THE EFFECT OF ANTIHYPERTENSIVE THERAPY ON HAEMODYNAMIC AND PLACENTAL MARKERS IN HYPERTENSIVE DISORDERS IN PREGNANCY

Submitted by

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Declaration

The work presented in this thesis is my own.

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ABSTRACT

The aim of this thesis was to investigate the effect of antihypertensive therapy on vascular function and placental markers in hypertensive disorders in pregnancy (HTD). We prospectively studied 208 women at the Homerton and University College London Hospitals. Vascular and serum markers were measured in 80 with HTD [51 pre-eclampsia (PE), 29 gestational hypertension (GH)] and 80 normotensive controls. The same markers were measured in placental samples from another 48 women (14 PE, 10 GH, 24 controls). Pulse wave analysis indices [augmentation pressure (AP) and augmentation index at heart rate 75/minute (AIx-75)], serum and placental concentrations of soluble fms-like tyrosine-kinase-1 (sFlt-1), soluble endoglin (sEng), placental growth factor (PlGF), vascular endothelial growth factor (VEGF), inhibin A, activin A, and uterine artery Doppler were measured before, and 24-48 hours after, initiating antihypertensive therapy. The three study groups were compared using ANOVA multiple comparisons with Bonferroni post hoc testing. Marker levels before and after antihypertensives were compared using paired t-test.

In both pre-eclampsia ($P<0.0001$) and gestational hypertension ($P<0.05$), serum sFlt-1 was increased and PlGF reduced ($P<0.001$) compared to controls. Serum sEng levels were also increased in pre-eclampsia. Placental sFlt-1 and sEng were significantly higher ($P<0.0001$), and PlGF lower ($P = 0.008$), in pre-eclampsia compared to controls and gestational hypertension. Antihypertensive therapy was associated with a significant fall in serum and placental sFlt-1 and sEng in pre-eclampsia only ($P<0.05$). In

pre-eclampsia, but not gestational hypertension, treatment was associated with significantly ($P< 0.05$) lower serum and placental inhibin A and activin A. In women with pre-eclampsia or gestational hypertension, both AP ($P<0.0001$ and $P<0.05$) and AIx-75 ($P<0.0001$ and $P<0.001$) were significantly higher than controls. Antihypertensive therapy resulted in a significant fall in both AP and AIx-75 in pre-eclampsia only ($P<0.0001$).

Antihypertensive drugs may have an effect on the pathophysiology of pre-eclampsia other than their known antihypertensive action.

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My supervisors, Mr Kevin Harrington and Professor Eric Jauniaux, created the idea for the thesis. I was solely responsible for the development of the idea, recruitment of women and performing the studies, including scanning women and data collection at recruitment and after delivery. I performed the analysis of the samples for measuring the various markers at the University College London research laboratory under the supervision of Dr Shanthi Muttukrishna, Lecturer in Reproductive Sciences, University College London Institute for Women's Health. I am indebted to Shanthi for teaching me the techniques of ELISA and placental protein extraction. I performed the statistical analysis, with guidance from Dr Derek Cooper, Senior Statistician at King's College London. I was responsible for the writing of the thesis and papers with advice from my supervisors.

My supervisors Kevin and Eric have provided me with constant support, encouragement and enthusiasm throughout the three years since I commenced this research. This has allowed me to work in an un-stressful environment whilst knowing that help was always available if required and for this I am extremely grateful.

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FIGURE LEGENDS

Figure 3.7. Augmentation pressure (AP) (a) and augmentation index at heart rate 75/min (AIx-75) (b) measurements in normotensives (controls), women with pre-eclampsia and women with gestational hypertension according to gestational age interval. Measurements before and after alpha methyldopa therapy are shown for women with pre-eclampsia and women with gestational hypertension.

Figure 3.8. Augmentation pressure (AP) (a) and augmentation index at heart rate 75/min (AIx-75) (b) measurements in women with early onset and late onset pre-eclampsia (PE), and in women with mild and severe pre-eclampsia.

Figure 3.9. Uterine artery Doppler mean Pulsatility Index before and after antihypertensive therapy in women with pre-eclampsia or gestational hypertension, and in normotensive controls, stratified according to gestation.

Figure 3.10. Uterine artery Doppler mean Resistance Index before and after antihypertensive therapy in women with pre-eclampsia or gestational hypertension, and in normotensive controls, stratified according to gestation.

Figure 3.11. Uterine artery Doppler mean Pulsatility Index in women with early compared with late onset, and severe compared with mild pre-eclampsia.

Figure 3.12. Uterine artery Doppler mean Resistance Index in women with early compared with late onset, and severe compared with mild pre-eclampsia.

Figure 3.13. Pulse wave analysis parameters according to gestation. Scatter plots of (a) augmentation pressure (AP), and (b) augmentation index at heart rate of 75/min (AIx-75) according to the gestational age in days (n = 541).

Figure 3.14. Monthly changes in augmentation index at heart rate of 75/min, central and peripheral blood pressure.

Figure 3.15. Longitudinal changes in pulse wave analysis parameters. Longitudinal data for the 45 women who had measurements taken at 12^{+0}-12^{+6} weeks, 23^{+0}-23^{+6} weeks, and 32^{+0}-32^{+6} weeks of gestation: (a) augmentation pressure (AP), and (b) augmentation index at heart rate 75 beats per minute (AIx-75).

TABLE LEGENDS

Table 3.1. Baseline characteristics of the study groups in whom serum levels of markers, pulse wave analysis and uterine artery Doppler were measured, according to gestational age at recruitment.

Table 3.2. Placental concentrations of sFlt-1, PlGF, sEng and VEGF (expressed per mg protein) in normotensive controls, pre-eclampsia and gestational hypertension, grouped according to whether they received antihypertensive therapy or not.

Table 3.3. Placental concentrations of inhibin A and activin A (expressed per mg protein) in women with pre-eclampsia, women with gestational hypertension and controls. Women with pre-eclampsia or gestational hypertension are grouped according to whether they received antihypertensive therapy or not.

Table 3.4. Heart rate, brachial and central haemodynamic measurements prior to antihypertensive therapy in women with pre-eclampsia, women with gestational hypertension and matched controls.

Table 3.5. Brachial and central haemodynamic measurements in women with pre-eclampsia and women with gestational hypertension. Measurements before and after alpha methyldopa therapy are compared.

Table 3.6. Baseline characteristics of pregnant and non-pregnant women.

Table 3.7. Haemodynamic parameters in pregnant and non-pregnant women.

ABBREVIATIONS

AFP	Alpha-fetoprotein
AIx	Augmentation index
AIx-75	Augmentation index at heart rate of 75 beats per minute
ANOVA	Analysis of variance
AP	Augmentation pressure
BMI	Body mass index
BP	Blood pressure
BSA	Bovine serum albumin
ELISA	Enzyme linked immuno-sorbent assay
FGR	Fetal growth restriction
FSH	Follicle stimulating hormone
GA	Gestational age
GH	Gestational hypertension
GnRH	Gonadotrophin releasing hormone
HCG	Human chorionic gonadotrophin
HELLP syndrome	Haemolysis, elevated liver enzymes, low platelets
OD	Optical density
PAPP-A	Pregnancy associated plasma protein A
PBS	Phosphate buffered saline
PE	Pre-eclampsia
PI	Pulsatility index

PlGF	Placental growth factor
PP	Pulse pressure
PP13	Placental protein 13
PWA	Pulse wave analysis
QC	Quality control
RI	Resistance index
SD	Standard deviation
SDS	Sodium dodecyl sulphate
sEng	Soluble endoglin
sFlt-1	Soluble serum fms-like tyrosine kinase-1
TGF	Transforming growth factor
UE3	Unconjugated estriol
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

PUBLICATIONS

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AWARDS (related to this research)

Best poster

British Maternal & Fetal Medicine Society, 2007

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CONTENTS **PAGE**

CHAPTER ONE

INTRODUCTION

INTRODUCTION

1.1 PRE-ECLAMPSIA

1.1.1 Background

Pre-eclampsia is a condition found only in human pregnancy. Its pathophysiology is still incompletely understood, so it has typically been defined as a triad of presenting symptoms and signs, namely hypertension, proteinuria, and oedema. In this thesis, the definition used is that of the International Society for the Study of the Hypertension in Pregnancy (ISSHP). This defines pre-eclampsia as "Two recordings of diastolic blood pressure ≥ 90 mm Hg, at least four hours apart; or one recording of diastolic BP ≥ 120 mm Hg, in a previously normotensive woman; and urine protein excretion ≥ 300 mg in 24 hours, or two readings of ++ or more on dipstick analysis of a midstream or catheter specimen of urine, if no 24 hour collection was available".[1]

1.1.2 Incidence

Pre-eclampsia occurs in 2-8% of all pregnancies and is a leading cause of maternal mortality and morbidity.[2-4] It is also responsible for considerable perinatal mortality and morbidity, as a result of both placental insufficiency and iatrogenic prematurity. Furthermore, pre-eclampsia carries long-term health implications for both mother and baby. Fetuses who suffered from intrauterine

growth restriction are at increased risk of hypertension and cardiovascular diseases later in life.[5-7] A recent meta-analysis has shown that women who suffer pre-eclampsia are at increased risk later in life of ischaemic heart disease (RR 2.16; 95% CI 1.86 to 2.52), hypertension (RR 3.7; 95% CI 2.7 to 5.05), stroke (RR 1.81; 95% CI 1.45 to 2.27) and venous thromboembolism (RR 1.79; 95% CI 1.37 to 2.33).[8] It is not known if this increased risk is a result of pre-eclampsia, or if factors predisposing women to pre-eclampsia also put them at increased risk of later cardiovascular disease. The only definitive treatment for pre-eclampsia is delivery of the fetus and placenta, regardless of the gestation. As a result, 15% of preterm births are secondary to early delivery for pre-eclampsia.[9]

1.1.3 Aetiology

Despite huge investment in terms of time, effort and money, the precise aetiology of pre-eclampsia has yet to be fully understood. The condition was formerly known as 'Toxaemia of pregnancy' because it was thought to result from a circulating toxin of fetal origin. Although a century later this idea of a circulating 'toxin' remains, it now seems certain that the source is the placenta rather than the fetus.[10-14] It is now widely accepted that abnormal trophoblastic invasion of maternal spiral arteries leads to placental ischaemia.[15-21] A placental factor is released into the maternal circulation where it has widespread effects on vascular endothelium, leading to the maternal syndrome of pre-eclampsia.[22-26] In addition, placental ischaemia may lead to intrauterine fetal growth restriction (FGR). As a result of endothelial damage, the maternal

syndrome can involve almost every major system of the body, particularly the brain, kidneys, liver and coagulation system.

1.1.4 The genetics of pre-eclampsia

The genetic aspects of pre-eclampsia are complex.[27-31] Women born after a pre-eclamptic pregnancy have double the risk of pre-eclampsia in their first pregnancy compared with women born of normotensive pregnancies (OR 2.2, 95% CI 2.0-2.4).[32] For men born after a pre-eclamptic pregnancy, the risk of pre-eclampsia in their partner's first pregnancy is also moderately increased, compared with men born after a pregnancy not complicated by pre-eclampsia (OR 1.5, 95% CI 1.3-1.7). Women and men born after pre-eclamptic pregnancies are also more likely to trigger *severe* pre-eclampsia in their own (or their partner's) pregnancy (OR 3.0, 95% CI 2.4-3.7 for mothers; and 1.9, 1.4-2.5 for fathers).

It seems likely that all these data are explained by the concept of gene transmission through both males and females. It is likely that some genes confer susceptibility to the development of pre-eclampsia in women, while others (transmitted to the fetus) are more likely to trigger pre-eclampsia in the mother. A man born of a pre-eclamptic pregnancy is more likely to possess, and therefore pass on to any fetus he fathers, genes which may trigger pre-eclampsia in the mother. Similarly, a woman born of a pre-eclamptic pregnancy may pass on these same pre-eclampsia triggering genes to her own fetus, thus making her more likely to develop pre-eclampsia during her own pregnancy. In addition, however, this mother may also have inherited

from her own mother a gene which makes her more susceptible to developing pre-eclampsia. Thus, women born of pre-eclamptic pregnancies themselves appear to be at the highest risk as they have both maternal and fetal predispositions.[32]

1.1.5 Immunological Factors

It seems certain that a failure of adequate trophoblast invasion of maternal spiral arteries in early pregnancy eventually leads to pre-eclampsia. However, it is far from clear what causes this failure. Some evidence suggests that it is a failure of the maternal immune system to 'tolerate' fetal antigens derived from its father.[33-35] Pregnancies associated with a greater trophoblast mass, e.g. multiple pregnancy, molar pregnancy, hydropic placenta, are associated with an increased risk of pre-eclampsia.[36]

Pre-eclampsia tends to be most severe in first pregnancies.[37] The maternal exposure to paternally derived fetal antigens which occurs during the first pregnancy may facilitate future tolerance of these genes. As a result, the prevalence (and severity) of pre-eclampsia tends to be less in subsequent pregnancies with the same partner. There is ample epidemiological evidence that prior exposure to paternal antigens protects against pre-eclampsia.[36,38] Furthermore, women becoming pregnant using donor embryos are at even higher risk of pre-eclampsia, presumably because the entire fetal genome is foreign.[39] Consistent with this theory is the observation that if women without

pre-eclampsia in their first pregnancy change partners for a subsequent pregnancy, there is a 30% increase in the risk of pre-eclampsia.[40]

1.1.5 The role of the placenta in the maternal syndrome

Over twenty years ago, Rodgers and colleagues[22] performed a series of elegant studies which supported the hypothesis that the hypoxic placenta released a factor or factors which circulated in the maternal circulation, and which ultimately led to endothelial cell damage. They showed that serum from women with pre-eclampsia was cytotoxic to cultured Human Umbilical Vein Endothelial Cells (HUVECs) when compared with control serum from normotensive pregnant women. They also showed that this cytotoxic effect declined rapidly when they used serum from pre-eclamptic women taken 24, then 48 hours after delivery. This suggested that the putative circulating factor(s) had a short half-life and was a product of the placenta (or fetus). The cytotoxic effect persisted when a mixture of sera from normal and pre-eclamptic women was used, suggesting that the effect was due to an active circulating factor, as opposed to a relative deficiency of a protective factor.

The authors suggested that these factors caused gross morphological injury and cell death. However, more recent work [23,24] suggests that the circulating factor(s) cause an alteration in endothelial cell *function* (as opposed to morphology). These studies show that, even in the presence of pre-eclamptic serum, endothelial cells proliferate, adhere, and spread normally. Moreover, endothelial cells continue to grow well in pre-eclamptic serum, and any

metabolic changes can be reversed by replacing the pre-eclamptic serum with standard culture medium.[25]

Other investigators have confirmed that it is a property of *plasma* from pre-eclamptic women, as opposed to any intrinsic abnormality of the endothelium or vessel wall, which leads to the endothelial dysfunction. Ashworth *et al* [26] incubated vessels isolated from normal pregnant women with plasma from women with pre-eclampsia. Using an isometric technique, they demonstrated an alteration in the endothelium dependent responses, such as the response to bradykinin, was markedly impaired. These blood vessels from normotensive pregnant women, in the presence of pre-eclamptic plasma, mimicked the behaviour of vessels isolated from women with pre-eclampsia.

1.1.6 Oxidative stress

Oxidative stress is a pathological state implicated in the aetiology of many disorders including atherosclerosis, in which pro-oxidants dominate over antioxidants. Recent evidence supports the hypothesis that abnormal placental perfusion (leading to hypoxia or hypoxia/reperfusion) results in the generation of reactive oxygen species [41] capable of damaging cell membranes, proteins and DNA.[42-46] Plasma levels of various antioxidants are lower in pre-eclamptic women compared with normotensive pregnant women; these include ascorbic acid, alpha–tocopherol and beta-carotene.[47-51] There is some evidence that increased levels of plasma endothelin-1 (ET-1) in women with pre-eclampsia may cause oxidative stress in the placenta.[52]

Increased superoxide generation caused by activation of NADPH oxidase is another possible cause of oxidative stress in the placenta in pre-eclampsia.[53]

In normal pregnancy, the onset of maternal blood flow into the placenta at around 10-12 weeks' gestation leads to an increase in oxygen tension.[54] Probably in response to this rise, there is a parallel rise in the levels of several antioxidants in the placenta. It is postulated [54] that a relative lack of this antioxidant activity could result in oxidative stress to the trophoblast causing trophoblastic degeneration. This in turn could contribute to impaired trophoblastic invasion of the spiral arteries. In other words, oxidative stress may play an important role early in the pathophysiology of pre-eclampsia.

All of this evidence suggested a potential role for antioxidants in the prevention of pre-eclampsia. Chappell and colleagues [55] carried out a prospective randomised placebo-controlled trial to examine the effects of vitamin C and E supplementation for women at increased risk of pre-eclampsia. They found a significant decrease in plasma markers of vascular endothelial cell activation and placental insufficiency. They also found a reduction in the occurrence of pre-eclampsia in the treated groups. As a result of this initial trial, a large multi-centre randomised trial, the Vitamins in Pregnancy Trial (VIP), was undertaken. Unfortunately, this failed to show any beneficial effects of vitamin C and E supplementation in these women.[56] Moreover, the VIP study found an *increased* incidence of low birthweight babies (< 2.5 kg) in the women taking vitamin supplements. Clearly these results were disappointing.

1.1.7 Trophoblast microparticles

The placenta is constantly growing but at the same time there is an ongoing process of apoptosis which leads to the release of syncytiotrophoblast microparticles into the maternal circulation. These microparticles are found in maternal blood in normal pregnancy, but found in significantly higher concentrations in the circulation of women with pre-eclampsia.[57] These microparticles can activate neutrophils and contribute to the systemic inflammatory response which characterises pre-eclampsia.[57]

1.1.8 Endothelial dysfunction in pre-eclampsia

The vascular endothelium is not merely an inert layer lining the vasculature but plays an active role in mediating acetylcholine-induced relaxation,[58] maintaining thrombo-resistance and participating in the inflammatory response.[59] The characteristic renal lesion known as Glomerular Endotheliosis, first described by Spargo in 1959,[60] is found in up to 80% women with pre-eclampsia, but disappears completely after delivery. It consists of glomerular capillary cells engorged with intracellular inclusions. This type of lesion is not found in any other form of hypertension, suggesting that it is unlikely to be a result of hypertension or hypoperfusion per se; rather, it is likely to result from the specific endothelial damage found in pre-eclampsia.

Many studies have described the hypercoagulability associated with pre-eclampsia.[61-65] Increased levels of fibronectin, factor VIII antigen, von

Willebrand's factor, tissue plasminogen activator [61,66] and circulating endothelin-1 [62,63] have been found in the plasma of women with pre-eclampsia. In addition, a reduction in platelet count [64] and an increase in the levels of plasma β-thromboglobulin [65] have also been described; these changes pre-date the clinical symptoms of the disease by several weeks.

There is a wealth of evidence that vascular sensitivity to pressor agents such as angiotensin II and noradrenaline is significantly reduced in normotensive pregnant women.[67-69] Women with pre-eclampsia demonstrate increased sensitivity to these pressor agents (similar in fact to non-pregnant women). A longitudinal study found that women destined to develop pre-eclampsia had increased sensitivity to infused angiotensin II from as early as 18 weeks of gestation.[67] Another study [68] confirmed this finding, showing that the responsiveness of vessels from pre-eclamptic women and non-pregnant women were similar. A similar increase in sensitivity to KCl or vasopressin in the omental arteries of pre-eclamptic women has also been described.[70]

A decreased response to pressor agents in normotensive pregnancy has also been demonstrated in animal models. Furthermore, removal of the endothelium has no significant effect on this reduction in response, suggesting that these changes seen in pregnancy may be neurologically mediated.[71]

While the responsiveness of pre-eclamptic small vessels to pressor agents is increased, their relaxation in response to relaxing agents is impaired. This has been demonstrated for acetylcholine,[72] bradykinin [73] and acetylcholine antihistamine.[74] This effect has been shown in subcutaneous resistance arteries,[72-74] omental arteries [70] and small myometrial arteries.[26]

1.1.9 Nitric Oxide in normal pregnancy and pre-eclampsia

The role of Nitric Oxide in the vascular changes in normal pregnancy and pre-eclampsia is debated. Nitric Oxide (NO) is synthesized from L-arginine in the reaction catalyzed by the enzyme Nitric Oxide Synthase (NOS), a reaction which requires at least four co-factors. There are at least three isoforms of NOS; endothelial (eNOS) and neuronal (nNOS) are the two constitutive forms and they are activated by an influx of calcium into cells. The third isoform, inducible NOS (iNOS), is functionally independent of calcium. iNOS is produced in response to immunological stimuli and is expressed in a wide variety of cells including macrophages, neutrophils and mast cells.[75]

Animal studies suggest that maternal vasodilation in normal pregnancy is mediated at least in part by increased nitric oxide synthesis.[76,77] However, the situation is not as clear in human pregnancy. Some investigators [76,77] found increased serum concentrations of nitrite (a stable end product of NO metabolism) in normotensive pregnant women compared with non-pregnant women. In contrast, other investigators have found no difference in plasma or urinary nitrate (the second stable end product of NO metabolism) or in exhaled NO in normal pregnancy.[78-80] Studies in pre-eclamptic pregnancies also conflict; some report increased levels,[81-83] others finding decreased levels [84] and some reporting no difference.[78] These conflicting results are probably explained by differences in

methodology, dietary intake of nitrate and glomerular filtration rate (which is commonly reduced in pre-eclampsia). In an attempt to overcome these limitations, a longitudinal study [85] was performed in women whose dietary intake of nitrate was strictly controlled and 24-hour urine collection samples analysed. No increase in plasma or urinary nitrate or nitrite was found in normal pregnancy, but there was a modest reduction in urinary excretion of these two metabolites of NO in pre-eclampsia.

Another possible explanation for these findings is that in normal pregnancy and pre-eclampsia nitric oxide production may vary in local vascular beds, whereas global production may not be significantly changed.

There is increasing evidence that NO synthesis may be regulated by the production of endogenous inhibitors of nitric oxide synthesis such as asymmetric dimethyl arginine (ADMA). The fall in blood pressure in normal pregnancy is mirrored by a significant fall in plasma concentration of ADMA and, later in pregnancy (as blood pressure rises again), ADMA concentration also rises.[86] Concentrations of ADMA are also significantly raised in pre-eclamptic women compared with non-pregnant or normotensive pregnant controls.[86] The authors of this study postulated that this excess ADMA might suppress NO synthesis, thus impairing NO mediated vasorelaxation.

However, it seems that the role of nitric oxide in the vasorelaxation of normal pregnancy may be limited. Various researchers studying small vessel relaxation described an endothelium dependent, NO independent vasorelaxation in subcutaneous arteries,[72] omental small arteries,[70] and myometrial resistance arteries.[26] Furthermore, impaired endothelium dependent relaxation has been described in vessels from women with pre-eclampsia, again suggesting that the role of NO is limited.

1.1.10 Pre-eclampsia as an inflammatory state

Normal pregnancy can be seen as an inflammatory process in which the endothelium plays a key role.[87] Monocytes, granulocytes and the endothelium are activated,[57] and there is evidence of increasing oxidative stress with advancing gestation.[88] All the inflammatory changes of normal pregnancy are exaggerated in pre-eclampsia. These include increased leucocytosis,[89] leucocyte activation,[90] complement activation,[91] activation of the clotting system and platelets (as described above), increased markers of endothelial activation,[92] and increased circulating pro-inflammatory cytokines, including tumour necrosis factor-α,[93] interleukin-6,[94] and interleukin-8.[95] In a recent review, Redman and Sargent [96] argue that although the consequences of dysfunctional endothelium are responsible for the most striking clinical features of pre-eclampsia, the other components of the systemic inflammatory network are also involved.

1.2 VASCULAR AND PLACENTAL MARKERS

1.2.1 Background

The vascular endothelial growth factor (VEGF) super-family is key in angiogenesis. It includes VEGF-A to F, and placental growth factor (PlGF). All members of the VEGF family (including PlGF) share a common structure of eight characteristically spaced cysteine residues.[97-99] VEGF-A is a key molecule in angiogenesis and vasculogenesis in a wide variety of situations including embryogenesis, corpus luteum formation, tumour growth and wound healing.[100] It mediates its responses primarily through the receptors VEGFR-1 and VEGFR-2.[97,101] It also increases blood vessel permeability and has an anti-apoptotic effect.[102-106] VEGF-A is highly expressed in the placenta, in both villous and extra-villous trophoblast cells.[107-110]

1.2.2 Placental Growth Factor

Human placental growth factor (PlGF) is structurally similar to the other members of the VEGF family,[111-113] and can potentiate their angiogenic effects.[114,115] PlGF is synthesized not just by cells of placental origin, but in many other tissues including human thyroid, brain, lung and skeletal

muscle.[116-120] Cross-sectional studies show that, in normal pregnancy, PlGF levels rise during the second trimester, reaching a peak in the early third trimester, probably reflecting growth of the placenta and the need to recruit and maintain an adequate placental circulation.[121] Levels then fall, reaching lower levels by the time of delivery. This gradual fall may be associated with slowly increasing placental hypoxia linked to increasing myometrial tone in the third trimester and, in particular, associated with uterine contractions during labour.

VEGF receptor-1 (VEGFR-1) is also known as FMS-like tyrosine kinase 1 (Flt-1). It is found in trophoblast but also in pericytes, osteoblasts, monocytes, renal mesangial cells and in some haematopoietic stem cells.[122,123] Flt-1 is stimulated by PlGF, VEGF-B and VEGF-A. Flt-1 is up-regulated during angiogenesis and also by hypoxia.

1.2.3 Soluble serum FMS-like tyrosine kinase 1 (sFlt-1)

Soluble serum FMS-like tyrosine kinase 1 (sFlt-1, also known as sVEGFR-1) is a splice variant of VEGFR-1 (Flt-1). sFlt-1 consists of the extracellular domain of Flt-1 and can bind to both VEGF and PlGF in maternal serum. In this way, it reduces the maternal serum concentration of free (i.e. bio-active) VEGF and PlGF, resulting in impaired angiogenesis and impaired endothelial cell proliferation;[124-127] in other words, sFlt-1 is an effective antagonist of VEGF and PlGF.[128-130] sFlt-1 is produced by a number of tissues including the placenta, yet its physiological role is as yet undefined.[131] Recently, evidence has emerged that sFlt-1 plays an important role in the pathophysiology of pre-eclampsia. Initial studies using in situ hybridisation showed that trophoblast

expresses sFlt-1 mRNA.[132] Soon after, it was discovered that serum from pregnant women contains a VEGF binding protein, later confirmed to be sFlt-1.[126,133] Since then, several studies have confirmed that maternal serum sFlt-1 levels, placental sFlt-1 expression and amniotic fluid sFlt-1 levels are all elevated in pre-eclampsia. [124-127,134-140]

In vitro evidence

Using the HUVEC (human umbilical vein endothelial cells) model, Maynard et al [134] demonstrated that the serum of women with pre-eclampsia has profound anti-angiogenic effects in-vitro. They also showed a similar effect when normotensive maternal serum with added sFlt-1 was used. Levels of circulating sFlt-1 similar to those observed in pre-eclampsia blocked the vasodilatation normally induced by VEGF or PlGF, suggesting that sFlt-1 might oppose physiological vasorelaxation, thus contributing to the hypertension in pre-eclampsia. The authors showed that, in pre-eclampsia, excess sFlt-1 is released by the placenta into the maternal circulation where it binds to VEGF and PlGF, leading to widespread endothelial dysfunction and the maternal syndrome.

Interestingly, several earlier studies had shown that maternal serum VEGF levels are *increased*, while others have reported *reduced* levels in women with pre-eclampsia.[141-147] On further analysis, however, it is clear that all the studies reporting increased VEGF levels measured *total* (both bound and unbound) VEGF, while those reporting reduced levels all used a commercially available ELISA kit (R & D systems) which measures *free* (unbound and therefore bioactive) VEGF levels. This difference is pivotal in

interpreting the results in pre-eclampsia, when *free* VEGF may be reduced (because of sFlt-1 binding) while *total* VEGF may be unchanged.

In vivo effects of sFlt-1

In pregnant rats the administration of sFlt-1 causes a reaction similar to that of human pre-eclampsia including hypertension, proteinuria and glomerular endotheliosis.[134] Similar changes were observed in non-pregnant rats injected with sFlt-1, suggesting that its systemic effects do not actually require the presence of a fetus or placenta. These findings are all the more remarkable, given that pre-eclampsia is not observed in mammals other than humans.

More recently, a case control study using blood samples from the Calcium for Pre-Eclampsia Prevention trial (CPEP) found that, in normotensive controls, sFlt-1 levels rose and PlGF levels fell only in the last two months of pregnancy.[135] In contrast, in women who later developed pre-eclampsia, these changes occurred earlier and were more pronounced. The rise in sFlt-1 levels can be detected around five weeks before the onset of clinical pre-eclampsia. PlGF levels were significantly lower in women who went on to develop pre-eclampsia than in controls. This difference could be detected as early as 13 weeks' gestation, and became more marked as the onset of pre-eclampsia approached. The rise in sFlt-1 levels was proportional to the fall in PlGF. This is consistent with the hypothesis that in pre-eclampsia there is increased release of sFlt-1 which binds to circulating PlGF, and suggests that a rise in sFlt-1 and fall in PlGF could predict pre-eclampsia.

The work of other groups has confirmed these findings.[134] In women who subsequently develop pre-eclampsia, lower PlGF levels have been

demonstrated in the second trimester,[138,148] and even as early as the first trimester, although they are less marked at this stage.[121,149,150] Unfortunately, attempts at predicting pre-eclampsia using either first or second trimester *serum* PlGF have proved disappointing, with poor sensitivity and specificity.[151,152]

Interestingly, although rats given exogenous sFlt-1 developed a typical pre-eclamptic phenotype, none developed thrombocytopenia or frank HELLP syndrome (haemolysis, elevated liver enzymes and low platelets).[134] Recent work suggests that endoglin may play a role in HELLP syndrome (see below).[153,154]

1.2.4 Endoglin

Endoglin (also known as Eng RCD105) is a trans-membrane glycoprotein found on cell surfaces, and highly expressed in endothelial cells and syncytiotrophoblasts.[154-156] A soluble form of endoglin, known as sEng, is found in serum. Evidence suggests that sEng is an N-terminal cleavage product of full length Eng.[154] Mutations in the gene encoding Eng are the underlying cause of hereditary haemorrhagic telangiectasia (HHT1). Eng plays a role in both cardiovascular development and subsequently vascular homeostasis.[157,158]

sEng and pre-eclampsia

sEng was recently identified in the serum of pregnant women.[154,155] It has a molecular weight of 65 kDa and is derived from the placenta. In non-pregnant

women, serum levels of sEng are barely detectable and in normotensive pregnant women levels are low.[134] Placental expression of mRNA coding for Eng and placental concentrations of sEng are increased four-fold in women with pre-eclampsia compared with normotensive controls.[154]

The concentration of serum sEng seems to be related to the severity of pre-eclampsia, with concentrations of sEng 3 -, 5 -, and 10-fold higher in individuals with mild pre-eclampsia, severe pre-eclampsia and HELLP syndrome, respectively, compared to gestational age matched controls.[154] The increase in serum sEng is proportional to the increase in serum sFlt-1 levels, except in women with HELLP syndrome, in whom the rise in sEng is significantly greater than the rise in sFlt-1. Forty eight hours after delivery there is a 70% fall in the level of sEng compared with normal pregnant women, suggesting that sEng is derived mainly from the placenta.

Mice pre-treated with adenovirus expressing sFlt-1, sEng or both exhibit a similar degree of increased capillary permeability in lungs and kidneys.[154] However, a combination of sFlt-1- and sEng-expressing adenovirus had an additive effect in liver, inducing significant vascular damage and leakage. This combination may be instrumental in the development of HELLP syndrome.

In mice, the administration of sFlt-1 causes hypertension, severe proteinuria and renal endotheliosis. sEng leads to similar but less marked changes.[154] When both sFlt-1 and sEng are added, they cause severe hypertension, proteinuria and biochemical evidence of HELLP syndrome, as well as severe glomerular endotheliosis, placental infarction and fetal growth restriction in the mice litters. Liver histology demonstrates signs of ischaemia

and areas of necrosis, similar to those seen in the livers of women with HELLP syndrome. Interestingly, the administration of both sFlt-1 and sEng to non-pregnant rats led to signs of severe vascular damage. These findings suggest that sFlt-1 and sEng may play a significant role in the pathophysiology of pre-eclampsia and HELLP syndrome.

Caution should be exercised in the interpretation of rodent experiments and in their extrapolation to human pregnancy. The rat has many similarities to humans and its short gestation and rapid development make it practical and affordable to study.[159] In recent years, the ability to create knockout and transgenic mouse strains has facilitated the study of genetically altered mice in an attempt to shed light on human physiology and pathology. However, there are significant differences between rodent and human pathophysiology. For example, one study paradoxically showed *reduced* atherosclerotic lesions in genetically engineered hyperinsulinaemic, type 2 diabetic mice.[160] In contrast to humans, rats are atricial animals, born with a poorly developed CNS and autocrine system; much of its maturation takes place during weaning. Rats that are growth restricted at birth show no evidence of adult obesity.[161] The rat has different lipid profiles to humans (cholesterol is carried mainly as HDL), and calorie/nutrient restriction diets in pregnant rats do not produce the same altered lipid profiles in offspring as those seen in humans with the metabolic syndrome.

There are also some practical problems in assessing hypertension in rodents. In humans, the term 'hypertension' is defined as a specific level above the norm, which is associated with adverse outcomes. In rats, however, the term is often used to describe a statistically significant rise in blood

pressure above the control value.[162] This, combined with the fact that basal blood pressure varies widely in individual rats (particularly as the measurement process itself may cause stress to the animal) and in different strains of rat, means that it is difficult to set values which accurately define the term 'hypertension'. In one study, for example, systolic blood pressure measured in controls using tail cuff plethysmography ranged from 75 to 150 mmHg.[163]

Evidence in humans

Data in human pregnancy support a role for sEng and sFlt-1 in pre-eclampsia.[153] sEng levels in controls are stable until 33-36 weeks' gestation, then slowly increase every week until delivery. In women who subsequently developed *term* pre-eclampsia (>37 weeks), sEng levels increase slightly from 25-28 weeks, then more rapidly at 33-36 weeks. In women who subsequently develop *preterm* pre-eclampsia, this rise begins earlier (17-20 weeks) and is steeper. Interestingly, in women who subsequently develop gestational hypertension, levels of sEng at 33-36 weeks' gestation were significantly higher than in controls, but significantly lower than in women who subsequently developed term pre-eclampsia. However, after the onset of the gestational hypertension, levels of sEng were similar to those in women who had term pre-eclampsia. Based on these data, the authors speculate that gestational hypertension is a mild form of term pre-eclampsia. Levels of sEng in women who subsequently developed *preterm* pre-eclampsia (i.e. the most severe form) rise above levels in matched controls, beginning 9 to 11 weeks

before the onset of clinical signs and symptoms; levels increase as the onset of pre-eclampsia approaches.

Interestingly, serum sFlt-1 levels are lower, and PlGF higher, in smokers compared with non-smokers in normal pregnancy.[164,165] Given that smoking is associated with a reduced risk of pre-eclampsia, this protective effect might be mediated through the effects of nicotine on angiogenic factors.

Levine's group tried to predict pre-eclampsia using the ratio of sFlt-1: PlGF at 20 weeks' gestation in women in the highest quartile compared with the lower three quartiles. An increase in this ratio predicted a greatly increased risk of preterm pre-eclampsia (adjusted OR 6.1; 95% CI, 2.4 to 15.4); and of pre-eclampsia with a small for gestational age infant (adjusted OR 8.1; 95% CI, 2.6 to 24.8).

In summary, sEng may potentially be used as a biomarker or predictor of pre-eclampsia. sEng and sFlt-1, each of which causes endothelial dysfunction by a different mechanism, contribute to the syndrome of pre-eclampsia. Acting in concert, they can cause severe pre-eclampsia and HELLP syndrome, and also fetal growth restriction.

1.2.5 Inhibin and activin

Inhibins and activins are glycoproteins belonging to the transforming growth factor beta (TGFβ) super-family.[166] Identified in 1923 through their role as regulators in the hypothalamus-pituitary-gonadal axis, it was not until 1985 that inhibin was isolated from follicular fluid.[167] The inhibin molecule effectively interferes with the secretion of FSH from the anterior pituitary.[168] Isolated in 1986, activin is structurally related to inhibin but functionally antagonistic; it

stimulates the production and secretion of FSH from the anterior pituitary.[169-171]

Initially, inhibin and activin were thought to act only on the anterior pituitary but subsequently messenger RNAs coding for these proteins were identified in a wide variety of other tissues including placenta, adrenal glands, pituitary gland, bone marrow, kidneys, spinal cord and brain.[172] The actions of activins suggest that they should be considered as growth factors, which is one of the reasons they have been classified into the TGFβ family of proteins.[173,174]

Structure of activin and inhibin

Inhibins are dimers consisting of an α subunit and a β subunit, linked by a disulphide bond. The inhibin α- and β-subunits are synthesized as pro-proteins (pro-α N-αC and pro-β-β).[175] Two other inhibin-related α-subunit proteins have been identified: aN and pro-aC, which consist of the amino terminal segment and a disulfide-linked dimer of the pro- and carboxyl terminal region of the inhibin α-subunit precursor.

There are two types of β subunit, namely β A and β B. Inhibin A consists of one α subunit and one β A subunit; inhibin B consists of one α subunit and one β B subunit (Figure 1.1).[166] Although the α subunits circulate as monomers, they are biologically inert; only dimeric forms of inhibin are biologically active.

Activins and inhibins are products of the same precursors. Activins are dimers composed solely of β subunits (Figure 1.1). There are three types of

activin: activin A, activin AB and activin B. Activin A is composed of two β A subunits linked by a disulphide bond; activin AB is composed of one β A subunit and one β B subunit; activin B is composed of two β B subunits (Figure 1.1). Activin A is identical to erythroid differentiation factor, previously isolated from a human leukaemic cell line.[176]

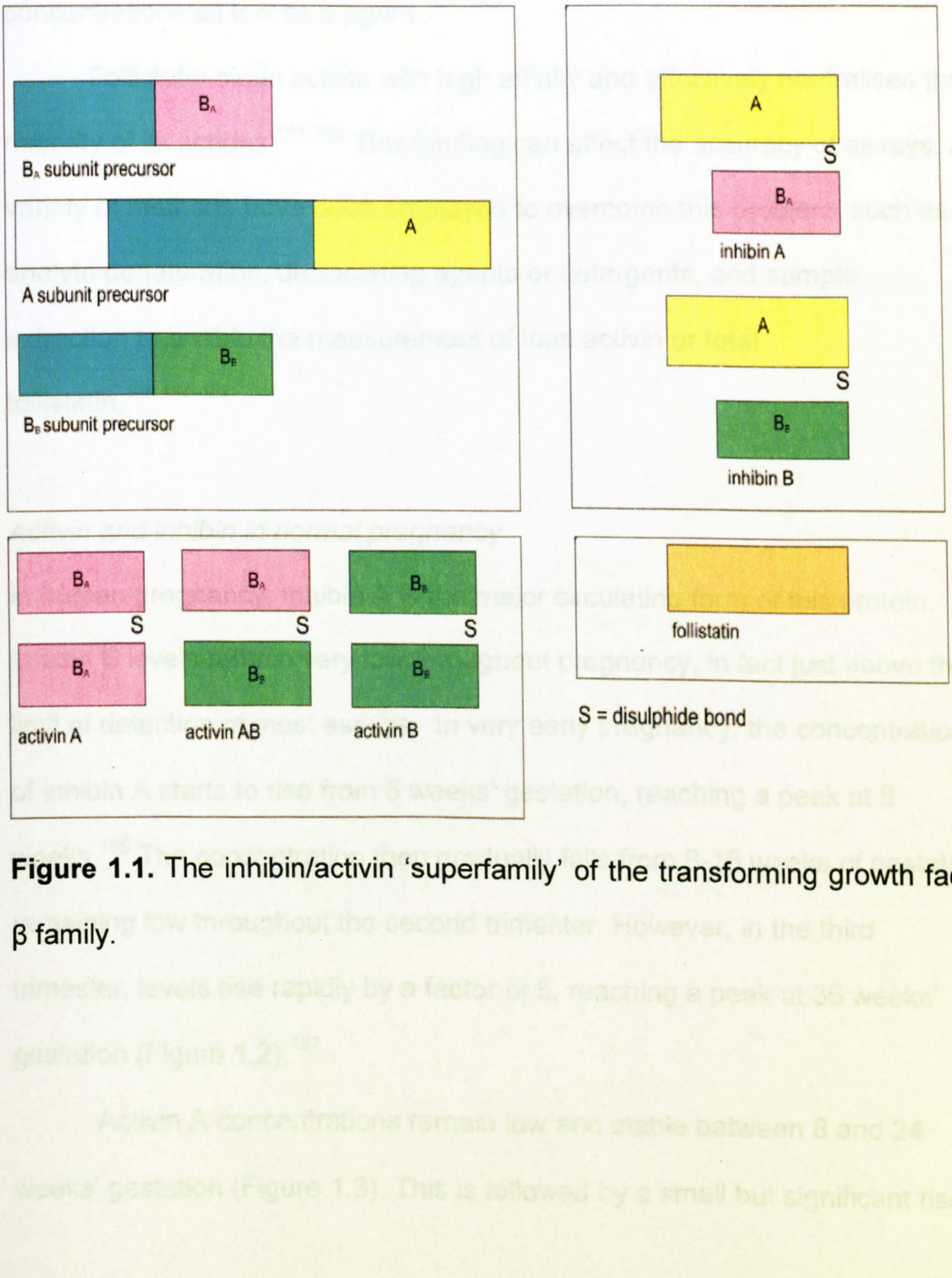


Figure 1.1. The inhibin/activin 'superfamily' of the transforming growth factor β family.

