). Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the human placenta. *Histochem Cell Biol*, **110,** 495-508.

Idriss, H.T., Naismith, J.H. (2000). TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc Res Tech*, **50,** 184-95.

Iio, S., Hayashi, N., Mita, E., Ueda, K., Mochizuki, K., Hiramatsu, N., Kanto, T., Sasaki, Y., Kasahara, A., Hori, M. (1998). Serum levels of soluble Fas antigen in chronic hepatitis C patients. *J Hepatol*, **29,** 517-23.

Irion, O., Masse, J., Forest, J.C., Moutquin, J.M. (1998). Prediction of pre-eclampsia, low birthweight for gestation and prematurity by uterine artery blood flow velocity waveforms analysis in low risk nulliparous women. *Br J Obstet Gynaecol*, **105,** 422-9.

Ishihara, N., Matsuo, H., Murakoshi, H., Laoag-Fernandez, J., Samoto, T., Maruo, T. (2000). Changes in proliferative potential, apoptosis and Bcl-2 protein expression in cytotrophoblasts and syncytiotrophoblast in human placenta over the course of pregnancy. *Endocr J,* **47,** 317-27.

Ishihara, N., Matsuo, H., Murakoshi, H., Laoag-Fernandez, J.B., Samoto, T., Maruo, T. (2002). Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. *Am J Obstet Gynecol,* **86,** 158-66.

Ishihara, N., Matsuo, H., Murakoshi, H., Laoag-Fernandez, J.B., Samoto, T., Maruo, T. (2000). Changes in proliferative potential, apoptosis and Bcl-2 protein expression in cytotrophoblasts and syncytiotrophoblast in human placenta over the course of pregnancy. *Endocr J,* **47,** 317-327.

Jackson, M.R., Walsh, A.J., Morrow, R.J., Mullen, J.B., Lye, S.J., Ritchie, J.W. (1995). Reduced placental villous tree elaboration in small-for-gestational-age pregnancies: relationship with umbilical artery Doppler waveforms. *Am J Obstet Gynecol*, **172,** 518-25.

Jacobson, S.L., Imhof, R., Manning, N., Mannion, V., Little, D., Rey, E., Redman, C. (1990). The value of Doppler assessment of the uteroplacental circulation in predicting preeclampsia or intrauterine growth retardation. *Am J Obstet Gynecol*, **162,** 110-4.

Jaffe, R., Jauniaux, E., Hustin, J. (1997). Maternal circulation in the first-trimester human placenta--myth or reality? *Am J Obstet Gynecol*, **176,** 695-705.

Jarad, G., Wang, B., Khan, S., DeVore, J., Miao, H., Wu, K., Nishimura, S.L., Wible, B.A., Konieczkowski, M., Sedor, J.R., Schelling, J.R. (2002). Fas activation induces renal tubular epithelial cell beta 8 integrin expression and function in the absence of apoptosis. *J Biol Chem*, **277,** 47826-33.

Jelkmann, W. (2001). Pitfalls in the measurement of circulating vascular endothelial growth factor. *Clin Chem*, **47,** 617-23.

Jiang, S.P., Vacchio, M.S. (1998). Multiple mechanisms of peripheral T cell tolerance to the fetal "allograft". *J Immunol*, **160,** 3086-90.

Johnstone, R. (1939). Toxaemias in pregnancy. In *A Textbook of Midwifery*. ed Comrie, J.D. pp. 252. Edinburgh.

Jonkman, E.J. (1980). Doppler research in the nineteenth century. *Ultrasound Med Biol*, **6,** 1-5.

Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkkinen, N., Alitalo, K. (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J*, **15,** 1751

Joza, N., Kroemer, G., Penninger, J.M. (2002). Genetic analysis of the mammalian cell death machinery. *Trends Genet*, **18,** 142-9.

Kabelitz, D. (1998). Apoptosis, graft rejection, and transplantation tolerance. *Transplantation,* **65,** 869-875.

Kang, D.H., Kim, Y.G., Andoh, T.F., Gordon, K.L., Suga, S., Mazzali, M., Jefferson, J.A., Hughes, J., Bennett, W., Schreiner, G.F., Johnson, R.J. (2001). Post-cyclosporine-mediated hypertension and nephropathy: amelioration by vascular endothelial growth factor. *Am J Physiol Renal Physiol*, **280,** F727-36.

Kang, S.M., Lin, Z., Ascher, N.L., Stock, P.G. (1998). Fas ligand expression on islets as well as multiple cell lines results in accelerated neutrophilic rejection. *Transplant Proc*, **30,** 538.

Kang, S.M., Schneider, D.B., Lin, Z., Hanahan, D., Dichek, D.A., Stock, P.G., Baekkeskov, S. (1997). Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction. *Nat Med*, **3,** 738-43.

Karsdorp, V.H., Dirks, B.K., van der Linden, J.C., van Vugt, J.M., Baak, J.P., van Geijn, H.P. (1996). Placenta morphology and absent or reversed end diastolic flow velocities in the umbilical artery: a clinical and morphometrical study. *Placenta*, **17,** 393-9.

Kaufmann, P., Bruns, U., Leiser, R., Luckhardt, M., Winterhager, E. (1985). The fetal vascularisation of term human placental villi. II. Intermediate and terminal villi. *Anat Embryol (Berl),* **173,** 203-14.

Kaufmann, P., Luckhardt, M., Leiser, R. (1988). Three dimensional representation of the fetal vessel system in the human placenta. *Trophoblast Res,* **3,** 113-37.

Kaufmann, P., Sen, D.K., Schweikhart, G. (1979). Classification of human placental villi. I Histology. *Cell Tissue Res,* **200,** 409-23.

Kauma, S., Matt, D., Strom, S., Eierman, D., Turner, T. (1990). Interleukin-1 beta, human leukocyte antigen HLA-DR alpha, and transforming growth factor-beta expression in endometrium, placenta, and placental membranes. *Am J Obstet Gynecol,* **163,** 1430-7.

Kauma, S.W., Huff, T.F., Hayes, N., Nilkaeo, A. (1999). Placental Fas ligand expression is a mechanism for maternal immune tolerance to the fetus. *J Clin Endocrinol Metab*, **84,** 2188-94.

Kendall, R.L., Thomas, K.A. (1993) Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci USA,* **90,** 10705– 9.

Kendall, R.L., Wang, G., Thomas, K.A. (1996). Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. *Biochem Biophys Res Commun*, **226,** 324-8.

Khaliq, A., Li, X.F., Shams, M., Sisi, P., Acevedo, C.A., Whittle, M.J., Weich, H., Ahmed, A. (1996). Localisation of placenta growth factor (PIGF) in human term placenta. *Growth Factors*, 13, 243-50.

Kilani, R.T., Mackova, M., Davidge, S.T., Guilbert, L.J. (2003). Effect of oxygen levels in villous trophoblast apoptosis. *Placenta*, **24,** 826-34.

Kim, Y.G., Suga, S.I., Kang, D.H., Jefferson, J.A., Mazzali, M., Gordon, K.L., Matsui, K., Breiteneder-Geleff, S., Shankland, S.J., Hughes, J., Kerjaschki, D., Schreiner, G.F., Johnson, R.J. (2000). Vascular endothelial growth factor accelerates renal recovery in experimental thrombotic microangiopathy. *Kidney Int*, **58,** 2390-9.

