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BRUCE RAMSAY BM MRCOG

KING'S COLLEGE HOSPITAL, LONDON

AND

CHELSEA AND WESTMINSTER HOSPITAL, LONDON

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UNIVERSITY OF SOUTHAMPTON, FACULTY OF MEDICINE

DOCTOR OF MEDICINE - ABSTRACT BY BRUCE RAMSAY

THE QUANTIFICATION OF NITRIC OXIDE PRODUCTION IN PREGNANT AND NON-PREGNANT WOMEN.

Nitric oxide (NO) is synthesised from L-arginine by the enzyme nitric oxide synthase (NOS). The role which NO plays in human pregnancy has not yet been widely explored, in part due to the difficulties of quantification *in vivo*. For this thesis I developed assays locally to allow indirect quantification of NO production *in vivo* in two ways: measuring NOS activity in tissue samples and measuring nitrite/nitrate levels in plasma samples. These techinques were then used, in conjunction with colour Doppler ultrasound, to explore the gestation related variations in NO production in 50 normal pregnancies in a cross-sectional study. Subsequently the study was extended to include some pregnancies complicated by abnormal Dopplers or fetal growth retardation. I also investigated the effect of NO donor drugs on utero-placental blood flow in early pregnancy and the effect of exogenous oestrogen on NO production in non-pregnant women. The main findings of the research were:-

1 Plasma nitrate levels rose in the second and third trimesters of pregnancy implying a systemic increase in NO production at that time.

2 Trophoblast NOS activity was highest in the first trimester of pregnancy and fell steadily towards term in normal pregnancies suggesting that trophoblast NO may play a significant role in the implantation process.

3 In pregnancies complicated by IUGR, trophoblast NOS activity was very low when compared to normals supporting the idea of a relative NO deficiency in such pregnancies.

4 NO donor drugs increased utero-placental diastolic blood flow in normal early pregnancy when prostacyclin did not, implying that NO plays a specific role in controlling local uteroplacental vascular tone.

5 Exogenous oestrogen increased plasma nitrate levels in women of child bearing age in a placebo controlled study, suggesting that NO is one of the mediators through which oestrogen exerts its cardiovascular effects.

CONTENTS

LIST OF ABREVIATIONS

ADMA	Asymmetric dimethyl-arginine
BP	Blood pressure
BPM	Beats per minute
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CV	Coefficient of Variation
CIA	Capillary ion analysis
E_2	Oestradiol
FSH	Follicle stimulating hormone
GTN	Glyceryl trinitrate
HPLC	High performance liquid chromatography
IUGR	Intra-uterine growth retardation
L-NMMA	N^G-monomethyl-L-arginine
LH	Luteinising hormone
MAP	Mean arterial pressure
NO	Nitric oxide
NO_2	Nitrite
NO_3	Nitrate
cNOS	Calcium dependent nitric oxide synthase
iNOS	Calcium independent nitric oxide synthase
PI	Pulsatility Index
PGI_2	Prostacyclin
RI	Resistance Index
SD	Standard Deviation
SEM	Standard Error of the Mean
TAV	Time averaged velocity
V_{mean}	Mean velocity

STATEMENT OF ORIGINALITY

The work presented in this thesis is original and was performed by the author with help from those mentioned in the acknowledgements.

STATEMENT OF ETHICAL APPROVAL

The work presented in this thesis was performed with full approval of the ethics committees at both Chelsea and Westminster Hospital and King's College Hospital, London.

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AIMS OF THIS THESIS

The primary aim of this thesis is to develop techniques to allow the quantification of nitric oxide production in humans so that the effect of normal pregnancy on NO production can be studied.

The secondary aims are to establish if there is a relationship between NO production and the presence of IUGR, the onset of labour , the changes in uteroplacental blood flow which occur in pregnancy or the exogenous administration of oestrogen or a nitric oxide donor drug.

HYPOTHESES

Primary

- That nitric oxide production is increased during normal human pregnancy and can be quantified.

Secondary

- That increased nitric oxide production is one of the factors responsible for local vasodilatation of the utero-placental circulation which occurs during early pregnancy.
- That nitric oxide is produced by the developing trophoblast.
- That there is a relative deficiency of nitric oxide production in pregnancies complicated by hypertension and fetal growth retardation
- That administration of a nitric oxide donor may be useful in the treatment of fetal growth retardation and/or preeclampsia.
- That nitric oxide production is related to oestrogen levels

CHAPTER 1 - BACKGROUND

1.1 THE ANATOMY AND PHYSIOLOGY OF EARLY HUMAN PREGNANCY.

1.1.1 Introduction

The process of embryonic development has been of great interest to anatomists and biologists for more than a century. However, due mainly to technical difficulties, most investigators have focused their attention on the anatomy and physiology of the human placenta of the second and third trimesters. There is now a growing body of scientific evidence of a close interaction between the conceptus and its mother as early as the blastocyst stage.

This interaction is of fundamental importance, since abnormalities of this process are associated not only with pregnancy loss but also with the subsequent development of serious complications such as fetal growth retardation, pre-eclampsia or preterm labour (De Wolf *et al* 1975; Frusca *et al* 1989; Strigini *et al* 1995) and even cardiovascular disease later on in adult life (Gennser *et al* 1988; Barker *et al* 1989; Barker *et al* 1990; Williams *et al* 1992).

There are two main processes which contribute to successful implantatiion and the development of a low resistance circulation in the placenta. The first is the remodelling of the spiral arteries by trophoblast infiltration, the second is the branching of the maturing villous vascular tree.

1.1.2 Implantation and trophoblast invasion

As soon as the blastocyst has hatched, the trophoectoderm layer attaches to the cell surface of the endometrium and by simple displacement early trophoblastic penetration within the endometrial stroma occurs (Boyd & Hamilton 1970; Ramsay & Donner 1980). Morphometric analyses have shown a tendency for maximal trophoblastic activity to occur at the centre of the implantation site and, subsequently, to extend centrifugally towards

the periphery (Pijnenborg *et al* 1980; Pijnenborg *et al* 1981). Progressively, the entire blastocyst will sink into maternal decidua and the migrating trophoblastic cells will encounter venous channels of increasing size, then superficial arterioles and, during the fourth week, the spiral arteries (Boyd & Hamilton 1970; Ramsay & Donner 1980).

The trophoblastic cells infiltrate the decidua and reach the deciduo-myometrial junction between 8 and 12 weeks of gestation (Pijnenborg *et al* 1980; Pijnenborg *et al* 1981; Brosens *et al* 1967). Once they reach the myometrium, some cytotrophoblastic cells fuse to form syncytial giant cells, the typical cells of the placental bed (Pijnenborg *et al* 1980; Pijnenborg *et al* 1981). The trophoblast "invasion" is thought to be limited by this fusion process, together with altered expression of collagen, fibronectin and laminin receptors by the cytotrophoblast cell as it descends into the decidua.

The extravillous trophoblast goes on to penetrate the inner third of the myometrium via the interstitial ground substance and alters its mechanical and electrophysiological properties to increase expansile capacity (Pijnenborg *et al* 1981). The process of trophoblastic infiltration of the myometrium is complete before 18 weeks of gestation in most normal pregnancies (Pijnenborg *et al* 1980; Brosens *et al*).

1.1.3 Remodelling of the spiral arteries

During early pregnancy the spiral arteries undergo major morphological change to become the uteroplacental arteries. These are distended, low resistance channels which allow a ten fold increase in blood supply to the feto-placental unit at term (Ramsay & Donner 1980). The morphological changes include swelling of endothelium, loss of myocytes from the media, loss of the internal elastic lamina and disruption of the architecture of the vessels (De Wolf *et al* 1979; Pijnenborg *et al* 1983).

1.1.4 Formation of villous tissue and the definitive placenta

The formation of primary villi starts between 13 and 15 days post ovulation corresponding to the beginning of the second month of gestation after the last menstrual period (Boyd & Hamilton 1970; Hamilton *et al* 1972; Moore 1982). Simultaneously, blood vessels appear in the extraembryonic mesoderm of the secondary yolk sac, the connecting stalk and the chorion (Hamilton *et al* 1972; Moore 1982). By 18 to 21 days post ovulation the villi become branched and the mesenchymal cells within the villous mesoblastic core differentiate into blood capillaries, forming an arteriocapillary venous network (Moore 1982). Around 28 days post ovulation the villous vasculature is connected with the primitive heart and the vascular plexus of the yolk sac by the developing vessels of the connecting stalk.

The early villous tree is essentially composed of long immature trunks, branching (mesenchymal) villi and sprouts. The villi are covered by the syncytio and cyto-trophoblast cells which form an epithelium-like surface layer separating the maternal blood flowing in the intervillous space from the villous core containing the fetal capillaries. The villous core is composed of loosely-packed mesenchymal cells organised in a delicate network of collagen fibres with fibre-free cavities containing macrophages or Hofbauer cells (Castellucci *et al* 1990; Jauniaux *et al* 1992). By nine weeks of gestation, the immature intermediate villi are conspicuous and reach a maximal development by the 16th week when the main trunks are transformed into stem villi (Castellucci *et al* 1990).

Up to the 10th week post-menstruation, which corresponds to the end of the embryonic period, villi cover the entire surface of the chorionic sac. As the gestational sac grows during fetal life, the villi associated with the decidua capsularis, surrounding the amniotic sac, become compressed and degenerate forming an avascular shell called the chorion laeve or smooth chorion (Boyd & Hamilton 1970; Moore 1982). Conversely, the

villous tissue portion associated with the decidua basalis proliferates forming the chorion frondosum or definitive placenta. The stimulus which causes this regression of two-thirds of the original placental ring is not known but it has been suggested that it may be due to a difference in nutritional supply after implantation (Moore 1982).

Morphometric analysis have demonstrated that in the first trimester the villi are quite large in diameter (170µm) and that they progressively decrease in size as term approaches to reach 40µm in diameter (Fox 1978). The number of branching villi increases gradually from 6 to 15 weeks of gestation (Jauniaux *et al* 1991). A decrease in this sprouting capacity is observed during the second trimester and so the villous sprouts are more numerous and longer (50-90µm) at the end of the embryonic period than in later pregnancy. By term the stem arteries have branched up to 14 times, to provide a vascular surface area of about $8m^2$. This branching also leads to a decrease in the mean thickness of the villous barrier and diffusion distance between the two circulations, further enhancing the efficiency of the placenta.

1.1.5 Development of the uteroplacental circulation

The existence of two separate circulatory systems is the only structural component that all placentas from different species of viviparous vertebrates have in common (Benirschke & Kaufmann1990). Continuous circulation of maternal blood inside the intervillous chamber is a dynamic process that requires a continuous adaptation of the individual placental cotyledon to the blood flow offered to it by the corresponding uteroplacental artery.

Classically it is taught that as soon as the blastocyst has implanted, a number of endometrial vessels are opened by phagocytic activity of trophoblast and maternal blood enters the intervillous space, establishing complete uteroplacental circulation (Boyd & Hamilton 1970; Ramsay and

Donner 1980; Hamilton *et al* 1972; Moore 1982). This hypothesis is challenged by the results of Hustin and Schaaps. Their investigations of the placenta in vivo by means of transvaginal sonography, intervillous hysteroscopy and phase contrast microscopy of chorionic villous sampling material, suggest that there is no real continuous blood flow in the intervillous space before 12 weeks of gestation (Hustin & Schaaps 1987; Hustin *et al* 1988). They have suggested that before this stage, the tips of the spiral arteries are obstructed by intravascular trophoblastic plugs and the intervillous space is bathed by a clear fluid possibly made of filtered plasma and uterine gland secretions. Around 12 weeks of gestation, the trophoblastic plugs are loosened and dislodged, allowing a continuous blood flow circulation to develop in the intervillous space.

Supporting evidence for this theory has come from pathological examination of hysterectomy specimens with in-situ pregnancies. Barium sulphate perfusion confirms that the growing embryo is totally isolated from the maternal circulation by the trophoblastic shell until 13 weeks of gestation (Jones & Fox 1991).

Further support for the idea of trophoblastic plugging during the first trimester has come from physiological studies of placental and endometrial pO_2. These show that between 8 and 10 weeks of gestation, placental pO_2 levels are significantly lower than endometrial pO2 levels, whilst between 12 and 13 weeks, pO2 levels are similar at both sites (Rodesch *et al* 1992).

Non-invasive studies have been conducted *in vivo* using Doppler ultrasound which have also been able to demonstrate major changes in the waveforms obtained from both the uterine and umbilical arteries during early normal gestation and in complicated pregnancies, these are discussed below in greater detail.

The mechanisms and mediators involved in the trophoblast invasion and

uterine vascular adaptation of normal early pregnancy remain to be fully elucidated.

1.2 PATHOPHYSIOLOGY OF PREGNANCIES COMPLICATED BY HYPERTENSION AND FETAL GROWTH RETARDATION.

1.2.1 Pre-eclampsia.

Pre-eclampsia is defined as blood pressure consistently higher than 140/90 with proteinuria greater than 1+ on dip-stick testing. It occurs in 2-5% of the obstetric population and is a leading cause of both fetal and maternal morbidity and mortality (Redman 1991). It is now recognised that pre-eclampsia is more than just pregnancy induced hypertension (Roberts & Redman 1993) and that widespread endothelial dysfunction is central to the disease process (Roberts *et al* 1989; Roberts *et al* 1994). It has been suggested that this damage is mediated by a serum factor since pre-eclamptic sera shows increased binding of IgG and IgM to endothelial cells in culture when compared to control sera (Rappaport 1990). The function of endothelial cells from pre-eclamptic women is, consequently, the subject of many current research projects.

1.2.2 Platelet Function

Platelet function is known to be altered in early normal pregnancy. Changes such as reduced angiotensin II binding sites may be found as early as 5-8 weeks gestation (Baker *et al* 1992). Later on , even in normal pregnancy, there is increased platelet turnover and intravascular coagulation as demonstrated by raised fibrin degradation products (FDPs), Beta-thromboglobulin, fibrinopeptide and platelet activating factor (PAF) levels in plasma (Gerbasi *et al* 1990). Whilst thrombocytopaenia is a well recognised sequelae of severe pre-eclampsia (Janes 1992) what is less well known is that platelet volume decreases for up to a week before the onset of clinically significant hypertension (Walker *et al* 1989). Whilst interesting, this observation is of little clinical use since there is an enormous overlap in the actual values of platelet volume when pre-eclamptics are compared to normotensives. Only serial values appear to be helpful.

Other more subtle tests of platelet function have therefore been investigated and it has been found that there is evidence of platelet dysfunction as early as 12 weeks gestation in pregnancies subsequently complicated by pre-eclampsia (Zemel *et al* 1990). This particular study by Zemel et al found an exaggerated increase of intraplatelet free calcium in response to arginine vasopressin in women who subsequently developed pre-eclampsia. However there does not appear to be a significant difference in either basal or stimulated cytosolic intraplatelet calcium concentrations between normotensive and hypertensive women in the third trimester (Van der Post *et al* 1993; Barr *et al* 1989). Spontaneous and stimulated (commonly by ADP) platelet aggregation *ex vivo* are additional ways of expressing platelet function and it is known that in other diseases, such as myocardial infarction, these indices may be helpful in predicting prognosis (Trip *et al* 1990).

At present there is no clear understanding of the mechanisms by which alterations in platelet function occur either in normal pregnancy or in pre-eclampsia.

1.2.3 Fetal growth retardation

Another frequent complication of pregnancy (either with or without pre-eclampsia) is fetal intra-uterine growth retardation (IUGR). Unfortunately there are problems of definition and identification of babies with IUGR. Birthweight below the 10th centile for gestational age is commonly used to define IUGR due to its ready availability and ease of measurement. In reality this identifies a group of babies which are small for gestational age (SGA), many of whom are "normal small" rather than growth retarded (Goodlin 1990). A better definition of growth retardation post-natally uses an index of body mass such as the Ponderal Index (birthweight / length^3) which is more closely related to perinatal morbidity and mortality than is the birthweight percentile alone (Miller 1972; Walther & Raemakers

1982). Antenatally it is possible to measure fetal head circumference (HC), abdominal circumference (AC) and femur length (FL) using ultrasound. Whilst use of these multiple parameters has now gained widespread use in the estimation of fetal weight (Hadlock *et al* 1985) the single most sensitive parameter to detect growth retardation is the AC percentile with a positive predictive value of 50%. To improve upon this pick-up rate investigators have used Doppler ultrasound to gain extra information on blood flow in the fetus and utero-placental circulation. The background to this is described in some detail in section 1.4. It is now widely accepted that the use of Doppler ultrasound in selected high risk cases will help distinguish the fetus with true growth restriction from the normally small fetus (Chang & Cheng 1994; Bates *et al* 1996; Harrington *et al* 1995).

1.2.4 Placental changes.

In pregnancies complicated by hypertensive disease and/or fetal growth retardation it has been shown that there are characteristic histological changes within the placental vasculature (Gerretsen *et al* 1981). These include proliferation of myo-intimal cells and necrosis of the medial cells. Fat accumulates within the myo-intimal cells to form the so-called foam cells which subsequently disintegrate to be engulfed by macrophages. Intraluminal fibrin and platelet deposition with luminal thrombosis are also present in many cases. These appearances were described by de Wolf, one of Brosen's group, as "acute atherosis of pregnancy" (De Wolf *et al* 1975). Khong, another member of the same group, went on to show using immunohistological techniques that this focal endothelial cell disruption seen in pre-eclampsia is caused by the trophoblast (Khong *et al* 1992).

In addition to this failure of trophoblast invasion there must also be a defect with the maturation of the villous tree in affected pregnancies, since the number of stem villous arteries is reduced in IUGR affected pregnancies with abnormal umbilical artery Doppler measurements (Giles

et al 1985; McCowan *et al* 1987).

The mechanisms underlying this picture of "acute atherosis" and poor placental development are not yet fully understood.

1.3.1 The use of ultrasound

As mentioned earlier, ultrasound may be used non-invasively to assess the developing uteroplacental circulation. Diagnostic ultrasound has been used widely in obstetric practice, without adverse effects, for many years now. Indeed, B-mode scanning is now a part of routine antenatal care in the UK. Over recent years technology has advanced rapidly and there is now widespread availability of Doppler ultrasound as well. However, since Doppler ultrasound uses higher energy levels than B-Mode scanning, its widespread introduction has re-opened the safety debate on ultrasound.

1.3.2 Safety

Diagnostic ultrasound has the theoretical potential to damage tissue through two physical processes; heating and cavitation. Although neither has been reported using current diagnostic techniques, it remains sensible to keep fetal exposure to a minimum. In practice this means using the lowest power setting for the shortest duration of time.

Several measurements of beam intensity are currently in use. The most relevant to the use of pulsed Doppler or colour flow imaging is the in situ Spatial Peak Temporal Average (SPTA) (De Wolf *et al* 1979). Currently the US Food and Drugs Administration (FDA) guidelines recommend a maximum intensity of 94mW/cm^2. All Doppler data presented here was obtained using colour flow directed, pulsed Doppler ultrasound from an Acuson 128 (Acuson, Mountain View, California) with a 7mHz transvaginal transducer or abdominally with a 5 mHz curvi-linear array with a high pass filter of 100 Hz. The high pass filter removes high amplitude, low frequency signals such as those arising from the vessel wall. The beam intensities on the Acuson 128 have been measured by the National Physical Laboratory as an SPTA of 36 mW/cm^2 at low (obstetric) power setting for colour Doppler and 76mW/cm^2 when pulsed

Doppler is used.

It has been shown that the use of colour imaging to place the sample volume gate accurately before recording pulsed Doppler signals has several advantages (Arduini *et al* 1990). The number of reliable recordings is increased, the intra and inter-observer variation reduced, the observation time shortened and the ultrasound energy exposure reduced. This was the technique used for all Doppler readings in this study.

1.3.3 Transvaginal versus transabdominal approach

In early pregnancy when the uterus lies within the pelvis the best images of the utero-placental vasculature are obtained using a vaginal transducer because the probe is closer to the structures being studied. This allows a higher frequency probe to be used, so improving resolution when compared to the abdominal view. In later pregnancy the trans-abdominal route is more appropriate as it allows greater depth of vision. Using the most appropriate technique minimises tissue ultrasound exposure without compromising image quality.

1.3.4 Definition of Doppler ultrasound

Doppler ultrasound may be defined mathematically as a sound wave of frequency "f", moving at a speed "c" which, on striking an object moving with velocity "v", gives rise to reflected waves of a different frequency. The reflected waves are said to have undergone a frequency change (Doppler shift) "dF" defined by the formula :-

$$dF = \frac{2 f v \cos\theta}{c}$$

where θ is the angle of insonation between the direction of motion and the incident beam, and the source and receiver are assumed to be very close

together. In practice this means using an angle of insonation less than 50^{o} whenever possible since beyond this any error will be magnified exponentially.

Continuous wave Doppler ultrasound was the first to be developed and is a constant beam of a single, fixed frequency. When reflected from blood cells moving through vessels it may be used to build up a spectral display known as a flow velocity waveform. The disadvantage of this technique is that reflected waves will be generated from structures at a variety of depths below the transducer. This means that it is difficult to be sure exactly where the signal is arising from.

Pulsed wave Doppler is a more recent development and is emitted in short pulses from a transducer which also acts as receiver. It is therefore possible, knowing the speed of ultrasound in tissue, to specify a given depth from which reflected waves will be recorded. This "sample volume gate" can then be displayed on a B-mode image and positioned over a specific vessel with confidence. In addition this will allow the angle of insonation of the Doppler beam on the blood vessel to be visualised so that accurate velocity measurements may be taken.

A further advance was the introduction of colour flow Doppler mapping which represents the Doppler shift by a colour spectrum. Each pixel of a B-mode image may then be assigned a colour to indicate speed and direction of movement relative to the transducer. This technique allows very small blood vessels to be identified reliably and the pulsed wave sample volume gate directed accurately to obtain meaningful results. The other advantage of colour flow imaging is that it is of a lower power intensity than pulsed wave Doppler and by using it to visualise first the desired vessel, the tissue energy exposure is minimised.

1.3.5 Calculated indices in common use and their reliability:-

A spectral display of the reflected pulse wave is reproducible and characteristic of the vessel from which the readings are taken. Unfortunately actual volume flow calculations are not so reliable because even small errors in measuring the vessel diameter or velocity will be much amplified in the calculation. Care must be taken when recording mean velocity too, since if the angle of insonation is greater than 50^{o}, the Cosine calculation will introduce large errors. For these reasons it is usual to calculate derived indices from the ratio of two or more velocities which therefore will be "angle independent".

The commonest in clinical use are:-

Systolic-Diastolic Ratio	S/D	= A/B	(Stuart & Drumm)
Resistance Index	RI	= (A-B)/A	(Pourcelot)
Pulsatility Index	PI	= $(A-B)/v_{mean}$	(Gosling & King)

where A is the peak systolic velocity, B the end diastolic velocity and v_{mean} the time averaged mean velocity.

The variability of Doppler measurements of uterine artery and umbilical artery blood flow from day to day is known to be small (Hastie *et al* 1988). It has also been found that the position of the sample gate along the length of the umbilical cord does not systematically alter the reading of pulsatility index (PI) obtained (Ruissen *et al* 1990). Intra and inter-observer effects have also been examined and are also known not to produce significant errors in the derived indices such as PI and RI although there is a greater tendency for variation in measurements of peak velocity (Thomas *et al* 1991; Tessler *et al* 1990). The PI in the umbilical artery is a reproducible measurement is known to correlate well with placental resistance to blood flow (Thompson *et al* 1986).

1.4 FETAL AND UTERINE ARTERY DOPPLER WAVEFORMS

1.4.1 Uterine artery Doppler waveforms

Measurement of uteroplacental blood flow using Doppler ultrasound was pioneered by Campbell in London (Campbell *et al* 1983) and Schulman in New York (Schulman *et al* 1986). In normal women they showed a marked fall in resistance during the second half of the menstrual cycle and a further fall during pregnancy. They also noted the disappearance of the early diastolic notch with advancing gestation, a feature characteristic of the flow velocity waveform obtained from the uterine artery in non-pregnant women.

Studies which have used Doppler ultrasound to non-invasively screen women for these changes have shown a definite link between abnormal flow velocity waveforms in the uterine arteries in the second trimester and the subsequent development of pre-eclampsia and IUGR (Campbell *et al* 1983 and 1986; Cohen-Overbeek *et al* 1985; Trudinger *et al* 1985a and 1985b; Arduini *et al* 1987; McCowan *et al* 1988; Steel *et al* 1990; Bewley *et al* 1991; Bower *et al* 1992; Fairlie *et al* 1992; Lin *et al* 1995). However, this association is not strong enough to support routine screening of a low risk obstetric population outside of research studies (Beattie & Dornan 1989; Dekker & Sibbai 1991; Davies *et al* 1992). This is particularly true since, as yet, there is no effective intervention to offer "at risk" women, even when identified early in pregnancy.

Nevertheless, with a positive predictive value of between 20 and 40%, utero-placental Doppler screening is a useful research tool to identify a group of high risk women (Arduini *et al* 1987; Beattie & Dornan 1989; McParland *et al* 1990; Dekker & Sibbai 1991; Bewley *et al* 1991; Harrington *et al* 1991). The abnormalities that best predict which women will subsequently develop pre-eclampsia or IUGR are, a uterine artery FVW with a steep upward slope to the systolic peak, an early diastolic notch, and a low end diastolic velocity (bilaterally). The widely agreed

definition for "abnormal" FVW of the main uterine arteries is an RI greater than 0.6 with bilateral post-systolic notching of the FVW persisiting beyond 20 weeks gestation.

There is a correlation between abnormal uterine artery blood flow and placental histological abnormalities. Investigators have clearly shown there is an increase in fibrinoid necrosis, vessel wall muscle thickness and placental infarction in the presence of abnormal uterine artery FVWs (Guzman *et al* 1995). Indeed in this study up to 60% of the placental mass was infarcted at delivery when abnormal uterine artery blood flow was noted, compared to 15% in normal controls.

1.4.2 Fetal Doppler

Doppler ultrasound has been increasingly used to evaluate fetal blood flow in the human since the early 1980s (Eik-nes *et al* 1980; Stuart *et al* 1980). Normal ranges have been established for PI, RI and mean velocity in many fetal vessels but those which have received the most attention are the umbilical artery, descending aorta, carotid artery and middle cerebral artery (See Appendix III for normal values).

Absence of end diastolic velocities in the umbilical cord is the single most widely investigated finding and is known to be associated with fetal hypoxia, acidosis and subsequent poor outcome (Nicolaides *et al* 1988, Trudinger *et al* 1985a and 1985b, Cohen-Overbeek *et al* 1985, Fairlie *et al* 1992). In addition IUGR babies "redistribute" blood flow in a brain sparing way, with increased middle cerebral artery flow (lowered resistance) and decreased aortic flow (raised resistance). Studies have shown that hypoxia is highly likely in a fetus which is showing this pattern of blood flow redistribution (Trudinger *et al* 1985a; Cohen-Overbeek *et al* 1985, Nicolaides *et al* 1988).

These findings prompted studies using Doppler as a screening test for

IUGR. It is clear from these studies that, in an unselected population, there is no benefit of routinely measuring umbilical artery blood flow (Newnham *et al* 1991; Davies *et al* 1992). However, in a selected population of SGA babies, Doppler monitoring has been shown to reduce intervention rates and improve outcome (Almstrom *et al* 1992; Pattinson *et al* 1994; Tyrell *et al* 1990). Normal ranges for middle cerebral artery, aortic and umbilical artery blood flow indices are given in Appendix 3.

1.5 NITRIC OXIDE AND VASCULAR TONE

1.5.1. Background

Up until the 1970's the vascular system was considered to be in a state of active vasoconstriction due to tonic sympathetic activity on arteriolar resistance vessels. Vasoactive substances which could alter this basal tone were all thought to be synthesised elsewhere and then transported in the blood to their site of action.

The human body contains a great deal of endothelial cells, around 1000-1500g on average, which would cover an area equal in size to 6 tennis courts (Henderson 1991) it was thought to be little more than an inert lining structure right up until the 1970's. However, in 1976 Moncada discovered prostacyclin (PGI_2), a vasodilator synthesised by cells of the vessel wall (Moncada *et al* 1976). It was subsequently shown to act by stimulating adenylate cyclase to increase levels of cyclic AMP (Tateson *et al* 1977). This finding stimulated research interest in the vascular endothelium and led on to the current understanding of the crucial role which the vascular endothelium plays in the control of vascular tone in man (Henrich 1991).

1.5.2 The role of endothelial cells in the control of vascular tone

In 1980 Furchgott and Zawadski published one of the key discoveries in vascular biology; the obligatory role of endothelial cells in the relaxation of arterial smooth muscle in response to acetylcholine (Furchgott & Zawadski 1980). This finding explained the paradox of acetylcholine behaviour in experimental preparations; namely that it had disparate effects, either vasodilatation or vasoconstrictor *in vitro*, yet acted as a vasodilator *in vivo*. While it would relax pre-contracted rabbit aortic rings it had no effect on aortic strips. Furchgott and Zawadski noticed the endothelial cell damage which occurred during the preparation of aortic strips and this led then to postulate the existence of an endothelial derived relaxing factor (EDRF) mediating the vasodilatation of

acetylcholine.

Subsequently it was found that a wide variety of substances could release EDRF including neurotransmitters, platelet products, coagulation products, local hormones and physicochemical stimuli (Henderson 1991; Ignarro 1990; Moncada *et al* 1991).

1.5.3 Production of nitric oxide by the nitric oxide synthases

Following its discovery, much effort was directed towards identifying the chemical nature of EDRF and in 1987 experimental evidence was produced showing it to be identical to nitric oxide (NO) (Palmer *et al* 1987). NO is produced *in vivo by* the enzyme nitric oxide synthase (NOS) using L-arginine as substrate (Palmer *et al* 1988). This process was first descibed in the vascular endothelial cell and is illustrated schematically in figure 1.1 whilst figure 1.2 gives the biochemical structures of the molecules involved.

NOS exists in two identifiable isoforms (Moncada *et al* 1991). The constitutive form (cNOS) was the first identified, in bovine endothelial cells and rat cerebellum. It requires calcium-calmodulin and NADPH in order to function and so has been called calcium dependent by some authors (Busse & Mulsch 1990; Nathan, 1992).

In 1987 Hibbs demonstrated that L-arginine was necessary in order for mouse macrophages to exert a cytotoxic effect against tumor cells (Hibbs, Vavrin & Taintor 1987). This effect was subsequently confirmed as being mediated through the synthesis of NO (Hibbs *et al* 1988) by a synthase enzyme not requiring calcium-calmodulin and only found after stimulation with cytokines (Moncada *et al* 1991). This enzyme, the inducible nitric oxide synthase (iNOS), can also be produced by cultured endothelial and smooth muscle cells following administration of lipopolysaccharide. The inducible enzyme is not controlled by calcium levels because, unlike the

cNOS, it binds calmodulin tightly at resting intracellular levels of calcium (Cho *et al* 1992). Instead it is activated by induction of new messenger RNA protein, hence the term inducible being used (Xie *et al* 1992). Both isoforms have been reported in a wide range of tissues and examples are shown in Table 1.1 below.

Table 1.1

Some of the sources and activators so far identified of constitutive and inducible nitric oxide synthases in man.

cNOS Enzyme	**iNOS Enzyme**
Endothelial cells, astrocytes, circulating neutrophils, mast cells, platelets, pancreatic islet cells, cardiac myocytes, some peripheral NANC neurones and some central neurones	Macrophages, Kupffer cells, hepatocytes, vascular smooth muscle cells, fibroblasts, endothelial cells, cardiac myocytes, activated neutrophils, articular chondrocytes

Agents which stimulate cNOS act immediately and have a short duration of action. In contrast iNOS activation, because it requires new protein synthesis, takes several hours and may persist for many hours or days (Xie *et al* 1992). How this production is turned off is not yet fully understood but it has been hypothesised that some form of negative feedback exists regulating total NO production (Moncada *et all* 1991).

Figure 1.1

Schematic representation of nitric oxide (NO) production from L-arginine by the enzyme nitric oxide synthase (NOS) in the vascular endothelial cell. The NO activates the soluble guanylate cyclase (SGC) to convert guanosine triphosphate (GTP) to cGMP and hence cause vasodilatation of the vascular smooth muscle cell.

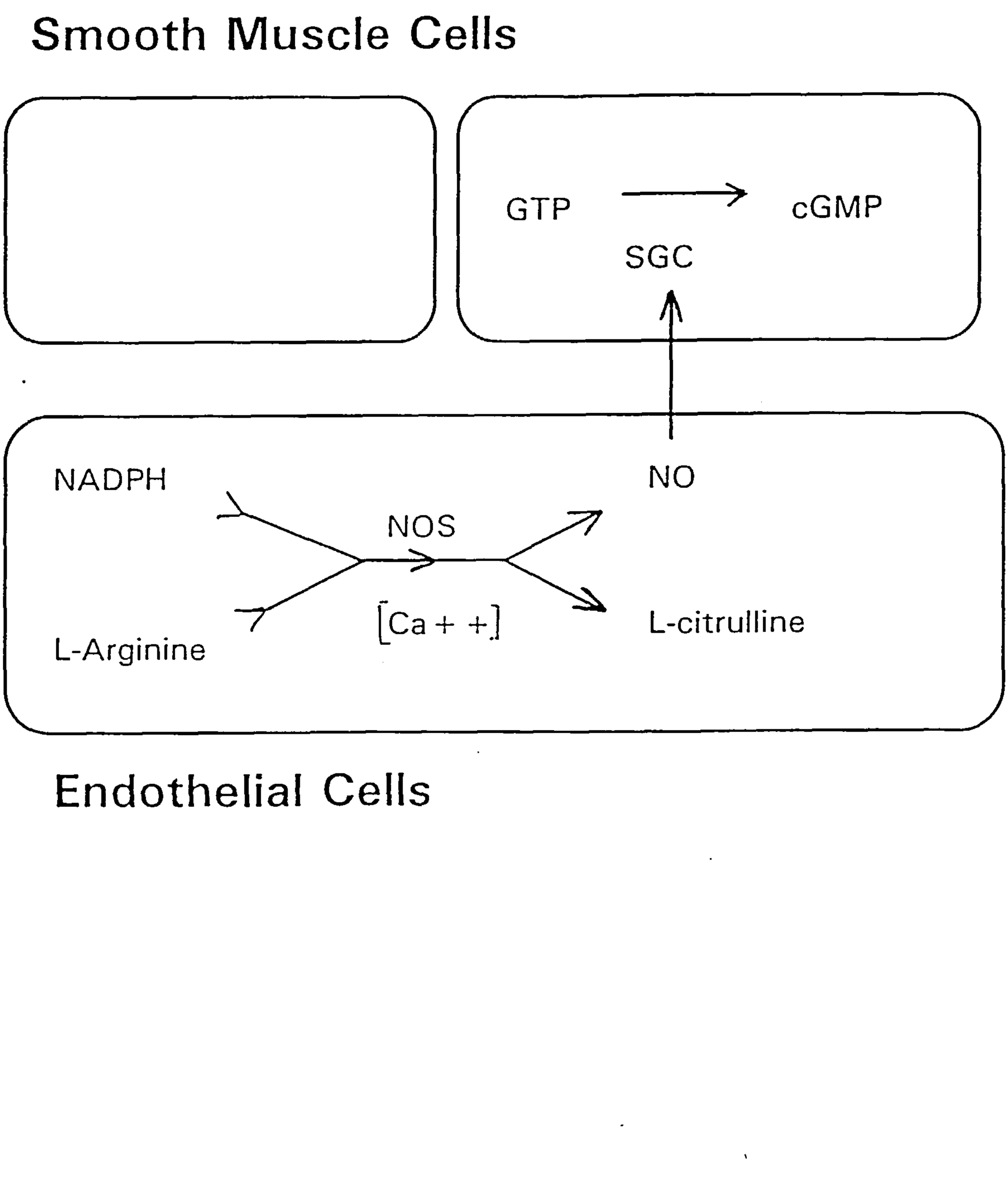
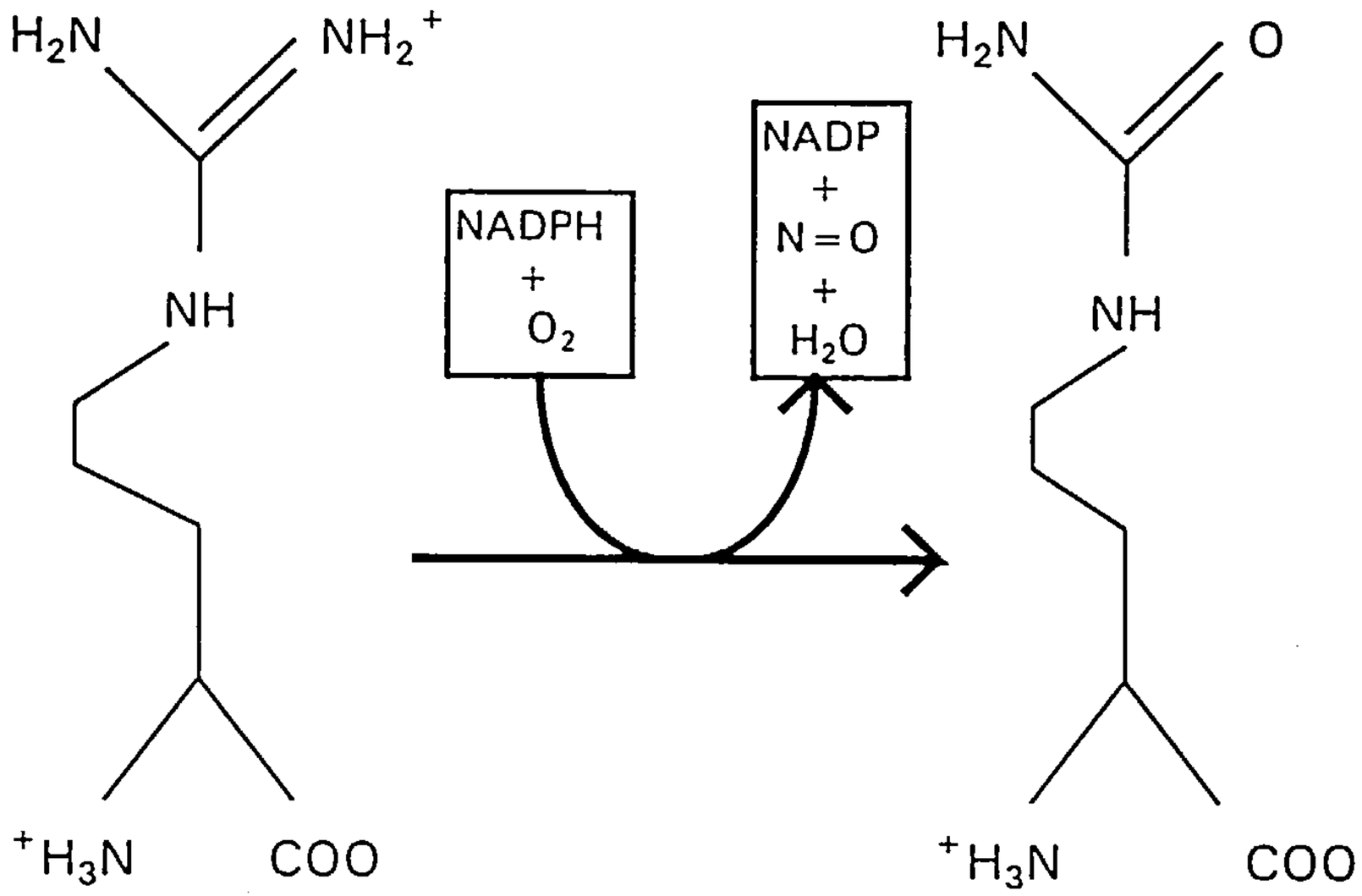


Figure 1.2

Diagrammatic representation of NO synthesis showing the biochemical structures of L-arginine and L-citrulline.



