THE PREDICTIVE POWER OF PROTEINURIA IN PREGNANCY

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by

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Abstract

The Predictive Power of Proteinuria in Pregnancy
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Background
This thesis examines the power of urinary protein excretion – both quantitative and qualitative – during pregnancy in predicting maternal and fetal outcomes. The independent effect of proteinuria in predicting adverse outcomes for women with chronic kidney disease (CKD) has not been well characterised. Secondly, pre-eclampsia is a leading cause of maternal and fetal morbidity and mortality worldwide. There are no tests to predict pre-eclampsia in clinical use.

Methods
Analysis of prospective data collected from pregnant women with CKD from multiple centres in the United Kingdom.
A longitudinal prospective clinical study of urine proteomics in early pregnancy in women at high risk of pre-eclampsia to identify putative predictive biomarkers.

Results
Data was analysed from 313 pregnancies in 256 women with CKD from 2005 to 2010. Urine ACR or PCR is accurate in quantifying proteinuria in women with CKD during pregnancy. Women in whom pregnancy-associated increases in proteinuria failed to return to baseline have increased progression of renal dysfunction compared to other women. Proteinuria at conception is not independently associated with pregnancy-associated accelerated loss of renal function but does predict preterm delivery. Nephrotic syndrome in pregnancy is not independently associated with adverse fetal outcomes.
145 participants were enrolled in a study of urine proteomics of whom 11 developed pre-eclampsia. A panel of 5 peptides was identified in urine collected prior to 20 weeks gestation that predicted the subsequent development of pre-eclampsia with 92% accuracy. A further peak was indentified in specimens collected from 20 to 25 weeks gestation with similar predictive performance.

Summary
Maternal proteinuria at conception is associated with preterm delivery in women with CKD. Increased proteinuria that fails to resolve postpartum is associated with a more rapid decline. Accurate prediction of the development of pre-eclampsia in women at high risk was achieved from urine proteomic analysis prior to 20 weeks gestation.

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Index of figures and tables

Abbreviations

µmol	micromole
ACE	angiotensin converting enzyme
ACR	Albumin:creatinine ratio
ADMA	asymmetric dimethylarginine
AFP	alphafetoprotein
ALT	alanine transferase
ANA	antinuclear antibody
ANCA	anti-neutrophil cytoplasmic antibody
ANN	artificial neural network
ANOVA	Analysis of variance
ASSHP	Australasian Society for the Study of Hypertension in Pregnancy
AST	aspartame transferase
AT2	angiotensin 2
AUC	Area under curve
C	celsius
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHCA	alpha-cyano-4-hydroxy cinnamic acid
CKD	Chronic kidney disease
COMT	catechol-O-methyltransferase
CV	coefficient of variance
CVA	cerebrovascular accident
Da	dalton
DBP	diastolic blood pressure
DIC	disseminated intravascular coagulation
dl	decilitre
DNA	deoxyribonucleic acid
EAM	energy absorbing matrix
EDTA	European Dialysis and Transplant Association
eGFR	Estimated glomerular filtration rate
ELISA	enzyme-linked immunosurbent assay
ENA	extractable nuclear antigen
ESA	Erythropoiesis stimulating agent
ESRD	End stage renal disease
ET_B	Endothelin receptor - type B
FH	family history
FSGS	Focal segmental glomerular sclerosis
g	gram

GFR	Glomerular filtration rate
GN	Glomerulonephritis
hCG	human chorionic gonadotrophin
HD	Haemodialysis
HELLP	haemolysis, elevated liver enzymes, low platelets
HKUPP	Human Kidney and Urine Proteome Project
HP	heavy proteinura (>3g/day)
HPLC	high performance liquid chromatography
HTN	hypertension
HuPO	Human proteome organisation
ICU	Intensive care unit
IgA	Immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
INR	international normalised ratio
IPP	inositol phosphoglycan P-type
IQR	Interquartile range
ISSHP	International Society for the Study of Hypertension in Pregnancy
IUGR	Intrauterine growth retardation
kDa	kilodalton
kg	kilogram
LBW	low birth weight
LFT	liver function test
LP	low proteinuria (<3g/day)
M	molar
M,C&S	microscopy, culture and sensitivities
m/z	mass / charge ratio
MALDI	matrix assisted laser desorption/ionisation
MCGN	Mesangiocapillary glomerulonephritis
mg	milligram
MHz	megahertz
ml	millilitre
mmHg	millimeters of mercury
mmol	millimole
MoM	multiples of median
MPO	myeloperoxidase
MS	mass spectrometry
NAG	N-acetyl glucosaminidase
NaOH	sodium hydroxide
NHANES	National Health and Nutrition Examination Survey

NHBPEP	National High Blood Pressure Education Programme
nJ	nanojoule
nm	nanometers
NND	neonatal death
NO	nitric oxide
NOS	nitric oxide synthetase
NPV	negative predictive value
NS	nephrotic syndrome
o/n	overnight
PALRF	Pregnancy associated accelerated loss of renal function
PAPP-A	pregnancy-associated plasma protein A
PCR	Protein:creatinine ratio
PD	Peritoneal dialysis
PE	pre-eclampsia
PGI	prostaglandin I
PI	pulsatility index
PIH	pregnancy-induced (gestational) hypertension
PlGF	placental growth factor
PMH	past medical history
PP-13	placental protein 13
PPV	positive predictive value
PR3	proteinase 3
ROC	Receiver Operating Curve
SBP	systolic blood pressure
SCr	serum creatinine
SD	Standard deviation
SDS	sodium dodecyl sulphate
SELDI	surface enhanced laser desorption/ionisation
SEM	standard error of the mean
s-Flt-1	soluble fms-like tyrosine kinase 1
SLE	Systemic lupus erythematosus
SVR	Systemic vascular resistance
TNF	tumour necrosis factor
TOF	time of flight
TRIS	2-amino-2-hydroxymethylpropane-1,3-diol
UK-CORD	United Kingdom Collaboration in Obstetrics and Renal Disease
UTI	urinary tract infection
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor

VLBM	Very low birth weight
VTE	Venous thromboembolism
VUR	Vesicouteric reflux

1. Introduction

1.1 Normal pregnancy

1.1.1 Physiological adaptations to normal pregnancy

1.1.1.1 The cardiovascular system in normal pregnancy

Changes in cardiovascular status occur early in pregnancy with an increase in circulating and extracellular fluid volume, increased cardiac output and decreased systemic vascular resistance (SVR) (1-3). This enables preserved uteroplacental perfusion in early pregnancy and acts to buffer blood loss associated with normal parturition or obstetric emergencies later in gestation (figure 1.1.1.1).

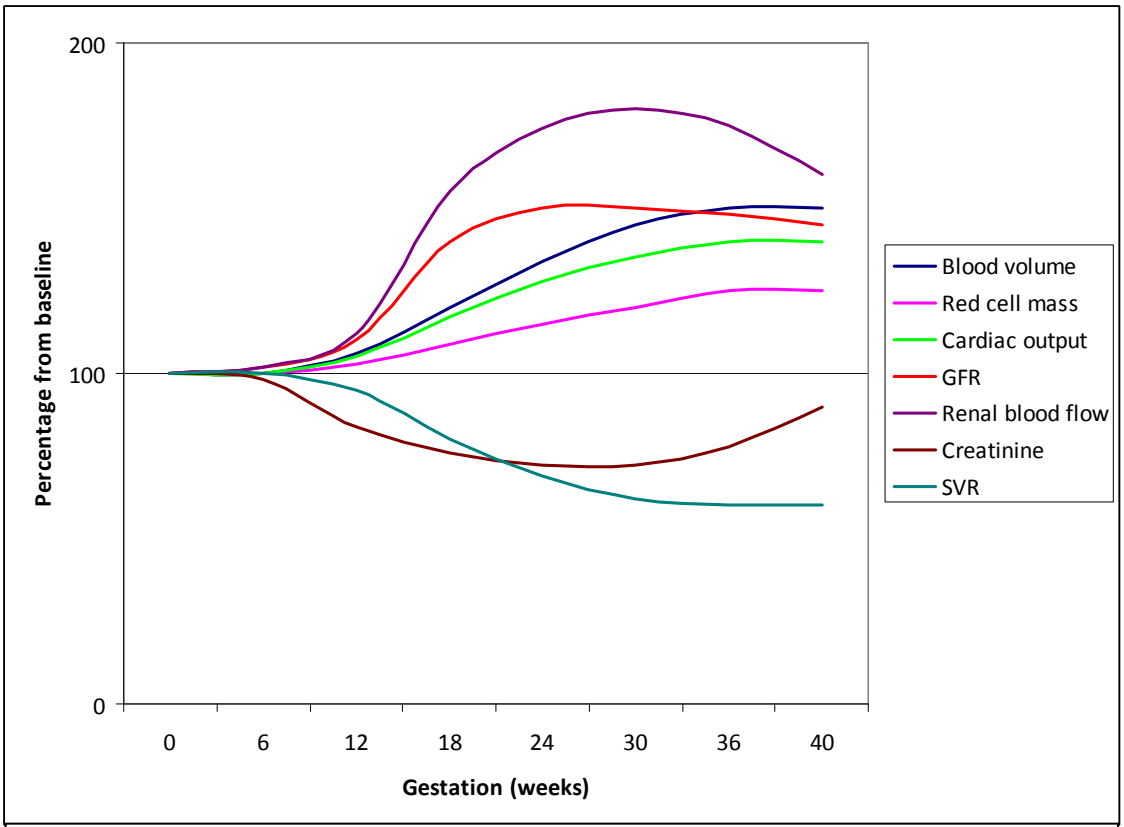


Figure 1.1.1.1 Physiological changes in normal pregnancy. GFR, glomerular filtration rate; SVR, systemic vascular resistance

Increased cardiac output and reduced SVR leads to increased renal blood flow and glomerular filtration rate (GFR) early in pregnancy. The reciprocal changes in cardiac output and SVR minimise alteration in blood pressure, however, subtle imbalances lead to a tendency for blood pressure to fall in the first two trimesters and gradually return to baseline as the pregnancy reaches term (4). Increased relaxin secretion and bioactivity acts on endothelin receptors (ET_B) which in turn up-regulate endothelial nitric oxide synthase to initiate vasodilation (1).

1.1.1.2 The renal system in normal pregnancy

Structural renal changes occur in pregnancy as a result of increased blood flow, urothelial smooth muscle relaxation and occasionally due to the obstructive impact of the gravid uterus on the collecting system. Kidney length increases by approximately 1cm (3). Dilation of the ureters, renal pelvis and calyces occur bilaterally but to a greater degree on the right. Differentiation between physiological changes and mechanical obstruction can be challenging.

Biochemical investigations show a fall in serum creatinine, urea, urate and potassium as a result of increased GFR and a relatively reduced increase in tubular reabsorption of some electrolytes (5). In later pregnancy serum urate climbs due to increased fetal urate production and decreased fractional excretion (5). Calcium excretion increases as a result of increased GFR and increased vitamin D 1α-hydroxylase activity, including within the placenta (5,6). Metabolic acidosis may occur as a result of bicarbonaturia but is ameliorated by progesterone-induced hyperventilation in later pregnancy (7). Glycosuria may develop in the absence of hyperglycaemia due to a reduced reabsorption threshold.

Increased sodium reabsorption exceeds the increase in filtered sodium. Extracellular fluid expansion occurs as a result of total net sodium retention of approximately 950mmol. This is mediated by the action of increased endogenous aldosterone, glucocorticoids and oestrogen. A complex interplay between increased renin-angiotensin-aldosterone activity, decreased osmotic thresholds for vasopressin release and increased GFR lead to a reduction in plasma sodium of 5mmol/l (8).

Increased GFR, increased capillary permeability and decreased reabsorption of filtered proteins in the proximal tubule lead to urine protein excretion increasing by up to 100% in normal pregnancy (see chapter 1.5). Proteinuria increases as pregnancy progresses. Mean protein excretion was 117mg/d in a study of 270 normal pregnancies (9) compared with 62.5mg/d in non-pregnant women aged 21 to 44 (10). An upper limit of normal proteinuria of 150mg/d in healthy non-pregnant females is supported by observational data and, in healthy pregnancies an upper limit of normal proteinuria of 260mg/d is suggested. Albuminuria accounts for approximately 10% of the excreted urine protein during pregnancy (9).

1.1.2 Epidemiology, outcomes and complications of pregnancy

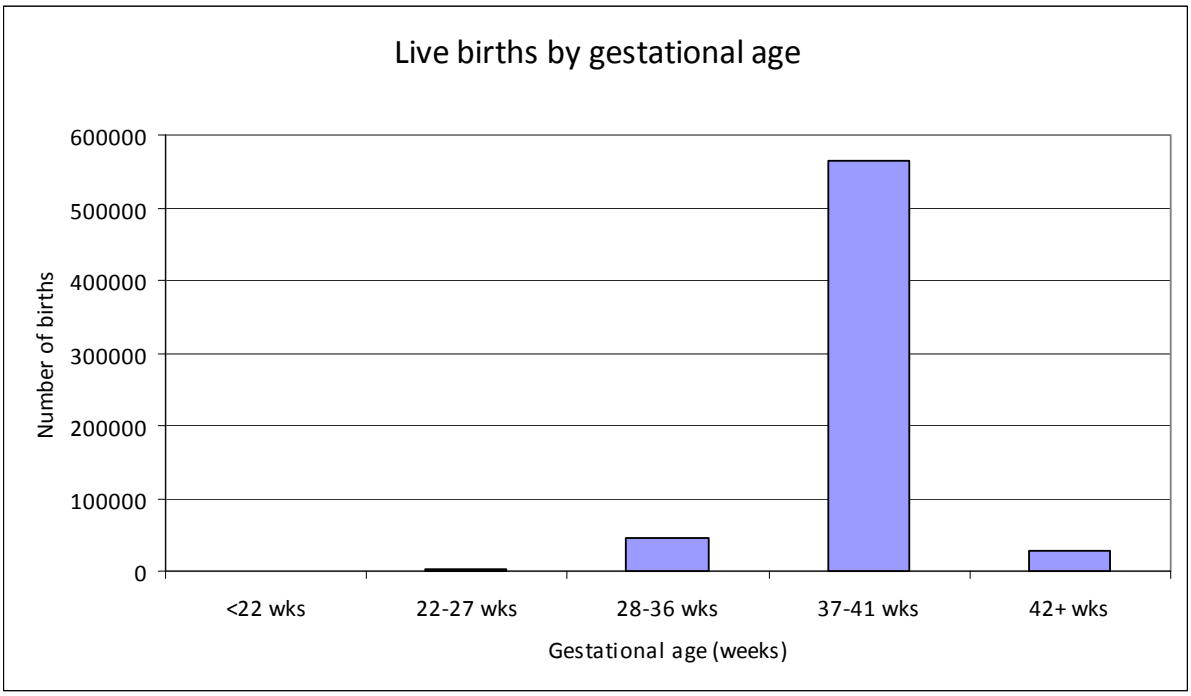
There are approximately 900000 conceptions per year in England and Wales leading to 700000 live births (78%), 190000 terminations (21%) and 3700 still births (0.4%) per year. In 2002, there were 3700 neonatal deaths (<30 days postpartum), of which 40% occurred in the first week of life (11). Data on rates of miscarriage are less reliable since as many as 50% of cases go unreported (12). Rates of miscarriage are higher with increasing maternal age (23% in mothers over 40) and previous miscarriage (43% following 3 previous miscarriages) (13). International data suggests that 15% of pregnancies end in miscarriage or stillbirth, 22% in termination and 63% live births (14).

Preterm delivery (less than 37 weeks) occurs in 6% of singleton pregnancies and 53% of multiple pregnancies (figure 1.1.1.1). Low birth weight (1500g – 2500g) occurs in 5.1% of pregnancies and very low birth weight (<1500g) in 1.1% (figure 1.1.1.2) (15,16). Birth weight is a composite end point of gestational age at delivery and adequacy of uteroplacental perfusion, therefore, the interpretation of arbitrary cut-offs to define outcomes in this way can be limited. Intrauterine growth retardation (IUGR) or small for gestational age (SGA) is defined according to growth centiles by gestation which can be customised to ethnicity and maternal characteristics (17). Infants born below the 10% centile can be defined in this way and such pregnancies reflect uteroplacental insufficiency.

Pre-eclampsia affects 2-8% of pregnancies, dependent on the diagnostic criteria used. Pre-eclampsia is discussed in chapter 3.1.

Maternal death is rare in the United Kingdom. The most recent Confidential Inquiry into Maternal and Child Health identified 295 maternal deaths out of over 2 million births over 3 years giving a maternal mortality rate of 14 per million pregnancies (18). Pre-eclampsia was directly implicated in 18 (4.5%) of these deaths.

Worldwide, maternal mortality is much greater as a result of undiagnosed hypertensive disorders of pregnancy, haemorrhage, malaria, other infection, mental illness and violence. Over the last 3 decades, maternal mortality has fallen from an estimated 529000 maternal deaths per year to 342900 per year. Global maternal mortality rate remains 251 per million pregnancies with 50% of all maternal deaths occurring in 6 countries (India, Nigeria, Pakistan, Afghanistan, Ethiopia, and the Democratic Republic of the Congo) (19).



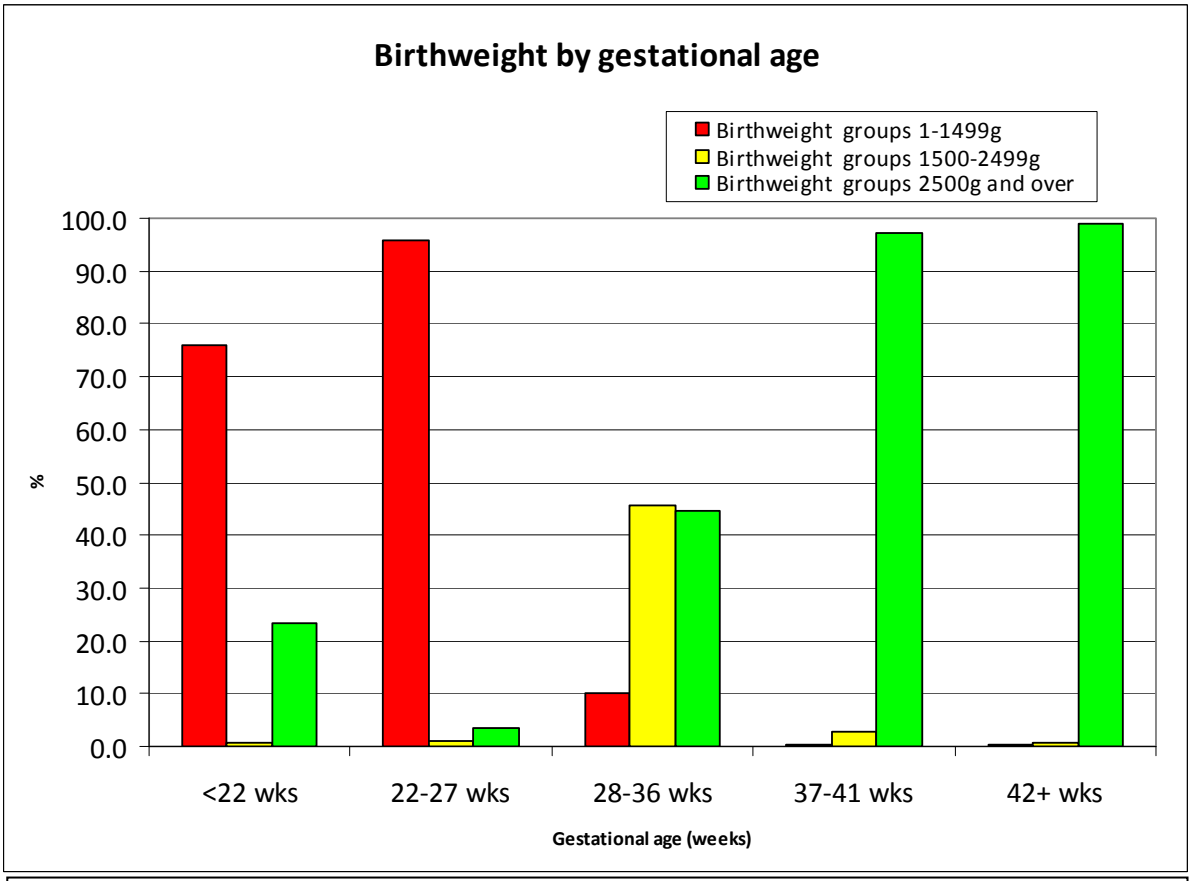


Figure 1.1.1.2. Preterm delivery and birthweight data for pregnancies in England and Wales (Office for National Statistics, 2009 (top panel), 2005 (bottom panel))

1.2 Pregnancy and chronic kidney disease (CKD)

“Children of women with renal disease used to be born dangerously or not at all – not at all, if their doctors had their way.”(20).