Kingdom, J. (1998) Adriana and Luisa Castellucci Award Lecture 1997. Placental pathology in obstetrics: adaptation or failure of the villous tree? *Placenta,* **19,** 347-51.

Kingdom, J.C., Burrell, S.J., Kaufmann, P. (1997). Pathology and clinical implications of abnormal umbilical artery Doppler waveforms. *Ultrasound Obstet Gynecol*, **9,** 271-86.

Kingdom, J.C., Kaufmann, P. (1999). Oxygen and placental vascular development, In Roach, R.C., ed. *Hypoxia: Into the Next Millennium,* Kluwer Academic/Plenum Publishing, New York. 259-75.

Kingdom, J.C.P., Kaufmann, P. (1997). Oxygen and placental villous development; Origins of fetal hypoxia. *Placenta,* **18,** 613-21.

Kiserud, T., Crowe, C., Hanson, M. (1998). Ductus venosus agenesis prevents transmission of central venous pulsations to the umbilical vein in fetal sheep. *Ultrasound Obstet Gynecol*, **11,** 190-4.

Klonoff-Cohen, H.S., Savitz, D.A., Cefalo, R.C. & McCann, M.F. (1989). An epidemiologic study of contraception and preeclampsia. *JAMA*, **262,** 3143-7.

Knight, M., Redman, C.W., Linton, E.A. & Sargent, I.L. (1998). Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. *Br J Obstet Gynaecol*, **105,** 632-40.

Knight, M., Redman, C.W., Linton, E.A. & Sargent, I.L. (1998). Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. *Br J Obstet Gynaecol*, **105,** 632-40.

Knipping, E., Krammer, P.H., Onel, K.B., Lehman, T.J., Mysler, E., Elkon, K.B. (1995). Levels of soluble Fas/APO-1/CD95 in systemic lupus erythematosus and juvenile rheumatoid arthritis. *Arthritis Rheum,* **38,** 1735-7.

Knock, G.A. & Poston, L. (1996). Bradykinin-mediated relaxation of isolated maternal resistance arteries in normal pregnancy and preeclampsia. *Am J Obstet Gynecol*, **175,** 1668-74.

Koelman, C.A., Coumans, A.B., Nijman, H.W., Doxiadis, II, Dekker, G.A. & Claas, F.H. (2000). Correlation between oral sex and a low incidence of preeclampsia: a role for soluble HLA in seminal fluid? *J Reprod Immunol*, **46,** 155-66.

Koenig, J.M., Chegini, N. (2000). Enhanced expression of Fas-associated proteins in decidual and trophoblastic tissues in pregnancy-induced hypertension. *Am J Reprod Immunol*, **44,** 347-9.

Koga, K., Osuga, Y., Yoshino, O., Hirota, Y., Ruimeng, X., Hirata, T., Takeda, S., Yano, T., Tsutsumi, O., Taketani, Y. (2003). Elevated serum soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) levels in women with preeclampsia. *J Clin Endocrinol Metab,* **88,** 2348-51.

Krammer, P.H. (1999). CD95(APO-1/Fas)-mediated apoptosis: live and let die. *Adv Immunol*, **71,** 163-210.

Krebs, C., Macara, L.M., Leiser, R., Bowman, A.W., Greer, I.A., Kingdom, J.C. (1996). Intrauterine growth restriction with absent end-diastolic flow velocity

in the umbilical artery is associated with maldevelopment of the placental terminal villous tree. *Am J Obstet Gynecol*, **175,** 1534-42.

Kuntz, T.B., Cristensen, R.D., Stenger, J., Duff, P., Koenig, J. (2001). Fas and Fas Ligand Expression in Maternal Blood and in Umbilical Cord Blood in Preeclampsia. *Ped Research,* **50,** 743-9.

Kurdi, W., Campbell, S., Aquilina, J., England, P., Harrington, K. (1998). The role of color Doppler imaging of the uterine arteries at 20 weeks' gestation in stratifying antenatal care. *Ultrasound Obstet Gynecol*, **12,** 339-45.

Kurz, H., Wilting, J., Sandau, K., Christ, B. (1998). Automated evaluation of angiogenic effects mediated by VEGF and PlGF homo- and heterodimers. *Microvasc Res*, **55,** 92-102.

Law, C.M., Barker, D.J., Bull, A.R., Osmond, C. (1991). Maternal and fetal influences on blood pressure. *Arch Dis Child*, **66,** 1291-5.

Leung, D.W., Cachianes, G., Kuang, W.J., Goeddel, D.V., Ferrara, N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*, **246,** 1306-9.

Levine, R.J., Maynard, S.E., Qian, C., Lim, K.H., England, L.J., Yu, K.F., Schisterman, E.F., Thadhani, R., Sachs, B.P., Epstein, F.H., Sibai, B.M., Sukhatme, V.P., Karumanchi, S.A. (2004). Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med*, **350,** 672-83.

Levy, R., Nelson, D. (2000). To be, or not to be, that is the question. Apoptosis in human trophoblast. *Placenta,* **21,** 1-13.

Levy, R., Smith, S.D., Chandler, K., Sadovsky, Y., Nelson, D.M. (2000). Apoptosis in human cultured trophoblasts is enhanced by hypoxia and diminished by epidermal growth factor. *Am J Physiol Cell Physiol*, **278,** C982-8.

Levy, R., Smith, S.D., Yusuf, K., Huettner, P.C., Kraus, F.T., Sadovsky, Y., Nelson, D.M. (2002). Trophoblast apoptosis from pregnancies complicated by fetal growth restriction is associated with enhanced p53 expression. *Am J Obstet Gynecol*, **186,** 1056-61.

Li, D.K. & Wi, S. (2000). Changing paternity and the risk of preeclampsia/eclampsia in the subsequent pregnancy. *Am J Epidemiol*, **151,** 57-62.

Liles, W.C., Kiener, P.A., Ledbetter, J.A., Aruffo, A., Klebanoff, S.J. (1996). Differential expression of Fas (CD95) and Fas ligand on normal human phagocytes: implications for the regulation of apoptosis in neutrophils. *J Exp Med,* **184,** 429-40.

Lim, K.H., Zhou, Y., Janatpour, M., McMaster, M., Bass, K., Chun, S.H. & Fisher, S.J. (1997). Human cytotrophoblast differentiation/invasion is abnormal in pre-eclampsia. *Am J Pathol*, **151,** 1809-18.

Liston, W.A. & Kilpatrick, D.C. (1991). Is genetic susceptibility to pre-eclampsia conferred by homozygosity for the same single recessive gene in mother and fetus? *Br J Obstet Gynaecol*, **98,** 1079-86.

Liu, J.H., Wei, S., Lamy, T., Epling-Burnette, P.K., Starkebaum, G., Djeu, J.Y., Loughran, T.P. (2000). Chronic neutropenia mediated by fas ligand. *Blood*, **95,** 3219-22.

Livingston, J.C., Chin, R., Haddad, B., McKinney, E.T., Ahokas, R., Sibai, B.M. (2000). Reductions of vascular endothelial growth factor and placental growth factor concentrations in severe preeclampsia. *Am J Obstet Gynecol*, **183,** 1554-7.