L-arginine

L-citrulline

1.5.4 Intracellular actions of nitric oxide

Smooth muscle contraction is initiated by free cytosolic calcium binding to calmodulin and the resultant complex activating myosin light chain kinase. This enzyme phosphorylates the myosin light chain permitting activation of the magnesium dependent ATPase on the myosin cross bridge of actin. Hydrolysis of ATP follows and tension develops by changes in the cross bridges producing relative movement of actin and myosin. Tension is regulated by the number and rate of turnover of active cross bridges.

In vascular smooth muscle the principle target for NO is the soluble guanylate cyclase (Ignarro 1990). Guanylate cyclase is activated by nitrosation of its haem moiety which then leads to a rise in intracellular cGMP. The cGMP probably enhances phosphorylation of key proteins involved in calcium handling to effect relaxation. The exact mechanism of action is still uncertain but research so far has shown that cGMP and its dependent protein kinase will inhibit:-

i) Calcium entry into cells through receptor operated channels (Godfraind 1980)
ii) Calcium release from sarcoplasmic reticulum (Kobayashi *et al* 1985)
iii) Production of inositol triphosphate (Rappaport 1986)
iv) Activation of sarcolemmal calcium ATPase (Popescu *et al* 1985)

In the macrophage the NO produced in response to cytokines will bind to Fe-S proteins (Nathan 1992; Hibbs *et al* 1990)and also react with superoxide anion. This latter reaction may lead to generation of powerful oxidants such as nitrogen dioxide or hydroxyl radicals which can in different circumstances be either protective or damaging (Beckman *et al* 1990). The reasons for this apparent paradox remain unknown.

1.5.5 Inhibitors of nitric oxide production

A wide range of substances have already been developed or discovered which can inhibit NOS. These include:-

I) Substrate inhibitors (L-arginine analogues) such as Nω-monomethyl-L-arginine (L-NMMA) or Nω-nitro-L-arginine methylester (L-NAME) which inhibit NO synthesis both *in vitro* and *in vivo* (Palmer *et al* 1988; Moncada *et al* 1991).

ii) Flavoprotein binders such as diphenylene iodinium which interferes with the co-factors necessary for the production of NO (Popescu *et al* 1985). This agent also binds other vital cellular flavoproteins so is non-specific and therefore not clinically useful.

iii) Calmodulin binders will also suppress NOS function but again are too general in their effects to be useful (Bredt & Snyder1990).

iv) Carbon monoxide (CO) can bind to the haem moiety in NOS and render it inactive (Nathan 1992).

v) The inducible enzyme can also be inhibited by corticosteroids (Di Rosa *et al* 1990) and certain cytokines such as interleukins 4 and 10 and transforming growth factor-β (Ding *et al* 1990). Experiments pre-treating macrophages with corticosteroids before introducing cytokines have shown reduced expression of iNOS (Radomski *et al* 1990a) and production of NO (Rees *et al* 1990).

In addition, there are known to be endogenous inhibitors of NOS in humans. The most widely studied being ADMA (MacAllister & Vallance 1993). Elevated ADMA levels were first shown in chronic renal failure (Vallance *et al* 1992). Subsequent studies have shown halving of basal ADMA activity in normal pregnancy and an increase in activity in cases of

pre-eclampsia (Fickling *et al* 1993). Whether this is a primary or secondary phenomenon is not known at present.

1.5.6 Physiological factors affecting NO production

The substrate inhibitors have been most useful in probing the role of NO and much of the initial research into control of vascular tone was performed using *in vitro* or *in vivo* animal studies with these agents or preparations of vessel without endothelial cells.

Experimental evidence has not only confirmed that NO is produced in response to a wide variety of chemical substances by activation of NOS (see table 1.2 below) (Moncada 1992; Moncada *et al* 1991). In addition studies have also demonstrated the effects on NO production of blood flow within a vessel (Pohl *et al* 1986; Rubanyi *et al* 1986). Indeed, shear stress may well be one of the most important stimuli to NO production in the luminal endothelium.

Table 1.2.

Activators of nitric oxide synthase leading to altered production of NO

cNOS Enzyme	iNOS Enzyme
Through calcium/calmodulin ACh, ADP, bradykinin, thrombin, endotoxin, leukotrienes, PAF, calcium ionophores, physicochemical stimulation and some amino acids	**Through induction of mRNA** Endotoxin, Lipopolysaccharide, interferon, TNF, IL-1

Flow induced vasodilatation, long known but unexplained by physiologists, is substantially accounted for by endothelial NO production (Henderson 1991). The concept of endothelium responding to shear stress was first put forward by Rodbard (Rodbard 1975) but not confirmed by experimental evidence until some years later. Studies confirmed that removal of endothelium attenuated the responsiveness of coronary arteries to changes in flow rate (Pohl *et al* 1986) and inhibited reactive hyperaemia (Inque *et al* 1989). In addition, methylene blue and haemoglobin (non-specific inhibitors of NO) have been shown to block flow dependent dilatation in dog femoral (Pohl *et al* 1986) and rabbit ear vessels (Griffith *et al* 1984). Interestingly, pulsatile flow further enhances release of NO (Rubanyi *et al* 1986; Pohl *et al* 1986) but experiments using cultured endothelial cells have shown that hydrostatic pressure alone does not alter NO production (Kelm *et al* 1991). It has been proposed that the endothelium senses shear stress through potassium channels sensitive to flow (Olesen *et al* 1988), although whether this then leads directly to NO release is unknown.

Oxygen tension is another factor which may play a role in controlling NO production. Whilst much of the vasodilatation seen in response to ischaemia is due to accumulation of metabolites, it is clear that endothelial derived vasoactive compounds also play an important role. NO, PGI_2 and PGE_2 are all released from hypoxic cultured endothelial cells (Roberts *et al* 1981) and have been shown to stimulate coronary vasodilatation in myocardial ischaemia (Park *et al* 1992).

There is also a growing body of evidence that oestrogen may directly affect NO production. This is an exciting possibility since it could explain some of the cardiovascular adaptations of pregnancy and the cardioprotective effects of HRT given to post-menopausal women. This is discussed in more detail later in section 1.6.2.

1.5.7 The physiological role of nitric oxide in man

Confirmation of the physiological role of the L-arginine - NO pathway in man came from studies using forearm venous occlusion plethysmography to show blood flow changes in response to L-NMMA infusion (Vallance 1989). This produced a 50% reduction in basal flow with an attenuation of acetylcholine induced vasodilatation.

In addition to its role as a vasodilatator, NO has profound effects on platelet adhesion and aggregation in man (Radomski *et al* 1987). Intraplatelet calcium levels appear to play a significant role in determining the final level of response (Radomski *et al* 1990b) as do intraplatelet cGMP levels (Pohl & Lamontagne 1991). Further confirmation of these observations has come from studies which show a decrease in platelet activation following administration of NO donor drugs (Benjamin *et al* 1991; Doni *et al* 1991; Zembowicz *et al* 1990).

Hibbs has demonstrated that activated macrophages produce NO which attacks the Fe-S groups of enzymes containing this non-haem Fe sub-unit (Hibbs *et al* 1990). The most important example of this type of enzyme is ribonucleotide reductase, the rate limiting step in DNA synthesis and he postulated that this interaction may explain the cytotoxicity of macrophages for some tumor cells (Hibbs *et al* 1988). Why this process should affect some cells but not others is a matter for speculation at present .

It is now appreciated that NO has significant effects not only as a vasodilator but also as an inhibitor of platelet aggregation, a neurotransmitter and mediator of host immunity (Moncada *et al* 1991).

1.5.8 Nitric oxide in pathological processes

Most research on NO in disease has concentrated on two main areas, namely endotoxic shock and hypertension/atherosclerosis. This has been helped by good experimental models for these diseases existing.

In the pathlogical condition of septic shock the endotoxins induce iNOS activity leading to an over production of NO and systemic hypotension which fails to respond to volume replacement or vasoconstrictor agents (Moncada *et al* 1991; Nathan 1992). Studies have shown increased levels of nitrite and nitrate in plasma from patients with endotoxic shock which is further evidence of the increased NO production (Ochoa *et al* 1991). It has been shown that these high levels of NO can cause local endothelial cell damage (Palmer *et al* 1992). Recently, reports of successful treatment with inhibitors of NO (such as L-NMMA) and steroids have appeared in the literature, which suggest a possible role for these drugs in the management of septic shock.

It is widely accepted that infusion of NO inhibitors into normotensive animals produces a rise in peripheral resistance and a consequent increase in blood pressure (Suzuki *et al* 1992; Baylis *et al* 1992). This is unsurprising since disordered endothelial response is known to be a feature of animal models of hypertension, atherosclerotic vascular disease and diabetes (Henderson 1991). Organ bath experiments have shown that atherosclerotic arteries produce less NO both in the basal state and following stimulation than normal control arteries (Chester *et al* 1990). Blockade of NO with L-NMMA in isolated perfused rabbit hearts and hindlimbs not only caused increases in vascular resistance but also increased platelet aggregation (Pohl & Lamontagne1991).

1.5.9 Quantification of nitric oxide activity

Nitric oxide, being a labile gas, has a very short half life (Ignarro 1991) and it has been suggested it may be inactivated through a variety of

pathways. These include rapid oxidisation to form nitrite and nitrate (Wennmalm *et al* 1992) and binding to both haemoglobin (Wennmalm *et al* 1992) and albumin (Stamler *et al* 1992).

Stamler proposed a reservoir of s-nitroso-serum albumin from which NO could be liberated to maintain vascular tone (Stamler *et al* 1992). However, whilst this is undoubtedly an abundant and long lived adduct there is no other evidence to support this hypothesis at present. Others have concentrated on the long established observation that haemoglobin will inhibit the action of NO. Work by Wennmalm demonstrated that whilst NO in plasma degrades rapidly to nitrite and nitrate in a 5:1 ratio, in whole blood it is converted, almost quantitatively, to nitrate with the additional formation of nitrosyl-haemoglobin and methaemoglobin (Wennmalm *et al* 1990). He also noted that the relative proportion of NO which is inactivated by each process varied with oxygen availability (Wennmalm *et al* 1992). Subsequent work by another group has confirmed that although in aqueous solutions NO is converted to nitrite, the presence of oxyhaemoglobin oxidises this almost completely to nitrate (Ignarro 1993).

This rapid and variable inactivation poses problems both for the measurement of NO activity *in vivo* and for the administration of NO in pharmacological studies.

Some investigators have measured NO directly using intra-cellular probes (Malinski & Taha1992) or chemiluminesence of exhaled breath (Gustafsson *et al* 1991; Leone *et al* 1994b). Whilst these methods work well under highly specialised conditions, they are less suitable for the study of NO in complex *in vivo* systems. Here, one is forced to measure one of the metabolites or oxidation products of NO, such as nitrite or nitrate.

The nitrogen balance of the body has been the subject of much study by

nutritionalists. It was initially noticed that external influences, particularly diet, can profoundly alter levels of both nitrite and nitrate in plasma (Tannenbaum *et al* 1978). Since the concentrations in drinking water vary widely from area to area, even this has to be taken into consideration (Hie & Young 1989).

The plasma levels of nitrite are known to be extremely low (approaching zero) in most normal people but can climb to 100 μmolar in cases of septic shock (Ochoa *et al* 1991). By comparison the basal plasma nitrate levels are higher than nitrite at around 25 to 75 μmolar (Lee *et al* 1986). These levels can rise further in disease states and are decreased by 50% following a 12 hour fast to between 20 and 40 μmolar. This level can be reduced still further by prolonged infusion of L-NMMA (A.M.Leone, personal communication) suggesting that plasma nitrate does reflect NO production in fasted subjects.

Further evidence to support this comes from large nutritional studies. These have shown that the total daily excretion of nitrate by humans ranges from 1000 to 2400 mol/day and comparing this to the total dietry intake over the same period leaves a mean excess of production of 870 mol/day (Lee *et al* 1986). It is likely that this excess nitrate production represents, at least in part, the basal NOS activity in the human body.

The conventional techniques for measurement of nitrite levels are based upon the Greiss reaction (Loquet *et al* 1991) in which nitrite is used to diazotise an aromatic amine such as sulphanilamide. The resultant diazo compound is coupled with N-ethylene diamine dihydrochloride to form a purple azo dye which can be quantified spectro-photometrically. Many different variations to the basic technique have been suggested over the past 40 years and have varied considerably in their complexity and sensitivity. One important adaptation allowed the quantification of nitrate levels by treating the sample with a powerful reducing agent such as

cadmium. This converts higher oxides of nitrogen to nitrite allowing measurement of NO_X. A simple subtraction of untreated nitrite levels will then give an estimate of nitrate concentration (Green *et al* 1982; Davison & Woof 1978). Unfortunately, all the techniques based on the Greiss reaction have a low sensitivity for nitrite and the nitrate and tend to produce variable results.

The most sensitive and specific methods available for the determination of nitrite and nitrate in plasma are the gas chromatography- mass spectrometry (GC-MS) based assays. However, mass spectrometry is rarely used routinely because of two main reasons. The first is that nitrite and nitrate are only measured after separate derivatisation and purification procedures which increases the risk of sample contamination and erroneous results. The second is the high cost of purchasing the necessary equipment and running it. As a result many groups have now moved on to apply high performance liquid chromatography (HPLC) for the measurement of nitrite and nitrate.

The determination of nitrate and nitrite ions by HPLC was initially done using conventional silica columns with aqueous standards.. Unfortunately there was a rapid deterioration of peak shape and reproduciblity of retention time when biological fluids such as plasma were used. The newer carbon based columns (hypercarb) overcame these difficulties and subsequently have been used successfully to quantify nitrite and nitrate (Wiklund *et al* 1993).

There is a comparatively new technique known as capillary ion analysis (CIA) which may also be applied to the measurement of nitrite and nitrate. This combines a high resolving power, minimal sample preparation requirements, fast analysis times and low sample volume consumption (1-50 nl injected). This is the technique which I used to measure nitrite and nitrate levels and the methodology together with some additional

background information is described in detail in section 2 of this thesis.

1.5.10 NO donor drugs

Since NO has a very short half life, in order to be administered pharmacologically it must be given as a compound drug which will give up the NO at the site of action in much the same way that haemoglobin carries oxygen. Such drugs are known as NO donors and have been available for many years in the form of inorganic nitrates, such as sodium nitroprusside or glyceryl trinitrate. Despite widespread usage their mechanism of action was poorly understood until in 1977 it was shown that sodium nitroprusside caused a dose dependent increase in smooth muscle cell cGMP (Schultz *et al* 1977; Katsuki *et al* 1977). It is now known that nitrate drugs release NO, which then acts on the soluble guanylate cyclase to produce this increase in cGMP (Feelisch & Noack 1987).

Glyceryl Trinitrate (GTN) is one such drug and is probably one of the most widely prescribed in cardiology. It has been shown by incubation of platelets with GTN in pharmacological doses that endothelial cells are necessary for inhibition of platelet aggregation to occur (Benjamin *et al* 1991; Doni *et al* 1991; Levin *et al* 1981). Further studies have confirmed this finding (Zembowicz *et al* 1990) and in addition shown increased plasma nitrite levels following administration (Benjamin *et al* 1991) without any alteration in prostacyclin production (De Caterina *et al* 1985).

Interestingly, different drugs appear to be metabolised by different pathways. Sodium nitroprusside requires reduction with NADPH or NADH in the microsomes (Fung *et al* 1991) whereas GTN is metabolised by a plasma membrane enzyme of the smooth muscle cell (Chung & Fung 1990). This may explain why NO donor drugs can vary in their effect profiles. For example, GTN is primarily a vasodilator without marked platelet effects *in vivo*, whereas molsidomine or nitrosoglutathione have

marked anti-platelet effects with less vasodilatation (Zembowicz *et al* 1990). Much pharmacological research is being directed towards the manufacture of NO donors with specific effect profiles for particular clinical applications.

1.6 NITRIC OXIDE IN PREGNANCY

1.6.1 Background

Following the identification of both endothelin and nitric oxide in the late 1980's, there was a rapid increase in research concerning endthelial cells. Since NO has profound effects on both vascular tone and platelet function, it was considered especially relevant to explore their role in the hypertensive disorders of pregnancy and fetal growth retardation. In recognition of this the National Institutes of Child Health and Human Development published research recommendations on endothelium derived vasoactive substances in perinatal research in 1991(Chaudhuri *et al* 1991). Subsequently, the possibility of NO mediating the cardio-protective effects of oestrogen has led to increased interest from gynaecological researchers studying the effects of hormone replacement therapy.

1.6.2 The role of NO in controlling vascular tone in pregnancy

Human studies from almost twenty years ago demonstrated elevate excretion of cGMP in the urine of pregnant women (Kopp 1977). The significance of this finding was not appreciated then, since NO was only identified later on in the 1980's. However, now the physiology is better understood, the quantification of plasma and urine levels of nitrite and nitrate and cGMP has become a standard tool for studying NO production in both animal and human models.

The urinary excretion of cGMP is known to be elevated in normal pregnancy in the rat (Conrad & Vernier 1989, Conrad *et al* 1993b). Further experiments using L-arginine analogues (e.g. L-NAME, L_NMMA) to block NO synthesis have suggested that NO is important in mediating the physiological decrease in systemic blood pressure that is seen in normal pregnancy (Chu & Beilin 1993).

The alterations in pressor sensitivity seen in pregnancy are well

documented in both animals and humans. Using isolated arterial rings from pregnant guinea pigs, Weiner was able to demonstrate that administration of L-NMMA would revert the pressor response to normal non-pregnant levels (Weiner *et al* 1992). This study also suggested that the uterine artery showed a more exaggerated response to L_NMMA than other arteries from the same animal. The involvement of NO in altering pressor responsiveness in normal pregnancy has now been confirmed by several other investigators (Molnar & Hertelendy 1992, St Louis & Sicotte 1992, Lubarsky *et al* 1995).

Since the sex steroid oestrogen is present in large amounts during pregnancy and is known to cause vasodilatation (Magness & Rosenfeld 1989), it has been hypothesised that it is involved in the regulation of NO synthesis. Indeed, the special sensitivity of the uterine artery to oestrogen is now thought to be due to a high concentration of oestrogen receptors in the nuclear fraction of the artery (Batra & Iosif 1987).

Using an instrumented sheep model Van Buren showed that the increase in uterine artery blood flow produced by oestrogen was reduced by 60% when L-NMMA was infused (Van Buren *et al* 1992). At much the same time, Hayashi was studying basal NO production in isolated aortic ring preparations from male, female and oophrectomised rabbits (Hayashi *et al* 1992). These studies showed that NO production in the female preparations was greater than that in the male preparations, but that this difference could be abolished by oophrectomy. More direct evidence has come from Weiner using the guinea pig model. Here, administration of oestrogen not only increased nitrate/nitrite production but also to cause increased expression of messenger RNA for the inducible form of NOS in skeletal and vascular smooth muscle cells (Weiner *et al* 1994).

The cardiovascular benefits of oestrogen in post-menopausal women are already well documented (Coditz *et al* 1987; Rosano *et al* 1993). The

vasodilatory effect of oestradiol in humans has been demonstrated *in vivo* at both a systemic (Magness & Rosenfeld 1989) and local level (Ganger *et al* 1991; Ginsberg *et al* 1989). It has been shown specifically, using Doppler ultrasound, that uterine artery blood flow is increased in association with high oestrogen levels during the second half of the menstrual cycle (Goswamy & Steptoe 1988; Ganger *et al* 1991) and in early pregnancy (Jauniaux *et al* 1992b). These findings coincide with an elevation in plasma nitrite/nitrate levels seen during normal follicular development (Rosselli *et al* 1994) and mid-cycle increase in exhaled NO (Kharitonov *et al* 1994).

Whilst most animal studies have been concerned with short term infusions of oestradiol, one detailed study infused oestrogen for 14 days into ovariectomised sheep (Magness *et al* 1993). This clearly showed that decreases in systemic vascular resistance and alterations in pressor sensitivity occurred promptly and were maintained throughout the experiment. Indeed, pressor responsiveness did not return fully to pre-treatment levels until 2 weeks following cessation of infusion. However, the uterine blood flow exhibited tachyphylaxis, with an initial sharp rise returning to baseline values within one week of the start of the experiment. This adds further weight to the concept of a receptor mediated process causing uterine artery vasodilatation, and suggests that systemic and uterine vascular responses to oestrogen may be mediated through different mechanisms.

Further *in vitro* work, examining omental arteries taken from normotensive pregnant women at the time of caearean section, has confirmed that the vasodilatory effect of oestrogen on these arteries is mediated through the endothelium (Belfort *et al* 1995).

1.6.3 Role of nitric oxide in complicated pregnancies

A relative deficiency of NO is an attractive hypothesis to explain the

pathogenesis of pre-eclampsia. It would not only explain the vasoconstriction and altered pressor responses but would also be consistent with the altered platelet function and endothelial cell damage which are characteristic of the end stage disease.

There is a variety of evidence to support an elevation of NO production in pregnant rats such as increased urinary nitrate and cGMP excretion which may be blocked by administration of NO inhibitors (Chu & Beilin 1993, Conrad *et al* 1993b). Such blockade also leads to development of a pre-eclampsia like illness in pregnant rats with raised blood pressure and growth retardation of fetuses (Yallampalli & Garfield 1993).

As described earlier, low oxygen concentration can inhibit NOS activity in both humans and animals (Kim *et al* 1993). Placental perfusion experiments have shown a prompt vasoconstrictiion in response to experimental hypoxia which is both reversible and repeatable (Howard *et al* 1987). Attempts to mimic IUGR using a sheep model have shown that in the presence of a mechanical reduction in uterine and umbilical blood flow, administration of oxygen will markedly improve oxygen delivery to the fetus (Paulick *et al* 1992). There are also reports of administration of oxygen to women with growth retarded fetuses producing improvement in Doppler indices of blood flow (Battaglia *et al* 1992). It is interesting to speculate that NO may mediate some of these utero-placental blood flow changes.

A cross-sectional study of a population of pregnant women in Ecuador has shown some indirect evidence for decreased nitric oxide synthesis in pre-eclampsia when compared to normal pregnancy (Lopez-Jaramillo *et al* 1992). The data shows a rise in urinary and cGMP excretion in normal pregnancy, which appears to be reduced or absent in pregnancies complicated by pre-eclampsia. Plasma nitrite levels were low (<1 μmolar) and not significantly different between groups. However, subsequent

studies of plasma nitrate/nitrite and cGMP levels have produced conflicting results (Cameron *et al* 1993, Seligman *et al* 1994). The finding of a halving of basal non-pregnant ADMA activity in normal pregnancy, but an increase in activity (above normal non-pregnant levels) in cases of pre-eclampsia (Fickling *et al* 1993) may help to explain some of these contradictions.

Whilst the existence of abnormal endothelial cell function in pre-eclampsia is widely accepted (Mc Carthy *et al* 1993), there is still controversy concerning the effect this has on NO synthesis. For example, in the animal studies mentioned earlier, the observed rise in BP produced by L-NMMA was reversible by concurrent administration of L-arginine in one study (Molnar & Hertelendy 1992) whilst in the other, GTN infusion failed to return BP to normal (Yallampalli & Garfield 1993). Since both drugs should work through the final common path of NO production it is difficult to explain this contradiction. In addition, experiments using helical strips of placental arteries (ie of fetal origin) from normal and pre-eclamptic women have failed to show any difference in responsiveness to a wide variety of endogenously produced vasoactive agents including serotonin, angiotensin II, norepinephrine, endothelin I, atrial naturetic factor and prostacyclin (Inayatulla *et al* 1993).

1.6.4. Preterm labour

Preterm delivery is the single most important contributor to perinatal morbidity and mortality in the developed world. Whilst a variety of causes have been identified, in the majority of cases the underlying mechanisms remain obscure. Our limited understanding is reflected in our inability to treat this condition effectively (The Canadian preterm labour investigation group 1992). However, there are two new pharmacological approaches which show promise. The first, an oxytocin receptor antagonist, Atosiban, is a logical development since it is known that myometrial sensitivity to oxytocin is enhanced in preterm labour (Takahashi *et al* 1980). The

second, a nitric oxide (NO) donor drug, glyceryl trinitrate, is more speculative since little is known about the role of NO in preterm labour. Reports so far having centred on clinical observations of its efficacy (Lees *et al* 1994; Greenspoon & Koviacic 1991).

Currently, the role of NO in the control of myometrial contractility is less clearly defined. It has been shown that administration of an NO donor drug will abolish contractions in rat uterine smooth muscle both *in-vitro* and *in-vivo* (Natuzzi *et al* 1993). Studies *in vitro* have shown a similar effect on human myometrium (Yallampalli *et al* 1993b). Interestingly, NO induced relaxation was less in samples of rat myometrium obtained at delivery than in those from mid-pregnancy, suggesting that NO may play a role in the complex control system which maintains uterine quiescence during pregnancy (Yallampalli *et al* 1994; Izumi *et al* 1993).

Elevated plasma nitrates have been reported by some investigators in the presence of premature rupture of the membranes and premature labour (Jaekle *et al* 1994). To explain this the authors have suggested that sub-clinical infection may cause a cytokine mediated increase in NO production by macrophages.

1.6.5. Summary of the case for further investigation

Nitric oxide has a wide variety of actions. There is already a reasonable body of evidence to support the notion that NO plays a role in mediating low resting vascular tone in the uteroplacental circulation in pregnancy. It also appears likely that oestrogen stimulates the production of NO in animals. At present there is less evidence on NO function in pregnancies complicated by pre-eclampsia and fetal growth retardation, however, the pathophysiological features of these conditions are well defined. These include endothelial cell damage and altered platelet function and it remains an attractive idea to link these pathologies together by invoking a relative deficiency of NO as the underlying problem.

CHAPTER 2 - GENERAL METHODOLOGY

2.1 ULTRASOUND TECHNIQUES

2.1.1 Transvaginal scans

The women were fully informed about the procedure before recruitment into the studies and written consent obtained. Scans were performed on a standard scanning couch in the presence of a chaperone. A disposable condom was used to cover the probe to minimise the risk of cross infection between cases. In addition the probe was wiped using the manufacturers recommended cleaning solution. The covered probe was coated with lubricating jelly and gently introduced into the vagina after parting the labia. First the uterus was identified and the crown-rump length of the fetus recorded and used to date the pregnancy accurately (Figure 2.1). Then, by directing the probe towards the pouch of Douglas and angling laterally the vessels of the broad ligament were visualised.

In B-Mode (Greyscale) scanning many veins can be seen at the level of the cervix together with the uterine arteries. The addition of colourflow imaging at this point made it easy to identify the main uterine artery and adjust the angle of insonation so that the vessel was seen to run vertically up the screen. The pulsed Doppler gate was then placed over the vessel and the size adjusted to equal the vessel diameter before obtaining a Doppler signal. This meant that exposure to the higher energy levels of pulsed Doppler ultrasound were kept to the minimum possible. When a good tracing was obtained, the RI, PI, maximum velocity and mean velocity were calculated using the machines inbuilt software, according to the equations given in the introduction. The presence or absence of a post-systolic notch was also noted (Figure 2.5).

2.1.2 Transabdominal Scans

In later pregnancy (ie beyond 14 weeks gestation) a transabdominal curvilinear array probe was used. This required a full bladder for good visualisation of the uterine contents at the beginning of the studies so that fetal size could be measured. Biparietal diameter, head circumference, abdominal circumference and femur length were all recorded (Figures 2.2 – 2.4) and plotted according to standard fetal growth charts (See Appendix 2).

The main uterine artery was identified in every case at the crossing point with the external iliac artery at the level of the cervix. This has several advantages over random sampling or measurement of arcuate artery flows higher up the uterus which have been dealt with in the introduction. Having identified the vessel the technique for recording Doppler flows was identical to that used transvaginally. An example of sample gate placement and Doppler signal obtained from the main uterine artery of a normal 20 week pregnancy is shown in Figure 2.6. This demonstrates the higher velocities and good diastolic flow which develop with advancing gestation and should be compared with the early pregnancy trace shown in figure 2.5. Examples of "abnormal" uterine artery blood flow velocity waveforms are given later in this thesis.

2.1.3 Fetal Doppler

Fetal Doppler measurements were restricted to umbilical artery flows in most cases. The arteries were identified using colour flow imaging and then a pulsed Doppler signal obtained which was purely from the artery without recording venous flow. This was to facilitate velocity measurements which would otherwise (using the Acuson software) have been erroneously high due to the inclusion of negative velocities in the calculation of the mean. The flow velocity waveforms obtained from early (first trimester) pregnancies characteristically showed low amplitude with absent end diastolic flow in the umbilical artery (Figure 2.7) whereas the

later pregnancies showed higher amplitude signals with good diastolic flow (Figure 2.8).

Those fetuses included as "growth retarded" for the purposes of this research project met two criteria: firstly that the abdominal circumference was on or below the 2.5th centile for gestational age (See fetal growth charts in Appendix) and secondly that there should be evidence of fetal blood flow "redistribution". This meant an abnormally high umbilical artery PI for gestational age together with a raised PI in the descending thoracic aorta and a lowered PI in the middle cerebral artery (Figure 2.7, 2.8 and 2.9). The additional Doppler criteria allowed only those small babies which were growth retarded (rather than normally small) to be selected for further study. The background to this has already been discussed in the introduction.

The typical umbilical artery Doppler signal from a normal third trimestr pregnancy is shown in figure 2.9 with an example of the FVW form a growth retarded fetus in figure 2.10 for comparison. Examples of normal and abnormal middle cerebral artery and descending thoracic aorta Doppler signals (defined according to normal ranges given in appendix 3) are shown in figures 2.11 – 14.

2.1.4. Reproducibility of Doppler measurements

The data from the NO infusion study later in this thesis has been analysed to examine the variation in serial Doppler readings taken from the same patient. The "control" readings which were used to establish a baseline were repeated twice on each patient within a few minutes of each other so I have assumed that the "true" value of the Doppler indices did not change between these readings.

In this situation the variance of two observations is half the square of their difference. So, if the difference between two observations for the subject *i*

is d_i then the within-subject standard deviation s_w , for n subjects, is given by

$$s_w^2 = \frac{1}{2n} \sum d_i^2$$

Taking the measurement error as sw , the difference between the subject's measurement and the "true" value will be less than $1.96s_w$ for 95% of observations. This may also be expressed as the repeatability which is $\sqrt{2} \times 1.96s_w$ or, more simply, $2.77s_w$. The repeatability shows the maximum difference which would be expected between two pairs of observations on the same subject in 95% of cases (Bland & Altman 1996).

The Doppler indices obtained from the main uterine artery FVWs in this thesis show repeatablities of 0.037 for resistance index, 0.061 for pulsatility index and 2.25 for time averaged mean velocities in the normal range.

Figure 2.1

Transvaginal scan showing measurement of crown rump length (CRL).

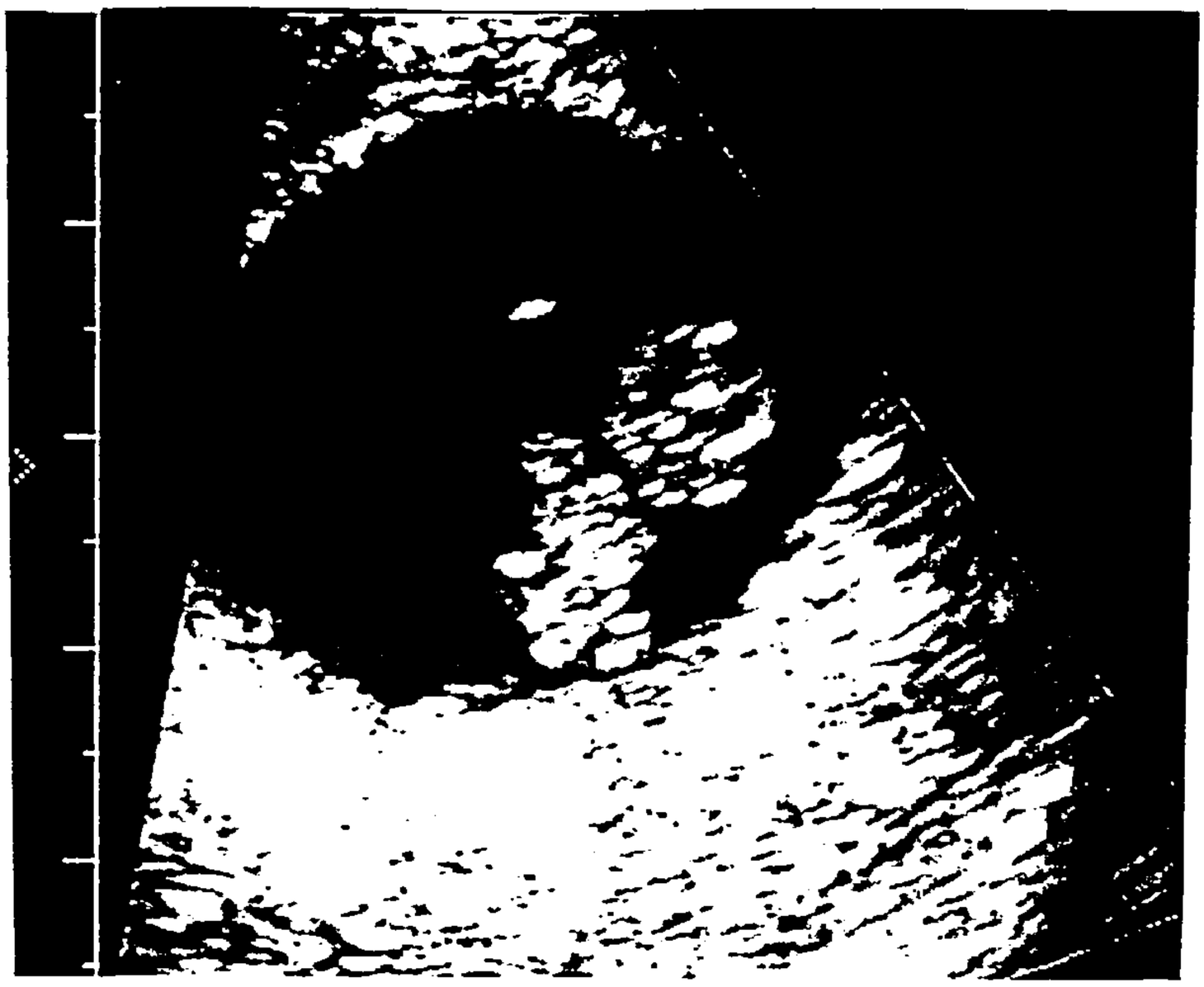


Figure 2.2

Transverse section through fetal head showing biparietal diameter and head circumference measurements.