This comment, published in the *Lancet* in 1975, followed a series of reports over the preceding decade which portrayed a dismal prognosis for women with kidney disease contemplating pregnancy. Perhaps the most extreme of these was by Mackay in 1963 in which observation of 6 women with plasma urea greater than 21.4mmol/l (60mg/dl) at conception resulted in 6 maternal and 6 fetal deaths (21). Since then, improvements in obstetric and renal disease management have led to much better prospects for women with renal disease.

1.2.1 Epidemiology of pregnancy and kidney disease

Pregnancy complicated by CKD is rare. Significant renal dysfunction (CKD stage 3 to 5, estimated GFR<60ml/min, table 1.2.1.1) is found in 0.03% of pregnancies in the United Kingdom. Less advanced CKD (CKD stage 1 and 2, estimated GFR>60ml/min with haematuria, proteinuria or structural abnormalities) is found in a further 0.12% of pregnancies. This incidence may be increasing as a result of the increased prevalence of diabetes mellitus and obesity, more supportive advice from nephrologists and obstetricians, renal transplantation and more effective treatment of immunological and structural renal disease (22).

Stage	Estimated GFR	
1	>90 ml/min	plus haematuria, proteinuria or structural abnormalities
2	60-90 ml/min	
3	30-59 ml/min	
4	15-29 ml/min	
5	≤15 ml/min	

Table 1.2.1.1 National Kidney Foundation K/DOQI classification of chronic kidney disease

1.2.2 Aetiology of CKD in pregnancy

In general nephrology, most cases of CKD are caused by diabetes mellitus, hypertension and chronic glomerulonephritis. In general, patients who are considering becoming pregnant or successfully conceive are younger and consequently have a different aetiology of disease compared to patients reaching end stage renal disease (figure 1.2.2.1).

The incidence of diabetic nephropathy and renal vascular disease is low in women contemplating pregnancy since the development of renal impairment with these common diseases is dependent on duration of disease exposure and age-related comorbidity. Other diagnoses include other structural abnormalities (such as medullary sponge kidney), functional defects (such as Gitelman syndrome) and other hereditary diseases (such as Fabry's disease).

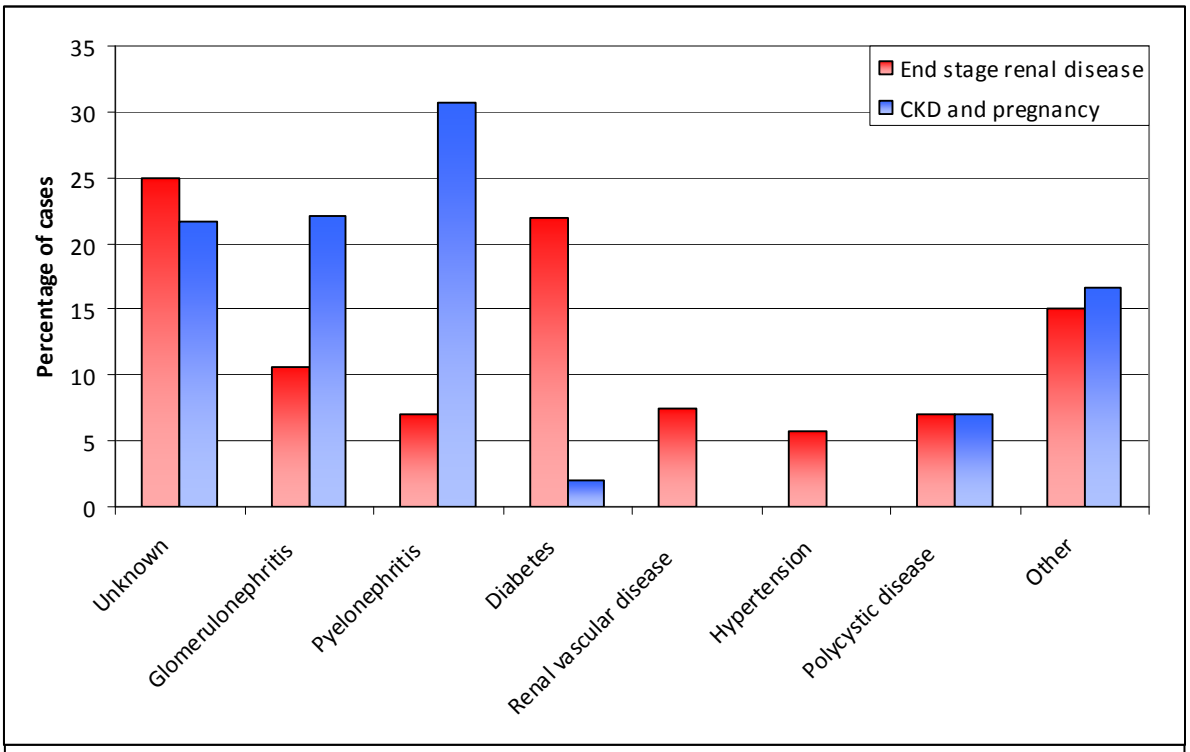


Figure 1.2.2.1. Aetiology of CKD in patients with end stage renal disease in the UK and those attending renal-obstetric clinics. End stage data from the Renal Registry report 2008 (n=43650). CKD data from the United Kingdom Collaboration in Obstetrics and Renal Disease (2004-2010, n=313)

1.2.3 Outcomes of pregnancy with CKD

Pregnancy in patients with CKD is associated with increased risk of premature delivery, low birth weight infants, Caesarean section, pre-eclampsia, acute kidney injury and sustained loss of kidney function (23). Successful pregnancy outcome for patients with CKD can be defined as the likelihood of taking home a live healthy infant without a longstanding detrimental effect on maternal health. Pregnancy and CKD can have a reciprocal detrimental effect on mother and baby.

Pregnancy outcome in patients with CKD has improved over the last 40 years, however, because of the paucity of published data, it is difficult to quantify these improvements. Compared with MacKay's report (from 1952 to 1962) in which a maternal serum urea >21.4mmol/l at conception was associated with 100% maternal and fetal mortality in 6 women (21), Imbasciati et al described 49 pregnancies (from 1977 to 2004) in women with estimated GFR <60ml/min at conception and reported no maternal deaths, 1 (2%) stillbirth, 1 (2%) neonatal death, 29 (59%) low birth weight and 31 (63%) preterm deliveries (24). A study of 120 pregnant women with CKD stages 1 to 5 from 2000 to 2009 reported no maternal deaths, 7 (6%) spontaneous abortions, 15 (17%) small for gestational age infants and 40 (44%) preterm deliveries (25). Many adverse pregnancy outcomes in patients with CKD are associated with pre-eclampsia, however, because variable definitions of pre-eclampsia and superimposed pre-eclampsia have been used, it is not possible to quantify the increased risk.

1.3 End stage renal disease and pregnancy

1.3.1 Dialysis

End stage renal disease (ESRD) reduces maternal fertility and conception is rare in patients receiving dialysis. In a large registry study of pregnancy amongst women on dialysis, conception rate was approximately 0.5% per year (26), compared with 8% in the general population.

Furthermore a large proportion of pregnancies result in first trimester miscarriages although the incidence of this is difficult to quantify. Of those pregnancies reaching the second trimester, over 90% will result in preterm delivery and intrauterine growth restriction and 50% will result in fetal death (23). It is estimated that an average sized renal unit in the United Kingdom will see one case of dialysis and pregnancy per 4 years (26,27).

1.3.3.1 Confirmation of pregnancy

Patients on dialysis are often anuric and, due to uraemic disturbance of the hypothalamic-pituitary-ovarian axis, menstruation is often erratic or absent. Diagnosis of pregnancy by amenorrhoea and semiquantative measurement of urine βHCG is therefore invalid.

Levels of serum βHCG in early pregnancy are up to 6 times higher in patients on dialysis than in women with normal renal function (28,29). If serum βHCG is elevated but no fetal heart sound is visible on ultrasonography this may represent either an unviable pregnancy or fetal immaturity. Repeat scanning in one to two weeks should be performed to confirm pregnancy.

1.3.3.2 Pre-eclampsia and dialysis

Superimposed pre-eclampsia is reported in 75% of pregnancies in women on dialysis (23), and usually presents as progressive increases in blood pressure refractory to medication or ultrafiltration. After preterm labour, pre-eclampsia is the commonest reason for fetal delivery in women on dialysis.

Diagnosis of superimposed pre-eclampsia in patients on dialysis is predominantly a clinical diagnosis based around the criteria of the National High Blood Pressure Education Programme (NHBPEP) (see chapter 3.1.1) (30). Treatment requires attempted control of the blood pressure and serial monitoring of the mother and fetus to predict impending eclampsia or fetal distress and permit timely delivery.

All patients with chronic kidney disease should receive aspirin as prophylaxis against pre-eclampsia although the benefits have not been confirmed amongst women on dialysis (31,32).

1.3.3.3 Blood pressure and anaemia management

A large proportion of patients on dialysis require medication to optimise their blood pressure. Agents that inhibit the renin-angiotensin system are associated with cranial and renal malformations, particularly when used in the second and third trimesters (33), and should be stopped prior to, or on confirmation of pregnancy. In a cohort study of 29507 infants from Tennessee, first trimester exposure to angiotensin converting enzyme inhibitors (ACEi) was associated with a 3.7-fold increased risk of cardiovascular system malformations and 4.4-fold increased risk of central nervous system malformations (34) . There is

considerable experience of using labetalol, methyldopa, hydralazine and nifedipine during pregnancy and these agents appear to be safe.

A target blood pressure of 140/90 mmHg has been recommended for women with chronic kidney disease during pregnancy by the Royal College of Obstetricians and Gynaecologists (35). “Over-treatment” of hypertension to diastolic blood pressure less than 80mmHg is associated with intrauterine growth retardation (36).

Changes on “dry weight” – that is, the estimated weight at which the patient is euvolaemic - need to be carefully monitored by clinical evaluation during pregnancy. As a guide, “dry weight” should be increased by 1.5kg over the first trimester and 0.2kg to 0.4kg per week until delivery (37).

Uncontrolled anaemia is associated with adverse fetal outcomes (38). Intravenous iron and erythropoiesis stimulating agents (ESA) appear safe in pregnancy. ESA requirement increases by 40% from baseline at 14 weeks gestation and 85% from baseline at 28 weeks gestation (39). A target haemoglobin of 10 – 11 g/dl during pregnancy in women on dialysis has been suggested (38).

1.3.3.4 Peritoneal dialysis and pregnancy

Pregnancy is neither an indication nor contra-indication to peritoneal dialysis (PD), although there are fewer reported pregnancies in women on PD than haemodialysis (HD). Registry studies suggest that conception rates are lower for women on PD than HD (29) but pregnancy outcomes are similar (40,41).

There are no trials to guide the optimum prescription of PD during pregnancy. Patients should continue their normal PD regime on confirmation of pregnancy. as

As pregnancy progresses however, women may need to increase the exchange frequency and use smaller dwell volumes to maintain efficient dialysis.

PD peritonitis should be suspected and diagnosed in pregnant patients in the same way as for non-pregnant patients; symptoms of abdominal pain, vomiting, fever or diarrhoea; cloudy PD effluent; and elevated PD effluent white cell count. Confirmed peritonitis can be treated with intraperitoneal antibiotics with or without oral or intravenous antibiotics. Vancomycin, gentamicin, ceftazidime, flucloxacillin and rifampicin may all be used as indicated but quinolones should be avoided.

Bloody PD effluent is associated with severe obstetric complications including uterine haemorrhage, placental abruption and fetal loss (40,42) and urgent obstetric assessment is indicated.

1.3.3.5 Haemodialysis and pregnancy

Previous registry data identified a conception rate of 0.5% per year for women on HD and an association between increased dialysis time (>20 hours per week) and improved fetal survival (26). More recent data have shown remarkable pregnancy outcomes in women on nocturnal haemodialysis receiving over 40 hours dialysis per week with a conception rate of 3.1% per year (39). Fetal survival was 87% with 3 of 6 successful pregnancies reaching term (>37 weeks gestation) and only 2 of 6 pregnancies resulting in low birth weight infants (as compared with data in figure 1.4.2.1).

There are no randomised controlled trials to guide the optimum prescription of HD in pregnancy. Severe prematurity (<32 weeks gestation) and very low birth weight infants (<1.5kg) are associated with pre-dialysis urea greater than 17mmol/l (43) and patients with the best reported pregnancy outcomes had pre-dialysis urea <10mmol/l (39).

Although increased duration of dialysis is associated with improved pregnancy outcomes, it is limited by patient tolerance and practicality. It is reasonable, however, to recommend that pregnant patients receive at least 20 hours dialysis per week to maintain pre-dialysis urea less than 17mmol/l, and that the greatest duration of dialysis that is tolerated by the patient and feasible by the dialysis service is provided.

1.3.2 Kidney transplantation and pregnancy

The first successful pregnancy in a recipient of a kidney transplant occurred in March 1958. Over 15000 children have been born to mothers with renal transplants since then.

Following renal transplantation, outcomes are improved as compared to patients on dialysis with a 95% chance of success (44-47). It is generally recommended that women wait 2 years following transplantation before embarking on pregnancy. Recent reports have suggested that a shorter period is safe and some suggest that pregnancy is safe 12 months (or even 6 months) after transplantation (48). Transplant rejection and function are not affected by pregnancy assuming the following are met:

- At least 12 months post-transplant.
- Stable renal function
- Proteinuria <1g/d
- Minimal or well-controlled hypertension
- No recent or on-going transplant rejection
- Minimal levels of appropriate immunosuppression

Baseline kidney function predicts pregnancy outcomes following renal transplantation. Persistent postpartum impairment in renal function occurs in 15% of transplant patients (49).

For mothers with baseline creatinine less than 125µmol/l, successful pregnancy occurs in 97% of cases reaching the second trimester. The incidence of preterm delivery, intrauterine growth restriction and pre-eclampsia is greater than the general population and 30% of pregnancies may be affected.

With more severe baseline renal dysfunction the incidence of adverse fetal outcome increases. The likelihood of accelerated maternal renal decline is also higher. In one study all transplant patients with creatinine greater than 200µmol/l at conception progressed to dialysis within 2 years (50).

There is no evidence of delayed development in children born to mothers with a renal transplant, independent of complications associated with preterm delivery.

Normal vaginal delivery is not contraindicated following renal transplantation. If Caesarean section is indicated (for obstetric reasons) then a lower segment approach may be difficult due to the course of the transplanted ureter.

1.4 Predictors of pregnancy outcomes in patients with CKD

Practicing obstetricians and nephrologists are frequently asked to give advice to patients with renal disease on the likely outcomes of a planned or current pregnancy. Almost all data describing the outcomes of pregnancy in patients with CKD are based on retrospective observational data. Subsequently, although associative patterns can be identified to aid patient counselling, causality and the relative importance of isolated parameters is more difficult to clarify.

1.4.1 Hypertension and pregnancy outcome in CKD

A retrospective study of 763 pregnancies in women with chronic hypertension but *no known CKD* found that preterm delivery occurred in 18%, IUGR in 23% and perinatal death in 4.6% (51) compared with 6%, 5% and 0.8% respectively in the general population.

In an analysis of 43 pregnancies in 30 women *with CKD* (serum creatinine 110 to 490 µmol/l) from 1975 to 1994 (52), hypertension was present from conception in 26 (60%). Logistic regression identified uncontrolled hypertension at conception as an independent risk factor for fetal death. The adjusted relative risk of fetal death for women with mean arterial blood pressure greater than 105mmHg at conception was 10.5 (95% confidence interval 1.6-69.4, $p<0.02$). Accelerated loss of maternal renal function was found in 7 patients, all of whom had hypertension, although the independence of hypertension in predicting this outcome was not ascertained in this study.

Pregnancies in 49 women from 1977 to 2004 with pre-conception estimated GFR less than 60ml/min were studied by Imbasciati et al (24). In contrast, hypertension was not identified as an independent risk factor for fetal loss or IUGR. Neither did they note any independent association between hypertension and accelerated loss of maternal renal function.

A retrospective analysis of 400 pregnancies in 358 women by the United Kingdom Collaboration of Obstetrics and Renal Disease found maternal hypertension in early pregnancy to be independently associated with neonatal death (diastolic

blood pressure >90mmHg versus <70mmHg, odds ratio 20.9) and preterm delivery (diastolic blood pressure >90mmHg versus <90mmHg, odds ration 1.7) (53).

In patients with IgA nephropathy, adverse pregnancy outcome was associated with chronic hypertension, albeit in univariate analysis only. Perinatal death (33% versus 1%, $p<0.001$) and abnormal delivery (80% versus 29%, $p<0.001$) were more common in patients with preconception blood pressure >140/90mmHg than <140/90mmHg (54).

Although the above data are generally supportive that elevated blood pressure is associated with adverse fetal outcomes in patients with CKD, there is no evidence that antihypertensive treatment ameliorates these outcomes. Outside of pregnancy, uncontrolled hypertension is associated with progression of CKD (55,56), however, given that there are potential risks of aggressive treatment of hypertension in pregnancy (36), the role of hypertension treatment in pregnancy and CKD is not fully elucidated.

1.4.2 Severity of renal disease and pregnancy outcome in CKD

It is intuitive that women with increasingly severe CKD are likely to have worse pregnancy outcomes and this is borne out in numerous observational series (24,52,57-63) summarised by Williams and Davison (figure 1.4.2.1) (23). A more recent study has shown that even early kidney disease, in which excretory renal capacity is preserved (CKD stages 1 and 2), is associated with an increased risk of preterm delivery, Caesarean section, low birth weight and requirement for neonatal intensive care than controls (figure 1.4.2.2) (25).