Lorentzen, B., Endresen, M.J., Hovig, T., Haug, E. & Henriksen, T. (1991). Sera from preeclamptic women increase the content of triglycerides and reduce the release of prostacyclin in cultured endothelial cells. *Thromb Res*, **63,** 363-72.

Lucas, A. (1991). Programming by early nutrition in man. In *"The childhood environment and adult disease" (Eds GR Buck and J Whelan).* Ciba foundation 156. John Wiley Chichester 1991.

Luckhardt, M., Leiser, R., Kingdom, J., Malek, A., Sager, R., Kaisig, C., Schneider, H. (1996). Effect of physiologic perfusion-fixation on the morphometrically evaluated dimensions of the term placental cotyledon. *J Soc Gynecol Investig*, **3,** 166-71.

Lunell, N.O., Nylund, L.E., Lewander, R., Sarby, B. (1982) Uteroplacental blood flow in pre-eclampsia measurements with indium-113m and a computer-linked gamma camera. *Clin Exp Hypertens B*, **1,** 105-17.

Luttun, A., Carmeliet, P. (2003). Soluble VEGF receptor Flt1: the elusive preeclampsia factor discovered? *J Clin Invest*, **111,** 600-2.

Lyall, F., Greer, I.A., Boswell, F., Fleming, R. (1997). Suppression of serum vascular endothelial growth factor immunoreactivity in normal pregnancy and in pre-eclampsia. *Br J Obstet Gynaecol,* **104,** 223-8.

Lyall, F., Young, A. & Greer, I.A. (1995). Nitric oxide concentrations are increased in the fetoplacental circulation in preeclampsia. *Am J Obstet Gynecol*, **173,** 714-8.

Lyall, F., Young, A., Boswell, F., Kingdom, J.C., Greer, I.A. (1997). Placental expression of vascular endothelial growth factor in placentae from pregnancies complicated by pre-eclampsia and intrauterine growth restriction does not support placental hypoxia at delivery. *Placenta,* **18,** 269-76.

Lyttle, D.J., Fraser, K.M., Fleming, S.B., Mercer, A.A., Robinson, A.J. (1994). Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus. *J Virol,* **68,** 84-92.

Macara, L., Kingdom, J.C., Kaufmann, P., Kohnen, G., Hair, J., More, I.A., Lyall, F., Greer, I.A. (1996). Structural analysis of placental terminal villi from growth-restricted pregnancies with abnormal umbilical artery Doppler waveforms. *Placenta*, **17,** 37-48.

MacCumber, M.W., Ross, C.A., Glaser, B.M. & Snyder, S.H. (1989). Endothelin: visualization of mRNAs by in situ hybridization provides evidence for local action. *Proc Natl Acad Sci U S A*, **86,** 7285-9.

Maglione, D., Guerriero, V., Viglietto, G., Delli-Bovi, P., Persico, M.G. (1991). Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc Natl Acad Sci U S A,* **88,** 9267-71.

Maglione, D., Guerriero, V., Viglietto, G., Ferraro, M.G., Aprelikova, O., Alitalo, K., Del Vecchio, S., Lei, K.J., Chou, J.Y., Persico, M.G. (1993). Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. *Oncogene,* **8,** 925-31.

Magness, R.R. & Gant, N.F. (1994). Control of vascular reactivity in pregnancy: the basis for therapeutic approaches to prevent pregnancy-induced hypertension. *Semin Perinatol*, **18,** 45-69.

Manning, F.A., Hill, L.M., Platt, L.D. (1981). Qualitative amniotic fluid volume determination by ultrasound: antepartum detection of intrauterine growth retardation. *Am J Obstet Gynecol*, **139,** 254-8.

Manning, F.A., Morrison, I., Lange, I.R., Harman, C.R., Chamberlain, P.F. (1985). Fetal assessment based on fetal biophysical profile scoring: experience in

12,620 referred high-risk pregnancies. I. Perinatal mortality by frequency and etiology. *Am J Obstet Gynecol*, **151,** 343-50.

Manning, F.A., Platt, L.D., Sipos, L. (1980). Antepartum fetal evaluation: development of a fetal biophysical profile. *Am J Obstet Gynecol,* **136,** 787-95.

Martinez-Lorenzo, M.J., Alava, M.A., Anel, A., Pineiro, A., Naval, J. (1996). Release of preformed Fas ligand in soluble form is the major factor for activation-induced death of Jurkat T cells. *Immunology*, **89,** 511-7.

Martinez-Lorenzo, M.J., Anel, A., Gamen, S., Monle, n. I., Lasierra, P., Larrad, L., Pineiro, A., Alava, M.A., Naval, J. (1999). Activated human T cells release bioactive Fas ligand and APO2 ligand in microvesicles. *J Immunol*, **163,** 1274-81.

Mason, G.C., Lilford, R.J., Porter, J., Nelson, E., Tyrell, S. (1993). Randomised comparison of routine versus highly selective use of Doppler ultrasound in low risk pregnancies. *Br J Obstet Gynaecol*, **100,** 130-3.

Mastrogiannis, D.S., O'Brien, W.F., Krammer, J. & Benoit, R. (1991). Potential role of endothelin-1 in normal and hypertensive pregnancies. *Am J Obstet Gynecol*, **165,** 1711-6.

Masuda, Y., Shimizu, A., Mori, T., Ishiwata, T., Kitamura, H., Ohashi, R., Ishizaki, M., Asano, G., Sugisaki, Y., Yamanaka, N. (2001). Vascular endothelial growth factor enhances glomerular capillary repair and accelerates resolution of experimentally induced glomerulonephritis. *Am J Pathol*, **159,** 599-608.

Maynard, S.E., Min, J.Y., Merchan, J., Lim, K.H., Li, J., Mondal, S., Libermann, T.A., Morgan, J.P., Sellke, F.W., Stillman, I.E., Epstein, F.H., Sukhatme, V.P., Karumanchi, S.A. (2003). Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinurea in preeclampsia. *J Clin Invest*, **111,** 649-58.

McCarthy, A.L., Taylor, P., Graves, J., Raju, S.K. & Poston, L. (1994). Endothelium-dependent relaxation of human resistance arteries in pregnancy. *Am J Obstet Gynecol*, **171,** 1309-15.

McKeeman, G.C., Ardill, J.E., Caldwell, C.M., Hunter, A.J., McClure, N. (2004). Soluble vascular endothelial growth factor receptor-1 (sFlt-1) is increased throughout gestation in patients who have preeclampsia develop. *Am J Obstet Gynecol*, **191,** 1240-6.

McMaster, M.T., Bass, K.E., Fisher, S.J. (1994). Human trophoblast invasion. Autocrine control and paracrine modulation. *Ann N Y Acad Sci,* **734,** 122-131.

Medchill, M.T., Peterson, C.M., Kreinick, C., Garbaciak, J. (1991). Prediction of estimated fetal weight in extremely low birth weight neonates (500-1000 g). *Obstet Gynecol*, **78,** 286-90.

Medema, J.P., Toes, R.E., Scaffidi, C., Zheng, T.S., Flavell, R.A., Melief, C.J., Peter, M.E., Offringa, R., Krammer, P.H. (1997). Cleavage of FLICE (caspase-8) by granzyme B during cytotoxic T lymphocyte-induced apoptosis. *Eur J Immunol*, **27,** 3492-8.