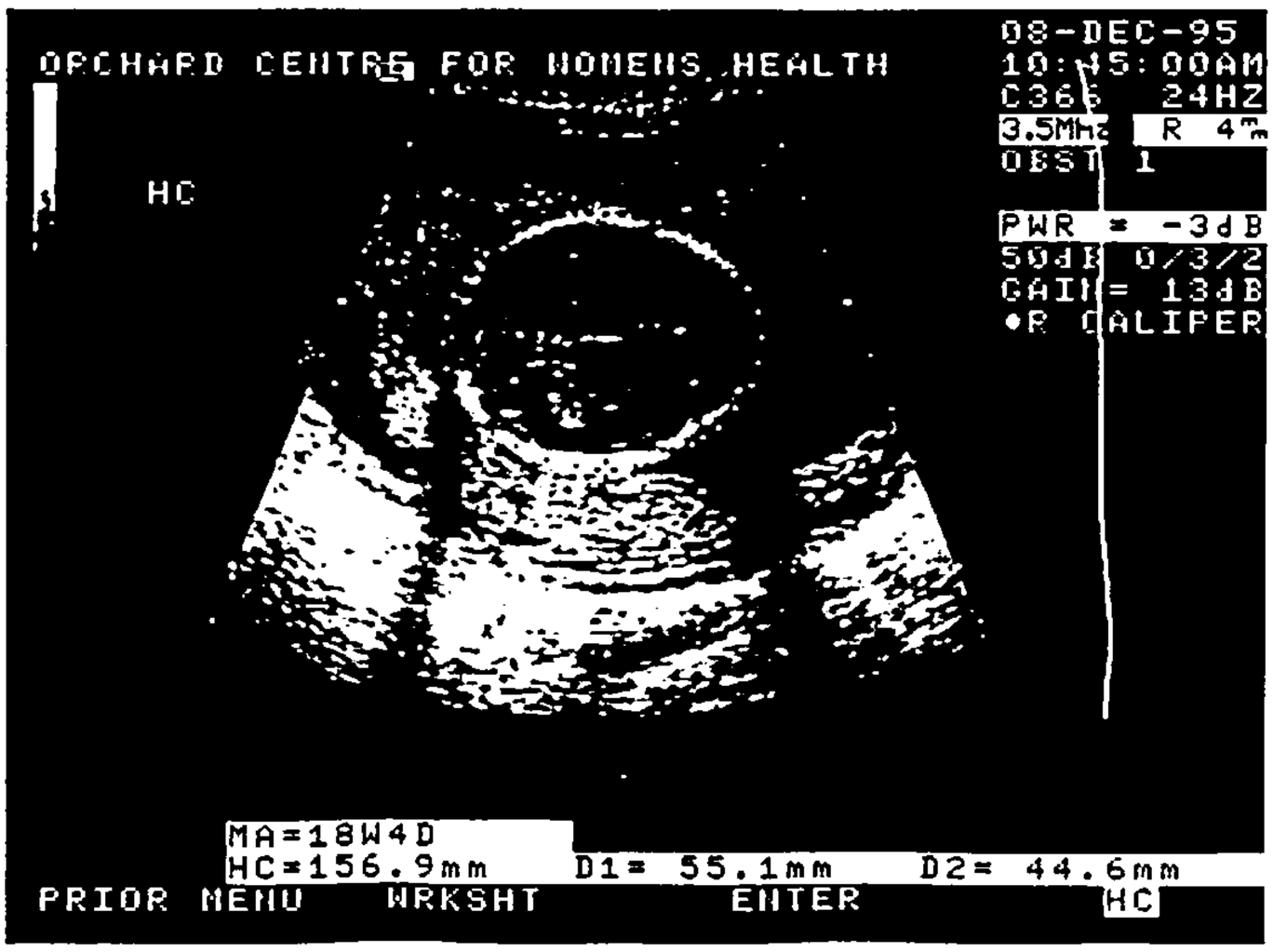


Figure 2.3

Transverse section through fetal abdomen showing abdominal circumference measurement.

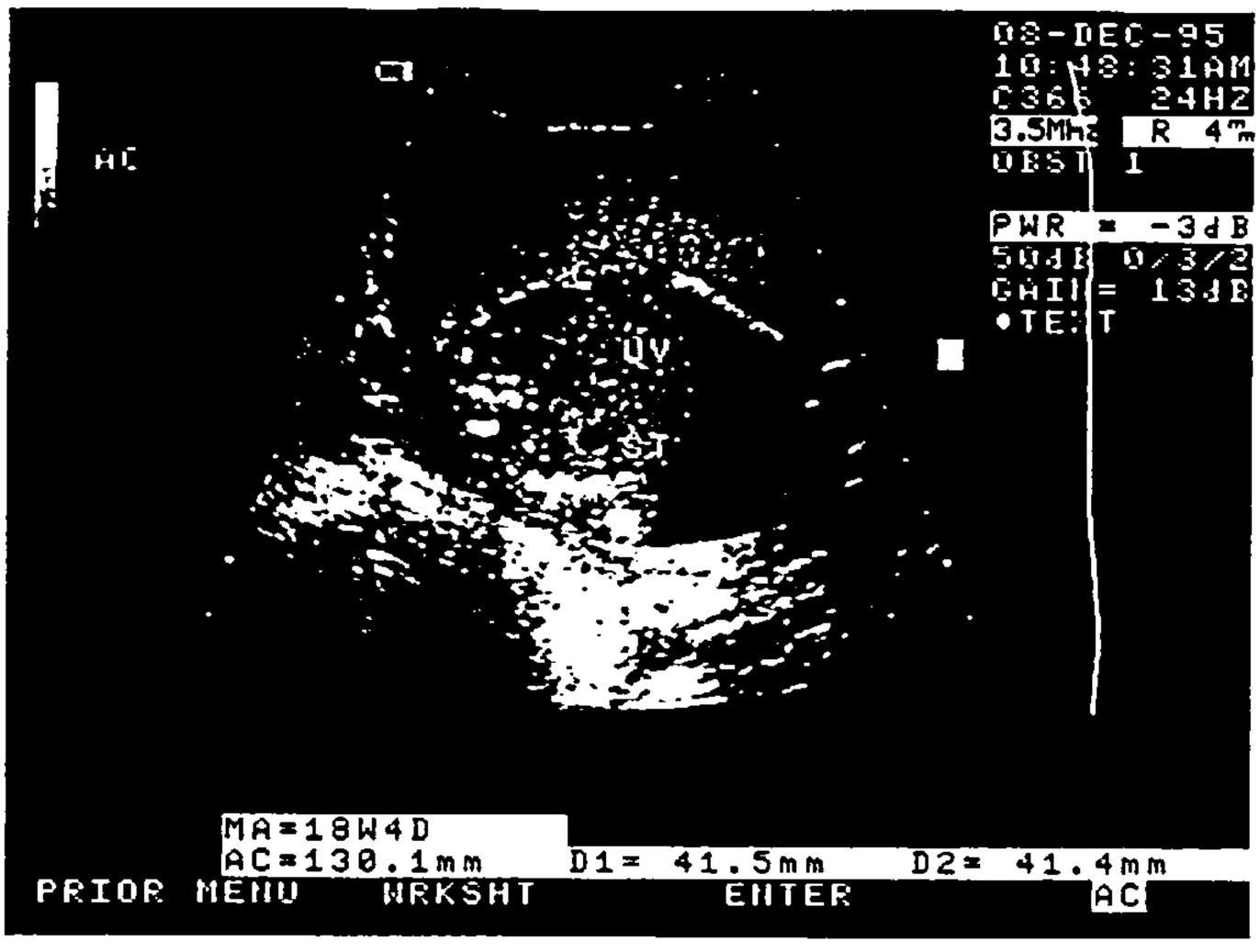


Figure 2.4

Fetal femur showing femur length measurement.

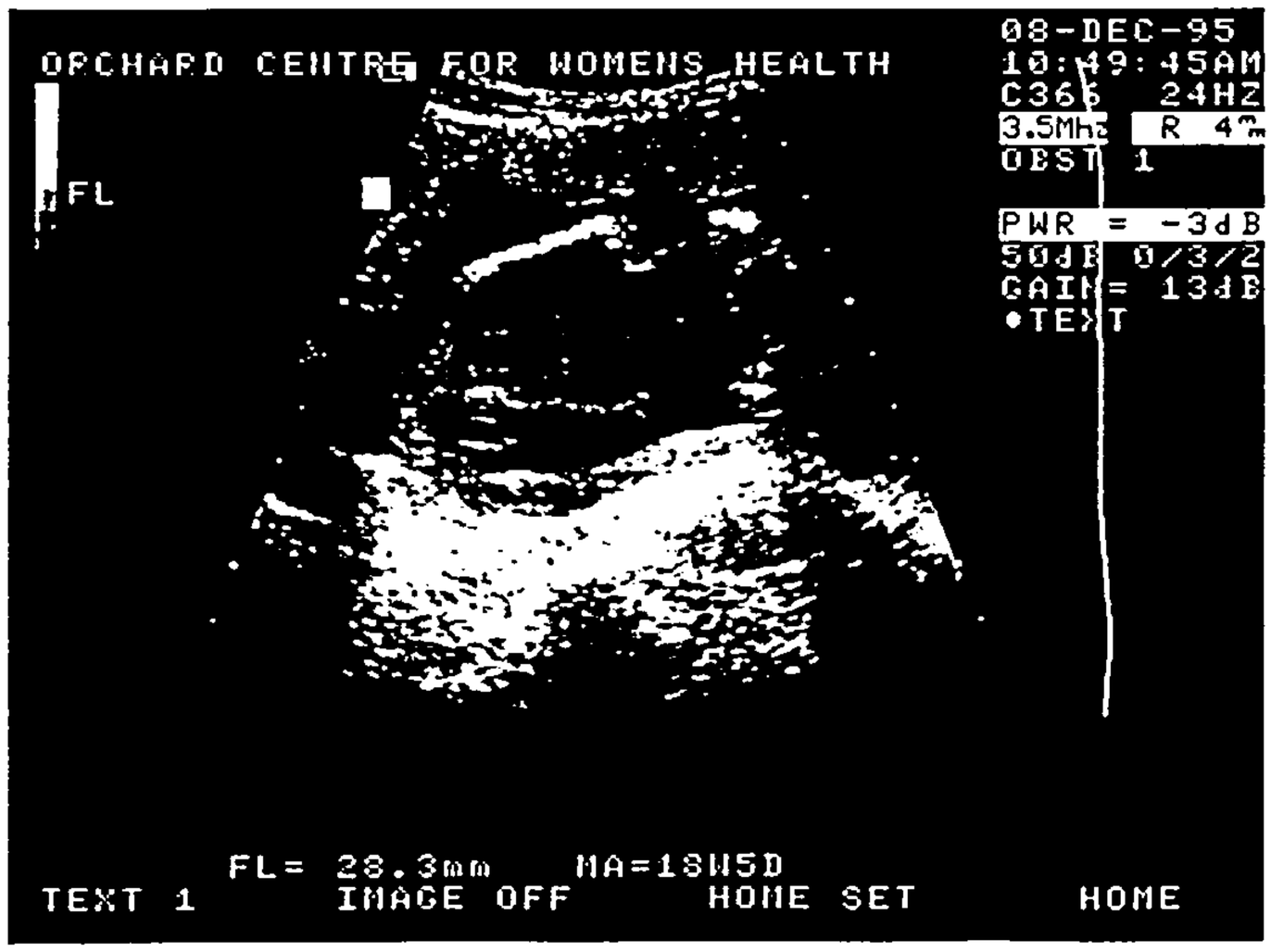


Figure 2.5

The main uterine artery visualised using colour Doppler imaging on a transvaginal scan with the flow velocity waveform below showing the low velocities and high resistance typical of the first trimester.

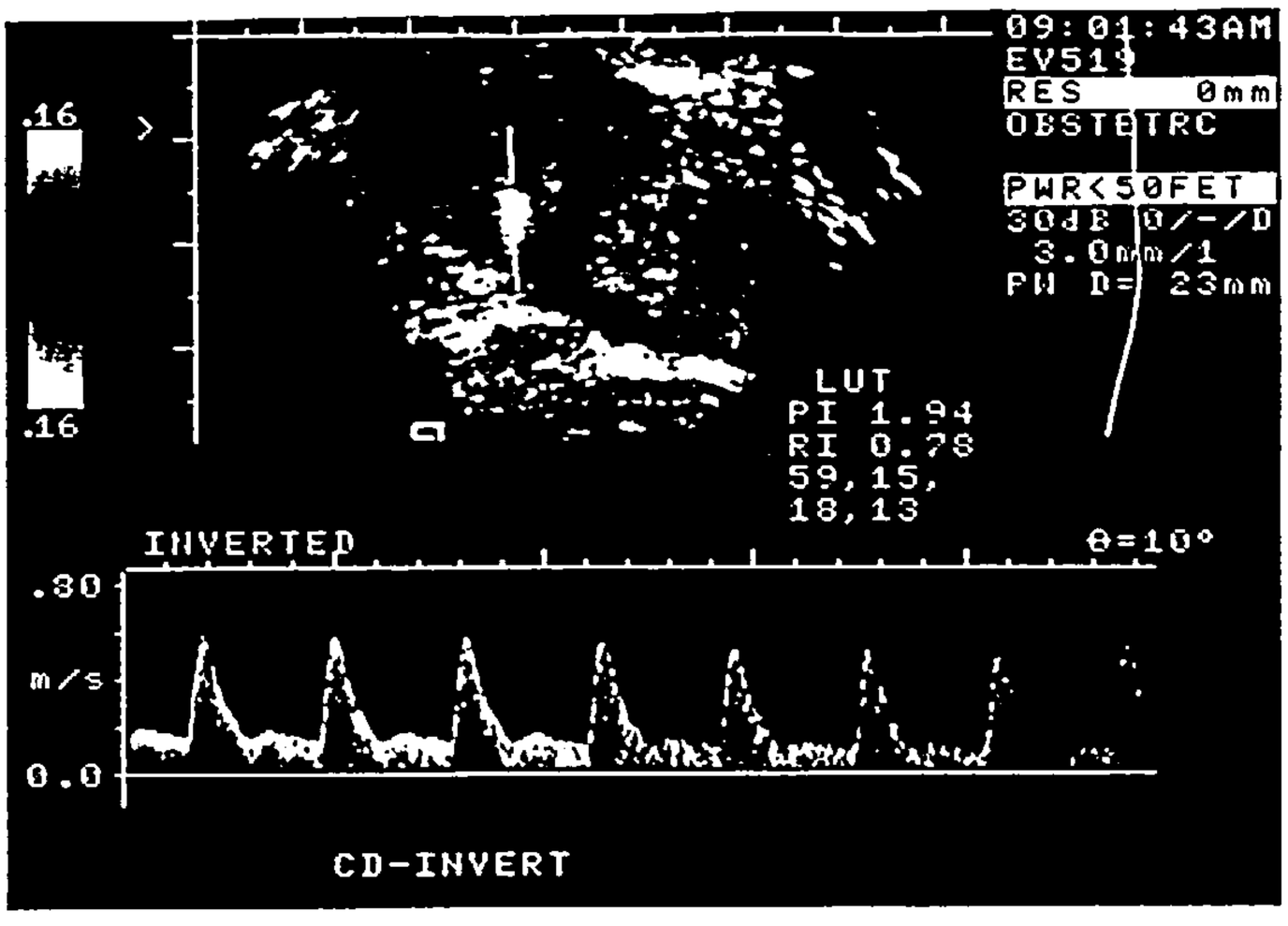


Figure 2.6

The main uterine artery visualised using colour Doppler imaging on a transabdominal scan with the flow velocity waveform below showing the higher velocities and lower resistance typical of this stage of pregnancy.

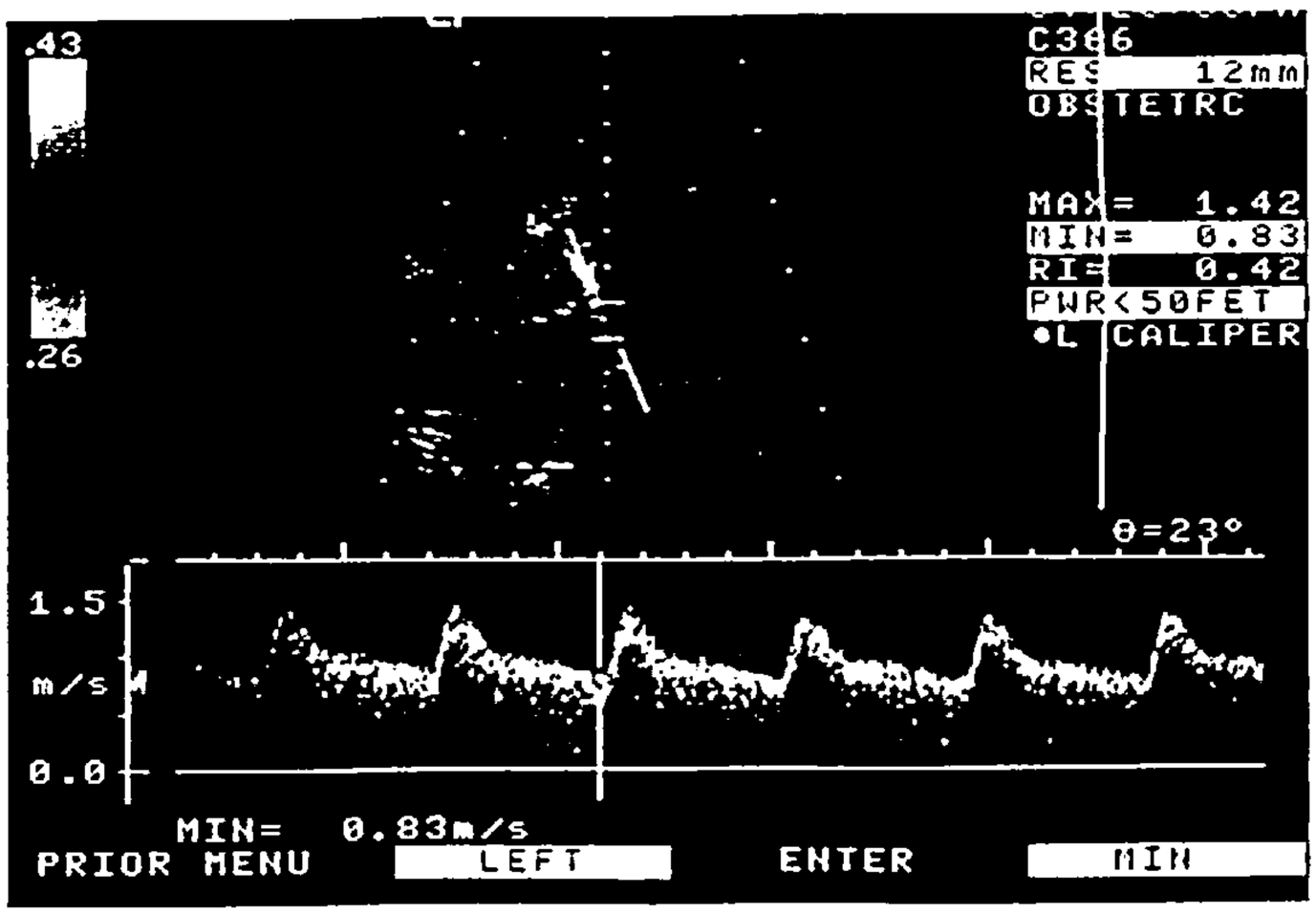


Figure 2.7

Umbilical artery Doppler from early pregnancy showing minimal diastolic blood flow to demonstrate gestational related changes in blood flow.

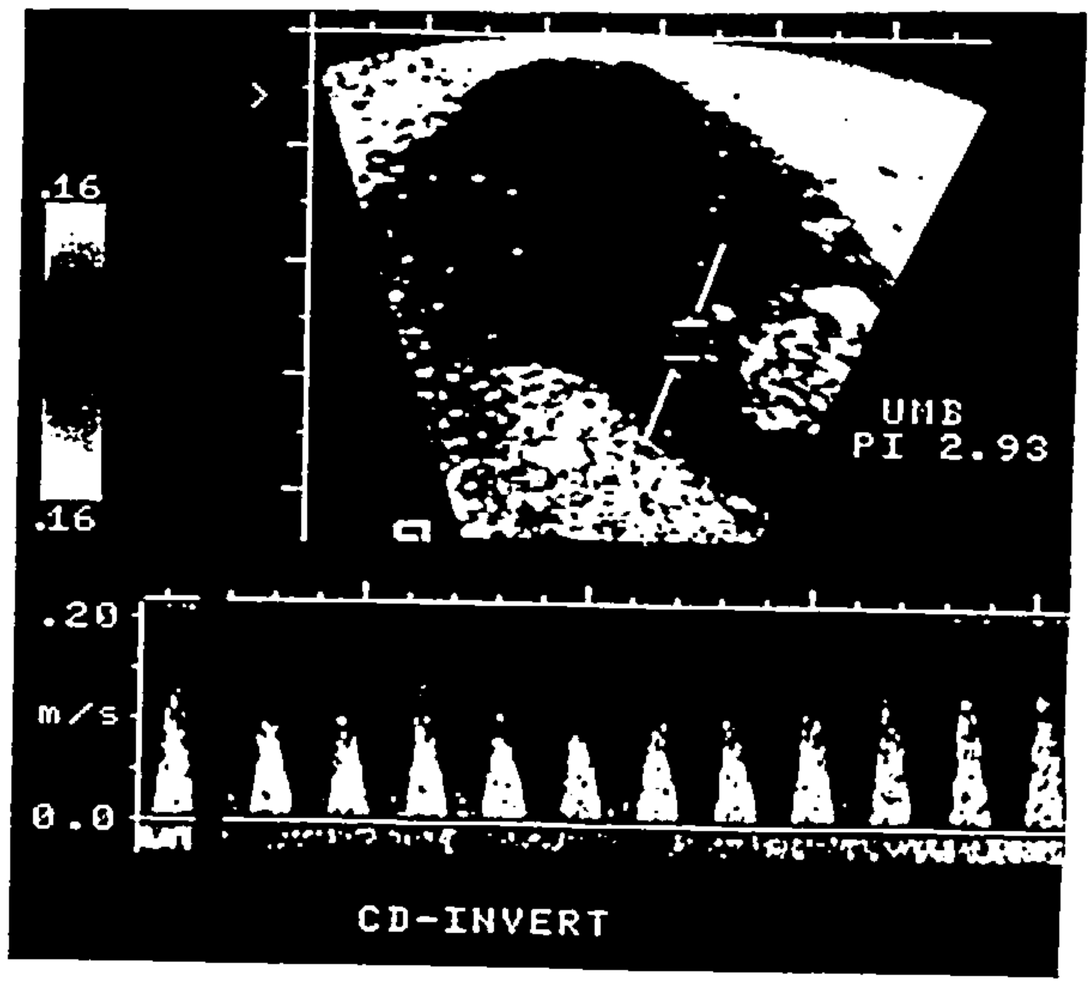


Figure 2.8

Example of umbilical artery Doppler from 20 week gestation pregnancy showing good diastolic blood flow to illustrate the gestational related change in blood flow which occurs.

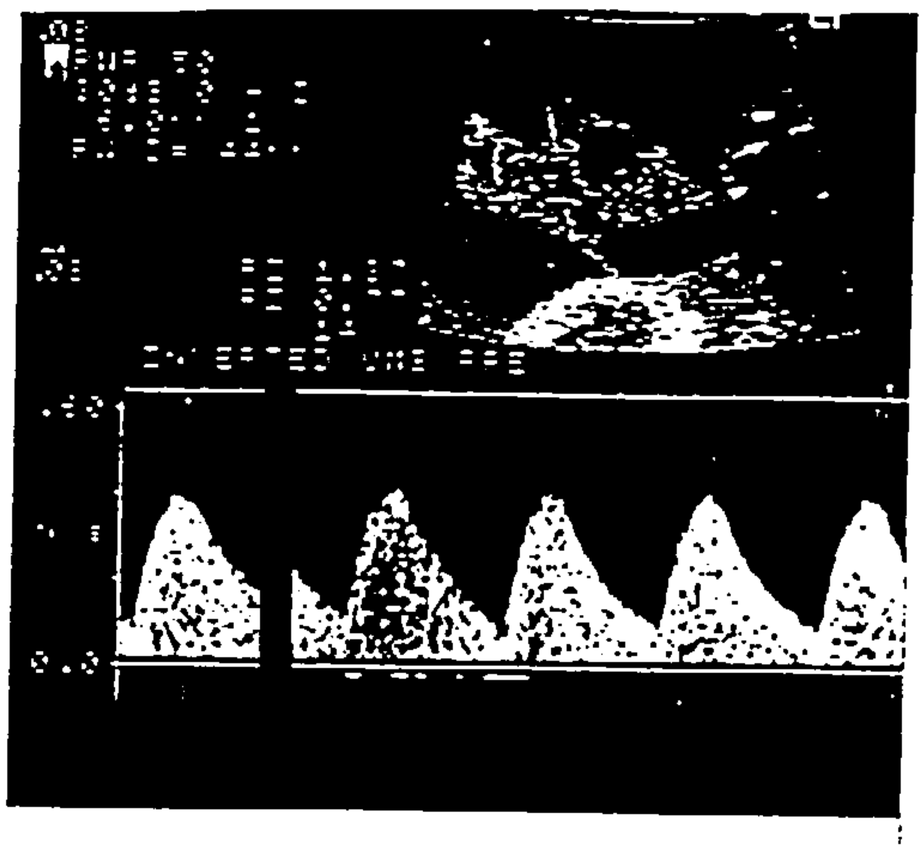


Figure 2.9

Umbilical artery Doppler from a normal third trimester pregnancy

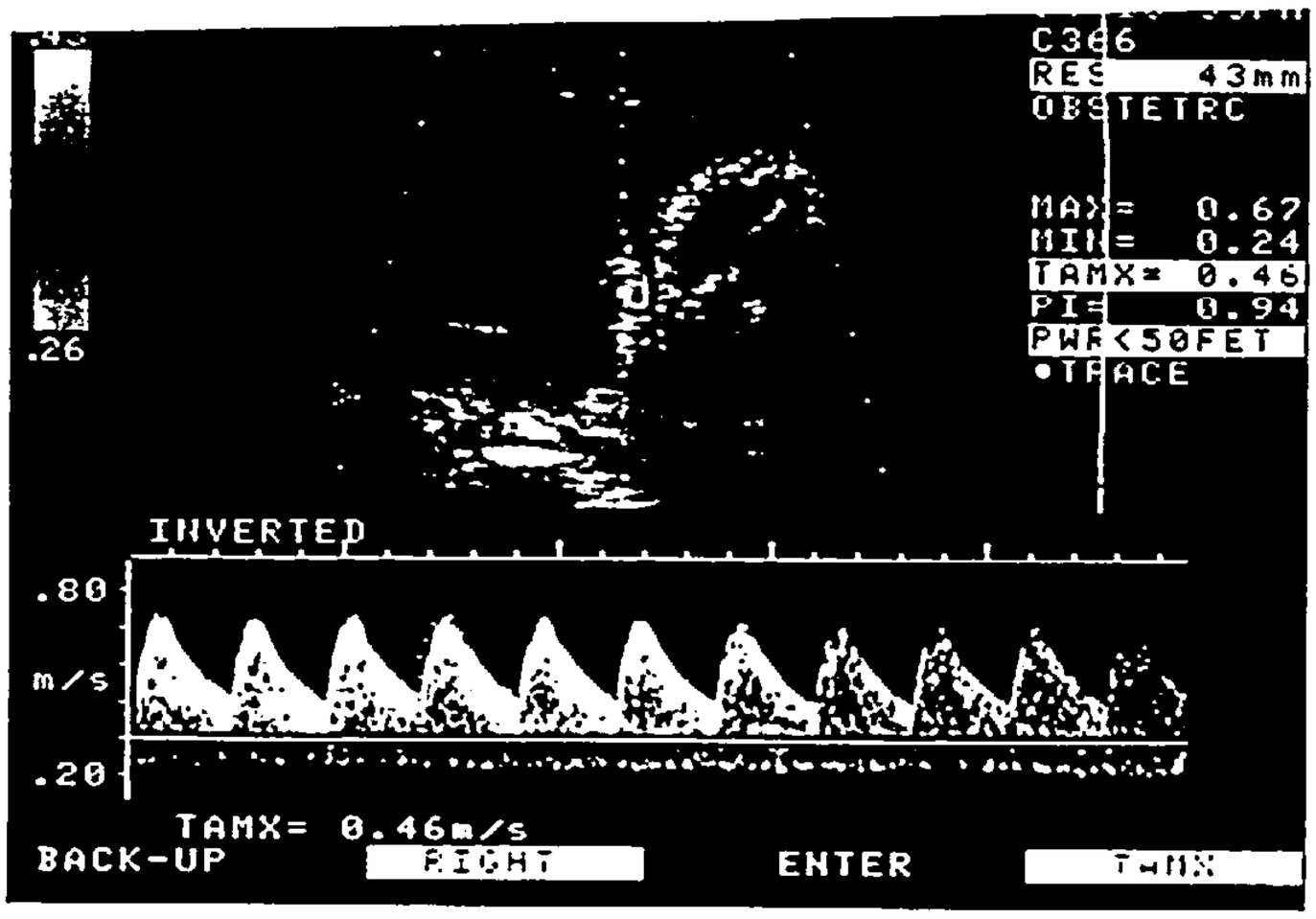


Figure 2.10

Umbilical artery Doppler from third trimester pregnancy complicated by fetal growth retardation showing reduced end diastolic blood flow.

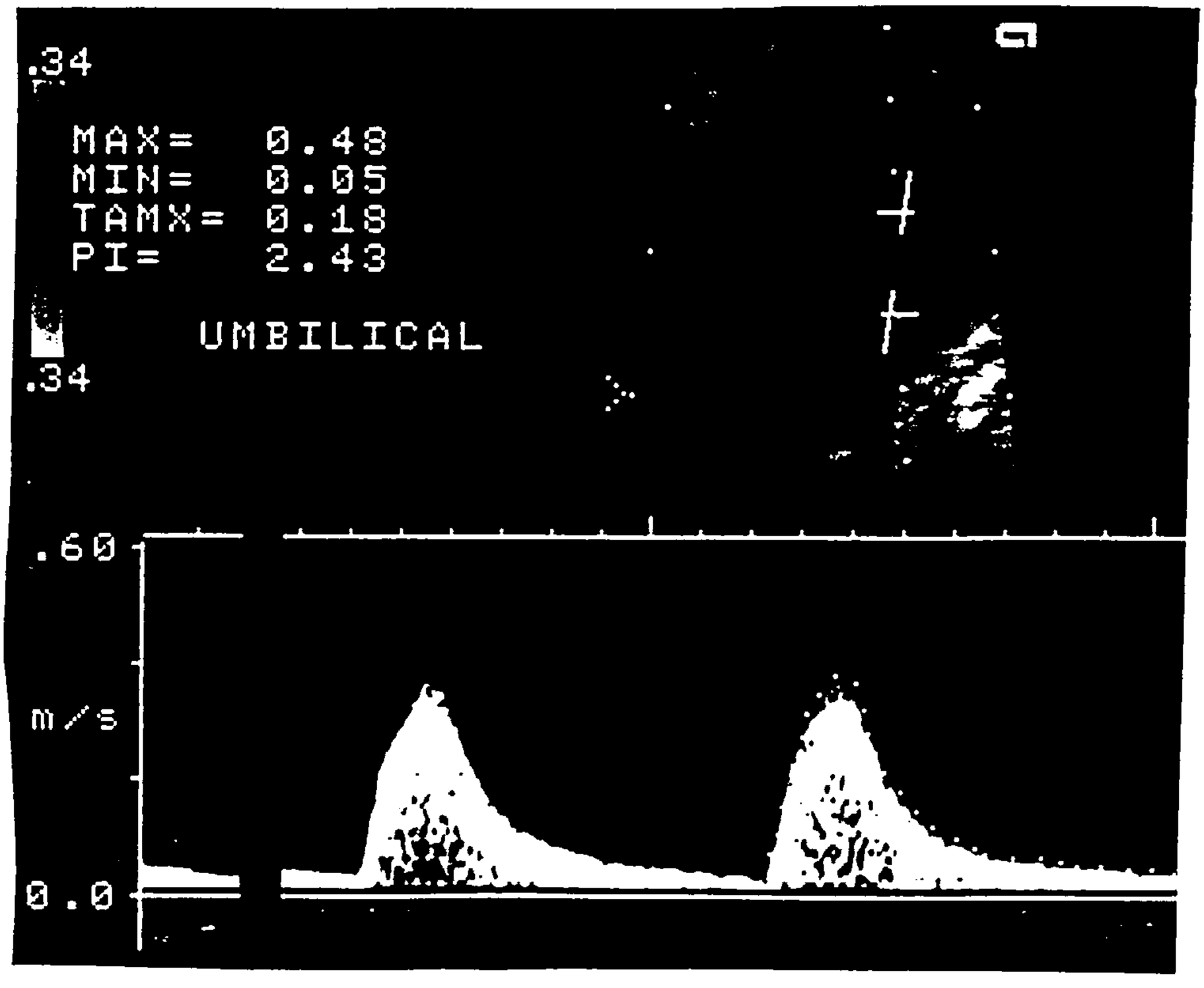


Figure 2.11

Fetal middle cerebral artery Doppler from normal third trimester pregnancy

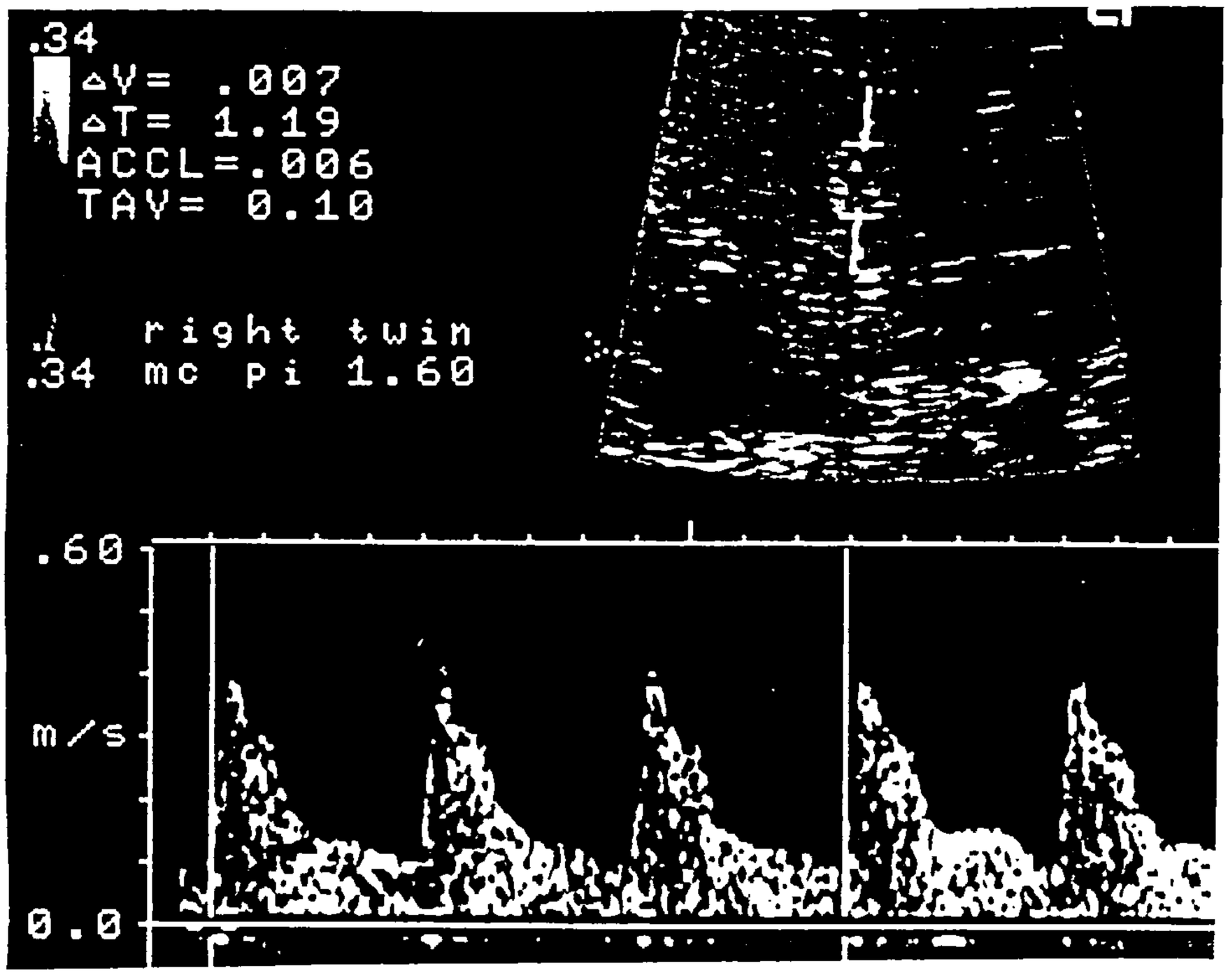


Figure 2.12

Fetal middle cerebral artery Doppler from third trimester pregnancy complicated by fetal growth retardation showing increased diastolic flow.

