The Use of Inositol Phosphoglycans as a Diagnostic Tool in Pregnancy

Malcolm Paine

MD Thesis

Molecular Medicine Unit
Department of Immunology and
Molecular Pathology
University College London

March 2004

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Table of Contents

Abstract

Preeclampsia is a common and well-recognised complication of human pregnancy. It remains one of the main causes of maternal and fetal mortality and morbidity worldwide and no single cause has been identified, though there are many known risk factors. It is a multi-system disorder that affects the vascular endothelium and appears to originate from the placenta. No treatment exists, save the delivery of the fetus and placenta. There is no single diagnostic test for preeclampsia and it remains a clinical diagnosis only. A reliable diagnostic or predictive test could lead to new treatments and this would be of great clinical benefit as it would allow both more effective antenatal surveillance of high-risk pregnancies and also facilitate further research into the aetiology and treatment of preeclampsia.

Previously published work has indicated a potential link between preeclampsia and IPGs. The nature of Inositol Phosphoglycans (IPGs) and their involvement (actual and theorised) in several pathologies is described. Pilot clinical data is presented to evaluate the utility of an ELISA assay for IPG-P in the screening and diagnosis of preeclampsia. The assay demonstrates a correlation between IPG-P levels in maternal urine and amniotic fluid in normal women but not in preeclampsia. The levels in preeclamptic women suggest a correlation with clinical severity of the disease. A hypothesis is presented suggesting disruption of maternal-fetal equilibrium in the preeclamptic disease state.

Total IPG-P bioactivity assays of serum samples show no difference between preeclamptic and normal women, and it may therefore play no role in the condition. Alternatively, there is the possibility that a sub-fraction or an unidentified, abnormal IPG-P form is part of the process.

Additionally, the ELISA assay demonstrates increased urinary IPG-P levels in *normotensive* labouring women. A second hypothesis is presented to suggest potential links between the onset of normal labour and the pathophysiology of preeclampsia. Finally, proposals for further work are discussed.

List of Tables

Page

Chapter 1

Chapter 2

Chapter 3

Chapter 4

(No Tables)

Chapter 5

Chapter 6

Chapter 7

(No Tables)

Appendix 1

List of Figures

Page

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Appendix 1

List of Abbreviations Used

AF	Amniotic fluid
AFI	Amniotic fluid index
ACU	Assisted Conception Unit
AID	Artificial insemination by donor
ALT	Alanine aminotransferase
ANC	Antenatal clinic
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CFA	Complete Freund's adjuvant
cPIP	Prostaglandylinositol cyclic phosphate
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
GPI	Glycosyl phosphatidyl inositol
GPI-PLD	GPI phospholipase D
HDL	High-density lipoprotein
HRPO	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
ICAM	Intercellular adhesion molecule
IFA	Incomplete Freund's adjuvant
IGF	Insulin-like growth factor
IL	Interleukin
IPG	Inositol phosphoglycan
IPG-P	P-type inositol phosphoglycan
IUGR	Intra-uterine growth restriction
IVF	In vitro fertilisation
LDL	Low-density lipoprotein

LSCS	Lower segment caesarean section
MAP	Mean arterial (blood) pressure
MoM	Multiples of the median
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NI	Notch index
NO	Nitric oxide
PET	Pre-eclampsia
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCOS	Polycystic Ovarian Syndrome
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
PG	Prostaglandin
PI	Pulsatility index
PI-PLC	Phosphatidylinositol Specific Phospholipase C
PlGF	Placental growth factor
sFlt1	Soluble fms-like tyrosine kinase 1
STB	Syncytiotrophoblast
TEMED	Tetramethyl-ethylene diamine
TMB	Tetra methyl benzidine
TNF	Tumour necrosis factor
TPP	Thiamine pyrophosphate
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein

Chapter 1

Introduction

1.1 Inositol Phosphoglycans

The name 'Inositol Phosphoglycan' (IPG) refers to a diverse family of molecules implicated as second messengers related to the cell membrane (Rademacher et al. 1994; Gaulton and Pratt 1994). IPGs were first purified from bovine liver cell membranes in 1986 and identified to mimic some of the actions of insulin (Saltiel and Cuatrecasas 1986). They have been shown to be present in many tissues in mammals including muscle (Katz et al. 1996), adipose tissue, liver (Caro et al. 1997), placenta (Kunjara et al. 2000a) and T Lymphocytes (Gaulton et al. 1988). They are also found in other diverse groups of organisms from Trypanosomes (Misek and Saltiel 1992) to mycobacteria. IPGs may be involved in the pathophysiology of diabetes (Shashkin et al. 1997), preeclampsia (Kunjara et al. 2000a) and polycystic ovarian syndrome (PCOS) (Nestler 1998). Early evidence suggested the existence of a family of IPGs due to the multiple different effects seen (Huang et al. 1993). They were broadly divided into two groups, listed below with examples of differential functions:

1. **'P-Type'** – Activate Pyruvate Dehydrogenase (Lilley et al. 1992); activate glycogen synthase phosphatase (Jones and Varela-Nieto 1998)
2. **'A-Type'** – Inhibit cAMP-dependent Protein Kinase (Thompson et al. 1984; Rademacher et al. 1994); inhibit adenylate cyclase (Malchoff et al. 1987)

These groups are derived from the products of the extraction procedure by acid-elution at different pH from anion-exchange resin columns (Chapter 2, Laboratory Methods for a more detailed description of this process). The title IPG-'P' type is given to the fraction eluted from the column at pH 2.0 and the title IPG-'A' type given to the fraction eluted at pH 1.3. Recently Larner published the first structural information regarding the 'P-Type' IPG (IPG-P) (Larner et al. 2003) (Section 1.1.1).

1.1.1 IPG Structure

The structure of membrane glycosyl phosphatidyl inositol (GPI), from which IPG-A may be derived (Figure 1.2) is highly variable (Low and Saltiel 1988). Early work centred on function and relatively scant structural information was known (Gaulton

and Pratt 1994). Indeed, initial identification of a putative insulin mediator was as a small peptide (Cheng and Larner 1985). Mato and colleagues described the partial structure of an insulin-sensitive glycophospholipid, derived from H35 hepatoma cells and rat liver membranes, containing a phosphatidyl-chiro-inositol moiety, glycosidically linked to a non-N-acetylated glucosamine (Mato et al. 1987a; Mato et al. 1987b). The release of the active compound following treatment of the membranes with bacterial (*S aureus*) phosphatidylinositol specific phospholipase C (PI-PLC), implies the presence of inositol phosphate. Around the same time, Saltiel and colleagues identified active compounds also generated by PI-PLC hydrolysis (or insulin stimulation) of intact hepatic plasma membranes capable of altering the activity of cAMP phosphodiesterase (a cytosolic enzyme). Radio-labelling indicated equal quantities of inositol and glucosamine (Saltiel et al. 1986). Subsequently, evidence was published identifying mediators containing galactosamine and D-chiro-inositol (Larner et al. 1988) and myo-inositol in the A-type plus glucosamine (Rademacher et al. 1994)

Recently, Larner et al have published detailed structural information for the first time of IPG-P isolated from beef liver (Larner et al. 2003). The D-chiro-inositol-containing mediator, termed by this group INS-2, was determined to be a novel inositol glycan pseudo-disaccharide Mn^{2+} chelate (Figure 1.1). The D-chiro-inositol is present as pinitol.

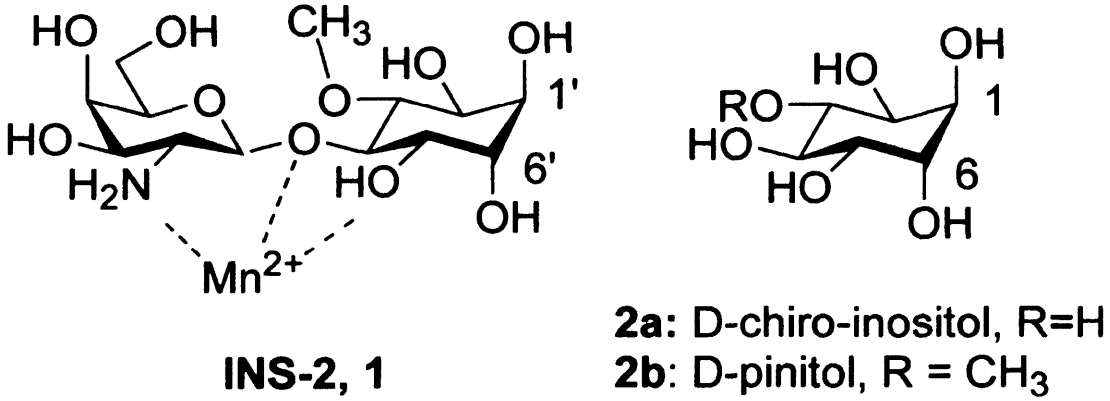


Figure 1. 1. Structures of putative insulin mediator **1** (INS2), D-chiro-inositol **2a**, and D-pinitol **2b**. (Reproduced from Larner et al. 2003)

This confirms the presence of Mn^{2+} in the P-type IPG. Only when this complexing was present did the chelate show PDH phosphatase activation (in vitro) in a dose dependent manner (Larner et al. 2003). Zinc may be complexed with the A-type (Rademacher et al. 1994).

Despite the initial lack of clarity and extensive variability, the use of structurally defined synthetic analogues of IPGs has confirmed that these molecules can affect the activities of key enzymes that are implicated in insulin action (Zapata et al. 1994; Varela-Nieto et al. 1996). There is also evidence that IPGs are related to, but not derived from the GPI protein anchors (Parpal et al. 1995).

1.1.2 Biological Origins

Insulin was first identified to cause the hydrolysis of a membrane glycolipid (later to be named as IPG) in 1986 (Saltiel et al. 1986). As mentioned above, early structural analysis indicated the presence of a phosphatidylinositol linked to glucosamine and an unidentified glycan moiety (Mato et al. 1987b). Insulin binding to the target cell receptor tyrosine kinase leads to cleavage of a GPI by the action of phosphatidylinositol-specific phospholipase C/D to release an IPG and phosphatidic acid or Diacylglycerol (Larner 1988; Suzuki et al. 1993). Subsequently, other growth factors have been identified to use this signalling pathway. Insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF) (Farese et al. 1988), erythropoietin (Devemy et al. 1994), and Transforming Growth Factor β1 (TGF-β1) (Vivien et al. 1994) have also been shown to regulate GPI hydrolysis. Interestingly, hormones involved in the female reproductive cycle and pregnancy are also known to utilise GPI signal transduction. Namely, follicle stimulating hormone (FSH) and human chorionic gonadotrophin (hCG) (Fanjul et al. 1993a) and also prolactin (Fanjul et al. 1993b). Thus IPGs are derived from membrane-associated Glycosyl phosphatidyl inositol (GPI) (Figure 1.2) and are cleaved from the cell membrane by GPI-specific Phospholipase D (GPI-PLD) (Rademacher et al. 1994). Heterogeneity of GPI hydrolysis may give rise to specific IPGs in specific cell environments (Jones and Varela-Nieto 1998). The IPG is released outside the cell (Caro et al. 1996) and

internalised (Anderson 1993; Rademacher et al. 1994) across the cell membrane. This may be closely associated with caveolae (Section 1.1.4).

The GPI is anchored in the cell membrane by the diglyceride tail attached to the phosphatidyl-inositol, which connects to the glucosamine and the glycan moiety.
A different family of GPI's also act as membrane anchors for many cell surface proteins such as the folate receptor (Rothberg et al. 1990; Smart et al. 1996), CD14 (Wang et al. 1995) and prion (Harmey et al. 1995).

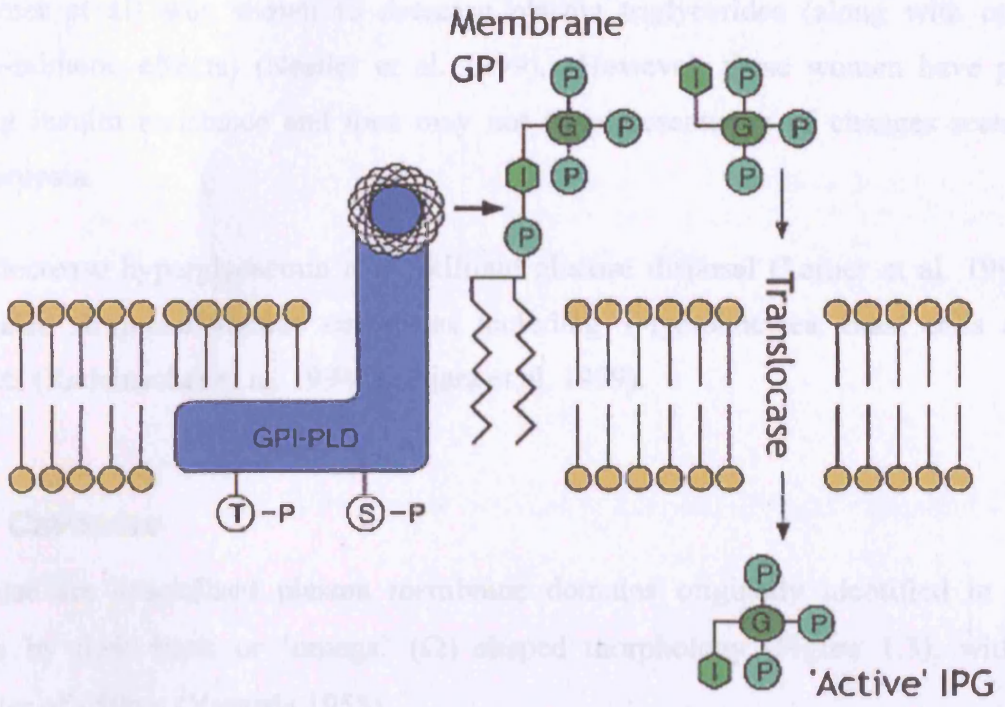


Figure 1. 2. Simple model of GPI cleavage and translocation into the cell.

GPI = Glycosyl phosphatidyl inositol. GPI-PLD = GPI-specific phospholipase D. I = Inositol, G = Glycan group, P = Phosphate, T = Threonine, S = Serine Activation of GPI-PLD is by G proteins. (Modified from Rademacher et al. 1994)

1.1.3 Actions of IPGs

In the main these are related to insulin signalling (Rademacher et al. 1994) including stimulation of lipogenesis, activation of acetyl-CoA carboxylase and inhibition of cAMP dependent protein kinase (Villalba et al. 1988; Kunjara et al. 1999). IPGs antagonized glucagon-dependent activation of glycogen phosphorylase in a dose-dependent manner, as well as the inactivating pyruvate kinase (Alvarez et al. 1987).

It is interesting to note at this point that one report has commented on the increased accumulation of triglycerides in endothelial cells exposed to preeclamptic women's sera in vitro (Lorentzen et al. 1991). Further work showed increased lipolytic activity and a higher ratio of free fatty acids to albumin in the sera of preeclamptic women (Endresen et al. 1992). There is no specific data regarding IPG-P and effects on triglycerides in preeclampsia. In a small study of obese women with polycystic ovary syndrome, oral administration of D-chiro-inositol (that shown to be in IPG-P by Larner et al) was shown to decrease plasma triglycerides (along with other insulin-mimetic effects) (Nestler et al. 1999). However, these women have pre-existing insulin resistance and thus may not be representative of changes seen in preeclampsia.

IPGs decrease hyperglycaemia and facilitate glucose disposal (Larner et al. 1998). They also stimulate various cell types including T-lymphocytes, mast cells and platelets (Rademacher et al. 1994; Kunjara et al. 1999).

1.1.4 Caveolae

Caveolae are specialised plasma membrane domains originally identified in the 1950's by their flask or 'omega' (Ω) shaped morphology (Figure 1.3), with a diameter of ~50nm (Yamada 1955).

Classically, invagination of the caveolae occurs to form intracellular vesicles and they are thus involved in endocytosis, exocytosis and transcellular movement of molecules across endothelial cells (Anderson 1998). Caveolae have been identified in many cell types and are now defined biochemically by a marker protein, caveolin (specifically Caveolin-1), and a specific membrane lipid composition, rich in glycosphingolipids (GSLs), and cholesterol (Brown and Rose 1992). They have a high prevalence of receptor and signal transduction molecules and other GPI-anchored proteins which are dynamically associated with the caveolae and appear to actually interact with the caveolin-1 (Anderson 1993; Anderson 1998). See also Table 1.1 for some molecules enriched in caveolae.

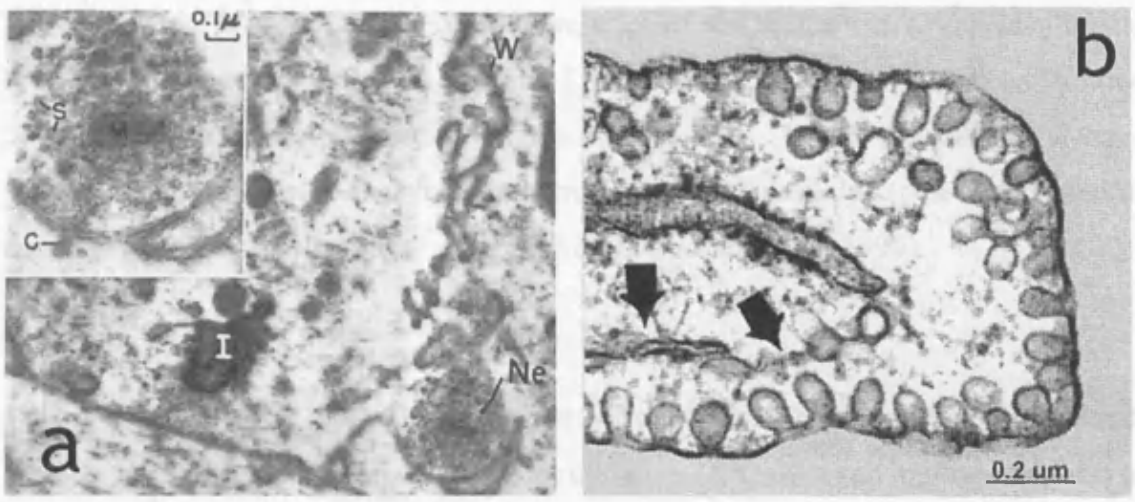


Figure 1. 3. The caveola cellularis.

(a) Mouse gall bladder epithelium. The inset, top left shows a mitochondrion, (M) a synaptic vesicle (s), and the caveola (c) (Reproduced from Yamada 1955). **(b)** Thin-section electron microscopy image of fibroblast caveolae. Arrows point to endoplasmic reticulum near invaginated caveolae (Reproduced from Anderson 1998).

Table 1. 1 Partial list of molecules enriched in caveolae (From Anderson 1998)

Lipid
Sphingomyelin
Diacylglycerol (DAG)
Cholesterol
Acylated Protein
eNOS (endothelial Nitric Oxide Synthase)
Caveolin
GPI-Anchored Protein
Folate Receptor
Alkaline Phosphatase
Prion
Membrane Receptor
Insulin
Endothelin
Membrane Transporter
Ca^{2+} ATPase
Second Messenger
Inositol Phosphoglycan (IPG)

It is interesting to note in particular the presence of eNOS, Ca^2+ ATPase, insulin and IPGs have been associated with caveolae (Anderson 1998). IPGs have been isolated in caveolae from insulin-stimulated cells (Parpal et al. 1995). Dorahy et al have presented some evidence that platelet membrane micro domains (possibly related to caveolae) may be involved in platelet activation (Dorahy et al. 1996).

'Potocytosis'

This is the process, found within caveolae, of concentration and movement of small molecules into the cell and endoplasmic reticulum (ER) or receiving/transmission of various kinds of cellular signals (Anderson et al. 1992). Anderson et al summarise how various molecules may be manoeuvred in this way, including Ca^{2+}, insulin and interleukin-2, in part by IPG intermediates formed from GPI-anchored molecules. They go on to suggest that this process can be controlled or compartmentalised by virtue of its location within the caveolae. This helps to solve the problem of potential loss of the IPG to the extracellular space after it is cleaved from the external side of the membrane. Examples cited of different pathways of potocytosis can be seen in figure 1.4.

In summary, caveolae are dynamic membrane domains in constant flux, active in cell transport and signalling processes in which are found several molecules of particular interest to the topic of preeclampsia. Additionally, caveolae may also be susceptible to oxidative stress, specifically oxidised cholesterol, containing receptors that bind HDL, LDL and oxidised lipoproteins (Babitt et al. 1997).

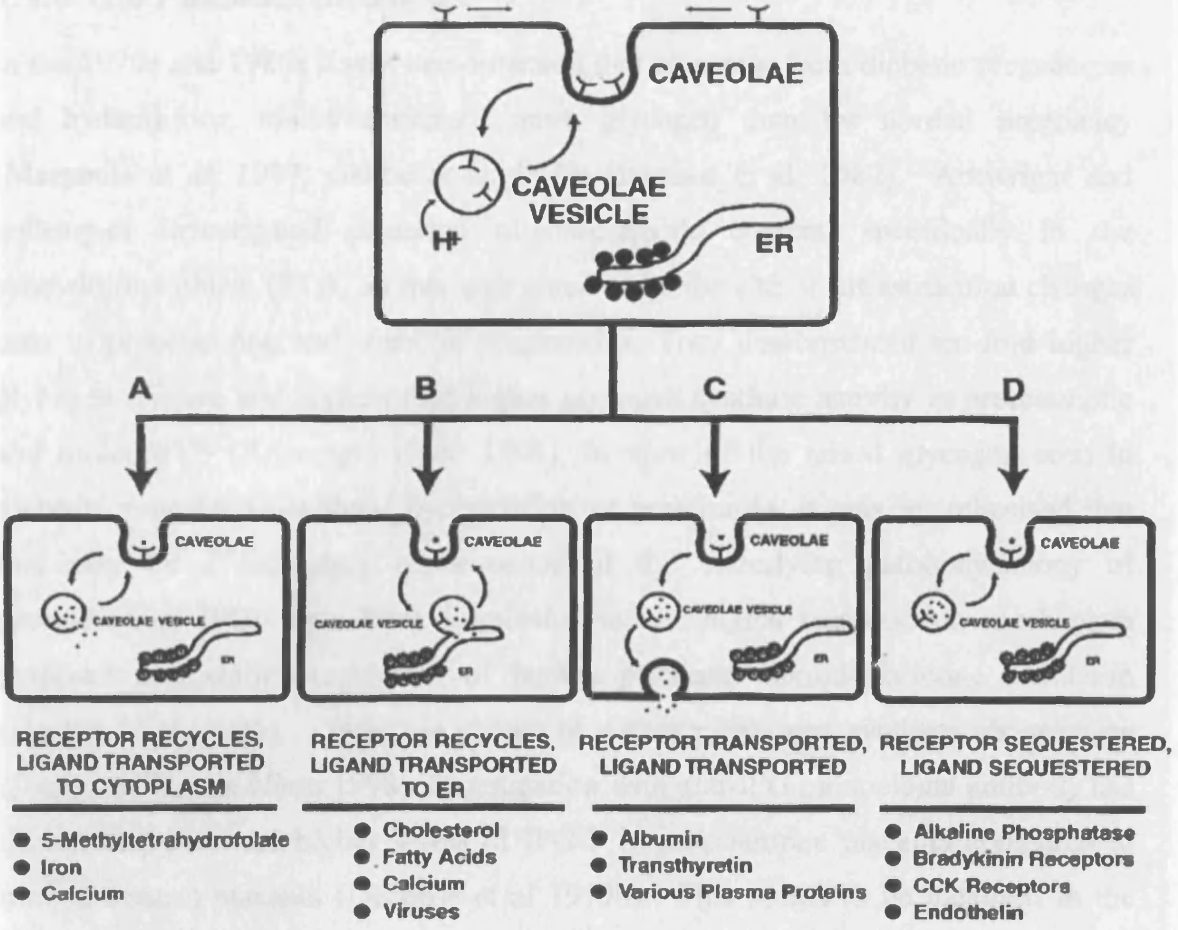


Figure 1. 4. Multiple pathways of potocytosis.

ER = endoplasmic reticulum. Molecules and receptors internalised by caveolae can have one of four fates. (A) The ligand is delivered to the cytoplasm while the receptor recycles back to the surface. (B) The ligand is delivered to the ER while the receptor recycles back to the surface. (C) The ligand is transported across the cell, and the receptor recycles, (D) Both the ligand and the receptor remain in a vesicular caveolae compartment. Examples of molecules that follow these routes are listed. (From Anderson 1998)

1.1.5 Diabetes

Much of the work on IPGs has focused on their role in type II diabetes. They were initially identified as insulin-mimetic factors (Saltiel and Cuatrecasas 1986) and have been identified as second messengers intimately involved in insulin metabolism (Rademacher et al. 1994). They are formed in response to insulin itself (Parpal et al. 1995). Recent work has revealed that levels of IPG-A and IPG-P are altered in relation to one another and correlated with body mass index (BMI) that this may be involved in the aetiology of diabetes itself (Kunjara et al. 1999).

1.1.6 The Placenta and IPGs

In the 1970s and 1980s it was demonstrated that placentae from diabetic pregnancies and hydatidiform moles contained more glycogen than for normal pregnancy (Maeyama et al. 1977; Gabbe et al. 1972; Diamant et al. 1982). Arkwright and colleagues investigated placental oligosaccharide content, specifically in the syncytiotrophoblast (STB) as this was noted to be the site of ultrastructural changes seen in preeclamptic and diabetic pregnancies. They demonstrated ten-fold higher glycogen content and sixteen-fold higher glycogen synthase activity in preeclamptic and molar STB (Arkwright et al. 1993). In view of the raised glycogen seen in diabetic pregnancies without hypertension or proteinuria, it was hypothesised that this may be a secondary consequence of the underlying pathophysiology of preeclampsia. IPGs have been implicated as the signal transduction mechanism involved in insulin's regulation of human placental steroid hormone formation (Nestler et al. 1991). IPGs are shown to activate glycogen synthase phosphatase (Jones and Varela-Nieto 1998). Investigation with anti-IPG monoclonal antibody has shown that there are higher levels of IPG-P in preeclamptic placenta compared to normal control placenta (Deborde et al 1999b). This seems to be localised in the microvillous brush border of the syncytiotrophoblast (Deborde et al 1999a). Thus there is evidence that in preeclampsia, placental glycogen metabolism is abnormal, and that both glycogen and IPG-P are abnormally raised in the syncytiotrophoblast. It will be discussed later how this may be relevant to the pathophysiology of the condition.

1.1.7 The Detection of IPGs in Urine

As is noted above, IPGs are found in the placental microvillous border (on the maternal side of the placenta) and are increased in preeclampsia. Likewise, placental fragmentation and shedding is increased in the preeclamptic condition (Knight et al. 1998). Potentially, therefore, large quantities of IPG-P are released into the maternal circulation. This would be abnormal as IPGs usually act at the autocrine (cell-to-cell) level. Increased urinary excretion of IPGs might therefore be expected as they are cleared from the maternal circulation. No data yet exists on maternal serum IPG levels in preeclampsia or normal pregnancy. However, data does exist on urinary IPG levels. Kunjara et al revealed that excretion of P-Type IPGs in the urine is

increased 2 to 3 fold in preeclampsia (Kunjara et al. 2000a). This paper used specific bioassay procedures to show IPG activity. The IPG-P bioassay involves the activation of pyruvate dehydrogenase (PDH) complex. The IPG-A bioassay involves the inhibition of Protein Kinase A. There is also evidence in this work of close correlation with traditional markers of disease severity in preeclampsia, namely proteinuria, plasma aspartate transaminase and platelet count (Kunjara et al. 2000a). Unpublished data shows that a P-type inositol phosphoglycan can also be identified in the urine of preeclamptic women using a polyclonal-based Enzyme Linked Immunosorbent Assay (ELISA)(Rodaris Pharmaceuticals, Oxford, England). It may be that this is an abnormal form of IPG-P with an extra lipid group on the inositol. Due to this consequent amphiphilic structure, the IPG forms micelles in the urine, which adhere to the solid phase of the ELISA assay (Also Appendix 1). Further unpublished data from a series of 38 matched pairs of preeclamptic and normal controls shows that this test seems to have no false positives and a 100% positive predictive value for preeclampsia. In a series of over 180 women that the test became positive up to 50-60 days before the clinical diagnosis and could thus be used to predict the onset of preeclampsia before symptoms and signs appeared (Rodaris Pharmaceuticals; unpublished observations).

Validation of the Polyclonal-Based ELISA and IPG extraction/bioassay procedure

This will be discussed fully in Chapter 2 – Laboratory Methods

1.1.8 The IPG Polyclonal-based ELISA vs. the 'Bioassay'

IPG Extraction and Bioassay

The IPG extraction process used in this work is based on heat denaturation of protein, charcoal adsorption of nucleotides and anion exchange resin chromatography, yielding both 'P-type' (pH2.0 eluate) and 'A-type' (pH1.3 eluate) fractions of IPG separately and was first described by Larner and colleagues in 1988 (Larner et al. 1988), from the method of Cheng et al (Cheng et al 1984). The bioassay then relies on activation of the Pyruvate Dehydrogenase (PDH) complex for the 'P-type' fraction and inhibition of Protein Kinase A (PKA) for the 'A-type'

fraction. For IPG extraction from serum (from diabetic subjects), Shashkin et al have shown no crossover activity of these two fractions (Shashkin et al. 1997). Kunjara and colleagues showed a 2-fold increase in bioactivity of P-IPG in urine of pregnant women compared to non-pregnant controls and a further 3-fold increase in preeclamptic urine (Kunjara et al. 2000a). For extraction from urine, larger volumes are required (at least 50ml) than for serum (1ml). For this reason, I decided it was not feasible to perform bioextraction for urine samples in this study. As second and third trimester amniotic fluid is composed largely of fetal urine, it was treated in the same way as urine samples accordingly. No data have yet been published on serum IPG levels in preeclampsia using this same bioassay.

Polyclonal-based ELISA for IPG-P

The Polyclonal serum used for the ELISA was developed by Rodaris Pharmaceuticals Ltd, Oxford, UK using 150ml of urine from a severely ill preeclamptic woman. Unfortunately, it has not been possible to obtain clinical data of this index patient. The then best purification procedure was used for the extraction, but the antigen has never been fully characterised. It is indicated from Rodaris' data that the ELISA assay shows strong positivity when used on urine of preeclamptic women, with up to a fifty-fold difference in signal (Section 2.1.1). Thus there is an order of magnitude difference between the ELISA and the bioassay in terms of how they distinguish between the normal and preeclamptic states. It is possible that this may be because the ELISA relies partly on the physical properties of the IPG-P (Appendix 1) and the bioassay on the biological activity. Equally, there is no reason why PDH-complex bioactive material (IPG-P) and the IPG measured in the diagnostic have to be one and the same. Although the ELISA is to be assessed as a clinical tool questions remain regarding its preparation, which will need to be answered in future, work.

Unpublished data from Rodaris Pharmaceuticals show no difference between preeclamptic and normal sera when assayed with the ELISA. Thus for the purposes of this research, the polyclonal-based ELISA assay was used for urine samples and the bioassay for the serum samples. See Chapter 2 – Laboratory Methods for further details of the assay protocols.

1.2 Preeclampsia

1.2.1 History and Epidemiology

De Sauvages first differentiated eclampsia from epilepsy in 1793 although preeclampsia had previously been recognised by others such as the ancient Greeks and Galen (Chesley 1984). It is a common and well-recognised complication of human pregnancy (Roberts and Redman 1993), occurring in 3-10% of all pregnancies (NHBPEP 1990; Williams and de Swiet 1997). It remains one of the main causes of maternal and fetal mortality and morbidity in Great Britain (Department of Health. 2001), the USA (Kaunitz et al. 1985), Scandinavia (Augensen and Bergsjo 1984) and the Developing World (Duley 1992) despite continued and widespread research. Direct maternal deaths due to hypertensive disorders of pregnancy in the United Kingdom have decreased to 15 over the last triennium (Department of Health. 2001), a rate of 7.1 per million maternities. Direct maternal deaths are those resulting from conditions or complications or their management that are unique to pregnancy, and preeclampsia remains the second leading cause of deaths in this category, behind thromboembolic disease. Worldwide, 10% of the total maternal deaths are due to preeclampsia/eclampsia (Duley 1992) - 50,000 women each year.

Table 1. 2 Some Risk Factors for Preeclampsia

Risk	Reference
Primiparity/primipaternity	(Discussed below)
Multiple pregnancy	(Long and Oats 1987)
Obesity	(Sibai et al. 1995)
Pre-existing Vascular pathology - Type I Diabetes Hypertension	(Siddiqi et al. 1991)
Family history	(Chesley et al. 1968)
Non-smoker	(Klonoff-Cohen et al. 1993)
Molar Pregnancy	(Discussed in Chesley 1984)
Extremes of age	
Barrier contraception	(Klonoff-Cohen et al. 1989)
Donor Gametes	(Serhal and Craft 1989; Abdalla et al. 1998)
High Altitude	(Palmer et al. 1999)

The precise aetiology of preeclampsia remains obscure. It is unique in that it only occurs in human gestation and only in pregnancy (Williams and de Swiet 1997). It is

a multi-system disorder (Roberts and Redman 1993) that affects vascular endothelium membranes and appears to originate from the placenta. There are many risk factors such as nulliparity and those with multiple pregnancy (Table 1.2). Many theories exist as to the cause of preeclampsia, which will be discussed in more detail below. Because the pathophysiology remains poorly understood, no diagnostic test or treatments exist (Higgins and Brennecke 1998) . Great potential clinical benefit seems likely if a diagnostic test and, subsequently, a treatment can be provided.

1.2.2 Aetiology and Pathophysiology

As stated above, the aetiology and pathophysiology of preeclampsia is complex and multifactorial and it has been dubbed 'The Disease of Theories' (Chesley 1984). Despite this however, it is generally agreed that the signs of preeclampsia are thought to arise from maternal endothelial dysfunction caused by circulating factors of placental origin (Kertesz et al. 1999).

1.2.3 The Normal Placenta

Before understanding the pathology behind preeclampsia, it is necessary first to describe the process of normal placentation. The placenta is the fetal lifeline, the source of oxygen and nutrients and waste removal. It develops from embryonic trophoblastic cells, which invade the decidual lining of the uterus in the first few weeks of the pregnancy. The extra-villous trophoblast migrates along the uterine spiral arterioles, breaking down the muscularis.

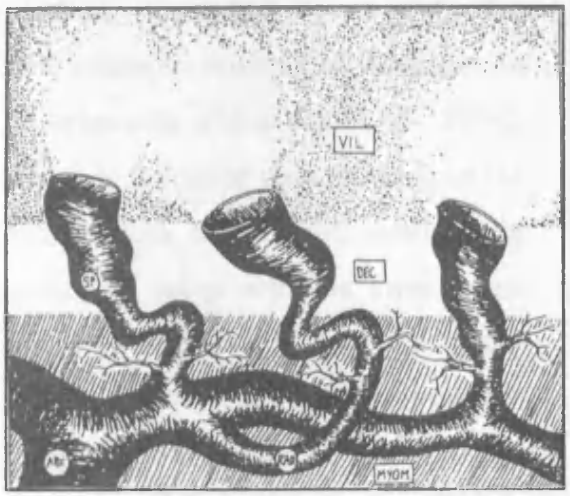


Figure 1. 5. Diagram of normal placenta illustrating dilated spiral arteries. (Reproduced from van Beek and Peeters 1998) Copyright © 1998 Williams and Wilkins.

Consequently, these vessels lose their narrow architecture and become the wide-bored, low-resistance vessels that allow adequate maternal-fetal exchange. This

process is completed by the middle of the second trimester of the pregnancy. Normal placentation involves trophoblast migration along firstly the decidual and secondly the myometrial portions of the spiral arteries. It is usually said that it is this second phase of placental invasion that fails or is inadequate in preeclampsia (see below and Figures 1.6 and 1.7). Classically this is thought to occur at 14-16 weeks' gestation (Clark 1994).

1.2.4 The Placenta and Preeclampsia

Evidence that the placenta is key in the aetiology of preeclampsia is compelling. It has been known for some time that placental histology is grossly abnormal in preeclamptic pregnancies. Robertson, Brosens and Dixon described the abnormal placental histology seen in preeclampsia three decades ago (Brosens et al. 1972; Robertson et al. 1975). The endovascular trophoblastic invasion is restricted to the decidual portion of the spiral arteries (rather than continuing into the myometrial portion) and consequent 'acute atherosis' is likely to develop in the myometrial portion. Meekins and colleagues examined a series of placental bed biopsies (21 normal, 24 preeclamptic taken at caesarean section, identifying defective endovascular trophoblast invasion in preeclampsia (Meekins et al. 1994). There is failure of the migration of the extra-villous trophoblast, resulting in persisting spiral arteriole architecture and consequent inadequate blood supply and fetal compromise. Preeclampsia occurs in hydatidiform molar pregnancies when only 'placental' (trophoblast) tissue is present (Berkowitz and Goldstein 1996).

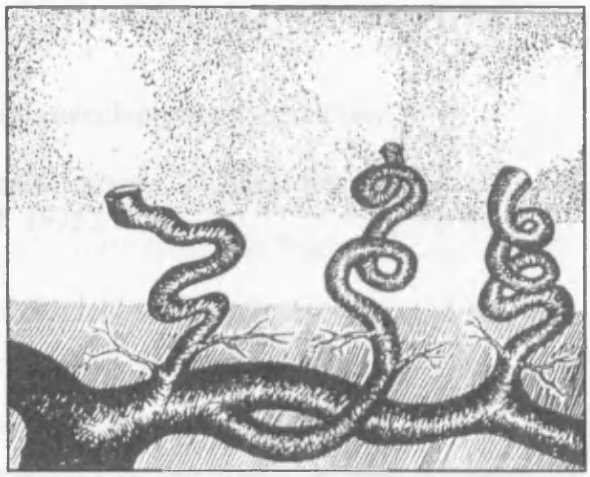


Figure 1. 6. Diagram of preeclamptic placenta illustrating narrow spiral arteries leading to the high-resistance flow seen in the disorder (Reproduced from van Beek and Peeters 1998). Copyright © 1998 Williams and Wilkins.

Interestingly, 90% of complete moles contain entirely paternal DNA (Kajii and Ohama 1977). Also, preeclampsia improves when and only when the placenta is removed at delivery.

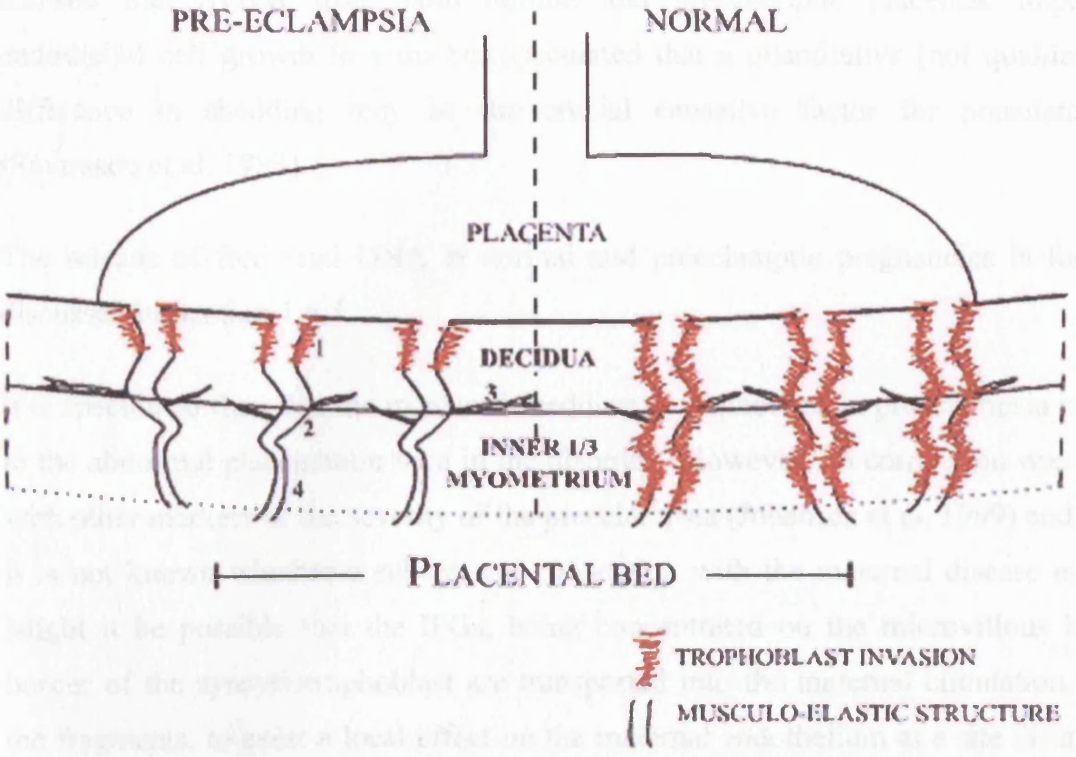


Figure 1. 7. Diagram comparing normal and preeclamptic placentation.

1 – Spiral artery. 2 – Myometrial spiral artery. 3- Basal arteriole, 4 – Radial artery (Adapted from Robertson et al. 1975).

It has been demonstrated with both morphological studies (Jaameri et al. 1965) and monoclonal antibody (JMB2) labelling (Chua et al. 1991) that shedding of placental (trophoblast) tissue into the maternal circulation occurs in normal and preeclamptic pregnancy. Trophoblast cells were first found in the lungs at post mortems of women who had died following eclampsia (Schmorl 1893) and more recently following preeclampsia (Attwood and Park 1961). It is probable that most of these fragments do not reach the maternal arterial circulation due to their size and consequent entrapment in the pulmonary circulation. It has been confirmed that the quantity of trophoblast fragment shedding is increased in preeclamptic pregnancy compared to normal controls though it is not related to disease severity (Chua et al. 1991; Knight et al. 1998; Johansen et al. 1999). Additionally, it has been shown that

these membrane fragments impair maternal vascular endothelial function in vitro (Cockell et al. 1997). This group used isolated maternal resistance arteries perfused with syncytiotrophoblast microvillous (STBM) membrane vesicles to show consequently reduced vasodilatation to acetylcholine. Smarason and colleagues also showed that STBM from both normal and preeclamptic placentae impaired endothelial cell growth in vitro but speculated that a quantitative (not qualitative) difference in shedding may be the crucial causative factor for preeclampsia (Smarason et al. 1993).

The release of free fetal DNA in normal and preeclamptic pregnancies is further discussed in Section 1.4.5.

It is speculated then that the increased shedding of trophoblast in preeclampsia is due to the abnormal placentation seen in the disorder. However, no correlation was seen with other markers of the severity of the preeclampsia (Johansen et al. 1999) and thus it is not known whether a subsequent connection with the maternal disease exists. Might it be possible that the IPGs, being concentrated on the microvillous brush border of the syncytiotrophoblast are transported into the maternal circulation with the fragments, to exert a local effect on the maternal endothelium at a site distant to the uterus?

1.2.5 Preeclampsia, Diabetes and Insulin Metabolism

Type I Diabetes is an independent risk factor for preeclampsia (Siddiqi et al. 1991) and an increased incidence of preeclampsia is seen with poor control of diabetes in early pregnancy (Hanson and Persson 1998). It has been mentioned previously that preeclamptic placenta, like placentae from diabetic pregnancies shows increased deposition of glycogen. (Arkwright et al. 1993)

Plasma glucose levels are not raised in preeclamptic pregnancies as compared to normal pregnancy. However, there is evidence that there may be insulin resistance (Kaaja et al. 1999) or frank hyperinsulinaemia in these women (Bauman et al. 1988; Martinez et al. 1996). Bauman's study did not control for maternal body mass index. The latter investigation was a well-controlled study with properly matched, but small series (10 each) of normal and preeclamptic women (Martinez et al. 1996).

Hyperinsulinaemia was demonstrated in both the fasting and post-glucose load states. The sympathomimetic effect of insulin may then contribute to the rise in blood pressure. Obesity is a common cause of insulin resistance and is an independent risk factor for preeclampsia (Sibai et al. 1995). It is also possible that diabetes is a risk factor due to the pre-existing vascular disease. I have also mentioned that glycogen deposits are greater in both the diabetic and preeclamptic placenta, although different mechanisms have been proposed (Arkwright et al. 1993).

1.2.6 Amniotic Fluid (AF) and Preeclampsia

AF at the most basic level is composed largely of fetal urine from 16-18 weeks of pregnancy onwards (Cunningham et al 1997). It is intimately involved with placental function, being the site of exchange for fluid, nutrients and metabolites between mother and fetus. It is well known that a reduction in placental function can lead to a reduction in the AF volume, presumably due to reduced fetal renal perfusion and therefore fetal renal output. This is usually measured as the 'Amniotic Fluid Index' (AFI), a function of the deepest pools of liquor measured on ultrasound scan.

Table 1. 3 Amniotic Fluid Factor Changes In Preeclampsia

Factor	Change	Sample Size (PE)	Reference
Prostacyclin	Decreased	27	**(Ylikorkala et al. 1981)***
Thromboxane	No Change	27	**(Ylikorkala et al. 1981)***
6-Keto-PGF1$_\alpha$, TXB_2	No Change	19	**(Moodley et al. 1984)**
Fetal fibronectin	Increased	20	**(Kupferminc et al. 1995)**
Soluble VEGF Receptor-1**	Increased	33	**(Vuorela et al. 2000)**
Interleukin-6	Increased,	15	**(Nakabayashi et al. 1998)**
	Decreased or No Change	9	**(Silver et al. 1993)** **(Heikkinen et al. 2001)**
Oxytocinase Activity	Decreased	80	**(Roy et al. 1993)**
cGMP	Decreased	20	**(Rizzo et al. 1996)**

PE = Preeclampsia, PG = Prostaglandin, TX = Thromboxane, VEGF = Vascular Endothelial Growth Factor, cGMP = Cyclic Guanosine Monophosphate
*No definition of 'preeclampsia' given; ** sFlt1

It must be remembered that increased levels of a marker could be due to a decreased AFI and the liquor being correspondingly concentrated. Some authors have

expressed results of AF per micromol of creatinine (For example Di Iorio et al. 1998).

Amniocentesis is commonly used to aspirate fluid for the analysis of fetal cells (for karyotyping), bilirubin measurement (management of fetal haemolysis) and other reasons. Being relatively easy to acquire therefore it has been an obvious target for research into preeclampsia and its pathophysiology.

Bjorkhem et al found amniotic total cortisol levels to increase with gestation (most significantly after 40 weeks) and, independently, in preeclampsia (Bjorkhem et al. 1978). The free cortisol changed much less so. Historically it was suggested that cortisol might have a role to play in the onset of labour, though this now thought to be only true in some mammals other than humans. Other groups have also looked at other markers (Table 1.3).

Of course, the presence of a substance in amniotic fluid may be the result of one or a combination of several pathways. For example, guanylate cyclase activity is present in the placenta and membranes (Rizzo et al. 1996) and any change in the AF levels may be a result of changes in either or both of these locations. Any substance that is physiologically present in the maternal system may potentially be transported across the placenta. Alternatively fetal production may be the main source. The difficulty with preeclampsia remains the lack of reliable and comparable animal models, as novel metabolic pathways may be present in the disease state.

1.2.7 Preeclampsia and Other Hormonal Links

A small study with age and gestation-matched women, using commercial RIA and ELISA showed raised testosterone (but not other androgens) in preeclampsia (Acromite et al. 1999) – The authors suggested a link to inhibin as this increases androgen production from ovarian theca cells in non-pregnant subjects with polycystic ovarian syndrome (Pigny et al. 1997), in which abnormal insulin metabolism is also found (Hopkinson et al. 1998). Inhibin, human chorionic gonadotrophin and alpha feto-protein are also raised in preeclampsia and this is discussed in Chapter 1.4.4.