Meekins, J.W., Luckas, M.J., Pijnenborg, R. & McFadyen, I.R. (1997). Histological study of decidual spiral arteries and the presence of maternal erythrocytes in the intervillous space during the first trimester of normal human pregnancy. *Placenta*, **18,** 459-64.

Meekins, J.W., Pijnenborg, R., Hanssens, M., McFadyen, I.R. & van Asshe, A. (1994). A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies. *Br J Obstet Gynaecol*, **101,** 669-74.

Mellor, A.L., Munn, D.H. (2000). Immunology at the maternal-fetal interface: lessons for T cell tolerance and suppression. *Annu Rev Immunol*, **8,** 367-91.
Merrill, D.C., Weiner, C. (1992). Fetal medicine. *Curr Opin Obstet Gynecol*, **4,** 273-9.
Merviel, P., Challier, J.C., Carbillon, L., Foidart, J.M., Uzan, S. (2001). The role of integrins in human embryo implantation. *Fetal Diagn Ther*, **16,** 364-71.

Michel, T. & Smith, T.W. (1993). Nitric oxide synthases and cardiovascular signaling. *Am J Cardiol*, **72,** 33C-38C.
Midis, G.P., Shen, Y., Owen-Schaub, L.B. (1996). Elevated soluble Fas (sFas) levels in nonhematopoietic human malignancy. *Cancer Res*, **56,** 3870-4.
Mikhail, M.S., Anyaegbunam, A., Garfinkel, D., Palan, P.R., Basu, J. & Romney, S.L. (1994). Preeclampsia and antioxidant nutrients: decreased plasma levels of reduced ascorbic acid, alpha-tocopherol, and beta-carotene in women with preeclampsia. *Am J Obstet Gynecol*, **171,** 150-7.
Mili, F., Edmonds, L.D., Khoury, M.J., McClearn, A.B. (1991). Prevalence of birth defects among low-birth-weight infants. A population study. *Am J Dis Child*, **145,** 1313-8.
Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., Moller, N.P., Risau, W., Ullrich, A. (1993). High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell*, **72,** 835-46.
Misra, D.P. & Kiely, J.L. (1997). The association between nulliparity and gestational hypertension. *J Clin Epidemiol*, **50,** 851-5.
Mor, G., Gutierrez, L.S., Eliza, M., Kahyaoglu, F., Arici, A. (1998). Fas-fas ligand system-induced apoptosis in human placenta and gestational trophoblastic disease. *Am J Reprod Immunol*, **40,** 89-94.
Morbidelli, L., Chang, C.H., Douglas, J.G., Granger, H.J., Ledda, F., Ziche, M. (1996). Nitric oxide mediates mitogenic effect of VEGF on coronary venular endothelium. *Am J Physiol,* **270,** H411-5.
Morris, N.H., Carroll, S., Nicolaides, K.H., Steer, P.J. & Warren, J.B. (1995). Exhaled nitric oxide concentration and amniotic fluid nitrite concentration during pregnancy. *Eur J Clin Invest*, **25,** 138-41.
Morrow, R.J., Adamson, S.L., Bull, S.B., Ritchie, J.W. (1989). Effect of placental embolization on the umbilical arterial velocity waveform in fetal sheep. *Am J Obstet Gynecol*, **161,** 1055-60.
Nagata, S. (1994). Fas and Fas ligand: a death factor and its receptor. *Adv Immunol,* **57,** 129-135.
Nelson, S.H., Steinsland, O.S., Suresh, M.S. & Lee, N.M. (1998). Pregnancy augments nitric oxide-dependent dilator response to acetylcholine in the human uterine artery. *Hum Reprod*, **13,** 1361-7.
Nicolaides, K., Rizzo, G., Hecher, K. (2000). Doppler studies in fetal hypoxemic hypoxia. In *Placental and fetal Doppler,* eds Nicolaides, K., Rizzo, G., Hecher, K. pp 67-88. New York, London. Parenthon Publishing.
Nicolaides, K., Rizzo, G., Hecher, K. (2000). Placental and fetal Doppler. In *Placental and fetal Doppler,* eds Nicolaides, K., Rizzo, G., Hecher, K. pp 89-104. Parenthon Publishing.
Nicolaides, K.H,, Bilardo, C.M., Soothill, P.W., Campbell, S. (1988). Absence of end diastolic frequencies in umbilical artery: a sign of fetal hypoxia and acidosis. *BMJ*, **297,** 1026-7.

Nicolaides, K.H., Economides, D.L., Soothill, P.W. (1989). Blood gases, pH, and lactate in appropriate- and small-for-gestational-age fetuses. *Am J Obstet Gynecol*, **161,** 996-1001.

Nobunaga, T., Tokugawa, Y., Hashimoto, K., Kimura, T., Matsuzaki, N., Nitta, Y., Fujita, T., Kidoguchi, K.I., Azuma, C. & Saji, F. (1996). Plasma nitric oxide levels in pregnant patients with preeclampsia and essential hypertension. *Gynecol Obstet Invest*, **41,** 189-93.

North, R.A., Ferrier, C., Long, D., Townend, K., Kincaid-Smith, P. (1994). Uterine artery Doppler flow velocity waveforms in the second trimester for the prediction of preeclampsia and fetal growth retardation. *Obstet Gynecol*, **83,** 378-86.

Norwitz, E.R., Schust, D.J., Fisher, S.J. (2001). Implantation and the survival of early pregnancy. *N Engl J Med,* **345,** 1400-8.

Oguogho, A., Aloamaka, C.P. & Ebeigbe, A.B. (1996). Depressed endothelium-dependent relaxation responses to acetylcholine and histamine in isolated human epigastric arteries from pre-eclamptic women. *Clin Auton Res*, **6,** 153-5.

Okuyama, M., Yamaguchi, S., Nozaki, N., Yamaoka, M., Shirakabe, M., Tomoike, H. (1997). Serum levels of soluble form of Fas molecule in patients with congestive heart failure. *Am J Cardiol*, **79,** 1698-701.

Olander, J.V., Connolly, D.T., DeLarco, J.E. (1991). Specific binding of vascular permeability factor to endothelial cells. *Biochem Biophys Res Commun*, **175,** 68-76.

Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Pettersson, R.F., Alitalo, K., Eriksson, U. (1996). Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci U S A*, **93,** 2576-81.

O'Reilly, M.S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R.A., Moses, M., Lane, W.S., Cao, Y., Sage, E.H., Folkman, J. (1994). Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell*, **79,** 315-28.

Ostendorf, T., Kunter, U., Eitner, F., Loos, A., Regele, H., Kerjaschki, D., Henninger, D.D., Janjic, N., Floege, J. (1999). VEGF(165) mediates glomerular endothelial repair. *J Clin Invest*, **104,** 913-23.

Paavonen, K., Horelli-Kuitunen, N., Chilov, D., Kukk, E., Pennanen, S., Kallioniemi, O.P., Pajusola, K., Olofsson, B., Eriksson, U., Joukov, V., Palotie, A., Alitalo, K. (1996). Novel human vascular endothelial growth factor genes VEGF-B and VEGF-C localize to chromosomes 11q13 and 4q34, respectively. *Circulation*, **93,** 1079-82.