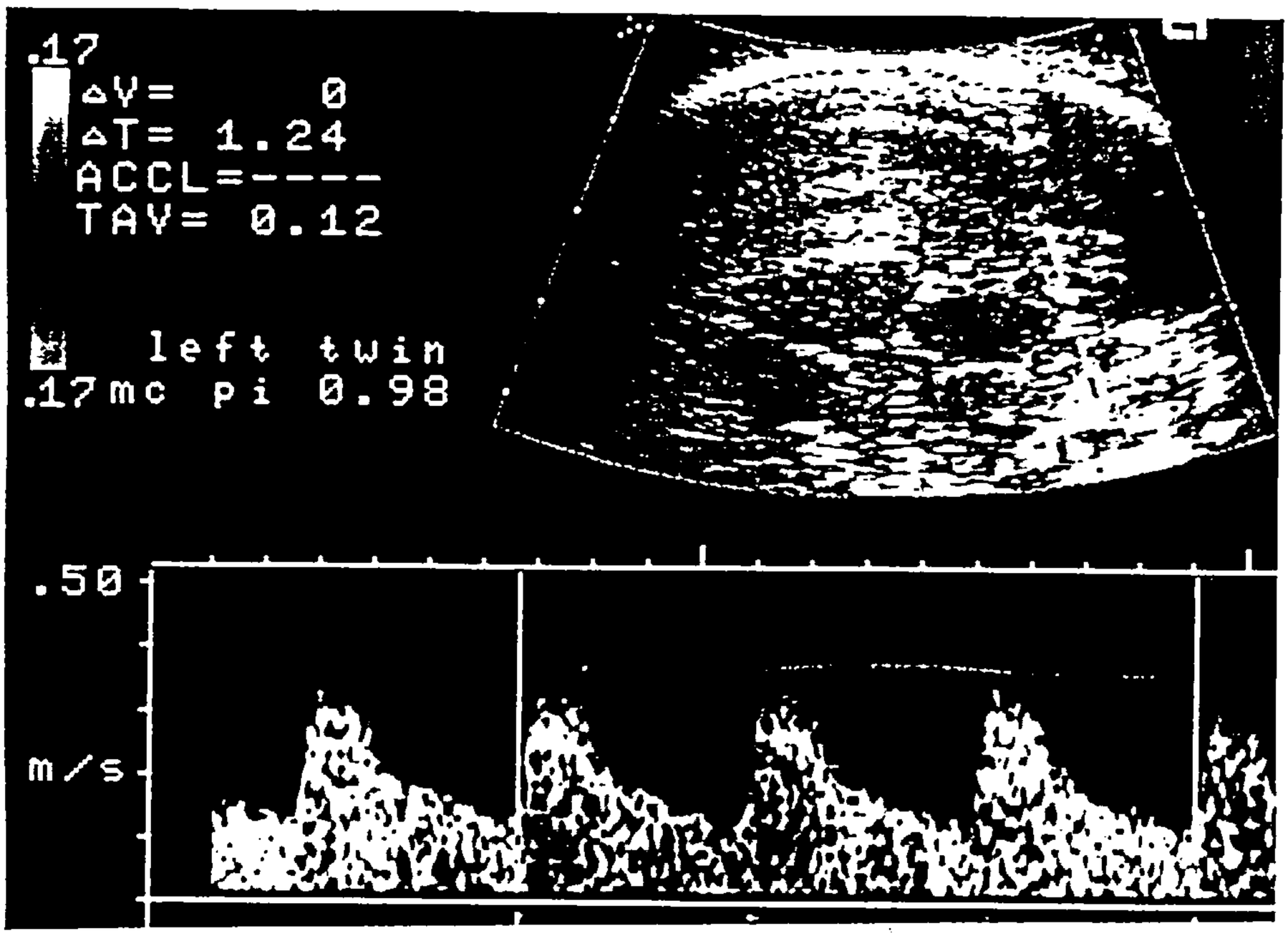


Figure 2.13

Fetal descending thoracic aorta Doppler from normal third trimester pregnancy.

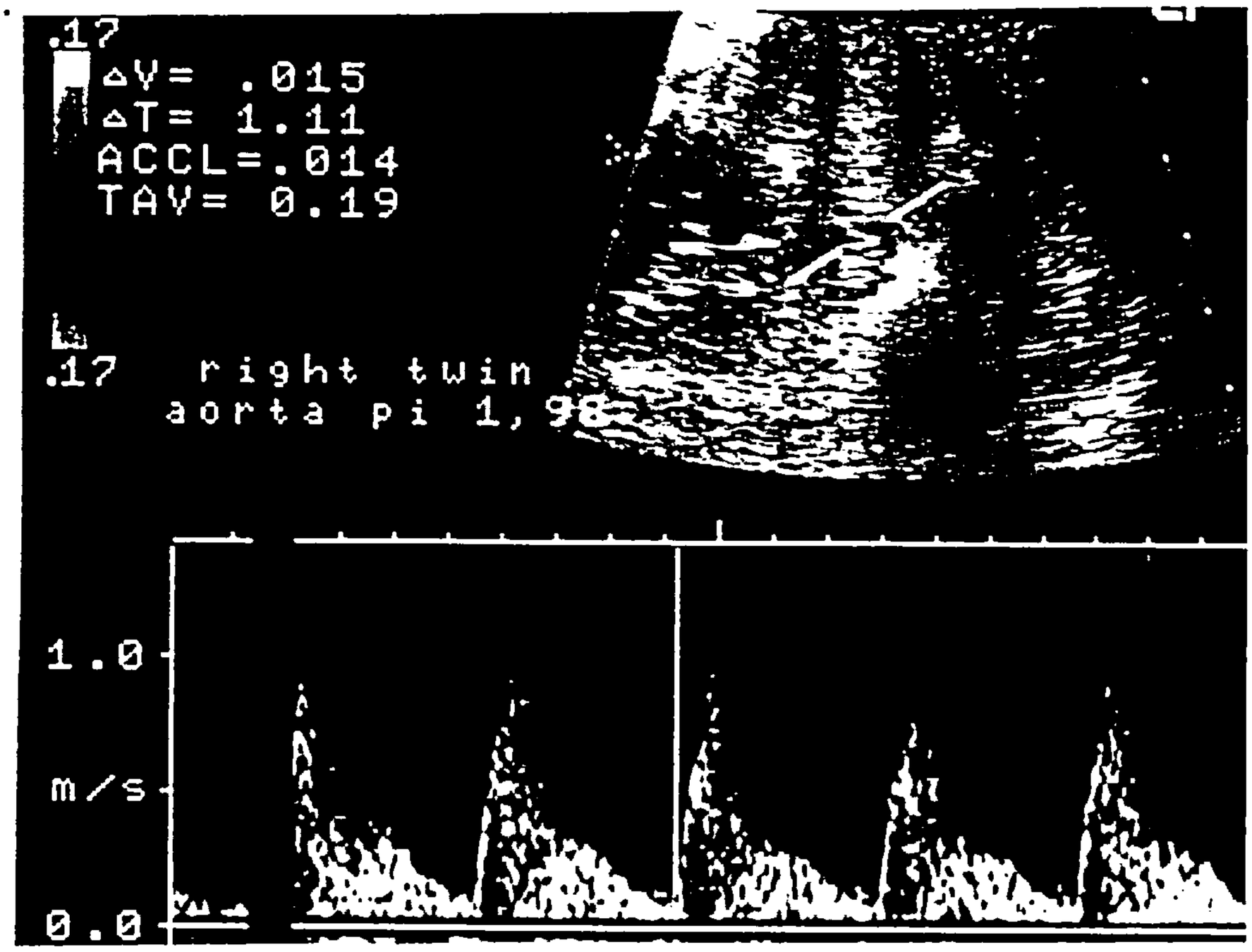
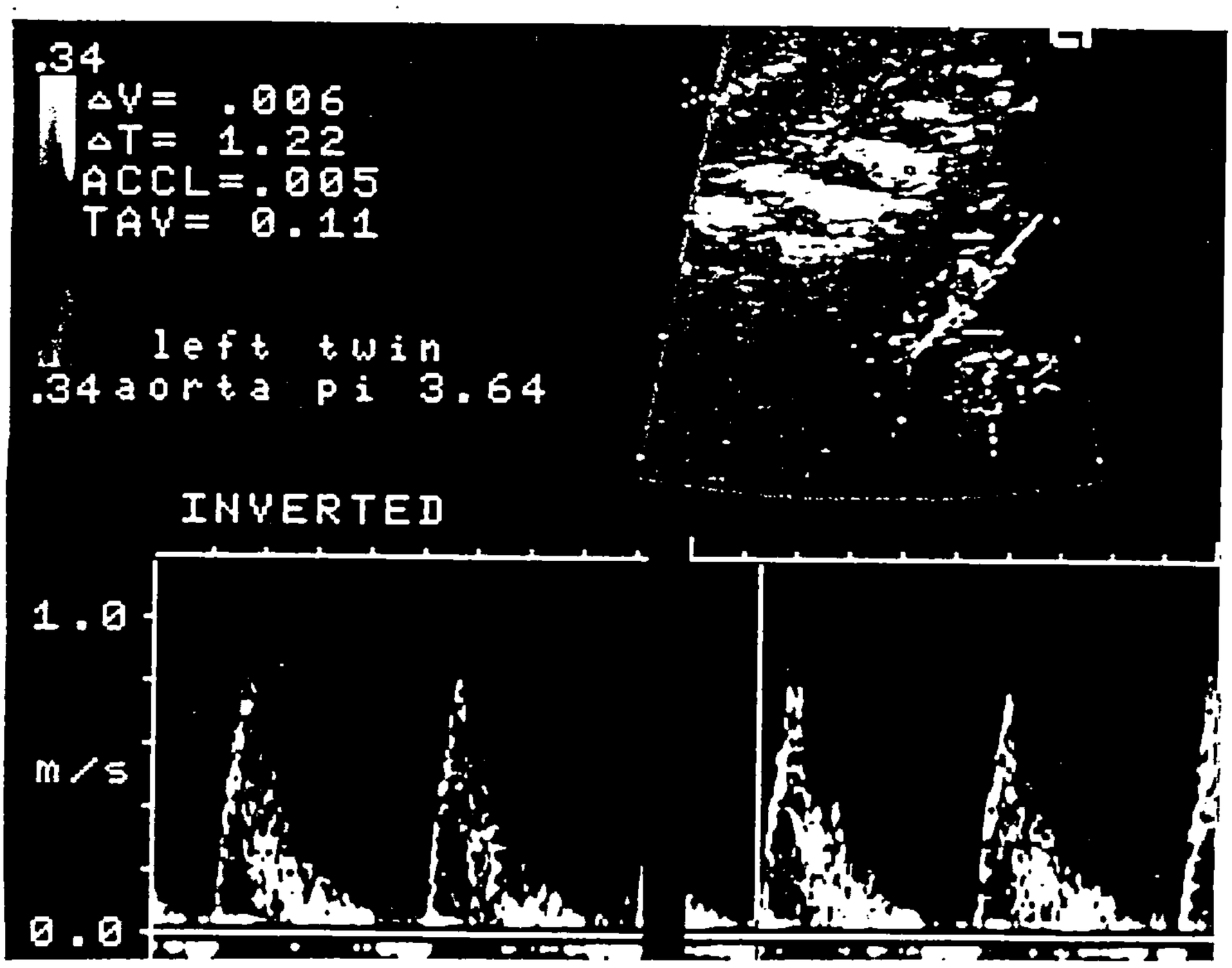


Figure 2.14

Fetal descending thoracic aorta Doppler from third trimester pregnancy complicated by fetal growth retardation showing reduced diastolic flow.



2.2 NITRITE / NITRATE ASSAY BY CAPILLARY ION ANALYSIS.

2.2.1 Background

Although many different approaches have been used to assess NO production (including quantification of nitrosyl-haemoglobin, nitroso-thiols and nitro-aromatics) the literature to date has been dominated by the Greiss reaction and chromatographic assays for the oxidation products of NO, namely nitrite and nitrate. The historical background to this has already been discussed in the introduction. I chose to use the technique of capillary ion analysis (CIA) because it is a relatively easy, reproducible technique which requires only small sample volumes.

CIA was first suggested as a separation technique in 1967, but published experiments did not appear until 1979. Commercial instruments first appeared in 1988 and since then the number of publications utilising the techniques has increased rapidly. It offers the ease and speed of high performance liquid chromatography (HPLC) but uses only a fraction of the sample volume (Raij 1994). The method I used was that of Leone (Leone *et al* 1994a), which in turn was based upon modifications of previously reported methods using both CIA (Molnar *et al* 1994; Franchi *et al* 1994) and HPLC (Lee *et al* 1986).

2.2.2 Instrumentation

The CIA instrument consists of a detector, dual high voltage power supplies and an electrophoresis compartment containing a capillary connecting two buffer reservoirs (Figure 2.15). Molecules are separated as electrical force drives them through the capillary at different rates. They then pass through a detector measuring changes in the transmission of ultraviolet light. These changes are displayed as deflection peaks on an "electropherogram". An example of the portion of an electropherogram showing a standard nitrite and nitrate peak is shown in Figure 2.16. This is an unsmoothed curve representing the raw data and shows the good separation which is obtained using this technique.

Figure 2.15

Schematic representation of capillary ion analysis showing two buffer reservoirs connected by capillary tube with electrical potential between capillary ends. Detector unit sits across capillary to record electropherograms as ions pass through capillary with time.

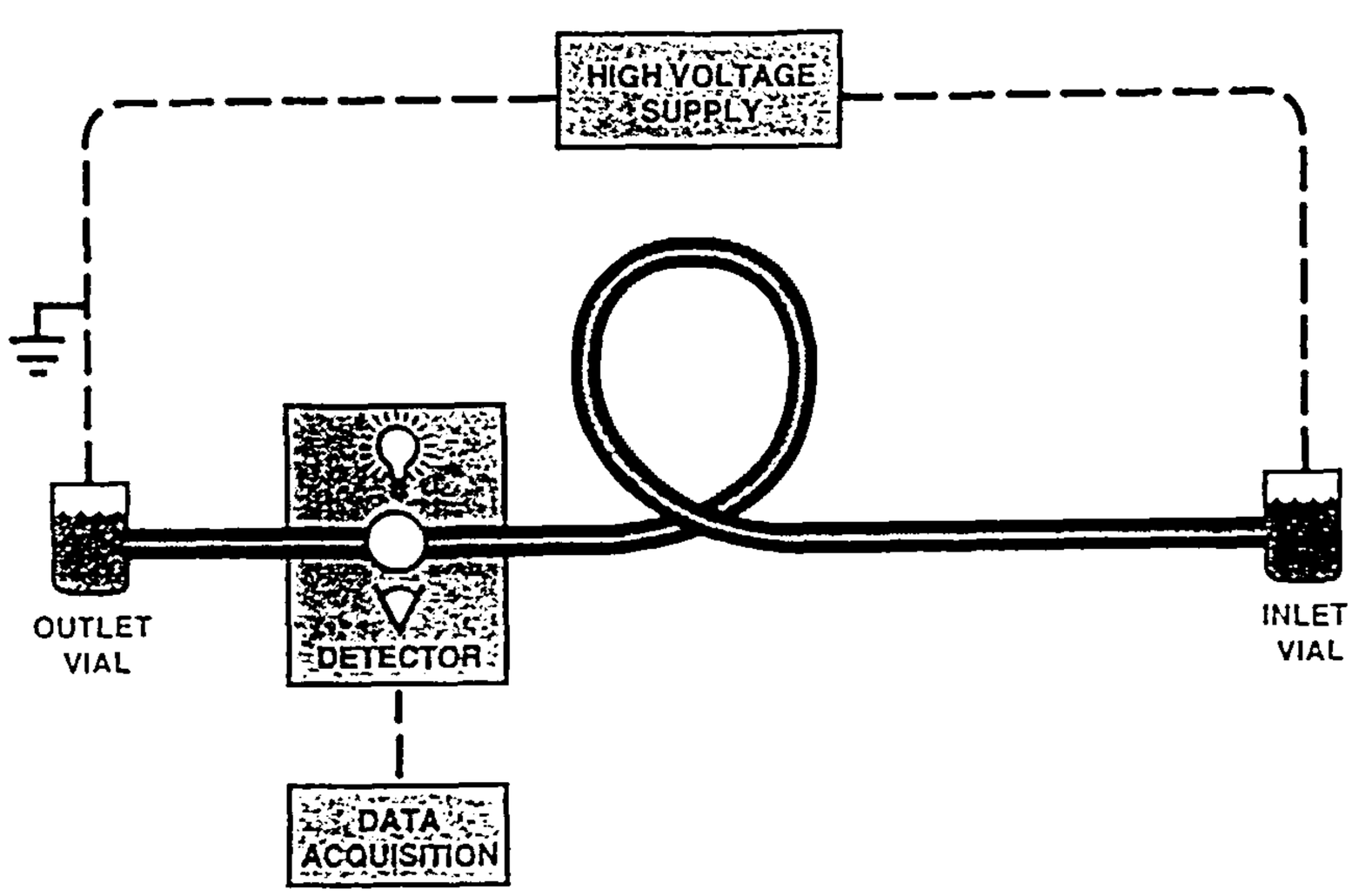
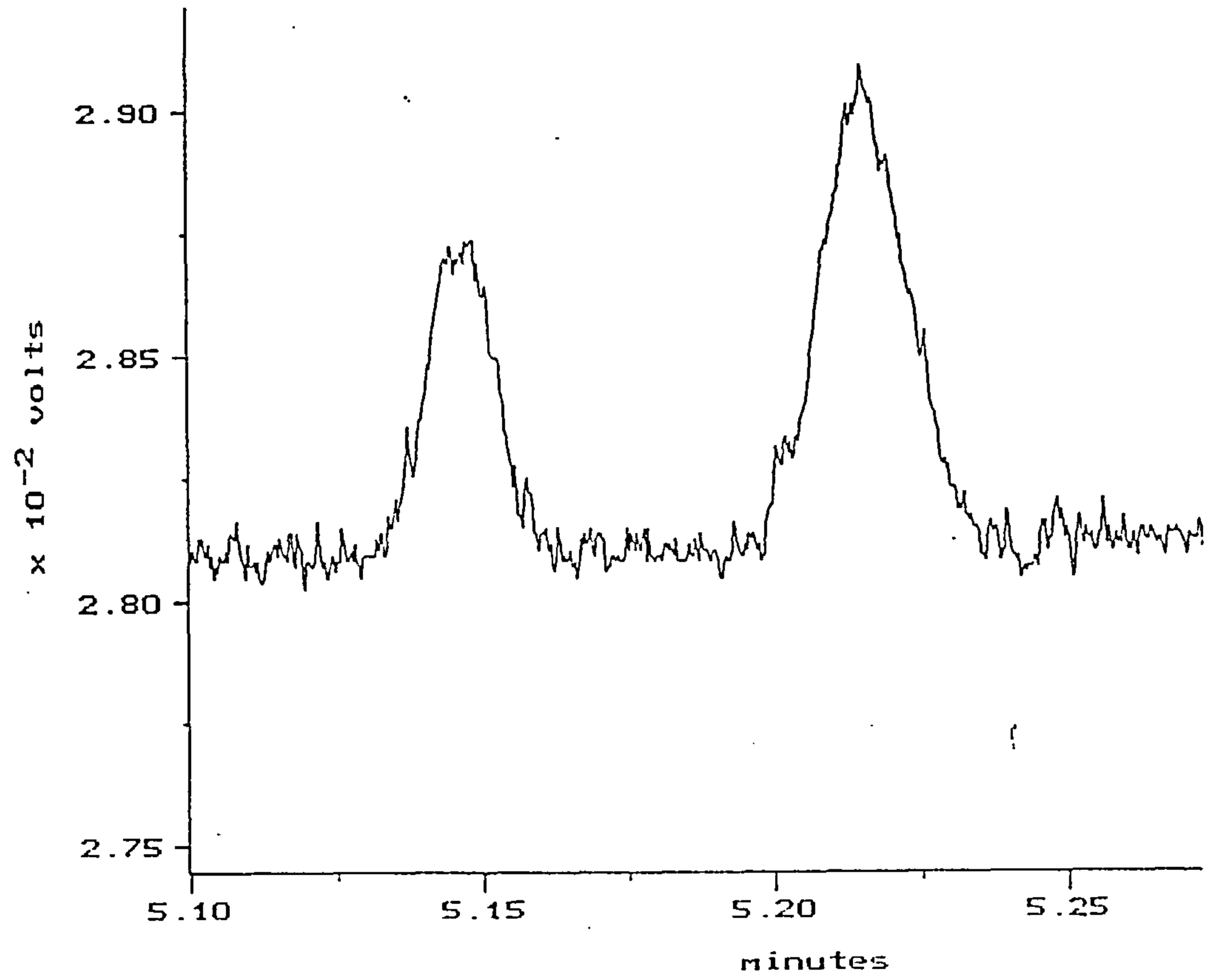


Figure 2.16

Example of electropherogram obtained from CIA machine showing peaks of nitrite and nitrate from spiked plasma samples. This is an unsmoothed curve. The dotted horizontal line is placed manually across the base of the peaks to allow area calculations to be carried out.

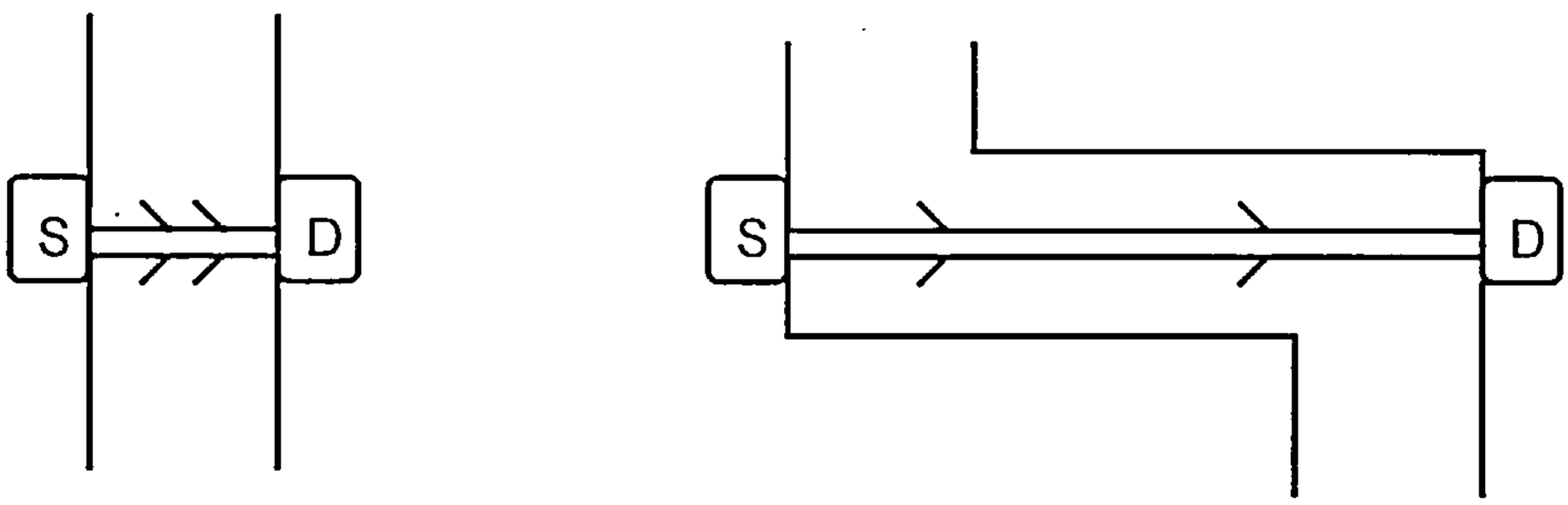


The final version of the assay described below was refined over a period of weeks to give the best possible results. Various concentrations of buffer and different capillary tubes were tried before settling on this combination. In order to give comparable data no further development of the assay was carried out or modifications made during the period of the studies reported here.

When CIA was first developed, the short distance which the light travelled through the capillary between the source and detector led to low sensitivities being reported (Stamler & Loscalzo 1992), however, this problem has been addressed subsequently by the development of the "Z-cell". By putting a kink in the capillary (the "Z") the optical path length is extended to 3mm, a 40-fold increase in length of light path over the internal diameter of a standard 75µm capillary (Figure 2.17). This improves sensitivity for nitrate/nitrite by 10-fold and allows reliable results to be obtained (Leone *et al* 1994a).

Figure 2.17

Diagrammatic representation (not to scale) of the increased light path between ultraviolet source (S) and detector (D) provided by Z-cell modification of a standard capillary.



The studies presented in this thesis used a Waters Quanta 4000 capillary electrophoresis system (Waters Chromatography Division, Millipore Corporation, Milford, MA, USA) fitted with a Z-Cell capillary. The 100cm x 75μm capillary tube was made of fused silica and coated with a layer of polymer for extra strength. It was manufactured in a cartridge format which not only allowed easy changing of the capillary in cases of blockage or damage but also ensured accurate alignment of the light source and detector. The capillary tube was primed by filling with an electrolyte solution made up of 10mM sodium sulphate (BDH Chemicals, Merk Ltd, Lutterworth, UK) containing 5% NICE-Pak OFM Anion-BT (Waters propietary osmotic flow modifier) in Milli-Q^+ water.

The end of the capillary was placed in the prepared sample (see sample preparation below) and a 30kV potential applied across its length for 30 seconds to preferentially draw the negative ions into the capillary tube. This includes not only nitrite and nitrate but also significant amounts of carbonate and chloride ions as well. Whilst this did not cause a problem for plasma measurements since the levels of chloride and sulphate are relatively constant it does make this technique less suitable for analysis of urine where the difference in chloride levels is substantial between subjects.

A steady 5kV potential was used for the next 7 minutes to allow slow electromigration of the negative ions along the capillary. The speed of migration was governed by the molecular weight and electrical charge of the different ions. When the ions passed through the portion of the tube with the optical detector a deflection was seen in the transmitted light level.

Data was acquired at 214nm (band width 2nm) in the ultraviolet range. This was chosen because although the UV maxima for nitrite and nitrate are actually less than this, the sample noise is significantly increased at

wavelengths below 200nm. Hence a narrow bandwidth at 214nm offered the optimum signal to noise ratio. The magnitude of alteration in the transmitted light during the 7 minute transit time was recorded on the electropherogram. The size of peaks obtained on the electropherogram was proportional to the concentrations of different anions present and peak area was easily measured using the supplied software. Samples spiked with standard solutions of nitrite and nitrate allowed identification of migration times for particular molecules and molar quantification based on peak area.

2.2.3 Preparation of samples and standards

The samples were diluted 1:10 with MilliQ+ water (Millipore, Bedford, MA, USA) in polypropylene vials which had been rinsed twice with MilliQ+ water. Ultrafree MC filters (Millipore) with a nominal molecular weight cut off of 5kD were then used to ultrafilter the samples at 5000g having first been rinsed with MiliQ+ water. The purified plasma was further diluted before reading by placing 100ul of filtered plasma with 100ul water in the vial for reading in the CIA analyser. Vials were prepared in batches of 20 (8 samples in duplicate with 4 standards) which then took 3 hours for analysis.

Standards were prepared using potassium nitrite and sodium nitrate (Analar grade, BDH Chemicals, UK). These had to made freshly at each analysis and then diluted to 5, 10, 50 and 100 μmol/l concentrations for analysis.

2.2.4. Assay Reliability

Standard curves constructed using the 5 -100 μmol/l solutions of nitrite and nitrate gave regression co-efficients of 0.98 and 0.99 respectively. The intra-assay co-efficients of variance (CV) were calculated for 10 repeated assays on fresh samples. The results showed CVs of 10% for basal nitrite, 5% for 50 μmol/l nitrite, 6% for basal nitrate and 2% for 50

μmol/l nitrate. The inter-assay CVs for basal and spiked samples were 10%.

Experiments were also carried out to establish if freezing and storage altered the readings. This was done by using alliquoted blood samples from the laboratory staff which were assayed immediately after 1 week and again after 1 month. The mean (SEM) results are shown below (n = 5).

	Immediate	Frozen 1 week	Frozen 1 month
Nitrate μmol/l	36(4)	35(4)	37(4)
Nitrite μmol/l	4(1)	4(1)	4(1)

These results showed no significant differences when compared using a paired Students t-test and this confirms that immediate freezing and subsequent storage at -70°C does not affect readings of nitrite and nitrate obtained by CIA. This is a most important step, since sample collection and batch analysis would have been impossible without it.

2.3 CITRULLINE ASSAY

2.3.1 Background

The activity of nitric oxide synthase in a tissue can be estimated from the production of citrulline *in vitro*. This is because molar equivalent quantities of L-citrulline are produced when NO is produced from L-arginine by NO synthase. The activities of the two forms of the NOS enzyme, iNOS and cNOS, may be calculated separately by performing parallel assays in the presence and absence of calcium/calmodulin according to the method of Salter (Salter *et al* 1991). Tissue can be from any source but requires freezing rapidly in liquid nitrogen to -70°C for storage prior to assay. An additional assay is used to quantitate the protein content of the tissue under study (Lowry *et al* 1951). This allows the final result to be standardised as mmol of citrulline formed per minute per mg of tissue protein so that different tissues can be meaningfully compared.

2.3.2 Assay materials

Dithiothreitol, phenylmethylsulphonyl fluoride (PMSF), soybean inhibitor, aprotinin and 1mM N^G-monomethyl-L-arginine (L-NMMA) were obtained from Sigma Chemical Co Ltd., Poole, Dorset. Dowex-50 (200-400), 8% cross-linked H^+ -form was purchased from BDH Chemicals Ltd., Poole, Dorset and L-[U-^{14}C]arginine was from Amersham International Plc, Amersham, Bucks.

2.3.3 Preparation of homogenates

Placental villi were obtained, weighed, wrapped in aluminium foil and labelled. The samples were then frozen in liquid nitrogen within 15 minutes. Samples were stored at -70°C for up to 4 weeks prior to being processed in batches. All subsequent procedures were carried out between 0 - 4°C.

5 volumes of 0.25 mol/l sucrose containing 50 mmol/l Tris-HCl at pH 7.0,

1 mmol/l dithiothreitol, 1 mmol/l EDTA, 100 µg/ml PMSF, 10 µg/ml of soybean inhibitor and 2 µg/ml of aprotinin were added and homogenised using an Ultra-Turrax homogeniser in 4 bursts of 15 seconds over 2 minutes. The resultant homogenate was then centrifuged for 5 minutes at 11000g, alliquoted into two parts and kept on ice for no longer than 15 minutes prior to assay. One aliquot of homogenate was dispensed into 0.1 mol/l NaOH and kept at 4°C for protein determination.

2.3.4 Protein Determination

This was done using the method of Lowry (Lowry *et al* 1951).

0.5ml of 1% $CuSO_4$ was mixed with 0.5ml of 2% sodium tartrate. The resultant solution was then added to 50ml of 2% Na_2CO_3 in 0.1mol/l NaOH to form solution A. Protein standards were prepared from 1.6mg/ml albumin in water. A 1:20 dilution of the concentrate produced a standard of 80µg/ml which was then further diluted to form standards of 40,20 and 10µg/ml.

1ml of prepared homogenate (or standard) was mixed with 5ml of solution A and allowed to stand at room temperature for 15 minutes. 0.5ml of Folin-Ciocalteu's Reagent (BDH) was then added and a further 30 minutes of standing allowed. At the end of this time the sample was read in a spectrophotometer at 720nm.

2.3.5 Citrulline assay

Assays were performed at 37°C according the method of Salter (Salter *et al* 1991). The assay solution consisted of 12.5 mmol/l HEPES at pH 7.3 with 1.2 mmol/l $MgCl_2$, 0.96 mmol/l $CaCl_2$, 60 mmol/l L-valine, 1.2 mmol/l L-citrulline, 0.024 mmol/l L-arginine, 150,000 dpm L-[U-^{14}C]arginine and 0.12 mmol/l beta-NADPH. For each sample one tube was prepared with assay solution only, one with assay solution plus 1.5 mmol/l EGTA, one with assay solution plus 1 mmol/l L-NMMA and one with assay solution

plus both 1.5 mmol/l EGTA and 1 mmol/l L-NMMA. The reaction mixture was pre-incubated at 37°C in 3 ml borosilicate tubes and the reaction initiated with 0.02 ml of homogenate. The reaction was stopped at 0 and 8 minutes (for trophoblast samples) and 16 minutes (for myometrial samples) with 1.0 ml of water/Dowex Na^+-form and mix. All assays were performed in duplicate.

The Na^+-form was prepared by washing the H^+-form of the resin with 1 mol/l NaOH four times and then washing with water until the pH was less than 7.5. In practice this usually meant more than six washes. A 3:1 volume water/Dowex Na^+ -form mixture was made for termination of the reaction. 1.0 ml of water was then added, mixed and left to settle for 10 minutes and a further 1.0 ml of water/Dowex was added to the supernatant, mix and centrifuge. 2.0ml of supernatant was added to 2.0ml of Ultima gold XR (Canberra Packard, Berks) and the amount of [^{14}C]citrulline counted by liquid scintillation counting. The first wash reduced radioactivity of samples by approximately 99.2% and the second wash removed a further 60% of the remaining label.

The activity of the calcium-dependent NOS was determined from the difference between the [^{14}C]citrulline produced in the control tubes and those containing 1 mmol/l L-NMMA. The activity of the calcium-independent NOS was determined from the difference between the [^{14}C]citrulline produced in the tubes with 1.5 mmol/l EGTA and those containing 1.5 mmol/l EGTA plus 1 mol/l L-NMMA.

2.3.6 Validation of the Assay

Several of the samples collected were divided into two and stored at -70°C so they could be measured at the Wellcome laboratories (Results marked by W) as well as at the Chelsea and Westminster hospital (Results marked by C) where the assay had only recently been set up. The results obtained are given in table 2.1. These data show that the new

assay at Chelsea and Westminster produced a slightly higher mean reading than the established assay at Wellcome but that the relative amounts of iNOS and cNOS were relatively reproducible.

Initially, incorrect readings were obtained due to an inadequate concentration of EGTA being used in the assay. This was brought out by the comparison and saved a great deal of time in setting up a good method. All data presented in this thesis used the method outlined above to allow comparison between different groups.

The assay was also verified using rat cerebellum.which formed 402.4 ± 36.7 μmol citrulline/min/g protein in the presence of calcium (cNOS) and 62.2 ± 3.1 μmol citrulline/min/g protein without calcium (iNOS). The limit of detection of both the calcium-dependent and -independent NOS activities was approximately 0.1 μmol citrulline/min/g of protein.

Table 2.1

NOS activities (μmol citrulline/min/g of protein) obtained from aliquots of the same tissue sample analysed by different operators performing the citrulline assay in two different laboratories (n=10). (W) represents results from Wellcome Research Laboratories and (C) represents results from Chelsea and Westminster Hospital.

cNOS(W)	iNOS(W)	cNOS(C)	INOS(C)
4.6	0.9	4.5	4.0
0.9	2.2	1.7	6.7
6.0	8.3	10.7	12.6
15.0	14.2	14.5	16.8
4.4	0.9	6.0	2.1
1.5	9.2	1.7	10.7
3.6	1.3	5.8	3.7
3.6	3.6	7.9	8.8
4.2	3.1	7.9	7.4
1.7	0.7	0.5	1.0

2.4 ASSAY OF OESTRADIOL, FSH AND LH

FSH, LH and Oestradiol were measured using ELISA assays on an ES 600 Analyser (Boehringer Mannheim GmbH).

2.4.1 LH Assay

The Luteinising Hormone assay measures in the range 0-150 mIU/ml with a lower detection limit below 0.5 mIU/ml. There is no measurable cross reactivity *in vitro*. Inter-assay precision throughout the range of the assay is shown in the table below with SD being standard deviation and CV being coefficient of variation.

	Low levels	Medium levels	High levels
Mean (mIU/ml)	3.64	20.8	84.6
SD	0.67	0.75	3.29
CV (%)	18.5	3.6	3.9

2.4.2 Oestradiol assay

The oestradiol assay measures in the range 0-4770 pmol/l with a lower detection limit of 37 pmol/l. Samples assayed above the upper limit were diluted to obtain a result ie second and third trimester of pregnancy samples. Cross reactivity *in vitro* (referred to oestradiol = 100%) is mainly with oestrone (1.1%) and oestriol (0.3%). The inter-assay precision throughout the range of the assay is shown below.

	Low levels	Medium levels	High levels
Mean (pmol/l)	118	368	1336
SD	17.5	29.3	98.2
CV (%)	14.9	8.0	7.4

2.4.3 FSH assay

The follicle stimulating hormone assay measures in the range 0-150 mIU/ml with a lower detection limit below 0.5 mIU/ml. There is no measurable cross reactivity *in vitro*. Inter-assay precision throughout the range of the assay is shown in the table below.

	Low levels	Medium levels	High levels
Mean (mIU/ml)	2.9	26.4	97.4
SD	0.57	0.66	3.49
CV (%)	15.3	3.2	4.1

CHAPTER 3 - NITRIC OXIDE PRODUCTION DURING PREGNANCY

3.1 INTRODUCTION

3.1.1. Control of vascular tone

In normal human pregnancy there is a fall in systemic blood pressure despite an increase in cardiac output and circulating plasma volume. This implies profound vasodilatation of the maternal circulation. Since NO plays a significant role in maintaining resting vascular tone in man (Vallance *et al* 1989) it is highly likely that there will be alterations in NO production during pregnancy.