1.2.8 Aetiological Theories

Once it is accepted that the placenta is central to the problem, it is helpful to define several separate, though partially overlapping areas, which need to be considered when discussing the aetiology of preeclampsia.

Immunological – From Primipaternity to HLA-G

Normal pregnancy is an immunologically intriguing event. It is known to be a stimulation of the immune system towards the tolerance pathway, rather than immune-suppression. It has been suggested that preeclampsia is a manifestation of a malfunction in this process.

It has long been recognised that women in their first pregnancy are at greater risk of preeclampsia compared to multiparous women, Mauriceau commenting in 1694 that primigravidae were more likely to develop convulsions than multiparae (Chesley 1984). It has been postulated that this is due to the maternal immunological sensitisation to paternal DNA either pre-conception (for example in the form of sperm antigens) or as the paternal component of fetal DNA in the trophoblast. Klonoff-Cohen et al noted the apparent increased risk of preeclampsia in women who used barrier contraception, resulting in fewer episodes of sperm exposure (Klonoff-Cohen et al. 1989). A dose-response gradient was observed. More recently, a Dutch group showed a reduced risk of preeclampsia related to oral sex and swallowing of sperm (Koelman et al. 2000). These effects may both potentially be secondary to induction of allogenic tolerance to paternal HLA.

Alternatively the actual duration of sexual cohabitation may affect the preeclampsia risk (Robillard and Hulsey 1996). A controversial explanation has been offered by the same authors in reference to genomic imprinting of the sperm (Robillard et al. 1999). Specifically, the higher incidence of preeclampsia in short-term relationships may be because "*...males would imprint their sperm more aggressively when they do not expect to have another offspring with that female (or if a mother already has existing children by another father).*"

Robillard has furthermore expounded a new theory of 'Primipaternity' as being more relevant (Robillard et al. 1998; Robillard et al. 1999). The theory focuses on the risk for preeclampsia being associated not with the mother's first pregnancy but with the first pregnancy with any given partner. Namely, multigravidae share the risk with primigravidae in case of a subsequent conception with a new partner (Robillard et al. 1998).

More recently, Skjærven et al. using retrospective data for a large cohort of women (over 750 000) from the Medical Birth Registration of Norway concluded that the protective effect of previous pregnancy against preeclampsia is transient (Skjaerven et al. 2002). Their data indicated that the risk of preeclampsia following a pregnancy increased with time, returning to that of a primigravida after 10 years. After adjustment for inter-birth interval, a change of partner was not associated with an increased risk of preeclampsia. This would seem to contradict the primipaternity theory. The group offers no explanation for this in immunological terms. Presumably, a mechanism might be similar to that of a primary inoculation with Hepatitis antigen - requiring a booster after 6 months to maintain a persistent immune response. A major problem with this study however is that retrospective use of birth records for paternity data is confounded by a potential 30% false paternity rate. One must therefore call the validity of the data into question. It was recognised by the authors that the data might also be confounded by the lack of data on non-smoking status and obesity (both associated with preeclampsia).

Logically, if immune tolerance plays a part in the causation of preeclampsia, the presence of immunologically foreign material should increase the incidence of the disorder. Fertility treatment involving donated gametes is such a situation. A large retrospective analysis of 232 ovum donation pregnancies, (leading to 140 deliveries) has shown increased incidence of non-proteinuric hypertension (11%) and preeclampsia (11%) (Abdalla et al. 1998). A smaller series from Italian ovum donation program suggests that there is no increased risk of preeclampsia, quoting 13% (6 of 44 clinical pregnancies) (Antinori et al. 1995). However, there is no definition given of what is meant by 'gestational hypertension', a misleading terminology. Secondly, the rate of this hypertension should be expressed as a percentage of the live-birth rate, namely 6 of 34 deliveries – 18%. This is

significantly higher than in spontaneous gestations. Similar preeclampsia rates were found in a more recent retrospective study of 72 women with donated gametes (Salha et al. 1999) though much higher rates (38%) have been reported in oocyte donation cases (Serhal and Craft 1989). A further paper by Makhseed and colleagues also shows increased rates of 'PIH' in multiple pregnancies following IVF and ICSI treatment (Makhseed et al. 1998). However, this paper is also flawed in that neither does it define the term 'pregnancy induced hypertension' nor was it primarily designed to look at this as an outcome measure. A history of infertility or in vitro fertilisation alone do not increase the risk of preeclampsia (Ros et al. 1998; Skupski et al. 1996).

It is all very well to have epidemiological evidence, but is this backed up by evidence of an immunological process?

Human Lymphocyte Antigen-G (HLA-G) is a non-classical class I gene of the major histocompatibility complex (MHC) with restricted tissue distribution, including expression on trophoblast in maternal spiral arteries (Geraghty et al. 1987). Involvement of this gene would be consistent with the fetal-paternal DNA component described by Lie et al below. It is possible that reduced or absent HLA-G expression exists in preeclamptic cases (Goldman-Wohl et al. 2000) and differences in HLA-G genotype between control and preeclamptic samples are an important component in the susceptibility to or development of preeclampsia (O'Brien et al. 2000).

Genetic Component

There is a strong familial association with a history of preeclampsia in first-degree relatives with a four- to eight-fold increased risk for women with a previously affected sister or mother (Williams and de Swiet 1997). Various models of inheritance have been proposed based on population data, including a single recessive or dominant maternal gene (Arngrimsson et al. 1990) or homozygosity for a single recessive gene shared by mother and fetus (Liston and Kilpatrick 1991). However the precise nature of the genetic link is not known. One study using retrospective data and patient interviews shows a familial predisposition in daughters of eclamptics/preeclamptics (Arngrimsson et al. 1990).

Table 1. 4. Single gene candidate studies

Blood Pressure	
eNOS	No link [1]
T_{235} Angiotensinogen Variant	Possible link (variable between different populations)[2,3]
Thrombogenesis	
Factor V Leiden	Many mixed results
Prothrombin	
MTHFR	
Placentation	
Adhesion molecules	
TNFα	No proven link[4,5]

[1](Lade et al. 1999)[2] (Ward et al. 1993)[3](Morgan et al. 1999)[4](Lachmeijer et al. 2001)[5](Livingston et al. 2001)

Retrospective analysis of birth records seems to reveal a low male/female sex ratio in the daughters compared to the daughters-in law of preeclampsia/eclampsia sufferers (Arngrimsson et al. 1993). Various candidate gene studies have been carried out looking at vascular and placental factors, some of which are summarised in Table 1.4.

There is also a single gene for GPI-PLD (which is involved in the production of IPG) in humans on chromosome 6p22 (Schofield and Rademacher 2000). Although no data exists as to prevalence of different alleles in preeclamptic and normal placentae, linkage studies have not demonstrated a connection with chromosome 6p22. Opinion is moving away from a single gene theory, with most inclined to believe in the involvement of a fetal paternal genetic component (i.e. separate to that from maternal DNA). For example, a woman's risk of preeclampsia a given pregnancy is increased if her partner has already fathered a preeclamptic pregnancy with another woman (Lie et al. 1998). The most likely situation may be one of a genetic predisposition/susceptibility to the disease process itself.

Placental

As already mentioned, the placenta is inextricably involved in the aetiology of preeclampsia. Poor utero-placental perfusion is usually found in preeclampsia and this is accompanied by changes in Doppler waveforms on ultrasound scan (Bower et al. 1993b) showing high resistance. Abnormal placentation leads to placental

ischaemia (Williams and de Swiet 1997) but it is not clear what the origins of the initial abnormality are.

Placental growth factor (PlGF), is an angiogenic factor whose levels are down-regulated by lowered oxygen tension (Ahmed et al. 2000). It is a marker of placental function and is decreased in the serum of preeclamptic women from the second trimester (Torry et al. 1998; Levine et al. 2004b) This can even be shown in high-risk women before the onset of clinical disease (Chappell et al. 2002).
Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), a placental growth-enhancing agent was reduced in the serum of preeclamptic subjects (Gratacos et al. 1998). The authors acknowledged that this might not represent the situation at the maternal-fetal interface however.

Vascular/Endothelial – Inflammatory response and Platelets

It is thought that the maternal syndrome of preeclampsia is an endothelial disorder (Roberts and Redman 1993; Williams and de Swiet 1997). Women with pre-existing microvascular disease (e.g. diabetics) or hypertension are more at risk (Roberts and Redman 1993).

It has been shown that there are increased cytokine levels (Conrad and Benyo 1997; Roberts et al. 1989a) as well as endothelial cell damage (Roberts et al. 1989b). Platelet activation (Greer et al. 1988; Redman 1990) has also been demonstrated.
Vascular endothelial growth factor (VEGF), an angiogenic growth factor, is expressed in placental tissue (Jackson et al. 1994). Recently, Maynard et al demonstrated that soluble fms-like tyrosine kinase 1 (sFlt1), a potent VEGF and PlGF antagonist is increased in maternal serum in preeclampsia and that it produces a preeclampsia-like syndrome in a rat model (Maynard et al. 2003). Further work by the same group demonstrated that it is also increased before the onset of clinical symptoms and that PlGF levels are correspondingly decreased (Levine et al. 2004a). Brockelsby indicated that VEGF levels may be increased in preeclampsia and that it parallels some of the effects of preeclamptic plasma in vitro (Brockelsby et al. 2000). This study measured plasma (not serum) VEGF levels and used both total and free (not bound to sFlt-1 receptors) levels - both important factors to consider when investigating VEGF. It is also an in vitro study and as Jelkmann notes in his review of VEGF, platelets and leukocytes both release VEGF during blood clotting

(Jelkmann 2001). In fact, free circulating levels of VEGF may actually be decreased in preeclampsia (Maynard et al. 2003) and this was hypothesised to be due to scavenging of the free component by sFlt1.

Table 1. 5. Summary of Vascular Factors Altered in Preeclampsia

Raised	Decreased
Plasma P-Selectin [1]	Endothelial Prostacyclin [3]
Total VEGF [2]	Nitric Oxide (bioavailability) [3]
Thromboxane [4]	Free VEGF[7]
TNFα[5]	
ICAM-1 and VCAM-1 [6]	

1. (Bosio et al. 2001) 2. (Brockelsby et al. 2000) 3. (Dekker and Sibai 1998) 4. (Friedman 1988) 5. (Conrad and Benyo 1997) 6. (Krauss et al. 1997) 7. (Maynard et al. 2003)

A longitudinal study of 70 women showed levels of plasma P-Selectin, an adhesion cell molecule were raised by 10-14 weeks in women who later developed preeclampsia (Bosio et al. 2001). The data is from a subset of non-matched women (preeclampsia, gestational hypertension and non-hypertensive pregnant controls) but shows quite significant rises in plasma P-Selectin from the first trimester, supporting the inflammatory model.

Pro-thrombotic Tendency

Pregnancy is a hypercoagulable state and this is increased in preeclampsia. There is increased thromboxane and platelet activity in preeclampsia (Friedman 1988). Although a major randomised trial failed to show benefit from aspirin, an anti-platelet agent (CLASP 1994), detection of this increased activation in the first trimester (CD63 expression) has been suggested as a way of predicting preeclampsia (Konijnenberg et al. 1997). Prostacyclin (PGI_2), which is mainly produced by endothelial cells, is a potent vasodilator and inhibitor of platelet aggregation. A Scottish group showed sera from preeclamptic women lowered PGI_2 in peripheral blood mononuclear cells (Chen et al. 1993). It has been shown that cell adhesion molecules may be raised early in pregnancy in those women who will subsequently develop preeclampsia (Krauss et al. 1997).

Oxygen Free Radicals – Oxidative Stress and Vitamin C

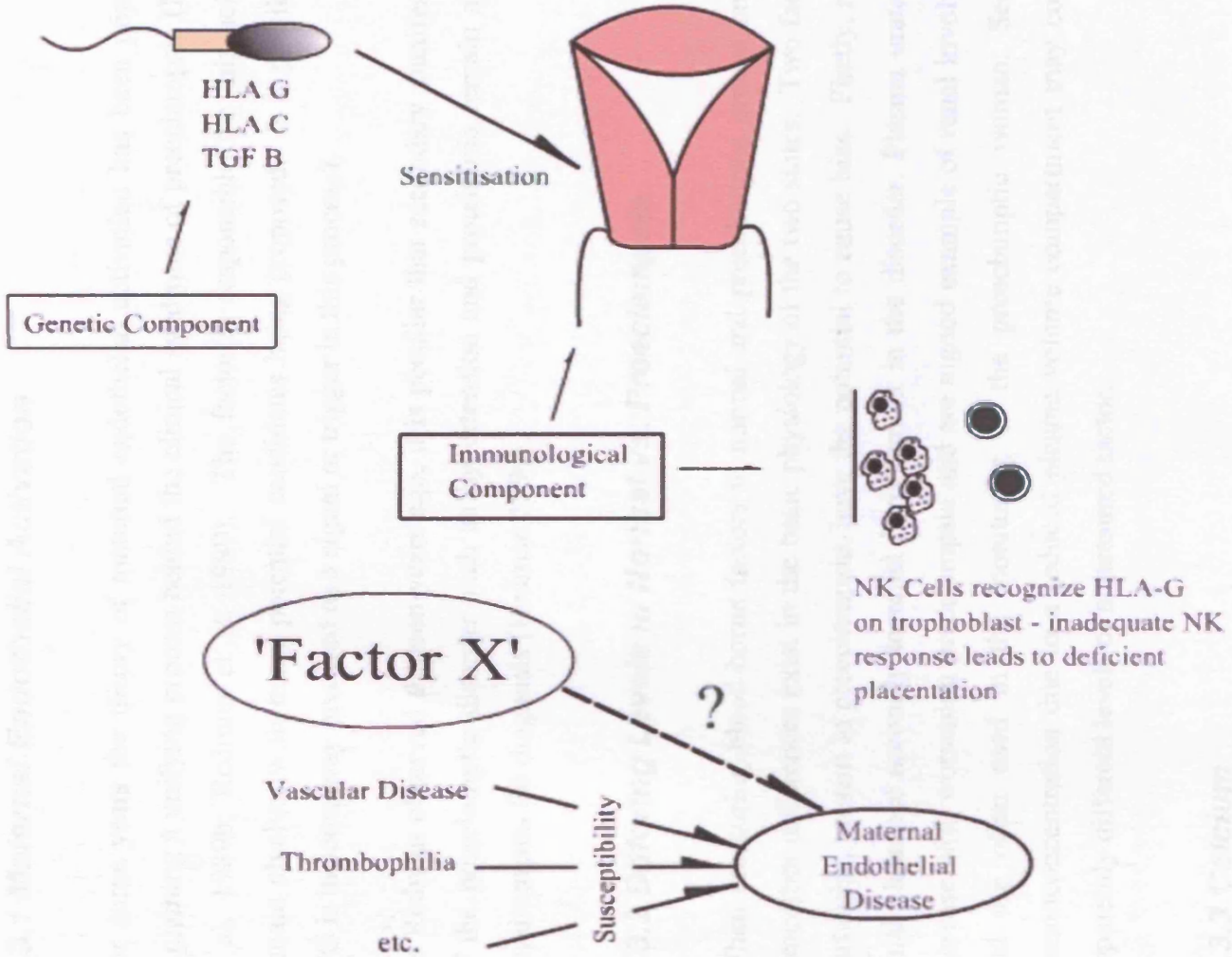
'Oxidative stress', a pathological state in which pro-oxidants dominate over anti-oxidants, has been implicated in the pathophysiology of both preeclampsia and atherosclerosis (Chappell et al. 1999b; Roberts and Hubel 1999), as well as other disease states. Reduced levels of antioxidants are present in preeclamptic women (Mikhail et al. 1994). Free radicals have emerged as likely promoters of maternal vascular endothelial damage (Dekker and Sibai 1998) and reactive oxygen species induce endothelial activation through many pathways (Davidge 1998). A trial of 238 women with 'high-risk' pregnancies (as determined by uterine artery Doppler or previous history of preeclampsia) suggested that prophylaxis with the antioxidant vitamins C and E may be effective for these selected cases (Chappell et al. 1999b). More recently, the same group have shown decreased ascorbic acid levels in preeclamptic pregnancies, as early as the second trimester (Chappell et al. 2002).

1.2.8 Summary

It is likely that the aetiology of preeclampsia is multifactorial, a series of events in a susceptible mother. One summary of this overall aetiological process has been presented by Williams and de Swiet (Williams and de Swiet 1997):

> *'It seems that susceptible women develop placental ischaemia triggering maternal endothelial cell dysfunction. This then leads to vasoconstriction, plasma volume contraction, maternal organ hypoperfusion and thus further impairment of placental blood flow.'*

Figure 1. 8. Possible pathophysiology of preeclampsia



1.3 The Possible Role of IPG-P in Preeclampsia and Labour

1.3.1 Maternal Endothelial Activation

For some years the theory of maternal endothelial activation has been popular as explaining a unifying process behind the clinical syndrome of preeclampsia (Roberts et al. 1989b; Redman et al. 1999). The factor(s) responsible for this activation remain elusive, with many potential candidates being proposed. Is it possible that IPG is in some way involved as a signal or trigger in this process?

As Redman observed thirteen years ago, it is peculiar that secondary manifestations of the preeclamptic disorder such as hypertension and proteinuria remain absolute requirements for diagnosis (Redman 1990).

1.3.2 Differing Levels in Normal vs. Preeclampsia

When measuring blood-borne factors in normal and preeclamptic groups one must remember differences exist in the basic physiology of the two states. Two factors in particular, present in preeclampsia, have the potential to cause bias. Firstly, reduced renal clearance secondly to renal involvement in the disorder. Plasma urate levels increase with worsening preeclampsia and are a good example of renal involvement and are often used in the monitoring of the preeclamptic woman. Secondly, haemoconcentration due to a reduced plasma volume compartment may confound apparently different levels of a measured factor.

1.3.3 Calcium

A significant increase in the response of intracellular free calcium to arginine vasopressin in maternal platelets predates the onset of preeclampsia (Zemel et al. 1990). Other authors have noted that platelet membrane-bound and free-intracellular calcium levels are increased in hypertension (Cooper et al. 1989) and preeclampsia (Haller et al. 1989) respectively.

IPGs have been implicated reducing calcium entry to rat hepatocytes by inhibiting calcium oscillations (Sanchez-Bueno et al. 1997).

A recent investigation indicated that plasma calcium concentrations may be decreased in preeclampsia compared to pregnant and non-pregnant controls (Kosch et al. 2000). The same paper demonstrated increased membrane calcium in erythrocytes, suggesting altered membrane ion transport. The authors did comment that only the free plasma calcium was measured and so it would be possible for protein binding to be altered in the preeclamptic state. It should be noted that some other authors have not found the plasma levels to be decreased.
There may also be a reduced calcium/creatinine ratio in urine of preeclamptic women (Rodriguez et al. 1988).

Caveolae, which have been identified as a key location for IPGs (as discussed in the Introduction), are also involved in calcium storage and entry (Anderson 1998). Is it possible that there is a link between abnormal IPG metabolism in caveolae and the observed changes in calcium seen in preeclamptic women?
Other factors altered in preeclampsia and affecting ion transport have also been identified, such as sodium pump inhibitors (Lopatin et al. 1999).

1.3.4 Zinc

Zinc has been described as an in vivo insulin mimetic in some of its functions (Ilouz et al. 2002). Zinc has been proposed as an important component of IPGs although the exact role has not been determined.

1.3.5 Preeclampsia, Lipid Metabolism and Plasma Leptin

Preeclampsia and obesity share certain metabolic characteristics, namely dyslipoproteinemia, insulin resistance and glucose intolerance (McCarthy et al. 1999). Obesity is also an independent risk factor for preeclampsia and pregnancy induced hypertension (Sibai et al. 1995). It was discussed in Chapter 1 that IPGs are ubiquitous in insulin metabolism. It is also known that human Placental Lactogen (a hormone with a lipolytic component) is increased in preeclampsia (Murai et al. 1997).

Leptin is the product of the Lep gene (the so-called human obesity gene). It is a circulating hormone produced by adipose tissue in the non-pregnant state, which responds rapidly to the availability of nutrients. Maternal leptin levels are raised in normal pregnancy by a factor of 3 or 4 compared with non-pregnant controls and correlate with maternal body mass index (Geary et al. 1999) and inversely with placental weight (Schubring et al. 1996). Umbilical cord levels also correlate with birth weight (Schubring et al. 1996; Geary et al. 1999). These two findings suggested a possible role for leptin as a regulator of maternal weight and fetal intra-uterine growth.

Plasma leptin levels are raised in preeclampsia (McCarthy et al. 1999; Ramsay et al 2003) and may possibly be used as part of a screening tool in the second trimester (Chappell et al. 2002). McCarthy and colleagues showed raised maternal plasma leptin levels in comparison to controls in a group of preeclamptic women in the third trimester. Subjects were matched for BMI and fetal gestational age. At the same time, there was a correlation in the preeclamptic cases between maternal and cord levels of Leptin. Unfortunately the cord bloods were mixed arterial and venous samples. More recently, Ramsay et al also showed raised fasting leptin, VLDL-C and triglyceride levels, as well as certain inflammatory markers (VCAM-1 and IL6), compared to women with intrauterine growth restriction (IUGR). Chappell's group showed elevated leptin and lowered placental growth factor in women identified as high risk for preeclampsia by personal history or abnormal second trimester uterine artery Doppler waveforms. It is possible that Leptin may be a significant factor in preeclamptic metabolism.

IPG-A has been demonstrated to inhibit leptin release from rat adipocytes in vitro contrasting with the action of insulin (stimulation) and IPG-P (no effect) (Kunjara et al. 2000b). If IPG-A levels were shown to be lower in preeclampsia, this would result in a correspondingly diminished inhibitory effect.

1.3.6 Nitric Oxide

Nitric oxide is synthesised from L-arginine by the enzyme nitric oxide synthase, which is found in endothelial cells, platelets and the placenta. The latter is an important source of nitric oxide (NO) during pregnancy (Norris et al. 1999).

Unfortunately, the evidence as to the status of Nitric Oxide in preeclampsia is contradictory and confusing. It was shown recently in a small series of age-matched preeclamptic and normal controls that NO was higher in the uteroplacental, fetoplacental and peripheral circulations in the preeclamptic cases (Norris et al. 1999). Some of the other conflicting results are summarised in Table 1.6 below.

Table 1. 6. Summary of Nitric Oxide (NO) Study Results

Sample and Location	Sample Size		Change in NO/ NO Synthase	Authors & Date
	PE	C		
Nitric Oxide				
Mat. Peripheral, Uterine Vein	15	15	Increased Increased	**(Norris et al. 1999)**
Mat. Peripheral	23	393	Increased	**(Nobunaga et al. 1996)**
Mat. Peripheral	20	32	Increased	**(Smarason et al. 1997)**
Mat. Peripheral	26	26	Decreased	**(Seligman et al. 1994)**
Mat. Peripheral, Urine	14	20	Unchanged Decreased	**(Davidge et al. 1996)**
NO Synthase				
Placenta	16	29	Decreased	**(Brennecke et al. 1997)**
Placenta	6	17	Decreased	**(Morris et al. 1995)**

PE = Preeclampsia, C = Control (pregnant and non-pregnant), Mat. = Maternal

Both sides of the apparent conflict have been explained in seemingly convincing ways. Namely if the NO levels/activity is increased, this is seen as either a compensatory mechanism for the effects of the preeclampsia or a reflection of decreased renal elimination. In either case, the increased NO may be a cause for endothelial cell injury. If it is decreased, this is seen as part of the disease process itself, causing the subsequent pathophysiological changes, principally the high-resistance blood flow patterns seen.

It is difficult to explain the different results as all the authors use similar collection and processing methods for the samples. Dietary differences are known to alter nitric oxide metabolite levels in the short-term and only Nobunaga et al control for this by taking fasting samples. However, the same group is the only one not to have made an attempt at matching preeclamptic and control subjects to some degree. The decreased urinary levels observed by Davidge et al would correspond to the raised

peripheral levels being as a result of reduced excretion. Magnesium sulphate may also affect NO metabolism.

Reduced activity of placental nitric oxide synthase would seem to be a possible cause of the high resistance fetoplacental circulation seen in preeclampsia (Morris et al. 1995; Brennecke et al. 1997). This would correspond to low measured maternal levels, though the local situation in the placenta is of course under local control and thus potentially isolated from the mother's periphery.

The situation is made more interesting by Cyclic Guanosine Monophosphate (cGMP) which can be used as a surrogate marker for nitric oxide production as NO stimulates guanylate cyclase. Reduced levels of amniotic fluid cGMP (Rizzo et al. 1996) have been discussed already in Chapter 6 but increased levels have also been reported

So where is this leading us? Caveolae have already been discussed in relation to IPG signalling. Various techniques have been employed to show that the majority of cell surface endothelial nitric oxide synthase (eNOS) is located in caveolae and Anderson has written that 'this suggests that caveolae are the site of NO production.' (Anderson 1998). Anderson goes on to suggest that caveolae are the location for signal integration between some of these signalling molecules, which, it must be remembered, is what IPGs function as at the most basic level.

Is it possible that abnormal IPG levels are affecting eNOS activation and thus causing the raised NO levels observed by some authors?

1.3.7 Are IPGs the missing 'Factor-X'?

There is a significant body of evidence to support the existence of a so-called 'Factor-X' (Walker et al. 1994) released from the placenta into the maternal circulation, leading to the maternal symptoms seen in preeclampsia (van Beek and Peeters 1998).

Studies to show the direct effects of preeclamptic serum on several features of preeclampsia are summarised in Table 1.7, and compared on the right to evidence of some of the effects of IPGs.

Table 1. 7. Effects of Preeclamptic Serum Factor(s) and IPGs

EFFECT OF SERUM FACTOR(S)	KNOWN EFFECTS OF IPGs
Cytotoxic to human endothelial cells **(Rodgers et al. 1988)**	
Mitogenic to fibroblasts **(Musci et al. 1988)**	Fibroblast proliferation **(Vasta et al. 1992)**
Stimulating synthesis of platelet-derived growth factor (PDGF) **(Taylor et al. 1991b)**	
Selectively activating endothelial cell procoagulant products **(Taylor et al. 1991a)**	
(Activated platelets in preeclampsia) **(Harlow et al. 2002)**	Human platelet activation **(Bruni et al. 1991)**
(Progesterone levels may be raised in preeclampsia) **(Zamudio et al. 1994)**	Insulin-dependent progesterone synthesis **(Romero et al. 1993)**

It can be seen therefore that there is some overlap with known properties of IPGs.

Different groups have suggested a proteinaceous cytotoxic factor as the culprit (Tsukimori et al. 1992b; Tsukimori et al. 1992a). Smarason and colleagues demonstrated a factor cytotoxic to human umbilical vein endothelial cells (HUVECs) that was present in plasma from preeclamptic women but was absent from their sera (Smarason et al. 1996), in contrast to the data from Rodgers et al and Tsukimori et al. However the latter two groups used a different methodology (release of 51Cr from HUVECs) to Smarason's group (uptake of 3H-Thymidine – taken up during spontaneous proliferation), which may explain this. Of course, this is all work in vitro and there is also the possibility that more than one factor is being released.

An alternative explanation would be that an alteration in maternal metabolism represents the 'Factor-X' rather than it being a distinct molecule released from the placenta.

1.4 Current Screening and Diagnosis of Preeclampsia

1.4.1 Methods of Diagnosis and Classification

The clinical syndrome of preeclampsia is classically defined as one of hypertension and proteinuria with or without oedema. However, classifications of hypertension in pregnancy are multiple and indeed there is much confusion in the available literature with overlap of terminology (such as 'Pregnancy Induced Hypertension')(Chappell et al. 1999a). Recently the International Society for the Study of Hypertension in Pregnancy (ISSHP) produced a statement to clarify the situation (Brown et al. 2001). For the purposes of this project, the definitions used in this statement will be used universally to avoid confusion.

The ISSHP considered several published reports:

1. Australian Society for the Study of Hypertension in Pregnancy (ASSHP), 2000 (Brown et al. 2000)
2. National High Blood Pressure Education Program (NHBPEP), 2000 (NHBPEP 2000)
3. Previous ISSHP report, 1998 (Davey and MacGillivray 1988)
4. World Health Organisation (WHO), 1987 (World Health Organisation Group 1987)
5. Canadian Hypertension Society, 1997 (Helewa et al. 1997)

Following from this, Table 1.8 below summarises the definitions used and using these definitions, the consensus for defining the hypertensive disorders of pregnancy is then shown in Table 1.9.

Table 1. 8. Definitions of hypertensive disease in pregnancy used here.

• Hypertension in Pregnancy	A systolic blood pressure (BP) of ≥ 140 mmHg and/or a diastolic BP ≥ 90 mmHg (Korotkoff 5 †)
• Proteinuria	Ideally measured in a 24-hour collection as ≥ 300mg/day. Dipstick testing - if the only test available, 1 + (30mg/dl) is often, but not always associated with ≥ 300mg/day proteinuria.

†Note on Blood Pressure readings: Korotkoff 5 (disappearance) should be used and K 4 (muffling) only utilised when K5 is absent. K4 shows significant inter-observer variability.

Table 1. 9. ISSHP Classification of hypertensive disorders of pregnancy

• Chronic Hypertension	Known hypertension before pregnancy or rise in blood pressure to ≥ 140/90 mmHg before 20 weeks' amenorrhoea.
• Preeclampsia/Eclampsia	De novo hypertension accompanied by proteinuria (both as defined in Table 1.8) occurring after 20 weeks' gestation and returning to normal post-partum. Eclampsia is the occurrence of seizures in a patient with preeclampsia.
• Preeclampsia superimposed on chronic hypertension	The combination of chronic hypertension and de novo proteinuria. †
• Gestational hypertension	Rise in blood pressure detected for the first time after 20 weeks gestation, without proteinuria. May be changed to "transient" when pressure normalises post-partum.

†A sudden increase in the magnitude of hypertension, the appearance of thrombocytopenia and/or abnormal levels of transaminases are labelled as highly likely of superimposed preeclampsia (NHBPEP 2000)

It is interesting to note that even within the current consensus there lies a certain degree of debate on the difference between so-called 'Clinical' (broader) and 'Research' (more restrictive) definitions of preeclampsia. The ISSHP statement recommended a research effort to investigate if the research criteria need to be broadened to approach the clinical criteria defined by the ASSHP.

`1.4.2 Methods of Screening

It could be imagined that a disorder seemingly so difficult to even classify would be somewhat enigmatic in its origins. Indeed, preeclampsia was first called the 'Disease of Theories' by Zweifel in 1916 (Quoted in Dekker and Sibai 1991) and whilst a more accurate picture of the subsequent pathophysiology is emerging, no definite evidence of the initial trigger exists.

There are more than 100 separate 'tests' for the screening or prediction of preeclampsia (Dekker and Sibai 1991) and yet no single predictive or diagnostic test exists (Grunewald 1997). In addition to this, the only definitive treatment that exists is to remove the placenta, leading to more than 40% of preterm iatrogenic deliveries (Meis et al. 1998).

The Screening Test

Before discussing individual pathologies and the screening tests that are available, it is first necessary to define the nature of the screening test itself. Many tests are used to predict if a patient has, or will develop preeclampsia. These range from simple blood tests such as urate levels (Chesley & Williams 1945) and platelet count to invasive tests. Initially it is necessary to look at the criteria for a valid screening test as set down by the WHO (Wilson and Jungner 1968), (Table 1.10). These criteria can be applied to each screening test as it is proposed and, of course, very few if any will meet the 'gold standard'.

There are four broad categories of screening tests used at this time, which are illustrated with examples in the table below (Table 1.11). For the purposes of this introduction, screening as it exists for preeclampsia falls in to the first category.

Table 1. 10. Criteria for an ideal screening test (Modified from Wilson and Jungner 1968)

• The condition should be an important health problem
• There should be an acceptable treatment for patients with recognised disease
• Facilities for diagnosis and treatment should be available

- There should be a recognisable latent or early symptomatic stage
- There should be a suitable test or examination
- The test should be acceptable to that population
- The natural history of the condition, including development from latent to declared disease should be adequately understood
- There should be an agreed policy concerning whom to treat as patients
- The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole
- Case finding should be a continuous process and not a "once and for all" project

Table 1. 11. Categories of screening tests

Reason	**Example**
1. Screening to detect early disease, which can then be treated.	Breast Cancer, Cervical Intraepithelial neoplasia
2. Screening as individual may be a risk to others	Hepatitis B (blood donors, health care workers)
3. Screening because special care may be beneficial	Screening children for hearing impairment following premature delivery/ Serum screening for Down's and neural tube defects
4. Screening to modify risk factors	Smoking, raised plasma lipids

At the present time, there is no cure for preeclampsia other than delivery of the infant and placenta. Additionally, there is no antenatal diagnostic test, other than clinical criteria. Placental histology can be used to confirm diagnosis but obviously only in the postnatal period. These factors therefore may restrict the clinical usefulness of any screening test. However, earlier identification of a pregnant woman with preeclampsia can be used to focus management of her care to allow earlier detection of clinical signs and symptoms. If an effective treatment is established, then a

screening test could potentially allow initiation of this treatment before completion of placentation. This will be discussed again later on.

Screening for preeclampsia also falls into the second category in table 1.11 above as treating the mother also benefits the fetus.

The 2 By 2 Table

In any population, a screening test is trying to identify two groups of individuals: those with the condition (or risk factor) being screened for and those without. The test itself will split the population into those who screen positive and those who screen negative. It is the efficiency with which any given test does this that tells us how good a test it is. An example of a 2 by 2 table is given below:

	Disease	
Test	***Yes***	***No***
Positive	a	c
Negative	b	d

a) True Positives – Those with disease correctly identified as positive
b) False Negatives – Those with the disease incorrectly screened as negative
c) False Positives – Those without disease incorrectly screened as positive
d) True Negatives – Those without the disease correctly screened as negative

Statistically this may then be utilised to form a picture of the effectiveness of the screening. The five main formulae used are:

- **SENSITIVITY = a/(a+b)%**

  The percentage of those with the disease who were correctly identified as positive by the screening test

- **SPECIFICITY = d/(c +d)%**

  The percentage of those without the disease who were correctly identified as negative by the screening test

- **POSITIVE PREDICTIVE VALUE = a/(a+c)%**

The proportion of those with a positive test who actually had the disease

- **NEGATIVE PREDICTIVE VALUE = d/(b+d)%**

  The proportion of those with a negative test who actually did not have the disease

- **PREVALENCE = (a+b)/(a+b+c+d)**

  The proportion of those in the screened population who actually have the disease

- **LIKELIHOOD RATIO:**

  The likelihood that a given test result would be expected in a patient with a disease compared to the likelihood that the same result would be expected in a patient without that disease.

  Likelihood ratios tell us how much we should shift our suspicion for a particular test result. Because tests can be positive or negative, there are at least two likelihood ratios for each test. The "positive likelihood ratio" (LR+) tells us how much to increase the probability of disease if the test is positive, while the "negative likelihood ratio" (LR-) tells us how much to decrease it if the test is negative. The formula for calculating the likelihood ratio is:

$$LR = \frac{\text{Probability of an individual } \textbf{with} \text{ the condition having the test result}}{\text{Probability of an individual } \textbf{without} \text{ the condition having the test result}}$$

  Thus, the positive and negative likelihood ratios are respectively:

$$LR+ = \frac{\text{Probability of an individual } \textbf{with} \text{ the condition having a positive test}}{\text{Probability of an individual } \textbf{without} \text{ the condition having a positive test}}$$

$$LR- = \frac{\text{Probability of an individual } \textbf{with} \text{ the condition having a negative test}}{\text{Probability of an individual } \textbf{without} \text{ the condition having a negative test}}$$

  One can also define the LR+ and LR- in terms of sensitivity and specificity:

  LR+ = sensitivity / (1-specificity)

  LR- = (1-sensitivity) / specificity

Any given screening test will have a fixed sensitivity and specificity (independent of prevalence) and the ideal would obviously be 100% for both. However, this is not practically possible and often, such as in the case of serum screening for neural tube

defects with serum AFP (Alpha feto-protein) levels, a trade-off between the two is achieved. That is to say, as one increases the sensitivity, the specificity will drop and vice versa. The positive and negative predictive values are dependent partially on the prevalence of the disease or risk factor in the screened population and will rise in proportion to this. The likelihood ratio is independent of disease prevalence within a population and thus has particular value in assessing clinical usefulness of a screening test.

It can be seen, therefore, that there are obvious benefits to screening for preeclampsia and this why so much research has been devoted to the area. The diagnosis of both conditions can be difficult and much often hangs on getting it right. There is also a great incentive for an accurate diagnostic test for preeclampsia for these reasons.

1.4.3 Assays Used in Diagnosis and Management of Preeclampsia

Some tests available are routinely used in management of preeclampsia and function as diagnostic or clinical tools rather than screening tests. The most common examples would be:

a) Platelet count

Lowered platelet count may be a sign of worsening disease or even HELLP Syndrome (Haemolysis, Elevated Liver Enzymes and Low Platelets). There is some evidence to suggest that the rate of a drop in the platelet count may be an indicator of the severity of the disorder.

b) Serum Urate Levels

Hyperuricemia has been used as a marker of preeclamptic disease for several decades and is associated with the severity of preeclampsia and with fetal outcome (Liedholm et al. 1984). Traditionally the high uric acid concentration in preeclampsia has been attributed solely to renal dysfunction. It has been suggested by Many and colleagues (Many et al. 1996) that other factors are involved. Purines (xanthine and hypoxanthine) act as a substrate for xanthine dehydrogenase/oxidase to produce uric acid. Uric acid production is coupled with formation of reactive oxygen species when

the enzyme is in the oxidase form. Several factors can increase the holoenzyme activity and the conversion of xanthine dehydrogenase/oxidase to its oxidase form. These factors include

1. Hypoxia-reperfusion
2. Cytokines
3. Increased substrate availability.

Preeclampsia is characterized by hyperuricemia and signs of increased formation of reactive oxygen species (such as free radicals) and decreased levels of antioxidants, producing elevated oxidative stress.

Preeclampsia is also characterized by shallow implantation, producing a relatively hypoxic maternal-fetal interface, and increased turnover of trophoblast tissue, which can result in higher xanthine and hypoxanthine concentrations and higher levels of circulating cytokines. These mechanisms can lead to increased production of uric acid and free radicals and contribute to the hyperuricemia and increased oxidative stress present in preeclampsia (although xanthine oxidase activity has not been demonstrated in the placenta). It is also noted that the antioxidant properties of urate may have a protective role in preeclampsia.

1.4.4 Currently Used Screening for Preeclampsia

As mentioned before, certain risk factors for preeclampsia provide an indication of increased risk at the time of pregnancy booking. Examples include raised diastolic (>80 mmHg) or systolic (>130 mmHg) blood pressure or the presence of anticardiolipin antibodies or lupus anticoagulant. There are then specific screening tools, examples of which are discussed below.

a) Uterine Artery Doppler Ultrasound

Doppler ultrasound is currently the most widely used early-pregnancy screening technique for preeclampsia and growth restriction. It is known that an increase in impedance to blood flow in the uterine artery occurs in preeclamptic pregnancies (Campbell et al. 1983). This is due to failure of invasion of trophoblast into the maternal spiral arteries. Campbell first identified the use of Doppler ultrasound as a potential screening test for high-risk pregnancies over 17 years ago (Campbell et al. 1986).

$$RI = \frac{\text{Systolic Peak} - \text{End-diastolic trough}}{\text{Systolic Peak}}$$

An elegant paper by Bower et al revealed the power of Doppler ultrasound as a screening tool using strict diagnostic criteria for preeclampsia and including early diastolic notching in the definition of an abnormal flow velocity waveform (FVW) in the uterine artery (Bower et al. 1993b). Strong positive predictive and negative predictive values were achieved when screening for 'moderate to severe' preeclampsia in particular. Subsequent further research by the same group used the criteria to screen for high-risk women by Doppler ultrasound at 18-22 and 24 weeks (Bower et al. 1996). This study showed the potential for Aspirin (an anti-platelet agent) to prevent some cases of severe preeclampsia in women identified as high risk by the presence of persistent uterine artery notching. In so doing, this study seemed to demonstrate a therapeutic effect of low-dose aspirin (in selected, 'high-risk' women) where the CLASP trial (CLASP 1994) (with an unselected population) did not. More recently, Doppler ultrasound has been used in combination with other tests to improve the positive predictive value. A preliminary study on a large group of women (689 women, with a preeclampsia rate of 5.5%) indicated that a combination of Doppler at 20 weeks' gestation and second trimester (15-19 weeks)

serum Inhibin levels could be used (Aquilina et al. 2001). They showed a Positive Predictive Value (PPV) of 38.5% (Sensitivity 71.4%, Specificity 93.4). This is as powerful and at a significantly earlier stage in the pregnancy than previous screening - Doppler alone at 24-26 weeks (Bower et al. 1993a)- but does rely on a dual-stage test. This confers some complexity and additional expense.

b) Beta Human Chorionic Gonadotrophin (βhCG)

Human Chorionic Gonadotrophin is a placental hormone with alpha and beta sub-units. There has been interest in serum βhCG as a potential screening test and various groups have investigated levels in the first (Haddad et al. 1999), second (Sorensen et al. 1993; Wenstrom et al. 1994; Ashour et al. 1997) and third (Said et al. 1984) trimesters. Said et al found significantly higher maternal serum βhCG levels in 41 primigravid patients with 'proteinuric preeclampsia' compared with those in 41 primigravid normotensive women matched for age and gestation (although an archaic definition of 'proteinuric preeclampsia' was used with 0.25g/litre of urine). The more recent studies by Sorensen et al and Ashour et al (the larger series of the two) both used a retrospective examination of samples taken for serum screening purposes. Using a cut-off of 2.0 MoM (Multiples of the median) both groups concluded that second trimester βhCG levels could be used to screen for women at high risk of pregnancy related hypertension. Both groups found higher risk ratios relating to proteinuric (compared to non-proteinuric) hypertension as would be expected. Sorensen's apparently more impressive results may be due to the inclusion process and the smaller sample size. As with any screening program this could then lead to closer surveillance of the high-risk group with potential therapeutic intervention.

The problem with second trimester intervention for the prevention of preeclampsia is that success may be limited as placental invasion and vessel formation is complete by 16 weeks. Thus a screening/diagnostic test would be of more clinical use.
Unfortunately, the study by Haddad et al failed to show a significant difference in serum βhCG levels in the first trimester (samples taken in 236 singleton IVF pregnancies between day 13 and 35 post-conception). Preeclampsia (ISSHP research definition used) was a primary outcome measure but is possible that the sample size was too small to detect differences at this early stage.

c) Inhibin/ Activin

Inhibins are heterodimeric proteins consisting of αβA (inhibin A) and αβB (inhibin B) subunits. Activins are homodimers with the subunits linked by disulphide bridges. Like βhCG these hormones are derived from the trophoblast, with both inhibin and activin subunits being expressed in human placenta (Vale et al 1994).

Inhibin A has been established in screening programmes for Down's Syndrome (Wald et al. 1999; Gilbert et al. 2001). Interestingly, it has also been shown that maternal serum alpha-fetoprotein (AFP), another component of this screening test is raised in preeclamptic pregnancies too (Waller et al. 1996). In some institutions a high AFP observed at the time of routine serum screening, in the absence of a karyotypic abnormality, is now used as an indication to perform further screening (for poor pregnancy outcome) by the use of Doppler ultrasound as described above.

It was demonstrated convincingly in a small retrospective study in the second half of pregnancy that serum inhibin A is raised in preeclampsia compared to age and gestation-matched control subjects (Muttukrishna et al. 1997). A further retrospective study of early second trimester Inhibin A levels, with preeclamptic subjects identified by lab records of proteinuria, indicated the predictive potential of the marker (Cuckle et al. 1998). A cross-sectional study found both Inhibin A and Activin A were raised in preeclamptic subjects and Activin A alone was raised in gestational hypertension (Silver et al. 1999). All three studies use the same, highly specific two-site immuno-assay (Serotec, Oxford, UK).

Inhibin B on the other hand may fall in preeclamptic pregnancies compared to normal (Petraglia et al. 1997). This group showed that Inhibin B levels rise with gestation but that it is not the predominant form of inhibin in the maternal circulation.

1.4.5 Historic and Potential Future Screening Tests

Two historic tests exist that are applicable in a practical way only in a research setting.

a) Angiotensin Sensitivity Test

Abdul-Karim and Assali first identified refractoriness to pressors in pregnant women in the 1960s (Abdul-Karim and Assali 1961). Subsequently, it was determined that

this was lost in those women who develop preeclampsia (Talledo 1966). This increased sensitivity to angiotensin II may arise several weeks before the onset of preeclampsia (Oney and Kaulhausen 1982). Kyle and colleagues have shown this to be ineffective as a screening test (Kyle et al. 1995) and it is also too complicated and time-consuming to be used in clinical practice (Dekker and Sibai 1991).

b) Supine Pressor Test:

Physiologically related to the angiotensin sensitivity test, in that it assesses the pressor response in pregnancy, this test was first described nearly thirty years ago (Gant et al. 1973). The method is less invasive than infusing angiotensin II, merely involving the measurement of blood pressure in the right arm of the patient lying on the left side, then supine. Although initially promising, Gant et al describe a very high prevalence (44.7%) of preeclampsia for an unselected group of primigravidae (Gant et al. 1973). Subsequent authors have failed to reproduce these results and again this test is of little clinical use.

Furthermore, assays for several markers have been proposed which may become useful in the future as part of a screening tool for preeclampsia prior to the onset of clinical symptoms or signs.

c) Free Fetal DNA

As well as the discovery of trophoblast tissue in the maternal circulation (Chua et al. 1991), several groups have identified free fetal DNA in maternal plasma. A group from London has demonstrated fetal DNA to be present throughout pregnancy from the first few weeks.(Thomas et al. 1995) The principle is one of demonstrating Y-chromosome DNA (from a male pregnancy) by means of PCR (polymerase chain reaction) amplification. Bianchi et al examined a series of peripheral venous samples taken from women undergoing prenatal cytogenetic diagnosis (amniocentesis and chorionic villous sampling) (Bianchi et al. 1997). Using PCR quantitation from a 16ml blood sample, detection of male DNA was possible in 99.3% of male pregnancies. More interestingly, there is an elevation of fetal cell DNA equivalents with pregnancies of abnormal karyotype (specifically, Trisomy 21). This difference has been shown significantly but to a lesser degree, using real time PCR techniques by Lo et al.(Lo et al. 1999a) Quantitative analysis shows an increase with gestation.(Lo et al. 1998) More relevant to the subject matter of this thesis is data

from the same group indicating a similar rise (Lo et al. 1998; Lo et al. 1999b) in the circulating fetal DNA in preeclamptic pregnancies. This study used gestation-matched groups of preeclamptic and non-hypertensive women (32 and 33 weeks mean respectively) with investigator blinding for analysis, once again by real time PCR.

d) First trimester Biochemical Markers

Leptin, a circulating hormone produced by adipose tissue in the non-pregnant state, may be raised in preeclampsia. It was shown in a group of preeclamptic women in the third trimester that maternal plasma leptin levels were raised in comparison to controls matched for BMI and fetal gestational age (McCarthy et al. 1999). At the same time, there was a correlation in the preeclamptic cases between maternal and cord levels of Leptin. Unfortunately the cord bloods were mixed arterial and venous samples. It is possible that Leptin may be a significant factor in preeclamptic metabolism. See also Chapter 1.3.5 regarding leptin.

e) Immunological Markers

1. **Interleukin-2 (IL-2)**

   A small prospective study of non-matched preeclamptic and normal pregnant controls indicated raised IL-2 activity in peripheral blood mononuclear cells (Chen et al. 1995). The group suggested that IL-2 may play a role in induce reactive oxygen species in preeclampsia. More recently, it was shown by a separate group that elevated IL-2 receptor levels were present in the first trimester in women who subsequently developed preeclampsia (Eneroth et al. 1998). This particular study was small (10 preeclamptic and 10 normal controls matched for age and parity).