Problems with measuring inhibin, activin and follistatin levels in serum

Earlier assays for inhibin such as the Monash radio-immuno assay were unable to discriminate between the bioactive dimeric form of inhibin and the free biologically inactive α subunits. However, more recent enzyme linked immuno-sorbent assays (ELISA) measure inhibin A and inhibin B (as well as activin A and activin AB) with a very high level of specificity and sensitivity, to concentrations as low as 5 pg/mL.[177-180]

Follistatin binds activin with high affinity and effectively neutralises the majority of its actions.[181,182] This binding can affect the accuracy of assays. A variety of methods have been employed to overcome this problem, such as analyte denaturation, dissociating agents or detergents, and sample extraction to enable the measurement of total activin or total follistatin.[180,183-186]

Activin and inhibin in normal pregnancy

In human pregnancy, inhibin A is the major circulating form of this protein. Inhibin B levels remain very low throughout pregnancy, in fact just above the limit of detection of most assays. In very early pregnancy, the concentration of inhibin A starts to rise from 5 weeks' gestation, reaching a peak at 8 weeks.[175] The concentration then gradually falls from 8-16 weeks of gestation, remaining low throughout the second trimester. However, in the third trimester, levels rise rapidly by a factor of 5, reaching a peak at 36 weeks' gestation (Figure 1.2).[187]

Activin A concentrations remain low and stable between 8 and 24 weeks' gestation (Figure 1.3). This is followed by a small but significant rise

from 24 to 28 weeks. There is a then slight plateau, followed by a steep rise from 34 weeks to a peak at 38-39 weeks' gestation.[188] There has been considerable debate about whether the primary source of inhibin A and activin A in pregnancy, particularly in early pregnancy, is the corpus luteum or the feto-placental unit.[189,190] However, there is now convincing evidence from studies using immuno-localisation, in situ hybridisation for mRNA, and immuno-histochemistry that the placenta is the primary source throughout *most* of pregnancy.[186,191-200]

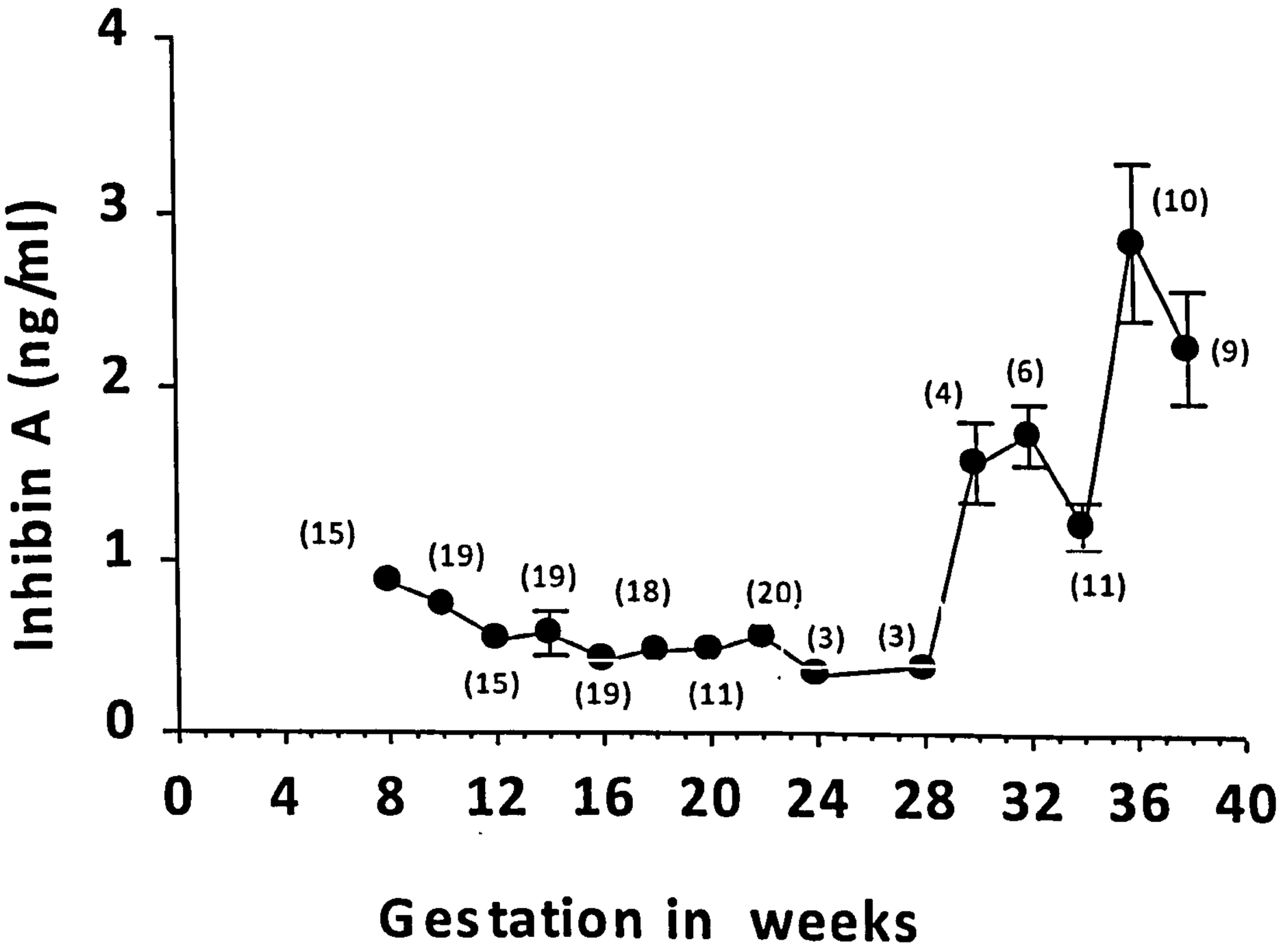


Figure 1.2. Concentration of inhibin A throughout pregnancy.[187]

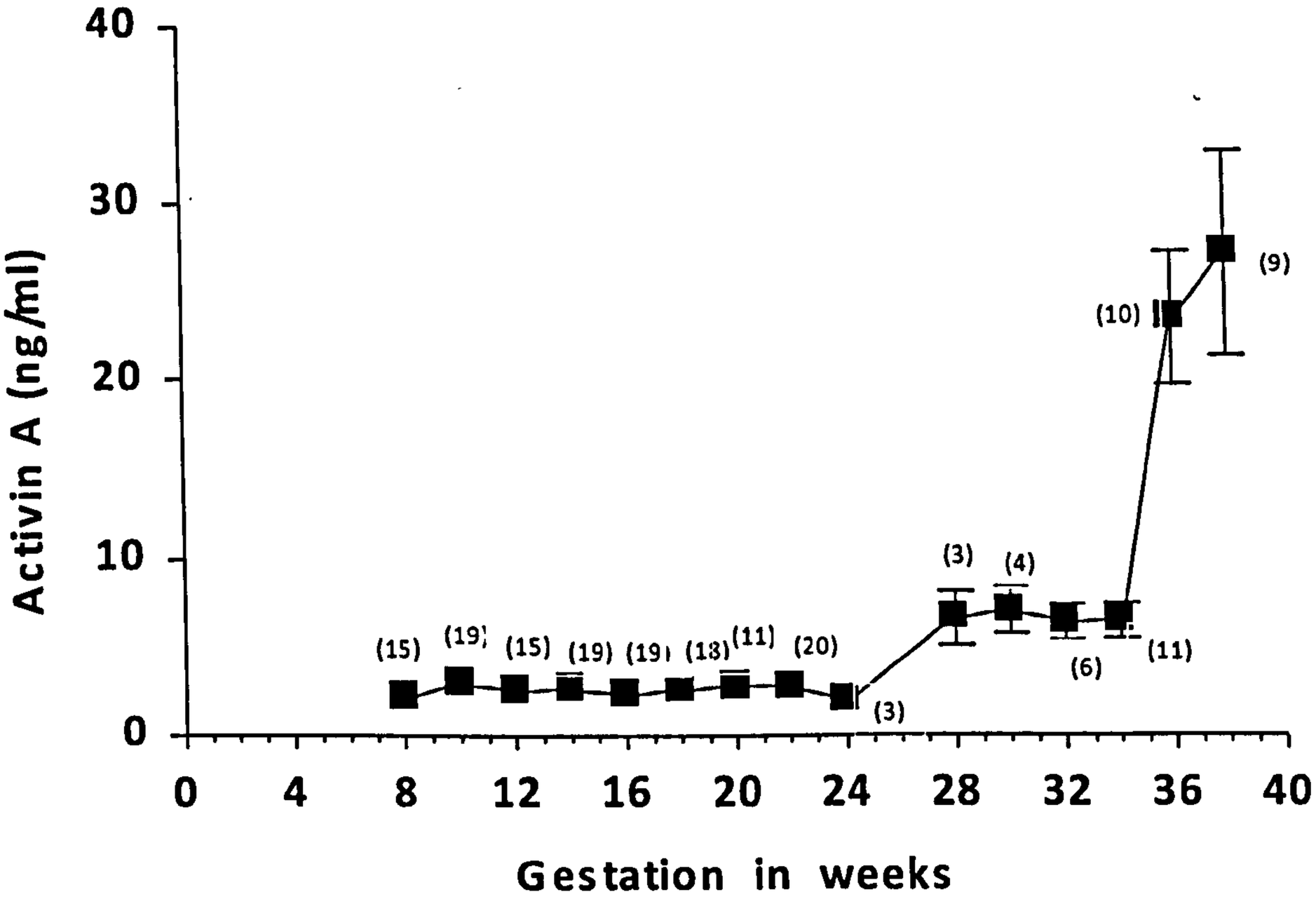


Figure 1.3. Concentration of activin A throughout pregnancy.[188]

The role of activin and inhibin in pregnancy remains uncertain. It has been shown that they increase placental production of gonadotrophin releasing hormone and progesterone, suggesting a hormonal role supporting pregnancy.[201] Activin A also has an immuno-modulating effect which may help to protect the fetus from rejection by its mother's immune system in early pregnancy.[202] Activin and inhibin may also play a paracrine role in early pregnancy recognition, i.e. acting locally within the ovary.[203] Inhibin A and activin A levels rise markedly close to term, suggesting that they may play a role in the initiation of labour.[187,204]

Established hypertensive disorders of pregnancy

It is clear from a plethora of publications over the past decade that levels of inhibin A and activin A are significantly increased in women with established pre-eclampsia compared with normotensive controls.[205-214] Most authors consider this to be further evidence of trophoblastic dysfunction in pre-eclampsia. The concentration of pro-alpha C (a precursor of inhibins) is also increased in women with pre-eclampsia.[206,207,209] It has also been shown that, in women with pre-eclampsia, the serum concentration of activin A and inhibin A is proportional to the degree of proteinuria.[206] Some studies distinguished the different types of hypertensive disorders of pregnancy;[210,215,216] levels of activin A and inhibin A were significantly raised in pre-eclampsia, even higher in HELLP syndrome, but in chronic hypertension levels remained normal. It seems that activin A shows the greatest increase in concentration in pre-eclampsia so is potentially a good serum screening marker.[211,212]

Placental expression of the genes encoding for the inhibin α subunits and inhibin/activin β subunits is increased in women with pre-eclampsia.[217] However, the mechanism leading to this increase has not yet been defined.

Urinary inhibin A, but not activin A, is significantly increased in women with pre-eclampsia.[218] There is also an increased *fractional* renal excretion of inhibin A, making it the best discriminator between women with pre-eclampsia and normotensive controls.

When in-vitro trophoblast preparations are rendered hypoxic, the production of inhibin A and activin A falls significantly.[219,220] This is perhaps

not what would have been expected, given the increased serum levels in pre-eclampsia. These findings suggest that low oxygen tension may not be the direct mechanism leading to increased serum concentrations of these proteins in women with pre-eclampsia.

Prediction of pre-eclampsia - second trimester

An increased serum concentration of inhibin A in the second trimester is associated with an increased risk of developing pre-eclampsia later in pregnancy, and this increase occurs on average 22 weeks before clinical diagnosis of the disease.[208,221-224] Inhibin A predicts pre-eclampsia better than hCG; for a specificity of 90%, inhibin A predicted 49% of cases of pre-eclampsia, compared with 31% predicted by hCG alone.[223] The addition of inhibin A to uterine artery Doppler indices improved the prediction of pre-eclampsia from 60% to 71% for a false positive rate of 7%.[225]

The serum markers, which include inhibin A, used in the second trimester quadruple screening test for trisomy, can identify 40% of women who later develop pre-eclampsia with a false positive rate of 6%.[226] In 2006 the performance of various potential markers for the prediction of pre-eclampsia was examined.[227] For a false positive rate of 5%, detection rates at 22^{+0} to 24^{+6} weeks were as follows:

Uterine artery mean PI	50%
PAPP-A	5%
Free β hCG	10%
Inhibin A	35%
Activin A	44%

The best combination was uterine artery mean PI, maternal serum activin A and inhibin A, which had a detection rate of 75% for a false positive rate of 5%, and a detection rate of 92% for a false positive rate of 10%.

The current consensus is that the addition of inhibin A and activin A affords at best a modest improvement on current screening methods (primarily uterine artery Doppler indices alone).

Prediction of pre-eclampsia - first trimester

Several studies have found increased serum concentrations of inhibin A and activin A in the first trimester in women who subsequently developed pre-eclampsia, when compared with normotensive controls.[228,230] Using a specific cut-off value for inhibin A, the odds ratio for subsequent pre-eclampsia was 4.9 (95% CI: 1.8 to 13.3).[231]

While inhibin A, PAPP-A, PlGF and activin A appear to be the best first trimester serum markers for later development of pre-eclampsia, their predictive values are so poor that they currently cannot be considered useful in clinical practice.[228]

Screening for Down's syndrome

In the mid 1990's, five studies showed that adding maternal serum inhibin A levels to the existing second trimester 'triple test' (AFP+ UE3+ β hCG) significantly improved the detection rate for Down's syndrome.[232-236] Inhibin A is of particular value because its level in maternal serum changes little between 15 and 18 weeks of gestation.

In the first trimester there is no significant difference in maternal serum concentrations of inhibin A between pregnancies affected by Down's syndrome and normal controls.[235,237,238]

Small for gestational age

No difference has been found in maternal serum levels of either activin A or inhibin A at the time of diagnosis of a small for gestational age fetus.[213] However, levels of activin A early in pregnancy were slightly higher in those who later developed a small for gestational age fetus, suggesting the potential role of this marker in its prediction.[213]

One study which confirmed the increased maternal serum levels of inhibin A and activin A in pre-eclampsia found that the presence of fetal growth restriction did not significantly modify these concentrations.[239]

Preterm labour

Several studies have shown that inhibin A and activin A levels rise markedly close to term.[187,204] This naturally led to speculation that they might also play a role in preterm labour. Data on this possible association are limited [240] and only few studies reported increased levels of activin A in the serum of women who subsequently went into preterm labour.[241,242]

1.2.6 Summary: serum and placental markers

There is a wealth of publications in the medical literature describing changes in many different serum and placental markers in pre-eclampsia. At the time that our research was being initiated, the most promising were felt to be sFlt-1, PlGF, sEng, VEGF, inhibin A and activin A, so these were the serum and placental markers we chose to investigate for the purposes of this MD thesis.

1.3 ARTERIAL PULSE WAVE ANALYSIS

1.3.1 Historical Perspective

The importance of the arterial pulse has been appreciated for many centuries; in 200 BC in 'The Yellow Emperor's Classic of Internal Medicine',[243] the following quote can be found: 'a hardening of the pulse...suggests disease of the kidney'. In 1872, Mohamed stated that 'the pulse ranks the first among our guides; no surgeon can despise its counsel, no physician shut his ears to its appeal'.[244] The development of the sphygmograph by Marey, however, provided for the first time an objective method of recording and assessing the character of the arterial pulse at the wrist. Mohamed reported significant differences in the shape of the radial artery waveform occurring with age and the pre-albuminuric stage of Bright's disease (now thought to be untreated essential hypertension): '*The most constant of these indications is the*

prolongation of the tidal wave; any one or all of the other characteristics may under certain conditions be absent'.

In 1896, the mercury sphygmomanometer was invented by Riva Rocci and was rapidly adopted by physicians as it provided numbers which could be more easily assessed. Ironically, the use of sphygmomanometry - which provided only the peak pressure of systole and the nadir of diastole - meant that much of the information contained in the shape of the arterial waveform was lost or ignored. The resurgence of arterial pulse wave analysis has been stimulated by the advance in computer technology, allowing computerised analysis of the waveform.[245]

1.3.2 The Arterial Pulse Wave

Arterial diameter ranges from several centimetres at the aorta to less than 200μ for a typical resistance vessel. Arteries with a diameter of > 200μ are described as large arteries, and include the elastic arteries such as the aorta and muscular arteries such as the brachial and iliac. The pulse waveform does not simply pass down the arterial tree unchanged, but is altered significantly because of the elasticity of the vessel walls and reflection of the wave at several points.

During systole, the left ventricle ejects blood into the large arteries whose elastic walls distend to accommodate it. This attenuates the height of the systolic peak. As pressure in the arteries begins to fall in diastole, the walls recoil, maintaining pressure during diastole. The net effect is to increase end-diastolic pressure and prolong the duration of forward blood flow. This

increase in end-diastolic pressure is particularly important as this is when the coronary arteries are perfused. Thus, elastic recoil of the large arteries smoothes the total waveform, reducing the height of the systolic peak but increasing the height of the diastolic part of the wave.

Wave reflection is equally important. Each heartbeat generates a pulse wave which travels away from the heart along the arterial tree. This waveform is reflected from bifurcations in the arterial tree and from the junctions of the pre-resistance and resistance vessels. The reflected wave travels back towards the heart (Figure 1.4) and meets the advancing wave. Thus, the height of the pulse wave at any point in the arterial tree is the net combination of the advancing and reflected waves (Figure 1.4). Generally, the reflected wave reaches the aorta during diastole, boosting the height of the diastolic portion of the wave. This also helps to maintain coronary artery perfusion.

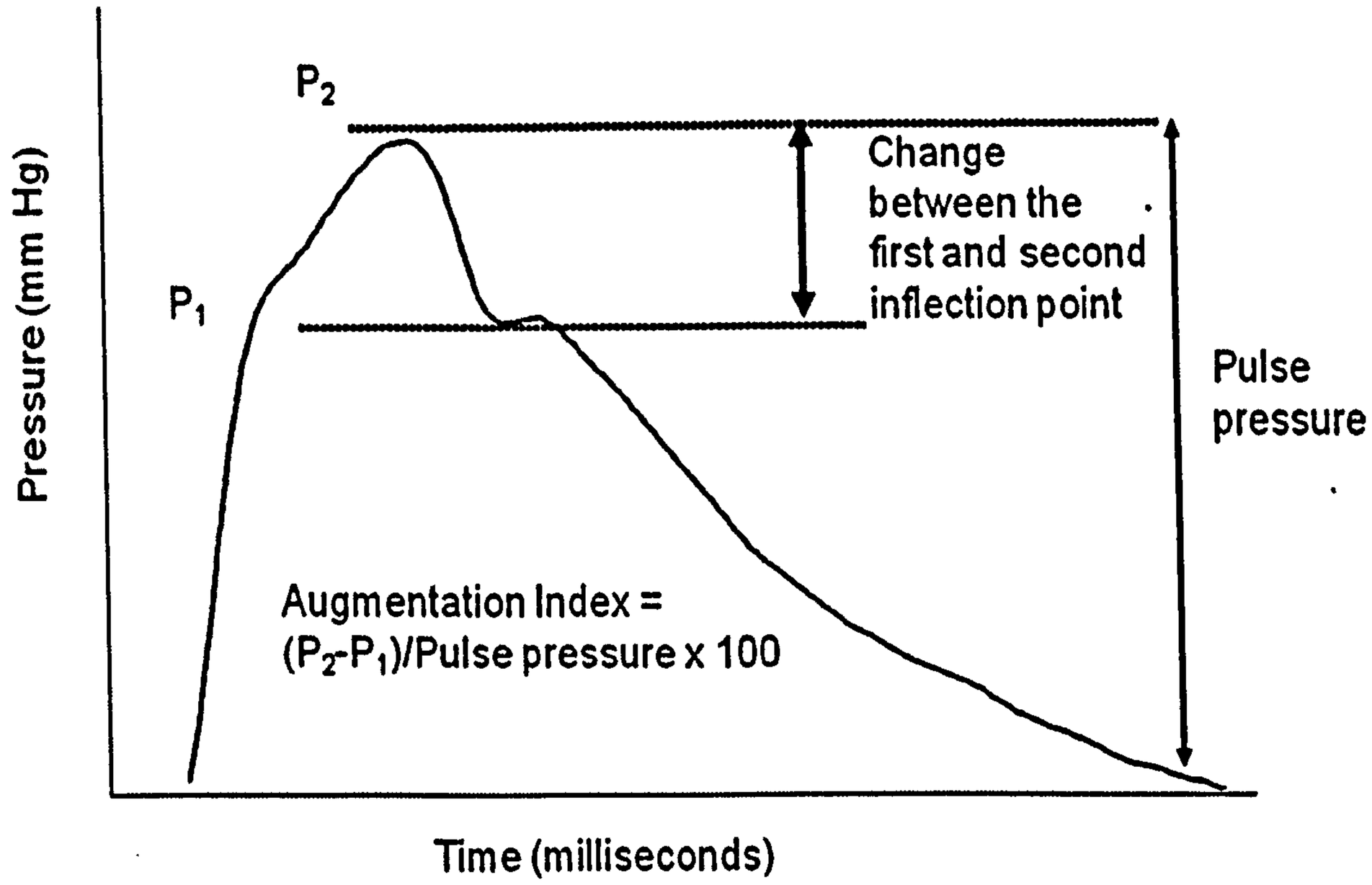


Figure 1.4. The aortic waveform. The first systolic peak (P1) is the maximum pressure created by the advancing pressure wave. The second systolic peak (P2) is a composite of the advancing and reflected waveforms. Augmentation pressure (AP) is calculated as P2 – P1 (ΔP). Augmentation index (AIx) is AP expressed as a percentage of aortic pulse pressure (PP).

Diastolic pressure and mean arterial pressure change little down the arterial tree (the slight fall of 2-3 mm Hg in mean arterial pressure from heart to peripheries is enough to maintain forward flow). However, systolic pressure, and therefore pulse pressure (which is systolic minus diastolic pressure) actually *increases* down the arm. This is a result of increasing arterial stiffness moving from heart to periphery and is due to the reflected

wave. As a result, resting radial systolic pressure exceeds aortic systolic pressure by around 10 mm Hg on average.[246]

Stiffening of the walls of large arteries is seen with aging and in pathological conditions such as diabetes and hypertension; these conditions cause damage to the elastic fibres in the wall, resulting in increased arterial stiffness. This increased arterial stiffness causes a rise in arterial pulse pressure in two ways:

a) The buffering capacity of the large arteries (described above) is lost. This means that systolic pressure is higher and diastolic lower.

b) The pulse wave travels more rapidly along the arterial tree, and the reflected wave travels more rapidly back towards the heart. Consequently, the reflected wave reaches the advancing wave earlier in systole, resulting in a higher (combined) peak.

This increased pulse pressure increases cardiac afterload which in the long term could lead to left ventricular hypertrophy.[247] It also causes a reduction in coronary artery perfusion due to the fall in diastolic pressure.

1.3.3 Assessment of Arterial Stiffness

The simplest way of assessing arterial stiffness is by measuring the pulse pressure, usually in the brachial artery using a conventional sphygmomanometer.[248] However, peripheral pulse pressure depends on both arterial stiffness and cardiac output so is an inaccurate surrogate for stiffness.

Another limitation is that there is a difference between aortic (central) and peripheral (brachial) pulse pressure. It might be assumed that peripheral pulse pressure could be used as a surrogate for central pulse pressure. However, the relationship between the two is not fixed; it varies according to a variety of factors such as exercise and posture.[249] Given that it is *central* rather than peripheral pressure which is 'seen' by the important organs (the heart, brain and kidneys), brachial artery sphygmomanometry is clearly suboptimal. Central blood pressure can be measured using an intra-arterial catheter but this is an invasive technique.

1.3.4 Analysis of the Arterial Pulse Wave

The technique of pulse wave analysis (PWA) was developed and reported in 1996.[220] This technique involves three principal steps:

1. Recording the radial artery pulse waveform using a tonometer.
2. Applying a generalised transfer function to the radial artery waveform in order to derive the *aortic* waveform.
3. Analysing the aortic waveform to provide measures of arterial wall stiffness.

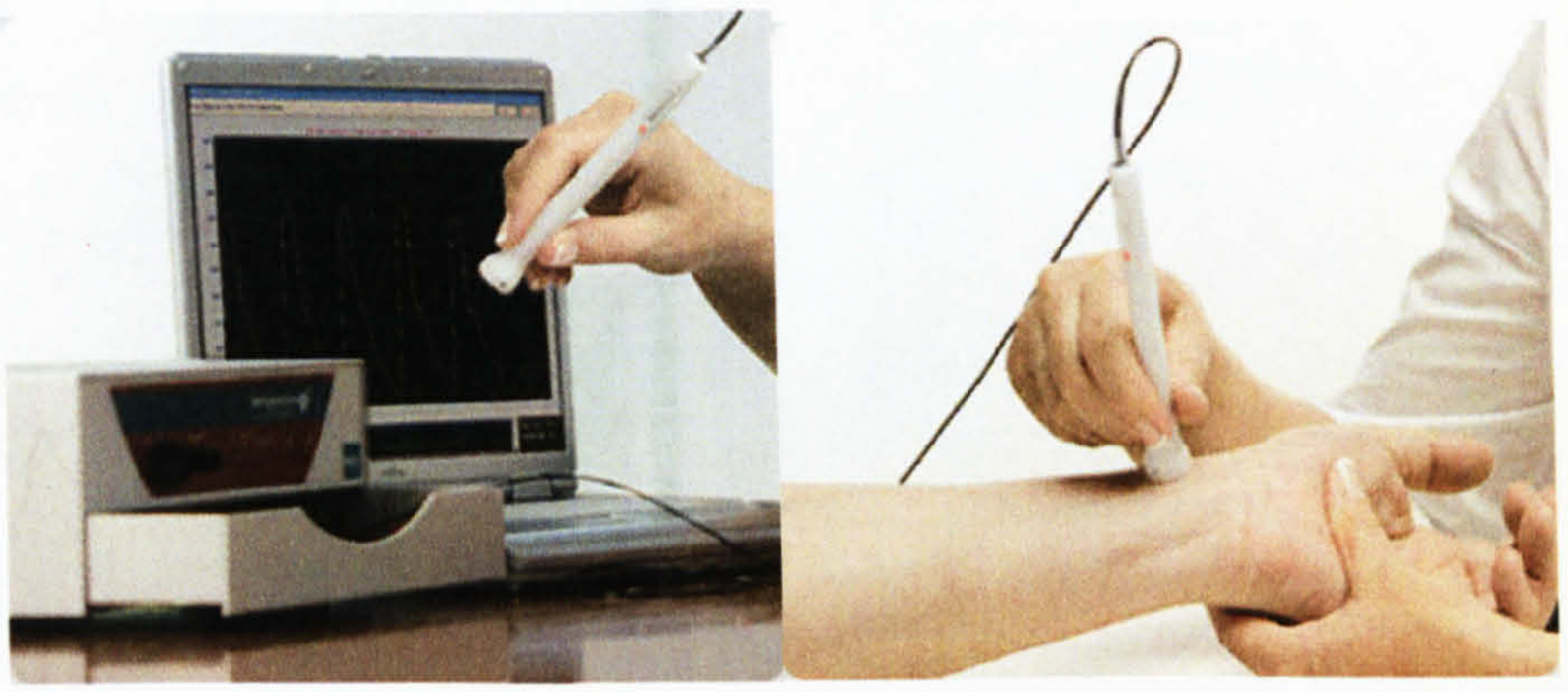


Figure 1.5. The radial artery waveform is measured using a tonometer.

A tonometer is a pencil-like probe containing a high fidelity micromanometer capable of accurately recording intra-arterial pressures (Figure 1.5). Originally developed to measure intraocular pressure, tonometers were subsequently adapted for measuring intravascular waveforms.[250,251] The flat surface of the tonometer is gently pressed against the radial artery, slightly compressing the artery against the underlying bone and tissue (Figure 1.6). This flattens the curved surface of the artery, equalising circumferential pressures, and allowing accurate recording of intra-luminal pressure.[252,253]

The radial artery pressure waveforms are recorded electronically and an averaged waveform assembled by the software program (Sphygmocor®). A generalised transfer function is then applied to this radial artery waveform in order to derive the corresponding aortic waveform (Figure 1.7).

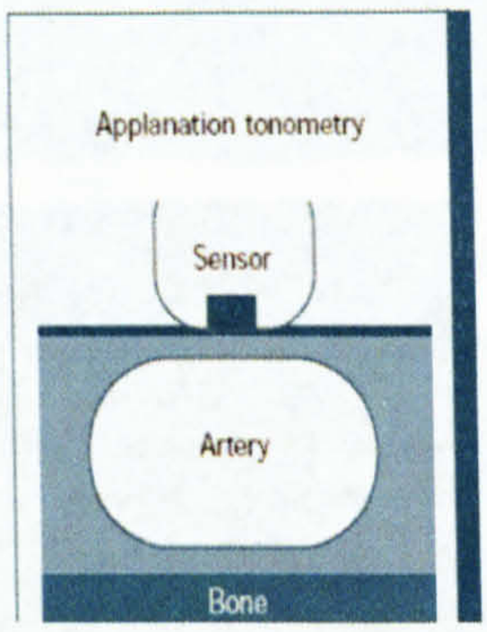
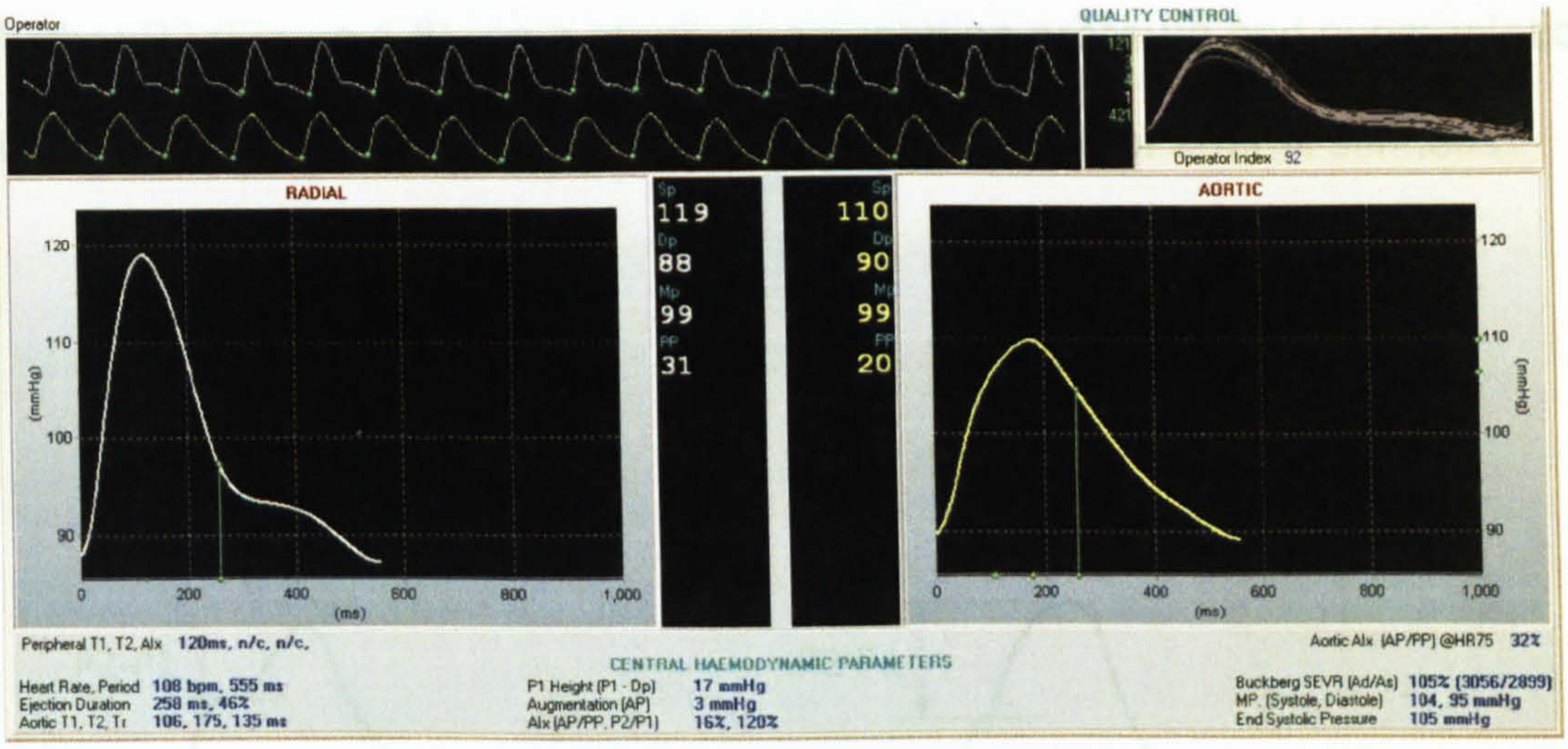


Figure 1.6. Schematic diagram of a tonometer in use. The artery is gently compressed against the underlying bone and tissue.

Figure 1.4 shows a typical central aortic pressure waveform, which has two systolic peaks: P1 and P2. P1 is the maximum pressure generated in the wave advancing from the heart. The second peak (P2) is the result of the combination of the advancing and reflected waves. The Augmentation Pressure (AP) is the difference between P2 and P1, and may be positive or negative depending on the relative heights of the two peaks. Augmentation Index (AIx) is defined as the augmentation pressure expressed as a percentage of pulse pressure.

(a)



(b)

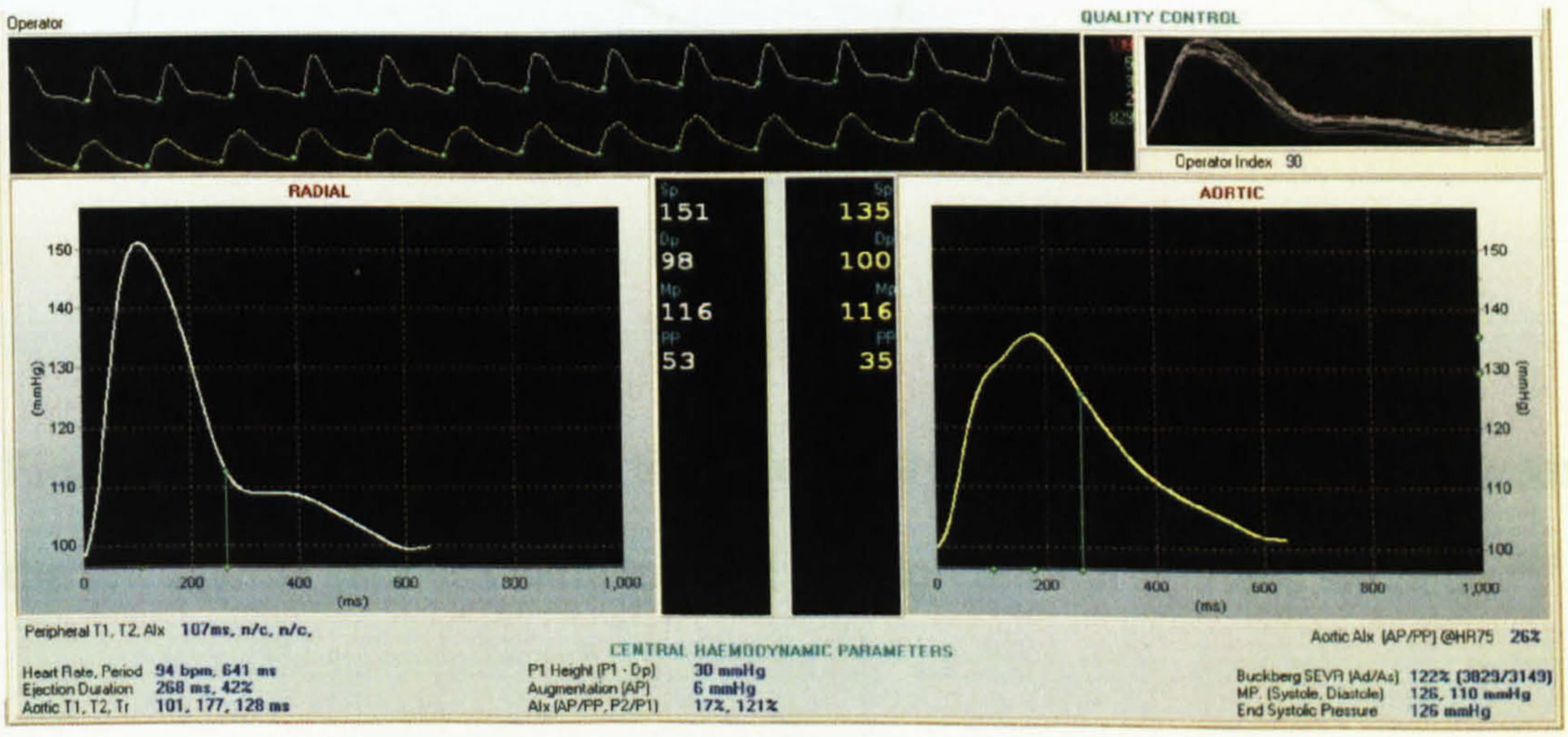


Figure 1.7. A radial artery waveform (left) and the corresponding aortic waveform (right): (a) normotensive woman and (b) hypertensive patient.

As described earlier, when arterial stiffness is increased, both the advancing and reflected waves travel faster; as a result, the reflected wave arrives back in the aorta at an earlier point in the advancing wave, i.e. early in

systole. This results in greater augmentation of the advancing wave, i.e. a higher P2 (Figure 1.8). As a result, augmentation pressure and augmentation index are increased. Thus, AP and AIx are measures of arterial stiffness.

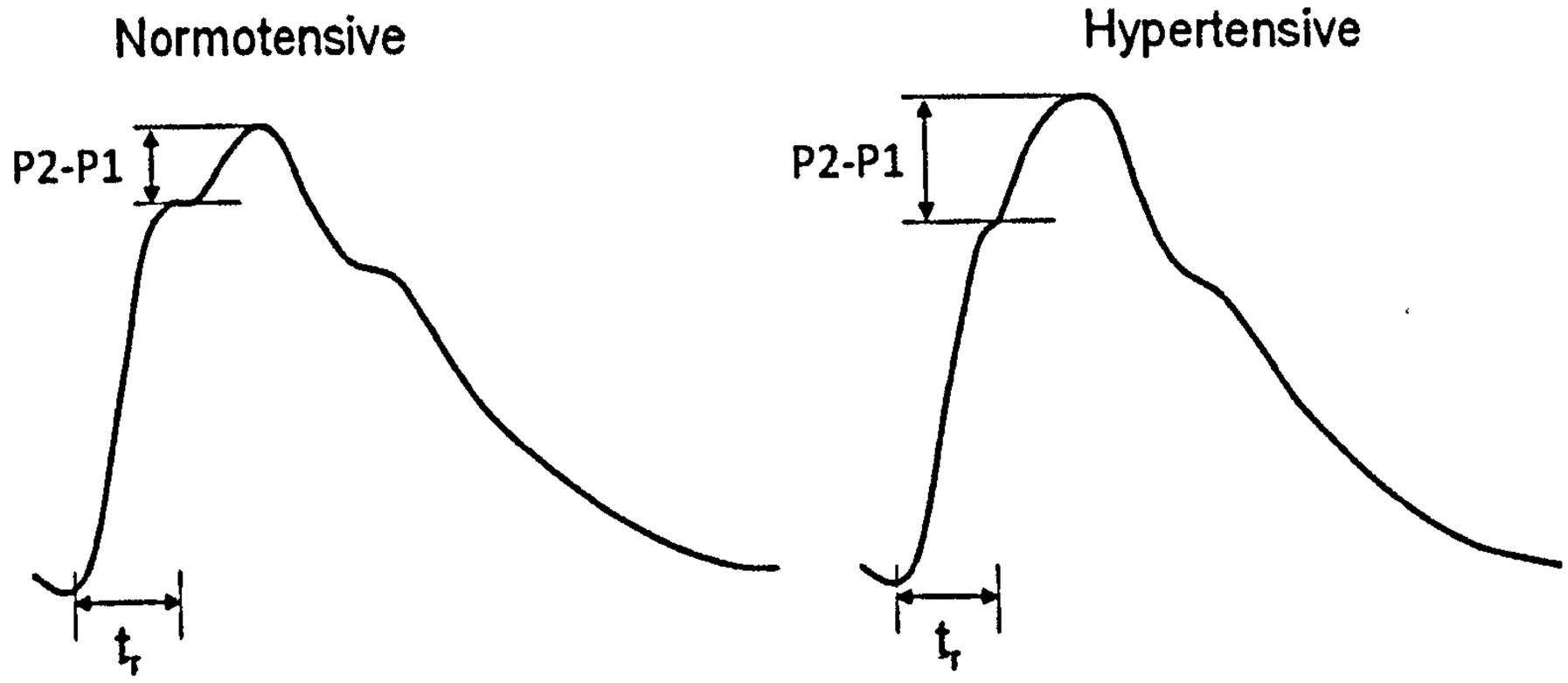


Figure 1.8. This figure shows the difference in the aortic waveform between the low arterial stiffness situation and the high arterial stiffness situation.[254]

1.3.5 Relationship between Heart Rate and Augmentation Index

Augmentation index is affected by changes in heart rate. An increase in heart rate shortens the duration of systole. As a result, the reflected wave reaches the advancing wave later in diastole than usual, resulting in a lower P2, i.e. reduced augmentation index. Using PWA, an elegant study [255] examined patients with in-dwelling cardiac pacemakers, paced at different heart rates.