The evidence from animal studies already suggests a role for NO in pregnancy (Chu & Beilin 1993; Weiner *et al* 1992; St Louis & Sicotte 1992; Conrad *et al* 1993b). Such animal models have now been adapted to examine the role of NO in pregnancies complicated by hypertension and/or IUGR (Yallampalli & Garfield 1993, Miller 1995. Diket *et al* 1995).

It is well known that the uteroplacental circulation, particularly in later pregnancy, presents a very low resistance to vascular flow (Schulman *et al* 1986; Yallampalli *et al* 1994; Bower *et al* 1992). This facilitates high blood flows and easy exchange of nutrients between mother and fetus. It is also widely accepted that there is an increase in uteroplacental vascular resistance to perfusion in pre-eclampsia and IUGR (Campbell *et al* 1986; Nylund *et al* 1983; Bewley *et all* 1991; McCowan *et al* 1988; Cohen-Overbeek *et al* 1985). In the most severely affected cases (e.g. reversed Doppler blood flow through the umbilical artery) this is known to be associated with fetal hypoxia and acidosis (Nicolaides *et al* 1988). In the 1970s and 1980s much work was carried out on prostacyclin, another endothelial derived vasodilator, to determine its relationship with IUGR and alterations in endothelial production of vasoactive substances such as prostacyclin (McLaren *et al* 1987), but more recently there has been increased interest in NO.

Since the vessels within the placenta have no autonomic nerve supply, vascular resistance must be determined by anatomical factors (Brosens *et al* 1967; Brosens *et al* 1977) and vasomotor tone modulated through endocrine or paracrine factors (Reilly & Russell 1977; Hardy *et al* 1994). Such a control mechanism is likely to be multifactorial and complex since there are many vasoactive substances which may participate. Several investigators have already demonstrated that NO is able to influence the placental vasculature through *in vivo* animal studies (Van Buren *et al* 1992) and *in vitro* human studies (Myatt *et al* 1992). There is also some evidence from *in vitro* work using human uterine artery tissue, to suggest a role for NO in mediating the changes in pressor responsiveness which are seen in normal pregnancy (Helmbrecht *et al* 1995).

Many groups have studied platelet function in pregnancy, particularly those pregnancies complicated by pre-eclampsia and IUGR. There is known to be increased platelet turnover with deposition in the placental bed leading to increased uteroplacental vascular resistance (Van der Post *et al* 1993; Barr *et al* 1989; Zemel *et al* 1990; Walker *et al* 1989; Trip *et al* 1990). Since NO is central to the regulation of platelet aggregation (Radomski *et al* 1987; Radomski *et al* 1990b; Benjamin *et al* 1991; Vallance *et al* 1992) it is possible that a relative deficiency of NO production by the placenta could contribute to the problem. Although animal studies have already demonstrated IUGR resulting from pharmacological inhibition of NOS during pregnancy in rats (Diket *et al* 1995) there is no data available as yet in humans on this subject.

3.1.2. Implantation

During the early stages of pregnancy the trophoblast is at its most active, invading deeply into the decidua and myometrium (Gerretsen *et al* 1981). In addition to these structural changes (discussed in detail in the main introduction), the trophoblast must stimulate local vasodilatation and at

the same time suppress maternal rejection of the fetal tissue. Since NO is known to have a regulatory effect on the immune system, it is possible that trophoblast derived NO may have other functions beyond simple vasodilatation in early pregnancy.

3.1.3. Maintenance of myometrial quiesence

It has been shown that administration of an NO donor drug will abolish contractions in rat uterine smooth muscle both *in-vitro* and *in-vivo* (Natuzzi *et al* 1993). Studies *in vitro* have shown a similar effect on human myometrium (Yallampalli *et al* 1993) and there have also been cases reported of NO donor drugs being used to suppress human myometrial activity *in vivo* (Lees *et al* 1994; Greenspoon & Koviacic 1991). Whilst it is possible that NO may help to maintain uterine quiesence during pregnancy, there is as yet little data available on NO production by the myometrium or placenta in humans in relation to onset of labour. Since premature labour is a major cause of perinatal morbidity and mortality this is an important area for further investigation.

3.1.4. Amino acid levels in placental tissue

Placental amino acid transfer is essential for the normal development of the fetus during pregnancy to provide adequate nitrogen for urea production and new tissue growth. In relation to NO function the substrate for NOS is L-Arginine, a non essential amino acid. It is important to know the availability of this substrate before being able to draw conclusions about NOS activities in placental tissue.

Many investigators have quantified amino acids in placental villous tissue (Pearse & Sornson 1969; Valazquez *et al* 1976; Phillips *et al* 1978) and several studies have measured fetal and maternal plasma amino acid concentrations (McIntosh *et al* 1984; Soltesz *et al* 1985; Bernardini *et al* 1991). These measurements will reflect not only transport across the placenta, but also placental amino acid metabolism. Since placental

tissue possesses several different transport systems (Enders *et al* 1976; Yudelovich & Sweiry 1985) and has the ability to synthesise several non-essential amino acids itself (Marconi *et al* 1989; Cetlin *et al* 1991) the relative tissue and plasma levels must be interpreted with caution.

Unfortunately there are several weaknesses with the existing data since virtually all the work on this subject was carried out 10-20 years ago. Now that technology has advanced it is possible to measure more than the 20 amino acids quoted in the original publications both more accurately and using smaller sample volumes of tissue. In addition it is now possible to easily quantify the total protein content of the tissue and express the individual amino acid concentrations relative to this rather than the weight of the tissue as a whole.

3.1.5. Aims of study

Systemic NO production during pregnancy can only be studied indirectly, through the quantification of breakdown products such as nitrite and nitrate. Therefore I collected samples of blood from women throughout pregnancy to assess the relationship of NO to the vascular adaptations which are known to occur in normal pregnancy. In addition I was able to collect blood from several women with complicated pregnancies for comparison. Blood flow measurements were made with Doppler ultrasound from the uteroplacental circulation to see if a correlation existed between NO production and blood flow.

Recently the calcium-dependent isoform of NOS has been isolated from term human placenta from both the syncytiotrophoblast (Conrad *et al* 1993a) and villous vascular tree (Myatt *et al* 1993). It may be that the NO produced by placental NOS (or by NOS in the myometrium) regulates the placental vascular resistance to blood flow or helps modulate myometrial contractility.

At present, there are no reports of the relationship between onset of labour or uteroplacental blood flow and NO production in human pregnancy. To investigate this I measured NOS activities in human trophoblast throughout gestation and in myometrium obtained from non-pregnant women and pregnant women before and after the onset of labour.

To assess the role of substrate availability I measured amino acid levels in the placental tissue, specifically L-Arginine.

3.2 METHODS

3.2.1 Study design

The study was intended to explore the potential role for NO in controlling the cardiovascular adaptations to pregnancy, uteroplacental blood flow and onset of labour. I collected maternal venous blood, fetal blood, placental tissue and myometrium from several groups of women as described below together with additional data on:-

- Details of gestational age, maternal age, smoking habit and racial origin.
- Doppler ultrasound measurements of uterine and umbilical blood flow.
- Maternal pulse and blood pressure.
- Fetal heart rate.

3.2.2. Patient Recruitment

Several groups of women were approached to help with this study and informed written consent obtained in every case.

- **Non-pregnant women.**

10 non-pregnant women were recruited from within the department. This allowed me to take blood at approximately the same stage of the menstrual cycle for all women (day 7-10). All had regular cycles, were non-smokers and did not use oral contraceptives. They took no medication in the week prior to sample collection and had the blood taken first thing in the morning following an overnight fast.

- **Normal pregnancies throughout gestation.**

50 women with uncomplicated pregnancies were recruited throughout gestation. 27 women in the first trimester (mean gestation 9.8 ± SD 1.4 weeks) and 11 women in the second trimester (17.6 ± 2.5 weeks) who were all attending the hospital for termination of pregnancy. 12 women in the third trimester (38.5 ± 1.3 weeks) were recruited at the time of elective

caesarean section for breech presentation. All had blood taken following an overnight fast and had not taken any medication in the week prior to this. Placental tissue was obtained at the time of surgery in each case.

In addition to the maternal samples, fetal blood was collected from a double clamped length of umbilical cord at the time of caesarean section for the term pregnancies and handled in exactly the same way as the maternal samples.

- **Complicated pregnancies at delivery**

These 8 women were recruited from the fetal assessment unit at King's College Hospital. They were all known to have ultrasound evidence of fetal growth retardation (AC < 2.5th centile for gestational age) with abnormal Dopplers. All had an umbilical artery PI above the normal range for gestational age with 5 of the 8 showing absent or reversed end diastolic velocities. In addition they all showed evidence of fetal blood flow redistribution (Middle Cerebral Artery PI below the normal range and thoracic aorta PI above the normal range for gestational age) together with relative oligohydramnios. All 8 were advised to undergo elective caesarean section at which time the blood samples were taken and placental tissue obtained. Not surprisingly, these operations were performed at an earlier gestation (range 31 - 37 weeks) than those for the women in group 2.

In addition to the maternal samples, fetal blood was collected from a double clamped length of umbilical cord at the time of caesarean section and handled in exactly the same way as the maternal samples.

- **Abnormal uterine artery Doppler at 26 weeks with normal controls.**

All women at King's College Hospital have uterine artery Doppler recordings taken at the time of the 20 week anomaly scan. All abnormals were then rescanned by me between 24 and 26 weeks gestation. 10

women with persistently abnormal uterine artery Dopplers at 24-26 weeks gestation were recruited for this study and had blood taken. The technique for obtaining Doppler signals from the main uterine artery has already been discussed in the general methods section. A reading was considered abnormal if the mean RI was greater than 0.6 with bilateral post-systolic notching of the waveform.

For comparison I selected 10 women who had normal Dopplers at the 20 week scan but who required rescanning at 26 weeks for another reason. Examples of this included confirmation of growth velocity before altering dates or fetal position at the time of the initial scan making assessment of certain structures (e.g. face and lips) difficult. All fetuses in this group were structurally normal and appropriately grown.

- **Women undergoing hysterectomy.**

Myometrial samples were obtained from 5 non-pregnant multiparous women of child bearing age (median age 42 years, range 34 - 48 years) undergoing hysterectomy for utero-vaginal prolapse. Women with dysfunctional bleeding, fibroids, malignancy, endometriosis or other pathology were excluded since it is not known if these conditions affect NOS activities.

- **Normal pregnancies at delivery.**

Myometrial samples were also obtained from 14 pregnant women at the time of term caesarean section following an uncomplicated pregnancy. 7 of these were elective operations before the onset of labour for breech presentation and 7 were performed after a period of spontaneous labour, 4 for abnormal CTG and 3 for failure to progress.

3.2.3. Collection of blood samples for nitrite / nitrate analysis

For maternal samples 5mls of venous blood was collected from an antecubital vein through an 18 gauge sterile needle into a 10ml syringe.

For fetal samples 5 mls of blood was collected from the umbilical artery immediately following delivery. In each case the syringe was pre-washed with de-ionised water to prevent possible contamination with nitrates and nitrates adherent to the plastic. The blood was immediately transferred into a washed 5ml plastic bottle and put into ice for transfer to the laboratory. All samples were centrifuged at 2000g for ten minutes within 30 minutes of the initial collection. This was logistically difficult but considered important because of the ongoing metabolism of NO, NO_2 and NO_3 which occurs in *ex-vivo* whole blood. Ideally the centrifuge would be in the same room as the patient but in my case it was at the other end of the hospital and shared by other users.

After centrifugation the resultant plasma was alliquoted into a series of 2ml eppendorf tubes, clearly labelled with a study number and then placed in liquid nitrogen for "snap" freezing. After 10 minutes these were then put in the -70°C freezer to await batch analysis at a later date.

3.2.4. Collection of placental samples for quantification of NOS activities

Placental villi were obtained at the time of first and second trimester terminations of pregnancy and term caesarean sections before the onset of labour. The gestational ages of first and second trimester pregnancies were determined by measurement of fetal crown-rump length immediately prior to the procedure and third trimester pregnancies were dated from the last menstrual period (confirmed at a 20 week ultrasound scan).

For the termination cases this involved identification of the placental tissue following vacuum aspiration of the uterine contents. For the caesarean section cases it involved the sharp dissection of placental tissue immediately following delivery. The samples of placental villous tissue were placed onto small squares of aluminium foil, labelled and then weighed dry before immersion in liquid nitrogen for 10 minutes to “snap

freeze". They were then stored at -70°C to await batch analysis.

3.2.5. Collection of myometrial samples for quantification of NOS activities

For the non-pregnant women the samples of myometrium were obtained following hysterectomy for utero-vaginal prolapse. For the pregnant group the myometrium was obtained from the upper edge of the transverse lower segment uterine incision used to perform caesarean section. The volume of tissue removed was approximately 1-2cm^3 in each case. The myometrium was handled in the same way as the placental tissue for NOS estimation, being "snap frozen" and subsequently stored at -70°C.

3.2.6. Ultrasound measurements

Ultrasound measurements of fetal growth, liquor volume and Doppler waveforms from uteroplacental circulation were carried out as described in the general methods section.

3.2.7. Quantification of plasma nitrite and nitrate

This was carried out using capillary ion analysis (CIA) as described in the general methods section.

3.2.8. Quantification of NOS activity

This was carried out using the citrulline assay as described in the general methods section.

3.2.9. Amino acid analysis

Dithiothreitol, phenylmethylsulphonyl fluoride, soybean inhibitor, aprotinin and s-aminoethylcysteine were obtained from Sigma Chemical Co Ltd., Poole, Dorset. All other laboratory reagents were of the purest grade available and were either from Sigma or BDH Chemicals Ltd., Poole, Dorset.

Frozen tissue was defrosted and all subsequent procedures carried out at 4°C. 5 volumes of 0.25M sucrose containing 50mM Tris-HCl, pH 7.0, 1mM dithiothreitol, 1mM EDTA, 100µg/mL phenylmethylsulphonyl fluoride, 10µg/mL of soybean inhibitor and 2µg/mL of aprotinin were added and homogenised using an Ultra-Turrax homogeniser (top speed) in 4 bursts of 15 seconds over 2 minutes at 4°C.

After centrifugation for 5 minutes at 11 000g, 40 mol of s-aminoethylcysteine was added to 0.18mL of placental homogenate to a final volume of 0.2mL. Next, 0.02mL of 35% (w\v) sulphosalicylic acid was added, mixed and left to stand for 5 minutes. After further centrifugation for 5 minutes at 11 000g, 0.1mL was diluted with an equal volume of Beckman loading buffer, pH 2.2, mixed and re-centrifuged. 0.05mL of the resultant supernatant was applied to the column of a Beckman 6300 HPLC ion exchange amino acid analyser.

Following application of the prepared sample the column was eluted with a 3 buffer system (Beckman lithium buffers A, B, C). Peaks were integrated using the Beckman 406 analogue to digital converter and Beckman "System Gold" software as supplied with the analyser. An automated print out of results was obtained for each sample of 22 amino acids including L-Arginine.

Total protein content of the tissue was also calculated using the same method as described in the general methods section. Results were then

expressed as nmol of amino acid / mg of tissue protein.

3.2.10. Statistical Methods

The results of plasma nitrite and nitrate for matched fetal / maternal samples were compared using a Wilcoxon signed-ranks test for non-parametric data.

All other comparisons used either a Mann-Whitney U or Kruskall-Wallis Analysis of Variance tests for non-parametric data.

3.3 RESULTS

3.3.1 Nitrate and nitrite levels in plasma

The results for plasma nitrate and nitrite are shown in tables 3.1 and 3.2 with the comparison data shown in table 3.3.

The levels of nitrite were low (range 0-7 µmol/L) in all women with no significant differences between groups. Fetal levels were also low and corresponded closely with the maternal levels. Growth retarded fetuses had similar nitrite levels to their normally grown counterparts.

Nitrate levels were higher (range 26-46 µmol/L) than the nitrite levels and in agreement with those published previously in humans. The lowest levels were found in the non-pregnant women and were significantly less than those seen in the second and third trimesters of pregnancy. There was no difference between those women with abnormal Dopplers at 26 weeks gestation and those with normal uteroplacental blood flow.

Mothers with growth retarded babies had similar nitrate levels to normal pregnancy as did the fetuses themselves. There was a tendency for fetal nitrate to be marginally higher than the maternal level. This just achieved statistical significance in the IUGR group ($p=0.04$) but not in the normal pregnancy group ($p=0.064$).

3.3.2. Other data on the normal pregnancies

These data are shown in table 3.4. Advancing gestation brought significant decreases in maternal blood pressure and haemoglobin levels as well as fetal heart rate. Doppler indices of uteroplacental vascular resistance fell over the same period indicating increased blood flow. The white cell count and oestrogen levels increased significantly with advancing gestation. There was no significant change in resting maternal pulse or platelet numbers.

3.3.3. NOS activities in trophoblast

These data are shown in table 3.5 and compared in table 3.6. The highest levels of NOS activity were found in first trimester villi with a fall towards term. Whilst this was true for both calcium dependent and independent activities, iNOS activities appeared to fall earlier than the cNOS activities. This is illustrated in figures 3.1 and 3.2.

Placental villi from IUGR pregnancies showed very little NOS activity at all. The levels were significantly lower than those found in normal second and third trimester placentae.

Due to the strong correlation of gestational age with NOS activities in the trophoblast a multiple regression analysis was used to investigate the relationship between NOS and the other parameters. This demonstrated a weak correlation between trophoblast NOS activities and the Doppler indices of uteroplacental blood flow.

3.3.4. Analysis of pregnancy data according to ethnicity.

The pregnancy data was re-analysed according to ethnicity and the results are shown in table 3.7. There were no significant differences in nitrite, nitrate, NOS activities, Doppler indices or other parameters between Caucasian and non-Caucasian women.

3.3.5. Analysis of pregnancy data according to smoking habit

The pregnancy data was re-analysed according to smoking habit and the results are shown in table 3.8. It was more common for the women attending for termination in the first trimester of pregnancy to smoke than it was for women at term undergoing caesarean section. This was reflected in the significant difference between the median gestations of the two groups. For this reason many of the parameters related to gestation showed significant differences between smokers and non-smokers.

To minimise these difficulties each trimester was then analysed separately using a Kruskall-Wallis ANOVA and the results for the first trimester are shown in table 3.9. The oestrogen level was the only factor which differed between the smokers and non-smokers in this analysis (p=0.038).

3.3.6. NOS activities in myometrium

The results are shown in table 3.10, with comparisons between sub-groups in table 3.12. These comparisons are illustrated in figure 3.3 and 3.4.

In the myometrium both cNOS and iNOS activities were at a much lower level than that seen in the trophoblast samples. The cNOS activities were statistically similar in all sub-groups whereas the iNOS activities were significantly lower in the pregnant group than in the non-pregnant group (p=0.007). The lowest values were obtained from the non-labour group which were significantly less than those of the labour group (p=0.011).

3.3.7. Amino acids in trophoblast

Concentrations of 22 amino acids were measured and the results are shown in table 3.12. Asparagine, citrulline, cystathionine and homocystine were also measured but none was detected in the homogenates.

The protein concentrations of first and third trimester placental villous homogenates were 4.1 ± 0.6 and 6.2 ± 0.9 mg/mL respectively (amino acid concentrations have been expressed as nmol/mg of homogenate protein±SEM). Previously amino acids data were presented per gram wet weight of tissues (Pearse & Sornson 1969; Phillips *et al* 1978), which is not such a reliable index.

The amino acids of interest to my study were citrulline and arginine.

There was no measurable level of citrulline in the tissues. However arginine levels showed a significant reduction with advancing gestation (10.2 vs 4.6 nmol/mg protein, p=0.04). IUGR arginine levels were similar to those obtained from normal first trimester pregnancies but significantly different to those obtained from normal term pregnancies (10.4 vs 4.6 nmol/mg protein, p=0.04).

Table 3.1

Descriptive statistics for plasma nitrate levels (μmol/l) from non-pregnant women; normal pregnant women in first, second and third trimesters; pregnant women with IUGR and pregnant women at 26 weeks gestation with normal or abnormal uterine artery Dopplers. Values are also shown for the matched fetal values from the term caesarean sections and the IUGR caesarean sections.

	Mean Gestation (weeks)	n	Min	25th centile	Median	75th centile	Max
Non-Pregnant	---	10	26	28.5	31	33.5	35
1st Trimester	10	27	24	29.0	34	38.0	46
2nd Trimester	18	11	29	35.0	38	40.0	42
3rd Trimester	38	12	31	34.5	37	39.0	43
Normal Fetal	38	12	32	33.5	38	39.5	44
IUGR Maternal	33	8	30	33.5	36	39.0	42
IUGR Fetal	33	8	34	38.0	39	41.0	46
Normal 26 week	26	10	28	35.0	37	39.0	41
Notch 26 week	26	10	30	35.0	37	39.5	42

Table 3.2

Descriptive statistics for plasma nitrite levels (μmol/l) from non-pregnant women; normal pregnant women in first, second and third trimesters; pregnant women with IUGR and pregnant women at 26 weeks gestation with normal or abnormal uterine artery Dopplers. Values are also shown for the matched fetal values from the term caesarean sections and the IUGR caesarean sections.

	Mean Gestation (weeks)	n	Min	25th centile	Median	75th centile	Max
Non-Pregnant	----	10	0	0	2.0	3.0	4
1st Trimester	10	27	0	0	1.0	4.0	7
2nd Trimester	18	11	0	2.0	2.0	3.0	5
3rd Trimester	38	12	0	1.5	2.0	3.0	4
Normal Fetal	38	12	0	1.5	2.0	3.0	5
IUGR Maternal	33	8	0	1.5	2.0	3.5	5
IUGR Fetal	33	8	0	2.0	3.0	4.0	6
Normal 26 week	26	10	0	2.0	2.0	4.0	6
Notch 26 week	26	10	0	2.0	2.0	3.0	5

Table 3.3

Actual p values obtained by comparison of plasma nitrite and nitrate levels (μmol/l) between the different groups. All values were compared using a Kruskall-Wallis ANOVA for non-parametric data to allow for multiple comparisons except for those marked * which were treated as paired data (i.e. mother and fetus) and analysed using a Wilcoxon Matched Pairs Signed-Ranks test for non-parametric data.

Comparisons	Nitrite	Nitrate
	p	p
1st vs 2nd Trimesters	0.4083	0.0448
1st vs 3rd Trimesters	0.9641	0.0748
2nd vs 3rd Trimesters	0.4491	0.6075
Non-pregnant vs 1st	0.9886	0.2157
Non-pregnant vs 2nd	0.2816	0.0006
Non-pregnant vs 3rd	0.9229	0.0004
Notch vs Normal Doppler	0.6290	0.6237
Normal fetal vs 3rd	0.3884*	0.0637*
IUGR maternal vs 2nd	0.5002	0.4609
IUGR maternal vs 3rd	0.5385	0.5654
IUGR fetal vs normal fetal	0.2939	0.0934
IUGR fetal vs IUGR maternal	0.1443*	0.0404*

Table 3.4 Results of other variables examined in 50 normal pregnant women analysed according to trimester of pregnancy showing median (range) for each variable and comparing across gestation using Kruskall-Wallis ANOVA.

	First n=27	p 1st vs 2nd	Second n=11	p 2nd vs 3rd	Third n=12	p 1st vs 3rd
Gestation (weeks)	9.9 (4.3)	----	18.0 (6.3)	----	38.0 (10)	----
Diastolic BP (mm Hg)	70 (40)	0.6235	70 (40)	0.0083	80 (25)	0.0033
Systolic BP (mm Hg)	110 (60)	0.6202	110 (40)	0.0848	120 (40)	0.0102
Maternal pulse (bpm)	76 (18)	0.7119	80 (10)	0.0902	80 (16)	0.0562
Fetal Heart Rate (bpm)	180 (42)	0.0000	156 (24)	0.0005	138 (32)	0.0000
Haemoglobin (g/dl)	12.3 (3.8)	0.5133	12.1 (2.7)	0.0103	10.8 (4.8)	0.0002
White Cell Count (10^9/l)	9.0 (7.8)	0.9863	8.3 (7.3)	0.0016	12.6 (6.8)	0.0000
Platelet Count (10^9/l)	223 (187)	0.0205	190 (103)	0.3252	201 (123)	0.1012
Oestrogen (pmol/l)	1200 (4100)	0.0000	16500 (16800)	0.0003	45600 (57000)	0.0000
Uterine Artery PI	2.40 (4.14)	0.0292	1.39 (2.54)	0.0008	0.54 (1.30)	0.0000
Uterine Artery RI	0.84 (0.47)	0.0195	0.66 (0.46)	0.0195	0.42 (0.40)	0.0000
Umb Art PI	2.54 (1.90)	0.0000	1.68 (0.77)	0.0005	1.12 (1.03)	0.0000

Table 3.5

Descriptive statistics for calcium-dependent (cNOS) and calcium-independent (iNOS) activities (nmol L-citrulline formed / min/ g tissue protein) in placental villi throughout normal gestation and from IUGR pregnancies at delivery.

	Group	n	Min	25th centile	Median	75th centile	Max
cNOS	1st Trimester	27	2.96	7.40	10.55	17.02	29.23
	2nd Trimester	11	1.72	7.65	8.56	14.82	20.30
	3rd Trimester	12	2.51	2.94	6.26	8.35	8.95
	IUGR	8	0.42	1.27	1.61	2.98	8.53
iNOS	1st Trimester	27	2.49	5.17	7.40	12.97	22.97
	2nd Trimester	11	2.61	4.22	7.32	15.09	16.20
	3rd Trimester	12	2.51	2.94	6.26	8.35	8.95
	IUGR	8	0.63	0.98	1.78	2.56	9.10

Table 3.6 Comparison of calcium-dependent (cNOS) and calcium-independent (iNOS) activities (nmol L-citrulline formed / min/ g tissue protein) in placental tissue throughout normal pregnancy and from IUGR pregnancies at delivery. Comparisons across gestation use a Kruskall-Wallis ANOVA. Comparisons between normal pregnancies and those affected by IUGR use a Mann-Whitney U Test.

Group	cNOS p	iNOS p
1st vs 2nd Trimesters	0.1764	0.0096
1st vs 3rd Trimesters	0.0004	0.0001
2nd vs 3rd Trimesters	0.0422	0.4233
IUGR vs 2nd Trimester	0.0001	0.0001
IUGR vs 3rd Trimester	0.0002	0.0002

Table 3.7

Results of other variables examined in 50 normal pregnant women analysed by ethnicity showing median (range) and actual p value for comparisons using Kruskall-Wallis ANOVA.

	Caucasian n=37	Non-Caucasian n=13	p
Gestation (weeks)	11.8 (33.3)	11.5 (31.3)	0.6421
Diastolic BP (mm Hg)	70 (45)	70 (30)	0.6101
Systolic BP (mm Hg)	110 (50)	120 (60)	0.3387
Maternal pulse (bpm)	78 (36)	79 (28)	0.6825
Fetal heart rate (bpm)	168 (62)	170 (61)	0.7164
Haemoglobin (g/dl)	12.1 (4.4)	11.7 (3.2)	0.0998
White cell count ($x10^9$/l)	9.4 (11.5)	10.7 (8.0)	0.8008
Platelets ($x10^9$/l)	216 (209)	216 (174)	0.7392
Oestrogen (pmol/l)	2800 (53600)	1600 (60400)	0.9758
Plasma nitrite (μmol/l)	2.0 (6.0)	2.0 (7.0)	0.2581
Plasma nitrate (μmol/l)	35.0 (22.0)	36.5 (18.0)	0.1372
Placental cNOS (nmol L-citrulline formed / min/ g tissue protein)	8.40 (27.15)	7.87 (18.53)	0.7197
Placental iNOS (nmol L-citrulline formed / min/ g tissue protein)	9.62 (20.51)	8.70 (19.16)	0.9579
Uterine Artery PI	1.70 (4.51)	2.36 (3.44)	0.9036
Uterine Artery RI	0.73 (0.64)	0.84 (0.61)	0.5998
Umb Art PI	2.12 (2.66)	2.00 (2.70)	0.8638

Table 3.8

Results of other variables examined in 50 normal pregnant women analysed by smoking habit showing median (range) and actual p value for comparisons using a Kruskall-Wallis ANOVA.

	Smokers n=24	Non-smokers n=26	p
Gestation (weeks)	10.2 (33.3)	18.3 (31.0)	0.0068
Diastolic BP (mm Hg)	70 (45)	70 (40)	0.0218
Systolic BP (mm Hg)	110 (45)	120 (50)	0.0308
Maternal pulse (bpm)	76 (28)	79 (28)	0.0271
Fetal Heart (bpm)	177 (61)	156 (62)	0.0148
Haemoglobin (g/dl)	12.2 (3.8)	11.5 (4.8)	0.0653
White cell count ($x10^9$/l)	9.1 (9.0)	11.0 (10.9)	0.0796
Platelets ($x10^9$/l)	219 (209)	216 (174)	0.8658
Oestrogen (pmol/l)	14000 (44600)	17500 (60100)	0.0005
Plasma nitrite (μmol/l)	2.0 (6.0)	2.0 (7.0)	0.6576
Plasma nitrate (μmol/l)	35.0 (22.0)	36.0 (20.0)	0.9219
Placental cNOS (nmol L-citrulline formed / min/ g tissue protein)	9.72 (27.51)	7.81 (25.82)	0.3758
Placental iNOS (nmol L-citrulline formed / min/ g tissue protein)	9.92 (21.71)	8.28 (22.86)	0.4342
Uterine Artery PI	1.73 (4.41)	1.44 (2.92)	0.0407
Uterine Artery RI	0.77 (0.57)	0.72 (0.59)	0.0453
Umb Art PI	2.40 (2.47)	1.68 (2.90)	0.0493

Table 3.9

Results of 27 normal first trimester pregnancies showing median (range) and actual p value for comparisons between smokers and non-smokers using a Kruskall-Wallis ANOVA.

	Smokers n=17	Non-smokers n=10	p
Gestation (weeks)	9.5 (4.3)	10.3 (2.8)	0.0649
Diastolic BP (mm Hg)	70 (30)	70 (40)	0.0969
Systolic BP (mm Hg)	110 (40)	120 (50)	0.1052
Maternal pulse (bpm)	76 (26)	80 (36)	0.0584
Fetal heart rate (bpm)	180 (17)	178 (42)	0.3269
Haemoglobin (g/dl)	12.4 (3.8)	12.2 (2.3)	0.8926
White cell count ($x10^9$/l)	8.3 (7.8)	11.0 (10.9)	0.0796
Platelets ($x10^9$/l)	224 (187)	216 (146)	0.9821
Oestrogen (pmol/l)	900 (4100)	1300 (3300)	0.0381
Plasma nitrite (μmol/l)	0.5 (9.0)	2.0 (8.0)	0.3691
Plasma nitrate (μmol/l)	34.5 (21.0)	35.0 (20.0)	0.6356
Placental cNOS (nmol L-citrulline formed / min/ g tissue protein)	12.39 (27.51)	15.01 (19.69)	0.4720
Placental iNOS (nmol L-citrulline formed / min/ g tissue protein)	10.94 (25.79)	16.15 (21.63)	0.1250
Uterine Artery PI	2.88 (4.14)	2.35 (2.33)	0.3012
Uterine Artery RI	0.90 (0.47)	0.83 (0.30)	0.2802
Umb Art PI	2.52 (1.66)	2.40 (2.80)	0.4999

Table 3.10

Descriptive statistics for calcium dependent (cNOS) and calcium independent (iNOS) activities (nmol L-citrulline formed / min/ g tissue protein) in myometrial samples obtained from non-pregnant women and from pregnant women before and after the onset of spontaneous labour at term.

		Min	25th centile	Median	75th centile	Max
Non-pregnant (n=5)	iNOS	0.35	0.66	1.01	1.39	1.55
	cNOS	0.56	0.60	0.67	1.55	1.92
All pregnant (n=14)	iNOS	0.00	0.23	0.40	0.71	0.91
	cNOS	0.00	0.09	0.43	0.83	1.50
In labour (n=7)	iNOS	0.20	0.31	0.69	0.87	0.91
	cNOS	0.15	0.30	0.69	1.02	1.11
Not in labour (n=7)	iNOS	0.00	0.00	0.30	0.49	0.60
	cNOS	0.00	0.04	0.10	0.72	1.50

Table 3.11

Actual p values obtained by comparison of myometrial NOS activities between non-pregnant women and pregnant women both before and after the onset of spontaneous labour at term using a Mann-Whitney U Test.

		U	p
Non-Pregnant vs All Pregnant	iNOS	7.0	0.0072
	cNOS	19.0	0.1560
Non-Pregnant vs Non-Labour	iNOS	2.0	0.0101
	cNOS	7.0	0.1061
Non-Pregnant vs Labour	iNOS	5.0	0.0480
	cNOS	12.0	0.4318
Labour vs Non-Labour	iNOS	5.5	0.0111
	cNOS	13.0	0.1417

Table 3.12

The amino acid concentrations (nmol / mg tissue protein) of normal first trimester, normal third trimester and IUGR placental villous tissue expressed as mean ± SEM.

Amino Acid	First Trimester n=10	Third Trimester n=10	IUGR Pregnancies n=8
Taurine	95.4 ± 11.0	98.7 ± 9.3	80.4 ± 7.5
Aspartic acid	65.3 ± 8.5	48.1 ± 3.5	40.9 ± 4.4
Threonine	18.0 ± 1.8	12.7 ± 1.6	17.6 ± 3.5
Serine	18.5 ± 1.6	12.7 ± 0.9	22.3 ± 5.6
Glutamic acid	96.5 ± 9.7	71.8 ± 9.7	80.7 ± 10.0
Glutamine	47.8 ± 2.9	20.0 ± 4.3	18.3 ± 3.8
Proline	17.2 ± 1.6	7.4 ± 0.7	14.8 ± 3.4
Glycine	41.6 ± 6.2	29.5 ± 3.0	45.5 ± 9.3
Alanine	46.8 ± 7.4	26.2 ± 2.7	40.0 ± 7.5
Valine	21.0 ± 2.1	10.1 ± 1.0	14.4 ± 3.0
Ethanolamine	8.5 ± 1.2	50.2 ± 6.1	47.1 ± 6.0
Phosphoethanolamine	52.9 ± 6.9	3.1 ± 1.7	5.8 ± 2.0
Methionine	4.4 ± 0.5	2.2 ± 0.2	3.4 ± 0.8
Isoleucine	5.2 ± 0.5	3.0 ± 0.3	5.5 ± 1.2
Leucine	20.6 ± 1.8	9.4 ± 1.1	12.7 ± 2.7
Tyrosine	5.6 ± 0.4	2.9 ± 0.4	5.0 ± 1.1
Phenylalanine	7.9 ± 0.6	11.6 ± 5.4	27.5 ± 4.3
Tryptophan	1.6 ± 0.4	0.3 ± 0.1	0.1 ± 0.1
Ornithine	4.1 ± 0.4	2.0 ± 0.3	7.0 ± 1.5
Lysine	36.5 ± 3.8	14.5 ± 2.2	21.7 ± 4.8
Histidine	14.0 ± 1.9	5.9 ± 1.3	4.5 ± 0.9
Arginine	10.2 ± 2.1	4.6 ± 1.0	9.8 ± 2.2

Figure 3.1

Calcium dependent NOS activity in trophoblast samples from normal first, second and third trimester placental villi. The fall across gestation is statistically significant (see text for details).