2. **Interleukin-6 (IL-6)**

   IL-6 was initially thought to be decreased in the amniotic fluid of preeclamptic pregnancies (Silver et al. 1993). Other authors have subsequently shown that the opposite may be true (Nakabayashi et al. 1998). Neither the source nor the function of amniotic fluid IL-6 is known, though Silver et al suggest increased levels observed in preterm delivery may be due to an infective process.

f) Other Candidates – More Factors Altered in Preeclampsia:

It has been demonstrated that magnesium levels rise with gestation in normal pregnancy but that total magnesium levels decrease early in preeclamptic pregnancies relative to the normal range (Standley et al. 1997). The authors comment that previously this has been related to the development of insulin resistance. Other altered factors include increased intra-cellular calcium levels (Haller et al. 1989), a low urinary kallikrein : creatinine ratio (Millar et al. 1996) and urinary N-acetyl glucosamine.

1.5 Summary and the Aim of this Thesis

Preeclampsia is a common and well-recognised complication of human pregnancy. It remains one of the main causes of maternal and fetal mortality and morbidity despite continued and widespread research. The cause remains an enigma although recent advances indicate that we are getting closer to an answer. It is unique in that it only occurs in human gestation. It is a multi-system disorder that affects endothelial membranes and appears to originate from the placenta. Many theories exist as to the cause of preeclampsia, from immunological through genetic. No treatment exists, save the delivery of the fetus and placenta. Great potential clinical benefit seems likely if a diagnostic test and, subsequently, a treatment can be provided.

The nature of IPGs and their involvement (actual and theorised) in several pathologies has been described above. Previously published work has indicated a potential link between preeclampsia and Inositol Phosphoglycans. The aim of this project was to further clarify the role of IPGs in specific relation to normal and preeclamptic pregnancy and labour. It was hoped that the work would cast further light on the role of IPGs in the pathophysiology of preeclampsia. In the future this may lead to a diagnostic test and potential treatment for the condition. Over the time of the project, specific clinical studies have been carried out with this in mind (Table 1.12)

At present there is no single diagnostic test for preeclampsia that can be used in a clinical setting. Early risk assessment has been proposed to identify women at high-risk of morbidity using a combination of clinical features and/or standard lab assays (urate, creatinine, proteinuria and liver enzymes) (Martin, Jr. et al. 1999; Chappell et al. 2002).

1.5.1 Preeclampsia

The aim was to further reveal the role of P-Type IPGs in the pathophysiology and pathogenesis of the disease process. This involved both a longitudinal and a cross-sectional study looking at women both before and after the onset of the clinical symptoms. Assessment was with a polyclonal-based ELISA assay, and also

previously validated extraction and bioactivity assays. Specimens collected were urine, cord blood, amniotic fluid, placenta and maternal blood from the peripheral circulation and also from the uterine vein.

1.5.2 Labour

The aim was to formally establish any change in urinary IPG-P levels in non-preeclamptic subjects before and after the onset of labour. This involved a prospective study of non-preeclamptic subjects recruited in the third trimester of pregnancy and followed to the time of delivery. Urine samples and clinical data were collected at recruitment and in labour. Assessment of samples was with a polyclonal-based ELISA assay.

Table 1. 12. Summary of Clinical Studies

Title	Aim
The role of IPGs in Preeclampsia	Longitudinal study to detect early pregnancy changes in urinary IPGs in subsequently preeclamptic women.
The Presence of IPGs in Normal Labour	To detect any increase in urinary IPG levels in normal labour in the absence of raised blood pressure
The Presence of IPGs in Preeclamptic tissue	To ascertain levels if IPGs in various body fluids in known preeclamptics at caesarean section

Chapter 2

Laboratory Methods

2.1 Urine Samples

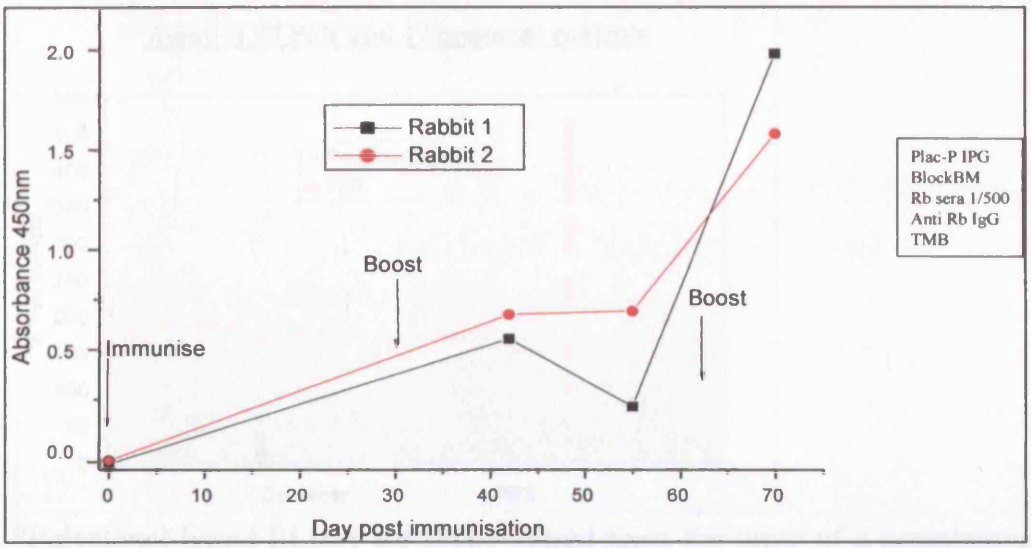
Polyclonal-Based Enzyme-Linked Immunosorbent Assay (ELISA) for detection of IPG-P

2.1.1 Development of the ELISA

The Polyclonal serum used for the ELISA was developed by Rodaris Pharmaceuticals Ltd, Oxford, UK and kindly donated for the purposes of this research. IPG-P was prepared from 150ml of urine from a single preeclamptic woman. This was dissolved in 500ml of water, to form a stock solution. 75µl of this stock was mixed with 175µl water and 750µl Freund's Complete Adjuvant (FCA). Two rabbits received a 1ml inoculation each in 5 sites.

Boosts (stock solution in Freund's Incomplete Adjuvant) were given on days 30 and 62 with 75% of antigen used in the initial immunisation (Figure 2.1). Exsanguination was carried out on day 70. Pre- and test bleeds were screened against human placental IPG-P extract. No data were provided on the purity of this extract but unpublished data from Rodaris indicated no correlation of the ELISA positivity with urine protein content. Sera from rabbits inoculated with FCA alone showed no reactivity.

Figure 2. 1. Screening of Sera against IPG-P*



*Data from Rodaris validation document

Data From Rodaris Product Profile

The polyclonal was evaluated with a total of 32 urines-16 patients with severe pre-eclampsia and 16 gestation-matched controls. The data with this kit show 100% (16/16) specificity and 100% sensitivity (16/16) with clear separation between patients and controls (Figure 2.2). A capture ELISA, using the polyclonal rabbit anti IPG-P showed significant difference between preeclamptic and normal pregnant controls (Figure 2.3).

Figure 2. 2. ELISA assay of 16 matched pairs of urines.

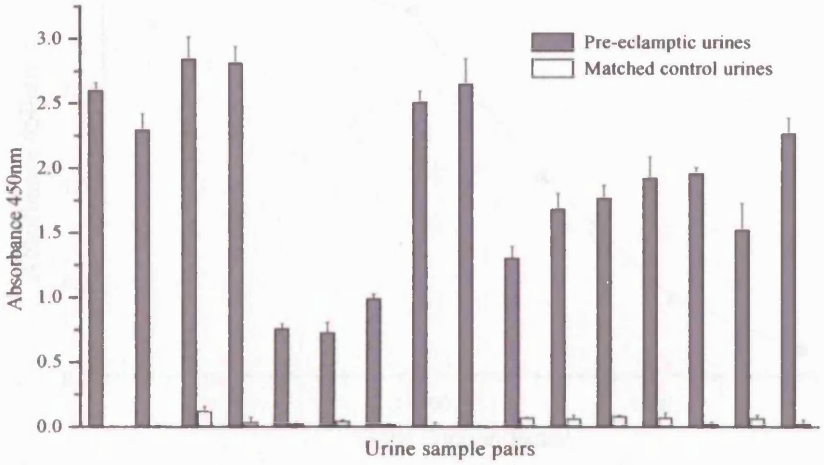
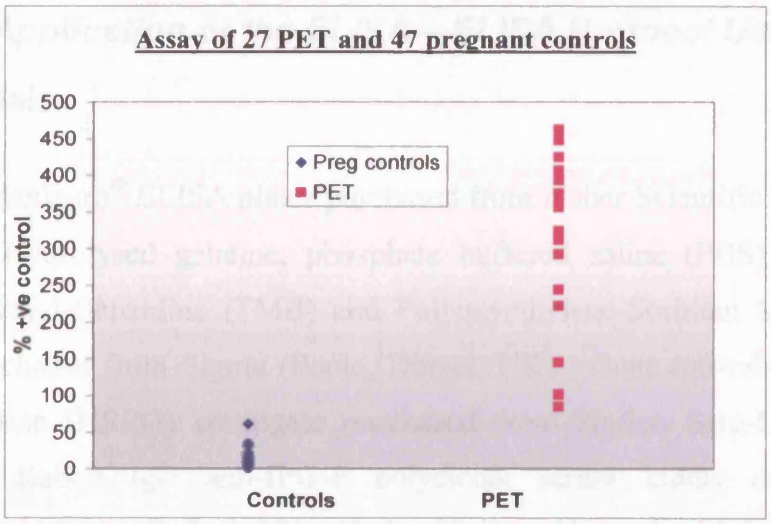


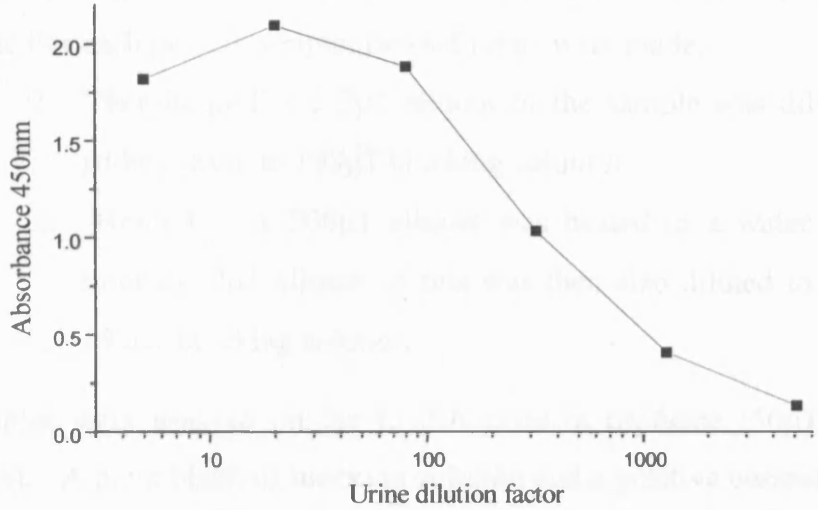
Figure 2. 3. Capture ELISA Normal and Preeclamptic Women (displayed as a percentage of a positive control*)



*Polyclonal-based ELISA for IPG-P raised from the urine of a preeclamptic woman. Data from Rodaris validation document

A serial dilution of one of the positive pre-eclamptic urines with the polyclonal serum showed that IPG could still be detected at a 5,000-fold dilution (Figure 2.4). This indicates that positive samples can still be detected at high dilutions.

Figure 2.4. Serial dilution of representative pre-eclamptic urine with prototype ELISA kit. Data from Rodaris Pharmaceuticals Ltd.



2.1.2 Application of the ELISA – ELISA Protocol Used

Materials

Nunc Maxisorb® ELISA plates purchased from Fisher Scientific Ltd (Loughborough, UK). Hydrolysed gelatine, phosphate buffered saline (PBS) tablets, 3,3', 5-5'-Tetramethyl-Benzidine (TMB) and Polyoxyethylene-Sorbitan Monolaurate (Tween 20) purchased from Sigma (Poole, Dorset, UK). Goat anti-rabbit IgG Horseradish Peroxidase (HRPO) conjugate purchased from Harlan Sera-Lab (Loughborough, UK). Rabbit IgG anti-IPG-P polyclonal serum kindly donated by Rodaris Pharmaceuticals (Oxford, UK). Hydrochloric acid purchased from BDH Laboratory supplies (Poole, Dorset, UK).

Protocol

Urine samples to be tested were defrosted immediately prior to assay. 1% gelatin solution was used as the capture and also the blocking agent. A solution of 1% hydrolysed gelatine in phosphate buffered saline (PBS) was made. A Maxisorb plate was blocked with 200µl of the blocking reagent in each plate well. The plate was stored in a sealed plastic container lined with a damp tissue to maintain humidity and incubated at 37°C for 20 min.

Blocking reagent was flicked out and the plate was blotted dry on tissue paper. Urine For each patient sample, two solutions were made:

1. '**Non-heated**' - a 2µl aliquot of the sample was diluted to 1 in 100 by adding urine to 198µl blocking solution.
2. '**Heated**' - A 200µl aliquot was heated in a water bath at 85°C for 5 minutes. 2µl aliquot of this was then also diluted to 1 in 100 by adding 198µl blocking solution.

Samples were assayed on the ELISA plate in triplicate (50µl in each of 3 plate wells). A plate blank of blocking solution and a positive control urine sample (from the same preeclamptic woman for all the plates) at 1 in 5000 dilution (in blocker) were also assayed in triplicate on each plate. The plate with the diluted urine samples was incubated for 40 min at 37°C. After the incubation, the liquid was flicked out. The plate was washed 3 times with 0.05% PBS/Tween 20 solution and was blotted dry on tissue paper. Figure 2.5 illustrates a sample plate template.

Polyclonal rabbit IgG anti-IPG was diluted 1/10,000 in blocking solution (by adding 1µl of polyclonal to 10ml of the 1% gelatine/PBS). 50µl of this polyclonal solution is then added per well and incubated at 37°C for 30 min. Once again, the plate was washed 3 times with 0.05% PBS/Tween 20 solution and was blotted dry on tissue paper.

Goat anti-rabbit IgG-HRPO was diluted 1/6000 as for the polyclonal above. 50µl of this solution was added to each plate well and the plate was incubated at 37°C for 20 min. The plate was washed 5 times with 0.05% PBS/Tween 20 solution and was blotted dry on tissue paper. TMB solution was pre-warmed to room temperature and

50µl of this was then added to each well. This was incubated at room temperature for 10 minutes.

The colour reaction was stopped with the addition of 50µl of 1M HCl per well. This resulted in a blue to yellow colour change, additionally increased the sensitivity of the plate reading. Any bubbles in the wells were burst with a fine needle and the base of the plate was wiped with a dry tissue. Absorbance was read at 450nm using a standard ELISA plate reader ThermoMax Optical Plate Reader (Molecular Devices, Wokingham, UK). See Fig 2.6 (b) for image of sample ELISA plate prior to plate reading

Figure 2. 5. Sample ELISA Plate Template

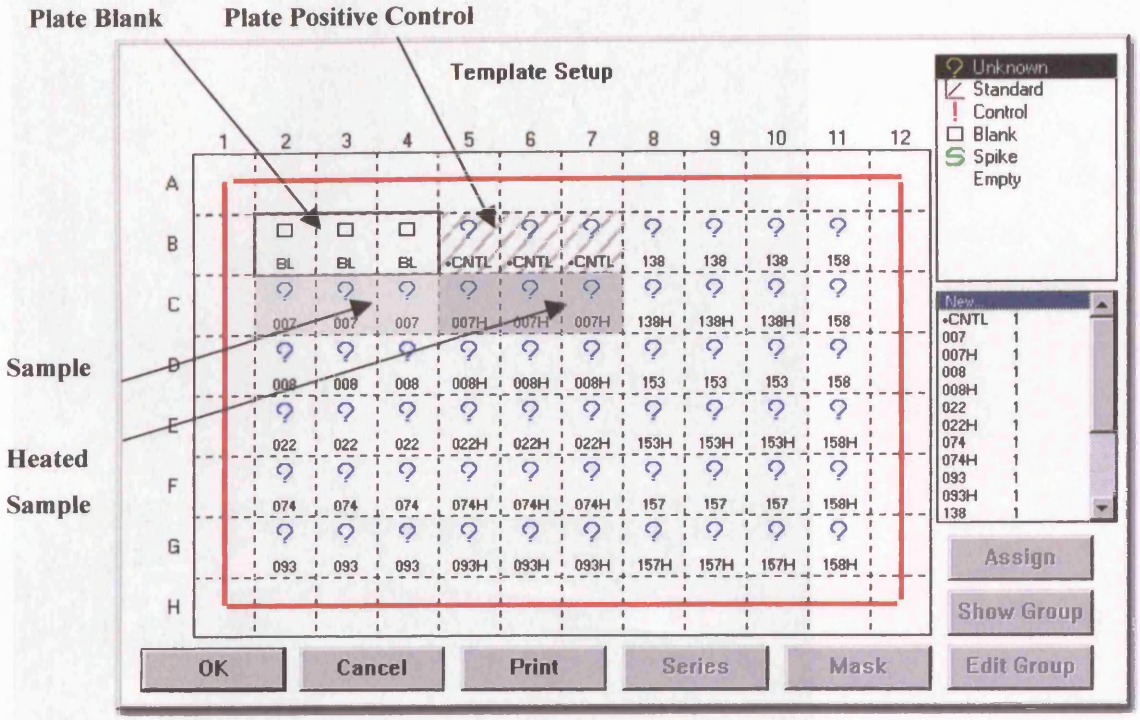
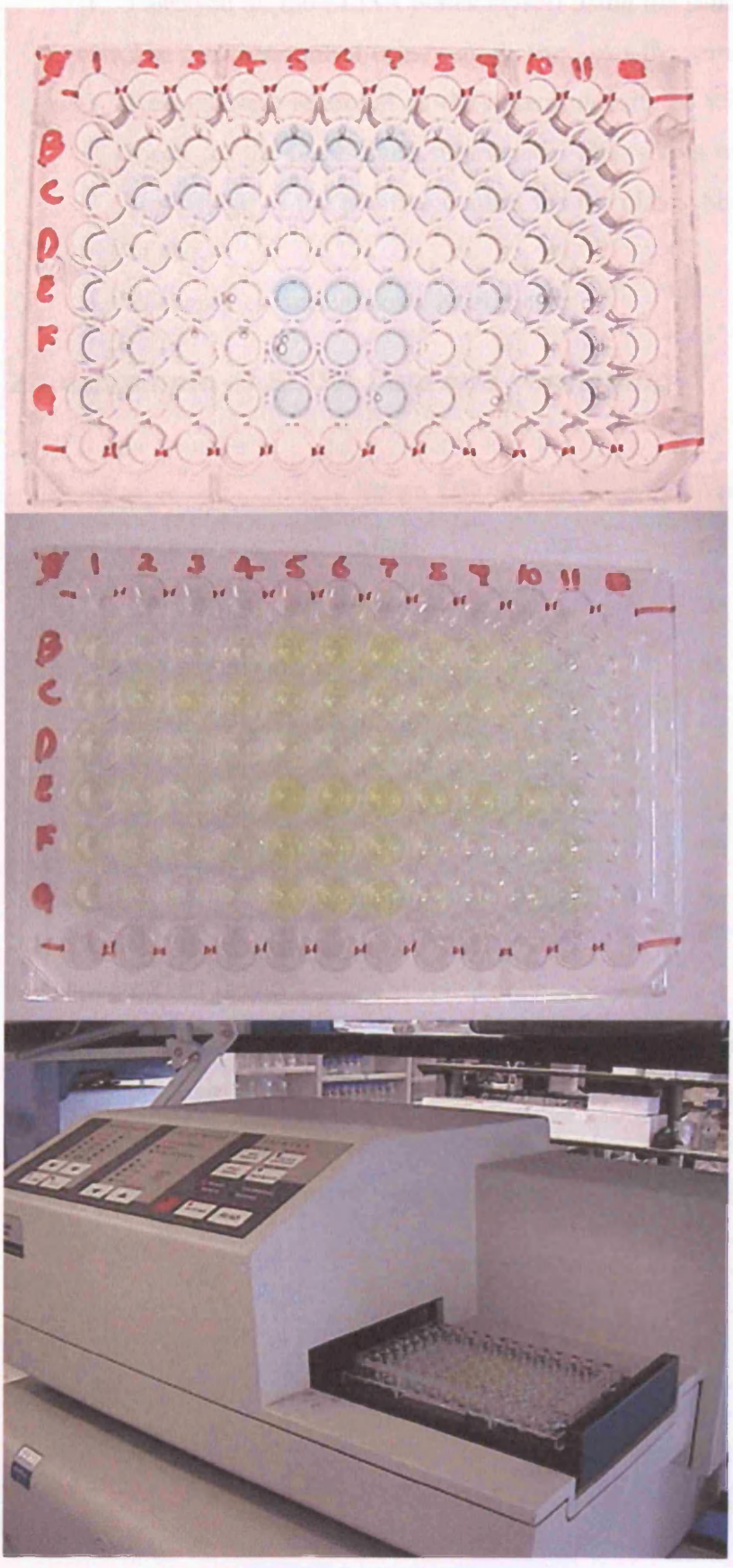


Figure 2. 6. ELISA plates (a) after incubation with TMB, (b) after addition of HCL and (c) in situ on plate reader.



a

b

c

2.1.3 Standardisation and error minimisation for the ELISA assay.

1. I assayed all the ELISA plates myself using the same lab equipment.
2. The positive control urine sample used was the same for all ELISA plates.
3. Results were recorded as the optical density at 450nm (OD450) with the mean for the plate blank subtracted. The results were then expressed as a percentage of the positive control for the plate. See below for an example of this.
4. All samples were assayed in triplicate

2.1.4 Example of ELISA Data and Formatting

Sample	**Mean OD**	**SD**	Well	**OD**
Plate Blank	**0.098**[1]	0.003	B2	0.101
			B3	0.095
			B4	0.099
Plate Positive Control	**0.440**[2]	0.034	B5	0.453
			B6	0.402
			B7	0.466
254	0.154	0.013	C2	0.141
			C3	0.166
			C4	0.155
255	0.796	0.041	C5	0.841
			C6	0.759
			C7	0.789

Subtract the plate blank mean value (1 above) from all mean OD values, including the plate positive control. The result for each sample mean is expressed as a percentage of the plate positive control mean (2 above). A cut-off of 80% or greater has been defined as a 'positive' result according to the Rodaris validation document.

E.g.

For sample 254

Sample mean	0.154	-- x
Subtract plate blank mean	0.098	-- b
	0.056	

Express this figure as a percentage of the mean plate positive control (after also subtracting the blank from this value) -- z

$0.056 \div (0.440\text{-}0.098)\%$

$= 16\%$ **i.e. A 'Negative' Result**

For sample 255

Sample mean	0.796
Subtract plate blank mean	0.098
	0.698

Express this figure as a percentage of the plate positive control (after also subtracting the blank)

$0.698 \div (0.440\text{-}0.098)\%$

$= 204\%$ **i.e. A 'Positive' Result**

To summarise, the formula for calculating the results is:

$$R = \left(\frac{\overline{x} - \overline{b}}{\overline{c} - \overline{b}} \right) \times 100\%$$

Where R is the final result for a given urine sample

$\overline{x}$ is the mean from triplicate wells for the sample

$\overline{b}$ is the mean of the plate blank wells

$\overline{c}$ is the mean of the positive control wells

This method of expressing the data will be used for all the clinical studies on the following chapters.

2.1.5 Standardising Urine Samples

Creatinine Correction and Creatinine Assay

The standard correction/standardisation for 24-hour urine collection is the creatinine content of the sample. Creatinine is a nitrogenous waste product and 24-hour production is proportional to body muscle mass. The molecular mass is 113.1 (Figure 2.7).

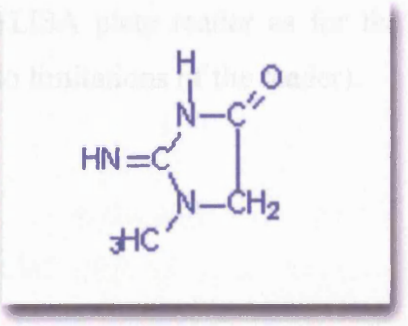


Figure 2. 7. Creatinine

It was possible to use this as an independent correction factor for the samples as the Coomassie Blue protein stain does not pick up creatinine in the samples due to the lack of free amino groups on the molecule.

Samples were therefore assayed for creatinine content using a standard lab assay (Roche Integra) at the Chemical Pathology Department, University College London Hospitals NHS Trust (Windeyer Building). All samples were anonymised prior to hospital lab analysis.

Protein Assay

Samples were assayed by using Bio-Rad Protein Assay (purchased from Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), which is based on the shift in the absorbance maximum for Coomassie Brilliant Blue G-250 from 465nm to 595nm when binding to protein (stoichiometrically, based on charged groups, for most proteins and polypeptides with MW >5000).

An Albumin Standard solution of an initial 2mg/ml was used (purchased from Micro BCA Protein Assay Reagent Kit, Perbio Science UK, Tattenhall, Cheshire, UK) to

make the required dilutions for the standard curve. Dilutions of 1, 5, 10, 15, 20 and 25 μg/ml were made and 1ml aliquots of each dilution were stored at -20°C until use. Urine samples were defrosted and assayed at a 1 in 10 dilution to read in the linear portion of the absorbency spectrum. 80 μl of urine sample, 720 μl of distilled H2O and 200 μl (1ml total volume) of the Protein Assay solution were well mixed in Eppendorf tubes. 100 μl of this solution was pipetted in triplicate into the ELISA plate wells. Each plate was assayed with triplicate blank wells (H_2O and Protein Assay Solution only) and each of the dilutions for the standard curve plus 25 urine samples. The plates were read on the same ELISA plate reader as for the urine ELISAs at absorbencies of 565 and 650nm (due to limitations of the reader).

2.2 Serum Samples

2.2.1 Collection and Processing of Blood Samples

Blood samples were collected from preeclamptic and normal women recruited at King Edward VIII Hospital, Durban, South Africa (Chapter 6, IPGs in Established Preeclampsia). Local Research Ethics Committee approval had been given for the study. As described in chapter 6, the samples were from

1.Peripheral (antecubital) vein
2.Uterine broad ligament vein
3.Umbilical artery
4.Umbilical vein

Blood samples were taken by syringe using a 21G (green) needle and transferred to plain Vacutainer tubes (Beckton-Dickinson, Oxford, England) and processed within 3 hours of sampling time. Samples were spun in an Eppendorf 5804R centrifuge (Eppendorf, Hamburg, Germany) for 5 minutes at 5000rpm (≈4200g) at room temperature. Serum was then stored at -20°C at the King Edward VII Hospital until transport to the UK.

2.2.2 Transport of Samples

Sample tubes were taken on dry ice via overnight air flight and delivered to University College London. Total transit time was 24 hours and samples remained at or below -20°C at all times. Samples then remained frozen at University College London until analysis.

2.2.3 IPG Extraction Protocol

The method for the IPG extraction has been adapted from Shashkin et al (Shashkin et al. 1997), as originally described by Larner, (Larner et al. 1988), Asplin (Asplin et al. 1993) and others, originally from the method of Cheng et al (Cheng et al 1984). In summary, the method uses separation of the IPG fractions from human serum using anion (Cheng et al 1984) exchange columns and elution with HCl.

2.2.4 Anion Exchange Resin

The basis of the extraction is an anion exchange resin. The AG (analytical grade) resin is a strongly basic anion exchanger. It is capable of exchanging anions of acidic, basic and neutral salts on the basic side if its pI. The formate form of the resin is used in the extraction. Relative selectivities of the AG 1 resin are shown in Table 1.

Table 2. 1. Relative Selectivity of Various Counterions*

Counterion	Relative Selectivity
OH^-	1.0
Cl^-	22.0
HPO_4^-	5.0
Formate	4.6

*From Product Information, supplied by Bio-Rad

The separation relies on the phosphate groups on the IPGs to be attracted to the resin. It can then be eluted off by use of increasing concentrations of Cl^- in hydrochloric acid.

2.2.5 Materials

Formic Acid and Hydrochloric Acid were purchased from BDH Laboratory supplies (Poole, Dorset, UK). 2-mercaptoethanol, Ethylenediamine Tetraacetic Acid (EDTA, Tetrasodium Salt), charcoal and ammonia solution were purchased from Sigma (Poole, Dorset, UK). Anion exchange resin (AG 1-X8, hydroxide form) and Polyprep chromatography columns were purchased from Bio-Rad Laboratories (Hemel Hempstead, UK). Nalgene® Polycarbonate centrifuge tubes were purchased from Nalge Company (Rochester, New York, USA).

2.2.6 Conversion of Resin

The AG1-X8 resin in **hydroxide** form was converted to the **formate** form. This was achieved by passing 2N Formic acid through the resin column to a pH of 2.0. Distilled water was then passed through the column until a pH of 4.0 was achieved.

2.2.7 Extraction Process

1ml serum was mixed with 3 ml extraction solution (Formic Acid 50 mmol/l, 2-mercaptoethanol 1mmol/1, EDTA 1mmol/l (final concentrations). The samples were heated at 100°C for 5 min in an oven and then cooled on ice. Following this, 20mg of charcoal was added to each sample. After 10 minutes the samples were centrifuged at 40000 × g for 30 minutes. (Beckman UltraCentrifuge, Model J2-21, 18,000 rpm, rotor type JA20).

The supernatants were neutralised with NH_4OH to pH6.0 and centrifugation repeated for a further 30 minutes. Anion exchange resin (formate form, 1.6ml) was added to each supernatant. The slurry was kept on ice in a fridge cabinet for approximately 20h with agitation. Each sample was then poured into a PolyPrep column.
Stepwise elution was then carried out with 3 bed volumes (i.e.4.8ml) of distilled water, 5 bed volumes (8ml) of HCl pH 2.0 (10 mmol/l) – (Eluate A) and 5 bed volumes (8ml) of HCl pH 1.3 (50 mmol/l) (Eluate B). Eluate A was neutralised with NH_4OH to pH4.0.

Both eluates A & B were frozen with liquid nitrogen and lyophilised, reconstituted with 500µl distilled H_2O and re-lyophilised in Eppendorf tubes. Extracts were then frozen at –20°C until time of the bioassay. At the time of the bioassay, samples were reconstituted as described below.

After the extraction of a proportion of the samples, it was decided to reduce the extraction volume by half due to the small quantity of some of the available samples. Thus, for these samples, 0.5ml serum was mixed with 1.5 ml extraction solution and the samples were heated at 100°C for 5 min in an oven and then cooled on ice. Following this, 10mg of charcoal was added to each sample. Centrifugation and neutralisation were the same, but only 0.8ml of the anion exchange resin was added. Similarly, on day 2 of the extraction, in the columns stepwise elution was carried out

with 3 bed volumes (i.e.2.4ml) of distilled water, 5 bed volumes (4ml) of HCl pH 2.0 (10 mmol/l) – (Eluate A) and 5 bed volumes (4ml) of HCl pH 1.3 (50 mmol/l) (Eluate B). Eluate A was again neutralised with NH_4OH to pH4.0.
Freezing and lyophilisation were as above.

A. pH 2.0 fraction activates PDH phosphatase = pH 2.0 IPG-P
B. pH 1.3 fraction inhibits PKA = pH 1.3 IPG-A
} No crossover activity seen

2.3 Amniotic Fluid Samples

Amniotic fluid samples were collected at caesarean section from preeclamptic and normal women recruited at King Edward VIII Hospital, Durban, South Africa (see Chapter 6, IPGs in Established Preeclampsia). 5ml samples were collected as detailed in the study protocol with a syringe and frozen within 3 hours of collection. Transport of the samples was as for serum and assay with the ELISA was performed as for the urine samples.

2.4 Validation of Extraction Procedure

The extraction procedure as described here, using heat denaturation and charcoal treatment at acid pH, followed by anion-exchange column chromatography and acid elution was first described in 1988 (Larner et al. 1988), from the method of Cheng and colleagues (Cheng et al 1984). It is based on heat denaturation of protein, charcoal adsorption of nucleotides and anion exchange resin chromatography, yielding both fractions of IPG separately.

This extraction method has been used by Caro et al (Caro et al. 1997) to characterise the different IPG fractions. In this paper, the pH2.0 fraction from the extraction was shown to stimulate PDH phosphatase and the pH1.3 fraction stimulated lipogenesis. There was no cross-over reactivity shown (Caro et al. 1997). In the same work, human liver-extracted IPG A and P type were shown to be structurally different and to have distinct insulin-like effects.

2.5 IPG Bioassay

2.5.1 IPG-P

The bioassay of the IPG-P type fraction involves the activation of Pyruvate Dehydrogenase complex (PDC), as first described in 1992 (Lilley et al. 1992) and subsequently applied by Caro et al (Caro et al. 1997). The method described is similar to that described by Caro et al, modified to allow multiple simultaneous assays to be read in a multiwell plate reader.

The PDC and the Pyruvate Dehydrogenase (PDH) phosphatase were kindly donated by Dr S Kunjara (Rodaris Pharmaceuticals, Oxford, UK) having been prepared as described (Lilley et al. 1992) and stored at -80°C until use. The assay for the PDH phosphatase, in the presence or absence of insulin mediator, was based upon the initial rate of the activation of the inactivated phosphorylated PDH complex.

The initial activity of the PDC was 25 units/ml (where 1 unit of enzyme produces 1μmol NADH/min). After inactivation with ATP, this value was reduced to 1% of the total activity.

A two-stage assay was used to quantitate the phosphatase activity. All samples were assayed blind and in duplicate on adjacent plate wells. Standards and water blanks were used on each plate.

A sample of inactivated PDC was pre-incubated at 30°C with 1mg/ml fat-free bovine serum albumin (BSA) containing 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM dithiothreitol and 20 mM potassium phosphate buffer, pH 7.0 for 3 min.

At this time, IPG-P extracts were reconstituted in 24 μl distilled H_2O to form stock solutions. 2 μl of each solution (equivalent to 83μl of serum) was added to the centre of a 96-well plate (Nunc Maxisorb® ELISA plate, Fisher Scientific Ltd Loughborough, UK). 25 μl of the PDC mixture was then added to each well. The reaction was initiated by the addition of 2 μl of PDH phosphatase and incubated for 3mins. At the end of this time 14 μl of chilled 0.3M NaF was added to each well to stop the reaction.

The activated PDH was determined at the second stage spectrophotometrically by measuring the rate of production of NADH. 200 µl of reaction mixture containing 50 mM potassium phosphate buffer at pH 8.0, 2.5 mM of the oxidised form of NAD^+, 0.2 mM thiamine pyrophosphate (TPP) 0.13 mM coenzyme A, and 0.32 mM dithiothreitol was added. This was followed by 20 µl 2 mM sodium pyruvate for each plate. The production of NADH was followed using an absorbance at 340 nm for 7 minutes. One unit of IPG PDH activity is the amount required to increase the basal rate by 50%.

2.5.2 IPG-A

Due to time constraints the IPG-A activity was not carried out at this time.

2.6 Validation of IPG-P Bioassay Procedure

The assay of IPG-P activation of the pyruvate dehydrogenase complex has been established for a number of years as referenced above. The Pyruvate Dehydrogenase (PDH) complex is a mitochondrial multi-enzyme complex with 3 active site regions, which converts pyruvate to Acetyl-CoA and as such is key in glucose metabolism. Multiple factors affect its activity through kinase (inactivating) and phosphatase (activating) enzymes, which may be tissue-specific. It has been shown that IPG-P stimulates PDH phosphatase in certain tissues (Gottschalk and Jarett 1988; Macaulay and Larkins 1990) and thus this has been used as a measure of IPG-P activity. Figure 2.8 illustrates the role of IPG-P in modulation of the PDH complex.

Although not commercially prepared, the PDC preparation was standardized as described in Chapter 2.5.1 above so initial activity of the preparation was always identical.

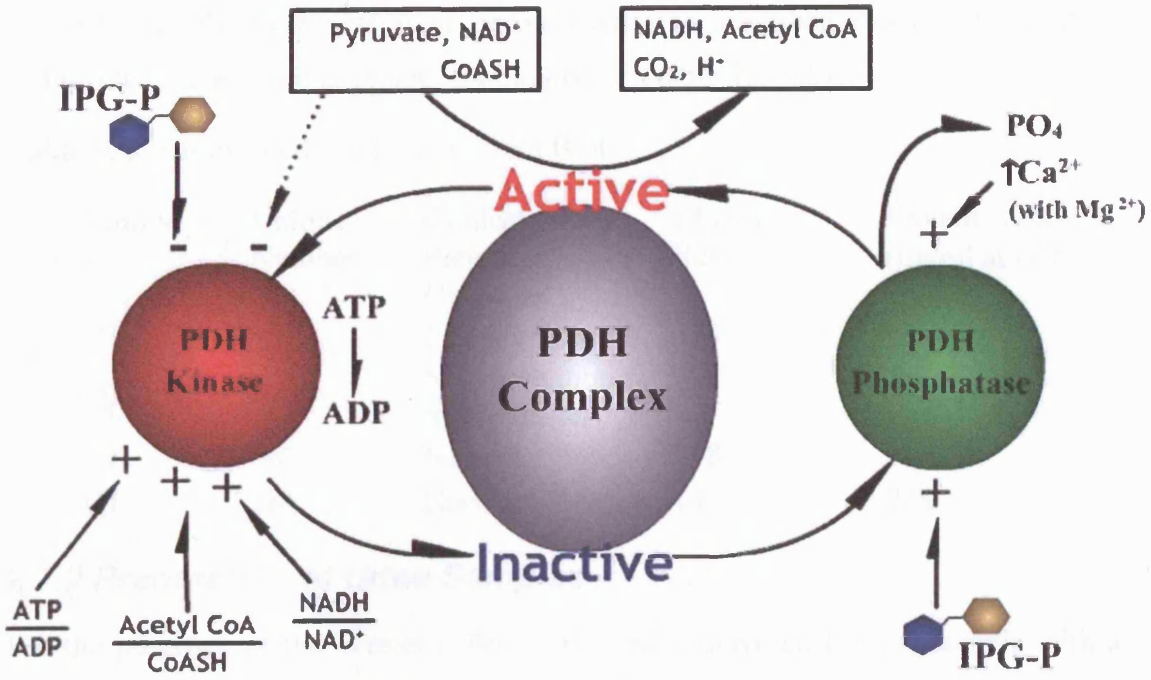


Figure 2. 8. Control of the pyruvate dehydrogenase (PDH) complex. IPG-P binds and lowers Ca^{2+} binding coefficient (KD), leading to increased Ca^{2+} binding which activates the phosphatase enzyme. NB kinases and phosphatases seem to be tissue-specific

2.7 Western Blot

The Western Blot of 4 urine samples was carried out at the end of the research period to determine what protein fractions might be being detected by the polyclonal serum used in the ELISA assays of the urine samples. It was decided that this might shed some light on the quality of the preparation used in the inoculation of the rabbits in the preparation of the polyclonal serum. This is discussed more fully in Chapter 7.

2.7.1 Selection of Urine Samples

Due to limitations of equipment available at the time of the experiment (the number of lanes that could be created with the comb for the gel) 4 urine samples could be assayed. I randomly selected two urine samples from women with non-hypertensive pregnancy outcome (the subject identities of which were not known to me at the time), one with a positive ELISA result, and one negative. One sample was selected from a woman with clinically diagnosed preeclampsia and one sample from a subject with hypertension but no proteinuria on dipstick. All samples had previously been

assayed using the ELISA assay and protein assay as described above. The samples and results of these two assays are summarised in table 2.2 below:

Table 2. 2. Samples selected for Western Blot

Sample	Unique Number	Clinical Pregnancy Outcome	ELISA Result	Protein Assay (μg/ml at OD 565)
①	321	Preeclampsia	313	630
②	432	Non-proteinuric hypertension	212	82
③	262	Normal	208	367
④	264	Normal	14	228

2.7.2 Preparation of Urine Samples

For the purposes of the Western Blot, it is ideal to have each urine sample with a similar quantity of protein at the time of the experiment. The ideal value is 25-50 μg protein in 50 μl of sample. Thus aliquots of each urine sample were spun in 10,000MW Vivaspin concentrator tubes (Purchased from Vivascience AG, Hanover, Germany) at 3000rpm in a Sorvall RT7 Centrifuge for 5 – 15 minutes until the correct volume (50 μl) was reached. The aliquots were removed and an equal volume of loading buffer (Laemmli – Sigma, Poole, Dorset, UK) to each one. The 100 μl samples were then frozen at -20°C for 48 hours until the rest of the experiment.

At the time of the experiment, the samples were defrosted and heated at 100°C with the added buffer for 3 minutes.

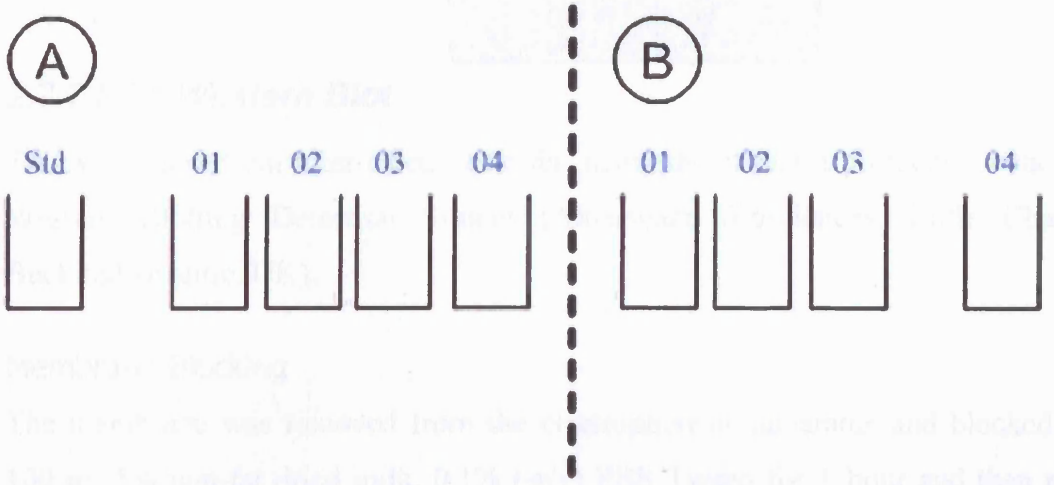
2.7.3 Gel Polymerisation

The gel was prepared with the Protogel System (Purchased from National Diagnostics, Atlanta, Georgia, USA). The following reagents were mixed to make 100ml of gel: Protogel (26.7ml) Protogel Buffer (26.0ml) deionised H_2O (46.2ml), Tetramethyl-Ethylene Diamine (TEMED, Purchased from Sigma, Poole, Dorset, UK) (100 μl) and Ammonium Persulphate (1ml). The gel was injected between the spaced glass plates and overlaid with isobutanol to facilitate polymerisation. The overlay was poured off and the stacking gel prepared in a similar way.

The following reagents were mixed to make 20ml of the stacking gel: Protogel (2.6ml), Protogel Stacking Buffer (5.0ml), deionised H_2O (12.2ml), TEMED (20 µl), Ammonium Persulphate (0.1ml). The Teflon comb was inserted into the stacking gel and it was allowed to set. After setting, the comb was removed and the wells washed with buffer to remove unpolymerised acrylamide.

The two times four 100 µl samples loaded, along with a protein standard mixture for molecular weights 30,000-200,000 (Sigma, Poole, Dorset, UK) as shown below:

Figure 2. 9. Western Gel Set-up



2.7.4 Electrophoresis

The electrophoresis apparatus was assembled and attached to power supply at 100V (8 V/cm). After running the gel for 90 minutes, this was increased to 200V (15 V/cm) and run until the bromophenol reached the bottom of the gel. When the glass plates had been removed, the gel was divided along the dotted line depicted in Figure 2.9 above.

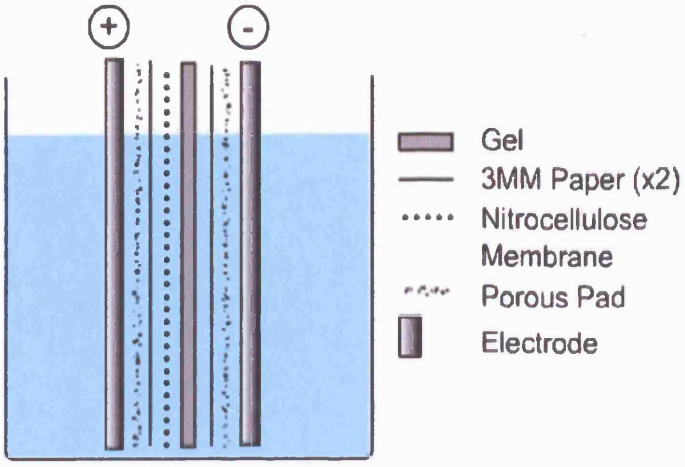
2.7.5 Staining and Destaining of Gel

Side 'A' of the gel was stained with Coomassie brilliant Blue (0.25g) in 100 ml Methanol: H_2O: Acetic Acid mixture (proportions of 45:45:10 respectively) overnight at room temperature on an agitator plate. Destaining in the above solution without the dye was carried out the next day and the gel was dried with a vacuum drier.

2.7.6 Electro-blotting of Gel

The electro-transfer apparatus was set up as in Figure 2.10, using a Hybond® ECL nitro-cellulose membrane (Purchased from Amersham Biosciences) and run overnight (with on a stirrer plate) at 4°C at 120 mA current.

Figure 2. 10. Electro-transfer apparatus



2.7.7 ECL Western Blot

This was carried out after electro-transfer, using the standard protocol for the ECL Western Blotting Detection System (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Membrane Blocking

The membrane was removed from the electrophoresis apparatus and blocked with 100 ml 5% non-fat dried milk, 0.1% (w/v) PBS Tween for 1 hour and then rinsed twice in PBS-Tween wash buffer.

Primary Antibody Incubation

The membrane was incubated in 1:6000 dilution of the IPG-P polyclonal serum (Rodaris Pharmaceuticals, Oxford, UK) for 1 hour at room temperature and rinsed with the wash buffer.

Secondary Antibody Incubation

The membrane was incubated with goat anti-rabbit HRP conjugate (Harlan Sera-Lab, Loughborough, UK), diluted 1 in 2000, for 1 hour at room temperature and rinsed for a third time in the wash buffer.

2.7.8 Detection

Detection was with the ECL + Plus Western Blotting Detection System (Purchased from Amersham Biosciences). The membrane was used to expose radiograph film.

Chapter 3

Urinary IPG-P in Normal Pregnancy

3.1 Introduction

At this point in time, no prospective data exist on the levels of IPG in normal pregnancy. Kunjara et al have used a PDH activation bioassay to show increased levels of IPG-P in preeclamptic urine (Kunjara et al. 2000a). This study looked at 24-hour urine samples from only a small number of women (four). However, whilst this could be viewed as a current gold standard, in an outpatient clinic setting the 24-hour urine collection is not practical.