This approach was preferable to pharmacological manipulation of heart rate as many of these drugs may also have effects on the vascular wall. For every 10 beats per minute increase in heart rate, there was a 4% decrease in aortic wave augmentation. For this reason, augmentation index is adjusted for heart rate; a standardised measure of AIx-75 (augmentation index at heart rate 75 beats per minute) is used. The Sphygmocor® software automatically calculates the value of AIx-75.

1.3.6 Validation of the technique of Arterial Pulse Wave Analysis

Validation of the technique of arterial pulse wave analysis was necessary primarily in three areas:

a) Whether the technique of applanation tonometry could produce a consistent radial artery waveform.

b) Whether the radial artery waveform measured by applanation tonometry accurately reflects the true radial artery waveform.

c) Whether the generalised transformation function used to derive the aortic waveform from the recorded radial artery waveform is valid.

Reproducibility

Accurate tonometry requires that the artery be applanated (flattened) beneath the sensor. The technique of applanation tonometry is usually quick and easy to learn, and several validation studies have shown low inter- and intra-observer variation.[256-258]

Accuracy of applanation tonometry

The waveform obtained from the radial artery using applanation tonometry has been compared with that obtained from invasive monitoring, and a close correlation between the two has been confirmed.[253,259]

Validation of the generalised transfer function

In order to calculate the aortic pressure waveform, a transfer function must be applied to the measured radial artery waveform. It might be expected that the use of a *generalised* transfer function (i.e. one that could be used for all subjects) would not be appropriate. After all, vascular dimensions depend on body size and vascular properties vary with age, arterial pressure and vasoactive drugs. Use of a generalised transfer function would assume a constant relationship between the aortic and radial artery waveforms in all subjects under all conditions, which seems unlikely.

However, results from clinical and theoretical studies consistently confirm that the use of a generalised transfer function produces surprisingly good calculations of the aortic waveform, approaching over 90% accuracy in most situations.[250,260-262] The explanation for this is likely to be that upper arm length varies little between different adults, and upper limb pulse wave velocity changes little with age,[250,260] with elevation of arterial pressure,[250,261] or with the types of vasoactive therapy commonly used in clinical practice.[250,262]

A generalised transfer function was first derived in a study comparing calculated aortic pressure waveform with that measured invasively at cardiac catheterisation in 14 patients.[262] The generalised transfer function held true

for all 14 patients under various conditions, including following administration of sublingual glyceryl trinitrate. This generalised transfer function was subsequently rigorously tested in a further 20 patients at cardiac catheterisation at John Hopkins Hospital in the US.[246] Recordings were made during one or more of the following haemodynamic transient manoeuvres: Valsalva manoeuvre, manual compression of the upper abdomen, intravenous bolus of 200 mg nitro-glycerine, or balloon obstruction of inferior vena cava flow. The use of a generalised transfer function produced an aortic waveform which was remarkably similar to the measured aortic waveform, both in amplitude and contour, in all these situations. Using the measured peripheral and central waveforms, an *individualised* transfer function was calculated for each subject. Application of this individualised transfer function to the same radial pressure data did not significantly improve the accuracy of the derived aortic waveform. Thus, the authors concluded that the use of a generalised transfer function '*rather surprisingly*' works remarkably well in a variety of individuals in a variety of haemodynamic situations; and that '*equally surprisingly*', the use of an individualised transfer function produced only a very small additional improvement in the prediction of the aortic waveform.

In 2004, the data from more than 400 patients used to calculate the original generalised transfer function were published.[263] This is the transfer function used in the Sphygmocor® software. Earlier (in 2001), the Sphygmocor® device had gained approval from the US Food and Drug Administration; this approval was based on the data subsequently published,

all of which assessed the transfer function by direct comparison with intra-arterial measures used as the gold standard.[263,264]

This generalised transfer function has not been specifically validated for pregnancy; this is because cardiac catheterisation is a rare event in pregnant women. However, as described above, it has been validated in non-pregnant subjects in a variety of circumstances relevant to pregnancy, such as Valsalva manoeuvre, manual compression of the upper abdomen, balloon obstruction of inferior vena cava flow, and during the administration of a variety of vasodilating treatments.[246,262] Given its remarkable consistency in a wide variety of subjects in a wide variety of clinical situations, it seems likely (although not yet proven) that the transfer function also holds true in pregnant women.

1.3.7 Use of Pulse Wave Analysis in non-pregnant subjects

Pulse Wave Analysis is used for the assessment of arterial function in patients with cardiovascular and renal disease, and diabetes. It has also been used for the assessment of vaso-active medications, particularly antihypertensives. For example, a blinded comparison of atenolol and ramipril found that both drugs caused a similar reduction in both central and peripheral diastolic pressure. The fall in *peripheral* systolic pressure was also similar for both drugs. However, the fall in *central* systolic pressure (and hence pulse pressure) was significantly greater with ramipril compared with atenolol.[265] PWA showed that this difference was due to the greater reduction in arterial stiffness caused by ramipril. It is likely that this difference explains the greater long-term benefits

of ramipril. In the REASON study,[266,267] PWA established similar benefits of a perindopril-indapamide combination over atenolol. These examples suggest that PWA may have a potentially important role to play in the assessment of new and existing cardiovascular medications so that central - as well as peripheral - effects can be determined.

Arterial PWA has been used to examine the effects of ageing on arteries. Because of the increase in arterial stiffness, aortic wave velocity more than doubles between ages 20 and 100 years.[250,260]This effect is offset to a certain extent by physical fitness.[268] PWA is also used in clinical practice to assess patients with cardiac failure [269] and end-stage renal failure.[270] Arterial stiffness has been shown to predict the risk of cardiovascular events in patients with diabetes,[271] end stage renal disease [272] and hypertension.[273] Menstrual cycle fluctuations in arterial distensibility have been described in premenopausal women.[261]

Similarly, other studies have shown that vasodilator drugs such as glyceryl trinitrate cause a significant fall in central pressures, despite the relative maintenance of peripheral systolic pressure;[274,275] this suggests that arterial pulse wave analysis provides a more accurate assessment of the effect of this type of vasodilator. Beta-blockers tend to increase arterial wave reflection.[276] Calcium channel antagonists reduce aortic augmentation index (AIx) because of their vasodilator action; clearly this is a beneficial effect.[250] PWA is increasingly being used in clinical practice in the diagnosis and management of patients with hypertension, cardiac failure, diabetes mellitus, nephropathy and angina.[277]

1.3.8 Vascular endothelial factors and arterial stiffness

A series of in vivo investigations using nitric oxide synthase inhibitors showed that basal nitric oxide production appears to regulate large artery stiffness, and that stiffness is reduced by exogenous nitric oxide donors and agonists that stimulate endothelial nitric oxide production.[278,279] This evidence suggests that arterial stiffness is not merely dependent on the intrinsic structure of the arterial wall but can be affected by local or circulating agents.

1.3.9 Arterial Pulse Wave Analysis in pregnancy

There are few reports in the literature of the use of arterial pulse wave analysis in pregnancy - the first appeared in 2004.[280-282] Smith and colleagues[281] studied a total of 53 normotensive women and 10 non-pregnant controls. They found that augmentation pressure and augmentation index were lower in pregnancy. However, none of the recruited women were studied in the first trimester (the earliest was 17 weeks' gestation) and only 20 women were studied in all three defined gestation groups (17-20 weeks; 25-28 weeks; and 33-36 weeks).

In the same year, another study compared 51 normotensive pregnant women, 38 hypertensive pregnant women and 33 women with pre-eclampsia.[280] In both hypertensive and pre-eclamptic pregnancies, AIx was significantly higher than in normotensive pregnant women. Within the pre-eclamptic group, there was no significant difference in haemodynamic parameters between those women on treatment and those not. However, the

numbers were small (16 versus 17) and no patient was studied both before and after starting anti-hypertensive medication. A single measurement was taken from each individual and the means of each group (normotensive, hypertensive and pre-eclamptic) compared. No comparison between individual values was made and no longitudinal studies of individual women were carried out (although the authors suggest that this 'would be valuable').

In 2005, another study compared 27 women with pre-eclampsia, 33 with gestational hypertension and 39 normotensive pregnant controls.[283] Each woman was studied once and no women with essential hypertension were included. AIx was increased in gestational hypertension but increased even more in women with pre-eclampsia. In the same year, a cross-sectional case control study compared four groups using PWA: 26 with pre-eclampsia, 26 normotensive pregnant, 22 non-pregnant normotensive but previously pre-eclamptic women, and 22 non-pregnant controls.[284] All women were between 30 and 42 weeks' gestation. Each woman was studied once with PWA and the means of each group compared. AIx was significantly increased in pre-eclampsia compared with normotensive pregnancy. Interestingly, in the pre-eclampsia group, AIx did not correlate with the degree of proteinuria. When non-pregnant controls were compared with the non-pregnant previously pre-eclamptic women, no significant differences in PWA parameters were found. The authors concluded that, although mean arterial pressure correlated with AIx in both pregnant and non-pregnant women (reflecting the fact that, by distending the arterial walls, elevated blood pressure *per se* causes functional stiffening of the large arteries), nevertheless pre-eclampsia was a strong independent determinant of AIx. This suggested that the pathological process

of pre-eclampsia contributes significantly to an increase in arterial stiffness over and above the increase caused by hypertension *per se*.

Arterial PWA has been used to measure pulse wave velocity (PWV) between the carotid and femoral arteries in 50 normotensive women.[285] Increased arterial stiffness leads to more rapid transit of the pulse wave, i.e. greater pulse wave velocity. Pulse wave velocity was found to be inversely proportional to birth weight centile, regardless of blood pressure. This suggests that increased arterial stiffness (represented by increased pulse wave velocity) may reflect the inadequate plasma volume expansion associated with fetal growth restriction.

In conclusion, there are few studies of the use of arterial pulse wave analysis in normal or pathological pregnancy. There are no longitudinal studies, no studies of the effect of antihypertensive treatment on an individual, none attempting to predict pre-eclampsia and none of women with the most severe form (usually early onset) of pre-eclampsia.

1.4 DOPPLER ULTRASOUND

1.4.1 Background

The Doppler principle, first described by Christian Doppler in 1843, states that the frequency of a wave (light or sound, for example) emitted from a moving source was altered by the speed and direction of movement of the source itself. Ultrasound is simply sound which cannot be heard by the human ear and has a frequency above 20 000 Hz (20 kHz). The velocity (v) of sound waves is constant at 1540 m/s. The wavelength (λ) multiplied by the frequency (f) equals the constant v:

$$\lambda = v/f$$

In Doppler ultrasound a source directs ultrasound waves into tissue. Tissue reflects these waves to a greater or lesser degree, largely depending on its density, and the reflected sound is detected by the ultrasound probe. The frequency of the sound reflected by static tissue is unchanged. However, the frequency of ultrasound reflected by a moving particle is changed; this change is known as the 'frequency shift' (or the 'Doppler frequency'). The frequency shift depends on how fast the object is moving and whether it is moving towards or away from the probe.

For pulsed wave Doppler and the majority of the colour flow imaging techniques, the equation which describes this relationship is:

$$\text{Doppler frequency} = 2v f_t \cos\theta/c$$

f_t = the frequency of the transmitted ultrasound wave

v = the velocity of the blood

θ = the angle of insonation (between the beam and the direction of flow)

c = speed of sound in tissue

1.4.2 Blood Flow Measurement using Doppler Ultrasound

Indices of Resistance

Several different indices of resistance to blood flow have been derived but those most commonly used are the Resistance Index (RI) and Pulsatility Index (PI). All are calculated from a measured Doppler shift waveform.

Pourcelot's Resistance Index

Described in 1974, Pourcelot's Resistance Index (RI) is defined as peak Systolic height minus minimum Diastolic height divided by peak Systolic height:

$$RI = (S-D) / S$$

As diastolic flow falls, value of RI increases; if diastolic flow is absent, then RI equals one. RI values greater than one are possible, if there is negative diastolic flow.

Pulsatility Index

The Pulsatility Index (PI) is defined as peak Systolic Height minus minimum Diastolic Height divided by mean waveform height (N):

$$PI = (S-D) / N$$

The PI has the advantage that it can describe a range of waveform shapes when there is no end-diastolic flow.

In general, a low pulsatility index indicates that resistance distal to the point of measurement is low; high pulsatility index suggests high vascular resistance distal to the vessel being examined. It is important to remember that these indices are not an absolute measure of either upstream or downstream factors. For example, alterations in heart rate can change the shape of the waveform, leading to significant changes in the value of these indices.[286]

Qualitative analysis

The waveform can be analysed for particular features, especially the presence or absence of an early diastolic notch (useful in uterine artery waveform analysis) or the presence or absence of end-diastolic flow (particularly useful when assessing the umbilical artery waveform). These features are useful measures of resistance (Figure 1.9).

1.4.3 Doppler ultrasound characteristics in physiological and pathological conditions

In non-pregnant women the uterine artery resistance (as measured by PI or RI) tends to be raised (compared with pregnant women) and the waveform has an early diastolic notch. In pregnancy, the uterine arteries gradually transform from a low volume, high resistance system to a high volume, low resistance circulation. This fall in vascular resistance in the uterine arteries

can be detected as early as five to seven weeks' gestation.[287,288] The initial increase in uterine artery flow is slow (from 77 mls per minute at five weeks to 159 mls at ten weeks) but accelerates between 10 and 12 weeks, reaching 665 mls per minute at 16 weeks of gestation.[289] The fall in utero-placental resistance continues until around 24 to 26 weeks.[290,291]

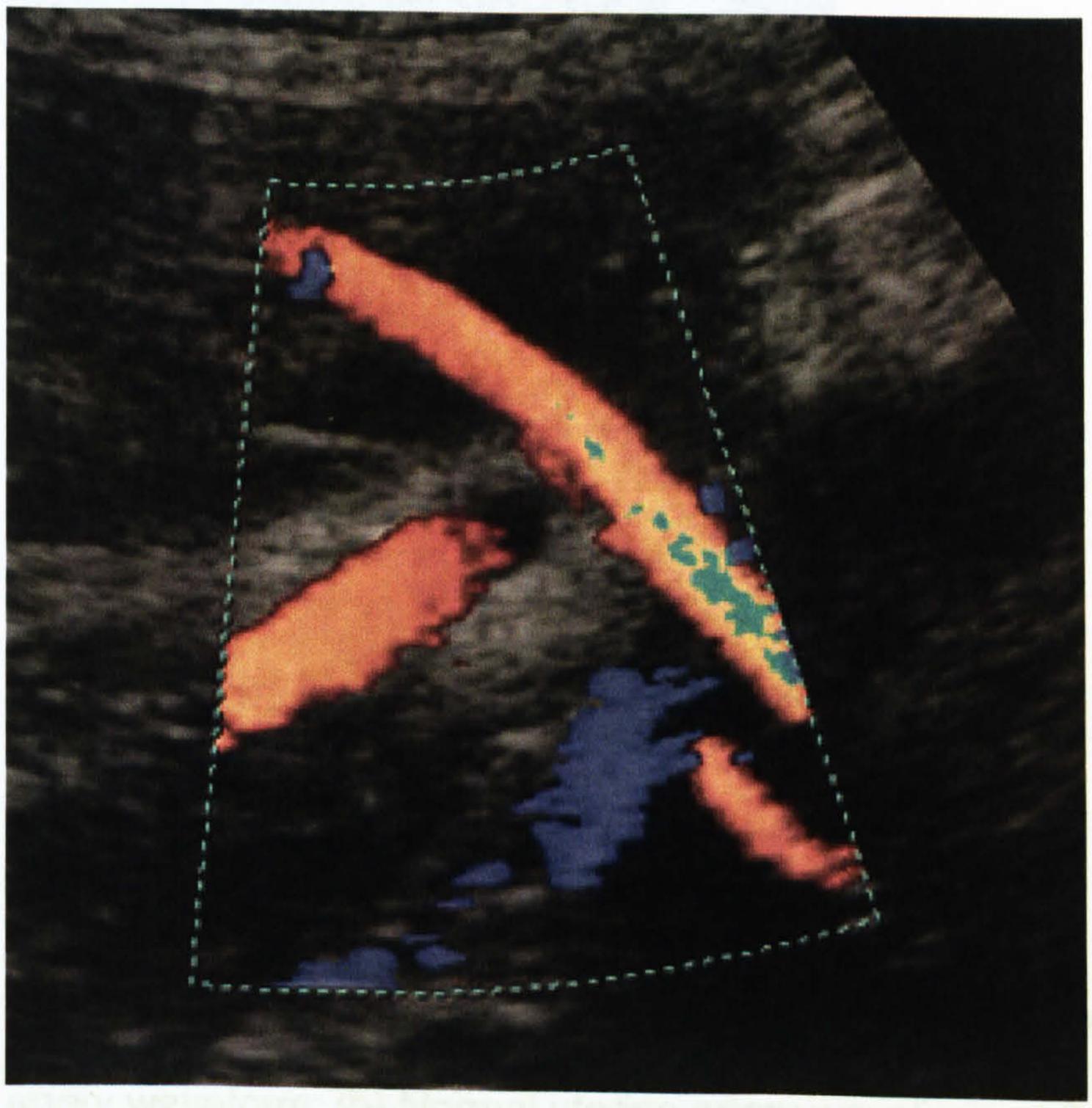
These changes can be detected by uterine artery Doppler examination (Figure 1.9a). The early diastolic notch usually disappears in the early second trimester (Figure 1.9b). This low resistance system (represented by loss of the early diastolic notch) is typical of pregnancy but has also been seen during the puerperium and after the menopause.[292,293] By 24 weeks' gestation, only 5% of women will still show uterine artery notches in both uterine arteries.[294] Persistence of this notch and/or raised PI or RI as gestation advances is an indicator of impaired uterine artery blood flow (Figure 1.9c).[295]

1.4.4 Uterine artery Doppler and pregnancy complications

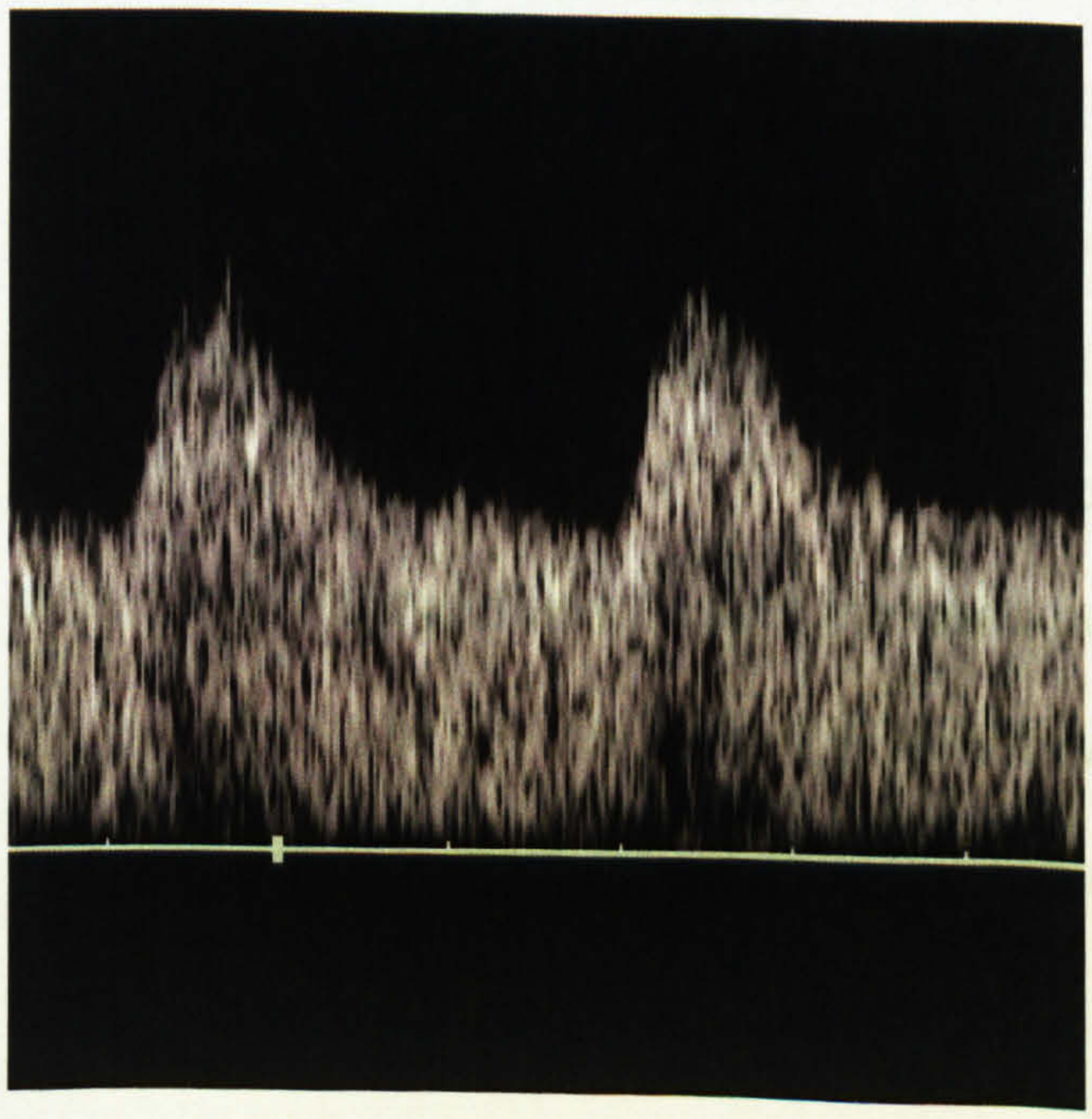
In the late 80's, several studies [296,297] found that impedance to flow in the uterine arteries is increased in pregnancies affected by pre-eclampsia or fetal growth restriction. These early findings triggered a wave of studies which attempted to identify women at increased risk of developing pre-eclampsia and/or fetal growth restriction before symptoms of clinical disease could be detected.

Figure 1.9. Uterine artery Doppler waveform.

(a)



(b)



(c)

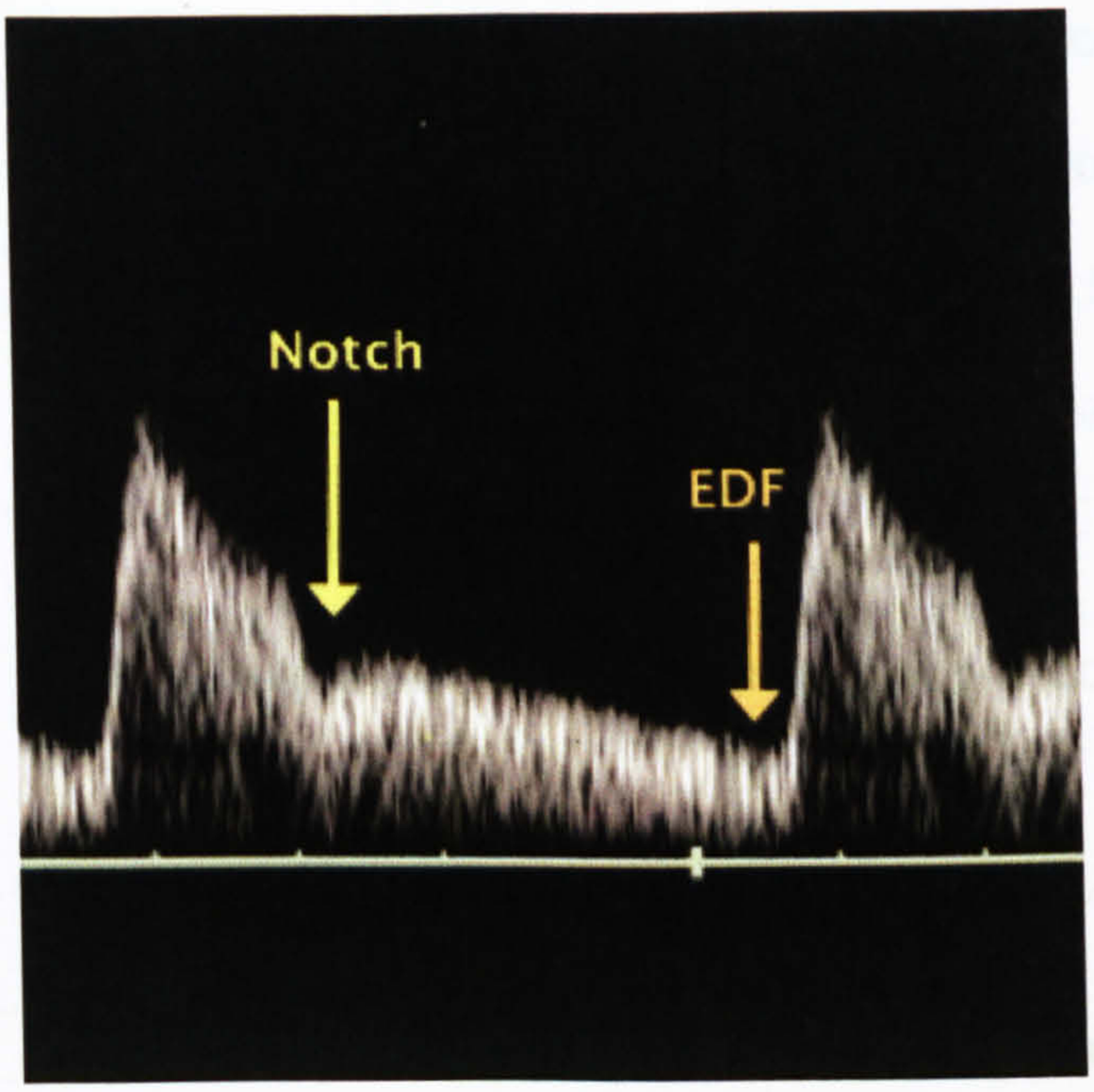


Figure 1.9. Uterine artery Doppler waveform: (a) Measurement of the uterine artery waveform; (b) Normal uterine artery waveform at 24 weeks' gestation; (c) Uterine artery waveform showing notching and the point at which end diastolic flow (EDF) is measured.

Papageorghiou *et al* [298] reviewed 15 good quality studies investigating the use of second trimester uterine artery Doppler to predict pre-eclampsia and fetal growth restriction.[225,294,299,300-309] Although conclusions are drawn from pooled data in this review, these studies are quite heterogeneous in several ways:

- The earlier studies used continuous waveform Doppler with all its limitations; more recent studies used real time ultrasound and pulsed wave Doppler.

- ❖ The criteria for defining abnormal Doppler waveforms vary (PI or RI above a certain centile, or the presence of an early diastolic notch).
- ❖ The populations examined differed. For example, the prevalence of pre-eclampsia ranged from 1.4% to 5.5%.
- ❖ The gestational age at which women were studied varied, ranging from 16 to 24 weeks.
- ❖ The criteria for the diagnosis of pre-eclampsia or fetal growth restriction differed.

Four of these studies used a two stage screening program; the initial screen was between 18 and 20 weeks using continuous wave Doppler. Those with increased resistance had a repeat Doppler study at 24 weeks.[294,304,305] The results are interesting. Overall, women with increased uterine artery resistance in the second trimester were six times as likely to develop pre-eclampsia as women with normal second trimester Dopplers. Conversely, normal uterine artery Dopplers gave a likelihood ratio for pre-eclampsia of 0.5. As expected perhaps, the ability of mid-trimester Dopplers to predict pre-eclampsia is better for the more severe forms of the disease. For example, one study [309] found that an increased second trimester PI predicted 44% of women who later developed pre-eclampsia at any stage but that the sensitivities increased to 54%, 70%, 81% and 90% for those who develop pre-eclampsia requiring delivery before 38, 36, 34 and 32 weeks of gestation respectively. The likelihood ratio for subsequent development of fetal growth restriction in women with increased uterine artery resistance in

the second trimester was 3.7. Again, the more severe the growth restriction, the better the predictive value of the test.

First Trimester Uterine Artery Doppler

Earlier studies showed a promising role of Doppler ultrasound examination of the uteroplacental circulation in the first trimester.[310] Early diastolic notching is present in 55% of the general pregnant population in the first trimester,[311] which means it is of limited use at this gestation. Generally, uterine artery PI above the 95th percentile has been used as the threshold. Examination of the uterine arteries is feasible in the first trimester; in one study the examination was successfully completed in 96% of women at 11 to 14 weeks' gestation.[311]

A study of 3,300 low risk women attending for routine antenatal care found that first trimester uterine artery PI above the 95th percentile had 27% sensitivity for later development of pre-eclampsia. However, sensitivity improved in proportion to the severity of the disease; the sensitivities for predicting pre-eclampsia requiring delivery before 36, 34, and 32 weeks of gestation were 40%, 50%, and 60% respectively. Another study examined women at increased risk of developing pre-eclampsia based on their personal or family history.[312] This study found that bilateral uterine artery notching at 12 to 14 weeks of gestation predicted pre-eclampsia with a sensitivity of 91% but with poor specificity (46%). Nevertheless, the positive predictive value was high (23%) in this high risk population.

In general, prediction rates for pre-eclampsia or fetal growth restriction are not as good in the first trimester as in the second. For a false

positive rate of 5%, around 40% of women requiring delivery for pre-eclampsia at less than 36 weeks, and 60% of those requiring delivery at less than 33 weeks' gestation, will be identified by first trimester Doppler.[311]

Combination of First Trimester Uterine Artery Doppler and Serum Markers

Nicolaides *et al.*[313] combined the use of serum placental protein 13 (PP13) with uterine artery Doppler PI, both measured in the first trimester (11^{+0} to 13^{+6} weeks' gestation). Using a nested case control model, they matched 10 women who subsequently developed pre-eclampsia requiring delivery before 34 weeks' gestation with 423 unaffected women. Median PP13 was lower and uterine artery PI higher in women who subsequently developed pre-eclampsia. Using logistic regression analysis, the authors predicted that, for a detection rate of 90%, PP13 alone would have a false positive rate of 12%, uterine artery PI alone a false positive rate of 31%, and a combination of the two a false positive rate of just 9%. They suggested the possibility of contingency screening. They calculated that if all women were first screened by serum PP13, with only those at highest risk (14%) undergoing Doppler examination, a detection rate of 90% could be achieved for an overall false positive rate of 6%. These findings have yet to be confirmed by larger prospective studies.[314]

First trimester uterine artery Doppler has also been combined with first trimester sFlt-1, Plasminogen Activator Inhibitor 1 (PAI1) to PAI2 ratio, and F-2 Isoprostane.[315] In this study, for a 5% false positive rate, first trimester Doppler alone predicted 25% of pre-eclampsia and 67% of severe pre-

eclampsia (requiring delivery before 35 weeks). None of the biochemical markers significantly improved the screening performance.

1.5 ALPHA METHYLDOPA

In UK clinical practice, alpha methyldopa is the antihypertensive drug most commonly used in pregnancy. Alpha methyldopa acts on α2-adrenergic receptors, primarily in the central nervous system, although an effect on peripheral α2-adrenoreceptors may also play a part.[316,317] Stimulation of pre-synaptic α2-adrenoreceptors in the central nervous system leads to a reduction of central sympathetic outflow and a reduction in blood pressure.[318] Methyldopa crosses the placental barrier and appears in cord blood and breast milk. However, there is no evidence that it causes any harm to the fetus or neonate, and it is considered safe in pregnancy.[319-321] Many women experience drowsiness for two or more days after commencing methyldopa. Women (10-20%) taking methyldopa will develop a positive Coomb's test.

We investigated whether alpha methyldopa has an effect on haemodynamic and placental markers over and above its known antihypertensive action in hypertensive disorders in pregnancy.

1.6 SUMMARY AND AIMS

In spite of decades of research, pre-eclampsia remains the 'disease of theories'. The nature of the placental factor(s) which lead to the multisystem maternal syndrome, and its precise effect on maternal vascular structure and function, remain poorly understood. The importance of pro-angiogenic and anti-angiogenic factors, including sFlt1, sEng, VEGF and PlGF, has recently been recognised, but their precise role in women with abnormal placentation is still not defined.

Pulse wave analysis, which can assess arterial stiffness, has been widely investigated outside pregnancy and proved useful in the management of cardiovascular diseases. However, as yet there are few reported studies in pregnancy, and the numbers of women remain small. Normal values throughout pregnancy have not been established.

Antihypertensive drugs are commonly used in women with hypertensive disorders of pregnancy. It is not known whether these drugs affect maternal serum levels of angiogenic factors or arterial stiffness, as measured by pulse wave analysis. If there is such an effect (and if it is independent of the antihypertensive effect of the drugs), this would imply an effect of the drug on the disease process itself, over and above its antihypertensive effect.

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

1. To evaluate the effect of antihypertensive therapy on angiogenic factors including sFlt-1, soluble endoglin, PlGF and VEGF in maternal serum and placenta.
2. To investigate the effect of antihypertensive therapy on the levels of inhibin A and activin A in maternal serum and placenta.
3. To determine effect of antihypertensive therapy on pulse wave analysis.
4. To ascertain the effect of antihypertensive therapy on uterine artery Doppler indices.
5. To establish normal values for pulse wave analysis parameters throughout pregnancy and investigate the effect of ethnicity.

CHAPTER TWO

METHODS

METHODS

2.1 ETHICS APPROVAL

Prior to commencing the study, ethics approval was obtained from the Camden & Islington Community Local Research Ethics Committee. Full written information in lay language describing the research project was provided and written consent obtained from each woman who agreed to participate. Each patient's General Practitioner was informed of her participation in the study.

2.2 SUBJECTS

2.2.1 Setting

Participants were recruited between January 2006 and June 2007 at the Homerton University Hospital (an associate teaching hospital in an urban setting in London). During this period, approximately 6,000 deliveries took place. The population in the catchment area of this hospital has a high Afro-Caribbean ethnic mix and a high incidence of pre-eclampsia.

Women and staff's awareness of the studies was raised by means of posters and fliers in antenatal clinic, the maternal-fetal assessment unit (MFAU), antenatal wards and labour ward. In the antenatal clinic, women

were assessed for eligibility for the 'normal values of PWA' study, and all eligible women were approached. Women diagnosed with pre-eclampsia or gestational hypertension, usually in the antenatal clinic, MFAU, antenatal and labour wards, were similarly assessed and eligible women approached. All women were given verbal and written information about the study for which they were eligible. Written informed consent was obtained from those who agreed to participate.

2.2.2 Studies of the effect of antihypertensive therapy

Women with a singleton pregnancy were prospectively recruited in the second and third trimesters. Exclusion criteria included multiple pregnancy, a history of hypertension, diabetes, renal disease or immune disorders, or women taking medication which could affect blood pressure.

The study group in whom uterine artery Doppler, pulse wave analysis and maternal serum marker levels were measured are described in detail (including a COHORT flow diagram) in Section 3.1. They included 51 women presenting with pre-eclampsia, 29 with gestational hypertension and 80 controls. All of these women had measurement of uterine artery Doppler, pulse wave analysis and serum levels of sFlt-1, sEng, PlGF, VEGF, inhibin A and activin A at the time of recruitment. The hypertensive group (n=80) had these measurements repeated 24-48 hours after starting antihypertensive therapy. Another group of 48 women were recruited for measurement of placental levels: 24 with hypertensive disorders in pregnancy (14 pre-

eclampsia, 10 gestational hypertension), and 24 controls matched for maternal age, gestational age and parity.

Blood pressure was measured in duplicate using a standard mercury sphygmomanometer and the average of two readings taken. All readings were taken by the same investigator (AK) with the subject in the sitting position. Korotkoff sounds 1 and 5 were used to define systolic and diastolic BP respectively. Mean BP was calculated as diastolic BP + $^1/_3$ pulse pressure.

Pre-eclampsia (PE) was defined according to the guidelines of the International Society for the Study of Hypertension in Pregnancy.[1] Diagnosis required two recordings of diastolic blood pressure ≥ 90 mm Hg, at least four hours apart; *or* one recording of diastolic BP ≥ 120 mm Hg, in a previously normotensive woman; *and* urine protein excretion ≥ 300 mg in 24 hours, *or* two readings of ++ or more on dipstick analysis of a midstream or catheter specimen of urine, if no 24 hour collection was available.

Severe pre-eclampsia was defined as severe hypertension (diastolic blood pressure ≥110 mmHg) and mild proteinuria, or mild hypertension and severe proteinuria (a 24-hour urine sample that contained ≥3.5 g protein or a urine specimen ≥3+ protein by dipstick measurement). Patients with abnormal liver function (aspartate aminotransferase >70 IU/L) and thrombocytopenia (platelet count <100,000/cm^3) were also classified as having severe pre-eclampsia.

Gestational hypertension (GH) was defined as a diastolic blood pressure ≥ 90 mm Hg on at least two consecutive occasions in the second half of pregnancy, without proteinuria, in a previously normotensive woman.[322]

Fetal growth restriction (FGR) was defined as birth weight less than the 5th centile for gestational age.

The controls consisted of 80 normotensive women matched for maternal age (± 3 years) and parity (none or one to two deliveries). All women in the control group had uncomplicated pregnancies. They had no history of cardiovascular disease, hypertension, proteinuria or fetal growth restriction, and were not taking medication that could affect blood pressure.

We collected blood samples at similar gestational ages (± 4 days) from the hypertensive patients and matched controls. The diastolic blood pressure of all women with pre-eclampsia or gestational hypertension was higher than 95 mm Hg. They received oral antihypertensive therapy in the form of alpha methyldopa 750-1500 mg/day for clinical indications according to local clinical guidelines. In accordance with local protocols, co-existing fetal growth restriction did not influence the decision to institute antihypertensive therapy. Venous blood was collected before and after (24-48 hours) antihypertensive therapy was commenced. A single venous blood sample was collected from controls.

Placental samples were collected from another group of women (n = 48) undergoing Caesarean section before the onset of labour, including 14 presenting with pre-eclampsia, 10 with gestational hypertension and 24 normotensive controls matched for gestational age (± 4 days), maternal age (± 3 years) and parity (none or one to two deliveries), and who were delivered by Caesarean section for obstetric reasons other than hypertension (e.g. preterm labour with an abnormal presentation or breech presentation at term). The hypertensive group included 12 women who received antenatal

antihypertensive therapy (alpha methyldopa 750-1500 mg/day). They were matched for gestational age with 12 women who were not taking antihypertensive treatment. Four to five placental biopsies were obtained at random from the maternal surface of the placenta, free of placental membranes.

2.2.3 Normal values of pulse wave analysis in pregnancy

For this prospective study, a different group of women with singleton pregnancies (n = 665) attending for routine antenatal care and non-pregnant women (n = 44) who were members of staff were incidentally recruited to participate in the study. None of these women (pregnant or non-pregnant) had a prior history of cardiovascular disease, chronic hypertension, diabetes, renal disease or immune disorders, and at the time of recruitment none was using medication which could affect blood pressure. All measurements in the non-pregnant women were taken during the follicular phase of their cycle. None was using hormonal contraception.

Each of these women was followed up through pregnancy and had one or more (depending on gestation at recruitment) assessments with arterial pulse wave analysis. Women who developed pre-eclampsia, gestational hypertension, fetal growth restriction, spontaneous preterm labour, gestational diabetes, fetal abnormalities, or who had a miscarriage or termination of pregnancy, were excluded from analysis in this part of the study.

2.3 DATA COLLECTION

Demographic and clinical data including age, body mass index (BMI), ethnicity, parity, blood pressure and gestational age (GA) were recorded for all recruited women. Gestational age was established on the basis of menstrual date and/or ultrasonographic examination prior to 20 weeks of gestation. All women were followed up until after delivery, and fetal and maternal outcomes were obtained from the women's medical records and labour ward records.

2.4 UTERINE ARTERY DOPPLER ULTRASOUND

Uterine artery Doppler indices were measured in the hypertensive and control groups at the time of recruitment. In women who received antihypertensive therapy, Doppler measurements were taken before and after (24-48 hours) therapy was initiated. The uterine artery was identified by a combination of real-time and colour Doppler techniques (iU22 Ultrasound System, Philips Medical Systems, Bothell, WA, USA). Blood velocity waveforms were recorded by the pulsed Doppler method (3.5 MHz curved probe; 120 Hz high-pass filter).