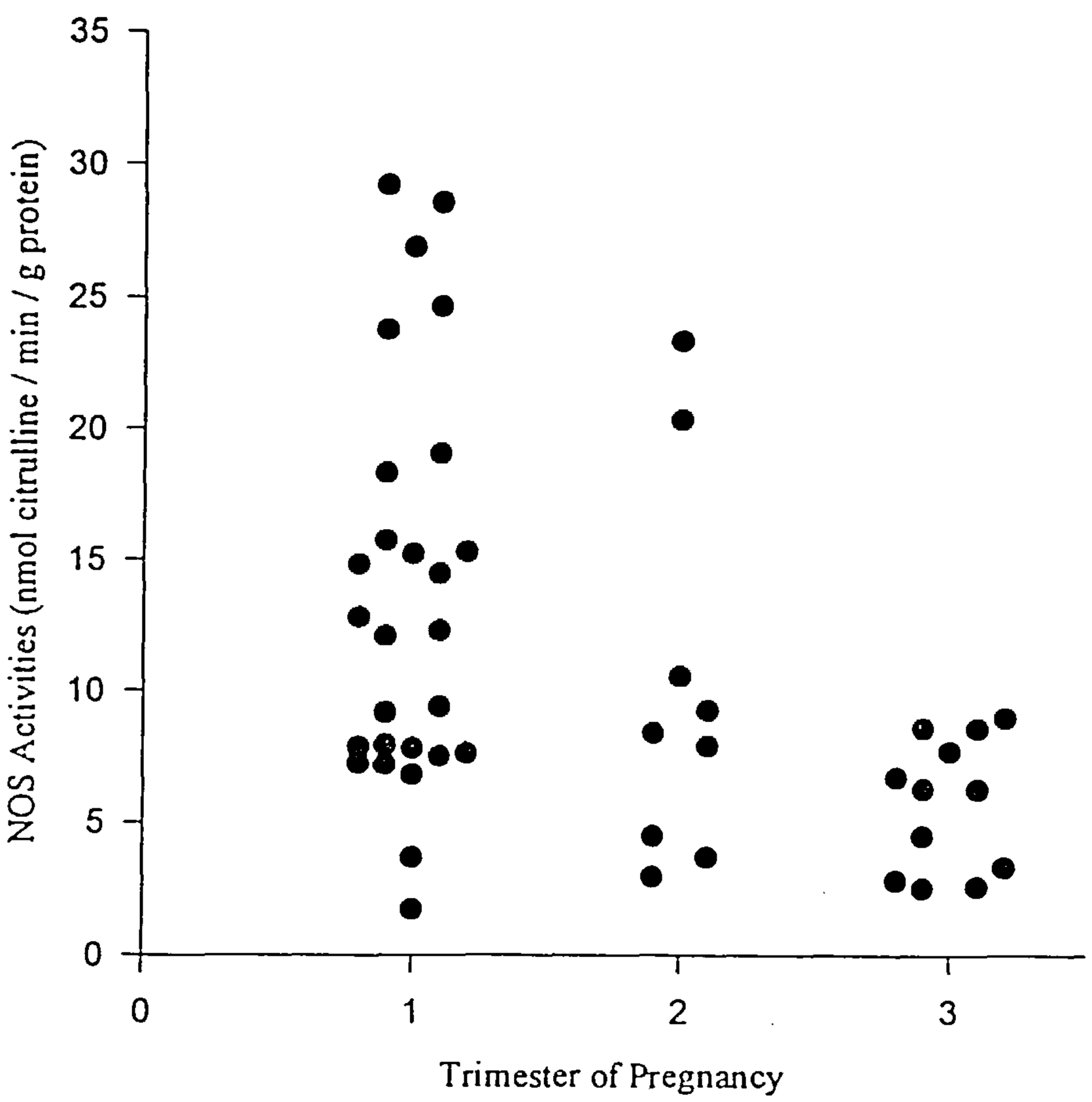


Figure 3.2

Calcium independent NOS activity in trophoblast samples from normal first, second and third trimester placental villi. The fall across gestation is statistically significant (see text for details).

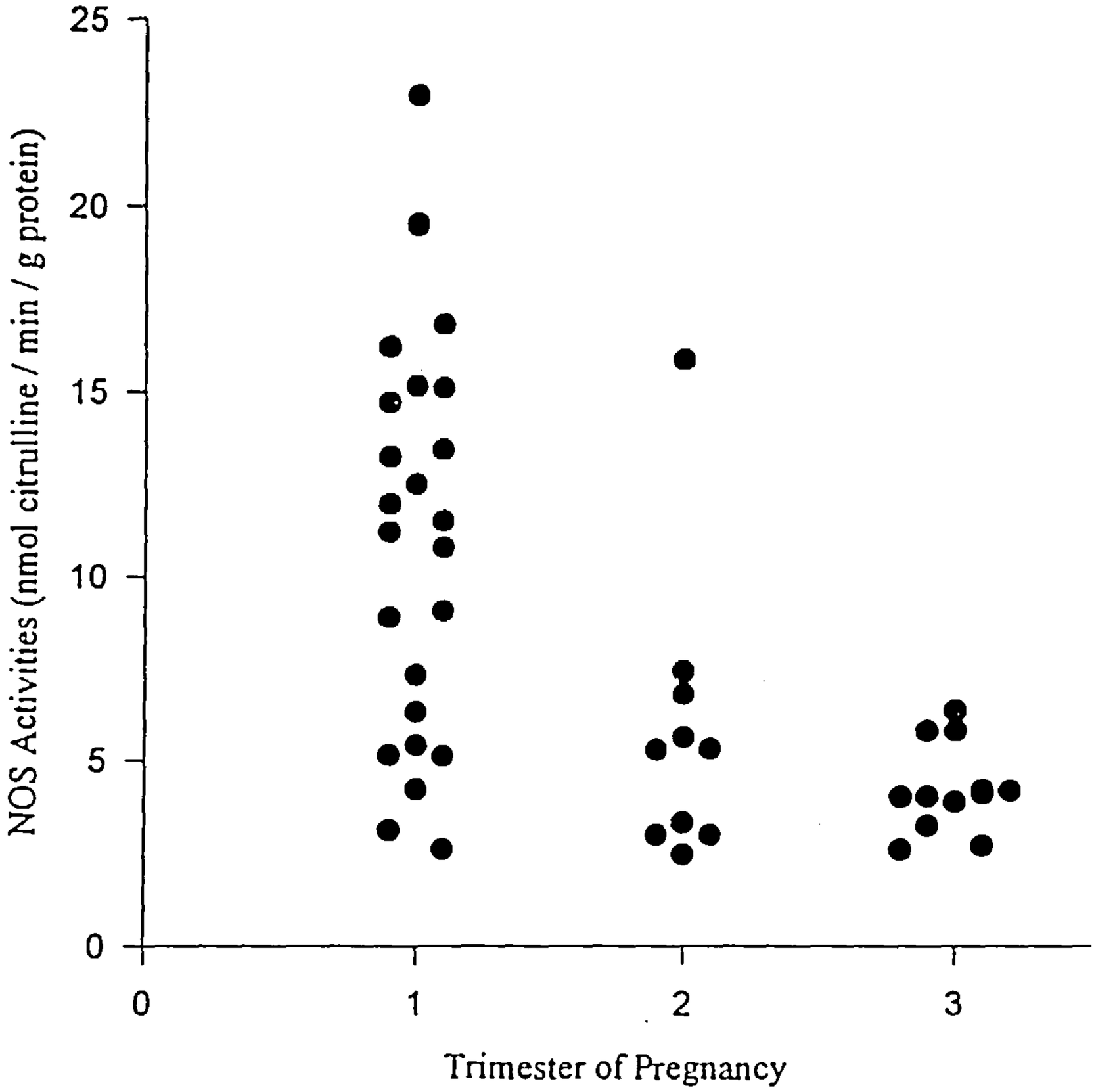


Figure 3.3

Calcium dependent NOS activity in the myometrial samples from non-pregnant, labouring and non-labouring women at term. The cNOS activities are not significantly different between groups (see text for details).

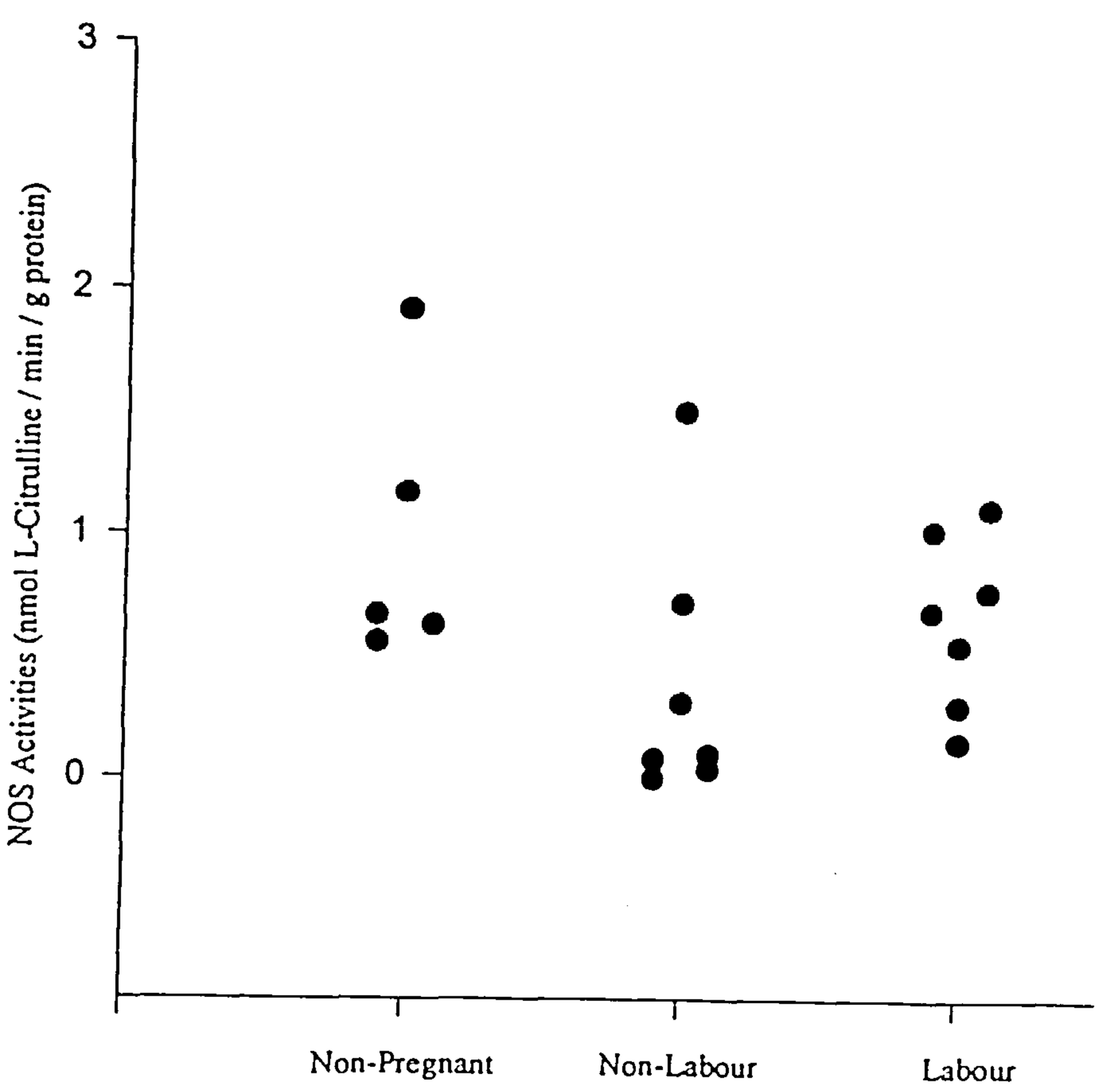
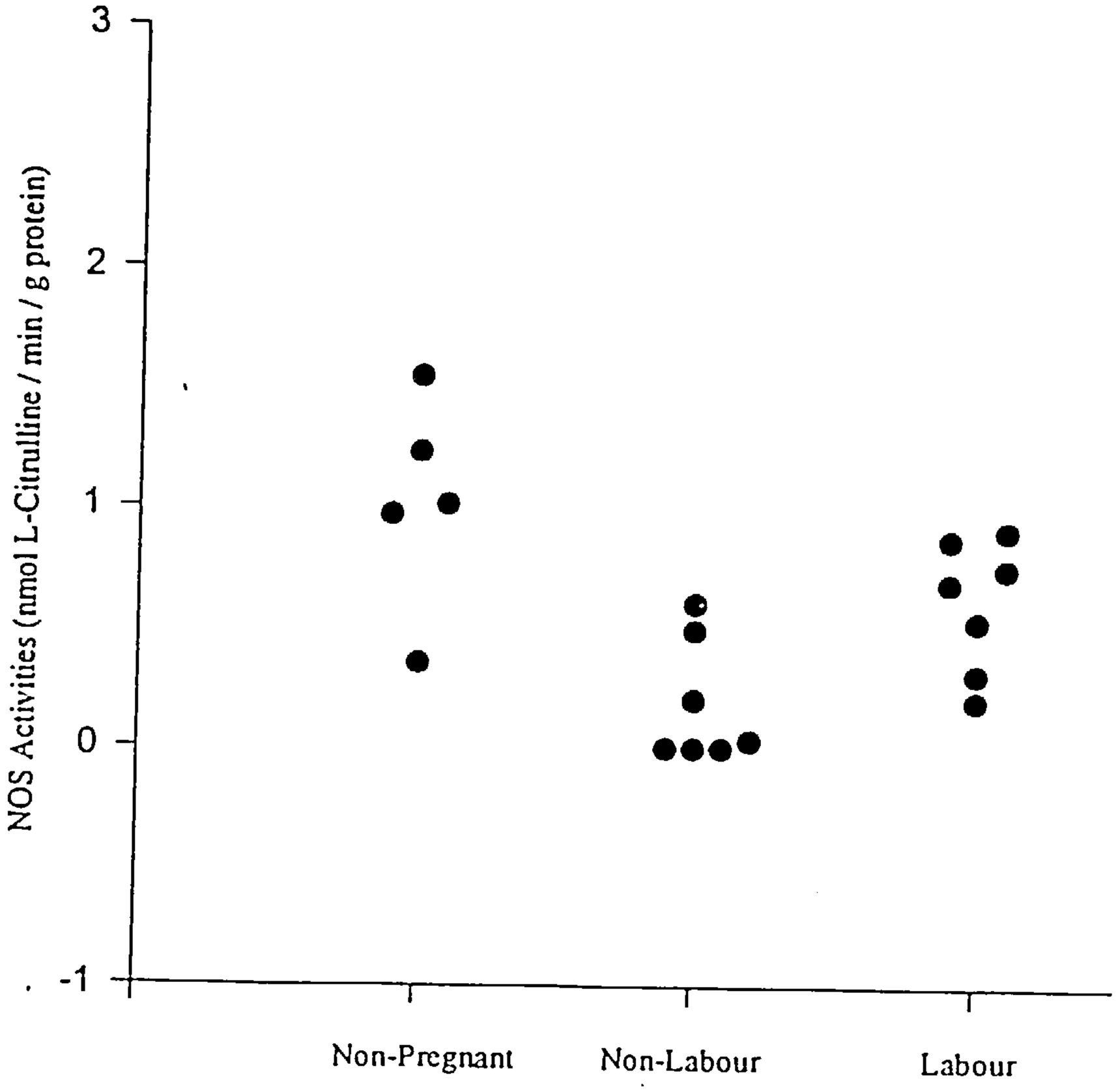


Figure 3.4

Calcium independent NOS activity in the myometrial samples from non-pregnant, labouring and non-labouring women at term. The iNOS activities are significantly lower in pregnancy than in non-pregnancy but unaffected by onset of labour (see text for details).



3.4. Discussion

3.4.1. Plasma nitrate and nitrite levels

The finding of low levels of nitrite in all samples is in accordance with previous studies of non-pregnant humans. It is therefore unsurprising that no significant differences could be demonstrated between groups.

In contrast, the nitrate levels showed a significant increase in pregnancy compared to non-pregnancy. Whilst this could be consistent with increased production of NO by the vascular endothelium causing the characteristic vasodilatation which occurs in pregnancy, it is puzzling that the rise in nitrate levels does not occur earlier. Indeed, the nitrate levels during the first trimester were not significantly different to those found in non-pregnant women, only those found in second and third trimesters. Remembering that the total plasma nitrate is heavily dependent on dietry intake it is interesting to postulate a difference in nitrate handling during the first trimester as a cause for this discrepancy. I obtained all plasma samples in the morning after an overnight fast to try and minimise this type of problem. However, early pregnancy is a time of great change and a number of compounding factors may influence nitrate levels. It is conceivable that uptake of excess dietry protein by the fetus for organogenesis would lead to less nitrate production from dietry protein. It is also possible that the increase in glomerular filtration rate leads to increased urinary excretion of nitrate by the mother to balance out any increase in nitrate production occurring secondary to NO production. These points indicate the difficulty of using indirect measurement of NO function.

The 26 week gestation pregnancies with abnormal uterine artery Dopplers showed no difference in nitrate levels from those with normal Dopplers. This is unsurprising since although these women are at increased risk of developing pre-eclampsia and fetal IUGR, they have none of the systemic effects apparent at this stage. Even if NO were a factor in the different

Doppler flow velocity waveform patterns, it is only locally active on the utero-placental circulation at this stage and any effect on systemic nitrate levels would be dwarfed by baseline production.

3.4.2. Trophoblast NOS activity throughout gestation

In the placental villi both cNOS and iNOS activities were present throughout pregnancy. The highest levels of activity were seen in the early gestations, before 12 weeks, where the tissue levels of the enzyme approach those reported in human disease states (De Belder *et al* 1993; Hamid *et al* 1993). First trimester trophoblast tissue gave the highest activities of both iNOS and cNOS which fell with advancing gestational age to levels at term similar to those reported previously by other investigators in term placentae (Myatt *et al* 1993; Conrad *et al* 1993a).

The measured cNOS activity probably derives mainly from the small vessels in the samples of villi. If this is indeed the case, then the decrease in activity seen with advancing gestation may reflect vascular changes which are known to occur in the placenta. Vessel dilatation and areas of infarction could combine to decrease the number of endothelial cells in any given weight of wet tissue and hence lead to an apparent reduction in NOS activity.

The finding of iNOS activity is more surprising since this has previously only been found in response to pathological stimuli and not in a physiological setting such as this.

During early pregnancy, the trophoblast is at its most active, invading deeply into the decidua and myometrium. At this time the uterus is infiltrated by large granular lymphocytes (King & Loake 1990) and indeed, the maternal response to implantation has been likened to an inflammatory process by several authors (Jauniaux *et al* 1992). Although it is possible that the high levels of iNOS activity shown by the first

trimester samples may simply reflect a number of these cells being included in the homogenates this is not supported by immunocytochemical data (A.M.Leone personal communication). This suggests the majority of the iNOS activity is localised to the cytotrophoblast itself.

The initially high NOS activity may be related to the rapid increase in oestrogen levels which occurs in early pregnancy. There is supporting evidence for this notion from animal studies which have shown in guinea pigs that oestrogen can induce NOS activity (Weiner *et al* 1994). It is widely recognised that oestrogen can cause systemic cardiovascular effects but it seems that the uterine circulation is particularly sensitive (Magness & Rosenfeld 1989).

From my data it is clear that the initial rise in trophoblast NOS activity occurs early on in pregnancy and although the oestrogen levels continue to rise, there is no further increase in NOS activity. Animal studies in oophrectomised ewes have shown that the effect of oestrogen on uterine blood flow occurs after only two hours of intravenous administration (Magness, Parker & Rosenfeld 1993). However, whereas systemic vascular changes persisted for the duration of the study (6 weeks), the increase in uterine blood flow returned to normal after two weeks. Indeed, after this time the uterine vascular resistance actually showed a rebound increase with consequent decrease in blood flow. The authors postulate that this may be due to receptor tachyphylaxis and therefore suggest that the increase in uterine blood flow is mediated through a different mechanism to the systemic effects.

In addition to vascular changes it would also be advantageous for the trophoblast at implantation to suppress uterine activity and the maternal immune response. A role for NO in the modulation of some or all of these processes would be consistent with the higher levels of NOS activity

found in first trimester villi. The fall in trophoblast derived NOS activity which occurred with advancing gestational age took place against a background of decreasing utero-placental resistance to blood flow. This observation would support the notion of trophoblast derived NO having other roles beyond simple vasodilatation of the uteroplacental circulation and suggest a physiological role for NO in the early stages of pregnancy. Further work is needed to establish the purpose of the NO produced.

3.4.3. Myometrial NOS activity

All the myometrial NOS activities were significantly lower than those from villous trophoblast, with calcium independent activities additionally showing some variation in relation to the onset of labour. The iNOS activities were less in pregnant myometrium than non-pregnant, with the lowest values being obtained prior to the onset of labour rather than during labour. Whilst these data would suggest that NO production in the myometrium is unlikely to have a role in the maintenance of myometrial quiescence, there are several complicating factors to take into account.

Firstly, it is possible that the myometrium from the labour subgroup was not truly "normal". All the women in this group were undergoing Caesarean section (following a period of spontaneous labour at term) for a clinical indication. The indication was "abnormal CTG" in 4 cases but "failure to progress" in the 3 others. It is therefore possible that the characteristics of myometrium taken from these women were abnormal. This point is difficult to answer since the only truly "normal" labours ending in caesarean occur in the unusual circumstance of a woman already booked for surgery (e.g. primigravid with breech presentation) arriving at the hospital in spontaneous labour.

Secondly, there may be enhanced responsiveness of the myometrium to NO during pregnancy in much the same way as there is to oxytocin.

There is no data at present to support or refute this notion but it is a question which needs to be answered by future work.

Finally, it is possible that NO released by the high levels of trophoblast NOS activity could exert a paracrine effect on the myometrium and thus may contribute to the maintenance of uterine quiescence in this way. Animal studies have already shown a reduction in decidual NOS activity with advancing gestation (Sladek *et al* 1993). This reaches a nadir on the day of delivery and is paralleled by a decrease in myometrial cGMP levels (the second messenger of NO) (Kishikawa, 1981). It is possible that high levels of trophoblast derived NO within the uterus actually influence the expression of the NOS in the myometrium by negative feedback. This would explain the lower levels in pregnant specimens than in those taken at routine hysterectomy. However it is unlikely that the NO produced by the trophoblast could exert a paracrine action on the myometrium because of the very short half-life *in-vivo*.

The relatively low levels of NOS in myometrium and the decline in activity between non-pregnant and Caesarean section samples make it unlikely that NO derived from the myometrium has any role in the maintenance of uterine quiescence although it is still possible that trophoblast derived NO may influence uterine contractility.

3.4.4. Amino acid levels in trophoblast

The arginine levels in the term placentae were significantly lower than those in first trimester trophoblast tissue ($p=0.042$) and this paralleled the decrease in NOS activities. In contrast, the IUGR pregnancies (which had the lowest NOS activities) had high levels of arginine equivalent to those found in normal first trimester trophoblast. It should be remembered that the IUGR pregnancies were delivered at earlier gestations than the normals (32-37 weeks vs 37-39 weeks) but it seems unlikely that this alone could explain the large observed difference in arginine levels.

The placentae of IUGR fetuses are presumed to function poorly and have impaired transport mechanisms. The amino acid data presented here would be consistent with local deficiency of NOS activity contributing to the high vascular resistance and platelet deposition seen in affected pregnancies. The demonstration of high arginine levels in association with low NOS activities implies that any deficiency of placental NO production in these IUGR pregnancies was due to an enzyme problem rather than a shortage of substrate.

However it should be remembered that the trophoblast levels of arginine only represent a snapshot in time and that most of the measured arginine within the placenta is likely to be in transit through it, rather than in use by it. If this is indeed the case, then the higher levels of arginine in IUGR placentae may indicate one of two possibilities. The first possiblity is an increased supply to the fetus for NO production as part of the blood flow redistribution which occurs in IUGR. The second is an impairment of efferent transport of arginine from the placenta to the fetus.

At first glance the plasma nitrate data would lend more support to the notion of increased fetal utilisation since the IUGR fetal plasma nitrates were significantly higher than those in the matched maternal blood samples, moreso than in the normal pregnancies. However, this difference could equally reflect an impaired ability of the fetus to "excrete" nitrate across a compromised feto-placental interface.

I believe it is likely that impaired placental utilisation of arginine due to low NOS activity and impaired transport across the feto-placental interface combine to produce the elevated levels of arginine demonstrated in the placentae of IUGR pregnancies.

CHAPTER 4 - OESTROGEN STUDY

4.1 INTRODUCTION

4.1.1 The need for a controlled trial

The observational data on oestrogen levels and nitrate/nitrite levels in early pregnancy presented earlier in this thesis fails to demonstrate a significant correlation between oestrogen and NO production as quantified in terms of plasma nitrate and nitrite levels. This was despite the very high levels of oestrogen found in pregnant women; up to a 200 fold increase at term when compared to the non-pregnant state. However, there are several complicating factors which may have prevented the observational study from demonstrating a correlation. These have already been discussed in some detail with the initial results and include:-

- Cross-reactivity of the oestradiol ELISA assay with oestriol in pregnancy.
- Large inter-subject variation of values for oestrogen during pregnancy.
- Relatively low and stable levels of both nitrite and nitrite.
- Dilutional and other haemodynamic changes affecting plasma levels.
- Increased glomerular filtration rate in pregnancy speeding excretion.
- Dietry and protein metabolism changes which occur during pregnancy.

I therefore attempted to design a study which would address some of these problems and allow the nul hypothesis to be further explored.

4.1.2 Study Design

In normal pregnancy oestriol is present in milligram quantities so that even a tiny percentage of cross-reactivity in the ELISA assay for oestradiol will greatly influence the results obtained. By using non-pregnant women there would be a relatively small and constant amount of oestriol to cause such problems. In addition, oestradiol levels, pulse,

blood pressure, cardiac output, renal function and circulating plasma volume would be much more stable when non-pregnant, since all are heavily influenced by advancing gestational age.

The initial intention was to administer supra-physiological (i.e. pharmacological) doses of oestrogen to normally menstruating women and observe the effect. However, oestradiol levels still vary significantly throughout the menstrual cycle (range 110-1650 pmol/l using the ELISA assay in our laboratory), so the problem with large inter and intra-subject variation would remain. This problem was addressed in two ways. The first was to conduct a longitudinal study using a cohort of volunteers. The second to down-regulate the menstrual cycle using a gonadotrophin releasing hormone (GnRH) agonist. Suppression of circulating oestradiol levels to the menopausal range (<100 pmol/l) and abolishing the menstrual cycle had several benefits:-

- Rather than administer pharmacological doses of oestrogen to women of child bearing age it allowed physiological (hormone replacement therapy) doses to be used.
- A more constant and predictable rise in oestradiol from a much tighter baseline resulting in decreased inter-subject variability.
- Facilitation of a longitudinal comparison, allowing the effect of an isolated change in oestradiol level to be studied without fluctuating levels of other hormones, such as LH, FSH or progesterone, confusing the interpretation of results.
- A longitudinal study design comparing oestradiol therapy with a placebo has the additional benefit of increasing the statistical power of the study.

For these reasons the study was designed as a double blind, prospective, randomised, placebo-controlled, crossover trial of oral oestrogen and placebo. This was approved by the hospital ethics committee.

4.2 METHODS

4.2.1 Recruitment of patients

30 non-pregnant women of child bearing age were recruited for the study from staff and students working at the hospital. Informed written consent was obtained in every case. All were in good health and taking no medication and did not smoke for the duration of the study.

Following recruitment an intra-muscular depo injection of the GnRH agonist Decapeptil (Ipsen Biotech, Paris, France) was administered into the buttock in accordance with the manufacturer's instructions. The dose used of 3.75mg was that recommended for the suppression of ovarian function in the short term treatment of endometriosis, ie sufficient to down regulate the hypothalamo-pituitary axis for a period of one month. The clinical effect of the GnRH analogue was to render the women menopausal.

4.2.2 Follow up visits and sample collection

The women attended the hospital 4 weeks later when a blood sample was taken to confirm down-regulation of their menstrual cycle. This was analysed for plasma levels of oestradiol (E_2), luteinising hormone (LH) and follicle stimulating hormone (FSH) as described briefly below. At the same time they were given a further injection of GnRH agonist and entered into the trial. This involved randomisation to treatment with either oestradiol valerate 2mg tablets daily or placebo. Block randomisation was used to allocate the initial treatment option and the study conducted double blind.

At the end of the first week subjects attended the laboratory pre-starved for 8 hours, usually first thing in the morning. A venous blood sample was taken for E_2, FSH and LH measurement and the assay of nitrite/nitrate. The blood was immediately centrifuged at 2000g for 10 minutes and the plasma snap-frozen at -70^{o}C to await batch analysis. The women were

then issued with a weeks supply of the other arm of the crossover trial which they had not already received. At the end of the second week the blood taking process was repeated and all samples stored for later analysis.

4.2.3 Assay of blood samples

Nitrite and nitrate were assayed using the technique of capillary zone electrophoresis as described earlier.

FSH, LH and Oestradiol were measured using an ELISA assay on an ES 600 Analyser (Boehringer Mannheim GmbH) as described earlier.

4.2.4 Exclusion criteria

Following initial recruitment to the study of 30 women, complete data sets were obtained from 26. Reasons for exclusion were:- one woman had only one blood sample taken, one had gastroenteritis at the second visit with consequently very elevated nitric oxide activity and two failed to respond to the GnRH analogue, having LH and FSH levels persistently within the reproductive range and continued menstrual bleeding. All 26 subjects included in the trial had LH levels less than 1mIU/ml at both visits and had amenorrhoea.

4.3 RESULTS

The descriptive statistics of the data obtained from oestrogen treated and placebo treated phases of the trial are shown in table 4.1. The data from oestrogen and placebo phases are then compared in table 4.2 using a paired Student's t-test. This assumes the data (or at least the sample of the data) to be normally distributed. The descriptive statistics would support this assumption but to confirm the finding I performed a logarithmic transformation of the data and repeated the analysis. This produced almost identical results (i.e. $t=4.03$, $p<0.001$ for the nitrate comparison).

Table 4.1

Actual results obtained for plasma oestradiol, FSH, nitrite and nitrate during oestrogen and placebo treated phases of the trial.

	Phase	Mean	SEM	SD	Min	Max
Oestradiol	Placebo	83.88	11.49	58.58	37.0	266.0
(pmol/l)	Oestrogen	230.50	30.31	154.56	37.0	644.0
FSH	Placebo	3.12	0.33	1.68	1.0	8.1
(mIU/ml)	Oestrogen	2.57	0.35	1.77	1.0	9.9
Nitrite	Placebo	2.77	0.52	2.64	0.0	8.0
(µmol/l)	Oestrogen	2.85	0.68	3.45	0.0	11.0
Nitrate	Placebo	29.85	0.83	4.22	21.0	38.0
(µmol/l)	Oestrogen	33.77	1.16	5.93	23.0	43.0

Table 4.2

Difference in means of oestradiol, FSH, nitrite and nitrate between oestrogen and placebo treated phases of the study. Comparison is using a paired Student's t-test following log transformation (n=26).

	Oestrogen Phase (SD)	**Placebo Phase (SD)**	**Mean difference (95% CI)**	**t**	**p**
Oestrogen (pmol/l)	230.5 (154.6)	83.9 (58.6)	146.6 (84.8 - 208.4)	4.89	<0.001
FSH (U/l)	2.56 (1.77)	3.11 (1.68)	-0.55 (-1.18 - 0.07)	-1.82	0.08
NO_2 (μmol/l)	2.85 (3.45)	2.77 (2.64)	0.08 (-1.4 - 1.5)	0.92	0.11
NO_3 (μmol/l)	33.77 (5.93)	29.84 (4.22)	3.92 (1.92 - 5.93)	4.02	<0.001

4.4 Discussion

4.4.1 Suppression of the menstrual cycle

LH values were less than 1mIU/ml in every case. FSH levels were low and not significantly different between the oestrogen and placebo phases. All 26 women included in the study had amenorrhoea and some reported minor menopausal side effects. This confirms the suppression of normal cyclical variation of hormones by the GnRH agonist, as was intended.

4.4.2 Response to oestrogen

The plasma oestradiol levels were significantly higher during the oestrogen treated phase, as would be expected (230.5 vs 83.9 pmol/l, $p<0.001$), although it is worth noting that the range for each group was considerable.

The lower limit of detection for oestradiol using the ELISA assay was 37 pmol/l. There were 3 samples in the placebo group and 1 in the oestrogen treated group which fell below this limit. This is not an uncommon finding in women taking GnRH agonists. Rather than exclude these cases from the study I chose to enter the actual value as 37 pmol/l. While it is possible in theory that this artificially increased the mean plasma oestradiol value in the placebo treated group, this will not have materially altered the results of the study.

Nevertheless, the one woman with plasma oestradiol levels below the assay detection limit when taking oral oestrogen does raise the question of compliance. However, compliance was not really a problem generally because of the study design. I used volunteers who worked in the hospital so they were motivated, understood the importance of the work and travelling was not a problem for them. In addition the short period for which they had to take tablets (2 x 7 days in total with further contact and reinforcement after the first week) made things easy. Interestingly three women opted to continue taking oral oestradiol for a month after the study

had been completed while waiting for the GnRH analogue to wear off.

The mean level of oestradiol obtained on oral therapy was 230.5pmol/l and is at the lower end of the normal range (see table 4.3). It may be that had a larger dose of oestrogen been given and plasma levels of over 500 pmol/l achieved, the differences between the two phases of the trial would have been more marked. In retrospect this low level of response to 2mg orally is probably just a sign of the extreme "down regulation" which GnRH agonists can produce in young women.

Table 4.3

Normal ranges for oestradiol during various stages of the menstrual cycle and pregnancy obtained using the ELISA technique on the SS 600 analyser (Boehringer Mannheim GmbH).

	Oestradiol (pmol/l)
Post-menopausal	<100
Early follicular	110-183
Mid-cycle	550-1650
Secretory	550-845
First trimester	400-5000
Second Trimester	2500-62500
Third trimester	8000-89000

4.4.3 Changes in plasma levels of nitrite and nitrate

When NO is inactivated it forms both NO_2 and NO_3. Whilst cell culture experiments result primarily in increased NO_2 because they are performed in aqueous solution, *in vivo* studies and those using oxygenated whole blood *in vitro* have shown quantitative conversion of NO to NO_3(Wennmalm, Lanne & Petersson 1992; Ignarro *et al* 1993). The plasma levels of each in this study are in close agreement with those reported previously in other human studies(Moncada, Palmer & Higgs 1991). Unfortunately, the normal NO_2 level is not only close to zero but also subject to wide inter-subject variation. In addition, any delay in centrifugation of the plasma allows further *ex vivo* oxidation of NO_2 to NO_3. Therefore, it is not surprising that no significant alteration in NO_2 could be demonstrated between phases of the study.

In contrast, the plasma NO_3 levels were significantly raised during the oestrogen treatment phase. Since other factors remained unchanged it is reasonable to assume that this resulted from increased NO production (as discussed in chapter 1). Whilst statistically significant, the magnitude of this increase was small (12% of baseline value) which may be due to the relatively low plasma levels of oestradiol which were attained. A deliberate attempt was made to maintain oestradiol levels within the physiological range during this study, rather than the pharmacological, but although the 2mg daily dose did produce a significant increase in plasma oestradiol the levels achieved were still low in comparison to those of a spontaneous cycle. It may be that a higher dose of oestrogen would have produced a greater magnitude of change in NO_3 levels.

5.1 INTRODUCTION

5.1.1 The uteroplacental circulation in high risk pregnancy

Using Doppler ultrasound to record flow velocity waveforms from the main uterine artery it is possible to identify a group of women in early pregnancy at high risk of subsequently developing pre-eclampsia and/or fetal growth retardation. It is a widely accepted view that both of these conditions share a common underlying pathology and may even represent two ends of the spectrum of the same disease process. Both conditions are characterised by placental changes including poor blood flow to the placenta with platelet deposition leading on to placental infarction in advanced disease. One of the hypotheses for this thesis is that these changes may be explained by a relative deficiency in nitric oxide production in pregnancies affected by hypertension or fetal growth retardation. If this is the case then therapy with a nitric oxide donor drug may well be beneficial in the early stages of the disease and lead to improved outcome for both mother and fetus.

5.1.2 Pharmacological alteration of uteroplacental blood flow

The concept of improving uteroplacental blood flow is not new, indeed specific therapy with prostacyclin has been tried previously (Steel & Pearce 1988). The choice of prostacyclin was made because of the imbalance in thromboxane/prostacyclin production which is known to exist in pre-eclampsia. No increase in uteroplacental blood flow was shown in either of the two pregnancies reported despite doses of 4ng/kg/min which caused significant maternal side effects including hypotension. A further study of 13 women treated with up to 8ng/kg/min prostacyclin also failed to show a benefit in terms of outcome(Jouppila *et al* 1985a). Indeed, there was a suggestion that intervillous blood flow was decreased by the infusion. Nevertheless, other investigators have shown that intermittent prostacyclin infusion, whilst not improving blood flow, can

still have a beneficial effect on platelet count (De Belder *et al* 1992). In this report a woman with severe thrombocytopaenia associated with pre-eclampsia had pregnancy prolonged by some weeks by intermittent infusions. It was interesting to note that the increase in platelet count persisted for over a week following infusion.

Most other studies which have examined the effect of a drug on uterine artery blood flow have been in the context of assessing the adverse effects of anti-hypertensive medication. Whilst it is clear that frank hypotension will reduce uteroplacental blood flow and induce fetal distress most of the reported changes were more subtle than this. In particular, none of the studies has shown that treatment significantly increased uteroplacental blood flow. Rat studies have shown that nifedipine causes vasodilatation of the reproductive organs (Ahokas *et al* 1988) but in humans, even doses causing systemic drop in blood pressure do not affect the placental blood flow (Lindow *et al* 1988; Puzey *et al* 1991). Further studies in humans have been done with a wide variety of anti-hypertensive drugs as well as the beta-sympathomimetics used to treat premature labour. Measurements were made using either radioactive accumulation techniques or Doppler ultrasound to non-invasively assess placental blood supply. The results indicate that dihydralazine (Jouppila *et al* 1985b), methyldopa (Montan *et al* 1993), ritodrine (Ylikorkala *et al* 1983; Jouppila *et al* 1985c) and labetolol (Jouppila *et al* 1986) all have no effect on blood flow. Other drugs such as atenolol (Montan *et al* 1987) and salbutamol (Elnas *et al* 1977) have actually been shown to decrease uteroplacental blood flow in some studies.

GTN has been used as a vasodilator for over one hundred years but only recently has it been appreciated that it acts by undergoing metabolism to NO in the vessel wall (Feelisch *et al* 1991). This effect is most marked in veins because low endogenous NO production at this site leads to up

regulation of the guanylate cyclase and consequent supersensitivity to NO (Moncada *et al* 1991).