Furthermore at the commencement of this research project, no data was available on the clinical utility of the polyclonal-based ELISA assay, which had potential (on the basis of data provided by the company) to be a useful test in the diagnosis of preeclampsia. Thus it was decided not to use the bioassay for our urine samples for this prospective study, as the aim was to assess the clinical utility of the ELISA. Potentially it could become a simple bedside or outpatient clinic test and this prospective data would help to establish the future feasibility of this. Serial data from normal pregnancies was also lacking. Thus the aim of this portion of the research was to establish whether the ELISA assay could be used to:

- Establish a normal set of values for the ELISA over the course of normal pregnancy.
- Diagnose and/or predict preeclampsia in a group of subjects with 'low-risk' pregnancies – i.e. with no known pre-existing risk factors for preeclampsia (outlined in Chapter 1).

The study was set up with these two aims in mind. The standard protocol for the ELISA assay to be used in the study and supplied by Rodaris Pharmaceuticals has been discussed in Chapter 2.

Ideally, the data gathered would be for a group of 'low-risk' women from early in pregnancy, through to term (greater than 37 completed weeks of gestation from the last menstrual period).

3.2 Setting up the Longitudinal Studies

3.2.1 Aim

The aim of the longitudinal study was to collect pilot data by following a group of women from pre- or early pregnancy to collect serial urine samples for analysis with a novel ELISA assay for P-type inositol phosphoglycans. Subsequently, this data would be used to establish the profile of urinary IPG-P in women with a normal pregnancy. These data have never previously been established. It was planned to recruit as large a number as possible of women in the time available who might subsequently develop preeclampsia.

3.2.2 Recruitment Considerations

Due to the presence of only one investigator (myself) to carry out recruitment and follow-up of subjects as well as all the laboratory assays of the urine samples, it was decided to restrict recruitment to one site (University College London Hospitals NHS Trust). As this project was collecting pilot data, a formal power calculation was not performed as it was instead decided to try to recruit as many subjects as time and manpower would allow.

3.2.3 Chronology

Initially, the study was set up to allow recruitment from two areas. Firstly, pre-pregnancy subjects from the Assisted Conception Unit (ACU) at University College London Hospitals NHS Trust (Group A) and secondly a control group form the Early Pregnancy Unit at the hospital (Group B). These subjects were to be matched for age, parity, smoking habits, ethnicity and number of fetuses. The aim was to recruit 100 women pre-pregnancy in group A. Allowing for a quoted fecundity rate of 30%, this would yield 30 subject pregnancies to be followed. Correspondingly, 30 subjects were then to be matched for group B. It was anticipated that a significant proportion of the ACU subjects would have multiple pregnancies.

Full Research Ethical Committee approval was given on 7th April 2000 to commence recruiting for the study.

Unfortunately, it became clear as recruitment began that the numbers of subjects being recruited from the ACU would not achieve the planned targets. Additionally, it was noted that the time scale of the follow up was quite lengthy, following the initial recruitment.

For these two reasons, it was decided to table an amendment to the protocol (Amendment 1, 17th June 2000) to include recruitment of a 'high risk' group of women from two clinics at the hospital. A high-risk obstetrics clinic is run on the Tuesday a.m. and patients were to be recruited from here and also the multiple pregnancy ultrasound scanning clinic in the Fetal Medicine Unit. Inclusion criteria are outlined below in Table 3.1.

Table 3. 1. Inclusion Criteria for High-Risk Subjects after Amendment 1.

Multiple pregnancy
Previous preeclampsia/eclampsia or IUGR*
Pre-existing Vascular pathology -
Diabetes or Essential Hypertension
Previous abruptio placentae
Certain types of fertility treatment
Sickle Cell Trait
Renal Disease**

*Defined as fetal birthweight below 10th centile for gestation as per Chitty and colleagues (Chitty et al. 1994a; Chitty et al. 1994c; Chitty et al. 1994b)
**Previous renal transplant or demonstrated renal impairment with serum creatinine >100mmol/l

The amendment was approved on July 19th 2000 and the new recruitment commenced on 5th September 2000.

Furthermore, it was apparent that recruitment from the Early Pregnancy Unit would be neither possible (due to the restricted space and time available) nor appropriate in some cases. Thus in order to boost numbers of normal, low risk subjects the location of recruitment was switched to the antenatal clinic setting, where subjects would first attend at between 10 and 13 weeks of gestation. Additionally, to maximise numbers, subjects were not matched at the recruitment stage and simply recruited as a cohort.

3.3 The Study – Setting and Methods

3.3.1 Setting

It was established that a broad range of data points was desirable, covering gestations from the first to the third trimesters for a number of women. The local hospital is University College London Hospitals NHS Trust. The Trust has a busy obstetric unit within the Elizabeth Garret Anderson and Obstetric Hospital with an antenatal clinic.

A link with the hospital had been previously established and the first step was to discuss with the Clinical Director about recruiting patients from the antenatal clinic. Basic approval was given at this point and a full ethical committee application was made.

3.3.2 Recruitment of Patients and Patient Numbers

I attended the antenatal clinic at the Elizabeth Garret Anderson and Obstetric Hospital. 40 'low risk' women were recruited from the Monday a.m. clinic between November 2000 and September 2001. Low risk was defined as the absence of any inclusion criteria for the 'high risk' group for the other parts of the study (Table 3.2 and Chapter 5, High Risk Subjects). The subjects were recruited at their booking visit and were both primigravidae and multigravidae.

Table 3. 2. Exclusion Criteria for Low-Risk Subjects

Multiple pregnancy
Previous preeclampsia/eclampsia or IUGR*
Pre-existing Vascular pathology -
Diabetes or Essential Hypertension
Previous abruptio placentae
Certain types of fertility treatment
Sickle Cell Trait
Renal Disease**

*Defined as fetal birthweight below 10th centile for gestation as per Chitty and colleagues (Chitty et al. 1994a; Chitty et al. 1994c; Chitty et al. 1994b)

**Previous renal transplant or demonstrated renal impairment with serum creatinine >100mmol/l

At recruitment, the study was discussed in full with the subjects and they were instructed to read the Patient Information Sheet (Appendix 2). After further discussion and if informed consent to participate was given, the subject was asked to sign a consent form (the same form was used for all the clinical studies) – see Appendix 5.

The original aim at the time of submission of the study was to collect certain samples from all the subjects at the time of delivery (placenta, cord blood, uterine vein blood in the event of a caesarean section). It became clear as the study progressed that this would not be practical or desirable due to (a) limited freezer storage space and (b) lack of intent to analyse multiple blood or placental samples from non-preeclamptic women.

3.3.3 Collecting samples

The gold standard assay for protein analysis is currently the 24-hour urine collection. Although there is poor predictive value from urine dipstick testing, accuracy may be improved at higher thresholds (greater than 1+ proteinuria)(Waugh et al. 2004). For the purposes of the longitudinal study it was decided that dipstick testing was the most practical. A protein assay of each sample was carried out as defined in the Laboratory Methods (Chapter 2).

All patients were asked to provide a mid-stream urine sample (MSU) at the time of their clinic attendance. These samples were collected in MSU sample pots provided by the antenatal clinic solely for this purpose. I collected these samples after standard Albustix® (Bayer plc Diagnostics, Newbury, Berkshire, England) dipstick testing for protein, performed by the clinic staff or myself. Occasionally, if the initial sample provided was too small, or if the MSU sample needed to be sent for microbiological culture, the patient provided a second sample in an identical container.

3.3.4 Patient tracking and follow-up

Patients were anonymously logged in a record with details of initials and hospital number to allow tracking of future appointments via the hospital Patient Access System (PAS). A weekly check of the PAS system by myself for all the patients in

the study established those attending for appointments. It was hoped at the outset that patients would be seen at routine times during their pregnancy as set out in the antenatal care plan. However, due to changes in hospital policy throughout the course of the study, subjects were often seen at variable and infrequent intervals in the antenatal clinic. At other times, the GP or Community Midwife saw subjects in a community setting.

3.3.5 Case report form completion

At the time of recruitment, basic demographic details were logged for each subject, including date of birth, medical and obstetric history, last menstrual period (and thus gestation) and expected date of delivery. At each subsequent visit where a urine sample was collected, a further sheet was filled out, with initials, date of birth and study number for identification purposes and also blood pressure (where taken) urine dipstick reading and any symptoms or signs of preeclampsia.

Following delivery I carried out a retrospective review of the case notes for each subject. I defined the hypertensive pregnancy outcome strictly by the previously described criteria (Tables 1.8 and 1.9). This did not necessarily agree with any clinically documented diagnosis in the notes. Accordingly then, a final data sheet was filled, with the details of the labour and delivery, and the outcome code as defined in the study protocol (Table 3.3 below).

Table 3. 3. Outcome Codes for Subjects

1. Preeclampsia (hypertension and proteinuria)
2. Pregnancy Induced Hypertension (PIH)(no proteinuria)
3. Preeclampsia superimposed on chronic hypertension
i.e. Hypertension present before 20 weeks of pregnancy
4. No hypertension

3.3.6 Labelling, logging and Storage of samples

All samples were labelled with details as on the example of sample label details below.

Initials..... Study Number.....
Date..... Sample No. .....
Time of Collection.....

Key to Label:
Date = Date of sample collection
Study No. = Subjects were assigned a unique study number at the time of recruitment
Sample No. = nth sample for this subject

All samples were thus anonymous at this stage to preserve patient confidentiality. At the time the samples were brought for storage, a unique number was given to each one, allowing a sample log to be kept and ensuring that samples could be accurately identified and traced back to the source patient. 2ml aliquots of the samples were taken in apex tubes. These tubes were labelled solely with the unique sample number and thus were completely anonymised. This served a twofold purpose. Firstly, it added to the patient confidentiality and secondly, it provided operator blinding for the time the ELISA assay was carried out. The 2ml aliquots and remaining sample were frozen separately at -20°C within 4 hours of sample collection.

3.4 ELISA Assay

3.4.1 ELISA Protocol

This is described in some detail in Chapter 2, Laboratory Methods. The protocol does not need to be further discussed at this point save to say that the same operator assayed all samples (myself). Operator blinding was achieved by the method outlined in the paragraph above.

3.4.2 Result analysis

All samples were run in triplicate as discussed in Chapter 2, Lab Methods. The ELISA plate reader, reading at an optical density (OD) of 450nm expressed the ELISA results in the form of a mean and standard deviation of the mean (s.d.).

3.4.3 Expression of Results

Results of the ELISA were expressed as a ratio of the plate positive control per mmol of creatinine (Units/mmol, where 1 Unit equals 1% of the plate positive control), as discussed in Chapter 2. For the subjects with serial labour samples, a single mean value was calculated to compare with the recruitment level for the same subject and paired t-test analysis was used to compare recruitment with labour samples using statistical software packages: Microcal Origin 5.0 (Microcal Software Inc., Northampton, MA, USA) and SPSS 11.5 (SPSS Inc., Chicago, IL, USA).

3.5 Results

40 low-risk women were recruited from the antenatal clinic in the ten months between 27th November 2000 and 3rd September 2001. 3 women were lost to follow up due to moving address and/or delivery at other centres. Clinical and demographic details for the subjects are summarised in Table 3.4 below. Mean age of the subjects was 32 years and mean BMI was 23.4. Median gestation at the time of recruitment (which was usually at the booking visit) was 11.9 weeks (range 9.1-19.6 weeks).

Table 3. 4. Details for Recruitment Low-risk Subjects

Demographic		
	Mean	(sd)
Age (y)	32	(±5.0)
BMI	23.4	(±3.5)
Ethnicity	n	(%)
Caucasian	39	(97.5)
Asian	1	(2.5)
Parity		
Primiparous	29	(72.5)
Multiparous	11	(27.5)

BMI = Body Mass Index

4 (10%) of the women were smokers, 2 (5%) had a definite family history of preeclampsia in a first-degree relative (mother or sister). At the booking visit, mean blood pressure was 105 systolic over 65 diastolic (Mean Arterial Pressure, MAP = 92).

Delivery details for the 37 women followed through are summarised in table 3.5 below. Median gestation of delivery was 40.9 weeks (range 37.1-42 weeks). A median of five urine samples was collected for each subject between recruitment and delivery. 62% (n=23) of the low-risk women laboured spontaneously and 46% (n = 17) overall had a normal vaginal delivery, which was lower than expected. This may be due to small numbers but is in fact a higher vaginal delivery rate than for the high-risk groups (Table 5.7) and may in fact be a reflection on overall delivery demographics for an inner London teaching hospital. A further 21% had assisted vaginal delivery with the remainder delivering by elective or emergency caesarean section.

Table 3. 5. Details for Pregnancy, Labour and Delivery Low-risk Subjects

Pregnancy	Median	(Range)
Gestation at recruitment (weeks)	11.9	(9.1-19.6)
Gestation at delivery (weeks)	40.9	(37.1-42)
Samples per patient	5	(1-9)
Onset of Labour	**n**	**(%)**
Spontaneous	23	(62)
Induced	11	(30)
Caesarean Section	3	(8)
Delivery	**n**	**(%)**
Normal Vaginal	17	(46)
Assisted Vaginal		
Ventouse	6	(16)
Forceps	2	(5)
Elective Caesarean Section	2	(5)
Emergency Caesarean Section	10	(28)

Primary outcomes for the subjects are summarised in Table 3.6 below and are based on the ISSHP classification as discussed in Chapter 1 (Brown et al. 2000). The overall preeclampsia rate for the low-risk women was 7.5% (n=3) with a further

7.5% (n=3) developing non-proteinuric hypertension (Table 3.6). This is comparable with other studies regarding the population prevalence of the disorder.

Table 3. 6. Details of hypertensive outcome for low-risk subjects

	n	(%)
Preeclampsia (hypertension and proteinuria)	3	(7.5)
Pregnancy Induced Hypertension (no proteinuria)	3	(7.5)
Preeclampsia superimposed on chronic hypertension	0	
No hypertension	31	(77.5)
Lost to Follow-up	3	(7.5)

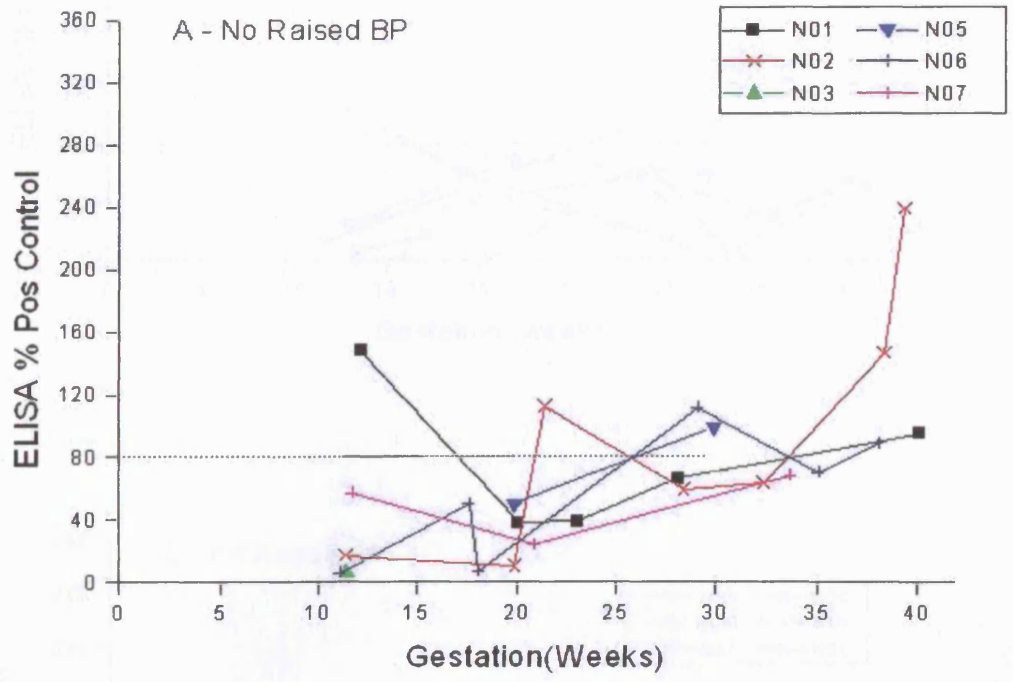
3.5.1 Normal Range for ELISA assay results

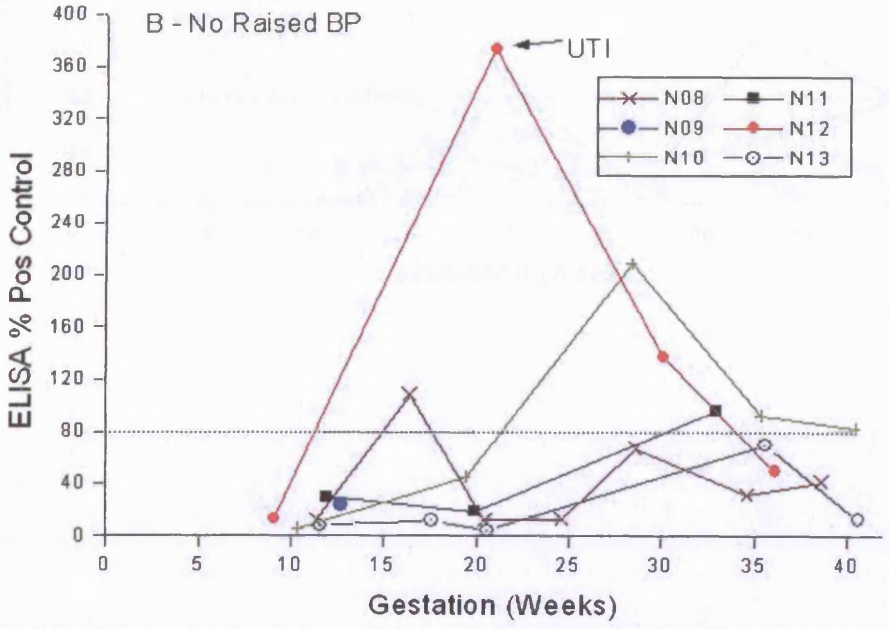
ELISA results for all subjects with no hypertension in pregnancy are displayed in figures 3.1-3.3 below. Serial values are shown for each subject, grouped numerically by study number. There is a large range of ELISA signal seen for these subjects. The median number of samples per subject was 5 (range 1-9).

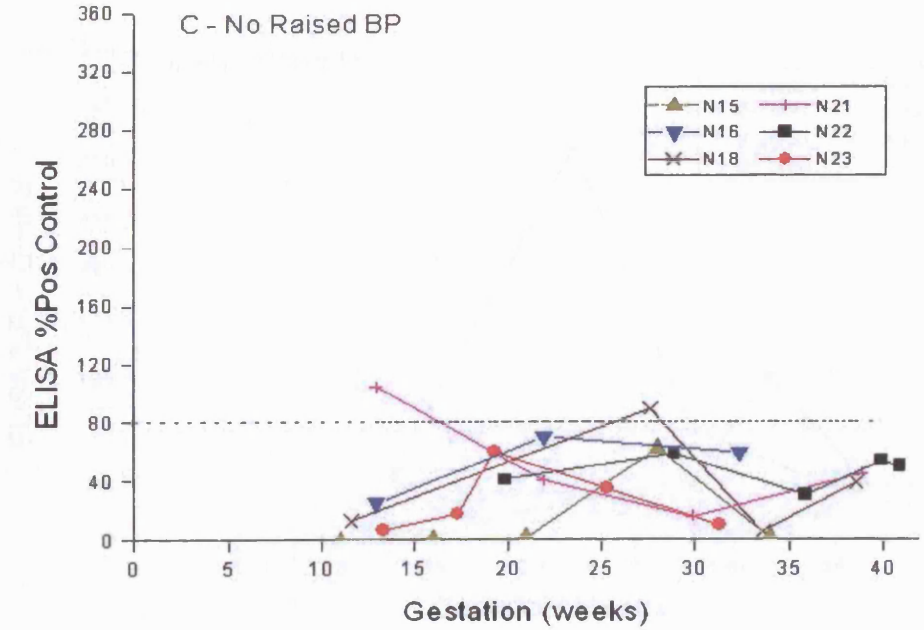
The pilot study design and resultant mix of single and serial samples from different study subjects does not make the creation of a true normal range possible. However, it is possible to take several messages from the individual plots below. Firstly, it can be seen that the majority of ELISA signal results from the samples lie below the nominal cut-off of 80% ppc (percent of the plate positive control) as established by the supplier of the ELISA protocol. Secondly however, it is noted that a certain number of 'false positive' ELISA results are seen at all gestations and for low- and high-risk subjects. Some can be possibly explained by the presence of a urine infection at the time of the sampling. For most of these false-positive results however, no obvious explanation exists.

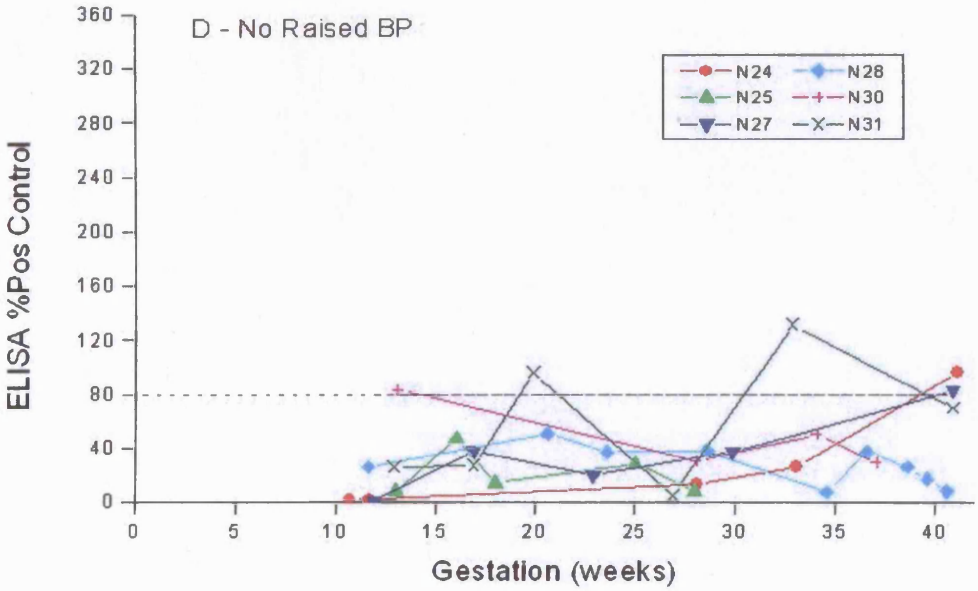
A certain false positive rate is usually accepted with any screening test (often 5%) and with a serious condition such as preeclampsia this may be an acceptable consequence.

Figure 3. 1. A-E Low-risk subjects. Individual serial ELISA-assay plots for all subjects with normotensive outcome, grouped by study number. ELISA values shown as a percentage of the plate positive control, as previously discussed. Dotted reference line shows cut off point for a 'positive test' (80% of plate positive control, ppc).






C - No Raised BP
ELISA %Pos Control
Gestation (weeks)
N15
N16
N18
N21
N22
N23
0
40
80
120
160
200
240
280
320
360
0
5
10
15
20
25
30
35
40


D - No Raised BP
ELISA %Pos Control
Gestation (weeks)
N24
N25
N27
N28
N30
N31
0
40
80
120
160
200
240
280
320
360
0
5
10
15
20
25
30
35
40

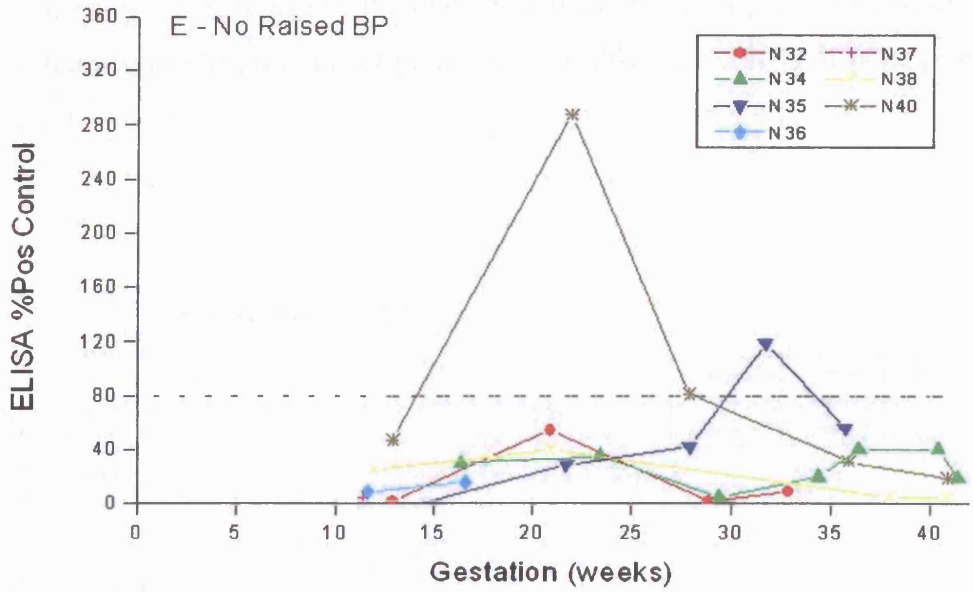
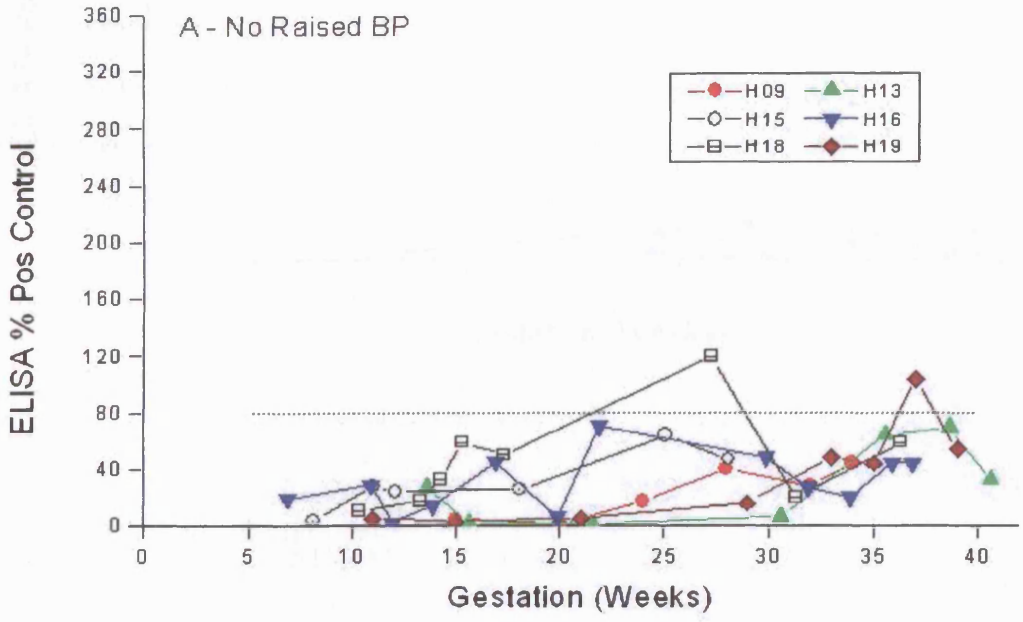
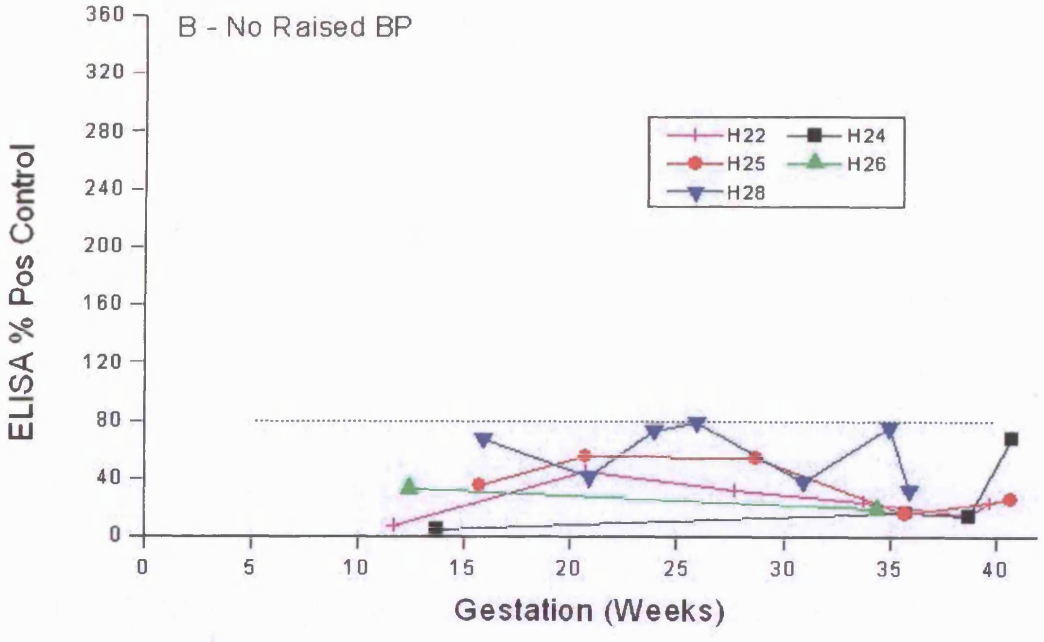

E - No Raised BP
N32
N37
N34
N38
N35
N40
N36
ELISA %Pos Control
360
320
280
240
200
160
120
80
40
0
0
5
10
15
20
25
30
35
40
Gestation (weeks)

Figure 3. 2. A-C High-risk subjects. Individual serial ELISA-assay plots for all subjects with normotensive outcome, grouped by study number. ELISA values shown as a percentage of the plate positive control, as previously discussed. Dotted reference line shows cut off point for a 'positive test' (80% of plate positive control, ppc).





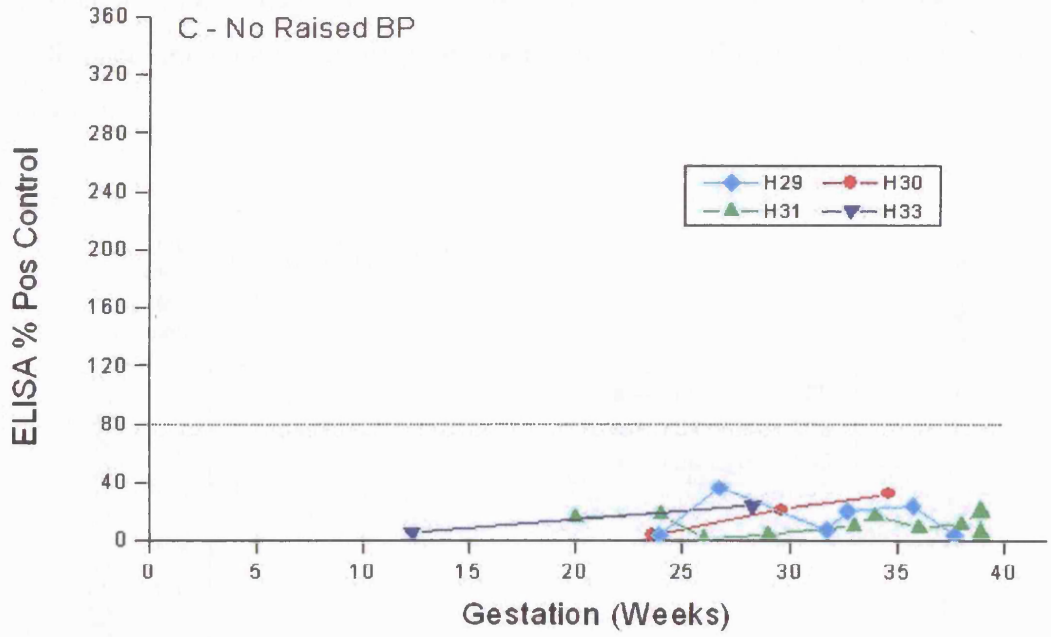
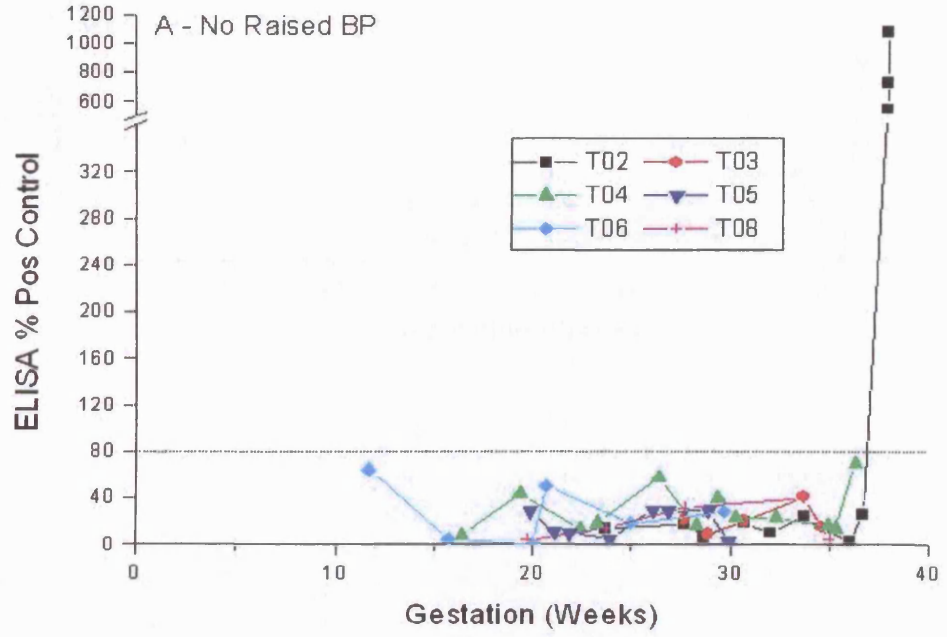
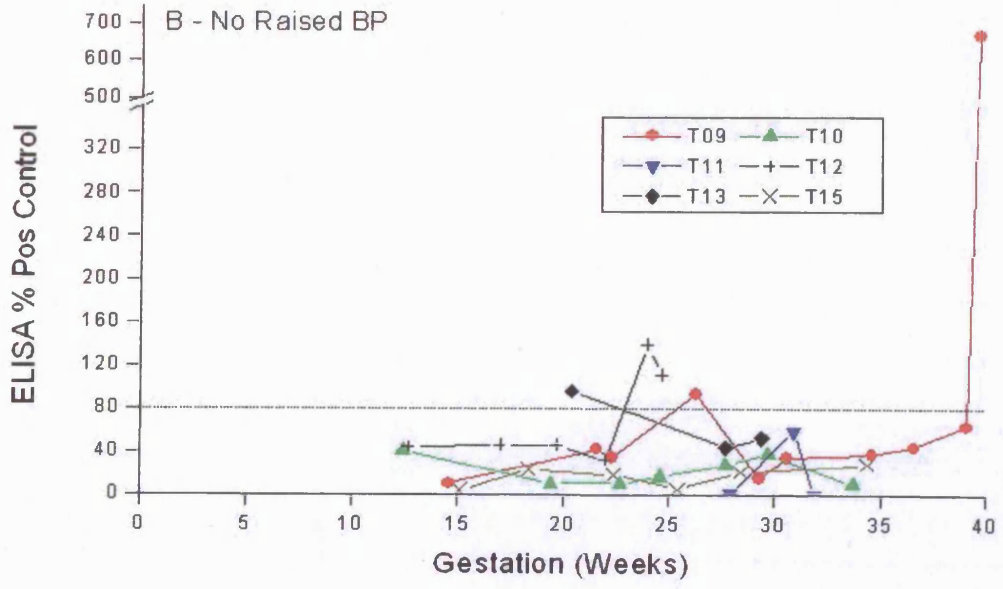
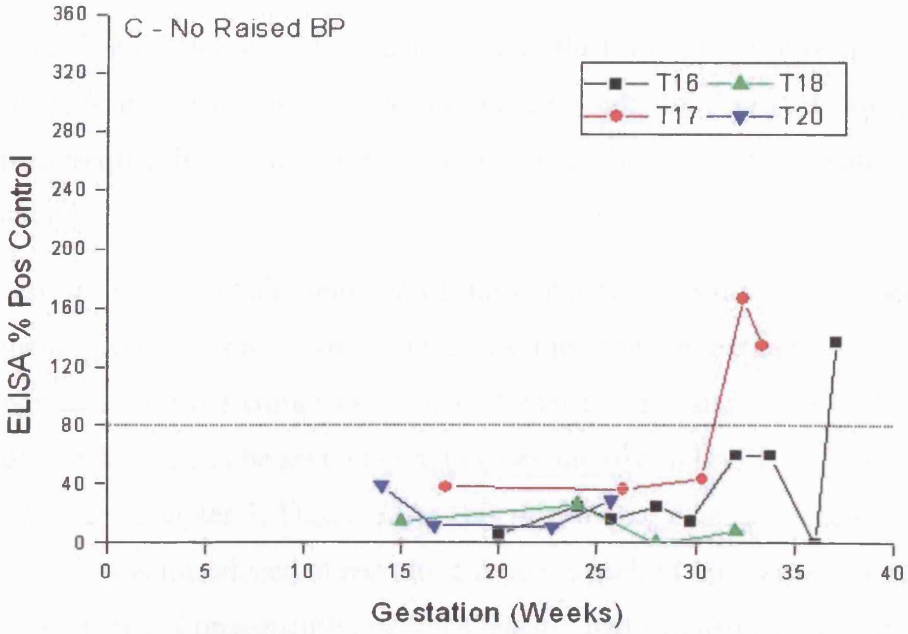

C - No Raised BP
ELISA % Pos Control
Gestation (Weeks)
H29
H30
H31
H33
360
320
280
240
200
160
120
80
40
0
0
5
10
15
20
25
30
35
40

Figure 3. 3. A-C Multiple pregnancy subjects. Individual serial ELISA-assay plots for all subjects with normotensive outcome, grouped by study number. ELISA values shown as a percentage of the plate positive control, as previously discussed. Dotted reference line shows cut off point for a 'positive test' (80% of plate positive control, ppc).






C - No Raised BP
T16
T18
T17
T20
ELISA % Pos Control
360
320
280
240
200
160
120
80
40
0
0
5
10
15
20
25
30
35
40
Gestation (Weeks)

3.6 Conclusions/Discussion

This is the first collection of longitudinal data illustrating the use of this polyclonal-based ELISA in normal pregnancy for the detection of IPG-P. Corresponding in women subsequently developing hypertensive complications of pregnancy are shown in Chapter 5.

It is easy to see a certain number of false positive results for women with no subsequent preeclampsia. In some cases this can be explained by concurrent pathology affecting the composition of maternal urine – such as a UTI (Figure 3.1 B). Additionally, it can be seen in certain cases the rise in IPG-P ELISA signal at the time of labour (Chapter 3, Figure 3.3). This mirrors the data later seen in Chapter 4. Possible bias was introduced at recruitment due to lack of interpreter for some ethnic minority patients. Consequently, patients usually had English as their first language or else were fluent in English.

3.6.1 Practical Problems of Sample Collection

Ideally, urine samples would be collected for each subject at each visit during her pregnancy, whether in the community or at the hospital. However, certain conditions stipulated by Rodaris Pharmaceuticals for the collection of the samples made this impossible (or at the very least highly impractical and expensive for a single investigator). The urine samples had to be frozen at -20°C within 4 hours of collection and to remain frozen until analysis. This prevented the patients posting samples to the investigator if they were not attending the hospital and courier transport of the samples would have been prohibitively expensive.

Practically, this meant that I would have to attend each community appointment for each patient and physically transport the samples back immediately to be frozen. When following over 100 patients this was obviously not possible. In fact, I took this approach for part of the study and this will be discussed further in Chapter 5. Low-risk subjects often only attended the antenatal clinic for booking and in the last month of the pregnancy and due to the above limitations, sample collection was unfortunately often infrequent and irregular. This may have limited the value of the study and certainly complicates its analysis.

Chapter 4

Urinary IPG-P in Labour and the Post-natal Period

4.1 Background & Introduction

The precise mechanism for the onset of labour remains obscure and in humans, unlike other species, its timing is quite variable (Slattery and Morrison 2002). Up to 20% of women with a normal singleton pregnancy will not labour at term (40 weeks ±2 weeks from date of last menstrual period) and in addition premature labour is a cause of a large percentage of maternal and neonatal morbidity and mortality (Slattery and Morrison 2002).

It is widely accepted that prostaglandins (PG) or other hormones such as oxytocin are involved in labour onset and are used in the artificial induction of labour. Prostaglandin gradients of PGE_2 and $PGF_{2\alpha}$ between uterine vein and peripheral artery are increased in labour (Davidson et al. 1987). Both uterotonic and uterorelaxant pathways have been characterized in mammalian and non-human primate systems but the precise sequence of events in humans is not established (Slattery and Morrison 2002). The non-human studies however all implicate modulation of intracellular calcium concentrations or calcium sensitisation as the key events in regulating myometrial contraction and relaxation. Placenta derived PGE_2 inhibits CTLL-2 T cells and may be involved in the immune response to the fetus during pregnancy suggesting that its primary role may not be in regulating labour onset per se (Kvirkvelia et al. 2002). However it has recently been shown that PGE may exist chemically bound to inositol cyclic phosphate, prostaglandylinositol cyclic phosphate (cPIP) which functions as a second messenger of insulin action similar to the inositol phosphoglycan (IPG) class insulin mediators (Shashkin et al. 2001). The latter class of insulin mediators have been shown to inhibit the calcium oscillations in liver cells (Sanchez-Bueno et al. 1997). Recent studies suggest that calcium signalling does not act as a simple on/off switch, but the transient oscillation number is used to differentially regulate and temporally coordinate different calcium-dependent processes (Ducibella et al. 2002).

Further, IPG-P has recently been demonstrated to interact with calcium sensitive transducers, such as in the pyruvate dehydrogenase phosphatase reaction to regulate the K_m for calcium (Kunjara et al. 2002). IPGs or cPIP type molecules therefore

would be able to mediate events, both increases in intracellular calcium or calcium sensitisation, which could initiate the onset of labour.

Previous work (unpublished data) indicated possible raised levels of urinary P-type inositol phosphoglycans (IPG-P) in labour and we hypothesized that a potential link might exist. A pilot study was initiated to further investigate urinary IPG-P in normal pregnant women in the 3rd trimester and during labour.

4.2 Methods

4.2.1 Recruitment

Recruitment of subjects was from a large central London teaching hospital antenatal clinic. The proposal was approved by the Joint UCL/UCLH Committees on the Ethics of Human Research. Subjects were consented to provide urine samples antenatally and agreed to provide further samples when in labour. Mid-stream urine samples were obtained from 37 'low-risk' non-labouring women in the third trimester of pregnancy. Serial urine samples were then obtained after admission in labour. Two patients were excluded from the study due to the development of pregnancy-induced hypertension/preeclampsia subsequent to recruitment. 11 patients were lost to follow-up due to delivery at other units (n=11) in the London area. This prevented sample collection due to the strict stipulations regarding sample freezing times as previously described (Paragraph 3.6.1). 1 patient withdrew consent. All urine samples were stored at -20°C until analysis. All samples were assayed using the polyclonal antibody-based ELISA for IPG-P (Rodaris Pharmaceuticals Ltd., Oxford, England) and also for total protein and creatinine content as described in Chapter 2 – Lab Methods.

4.2.2 Expression of Results

Results of the ELISA were expressed as a ratio of the plate positive control per mmol of creatinine (Units/mmol, where 1 Unit equals 1% of the plate positive control). For the subjects with serial labour samples, a single mean value was calculated to compare with the recruitment level for the same subject and paired t-test analysis was

used to compare recruitment with labour samples using a statistical software package (Microcal Origin).

4.3 Results

23 non-labour and 59 labour samples were obtained from 23 women with uncomplicated singleton pregnancies (median 2 labour samples for each subject). The mean gestation at recruitment was 35.8 weeks (range 34.1-38.7). 16 women were primigravidae, 7 were multigravidae. 17 women laboured spontaneously, 6 women had induction of labour with prostaglandins (Prostin E2 Vaginal Gel, Pharmacia & Upjohn). Reasons for induction of labour were post-dates (n=2), premature rupture of membranes, low liquor volume, maternal request and systemic lupus erythematosus (1 each). 13 women (57%) achieved a normal vaginal delivery, 7 women had ventouse extraction (30%) and the remaining three (13%) underwent caesarean section. The ELISA showed that urinary IPG-P levels in labour were significantly higher (median 9.64 Units/mmol) when compared to paired samples from the same women prior to labour (non-labouring group, median 2.31 Units/mmol) $p<0.00001$, see Figure 4.1. There was no significant difference between the non-labour and labour samples for protein ($p = 0.13$) or creatinine ($p = 0.09$) content, Figure 4.2. No correlation was found between IPG levels and vaginal delivery rate or length of labour though larger numbers are needed to confirm this, as it was not a defined primary outcome measure of the study. No difference in outcome was seen between primigravidae and multigravidae, but this was not a defined outcome measure so the study is insufficiently powered to examine this properly. Figure 4.3 shows the urinary ELISA values for women (n=17) with spontaneous labour and suggests that the IPG values start to rise around 18-24 hours before birth consistent with Figure 4.2. Interestingly the data suggests that peak levels occur prior to delivery suggesting that the raised levels may not simply be the consequence of increased myometrial activity but more consistent with a possible role in either the priming or actual induction of labour. Figure 4.4 shows the values for 6 women undergoing induction of labour with vaginal prostaglandins. These women relative to the time of delivery also show raised levels occurring around 18-24 hours prior to delivery. Clearly further studies are required to detail the exact timing of the rise in IPG levels.

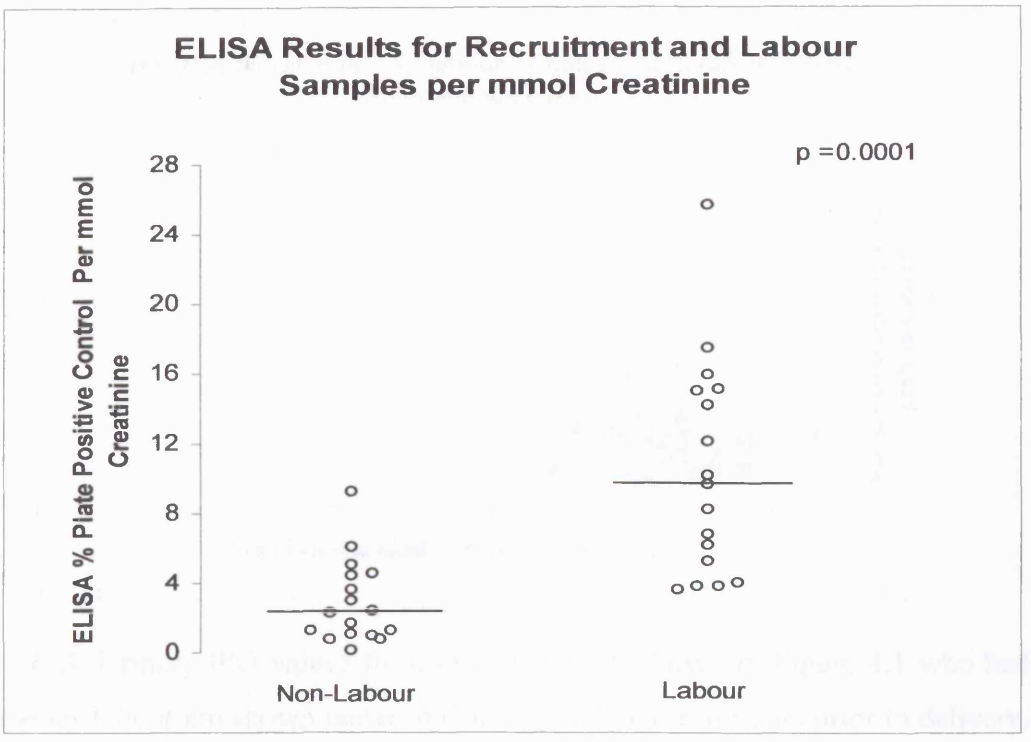


Figure 4. 1. Urinary samples were obtained from 17 women prior to spontaneous labour (all third trimester) and during labour. When more than one value was obtained during labour the mean value is reported. Values are expressed per mmol urinary creatinine.