The transducer was placed over the iliac fossa and the course of the uterine artery followed from the lateral pelvic wall across the external iliac artery (Figure 1.9a). Pulsed Doppler was then applied 1 cm medial to the crossover point. The angle of insonation used was less than 30 degrees. Five

consecutive flow velocity waveforms of good quality were recorded, and the pulsatility index and resistance index derived.

The presence or absence of a diastolic notch in each uterine artery was noted (Figure 1.9c). A notch was considered to be present when there was a clearly defined upturn in the flow velocity waveform at the beginning of diastole, which was present in all recorded waveforms.[294]

2.5 ARTERIAL PULSE WAVE ANALYSIS

All pulse wave analysis (PWA) measurements were performed in the same room at the same temperature (23 °C), after a period of rest of at least 10 minutes. The women were asked to refrain from caffeine intake on the day of the study. During measurements, participants did not move or speak. Brachial artery blood pressure was measured in the non-dominant upper arm using a calibrated standard mercury sphygmomanometer. Brachial artery systolic BP was defined by the first Korotkoff sound and brachial diastolic BP by the fifth Korotkoff sound.[323] For each woman, the mean of two readings was used. Pulse pressure (PP) was defined as systolic minus diastolic pressure. The radial artery waveform was recorded using applanation tonometry, and the Sphygmocor® system (Atcor Medical, West Ryde, Australia) was used to analyse the radial artery wave contour. [277,324] The tip of the tonometer (a high fidelity pressure sensor) was pressed gently against the radial artery at the site of maximum pulsation at the wrist. This micromanometer precisely records pressure within the artery (Millar instruments, Houston, Texas.

USA).[252,253] A generalised transfer function [246,262,325] was applied to the radial artery waveform in order to derive the aortic waveform.

After an initial learning period (approximately 20 repeated measurements), satisfactory reproducibility was achieved (< 5% variability between duplicate measurements in the same woman). The Sphygmocor® software incorporates a quality control feature (operator index) which is displayed on the screen, and which ensures that all recorded waveforms from the same patient are similar. Recordings were considered acceptable for the study when the operator index was > 90%. Reasonable confidence is gained if the pressure waveforms are consistent; the Sphygmocor® software compares waveforms and accepts a series only if similarity is within prescribed limits. Because there is a linear relationship between AIx and heart rate, AIx was standardised to a heart rate of 75 beats per minute (AIx-75).[255]

2.6 COLLECTION OF BLOOD SAMPLES

Venous blood was collected from consenting participants. Ten mls of blood were collected into a vacuum tube and allowed to clot at room temperature for 30 minutes. Serum was separated by centrifugation at 3,000 rpm for 10 minutes and frozen in aliquots at -80 °C for further assay (see below).

2.7 COLLECTION OF PLACENTAL SAMPLES

Four to five random placental biopsies were obtained from the maternal surface of the placenta, free of placental membranes. Placental samples were collected only from women who underwent Caesarean delivery (both cases and controls) in order to avoid the potential effect of labour on placental expression of the markers to be studied. The samples (placental villi, 164-559 mg wet weight) were collected immediately after delivery and rinsed in sterile phosphate buffered saline (PBS), then snap frozen.

2.8 ASSAYS FOR TGF BETA PROTEINS IN SERUM AND PLACENTA

2.8.1 Assays

All markers (sFlt-1, sEng, PlGF, VEGF, inhibin A and activin A) were assayed using the same principle - a quantitative sandwich enzyme immunoassay technique (Figure 2.1). After appropriate sample preparation (see below) and validation of the technique for placental assay, placental samples were processed in the same way. A monoclonal antibody specific for the marker was pre-coated onto a microplate. Standards and samples were then pipetted into the wells on the plate and any antigen present became bound by the immobilised antibody. After washing away any unbound substances, an enzyme linked polyclonal antibody specific for the marker being assayed was

added to the wells. Following a wash to remove any unbound antibody-enzyme reagent of antigen bound in the initial step, the colour development was stopped and the intensity of the colour measured. The optical density (OD) of each well was determined within 30 minutes, using a microplate reader set to either 490 nm (for inhibin A and activin A) or 540 nm (for sFlt-1, sEng, PlGF, VEGF).

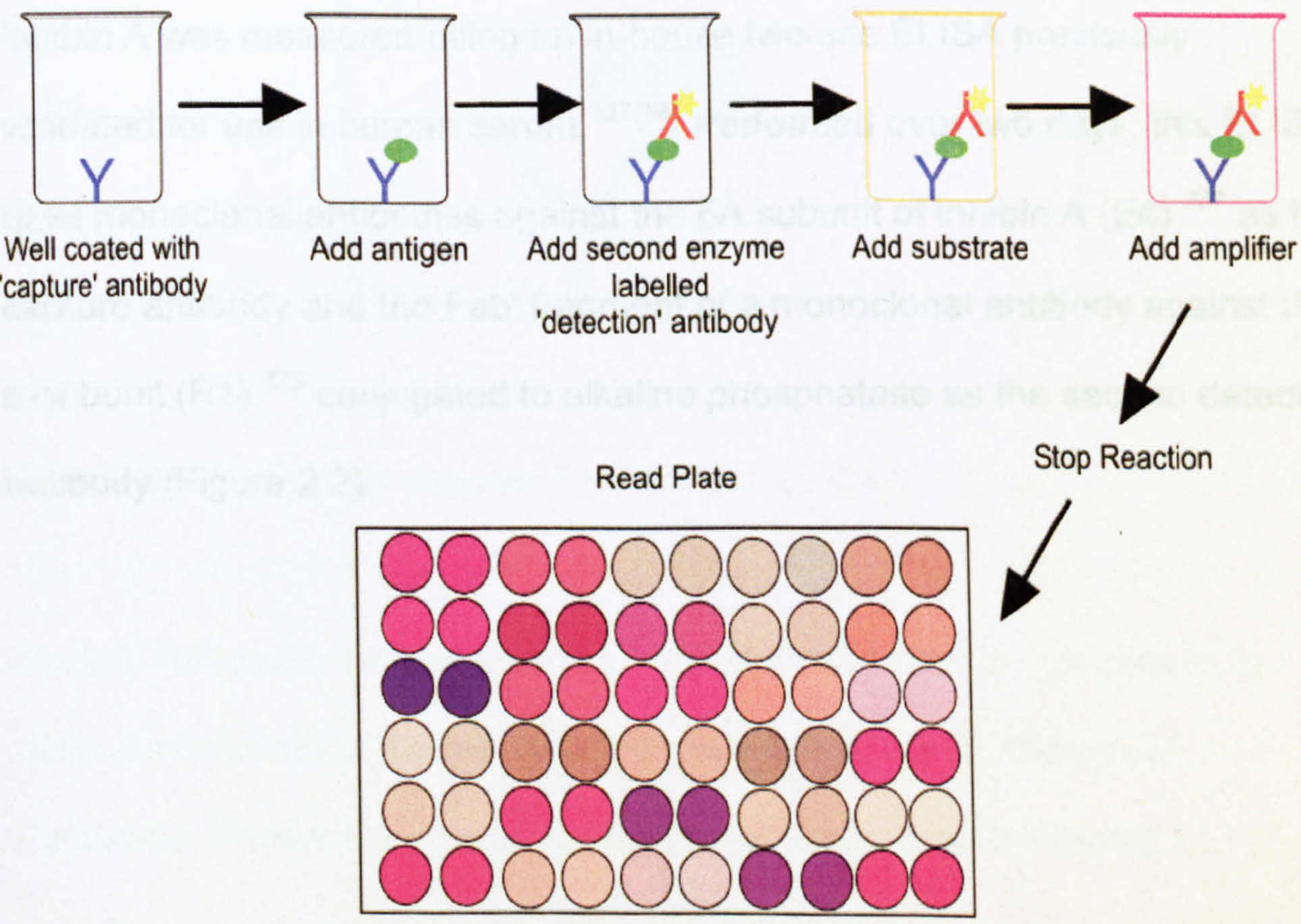


Figure 2.1. Schematic representation of a sandwich ELISA assay.

2.8.2 Serum samples

Inhibin A and activin A were measured using an in-house enzyme immune assay (ELISA) using monoclonal antibodies as both capture and detection antibodies. These assays are two-site 'sandwich' assays that use an ultra-sensitive detection system with alkaline phosphatase as the label (Figure 2.2).

Inhibin A ELISA

Inhibin A was measured using an in-house two-site ELISA previously validated for use in human serum.[187,326] Performed over two days, this ELISA uses monoclonal antibodies against the βA subunit of inhibin A (E4) [327] as the capture antibody and the Fab' fragment of a monoclonal antibody against the α-subunit (R1) [328] conjugated to alkaline phosphatase as the second detection antibody (Figure 2.2).

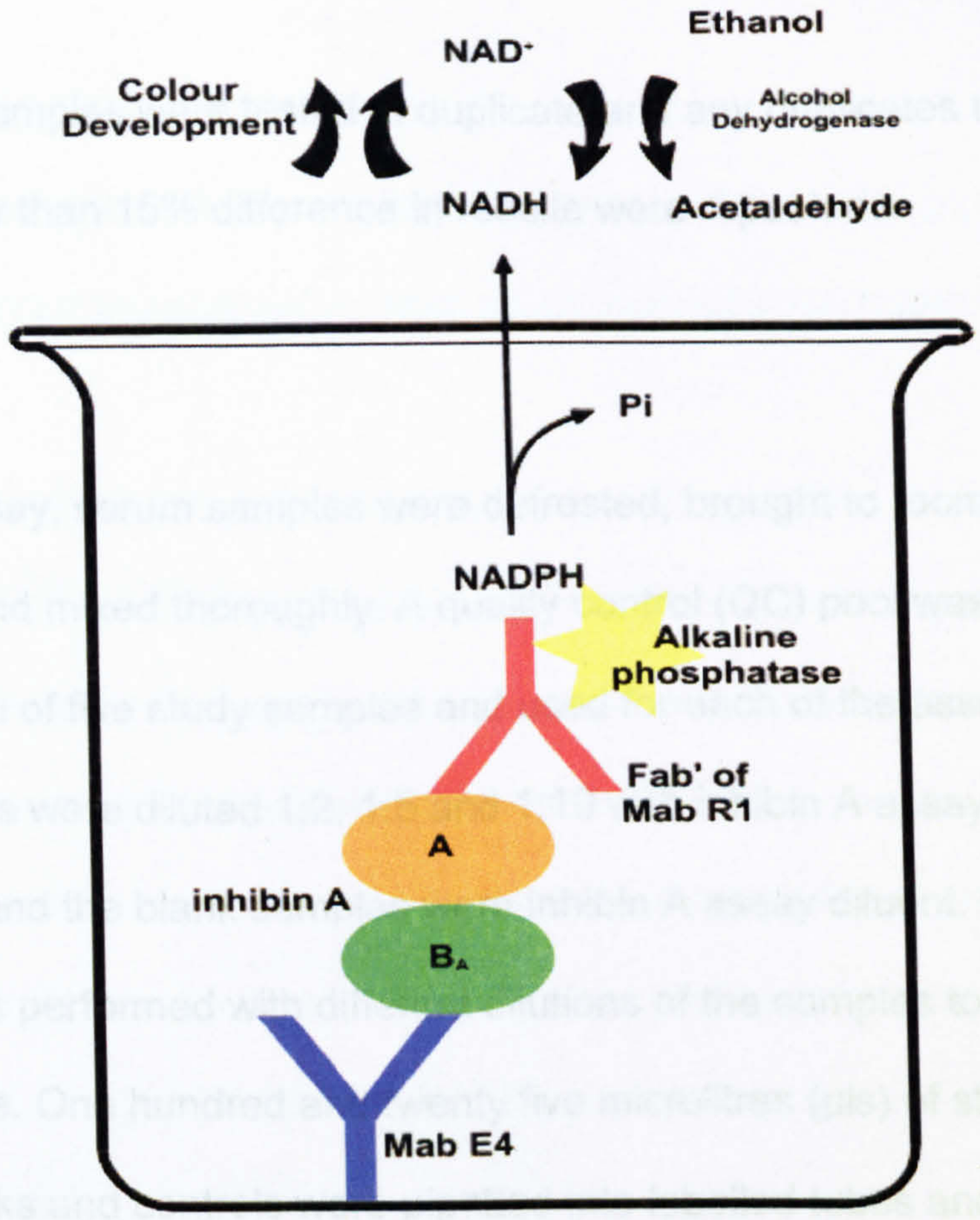


Figure 2.2. Schematic representation of the inhibin A ELISA.

Sample standards were prepared in inhibin A assay diluent (Appendix 1) using human recombinant inhibin A (National Institute for Biological Standards, Potters Bar, Herts, UK) in the following concentrations:

500 pg/ml: 250 pg/ml: 125 pg/ml: 62.5 pg/ml:
31.3 pg/ml: 15.6 pg/ml: 7.8 pg/ml: 3.6 pg/ml

The minimum detection limit of this assay for human recombinant inhibin A was 2pg/ml. Inter- and intra-assay variability were 4.8% and 5.2%

respectively. Samples were tested in duplicate and any duplicates that showed greater than 15% difference in results were repeated.

DAY 1

Prior to the assay, serum samples were defrosted, brought to room temperature and mixed thoroughly. A quality control (QC) pool was prepared from the serum of five study samples and used for each of the assay plates. Quality controls were diluted 1:2, 1:5 and 1:10 with inhibin A assay diluent (Appendix 1) and the blank samples were inhibin A assay diluent. Initially, a test assay was performed with different dilutions of the samples to derive the standard curve. One hundred and twenty five microlitres (µls) of standards, samples, blanks and controls were pipetted into labelled tubes and 125 µls inhibin A assay diluent was added to each sample tube. 125 µls fetal calf serum was added to the standards and blanks (to overcome the matrix effect), following which 50 µls of 10% hydrogen peroxide (H_2O_2) was added to each well. Samples were mixed well and incubated for 15 to 30 minutes. 100 µls blanks, samples, standards and controls were transferred into duplicate wells from an ELISA E4 coated plate. The plate was then covered, placed in a moist box and incubated overnight at 4 °C.

DAY 2

Any unbound sample was discarded. The plates were then washed ten times with ELISA wash buffer (Appendix 2) using a Wellwash 4 Mk II plate washer (Thermo Electron Corp, Bioscience Technologies, Basingstoke, UK) and bang dried on paper towelling. 50 µls of anti-α subunit antibody conjugated to alkaline phosphatase (R1-alk phos) was added to each well and the plate

incubated for two hours at room temperature in a moist box. Any unbound antibody was then discarded and the plate washed 15 times on a plate washer followed by two manual washes using manual wash buffer (Appendix 3).

50 µls substrate (ELISA Amplification System, Invitrogen Life Technologies) was added to each well. After one hour of incubation at room temperature, 50 µls amplifier was added to each well in the same sequence and timing as the substrate. When the blanks started to develop colour (after approximately ten minutes) the reaction was stopped with 50 µls per well of Stop solution (Appendix 4) and the plate was shaken for a few minutes. The plate was then read at 490 nm on an MRX 2 Microplate Reader (Dynex Technologies, Chantilly, VA, USA).

Activin A ELISA

The activin A ELISA is also a two-site assay specific for *total* activin A (follistatin-bound and unbound).[183,188] This assay uses the E4 monoclonal antibody as both the capture and detection antibody. The E4 used for detection is biotinylated; extravidin conjugated to alkaline phosphatase is incorporated into the assay as E4 cannot make a Fab' alkaline phosphate conjugate (Figure 2.3). A sodium dodecyl sulphate (SDS)/heat treatment is added which denatures follistatin to allow the measurement of total activin A and not just the free form. The detection limit of this assay for human recombinant activin A (Genentech, San Francisco, CA, USA) is 50 pg/ml. Intra- and inter-assay variations were 8.6% and 9.7% respectively. Any duplicate samples that showed greater than 15% variability were repeated.

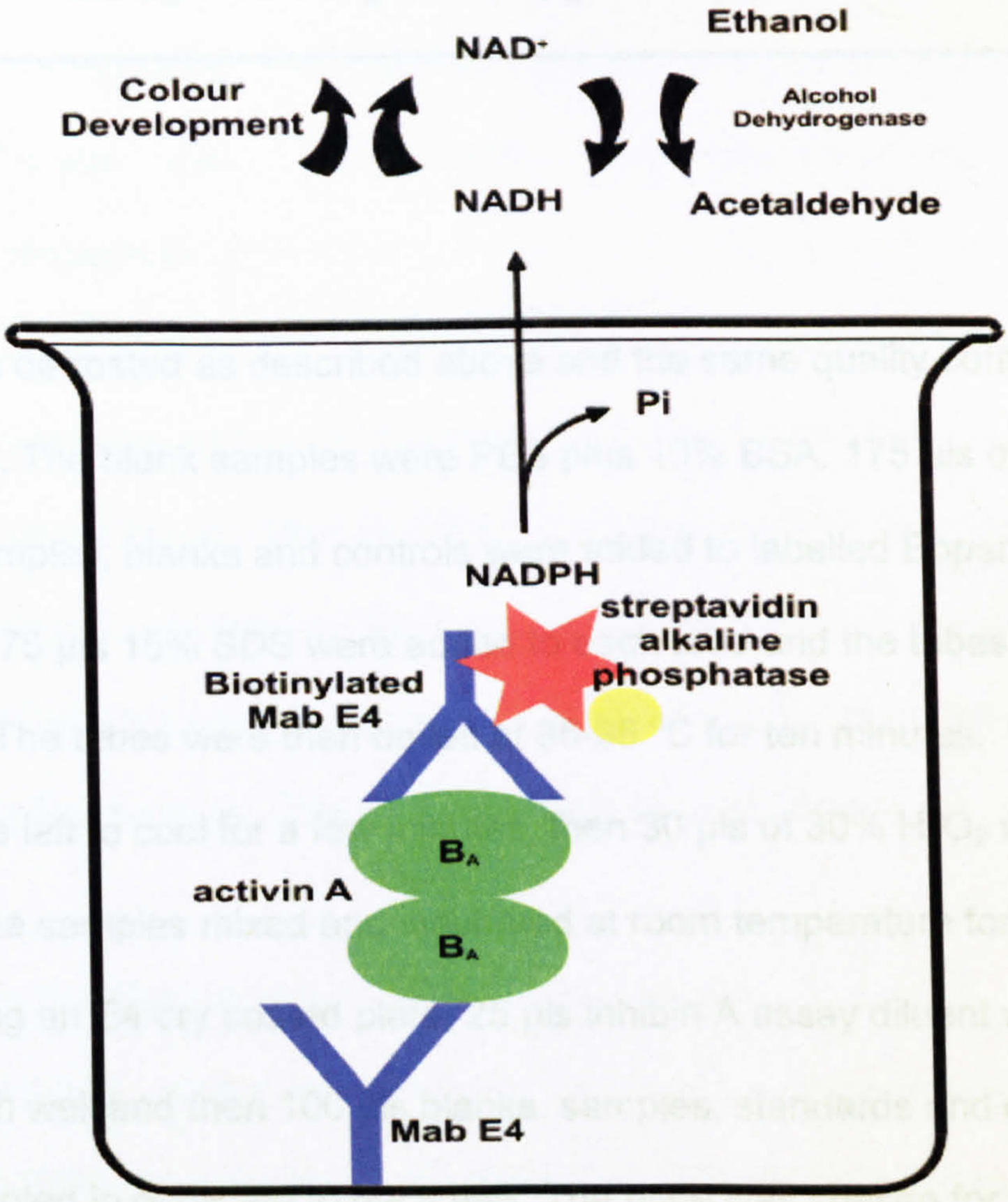


Figure 2.3. Schematic representation of the activin A ELISA.

An affinity purified human follicular fluid stock standardised against human recombinant activin A was used as standard. Standards were made up to the following concentrations using PBS plus 10% Bovine Serum Albumin (BSA) solution (Appendix 5):

10,000 pg/ml: 5,000 pg/ml: 2,500 pg/ml: 1,250 pg/ml: 626 pg/ml: 312 pg/ml: 156 pg/ml: 78 pg/ml

DAY 1

Samples were defrosted as described above and the same quality control samples used. The blank samples were PBS plus 10% BSA. 175 µls of standards, samples, blanks and controls were added to labelled Eppendorf tubes. Then 175 µls 15% SDS were added to each tube and the tubes mixed by inversion. The tubes were then boiled at 85-95 °C for ten minutes. Samples were left to cool for a few minutes, then 30 µls of 30% H_2O_2 was added, and the samples mixed and incubated at room temperature for 10-20 minutes. Using an E4 dry coated plate, 25 µls inhibin A assay diluent was added to each well and then 100 µls blanks, samples, standards and quality controls aliquoted in duplicate to each well. The plate was shaken for ten minutes on a plate shaker to allow mixing, then 25 µls of biotinylated E4 was added to all wells. The plate was covered and incubated at 4 °C in a moist box overnight.

DAY 2

Unbound sample was discarded and the plates washed ten times with ELISA wash buffer on a plate washer, then dried. To each well, 50 µls of extravidin conjugated to alkaline phosphatase (1 in 10,000 dilution) was added using a multi-head pipette. The plate was then incubated for two hours in a moist box

on a plate shaker. After incubation, the plate was washed 15 times on a plate washer, dried and then washed manually twice using manual wash buffer. 250 µls manual wash buffer was added to each well and the plate was incubated on the plate shaker for ten minutes. This process was repeated and the buffer discarded.

50 µls per well of substrate (ELISA amplification system, Invitrogen Life Technologies) was added and the plate incubated for one hour at room temperature in a moist box. After an hour, 50 µls of amplifier was added to each well. Once colour started to develop in the blank wells, the reaction was stopped with 50 µls per well of Stop solution and the plate shaken for a few minutes to mix the reagents. The plate was then read at 490 nm.

Serum soluble fms-like tyrosine kinase 1 (sFlt-1) ELISA

We used the Human Soluble VEGF R1/Flt-1 Immunoassay commercial Quantikine kit Catalogue Number DVR100B (R & D Systems, Minneapolis, Minnesota, USA) for assay of sFlt-1.

Sample and standard preparation

Samples were diluted up to 20-fold. To obtain 20-fold dilution, 20 µl samples were added to 380 µl calibrator diluent RD 6-10 (a buffered protein base with Sodium Azide and other preservatives).

The VEGFR-1 standard was prepared as follows: standard recombinant human VEGFR-1 in a buffer, lyophilised, was reconstituted with 1 ml of distilled water. This produced a stock solution of 20 ng/ml. The

standard was mixed to ensure complete reconstitution and was allowed to sit for a minimum of 15 minutes with gentle agitation prior to further dilution. 900 µl of calibrator diluent RD 6-10 was pipetted into the 2000 pg/ml tube, and 500 µl into the remaining tubes.

The stock solution was used to produce the following dilution series:

2000 pg/ml: 1000 pg/ml: 500 pg/ml: 250 pg/ml:
125 pg/ml: 62.5 pg/ml: 31.2 pg/ml

Each tube was mixed thoroughly before the next transfer. The blank tube (0 standard) contained only calibrator diluent RD 6-10.

Assay procedure

A plate plan was written prior to commencement of the assay, in order to record standards and samples assayed. All reagents and samples were brought to room temperature before use. All samples, controls and standards were assayed in duplicate.

100 µl of the assay diluent RD 1-68 (buffered solution) was added to each well. 100 µl of standard, control or sample was then added to each well. The plate was covered with an adhesive strip and incubated for two hours at room temperature on a horizontal microplate shaker set at 500 rpm.

The plate was then washed four times with a wash buffer (400 µl of buffered surfactant) using a plate washer. After the last wash, any remaining wash buffer was removed by aspirating, then inverting the plate and striking it against clean paper towels. 200 µl of VEGFR-1 conjugate (polyclonal antibody

against VEGFR-1 conjugated to horseradish peroxidase) was added to each well. The plate was then covered with a new adhesive strip and incubated for a further two hours at room temperature on a plate shaker. Following this, the plate was washed four times as before.

200 µl of substrate solution [a mixture of stabilised hydrogen peroxide and chromogen (Tetramethylbenzadine)] was then added to each well. The plate was incubated for 30 minutes at room temperature on the bench-top, protected from light. 50 µl of Stop solution (2N sulphuric acid) was then added to each well. The optical density of each well was then measured using a microplate reader and the concentration of sFlt-1 determined from the OD at 540 nm.

Serum soluble endoglin ELISA

We used the Human Endoglin/CD105 commercial Immunoassay DuoSet™ kit (Catalogue Number DY1097, R & D Systems, Minneapolis, Minnesota, USA) for assay of soluble endoglin.

Sample and standard preparation

Samples were prepared as described above for sFlt-1 assay. The endoglin standard used was recombinant human endoglin in a buffered protein base, lyophilised. The calibrator diluent used was RD5K. The stock solution was used to produce the following dilution series:

10 ng/ml: 5 ng/ml: 2.5 ng/ml: 1.25 ng/ml:
0.625 ng/ml: 0.313 ng/ml: 0.156 ng/ml

Assay procedure

Assay technique was similar to that described for sFlt-1. The assay diluent used was RD1S (buffered protein base). The assay antibody was endoglin conjugate (mouse monoclonal antibody against human endoglin, conjugated to horseradish peroxidase). The concentration of sEng was determined from the OD at 540 nm.

Serum PlGF ELISA

We used the Human PlGF Immunoassay commercial Quantikine kit (Catalogue Number DPG00, R & D Systems, Minneapolis, Minnesota, USA) for assay of placental growth factor.

Sample and standard preparation

Samples were prepared as described above for sFlt-1 assay. The PlGF standard used was recombinant human PlGF in a buffered protein base, lyophilised. The calibrator diluent used was RD6-11 (buffered protein base). The stock solution was used to produce the following dilution series:

500 pg/ml: 250 pg/ml: 125 pg/ml:
62.5 pg/ml: 31.2 pg/ml: 15.6 pg/ml

Assay procedure

Assay technique was similar to that described for sFlt-1. The assay diluent used was RD1-22 (buffered protein base). The assay antibody was PlGF

conjugate (polyclonal antibody against PlGF conjugated with horseradish peroxidase). The concentration of PlGF was determined from the OD at 540 nm as described before.

2.8.3 Placental samples

Preparation of placental samples

Placental biopsies were weighed in a sterile bottle and manually homogenised in four volumes (w/v) of Tris buffered saline containing EDTA-free serine-/cysteine-protease inhibitor cocktail, diluted according to the manufacturer's instructions (Roche Biochemicals) using a homogeniser. The protein extract was collected after centrifugation (3,000 rpm for 10 minutes) and stored at -80 °C until quantitative analyses were performed in batches.

Concentrations of placental markers are expressed per milligram of placental protein. Total placental protein concentration was determined using a commercial Coomassie Dry™ protein assay (Bradford, UK). Placental markers were assayed in the same way as serum samples, using a quantitative sandwich enzyme immunoassay technique.

Coomassie Dry Protein Assay

Principle of the assay

The assay as supplied already has the reagent dried in plate wells and prepared so that it dissolves rapidly when protein sample is added. The intensity of the blue colour produced by the reaction is proportional to the

concentration of protein present in the sample. This intensity can be measured in a microplate reader at 595 nm, and the optical density converted into protein concentration. The reagent dried in the wells is a modification of the Bradford reagent for total protein detection and quantification. Coomassie G-250 dye binds to protein and changes colour from reddish brown to blue, corresponding to an absorbance shift from 465 nm to 595 nm.

Technique

The standard was prepared using bovine serum albumin diluted in Phosphate Buffered Saline with the following dilutions:

1000 mcg/ml: 500 mcg/ml: 250 mcg/ml:
62.5 mcg/ml: 31.3 mcg/ml: 15.6 mcg/ml

Samples were then prepared by diluting in PBS (1 in 50). Before use, assay plates were equilibrated to room temperature. 100 µl per well of blank, standard and samples were assayed in triplicate. The plate was shaken vigorously for 60 seconds until the solid dye was completely dissolved. The plate was then read in a microplate reader at 595 nm. The standard curve was plotted and the protein content calculated. Consistent timing between sample addition, plate mixing and absorbance measurement was ensured.

Placental VEGF ELISA

We used R & D commercial DuoSet™ kits (Catalogue number DY293B, R & D Systems, Minneapolis, Minnesota, USA) for assay of placental VEGF. This assay employs the quantitative sandwich enzyme immuno-assay technique. The VEGF level in the serum samples was below the detectable levels.

Sample preparation

Placental samples were homogenised and the supernatant was stored at -80 °C. Serial dilutions of the homogenate were prepared (neat, 1:2, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200).

Plate preparation

The capture antibody used was mouse anti-human VEGF. This was reconstituted with 1 ml of PBS to 180 µg/ml. This was then diluted with 12 mls of PBS to give a 1 µg /ml (66.67 µl) solution. The plate was coated with 100 µl per well of capture antibody, then incubated overnight for 12-18 hours at room temperature in a moist box.

The following day, the antibody remaining on the plate was discarded. The plate was washed three times using a wash buffer solution [0.05% Tween 20 (Sigma) in PBS] 250 µl per well. The plate was then inverted and blotted dry against clean paper towels. 250 µl/well of blocking solution (1% BSA in PBS) was added to the plate which was left for a minimum of one hour at room temperature. After an hour, the plate was washed three times by repeated aspiration and washing.

Standard preparation

Recombinant VEGF was reconstituted with 0.5 mls of reagent diluent (1% BSA in PBS) to a concentration of 120 µg/ml. Prior to making the dilutions, the standard was allowed to sit for a minimum of 15 minutes with gentle agitation. The stock was subsequently diluted by a factor of 55 to give a top standard (10 µl stock standard + 540 µl reagent diluent). The standards were diluted to give the following concentrations:

2000 pg/ml: 1000 pg/ml: 500 pg/ml: 250 pg/ml: 125 pg/ml: 62.5 pg/ml: 31.3 pg/ml

Samples and quality control preparation

Samples and quality controls were diluted using reagent diluent as follows:

Up to 200-fold for samples from women with pre-eclampsia.

Up to 100-fold for samples from women with gestational hypertension and from controls.

These dilutions were derived from a dilution curve as previously described.

Assay procedure

A plate plan was written before starting the assay. After washing (blocking) the plate, 100 µl per well of the diluted standards, samples and quality controls were added in triplicate. The plate was then covered and incubated for two hours at room temperature. Following incubation, the plate was

washed three times by repeating the same cycle of aspiration and washing as before. 100 µl per well of detection antibody diluted reagent was added to the plate.

The stock of the detection antibody was prepared by reconstituting biotinylated goat anti-human VEGF with 1 ml of reagent diluent to a concentration of 9 µl/ml. After reconstitution, the aliquots were stored at -80 °C. The working concentration was 50 ng/ml (66.67 µl diluted in 12 mls of reagent diluent). The plate was covered and incubated for two hours at room temperature. Following incubation, the plate was washed three times as before. A 1 in 200 dilution preparation of streptavidin-HRP was prepared by adding 60 µl of streptavidin conjugated to horseradish peroxidase to 12 mls of reagent diluent. 100 µl/well of this streptavidin-HRP was added to the plate, which was then covered and incubated for 20 minutes at room temperature in the dark.

After 20 minutes, the plate was washed three times as before. A substrate solution was prepared as a 1:1 mixture of colour reagent A (H_2O_2) and colour reagent B (tetramethylbenzidine). 100 µg/well of substrate solution was added to the plate, which was then incubated for a further 20 minutes at room temperature in the dark.

50 µg/well of stop solution (2N H_2S0_4) was then added. The optical density of each well was determined using a microplate reader set to 540 nm.

Placenta soluble endoglin assay

Placental soluble endoglin was assayed using R & D commercial DuoSet™ human Endoglin (CD105) kits (Catalogue number DY1097).

Sample, standard and quality control preparation

Samples were prepared as described above for placental VEGF assay. The capture antibody used was mouse anti-human endoglin. The standard used was recombinant human endoglin in a buffered protein base, lyophilised. The assay diluent used was 1% BSA in PBS. The standards were diluted to give the following concentrations:

8000 pg/ml: 4000 pg/ml: 2000 pg/ml: 1000 pg/ml:
500 pg/ml: 250 pg/ml: 125 pg/ml: 62.5 pg/ml

The samples were diluted using reagent diluent as follows:

Up to 200-fold for samples from women with pre-eclampsia.

Up to 100-fold for samples from women with gestational hypertension and from controls.

These dilutions were derived from the dilution curve as previously described.

Assay Procedure

Assay technique was similar to that described above for placental VEGF. The assay diluent used was 1% BSA in PBS. The detection antibody was biotinylated goat anti-human endoglin. The concentration of sEng was determined from the OD at 540 nm as described before.

Placental PlGF assay

Placental PlGF was assayed using R & D commercial Duo Set™ human PlGF kits (Catalogue number DY264).

Sample, standard and quality control preparation

Samples were prepared as described above for placental VEGF assay. The capture antibody used was mouse anti-human PlGF. The PlGF standard used was recombinant human PlGF in a buffered protein base, lyophilised. The assay diluent was 1% BSA in PBS. The standards were diluted to give the following concentrations:

2000 pg/ml: 1000 pg/ml: 500 pg/ml: 250 pg/ml:
125 pg/ml: 62.5 pg/ml: 31.3 pg/ml

The samples and quality controls were diluted in reagent diluent up to two-fold for all samples (from women with pre-eclampsia, women with gestational hypertension and controls). The dilution was derived from the dilution curve as previously described.

Assay Procedure

Assay technique was similar to that described for placental VEGF. The assay diluent used was 1% BSA in PBS. The detection antibody was biotinylated goat anti-human PlGF. The concentration of PlGF was determined from the OD at 540 nm.

Placental sFlt-1 assay

We used the Human Soluble VEGF R1/Flt-1 commercial Immunoassay Quantikine kit (Catalogue number DVR100B, R & D Systems, Minneapolis, Minnesota, USA) for assay of sFlt-1. Sample and standard preparation, and assay procedure were identical to that used for serum sFlt-1 assay.

Sample, standard and quality control preparation

The samples were diluted using reagent diluent as follows:

Up to 200-fold for samples from women with pre-eclampsia.

Up to 100-fold for samples from women with gestational hypertension and from controls.

These dilutions were derived from the dilution curve as previously described.

Placental inhibin A assay

Placental inhibin A was measured using the assay described above for serum assay, which was previously validated for placental extracts.[329 187 326]

Sample, standard and quality control preparation

This was as described for serum inhibin A assay, but inhibin A assay diluent (Appendix 1) was used instead of FCS.

Placental samples were diluted using reagent diluent as follows:

1 in 10 for samples from women with pre-eclampsia.

1 in 5 for samples from women with gestational hypertension

1 in 2 for samples from controls.

Placental activin A assay

Placental activin A was measured using the assay described above for serum assay, which was previously validated for placental extracts.[183 330 329]

Sample, standard and quality control preparation

This was as described for serum activin A assay, but the sample preparation step was different, using PBS+5% BSA sample buffer and 10% SDS.

Placental samples were diluted using reagent diluent as follows:

1 in 20 for samples from women with pre-eclampsia.

1 in 10 for samples from women with gestational hypertension

1 in 5 for samples from controls.

Dilution curve

Different pools of samples were prepared according to the condition (pre-eclampsia, gestational hypertension and controls) and gestational age (< 34 weeks or ≥ 34 weeks of gestation). Test assays were performed on these pools with various chosen dilutions. Appropriate dilutions were chosen for the different groups of samples.

Validation

The samples were diluted parallel to the standard curve (Figure 2.4).

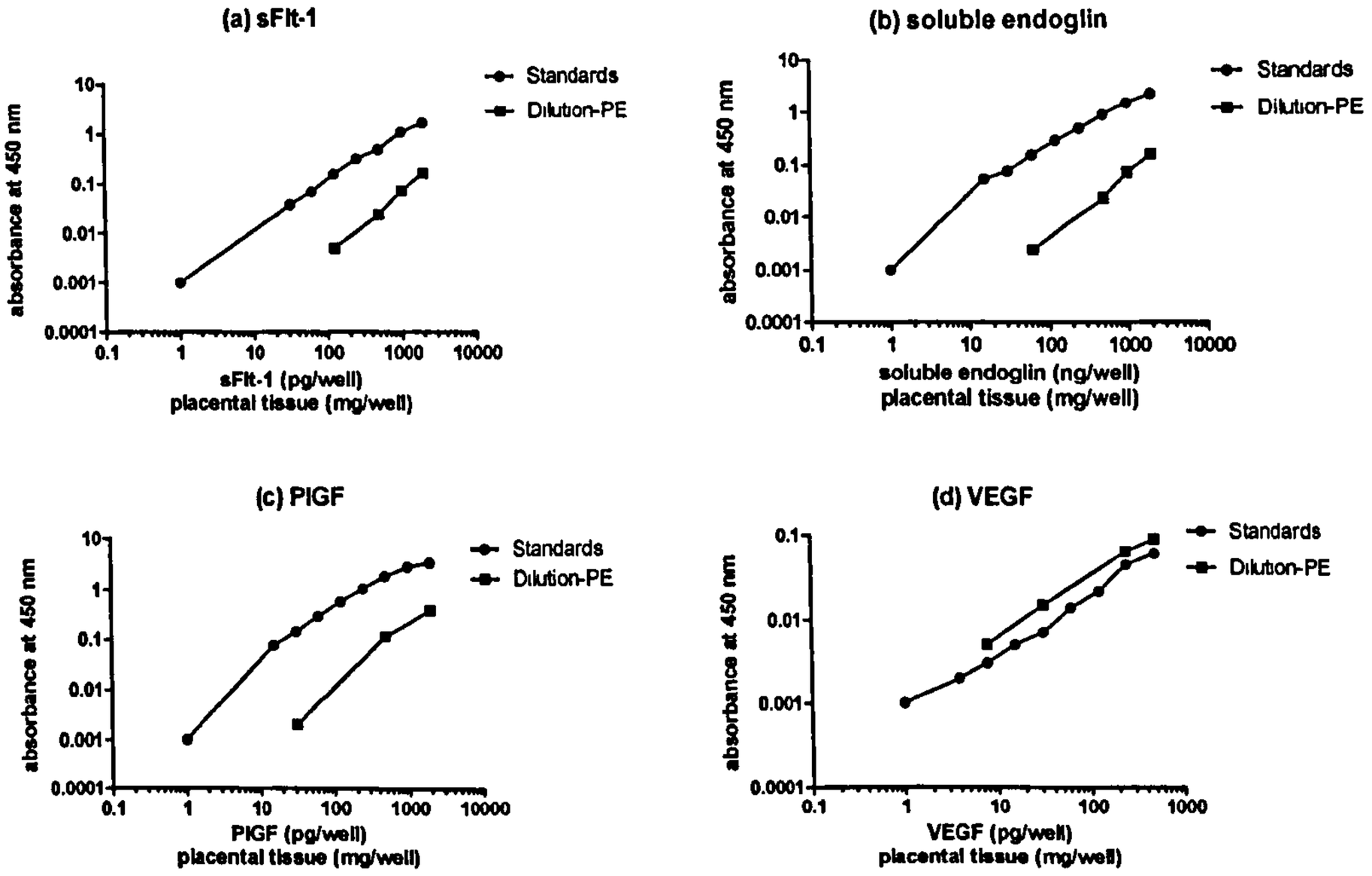


Figure 2.4. Dilution curves for pre-eclampsia placental samples for (a) sFlt-1, (b) soluble endoglin, (c) PlGF and (d) VEGF. Standard curves were obtained using the respective standards in the ELISA.

PE = pre-eclampsia

2.9 DATA AND STATISTICAL ANALYSIS

2.9.1 Power calculation

For the 'normal values of PWA' study, a power calculation was performed in advance, although recognising that this calculation was based on the limited existing data about normal values in pregnancy. It was calculated that 144 measurements in each trimester (including only one measurement per

woman) would provide the power to be 95% certain that the mean AIx75 within each trimester was estimated within 2 of the correct value.

There were even fewer data on which to base the power calculation for the studies examining the effects of antihypertensive therapy. There are no published studies reporting the effects of such therapy on levels of angiogenic and anti-angiogenic factors, inhibin or activin, either in pregnancy or in non-pregnant subjects. The data on the effect of antihypertensive therapy on uterine artery Doppler resistance are conflicting. The power calculation for these studies was therefore based on studies of the effect of antihypertensive therapy on arterial stiffness as measured by PWA in non-pregnant hypertensive subjects. It was calculated that 27 women were needed in each hypertensive group to have 90% power to detect a difference of 2.5 percentage points in augmentation index-75 at the 5% level.

2.9.2 Effect of antihypertensive therapy on levels of markers and uterine artery Doppler in pregnancies with hypertensive disorders

D'Agostino and Pearson Omnibus test was used to assess normality of continuous data. Analysis of variance (ANOVA) with Bonferroni post hoc tests were carried out to study the differences among the three groups. Data were analysed in two gestational age intervals: < 34 weeks (representing early onset disease) and ≥ 34 weeks (representing late onset disease). Unpaired *t* test was used to compare the markers levels between mild and severe, and between early onset and late onset pre-eclampsia. Paired *t* test was used to compare marker levels before and after antihypertensive therapy. Unpaired *t*

test was used to compare placental marker levels in those who received antihypertensive therapy and those who did not. The data were normally distributed after logarithmic transformation. Pearson correlation analysis was carried out to investigate the relationship between the parameters measured. Data were analysed using SPSS® (SPSS version 15, 2007, SPSS Inc., Chicago, Illinois, USA). GraphPad Prism® 5.0 for Windows (InStata, GraphPad Software Inc., San Diego, California, USA) was used to test the normality of data. Results were considered statistically significant at $P<0.05$.