The observation that GTN could alter uteroplacental blood flow was first made by Giles (Giles *et al* 1992) who administered 300µg sublingually. This had an almost immediate effect which persisted for just 30 minutes. He reported no fall in maternal blood pressure but an increase of 25% in pulse rate. The fetal heart rate did not appear to be affected.

Data from placental perfusion studies *in vitro* would also point to a role for NO in controlling basal tone in the placental vasculature, since inhibition of NO produces a marked increase in resistance (Gude *et al* 1990). The same authors subsequently demonstrated that infusion of ADP, which is known to elicit endothelium dependent relaxation through NO (Moncada 1991), caused vasodilatation in the perfused human placenta (Gude *et al* 1993).

Maternal hyper-oxygenation has been used in both animal and human studies to treat fetal growth retardation. In sheep it has been shown to reduce fetal acidosis without affecting the uteroplacental blood flow as measured using indwelling probes (Paulick *et al* 1992). This finding has been confirmed non-invasively in the human using Doppler ultrasound which has shown an improvement in fetal blood flow redistribution (a sign of fetal hypoxia previously discussed in the introduction and methods sections) without significant alterations in the uteroplacental and umbilical blood flows (Battaglia *et al* 1992). Smoking has been shown to have the reverse effect by increasing the resistance indices in the umbilical artery in the short term, again without affecting the uterine artery blood flow (Morrow *et al* 1988).

5.1.3 Study design and choice of infusions

By using Doppler ultrasound to identify patients with "abnormal" uteroplacental blood flow late in the second trimester of pregnancy I selected a group to be treated with an NO donor drug. This work was intended to quantify the changes in blood flow produced by a short term infusion of glyceryl trinitrate (GTN) under strictly monitored clinical conditions. GTN was chosen because it has been used for many years in pregnancy without reports of adverse effects. This is obviously of paramount importance when recruiting essentially "normal" healthy women for participation in a clinical trial.

The potential side effects of GTN include throbbing headaches, flushing dizziness or tachycardia. Interestingly, long term use (in non-pregnant people) has occasionally resulted in methaemoglobinaemia which would be consistent with Wennmalm's finding that NO forms not only NO3 but also methaemoglobin in human whole blood (Wennmalm, Lanne & Petersson 1992). Recommended infusion rates are 10-200μg/min for cardiac conditions. The infusion doses chosen for this study were at the extreme lower end of this range and administered under close clinical supervision of blood pressure pulse and fetal heart rate. An additional safeguard was to "test" the effect of low dose infusion using women requesting termination of pregnancy. Only when this proved successful was the work extended to include those women with ongoing pregnancies.

In order to test the hypothesis that NO was central in effecting any observed increase in uterine blood flow, a control infusion was needed. Prostacyclin was chosen for several reasons. It is another endothelium derived vasodilator, available in pharmacological preparations which has previously been used in pregnancy in an attempt to treat thrombocytopaenia found in association with pre-eclampsia. It differs from NO by causing vasodilatation through the second messenger cAMP rather than cGMP. I therefore infused prostacyclin as well as GTN in a

sub-group of women, in order to compare the two.

The half life of PGI_2 is only 3 minutes so it is always given by continuous infusion. Potentially, side effects include flushing, headache, bradycardia, sweating, pallor, hypotension and disturbance of platelet function (British National Formulary). To minimise the risks I gave it at the lowest recommended dose and for the shortest possible time with close clinical supervision of blood pressure, maternal pulse and fetal heart rate.

5.2 PATIENTS AND METHODS

5.2.1 Recruitment of patients for infusion study

The first group of patients studied were women undergoing voluntary termination of pregnancy at King's College Hospital, between 8 and 10 weeks gestation (Group 1). The women were seen in the counselling clinic after the decision to have a termination had been made. At this stage an ultrasound scan was performed trans-abdominally to confirm gestational age and a date booked for surgery. They were shown the information sheet relating to the study (Appendix 1) and those who agreed to participate were booked for an infusion on the morning before their operation. This arrangement required the women to make no extra visits to the hospital and also allowed the cannula from the infusion study to be used for administering anaesthetic drugs in the afternoon. Although these factors undoubtedly helped recruitment, there were disadvantages. The most frustrating of these was only being able to book one or two patients each week and relying very heavily on them attending as planned. The scans for this initial group of first trimester pregnancies were carried out transvaginally. Details of the ultrasound techniques used and an explanation of the indices derived can be found in the methods section of the thesis.

After gaining successful results from the first group of 10 patients I applied to the ethical committee to extend the trial to include those women with "abnormal" uterine artery blood flow between 24 and 26 weeks gestation with ongoing pregnancies. These women would therefore be at high risk of developing pre-eclampsia and/or fetal growth retardation later in the pregnancy. The positive predictive value from published work is in the region of 35% (see main introduction section of thesis).

In order to prospectively recruit these women I undertook screening of uterine artery blood flow, at the time of the routine 20 week scan, for all women booking at King's College Hospital over a 6 month period. This

was done using continuous wave equipment and a pencil probe to identify the main uterine artery flow velocity waveform. This equipment was freely available and not needed for other research projects like the large Acuson machine. Doppler techniques) and all those with bilateral notching and/or a resistance index (RI) > 0.6 were booked for repeat scan at 24 weeks gestation on the Acuson 128 ultrasound machine. Those found to have persistent notching of the waveform with high RI were then approached to enter the study and given the information sheet (Appendix 1).

This amounted to approximately 500 "screening" scans over the six month period in order to recruit enough women for this study, so whilst I have chosen to present the data in a compact format, the study was very time consuming to conduct.

5.2.2 Preparation of Intravenous Infusions

All infusions were made up freshly on the morning they were needed and kept refrigerated and protected from light until used.

Glyceryl Trinitrate (GTN)

40 ml of 0.9% saline for injection was drawn up into a 50 ml syringe. 10 ml of 0.5 mg/ml GTN (Tridil, Dupont) was then added to give a final concentration of 100 μg/ml. Infusions of GTN were given at two dose rates. Initially 6 ml/hr (equivalent to 10μg/min) and subsequently at 12ml/hr (equivalent to 20μg/min).

Prostacyclin (PGI_2)

Using the diluent provided (pH 10.5), 500μg of the powdered drug (Epoprostenol Flolan, Wellcome) was reconstituted according to the manufacturers instructions. 100μg of this was transferred to a 50ml syringe and topped up with 50ml 0.9% saline for injection to give a final concentration of 2μg/ml. Infusions were given at two doses. Initially at 3 ml/hr (equivalent to 0.1μg/min) and subsequently at 6 ml/hr (equivalent to

0.2μg/min).

5.2.3 Administration of Infusions

The subjects were semi-recumbent on a scanning couch in a temperature controlled room for 20 minutes before baseline readings were taken. Pulse and blood pressure were measured continuously during infusions using a dymomap recorder. The initial readings were also checked manually using a mercury sphygmomanometer to ensure accuracy.

An 18G intravenous cannula (Venflon) was inserted into a forearm vein using local anaesthetic. Those patients providing blood samples for other investigations had the blood taken from the cannula at this time. A 50 ml syringe containing 0.9% saline for injection was connected to the cannula via a 1m long connecting tube and 3-way tap. Baseline Doppler readings were taken at this point as described earlier.

For those patients receiving only GTN, the syringe containing GTN infusion was then connected to the tubing in place of the saline and the tubing purged by opening the spare port of the 3-way tap. The syringe was then put into a pump driver set to deliver 6ml/hr. This provided a continuous infusion rate of 10μg/min GTN. The infusion syringe was kept out of sunlight in an opaque bag as recommended by the manufacturers. After a period of stabilisation for 20 minutes the Doppler readings were repeated. The dose of GTN was then doubled and after a further 20 minutes more readings taken. Finally, the GTN was replaced by the syringe containing saline for 20 minutes and a last set of readings taken.

The initial infusions on patients undergoing termination of pregnancy were all done by me alone in an unblinded manner. This was to validate the technique and obtain some preliminary data. The results, shown below, appeared very positive so the subsequent work on the later pregnancy group was carried out with some assistance. By getting another doctor to

administer the infusions and check pulse and blood pressure, the infusions could be administered in a double blind manner so that neither I nor the patient were aware what they were receiving at any one time. We were able to vary the order in which infusions of saline, GTN or PGI_2 were given because of the short half-lives of the drugs being used. This also allowed me more time to scan so that I was able to image other vessels in addition to the uterine arteries. The umbilical artery was an obvious choice to assess fetal well being and in addition I examined the maternal carotid artery.

5.2.4 Statistical Analysis

Since the number of patients available to me was going to be small I took statistical advice at the planning stage. I knew that the range of values for RI, PI and mean velocity would show wide variation throughout the study population (inter-subject variation) as well as from vessel to vessel. This meant that a simple comparison of means would fail to show any significant changes. It was important to analyse the data as paired samples and try to present the findings in an easily understandable format. I therefore decided to treat the baseline reading as 100% and express all subsequent readings as a percentage of this. The "percentages" could then be compared using a paired Students t-test to assess significance.

The duplicate measurements used to establish baseline levels were also used to assess the reproducability of the ultrasound Doppler measurements as described in the main methods section.

5.3 RESULTS

The raw data obtained from this study was in the form of a series of RI, PI and mean velocity measurements from each vessel imaged together with recordings of pulse and blood pressure. The results obtained during each infusion of GTN, prostacyclin and saline were converted to a percentage of the baseline values and are summarised in tables 5.1, 5.2 and 5.3 below.

5.3.1. Actual baseline readings

The baseline readings of pulse and blood pressure for groups 1, 2a and 2b respectively, were not significantly different (pulse 78 vs 82 vs 80, blood pressure 122/72 vs 118/68 vs 118/70). Actual mean uterine RIs for groups 1, 2a and 2b respectively were 0.89 vs 0.68 vs 0.66 with actual mean PIs of 2.28 vs 1.64 vs 1.72. These baseline uterine Doppler readings demonstrate the higher resistance to flow seen in early pregnancy (group 1) compared with later pregnancy (groups 2a and 2b). There were no significant differences between subgroups 2a and 2b in baseline uterine Doppler indices.

5.3.2. GTN infusions

In group 1 (8-10 weeks gestation), GTN infusion produced a significant fall in both the resistance and pulsatility indices of the uterine artery ($p<0.01$) at both dose rates used. There was a concomitant rise in mean velocity, although this only became significant when GTN was infused at the higher rate of 20 µg/min. In addition, 8 of the 10 women showed a reduction of post-systolic notching of the FVW with disappearance of the notch in 4 cases. Examples of the changes seen are shown in figure 5.1.

In group 2a (24-26 weeks gestation), the infusion of GTN produced a similar decrease in the resistance and pulsatility indices of the uterine artery but without significant alteration in velocity. There were no significant changes in heart rate, systemic blood pressure or the indices

derived from either the umbilical or carotid arteries.

5.3.3 Prostacyclin infusions

Patients in group 2b showed a similar fall in resistance indices with GTN infusion to those women in group 2a. However, the decrease in uterine artery RI and PI with PGI_2 infusion was much less than that produced by GTN infusion and was not statistically significant at the 5% level. In the interests of safety the dose of PGI_2 could not be increased further because of the systemic effects on pulse and blood pressure which occurred at the lowest dose (8% fall in BP, 12% rise in pulse).

Table 5.1

Results from group 1, 10 normal pregnancies of 8-10 weeks gestation. Doppler results are expressed as a percentage of baseline value with standard deviation in brackets. Comparison of means uses a paired Students t-test to show differences between saline and active infusions with * = $p<0.01$, ** = $P<0.05$

	Saline Control	GTN 10 µg/min	GTN 20 µg/min
Uterine			
PI%	107.3 (SD 14.6)	85.4 (SD 17.3)*	70.9 (SD 14.6)*
RI%	100.8 (SD 5.4)	91.6 (SD 6.4)*	82.4 (SD 6.8)*
V%	103.6 (SD 27.0)	120.8 (SD 40.0)	144.1 (SD 36.0)*

Table 5.2

Results from group 2a, 10 pregnancies of 24-26 weeks gestation with abnormal uterine artery Doppler waveforms given infusions of GTN. Doppler data is from the main uterine, umbilical and maternal carotid arteries. Pulse and mean arterial pressure (MAP) are shown at the foot. Doppler results are expressed as a percentage of baseline value with standard deviation in brackets. Comparison of means uses a paired Students t-test to show differences between saline and active infusions with * = p<0.01, ** = P<0.05

	Saline Control	GTN 10 µg/min	GTN 20 µg/min
Uterine			
PI%	100.2 (SD 17.2)	84.6 (SD 21.1)**	78.9 (SD 17.0)*
RI%	104.9 (SD 6.6)	92.8 (SD 9.2)*	85.8 (SD 7.9)*
V%	103.1 (SD 17.7)	99.6 (SD 22.0)	103.2 (SD 24.4)
Carotid			
PI%	107.2 (SD 13.2)	116.4 (SD 15.4)	103.1 (SD 14.2)
RI%	101.5 (SD 6.8)	105.7 (SD 9.4)	99.0 (SD 10.4)
V%	100.1 (SD 19.2)	101.0 (SD 22.6)	93.7 (SD 17.5)
Umbilical			
PI%	96.6 (SD 14.8)	96.4 (SD 15.4)	93.3 (SD 21.1)
RI%	95.8 (SD 6.6)	95.8 (SD 8.6)	91.3 (SD 9.0)
V%	100.1 (SD 19.2)	105.1 (SD 21.7)	108.4 (SD 16.9)
Pulse (bpm)	99.2 (SD 5.2)	103.5 (SD 5.7)	103.3 (SD 5.6)
MAP (mmHg)	102.9 (SD 8.3)	104.3 (SD 7.9)	103.2 (SD 7.4)

Table 5.3

Results from group 2b, 5 pregnancies of 24-26 weeks gestation with abnormal uterine artery Doppler waveforms given infusions of both GTN and prostacyclin. Doppler data is from the main uterine arteries. Pulse and mean arterial pressure (MAP) are shown at the foot. Doppler results are expressed as a percentage of baseline value with standard deviation in brackets. Comparison of means uses a paired Students t-test to show differences between saline and active infusions with * = $p<0.01$, ** = $P<0.05$

	Saline Control	GTN 10 µg/min	PGI_2 100 ng/min
Uterine			
PI%	103.3 (SD 15.1)	86.2 (SD 18.8)**	94.9 (SD 19.2)
RI%	98.5 (SD 6.4)	91.1 (SD 8.7)**	95.1 (SD 9.3)
V%	107.6 (SD 17.4)	98.2 (SD 21.6)	92.3 (SD 23.6)
Pulse(bpm)	99.1 (SD 5.1)	102.4 (SD 5.7)	111.8 (SD 5.3)*
BP (mmHg)	103.5 (SD 8.3)	104.3 (SD 8.1)	92.2 (SD 7.9)**

Figure 5.1

Normal uterine artery blood flow in the first trimester showing little end-diastolic flow and a post-systolic notch.

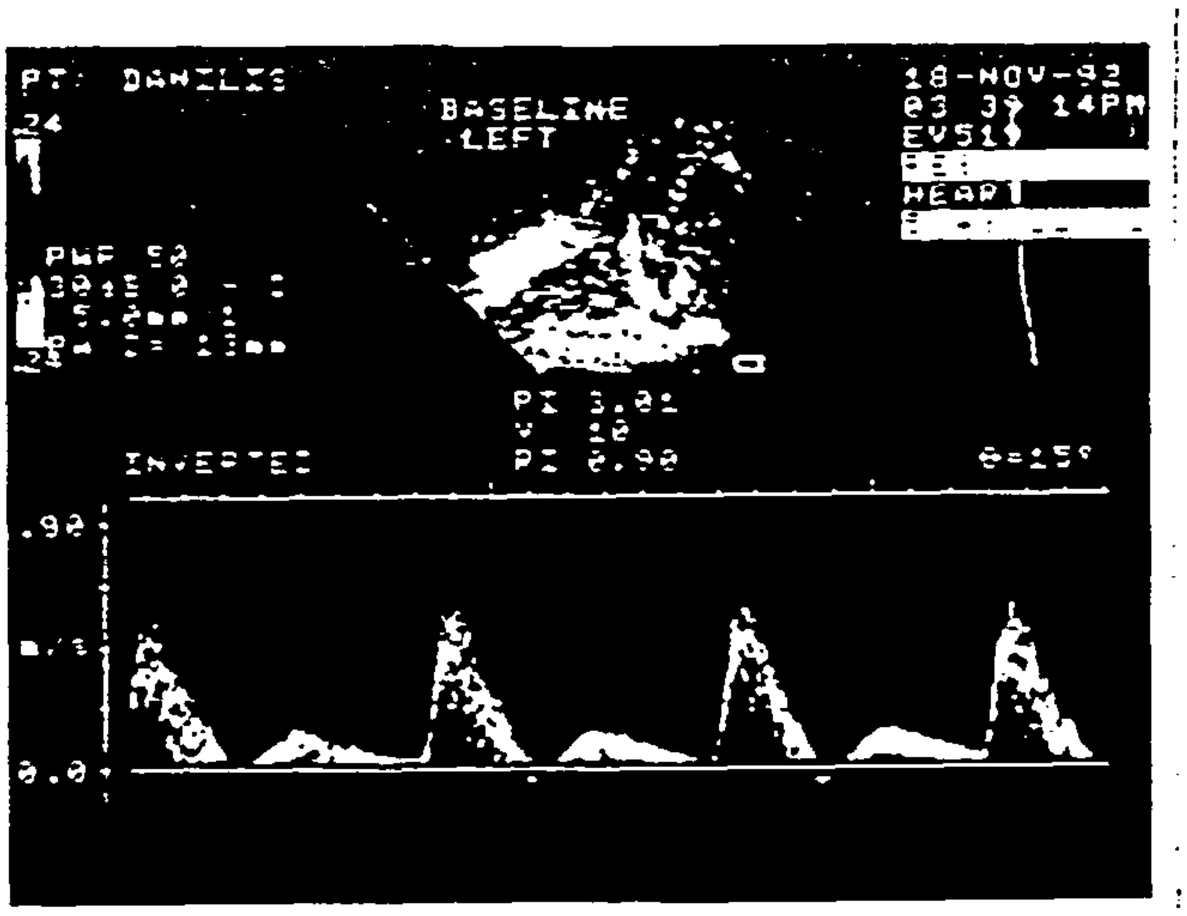


Figure 5.2

Uterine artery blood flow in the same patient as figure 5.1 following infusion of GTN.

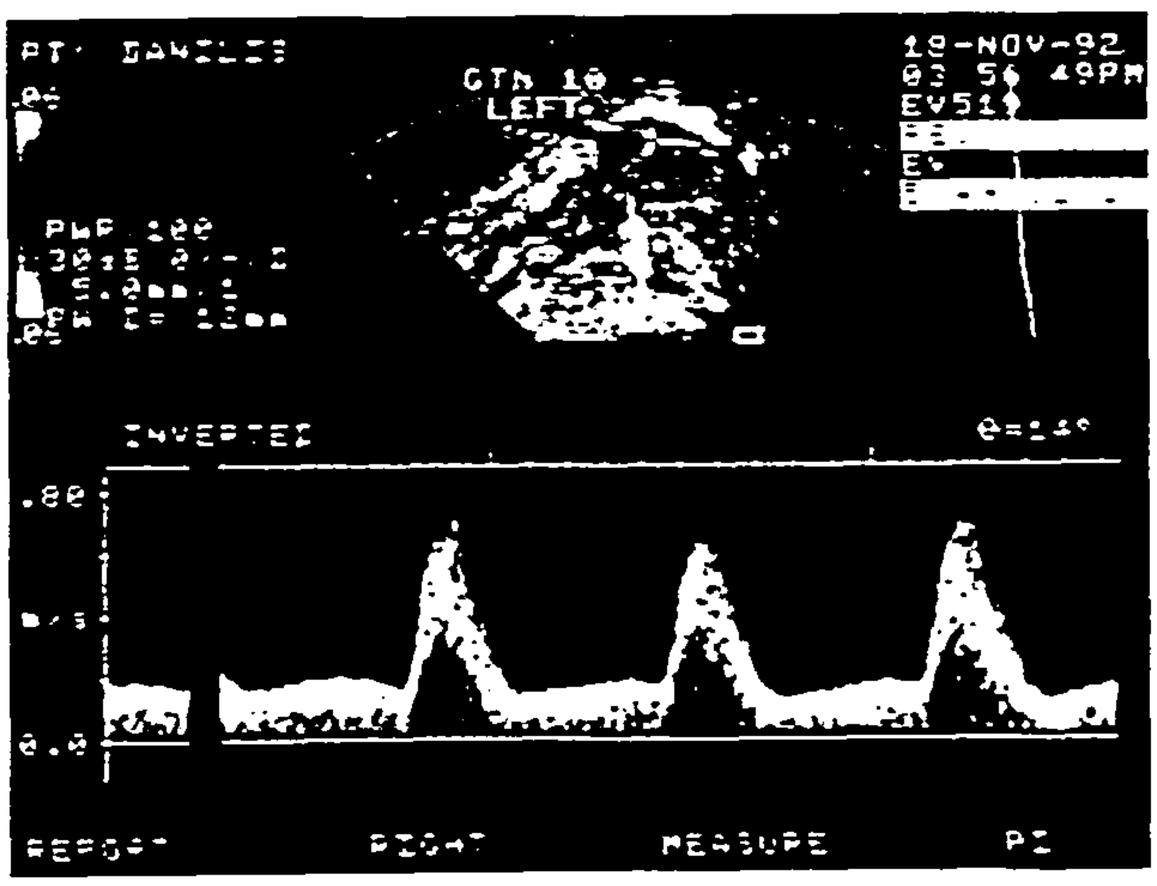


Figure 5.3

Uterine artery blood flow which would be considered abnormal in the second trimester showing reduced diastolic flow (mean RI > 0.6) and a post-systolic notch.

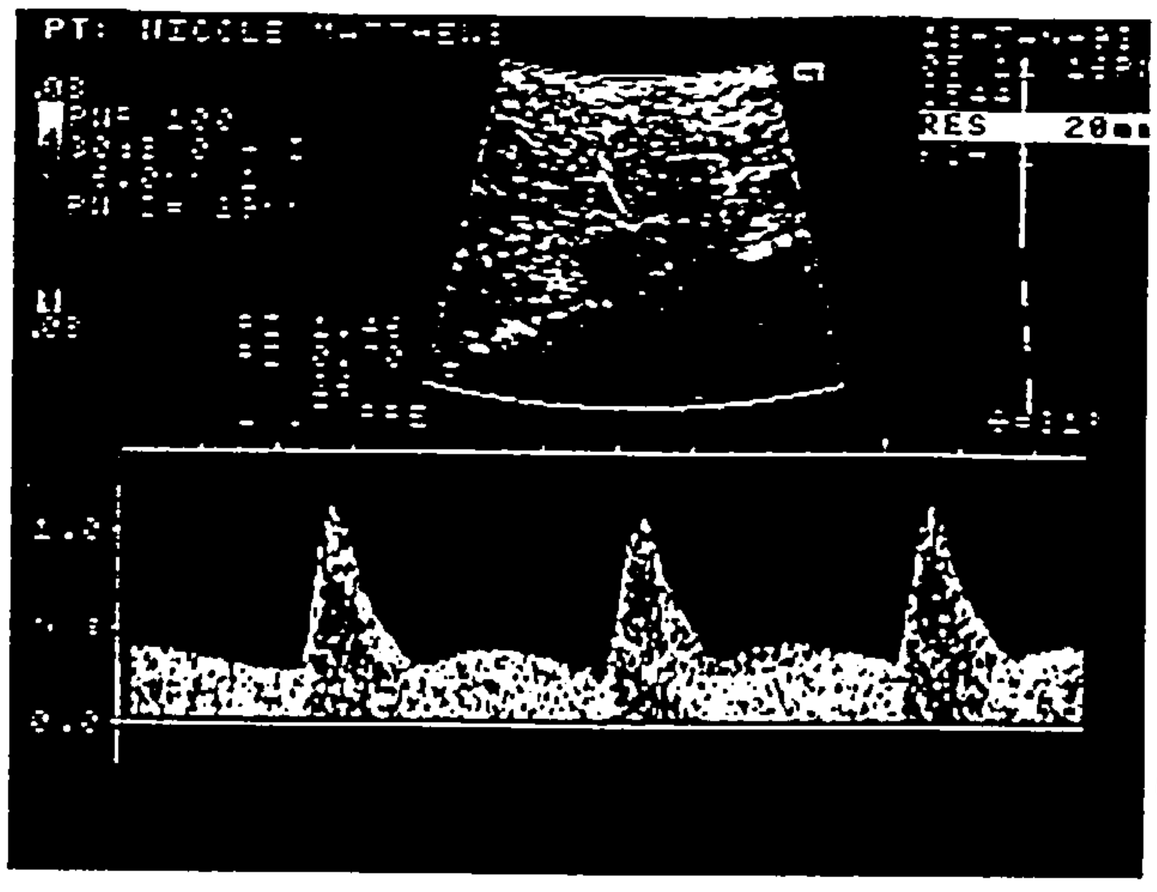
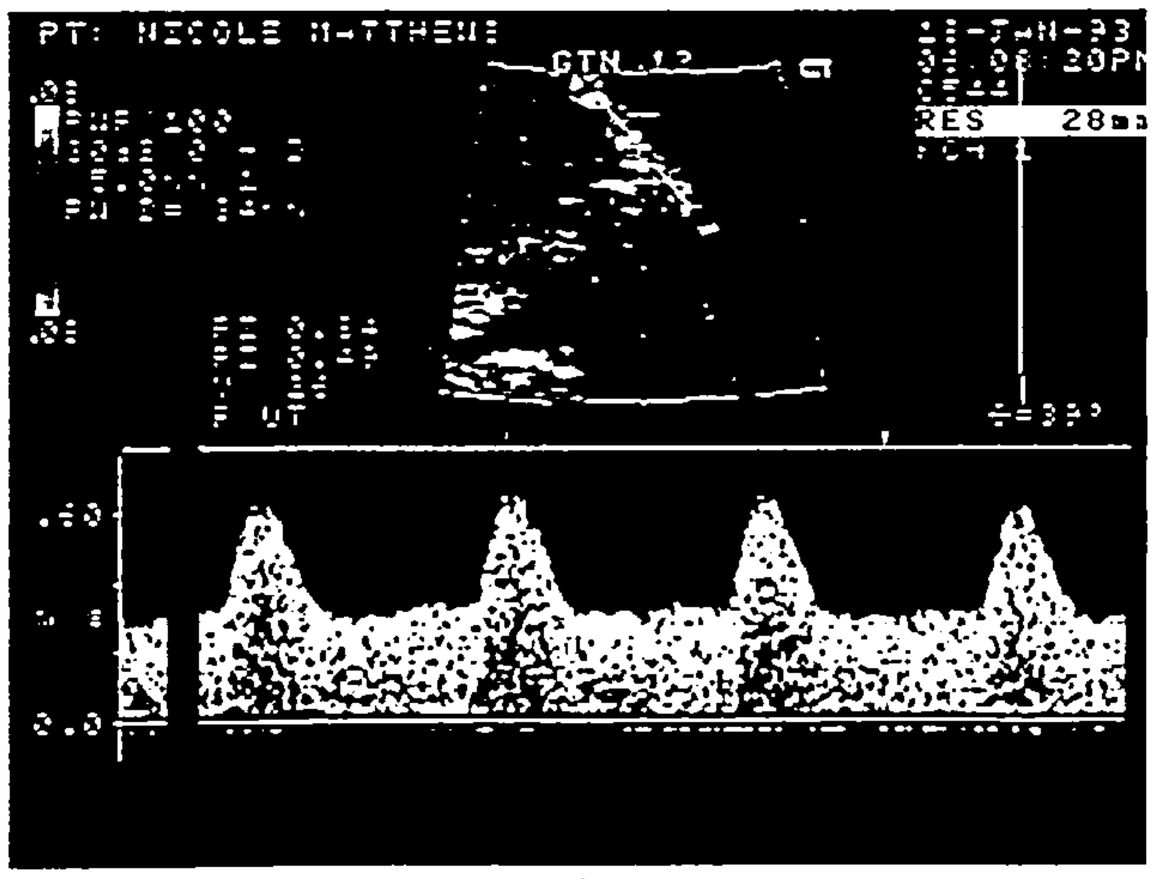


Figure 5.4

Uterine artery blood flow in the same patient as figure 5.3 following infusion of GTN.



5.4 Discussion

5.4.1 Baseline Results

Doppler ultrasound offers a reproducible method for non-invasively assessing uteroplacental blood flow. The fact that the results for saline infusion are so close to 100% of the baseline value confirm this and also offers support to the initial assumption that both GTN and PGI_2 have only transient effects on the circulation due to short half-lives. The lower resistance indices in group 2 when compared to group 1 are to be expected due to the physiological changes of advancing gestational age.

5.4.2 GTN infusions in the first trimester

In the first trimester study on women undergoing termination of pregnancy there was an obvious fall in uteroplacental vascular resistance. Whilst the rise in mean velocity in association with this is suggestive of an increase in uteroplacental blood flow it must be remembered that this is an interpretation of the Doppler FVW and not a direct measurement of volume flow. It may be better to say simply that the GTN infusion mimicked the gestation related changes that would have occurred over the coming month. The disappearance of the post-systolic notch and 18% reduction in RI would equate with this.

One disadvantage of collecting data from women undergoing termination is that there will be no outcome data for the pregnancy. By chance, one of the ten women in this group may have been destined to become hypertensive had the pregnancy been allowed to continue. Unfortunately it is impossible to say which (if any) of the women this would have been. Nevertheless it is interesting to postulate that a failure to respond locally to GTN at this stage would identify those with poor trophoblast invasion who are likely to subsequently develop pre-eclampsia or growth retardation.

5.4.3 GTN and PGI_2 infusions in women with abnormal Dopplers

The uterine artery FVW derived indices of resistance fell significantly after GTN administration in the later pregnancy group. Indeed, this improvement was significant enough in some cases to have changed a woman from being defined as "high risk" (in terms of uterine artery RI and a post-systolic notch) to "low risk" of subsequently developing pre-eclampsia.

The umbilical artery PI showed a similar trend to the uterine artery, with a fall in resistance indices and rise in velocity in response to GTN. However, both these changes failed to achieve statistical significance at the 5% level due to high SDs. Other investigators have given higher doses of GTN to pregnant women and shown a marked effect on umbilical artery Doppler signals. An Australian group showed that following 300μg sublingual GTN the umbilical S/D ratio fell by 20%, returning to normal over 30 minutes(Giles *et al* 1992). However this was based on uncontrolled observations of only 5 women and in addition, the dose of GTN given caused a significant maternal tachycardia. The other problem with only measuring S/D ratios without any indication of velocities is that it does not allow any comment to be made on volume blood flow.

Vasodilatation of the umbilical or uterine artery will decrease Doppler resistance indices but probably not increase the actual blood flow through the vessel. What is required for a true "improvement" in blood supply is a fall in resistance accompanied by a rise in velocity, indicating vasodilatation of the distal vascular resistance vessels, i.e. the placenta.

It is reasonable to deduce the observed effect was mediated mainly by NO because of the failure of prostacyclin infusion to achieve the same result. Investigators have already used perfused preparations of term placentae to demonstrate the role of NO in maintaining low resting vascular tone (Myatt *et al* 1992; Van Buren, Yang & Clark 1992). The

data presented here confirms the importance of the L-Arginine-Nitric oxide-cGMP pathway in the control of placental blood flow *in vivo*.

5.4.4 Possible clinical applications

The improvement of uterine artery blood flow in the absence of systemic side effects is encouraging. This means it may be possible to administer GTN (or another NO donor) therapeutically in the future. Since high resistance to flow in the uteroplacental circulation is associated with the subsequent development of pre-eclampsia and growth retardation, the decrease in resistance produced by GTN may be beneficial in affected pregnancies. In addition GTN would have an anti-thrombotic action to combat platelet deposition in the placental bed. However , it must be remembered that the flow velocity waveforms only provide an indirect measurement of resistance and it is impossible to comment on volume flow from these data. This means that although diastolic flow may be increased, if the actual pressure is also reduced there will not be an increase in blood supply to the fetus, so limiting any therpeutic benefit.

Further investigation is needed to see if NO donors have any role to play in the treatment, or indeed prophylaxis, of pre-eclampsia or fetal growth retardation.

CHAPTER 6 - CONCLUSION

6.1 GENERAL OVERVIEW OF RESULTS

6.1.1 Plasma nitrate and nitrite levels

The level of nitrite in all plasma samples was extremely low with no significant differences between any of the groups examined. This leads me to conclude that nitrite estimation is of very limited value in assessing NO production *in vivo*. This is consistent with previously published work which suggests that NO is converted to nitrate rather than nitrite in the presence of oxyhaemoglobin (Wennmalm *et al* 1992, Ignarro *et al* 1994).

Plasma nitrate levels were higher during the second and third trimesters of pregnancy (38 and 37 μmol/l respectively) than in non-pregnant women or during the first trimester of pregnancy (31 and 34 μmol/l respectively), despite the haemodilution which is known to occur in later pregnancy. This finding supports the notion of increased NO production in normal pregnancy and would fit well with the alterations in systemic blood pressure which are known to take place during pregnancy.

There was no clear correlation with Doppler blood flows since uteroplacental vascular resistance falls progressively towards term, not reflecting the increase in systemic blood pressure characteristic of normal third trimester pregnancies. Similarly, abnormal Doppler flows in the uterine artery at 26 weeks gestation did not correlate with alterations in nitrate levels. It is likely that any sublte changes to local pathology would be dwarfed by the systmic production of NO by the rest of the vascular system.

Fetal nitrate levels were closely related to matched maternal levels, tending to be slightly higher. This difference only achieved statistical significance in the IUGR pregnancies. This may reflect increased NO production by the fetus as part of a compensation process or may indicate impaired transport across the feto-placental barrier.

6.1.2. Nitric oxide synthase activities in trophoblast.

My finding of cNOS activity within the trophoblast from term placentae is consistent with other reports in the literature (Myatt *et al* 1992, Buttery *et al* 1994). However, the relative amounts of calcium dependent and independent activity which I have found (55% cNOS vs 45% iNOS) are different to those previously reported (95% cNOS vs 5% iNOS) (Myatt *et al* 1993). This apparent contradiction can be explained by different methods of data handling between publications. Some authors subtract calcium dependent form calcium independent activities to arrive at an "inducible" enzyme activity. This leads to very small values of "iNOS" being reported. I actually quote the calcium dependent and independent acitivies as the raw data. Since the nomenclature is constantly being updated care must be taken when comparing publications.

I found the total NOS activity to be highest in the first trimester trophoblast. This is a new finding and is particulalry interesting because of the relativley high contribution of iNOS acitivity to the total. Whilst cNOS activities fell most sharply in the third trimester, the iNOS activities fell earlier, at the end of the first trimester. This suggests a role for trophoblast derived calcium independent NOS in early pregnancy during the process of implantation.

It has been shown that the cytosolic guanyate cyclase found in human placenta is only activated by NO around one fifth as much as the equivalent bovine enzyme (Idriss *et al* 1992). The authors suggested tis may be due to a hexa-coordinate haem prosthetic group in the bovine enzyme making it less available to bind withNO. Whilst this insensitivity of guanylate cyclase to NO may offer an explanation for the high levels of NOS activity seen in trophoblast, it does not explain the fall in activity seen with advancing gestation.

Trophoblast from IUGR placentae showed extrremely low levels of both

iNOS and cNOS activity at the time of delivery. This means there is likely to be a relative deficiency of NO in such placentae and is consistent with the platelet deposition and high resistance to blood flow which occurs within the feto-placental circulation of pregnancies complicated by IUGR.

In normal pregnancies the concentration of L-arginine in the trophoblast fell throughout gestation in parallel with the decrease seen in both iNOS and cNOS activities. This fall did not occur in pregnancies complicated by IUGR despite these placentae showing the lowest NOS activities of all. Although this may simply reflect a problem with the NOS enzyme in the trophoblast this seems unlikely. Much of the measured L-arginine may have been in transit through the trophoblast tissue at the time the sample was taken, rather than actually being used by the trophoblast itself. Hence, the picture could also be explained by accumulation within the placenta due to impaired transport at the feto-placental interface or an impaired ability of the fetus itself to utilise the arginine. The plasma nitrate data showing slightly higher levels in the fetus than the mother means that impaired utilisation of arginine is unlikely. Whilst this would support the idea of impaired transport between fetus and placenta leading to accumulation of nitrate on one side and arginine on the other, this view is too simplistic. The data on other amino acids clearly shows that although some amino acid levels are lower in IUGR placentae than normals, there are some which are higher. Without knowing the precise utilisation of these amino acids and fetal and maternal blood levels, it is difficult to comment further on these results.