Page, N.M., Woods, R.J., Gardiner, S.M., Lomthaisong, K., Gladwell, R.T., Butlin, D.J., Manyonda, I.T. & Lowry, P.J. (2000). Excessive placental secretion of neurokinin B during the third trimester causes pre-eclampsia. *Nature*, **405,** 797-800.

Panigel, M., Myers, R.E. (1972). Histological and ultrastructural changes in rhesus monkey placenta following interruption of fetal placental circulation by fetectomy or interplacental umbilical vessel ligation. *Acta Anat (Basel),* **81,** 481-506.

Papageorghiou, A.T., Yu, C.K., Bindra, R., Pandis, G., Nicolaides, K.H., Fetal Medicine Foundation Second Trimester Screening Group. (2001). Multicenter screening for pre-eclampsia and fetal growth restriction by transvaginal uterine

artery Doppler at 23 weeks of gestation. *Ultrasound Obstet Gynecol*, **18,** 441-9.

Pardi, G., Cetin, I., Marconi, A.M., Bozzetti, P., Buscaglia, M., Makowski, E.L., Battaglia, F.C. (1992). Venous drainage of the human uterus: respiratory gas studies in normal and fetal growth-retarded pregnancies. *Am J Obstet Gynecol*, **166,** 699-706.

Pardi, G., Cetin, I., Marconi, A.M., Lanfranchi, A., Bozzetti, P., Ferrazzi, E., Buscaglia, M., Battaglia, F.C. (1993). Diagnostic value of blood sampling in fetuses with growth retardation. *N Engl J Med*, 328, 692-6.

Parent, A.,Schiffrin, E. L. & St-Louis, J. (1990). Role of the endothelium in adrenergic responses of mesenteric artery rings of pregnant rats. *Am J Obstet Gynecol,***163**, 229-34.

Pascoal, I.F. & Umans, J.G. (1996). Effect of pregnancy on mechanisms of relaxation in human omental microvessels. *Hypertension*, **28,** 183-7.

Pascoal, I.F., Lindheimer, M.D., Nalbantian-Brandt, C. & Umans, J.G. (1998). Preeclampsia selectively impairs endothelium-dependent relaxation and leads to oscillatory activity in small omental arteries. *J Clin Invest*, **101,** 464-70.

Payne, S.G., Smith, S.C., Davidge, S.T., Baker, P.N., Guilbert, L.J. (1999). Death receptor Fas/Apo-1/CD95 expressed by human placental cytotrophoblasts does not mediate apoptosis. *Biol Reprod,* **60,** 1144-50.

Peeters, L.L., Sheldon, R.E., Jones, M.D. Jr., Makowski, E.L., Meschia, G. (1979). Blood flow to fetal organs as a function of arterial oxygen content. *Am J Obstet Gynecol*, **135,** 637-46.

Peters, K.G., De Vries, C., Williams, L.T. (1993). Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. *Proc Natl Acad Sci U S A,* **90,** 8915-9.

Pijnenborg, R., Bland, J.M., Robertson, W.B. & Brosens, I. (1983). Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. *Placenta*, **4,** 397-413.

Pijnenborg, R., Dixon, G., Robertson, W.B. & Brosens, I. (1980). Trophoblastic invasion of human decidua from 8 to 18 weeks of pregnancy. *Placenta*, **1,** 3-19.

Pijnenborg, R., Luyten, C., Vercruysse, L. & Van Assche, F.A. (1996). Attachment and differentiation in vitro of trophoblast from normal and preeclamptic human placentas. *Am J Obstet Gynecol*, **175,** 30-6.

Pinkoski, M.J., Brunner, T., Green, D.R. Lin, T. (2000). Fas and Fas ligand im gut and liver. *Am J Physiol,* **278,** G354-G366.

Pinkoski, M.J., Green, D.R. (2000). Cloak and dagger in the avoidance of immune surveillance. *Curr Opin Genet Dev,* **10,** 114-9.

Pourcelot, L. Applications clinique de l'examen Doppler transcutaine. In. Peronneau P (eds). (1974). *Velocimetric Ultrasonor Doppler*. Paris: INSERM **34,** 213-40.

Prefumo, F., Bhide, A., Sairam, S., Penna, L., Hollis, B., Thilaganathan, B. (2004). Effect of parity on second-trimester uterine artery Doppler flow velocity and waveforms. *Ultrasound Obstet Gynecol*, **23,** 46-9.

Pryor, J.E. Physical and developmental status of pre-school small for gestational age children: a comparative study. *J Paed Child Health,* **28,** 162-7.

Redman, C.W., Bonnar, J. & Beilin, L. (1978). Early platelet consumption in pre-eclampsia. *Br Med J*, **1,** 467-9.

Redman, C.W., Denson, K.W., Beilin, L.J., Bolton, F.G. & Stirrat, G.M. (1977). Factor-VIII consumption in pre-eclampsia. *Lancet*, **2,** 1249-52.

Redman, C.W., Sacks, G.P., Sargent, I.L. (1999). Preeclampsia: an excessive maternal inflammatory response to pregnancy. *Am J Obstet Gynecol*, **180,** 499-506.

Reed, K.L., Anderson, C.F., Shenker, L. (1987). Fetal pulmonary artery and aorta: two-dimensional Doppler echocardiography. *Obstet Gynecol*, **69,** 175-8.

Reilly, R.D., Russell, P.T. (1977). Neurohistochemical evidence supporting an absence of adrenergic and cholinergic innervation in the human placenta and umbilical cord. *Anat Rec,* **188,** 277-86.

Reuss, M.L., Rudolph, A.M. (1980). Distribution and recirculation of umbilical and systemic venous blood flow in fetal lambs during hypoxia. *J Dev Physiol*, **2,** 71-84.

Reuss, M.L., Rudolph, A.M., Dae, M.W. (1983). Phasic blood flow patterns in the superior and inferior venae cavae and umbilical vein of fetal sheep. *Am J Obstet Gynecol*, **145,** 70-8.

Reuvekamp, A., Velsing-Aarts, F.V., Poulina, I.E., Capello, J.J., Duits, A.J. (1999). Selective deficit of angiogenic growth factors characterises pregnancies complicated by pre-eclampsia. *Br J Obstet Gynaecol,* **106**, 1019-22.

Rizzo, G., Capponi, A., Soregaroli, M., Arduini, D., Romanini, C. (1995). Umbilical vein pulsations and acid-base status at cordocentesis in growth-retarded fetuses with absent end-diastolic velocity in umbilical artery. *Biol Neonate*, **68,** 163-8.

Roberts, J.M. & Hubel, C.A. (1999). Is oxidative stress the link in the two-stage model of pre-eclampsia? *Lancet*, **354,** 788-9.

Roberts, J.M. & Redman, C.W. (1993). Pre-eclampsia: more than pregnancy-induced hypertension. *Lancet*, **341,** 1447-51.

Roberts, J.M., Cooper, D.W. (2001). Pathogenesis and genetics of pre-eclampsia. *Lancet*, **357,** 53-6.

Roberts, J.M., Edep, M.E., Goldfien, A. & Taylor, R.N. (1992). Sera from preeclamptic women specifically activate human umbilical vein endothelial cells in vitro: morphological and biochemical evidence. *Am J Reprod Immunol*, **27,** 101-8.