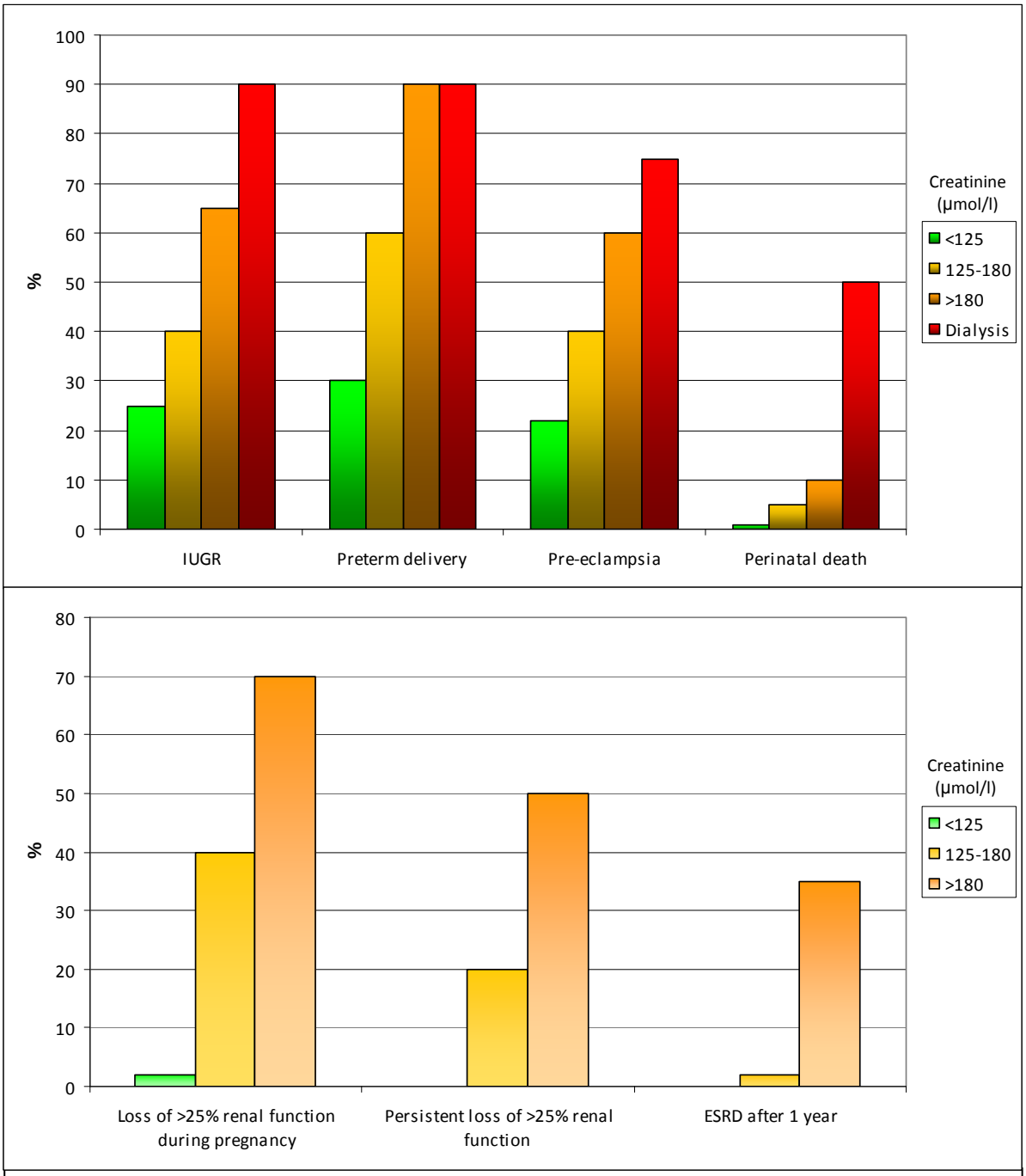


Figure 1.4.2.1 Incidence of maternal and fetal morbidity in pregnancy and kidney disease by serum creatinine at conception. IUGR, intrauterine growth restriction; ESRD, end stage renal disease. (From Williams and Davison, 2008 (14))

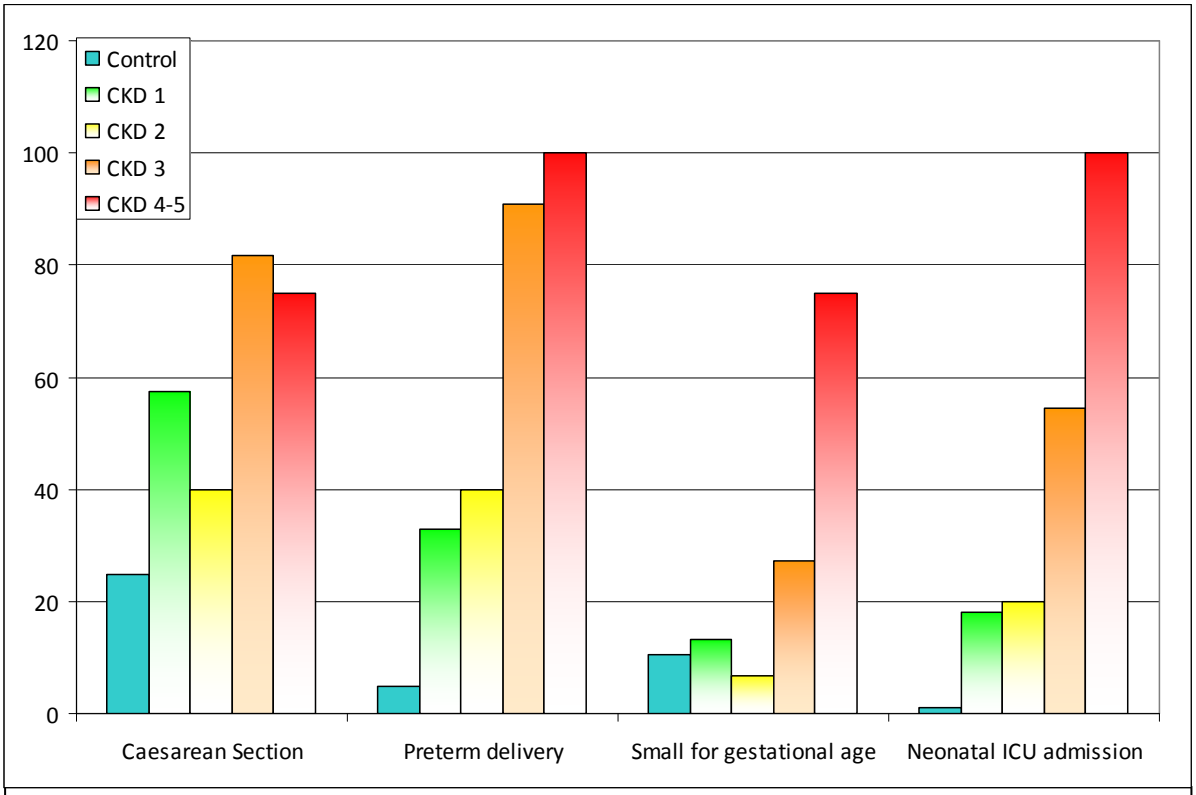


Figure 1.4.2.2 Pregnancy outcome according to CKD stage at conception. From Piccoli et al, 2010 (16)

1.4.3 Aetiology of renal disease and pregnancy outcome in CKD

A review in 1991 compared maternal and fetal outcomes in a range of primary and secondary renal diseases as shown in table 1.4.3.1. Although some differences between outcomes and conditions are noted, this analysis failed to account for differences in other potentially causative parameters, notably hypertension and renal function (64).

	Perinatal loss	Preterm delivery	Renal function decline	Permanent blood pressure increase
FSGS (n=85)	23%	32%	13%	10%
Membranous GN (n=110)	4%	35%	3%	3%
IgA nephropathy (n=268)	15%	21%	12%	12%
MC GN (n=278)	12%	9%	2%	7%
Diabetic nephropathy (n=97)	6%	36%	32%	58%
Polycystic disease (n=464)	3%	10%	3%	14%
Reflux nephropathy (n=137)	7%	15%	0.7%	11%

Table 1.4.3.1 Comparison of pregnancy outcomes by aetiology of CKD. FSGS, focal segmental glomerulosclerosis; GN, glomerulonephritis; MC, mesangiocapillary (from (64)).

More recent studies have not identified an independent role of aetiology of CKD in determining pregnancy outcomes (24,25,53) although non-renal manifestations of systemic diseases associated with secondary renal disease may independently affect pregnancy outcomes.

Systemic lupus erythematosus (SLE) is a multisystem disease with renal involvement in approximately 50% of cases, classically affecting women of childbearing age. Lupus nephritis may present with non-specific and isolated urine dipstick abnormalities, nephrotic syndrome or rapidly progressive renal involvement. Proliferative lupus nephritis presents with hypertension, proteinuria (and invisible haematuria) with impairment of renal function. Such flares of

disease can be clinically indistinguishable from pre-eclampsia if they occur during pregnancy. SLE may also be associated with the antiphospholipid syndrome – a profound risk factor for pre-eclampsia and early miscarriage – and anti-Ro antibodies which can lead to fetal complete heart block. Small observational studies over 4 decades fail to confirm whether pregnancy with lupus nephritis is associated with increased risks independent to those expected as a result of hypertension and renal dysfunction, and the specific immunological-based complications above (65-67). Nevertheless, it is accepted that pregnancy is not recommended in the presence of active disease and should be postponed until immunosuppression has been minimised (68).

Vesicoureteric reflux (VUR) affects approximately 1% of the population with significant familial clustering. Progressive renal scarring occurs following retrograde flow of urine during micturition, usually associated with recurrent urinary tract infection. The disease may be unilateral or bilateral, with the latter being more often associated with progressive renal dysfunction and hypertension. Similar to other causes of CKD, adverse pregnancy outcomes in patients with VUR seem to be associated with level of renal function at conception and the presence of hypertension (69,70). Asymptomatic bacteruria in pregnancy is 4 times more likely to lead to pyelonephritis than outside of pregnancy (71) and is more common in patients with VUR. It is generally accepted that asymptomatic bacteruria and overt infection are treated during pregnancy to prevent pyelonephritis and preterm delivery (72), although recent reports suggest no

increased incidence of adverse pregnancy outcomes in patients with urinary tract infections (73,74).

1.4.4 Proteinuria and pregnancy outcome in CKD

Proteinuria (see chapter 1.5) is an independent risk factor for progressive renal disease in non-pregnant subjects (75,76). The independent impact of proteinuria on pregnancy outcomes in patients with CKD is less clear.

In a retrospective study of 121 pregnancies in women with CKD, Katz identified that proteinuria doubled in 57 of 121 (47%) pregnant women with kidney disease but outcomes dependent on proteinuria were not described (77).

Stettler and Cunningham reviewed outcomes in pregnant patients found to have proteinuria in the absence of known kidney disease or pre-eclampsia (78). Chronic kidney disease was subsequently identified in 74%. Adverse outcomes were not dependent on proteinuria unless associated with renal insufficiency or hypertension. Franceschini et al investigated the risk of preterm delivery in patients without known kidney disease according to the albumin:creatinine ratio (ACR) in the second trimester. Although an ACR>3mg/mmol gave an odds ratio of 1.9 for preterm delivery, and an ACR>20 gave an odds ratio of 4.7 for preterm delivery, this effect was abolished after excluding patients with diabetes and hypertension (79).

In patients with known primary renal disease and moderate renal dysfunction (serum creatinine greater than 1.4mg/dl (124µmol/l)), preconception proteinuria greater than 3g/d was found in 27% and a gestational change in proteinuria was noted in 29% of patients, however, the presence of high grade proteinuria

(>3g/day) at any time during pregnancy had no effect on the outcome of pregnancy (60).

No influence of proteinuria on the likelihood of successful outcomes was identified in a study of 112 pregnancies with primary renal disease, diabetic nephropathy or renal transplants (57). In contrast, multiple regression analysis of retrospective data from 19 patients with renal disease who became pregnant and 31 non-pregnant patients with renal disease identified pregnancy and initial proteinuria as predictors of the rate of renal loss. Patients who had accelerated decline in renal function following pregnancy had 2.5-fold higher proteinuria prior to conception with no difference in serum creatinine (80).

In the largest prospective study of pregnancy outcomes in patients with chronic kidney disease, Imbasciati et al found that neither proteinuria (greater than 1g/d) nor more advanced renal disease (eGFR<40ml/min) were independently predictive of pregnancy induced renal decline but the combination of the two was (hazard ratio 5.2 for shorter renal survival) (24).

Therefore, there appears to be a dichotomy that elevated urine protein excretion is found in patients with adverse pregnancy outcomes but there is limited evidence that proteinuria is an independent predictor for such outcomes.

1.4.4.1 Nephrotic syndrome

In general nephrology, nephrotic syndrome is defined as proteinuria greater than 3g/day, serum albumin less than 30g/l and oedema. It is associated with an adverse lipid profile and increased risk of venous thromboembolism due to urinary loss of antithrombotic factors.

In pregnancy, the same definition is commonly used, however, it is important to remember that normal physiological changes of pregnancy lead to a dilutional fall in serum albumin and a doubling of urine protein excretion (9). Oedema is also common in normal pregnancy following salt and water retention and decreased peripheral venous return as a result of the gravid uterus.

Nephrotic syndrome affects about 0.025% of pregnancies and may occur as a result of *de novo* GN, exacerbation of chronic glomerular disease or in association with pre-eclampsia (81).

Pre-eclampsia is the most common aetiology of nephrotic syndrome in the second half of pregnancy. In a case series of 100 renal biopsies performed during pregnancy, 27 patients had nephrotic range proteinuria (greater than 3.5g/day) of whom 23 (85%) had pre-eclampsia (82). In patients with hypertension and nephrotic syndrome, 11 of 13 (85%) patients undergoing renal biopsy were found to have pre-clampsia (83). Imasawa et al report a case of nephrotic syndrome attributed to pre-eclampsia at 15 weeks gestation diagnosed by clinical features and renal biopsy (84), however, the International Society for the Study of Hypertension in Pregnancy define pre-eclampsia as the onset of *de novo* hypertension and proteinuria after 20 weeks (85).

Glomerular endotheliosis, the pathognomonic histological lesion of pre-eclampsia, is characterised by endothelial cell oedema, diffuse cellular proliferation, splitting of the GBM and capillary lumen narrowing. Pre-eclamptic patients progressing to nephrotic syndrome have more severe histological changes than non-nephrotic presentations and worse fetal outcomes; First et al reported a perinatal mortality of 33% and low birth weight in 63% (83). Nephrotic range proteinuria is independently associated with adverse maternal and fetal outcomes in pre-eclampsia (86). Nevertheless, placental histological changes are almost always wholly reversible following delivery of the fetus and persistent renal disease is rare (87).

Nephrotic syndrome caused by GN should be suspected if heavy proteinuria is noted in early pregnancy, or if it fails to resolve after fetal and placental delivery. Maternal and fetal prognosis following the development of nephrotic syndrome in early pregnancy is less favourable then when caused by pre-eclampsia. Numerous case reports describe second trimester intrauterine death or termination of pregnancy with resolution of nephrotic syndrome post-partum (81)(84)(88)(89,90). McLigeyo et al reported 5 cases of nephrotic syndrome in early pregnancy with successful fetal outcomes, however, two maternal deaths (40%) occurred within 5 years of delivery (91). In 13 women with nephrotic syndrome and known renal disease 4 pregnancies ended in therapeutic termination, 1 with preterm delivery and 8 completed successfully at full term. Two mothers (15%) progressed to end stage renal disease within 5 years. Yao et al describe 40 cases of nephrotic syndrome in pregnancy of which 6 patients

developed the condition prior to 20 weeks gestation. Overall, fetal mortality was 42% and maternal mortality 2.5% (92).

Loss of antithrombotic factors through urinary excretion in nephrotic syndrome is associated with increased risk of venous thromboembolism (VTE) (93) and exacerbates the prothrombotic state of pregnancy. Population studies of thromboembolic risk factors in pregnancy have not evaluated proteinuria independently and are not included in risk calculators (94). Nevertheless, given that nephrotic syndrome is associated with a relative risk of VTE of 1.7 (93) and pregnancy with a relative risk of VTE of 4.3 (95), pregnant patients with nephrotic syndrome are at particularly high risk and prophylactic therapy with low molecular weight heparin should be considered during pregnancy and the puerperium (35).

Nephrotic syndrome may exacerbate iron and vitamin D deficiency due to loss of transferrin and vitamin D binding protein in the urine.

1.5 Proteinuria

1.5.1 Mechanisms of proteinuria

Proteinuria is associated with an increased risk of progressive renal disease in patients with diabetes mellitus and chronic kidney disease and is an independent prognostic factor in cardiovascular disease (56,75,76,96). In health, less than 150mg per day of urinary protein is excreted, predominantly as a result of tubular secretion of Tamm-Horsfall and other epithelial proteins (10). Increased protein excretion occurs as a result of glomerular protein overload (such as that seen with plasma cell dyscrasias), tubular dysfunction leading to impaired reabsorption of filtered proteins or glomerular proteinuria. Plasma proteins are prevented form entering the glomerular filtrate as a result of a highly specialised filter consisting of fenestrated capillary endothelium, the slit diaphragm and intercalated foot processes of podocytes. Disruption of the glomerular basement membrane by immunological insult, endothelial dysfunction, or podocyte dysfunction can increase its permeability to plasma protein. When the tubular threshold for protein reabsorption is breached, plasma proteins – predominantly albumin – are detectable in the urine.

The concept of the glomerular basement membrane as a highly selective and generally impermeable barrier to plasma proteins was challenged recently by a set of experiments reporting that nephrotic range protein concentrations crossed the membrane followed by rapid tubular reabsorption (97). Repeated investigation using similar technology but increased signal resolution resulted in protein

filtrations rates more congruent with previous estimates however and has brought the significance of the initial findings into doubt (98).

1.5.2 Renal handling of protein in pregnancy

In normal pregnancy, urinary protein excretion increases by almost 100% to an upper limit of normal of 260mg per day (9) predominantly as a result of increased glomerular permeability and, to a lesser extent, increased renal blood flow (99). International diagnostic criteria for the diagnosis of pre-eclampsia define proteinuria as excretion greater than 300mg per day (85).

Changes in the qualitative nature of proteinuria in pregnancy have not been well characterised.

1.5.3 Measuring proteinuria

1.5.3.1 Quantitative measurement of proteinuria

Measurement of proteinuria has traditionally used a 24 hour urine collection. However this is cumbersome for patients and commonly incomplete and inaccurate due to missed micturition or unsuitable storage conditions. In a recent study of the completeness of 24 hour urine collection for the assessment of proteinuria in pregnancy, up to 54% of collections were incomplete (100). This has led to the assessment and validation of the protein:creatinine ratio (PCR) or albumin:creatinine ratio (ACR) in a spot urine sample as a surrogate marker of diurnal protein excretion. The protein concentration in a random spot urine sample will vary considerably dependent on the concentrating status of the renal collecting duct. Assuming there is a constant production and excretion of creatinine, the ratio of protein (or albumin) to creatinine concentration in the urine will correct for the urine water content.

Previous data have confirmed a close correlation of PCR and ACR to urine 24 hour protein excretion in diabetes (101), pregnancy (79,102) and chronic kidney disease (103), and subsequently these measurements have been accepted in international guidelines for the management or diabetes (104), hypertensive disorders of pregnancy (85) and chronic kidney disease (105).

As above, renal handling of protein is affected by both renal disease and pregnancy. The impact of these two (patho)physiological states on quantitative measurement of protein excretion is not clear.

1.5.3.2 Qualitative assessment of proteinuria

In clinical practice, proteinuria is usually described in terms of the total quantity of protein excreted in the urine. Isolated measurement of albumin excretion or free light chains has clinical utility in the early detection and monitoring of response to treatment in diabetic nephropathy and myeloma respectively, but the diagnostic potential of changes in the qualitative nature of proteinuria has only been identified recently.

Proteomics is the study of the entire complement of proteins, including the modifications made to a particular set of proteins, produced by an organism or system (106). Using technologies offering increased sensitivity of detection of proteins in biological systems, the depth of complement of proteins identified in urine has increased. For example, Adachi et al. identified over 1500 proteins in healthy urine using linear trap quadropole Fourier transform and Orbitrap® systems (107).

Urine proteomics, its limitations and its application to pregnancy and pre-eclampsia are discussed in detail in chapters 3.1.8 to 3.1.10.

1.6 Study aims

(1) To elucidate the independent impact of proteinuria on maternal and fetal outcomes in pregnant women with CKD.

(2) To identify a unique urine protein “fingerprint” in pregnancy that is predictive of the subsequent development of pre-eclampsia in women at high risk of the disease.

2 Proteinuria and pregnancy in patients with CKD

2.1 Background

The United Kingdom Collaboration in Obstetrics and Renal Disease (UK-CORD) was formed with the aim to collate clinical experience and data on patients with kidney disease in pregnancy. Since CKD complicates less than 0.5% of pregnancies and that it is rarely feasible to conduct randomised controlled trials in pregnancy, obtaining a large body of observational data is crucial to assist with patient counselling and management. Patients referred to specialist renal-obstetric services are included in the database except for those erroneously referred with no evidence of renal disease.

Nephrologists and obstetricians from Leicester General Hospital, Queen Elizabeth and Birmingham Women's Hospital, Birmingham and Queen Mary and Hammersmith Hospitals in London initially collected data retrospectively on pregnancies under their care from 1999 to 2002. In 2003, a purpose-specific database was constructed to be used by all contributing centres and prospective data collection began. The 3 contributory centres have a combined delivery rate of 22000 babies per year.

UK-CORD is the only pregnancy register of renal disease in the UK with plans to expand its data collection to other centres running similar services. The register is not funded. Data collection, database management and analysis are performed by clinicians at each centre.

During this research fellowship I redesigned the database through numerous iterations to enable more effective data entry and analysis. In addition to

inputting data from Leicester patients, I maintained previous records with 6 monthly post-partum data and retrospective searches for missing data. Further data base modification was required to merge data from all three centres. I designed data searches using Microsoft Access Query Wizard and adapted via Structured Query Language (SQL). Output data was analysed using Microsoft Excel 2003 and SPSS Statistics versions 15.0 to 18.0.

2.2 Data collection

Patients for inclusion in UK-CORD are identified from renal-obstetric specialist clinics held in each of the three centres. Preconception data on baseline renal function, blood pressure, medication and past medical history were obtained from retrospective review of the patient's notes, then data recorded prospectively during and following pregnancy. Patient records were updated every 6 months post-partum as data are available. The full data set and relationships are listed in the Appendix.

As an observational audit tool, collection of data does not influence patient care; therefore not all fields are completed for all patients at all visits. Missing data may reflect differences in clinical practice or a selection bias depending on clinical progress. For example, proteinuria may not be quantified with a protein:creatinine ratio or 24 hour urine collection for a patient with a negative urine dipstick result. The range of quantified proteinuria recorded will thus be falsely elevated from the true range. Interpretation of these analyses is therefore limited.