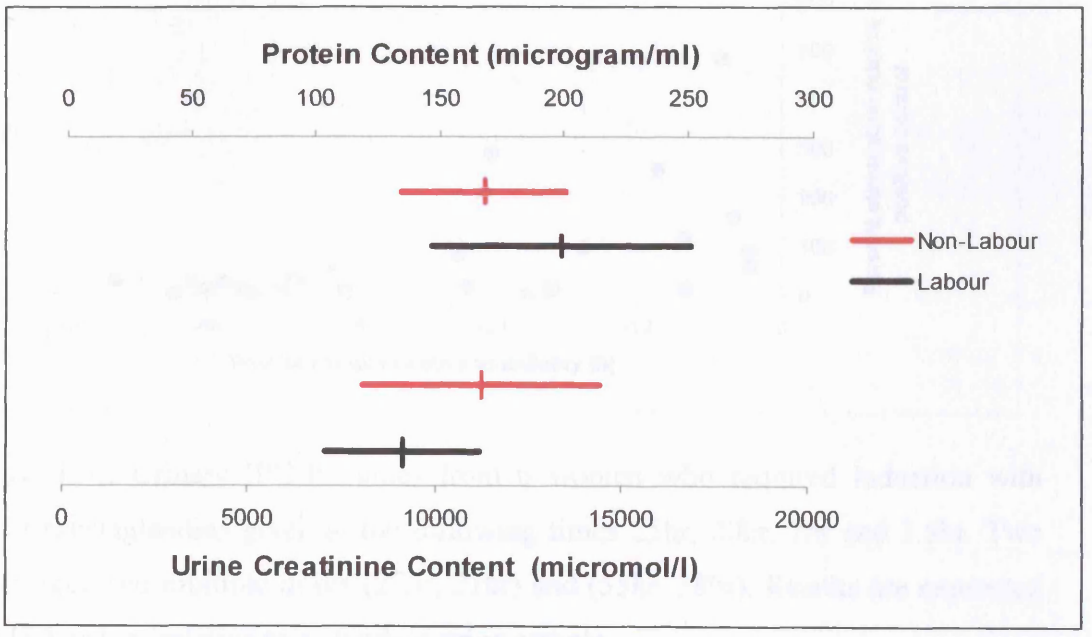


Figure 4. 2. Protein and Creatinine content for non-labour (recruitment) and labour samples. Bars represent mean ± 95% confidence intervals

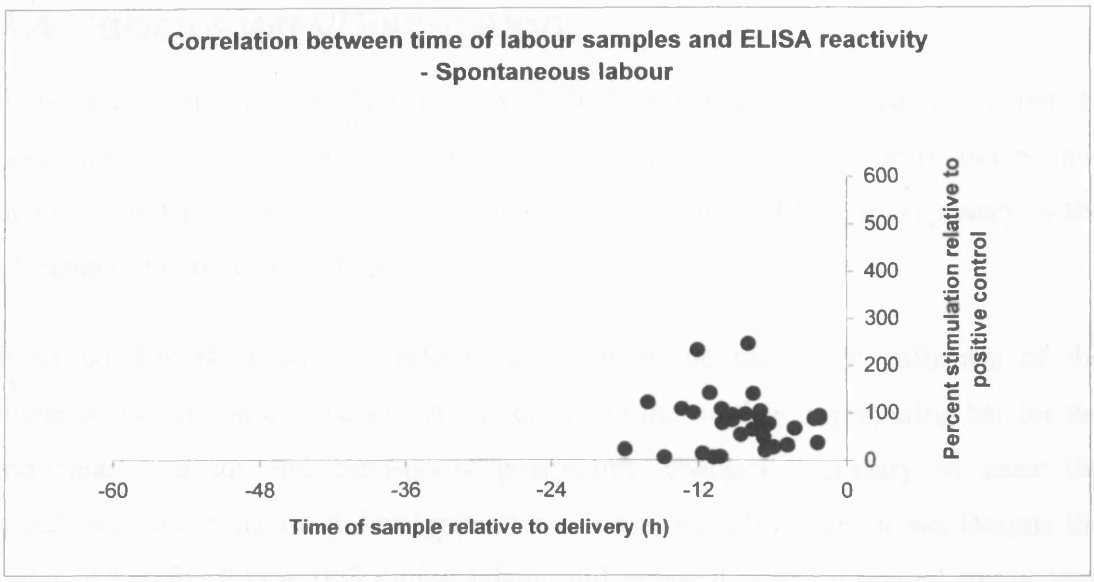


Figure 4. 3. Urinary IPG values from the 17 women shown in Figure 4.1 who had spontaneous labour are shown between delivery and eighteen hours prior to delivery. Results are expressed as ELISA values relative to a standard urine sample.

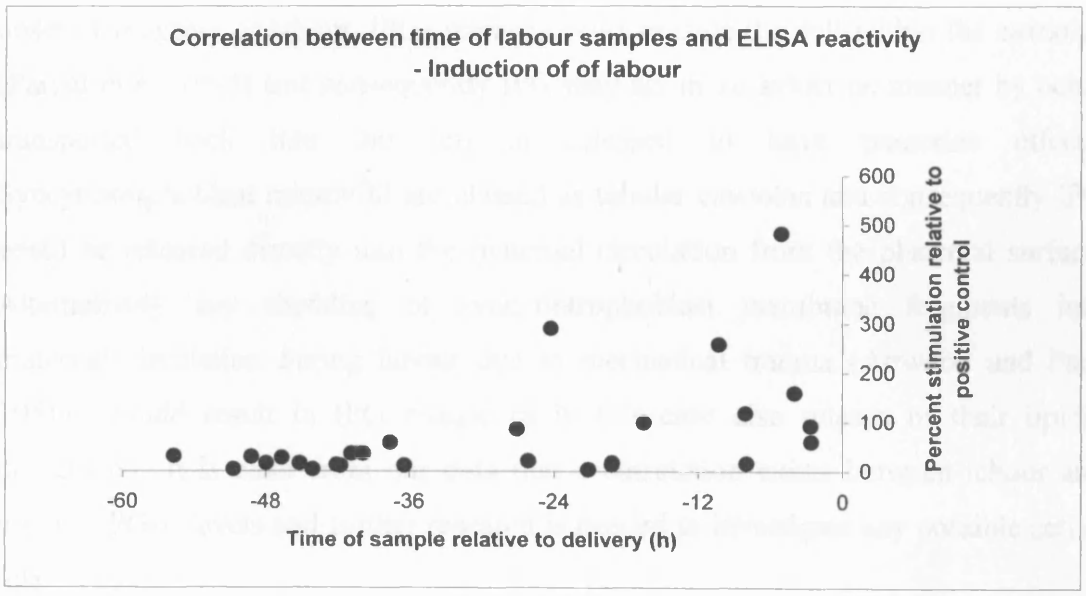


Figure 4. 4. Urinary IPG-P values from 6 women who required induction with vaginal prostaglandins given at the following times 25hr, 20hr, 7hr and 3.5hr. Two women received multiple doses (27hr, 21hr) and (55hr, 38hr). Results are expressed as ELISA values relative to a standard urine sample.

4.4 Conclusions/Discussion

It is thus demonstrated that urinary IPG-P levels are significantly higher in normotensive labouring women than in non-labouring women. Previous studies have demonstrated that the most likely source of these urinary IPGs in pregnancy is the placenta (Kunjara et al. 2000a).

Placental factors which are able to affect both the uterotonic pathways of the myometrium and also have a systemic effect on the mother in preparing her for the immediate partum and subsequent postpartum changes necessary to enter the lactation phase of neonatal development would make evolutionary sense. Despite the rapid rise in the P-type IPG during labour and removal of the placental source they persist into the circulation during the postpartum period for a considerable period, which may extend to weeks (Kunjara et al. 2000a).

Raised levels in labouring women compared to non-labour may indicate a role in the onset or progress of labour. IPGs release occurs outside the cell within the caveolae (Parpal et al. 1995) and consequently IPG may act in an autocrine manner by being transported back into the cell or released to have paracrine effects. Syncytiotrophoblast microvilli are classed as tubular caveolae and consequently IPG could be released directly into the maternal circulation from the placental surface. Alternatively any shedding of syncytiotrophoblast membrane fragments into maternal circulation during labour due to mechanical trauma (Attwood and Park 1961) would result in IPG release or in this case also release of their lipidic precursors. It is clear from our data that a correlation exists between labour and urinary IPG-P levels and further research is needed to investigate any possible active role.

Chapter 5

IPGs and the Prediction of Preeclampsia

5.1 Introduction

5.1.1 Aim

The data presented in this chapter form a parallel set to those from Chapter 3. The aim was to try to recruit as large a number as possible of women in the time available who might subsequently develop preeclampsia. 'High-risk' groups of patients were therefore recruited. Subsequently, this data would be used to establish the profile of urinary IPG-P in women with a hypertensive pregnancy outcome. These longitudinal data have never previously been collected.

5.1.2 Recruitment Sites

The following three sites were identified as locations where women with a higher than average risk of developing preeclampsia might be found.

1. Assisted Conception Unit
2. High-risk Obstetric Antenatal Clinic
3. Multiple Pregnancy Ultrasound Clinic

As this project was collecting pilot data, a formal power calculation to calculate subject numbers was not performed as it was instead decided to try to recruit as many subjects as time and manpower would allow. As with the low-risk women in Chapter 3, the sites were all favourable in respect to their location within the University College London Hospital location. Each group will now be discussed separately as regards the recruitment

5.1.3 Assisted Conception Unit

I saw this site as an advantageous recruitment location for two reasons. Firstly, pre-pregnancy urine samples could be obtained from women who would become pregnant in the near future. This was combined with the favourable fecundity rate reported by the Unit (see results). Secondly, certain forms of assisted conception (principally those with donor gametes, but also multiple pregnancies) carry a reportedly higher incidence of preeclampsia (Serhal and Craft 1989; Abdalla et al. 1998; Salha et al. 1999). The potential reasons for this have been discussed in Chapter 1.

5.1.4 High Risk Obstetric Antenatal Clinic

Women identified as being 'high-risk' are referred for the booking visit in this clinic. Certain of these women had risk factors for preeclampsia, detailed in Table 5.1 below. Women were identified from case notes as having one or more of the risk factors and the study was then discussed with them.

Table 5. 1. Inclusion Criteria for High-Risk Subjects after Amendment 1.

Multiple pregnancy
Previous preeclampsia/eclampsia or IUGR*
Pre-existing Vascular pathology -
Diabetes or Essential Hypertension
Previous abruptio placentae
Certain types of fertility treatment
Sickle Cell Trait
Renal Disease**

*Defined as fetal birthweight below 10th centile for gestation as per Chitty and colleagues (Chitty et al. 1994a; Chitty et al. 1994c; Chitty et al. 1994b)
**Previous renal transplant or demonstrated renal impairment with serum creatinine >100mmol/l

5.1.5 Multiple Pregnancy Ultrasound Clinic

All women booking at University College London Hospital were offered a booking/dating ultrasound scan. Those identified as having a multiple pregnancy were subsequently scanned in the specialist multiple pregnancy ultrasound clinic in the Fetal Medicine Unit for their anomaly scan in the second trimester. It was at this point that I identified these women and discussed the study with them.

For each of these three groups of women, the study was discussed and full informed consent gained as per the Amendment 1 to the study protocol.

5.1.6 Sample Collection and Patient Tracking/Follow-Up

The process was as described for the low-risk subjects in Chapter 3.

5.1.7 CRF completion

At the time of recruitment, basic demographic details were logged for each subject, including date of birth, medical and obstetric history, last menstrual period (and thus

gestation) and expected date of delivery. At each subsequent visit where a urine sample was collected, a further sheet was filled out, with initials, date of birth and study number for identification purposes and also blood pressure (where taken) urine dipstick reading and any symptoms or signs of preeclampsia.

At the time of delivery, a final sheet was filled, with the details of the labour and delivery, and the outcome code as defined in the study protocol (Chapter 3, Table 3.3).

5.2 ELISA Assay

5.2.1 ELISA Protocol

This is described in some detail in Chapter 2, Laboratory Methods. The protocol does not need to be further discussed at this point save to say that the same operator assayed all samples (myself). Operator blinding was achieved by the method outlined in the paragraph above.

5.2.2 Result analysis

All samples were run in triplicate as discussed in Chapter 2, Lab Methods. The ELISA plate reader, reading at an optical density (OD) of 450nm expressed the ELISA results in the form of a mean and standard deviation of the mean (s.d.). These results were analysed following subtraction of the plate blank, as a percentage of the plate positive control (as discussed in Chapter 2).

5.3 Results

In total, 55 women were recruited in the antenatal period and 46 non-pregnant women were recruited from the Assisted Conception Unit (ACU). In the latter group, 25 pregnancies were recorded but 8 of these miscarried in the first trimester. Of the pregnancies that were ongoing beyond the 20th week, three were multiple and fourteen were singleton. One of the three multiple pregnancies was a triplet pregnancy with a fetal reduction in the early second trimester to a twin pregnancy carried out in the second trimester. This pregnancy continued to 38 weeks, when a

vaginal delivery of both twins was achieved. The other two were also twin pregnancies. See Table 5.2 for details of mode of conception for the ACU subjects.

Table 5. 2. Mode of conception for Assisted Conception Unit patients

Mode of Pregnancy	n
In-vitro Fertilisation (IVF)	7
IUI/Superovulation	1
Gamete Intra-Fallopian Transfer	1
Male Factor	
Intra-Cytoplasmic Sperm Injection (ICSI)	4
ICSI/PESA	1
ICSI/TESA	1
IVF with Pre-Implantation Genetic Diagnosis (PGD)	1
Spontaneous	1

PESA = Percutaneous epididymal sperm aspiration, TESA = Testicular Sperm Aspiration

35 women were recruited from the high-risk antenatal clinic and 20 from the multiple pregnancy clinic. Three of these women were lost to follow up due to mid-trimester miscarriage (n=1) or change of address (n=2). Clinical and demographic details for the subjects are summarised in Table 5.3. The mean age of the high-risk antenatal subjects was 35.6 years and mean Body Mass Index (BMI) was 27.7. Both values were significantly higher than for the low risk women ($p = 0.002$ and 0.007 respectively). No difference was seen for the values of the women with multiple pregnancies (Mean age 33.9y, BMI 23.6).

A breakdown of the risk factors for preeclampsia for those women recruited in the high-risk clinic can be seen in table 5.4. The most common inclusion criteria were a history of thrombophilia (n=11) and/or a previous history of preeclampsia (n=9) or a combination of one of these plus (an)other risk factor(s). Often, an adverse event in a previous pregnancy led to investigation and diagnosis of an underlying predisposing factor. All the women seen in antenatal clinic with thrombophilia were commenced on low-dose aspirin (75mg) plus low-molecular weight heparin (LMWH) daily by the clinical team or by the subject's own general practitioner. Several of the other subjects had also been (or were subsequently) commenced on low dose aspirin only (Table 5.4).

Table 5. 3. Details for Recruitment High-risk Subjects

	ACU (n = 17*)		High-Risk Antenatal (n = 32)		Multiple (n = 20)	
	Mean	(±2s.d)	Mean	(±2s.d)	Mean	(±2s.d)
Age (y)			35.6	(6.6)	33.9	(9.2)
BMI			27.7	(12.2)	23.6	(7.4)
Ethnicity	n	(%)	n	(%)	n	(%)
Caucasian	14	(82)	22	(67)	18	(90)
Afro-Caribbean	-	-	4	(12)	-	-
Black African	2	(12)	2	(6)	1	(5)
Asian	-	-	1	(3)	1	(5)
Indian Sub-Continent	1	(6)	1	(3)	-	-
Other	-	-	3	(9)	-	-
Parity						
Primiparous	17	(100)	13	(40)	13	(65)
Multiparous	0	(0)	20	(60)	7	(35)

ACU = Assisted Conception Unit. *Numbers shown for pregnancies achieving >20 weeks

Table 5. 4. Risk Factors for preeclampsia at recruitment

Risk Factor	n
Thrombophilia*‡	11
Previous Severe Preeclampsia/Eclampsia	9
Essential Hypertension	2
Previous Abruptio Placentae at <37 weeks‡‡	2
IVF, Gamete donation	1
Previous cerebro-vascular accident	1
Sickle Trait	1
Diabetes	1
Other	
Previous abruption + history of essential hypertension	1
Essential Hypertension + family history of preeclampsia	1
Factor V Leiden + previous HELLP syndrome‡	1
Increased hCG at Serum Screening	1

*e.g. Anti-phospholipid syndrome (APS), Factor V Leiden, Prothrombin gene mutation heterozygote, Positive Lupus Anticoagulant, Anti-phospholipid antibodies, anticardiolipin antibody

‡Of which all treated with low dose aspirin ± LMWH (Low Molecular Weight Heparin)

‡‡Treated with low dose aspirin

No delivery details were available for a further four subjects in the high-risk group and one of the multiple pregnancies. Details of the onset of labour can be seen in Table 5.5 below (cf. for low risk women, Chapter 3, Table 3.5). Chi Squared analysis indicates that the high-risk antenatal group were significantly more likely to have a caesarean section than the low risk group, (χ^2= 7.93, $p<0.05$) though this was not the case for the multiple pregnancy group.

Table 5. 5. Details for Onset of Labour High-risk Subjects

	ACU		High-Risk Antenatal		Multiple	
	(n = 17*)		(n = 29)		(n = 19)	
	n	(%)	n	(%)	n	(%)
Onset of Labour						
Spontaneous	8	(47)	7	(24)	5	(26)
Induction	1	(6)	10	(34)	3	(16)
ELSCS	4	(24)	8	(28)	11	(58)
EmLSCS	0		4	(14)	0	
Not Known	4	(24)				

ACU = Assisted Conception Unit. ELSCS = Elective Lower Segment Caesarean Section, EmLSCS = Emergency Caesarean Section (before labour). *Numbers shown for pregnancies achieving >20 weeks

Overall, in these high-risk women, there were 6 cases of preeclampsia, an overall rate of 7.6%. However, the rate varied between the 3 recruitment sites. The highest rate being in those women recruited from the antenatal clinic at 15% (n=5), with 5% (n=1) in those with multiple pregnancies. This was lower than expected and indeed lower than that in those women with no identified risk factor at booking (7.5%, n=3). No cases of preeclampsia were identified in the women from the Assisted Conception Unit (Table 5.6). However, Chi Squared analysis of the distribution of hypertensive outcomes revealed no significant difference between the low risk women and any of the high-risk groups (χ^2 value = 6.24, $p>0.1$) but this may be due to the small numbers.

Booking blood pressure was significantly higher in the high-risk group compared to the low risk group ($p=0.002$ for Mean Arterial Pressure, MAP, using Student's T Test), but not in the multiple pregnancy group. These women also had a significantly higher age ($p = 0.001$) and BMI ($p = 0.007$) than the low risk group.

It seems that although multiple pregnancy is a risk factor for preeclampsia, that this is due to the placental volume rather than possible vascular risk factors (including age) that are found in the other women identified as high-risk.

Table 5. 6. Comparison of low risk and different high-risk groups in terms of selected factors and outcomes.

	'Low Risk' (n = 40)		ACU (n = 17)			'High Risk' ANC (n = 33)			Multiple (n = 19)		
	Mean	(±2s.d)	Mean	(±2s.d)		Mean	(±2s.d)	p	Mean	(±2s.d)	p
Age (y)	32.3	(10)	36.6	(8.8)		35.6	(6.6)	0.002*	33.9	(9.2)	0.16*
BMI (at booking)‡	22.8	(7.6)	23.9	(7.7)		27.7	(12.2)	0.007*	23.6	(7.4)	0.84*
Booking BP											
Systolic	105	(25.3)	111	(24.2)	0.118*	116	(29.8)	0.002*	109	(22.8)	0.21*
Diastolic	65	(18.0)	72	(18.5)	0.018*	72	(26.2)	0.01*	68	(15.4)	0.21*
MAP	92	(21.1)	98	(20.5)	0.05*	101	(27.8)	0.002*	95	(18.8)	0.17*
Gestation (Weeks)	Median	(Range)	Median	(Range)		Median	(Range)		Median	(Range)	
Recruitment	11.9	(9.1-19.9)	-	-		13.6	(6.9-33.3)		17.9	(11.7-27.9)	
Delivery	40.3	(36-42)	38	(23.6-41.6)		38.3	(28.7-41.4)		37.3	(27.3-38.9)	
Delivery Type	**n**	**(%)**	**n**	**(%)**		**n**	**(%)**		**n**	(%)	
NVD	17	(46)	5	(29)		12	(41)		2	(11)	
Assisted Vaginal	8	(21)	4	(24)		4	(14)		3	(17)	
Elective Caesarean	2	(5)	4	(24)		8	(28)		10	(55)	
Emergency Caesarean	10	(28)	1	(6)		5	(17)		3	(17)	
NK/Lost to f-u	-		3	(17)		-			-		
Outcome											
I – Preeclampsia	3	(7.5)	0			5	(15)		1	(5)	
II – PIH (no Proteinuria)	3	(7.5)	1	(4)		7	(21)		2	(10)	
III – PE on Chronic	0		0			1	(3)		0		
IV – No Hypertension	31	(77.5)	16	(64)		15	(45)		15	(75)	
Lost to Follow Up**	3	(7.5)	8	(32)‡‡		5	(15)		2	(10)	

ACU = Assisted Conception Unit, ANC = Antenatal Clinic, BP = Blood Pressure, PE = Preeclampsia. MAP = Mean Arterial Blood Pressure. NK = Not known Outcomes as also defined in text of chapter. I – Preeclampsia, II - Pregnancy Induced Hypertension (no proteinuria), III - Preeclampsia superimposed on chronic hypertension, IV - No hypertension. P values by *Student's t-test ‡ACU subjects BMI recorded pre-pregnancy. **Early pregnancy loss or delivery at other site. ‡‡ Early pregnancy loss

5.3.1 Raw ELISA Data

The longitudinal data are presented below grouped by hypertensive outcome, as defined in Chapter 3.3.5 (Table 3.3) and by recruitment group. Figure 5.1 shows low risk subjects, Figure 5.2 the high-risk and Figure 5.3 the multiple pregnancy subjects.

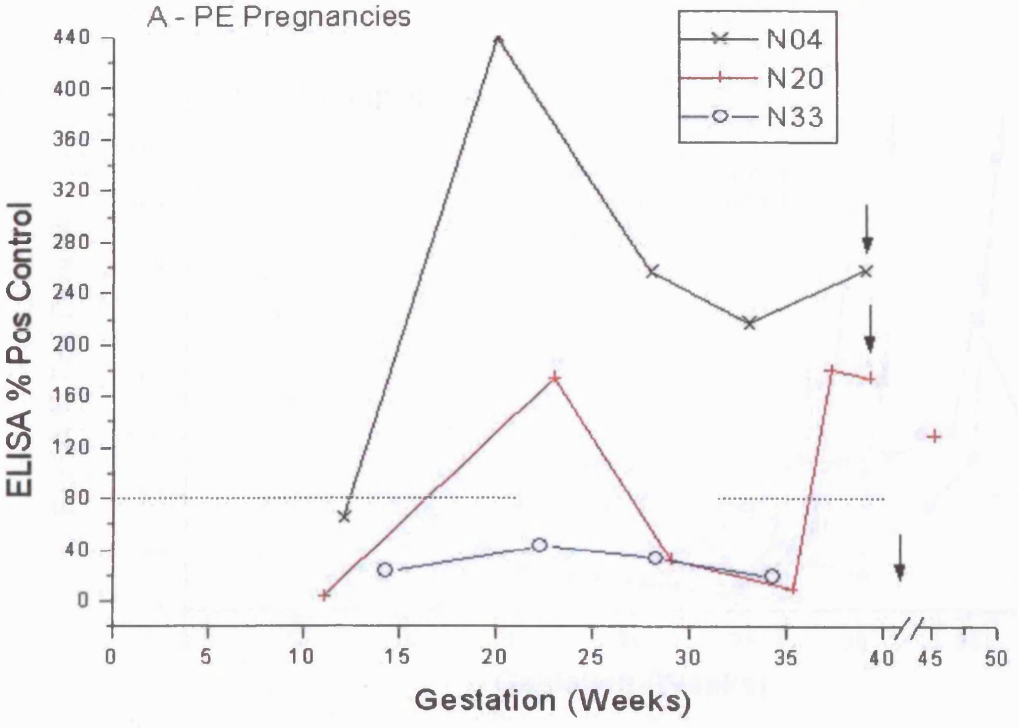
It can be seen that there are positive test results in the women with subsequent preeclampsia, sometimes from the second trimester (as early as 20 weeks, Figure 5.1 A). This appears to be a different pattern in the low-risk subjects to the non-proteinuric hypertension (PIH) group. No positive results were seen in this group until late in the third trimester (Figure 5.1 B).

In the high-risk group, the majority of the subjects with preeclampsia had positive results before or at the time of clinical diagnosis. However, only two displayed positive results at an earlier stage in the pregnancy (Figure 5.2 A). Once again, in general the subjects with subsequent PIH did not display positive results (if at all) until shortly before delivery (Figure 5.2 B). This was the same as in the subject with preeclampsia superimposed on chronic hypertension (Figure 5.2 C).

In the single multiple pregnancy subject with preeclampsia, the ELISA only showed a positive result at the time of diagnosis of preeclampsia (Figure 5.3 A). The clinical team expedited delivery shortly afterwards. In the multiple pregnancy subjects with PIH (i.e. non-proteinuric) there were positive results for the ELISA several weeks before delivery, which is different to the low-risk and high-risk groups as above (Figure 5.3 B).

It is also possible that the small numbers in the study may explain the differences.

Figure 5. 1. Low-risk subjects. Individual serial ELISA-assay plots for all subjects grouped by hypertensive pregnancy outcome and study number. ELISA values shown as a percentage of the plate positive control, as previously discussed. ↓ = Clinical diagnosis of preeclampsia. | = Delivery of PIH subjects. Dotted reference line shows cut off point for a 'positive test' (80% of plate positive control, ppc). PE = Preeclampsia. PIH = Non-proteinuric pregnancy induced hypertension



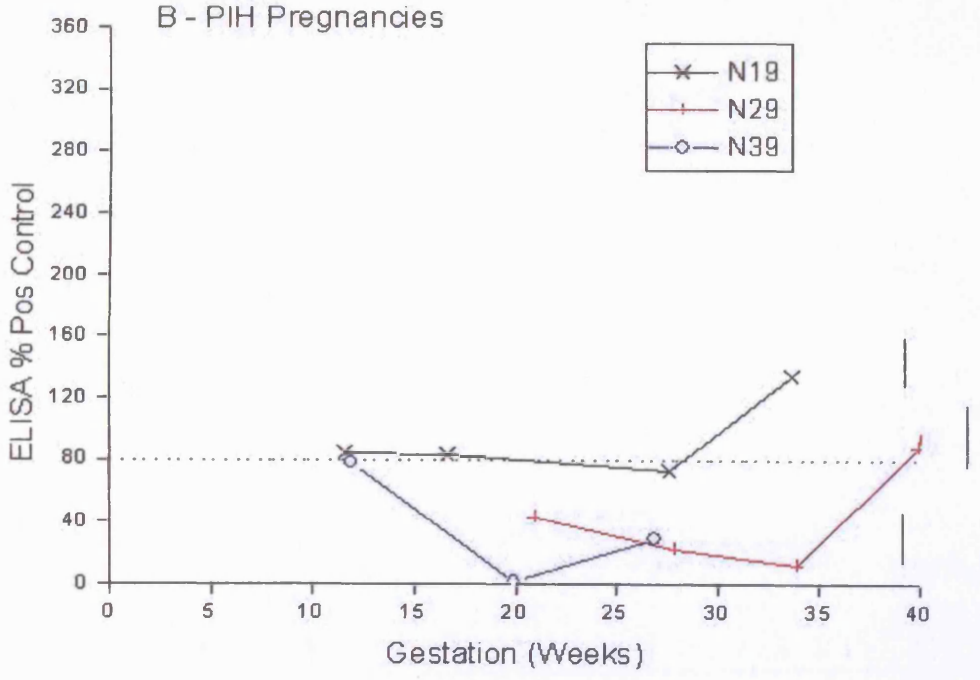
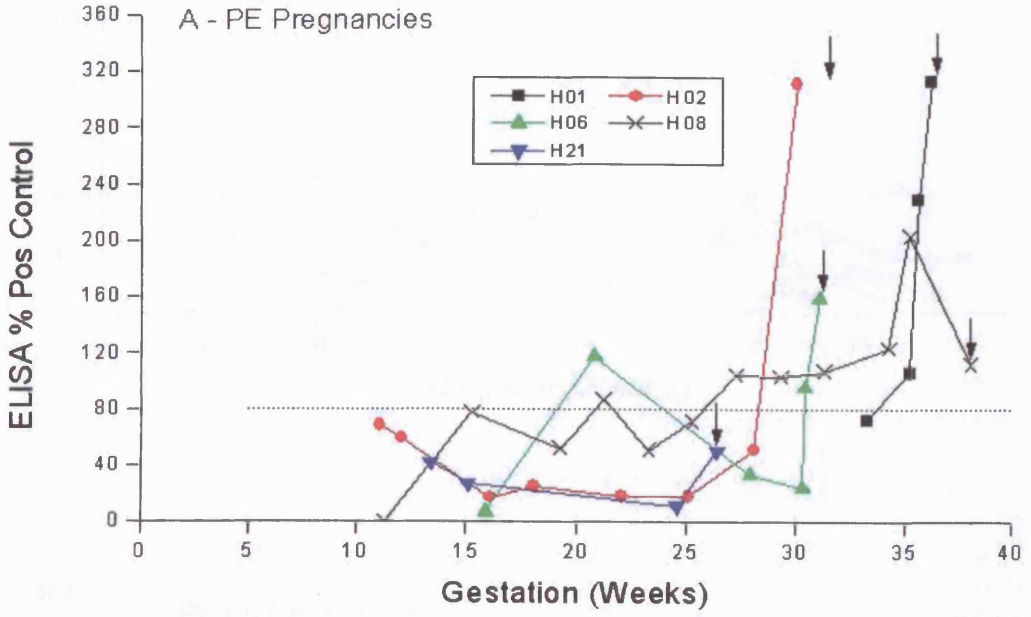
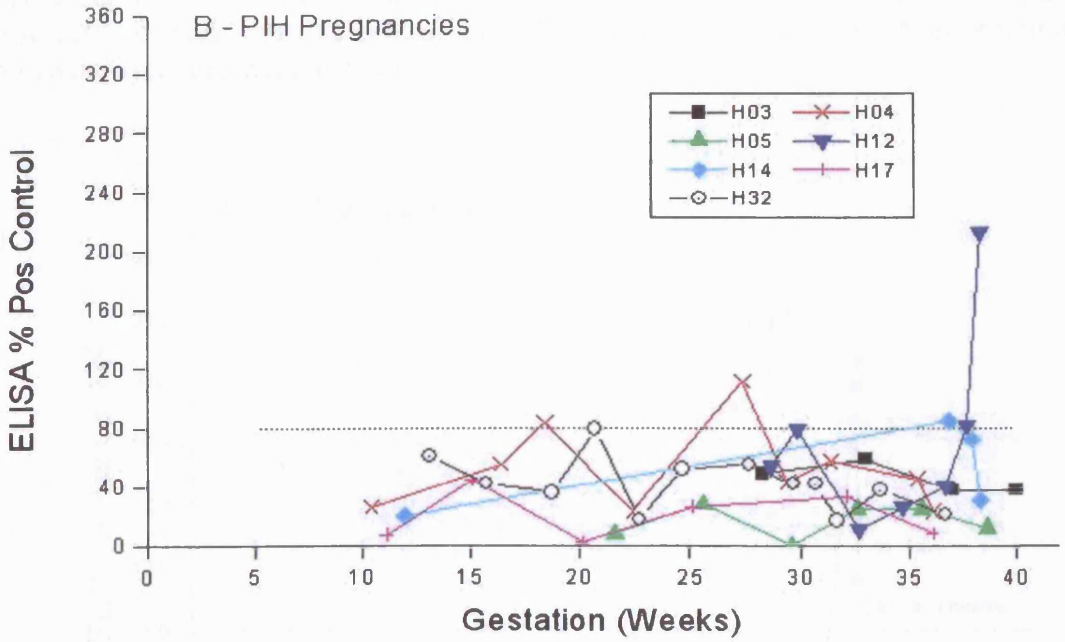


Figure 5. 2. High-risk subjects. Individual serial ELISA-assay plots for all subjects grouped by hypertensive pregnancy outcome and study number. ELISA values shown as a percentage of the plate positive control, as previously discussed. ↓ = Clinical diagnosis of preeclampsia. Dotted reference line shows cut off point for a 'positive test' (80% of plate positive control, ppc). PE = Preeclampsia. PIH = Non-proteinuric pregnancy induced hypertension




B - PIH Pregnancies
H03
H04
H05
H12
H14
H17
H32
ELISA % Pos Control
Gestation (Weeks)
0
40
80
120
160
200
240
280
320
360
0
5
10
15
20
25
30
35
40

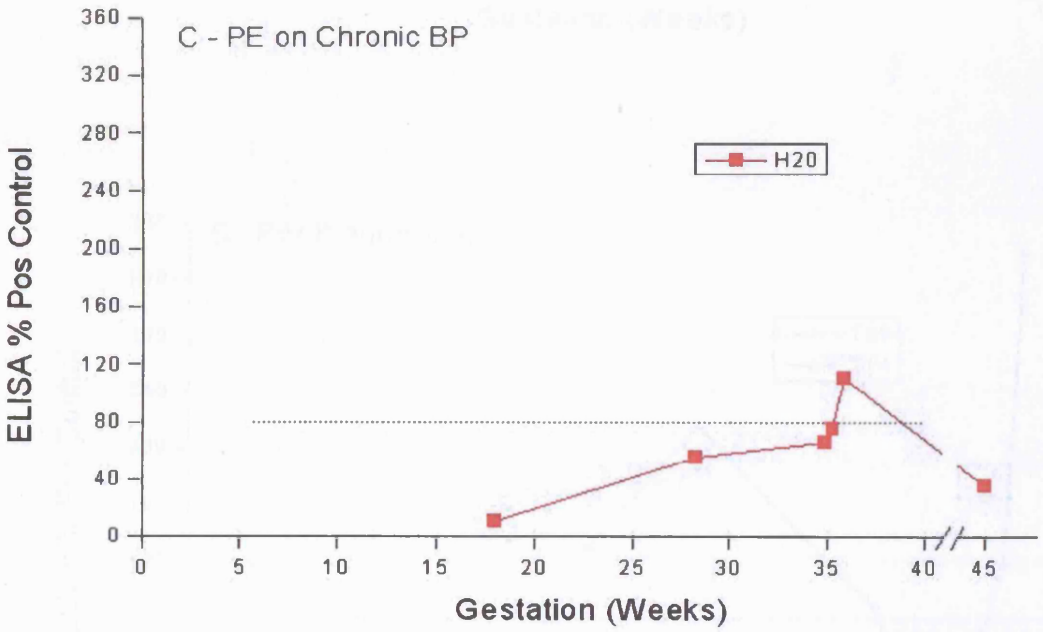
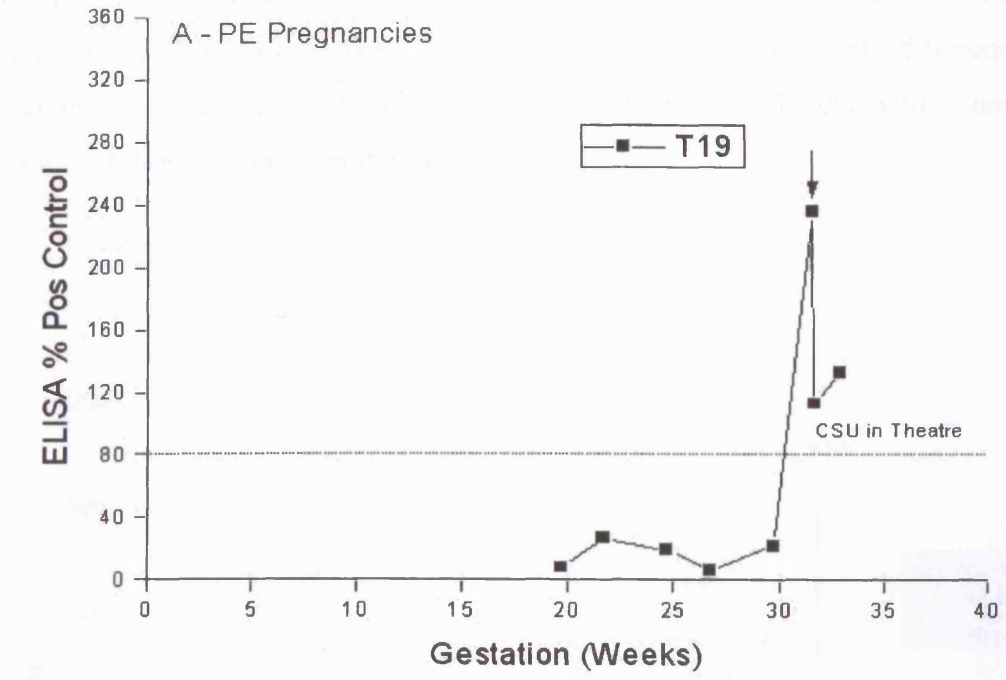
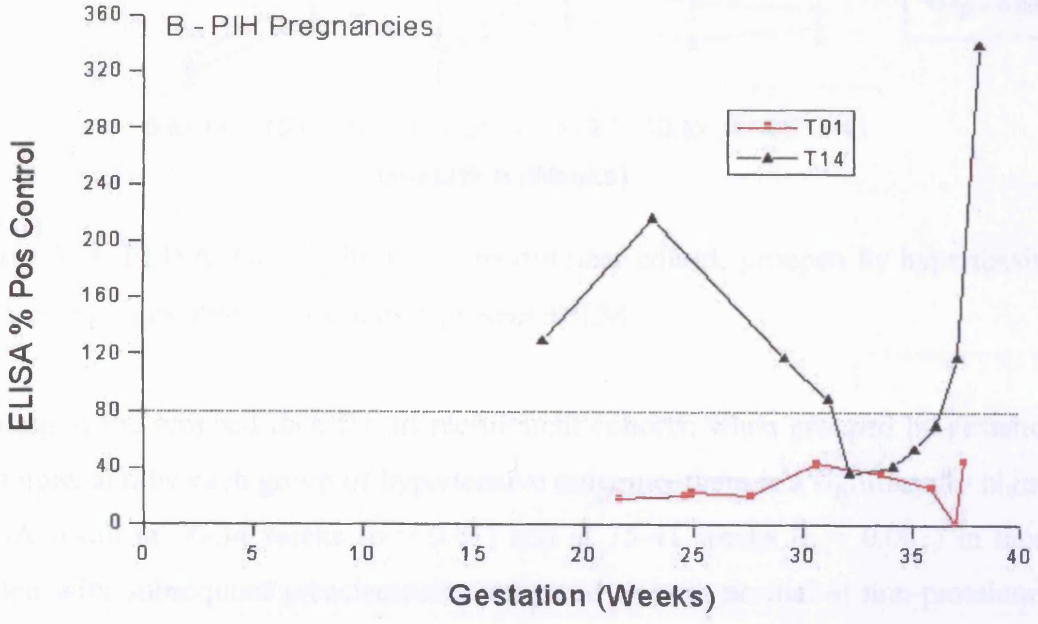

C - PE on Chronic BP
H20
ELISA % Pos Control
Gestation (Weeks)
0
40
80
120
160
200
240
280
320
360
0
5
10
15
20
25
30
35
40
45

Figure 5. 3. Multiple pregnancy subjects. Individual serial ELISA-assay plots for all subjects grouped by hypertensive pregnancy outcome. ELISA values shown as a percentage of the plate positive control, as previously discussed. ↓ = Clinical diagnosis of preeclampsia. Dotted reference line shows cut off point for a 'positive test' (80% of plate positive control, ppc). PE = Preeclampsia. PIH = Non-proteinuric pregnancy induced hypertension





5.3.2 Grouped ELISA Data

The ELISA data were grouped by hypertensive outcome, as seen below. For the group of women who were high-risk at recruitment, when grouped by gestation of sample, and by each group of hypertensive outcome, there is a significantly higher ELISA result at booking ($p = 0.03$), at 30-34 weeks ($p = 0.01$) and at 35-41 weeks ($p = 0.008$) in those women with subsequent preeclampsia, compared to both normal at non-proteinuric (gestational) hypertension outcomes (Figure 5.4). No difference is seen at other gestations. No difference is seen between subjects with a normal outcome and those with non-proteinuric hypertension.

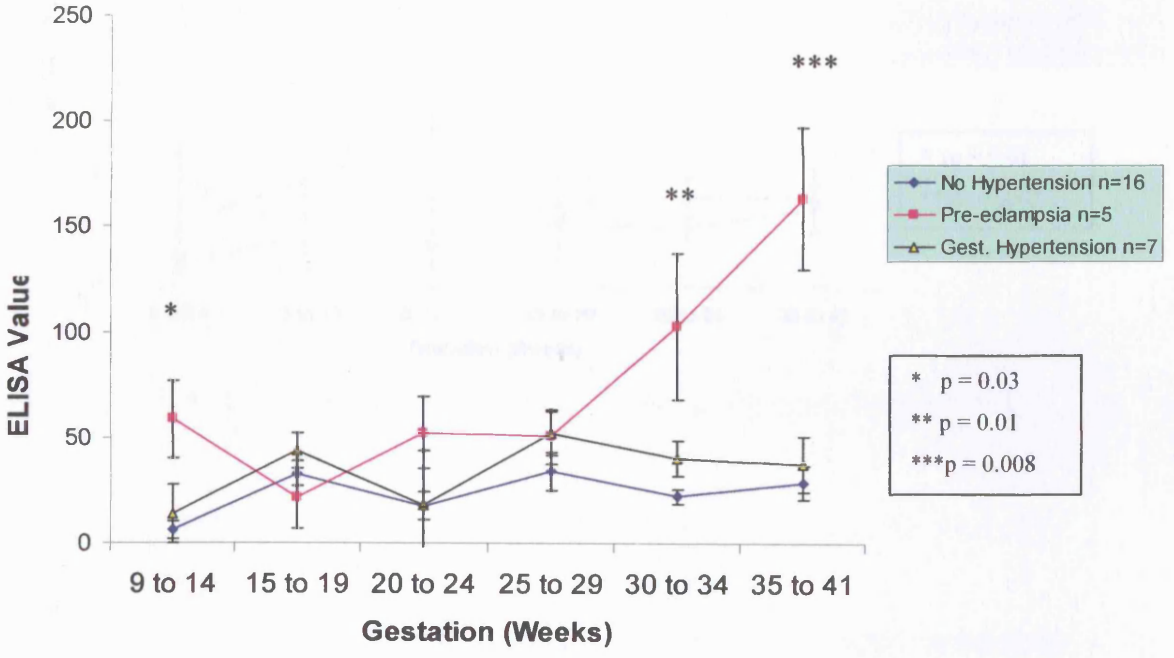
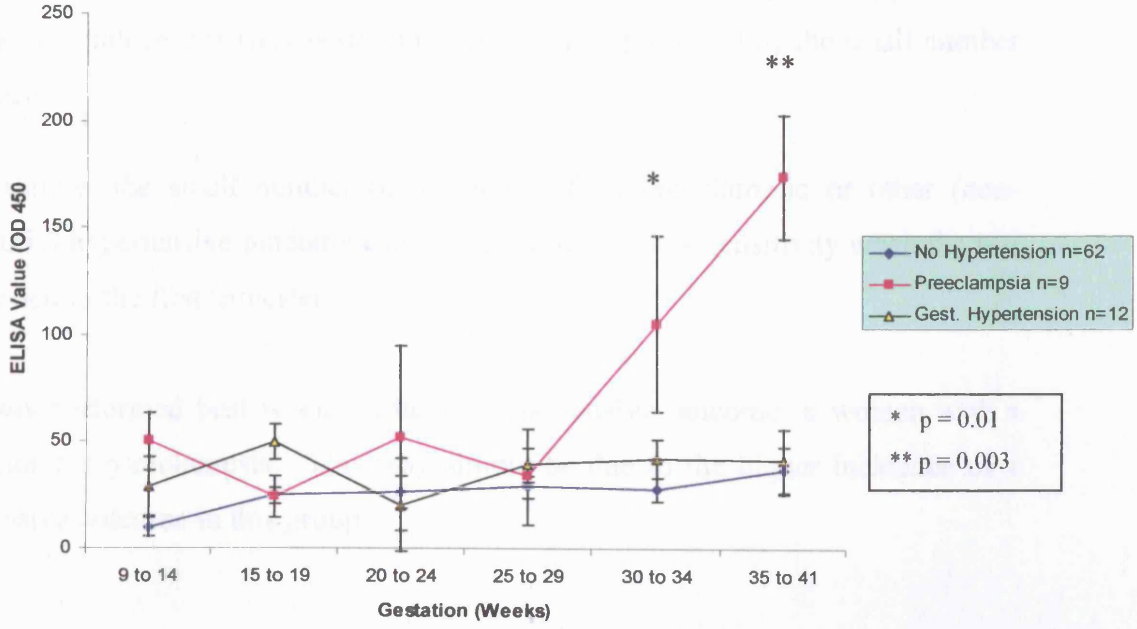


Figure 5. 4. ELISA data for high-risk recruitment cohort, grouped by hypertensive outcome with gestation. Error bars represent ±SEM.

Looking at the grouped data for all recruitment cohorts, when grouped by gestation of sample, and by each group of hypertensive outcome, there is a significantly higher ELISA result at 30-34 weeks ($p = 0.01$) and at 35-41 weeks ($p = 0.003$) in those women with subsequent preeclampsia, compared to both normal at non-proteinuric hypertension outcomes (Figure 5.5). No difference is seen at other gestations.

No difference is seen between subjects with a normal outcome and those with non-proteinuric (gestational) hypertension.

Figure 5. 5. ELISA data for all subjects, grouped by hypertensive outcome with gestation. Error bars represent ±SEM.



5.3.3 The IPG-P ELISA as a Screening Test

The ELISA assay was analysed using 2 by 2 table analysis (Figure 5.7), where a 'positive' result was taken as equal to or greater than 80% of the plate positive control, as described in Chapter 2. Likewise, a negative result was taken as less than 80% of the plate positive control.

Due to study design, it was necessary to analyse individual samples for subjects grouped by gestational age at the time of the sample and by the hypertensive outcome. Overall results were poor and were also compromised by the small number of subjects.

Unfortunately, the small number of subjects with a preeclamptic or other (non-proteinuric) hypertensive outcome contributed to a very low sensitivity when the test was applied in the first trimester.

The assay performed best when predicting hypertensive outcome in women with a risk factor for preeclampsia. This may simply be due to the higher incidence of a hypertensive outcome in this group.