2.9.3 Effect of antihypertensive therapy on Pulse Wave Analysis in pregnancies with hypertensive disorders

Baseline characteristics were compared using Chi-square test (Fisher's exact test when appropriate) for categorical variables and independent *t*-test for continuous variables. Although cases were matched to controls for maternal age, gestational age and parity, when comparing any two of the three groups (pre-eclampsia, gestational hypertension and controls), we chose to use unpaired *t*-test because of concerns that other differences between the groups in BMI, smoking status and ethnicity (which were not matched) could potentially bias the results. For comparison of all three groups, ANOVA with Dunnett's post-hoc test was used. Unpaired *t* test was used to compare the measurements between mild and severe, and between early onset and late onset pre-eclampsia. Measurements before and after starting antihypertensive therapy were compared using paired *t*-test. Data were

analysed using SPSS® (SPSS version 14.0, 2005, SPSS Inc., Chicago, IL, USA). Multiple linear regression models were used to analyse the associations of AIx-75 with baseline characteristics and with haemodynamic parameters.

2.9.4 Pulse Wave Analysis in normal human pregnancy and the effect of ethnicity

Baseline characteristics were compared between pregnant and non-pregnant, and between the two ethnic groups, using Chi-square test (Fisher's exact test when appropriate) for categorical variables and independent *t*-test for continuous variables. Independent *t*-test was used to compare haemodynamic parameters between pregnant and non-pregnant, and between the two ethnic groups. For comparison of the three gestational age intervals, we used ANOVA multiple comparisons with Bonferroni post hoc testing. Multiple linear regression models were used to analyse the association of Pulse Wave Analysis parameters (AP and AIx-75) with baseline characteristics and with other haemodynamic parameters, such as blood pressure. A value of $P<0.05$ was considered to be statistically significant. All *P* values were two-tailed. Data were analysed using SPSS® (SPSS version 14.0, 2005, SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (InStata, GraphPad, San Diego, CA, USA).

Linear regression models were used for comparison of haemodynamic parameters between the study groups, adjusted for BP. When correcting for

BP between groups, mean pressure was the preferred parameter, as it is assumed to be constant throughout the arterial tree.[225]

CHAPTER THREE

RESULTS

RESULTS

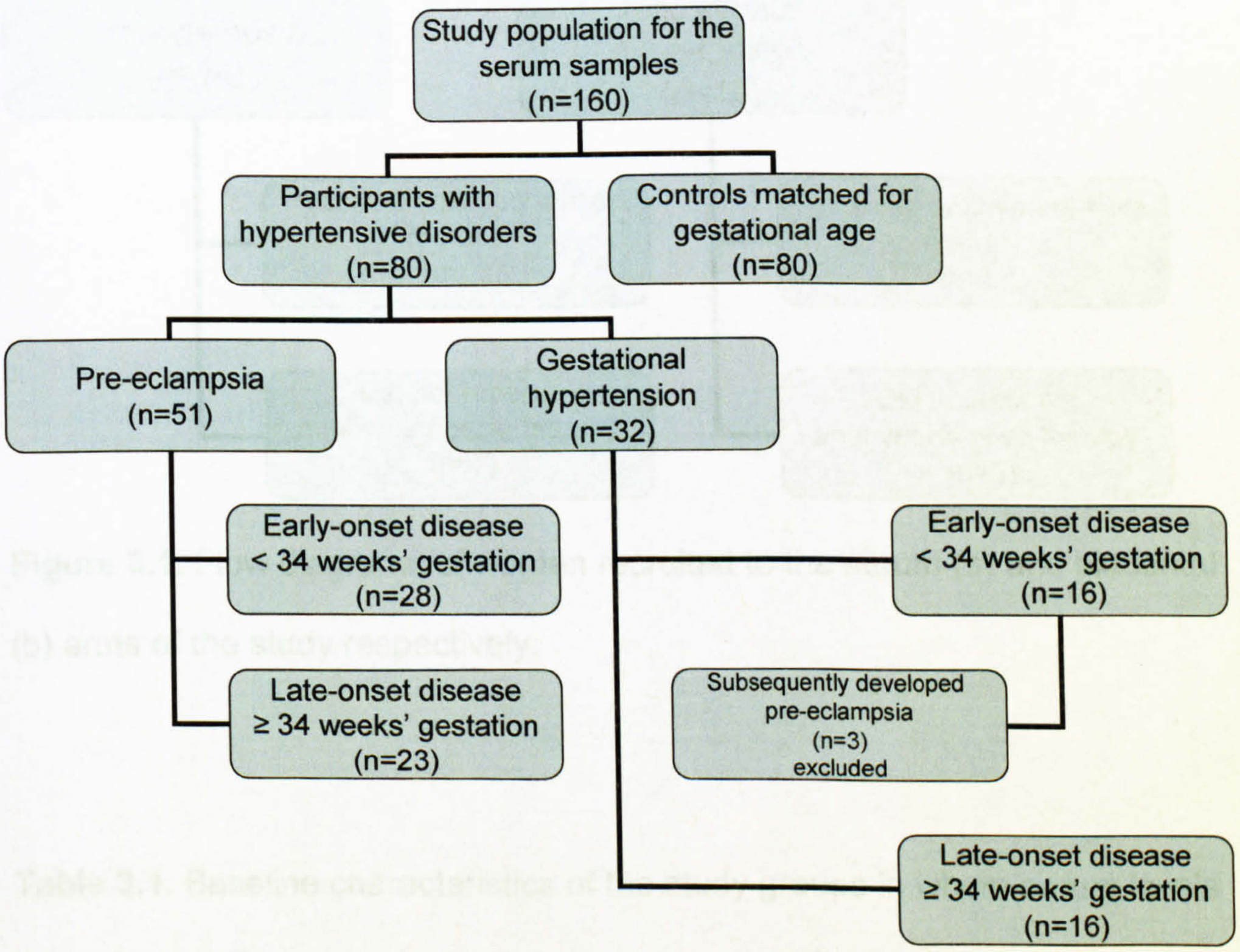
3.1 OVERVIEW

Figures 3.1 (a) and (b) are flow diagrams describing the women recruited to the serum and placental arms of the study respectively. The baseline characteristics of the serum study groups are shown in Table 3.1. The time interval between the two measurements or sampling in the hypertensive women is also shown in Table 3.1. Among the 51 women with pre-eclampsia in this arm of the study, 16 (31%) had associated fetal growth restriction (FGR) and 8 (16%) had severe pre-eclampsia. All the severe pre-eclampsia cases were in the early-onset group.

Of the 32 women recruited with gestational hypertension (16 < 34 weeks and 16 ≥ 34 weeks' gestation), 3 subsequently developed pre-eclampsia (3 in the gestational hypertension group recruited < 34 weeks and none in the gestational hypertension group recruited ≥ 34 weeks' gestation) and were excluded.

Figure 3.1

(a)


Study population for the serum samples (n=160)
Participants with hypertensive disorders (n=80)
Controls matched for gestational age (n=80)
Pre-eclampsia (n=51)
Gestational hypertension (n=32)
Early-onset disease < 34 weeks' gestation (n=28)
Late-onset disease ≥ 34 weeks' gestation (n=23)
Early-onset disease < 34 weeks' gestation (n=16)
Subsequently developed pre-eclampsia (n=3) excluded
Late-onset disease ≥ 34 weeks' gestation (n=16)

(b)

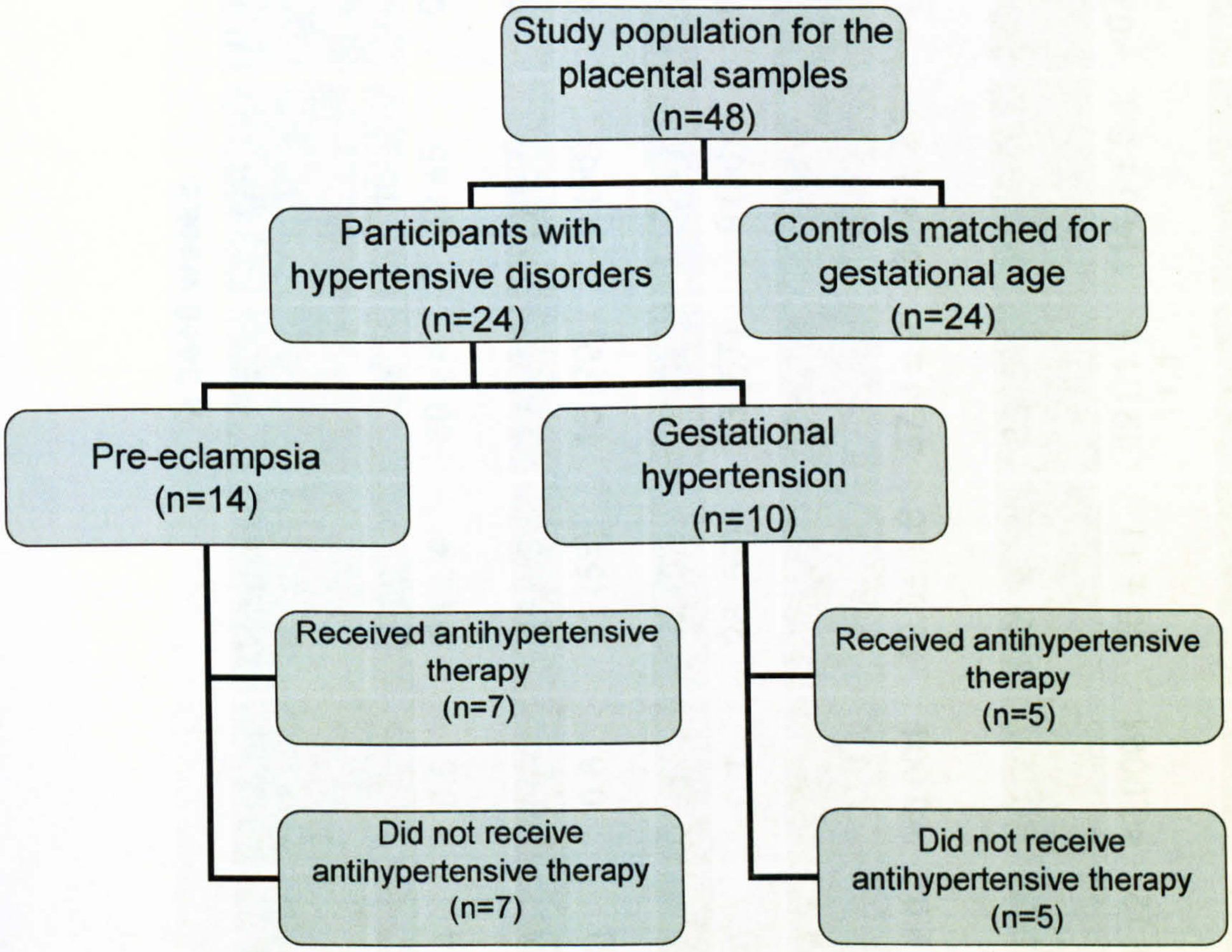


Figure 3.1. Flow diagrams of women recruited to the serum (a) and placental (b) arms of the study respectively.

Table 3.1. Baseline characteristics of the study groups in whom serum levels of markers, pulse wave analysis and uterine artery Doppler were measured, according to gestational age at recruitment.

BMI = body mass index GA = gestational age

Mean BP = mean blood pressure PE = pre-eclampsia

GH = gestational hypertension

† Data presented as mean ± SD and analysed by one-way ANOVA with Bonferroni post hoc analysis.

Table 3.1. Baseline characteristics of the study groups.

	< 34+0 weeks				≥ 34+0 weeks			
	Controls	**PE**	**GH**	*P* value	**Controls**	**PE**	**GH**	*P* value
n	41	28	13		39	23	16	
Age (years) †	31 ± 4	30 ± 5	32 ± 4	0.6	31 ± 4	30 ± 4	32 ±5	0.2
BMI (kg/m^2) †	27 ± 4	30 ±4	27 ± 4	0.07	28 ± 3	27 ± 5	29 ± 5	0.2
Nulliparity [n (%)]	25 (61)	20 (71)	7 (54)	0.6	21 (53)	16 (70)	9 (56)	0.3
Current smoker [n (%)]	1 (2)	0 (0)	0 (0)	1	2 (5)	1 (4)	0 (0)	1
Caucasian [n (%)]	20 (48)	13 (46)	7 (54)	0.7	23 (59)	13 (57)	9 (56)	0.4
GA at recruitment (days) †	30 ± 1.3	30 ± 0.4	30.4± 0.8	0.5	36.6 ± 2.4	36 ± 2.3	36.4 ± 2	0.4
GA at delivery (days) †	39.7 ± 2.3	33 ± 1.7	36.7 ± 2.9	<0.001	39.8± 1.6	37.3 ± 2	38.6 ± 2.3	<0.001
Birth weight (grammes) †	3398 ± 529	1685 ± 204	2725± 198	<0.001	3405 ± 526	2896 ± 689	3174 ±591	<0.001
Mean BP (mmHg)	85 ± 12	126.6 ± 12	125.1 ± 12	<0.0001	85 ± 11	121.1 ± 11.1	114.5 ± 6.1	<0.0001
Interval between measurements (hours)		32 ± 4	34 ± 3			38 ± 6	39 ± 7	

3.2 EFFECT OF ANTIHYPERTENSIVE THERAPY WITH ALPHA METHYLDOPA ON LEVELS OF ANGIOGENIC FACTORS IN PREGNANCIES WITH HYPERTENSIVE DISORDERS

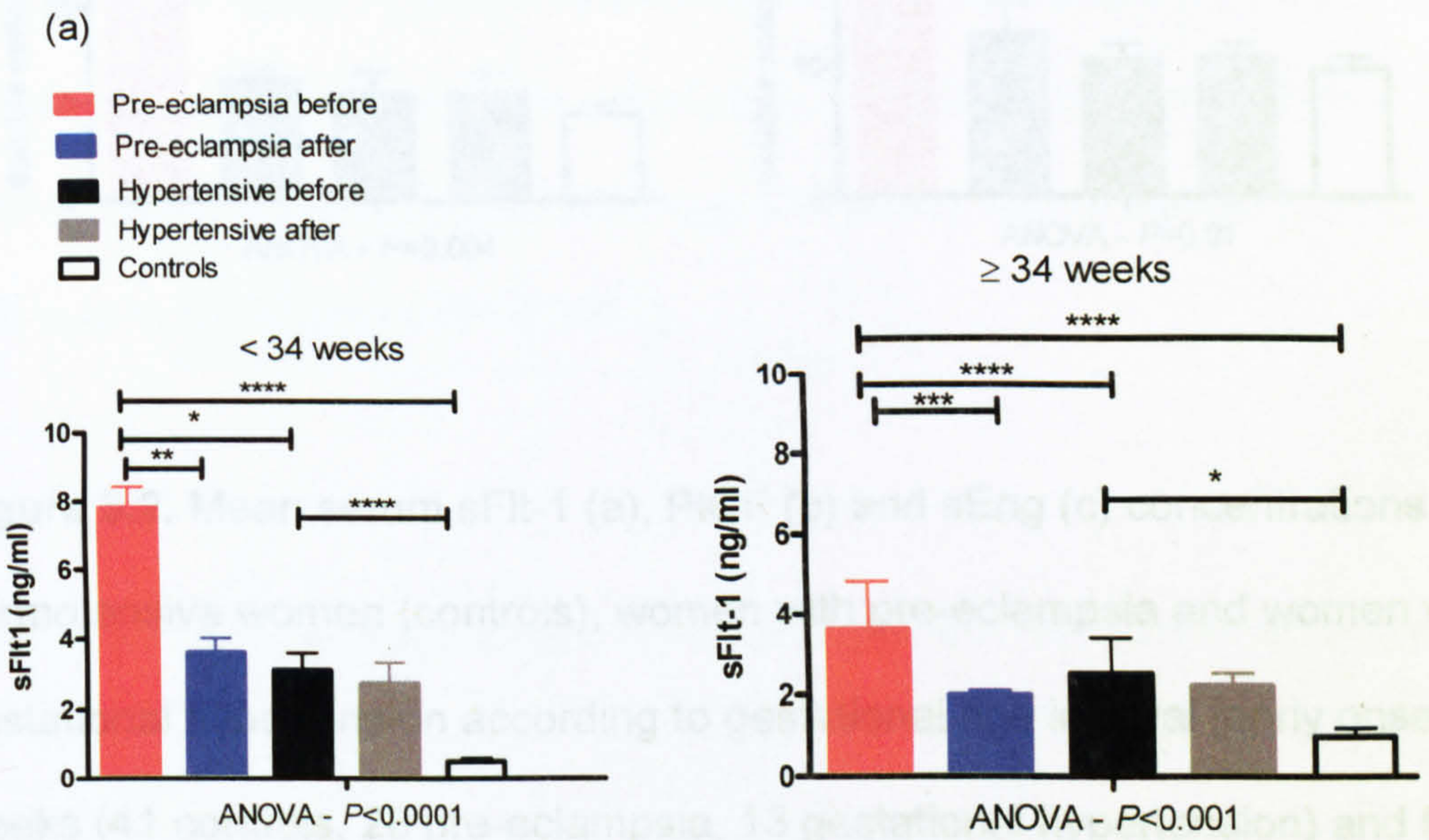
3.2.1 Results

Serum levels prior to treatment

Figure 3.2 shows the serum levels of angiogenic markers before and after antihypertensive therapy. Prior to treatment in women with pre-eclampsia, serum levels of sFlt-1 (Figure 3.2a) were significantly higher than normotensive controls (P <0.0001 both before 34 weeks and ≥34 weeks), and higher than women with gestational hypertension (before 34 weeks, P <0.05; ≥34 weeks, P<0.0001). Serum sFlt-1 levels were also higher in gestational hypertension compared with controls (before 34 weeks, P <0.0001; ≥34 weeks, P<0.05). Figure 3.2b shows that, prior to treatment in women with pre-eclampsia, serum PlGF levels were significantly lower than in controls (before 34 weeks, P <0.0001; ≥34 weeks, P<0.01) but not significantly different from women with gestational hypertension. Levels in gestational hypertension were also significantly lower than in controls (before 34 weeks, P <0.0001; ≥34 weeks, P<0.05). Figure 3.2c shows that, prior to treatment in women with pre-eclampsia, serum sEng was significantly higher than in normotensive controls (before 34 weeks, P <0.0001; ≥34 weeks, P <0.01), and higher than women with gestational hypertension (before 34 weeks, P <0.0001; ≥34 weeks,

P<0.05). Serum levels of sEng were not significantly different between gestational hypertension and normotensive controls.

Figure 3.2. Mean serum sFlt-1 (a), PlGF (b) and sEng (c) concentrations.



(c)

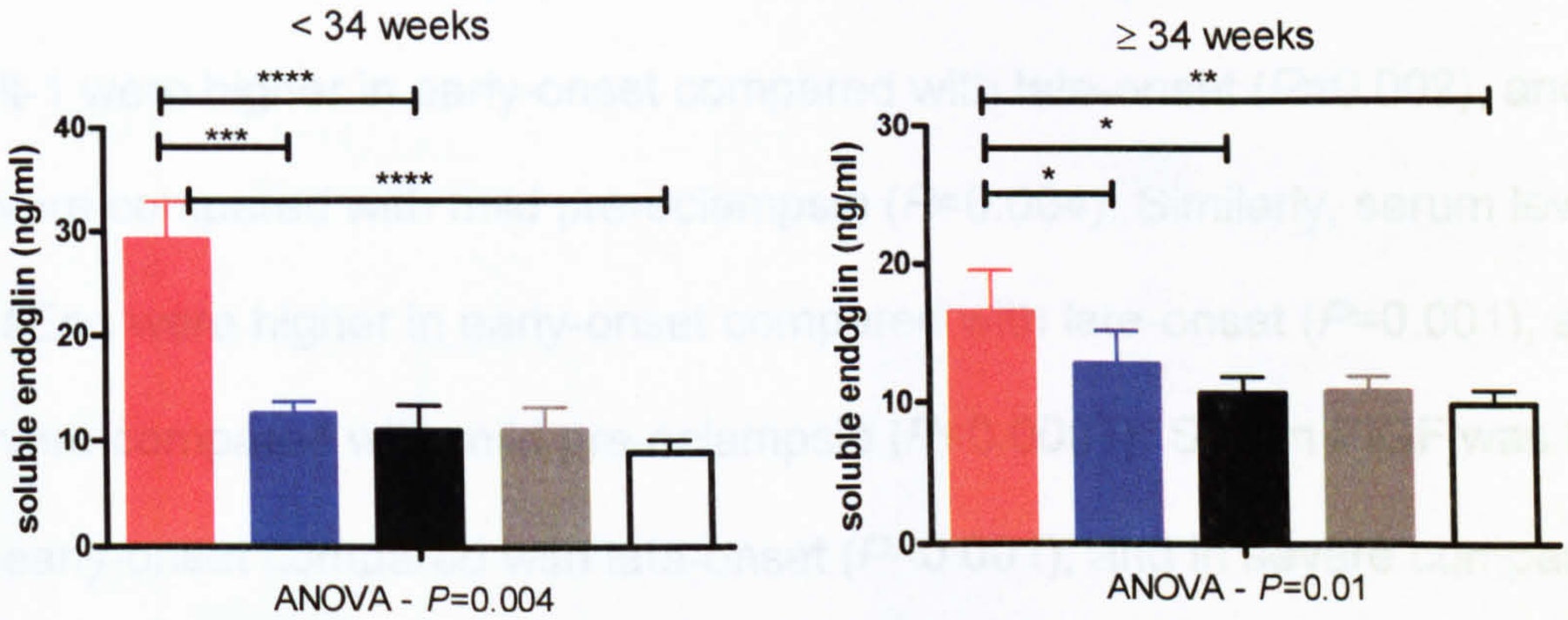
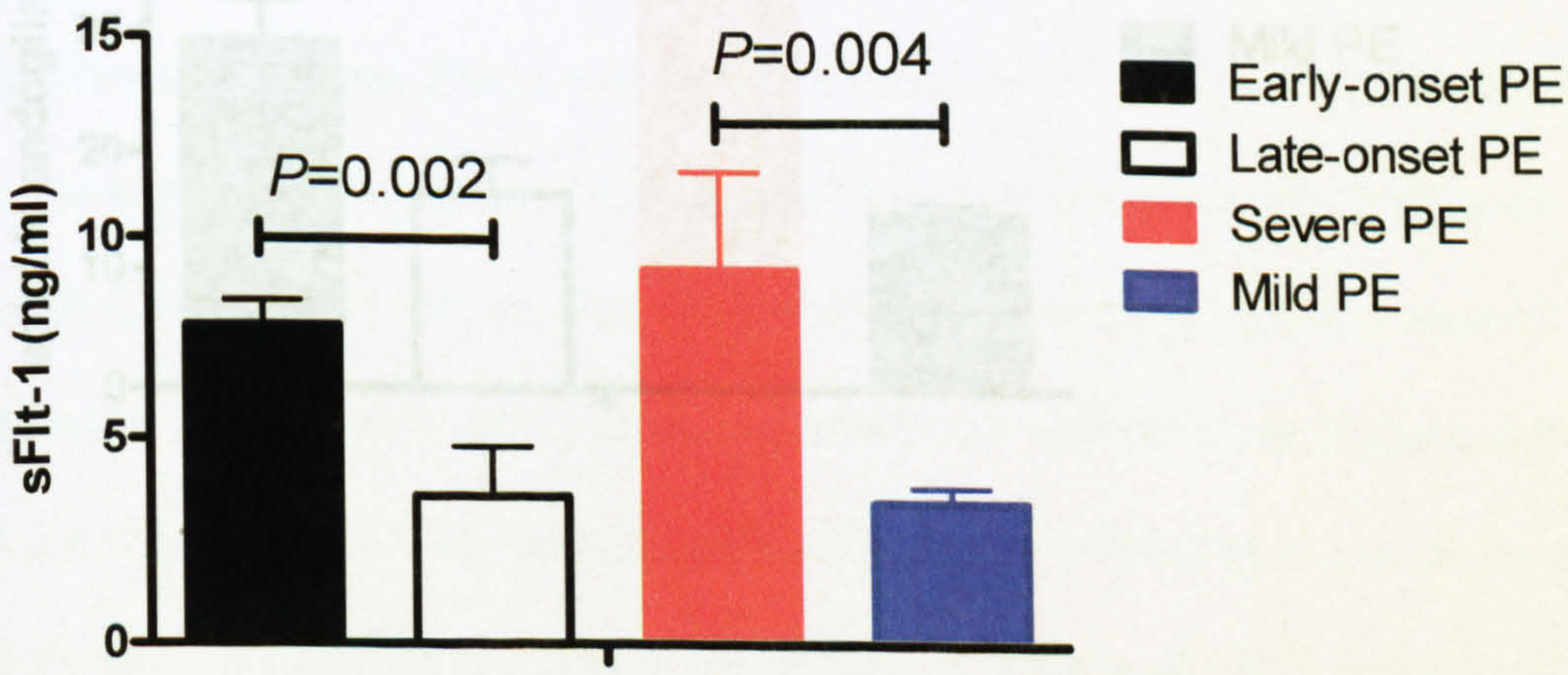


Figure 3.2. Mean serum sFlt-1 (a), PlGF (b) and sEng (c) concentrations in normotensive women (controls), women with pre-eclampsia and women with gestational hypertension according to gestational age interval [early onset <34 weeks (41 controls, 28 pre-eclampsia, 13 gestational hypertension) and late onset ≥ 34 weeks (39 controls, 23 pre-eclampsia, 16 gestational hypertension)]. Error bars represent standard errors. Comparison of controls and cases (with pre-eclampsia or gestational hypertension) was performed after logarithmic transformation. Levels before and after alpha methyldopa therapy are shown for women with pre-eclampsia and women with gestational hypertension. The levels in the three groups were compared using ANOVA with Bonferroni Dunn's posthoc tests. The levels before and after antihypertensive therapy are compared using the paired *t* test. ****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$. *P* values are shown only for differences which are statistically significant.

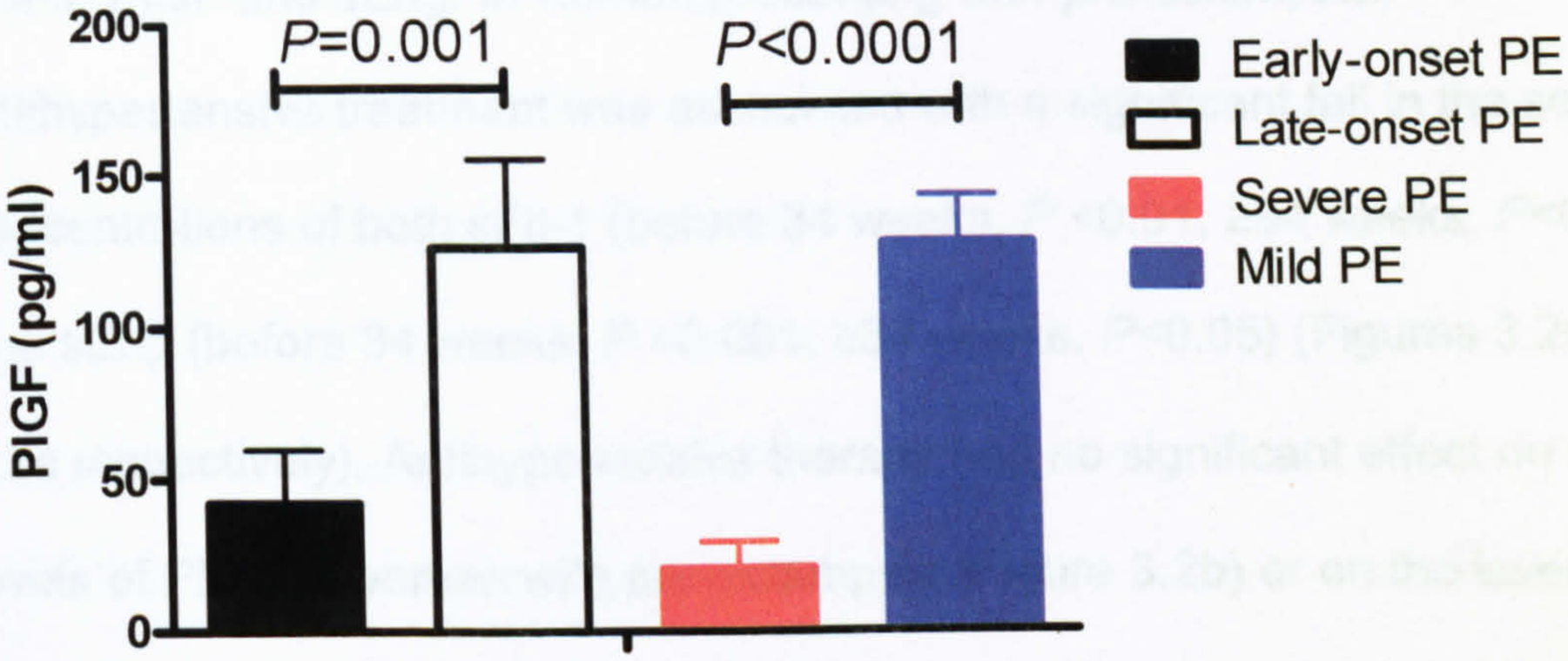
Figure 3.3 shows serum levels of these markers in early compared with late onset, and mild compared with severe pre-eclampsia. Serum levels of sFlt-1 were higher in early-onset compared with late-onset (P=0.002), and in severe compared with mild pre-eclampsia (P=0.004). Similarly, serum levels of sEng were higher in early-onset compared with late-onset (P=0.001), and in severe compared with mild pre-eclampsia (P<0.0001). Serum PlGF was lower in early-onset compared with late-onset (P=0.001), and in severe compared with mild pre-eclampsia (P<0.0001).

Figure 3.3. Early onset and late onset, and mild and severe pre-eclampsia.

(a)



(b)



(c)

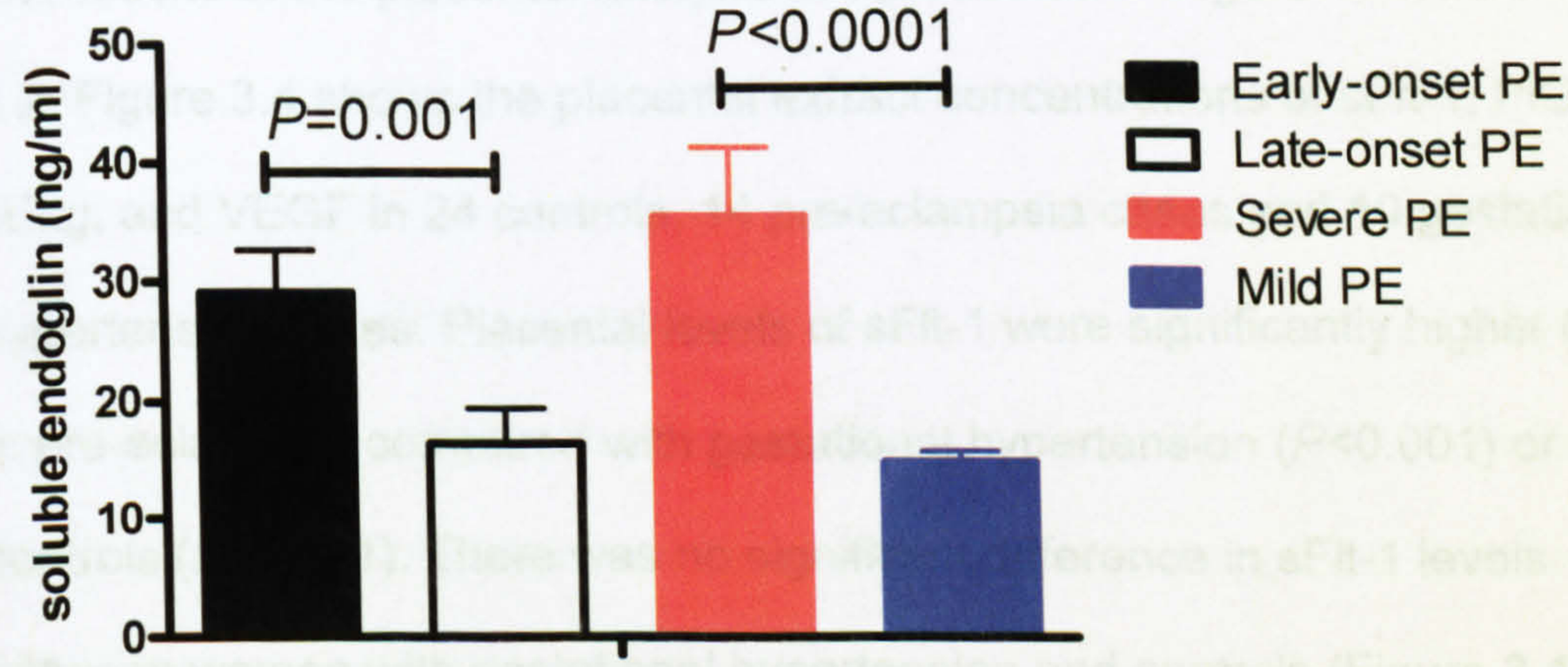


Figure 3.3. Mean maternal serum concentrations of sFlt-1 (a), PlGF (b) and soluble endoglin (c) in women with early onset and late onset pre-eclampsia (PE), and in women with mild and severe pre-eclampsia. Error bars represent standard errors. Early-onset were compared with late onset, and mild with severe pre-eclampsia after logarithmic transformation using unpaired *t* test.

Effect of treatment on serum levels

Figure 3.2 shows the effect of treatment with methyldopa on serum levels of sFlt-1, PlGF and sEng. In women presenting with pre-eclampsia, antihypertensive treatment was associated with a significant fall in the serum concentrations of both sFlt-1 (before 34 weeks, P <0.01; ≥34 weeks, P<0.001) and sEng (before 34 weeks, P <0.001; ≥34 weeks, P<0.05) (Figures 3.2a and 3.2c respectively). Antihypertensive therapy had no significant effect on serum levels of PlGF in women with pre-eclampsia (Figure 3.2b) or on the level of any of these proteins in women with gestational hypertension.

Placental levels in hypertensive disorders

The results of the placental analysis are presented in Figure 3.4 and Table 3.2. Figure 3.4 shows the placental extract concentrations of sFlt-1, PlGF, sEng, and VEGF in 24 controls, 14 pre-eclampsia cases and 10 gestational hypertension cases. Placental levels of sFlt-1 were significantly higher (5-fold) in pre-eclampsia compared with gestational hypertension (P<0.001) or controls (P<0.001). There was no significant difference in sFlt-1 levels between women with gestational hypertension and controls (Figure 3.4a). Placental levels of PlGF were significantly lower (P=0.01) in pre-eclampsia compared to controls (Figure 3.4b). Placental levels of PlGF were lower in gestational hypertension compared with controls but this difference did not achieve statistical significance (P=0.5). Placental concentrations of sEng were significantly higher (P<0.0001) in women with pre-eclampsia compared to gestational hypertension or normotensive controls (Figure 3.4c). There was no significant difference in placental sEng between controls and women with

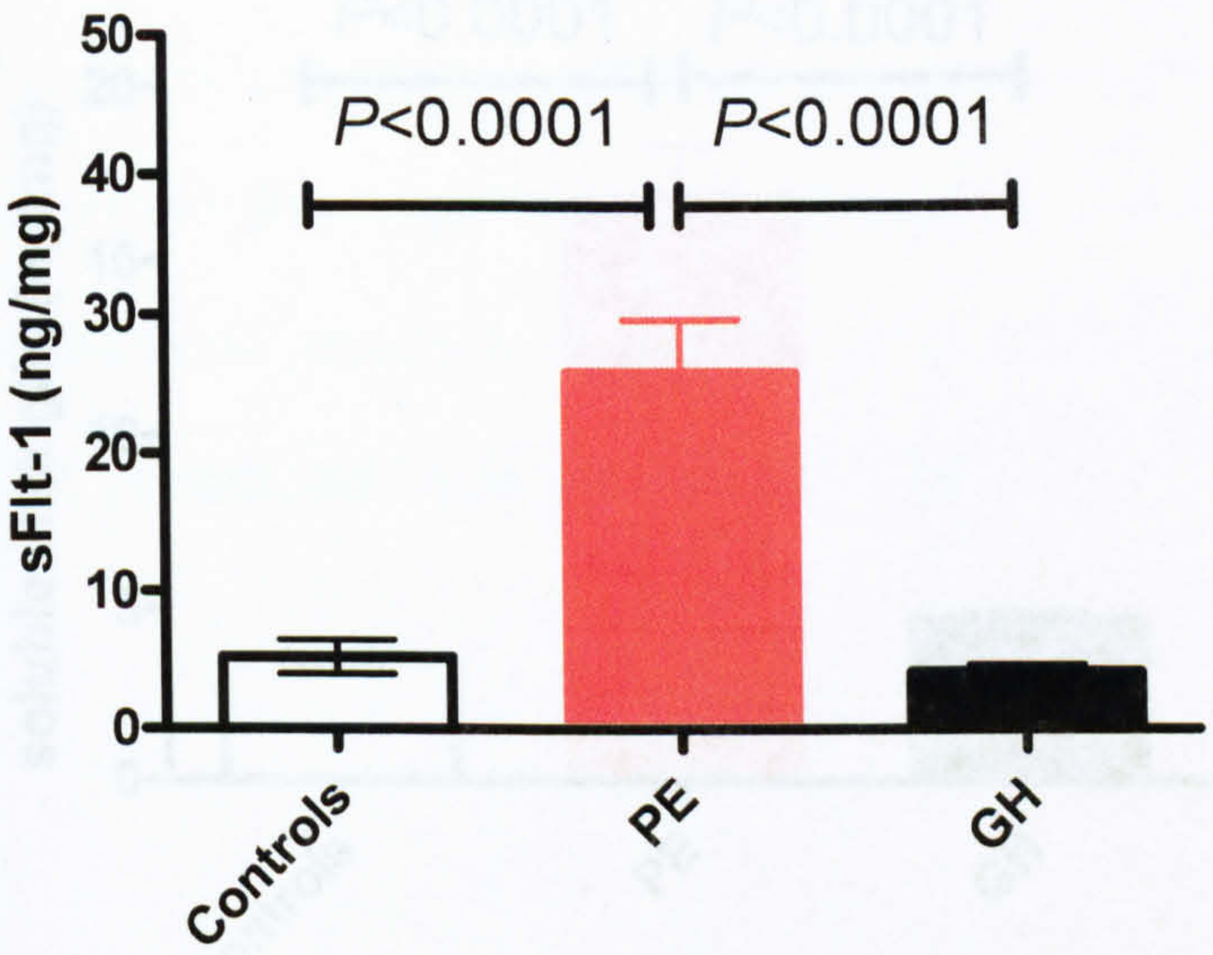
gestational hypertension (*P*=0.07). Placental VEGF was significantly higher in women with either pre-eclampsia or gestational hypertension (*P*<0.0001) compared with normotensive controls (Figure 3.4d). There was no significant difference in placental VEGF between women with pre-eclampsia and women with gestational hypertension (*P*=0.6).

Antihypertensive treatment and placental levels

Table 3.2 shows the placental concentrations of the same four markers in women with pre-eclampsia and gestational hypertension, grouped according to whether they received antihypertensive therapy (all with methyldopa) or not. In women with pre-eclampsia, treatment with methyldopa was associated with a significantly (almost 50%) lower placental concentration of sFlt-1 (*P* = 0.01). In women with gestational hypertension, treatment was also associated with a lower placental sFlt-1 concentration but this did not achieve statistical significance (*P*=0.06). Antihypertensive treatment was also associated with significantly (*P* =0.02) lower placental sEng in women with pre-eclampsia, but not in gestational hypertension. Treatment with methyldopa did not affect placental levels of PlGF or VEGF.

Figure 3.4. Concentrations of sFlt-1(a), PlGF (b), soluble endoglin (c) and VEGF (d) in placental tissue.