2.3 Summary of patient characteristics and outcomes

Prospective data on 313 pregnancies in 256 women with CKD have been collected. The mean age (± standard deviation, SD) of women at conception was 30.5±5.8 years (figure 2.3.1). Forty seven pregnancies (15%) were in patients treated for hypertension prior to pregnancy - a lower proportion than in other reported series of patients with more advanced CKD (52). Nine patients (2.9%) had diabetes mellitus. Eight pregnancies (2.6%) occurred in recipients of renal transplants. No pregnancies occurred in patients established on haemodialysis or peritoneal dialysis.

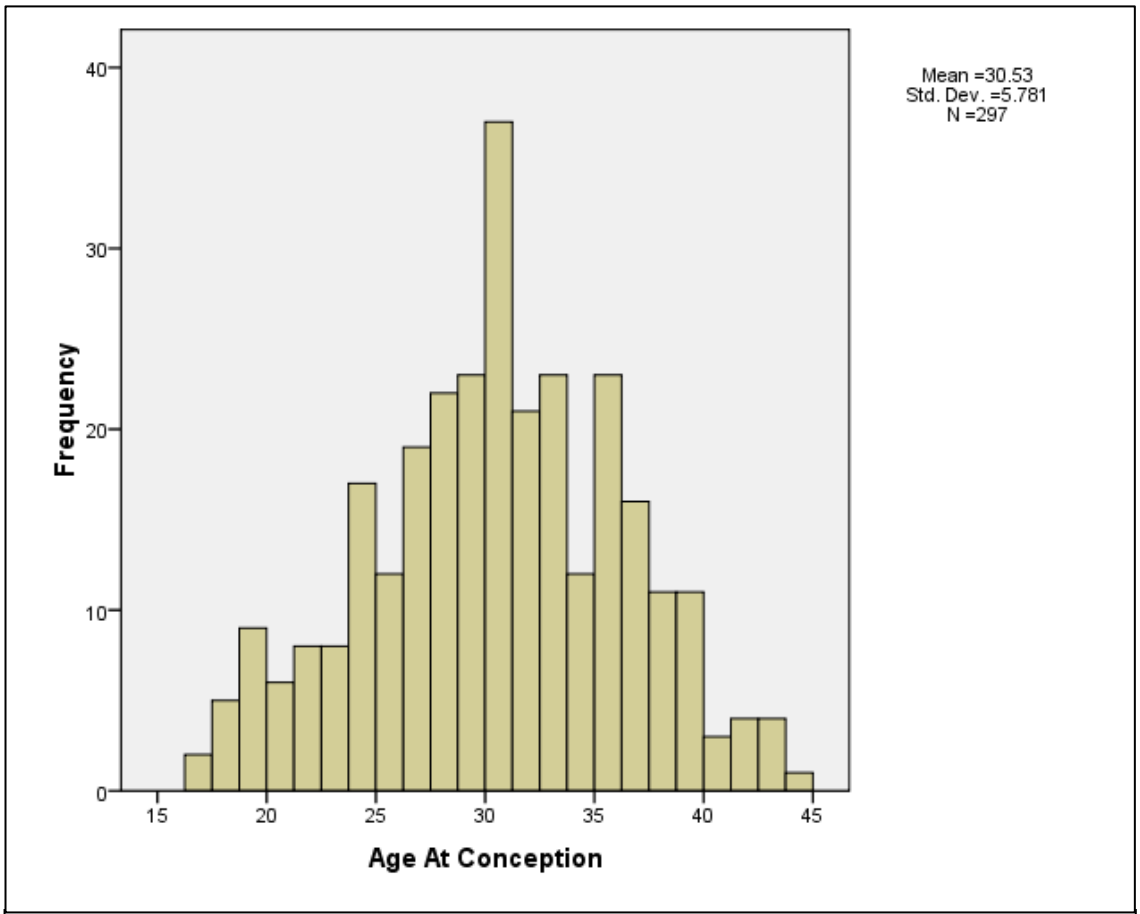


Figure 2.3.1. Maternal age at conception for pregnancies in UK-CORD. Data missing for 14 pregnancies.

Data on serum creatinine prior to conception were available for 110 patients with a mean (±SD) of 88±37µmol/l (figure 2.3.2). Fourteen women (13%) had a baseline serum creatinine greater than 110µmol/l. Mean serum creatinine in the first trimester (n=112) was 81±30µmol/l with 17 (15%) patients having a level greater than 110µmol/l.

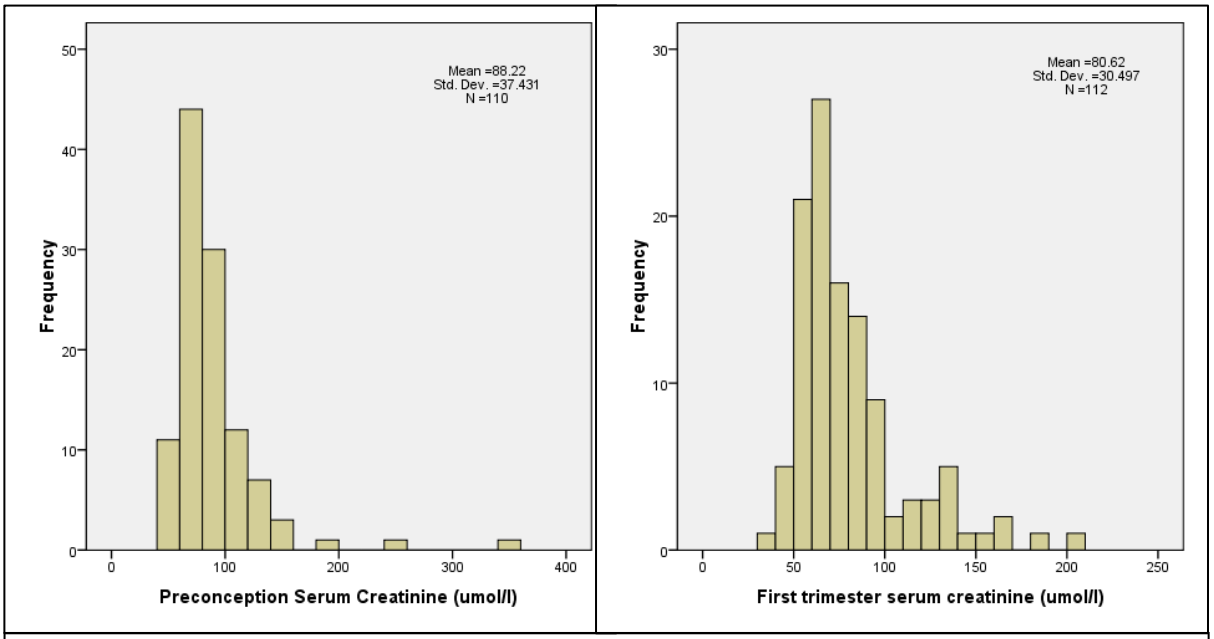


Figure 2.3.2 UK-CORD data. Serum creatinine prior to conception and during the first trimester.

Proteinuria data were available in 218 pregnancies on 950 occasions. There was weak linear correlation between gestation and proteinuria (Spearman's ρ for non-parametric data=0.177, $p<0.001$). Over half of the samples showed overt proteinuria (>300mg/day) (figure 2.3.3).

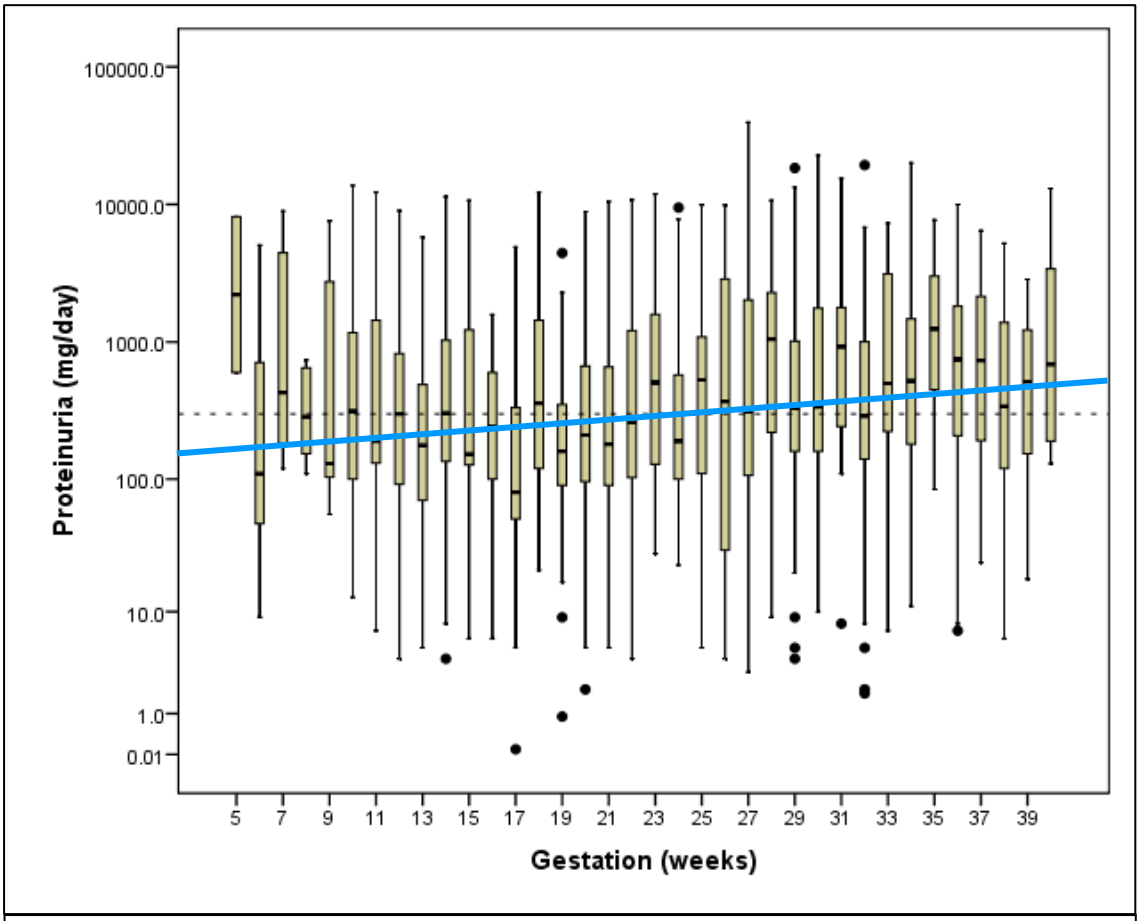


Figure 2.3.3. UK-CORD data: median proteinuria by gestation. Dashed line represents upper limit of normal excretion in pregnancy (260mg/day). Blue line represents linear correlation of median values (Spearman's ρ=0.177, p<0.001).

Pregnancy was successful in 199 of 213 (93%) pregnancies with outcome data, of which 5 infants were born with congenital anomalies or persistent neurological impairment. Of the 14 unsuccessful pregnancies, 4 ended in termination, 5 in miscarriage, 1 with a stillbirth and four early neonatal deaths (<7 days). The true rate of miscarriage and termination in CKD may be underestimated from this cohort since not all patients may have attended for renal-obstetric review prior to the end of pregnancy.

Preterm delivery (<37 weeks gestation) occurred in 60 (26%) of pregnancies. Infants were born with low birth weight (<2.5kg) in 50 pregnancies (22%) and very

low birth weight (<1.5kg) in 12 pregnancies (5%) (figure 2.3.4). Missing data for maternal biophysical parameters precluded reliable calculation of infant growth centiles at birth. Twenty seven infants (14%) were admitted to neonatal intensive care. Given that the majority of patients had preserved renal excretory function (preconception or first trimester serum creatinine <110µmol/l), the incidence of these complications is comparable to previous studies (23).

Figure 2.3.4. UK-CORD data – gestational age and infant weight at delivery

2.4 Measuring proteinuria in pregnancy and CKD

2.4.1 Introduction

As discussed above (chapter 1.4.4), the clinical importance of proteinuria in patients with chronic kidney disease during pregnancy is unclear. Maternal and fetal outcomes for patients with chronic kidney disease are predominantly related to baseline renal function and the presence of and control of hypertension (24,25,52,60,64,108).

Nevertheless, small increases in protein excretion in early pregnancy (albumin:creatinine ratio (ACR) 3-20mg/mmol) are associated with adverse pregnancy outcomes in retrospective analyses (79,80). A prospective study suggested that patients with impaired renal function (estimated glomerular filtration rate less than 40ml/min) and more than 1000mg per day proteinuria were at risk of increased rate of renal decline as a result of pregnancy, but neither parameter was independently predictive (24). Heavy proteinuria associated with nephrotic syndrome (greater than 3000mg/d) has been associated with preterm delivery, intrauterine growth restriction and maternal morbidity and mortality, particularly when mothers are symptomatic in the first trimester (91,92).

Quantitative assessment of proteinuria in patients with CKD is therefore of clinical interest. As above (chapter 1.5.3.1), 24 hour urine collections are considered the gold standard for quantifying proteinuria but are cumbersome and frequently inaccurate (100). Spot urine albumin:creatinine (ACR) or protein:creatinine ratios (PCR) have entered clinical practice in general medicine, diabetology, nephrology

and obstetrics, however, there are no data to support the validity of using ACR and PCR in patients with CKD and pregnancy.

The United Kingdom Collaboration in Obstetrics and Renal Disease (UK-CORD) has collected prospective observational data on patients with chronic kidney disease and pregnancy in three specialist centres since 2003. In this post-hoc analysis of data I assessed the validity of PCR and ACR in patients with chronic kidney disease and pregnancy.

2.4.2 Methods

2.4.2.1 Patients

Obstetric and renal management of patients were directed according to local physician expertise and preference and observational data obtained from clinical encounters, laboratory data systems and radiological reports. Proteinuria data, demographic data and fetal outcomes were extracted from the database retrospectively.

2.4.2.2 Urine specimens

Twenty four urine collections were requested by managing clinicians to assess baseline protein excretion or monitor clinical progress. Adequacy of 24 hour urine collection was assessed by 24 hour creatinine excretion (greater than 6.8mmol/d (0.6g/d)) and 24 hour urine volume (greater than 1000ml/d) (100).

PCR or ACR measured within 72 hours of the 24 hour urine collection were paired with the urine 24 hour protein excretion. Values of urine volume, albumin, protein and creatinine concentration were obtained from laboratories in each hospital, requested as part of routine clinical practice.

2.4.2.3 Statistical analysis

Correlation between methods of urine protein excretion quantification was assessed by Spearman ρ rank correlation co-efficient.

Receiver operator curves (ROC) were constructed to assess the optimum values of different methods of proteinuria quantification in defining arbitrary levels of 24

hour urine excretion used in clinical practice. ROC analysis plots the sensitivity and specificity of a parameter in predicting an outcome across the range of parameter values encountered in the study. The area under the curve (AUC) represents the practical utility of a parameter in defining an outcome with 1.0 representing a "perfect" discriminatory test and 0.5 representing a non-predictive test. An AUC > 0.7 describes a good test and >0.8 an excellent test (109,110).

The utility of different methods of quantification were then compared using arbitrary levels of 24 hour urine excretion used in clinical practice and the corresponding optimum cut off values identified by ROC analysis. Differences in the frequency of clinical endpoints between proteinuria thresholds were compared for each method of quantification using Fisher's exact test.

A p-value of <0.05 was considered statistically significant.

2.4.3 Results

2.4.3.1 Participants and samples

Two hundred and twelve paired urine specimens were obtained from 80 patients in 88 pregnancies. Urine collections were obtained from 5 to 40 weeks gestation (median (interquartile range, IQR) 28 (20-36) weeks) as part of routine clinical care. Maternal age was 19 to 44 years (mean±SD 30.8±4.8 years). Creatinine clearance was calculated from 24 hour urine creatinine excretion as part of routine care. Median creatinine clearance was 118ml/min (IQR 73-157 ml/min). One hundred and one samples (48%) came from patients with primary or secondary glomerular disease, 22 (10%) from patients with tubular or interstitial disease, 23 (11%) from patients with hereditary and genetic renal disease, 11 (5%) with structural renal disease and 55 (26%) with renal disease of unknown aetiology .

2.4.3.2 Adequacy of 24 hour urine collection

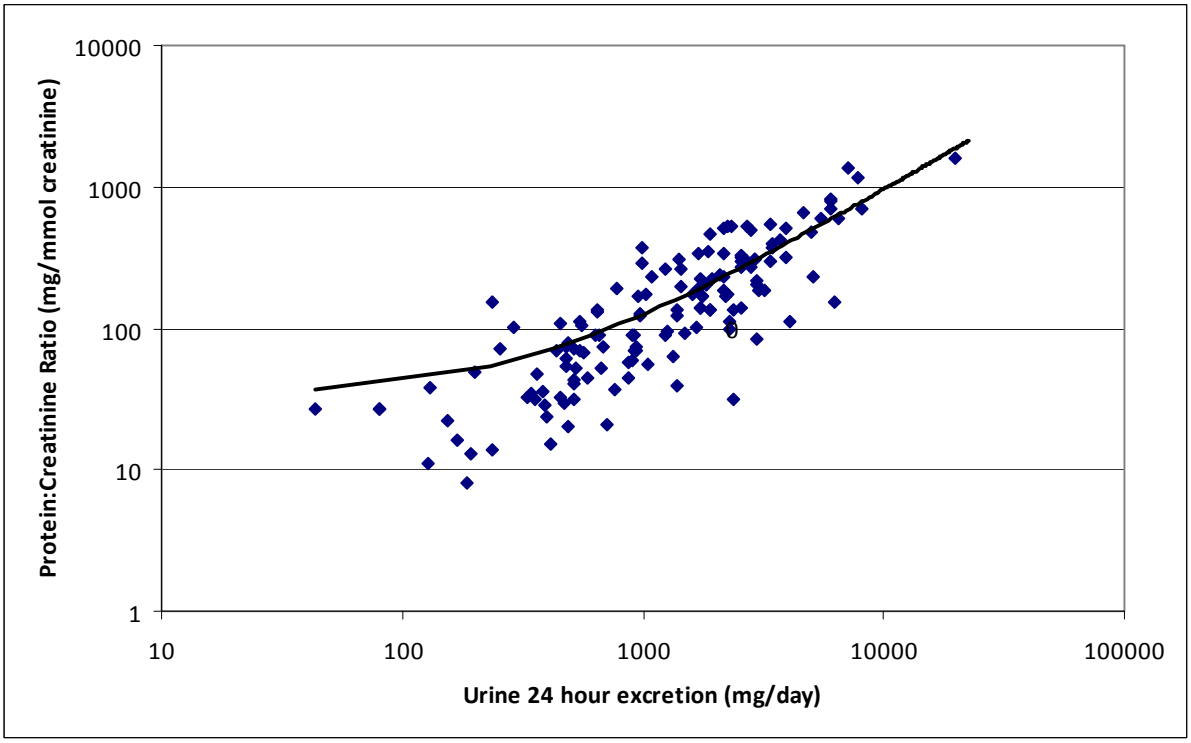
Urinary volume was available for 100% of collections and creatinine excretion in 71% of collections. Body weight was available for only 17% of patients with 24 hour urine collections so adequacy was not assessed according to creatinine excretion per kilogram body weight.

Mean (±SD) urine creatinine excretion was 13.0±8.2 mmol/d (1.15±0.72 g/d). Urine creatinine excretion was greater than 6.8mmol/d (0.6g/d) in 89.4% of collections.

Mean (±SD) urinary volume was 1726±725 ml. Urine excretion was greater than 1000ml in 85% of collections.

2.4.3.3 Correlation of PCR and ACR with urine 24 hour protein

One hundred and forty three 24 hour urine results had a paired PCR and 89 had a paired ACR. There were 64 results that had both ACR and PCR. PCR and ACR significantly correlated with each other (Spearman's ρ 0.91, $p<0.001$) and with measured 24 hour urine protein (Spearman's ρ 0.84, $p<0.001$ and Spearman's ρ 0.82, $p<0.001$ respectively, figure 2.4.3.1).