6.1.3. Nitric oxide synthase activities in myometrium

Myometrial NOS activity was much lower than that found in trophoblast tissue (0-2 vs 0.5-30 nmol L-citrulline/min/g tissue protein). Whilst the cNOS activity was similar in all groups, probably arising from the myometrial vasculature, the iNOS levels varied between groups. The highest levels were found in the non-pregnanct specimens and the lowest

in the term pregnant uterus not yet in labour. These findings make it unlikely that myometrial derived NO will contribute significantly to maintenance of either vasodilatation or myometrial quiescence in pregnancy. It is known that high concentrations of NO will usually inhibit iNOS activity by negative feedback, so it is possible that the fall in myometrial iNOS activity in pregnancy is partly a result of the high NOS activity in trophoblast tissue.

6.1.4. Oestrogen Study

The dat presented provide evidence for a link between NO production and circulating oestrogen levels in the human. The observed increase in plasma nitrate levels associated with administration of exogenous oestrogen was small (around 10%) but statistically significant. The higher levels were consistent with those seen in the second trimester of pregnancy. It seems logical to assume that the magnitude of rise which could be expected will always be relatively small, since increased renal excretion is likely to occur over the course of a month as a homeostatic function.

6.1.5. NO donor infusions

Infusion of GTN increased uterine artery diastolic blood flow in early normal pregnancy and also in those women with abnormal uterine artery Doppler blood flow velocity waveforms in the second trimester. Due to ethical considerations it was not possible to test whether GTN woudl have had a similar effect on normal second or third trimester pregnancies.

These findings suggest a role for NO in the control of diastolic blood flow in the uteroplacental circulation during normal pregnancy. In addition they would support the hypothesis of locally deficient NO production in the utero-placental circulation of women whose pregnancies are complicated by hypertension and IUGR.

6.2. THE NEED FOR FURTHER RESEARCH

The studies I have carried out have demonstrated an increase in plasma nitrate levels in pregnancy consistent with NO playing a role in mediating the systemic caridovascular changes of normal pregnancy. However, my data did not show a firm link between uteroplacental blood flow and systemic levels of nitrate despite the ability of NO donor infusion to increase diastolic blood flow. Whilst it may be that, at a local level, NO contributes to the low resting vascular tone in the uteroplacental circulation, it may in fact be more important in some of the other processes which occur in early pregnancy e.g. control of trophoblast invasion or prevention of rejection or platelet aggregation. This hypothesis would certainly be consistent with the fall in trophoblast NOS activity seen with advancing gestation whcih occurs against a background of falling vascular resistance. Further investigation will be needed to clarify the role of NO produced by trophoblast iNOS in the first trimester of pregnancy .

I have demonstrated deifferences in trophoblast NO production between normal pregnancies and those complicated by IUGR. It remains an attractive idea to link high resistance to blood flow and platelet deposition in the placental bed by invoking a relative shortage of NO. It is possible that decreased placental NOS activity, increased fetal production and impaired transport across the feto-placental barrier all combine to cause this relative deficiency. Further studies will be needed to clarify this exciting area, since if a deficiency were identified it would suggest the possibility of corrective therapy with NO donor drugs.

Finally, it may be that production of NO is one of the mechanisms by which oestrogen exerts its cardioprotective effect in women of child bearing age or mediates the cardiovascular changes of pregnancy. This is potentially the most important area for future research.

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APPENDIX 1 - Information Sheets for study participants.

PATIENT INFORMATION LETTER FOR GTN INFUSIONS IN EARLY PREGNANCY

When you come to hospital for your operation we would like to ask for your help with an important research project.

When some women are pregnant they develop high blood pressure which is not only dangerous for them but also reduces the blood flow to the womb, so slowing the growth of the fetus. Our work aims to find a treatment which will prevent these problems occurring.

There is a drug in use which can safely increase blood flow to the heart called GTN. We believe this same drug may increase blood flow to the womb in pregnancies affected by high blood pressure and so improve the outcome. However, before trying these drugs in ongoing pregnancies we wish to use them on women coming to hospital who are requesting termination.

You will require a drip for your operation and we can use this to give you the drug so there will be no extra injections required. The blood flow to your womb will be measured using a vaginal ultrasound scan which is not painful. The whole procedure should take about 30 minutes. the effects will wear off rapidly. If more information, then please talk to the Doctor arranging your admission. You are under no obligation to be given this drug and your care will not be affected in any way if you do not wish to participate in the study. Very rarely people get side-effects of headaches or hot flushes. If these things are a problem for you we can stop immediately and the effects will wear off very rapidly (5-10 minutes). Your care will not be altered in any way if you do not wish to participate but we do hope you will be able to help us.

PATIENT INFORMATION LETTER FOR GTN INFUSIONS IN PREGNANT WOMEN WITH ABNORMAL DOPPLERS

We would like to ask for your help with important research work we are carrying out here at King's.

Why Me?

You have been found to have signs of reduced blood flow to your womb (a "notch") which we know makes you more likely to develop problems with raised blood pressure or slow growth of the baby later in pregnancy.

What's the Problem?

During normal pregnancy the blood supply to the baby increases because of natural substances in the blood which relax the arteries. We believe that a shortage of these substances could be responsible for the problems which some women have with high blood pressure or slow growth of the baby. Currently there are no treatments available for this problem.

What can be done?

We have recently discovered that certain drugs which dilate arteries (such as GTN) actually work by releasing the same substances which occur naturally. GTN has been used for many years to treat angina without problems, so we are confident it is safe. Our work has already shown that we can improve the blood flow to the womb in early pregnancy but now we need to concentrate on those in later pregnancy with problems.

What do I need to do?

We measure your blood flow using the scanner as before. The GTN will be given for 30 minutes only, whilst watching the effect on blood flow. The GTN only lasts for 3 minutes in the blood so will have no long term problems or side effects. The whole procedure should take just over an hour.

Although you are under no obligation to receive this treatment we do hope that you decide to help us. This research could provide a major advance in understanding these important pregnancy problems and offer treatment to very small babies where currently there is none.

PATIENT INFORMATION LETTER FOR

EARLY PREGNANCY STUDY

Whilst you are in hospital for your operation we would like to ask for your help with a research project.

What is the research?

As you may know, many women suffer from raised blood pressure in late pregnancy and this can lead to major problems for both mother and baby. Our research aims to find out what causes this rise in blood pressure so that we may give better treatment.

Why me?

In order to understand the disease we must first study what controls blood pressure in normal pregnancy. Since you are in early pregnancy and already attending the hospital this will not cause you any extra inconvenience.

What is involved?

We would like to perform an ultrasound scan to measure blood flow in and around the womb before you have the operation. This takes no longer than 10 minutes and is done using a small probe in the vagina. We do the scan this way because it gives much better results. It is safe and is not painful.

Before you receive the anaesthetic it is necessary to have an injection. We would like to take a small sample of blood at this time. We will analyse this later, together with a sample of the placental tissue obtained during the operation.

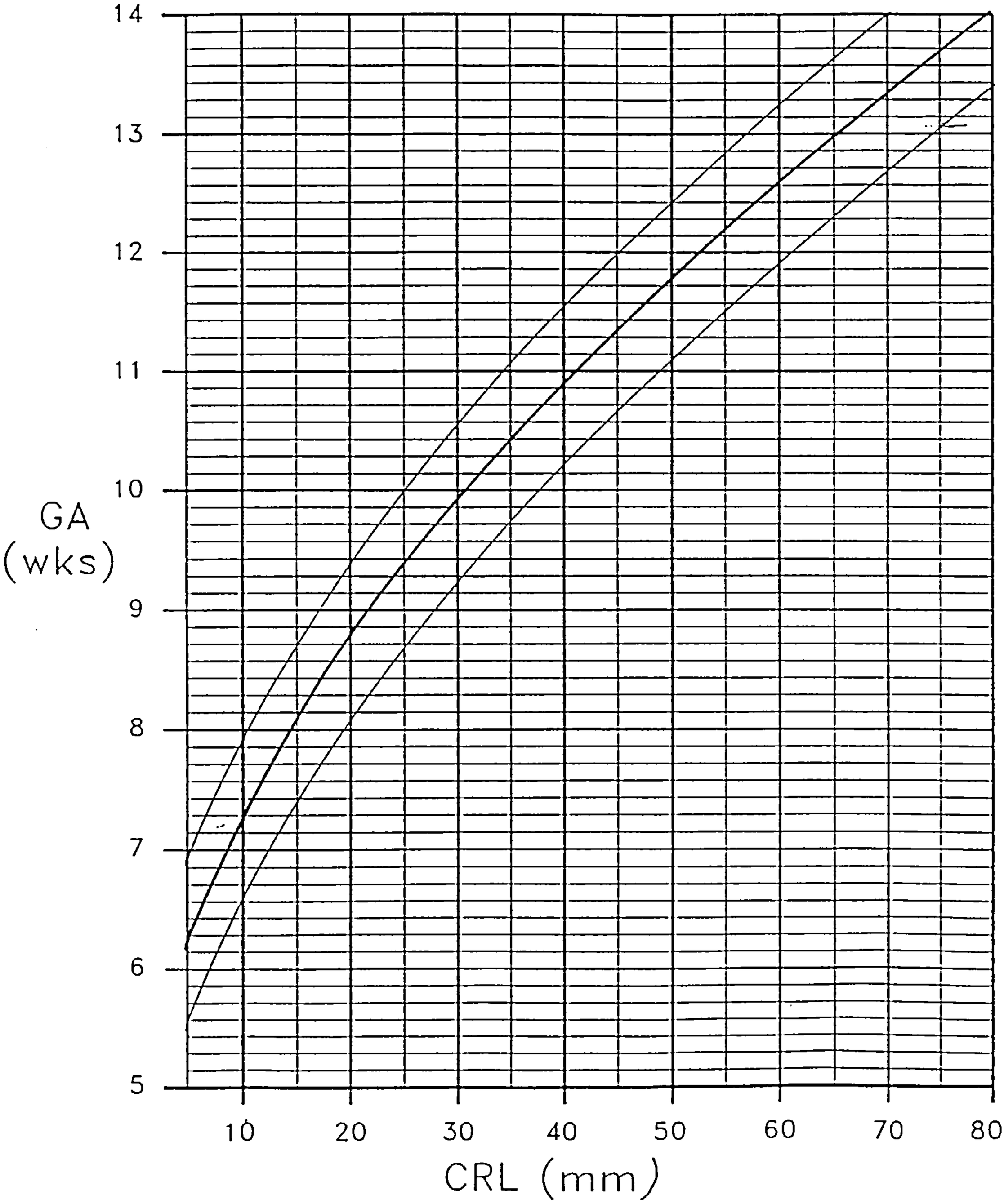
What now?

If you agree to help us with this important work it is necessary for you to sign the consent form on the reverse of this form. We will then perform the scan and collect the blood sample before you have the operation. If you decide not to participate then we can reassure you that your care will not be altered in any way by your decision.

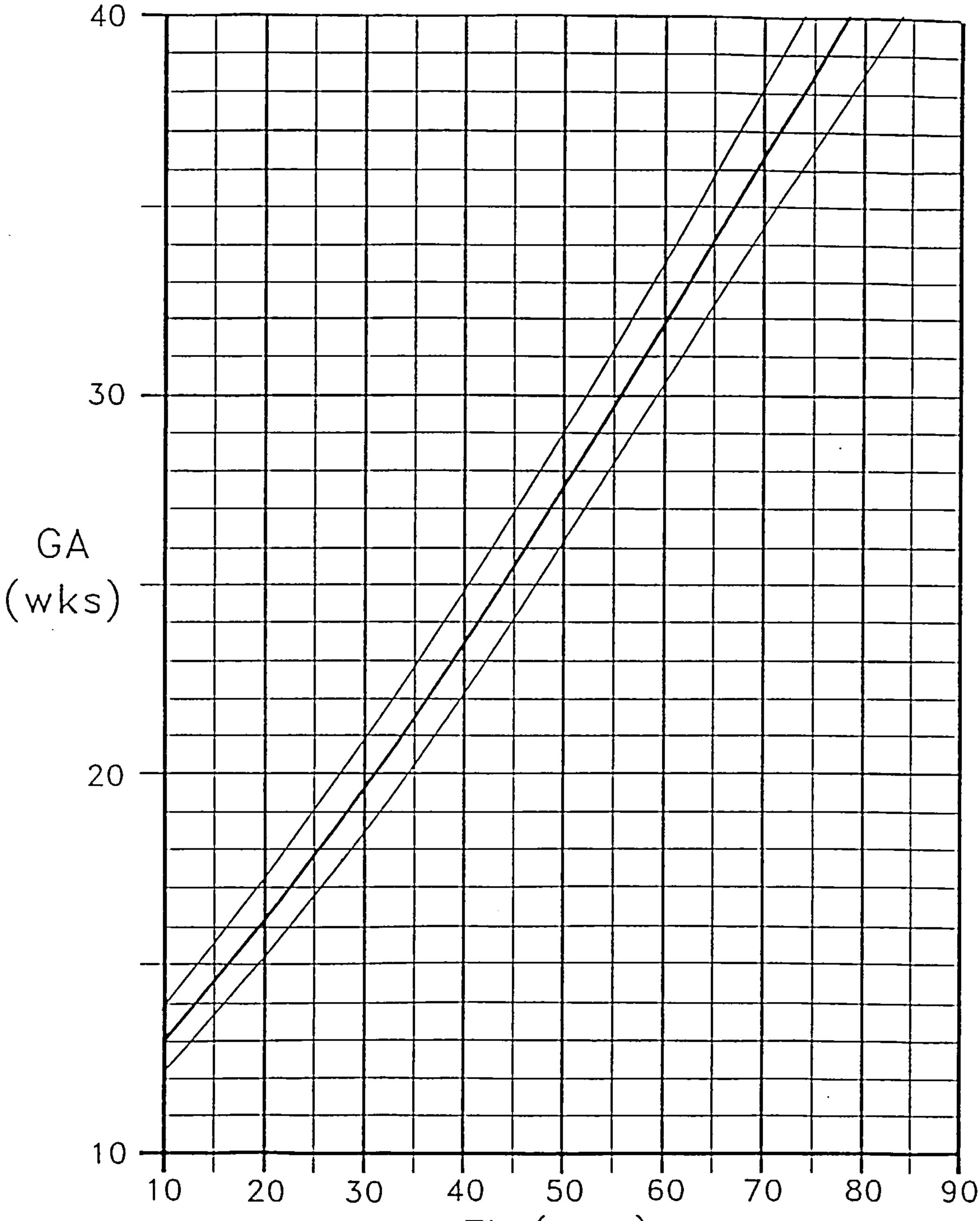
APPENDIX 2 - Normal values for fetal ultrasound measurements

(British Medical Ultrasound Society recommended data)

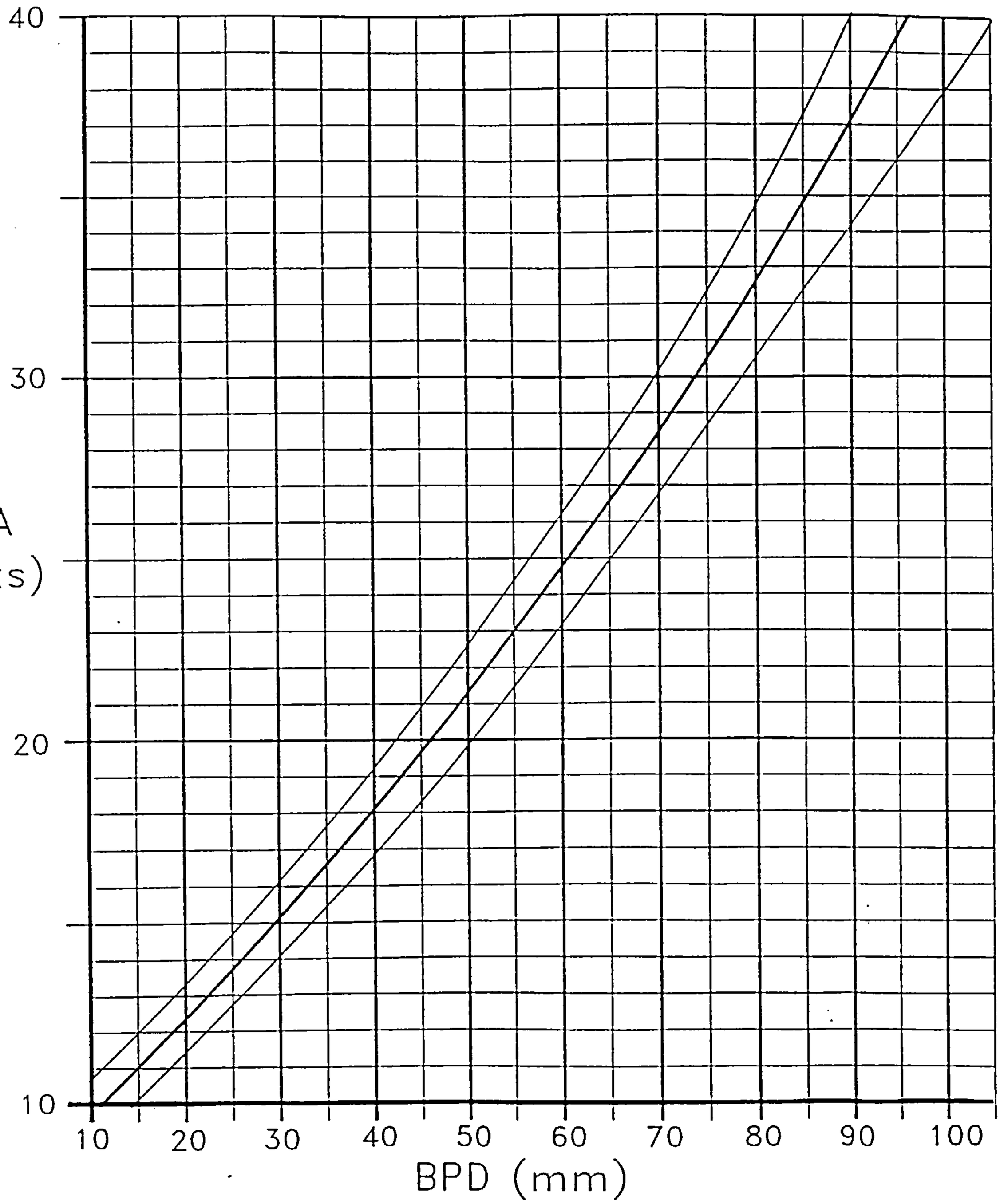
Crown-rump length versus gestational age



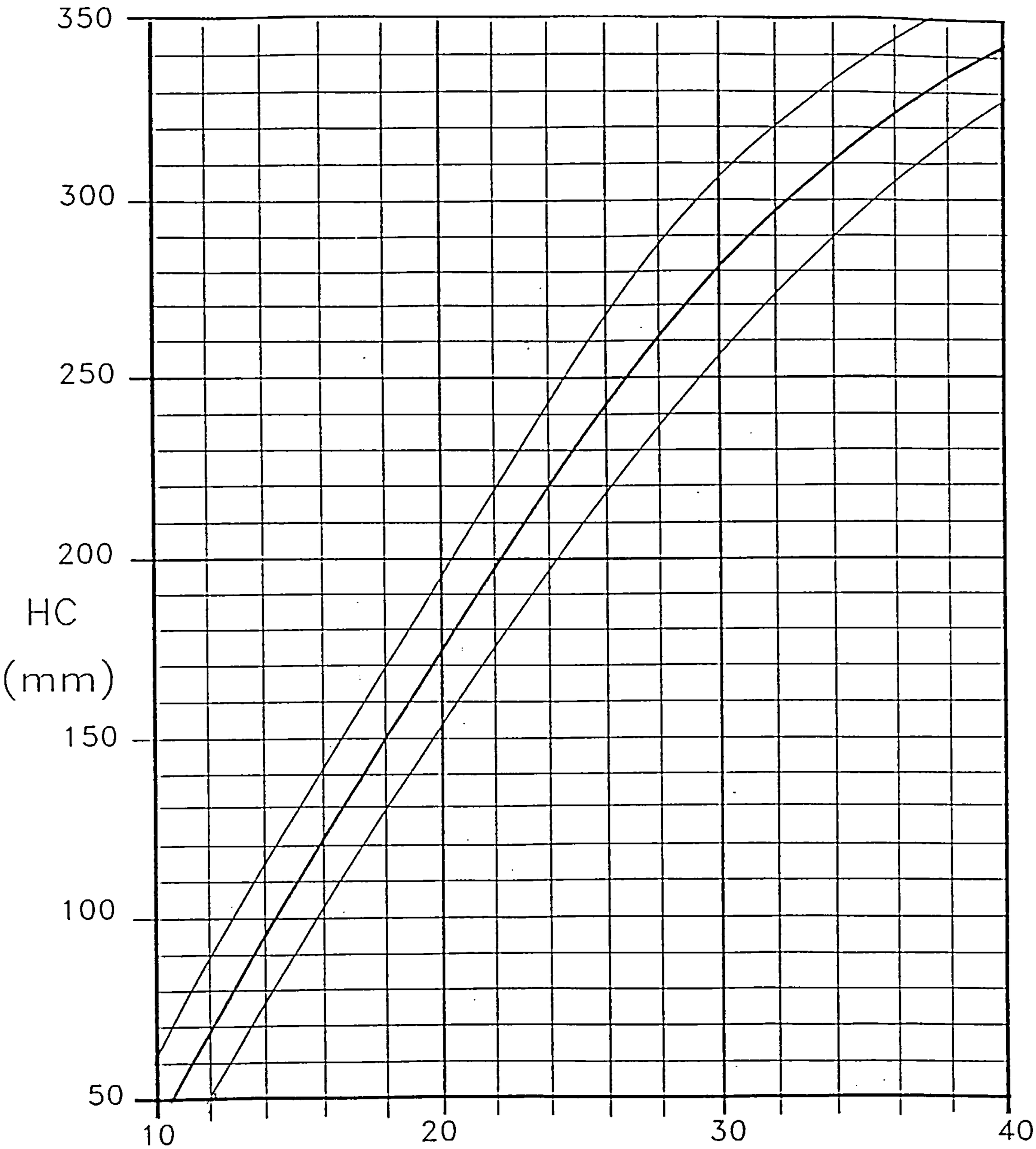
Femur length versus gestational age



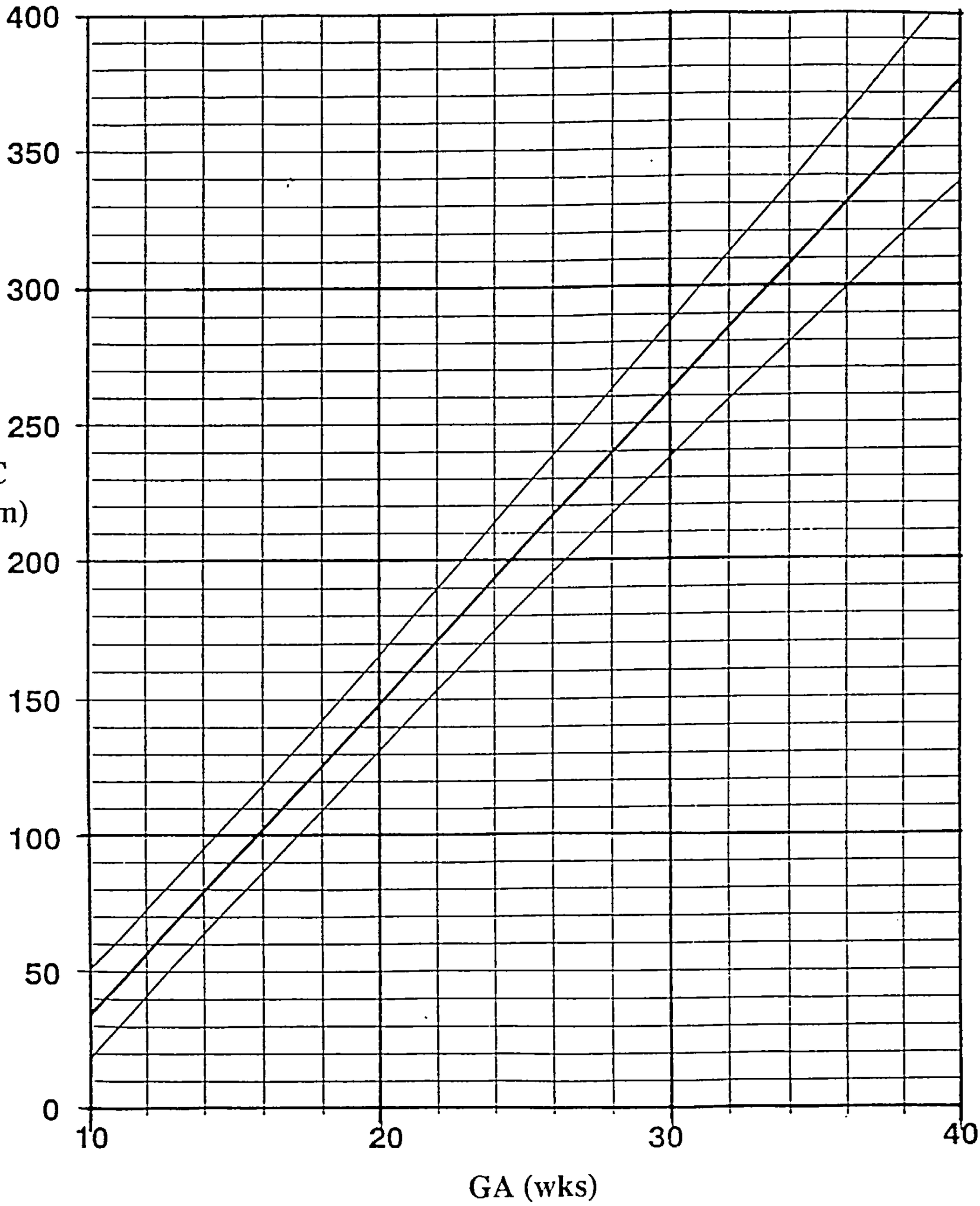
Biparietal diameter versus gestational age



Head circumference versus gestational age



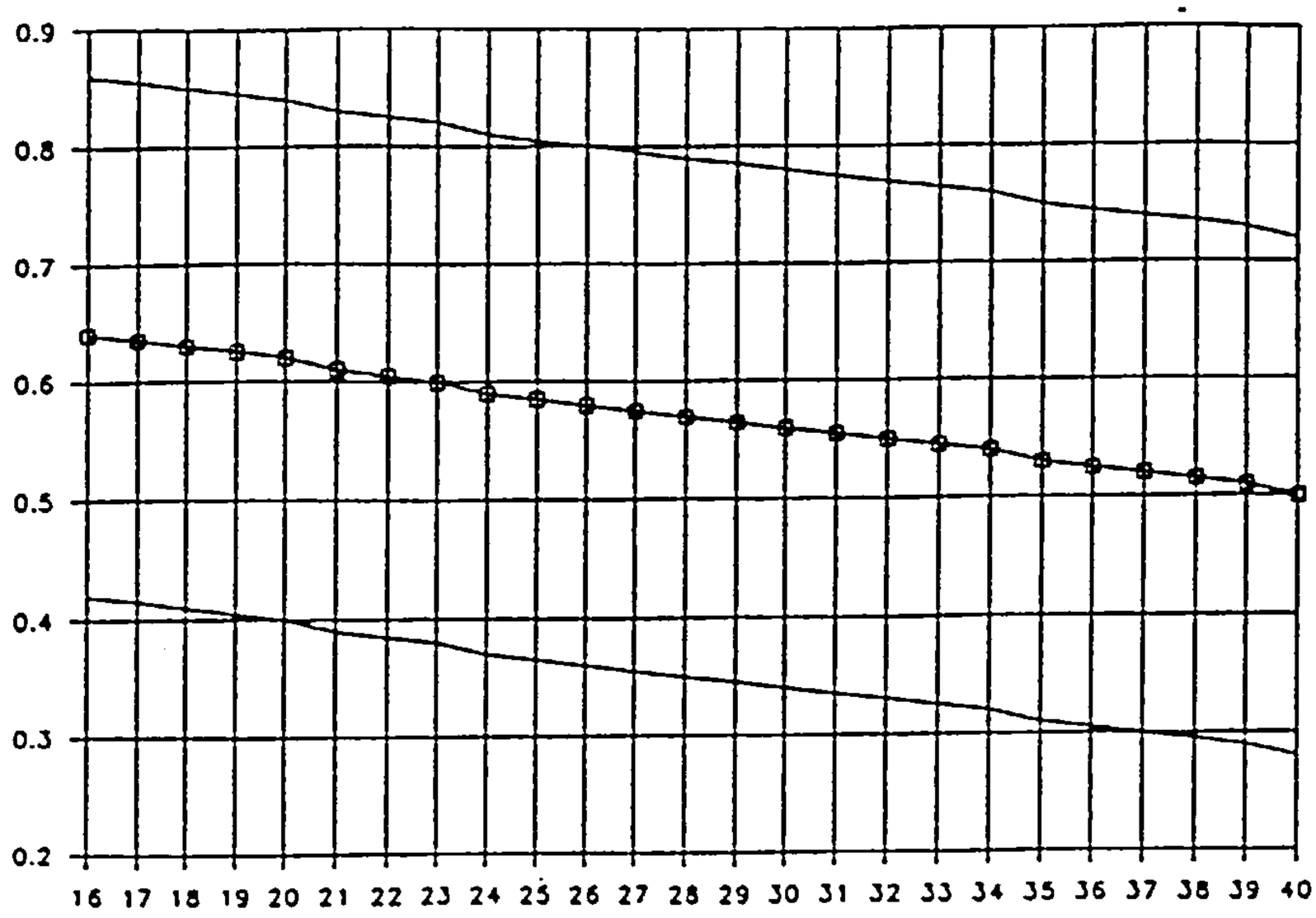
Abdominal circumference versus gestational age



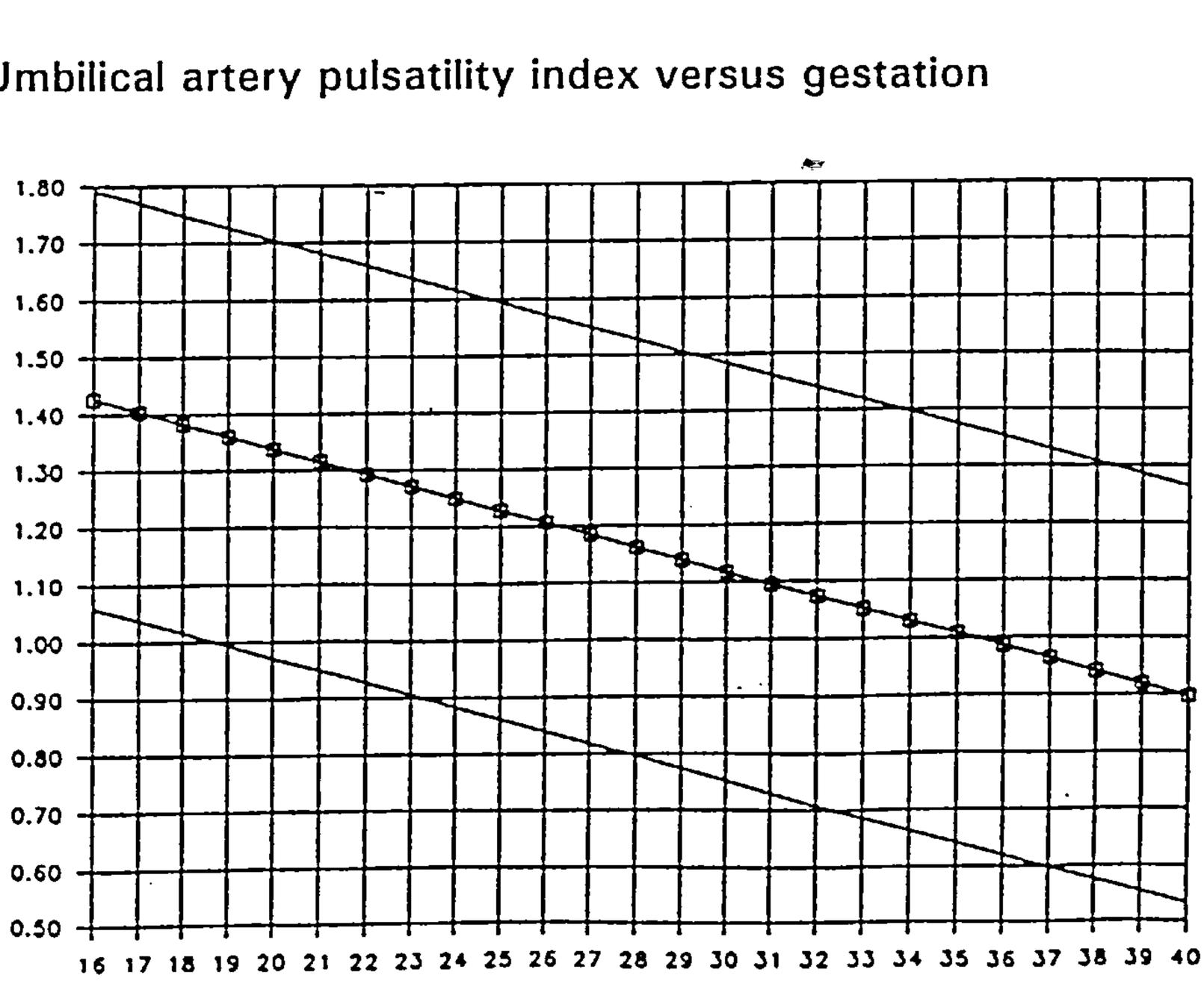
Appendix 3 - Normal values of Doppler indices

(Mean + 2 standard deviations)

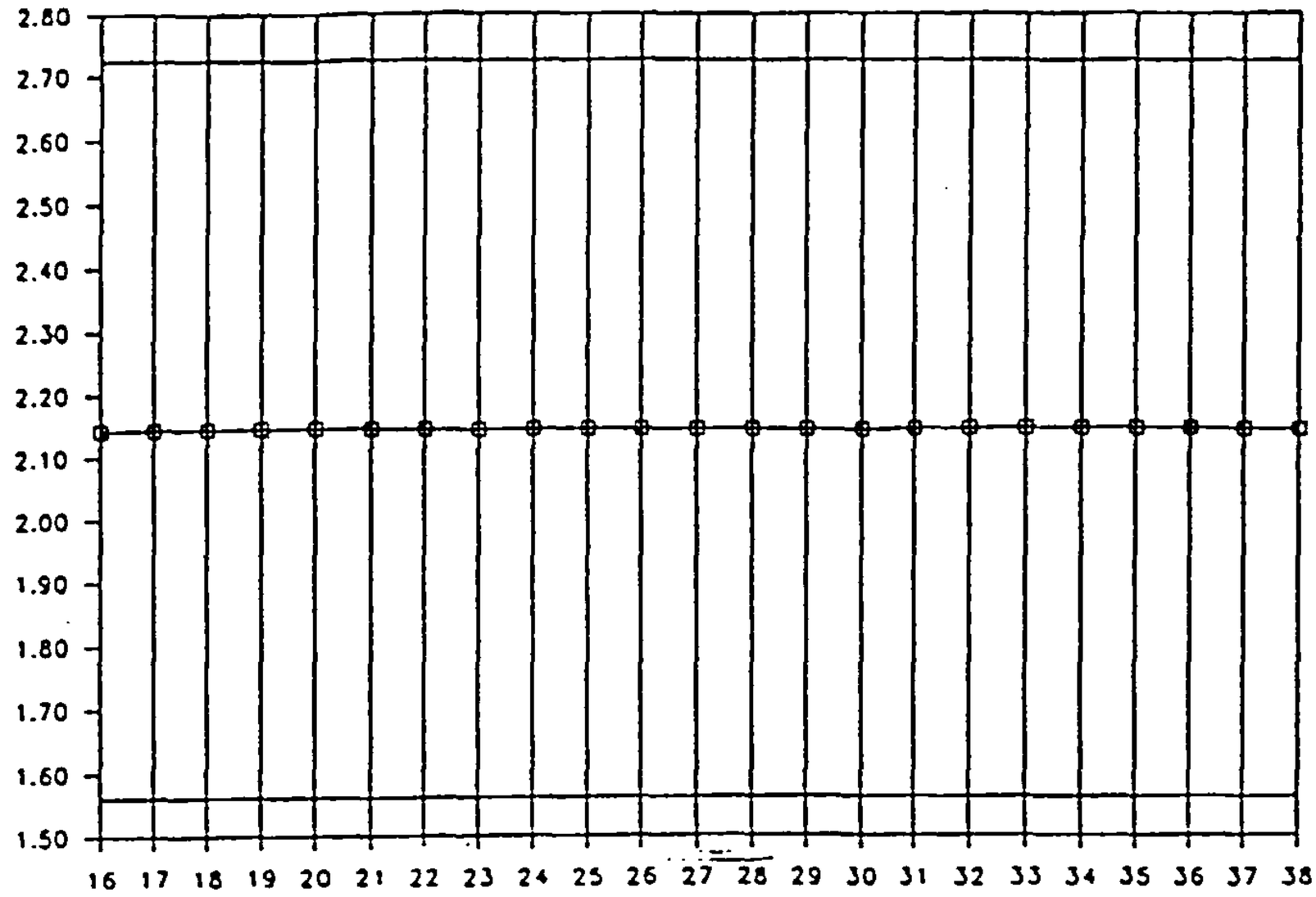
Uterine artery resistance index versus gestation



Umbilical artery pulsatility index versus gestation



Fetal descending thoracic aorta pulsatility index versus gestation



Fetal middle cerebral artery pulsatility index versus gestation