(a)



(b)

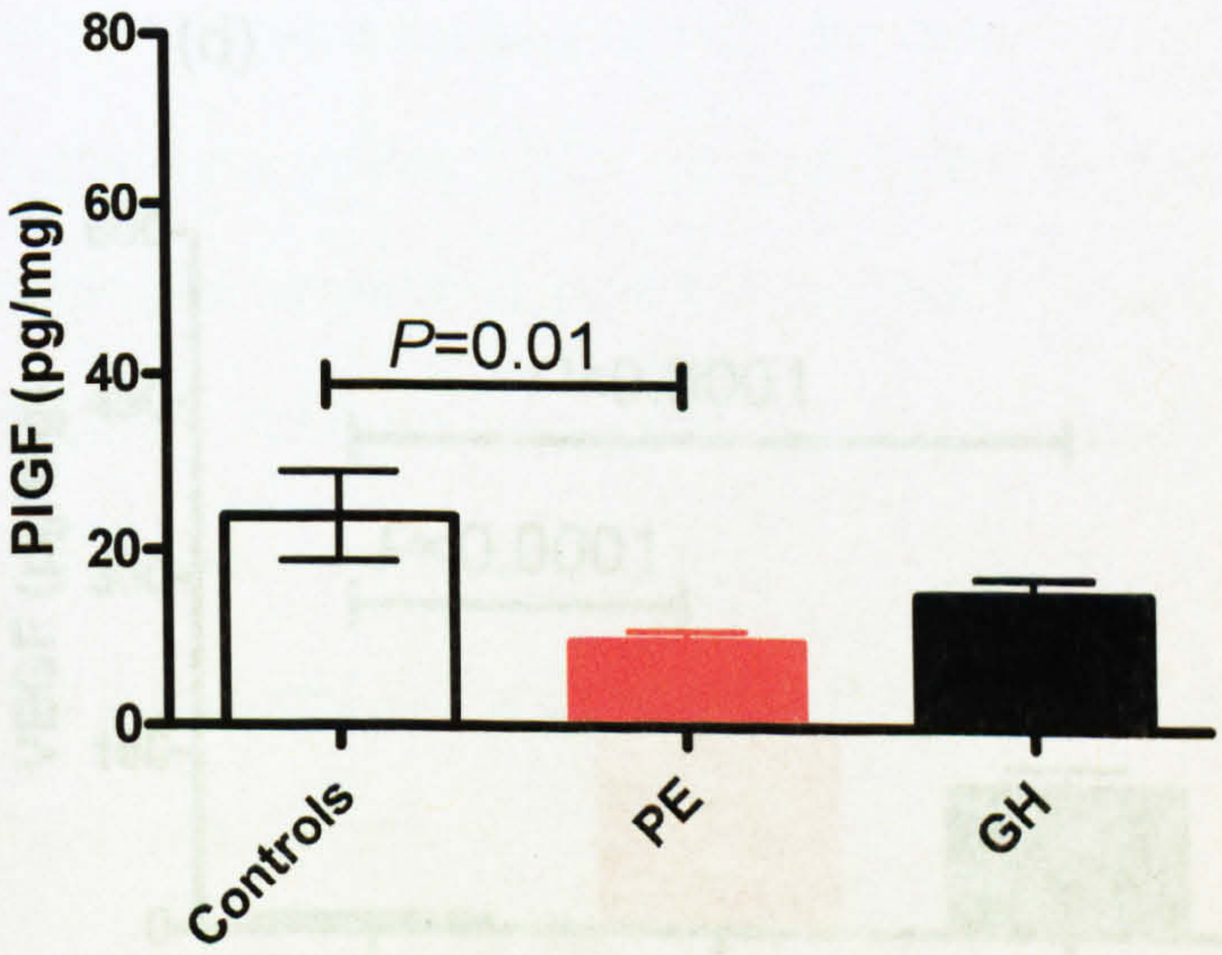
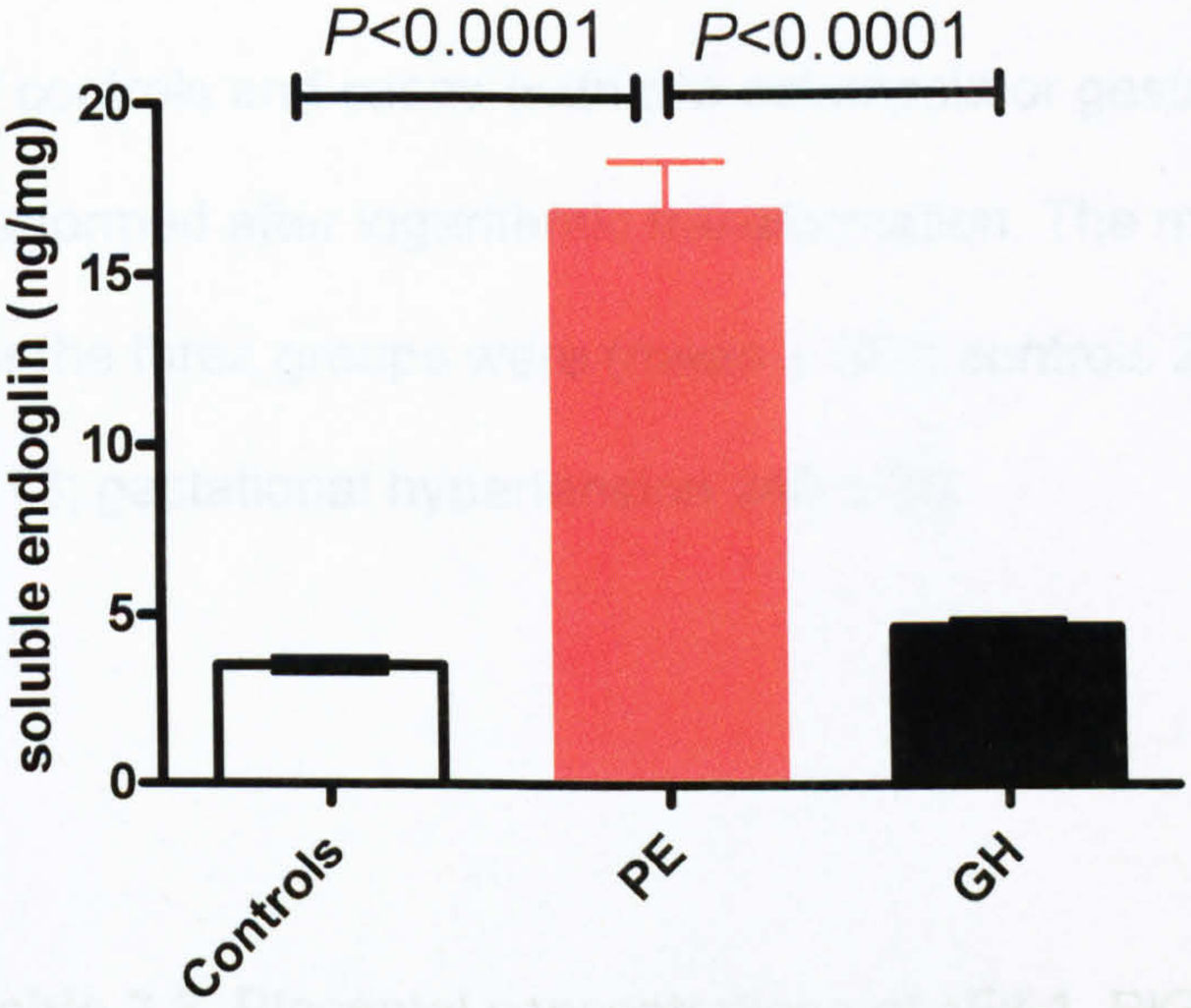


Figure 3.4. Concentrations of sFlt-1(a), PlGF (b), soluble endoglin (c) and VEGF (d) in placental tissue.

(c)



(d)

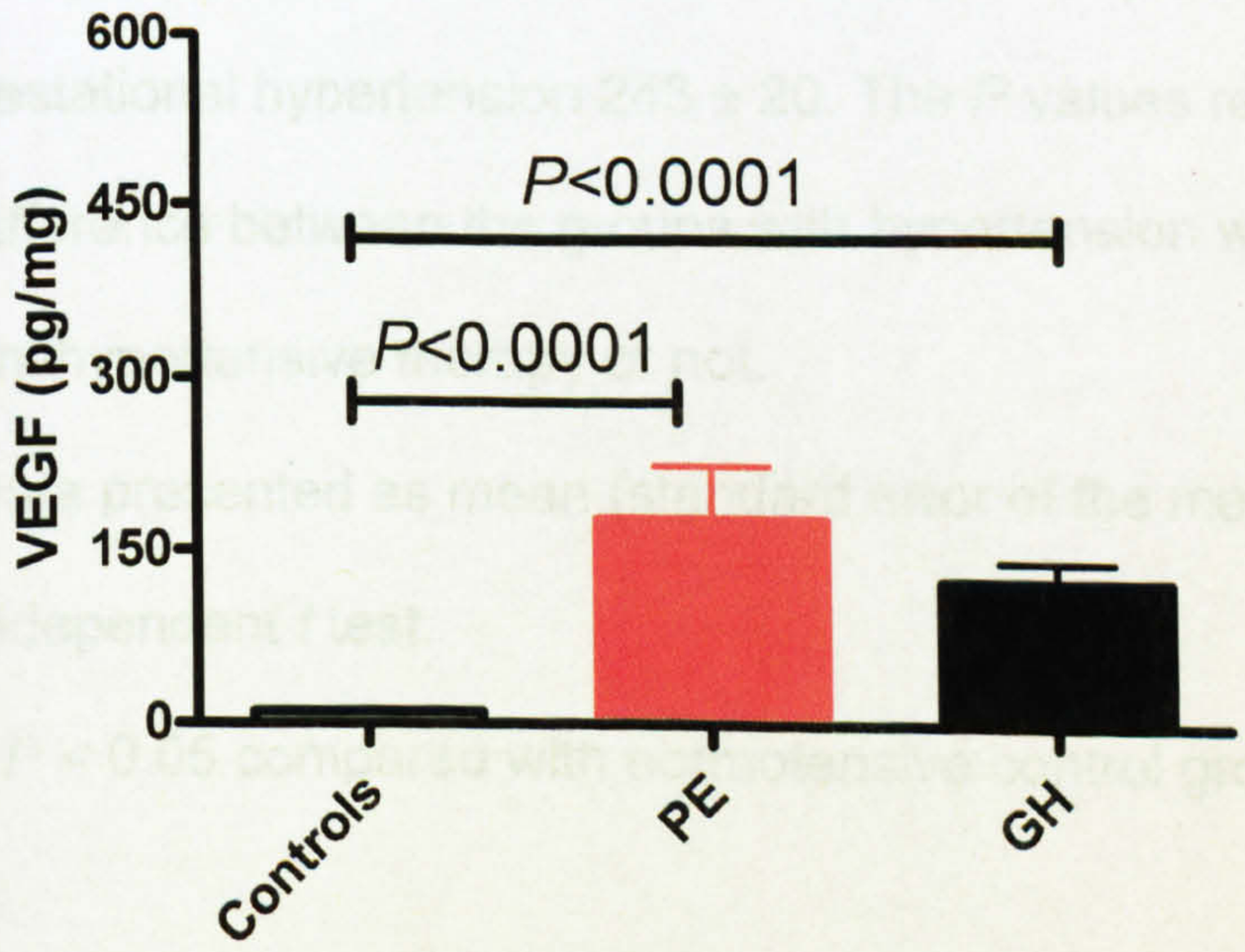


Figure 3.4. Concentrations of sFlt-1(a), PlGF (b), soluble endoglin (c) and VEGF (d) in placental tissue (expressed per mg protein) from normotensive (controls), n = 24; pre-eclampsia (PE), n = 14; and gestational hypertension (GH), n = 10 pregnancies. Error bars represent standard errors. Comparison of controls and cases (with pre-eclampsia or gestational hypertension) was performed after logarithmic transformation. The mean gestational ages (days) for the three groups were (mean ± SD): controls 242 ± 16; pre-eclampsia 238 ± 13; gestational hypertension 243 ± 20.

Table 3.2. Placental concentrations of sFlt-1, PlGF, sEng and VEGF (expressed per mg protein) in normotensive controls, pre-eclampsia and gestational hypertension, grouped according to whether they received antihypertensive therapy or not. The mean gestational ages for the three groups were (mean ± SD): controls 242 ± 16; pre-eclampsia 238 ± 13; gestational hypertension 243 ± 20. The *P* values represent the statistical difference between the groups with hypertension who received antihypertensive therapy or not.

Data presented as mean (standard error of the mean) and analysed by independent *t* test.

* $P < 0.05$ compared with normotensive control group

Table 3.2. Placental concentrations of sFlt-1, PlGF, sEng and VEGF (expressed per mg protein) in normotensive controls, pre-eclampsia and gestational hypertension, grouped according to whether they received antihypertensive therapy or not.

	Pre-eclampsia			**Gestational Hypertension**			**Controls**
	Anti-hypertensive (n = 7)	**No anti-hypertensive (n = 7)**	***P* value**	**Anti-hypertensive (n = 5)**	**No anti-hypertensive (n = 5)**	***P* value**	
sFlt-1 (ng/ml)	17.7 (3)*	33.9 (5)*	0.01	3.1 (0.4)	5.3 (0.8)	0.06	5.2 (1.2)
PlGF (pg/ml)	8.4 (1.8)*	7.7 (2)*	0.56	20.7 (3)	15.9 (4)	0.18	23.9 (5)
sEng (ng/ml)	13.1 (1.6)*	19.6 (1.7)*	0.02	3.8 (0.4)	4.3 (0.3)	0.35	3.5 (0.2)
VEGF (pg/ml)	107.5 (30)*	247 (82)*	0.09	108 (39)*	166 (32)*	0.1	10.4 (1.2)

3.2.2 Discussion

The data from this study confirm that, in both early and late onset pre-eclampsia, maternal serum levels of sFlt-1 and sEng are higher, and PlGF lower, in women presenting with pre-eclampsia.[135,153,154] In addition, we found that placental sFlt-1 and sEng were significantly increased, and PlGF decreased, in women with pre-eclampsia compared to controls. Our data suggest that in pre-eclampsia placental concentrations of sFlt-1, sEng and PlGF mirror the maternal serum changes. These findings are consistent with the view that the placenta is the main source of sFlt-1, sEng and PlGF during pregnancy.[331]

Circulating sFlt-1 can bind to PlGF and VEGF, effectively inhibiting their actions.[128,131] Soluble Flt-1 is therefore considered to be a circulating anti-angiogenic factor. In our study, consistent with previous reports,[134,135] levels of sFlt-1 were elevated and PlGF reduced in the serum of women with pre-eclampsia prior to treatment. The lower levels of free PlGF found in the serum of women with pre-eclampsia may be the result of impaired placental production or secretion, or due to increased binding by sFlt-1 in maternal serum.

We found that placental concentration of VEGF was significantly higher in pre-eclampsia compared with controls. Although some studies have reported lower placental VEGF in pre-eclampsia,[145,332] Trollmann *et al* [333] found increased VEGF mRNA expression in the placentas of women with pre-eclampsia (or women with acute fetal hypoxia) compared with gestational age matched controls. They also found increased in vitro expression of VEGF

mRNA in response to hypoxia. Immunohistological examination also demonstrated higher concentrations of VEGF protein in placental tissue, and particularly in placental vascular endothelial cells, of women with pre-eclampsia (or with acute fetal hypoxia) compared with controls. This points to a possible role for the VEGF system in the neovasculogenesis which occurs as an adaptive mechanism in response to placental hypoxia associated with pre-eclampsia. This is consistent with the finding that, in pre-eclampsia, placental VEGF protein expression increases in proportion to uterine artery resistance index.[334]

Our findings indicate that antihypertensive treatment with alpha methyldopa is associated with a significant fall in *serum* concentrations of both sFlt-1 and sEng in women presenting with either early onset or late onset pre-eclampsia. Methyldopa therapy had no significant effect on the *serum* levels of these markers in women presenting with gestational hypertension. Consistent with the trend in maternal serum, antihypertensive treatment with methyldopa was also associated with significantly lower *placental* concentrations of both sFlt-1 and sEng in pre-eclampsia, but not in gestational hypertension. These findings suggest that in pre-eclampsia alpha methyldopa may have a direct effect on placental synthesis and/or secretory functions and that this effect may not be simply the result of a reduction in maternal blood pressure and /or a change in utero-placental blood flow. However, sFlt-1 and sEng are also produced by vascular endothelial cells and we cannot exclude an endothelial cell effect of the medication in women with pre-eclampsia. The specific effect in pre-eclampsia (with no effect in gestational hypertension) indicates that methyldopa has a different effect on placental and/or endothelial

production and/or secretion of angiogenic factors depending on the pathophysiology of the hypertensive disorder. These findings support the concept of a difference in pathophysiology between gestational hypertension and the pathological endothelial toxic effect of pre-eclampsia.

It has been shown that serum and placental sFlt-1 levels are elevated in women with established pre-eclampsia,[124,126-128,135-140,335] and the rise in serum sFlt-1 can be detected around five weeks before the onset of clinical disease.[135] We have shown that treatment of pre-eclampsia with methyldopa is associated with a reduction in serum and placental concentrations of sFlt-1. Yet it has previously been established that methyldopa therapy does not prevent pre-eclampsia.[336] These findings combined do not support the hypothesis that sFlt-1 plays a direct role in the pathophysiology of pre-eclampsia.

Alpha methyldopa acts on α_2-adrenergic receptors, primarily in the central nervous system although an effect on peripheral α_2-adrenoreceptors may also play a part.[316,317] Its main active metabolite is alpha-methyl noradrenaline, which resembles noradrenaline in its effects. Stimulation of pre-synaptic α_2-adrenoreceptors in the central nervous system leads to a reduction of central sympathetic outflow. This causes a reduction in blood pressure.[337] α_2-adrenoreceptors have also been identified in a variety of other human tissues outside the central nervous system, including myometrium and placenta.[318,338] An almost universal effect of α_2-adrenoreceptor stimulation is the inhibition of adenylyl cyclase which leads to decreased production of

cAMP.[339,340] cAMP has been shown to be a strong inducer of Flt-1 expression in mice.[341,342]

In 2007, it was demonstrated that down-regulation of $\alpha_{2\beta}$-adrenoceptors in mice placenta resulted in increased levels of Flt-1 and sFlt-1,[343] suggesting that stimulation of $\alpha_{2\beta}$-adrenoceptors can suppress production of sFlt-1. Deletion of the gene encoding $\alpha_{2\beta}$-adrenoceptors resulted in upregulation of Flt-1 in spongiotrophoblast cells. These data support a direct link between adrenergic receptor signalling and angiogenic regulation by the VEGF system. This may be the mechanism by which alpha methyldopa leads to the reduction in sFlt-1 which our data support. Although this study [343] was done in mice, several functionally relevant polymorphisms that may potentially affect sFlt-1 expression and blood vessel formation have been identified in human adrenoceptor genes. This adds weight to the argument that methyldopa has an effect on maternal production of vasoactive substances: the fact that we see a different response in women with pre-eclampsia may reflect the finding that women with this disease are producing abnormal amounts of these substances in the first place.

Clinically, the need for antihypertensive treatment is a marker of disease severity; thus, prior to treatment, higher levels of sFlt-1 and sEng would be expected in the treatment group compared with the non-treatment group. Nevertheless, we found that antihypertensive treatment was associated with significantly lower levels of these two markers in the placentas of women treated with methyldopa compared to the placenta of untreated women.

It is not yet clear whether sFlt-1 and sEng are directly involved in the pathophysiology of pre-eclampsia or are simply markers of the disease process. Our data showing that antihypertensive treatment with alpha methyldopa is associated with a significant fall in their concentrations in both maternal serum and placenta is consistent with a positive effect on the control of disease progress. This finding supports the concept that pre-eclampsia combines an excessive maternal response to the presence of a pregnancy and placenta and progressive utero-placental insufficiency during the second half of pregnancy at the time of maximal fetal growth.

3.3 EFFECT OF ANTIHYPERTENSIVE THERAPY WITH ALPHA METHYLDOPA ON LEVELS OF INHIBIN A AND ACTIVIN A IN PREGNANCIES WITH HYPERTENSIVE DISORDERS

3.3.1 Results

Serum levels

In pre-eclampsia, serum inhibin A (before 34 weeks, $P<0.001$; ≥ 34 weeks, $P=0.001$) and activin A levels (before 34 weeks, $P<0.001$; ≥ 34 weeks, $P<0.001$) were significantly increased in both gestational age subgroups compared with controls (Figure 3.5). In gestational hypertension, serum inhibin A was significantly ($P=0.002$) higher than controls before 34 weeks but not ≥ 34 weeks, whereas activin A levels were significantly ($P<0.0001$) higher than controls in both subgroups. Serum levels of both inhibin A and activin A were significantly ($P<0.001$) higher in pre-eclampsia compared with gestational hypertension before 34 weeks but not ≥ 34 weeks. In both pre-eclampsia gestational age subgroups, treatment with α-methyldopa was associated with a significant decrease in the serum levels of both inhibin A (before 34 weeks, $P=0.04$; ≥ 34 weeks, $P<0.001$) and activin A (before 34 weeks, $P=0.01$; ≥ 34 weeks, $P=0.007$). In contrast, in gestational hypertension treatment did not have a significant effect on their serum levels in either gestational age subgroup (inhibin A: before 34 weeks, $P=0.2$; ≥ 34 weeks, $P=0.5$; and activin A: before 34 weeks, $P=0.06$; ≥ 34 weeks, $P=0.3$).

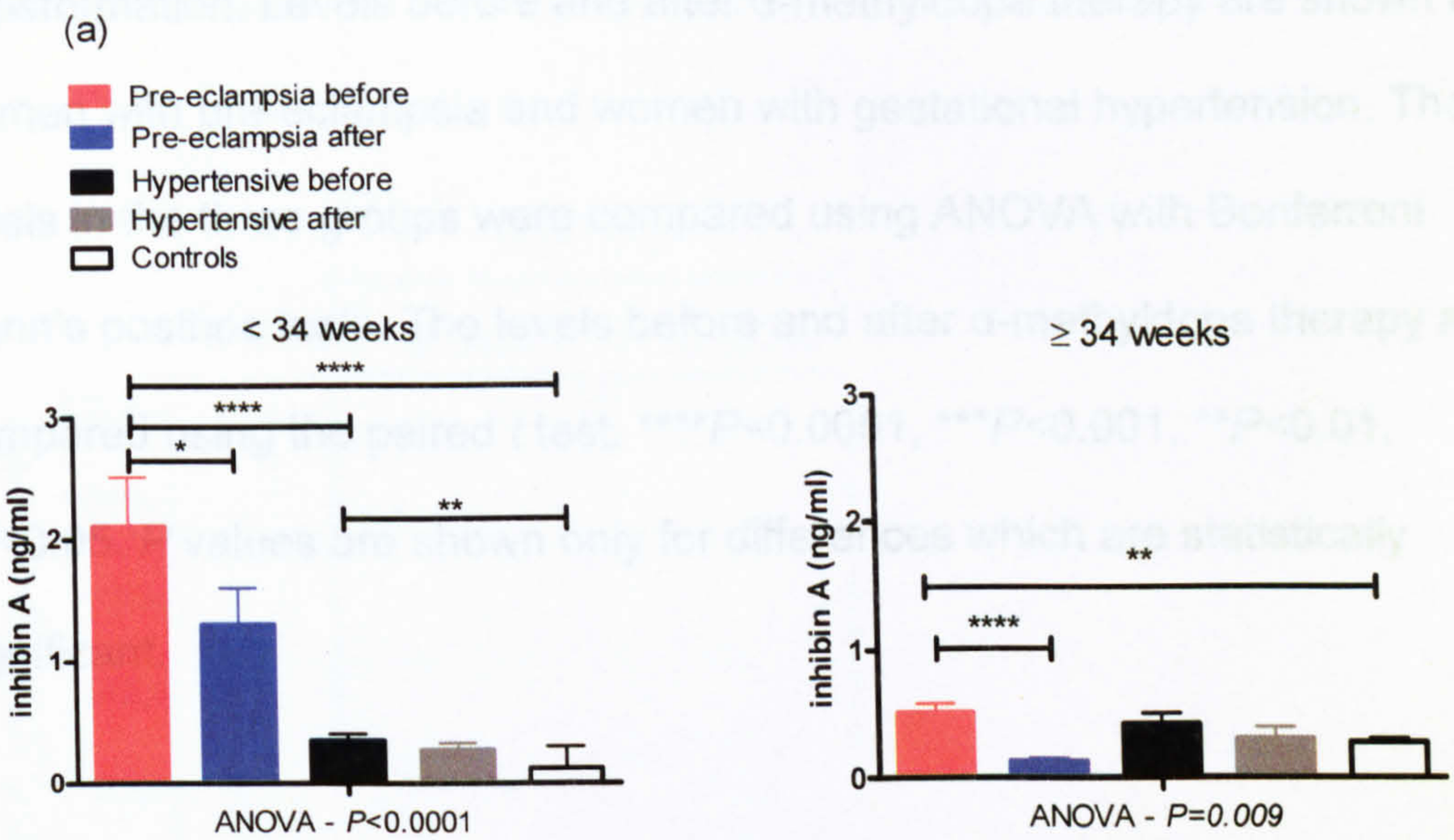


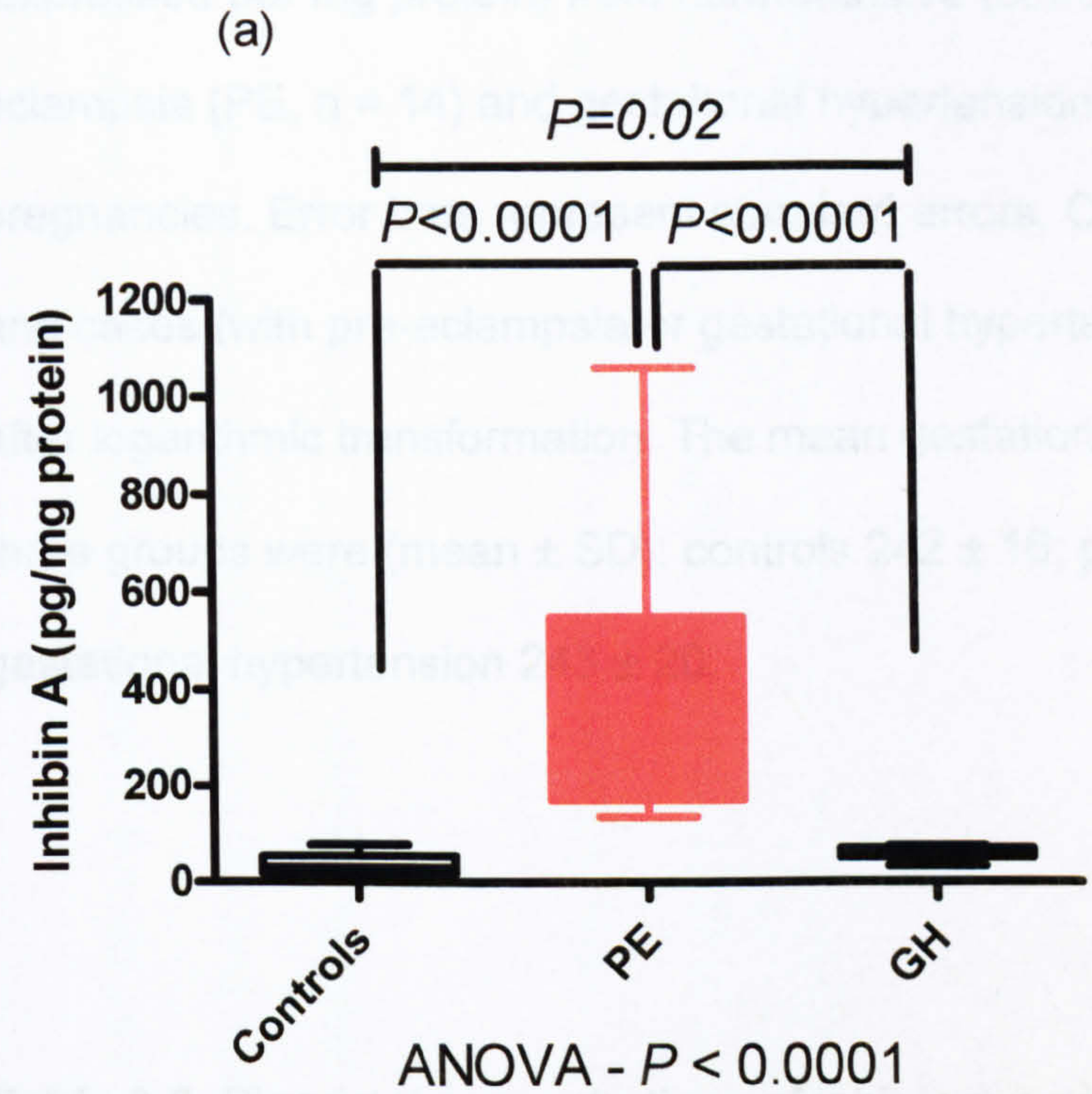
Figure 3.5. Mean serum inhibin A (a) and activin A (b) concentrations in normotensives (controls), women with pre-eclampsia and women with gestational hypertension according to gestational age interval [< 34 weeks (41 controls, 28 pre-eclampsia, 13 gestational hypertension) and ≥ 34 weeks (39 controls, 23 pre-eclampsia, 16 gestational hypertension)]. Error bars represent standard errors. Comparison of controls and cases (with pre-eclampsia or gestational hypertension) was performed after logarithmic

transformation. Levels before and after α-methyldopa therapy are shown for women with pre-eclampsia and women with gestational hypertension. The levels in the three groups were compared using ANOVA with Bonferroni Dunn's posthoc tests. The levels before and after α-methyldopa therapy are compared using the paired *t* test. ****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$. *P* values are shown only for differences which are statistically significant.

Placental levels

In both pre-eclampsia and gestational hypertension, placental concentrations of both inhibin A and activin A were significantly ($P<0.001$) higher than in controls (Figure 3.6). Placental levels of both markers were significantly ($P<0.05$) higher in pre-eclampsia compared to gestational hypertension. Significantly lower placental levels of inhibin A ($P=0.02$) and activin A ($P=0.04$) were found in women presenting with pre-eclampsia treated with α-methyldopa, compared with untreated women with pre-eclampsia (Table 3.3). After treatment, the levels of both hormones remained significantly ($P<0.05$) higher in pre-eclampsia than in controls. In gestational hypertension, treatment was not associated with a significant difference in the level of either hormone.

Figure 3.6. Placental concentrations of inhibin A (a) and activin A (b).



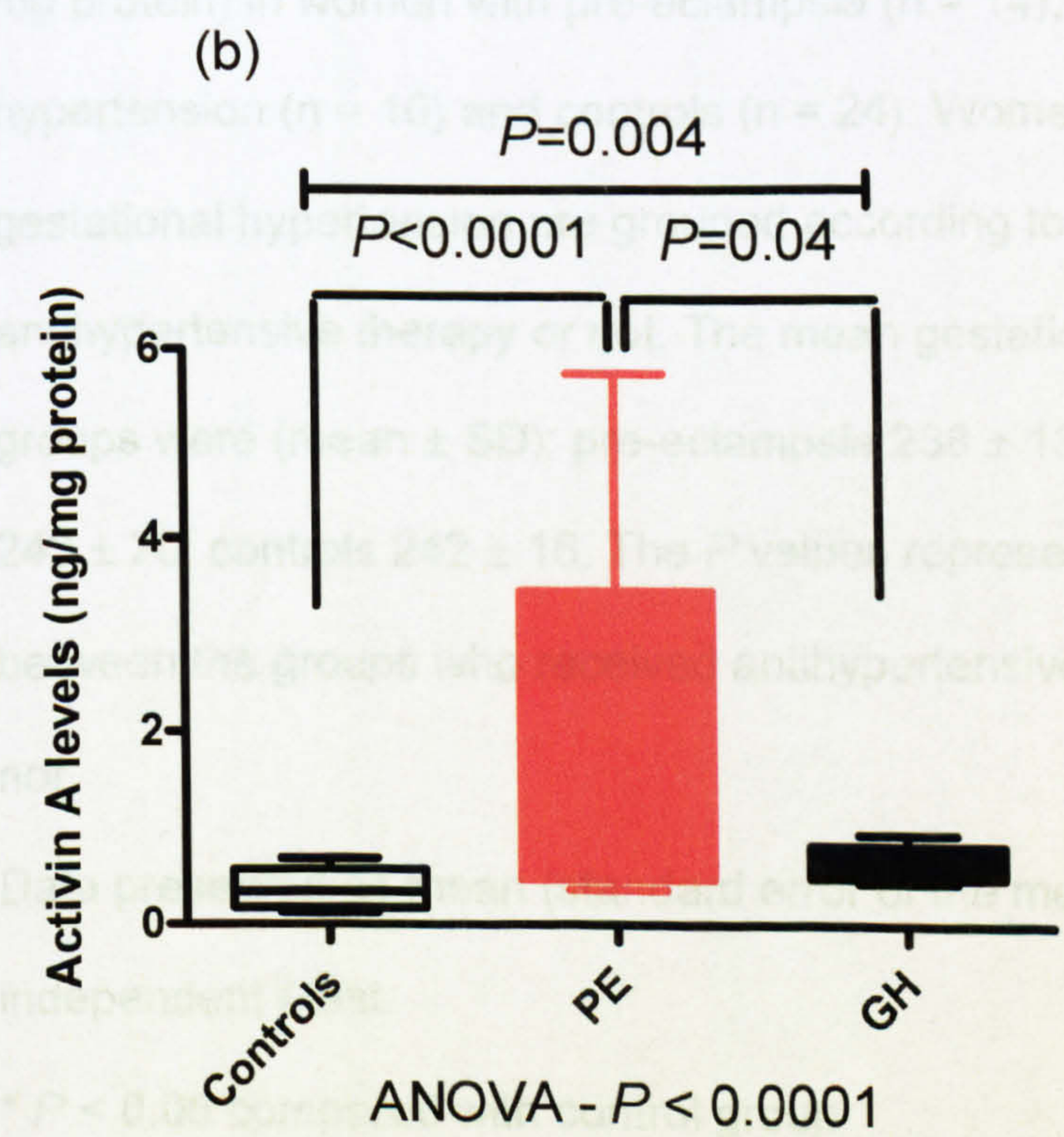


Figure 3.6. Concentrations of inhibin A (a) and activin A (b) in placental tissue (expressed per mg protein) from normotensive (controls, n = 24), pre-eclampsia (PE, n = 14) and gestational hypertension (GH, n = 10) pregnancies. Error bars represent standard errors. Comparison of controls and cases (with pre-eclampsia or gestational hypertension) was performed after logarithmic transformation. The mean gestational ages (days) for the three groups were (mean ± SD): controls 242 ± 16; pre-eclampsia 238 ± 13; gestational hypertension 243 ± 20.

Table 3.3. Placental concentrations of inhibin A and activin A (expressed per mg protein) in women with pre-eclampsia (n = 14), women with gestational hypertension (n = 10) and controls (n = 24). Women with pre-eclampsia or gestational hypertension are grouped according to whether they received antihypertensive therapy or not. The mean gestational ages for the three groups were (mean ± SD): pre-eclampsia 238 ± 13; gestational hypertension 243 ± 20; controls 242 ± 16. The *P* values represent the statistical difference between the groups who received antihypertensive therapy and those who did not.

Data presented as mean (standard error of the mean) and analysed by independent *t* test.

* $P < 0.05$ compared with control group

Table 3.3. Placental concentrations of inhibin A and activin A (expressed per mg protein) in women with pre-eclampsia (n = 14), women with gestational hypertension (n = 10) and controls (n = 24). Women with pre-eclampsia or gestational hypertension are grouped according to whether they received antihypertensive therapy or not.

	Pre-eclampsia			**Gestational Hypertension**			**Controls**
	Anti-hypertensive (n = 7)	**No anti-hypertensive (n = 7)**	***P* value**	**Anti-hypertensive (n = 5)**	**No anti-hypertensive (n = 5)**	***P* value**	
Inhibin A (pg/mg)	222 (28) *	600 (141) *	0.02	52.4 (6.4)	65.3 (4.6) *	0.2	41.8 (7.9)
Activin A (ng/mg)	1 (0.4) *	3.1 (0.8) *	0.04	0.7 (0.07) *	0.6 (0.1)	0.4	0.38 (0.04)

3.3.2 Discussion

Our data confirm that inhibin A and activin A synthesis and secretion are increased in women presenting with hypertensive disorders in pregnancy and indicate for the first time that in pre-eclampsia placental production and circulating serum levels of these proteins decrease within 48 hours of initiating antihypertensive treatment with alpha methyldopa.

Serum levels of inhibin A and activin A increase several fold in women with pre-eclampsia and the pattern of changes in these levels is directly linked with the severity of the disease.[208] Placental secretion of inhibin A has also been shown to be increased in pre-eclampsia.[329] In the current study, we found that placental concentrations of inhibin A and activin A were significantly higher in pre-eclampsia compared with gestational hypertension. In both gestational hypertension and pre-eclampsia, placental levels of these proteins were higher than in normotensive controls. In women presenting with pre-eclampsia or gestational hypertension, serum levels of inhibin A and activin A reflected those in placental samples, suggesting that placental production/secretion of these markers is increased in both conditions, but to a greater extent in pre-eclampsia. Compared to late onset pre-eclampsia, early onset pre-eclampsia was associated with higher levels of both markers, supporting the view that these are more likely to represent different degrees of severity of the disease rather than distinct pathological entities.

Overall, our data on the effect of antihypertensive therapy show that alpha methyldopa reduces the serum levels of inhibin A and activin A in both gestational age subgroups of pre-eclampsia but has no significant effect on

their levels in gestational hypertension at any gestation. In pre-eclampsia, placental levels of both hormones were also lower in women after antihypertensive therapy, although they remained higher than in normotensive controls (Table 3.3). These data suggest that the increased placental production/release of these markers in pre-eclampsia is partially, but not completely, reversed by alpha methyldopa therapy within the study period of 48 hours. However, this effect was not observed in women presenting with gestational hypertension. The significant fall in serum inhibin A and activin A levels in pre-eclampsia but not in gestational hypertension, despite a similar effect on blood pressure, supports the view that different placental pathologies may be involved. For obvious reasons, it was not possible to measure placental concentrations of these markers before and after antihypertensive medication was started, so instead we compared placental levels in women who had this therapy and those who did not (Table 3.3).

As discussed earlier (section 3.2.2), alpha methyldopa stimulates pre-synaptic α_2-adrenoreceptors in the central nervous system, reducing central sympathetic outflow, which leads to a fall in blood pressure.[316,317,337]

α_2-adrenoreceptors have been identified in a variety of other human tissues outside the central nervous system, including myometrium and placenta.[318,338] Stimulation of α_2-adrenoreceptors inhibits adenyl cyclase, leading to decreased production of cAMP.[339,340] cAMP has been shown to increase the production of inhibin A and activin A in cultured human ovarian granulosa-luteal cells.[344] It has also been shown in the same tissue that cAMP induces mRNA for both the α and β_A subunits of inhibin A and activin A.[345] In

fact, α subunit mRNA is induced via this cAMP-dependent pathway in a remarkably similar way in a variety of tissues in different species, including human.[346,347] It is therefore possible that alpha methyldopa stimulates placental α_2-adrenoreceptors, reducing the production of cAMP which in turn leads to reduced production of inhibin A and activin A.

It is also possible that antihypertensive medication directly affects trophoblasts to reduce production and/or secretion of inhibin A and activin A. Inflammatory cells produce activin A, and peripheral mononuclear cell secretion of activin A is increased in culture in the presence of inflammatory cytokines.[348-351] It has been shown that the inflammatory cytokines which are increased in pre-eclampsia stimulate placental trophoblast secretion of inhibin A and activin A.[352] Recently it was shown that antihypertensive treatment can alter cytokine release in vitro;[353-354] this may explain the effect of antihypertensive drugs on placental content of inhibin A and activin A via cytokines in an autocrine/paracrine manner. However, since alpha methyldopa treatment does not cause any significant changes in women presenting with gestational hypertension, the mechanism by which it leads to a fall in circulating levels of inhibin A and activin A in pre-eclamptic women remains to be elucidated.

Activin A has been implicated in the regulation of endothelial cell function and, in combination with TGF-β, may inhibit capillary endothelial cell growth.[355] Reduction in activin A levels by alpha methyldopa may therefore result in improved capillary endothelial cell function and growth. There is also evidence that activin A (with TGF-β) stimulates growth of vascular smooth

muscle cells [356,357] so it is possible that a reduction in activin A levels may contribute to a reduction in peripheral vascular resistance.

It is uncertain if inhibin A and activin A play a role in the pathophysiology of pre-eclampsia or are simply markers of the disease process. Nevertheless, the fact that antihypertensive treatment with alpha methyldopa is associated with a fall in their serum and placental concentrations is likely to reflect a beneficial effect on the disease evolution over and above its known antihypertensive action. It is not yet known whether these drugs act directly on trophoblast cells to reduce production and/or release of these markers, or whether this effect may be mediated through other placental molecules.

3.4 EFFECT OF ANTIHYPERTENSIVE THERAPY WITH ALPHA METHYLDOPA ON CENTRAL HAEMODYNAMICS IN PREGNANCIES WITH HYPERTENSIVE DISORDERS

3.4.1 Results

Prior to antihypertensive treatment

The haemodynamic parameters for each study group prior to treatment are presented and compared in Table 3.4. Mean arterial pressure, brachial and central systolic, diastolic and pulse pressures were all significantly ($P<0.05$) higher in both pre-eclampsia and gestational hypertension compared to the control group. There were no significant differences in brachial systolic, diastolic, pulse pressure or mean arterial pressure between the two hypertensive groups. Within each group (controls, pre-eclampsia, gestational hypertension) prior to treatment, there were no significant differences in augmentation pressure (AP) or augmentation index (AIx-75) between primigravid and multigravid women.

Table 3.4. Heart rate, brachial and central haemodynamic measurements prior to antihypertensive therapy in women with pre-eclampsia (n=51), women with gestational hypertension (n=29) and matched controls (n=80). The measurements in pre-eclampsia and gestational hypertension are compared using the Student *t* test (*P* value in the last column).

Data are expressed as means ± SD.

* represents $P< 0.05$ compared to the control group.