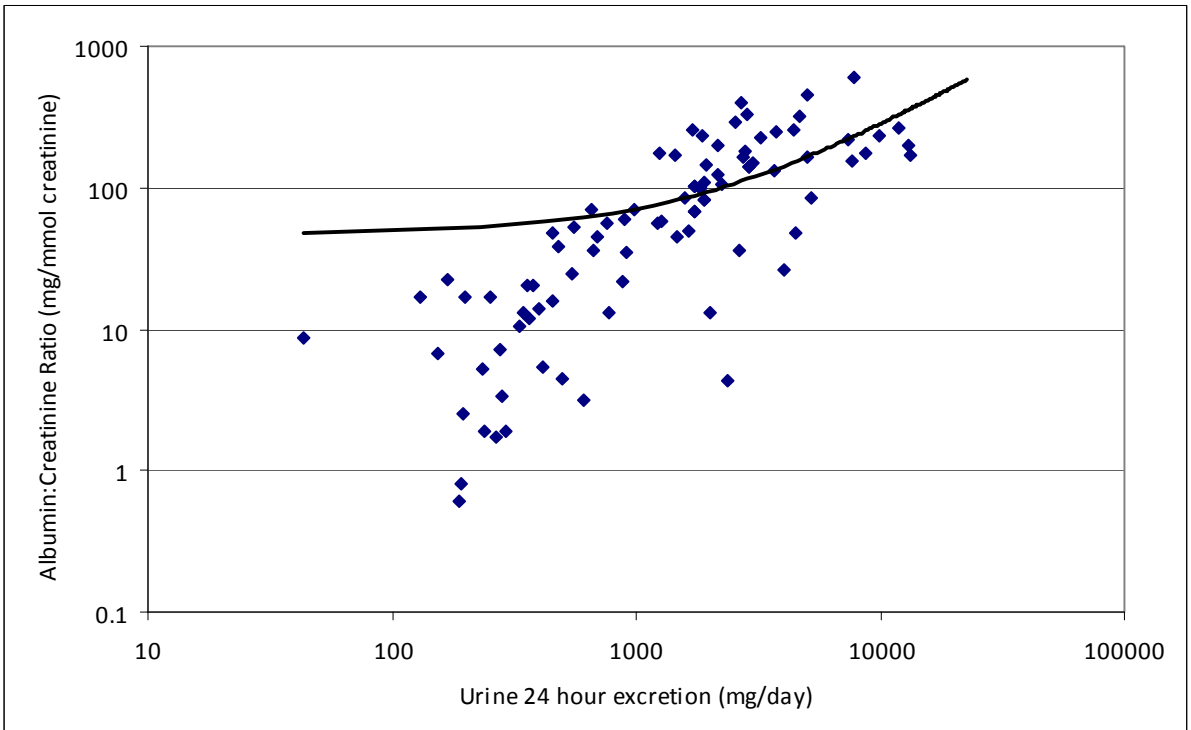


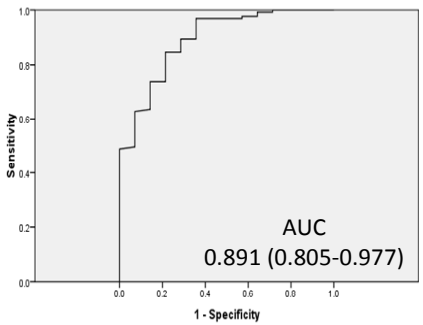
Figure 2.4.3.1. Correlation of urine 24 hour protein excretion to protein:creatinine ratio (Spearman's ρ 0.838, p<0.001) and albumin:creatinine ratio (Spearman's ρ 0.818, p<0.001) in patients with pregnancy and CKD.

2.4.3.4 Diagnostic potential of PCR and ACR

To assess the clinical utility of PCR or ACR, ROC curves were constructed to qualify the optimum sensitivity and specificity in defining arbitrary thresholds of proteinuria used in practice. Values of 300mg/d (diagnostic criterion for pre-eclampsia), 1000mg/d (heavy proteinuria), 3000mg/d (nephrotic range proteinuria) and 5000mg/d (severe pre-eclampsia) were used.

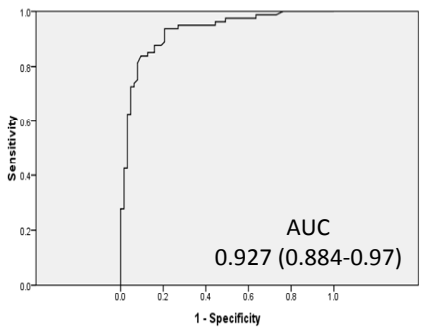
The area under curve (AUC) (± 95% confidence interval) was greater than 0.8 for PCR at all thresholds (figure 2.4.3.2). ACR performed better than PCR at detecting 300mg/d and 1000mg/d thresholds for proteinuria but less well at detecting 3000mg/d and 5000mg/d thresholds (figure 2.4.3.3). The optimum PCR and ACR representative for each proteinuric threshold were extracted from the ROC curves as that with the highest combined sensitivity and specificity. Values with 90% sensitivity and specificity were also extracted for potential use in clinical studies. The optimum PCR to identify patients with 24 hour urine protein excretion greater than 300mg/d was 50.5 mg/mmol (specificity 84.5%, sensitivity 78.6%), greater than 1000mg/d was 107.5 mg/mmol (87.5%, 82.5%), greater than 3000mg/d was 232.5 mg/mmol (82.6%, 80.8%) and greater than 5000mg/d was 472 mg/mmol (83.3%, 93.9%). The optimum ACR to identify patients with 24 hour urine protein excretion greater than 300mg/d was 18.5 mg/mmol (specificity 85.1%, sensitivity 86.7%), greater than 1000mg/d was 54.7 mg/mmol (86.3%, 89.5%), greater than 3000mg/d was 126.3 mg/mmol (83.3%, 80.3%) and greater than 5000mg/d was 153.2 mg/mmol (90.9%, 82.1%).

300mg/d



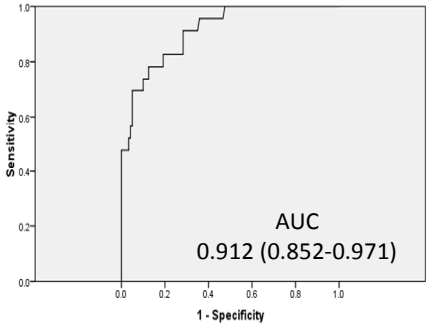
Optimum PCR	50.5 mg/mmol creat Specificity 84.5% Sensitivity 78.6%
90% sensitivity	36.5 mg/mmol creat
90% specificity	104 mg/mmol creat

1000mg/d



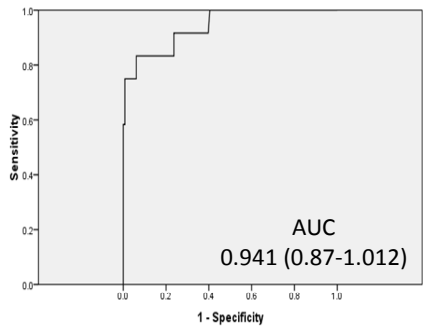
Optimum PCR	107.5 mg/mmol creat Specificity 87.5% Sensitivity 82.5%
90% sensitivity	97 mg/mmol creat
90% specificity	132.5 mg/mmol creat

3000mg/d



Optimum PCR	232.5 mg/mmol creat Specificity 82.6% Sensitivity 80.8%
90% sensitivity	185.5 mg/mmol creat
90% specificity	312.5 mg/mmol creat

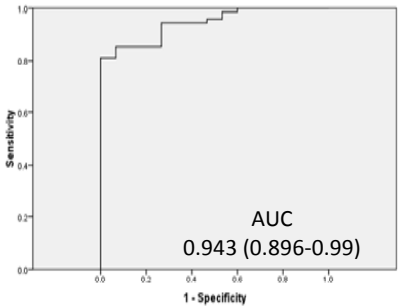
5000mg/d



Optimum PCR	472 mg/mmol creat Specificity 83.3% Sensitivity 93.9%
90% sensitivity	232.5 mg/mmol creat
90% specificity	361.5 mg/mmol creat

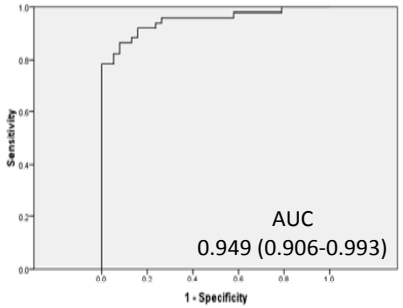
Figure 2.4.3.2. Receiver operator curves of protein:creatinine ratio and urine 24 hour protein excretion thresholds for diagnosis of pre-eclampsia (300mg/d), heavy proteinuria (1000mg/d), nephrotic range proteinuria (3000mg/d) and severe pre-eclampsia (5000mg/d). AUC, area under curve; PCR, protein:creatinine ratio, n=143

300mg/d



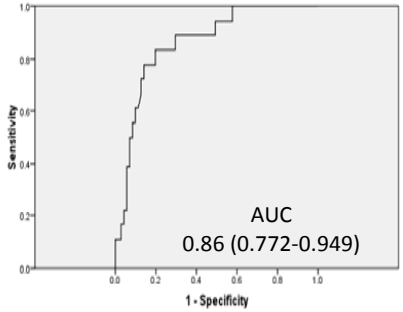
Optimum ACR	18.5 mg/mmol creat Sensitivity 85.1% Specificity 86.7%
90% sensitivity	13.1 mg/mmol creat
90% specificity	18.5 mg/mmol creat

1000mg/d



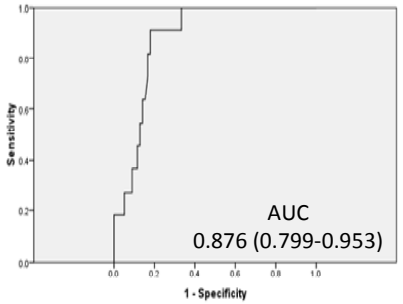
Optimum ACR	54.7 mg/mmol creat Sensitivity 86.3% Specificity 89.5%
90% sensitivity	46.2 mg/mmol creat
90% specificity	56.5 mg/mmol creat

3000mg/d



Optimum ACR	126.3 mg/mmol creat Sensitivity 83.3% Specificity 80.3%
90% sensitivity	46.2 mg/mmol creat
90% specificity	174 mg/mmol creat

5000mg/d



Optimum ACR	153.2 mg/mmol creat Sensitivity 90.9% Specificity 82.1%
90% sensitivity	153.2 mg/mmol creat
90% specificity	234.6 mg/mmol creat

Figure 2.4.3.3 Receiver operator curves of albumin:creatinine ratio and urine 24 hour protein excretion thresholds for diagnosis of pre-eclampsia (300mg/d), heavy proteinuria (1000mg/d), nephrotic range proteinuria (3000mg/d) and severe pre-eclampsia (5000mg/d). AUC, area under curve; PCR, protein:creatinine ratio, n=89

2.4.3.5 Clinical significance of PCR and ACR

Using the optimum thresholds obtained for PCR and ACR relating to urine 24 hour protein excretion, the association between PCR, ACR and urine 24 hour protein excretion and fetal outcomes were studied. Preterm delivery and low birth weight infants were associated with urine 24 hour excretion greater than 3000mg/d. A similar association was noted with the optimum threshold for ACR and PCR (table 2.4.3.1). The same pattern was noted for low birth weight infants. Very low birth weight infants were associated with urine 24 hour protein excretion greater than 5000mg/d but the association with ACR and PCR was less clear.

		Preterm	LBW	VLBW
U24Pro > 300mg/d	Yes	35.6%	31.9%	8%
	No	25%	20.8%	16.7%
	p	0.37	0.35	0.243
PCR > 50.5mg/mmol	Yes	33%	33.9%	8%
	No	35.5%	12.9%	9.7%
	p	0.832	0.026	0.723
ACR > 18.5mg/mmol	Yes	28.1%	29.7%	9.4%
	No	24%	12%	4%
	p	0.794	0.104	0.668

		Preterm	LBW	VLBW
U24Pro > 3000mg/d	Yes	62.1%	51.7%	15.5%
	No	24%	22.7%	6.5%
	p	<0.001	<0.001	0.057
PCR > 232.5mg/mmol	Yes	52.4%	50%	9.5%
	No	25.7%	20.8%	7.8%
	p	0.003	0.001	0.746
ACR > 126.3mg/mmol	Yes	44.8%	44.8%	17.2%
	No	18.3%	15%	3.3%
	p	0.011	0.004	0.035

		Preterm	LBW	VLBW
U24Pro > 1000mg/d	Yes	39.2%	34.6%	9.2%
	No	26.8%	24.4%	8.5%
	p	0.08	0.128	1
PCR > 107.5mg/mmol	Yes	34.6%	35.8%	8.6%
	No	32.3%	21%	8.1%
	p	0.859	0.065	1
ACR > 54.7mg/mmol	Yes	29.2%	29.2%	10.4%
	No	24.4%	19.5%	4.9%
	p	0.64	0.333	0.445

		Preterm	LBW	VLBW
U24Pro > 5000mg/d	Yes	73.7%	65.8%	23.7%
	No	25.9%	23%	5.7%
	p	<0.001	<0.001	0.002
PCR > 472mg/mmol	Yes	66.7%	55.6%	11.1%
	No	28.8%	25.6%	8%
	p	0.003	0.013	0.649
ACR > 153.2mg/mmol	Yes	50%	50%	16.7%
	No	18.5%	15.4%	4.6%
	p	0.006	0.002	0.082

Table 2.4.3.1. Association between thresholds of proteinuria measured by urine 24 hour protein (U24Pro) excretion, protein:creatinine ratio (PCR) and albumin:creatinine ratio (ACR) and fetal outcomes in patients with pregnancy and kidney disease. Preterm delivery and low birth weight (LBW) infants were associated with proteinuria greater than 3000mg/d. Very low birth weight (VLBW) infants were associated with proteinuria greater than 5000mg/d. Similar associations were found with thresholds for ACR and, to a lesser extent, PCR.

2.4.4 Discussion

Values obtained from PCR or ACR correlate closely to 24 urine protein excretion in pregnant patients with CKD. Accordingly, PCR and ACR perform well in identifying arbitrary levels of proteinuria used in clinical practice for the diagnosis of pre-eclampsia, heavy proteinuria, nephrotic syndrome and severe pre-eclampsia with ROC AUC values >0.80 in all cases.

AUC values for ACR were higher than PCR for lower levels of proteinuria detection (300mg/d and 1000mg/d) with the reciprocal true for higher levels (3000mg/d and 5000mg/d). Minor changes in glomerular permeability due to primary renal disease or pre-eclampsia increase the transfer of plasma proteins, notably albumin, to the urinary filtrate. Given that there is obligate protein loss from tubular secretion of Tamm-Horsfall and other proteins, albuminuria may be a more sensitive measure of early glomerular disease than total proteinuria. This has previously been shown in patients with diabetic nephropathy (101) and may explain the differences in performance noted.

These results show that both PCR and ACR perform adequately in the assessment of proteinuria in pregnancy and CKD and are comparable with results obtained in other patient populations (111,112). Importantly, clinical outcomes showed similar relationships against proteinuria thresholds obtained by any of the methods studied.

Together, these results suggest that PCR or ACR can be used in the assessment and monitoring of pregnant patients with CKD.

2.5 Patterns of proteinuria during pregnancy in women with CKD and associations with maternal and fetal outcomes

2.5.1 Introduction

In normal pregnancy, urine protein excretion increases from a mean of 62.5mg/d to 117mg/d (9,10). Patients with CKD often have proteinuria (>300mg/d) and the presence and severity of proteinuria is an independent predictive factor for decline in renal function (75). Inhibition of the renin-angiotensin system (RAS) is renoprotective for patients with proteinuric renal disease (76,113) but angiotensin converting enzyme inhibitors and angiotensin receptor blockers are contraindicated in pregnancy (34). Thus, during attempted conception and pregnancy (when agents inhibiting RAS have been discontinued), patients with CKD are potentially exposed to higher levels of proteinuria with potentially deleterious effects on their underlying renal disease.

This analysis investigated how proteinuria in patients with CKD changes during pregnancy, and the association of these changes with maternal and fetal outcomes.

2.5.2 Methods

Patients with data on baseline proteinuria and maternal renal function postpartum were included in a retrospective analysis of the prospective data in the UK-CORD database. Proteinuria was measured by albumin:creatinine ratio (ACR), protein:creatinine ratio (PCR) or 24 hour urine collection. Where 24 hour collections were not available, PCR or ACR values were multiplied by a factor of 10 to approximate 24 hour urine excretion in mg/d. A factor of 10 was selected based on linear regression analysis of data in the previous chapter (chapter 2.4) identifying regression coefficients of 8.7 ± 1.1 mmol/d for PCR and 13.3 ± 4.5 mmol/d for ACR.

Proteinuria was defined as an excretion of greater than 300mg/d. Patients were characterised according to the pattern of proteinuria they displayed during pregnancy in a post-hoc analysis (figure 2.5.2.1). Increased proteinuria was arbritrarily defined as a change of greater than 33% from baseline value (preconception or <12 weeks gestation) during pregnancy to accommodate the observed range of changes and the expected coefficient of variance on measured proteinuria quantification (figure 2.5.2.2).

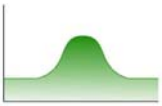
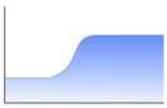
Group 1 Proteinuria prior to, during and after pregnancy without significant increase during pregnancy (<33%)	
Group 2 Increase in proteinuria during pregnancy (>33%) with return to baseline by 6 months postpartum	
Group 3 Increase in proteinuria during pregnancy (>33%) without return to baseline by 6 months postpartum	
Group 4 No proteinuria prior to, during or after pregnancy	

Figure 2.5.2.1. Patterns of proteinuria defined in post hoc analysis of UK-CORD data

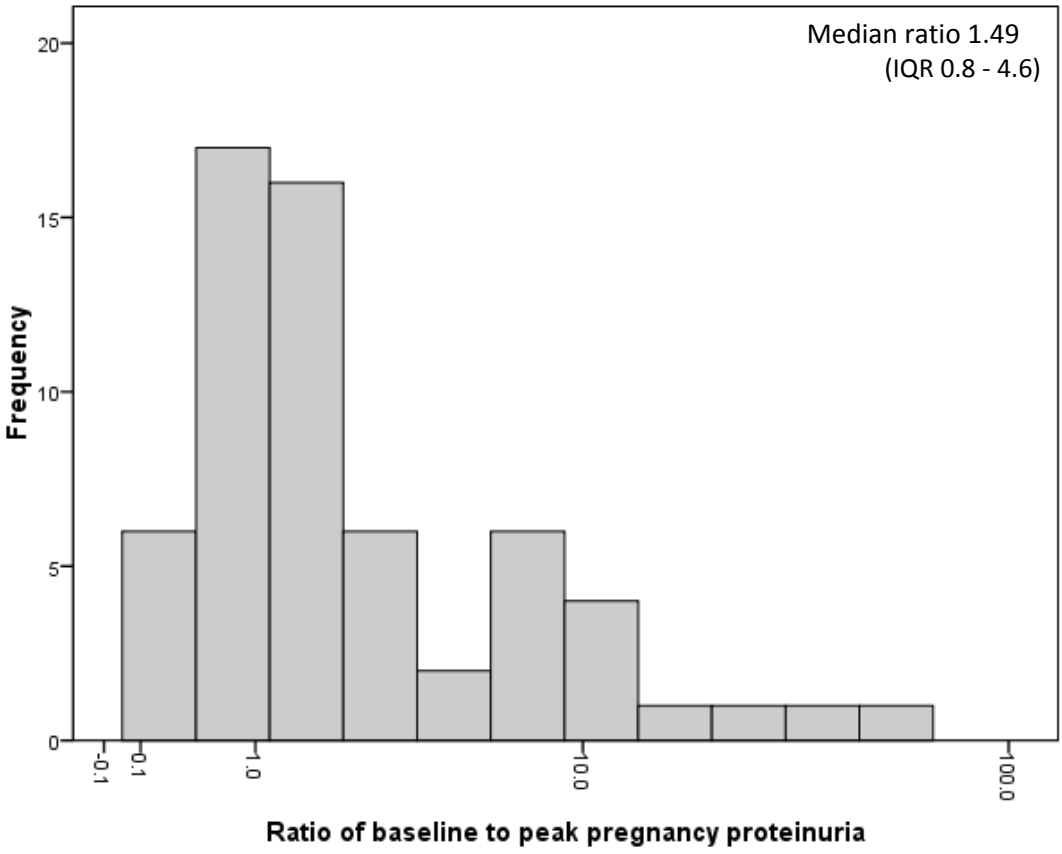


Figure 2.5.2.2. Range of changes in proteinuria from baseline to pregnancy. IQR, interquartile range.