5.3.4 The ELISA Assay as a Diagnostic Test in Population vs. High Risk Women

Thus we have seen that from these data the ELISA is not particularly clinically useful as a screening test in the first or second trimester.
For samples taken near to the time of delivery, the test does seem to show consistent positivity in the preeclamptic subjects. However there is a risk, in labouring subjects of this becoming confused with the positive tests seen for non-hypertensive women, seen in Chapter 4.

Table 5. 7. 2 By 2 Table analysis of IPG-P ELISA assay for groups of samples.

	Population Sub-Group	n	Specificity (%)	Sensitivity (%)	PPV (%)	NPV (%)	LR+	LR-
Preeclampsia vs. other Outcome	**Single ELISA Assay at:**							
	(a) Booking (<16 Weeks)							
	All Subjects	62	91.1	0.0	0.0	89.5	0.0	1.1
	Risk Factor for Preeclampsia	27	100.0	0.0	0.0	88.9	0.0	1.0
	Low risk subjects only	33	83.3	0.0	0.0	89.3	0.0	1.2
	(b) Second Trimester (16-28 Weeks)							
	All Subjects	74	80.3	50.0	23.5	93.0	2.5	0.6
	Risk Factor for Preeclampsia	40	82.9	40.0	25.0	90.6	2.3	0.7
	Low risk subjects only	32	75.9	66.7	22.2	95.7	2.8	0.4
Hypertensive vs. Other outcome	**Single ELISA Assay at:**							
	(a) Booking (<16 Weeks)							
	All Subjects	62	93.9	15.4	40.0	80.7	2.5	0.9
	Risk Factor for Preeclampsia	27	100.0	0.0	0.0	74.1	0.0	1.0
	Low risk subjects only	33	89.3	40.0	40.0	89.3	3.7	0.3
	(b) Second Trimester (16-28 Weeks)							
	All Subjects	74	84.2	47.1	47.1	84.2	3.0	0.6
	Risk Factor for Preeclampsia	40	89.3	45.5	62.5	80.6	4.2	0.6
	Low risk subjects only	32	76.9	50.0	33.3	87.0	2.2	0.7

LR+ = Likelihood Ratio for positive test, LR- = Likelihood ratio for negative test, NPV = Negative Predictive Value, PPV = Positive Predictive value

5.4 Conclusions/Discussion

Thus it is shown that as for the data from normal, non-hypertensive labour (seen in Chapter 4) there is a significant increase in the IPG-P ELISA signal in preeclamptic women. This is shown again in the single, paired urine samples (Chapter 6) and mirrors the increased urinary IPG-P bioactivity seen by Kunjara et al (Kunjara et al. 2000a). Examining the values for the subjects who developed preeclampsia, there appear to be two groups in terms of the ELISA positivity. Those that develop positive test results up to 15 weeks prior to the onset of clinical symptoms and those that develop test positivity around the time of clinical diagnosis. Numbers were too small to identify any difference in the clinical picture between these two groups. Additionally, a rise in the IPG-P ELISA signal can be seen at the time of labour in some of the samples. This parallels the data for the labouring urine samples discussed in Chapter 4.

It can be seen also that the ELISA does not stand alone as a screening test for preeclampsia. The specificity can be high, but the sensitivity is generally poor, and this would seem to be due to lack of ELISA positivity until the time of (or immediately before) the development of clinical preeclampsia in a proportion of the subjects. However, it might have application as one element of a two- or multiple-part test (such as doppler ultrasound, leptin, or nitric oxide) to improve overall sensitivity. Unfortunately, the minimally invasive design of this study prevented, for example, concurrent serum tests for other markers of preeclampsia.

The test may have some clinical use as a diagnostic assay, in combination with the clinical picture. This may be confused by test positivity at the time of labour in non-hypertensive women.

5.4.1 Follow up Problems

Certain problems were experienced with the follow up of subjects recruited at the Assisted Conception Unit. As a non-NHS unit, referrals to the ACU were from a wide area. This was exacerbated particularly as the Unit has a good fecundity rate and is involved in pioneering technologies including pre-implantation genetic diagnosis (PGD). Table 5.8 below shows subsequent booking hospitals for those subjects with pregnancies ongoing beyond the 20th week of gestation.

Table 5. 8. Booking hospitals for subjects recruited pre-pregnancy at the ACU

Within Greater London Area	**n**
Barnet Hospital, Wellhouse Lane, Barnet, Herts	4
University College London Hospitals NHS Trust	3
Portland Hospital, Great Portland Street, London	1
Chelsea and Westminster Hospital, Fulham Road, London	1
Kingston Hospital, Kingston upon Thames	1
Farnborough Hospital, Orpington, Kent	1
Outside Greater London Area	
Darenth Valley Hospital, Dartford, Kent	1
East Surrey Hospital, Redhill, Surrey	1
Ashford Hospital, Ashford, Middlesex, TW15	1
West Hertfordshire Hospitals NHS Trust	1
East Hertfordshire Hospitals NHS Trust	1
Outside UK	
Badhomburg, Germany	1

Understandably it was not practical to follow up these individuals as the protocol planned, due to manpower and time restrictions. In many cases, follow-up was limited to collection of delivery data and hypertensive pregnancy outcome code. In the event, none of these pregnancies resulted in an overt preeclamptic case (Table 5.6), despite the original aim of specifically recruiting higher risk cases. It was not possible to get further detailed data on certain aspects of labour care via telephone interviews with maternity staff (e.g. onset of labour data for four women, Table 5.5). As these did not affect primary outcome data (which were known), it was not felt appropriate in terms of time or financial resources to travel to distant maternity units and obtain ethical permission to examine case notes for these women.

5.4.2 Future Work

It is clear that the recruitment and numbers of preeclamptic cases were lower than hoped for and further studies are needed to increase these numbers.

Additionally, I would also hope to design a separate study to evaluate the test for women admitted with a hypertensive episode. That is, to properly assess its utility as a diagnostic assay in these cases – a potentially clinically useful application.

Chapter 6

IPGs in Established Preeclampsia

6.1 Introduction

I have discussed in previous chapters how the polyclonal antibody based ELISA has been used as a urine assay for the prediction/diagnosis of preeclampsia in a varied group of women. The assay relies on detecting the presence of a P-type IPG in the urine of these women. The presence of this product in urine proves nothing, however, in terms of the pathophysiology of the disorder. It could be argued that the IPG may be produced systemically in the maternal blood, renal or other tissues and may not relate to the preeclampsia or to the pregnancy at all.

On the other hand, previous work has implicated the placenta as a source of the IPG. It has been observed that glycogen accumulates in higher quantities in the placenta in preeclampsia (Arkwright et al. 1993). Investigation with anti-IPG monoclonal antibody has shown that there are higher levels of P-type IPG in preeclamptic placenta compared to normal control placenta (Deborde et al 1999b). This seems to be localised in the microvillous brush border of the syncytiotrophoblast (Deborde et al 1999a). As noted in Chapter 1, there is also evidence that this syncytiotrophoblast is shed into the maternal circulation and it could be that the IPGs 'hitch a lift' as it were from the placental surface to the maternal system. Alternatively, the IPG release may be in parallel to this cellular shedding/apoptosis.

Nothing is known at this time about the presence of IPGs on the fetal side of the equation. It is possible that they are released on the fetal side of the placenta also. Amniotic fluid is largely composed of fetal urine from around 16 to 18 weeks of pregnancy onwards (In Williams Obstetrics: Cunningham et al 1997). Waste products from the fetal circulation such as urea, creatinine and uric acid tend to be concentrated here (though osmolality is lower than fetal urine) and so may IPGs be also.

6.2 Objectives

The objective of this study was to assess IPG levels in various compartments on the maternal and fetal side of the placenta to attempt to further localise the source of the IPG production. The measurement of the IPGs was to be carried out by using a combination of techniques. Firstly, the polyclonal-based ELISA and secondly a well-established ion-

exchange column chromatography extraction followed by bioactivity assays of the extracted IPGs.

6.3 Design

A case-control study of women with active clinically diagnosed preeclampsia undergoing caesarean section before labour (cases) and non-hypertensive pregnant controls undergoing elective caesarean section was planned. Samples were to be taken at caesarean section. Initially, recruitment commenced at University College London Hospitals NHS Trust. The proposal was approved by the Joint UCL/UCLH Committees on the Ethics of Human Research. Recruitment was slower than expected, however, and it was decided to improve numbers by collaborating with a second institution. A British Council Higher Education Link existed between the Academic Departments of Obstetrics and Gynaecology at UCL and the Nelson R Mandela Medical School (University of Natal, Durban, South Africa). I contacted the head of department, Professor Moodley in Durban about recruitment. I arranged to travel to the University of Natal. The Medical School is attached to King Edward VIII Hospital, which has a large obstetric unit, with approximately 8000 deliveries per year. The research proposal was approved by the Research Ethics Committee at Nelson R Mandela Medical School prior to my trip in January-February 2002.

6.4 Subjects

All the data presented here is from recruitment at King Edward VIII Hospital, Durban, South Africa. Recruitment took place between 16th January and 8th February 2002. The study group included eleven women undergoing emergency caesarean section for clinically diagnosed preeclampsia or eclampsia (Table 6.1). Preeclampsia was defined by the criteria established by the International Society for the Study of Hypertension in Pregnancy (ISSHP) (Brown et al. 2001). Eleven normal, non-hypertensive pregnant control women were recruited in the same time period (Table 6.2). The subjects were matched for age and ethnicity with controls. It was obviously not possible to match for gestation, as preeclamptic subject requiring rapid delivery by caesarean would be of a less advanced gestation than controls requiring elective caesarean section. This problem has been encountered elsewhere (Chua et al. 1991).

Table 6. 1. Characteristics of eleven patients with severe preeclampsia/eclampsia delivered by caesarean section

Patient	Parity	Gestation at delivery (weeks)	Indication for delivery
1	0	39.6	Eclampsia
2	0	35.1	Preeclampsia, IUGR
3	6	36.0	HELLP, IUFD
4	2	31.0	Preeclampsia
5	0	31.0	Preeclampsia
6	2	32.6	Preeclampsia
7	1	37.0	Eclampsia
8	3	37.7	Preeclampsia
9	2	34.0	Hypertension, Raised Urate
10	1	36.4	Preeclampsia
11	2	30.7	Preeclampsia, Elevated liver enzymes

IUGR = Intra-uterine growth retardation; HELLP = Syndrome of haemolysis, elevated liver enzymes and low platelets; IUFD = Intra-uterine fetal death

Table 6. 2. Characteristics of eleven control patients with no hypertensive disease delivered by caesarean section

Patient	Parity	Gestation at delivery (weeks)	Indication for delivery
1	0	39.0	Post Dates, Fetal Distress
2	1	38.0	Previous CS
3	2	37.7	Term Breech
4	3	39.0	Previous CS x3
5	2	39.9	Previous CS x2
6	2	37.0	Previous CS x2
7	2	39.4	Poor Obstetric history
8	2	37.9	Previous CS x2
9	2	39.3	Previous CS x2
10	3	40.0	Coded patient
11	3	37.0	Previous CS x3

CS = Caesarean Section

6.4.1 Inclusion Criteria

Subjects:

- Attended King Edward VIII Hospital, Durban for antenatal care
- Were diagnosed with preeclampsia as defined by ISSHP classification (see Chapter 1)

- Underwent caesarean section before the onset of labour, as decided by the Obstetric Team on duty.
- Signed informed consent for the study
- Were willing to have the data of the pregnancy documented for study

Controls:

- No evidence of raised blood pressure
- Undergoing caesarean section before the onset of labour for reasons other than preeclampsia

6.4.2 Exclusion Criteria

- Under 18 years of age at the time of recruitment

6.4.3 Informed Consent

Informed consent was an important part of the recruitment process, having obviously been a key part of the Ethics approval and the study involving invasive testing. The majority of patients attending King Edward VIII Hospital did not have English as a first language. Upon commencing the study, a copy of the patient information sheet translated into the Zulu language was prepared by staff with experience in this at Nelson Mandela Medical School. If the subjects did not understand English, this Zulu Information Sheet was used. The local Research Ethics Committee approved the Zulu version of the information sheet.

Following consent, details were taken of the current pregnancy and past obstetrics and medical history.

6.5 Methods

6.5.1 Sample Collection

All samples were taken by myself (Table 6.3). The samples taken at the time of the caesarean section were done so with effort to avoid risk to the mother and baby and prolongation of the procedure itself.

Table 6. 3. Samples taken

Timing	Sample	Analysis
• Immediately Prior to Caesarean [§]	Peripheral venous blood (5ml)	Bioactivity assay
• At Caesarean Section	Catheter urine specimen (30ml)	ELISA for P-IPG
	Amniotic fluid (5ml)	ELISA for P-IPG
	Umbilical Venous Blood (5ml)	Bioactivity assay
	Umbilical Arterial Blood (5ml)	Bioactivity assay
	Uterine venous blood (5ml)	Bioactivity assay

[§] Within 30 minutes of the procedure

Urine This was collected as a catheter sample at the time of caesarean section and stored at -20°C initially.

Peripheral Venous Blood

This was collected at the time of or within thirty minutes prior to the caesarean. It was collected by syringe as a 5ml sample and transferred to a plain (unheparinised) tube.

Amniotic Fluid

At the time of caesarean section, a 5ml sample was aspirated with a sterile plastic syringe under direct vision after opening of the uterus and immediately after rupture of the membranes at the time of the operation. Care was taken to avoid contamination of the amniotic fluid with maternal blood. It had previously been planned to collect the fluid prior to membrane rupture, using a green needle to puncture the membranes and thereby avoid contamination with other fluids. However, the Ethics Committee decided that this would incur increased risk for the fetus and thus approval was given only for the samples to be taken without a needle on the syringe. Therefore this necessitated the samples to be taken after membrane rupture.

Cord Blood

Two 5ml samples were taken from the umbilical cord (one from the umbilical artery & and one from the vein) following delivery of infant and before or immediately after (within 5 minutes of) the separation of the placenta.

Uterine Vein Blood

The sampling procedure is based on the method described by Johansen and Redman (Johansen et al. 1999). At the time of caesarean section, after the peritoneum was opened but before the incision was made in the uterus, a sample of 5ml blood was taken with a 21-gauge (green) needle from one of the broad ligament veins. Any small risk of haematoma formation was minimised by pressure on the vein after taking the sample. Additionally, uterine contraction following delivery minimised further blood loss. No excess blood loss or haematoma formation was observed during the conduct of the study.

6.5.2 Sample Processing

All samples were collected using a plain syringe and transferred to plain Vacutainer tubes (Beckton-Dickinson, Oxford, England) and processed within 3 hours of sampling time. Blood samples were spun in an Eppendorf 5804R centrifuge (Eppendorf, Hamburg, Germany) for 5 minutes at 5000rpm (≈4200g) at room temperature. Serum was then removed and stored at -20°C at the King Edward VIII Hospital until transport to the UK for analysis. Sample tubes were taken on dry ice via overnight air flight and delivered to University College London. Total transit time was 24 hours and samples remained at or below -20°C at all times. Samples then remained frozen at University College London until analysis.

6.5.3 Materials and Lab Analysis

These are as described in Chapter 2 – Lab Methods.

6.5.4 Statistical Analysis

For matched samples, unpaired t-test analysis was carried out with a standard statistical and graphing package (Origin 5.0, Microcal Software Inc, Northampton, MA, USA).

6.6 Results

The mean maternal age for the preeclampsia/eclampsia (study) and control groups was not significantly different (28.1 y and 27.8 y respectively). The mean gestation for the study group was 34.6 weeks and mean birthweight was 1905g. For the control group, these were 38.6 weeks and 3021g respectively.

Urine and Amniotic Fluid

Urine was not available for one of the normal subjects. Amniotic fluid was not obtained from four of the preeclamptic subjects due to reduced amniotic fluid volume at the time of sample collection or contamination with blood. Overall ELISA values (expressed relative to a standard urine sample) for all samples were significantly higher in amniotic fluid (mean = 693 units, where 1 unit is equivalent to 1% of the signal with the standard sample) compared to urine (mean = 175 units, $p < 0.00001$). For the matched urine and amniotic fluid pairs, values were significantly higher for the preeclampsia/eclampsia group compared to the control group (Table 6.4 and Figure 6.1, $p = 0.002$ and 0.01 respectively).

Table 6. 4. IPG-P ELISA values for urine and amniotic fluid from normal and preeclamptic women.

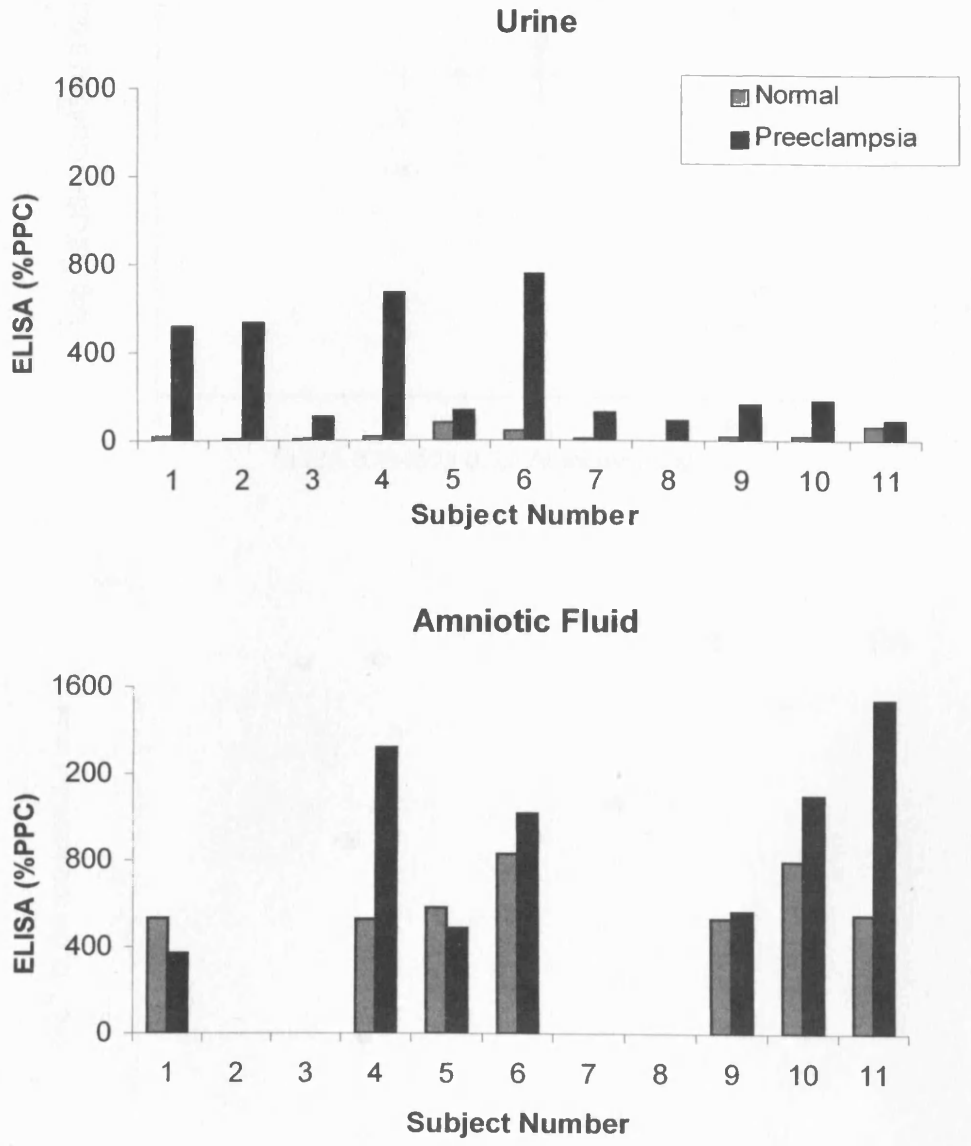
	Normal	**Preeclampsia**	**p**
	Urine		
	(n=10)	(n=11)	
Mean (SEM)	30 (7.7)	308 (77.6)	0.002*
Median (range)	18 (6-78)	168 (90-752)	
	AF		
	(n=11)	(n=7)	
Mean (SEM)	555 (43.0)	911 (134.6)	0.01*
Median (range)	529 (337-828)	1012 (370-1536)	
p	<0.001*	0.001*	

AF = Amniotic Fluid. P-values for means by unpaired Student's t-test.

There was also a correlation between amniotic fluid and urine ELISA values for the normal group ($r = 0.70$, $p = 0.02$), but this was not apparent in the preeclamptic group, (Figures 6.2a and b).

The maternal platelet count was inversely correlated to amniotic fluid ELISA values ($p = 0.01$) in the preeclamptic subjects (Figure 6.3a). No correlation was seen between maternal urinary ELISA and platelet levels (Figure 6.3b) or with other measured clinical parameters (gestation, maternal age, birthweight, serum urate or mean arterial blood pressure; data not shown).

Figure 6. 1. ELISA values for matched urine and amniotic fluid sample pairs sample pairs for preeclampsia/eclampsia and control groups. Results expressed as ELISA values relative to a standard urine sample.



(%PPC = Optical density (OD) expressed as percentage of plate control)

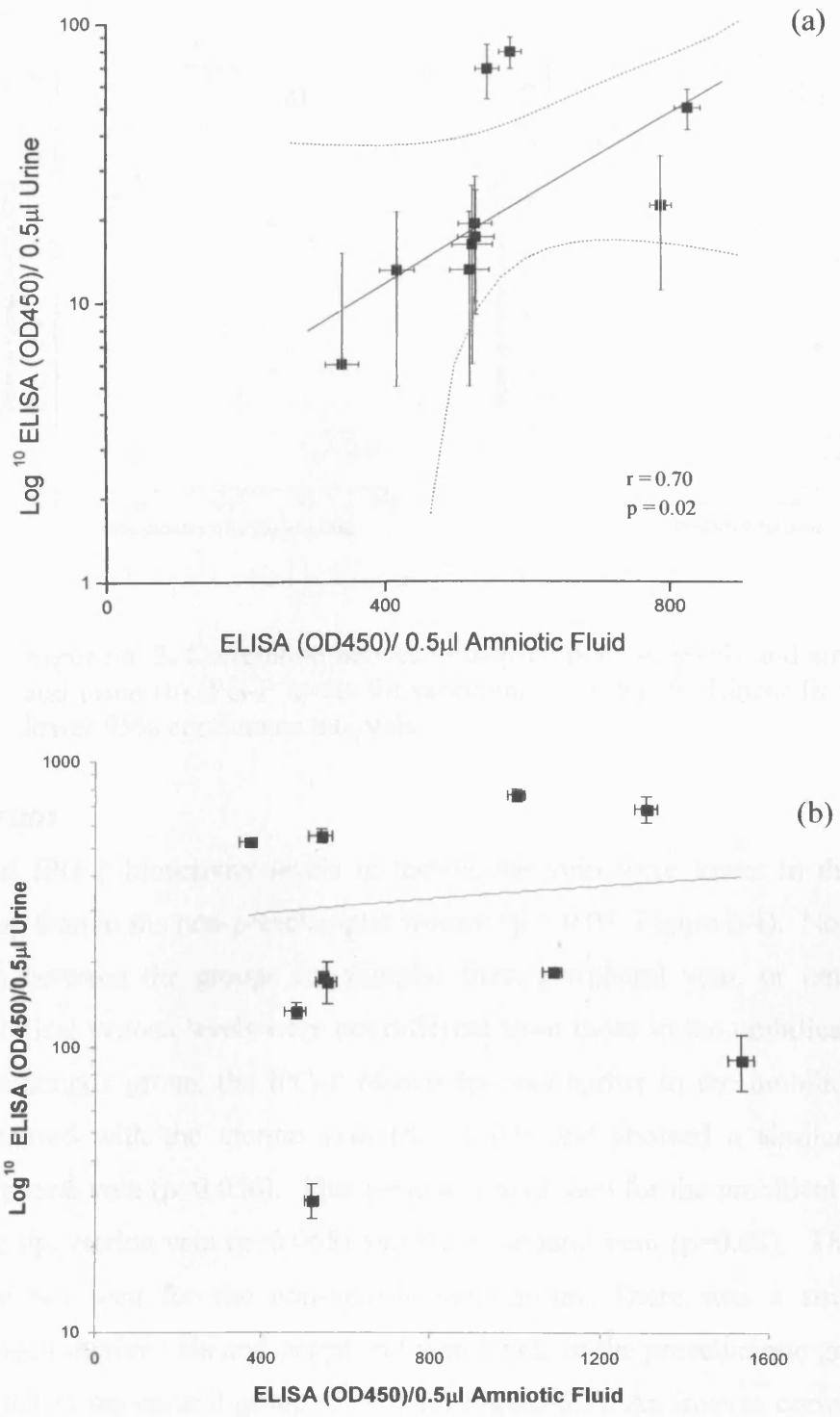


Figure 6. 2. Graph of ELISA values from maternal urine (shown on log^{10} scale) and amniotic fluid samples (expressed relative to a standard urine sample) for normal (a) and preeclamptic (b) women. Error bars represent SEM. Upper and lower 95% confidence intervals shown for fig 2a.

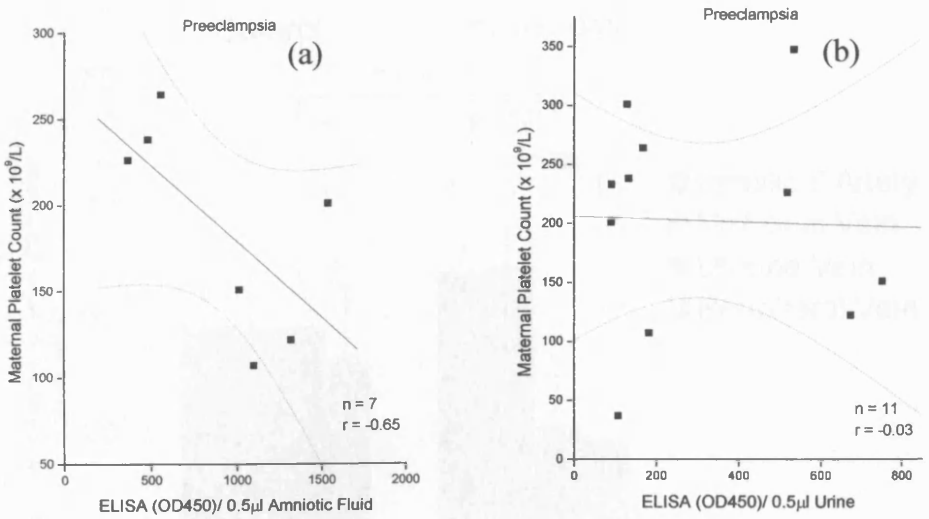


Figure 6. 3. Correlation between maternal platelet levels and amniotic fluid (a) and urine (b) IPG-P levels for preeclamptic subjects. Linear fit with upper and lower 95% confidence intervals

Serum

Total IPG-P bioactivity levels in the uterine vein were lower in the preeclamptic group than in the non-preeclamptic women ($p = 0.01$, Figure 6.4). No difference was seen between the groups for samples from peripheral vein, or umbilical vessels. Umbilical venous levels were not different from those in the umbilical artery. In the preeclamptic group, the IPG-P bioactivity was higher in the umbilical artery when compared with the uterine vein ($p = 0.02$) and showed a similar trend for the peripheral vein ($p=0.056$). This trend was also seen for the umbilical vein compared with the uterine vein ($p=0.065$) and the peripheral vein ($p=0.08$). These differences were not seen for the non-preeclamptic group. There was a strong correlation between uterine vein and peripheral vein levels in the preeclamptic group ($r^2 = 0.88$) but not in the control group ($r^2 = 0.41$, Figure 6.5). An inverse correlation was also seen between maternal platelet count and bioactive IPG-P in uterine and peripheral vein in the preeclamptic women (Figure 6.6, c & d). This was not seen for the umbilical serum samples (Figure 6.6, a & b). No correlation was seen with gestation for either preeclamptic or normal IPG-P levels for any of the values (data not shown).

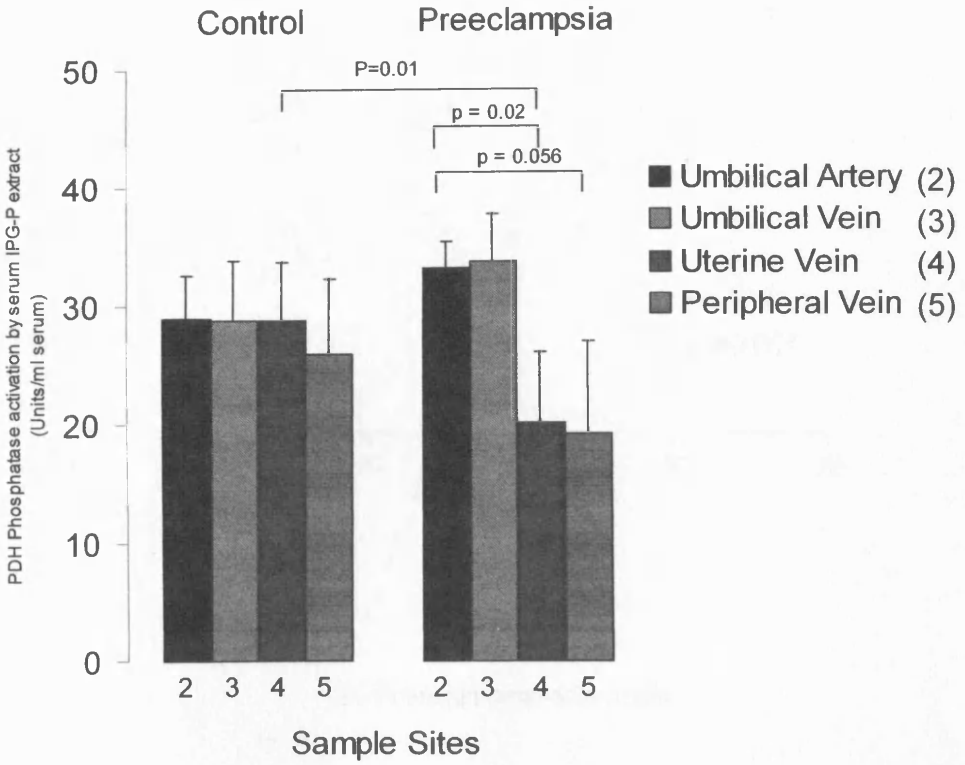


Figure 6. 4. Total serum IPG-P bioactivity for preeclampsia/eclampsia and control groups from different sample sites identified in Fig 3 Expressed as units of activity per ml of serum (where 1 unit of IPG PDH activity is the amount required to increase the basal rate by 50%). Error bars represent SEM. Non-significant differences not shown. Sample site numbers correspond to those in Figure 7.1.

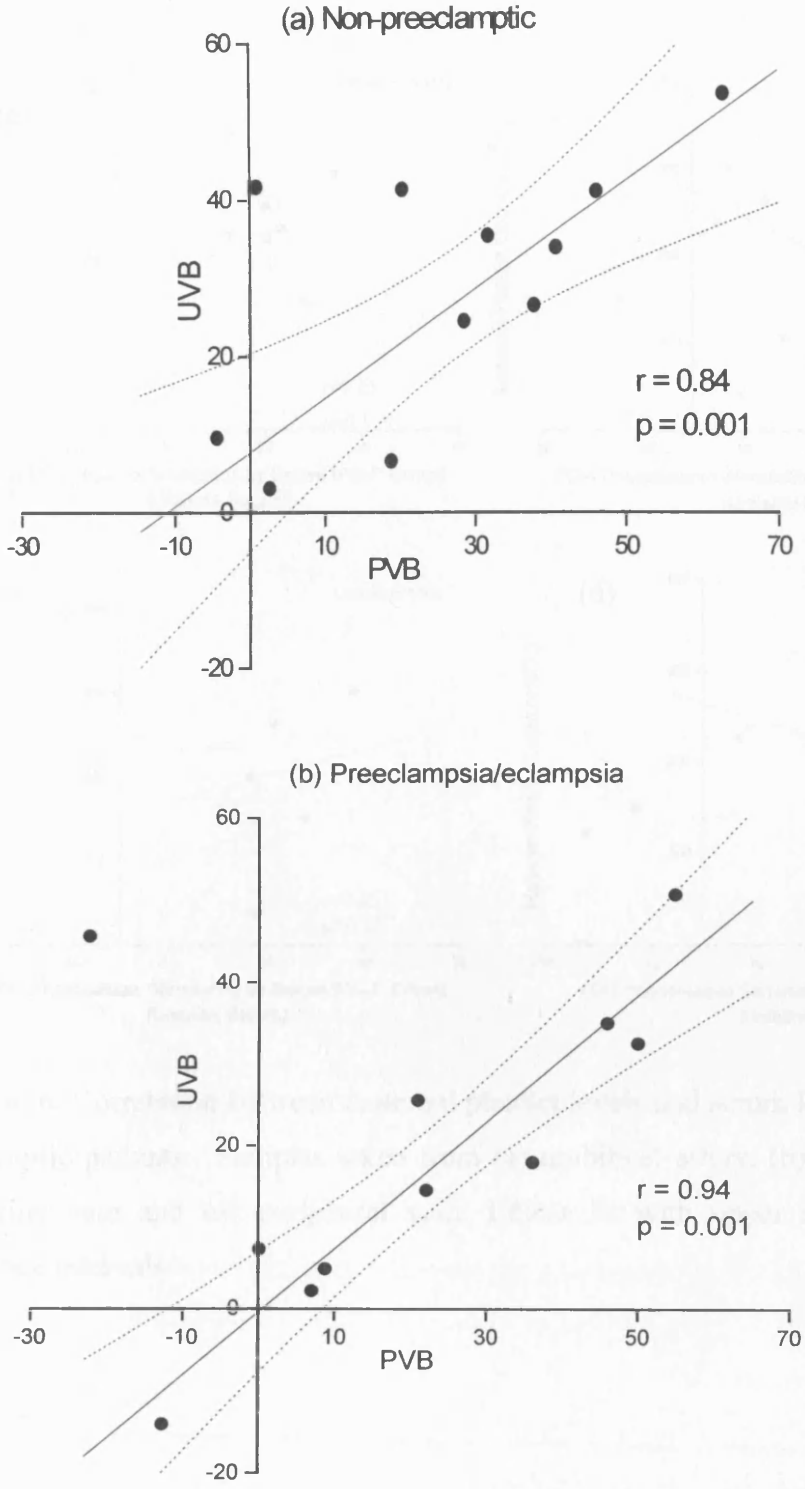


Figure 6. 5. Correlation between uterine vein (UVB) and peripheral vein (PVB) IPG-P levels in (a) normal and (b) preeclamptic subjects. Expressed as units of activity per ml of serum (where 1 unit of IPG PDH activity is the amount required to increase the basal rate by 50%).

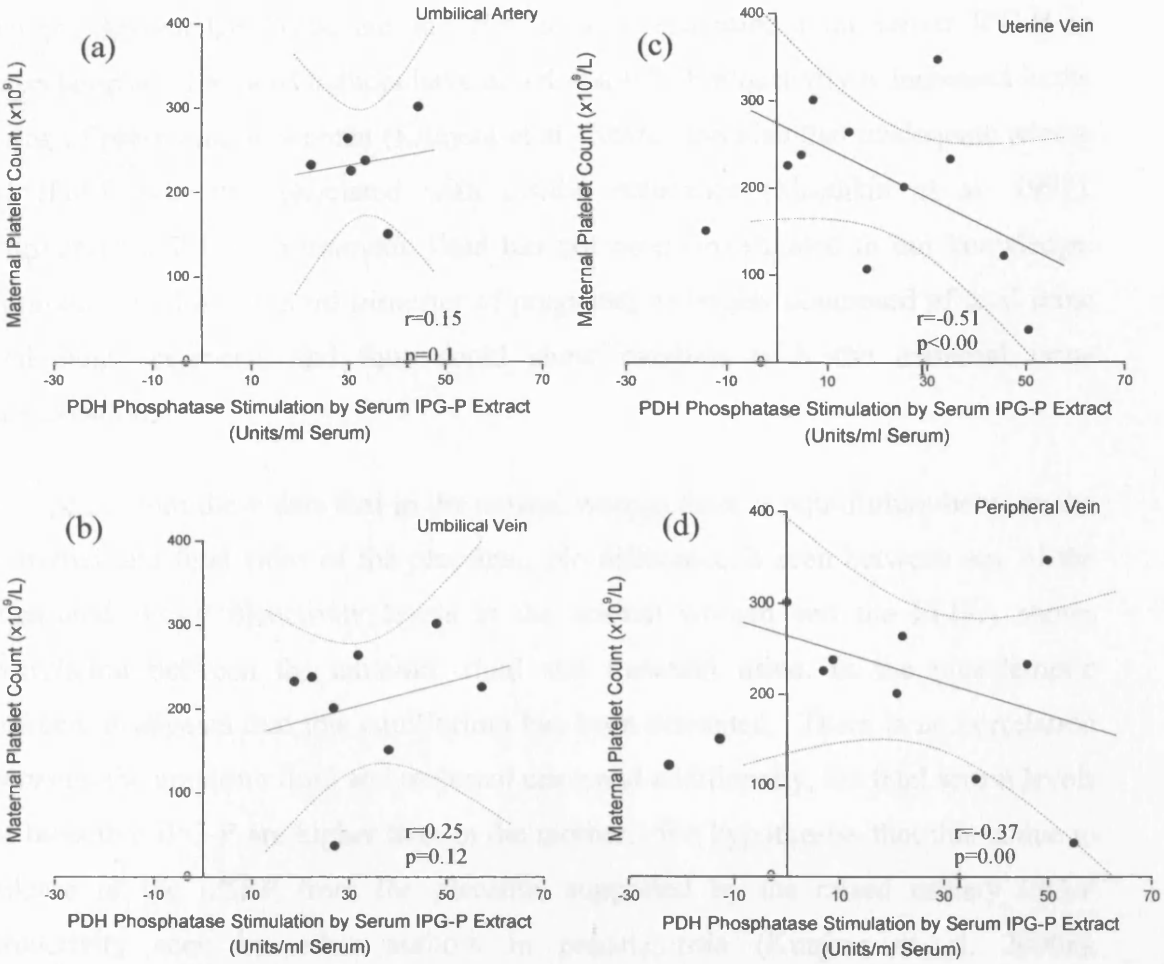


Figure 6. 6. Correlation between maternal platelet levels and serum IPG-P activity in preeclamptic patients. Samples taken from (a) umbilical artery, (b) umbilical vein, (c) uterine vein and (d) peripheral vein. Linear fit with upper and lower 95% confidence intervals.

6.7 Conclusions/Discussion

To my knowledge, these are the first data investigating total serum IPG-P in preeclampsia. Previous authors have noted that IPG-P bioactivity is increased in the urine of preeclamptic women (Kunjara et al. 2000a) and also that inadequate release of IPG-P may be associated with insulin resistance (Shashkin et al. 1997). Furthermore, IPG-P in amniotic fluid has not been investigated to our knowledge. Amniotic fluid in the third trimester of pregnancy is largely composed of fetal urine and lung secretions and thus could show parallels with the maternal urine composition.

It appears from these data that in the normal women there is equilibrium between the maternal and fetal sides of the placenta. No difference is seen between any of the measured IPG-P bioactivity levels in the normal women and the ELISA shows correlation between the amniotic fluid and maternal urine. In the preeclamptic women, it appears that this equilibrium has been disrupted. There is no correlation between the amniotic fluid and maternal urine and additionally, the fetal serum levels of bioactive IPG-P are higher than in the mother. We hypothesise that this is due to release of the IPG-P from the placenta, supported by the raised urinary IPG-P bioactivity seen by other authors in preeclampsia (Kunjara et al. 2000a). Alternatively, secretion from the maternal kidney is a possibility, though secretion and immediate excretion would make less biological sense.

The inverse correlation of the amniotic fluid (ELISA) levels and maternal serum (bioactivity) levels with maternal platelets in preeclampsia (Figure 6.3a) provide further evidence that the IPG-P may in some way be involved in the pathophysiology of preeclampsia. Platelet count is well established as a reliable clinical marker of disease severity and forms a component of the HELLP syndrome, a severe form of preeclampsia. This data mirrors the inverse relationship between urinary IPG-P bioactivity and platelet count, which has been shown previously (Kunjara et al. 2000a).

Previous work would suggest a placental source of the IPG-P production, with release into maternal and fetal circulations. However, the higher levels of measurable IPG-P in serum on the fetal side of the placenta in preeclampsia, and an

absence of any difference in umbilical arterial and venous levels might indicate several possibilities. Firstly, a potential fetal production source with breakdown of placental transport to mother. Neither umbilical arterial nor venous serum levels reached statistical significance in terms of being higher in preeclampsia than in normal subjects and therefore no backlog on the fetal side seems to be occurring. However, amniotic fluid ELISA signals were much higher in preeclamptic women, suggesting sequestration in this compartment. Alternatively there may a placental production source with release of an abnormal, lipidic form of IPG-P (a GPI) into the maternal circulation. This form would not be detected by the conventional extraction and bioassay procedures.

The significantly higher levels in urine from the preeclamptic women compared with the normal group indicate the potential for use of the ELISA as diagnostic assay. In the preeclamptic state it is possible that an abnormal form of IPG-P, as yet to be identified, may be causing high ELISA positivity but not being measured in the serum by the conventional assay we used here. Alternatively, we recognise that a renal source of IPG-P production may exist. However, it is key that the two tests are in fact measuring IPG-P in different ways. The bio-extraction relies on interaction with the anion exchange column whereas the ELISA assay may be selectively binding an abnormal form of IPG to the gelatin phase of the plate.

This leaves the possibility that a sub-fraction or an unidentified, abnormal IPG-P form is part of the pathophysiology of preeclampsia. In this case, the amount of IPG-P measurable by the conventional bioassay may exclude the abnormal, lipidic form. The bioactive half-life of IPG-P, either in conventional or an undescribed form is not known at this time.

It is possible that IPG levels do not differ in the serum between the normal and preeclamptic state and that a difference is seen in the urine and amniotic fluid due to concentration/sequestration in these fluid compartments. Alternatively, increased renal clearance may be occurring, alongside increased placental production. Previous work has indicated that the IPG release is on the maternal side of the trophoblast(Deborde et al 1999b; Deborde et al 1999a) and it is thus interesting to speculate on the source of the IPG on the fetal side of the placenta. In preeclampsia,

the umbilical arterial IPG-P levels are higher than in the maternal uterine vein, and probably the uterine artery; likewise the umbilical venous levels show a similar trend. It would be desirable to study larger numbers of patients to reinforce these data seen here.

We have previously demonstrated elevated urinary IPG-P in non-hypertensive labouring women (Paine et al. 2003). An earlier study had indicated the possibility of a common causal link between labour and preeclampsia (Adamsons and Wallach 1989) related to declining oxygen tension within the trophoblast. It is possible that this causes abnormal release or metabolism of IPG-P, which may then be involved in the pathophysiology and could explain why we have found similar urinary IPG-P values in preeclampsia and labour.

6.7.1 Amniotic Fluid IPG-P

Amniotic fluid (AF) seems to be a rich source of IPG-P, though we do not yet have data on bioactivity levels.

There are altered amniotic fluid metal levels in preeclampsia in the third trimester, for example raised magnesium levels (Kolasa et al. 1983) and depressed zinc and calcium levels (Dawson et al. 1999). The data for this comes from a series of 29 preeclamptic and 101 normal pregnancies. No published data exists regarding manganese levels in amniotic fluid in normal and preeclamptic pregnancy. In light of the published structure of IPG-P (Larner et al. 2003), which confirms the presence of complexed Mn^{2+}, it would be of interest to examine this in the future. Considering the suggested structure of IPG-A, containing complexed zinc, it would be valuable to perform IPG-A bioactivity assays on the normal and preeclamptic amniotic fluid. Time limitations prevented this being done as part of this project.

Chapter 7

Conclusions, Discussion and Further Research

7.1 Summary and Conclusions

7.1.1 Longitudinal data

It has been shown from the longitudinal studies that the ELISA is a qualitative test with a large spread. It is not a quantitative assay and its value is in the detection of a rapid rise or a high value, which will ring alarm bells for the clinician. Subtle differences and low values can be ignored. When expressed as a percentage of a standard positive control, a cut-off of 80% (as established by Rodaris, See Chapter 2.1.4) seems to be a useful point for a 'positive' versus 'negative' value. In terms of monitoring IPG-P levels over the full pregnancy, the polyclonal-based ELISA is not a useful screening tool. However, it may be useful as a diagnostic tool for clinical usage.

Potential Clinical Usage of a Diagnostic Assay for Preeclampsia

There are several key problems facing clinicians in the management of preeclamptic patients where the presence of a diagnostic assay would be clinically useful.

1. **Missed Diagnoses**

   Patients with underlying pre-pregnancy pathologies, including hypertension and renal dysfunction often mask the emergence of preeclampsia and patients may go undiagnosed with all of the attendant risks to mother and fetus. Preeclampsia is frequently then a retrospective diagnosis.

2. **Mis-diagnosis**

   Patients with transient hypertension, often detected at the antenatal clinic visit, are admitted, in many cases, almost immediately to hospital. Preeclampsia symptoms may develop within hours and it is essential that these patients be closely monitored. Often, however, the rise in blood pressure is the result of a non-associated event and hospital beds are filled needlessly because of the absence of a definitive test. This may be particularly useful in patients with pre-existing risk factors for preeclampsia.

3. Management of the Preeclamptic

Patients with a confirmed clinical diagnosis of preeclampsia present a difficult management conundrum for clinicians. With the absence of any effective therapy, patient management involves blood pressure control and seizure prevention, cardiotocography and ultrasound assessment of the fetus as described earlier.

For the grouped data, there is a significant increase in the ELISA signal in preeclampsia (Figure 5.4). However, in some of the individual cases, no rise was seen in the ELISA prior to the onset of preeclampsia. Similarly, a high proportion of false positive results were seen in subjects without a hypertensive episode in the pregnancy. For this reason, I propose that the ELISA would be more clinically effective as a discriminatory or diagnostic tool in women with a hypertensive episode than as a screening tool in the first or second trimester.

7.1.2 Urinary IPGs in Labour and the Postnatal Period

I have shown in Chapter 4 that urinary IPG-P is significantly raised in labour in non-preeclamptic women (Paine et al. 2003, Appendix 5). It was also mentioned that despite this rapid rise and the removal of the placental source they persist into the circulation during the postpartum period for a considerable period (Kunjara et al. 2000a). It is possible that these raised levels in labouring women compared to non-labour may indicate a role in the onset or progress of labour.

A previous study has indicated the possibility of a common causal link between labour and preeclampsia (Adamsons and Wallach 1989) related to declining oxygen tension within the trophoblast. It is possible that this causes abnormal metabolism or release of an abnormal IPG form, which may then be involved in the pathophysiology and could explain why we have found similar urinary IPG values in preeclampsia and labour.

Potential Clinical Usage of a Diagnostic Assay for Labour

1. **Diagnosis of Early Labour at Term**

   Such an assay would be useful for women admitted in possible early labour. Frequently, other currently used parameters – cardiotocography, clinical assessment of the cervix and pH testing for the presence of amniotic fluid – provide an equivocal result.

2. **Diagnosis of Premature Labour**

   Women admitted with abdominal discomfort or pain when not at term in the pregnancy may be submitted to invasive speculum examination (which may indeed provide a falsely reassuring negative result) and are often needlessly admitted or wrongly discharged. A definitive diagnostic assay would eliminate much clinical uncertainty, risk to fetal and maternal health, and unnecessary expense.