Haemodynamic variable	Controls	Pre-eclampsia	Gestational Hypertension	*P* value
N	80	51	29	
Heart rate (bpm)	82.0 (9.4)	81.0 (8.6)	82.7 (9.0)	0.4
Brachial systolic pressure (mmHg)	108.1 (11.6)	156.1 (10.6) *	151.9 (17.8) *	0.2
Brachial diastolic pressure (mmHg)	67.3 (8.0)	101.6 (7) *	99.3 (7.7) *	0.2
Brachial pulse pressure (mmHg)	40.8 (8.7)	54.4 (11.6) *	52.7 (12.6) *	0.5
Mean arterial pressure (mmHg)	80.6 (8.8)	116.3 (9.4) *	118.6 (18.9) *	0.5
Central systolic pressure (mm Hg)	94.4 (10.0)	143.6 (11.5) *	138 (15.9) *	0.07
Central diastolic pressure (mm Hg)	68.5 (8.2)	103.4 (6.8) *	101 (8) *	0.2
Central pulse pressure (mm Hg)	25.9 (5.6)	40.2 (11.3) *	37 (10.9) *	0.2

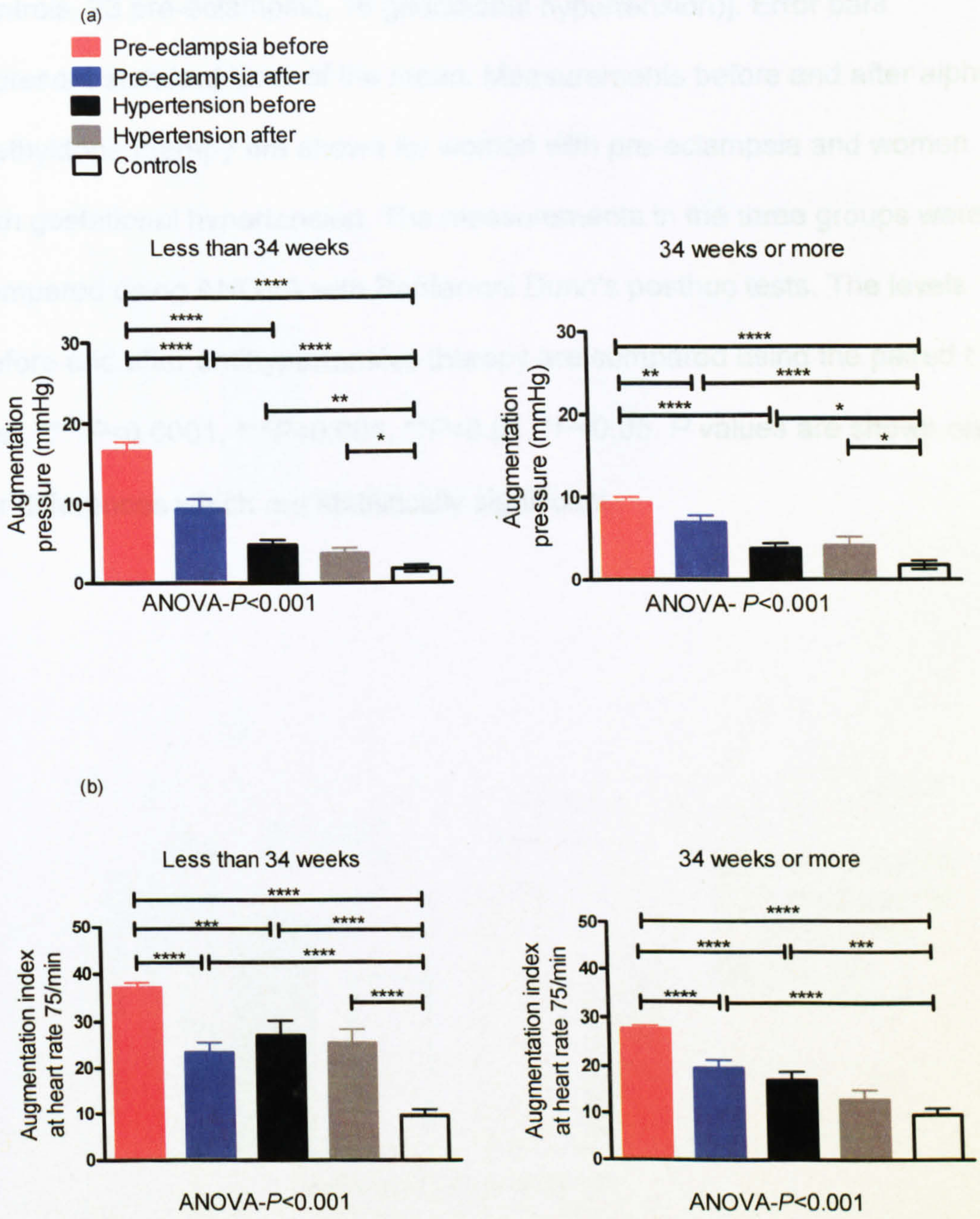


Figure 3.7. Augmentation pressure (a) and augmentation index at heart rate 75/min (AIx-75) (b) measurements in normotensives (controls), women with pre-eclampsia and women with gestational hypertension according to gestational age interval [early onset <34 weeks (41 controls, 28 pre-eclampsia, 13 gestational hypertension) and late onset ≥ 34 weeks (39

controls, 23 pre-eclampsia, 16 gestational hypertension)]. Error bars represent standard error of the mean. Measurements before and after alpha methyldopa therapy are shown for women with pre-eclampsia and women with gestational hypertension. The measurements in the three groups were compared using ANOVA with Bonferroni Dunn's posthoc tests. The levels before and after antihypertensive therapy are compared using the paired *t* test. ****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$. *P* values are shown only for differences which are statistically significant.

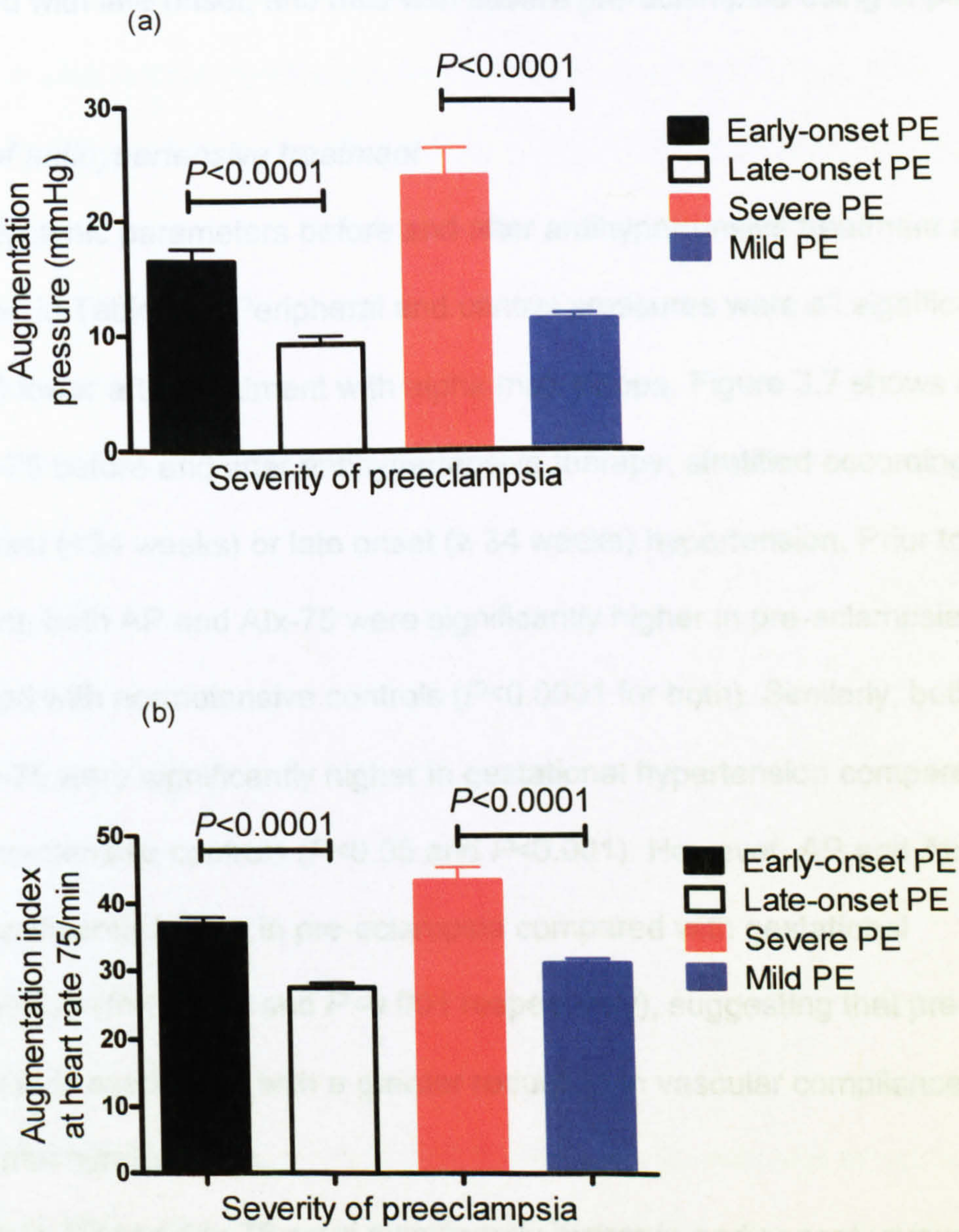


Figure 3.8. Augmentation pressure (a) and augmentation index at heart rate 75/min (AIx-75) (b) measurements in women with early onset (n=28) and late onset pre-eclampsia (n=23), and in women with mild (n=43) and severe pre-

eclampsia (n=8). Error bars represent standard deviation. Early onset were compared with late onset, and mild with severe pre-eclampsia using unpaired *t* tests.

Effects of antihypertensive treatment

Haemodynamic parameters before and after antihypertensive treatment are compared in Table 3.5. Peripheral and central pressures were all significantly ($P<0.05$) lower after treatment with alpha methyldopa. Figure 3.7 shows AP and AIx-75 before and after antihypertensive therapy, stratified according to early onset (<34 weeks) or late onset (≥ 34 weeks) hypertension. Prior to treatment, both AP and AIx-75 were significantly higher in pre-eclampsia compared with normotensive controls ($P<0.0001$ for both). Similarly, both AP and AIx-75 were significantly higher in gestational hypertension compared with normotensive controls ($P<0.05$ and $P<0.001$). However, AP and AIx-75 were significantly higher in pre-eclampsia compared with gestational hypertension ($P<0.0001$ and $P<0.001$ respectively), suggesting that pre-eclampsia is associated with a greater reduction in vascular compliance than gestational hypertension.

Both AP and AIx-75 were significantly higher in early-onset versus late-onset pre-eclampsia ($P< 0.0001$) and in severe versus mild pre-eclampsia ($P< 0.0001$) (Figure 3.8).

Treatment with methyldopa was associated with a significant fall in heart rate in both pre-eclampsia and gestational hypertension. In pre-eclampsia, in both gestational age intervals, both AP and AIx-75 were significantly ($P<0.0001$) lower after treatment (Figure 3.7). In gestational hypertension, however, there was no significant change after treatment in either AP (< 34

weeks, *P*=0.2; ≥ 34 weeks, *P*=0.7) or AIx-75 (< 34 weeks, *P*=0.7; ≥ 34 weeks, *P*=0.07). When we compared PWA indices between women with pre-eclampsia after antihypertensive therapy and the normotensive controls, a significant difference persisted for both AP and AIx-75 (*P* <0.0001 for both).

Table 3.5. Brachial and central haemodynamic measurements in women with pre-eclampsia (n=51) and women with gestational hypertension (n=29). Measurements before and after alpha methyldopa therapy are compared using the paired *t* test.

PE = pre-eclampsia GH = gestational hypertension

Data are expressed as means ± SD

Haemodynamic variable	PE before	PE after	*P* value	GH before	GH after	*P* value
N	51	51		29	29	
Heart rate (bpm)	81.0 (8.6)	77.1 (7)	<0.001	82.7 (9)	79.2 (8)	<0.001
Brachial systolic pressure (mmHg)	156.1 (10.6)	129.4 (15.2)	<0.001	151.9 (17.8)	138.2 (19.6)	0.023
Brachial diastolic pressure (mmHg)	101.6 (7.0)	84.8 (7.1)	<0.001	99.3 (7.7)	87.0 (8.1)	<0.001
Brachial pulse pressure (mmHg)	54.4 (11.6)	44.6 (10.6)	0.001	52.7 (12.6)	51.2 (15.2)	0.72
Central systolic pressure (mm Hg)	143.6 (11.5)	116.9 (13.7)	<0.001	138.0 (15.9)	124.1 (18.6)	0.01
Central diastolic pressure (mm Hg)	103.4 (6.8)	86.3 (7.4)	<0.001	101.0 (8.0)	88.8 (8.3)	<0.001
Central pulse pressure (mm Hg)	40.2 (11.3)	30.6 (8.7)	<0.001	37.0 (10.9)	35.3 (13.4)	0.6

3.4.2 Discussion

Using pulse wave analysis, we have confirmed that vascular compliance is decreased in women with hypertensive disorders of pregnancy, particularly in pre-eclampsia. In pre-eclampsia, but not in gestational hypertension, vascular compliance is improved by treatment with alpha methyldopa.

Each heartbeat generates a pulse wave which travels away from the heart. This waveform is reflected from bifurcations within the arterial tree and from the junctions of the pre-resistance and resistance vessels (see 1.3.2).[250,358,359] The reflected wave travels back towards the heart and meets the advancing wave, augmenting its height (Figure 1.4). Generally, the reflected wave reaches the aorta during diastole, boosting the height of the diastolic portion of the wave. When arterial wall stiffness is increased (as in hypertensive disorders of pregnancy) the arterial pulse wave travels faster, so the reflected wave reaches the advancing wave in systole, resulting in significant augmentation of the systolic peak. This can be measured as raised augmentation pressure and augmentation index (Figure 1.8).

Previous studies have demonstrated that, in normal pregnancy, aortic compliance increases in response to increased levels of oestrogen, mediated by increased circulating nitric oxide levels: resistance remains low until delivery.[281] The poor vascular compliance seen in women with hypertension probably reflects failure of the mother to adapt as well to the vascular challenge posed by the presence of a pregnancy. In a small observational study of 16 women with hypertensive disorders in pregnancy,[280] no difference in arterial stiffness was found between those receiving antihypertensive

treatment or not. However, haemodynamic parameters were compared between women who received treatment and those who did not; there are no data comparing women before and after treatment.

It is estimated that 15-25% of women who present with gestational hypertension progress to pre-eclampsia.[360] We found that arterial stiffness is increased in gestational hypertension compared with normotensive pregnant women, but increased significantly more in women with pre-eclampsia. The differences in vascular compliance may be partly explained by changes in maternal blood volume; in pre-eclampsia, plasma volume is contracted whereas in gestational hypertension it may be unchanged or increased. The fact that AP and AIx-75 are higher in early onset compared with late onset, and severe compared with mild pre-eclampsia is consistent with the view that early and severe pre-eclampsia are part of the spectrum of the disease, and not different pathophysiological entities. Consistent with this, in another study, we found that first trimester PWA predicts early onset pre-eclampsia with greater accuracy than late onset.[361]

In pre-eclampsia, the improvement in arterial stiffness brought about by alpha methyldopa is probably due in part to normalisation of blood pressure (blood pressure increases arterial stiffness).[362,363] Even after antihypertensive therapy, however, arterial stiffness remained higher when compared to normotensive pregnant women. This finding suggests that other factors are involved, such as poor vessel wall compliance and endothelial dysfunction, which contribute to the increased arterial stiffness but which are not corrected by antihypertensive treatment with alpha methyldopa.

Maternal heart rate fell significantly after treatment with methyldopa in both pre-eclampsia and gestational hypertension. This is consistent with previous reports.[364] After treatment, AIx-75 (which is adjusted for heart rate) was significantly reduced in pre-eclampsia but not gestational hypertension. The changes in AP (which is not adjusted for heart rate) after methyldopa treatment mirrored those in AIx-75, i.e. a fall in pre-eclampsia but no significant change in gestational hypertension. These findings suggest that the methyldopa-induced fall in maternal heart rate do not explain the changes we observed in arterial stiffness.

Alpha methyldopa stimulates pre-synaptic α2-adrenergic receptors, primarily in the central nervous system,[316,317] leading to a reduction of central sympathetic outflow and a reduction in blood pressure and heart rate.[318,337,338,364] Vasodilator drugs probably have little direct effect on large central elastic arteries, but their effects on peripheral muscular arteries include a reduction in the amplitude of wave reflection and markedly lower systolic and pulse pressures.[250,267,358,359,365-367] Therefore, the reflected wave takes longer to return, reaching the advancing wave later in diastole. The net result is less augmentation of the central waveform, which means that augmentation pressure and augmentation index are reduced.

We have already shown that antihypertensive therapy with alpha methyldopa in women with pre-eclampsia but not in gestational hypertension is associated with significantly reduced placental and serum levels of the anti-angiogenic factors sFlt-1 and soluble endoglin (Section 3.2).[368] The beneficial effect of this drug on arterial stiffness may be due to a direct effect on the

arterial wall or may be mediated through reduced circulating anti-angiogenic factors. These findings support the concept of a difference in pathophysiology between gestational hypertension and the pathological endothelial toxic effect of pre-eclampsia.

Another possible explanation for the differential effect of methyldopa on arterial stiffness in pre-eclampsia and gestational hypertension involves its effect on the sympathetic system. Pre-eclampsia is associated with increased sympathetic tone.[369,370] Alpha methyldopa acts on α2-adrenoreceptors in the CNS, leading to a reduction in central sympathetic outflow. Thus it might be expected to bring about a greater reduction in vascular sympathetic tone and arterial stiffness in pre-eclampsia compared with gestational hypertension.

Pulse wave analysis has previously been used to assess vaso-active medications, particularly antihypertensive drugs, in the non-pregnant population. For example, a randomised controlled study comparing atenolol and ramipril found that both cause a similar reduction in peripheral blood pressure.[265] However, the fall in *central* systolic pressure is significantly greater with ramipril. Pulse wave analysis showed that this difference is due to the greater reduction in arterial stiffness caused by ramipril, and probably explains its greater long-term benefits. Studies such as these have led to a significant change in guidelines for managing hypertension in the non-pregnant population.[277] Our findings suggest that pulse wave analysis may also have an important role to play in the assessment of new and existing antihypertensive medications used in pregnancy, so that central - as well as peripheral - effects can be determined.[274,275,359]

Ideally, we would have included in our study a group of women with a similar degree of pre-eclampsia who did not receive antihypertensive therapy and another group of normotensive women who did. However, because of ethical concerns about the potential effects of such management, this was not possible.

Our findings suggest that any future research into pulse wave analysis in hypertensive disorders of pregnancy should be mindful of possible effects of antihypertensive therapy. Further research is needed to evaluate the effect of prolonged antihypertensive therapy, and whether different antihypertensive drugs have differential beneficial effects on maternal central haemodynamics. Such research will improve our understanding of the pathophysiology of pre-eclampsia but may also lead to better therapeutic clinical protocols. Women who develop pre-eclampsia are at significantly increased risk, later in life, of cardiovascular disease such as ischaemic heart disease and stroke. It is not known whether the beneficial effect of antihypertensive treatment in pregnancy on pulse wave analysis could modify this long-term risk.

3.5 EFFECT OF ANTIHYPERTENSIVE THERAPY WITH ALPHA METHYLDOPA ON UTERINE ARTERY DOPPLER IN PREGNANCIES WITH HYPERTENSIVE DISORDERS

3.5.1 Results

Prior to treatment

Before 34 weeks' gestation, prior to treatment, mean (SD) uterine artery pulsatility index (PI) was significantly higher in women with pre-eclampsia than in women with gestational hypertension: 1.51 (0.45) versus 0.85 (0.19), $P<0.0001$; or controls: 1.51 (0.45) versus 0.84 (0.16), $P<0.0001$ (Figure 2a). However, before 34 weeks there was no significant difference between women with gestational hypertension and controls: 0.85 (0.19) versus 0.84 (0.16), $P=0.95$.

A similar pattern of results was seen ≥ 34 weeks' gestation prior to treatment (Figure 3.9). Mean (SD) PI in women with pre-eclampsia was significantly higher compared with women with gestational hypertension: 0.79 (0.12) versus 0.61 (0.11), $P<0.0001$; or controls: 0.79 (0.12) versus 0.62 (0.13), $P<0.0001$. Neither was there any significant difference between women with gestational hypertension prior to treatment and controls: 0.61 (0.11) versus 0.62 (0.13), $P=0.95$.

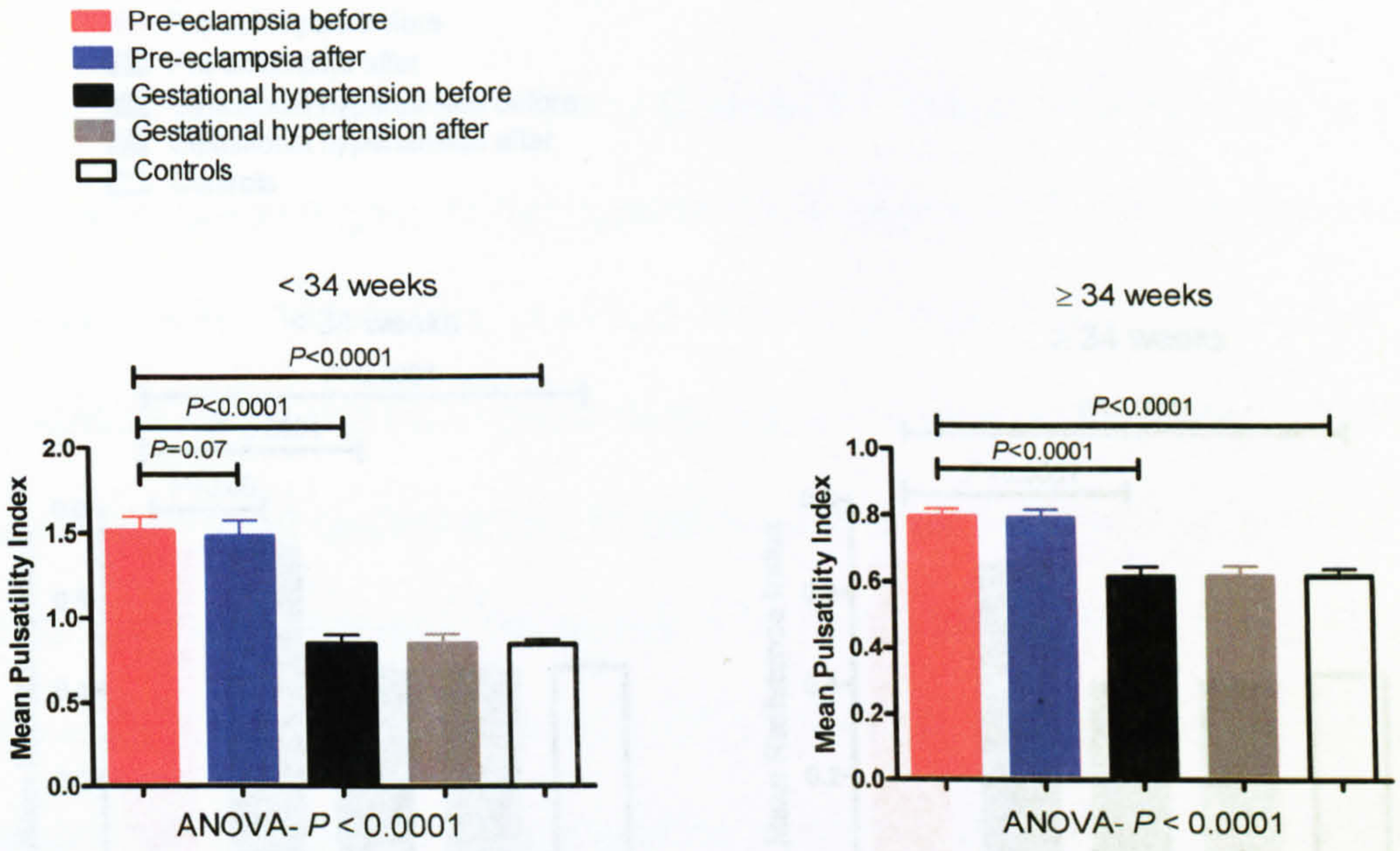


Figure 3.9. Uterine artery Doppler mean Pulsatility Index before and after antihypertensive therapy in women with pre-eclampsia or gestational hypertension, and in normotensive controls, stratified according to gestation.

Mean resistance index measurements followed a similar pattern (Figure 3.10). Prior to treatment before 34 weeks' gestation in women with pre-eclampsia, mean (SD) RI was significantly higher than in women with gestational hypertension: 0.72 (0.07) versus 0.44 (0.05), *P*<0.0001; or in controls: 0.72 (0.07) versus 0.45 (0.07), *P*<0.0001. Below 34 weeks' gestation, there was no significant difference between women with gestational hypertension prior to treatment and controls: 0.44 (0.05) versus 0.45 (0.07), *P*=0.6.

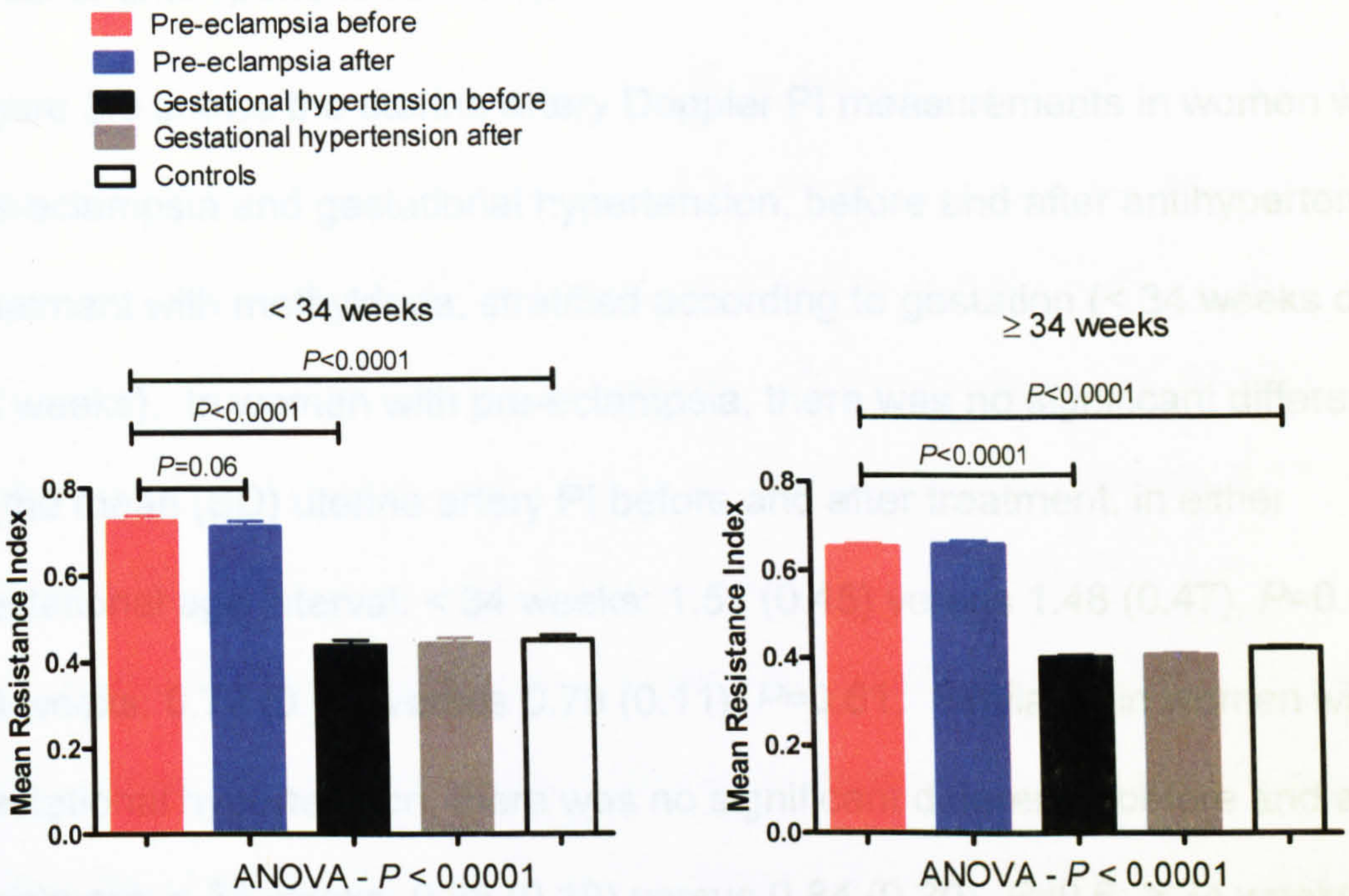


Figure 3.10. Uterine artery Doppler mean Resistance Index before and after antihypertensive therapy in women with pre-eclampsia or gestational hypertension, and in normotensive controls, stratified according to gestation.

After 34 weeks, the mean (SD) RI was significantly higher in women with pre-eclampsia prior to treatment than in women with gestational hypertension: 0.65 (0.03) versus 0.40 (0.02), $P<0.0001$; and in controls: 0.65 (0.003) versus 0.42 (0.03), $P < 0.0001$ (Figure 3.10). At this gestation, there was also no significant difference between women with gestational hypertension prior to treatment and controls: 0.40 (0.02) versus 0.42 (0.03), $P=0.4$.

Effect of antihypertensive therapy

Figure 3.9 shows the uterine artery Doppler PI measurements in women with pre-eclampsia and gestational hypertension, before and after antihypertensive treatment with methyldopa, stratified according to gestation (< 34 weeks or ≥ 34 weeks). In women with pre-eclampsia, there was no significant difference in the mean (SD) uterine artery PI before and after treatment, in either gestational age interval: < 34 weeks: 1.51 (0.45) versus 1.48 (0.47), *P*=0.07; ≥ 34 weeks: 0.79 (0.12) versus 0.79 (0.11), *P*=0.61. Similarly, in women with gestational hypertension, there was no significant difference before and after treatment: < 34 weeks: 0.85 (0.19) versus 0.84 (0.20), *P*=0.6; ≥ 34 weeks: 0.61 (0.11) versus 0.61 (0.12), *P*=0.4.

Antihypertensive treatment was not associated with any significant changes in mean RI in women with either pre-eclampsia or gestational hypertension, at any gestation (Figure 3.10). In women with pre-eclampsia prior to 34 weeks, the mean (SD) RI before and after treatment was: 0.72 (0.07) versus 0.71 (0.07), *P*=0.06. The equivalent figures for women with gestational hypertension were: 0.43 (0.05) versus 0.44 (0.04), *P*=0.2. After 34 weeks' gestation, mean (SD) RI in women with pre-eclampsia before and after treatment was: 0.65 (0.03) versus 0.66 (0.04), *P*=0.5. The equivalent figures for women with gestational hypertension after 34 weeks were: 0.40 (0.02) versus 0.40 (0.02), *P*=0.58.

Early onset versus late onset disease

We further analysed women with pre-eclampsia, comparing those with early onset versus late onset, and those with severe versus mild pre-eclampsia. The mean (SD) uterine artery PI was significantly higher in early onset compared with late onset pre-eclampsia: 1.51(0.45) versus 0.79 (0.12), *P*<0.0001; and in women with severe compared with mild disease: 1.86 (0.45) versus 1.06 (0.39), *P*<0.0001 (Figure 3.11).

A similar pattern was seen in resistance index measurements (Figure 3.12). Mean (SD) RI was significantly higher in women with early onset versus late onset pre-eclampsia: 0.72 (0.07) versus 0.65 (0.03), *P*<0.0001; and in women with severe versus mild pre-eclampsia: 0.80 (0.05) versus 0.67 (0.04), *P*<0.0001.

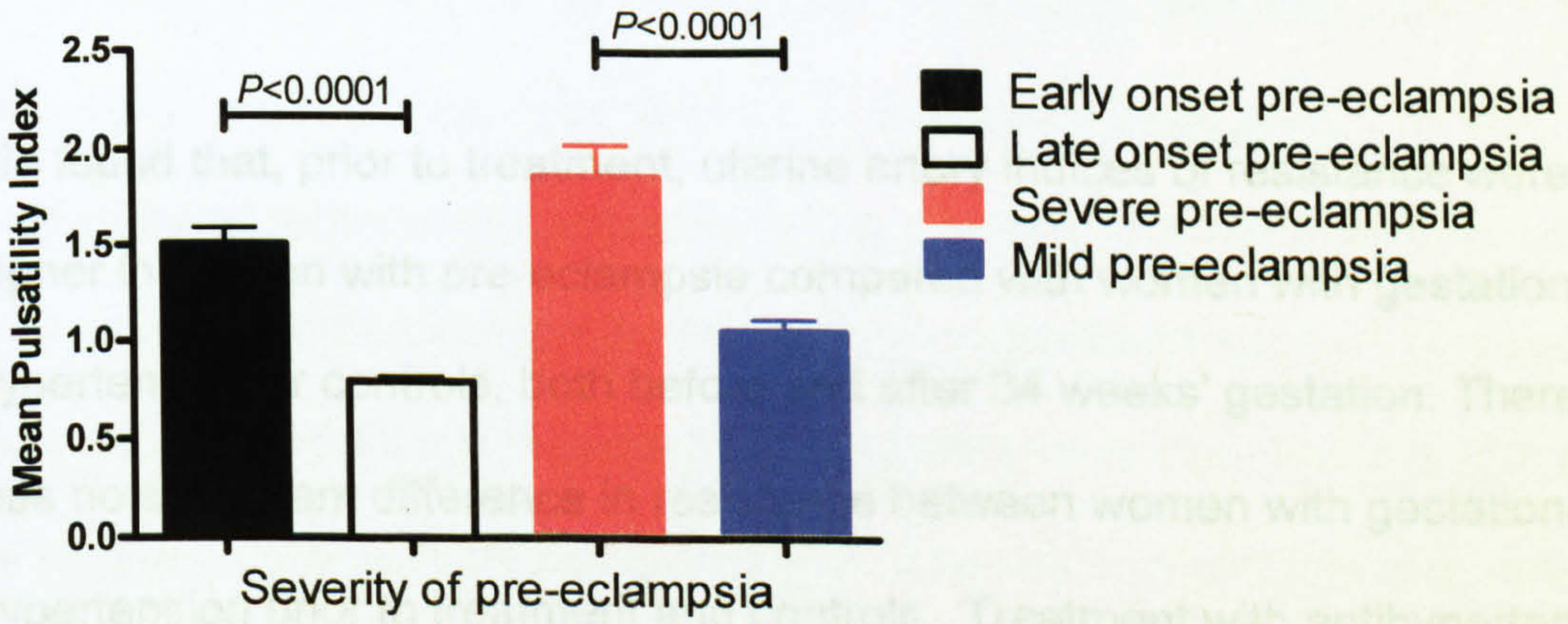


Figure 3.11. Uterine artery Doppler mean Pulsatility Index in women with early compared with late onset, and severe compared with mild pre-eclampsia.

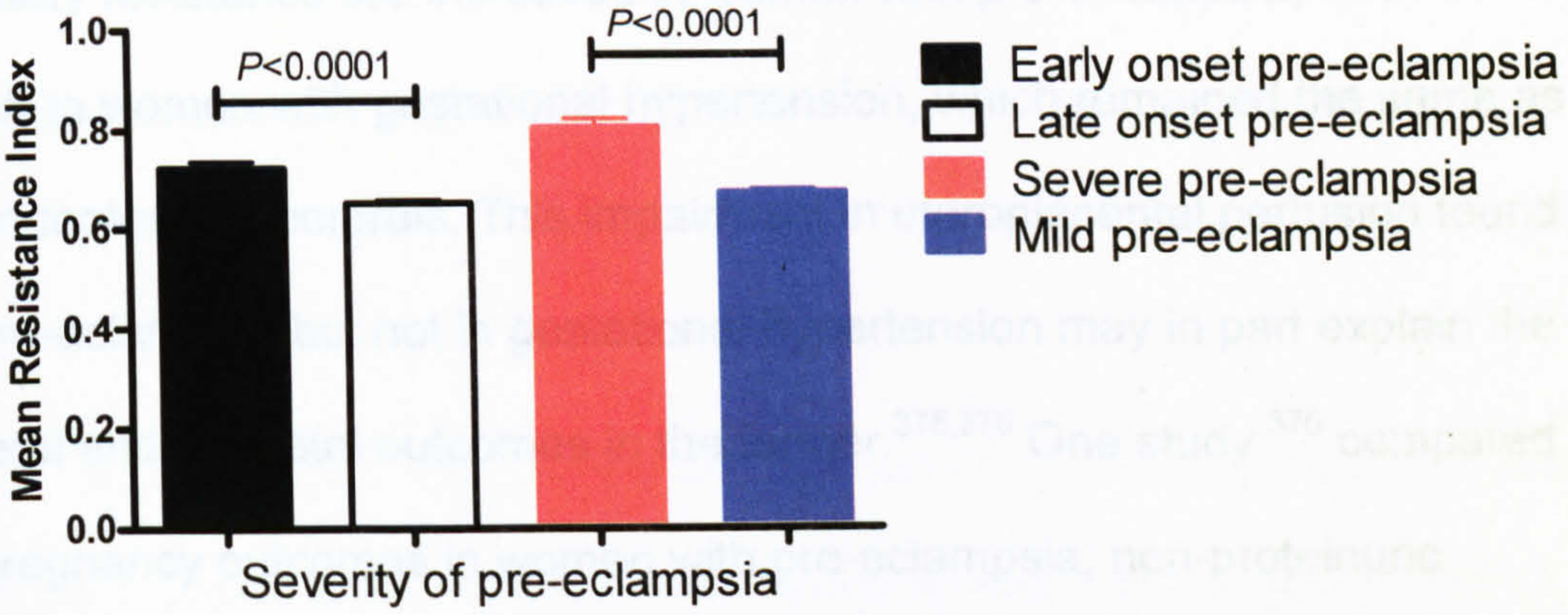


Figure 3.12. Uterine artery Doppler mean Resistance Index in women with early compared with late onset, and severe compared with mild pre-eclampsia.

3.5.2 Discussion

We found that, prior to treatment, uterine artery indices of resistance were higher in women with pre-eclampsia compared with women with gestational hypertension or controls, both before and after 34 weeks' gestation. There was no significant difference in resistance between women with gestational hypertension prior to treatment and controls. Treatment with antihypertensive medication did not significantly affect these indices in either pre-eclampsia or gestational hypertension, either before or after 34 weeks' gestation. Early onset and severe pre-eclampsia were associated with higher resistance indices.

Our study confirms the findings of other studies that the indices of uterine artery resistance are increased in women with pre-eclampsia,[296,297,371-374] but not in women with gestational hypertension, which remained the same as in normotensive controls. This impairment in uteroplacental perfusion found in pre-eclampsia but not in gestational hypertension may in part explain the poor fetal and neonatal outcomes in the former.[375,376] One study [376] compared pregnancy outcomes in women with pre-eclampsia, non-proteinuric gestational hypertension and chronic hypertension. Compared with non-proteinuric hypertensive patients, women with pre-eclampsia delivered significantly earlier (35.3 versus 38.6 weeks) with a lower birth weight (1997 versus 2922 gm) and higher rates of preterm delivery (58% versus 10%), small for gestational age infants (52% versus 18%) and perinatal death (13% versus 3%).

Nevertheless, it must be highlighted that adverse perinatal outcomes also occur in gestational hypertension.[377] It has also been confirmed that maternal and fetal complications are more common in hypertensive women with proteinuria than in hypertensive women without proteinuria.[378] However, in this study, even the non-proteinuric hypertensive women had a higher rate of maternal complications compared with normotensive controls (4% versus 13%), confirming that non-proteinuric hypertension is a pathological condition.

One study published in 2003 [379] showed that, as expected, abnormal uterine artery Doppler waveforms were common in women with pre-eclampsia (164/186) compared with women with gestational hypertension (71/158). However, in both groups (pre-eclampsia and gestational hypertension), the presence of abnormal uterine artery Dopplers was related to the risk of poor

pregnancy outcome. The authors of this study concluded that "patients with gestational hypertension without proteinuria but with abnormal uterine artery velocimetry have pregnancy outcomes that do not differ significantly from the outcomes of pre-eclamptic patients, in terms of week at delivery, birth weight, and frequency of growth restricted fetuses".

We found that methyldopa therapy had no significant effect on uterine artery Doppler resistance in women with pre-eclampsia or gestational hypertension. Four previous studies have examined the effect of methyldopa on uterine artery Doppler indices in women with hypertensive disorders of pregnancy.[372,380-382] These studies yielded mixed results. In a study [380] of 20 women with pre-eclampsia in the third trimester receiving 750 mg of methyldopa daily for a week, no significant effect was found on uterine artery PI. Another study by the same authors [381] examining the effects of isradipine and methyldopa did not find an effect on uterine artery PI. In contrast, a study by Rey *et al* [382] compared 25 women with pre-eclampsia (14 on treatment, 11 untreated), 43 women with chronic hypertension (14 on treatment, 29 untreated) and 22 normotensive controls. Women were treated with methyldopa 750 mg daily for a week. There was a significant decrease in placental artery PI in women taking methyldopa compared with those untreated, in both the pre-eclamptic and chronic hypertensive groups. The most recent of these studies [372] compared 24 women with pre-eclampsia treated with 1 g of methyldopa per day for a week, with 20 normotensive pregnant controls. Uterine artery PI, RI and S/D were significantly lower after antihypertensive therapy. Ours is the single biggest study of the effect of

methyldopa on uterine artery indices in women with hypertensive disorders of pregnancy. We found no significant effect in either pre-eclampsia or gestational hypertension, either before or after 34 weeks' gestation.