Non-parametric characteristics were compared with the Kruskal-Wallis test. Maternal and fetal outcomes were compared between the groups by ANOVA with Tukey post-hoc analysis for continuous variables and chi-squared test for categorical variables. Multiple regression analysis was performed to account for possible confounding factors including maternal age, blood pressure, serum creatinine and gravidity. Proteinuria data was logarithmically transformed for inclusion in linear models. Low birth weight was defined as less than 2.5kg at delivery, very low birth weight as less than 1.5kg at delivery and small for gestational age as <10% centile calculated from customised centile charts.

2.5.3 Results

2.5.3.1 Demographic and clinical characteresitics

Data were available for 79 pregnancies, 42 of which had no proteinuria prior to, during or after pregnancy. Baseline characteristics are shown in table 2.5.3.1. Patients without proteinuria (group 4) were less likely to have glomerular disease than groups 1 and 3.

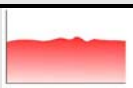
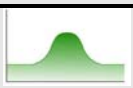
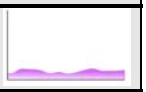
Group	1	2	3	4
Pattern of proteinuria				
n	4	24	9	42
Age	32.9	31.7	30.3	30.8
Gravidity	2.75	1.79	1.50	1.82
Chronic treated hypertension (%)	25	60	33.3	27.3
Treated hypertension during pregnancy (%)	25	60	55.6	30.9
Baseline SBP (mmHg)	133	122	131	122
Baseline DBP (mmHg)	83	75	80	80
Glomerular disease (%)*	75	31.8	66.7	26.2

Table 2.5.3.1. Baseline demographics and clinical characteristics. Values are means unless indicated. SBP, systolic blood pressure; DBP, diastolic blood pressure. ANOVA between groups comparison, *p<0.01.

2.5.3.2 Maternal renal outcomes

As expected by the defined groups, proteinuria was higher throughout pregnancy in group 1 than group 4, higher in group 2 during pregnancy, and group 3 in pregnancy and postpartum ($p<0.001$ for between group comparison at all time points, table 2.5.3.2).

No difference in serum creatinine was identified between groups at baseline ($p=0.354$), but higher in group 3 during pregnancy ($p=0.005$). Serum creatinine postpartum was higher in group 3 but did not reach statistical significance ($p=0.157$).

Comparison of the change in serum creatinine between baseline and over 6 months postpartum identified a significant difference in patients in with persistent proteinuria postpartum (group 3, figure 2.5.3.1). The rate of increase in serum creatinine was 7 nmol/l/d is group 1, 30 nmol/l/d in group 2, 89 nmol/l/d in group 3 and 0.49 nmol/l/d in group 4 ($p=0.02$, Tukey post-hoc analysis of ANOVA comparing group 3 to group 4). No difference in age, gravidity or blood pressure co-existed to account for this finding.

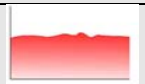
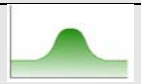
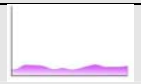
Group	1	2	3	4
Pattern of proteinuria				
Baseline data (<12 weeks gestation)				
Proteinuria (mg/day)	**4695 (280 - 13700)**	390(40 - 5190)	1000 (290 – 5000)	120 (10 – 260)
Serum creatinine (µmol/l)	88 (56 – 110)	79 (54 – 151)	94 (66 – 203)	73 (67 – 187)
Pregnancy data (12 weeks – delivery)				
Proteinuria (mg/day)	**500 (300 – 13300)**	**2208 (270 – 13090)**	**3700 (490 – 15440)**	203 (1 – 290)
Serum creatinine (µmol/l)	108 (58 – 114)	76 (52 – 155)	**134 (73 – 243)**	70 (48 – 221)
Post partum (>6 months post partum)				
Proteinuria (mg/day)	**3760 (270 – 10080)**	610 (20 – 4830)	**4930 (580 – 10560)**	104 (0 – 170)
Serum creatinine (µmol/l)	97 (70 – 145)	84 (50 – 150)	163 (63 – 277)	81 (55 – 111)

Table 2.5.3.2. Proteinuria and creatinine by pattern of proteinuria. Values are median (range). Figures in bold are significant between group differences ($p<0.05$).

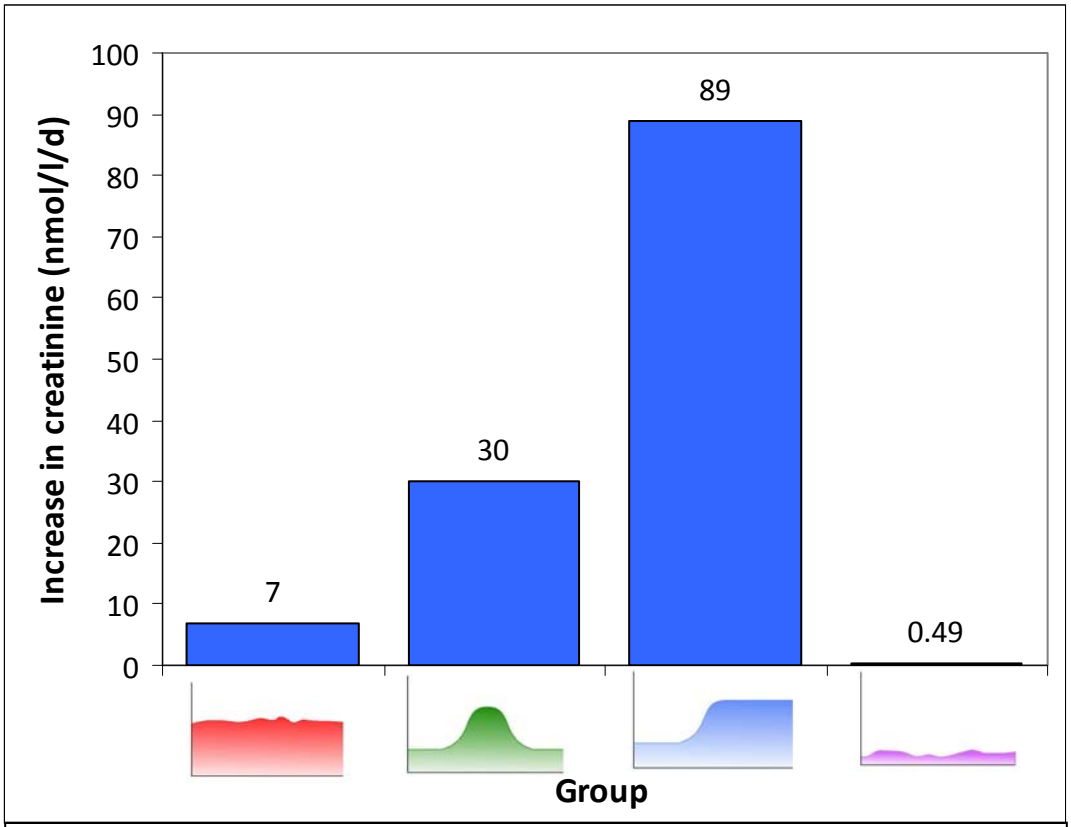


Figure 2.5.3.1. Rate of decline in renal function by pattern of proteinuria from baseline serum creatinine to >6 months post partum.

2.5.3.3 Infant outcomes

Of 79 pregnancies, there were 76 (96%) live births, 1 (1%) stillbirth and 2 (2%) miscarriages. One infant was born with persistent neurological impairment.

Low birth weight and small for gestational age infants were more common in groups with persistent gestational proteinuria (table 2.5.3.3, figure 2.5.3.2).

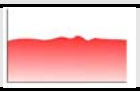
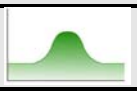
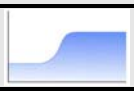
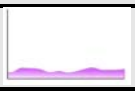
Group	1	2	3	4
Pattern of proteinuria				
n	4	24	9	42
Preterm delivery (%)*	50	50	66.6	7.1
LBW (%)*	50	12.5	66.7	11.9
VLBW (%)	0	16.6	11.1	0
SGA (%)*	50	27	67	23

Table 2.5.3.3. Frequency of fetal outcomes by pattern of proteinuria during pregnancy. Compared against group 4, *p<0.05 across groups. LBW, low birth weight; VLBW, very low birth weight. SGA, small for gestational age

Proteinuria and birth weight were correlated significantly (p=0.01, Spearman's ρ=-0.295). However, a general linear model of possible confounding factors identified that birth weight was independently predicted by gestational age at delivery and serum creatinine only; proteinuria had no independent effect on birth weight in this cohort (p=0.986).

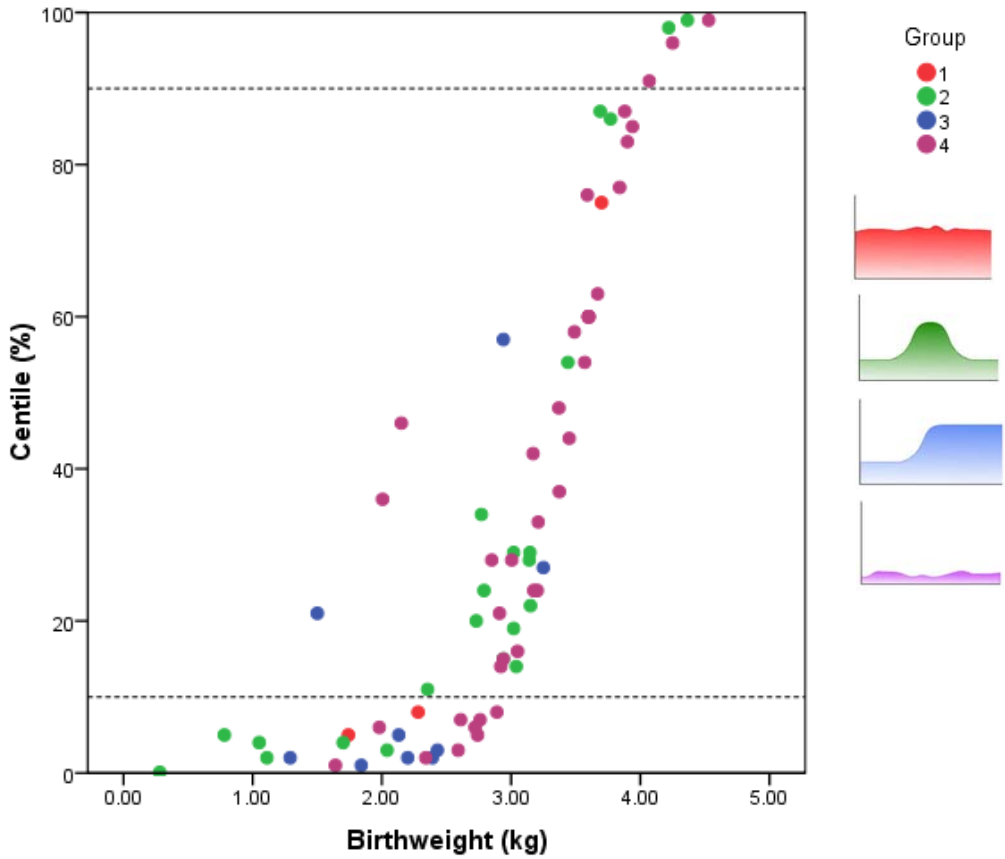


Figure 2.5.3.2. Birth weight and centile by proteinuric group

Preterm delivery was more common in groups associated with proteinuria during pregnancy. Proteinuria significantly correlated with gestational age at delivery (p=0.004, Spearman's ρ=-0.322). In a model including serum creatinine (p=0.475), maternal age (p=0.988), systolic blood pressure (p=0.418) and diastolic blood pressure (p=0.314), logarithm transformed proteinuria (p=0.028) was independently associated with gestational age at delivery.

2.5.4 Discussion

Development of proteinuria during pregnancy was associated with earlier delivery, lower birth weight and intrauterine growth retardation. Data from this study can not determine the rates of physician-induced delivery that may have contributed to the earlier deliveries noted, however intrauterine growth retardation was noted in a high proportion of proteinuric patients raising the possibility of potential or overt pre-eclampsia.

Persistent proteinuria after pregnancy was also associated with greater decline in maternal renal function 6 months post-partum, however the rate of change in renal function remained small and may not be of clinical significance. Of interest, patients who developed proteinuria during pregnancy which subsequently resolved did not display such a decline in renal function. Long term exposure of renal tubular epithelium to high loads of filtered protein is associated with progressive renal parenchymal damage, irrespective of the aetiology (103,113). It is therefore reassuring that transient proteinuria associated with pregnancy is not associated with loss of renal function in the short term. Post-partum follow-up of patients with proteinuria in pregnancy is supported by these results.

Proteinuria during pregnancy was noted in almost half of the patients included in this analysis, although selection bias is likely given the requirement for documented measurement of proteinuria before, during and after pregnancy.

As an observational retrospective analysis of the data there are likely to be unseen confounding factors contributing to the study findings, particularly given

the small data set. It is possible that the presence of persistent proteinuria during pregnancy prompted early physician-led delivery, and subsequently low birth weight infants, due to signs or symptoms of impending pre-eclampsia, not evaluated in this analysis. The use of an arbritrary cut-off value of 33% to define changes in proteinuria during pregnancy has not been subject to validation in other cohorts and may limit the reproducibility of these findings in other settings.

Nevertheless, the presence of proteinuria during pregnancy was associated with adverse maternal and fetal outcomes. This highlights the importance of monitoring urine protein excretion during pregnancy in women with CKD and also ensuring that postpartum follow-up of patients is arranged to identify progressive renal decline in pregnancies complicated by proteinuria.

2.6 Pregnancy-associated accelerated loss of renal function: is proteinuria a predictive factor?

2.6.1 Introduction

The results above suggest that proteinuria during pregnancy is associated with preterm delivery and decline in maternal renal function. When counselling women with CKD about the potential risks associated with a planned pregnancy the impact of proteinuria on outcomes has not been clearly elucidated (see chapter 1.4.4). It is clear that some women will suffer an accelerated loss of renal function due to pregnancy, particularly those with more advanced renal disease and hypertension. Observational data suggests that women with a pre-conception serum creatinine >180μmol/l have a 33% risk of requiring dialysis during or within 6 months of pregnancy (23).

This analysis studies the independent impact of proteinuria on predicting pregnancy-accelerated loss of renal function in women with CKD.

2.6.1 Methods

Patients included in the UK-CORD database were reviewed to identify those with data to calculate pre-conception rate of change in renal function, baseline demographic and clinical parameters and follow-up data at least 6 months post-partum.

The rate of decline in renal function prior to pregnancy was calculated from linear regression analysis of available estimated GFR values, with 95% confidence intervals. This rate of decline was extrapolated to at least 6 months post-partum and actual estimated GFR compared to that predicted from the regression function. Patients with estimated GFR below the lower 95% confidence interval were defined as having pregnancy-associated accelerated loss of renal function (PALRF) as illustrated in figure 2.6.1.1 with example data.

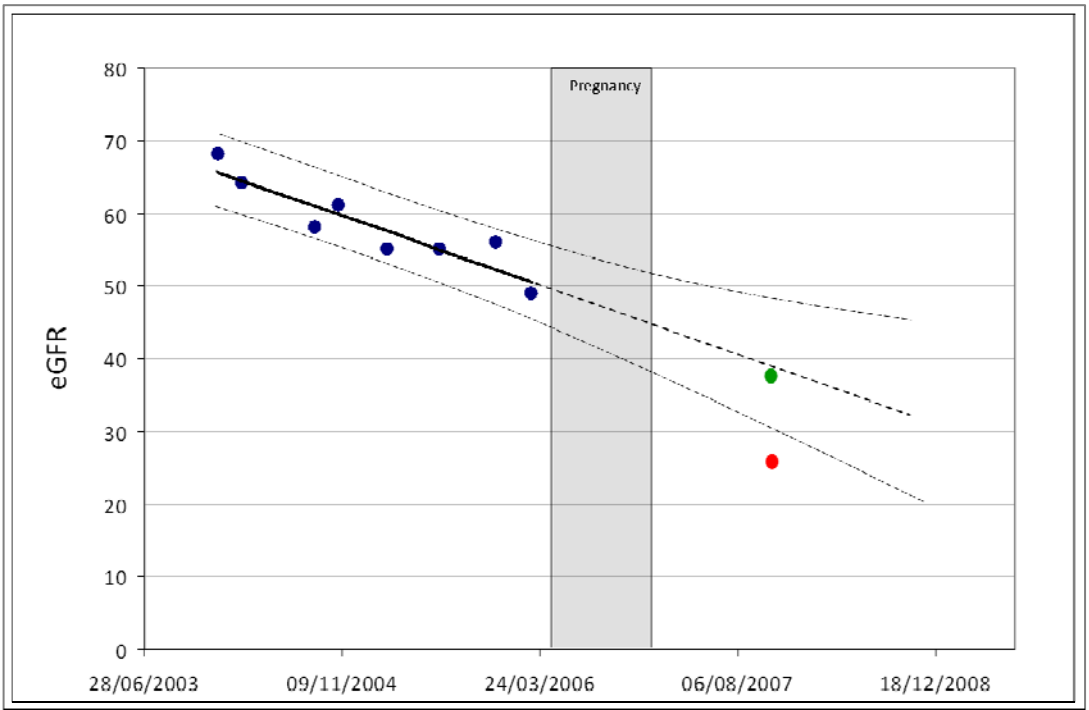


Figure 2.6.1.1. Example data illustrating pregnancy-associated accelerated loss of renal function (PALRF). Estimated GFR measured 6 months post-partum was compared to linear regression of preconception eGFR values (and calculated 95% confidence interval) to identify patients with renal decline greater than predicted (red circle) or as expected (green circle).

Baseline values for proteinuria, creatinine, medication and blood pressure were obtained retrospectively from results within 6 months of the patient's last menstrual period. Continuous variables were compared by Student's t-test or Mann-Whitney *U* test. Categorical variables were compared by Chi-squared or Fisher's Exact test. ROC analysis was performed to identify optimum thresholds of factors predictive of PALRF.

2.6.2 Results

Data were available for 27 patients of whom 6 (22%) developed PALRF. Baseline blood pressure and renal function were identified as predictive of PALRF, but not proteinuria (table 2.6.3.1, figure 2.6.3.2). Similarly, there was no significant increased risk of PALRF in patients with proteinuria >100mg/mmol creatinine (25.0% versus 18.2%, p=0.528) or >300mg/mmol creatinine (27.3% versus 0%, p=0.185). Rate of decline in renal function prior to conception was not associated with PALRF (0.44ml/min/year versus -0.50 ml/min/year, p=0.932).