Roberts, J.M., Taylor, R.N., Musci, T.J., Rodgers, G.M., Hubel, C.A. & McLaughlin, M.K. (1989). Preeclampsia: an endothelial cell disorder. *Am J Obstet Gynecol*, **161,** 1200-4.

Robillard, P.Y. & Hulsey, T.C. (1996). Association of pregnancy-induced-hypertension, pre-eclampsia, and eclampsia with duration of sexual cohabitation before conception. *Lancet*, **347,** 619.

Rodgers, G.M., Taylor, R.N. & Roberts, J.M. (1988). Preeclampsia is associated with a serum factor cytotoxic to human endothelial cells. *Am J Obstet Gynecol*, **159,** 908-14.

Rosenberg, K., Grant, J.M., Hepburn, M. (1982). Antenatal detection of growth retardation: actual practice in a large maternity hospital. *Br J Obstet Gynaecol*, **89,** 12-5.

Rosenberg, K., Grant, J.M., Tweedie, I., Aitchison, T., Gallagher, F. (1982). Measurement of fundal height as a screening test for fetal growth retardation. *Br J Obstet Gynaecol,* **89,** 447-50.

Roth, I., Fisher, S.J. (1999). IL-10 is an autocrine inhibitor of human placental cytotrophoblast MMP-9 production and invasion. *Dev Biol,* **205,** 194-204.

Runic, R., Lockwood, C.J., Ma, Y., Dipasquale, B., Guller, S. (1996). Expression of Fas ligand by human cytotrophoblasts: implications in placentation and fetal survival. *J Clin Endocrinol Metab*, **81,** 3119-22.

Salafia, C.M., Pezzullo, J.C., Minior, V.K., Divon, M.Y. (1997). Placental pathology of absent and reversed end-diastolic flow in growth-restricted fetuses. *Obstet Gynecol*, **90,** 830-6.

Salha, O., Sharma, V., Dada, T., Nugent, D., Rutherford, A.J., Tomlinson, A.J., Philips, S., Allgar, V. & Walker, J.J. (1999). The influence of donated gametes on the incidence of hypertensive disorders of pregnancy. *Hum Reprod*, **14,** 2268-73.

Sata, M., Suhara, T., Walsh, K. (2000). Vascular endothelial cells and smooth muscle cells differ in expression of Fas and Fas ligand and in sensitivity to Fas ligand-induced cell death: Implication in vascular disease and therapy. *Arterioscler Thromb Vasc Biol,* **20,** 309-16.

Schneider, P., Holler, N., Bodmer, J.L., Hahne, M., Frei, K., Fontana, A., Tschopp, J. (1998). Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. *J Exp Med*, **187,** 1205-13.

Schulze-Osthoff, K., Ferrari, D., Los, M., Wesselborg, S., Peter,M.E. (1998). Apoptosis signaling by death receptors. *Eur J Biochem*, **254,** 439-59.

Scott, J.R. & Beer, A.A. (1976). Immunologic aspects of pre-eclampsia. *Am J Obstet Gynecol*, **125,** 418-27.

Seligman, S.P., Buyon, J.P., Clancy, R.M., Young, B.K. & Abramson, S.B. (1994). The role of nitric oxide in the pathogenesis of preeclampsia. *Am J Obstet Gynecol*, **171,** 944-8.

Sharkey, A.M., Cooper, J.C., Balmforth, J.R., McLaren, J., Clark, D.E., Charnock-Jones, D.S., Morris, N.H., Smith, S.K. (1996). Maternal plasma levels of vascular endothelial growth factor in normotensive pregnancies and in pregnancies complicated by pre-eclampsia. *Eur J Clin Invest*, **26,** 1182-5.

Shibuya, M. (2001). Structure and function of VEGF/VEGF-receptor system involved in angiogenesis. *Cell Struct Funct,* **26,** 25-35.

Shibuya, M., Yamaguchi, S., Yamane, A., Ikeda, T., Tojo, A., Matsushime, H., Sato, M. (1990). Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. *Oncogene*, **5,** 519-24.

Shore, V.H., Wang, T.H., Wang, C.L., Torry, R.J., Caudle, M.R., Torry, D.S. (1997). Vascular endothelial growth factor, placenta growth factor and their receptors in isolated human trophoblast. *Placenta,* **18,** 657-65.

Sibai, B.M., Hauth, J., Caritis, S., Lindheimer, M.D., MacPherson, C., Klebanoff, M., VanDorsten, J.P., Landon, M., Miodovnik, M., Paul, R., Meis, P., Thurnau, G., Dombrowski, M., Roberts, J. & McNellis, D. (2000). Hypertensive disorders in twin versus singleton gestations. National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. *Am J Obstet Gynecol*, **182,** 938-42.

Skovron, M.L., Berkowitz, G.S., Lapinski, R.H., Kim, J.M., Chitkara, U. (1991). Evaluation of early third-trimester ultrasound screening for intrauterine growth retardation. *J Ultrasound Med*, **10,** 153-9.

Smarason, A.K., Allman, K.G., Young, D. & Redman, C.W. (1997). Elevated levels of serum nitrate, a stable end product of nitric oxide, in women with pre-eclampsia. *Br J Obstet Gynaecol*, **104,** 538-43.

Smarason, A.K., Sargent, I.L., Starkey, P.M. & Redman, C.W. (1993). The effect of placental syncytiotrophoblast microvillous membranes from normal and pre-eclamptic women on the growth of endothelial cells in vitro. *Br J Obstet Gynaecol*, **100,** 943-9.

Smith, G.N., Walker, M., Tessier, J.L. & Millar, K.G. (1997). Increased incidence of preeclampsia in women conceiving by intrauterine insemination with donor versus partner sperm for treatment of primary infertility. *Am J Obstet Gynecol*, **177,** 455-8.

Smith, S.C., Baker, P., Symmonds, E.M. (1997). Increased placental apoptosis in intrauterine growth restriction. *Am J Obstet Gynecol,* **177,** 1395-401.

Snijders, R.J., Nicolaides, K.H. (1994). Fetal biometry at 14-40 weeks' gestation. *Ultrasound Obstet Gynecol*, 4, 34-48.

Song, J., Sapi, E., Brown, W., Nilsen, J., Tartaro, K., Kacinski, B., Naftolin, F., Mor, G. (2000). Expression of Fas and Fas ligand during pregnancy, lactation and involution and its potential role during mammary gland remodelling. *J Clin Invest,* **106,** 1209-1224.

Soothill, P.W., Nicolaides, K.H., Bilardo, K., Hackett, G.A., Campbell, S. (1986). Utero-placental blood velocity resistance index and umbilical venous pO2, pCO2, pH, lactate and erythroblast count in growth-retarded fetuses. *Fetal Ther*, **1,** 176-9.

Soothill, P.W., Nicolaides, K.H., Rodeck, C.H., Campbell, S. (1986). Effect of gestational age on fetal and intervillous blood gas and acid-base values in human pregnancy. *Fetal Ther*, **1,** 168-75.

Spargo, B., McCartney, C. & Winemiller, R. (1959). Glomerular capillary endotheliosis in toxaemia of pregnancy. *Archives of Pathology*, **68,** 593-599.