Further Research

Due to the nature of the study design, the study carried out was unable to define IPG-P levels in early labour except in a small number of subjects undergoing induction of labour, which cannot be extrapolated to spontaneous labour. Further work needs to be carried out to obtain regular, timed urine samples leading up to labour. Additionally, more invasive studies could be designed to obtain regular urine samples from catheterised women in labour at more frequent intervals

7.1.3 IPGs in Established Preeclampsia

When analysing and comparing data from Established preeclampsia samples it must be stressed that the IPG-ELISA is likely to be measuring a different, high molecular weight form of IPG-P. The bio-extraction relies on interaction with the anion exchange column whereas the ELISA assay is likely to be selectively binding an abnormal form of IPG (possibly with a lipidic group attached) to the gelatin phase of the plate.

The data in Chapter 6 show that the overall IPG-ELISA values (expressed relative to a standard urine sample) for all samples were significantly higher in amniotic fluid

compared to urine. This suggests a build-up on fetal side of the placenta, possibly due to the re-circulation of fetal urine within the amniotic cavity or production within the fetal kidney. For the matched urine and amniotic fluid pairs, values were significantly higher for the preeclampsia/eclampsia group compared to the control group.

Amniotic Fluid and Urine

There was a positive correlation between amniotic fluid and urine IPG-ELISA values for the normal group, but this was not apparent in the preeclamptic group. This may be due to the disruption of the equilibrium (normally found in a healthy pregnancy) during or after the onset of the preeclamptic disease process.

Platelet count was inversely correlated to amniotic fluid IPG-ELISA values in preeclamptic women, but no correlation was seen with other measured clinical parameters (gestation, maternal age, birth weight, serum urate or mean arterial blood pressure) or between maternal urinary IPG-ELISA and platelet levels. An inverse correlation was also seen between maternal platelet count and bioactive IPG-P in uterine and peripheral vein in the preeclamptic women. This indicates that the IPG-P may be involved in the pathophysiology of preeclampsia. Platelet count is well established as a reliable clinical marker of disease severity (Redman et al. 1978) and forms a component of the HELLP syndrome, a severe form of preeclampsia. These data mirror the inverse relationship between urinary IPG-P bioactivity and platelet count that has been shown by Kunjara (Kunjara et al. 2000a).

Serum

Total IPG-P bioactivity levels in the uterine vein were lower in the preeclamptic group than in the non-preeclamptic women but no difference was seen between the groups for samples from peripheral vein, or umbilical vessels. Umbilical venous levels were not different from those in the umbilical artery. In the preeclamptic group, the IPG-P bioactivity was higher in the umbilical artery, and probably the umbilical vein when compared with the uterine vein and the same trend was seen when compared with the peripheral vein. These differences were not seen for the non-preeclamptic group.

There was a strong correlation between uterine vein and peripheral vein serum IPG-P levels in the preeclamptic group but not in the control group.
No correlation was seen with gestation for either preeclamptic or normal IPG-P levels for any of the values.

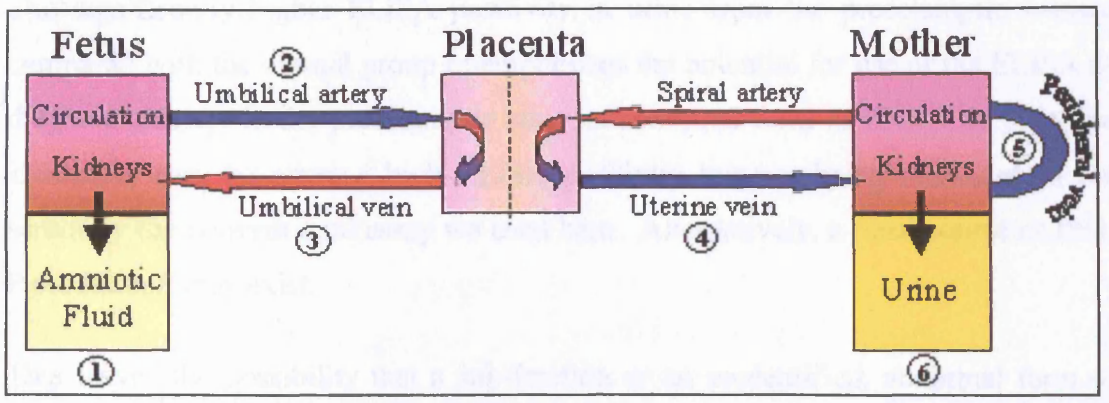


Figure 7. 1. Proposed equilibrium of IPG-P in non-preeclamptic women

The data would indicate equilibrium (Figure 7.1) in the normal women between the maternal and fetal sides of the placenta. No difference is seen between any of the measured IPG-P bioactivity levels in the normal women and the ELISA shows correlation between the amniotic fluid and maternal urine.

In the preeclamptic women, it appears that this equilibrium has been disrupted. Amniotic fluid and urine levels do not correlate and additionally, serum levels of bioactive IPG-P are higher on the fetal than the maternal side of the placenta. This may be due to active secretion of the IPG-P from the maternal kidney. This does not seem to occur in the fetal kidney due either to fetal immaturity or the fact that the amniotic sac is a closed compartment.

Previous work would suggest a placental source of the IPG-P production (Deborde et al. 1999b), with release into both maternal and fetal circulations (Kunjara et al. 2000a). However, the higher levels of measurable IPG-P in serum on the fetal side of the placenta in preeclampsia (Deborde et al. 1999a), and an absence of any difference in umbilical arterial and venous levels from this data might indicate an alternative situation. Firstly, a potential fetal production source with breakdown of placental transport to mother. Umbilical serum and amniotic fluid levels were significantly higher in preeclampsia than in normal and therefore a backlog on the fetal side may be occurring. Given these findings there may a placental production

source with release of an abnormal, form of IPG-P (Figure 7.2) into the maternal circulation. This form would not be detected by the conventional extraction and bioassay procedures.

The significantly higher ELISA positivity in urine from the preeclamptic women compared with the normal group re-emphasises the potential for use of the ELISA as diagnostic assay. In the preeclamptic state an abnormal form of IPG-P, as yet to be identified, may be causing high ELISA positivity but not being measured in the serum by the conventional assay we used here. Alternatively, a renal source of IPG-P production may exist.

This leaves the possibility that a sub-fraction or an unidentified, abnormal form of IPG-P is part of the pathophysiology of preeclampsia. In this situation, the amount of IPG-P measurable by the conventional bioassay may exclude an abnormal, lipidic form.

It is possible that IPG levels do not differ in the serum between the normal and preeclamptic state and that a difference is seen in the urine and amniotic fluid due to concentration/sequestration in these fluid compartments. Alternatively, increased renal clearance may be occurring, alongside increased production. Previous work has indicated that the IPG release is on the maternal side of the trophoblast(Deborde et al 1999b; Deborde et al 1999a) and it is thus interesting to speculate on the source of the IPG on the fetal side of the placenta. Amniotic fluid seems to be a rich source of IPG-P. It would be desirable to study larger numbers of patients to reinforce the data seen here.

7.2 Future Work

7.2.1 Labour Studies

Further studies need to be designed to obtain frequent urine samples for the days immediately preceding labour. Design would involve more effort for the subjects due to the constraints of freezing samples within 4 hours of collection and their subsequent transport. Additionally, more invasive studies could be designed to obtain

regular urine samples from catheterised women in labour at more frequent intervals. This would require consent by the subjects for urinary catheterisation and the consequent increased risk of urinary tract infection and urinary retention (post-catheter removal). This may represent an ethical barrier to such a study. Women with clinically indicated catheterisation in labour tend to be those with prolonged or obstructed labour – an abnormal sub-group and not necessarily representative.

The data I have presented here show parallels between preeclamptic pregnancy urine samples and non-preeclamptic labouring urine samples. Some early retrospective data have been shown to demonstrate that preeclamptic women are more easily induced into labour than non-preeclamptics (Bailey and Walton 2003). The effect was most marked at gestations below 37 weeks and in multiparous women. The authors do not state the method of induction used. Could this facilitation of induction of labour be related to elevated IPG-P levels? Conversely, Ferrazzani et al in a prospective study of a smaller series showed *lower* successful cervical ripening in the preeclamptic group compared to normals (Ferrazzani et al. 2003). The study design in this work was set to examine only non-preeclamptic women in labour and as such there is no comparable data. It would be valuable to include collection of delivery outcome data in a future prospective study collecting urine samples for ELISA in a larger cohort of preeclamptic and normal subjects requiring labour induction.

7.2.2 Preeclampsia

Ideally, more frequent and/or more regularly spaced samples should be collected to make the preeclamptic data set more robust and suitable for multiple regression analysis. It would be desirable to increase the numbers for the cross-sectional study looking at serum levels to elucidate any so far unseen differences in IPG-P within and between the preeclamptic and normal states. Furthermore, work on the IPG-A bioassay, which was not possible within the time frame of this MD would be ideal.

Acknowledgements

This work could not have been completed without the kindness of:

University College London:

Professor Thomas W Rademacher

Professor Charles H Rodeck

Dr Alan Jaques

Antonio Manuel Gomez-Galan

Nelson Mandela School of Medicine/King Edward VIII Hospital, Durban, South Africa

Professor Jack Moodley

Dr Michelle Singh

Dr Candice Roberts

Dr Sean Mould

Mrs Glynis Starling

Rodaris Pharmaceuticals, Oxford, UK

Dr Phil Williams,

Dr Betty Kunjara

All the labour ward, antenatal clinic and ward staff at University College Hospitals NHS Trust, London, UK and King Edward VIII Hospital, Durban, South Africa.

References

National High Blood Pressure Education Program Working Group Report on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol* 163: 1691-1712, 1990.

CLASP: a randomised trial of low-dose aspirin for the prevention and treatment of pre-eclampsia among 9364 pregnant women. CLASP (Collaborative Low-dose Aspirin Study in Pregnancy) Collaborative Group. *Lancet* 343: 619-629, 1994.

Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol* 183: S1-S22, 2000.

Abdalla HI, Billett A, Kan AK, Baig S, Wren M, Korea L and Studd JW. Obstetric outcome in 232 ovum donation pregnancies. *Br J Obstet Gynaecol* 105: 332-337, 1998.

Abdul-Karim R and Assali NS. Pressor response to angiotonin in pregnant and nonpregnant women. *Am J Obstet Gynecol* 1968: 88-96, 1961.

Acromite MT, Mantzoros CS, Leach RE, Hurwitz J and Dorey LG. Androgens in preeclampsia. *Am J Obstet Gynecol* 180: 60-63, 1999.

Adamsons K and Wallach RC. Single cause for initiation of labor and toxemia: a hypothesis. *Am J Perinatol* 6: 133-137, 1989.

Ahmed A, Dunk C, Ahmad S and Khaliq A. Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF) and soluble Flt-1 by oxygen--a review. *Placenta* 21 Suppl A: S16-S24, 2000.

Alvarez JF, Cabello MA, Feliu JE and Mato JM. A phospho-oligosaccharide mimics insulin action on glycogen phosphorylase and pyruvate kinase activities in isolated rat hepatocytes. *Biochem Biophys Res Commun* 147: 765-771, 1987.

Anderson RG. Caveolae: where incoming and outgoing messengers meet. *Proc Natl Acad Sci U S A* 90: 10909-10913, 1993.

Anderson RG. The caveolae membrane system. *Annu Rev Biochem* 67: 199-225, 1998.

Anderson RG, Kamen BA, Rothberg KG and Lacey SW. Potocytosis: sequestration and transport of small molecules by caveolae. *Science* 255: 410-411, 1992.

Antinori S, Versaci C, Panci C, Caffa B and Gholami GH. Fetal and maternal morbidity and mortality in menopausal women aged 45-63 years. *Hum Reprod* 10: 464-469, 1995.

Aquilina J, Thompson O, Thilaganathan B and Harrington K. Improved early prediction of pre-eclampsia by combining second-trimester maternal serum inhibin-A and uterine artery Doppler. *Ultrasound Obstet Gynecol* 17: 477-484, 2001.

Arkwright PD, Rademacher TW, Dwek RA and Redman CW. Pre-eclampsia is associated with an increase in trophoblast glycogen content and glycogen synthase activity, similar to that found in hydatidiform moles. *J Clin Invest* 91: 2744-2753, 1993.

Arngrimsson R, Bjornsson S, Geirsson RT, Bjornsson H, Walker JJ and Snaedal G. Genetic and familial predisposition to eclampsia and pre-eclampsia in a defined population. *Br J Obstet Gynaecol* 97: 762-769, 1990.

Arngrimsson R, Walker JJ, Geirsson RT and Bjornsson S. A low male/female sex ratio in offspring of women with a family history of pre-eclampsia and eclampsia. *Br J Obstet Gynaecol* 100: 496-497, 1993.

Ashour AM, Lieberman ES, Haug LE and Repke JT. The value of elevated second-trimester beta-human chorionic gonadotropin in predicting development of preeclampsia. *Am J Obstet Gynecol* 176: 438-442, 1997.

Asplin I, Galasko G and Larner J. chiro-inositol deficiency and insulin resistance: a comparison of the chiro-inositol- and the myo-inositol-containing insulin mediators isolated from urine, hemodialysate, and muscle of control and type II diabetic subjects. *Proc Natl Acad Sci U S A* 90: 5924-5928, 1993.

Attwood HD and Park WW. Embolism to the lungs by trophoblast. *J Obstet Gynaecol Br Commonw* 68: 611-617, 1961.

Augensen K and Bergsjo P. Maternal mortality in the Nordic countries 1970-1979. *Acta Obstet Gynecol Scand* 63: 115-121, 1984.

Babitt J, Trigatti B, Rigotti A, Smart EJ, Anderson RG, Xu S and Krieger M. Murine SR-BI, a high density lipoprotein receptor that mediates selective lipid uptake, is N-glycosylated and fatty acylated and colocalizes with plasma membrane caveolae. *J Biol Chem* 272: 13242-13249, 1997.

Bailey DJ and Walton SM. Term and pre-term induction of labour for pregnancy-induced hypertension [abstract]. Journal of Obstetrics and Gynaecology 2003;23(Supplement 1): S52.

Bauman WA, Maimen M and Langer O. An association between hyperinsulinemia and hypertension during the third trimester of pregnancy. *Am J Obstet Gynecol* 159: 446-450, 1988.

Berkowitz RS and Goldstein DP. Chorionic tumors. *N Engl J Med* 335: 1740-1748, 1996.

Bianchi DW, Williams JM, Sullivan LM, Hanson FW, Klinger KW and Shuber AP. PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies. *Am J Hum Genet* 61: 822-829, 1997.

Bjorkhem I, Lantto O, Lunell NO and Pschera H. Total and free cortisol in amniotic fluid during late pregnancy. *Br J Obstet Gynaecol* 85: 446-450, 1978.

Bosio PM, Cannon S, McKenna PJ, O'Herlihy C, Conroy R and Brady H. Plasma P-selectin is elevated in the first trimester in women who subsequently develop pre-eclampsia. *BJOG* 108: 709-715, 2001.

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

Bower S, Schuchter K and Campbell S. Doppler ultrasound screening as part of routine antenatal scanning: prediction of pre-eclampsia and intrauterine growth retardation. *Br J Obstet Gynaecol* 100: 989-994, 1993b.

Bower SJ, Harrington KF, Schuchter K, McGirr C and Campbell S. Prediction of pre-eclampsia by abnormal uterine Doppler ultrasound and modification by aspirin. *Br J Obstet Gynaecol* 103: 625-629, 1996.

Brennecke SP, Gude NM, Di Iulio JL and King RG. Reduction of placental nitric oxide synthase activity in pre-eclampsia. *Clin Sci (Lond)* 93: 51-55, 1997.

Brockelsby JC, Anthony FW, Johnson IR and Baker PN. The effects of vascular endothelial growth factor on endothelial cells: a potential role in preeclampsia. *Am J Obstet Gynecol* 182: 176-183, 2000.

Brosens IA, Robertson WB and Dixon HG. The role of the spiral arteries in the pathogenesis of preeclampsia. *Obstet Gynecol Annu* 1: 177-191, 1972.

Brown DA and Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* 68: 533-544, 1992.

Brown MA, Hague WM, Higgins J, Lowe S, McCowan L, Oats J, Peek MJ, Rowan JA and Walters BN. The detection, investigation and management of

hypertension in pregnancy: executive summary. *Aust N Z J Obstet Gynaecol* 40: 133-138, 2000.

Brown MA, Lindheimer MD, de Swiet M, Van Assche A and Moutquin J-M. The Classification and Diagnosis of the Hypertensive Disorders of Pregnancy: Statement From the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Hypertens Pregnancy* ix-xiv, 2001.

Bruni P, Vasta V, Berti L, Avila MA, Farnararo M and Varela-Nieto I. An inositol phosphoglycan stimulates glycolysis in human platelets. *Biochem Biophys Res Commun* 180: 1041-1047, 1991.

Campbell S, Diaz-Recasens J, Griffin DR, Cohen-Overbeek TE, Pearce JM, Willson K and Teague MJ. New doppler technique for assessing uteroplacental blood flow. *Lancet* 1: 675-677, 1983.

Campbell S, Pearce JM, Hackett G, Cohen-Overbeek T and Hernandez C. Qualitative assessment of uteroplacental blood flow: early screening test for high-risk pregnancies. *Obstet Gynecol* 68: 649-653, 1986.

Caro HN, Kunjara S, Rademacher TW, Leon Y, Jones DR, Avila MA and Varela-Nieto I. Isolation and partial characterisation of insulin-mimetic inositol phosphoglycans from human liver. *Biochem Mol Med* 61: 214-228, 1997.

Caro HN, Sheikh NA, Taverne J, Playfair JH and Rademacher TW. Structural similarities among malaria toxins insulin second messengers, and bacterial endotoxin. *Infect Immun* 64: 3438-3441, 1996.

Chappell L, Poulton L, Halligan A and Shennan AH. Lack of consistency in research papers over the definition of pre-eclampsia. *Br J Obstet Gynaecol* 106: 983-985, 1999a.

Chappell LC, Seed PT, Briley A, Kelly FJ, Hunt BJ, Charnock-Jones DS, Mallet AI and Poston L. A longitudinal study of biochemical variables in women at risk of preeclampsia. *Am J Obstet Gynecol* 187: 127-136, 2002.

Chappell LC, Seed PT, Briley AL, Kelly FJ, Lee R, Hunt BJ, Parmar K, Bewley SJ, Shennan AH, Steer PJ and Poston L. Effect of antioxidants on the occurrence of pre-eclampsia in women at increased risk: a randomised trial. *Lancet* 354: 810-816, 1999b.

Chen G, Wilson R, Cumming G, Walker JJ and McKillop JH. Production of prostacyclin and thromboxane A2 in mononuclear cells from preeclamptic women. *Am J Obstet Gynecol* 169: 1106-1111, 1993.

Chen G, Wilson R, Cumming G, Walker JJ and McKillop JH. Interleukin-2 and pregnancy induced hypertension. *J Obstet Gynaecol* 15: 27-30, 1995.

Cheng K and Larner J. Intracellular mediators of insulin action. *Annu Rev Physiol* 47: 405-424, 1985.

Cheng K, Thompson M and Larner J.In: Methods in Diabetes Research, edited by Larner J and Pohl S. New York, N.Y.: John Wiley and Sons, 1984, p. 81-89.

Chesley LC. History and epidemiology of preeclampsia-eclampsia. *Clin Obstet Gynecol* 27: 801-820, 1984.

Chesley LC, Annitto JE and Cosgrove RA. The familial factor in toxemia of pregnancy. *Obstet Gynecol* 32: 303-311, 1968.

Chitty LS, Altman DG, Henderson A and Campbell S. Charts of fetal size: 2. Head measurements. *Br J Obstet Gynaecol* 101: 35-43, 1994a.

Chitty LS, Altman DG, Henderson A and Campbell S. Charts of fetal size: 3. Abdominal measurements. *Br J Obstet Gynaecol* 101: 125-131, 1994b.

Chitty LS, Altman DG, Henderson A and Campbell S. Charts of fetal size: 4. Femur length. *Br J Obstet Gynaecol* 101: 132-135, 1994c.

Chua S, Wilkins T, Sargent I and Redman C. Trophoblast deportation in pre-eclamptic pregnancy. *Br J Obstet Gynaecol* 98: 973-979, 1991.

Clark DA. Does immunological intercourse prevent pre-eclampsia? *Lancet* 344: 969-970, 1994.

Cockell AP, Learmont JG, Smarason AK, Redman CW, Sargent IL and Poston L. Human placental syncytiotrophoblast microvillous membranes impair maternal vascular endothelial function. *Br J Obstet Gynaecol* 104: 235-240, 1997.

Conrad KP and Benyo DF. Placental cytokines and the pathogenesis of preeclampsia. *Am J Reprod Immunol* 37: 240-249, 1997.

Cooper R, Lipowski J, Ford E, Shamsi N, Feinberg H and Le Breton G. Increased membrane-bound calcium in platelets of hypertensive patients. *Hypertension* 13: 139-144, 1989.

Cuckle H, Sehmi I and Jones R. Maternal serum inhibin A can predict pre-eclampsia. *Br J Obstet Gynaecol* 105: 1101-1103, 1998.

Cunningham FG, MacDonald PC, Gant NF, Leveno KJ, Gilstrap LC, Hankins GDV and Clark AD. The Morphological and Functional Development of the Fetus. In: Williams' Obstetrics, edited by Cunningham FG, MacDonald PC, Gant NF, Leveno KJ, Gilstrap LC, Hankins GDV and Clark SL. Appleton and Lange, 1997, p. 151-190.

Das V, Bhargava T, Das SK and Pandey S. Microalbuminuria: a predictor of pregnancy-induced hypertension. *Br J Obstet Gynaecol* 103: 928-930, 1996.

Davey DA and MacGillivray I. The classification and definition of the hypertensive disorders of pregnancy. *Am J Obstet Gynecol* 158: 892-898, 1988.

Davidge ST. Oxidative stress and altered endothelial cell function in preeclampsia. *Semin Reprod Endocrinol* 16: 65-73, 1998.

Davidge ST, Stranko CP and Roberts JM. Urine but not plasma nitric oxide metabolites are decreased in women with preeclampsia. *Am J Obstet Gynecol* 174: 1008-1013, 1996.

Davidson BJ, Murray RD, Challis JR and Valenzuela GJ. Estrogen, progesterone, prolactin, prostaglandin E2, prostaglandin F2 alpha, 13,14-dihydro-15-keto-prostaglandin F2 alpha, and 6-keto-prostaglandin F1 alpha gradients across the uterus in women in labor and not in labor. *Am J Obstet Gynecol* 157: 54-58, 1987.

Dawson EB, Evans DR and Nosovitch J. Third-trimester amniotic fluid metal levels associated with preeclampsia. *Arch Environ Health* 54: 412-415, 1999.

Deborde S, Puan K, and Rademacher TW. GPI-PLD activity in the brush border membrane of normal human term placenta [abstract] Placenta 1999a;20: A.21.

Deborde S, Sooranna SR, Williams P, Mato J, and Rademacher TW. Higher detection of inositolphosphoglycans (IPG) in preeclamptic than in normal placenta by immunohistochemical staining [abstract] Placenta 1999b;20: A.21.

Dekker GA and Sibai BM. Early detection of preeclampsia. *Am J Obstet Gynecol* 165: 160-172, 1991.

Dekker GA and Sibai BM. Etiology and pathogenesis of preeclampsia: current concepts. *Am J Obstet Gynecol* 179: 1359-1375, 1998.

Department of Health. Why Mothers Die 1997-1999: The Fifth Report of the UK Confidential Enquiries into Maternal Deaths 2001London, RCOG Press.

Devemy E, Billat C, Sartelet H, Martiny L and Haye B. Erythropoietin stimulates glycosylphosphatidylinositol hydrolysis in rat erythroid progenitor cells and inositolphosphate glycan modulates their proliferation. *Cell Signal* 6: 523-529, 1994.

Di Iorio R, Marinoni E, Letizia C, Alo P, Villaccio B and Cosmi EV. Adrenomedullin, a new vasoactive peptide, is increased in preeclampsia. *Hypertension* 32: 758-763, 1998.

Diamant YZ, Metzger BE, Freinkel N and Shafrir E. Placental lipid and glycogen content in human and experimental diabetes mellitus. *Am J Obstet Gynecol* 144: 5-11, 1982.

Dorahy DJ, Lincz LF, Meldrum CJ and Burns GF. Biochemical isolation of a membrane microdomain from resting platelets highly enriched in the plasma membrane glycoprotein CD36. *Biochem J* 319 (Pt 1): 67-72, 1996.

Ducibella T, Huneau D, Angelichio E, Xu Z, Schultz RM, Kopf GS, Fissore R, Madoux S and Ozil JP. Egg-to-embryo transition is driven by differential responses to Ca(2+) oscillation number. *Dev Biol* 250: 280-291, 2002.

Duley L. Maternal mortality associated with hypertensive disorders of pregnancy in Africa, Asia, Latin America and the Caribbean. *Br J Obstet Gynaecol* 99: 547-553, 1992.

Endresen MJ, Lorentzen B and Henriksen T. Increased lipolytic activity and high ratio of free fatty acids to albumin in sera from women with preeclampsia leads to triglyceride accumulation in cultured endothelial cells. *Am J Obstet Gynecol* 440-447, 1992.

Eneroth E, Remberger M, Vahlne A and Ringden O. Increased serum concentrations of interleukin-2 receptor in the first trimester in women who later developed severe preeclampsia. *Acta Obstet Gynecol Scand* 77: 591-593, 1998.

Fanjul LF, Marrero I, Estevez F, Gonzalez J, Quintana J, Santana P and Ruiz de Galarreta CM. Follicle-stimulating hormone and human chorionic gonadotropin induced changes in granulosa cell glycosyl-phosphatidylinositol concentration. *J Cell Physiol* 155: 273-281, 1993a.

Fanjul LF, Marrero I, Gonzalez J, Quintana J, Santana P, Estevez F, Mato JM and Ruiz de Galarreta CM. Does oligosaccharide-phosphatidylinositol (glycosyl-phosphatidylinositol) hydrolysis mediate prolactin signal transduction in granulosa cells? *Eur J Biochem* 216: 747-755, 1993b.

Farese RV, Nair GP, Standaert ML and Cooper DR. Epidermal growth factor and insulin-like growth factor I stimulate the hydrolysis of the insulin-sensitive phosphatidylinositol-glycan in BC3H-1 myocytes. *Biochem Biophys Res Commun* 156: 1346-1352, 1988.

Ferrazzani S, Caruso A, De Carolis S, Martino IV and Mancuso S. Proteinuria and outcome of 444 pregnancies complicated by hypertension. *Am J Obstet Gynecol* 162: 366-371, 1990.

Ferrazzani S, De Santis L, Carducci B, Caliandro D, De Carolis S, Di Simone N and Caruso A. Prostaglandin: cervical ripening in hypertensive pregnancies. *Acta Obstet Gynecol Scand* 82: 510-515, 2003.

Friedman SA. Preeclampsia: a review of the role of prostaglandins. *Obstet Gynecol* 71: 122-137, 1988.

Gabbe SG, Demers LM, Greep RO and Villee CA. Placental glycogen metabolism in diabetes mellitus. *Diabetes* 21: 1185-1191, 1972.

Gant NF, Daley GL, Chand S, Whalley PJ and MacDonald PC. A study of angiotensin II pressor response throughout primigravid pregnancy. *J Clin Invest* 52: 2682-2689, 1973.

Gaulton GN, Kelly KL, Pawlowski J, Mato JM and Jarett L. Regulation and function of an insulin-sensitive glycosyl-phosphatidylinositol during T lymphocyte activation. *Cell* 53: 963-970, 1988.

Gaulton GN and Pratt JC. Glycosylated phosphatidylinositol molecules as second messengers. *Semin Immunol* 6: 97-104, 1994.

Geary M, Pringle PJ, Persaud M, Wilshin J, Hindmarsh PC, Rodeck CH and Brook CG. Leptin concentrations in maternal serum and cord blood: relationship to maternal anthropometry and fetal growth. *Br J Obstet Gynaecol* 106: 1054-1060, 1999.

Geraghty DE, Koller BH and Orr HT. A human major histocompatibility complex class I gene that encodes a protein with a shortened cytoplasmic segment. *Proc Natl Acad Sci U S A* 84: 9145-9149, 1987.

Gilbert RE, Augood C, Gupta R, Ades AE, Logan S, Sculpher M and Der Meulen JH. Screening for Down's syndrome: effects, safety, and cost effectiveness of first and second trimester strategies. *BMJ* 323: 423-425, 2001.

Goldman-Wohl DS, Ariel I, Greenfield C, Hochner-Celnikier D, Cross J, Fisher S and Yagel S. Lack of human leukocyte antigen-G expression in extravillous trophoblasts is associated with pre-eclampsia. *Mol Hum Reprod* 6: 88-95, 2000.

Gottschalk WK and Jarett L. The insulinomimetic effects of the polar head group of an insulin-sensitive glycophospholipid on pyruvate dehydrogenase in both subcellular and whole cell assays. *Arch Biochem Biophys* 261: 175-185, 1988.

Gratacos E, Filella X, Palacio M, Cararach V, Alonso PL and Fortuny A. Interleukin-4, interleukin-10, and granulocyte-macrophage colony stimulating factor in second-trimester serum from women with preeclampsia. *Obstet Gynecol* 92: 849-853, 1998.

Greer IA, Calder AA, Walker JJ, Lunan CB and Tulloch I. Increased platelet reactivity in pregnancy-induced hypertension and uncomplicated diabetic pregnancy: an indication for antiplatelet therapy? *Br J Obstet Gynaecol* 95: 1204-1206, 1988.

Grunewald C. Biochemical prediction of pre-eclampsia. *Acta Obstet Gynecol Scand Suppl* 164: 104-107, 1997.

Haddad B, Abirached F, Louis-Sylvestre C, Le Blond J, Paniel BJ and Zorn JR. Predictive value of early human chorionic gonadotrophin serum profiles for fetal growth retardation. *Hum Reprod* 14: 2872-2875, 1999.

Haller H, Oeney T, Hauck U, Distler A and Philipp T. Increased intracellular free calcium and sensitivity to angiotensin II in platelets of preeclamptic women. *Am J Hypertens* 2: 238-243, 1989.

Hanson U and Persson B. Epidemiology of pregnancy-induced hypertension and preeclampsia in type 1 (insulin-dependent) diabetic pregnancies in Sweden. *Acta Obstet Gynecol Scand* 77: 620-624, 1998.

Harlow FH, Brown MA, Brighton TA, Smith SL, Trickett AE, Kwan YL and Davis GK. Platelet activation in the hypertensive disorders of pregnancy. *Am J Obstet Gynecol* 187: 688-695, 2002.

Harmey JH, Doyle D, Brown V and Rogers MS. The cellular isoform of the prion protein, PrPc, is associated with caveolae in mouse neuroblastoma (N2a) cells. *Biochem Biophys Res Commun* 210: 753-759, 1995.

Heikkinen J, Mottonen M, Pulkki K, Lassila O and Alanen A. Cytokine levels in midtrimester amniotic fluid in normal pregnancy and in the prediction of pre-eclampsia. *Scand J Immunol* 53: 310-314, 2001.

Helewa ME, Burrows RF, Smith J, Williams K, Brain P and Rabkin SW. Report of the Canadian Hypertension Society Consensus Conference: 1. Definitions, evaluation and classification of hypertensive disorders in pregnancy. *CMAJ* 157: 715-725, 1997.

Higby K, Suiter CR, Phelps JY, Siler-Khodr T and Langer O. Normal values of urinary albumin and total protein excretion during pregnancy. *Am J Obstet Gynecol* 171: 984-989, 1994.

Higgins JR and Brennecke SP. Pre-eclampsia--still a disease of theories? *Curr Opin Obstet Gynecol* 10: 129-133, 1998.

Hopkinson ZE, Sattar N, Fleming R and Greer IA. Polycystic ovarian syndrome: the metabolic syndrome comes to gynaecology. *BMJ* 317: 329-332, 1998.

Huang LC, Fonteles MC, Houston DB, Zhang C and Larner J. Chiroinositol deficiency and insulin resistance. III. Acute glycogenic and hypoglycemic effects of two inositol phosphoglycan insulin mediators in normal and streptozotocin-diabetic rats in vivo. *Endocrinology* 132: 652-657, 1993.

Ilouz R, Kaidanovich O, Gurwitz D and Eldar-Finkelman H. Inhibition of glycogen synthase kinase-3beta by bivalent zinc ions: insight into the insulin-mimetic action of zinc. *Biochem Biophys Res Commun* 295: 102-106, 2002.

Irgens-Moller L, Hemmingsen L and Holm J. Diagnostic value of microalbuminuria in pre-eclampsia. *Clin Chim Acta* 157: 295-298, 1986.

Jaameri KE, Koivuniemi AP and Carpen EO. Occurrence of trophoblasts in the blood of toxaemic patients. *Gynaecologia* 160: 315-320, 1965.

Jackson MR, Carney EW, Lye SJ and Ritchie JW. Localization of two angiogenic growth factors (PDECGF and VEGF) in human placentae throughout gestation. *Placenta* 15: 341-353, 1994.

Jelkmann W. Pitfalls in the measurement of circulating vascular endothelial growth factor. *Clin Chem* 47: 617-623, 2001.

Johansen M, Redman CW, Wilkins T and Sargent IL. Trophoblast deportation in human pregnancy--its relevance for pre-eclampsia. *Placenta* 20: 531-539, 1999.

Jones DR and Varela-Nieto I. The role of glycosyl-phosphatidylinositol in signal transduction. *Int J Biochem Cell Biol* 30: 313-326, 1998.

Kaaja R, Laivuori H, Laakso M, Tikkanen MJ and Ylikorkala O. Evidence of a state of increased insulin resistance in preeclampsia. *Metabolism* 48: 892-896, 1999.

Kajii T and Ohama K. Androgenetic origin of hydatidiform mole. *Nature* 268: 633-634, 1977.

Katz A, Hultman E, Huang L, Villar-Palasi C and Larner J. No change in insulin mediators in human skeletal muscle during isometric contraction or recovery. *Horm Metab Res* 28: 545-548, 1996.

Kaunitz AM, Hughes JM, Grimes DA, Smith JC, Rochat RW and Kafrissen ME. Causes of maternal mortality in the United States. *Obstet Gynecol* 65: 605-612, 1985.

Kertesz Z, Hurst G, Ward M, Willis AC, Caro H, Linton EA, Sargent IL and Redman CW. Purification and characterization of a complex from placental syncytiotrophoblast microvillous membranes which inhibits the proliferation of human umbilical vein endothelial cells. *Placenta* 20: 71-79, 1999.

Klonoff-Cohen H, Edelstein S and Savitz D. Cigarette smoking and preeclampsia. *Obstet Gynecol* 81: 541-544, 1993.

Klonoff-Cohen HS, Savitz DA, Cefalo RC and McCann MF. An epidemiologic study of contraception and preeclampsia. *JAMA* 262: 3143-3147, 1989.

Knight M, Redman CW, Linton EA and Sargent IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. *Br J Obstet Gynaecol* 105: 632-640, 1998.

Koelman CA, Coumans AB, Nijman HW, Doxiadis II, Dekker GA and Claas FH. Correlation between oral sex and a low incidence of preeclampsia: a role for soluble HLA in seminal fluid? *J Reprod Immunol* 46: 155-166, 2000.

Kolasa F, Dec W, Szpakowski M and Krajewski J. [Magnesium content of the amniotic fluid in physiological pregnancy, prolonged pregnancy and EPH gestosis]. *Ginekol Pol* 54: 179-181, 1983.

Konijnenberg A, van der Post JA, Mol BW, Schaap MC, Lazarov R, Bleker OP, Boer K and Sturk A. Can flow cytometric detection of platelet activation early in pregnancy predict the occurrence of preeclampsia? A prospective study. *Am J Obstet Gynecol* 177: 434-442, 1997.

Konstantin-Hansen KF, Hesseldahl H and Pedersen SM. Microalbuminuria as a predictor of preeclampsia. *Acta Obstet Gynecol Scand* 71: 343-346, 1992.

Koopman MG, Krediet RT, Zuyderhoudt FJ, De Moor EA and Arisz L. A circadian rhythm of proteinuria in patients with a nephrotic syndrome. *Clin Sci (Lond)* 69: 395-401, 1985.

Kosch M, Hausberg M, Louwen F, Barenbrock M, Rahn KH and Kisters K. Alterations of plasma calcium and intracellular and membrane calcium in erythrocytes of patients with pre-eclampsia. *J Hum Hypertens* 14: 333-336, 2000.

Krauss T, Kuhn W, Lakoma C and Augustin HG. Circulating endothelial cell adhesion molecules as diagnostic markers for the early identification of pregnant women at risk for development of preeclampsia. *Am J Obstet Gynecol* 177: 443-449, 1997.

Kunjara S, Greenbaum AL, McLean P and Rademacher TW. Inositol phosphoglycans; a fine tuning mechanism to target calcium responses in the cell. *Nature* - Submitted: 2002.

Kunjara S, Greenbaum AL, Wang DY, Caro HN, McLean P, Redman CW and Rademacher TW. Inositol phosphoglycans and signal transduction systems in

pregnancy in preeclampsia and diabetes: evidence for a significant regulatory role in preeclampsia at placental and systemic levels. *Mol Genet Metab* 69: 144-158, 2000a.

Kunjara S, Wang DY, Greenbaum AL, McLean P, Kurtz A and Rademacher TW. Inositol phosphoglycans in diabetes and obesity: urinary levels of IPG A-type and IPG P-type, and relationship to pathophysiological changes. *Mol Genet Metab* 68: 488-502, 1999.

Kunjara S, Wang DY, McLean P, Greenbaum AL and Rademacher TW. Inositol phosphoglycans and the regulation of the secretion of leptin: in vitro effects on leptin release from adipocytes and the relationship to obesity. *Mol Genet Metab* 70: 61-68, 2000b.

Kupferminc MJ, Peaceman AM, Wigton TR, Rehnberg KA and Socol ML. Fetal fibronectin levels are elevated in maternal plasma and amniotic fluid of patients with severe preeclampsia. *Am J Obstet Gynecol* 172: 649-653, 1995.

Kvirkvelia N, Vojnovic I, Warner TD, Athie-Morales V, Free P, Rayment N, Chain BM, Rademacher TW, Lund T, Roitt IM and Delves PJ. Placentally derived prostaglandin E2 acts via the EP4 receptor to inhibit IL-2-dependent proliferation of CTLL-2 T cells. *Clin Exp Immunol* 127: 263-269, 2002.

Kyle PM, Buckley D, Kissane J, de Swiet M and Redman CW. The angiotensin sensitivity test and low-dose aspirin are ineffective methods to predict and prevent hypertensive disorders in nulliparous pregnancy. *Am J Obstet Gynecol* 173: 865-872, 1995.

Lachmeijer AM, Crusius JB, Pals G, Dekker GA, Arngrimsson R and ten Kate LP. Polymorphisms in the tumor necrosis factor and lymphotoxin-alpha gene region and preeclampsia. *Obstet Gynecol* 98: 612-619, 2001.

Lade JA, Moses EK, Guo G, Wilton AN, Grehan M, Cooper DW and Brennecke SP. The eNOS gene: a candidate for the preeclampsia susceptibility locus? *Hypertens Pregnancy* 18: 81-93, 1999.

Larner J. Insulin-signaling mechanisms. Lessons from the old testament of glycogen metabolism and the new testament of molecular biology. *Diabetes* 37: 262-275, 1988.

Larner J, Allan G, Kessler C, Reamer P, Gunn R and Huang LC. Phosphoinositol glycan derived mediators and insulin resistance. Prospects for diagnosis and therapy. *J Basic Clin Physiol Pharmacol* 9: 127-137, 1998.

Larner J, Huang LC, Schwartz CF, Oswald AS, Shen TY, Kinter M, Tang GZ and Zeller K. Rat liver insulin mediator which stimulates pyruvate dehydrogenase phosphate contains galactosamine and D-chiroinositol. *Biochem Biophys Res Commun* 151: 1416-1426, 1988.

Larner J, Price JD, Heimark D, Smith L, Rule G, Piccariello T, Fonteles MC, Pontes C, Vale D and Huang L. Isolation, structure, synthesis, and bioactivity of a novel putative insulin mediator. A galactosamine chiro-inositol pseudo-disaccharide Mn2+ chelate with insulin-like activity. *J Med Chem* 46: 3283-3291, 2003.

Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH, Sibai BM, Sukhatme VP and Karumanchi SA. Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* 350: 672-683, 2004b.

Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH, Sibai BM, Sukhatme VP and Karumanchi SA. Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* 350: 672-683, 2004a.

Lie RT, Rasmussen S, Brunborg H, Gjessing HK, Lie-Nielsen E and Irgens LM. Fetal and maternal contributions to risk of pre-eclampsia: population based study. *BMJ* 316: 1343-1347, 1998.

Liedholm H, Montan S and Aberg A. Risk grouping of 113 patients with hypertensive disorders during pregnancy, with respect to serum urate, proteinuria and time of onset of hypertension. *Acta Obstet Gynecol Scand Suppl* 118: 43-48, 1984.

Lilley K, Zhang C, Villar-Palasi C, Larner J and Huang L. Insulin mediator stimulation of pyruvate dehydrogenase phosphatases. *Arch Biochem Biophys* 296: 170-174, 1992.

Liston WA and Kilpatrick DC. Is genetic susceptibility to pre-eclampsia conferred by homozygosity for the same single recessive gene in mother and fetus? *Br J Obstet Gynaecol* 98: 1079-1086, 1991.

Livingston JC, Park V, Barton JR, Elfering S, Haddad B, Mabie WC, Quasney M and Sibai BM. Lack of association of severe preeclampsia with maternal and fetal mutant alleles for tumor necrosis factor alpha and lymphotoxin alpha genes and plasma tumor necrosis factor alpha levels. *Am J Obstet Gynecol* 184: 1273-1277, 2001.

Lo YM, Lau TK, Zhang J, Leung TN, Chang AM, Hjelm NM, Elmes RS and Bianchi DW. Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. *Clin Chem* 45: 1747-1751, 1999a.

Lo YM, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK, Haines CJ and Redman CW. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. *Clin Chem* 45: 184-188, 1999b.

Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, Wainscoat JS, Johnson PJ, Chang AM and Hjelm NM. Quantitative analysis of fetal DNA in

maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 62: 768-775, 1998.

Long PA and Oats JN. Preeclampsia in twin pregnancy--severity and pathogenesis. *Aust N Z J Obstet Gynaecol* 27: 1-5, 1987.

Lopatin DA, Ailamazian EK, Dmitrieva RI, Shpen VM, Fedorova OV, Doris PA and Bagrov AY. Circulating bufodienolide and cardenolide sodium pump inhibitors in preeclampsia. *J Hypertens* 17: 1179-1187, 1999.

Lorentzen B, Endresen MJ, Hovig T, Haug E and Henriksen T. Sera from preeclamptic women increase the content of triglycerides and reduce the release of prostacyclin in cultured endothelial cells. *Thromb Res* 63: 363-372, 1991.

Low MG and Saltiel AR. Structural and functional roles of glycosyl-phosphatidylinositol in membranes. *Science* 239: 268-275, 1988.

Macaulay SL and Larkins RG. Isolation of insulin-sensitive phosphatidylinositol-glycan from rat adipocytes. Its impaired breakdown in the streptozotocin-diabetic rat. *Biochem J* 271: 427-435, 1990.

Maeyama M, Matsuo I and Nakahara K. Glycogen metabolism in vesicles of hydatidiform mole in vitro. *Fertil Steril* 28: 851-855, 1977.

Makhseed M, Al Sharhan M, Egbase P, Al Essa M and Grudzinskas JG. Maternal and perinatal outcomes of multiple pregnancy following IVF-ET. *Int J Gynaecol Obstet* 61: 155-163, 1998.

Malchoff CD, Huang L, Gillespie N, Palasi CV, Schwartz CF, Cheng K, Hewlett EL and Larner J. A putative mediator of insulin action which inhibits adenylate cyclase and adenosine 3',5'-monophosphate-dependent protein kinase: partial purification from rat liver: site and kinetic mechanism of action. *Endocrinology* 120: 1327-1337, 1987.

Many A, Hubel CA and Roberts JM. Hyperuricemia and xanthine oxidase in preeclampsia, revisited. *Am J Obstet Gynecol* 174: 288-291, 1996.

Martin JN, Jr., May WL, Magann EF, Terrone DA, Rinehart BK and Blake PG. Early risk assessment of severe preeclampsia: admission battery of symptoms and laboratory tests to predict likelihood of subsequent significant maternal morbidity. *Am J Obstet Gynecol* 180: 1407-1414, 1999.

Martinez AE, Gonzalez OM, Quinones GA and Ferrannini E. Hyperinsulinemia in glucose-tolerant women with preeclampsia. A controlled study. *Am J Hypertens* 9: 610-614, 1996.

Mato JM, Kelly KL, Abler A and Jarett L. Identification of a novel insulin-sensitive glycophospholipid from H35 hepatoma cells. *J Biol Chem* 262: 2131-2137, 1987a.

Mato JM, Kelly KL, Abler A, Jarett L, Corkey BE, Cashel JA and Zopf D. Partial structure of an insulin-sensitive glycophospholipid. *Biochem Biophys Res Commun* 146: 764-770, 1987b.

Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, Epstein FH, Sukhatme VP and Karumanchi SA. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 111: 649-658, 2003.

McCarthy JF, Misra DN and Roberts JM. Maternal plasma leptin is increased in preeclampsia and positively correlates with fetal cord concentration. *Am J Obstet Gynecol* 180: 731-736, 1999.

Meekins JW, Pijnenborg R, Hanssens M, McFadyen IR and van Asshe A. A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies. *Br J Obstet Gynaecol* 101: 669-674, 1994.

Meis PJ, Goldenberg RL, Mercer BM, Iams JD, Moawad AH, Miodovnik M, Menard MK, Caritis SN, Thurnau GR, Bottoms SF, Das A, Roberts JM and McNellis D. The preterm prediction study: risk factors for indicated preterm births. Maternal-Fetal Medicine Units Network of the National Institute of Child Health and Human Development. *Am J Obstet Gynecol* 178: 562-567, 1998.

Mikhail MS, Anyaegbunam A, Garfinkel D, Palan PR, Basu J and Romney SL. Preeclampsia and antioxidant nutrients: decreased plasma levels of reduced ascorbic acid, alpha-tocopherol, and beta-carotene in women with preeclampsia. *Am J Obstet Gynecol* 171: 150-157, 1994.

Millar JG, Campbell SK, Albano JD, Higgins BR and Clark AD. Early prediction of pre-eclampsia by measurement of kallikrein and creatinine on a random urine sample. *Br J Obstet Gynaecol* 103: 421-426, 1996.

Misek DE and Saltiel AR. An inositol phosphate glycan derived from a Trypanosoma brucei glycosyl-phosphatidylinositol mimics some of the metabolic actions of insulin. *J Biol Chem* 267: 16266-16273, 1992.

Misiani R, Marchesi D, Tiraboschi G, Gualandris L, Pagni R, Goglio A, Amuso G, Muratore D, Bertuletti P and Massazza M. Urinary albumin excretion in normal pregnancy and pregnancy-induced hypertension. *Nephron* 59: 416-422, 1991.

Moodley J, Reddi K, Norman R and Naidoo JK. Amniotic fluid prostanoids in preeclampsia. *Obstet Gynecol* 64: 69-71, 1984.

Morgan L, Crawshaw S, Baker PN, Broughton PF and Kalsheker N. Maternal and fetal angiotensinogen gene allele sharing in pre-eclampsia. *Br J Obstet Gynaecol* 106: 244-251, 1999.