		PALRF		
		Yes	No	p value
n		6	21	
Maternal age (years)	Mean (SD)	33.0 (3.7)	30.4 (3,3)	0.103
Gravidity	Median (range)	3 (1-4)	3 (1-5)	0.057
Underlying glomerular disease	n (%)	2 (33%)	13 (69%)	0.357
Baseline serum creatinine (μmol/l)	Mean (SD)	111 (46)	81 (20)	**0.027**
Baseline eGFR (ml/min)	Mean (SD)	63 (28)	79 (16)	0.077
Baseline protein:creatinine ratio (mg/mmol creatinine)	Median (IQR)	29 (206)	46 (272)	0.345
Rate of decline in eGFR prior to conception (ml/min/year)	Median (IQR)	0.44 (4.15)	-0.50 (3.52)	0.932
Baseline systolic BP (mmHg)	Mean (SD)	143.1 (18.3)	136.6 (20.8)	0.08
Baseline diastolic BP (mmHg)	Mean (SD)	81.0 (4.7)	71.6 (9.7)	**0.009**
Receiving antihypertensives	n (%)	5 (83.3%)	7 (33.3%)	**0.03**

Table 2.6.3.1. Demographic and clinical parameters of study cohort

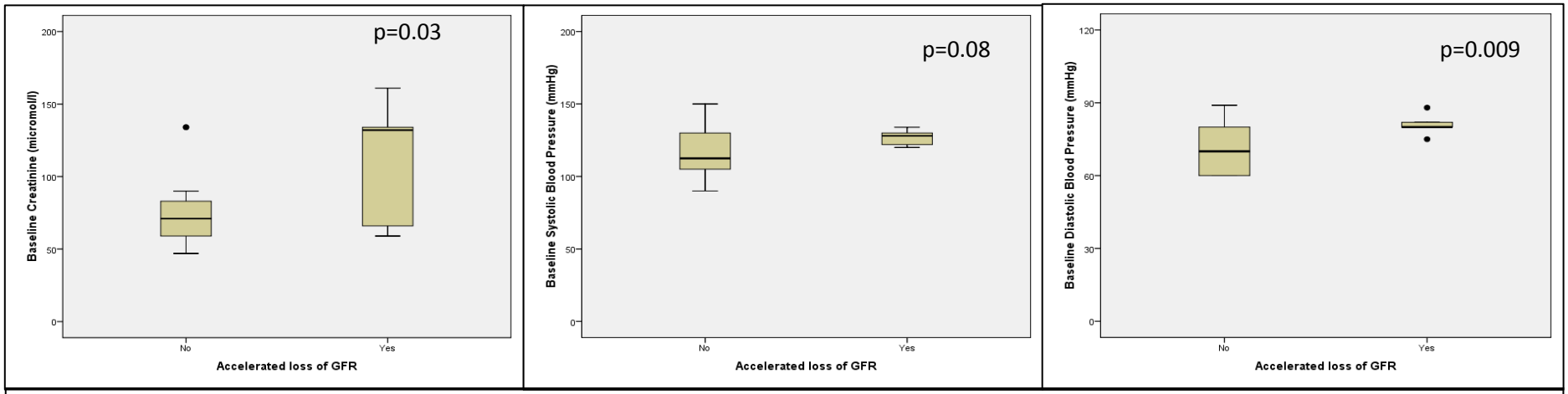


Figure 2.6.3.2. Pregnancy-related accelerated loss of renal function is associated with increased blood pressure and serum creatinine prior to conception

ROC analysis of baseline diastolic blood pressure identified 75mmHg as the optimum predictive cut-off as a predictor of PALRF giving sensitivity of 100% and specificity 62% (figure 2.6.3.3).

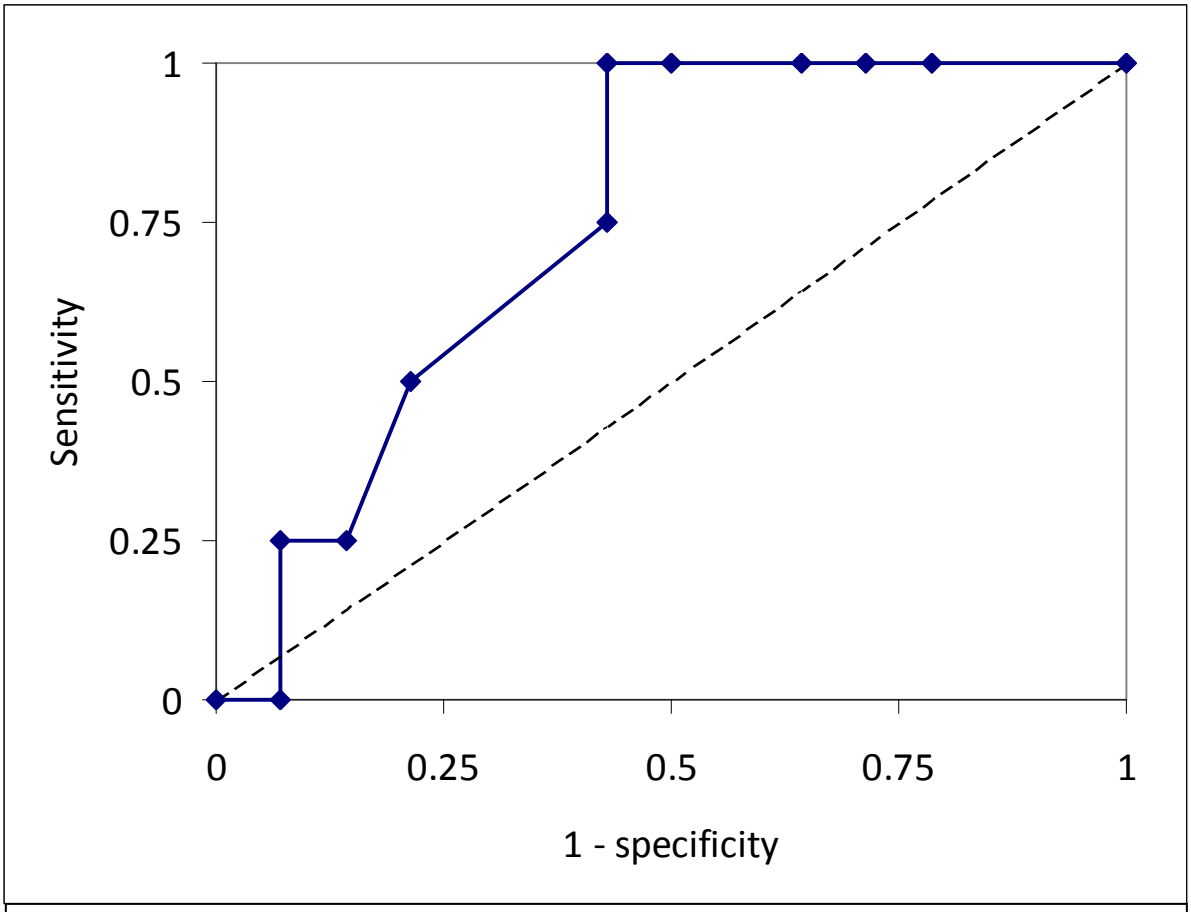


Figure 2.6.3.3 Receiver operating curve of diastolic blood pressure as predictor of PALRF. AUC=0.75, p=0.053

ROC analysis of baseline creatinine indentified 111 µmol/l as the optimum predictive cut-off as a predictor of PALRF giving sensitivity of 60% and specificity 89% however there was no clear threshold identified (AUC 0.69, p=0.21, figure 2.6.3.4).

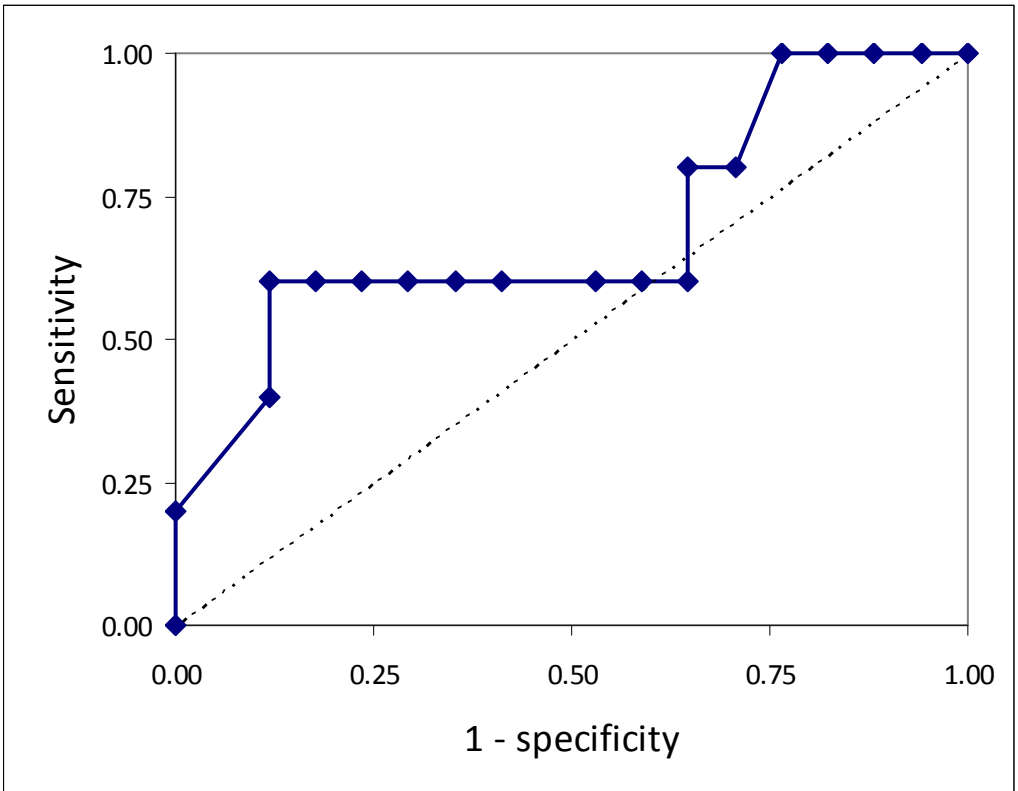


Figure 2.6.3.4. Receiver operating curve of baseline serum creatinine as predictor of PALRF. AUC 0.69, p=0.21

2.6.3 Discussion

These results support previous case series in identifying preconception blood pressure and renal function as key predictors of pregnancy-related accelerated loss of renal function (23,24,52). Proteinuria was not found to be predictive of accelerated decline in renal function. Interestingly, rate of renal decline prior to conception was not associated with loss of renal function above that expected by the natural history of their underlying disease.

Preconception counselling women with CKD can optimise pregnancy outcomes by making necessary adjustments to medication and outlining the maternal and fetal risks associated with a pregnancy. Intuitively, women with advanced renal disease are more likely to face risks to their health than those with mild disease. The prognosis for women with progressive but early renal disease has been less clear. Our results suggest that, for example, a woman with IgA nephropathy, heavy proteinuria and a decline in estimated GFR of 5ml/min/year, but stable blood pressure and an estimated GFR of 55ml/min is unlikely to suffer PALRF and, with close monitoring, may expect to complete pregnancy with little impact on the natural history of her renal disease. The alternative option of waiting until she reaches end stage renal disease and has stable functioning renal transplant may not occur for a decade or more, and therefore she could be advised to consider pregnancy now rather than later.

Although proteinuria was not found to be predictive of PALRF, it is important to remain aware that proteinuria may confer other risks to the mother, most notably venous thromboembolism (114).

2.7 Clinical and demographic factors predictive of adverse fetal outcomes: the independent impact of proteinuria

2.7.1 Introduction

Proteinuria during pregnancy may be associated with preterm delivery and low birth weight (chapter 2.5) and increased rates of fetal loss are noted in series of nephrotic syndrome in pregnancy (91,92). There is conflicting evidence for clinicians to base their advice to women with proteinuric CKD who are contemplating pregnancy on the possible fetal outcomes (see chapter 1.4.4).

This analysis aims to identify the independent impact of proteinuria on fetal outcomes.

2.7.2 Methods

Data were obtained from the UK-CORD database. Patients with quantification of proteinuria between 6 months preconception and 12 weeks gestation were included in the study. Where multiple values were available within this period, the peak proteinuria was used as baseline.

Preterm delivery was defined as <37 weeks and low birth weight as <2.5kg. Fetal loss included miscarriage, stillbirth and neonatal death.

Outcomes were compared by independent samples t-test for continuous variables and Fisher's Exact test for categorical variables. Backwards stepwise logistic regression analysis was used to assess independence of proteinuria as a predictor of adverse outcomes.

To assess the clinical utility of proteinuria as a predictor of outcomes receiver operating curve (ROC) analysis was performed (see chapter 2.4.2.3).

2.7.3 Results

Data were available for 59 pregnancies. Maternal characteristics are shown in table 2.7.3.1.

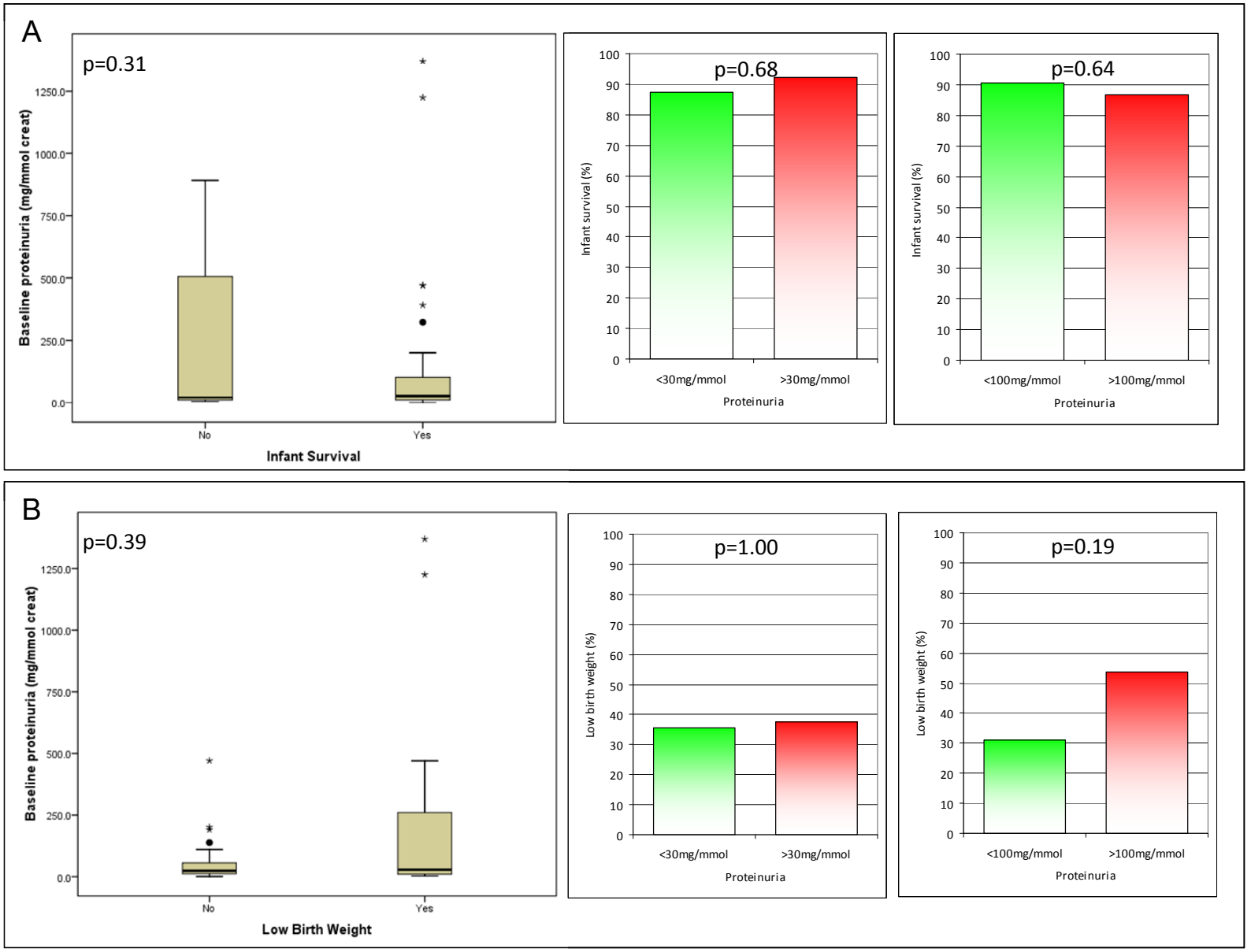
Maternal characteristics	
n	59
Maternal age (years)	31.5 ± 4.5
Renal transplant	2 (3.4%)
Diabetes mellitus	5 (8.5%)
Recurrent urinary tract infection	5 (8.5%)
Baseline serum creatinine (µmol/l)	87.3 ± 35.4
Serum creatinine >110µmol/l	9 (15.3%)
Baseline proteinuria (mg/mmol creatinine) (Median (IQR))	24.1 (77.0)
Proteinuria >30mg/mmol creatinine	26 (44%)
Proteinuria >100mg/mmol creatinine	15 (25%)
Treated hypertension	22 (37%)
Baseline systolic blood pressure (mmHg)	127.3 ± 17.2
Systolic blood pressure >140mmHg	13 (22%)
Baseline diastolic blood pressure (mmHg)	80.8 ± 11.8
Diastolic blood pressure >90mmHg	12 (20%)
Table 2.7.3.1. Maternal characteristics of patients included in this analysis. Values are presented as mean±SD unless stated.	

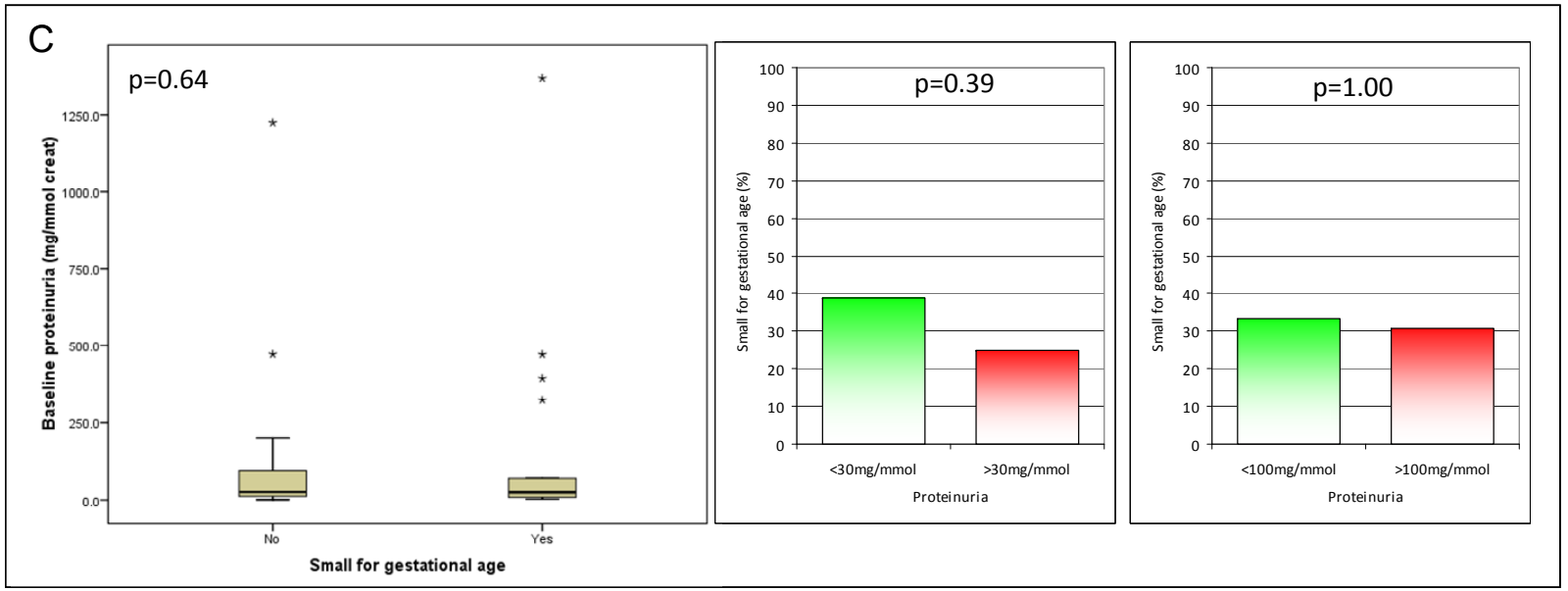
Pregnancy outcomes are summarised in table 2.7.3.2. Three pregnancies ended in miscarriage and 1 in neonatal death. Of the 55 remaining pregnancies, infant outcome was unknown for one pregnancy and 2 infants had persistent functional impairment.