It is clinically relevant that our study shows no adverse effects of methyldopa treatment on uteroplacental perfusion because there is a theoretical concern that antihypertensive treatment in women with pre-eclampsia might adversely affect uteroplacental perfusion by reducing blood pressure when there is already increased uterine artery resistance.[383,384] Furthermore, it has been suggested that antihypertensive treatment should be preceded by plasma expansion with intravenous fluids.[385] Our findings suggest that volume expansion prior to antihypertensive treatment with methyldopa in these women is not necessary; this therapeutic approach might be useful for women treated with vasodilators.

Conversely, treatment with methyldopa, the antihypertensive most commonly used in women with pre-eclampsia in the United Kingdom, does not *improve* uteroplacental perfusion. This observation is consistent with the findings of a systematic review that antihypertensive drug therapy for mild to moderate hypertension during pregnancy has no beneficial effect on the risk of fetal demise (relative risk 0.73, 95% CI 0.50 to 1.08), small for gestational age babies (relative risk 1.04, 95% CI 0.84 to 1.27) or on any other adverse fetal outcome.[336] Overall, the main benefit of antihypertensive treatment in these women is an improvement in *maternal* outcome, in particular a halving of the risk of developing severe hypertension (relative risk 0.50, 95% CI 0.41 to 0.61).

3.6 PULSE WAVE ANALYSIS: NORMAL VALUES IN PREGNANCY

3.6.1 Results

Of the 665 pregnant women studied, 24 (3.6%) developed pre-eclampsia, 36 (5.4%) non-proteinuric gestational hypertension and 17 (2.6%) fetal growth restriction. We also excluded 20 women (3.0%) with spontaneous preterm labour, 9 (1.3%) with gestational diabetes, 4 (0.6%) who had a miscarriage, 4 (0.6%) who had fetal abnormalities and underwent termination of pregnancy, and 10 (1.5%) whose outcomes were missing. This left 541 healthy normotensive pregnant women and 44 non-pregnant women eligible for subsequent analysis. Of these 541 women, 154 were recruited in the first and early second trimester (8^{+1} to 13^{+6} weeks), 209 in the second trimester (14^{+0} to 26^{+0} weeks), and 178 in the late second trimester and third trimester (26^{+1} to 39^{+0} weeks). Of the 154 women recruited in the first trimester, 45 had measurements taken at 12^{+0}-12^{+6} weeks, 23^{+0}-23^{+6} weeks, and 32^{+0}-32^{+6} weeks of gestation; these longitudinal data were analysed separately.

The baseline characteristics of the two normotensive study groups (pregnant and non-pregnant) are shown in Table 3.6. There were no statistically significant differences between the two groups. Similarly, there were no significant differences in baseline characteristics among women recruited in the three trimesters.

Table 3.6. Baseline characteristics of pregnant and non-pregnant women.

BMI = body mass index

Data are expressed as means ± SD or as percentages

	Pregnant	**Non-pregnant**	***P* value**
	N = 541	n = 44	
Age (years)	30.5 (6)	31 (6)	0.6
BMI (kg/m^2)	27 (5)	27 (5)	1
Nulliparity n (%)	242 (45)	17 (39)	0.4
Caucasian n (%)	229 (42)	18 (41)	0.9
Smokers n (%)	78 (14)	7 (14)	0.8

Table 3.7 shows the haemodynamic parameters for the same two study groups. Heart rate was significantly faster (P <0.001), and AP and AIx-75 significantly lower (P =0.004, P =0.01), in normotensive pregnant women compared with non-pregnant.

Table 3.7. Haemodynamic parameters in pregnant and non-pregnant women.

Parameter	Pregnant	Non-pregnant	*P* value
	n = 541	n = 44	
Brachial systolic BP (mmHg)	109 (16)	109 (17)	1
Brachial diastolic BP (mmHg)	68 (9)	70 (9)	0.1
Brachial pulse pressure (mmHg)	41 (12)	39 (11)	0.2
Mean BP (mmHg)	83 (11)	84 (12)	0.4
Heart rate (bpm)	87 (15)	75 (12)	< 0.001
Central systolic BP (mmHg)	97 (14)	99 (16)	0.3
Central diastolic BP (mmHg)	70 (9)	71 (9)	0.4
Central pulse pressure (mmHg)	27 (9)	28 (9)	0.5
AP (mmHg)	3.5 (4)	5.5 (4)	0.004
AIx-75 (%)	16 (11)	20.4 (12)	0.01

BP = blood pressure

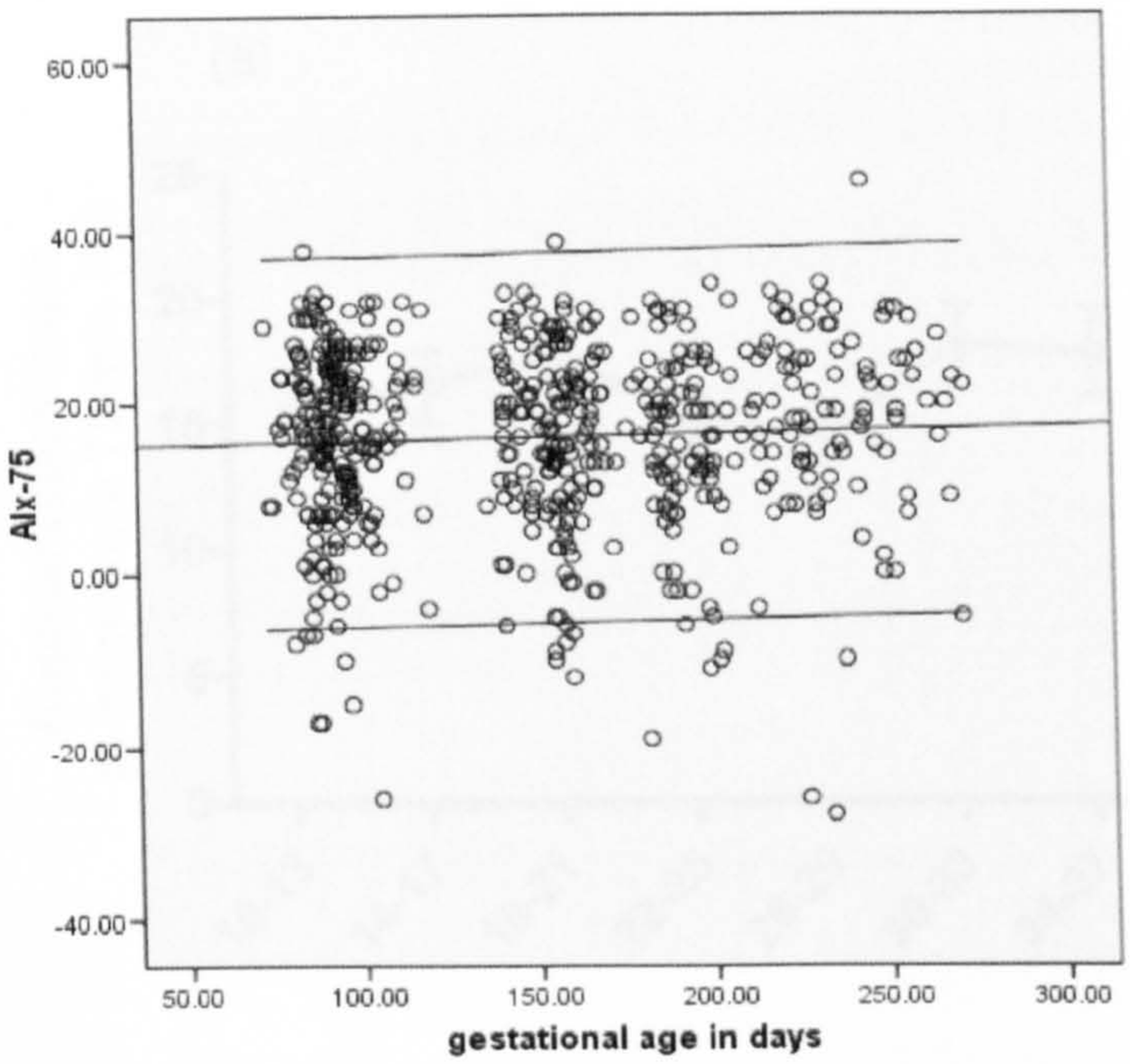
bpm = beats per minute

AP = augmentation pressure

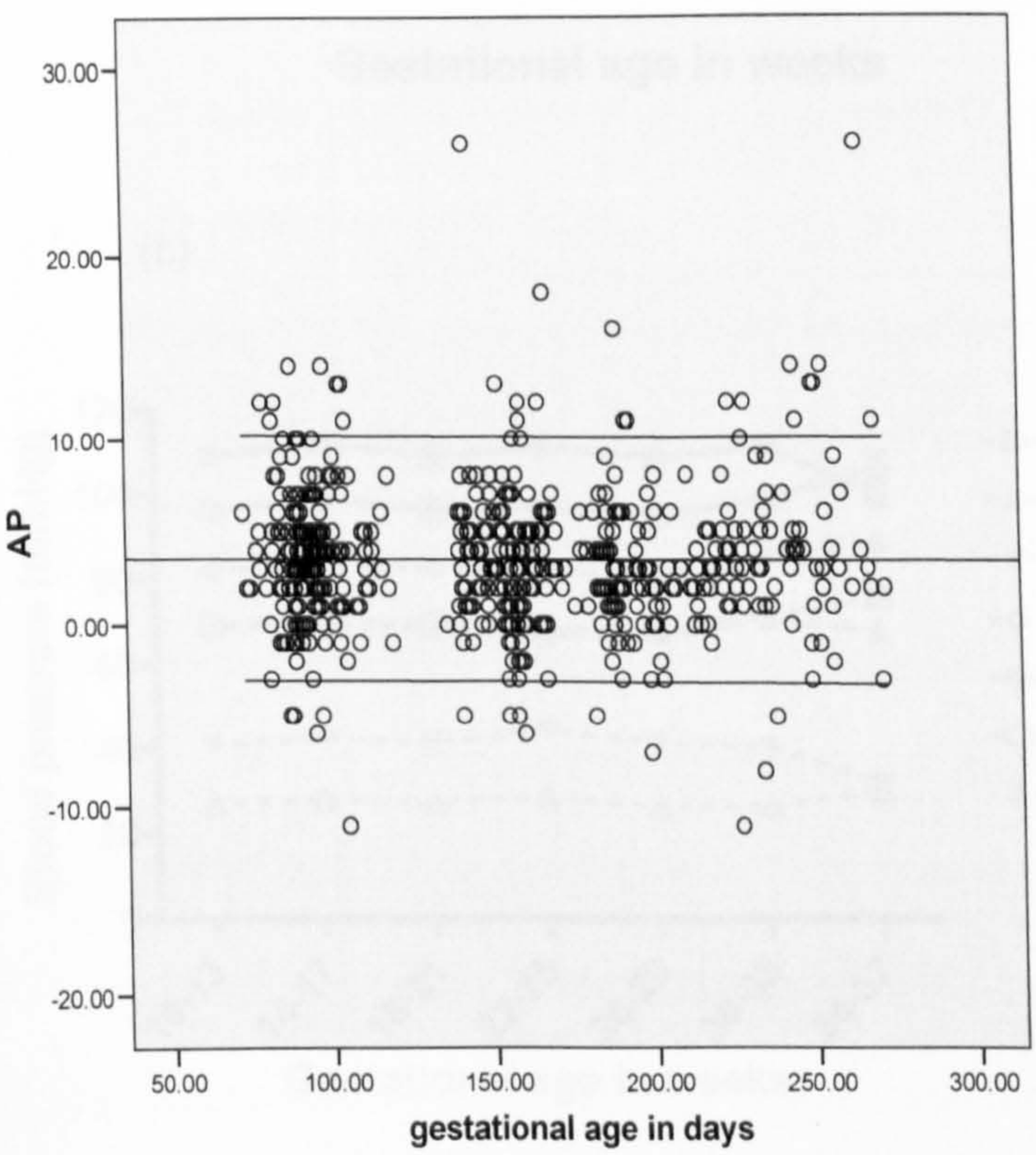
AIx-75 = augmentation index at heart rate 75 beats per minute

Data are expressed as means ± SD or as percentages

We compared the haemodynamic parameters for the normotensive pregnant women according to trimester. There were no significant changes in AP or AIx-75 as pregnancy progressed. There was no significant change in either brachial systolic or diastolic BP from trimester to trimester, but brachial pulse pressure was significantly lower in the third trimester compared with the second. Heart rate rose significantly from first to second (P <0.001) and second to third trimester (P <0.001). The changes in central diastolic blood pressure approached significance (P =0.055) and the post hoc comparison showed a significant difference between the second and third trimesters (P =0.045), rising from a mean (SD) of 68.8 (8.7) to 71.1 (10.5). The changes in AP and AIx-75 according to days of gestation are shown in Figure 3.13 (AP: r=-0.01, P =0.80; AIx-75: r=0.04, P =0.34). The monthly changes in AIx-75, brachial and central BP are presented in Figure 3.14.



(a)



(b)

Figure 3.13. Pulse wave analysis parameters according to gestation. Scatter plots of (a) augmentation pressure (AP), and (b) augmentation index at heart rate of 75/min (AIx-75) according to the gestational age in days (n = 541). The 5th and 95th centiles are shown.

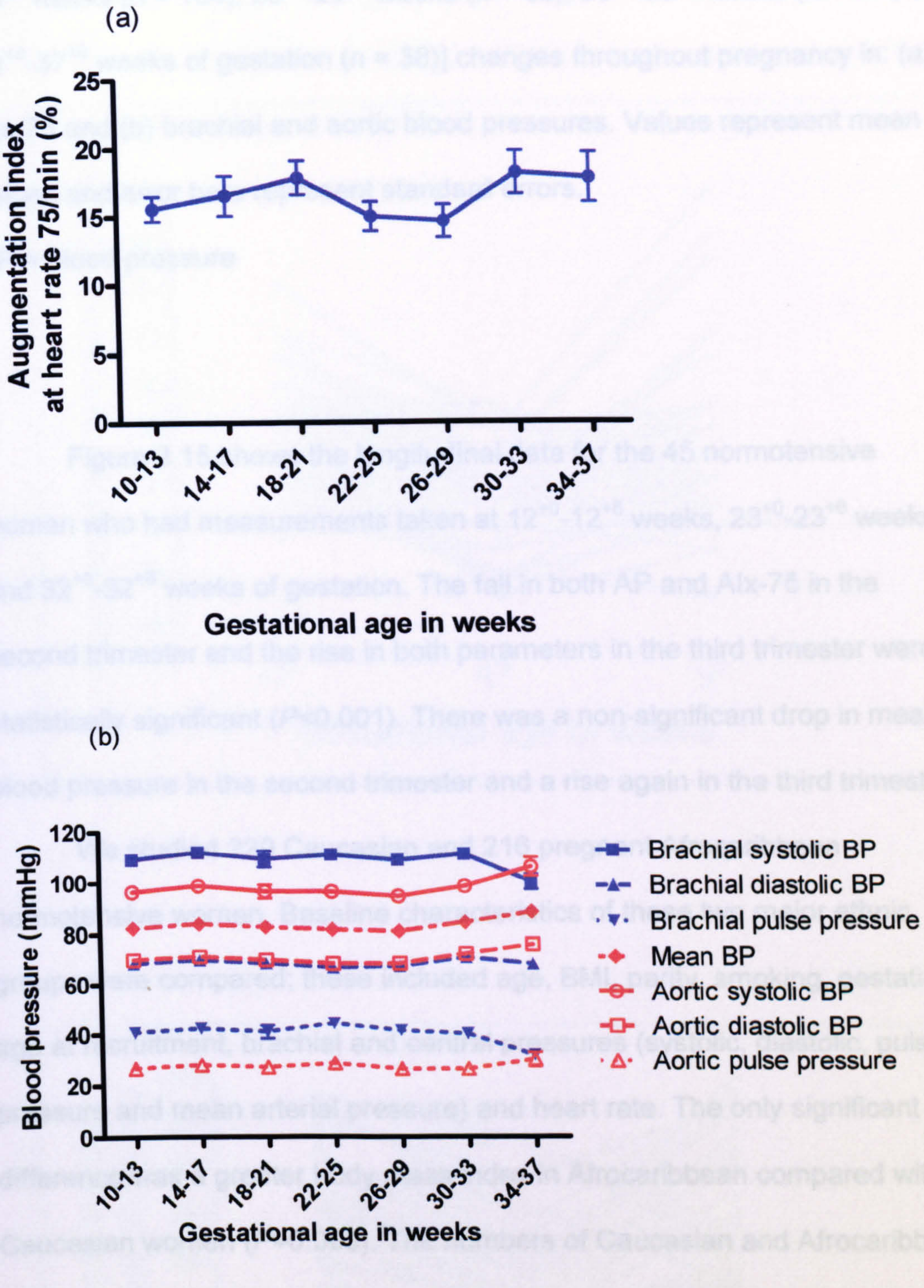


Figure 3.14. Monthly changes in augmentation index at heart rate 75/min (Alx-75), central and peripheral blood pressure. Monthly [10^{+0}-13^{+6} weeks (n = 145), 14^{+0}-17^{+6} weeks (n = 56), 18^{+0}-21^{+6} weeks (n = 55), 22^{+0}-

25^{+6} weeks (n = 104), 26^{+0}-29^{+6} weeks (n = 86), 30^{+0}-33^{+6} weeks (n = 57) and 34^{+0}-37^{+6} weeks of gestation (n = 38)] changes throughout pregnancy in: (a) AIx-75 and (b) brachial and aortic blood pressures. Values represent mean values and error bars represent standard errors.

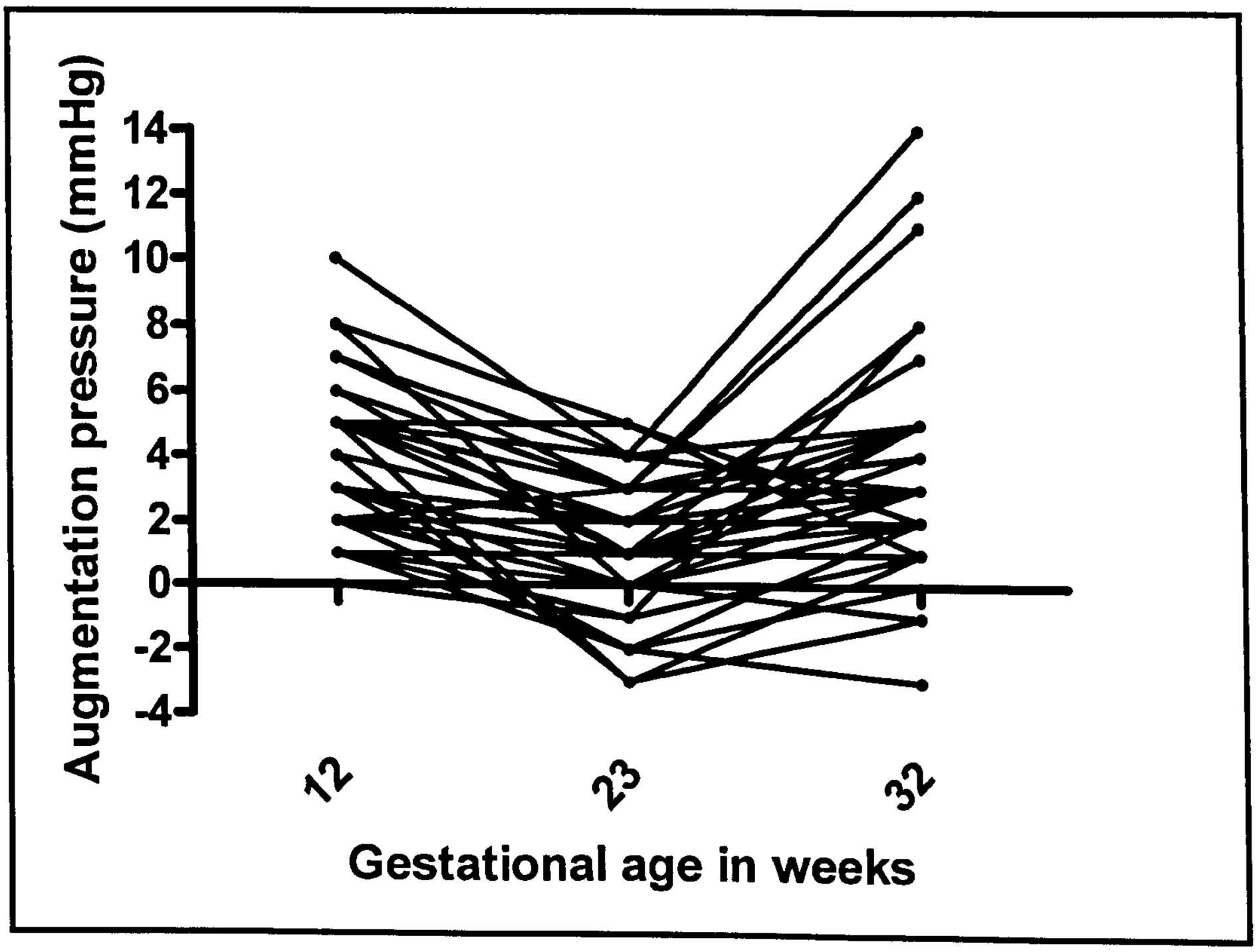
BP = blood pressure

Figure 3.15 shows the longitudinal data for the 45 normotensive women who had measurements taken at 12^{+0}-12^{+6} weeks, 23^{+0}-23^{+6} weeks, and 32^{+0}-32^{+6} weeks of gestation. The fall in both AP and AIx-75 in the second trimester and the rise in both parameters in the third trimester were statistically significant (P<0.001). There was a non-significant drop in mean blood pressure in the second trimester and a rise again in the third trimester.

We studied 229 Caucasian and 216 pregnant Afrocaribbean normotensive women. Baseline characteristics of these two major ethnic groups were compared; these included age, BMI, parity, smoking, gestational age at recruitment, brachial and central pressures (systolic, diastolic, pulse pressure and mean arterial pressure) and heart rate. The only significant difference was a greater body mass index in Afrocaribbean compared with Caucasian women (P=0.003). The numbers of Caucasian and Afrocaribbean women respectively recruited in each trimester were: 11^{+0} to 13^{+6} weeks, 68 and 60; 14^{+0} to 26^{+0} weeks, 87 and 88; 26^{+1} to 33^{+0} weeks, 74 and 68. There were no statistically significant differences in AP or AIx-75 between these two ethnic groups in any trimester.

Figure 3.15. Longitudinal changes in pulse wave analysis parameters.

(a)



(b)

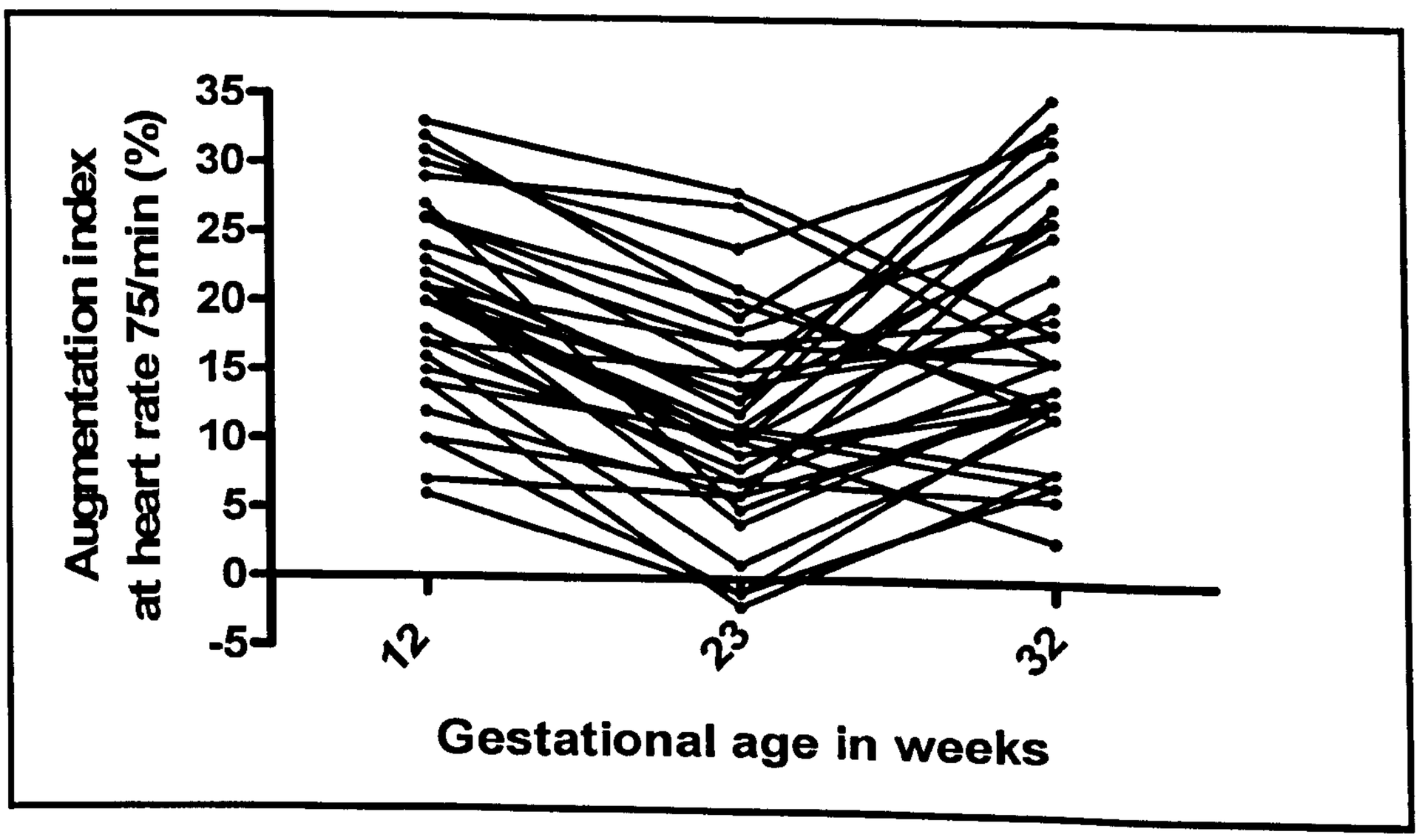


Figure 3.15. Longitudinal changes in pulse wave analysis parameters. Longitudinal data for the 45 women who had measurements taken at 12^{+0}-12^{+6} weeks, 23^{+0}-23^{+6} weeks, and 32^{+0}-32^{+6} weeks of gestation: (a) augmentation pressure and (b) augmentation index at heart rate 75 beats per minute (AIx-75).

3.6.2 Discussion

This study establishes the normal ranges for pulse wave analysis parameters in normal pregnancy. We found no significant differences in these parameters between the two main ethnic groups in our population. Arterial stiffness was significantly lower in pregnant compared with non-pregnant women, confirming the findings of previous studies.[281,282] This is also consistent with the known vasorelaxation of pregnancy caused by vasoactive agents such as progesterone and relaxin.[386-389]

Augmentation index has a linear relationship with heart rate,[255] highlighting the importance of controlling augmentation index for pulse rate and thus the need in pregnancy to use AIx-75 rather than AIx.

Our study confirms that, in normal pregnancy, aortic stiffness varies throughout pregnancy, reaching its nadir in the second trimester and rising again in the third.[282] Individual women followed longitudinally throughout pregnancy had a significant fall in arterial stiffness (AIx-75) in the second trimester, with a significant rise again in the third. These changes were not linked to variations in blood pressure, which in our population did not change significantly through pregnancy, or to changes in heart rate, which rose

consistently throughout pregnancy and was controlled for in AIx-75. These results suggest that changes in arterial stiffness may result from changes in the levels of vasoactive substances such as progesterone and relaxin, and volume expansion of pregnancy. When we pooled our data, we found no significant changes in mean AP and AIx-75 from one trimester to the next. However, we observed a trend towards a fall in AIx-75 in the second trimester and a rise in the third, consistent with the trend seen in the sub-group of women followed longitudinally.

Arterial pulse wave analysis is non-invasive and easy to learn. The equipment is inexpensive and portable and can be easily used in the outpatient setting. This study establishes normal values for pulse wave analysis parameters in all three trimesters and in Caucasian and Afrocaribbean women. These data may be used as the basis for further investigation into the role of pulse wave analysis in the assessment, management and perhaps prediction of pre-eclampsia.

3.7 SUMMARY OF FINDINGS

In pre-eclampsia, maternal serum and placental levels of sFlt-1 and sEng are higher, and PlGF lower, compared with controls. Antihypertensive treatment with alpha methyldopa is associated with significantly lower serum and placental concentrations of both sFlt-1 and sEng in women presenting with pre-eclampsia but not those with gestational hypertension.

Both maternal serum and placental concentrations of inhibin A and activin A were significantly higher in pre-eclampsia compared with levels in gestational hypertension, which in turn were higher than in normotensive controls. Alpha methyldopa therapy was associated with reduced serum and placental concentrations of inhibin A and activin A in pre-eclampsia but not in gestational hypertension.

Arterial stiffness is increased in women with gestational hypertension compared with normotensive pregnant women, but increased significantly more in women with pre-eclampsia. In pre-eclampsia, but not in gestational hypertension, arterial stiffness is improved by treatment with alpha methyldopa.

Prior to treatment, uterine artery indices of resistance were higher in women with pre-eclampsia compared with women with gestational hypertension or controls. There was no significant difference in resistance between gestational hypertension and controls. Treatment with alpha methyldopa did not significantly affect these indices in either pre-eclampsia or gestational hypertension.

We have established the normal ranges for pulse wave analysis parameters in normal pregnancy. There were no significant differences in these parameters between the two main ethnic groups in our population, namely Afrocaribbean and Caucasian. Arterial stiffness was significantly lower in pregnant compared with non-pregnant women. In normal pregnancy, aortic stiffness varies throughout pregnancy, reaching its nadir in the second trimester and rising again in the third.

CHAPTER FOUR

CONCLUSIONS

CONCLUSIONS

The aim of this thesis was to investigate the effect of antihypertensive therapy on vascular and placental function in hypertensive disorders in pregnancy. The null hypothesis was that antihypertensive therapy with alpha methyldopa would not have any significant effect on vascular function or placental markers in hypertensive disorders in pregnancy.

We found that therapy with methyldopa significantly reduced arterial stiffness (as measured by pulse wave analysis) and maternal serum and placental markers (sFlt-1, soluble endoglin, inhibin A and activin A) in pre-eclampsia, but not in gestational hypertension, thereby disproving the null hypothesis.

Our findings suggest that, within 48 hours of commencing treatment in women with pre-eclampsia, alpha-methyldopa reduces placental production of sFlt1, soluble endoglin, inhibin A and activin A, leading to a reduction in circulating levels; this has a beneficial effect on vascular endothelium and/or vascular wall, leading to a reduction in arterial stiffness.

We confirmed that, prior to treatment, levels of sFlt-1 and soluble endoglin in both serum and placenta are increased in women with gestational hypertension but increased significantly more in women with pre-eclampsia. The same is true of serum and placental inhibin A and activin A.

Antihypertensive treatment with alpha methyldopa is associated with a significant fall in both serum and placental concentrations of these four markers in women with pre-eclampsia, but not in gestational hypertension. These findings suggest that, in pre-eclampsia, alpha methyldopa may have a

direct effect on placental synthesis and/or secretory functions, leading to a reduction in circulating concentrations in maternal serum, and that this effect may not be simply the result of a reduction in maternal blood pressure and /or a change in utero-placental blood flow. As discussed in section 3.2.2, the inhibition of sFlt-1 by methyldopa may be mediated through the inhibition of adenylyl cyclase, leading to decreased production of cyclic AMP. Inhibition of cyclic AMP production may also be the mechanism by which alpha-methyldopa leads to reduced placental production of inhibin A and activin A (section 3.3.2). Alternatively, methyldopa may inhibit cytokine activity or release in trophoblasts which may in turn lead to a reduction in inhibin A and activin A secretion.

The fact that changes in serum and placental concentrations of these four markers mirror each other, both before and after antihypertensive treatment, supports the hypothesis that the placenta is their primary source. We examined the possibility that the apparent difference in effect of methyldopa on these markers in pre-eclampsia and gestational hypertension was simply due to the fact that marker levels prior to treatment were higher in pre-eclampsia, so that a similar proportional change would be more likely to result in a statistically significant *P* value. However, this was not the case: the proportional change following treatment in women with pre-eclampsia was significantly greater than the proportional change in women with gestational hypertension.

Marker levels were significantly higher in early onset compared with late onset, and in severe compared with mild pre-eclampsia. However, the improvement in marker levels in these groups before and after treatment was

similar; the difference was only a question of degree. It has been suggested that severe early onset pre-eclampsia might represent a different disease entity to the late onset (and usually milder) form.[390] However, our data suggest that severe and/or early onset pre-eclampsia is just a more severe form of the same disease process.

We confirmed that arterial stiffness is increased in gestational hypertension but increased even more in women with pre-eclampsia. We showed for the first time that, in women with pre-eclampsia but not in women with gestational hypertension, methyldopa brings about a significant improvement in arterial stiffness.

Arterial stiffness as measured by pulse wave analysis was significantly reduced following treatment in women with pre-eclampsia, but did not return to the same level as normotensive controls. This was in spite of adequate control of blood pressure, implying that the improvement in arterial stiffness was not due to a fall in blood pressure alone. This suggests that methyldopa also has an effect on maternal blood vessels, either improving vessel wall compliance or endothelial dysfunction. These benefits may be due to a direct effect on the vessel wall. Alternatively, they may be mediated through falls in the circulating maternal levels of sFlt1, soluble endoglin, inhibin A or activin A; our work shows that changes in the levels of these markers in pre-eclampsia and gestational hypertension mirror the changes in arterial stiffness before and after treatment with methyldopa. Because these changes take place over a relatively short space of time, it is tempting to speculate that the effect on vascular endothelium or vascular wall is a functional rather than a structural change.

In our study, methyldopa therapy had no significant effect on uterine artery Doppler indices in either pre-eclampsia or gestational hypertension. This is perhaps surprising, given that this drug has been shown to reduce vasospasm in other (maternal) arteries in women with pre-eclampsia. It may be that the increased resistance found in the uterine arteries of women with pre-eclampsia is not so much due to vasospasm as to the earlier failure of adequate trophoblastic invasion, thus impairing the development of the low resistance system usually found in pregnancy. The fact that methyldopa did not significantly affect Doppler indices suggests that the fall in placental levels of sFlt1, soluble endoglin, inhibin A and activin A was not due to an increase in uteroplacental perfusion. This supports the hypothesis that methyldopa has a direct effect on placental production and/or secretion of these markers.

The fact that maternal serum and placental markers, and arterial stiffness, were affected in women with pre-eclampsia but not in women with gestational hypertension, suggests that these represent two different pathophysiological entities.

We used multiple tools to study the effect of antihypertensive therapy, including anti-angiogenic and pro-angiogenic markers, inhibin A, activin A, uterine artery Doppler and pulse wave analysis. Some of these markers have previously been the subject of intensive research, while pulse wave analysis is considered novel in this field.

We studied a group of women who developed non-proteinuric hypertension as well as a group with pre-eclampsia. This added to our understanding of the differences between these two conditions; the two

groups responded differently to antihypertensive treatment despite a similar reduction in blood pressure.

We measured marker levels within 48 hours after the commencement of methyldopa therapy. Maximal antihypertensive effect from methyldopa is not gained until at least 48 hours following commencement of treatment, so it is possible that, with longer duration of treatment, a greater effect on markers levels might have been observed.

From the scientific point of view, it would have been interesting to include a control group of women with pre-eclampsia in whom treatment was indicated but withheld, and another group of normotensive women who did, but this was not considered ethically acceptable. However, it would be reasonable to expect that in such a control group, levels of maternal serum markers would either stay the same or increase still further over a 48-hour period. In contrast, in women treated with methyldopa over this time period, there was a significant fall in serum markers, suggesting that this is a genuine effect.

I believe that my work in preparing this thesis has greatly enhanced my practical and theoretical knowledge, specifically in the area of hypertensive disorders in pregnancy, but more broadly in research methodology in general. I now realise that measurement of peripheral blood pressure is a crude indicator of the changes taking place in the cardiovascular system, and that there is more information in the arterial pulse wave which should be appreciated. In the long-term, markers of the vascular changes taking place at the molecular level are likely to prove the best tools for understanding and managing these disorders.

I now feel that we should question our concept that antihypertensive therapy leads only to reduction in blood pressure and appreciate that it may potentially affect the pathophysiological changes in pre-eclampsia. This effect may vary according to the individual antihypertensive drug.

On a practical level, I have learned from scratch how to set up, run and complete a complex study, from devising a protocol and obtaining ethics approval, to recruiting and studying women, to processing serum and placental samples in the laboratory, to analysing, presenting and publishing my findings. My skills in ultrasound scanning and pulse wave analysis have also been honed along the way. I feel certain that all of this will benefit me in my future career in clinical and academic medicine.

In women with pre-eclampsia, levels of these four markers fell after treatment with methyldopa but, in spite of normalisation of blood pressure, levels did not fall to those of normotensive controls. It would be interesting to investigate whether more aggressive treatment could restore these levels to normal, perhaps representing better control of the disease process. A potential disadvantage of this approach is the possibility that more aggressive antihypertensive treatment might lead to lower blood pressure and impairment of uteroplacental perfusion. Doppler examination in this study suggests that the levels of treatment currently used do not produce any such impairment.

It would also be potentially valuable to explore the effect of other antihypertensive medications on these maternal serum markers. Other antihypertensives which produce similar effects on blood pressure might have a more beneficial effect on these markers, possibly representing better control of the underlying disease.

In the non-pregnant population, it has been shown that different antihypertensive drugs, while bringing about a similar reduction in peripheral blood pressure, result in different changes in central (aortic) haemodynamics as measured by arterial pulse wave analysis and this translates into better long-term clinical outcomes. Such studies have led to significant changes in the guidelines for managing hypertension in the non-pregnant population. Our findings suggest that similar studies using pulse wave analysis to assess the central effects of the different antihypertensives used in pregnancy may be valuable.

I believe that this work has disproved the null hypothesis that antihypertensive therapy with alpha methyldopa does not have any significant effect on vascular function or placental markers in hypertensive disorders in pregnancy, specifically in the case of pre-eclampsia.

Appendix 1

Inhibin A Assay Diluent

Reagents:

- 10% BSA (protease free)
- 5% mouse serum
- 5% Triton X-100
- 0.15 M NaCl
- 0.1% Sodium Azide
- 0.1 M Tris HCl buffer (pH 7.5)

To make up 500 mls:

- 50g BSA
- 25 mls mouse serum
- 25 mls Triton X-100
- 4.375 g NaCl
- 0.5 g Sodium Azide
- 45 mls 1M Tris made up to 450 mls with distilled water

Mix well for 30-60 minutes and filter diluent through cotton wool.

Appendix 2

ELISA Wash Buffer (X 20 Stock)

Reagents:

- 1M Tris base - 0.05M (x 20)
- 3M NaCl - 0.15M (x 20)
- 1% Sodium Azide - 0.05% (x 20)
- Tween20
-

To make up 2.5 litres stock buffer:

- Tris base – 302.75 g
- NaCl - 175.32 g
- Sodium Azide - 25 g

Dissolve in 2 litres of distilled H_2O, neutralise pH using concentrated HCl to pH 7.5.

Make up volume to 2.5 litres using distilled water.

Preparation of 10 litres normal wash buffer from stock:

For 10 litres:

- 500 mls stock buffer (pH 7.5)
- 9.5 litres distilled water.
- 5 mls Tween20.

Stir well for 30 minutes and store at room temperature.

Appendix 3

ELISA manual wash buffer

Reagents:

- 0.05M Tris HCl buffer
- 0.15M NaCl

To make up 500 ml:

- 25 mls of 1M Tris HCl buffer (pH 7.5)
- 475 mls distilled water
- 4.4 g NaCl

Mix well and store at 4 °C.

Appendix 4

ELISA Stop Solution

Reagents:

- Concentrated sulphuric acid (H_2SO_4)
- Distilled water

To make 1 Molar H_2SO_4:

Under the fume hood take 946.74 mls distilled water in a glass bottle and measure 53.26 mls concentrated H_2SO_4 with a glass pipette. Add the acid to the water and mix well.

To make 500 mls 0.3 Molar H_2SO_4-ELISA Stop solution:

Add 150.15 mls of 1 M H_2SO_4 to 349.85 mls distilled water.

Mix well and store at room temperature.

Appendix 5

Sample buffer for Activin A ELISA

Reagents:

- Phosphate Buffered Saline
- 10% BSA (protease free)
- 0.1% Sodium Azide

To make up 100 mls:

- Dissolve 1 PBS tablet in 100 mls distilled water
- 10 g BSA
- 100 mg Sodium Azide.

Mix well and store at 4 °C.

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