Steel, S.A., Pearce, J.M., McParland, P., Chamberlain, G.V. (1990). Early Doppler ultrasound screening in prediction of hypertensive disorders of pregnancy. *Lancet,* **335,** 1548-51.

Strand, S., Hofmann, W.J., Hug, H., Muller, M., Otto, G., Strand, D., Mariani, S.M., Stremmel, W., Krammer, P.H., Galle, P.R. (1996). Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells--a mechanism of immune evasion? *Nat Med.* **2,** 1361-6.

Szulman, A.E. & Surti, U. (1982). The clinicopathologic profile of the partial hydatidiform mole. *Obstet Gynecol*, **59,** 597-602.

Taylor, R.N. (1997). Review: immunobiology of preeclampsia. *Am J Reprod Immunol*, **37,** 79-86.

Taylor, R.N., Grimwood, J., Taylor, R.S., McMaster, M.T., Fisher, S.J., North, R.A. (2003). Longitudinal serum concentrations of placental growth factor: evidence for abnormal placental angiogenesis in pathologic pregnancies. *Am J Obstet Gynecol,* **188,** 177-82.

Taylor, R.N., Varma, M., Teng, N.N. & Roberts, J.M. (1990). Women with preeclampsia have higher plasma endothelin levels than women with normal pregnancies. *J Clin Endocrinol Metab*, **71,** 1675-7.

Tedesco, F., Narchi, G., Radillo, O., Meri, S., Ferrone, S., Betterle, C. (1993). Susceptibility of human trophoblast to killing by human complement and the role of the complement regulatory proteins. *J Immunol,* **151,** 1562-70.

Thadhani, R., Mutter, W.P., Wolf, M., Levine, R.J., Taylor, R.N., Sukhatme, V.P., Ecker, J., Karumanchi, S.A. (2004). First trimester placental growth factor and soluble fms-like tyrosine kinase 1 and risk for preeclampsia. *J Clin Endocrinol Metab*, **89,** 770-5.

Thompson, G.W., Hoover, D.B., Ardell, J.L. & Armour, J.A. (1998). Canine intrinsic cardiac neurons involved in cardiac regulation possess NK1, NK2, and NK3 receptors. *Am J Physiol*, **275,** R1683-9.

Thornton, J.G. & Macdonald, A.M. (1999). Twin mothers, pregnancy hypertension and pre-eclampsia. *Br J Obstet Gynaecol*, **106,** 570-5.

Todros, T., Sciarrone, A., Piccoli, E., Guiot, C., Kaufmann, P., Kingdom, J. (1999). Umbilical Doppler waveforms and placental villous angiogenesis in pregnancies complicated by fetal growth restriction. *Obstet Gynecol*, **93,** 499-503.

Torry, D.S., Wang, H.S., Wang, T.H., Caudle, M.R., Torry, R.J. (1998). Preeclampsia is associated with reduced serum levels of placenta growth factor. *Am J Obstet Gynecol*, **179,** 1539-44.

Tranchot-Diallo, J., Gras, G., Parnet-Mathieu, F., Benveniste, O., Marce, D., Roques, P., Milliez, J., Chaouat, G., Dormont, D. (1997). Modulations of cytokine expression in pregnant women. *Am J Reprod Immunol*, **37,** 215-26.

Trudinger, B.J., Giles, W.B., Cook, C.M., Bombardieri, J., Collins, L. (1985). Fetal umbilical artery flow velocity waveforms and placental resistance: clinical significance. *Br J Obstet Gynaecol*, **92,** 23-30.

Trupin, L.S., Simon, L.P. & Eskenazi, B. (1996). Change in paternity: a risk factor for preeclampsia in multiparas. *Epidemiology*, **7,** 240-4.

Tsatsaris,V., Goffin, F., Munaut, C., Brichant, J.F., Pignon, M.R., Noel, A., Schaaps, J.P., Cabrol, D., Frankenne, F., Foidart, J.M. (2003). Overexpression of the soluble vascular endothelial growth factor receptor in preeclamptic patients: pathophysiological consequences. *J Clin Endocrinol Metab*, **88,** 5555-63.

Tsukimori, K., Maeda, H., Shingu, M., Koyanagi, T., Nobunaga, M. & Nakano, H. (1992). The possible role of endothelial cells in hypertensive disorders during pregnancy. *Obstet Gynecol*, **80,** 229-33.

Uckan, D., Steele, A., Cherry, Wang, B.Y., Chamizo, W., Koutsonikolis, A., Gilbert-Barness, E., Good, R.A. (1997). Trophoblasts express Fas ligand: a proposed mechanism for immune privilege in placenta and maternal invasion. *Mol Hum Reprod*, **3,** 655-62.

Valensise, H., Bezzeccheri, V., Rizzo, G., Tranquilli, A.L., Garzetti, G.G., Romanini, C. (1993). Doppler velocimetry of the uterine artery as a screening test for gestational hypertension. *Ultrasound Obstet Gynecol*, **3,** 18-22.

Varani, J., Dame, M.K., Gibbs, D.F., Taylor, C.G., Weinberg, J.M., Shayevitz, J., Ward, P.A. (1992). Human umbilical vein endothelial cell killing by activated neutrophils. Loss of sensitivity to injury is accompanied by decreased iron content during in vitro culture and is restored with exogenous iron. *Lab Invest*, **66,** 708-14.

Villar, J., Belizan, J. (1982) The timing factor in the pathophysiology of intrauterine growth retardation. *Obstet Gynecol Surv*, **37,** 499-506.

Vinatier, D. & Monnier, J.C. (1995). Pre-eclampsia: physiology and immunological aspects. *Eur J Obstet Gynecol Reprod Biol*, **61,** 85-97.

Vintzileos, A.M., Gaffney, S.E., Salinger, L.M., Kontopoulos, V.G., Campbell, W.A., Nochimson, D.J. (1987). The relationships among the fetal biophysical profile, umbilical cord pH, and Apgar scores. *Am J Obstet Gynecol,* **157,** 627-31.

Von Dadelszen, P., Hurst, G. & Redman, C.W. (1999). Supernatants from co-cultured endothelial cells and syncytiotrophoblast microvillous membranes activate peripheral blood leukocytes in vitro. *Hum Reprod*, **14,** 919-24.

Von Dadelszen, P., Watson, R.W.G., Noorwali, F., Marshall, J.C., Parodo, J., Farine, D, Lye, S.J., Ritchie, J.W., Rotstein, O.D. (1999). Maternal neutrophil apoptosis in normal pregnancy, preeclampsia, and normotensive intrauterine growth restriction. *Am J Obstet Gynecol,* **181,** 408-14.

Vuckovic M, Ponting J, Terman BI, Niketic V, Seif MW, Kumar S. (1996). Expression of the vascular endothelial growth factor receptor, KDR, in human placenta. *J Anat*, **188,** 361-6.

Vuorela, P., Hatva, E., Lymboussaki, A., Kaipainen, A., Joukov, V., Persico, M.G., Alitalo, K., Halmesmaki E. (1997). Expression of vascular endothelial growth factor and placenta growth factor in human placenta. *Biol Reprod*, **56,** 489-94.

Vuorela, P., Helske,S., Hornig, C., Alitalo, K., Weich, H., Halmesmaki, E. (2000). Amniotic fluid--soluble vascular endothelial growth factor receptor-1 in preeclampsia. *Obstet Gynecol*, **95,** 353-7.