Morris NH, Sooranna SR, Learmont JG, Poston L, Ramsey B, Pearson JD and Steer PJ. Nitric oxide synthase activities in placental tissue from normotensive, pre-eclamptic and growth retarded pregnancies. *Br J Obstet Gynaecol* 102: 711-714, 1995.

Murai JT, Muzykanskiy E and Taylor RN. Maternal and fetal modulators of lipid metabolism correlate with the development of preeclampsia. *Metabolism* 46: 963-967, 1997.

Musci TJ, Roberts JM, Rodgers GM and Taylor RN. Mitogenic activity is increased in the sera of preeclamptic women before delivery. *Am J Obstet Gynecol* 159: 1446-1451, 1988.

Muttukrishna S, Knight PG, Groome NP, Redman CW and Ledger WL. Activin A and inhibin A as possible endocrine markers for pre-eclampsia. *Lancet* 349: 1285-1288, 1997.

Nakabayashi M, Sakura M, Takeda Y and Sato K. Elevated IL-6 in midtrimester amniotic fluid is involved with the onset of preeclampsia. *Am J Reprod Immunol* 39: 329-334, 1998.

Nestler JE. Inositolphosphoglycans (IPGs) as mediators of insulin's steroidogenic actions. *J Basic Clin Physiol Pharmacol* 9: 197-204, 1998.

Nestler JE, Jakubowicz DJ, Reamer P, Gunn RD and Allan G. Ovulatory and metabolic effects of D-chiro-inositol in the polycystic ovary syndrome. *N Engl J Med* 340: 1314-1320, 1999.

Nestler JE, Romero G, Huang LC, Zhang CG and Larner J. Insulin mediators are the signal transduction system responsible for insulin's actions on human placental steroidogenesis. *Endocrinology* 129: 2951-2956, 1991.

Nobunaga T, Tokugawa Y, Hashimoto K, Kimura T, Matsuzaki N, Nitta Y, Fujita T, Kidoguchi KI, Azuma C and Saji F. Plasma nitric oxide levels in pregnant patients with preeclampsia and essential hypertension. *Gynecol Obstet Invest* 41: 189-193, 1996.

Norris LA, Higgins JR, Darling MR, Walshe JJ and Bonnar J. Nitric oxide in the uteroplacental, fetoplacental, and peripheral circulations in preeclampsia. *Obstet Gynecol* 93: 958-963, 1999.

O'Brien M, Dausset J, Carosella ED and Moreau P. Analysis of the role of HLA-G in preeclampsia. *Hum Immunol* 61: 1126-1131, 2000.

Oney T and Kaulhausen H. The value of the angiotensin sensitivity test in the early diagnosis of hypertensive disorders in pregnancy. *Am J Obstet Gynecol* 142: 17-20, 1982.

Paine MA, Rodeck CH, Williams PJ and Rademacher TW. Possible involvement of inositol phosphoglycan-P in human parturition. *J Reprod Immunol* 59: 267-275, 2003.

Palmer SK, Moore LG, Young D, Cregger B, Berman JC and Zamudio S. Altered blood pressure course during normal pregnancy and increased preeclampsia at high altitude (3100 meters) in Colorado. *Am J Obstet Gynecol* 180: 1161-1168, 1999.

Parpal S, Gustavsson J and Stralfors P. Isolation of phosphooligosaccharide/phosphoinositol glycan from caveolae and cytosol of insulin-stimulated cells. *J Cell Biol* 131: 125-135, 1995.

Petraglia F, Luisi S, Benedetto C, Zonca M, Florio P, Casarosa E, Volpe A, Bernasconi S and Genazzani AR. Changes of dimeric inhibin B levels in maternal serum throughout healthy gestation and in women with gestational diseases. *J Clin Endocrinol Metab* 82: 2991-2995, 1997.

Pigny P, Desailloud R, Cortet-Rudelli C, Duhamel A, Deroubaix-Allard D, Racadot A and Dewailly D. Serum alpha-inhibin levels in polycystic ovary syndrome: relationship to the serum androstenedione level. *J Clin Endocrinol Metab* 82: 1939-1943, 1997.

Rademacher TW, Caro H, Kunjara S, Wang DY, Greenbaum AL and McLean P. Inositolphosphoglycan second messengers. *Braz J Med Biol Res* 27: 327-341, 1994.

Ramsay JE, Sattar N, and Greer IA. Leptin and body fat in preeclampsia and intrauterine growth restriction [abstract]. Journal of Obstetrics and Gynaecology 2003;23(Supplement 1): S16.

Redman CW. Platelets and the beginnings of preeclampsia. *N Engl J Med* 323: 478-480, 1990.

Redman CW, Bonnar J and Beilin L. Early platelet consumption in pre-eclampsia. *Br Med J* 1: 467-469, 1978.

Redman CW, Sacks GP and Sargent IL. Preeclampsia: an excessive maternal inflammatory response to pregnancy. *Am J Obstet Gynecol* 180: 499-506, 1999.

Rizzo G, Capponi A, Rinaldo D, Arduini D and Romanini C. Low cyclic guanosine monophosphate levels in the amniotic fluid of pre-eclamptic pregnancies. *Br J Obstet Gynaecol* 103: 834-837, 1996.

Roberts JM and Hubel CA. Is oxidative stress the link in the two-stage model of pre-eclampsia? *Lancet* 354: 788-789, 1999.

Roberts JM and Redman CW. Pre-eclampsia: more than pregnancy-induced hypertension. *Lancet* 341: 1447-1451, 1993.

Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA and McLaughlin MK. Preeclampsia: an endothelial cell disorder. *Am J Obstet Gynecol* 161: 1200-1204, 1989b.

Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA and McLaughlin MK. Preeclampsia: an endothelial cell disorder. *Am J Obstet Gynecol* 161: 1200-1204, 1989a.

Robertson WB, Brosens I and Dixon G. Uteroplacental vascular pathology. *Eur J Obstet Gynecol Reprod Biol* 5: 47-65, 1975.

Robillard PY, Dekker GA and Hulsey TC. Primipaternities in families: is the incidence of pregnancy-induced hypertensive disorders in multigravidas an anthropological marker of reproduction? *Aust N Z J Obstet Gynaecol* 38: 284-287, 1998.

Robillard PY, Dekker GA and Hulsey TC. Revisiting the epidemiological standard of preeclampsia: primigravidity or primipaternity? *Eur J Obstet Gynecol Reprod Biol* 84: 37-41, 1999.

Robillard PY and Hulsey TC. Association of pregnancy-induced-hypertension, pre-eclampsia, and eclampsia with duration of sexual cohabitation before conception. *Lancet* 347: 619, 1996.

Rodgers GM, Taylor RN and Roberts JM. Preeclampsia is associated with a serum factor cytotoxic to human endothelial cells. *Am J Obstet Gynecol* 159: 908-914, 1988.

Rodriguez MH, Masaki DI, Mestman J, Kumar D and Rude R. Calcium/creatinine ratio and microalbuminuria in the prediction of preeclampsia. *Am J Obstet Gynecol* 159: 1452-1455, 1988.

Romero G, Garmey JC and Veldhuis JD. The involvement of inositol phosphoglycan mediators in the modulation of steroidogenesis by insulin and insulin-like growth factor-I. *Endocrinology* 132: 1561-1568, 1993.

Ros HS, Cnattingius S and Lipworth L. Comparison of risk factors for preeclampsia and gestational hypertension in a population-based cohort study. *Am J Epidemiol* 147: 1062-1070, 1998.

Rothberg KG, Ying YS, Kolhouse JF, Kamen BA and Anderson RG. The glycophospholipid-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytic pathway. *J Cell Biol* 110: 637-649, 1990.

Roy AC, Viegas OA, Sen DK and Ratnam SS. Decreased levels of amniotic fluid oxytocinase activity in preeclampsia. *Gynecol Obstet Invest* 35: 166-168, 1993.

Said ME, Campbell DM, Azzam ME and MacGillivray I. Beta-human chorionic gonadotrophin levels before and after the development of pre-eclampsia. *Br J Obstet Gynaecol* 91: 772-775, 1984.

Salha O, Sharma V, Dada T, Nugent D, Rutherford AJ, Tomlinson AJ, Philips S, Allgar V and Walker JJ. The influence of donated gametes on the incidence of hypertensive disorders of pregnancy. *Hum Reprod* 14: 2268-2273, 1999.

Saltiel AR and Cuatrecasas P. Insulin stimulates the generation from hepatic plasma membranes of modulators derived from an inositol glycolipid. *Proc Natl Acad Sci U S A* 83: 5793-5797, 1986.

Saltiel AR, Fox JA, Sherline P and Cuatrecasas P. Insulin-stimulated hydrolysis of a novel glycolipid generates modulators of cAMP phosphodiesterase. *Science* 233: 967-972, 1986.

Sanchez-Bueno A, Greenwood MR, Varela-Nieto I, Marrero I, Gil B, Mato JM and Cobbold PH. Inositol-phosphoglycan inhibits calcium oscillations in hepatocytes by reducing calcium entry. *Cell Calcium* 21: 125-133, 1997.

Schmorl G. *Pathologisch-anatomische Untersuchungen über Publeraleklampsie.* Leipzig: Vogel, 1893.

Schofield JN and Rademacher TW. Structure and expression of the human glycosylphosphatidylinositol phospholipase D1 (GPLD1) gene. *Biochim Biophys Acta* 1494: 189-194, 2000.

Schubring C, Kiess W, Englaro P, Rascher W and Blum W. Leptin concentrations in amniotic fluid, venous and arterial cord blood and maternal serum: high leptin synthesis in the fetus and inverse correlation with placental weight. *Eur J Pediatr* 155: 830, 1996.

Seligman SP, Buyon JP, Clancy RM, Young BK and Abramson SB. The role of nitric oxide in the pathogenesis of preeclampsia. *Am J Obstet Gynecol* 171: 944-948, 1994.

Serhal PF and Craft IL. Oocyte donation in 61 patients. *Lancet* 1: 1185-1187, 1989.

Shashkin PN, Shashkina EF, Fernqvist-Forbes E, Zhou YP, Grill V and Katz A. Insulin mediators in man: effects of glucose ingestion and insulin resistance. *Diabetologia* 40: 557-563, 1997.

Shashkin PN, Wasner HK, Ortmeyer HK and Hansen BC. Prostaglandylinositol cyclic phosphate (cPIP): a novel second messenger of insulin action. Comparative analysis of two kinds of "insulin mediators". *Diabetes Metab Res Rev* 17: 273-284, 2001.

Sibai BM, Gordon T, Thom E, Caritis SN, Klebanoff M, McNellis D and Paul RH. Risk factors for preeclampsia in healthy nulliparous women: a prospective multicenter study. The National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. *Am J Obstet Gynecol* 172: 642-648, 1995.

Siddiqi T, Rosenn B, Mimouni F, Khoury J and Miodovnik M. Hypertension during pregnancy in insulin-dependent diabetic women. *Obstet Gynecol* 77: 514-519, 1991.

Silver HM, Lambert-Messerlian GM, Star JA, Hogan J and Canick JA. Comparison of maternal serum total activin A and inhibin A in normal, preeclamptic, and nonproteinuric gestationally hypertensive pregnancies. *Am J Obstet Gynecol* 180: 1131-1137, 1999.

Silver RM, Schwinzer B and McGregor JA. Interleukin-6 levels in amniotic fluid in normal and abnormal pregnancies: preeclampsia, small-for-gestational-age fetus, and premature labor. *Am J Obstet Gynecol* 169: 1101-1105, 1993.

Skjaerven R, Wilcox AJ and Lie RT. The interval between pregnancies and the risk of preeclampsia. *N Engl J Med* 346: 33-38, 2002.

Skupski DW, Nelson S, Kowalik A, Polaneczky M, Smith-Levitin M, Hutson JM and Rosenwaks Z. Multiple gestations from in vitro fertilization: successful implantation alone is not associated with subsequent preeclampsia. *Am J Obstet Gynecol* 175: 1029-1032, 1996.

Slattery MM and Morrison JJ. Preterm delivery. *Lancet* 360: 1489-1497, 2002.

Smarason AK, Allman KG, Young D and Redman CW. Elevated levels of serum nitrate, a stable end product of nitric oxide, in women with pre-eclampsia. *Br J Obstet Gynaecol* 104: 538-543, 1997.

Smarason AK, Sargent IL and Redman CW. Endothelial cell proliferation is suppressed by plasma but not serum from women with preeclampsia. *Am J Obstet Gynecol* 174: 787-793, 1996.

Smarason AK, Sargent IL, Starkey PM and Redman CW. The effect of placental syncytiotrophoblast microvillous membranes from normal and pre-eclamptic women on the growth of endothelial cells in vitro. *Br J Obstet Gynaecol* 100: 943-949, 1993.

Smart EJ, Mineo C and Anderson RG. Clustered folate receptors deliver 5-methyltetrahydrofolate to cytoplasm of MA104 cells. *J Cell Biol* 134: 1169-1177, 1996.

Sorensen TK, Williams MA, Zingheim RW, Clement SJ and Hickok DE. Elevated second-trimester human chorionic gonadotropin and subsequent pregnancy-induced hypertension. *Am J Obstet Gynecol* 169: 834-838, 1993.

Standley CA, Whitty JE, Mason BA and Cotton DB. Serum ionized magnesium levels in normal and preeclamptic gestation. *Obstet Gynecol* 89: 24-27, 1997.

Suzuki S, Taneda Y, Hirai S, Satoh Y and Toyota T. Insulin stimulates hydrolysis of plasmanylinositol-glycan and phosphatidylinositol-glycan in rat adipocytes. Insulin-induced generation of inositol glycan, alkylacylglycerol, and diacylglycerol. *Diabetes* 42: 988-994, 1993.

Talledo OE. Renin-angiotensin system in normal and toxemic pregnancies. I. Angiotensin infusion test. *Am J Obstet Gynecol* 96: 141-143, 1966.

Taylor RN, Casal DC, Jones LA, Varma M, Martin JN, Jr. and Roberts JM. Selective effects of preeclamptic sera on human endothelial cell procoagulant protein expression. *Am J Obstet Gynecol* 165: 1705-1710, 1991a.

Taylor RN, Musci TJ, Rodgers GM and Roberts JM. Preeclamptic sera stimulate increased platelet-derived growth factor mRNA and protein expression by cultured human endothelial cells. *Am J Reprod Immunol* 25: 105-108, 1991b.

Thomas MR, Tutschek B, Frost A, Rodeck CH, Yazdani N, Craft I and Williamson R. The time of appearance and disappearance of fetal DNA from the maternal circulation. *Prenat Diagn* 15: 641-646, 1995.

Thompson MP, Larner J and Kilpatrick DL. Purification and partial characterization of a putative mediator of insulin action on cyclic AMP-dependent protein kinase. *Mol Cell Biochem* 62: 67-75, 1984.

Torry DS, Wang HS, Wang TH, Caudle MR and Torry RJ. Preeclampsia is associated with reduced serum levels of placenta growth factor. *Am J Obstet Gynecol* 179: 1539-1544, 1998.

Tsukimori K, Maeda H, Shingu M, Koyanagi T, Nobunaga M and Nakano H. The possible role of endothelial cells in hypertensive disorders during pregnancy. *Obstet Gynecol* 80: 229-233, 1992a.

Tsukimori K, Maeda H, Shingu M, Koyanagi T, Nobunaga M and Nakano H. The possible role of endothelial cells in hypertensive disorders during pregnancy. *Obstet Gynecol* 80: 229-233, 1992b.

Vale W, River C and Vaughan J. Inhibins and Activins. In: The Physiology of Reproduction, edited by Knobil E and Neill JD. New York: Raven Press, 1994, p. 1861-1878.

van Beek E and Peeters LL. Pathogenesis of preeclampsia: a comprehensive model. *Obstet Gynecol Surv* 53: 233-239, 1998.

Varela-Nieto I, Leon Y and Caro HN. Cell signalling by inositol phosphoglycans from different species. *Comp Biochem Physiol B Biochem Mol Biol* 115: 223-241, 1996.

Vasta V, Bruni P, Clemente R, Vannini F, Ochoa P, Romero G, Farnararo M and Varela-Nieto I. Role of the glycosylphosphatidylinositol/inositol phosphoglycan system in human fibroblast proliferation. *Exp Cell Res* 200: 439-443, 1992.

Villalba M, Kelly KL and Mato JM. Inhibition of cyclic AMP-dependent protein kinase by the polar head group of an insulin-sensitive glycophospholipid. *Biochim Biophys Acta* 968: 69-76, 1988.

Vivien D, Bogdanowicz P, Boumediene K, Martiny L, Haye B and Pujol JP. Different phosphorylated forms of inositolphosphate glycan could be involved in the transforming growth factor-beta 1 (TGF-beta 1) signalling pathway. *Cell Signal* 6: 173-180, 1994.

Vuorela P, Helske S, Hornig C, Alitalo K, Weich H and Halmesmaki E. Amniotic fluid--soluble vascular endothelial growth factor receptor-1 in preeclampsia. *Obstet Gynecol* 95: 353-357, 2000.

Wald NJ, Watt HC and Hackshaw AK. Integrated screening for Down's syndrome on the basis of tests performed during the first and second trimesters. *N Engl J Med* 341: 461-467, 1999.

Walker JJ, Sargent I, Pijnenborg R, Visser GHA, Dekker GA, Pipkin FB, Davison JM, de Swiet M, Robson S, Howell P, Redman CW, Rubin PC and Duley L. Hypertension in Pregnancy - Report of a RCOG Meeting: 27 January 1994. *Br J Obstet Gynaecol* 101: 639-644, 1994.

Waller DK, Lustig LS, Cunningham GC, Feuchtbaum LB and Hook EB. The association between maternal serum alpha-fetoprotein and preterm birth, small for gestational age infants, preeclampsia, and placental complications. *Obstet Gynecol* 88: 816-822, 1996.

Wang PY, Kitchens RL and Munford RS. Bacterial lipopolysaccharide binds to CD14 in low-density domains of the monocyte-macrophage plasma membrane. *J Inflamm* 47: 126-137, 1995.

Ward K, Hata A, Jeunemaitre X, Helin C, Nelson L, Namikawa C, Farrington PF, Ogasawara M, Suzumori K, Tomoda S and . A molecular variant of angiotensinogen associated with preeclampsia. *Nat Genet* 4: 59-61, 1993.

Waugh JJ, Clark TJ, Divakaran TG, Khan KS and Kilby MD. Accuracy of urinalysis dipstick techniques in predicting significant proteinuria in pregnancy. *Obstet Gynecol* 103: 769-777, 2004.

Wenstrom KD, Owen J, Boots LR and DuBard MB. Elevated second-trimester human chorionic gonadotropin levels in association with poor pregnancy outcome. *Am J Obstet Gynecol* 171: 1038-1041, 1994.

Williams DJ and de Swiet M. The pathophysiology of pre-eclampsia. *Intensive Care Med* 23: 620-629, 1997.

Wilson JM and Jungner YG. [Principles and practice of mass screening for disease]. *Bol Oficina Sanit Panam* 65: 281-393, 1968.

World Health Organisation Group. The Hypertensive Disorders of Pregnancy 1987;Technical Report Series No. 758Geneva, WHO.

Yamada E. The fine structure of the gall bladder epithelium of the mouse. *J Biophys Biochem Cytol* 1: 445-458, 1955.

Ylikorkala O, Makila UM and Viinikka L. Amniotic fluid prostacyclin and thromboxane in normal, preeclamptic, and some other complicated pregnancies. *Am J Obstet Gynecol* 141: 487-490, 1981.

Zamudio S, Leslie KK, White M, Hagerman DD and Moore LG. Low serum estradiol and high serum progesterone concentrations characterize hypertensive pregnancies at high altitude. *J Soc Gynecol Investig* 1: 197-205, 1994.

Zapata A, Leon Y, Mato JM, Varela-Nieto I, Penades S and Martin-Lomas M. Synthesis and investigation of the possible insulin-like activity of 1D-4-O- and 1D-6-O-(2-amino-2-deoxy-alpha-D-glucopyranosyl)-myo-inositol 1-phosphate and 1D-6-O-(2-amino-2-deoxy-alpha-D-glucopyranosyl)-myo-inositol 1,2-(cyclic phosphate). *Carbohydr Res* 264: 21-31, 1994.

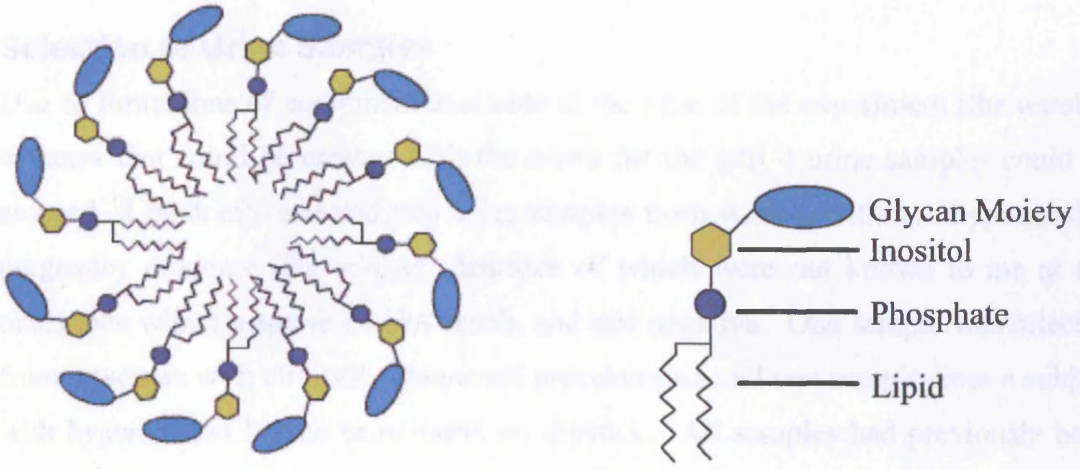
Zemel MB, Zemel PC, Berry S, Norman G, Kowalczyk C, Sokol RJ, Standley PR, Walsh MF and Sowers JR. Altered platelet calcium metabolism as an early predictor of increased peripheral vascular resistance and preeclampsia in urban black women. *N Engl J Med* 323: 434-438, 1990.

Zuijderhoudt FM, Kluitenberg WE, Satink JG and Huikeshoven FJ. Measuring low-grade albuminuria in pregnancy. *Br J Obstet Gynaecol* 96: 619-620, 1989.

Appendix 1: Comments on the IPG-ELISA

As stated previously, the polyclonal-based ELISA used here is effective due to a physical property of the IPG-P (possibly an abnormal, lipidic form) combining with the gelatin phase of the plate. The greatly raised positivity of the ELISA with preeclamptic urine and amniotic fluid samples indicates much higher levels of IPG-P in these compartments and/or an altered binding property of the IPG-P. It is possible that the IPG-P molecules may form micelles in the urine and amniotic fluid due to an amphiphilic property, (Figure A1.1).

Figure A1. 1. Schematic representation of GPI micelle showing hypothetical abnormal lipidic form.



Previous data does support the evidence of raised IPG-P bioactivity in maternal urine in preeclampsia (Kunjara et al. 2000a).

The IPG-P polyclonal ELISA assay was provided by kindness of Rodaris Pharmaceuticals Ltd (Oxford, UK). At the outset of the research, it was presented as a reliable assay for the diagnosis and possibly the prediction of preeclampsia. The company provided data of the inoculation protocol that was used to make the polyclonal. The IPG-P polyclonal antigen used as the immunogen in the preparation, was purified by the then-best purification procedure from the urine of a single woman with severe pre-eclampsia. However, no specific data were provided on the

A1.2 Protein Content of Urine Samples

As an adjunct to the above investigation, protein values for the non-labour and labour samples from non-preeclamptic subjects in the study discussed in Chapter 4 were plotted against ELISA signal values. These can be seen in figure A1.3 below. It is noted that a possible correlation exists, although the r-squared values are low (0.37 for labour and 0.54 for non-labour samples respectively). Therefore the option of expressing the ELISA values per unit of protein should be considered.

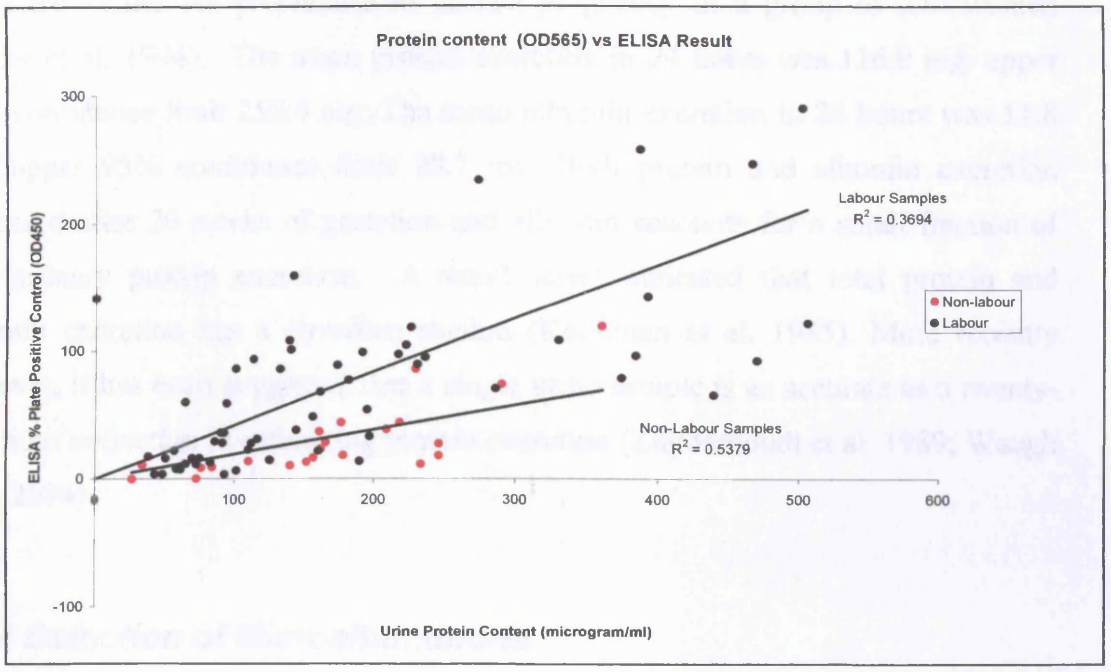


Figure A1. 3. Plot of urine protein content in μg/ml against ELISA value for non-labour and labour samples with respective trend lines and r-squared values.

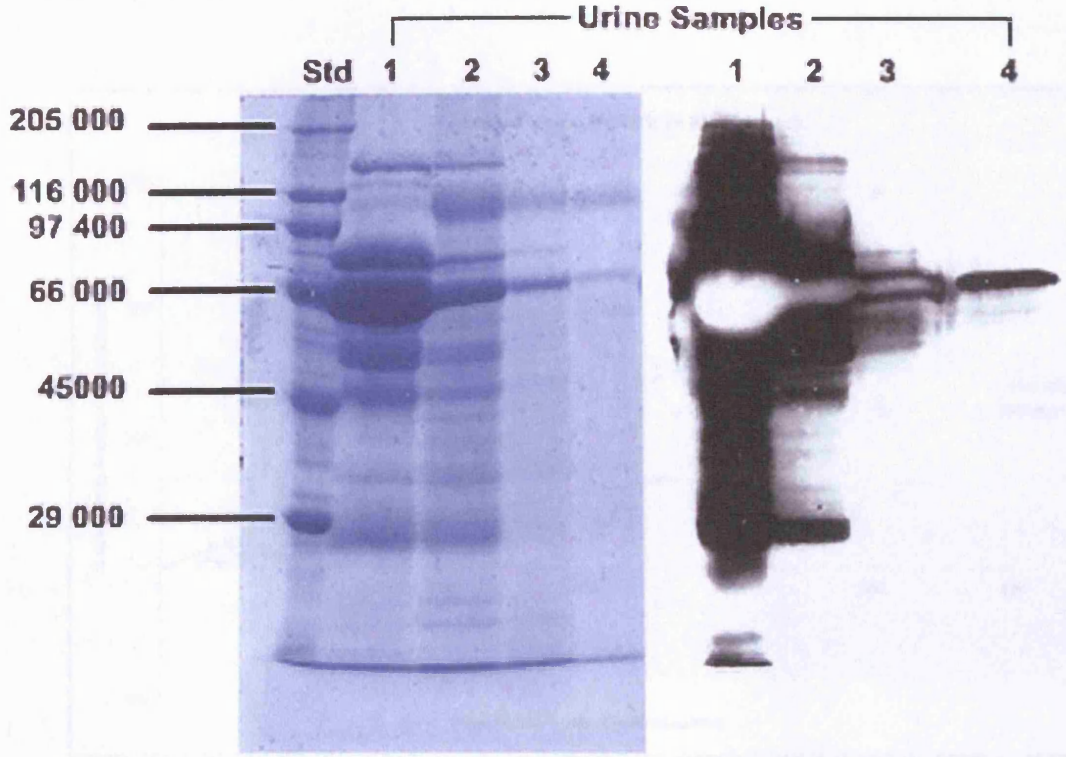
A1.3 Proteinuria and Microalbuminuria

Proteinuria can be a common finding in normal, non-preeclamptic pregnancy, due to increased filtration rate and morphological and/or functional changes in the glomerular capillary. However proteinuria also correlates with increasing perinatal mortality (Ferrazzani et al. 1990).

Results

See Figure A1.2 below.

Figure A1. 2. Western Blot of urine samples with polyclonal ELISA serum. Protein standard (left-hand lane) (Sigma, Poole, Dorset, UK) plus urine samples in corresponding lanes.



The above film reveals that the polyclonal serum showed reactivity with the full spectrum of proteins present in the preeclamptic sample, including human serum albumin (HSA, M_r = 68,000). This might indicate that the original stock solution used as the inoculate to prepare the serum was non-pure IPG-P. Consequently, the ELISA may then be detecting protein as well as IPG-P. However, it must be remembered that the Western gel will only detect proteins (and not carbohydrates/lipids).

purity of the IPG-P antigen and the antigen itself has never been fully characterised.

I speculated that the ELISA polyclonal antibody might be detecting other elements in the urine and amniotic fluid. Proteinuria is part of the definition of preeclampsia (Brown et al. 2001) and the extract was prepared from the urine of a preeclamptic woman (although clinical data of this index subject was not available from the company at the time).

A1.1 Western Blot of Urine Samples with Polyclonal Serum

Method

See Chapter 2 – Lab Methods

Selection of Urine Samples

Due to limitations of equipment available at the time of the experiment (the number of lanes that could be created with the comb for the gel) 4 urine samples could be assayed. I randomly selected two urine samples from women with non-hypertensive pregnancy outcome (the subject identities of which were not known to me at the time), one with a positive ELISA result, and one negative. One sample was selected from a woman with clinically diagnosed preeclampsia and one sample from a subject with hypertension but no proteinuria on dipstick. All samples had previously been assayed using the ELISA assay and protein assay as described above. The samples and results of these two assays are summarised in table A1.1 below:

Table A1. 1. Samples selected for Western Blot

Sample	Unique Number	Clinical Pregnancy Outcome	ELISA Result (OD 450)	Protein Assay (μg/ml at OD 565)
①	321	Preeclampsia	313	630
②	432	Non-proteinuric hypertension	212	82
③	262	Normal	208	367
④	264	Normal	14	228

Abnormal protein excretion in pregnancy is usually defined as >300mg in 24 hours but it is not clear from where this figure is derived. Early work involved small study groups or mixed normal and hypertensive subjects.
Importantly, microalbuminuria is distinct from total proteinuria and albumin accounts for a small fraction of urinary protein (less than 12%) (Higby et al. 1994) This may be significant as the protein used bovine serum albumin as the standard.

Twenty-four hour urine collection for total protein is the gold standard. Higby et al published values for proteinuria in normal pregnancy in a group of 270 women (Higby et al. 1994). The mean protein excretion in 24 hours was 116.9 mg, upper 95% confidence limit 259.4 mg. The mean albumin excretion in 24 hours was 11.8 mg, upper 95% confidence limit 28.7 mg. Both protein and albumin excretion increased after 20 weeks of gestation and albumin accounts for a small fraction of total urinary protein excretion. A small series indicated that total protein and albumin excretion has a circadian rhythm (Koopman et al. 1985). More recently however, it has been suggested that a single urine sample is as accurate as a twenty-four hour collection in estimating protein excretion (Zuijderhoudt et al. 1989; Waugh et al. 2004).

A1.4 Detection of Microalbuminuria

Various published techniques have been used to detect microalbuminuria in a clinical setting (Table A1.2)

Table A1. 2. Methods for assessing microalbuminuria compared with IPG-P ELISA

Method Used	Cut-off for Positive Test	Sensitivity %	Specificity %	PPV %	NPV %
Micral Test[1]	20 μg/ml	68	92	56	94
Radio ImmunoAssay[2]	11 μg/ml	50	82	26	93
Immunochemical [3]		61	87	61	87
IPG-P ELISA					
General Population	80% PPC*	42	88	31	92
High-Risk Population	80% PPC*	42	94	61	88

[1](Das et al. 1996); [2] (Rodriguez et al. 1988); [3] (Irgens-Moller et al. 1986)
*PPC = Percent of Plate Positive Control

Das et al showed in a small group of low-risk women that detection of microalbuminuria of greater than 20μg/ml might be useful as a screening test for preeclampsia (Das et al. 1996). However, the study group had a very high incidence of preeclampsia (14.6%), which would tend to increase the sensitivity of the assay.

Misiani et al (Misiani et al. 1991) showed level of daytime urinary albumin secretion was significantly lower than in the post-partum period or non-pregnant controls, possibly due to lower serum albumin levels in pregnancy. They showed that secretion increased in women with subsequent preeclampsia from the 28th week onwards and on average 9 weeks before the clinical onset of the disorder. Another group has shown an increase in urinary albumin secretion in normal pregnancy and no significant difference in those subsequently developing preeclampsia (Konstantin-Hansen et al. 1992).

Figure A1.4 shows the relationship of gestation vs. protein content for all samples from subjects with a non-preeclamptic pregnancy outcome. Preeclamptic subjects have been removed, as this would inevitably bias the set, as they will by definition have increased proteinuria after 28 weeks. The graph shows a wide variation in protein content

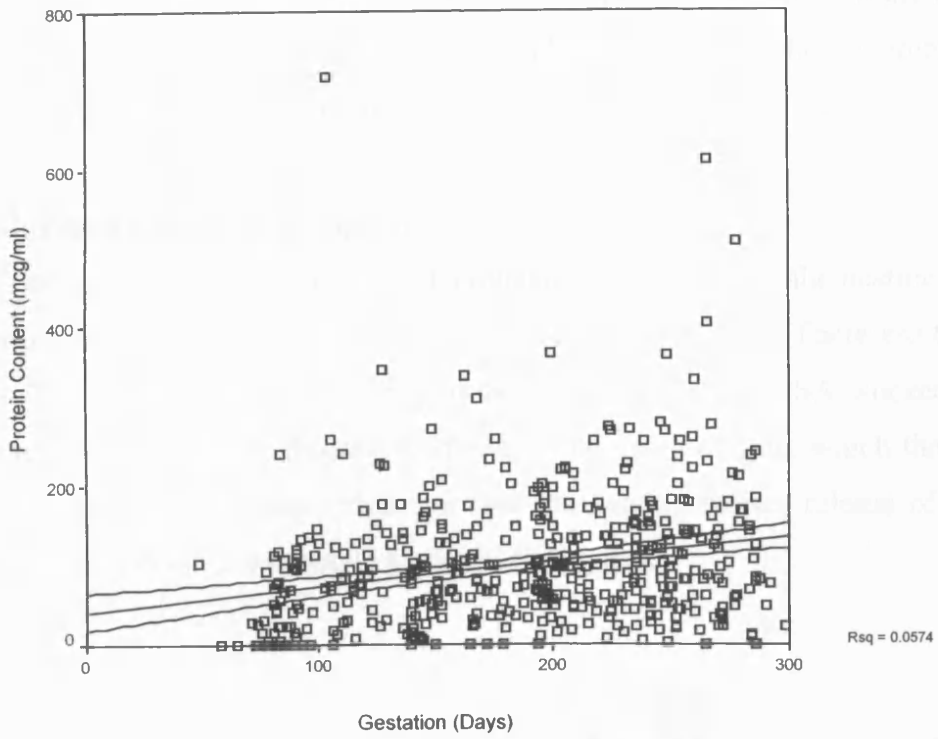


Figure A1. 4. Graph of Gestation vs. Protein Content for all non-preeclamptic subjects.

There are commercially available proteinuria tests such as Albustix® (Bayer Diagnostics) and the more sensitive (and more expensive) Micral Test II ® (Roche Diagnostics).

A1.5 Caveats Regarding the Polyclonal Serum

Given the above comments and that the polyclonal-based ELISA signal correlates to some degree with proteinuria, it is possible that certain problems exist regarding its original preparation.

Firstly, low molecular weight compounds (<3000 MW) are not very antigenic unless they are complexed with proteins. The IPG extract was not complexed in this way and was used as a raw material for the preparation of the serum. Secondly, the correlation with protein content is seen in the urine samples from normal women (and not just in preeclamptics). This does not mean that the ELISA is exclusively picking up protein however. Finally, the Western blot is seen to be picking up every

band of protein in the blot that is found in the preeclamptic (PE) urine (and not the one band that is not in the PE urine i.e. albumin). It must be remembered that of course gels only pick up protein.

Polyclonal Heated Samples

It is an interesting feature of the polyclonal protocol used that the heating of the urine samples alters (in the majority of cases) the ELISA signal. There are two obvious possibilities, related to the binding of the substrate to the ELISA blocked plate well. Either that the heating denatures protein in the urine sample, which then facilitates the capture by the gelatin phase, or that the heating causes release of bound IPG which then becomes detectable by the ELISA system.

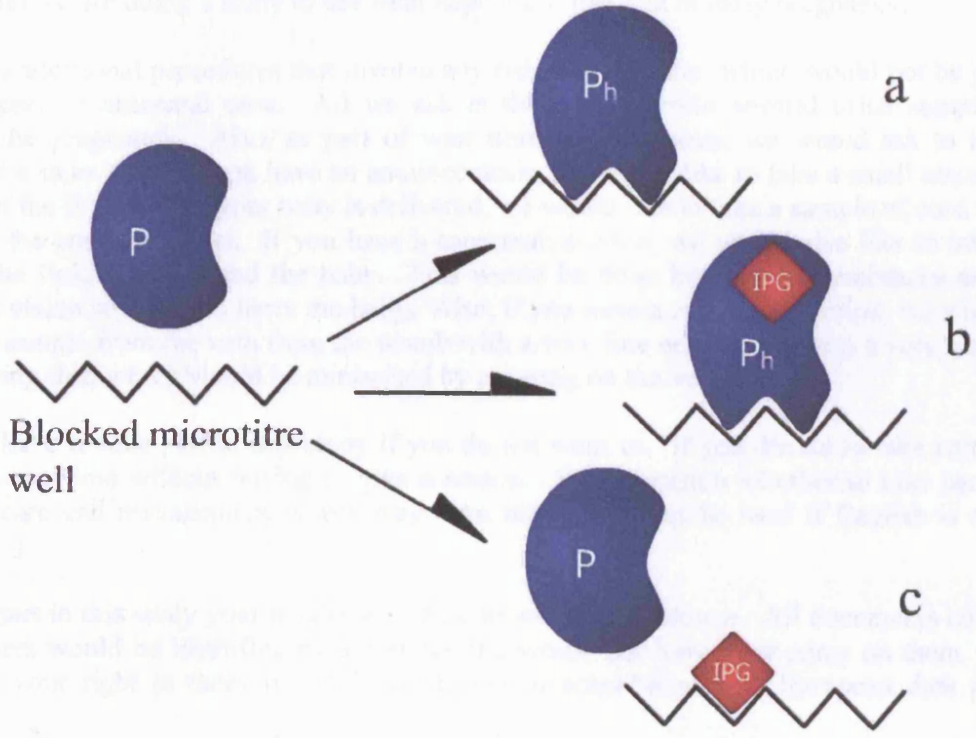


Figure A1. 5. Possible mechanisms for ELISA microtitre well capture following heating, showing (a) denatured protein (b) bound IPG enabling protein binding (possibly with reconfiguration) and (c) heating causing release of free IPG which binds to gelatin phase.

University College London Hospitals NHS
NHS Trust
Dr M A Paine
Elizabeth Garrett Anderson Hospital
Huntley Street
London WC1E 6DH

Telephone:

Appendix 2 – Patient Information Sheet (CSP-MAP-001)

Patient Information Sheet **Confidential**

Study title:
A prospective observational cohort study to determine the role of inositol phosphoglycans in the onset of pre-eclampsia in a population of women

You are invited to take part in some research at this hospital to look at pre-eclampsia. You may have heard of this condition, which results in high blood pressure during pregnancy and can affect up to 5% of all women during their pregnancy. At this time, the cause of pre-eclampsia is not known and there is no treatment except to deliver the baby. A new urine test is being developed to diagnose pre-eclampsia and we are doing a study to see what happens to this test in early pregnancy.

There are no additional procedures that involve any risk to your baby, which would not be performed already as part of antenatal care. All we ask is that you provide several urine samples for us throughout the pregnancy. Also, as part of your normal blood tests, we would ask to take small samples at the same time. If you have an amniocentesis, we would like to take a small amount (a few mls) extra of the fluid. After your baby is delivered, we would like to take a sample of cord blood and also part or the entire placenta. If you have a caesarean section, we would also like to take a small sample of the fluid from around the baby. This would be done before the membranes are broken, under direct vision so as not to harm the baby. Also, if you have a caesarean section, we would take a small blood sample from the vein from the womb with a very fine needle. There is a very small risk of bruising during this, which would be minimised by pressing on the vein.

You do not have to take part in this study if you do not want to. If you decide to take part you may withdraw at any time without having to give a reason. Your decision whether to take part will not affect your care and management in any way. An interpreter can be used if English is not a first language.

If you take part in this study your results would be treated in confidence. All documents collected by the researchers would be identified by a number and would not have your name on them. All such records and your right to them would be protected in accordance with European data protection legislation.

You are encouraged to request any further information about the study and you can contact:

Dr Malcolm Pain or (if Dr Paine is absent)
Clinical Research Fellow
Department of Obstetrics and Gynaecology
University College Hospital
86-96 Chenies Mews
London WC1
Aircall

Professor Rodeck
Department of Obstetrics & Gynaecology
University College Hospital
Telephone: 7

All proposals for research using human subjects are reviewed by an Ethics committee before they can proceed. This proposal was reviewed by the Joint UCL/UCLH Committees on the Ethics of Human Research.

University College London Hospitals NHS
NHS Trust
Dr M A Paine
Elizabeth Garrett Anderson Hospital
Huntley Street
London WC1E 6DH

Appendix 3 – Patient Information Sheet (CSP-MAP-004)

Patient Information Sheet **Confidential**

Study title:
An observational cohort study to determine the role of inositol phosphoglycans (P-IPG) in the onset of labour.

You are invited to take part in some research at this hospital to look at a new group of messenger compounds called Inositol Phosphoglycans or 'IPG's. These have been found in many parts of the body. They are involved in the way different cells in the body send messages to each other. Some previous work that has been done has shown that they may be increased in urine as part of the normal process of labour. We want to find out if this is true.

Taking part in the study would involve firstly answering some simple questions about your pregnancy and medical history and giving one urine sample in the antenatal clinic. Secondly, we would ask for several samples of urine during your labour. We will not interfere with your labour or delivery in any other way. There will be no procedures that will involve any risk to you or your baby.

You do not have to take part in this study if you do not want to. If you decide to take **part you may withdraw from the study at any moment without having to give a reason.** Your decision whether to take part will not affect your care and management in any way. An interpreter can be used if English is not a first language.

If you take part in this study your results would be treated in confidence. All documents collected by the researchers would be identified by a number and would not have your name on them. All such records and your right to them would be protected in accordance with European data protection legislation.
You are encouraged to request any further information about the study and you can contact:

Dr Malcolm Paine
Clinical Research Fellow
Department of Obstetrics and Gynaecology
University College Hospital
86-96 Chenies Mews
London WC1

All proposals for research using human subjects are reviewed by an Ethics committee before they can proceed. This proposal was reviewed by the Joint UCL/UCLH Committees on the Ethics of Human Research.

Version 2

University College London Hospitals NHS
NHS Trust
Dr M A Paine
Elizabeth Garrett Anderson Hospital
Huntley Street
London WC1E 6DH

Appendix 4 – Patient Information Sheet (CSP-MAP-005)

Patient Information Sheet **Confidential**

Study title:

A case-control study to determine levels of Inositol Phosphoglycans (IPGs) in pre-eclampsia.

You are invited to take part in some research at this hospital to look at pre-eclampsia. You may have heard of this condition, which results in high blood pressure during pregnancy and can affect up to 5% of all women during their pregnancy. At this time, the cause of pre-eclampsia is not known and there is no treatment except to deliver the baby. We have shown that IPGs are raised in pre-eclampsia in urine and this study is looking at where the levels are highest in order to discover more about the cause of pre-eclampsia. We are recruiting women with pre-eclampsia and also women with normal pregnancies for comparison.

The obstetric team has decided that the best way to deliver your baby (or babies) is by caesarean section. We would like to take some samples before and during the procedure. This should not prolong the operation significantly and there is no risk to your baby. The samples will be:

- **Before the Procedure:**
  - A urine specimen and a saliva sample
  - A blood sample just before the operation.
- **During the Caesarean Section:**
  1. A small sample of blood from the vein of the womb, taken with a fine needle before the delivery of the babies. There is a small risk of bruising from this, which will be minimised by pressing on the vein after taking the sample.
  2. A sample of fluid from around the baby (or babies), taken before the membranes are ruptured. This will be taken under direct vision, so that we can see that we will not harm the baby.
  3. Part of the placenta and some blood from the umbilical cord - after the delivery.

Finally, we would like to take a daily urine sample for three days after your operation.

You do not have to take part in this study if you do not want to. If you decide to take part you may withdraw at any time without having to give a reason. Your decision whether to take part will not affect your care and management in any way. An interpreter can be used if English is not a first language.

If you take part in this study your results would be treated in confidence. All documents collected by the researchers would be identified by a number and would not have your name on them. All such records and your right to them would be protected in accordance with European data protection legislation.

You are encouraged to request any further information about the study and you can contact:

Dr Malcolm Paine
Clinical Research Fellow
Department of Obstetrics and Gynaecology
University College Hospital
86-96 Chenies Mews
London WC1

Aircall 0800 679402
Number 'UCH 443'

All proposals for research using human subjects are reviewed by an Ethics committee before they can proceed. This proposal was reviewed by the Joint UCL/UCLH Committees on the Ethics of Human Research.

Version 2

University College London Hospitals NHS
NHS Trust
Dr M A Paine
Elizabeth Garrett Anderson Hospital
Huntley Street
London WC1E 6DH

Appendix 5 – Consent Form

Consent Form **Confidential**

Study Title:

Insert Study Title Here

Please ask the patient to complete the following:

	Please circle as necessary
Have you read the Patient Information Sheet?	Yes / No
Have you had an opportunity to ask questions and discuss the study?	Yes / No
Have you received satisfactory answers to all your questions?	Yes / No
Have you received enough information about the study?	Yes / No

To whom have you spoken?.....

Do you understand that you are free to withdraw from the study:

At any time?
Without having to give a reason for withdrawing?
And without affecting your future medical care? Yes / No

Do you agree to take part in this study? Yes / No

Signed..... Date.....

(Name in Block Letters).....

Signed (Researcher)..... Date.....