Vyas, S., Nicolaides, K.H., Bower, S., Campbell, S. (1990). Middle cerebral artery flow velocity waveforms in fetal hypoxaemia. *Br J Obstet Gynaecol*, **97,** 797-803.

Wang, Y. & Walsh, S.W. (1996). Antioxidant activities and mRNA expression of superoxide dismutase, catalase, and glutathione peroxidase in normal and preeclamptic placentas. *J Soc Gynecol Investig*, **3,** 179-84.

Wang, Y. & Walsh, S.W. (1996). Antioxidant activities and mRNA expression of superoxide dismutase, catalase, and glutathione peroxidase in normal and preeclamptic placentas. *J Soc Gynecol Investig*, **3,** 179-84.

Wang, Y., Walsh, S.W. & Kay, H.H. (1992). Placental lipid peroxides and thromboxane are increased and prostacyclin is decreased in women with preeclampsia. *Am J Obstet Gynecol*, **167,** 946-9.

Wang, Y.P., Walsh, S.W., Guo, J.D. & Zhang, J.Y. (1991). The imbalance between thromboxane and prostacyclin in preeclampsia is associated with an imbalance between lipid peroxides and vitamin E in maternal blood. *Am J Obstet Gynecol*, **165,** 1695-700.

Wang, Y.P., Walsh, S.W., Guo, J.D. & Zhang, J.Y. (1991). The imbalance between thromboxane and prostacyclin in preeclampsia is associated with an imbalance between lipid peroxides and vitamin E in maternal blood. *Am J Obstet Gynecol*, **165,** 1695-700.

Watanabe, Y., Dvorak, H.F. (1997). Vascular permeability factor/vascular endothelial growth factor inhibits anchorage-disruption-induced apoptosis in microvessel endothelial cells by inducing scaffold formation. *Exp Cell Res,* **233,** 340-9.

Weetman, A. (1999). The immunology of pregnancy. *Thyroid,* **9,** 643-6.

Wegmann, T.G., Guilbert, L.J. (1992). Immune signalling at the maternal-fetal interface and trophoblast differentiation. *Dev Comp Immunol*, **16,** 425-30.

Wegmann, T.G., Lin, H., Guilbert, L., Mosmann, T.R. (1993). Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today*, **14,** 353-6.

Weiner, C.P., Lizasoain, I., Baylis, S.A., Knowles, R.G., Charles, I.G. & Moncada, S. (1994). Induction of calcium-dependent nitric oxide synthases by sex hormones. *Proc Natl Acad Sci U S A*, **91,** 5212-6.

Wheeler, T., Elcock, C.L., Anthony, F.W. (1995). Angiogenesis and the placental environment. *Placenta,* **16,** 289-96.

Wheeler, T., Murrils, A. (1978). Patterns of fetal heart rate activity during normal pregnancy. *Br J Obstet Gynaecol,* **86,** 18-27.

Wilting, J., Birkenhager, R., Eichmann, A., Kurz, H., Martiny-Baron, G., Marme, D., McCarthy, J.E., Christ, B., Weich, H.A. (1996). VEGF121 induces proliferation of vascular endothelial cells and expression of flk-1 without affecting lymphatic vessels of chorioallantoic membrane. *Dev Biol*, **176,** 76-85.

Xiao, S., Jodo, S., Sung, S.S., Marshak-Rothstein, A., Ju, S.T. (2002). A novel signaling mechanism for soluble CD95 ligand. Synergy with anti-CD95 monoclonal antibodies for apoptosis and NF-kappaB nuclear translocation. *J Biol Chem,* **277,** 50907-13.

Yamada, Y., Nezu, J., Shimane, M,, Hirata, Y. (1997). Molecular cloning of a novel vascular endothelial growth factor, VEGF-D. *Genomics*, **42,** 483-8.

Yamaguchi, S., Iwata, K., Shibuya, M. (2002). Soluble Flt-1 (soluble VEGFR-1), a potent natural antiangiogenic molecule in mammals, is phylogenetically conserved in avians. *Biochem Biophys Res Commun,* **291,** 554–9.

Yang, D., Lang, U., Greenberg, S.G., Myatt, L. & Clark, K.E. (1996). Elevation of nitrate levels in pregnant ewes and their fetuses. *Am J Obstet Gynecol*, **174,** 573-7.

Yang, J.C. (2002). A randomizd double-blind placebo controlled trial of bevacizumab (anti-VEGF antibody) demonstrating a prolongation in time to progression in patients with metastatic renal cancer: ASCO meeting abstract. *Proc. Am. Soc. Clin. Oncol.* **12,** A15 (Abstr.).

Yoshimoto, S., Ishizaki, Y., Kurihara, H., Sasaki, T., Yoshizumi, M., Yanagisawa, M., Yazaki, Y., Masaki, T., Takakura, K. & Murota, S. (1990). Cerebral microvessel endothelium is producing endothelin. *Brain Res*, **508,** 283-5.

Yui, J., Garcia-Lloret, M., Brown, A.J., Berdan, R.C., Morrish, D.W., Wegmann, T,G., Guilbert, L.J. (1994). Functional, long-term cultures of human term trophoblasts purified by column-elimination of CD9 expressing cells. *Placenta,* **15,** 231-46.

Zeng, H., Dvorak, H.F., Mukhopadhyay, D. (2001). Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) peceptor-1 down-modulates VPF/VEGF receptor-2-mediated endothelial cell proliferation, but not migration, through phosphatidylinositol 3-kinase-dependent pathways. *J Biol Chem*, **276,** 26969-79.

Zhou, Y., Damsky, C.H. & Fisher, S.J. (1997a). Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? *J Clin Invest*, **99,** 2152-64.

Zhou, Y., Fisher, S.J., Janatpour, M., Genbacev, O., Dejana, E., Wheelock, M. & Damsky, C.H. (1997b). Human cytotrophoblasts adopt a vascular phenotype

as they differentiate. A strategy for successful endovascular invasion? *J Clin Invest*, **99,** 2139-51.

Zhou, Y., McMaster, M., Woo, K., Janatpour, M., Perry, J., Karpanen, T., Alitalo, K., Damsky, C., Fisher, S.J. (2002). Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. *Am J Pathol*, **160,** 1405-23.

Ziche, M., Maglione, D., Ribatti, D., Morbidelli, L., Lago, C.T., Battisti, M., Paoletti, I., Barra, A., Tucci, M., Parise, G., Vincenti, V., Granger, H.J., Viglietto, G., Persico, M.G. (1997). Placenta growth factor-1 is chemotactic, mitogenic, and angiogenic. *Lab Invest,* **76,** 517-31.

Zimmermann, P., Eirio, V., Koskinen, J., Kujansuu, E., Ranta, T. (1997). Doppler assessment of the uterine and uteroplacental circulation in the second trimester in pregnancies at high risk for pre-eclampsia and/or intrauterine growth retardation: comparison and correlation between different Doppler parameters. *Ultrasound Obstet Gynecol*, **9,** 330-8.

Zwiefel, P. (1916) Eklampsie. In; *Handbuch der Gegurtshilfe*, Vol II (ed) Dodelein, A Wiesbaden, Bergman. P672-723.