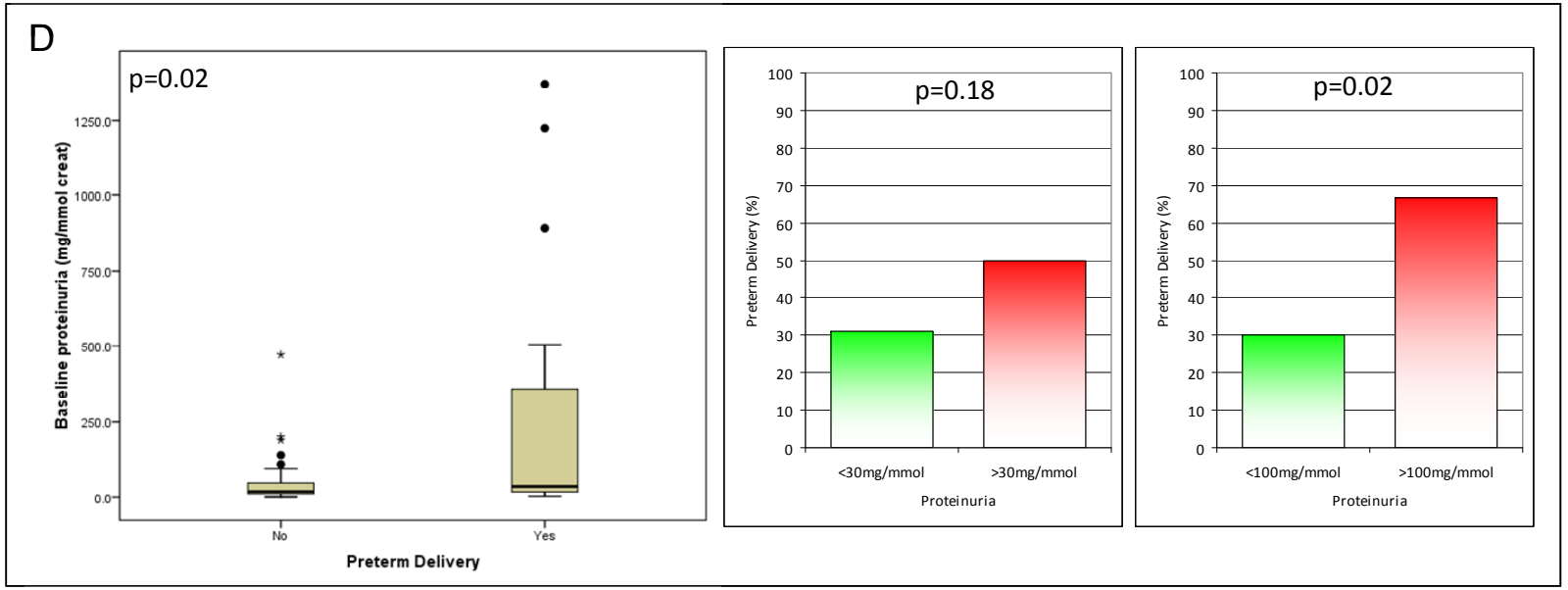
Pregnancy outcome	
n	59
Fetal loss	4 (6%)
Gestational age at delivery (weeks)	36.4 ± 5.6
Preterm delivery	24 (41%)
Birth weight (kg)	2.79 ± 0.84
Low birth weight	20 (34%)
Table 2.7.3.2. Outcomes from pregnancies included in this analysis. Values are presented as mean±SD unless stated.	

Pregnancy outcomes were compared to baseline proteinuria. Baseline proteinuria excretion was higher in pregnancies that ended in preterm delivery but there was no association with fetal loss or low birth weight (figure 2.7.3.1). Pregnancies in mothers with baseline proteinuria >100mg/mmol creatinine were more likely to end in preterm delivery than those with lesser degrees of proteinuria.

Figure 2.7.3.1. Baseline proteinuria and infant outcomes. Baseline proteinuria was not different in pregnancies ending with unsuccessful outcomes (A), low birth weight infants (B), or small for gestational age infants (C). Preterm deliveries were more common in women with baseline proteinuria >100mg/mmol creatinine (D).


A
p=0.31
Baseline proteinuria (mg/mmol creat)
1250.0
1000.0
750.0
500.0
250.0
0.0
No
Yes
Infant Survival
p=0.68
Infant survival (%)
100
90
80
70
60
50
40
30
20
10
0
<30mg/mmol
>30mg/mmol
Proteinuria
p=0.64
Infant survival (%)
<100mg/mmol
>100mg/mmol
Proteinuria
B
p=0.39
Baseline proteinuria (mg/mmol creat)
Low Birth Weight
p=1.00
Low birth weight (%)
p=0.19
Low birth weight (%)


C
p=0.64
Baseline proteinuria (mg/mmol creat)
1250.0
1000.0
750.0
500.0
250.0
0.0
No
Yes
Small for gestational age
p=0.39
Small for gestational age (%)
100
90
80
70
60
50
40
30
20
10
0
<30mg/mmol
>30mg/mmol
Proteinuria
p=1.00
Small for gestational age (%)
<100mg/mmol
>100mg/mmol
Proteinuria


D
p=0.02
Baseline proteinuria (mg/mmol creat)
1250.0
1000.0
750.0
500.0
250.0
0.0
No
Yes
Preterm Delivery
p=0.18
Preterm Delivery (%)
100
90
80
70
60
50
40
30
20
10
0
<30mg/mmol
>30mg/mmol
Proteinuria
p=0.02
Preterm Delivery (%)
<100mg/mmol
>100mg/mmol
Proteinuria

Univariate analysis identified an association between preterm delivery and creatinine, blood pressure and proteinuria but not maternal age or antihypertensive therapy. Backward stepwise logistic regression was used to assess the independence of proteinuria as a risk factor for preterm delivery. Proteinuria values underwent logarithmic transformation prior to analysis. Maternal age, baseline serum creatinine, systolic and diastolic blood pressure and antihypertensive treatment were included as covariates. Regression analysis identified only baseline serum creatinine (p=0.009) and proteinuria (p=0.012) as independent predictors of preterm delivery. Proteinuria was not associated with an increased incidence of Caeasrean section (figure 2.7.3.2).

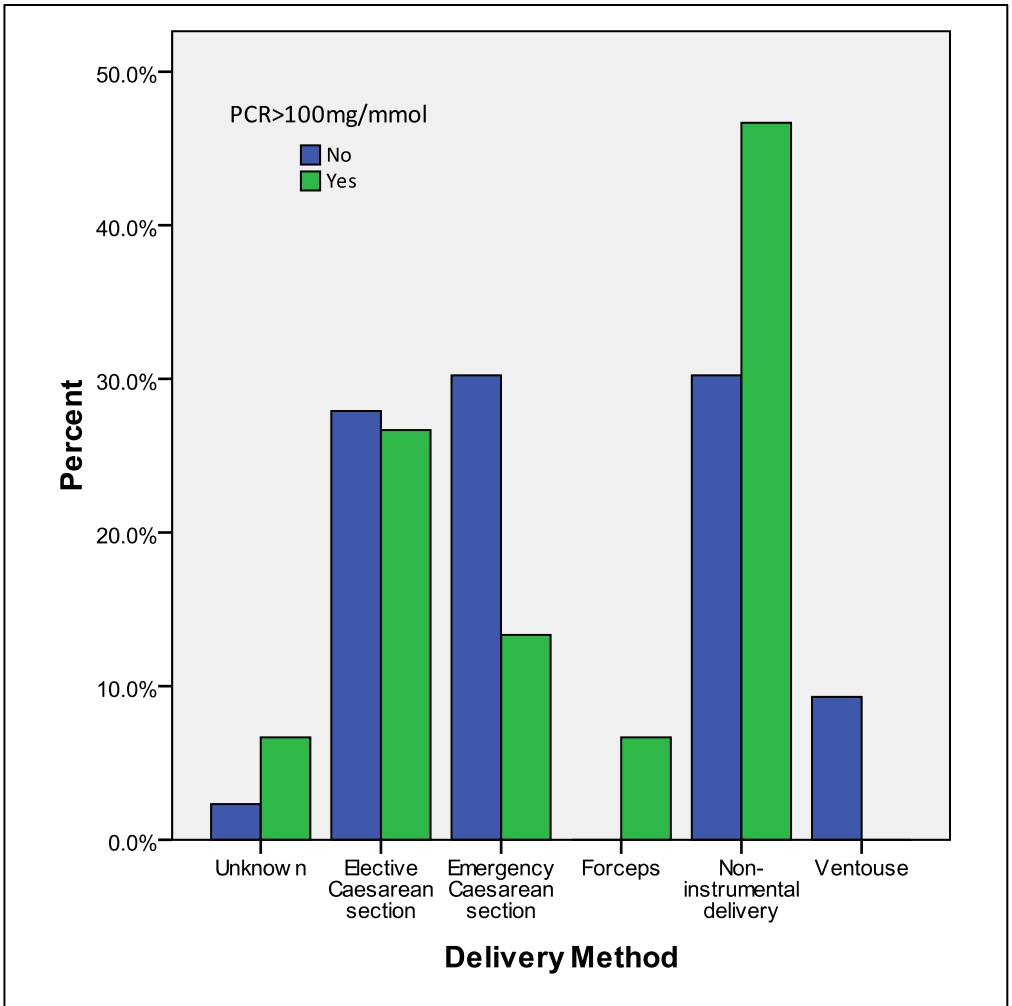


Figure 2.7.3.2 Delivery method according to baseline proteinuria (< or > 100mg/mmol creatinine)

As a clinically relevant independent predictive marker of preterm delivery, proteinuria performed poorly. Receiver operating curve analysis revealed an area under the curve of 0.66 (95% confidence interval 0.51-0.80, p=0.04) with no clear “shoulder” (figure 2.7.3.3).

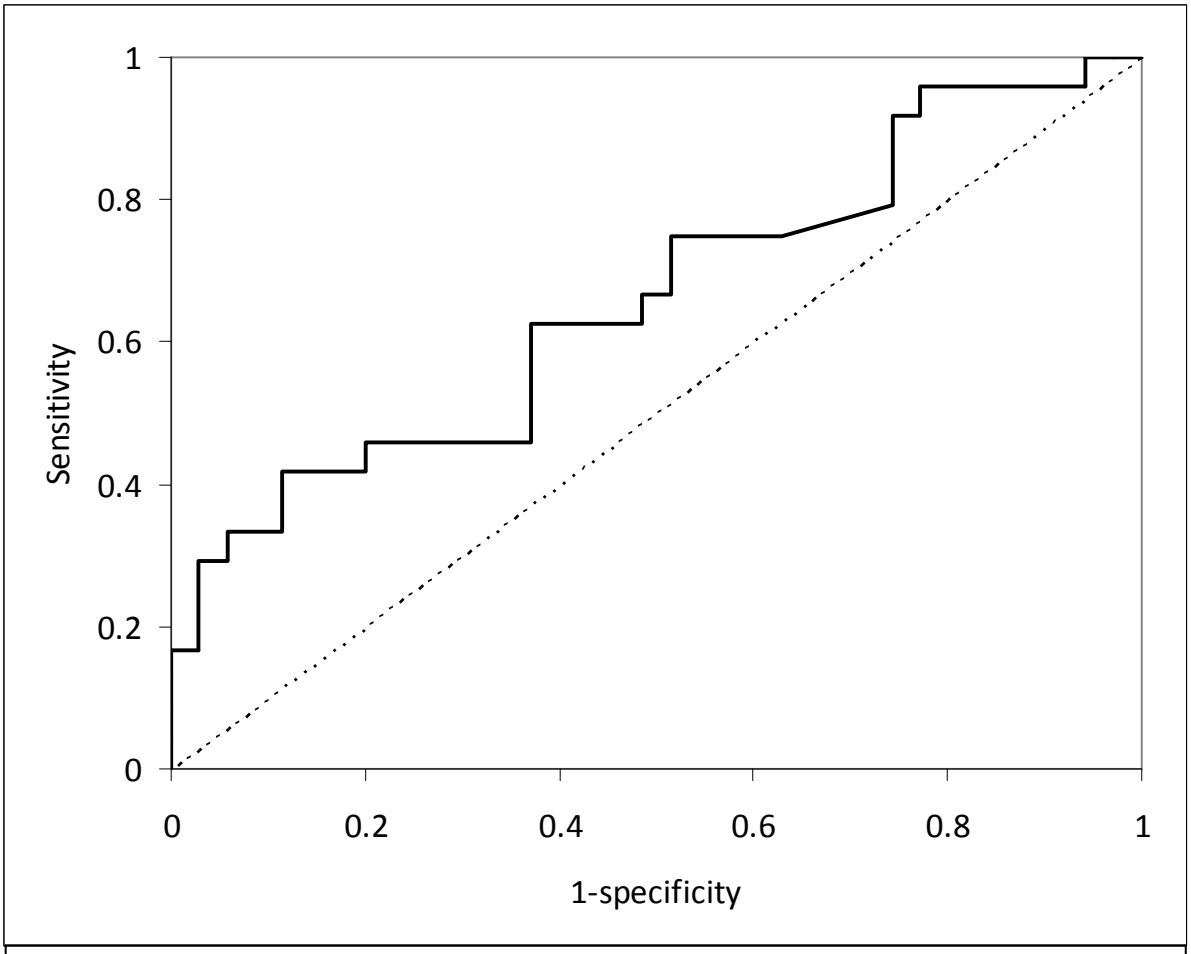


Figure 2.7.3.2. Receiver operating curve for baseline proteinuria in predicting preterm delivery. AUC=0.66 (0.51-0.80), p=0.04

2.7.4 Discussion

Baseline proteinuria in women with CKD is independently associated with preterm delivery but not fetal loss or low birth weight. Although no clear “cut off” of proteinuria was associated with an abrupt increase in risk of preterm delivery, pregnancies in women with a protein:creatinine ratio >100mg/mmol creatinine were 2.1 times more likely to end in preterm delivery than those <100mg/mmol creatinine.

This observational study does not permit causality to be assigned to proteinuria in this respect. Pregnancies complicated by proteinuria may have been led to physician-led early delivery due to concerns about impending pre-eclampsia, reduced fetal growth or complications related to heavy urinary protein loss. Heavy proteinuria is associated with an increased risk of thromboembolism (93). Baseline proteinuria was not associated with an increased incidence of Caesarean section.

Preconception counselling for women with CKD should nevertheless include the observation that preterm delivery is more likely for proteinuric patients than for non-proteinuric patients, even for those with preserved renal function.

2.8 Nephrotic syndrome and pregnancy: fetal outcomes

2.8.1 Introduction

As discussed in chapter 1.4.4.1, historical series reporting maternal and fetal outcomes for pregnancies complicated by nephrotic syndrome have been very poor. Fetal mortality rates of 40% were observed in the largest reported series (92). Maternal risks related to nephrotic syndrome in pregnancy, particularly thromboembolism (93,95), are well described, albeit not quantified (94).

Anecdotal reports from UK-CORD members suggested that fetal outcomes for patients with nephrotic syndrome in pregnancy were far better than those previously reported. In this analysis, fetal outcomes from pregnancies in the UK-CORD database in mothers with nephrotic syndrome are compared to non-nephrotic pregnancies.

2.8.2 Methods

Data were extracted from the UK-CORD database on all pregnancies with data relating to proteinuria during pregnancy. The incidence of preterm delivery (<37 weeks gestation), low birth weight (<2.5kg) and adverse fetal outcome (termination, miscarriage, stillbirth, neonatal death and persistent functional impairment) were compared in patients with nephrotic syndrome, heavy proteinuria and low or no proteinuria.

Definitions of nephrotic syndrome in pregnancy are controversial since (a) physiological changes lead to a doubling of urine protein excretion (9), (b) relative dilution of serum results in a drop in serum albumin and (c) peripheral oedema is common due to vasorelaxation and the compressive effects of the gravid uterus in normal pregnancy. In this analysis nephrotic syndrome was defined as serum albumin <30g/l and nephritic range proteinuria (>3g/day or protein:creatinine ratio > 300mg/mmol creatinine or albumin:creatinine ratio > 300mg/mmol creatinine). Heavy proteinuria was defined as nephrotic range proteinuria with preserved serum albumin; low proteinuria as other pregnancies.

Baseline maternal characteristics were compared between clinical groups by one-way analysis of variance with Bonferroni post-hoc analysis for continuous variables and chi-squared test for categorical variables. Fetal outcomes were compared between groups using multivariate analysis to identify a possible independent effect of nephrotic syndrome on outcome.

2.8.3 Results

Data were available for 239 pregnancies of which 208 had low proteinuria (LP), 14 had heavy proteinuria (HP) and 17 had nephrotic syndrome (NS). Patients with heavy proteinuria and nephrotic syndrome were more likely to have hypertension, glomerular disease and renal dysfunction (table 2.8.3.1). As expected, proteinuria was greater in HP and NS groups and serum albumin lower in the NS group. Superimposed re-eclampsia was diagnosed in 10 patients (4.1%) with a significantly increased incidence amongst patients with HP (p=0.003).

		LP	**HP**	**NS**	p value
n		208	14	17	
Age		30.3 (5.8)	32.4 (5.0)	29.6 (4.5)	0.359
Gravidity	Median (IQR)	2 (2)	2 (2)	2 (2)	0.753
Glomerular disease	n (%)	72 (34.6)	7 (50.0)	11 (64.7)	**0.03**
Treated hypertension	n (%)	29 (13.9)	3 (21.4)	7 (41.2)	**0.012**
Peak SBP (mmHg)		133 (20)	134 (11)	143 (16)	0.162
Peak DBP (mmHg)		82 (12)	86 (9)	90 (12)	**0.016**
Peak SCr (µmol/l)	Median (IQR)	71 (103)	93.5 (103)	95 (56)	**<0.001**
Peak PCR (mg/mmol creat)	Median (IQR)	28 (86)	406 (195)	733 (768)	**<0.001**
Minimum serum albumin (g/dl)		33 (6)	31 (3)	24 (5)	**<0.001**
Superimposed pre-eclampsia	n (%)	6 (2.9)	3 (21)	1 (5.9)	**0.003**

Table 2.8.3.1. Characteristics of study population. Values mean (SD) unless stated. SBP, systolic blood pressure; DBP, diastolic blood pressure; SCr, serum creatinine

Fetal outcomes were compared between the three groups. In contrast to the previous published case series (91,92), fetal survival was over 90% including pregnancies complicated by nephrotic syndrome. Low birth weight was more common in patients with heavy proteinuria or nephrotic syndrome and preterm delivery in those with nephrotic syndrome (figure 2.8.3.1).

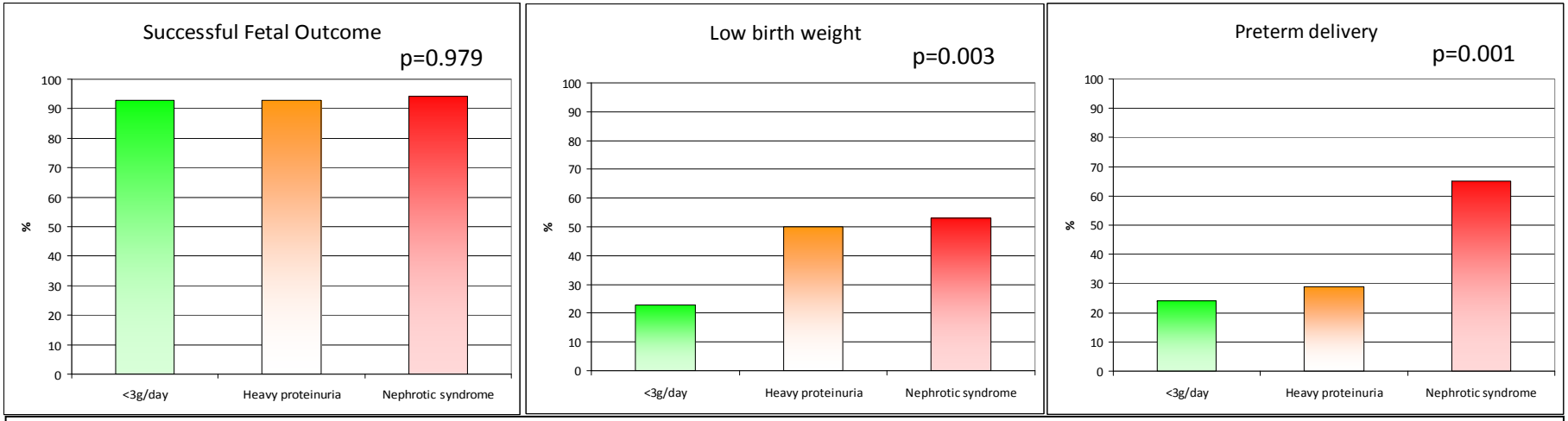


Figure 2.8.3.1. No difference in adverse fetal outcome was found between proteinuric groups. Low birth weight was associated with heavy proteinuria (n=14) and nephrotic syndrome (n=17) in univariate analysis as compared to those with low proteinuria (<3g/d, n= 208); preterm delivery was associated with nephrotic syndrome.

As shown in table 2.8.3.1, the clinical groups were not homogeneous at baseline. Multivariate analysis was performed to elucidate nephrotic syndrome as an independent risk factor for preterm delivery or low birth weight.

Using a model including maternal age, inverse of serum creatinine, systolic and diastolic blood pressure, antihypertensive treatment and the defined clinical groups as covariates, backward stepwise logistic regression did not identify nephrotic syndrome as an independent predictor of low birth weight (p=0.206). Serum creatinine (p=0.003) and diastolic blood pressure (0.003) were independent predictors of low birth weight.

A similar result was found for preterm delivery; nephrotic syndrome was not an independent predictor (p=0.20) whereas serum creatinine (p=0.002) and diastolic blood pressure (p=0.003) were.

Conversely, serum albumin concentration was independently associated with preterm delivery (p=0.018) but not low birth weight (p=0.167). A similar association was found for proteinuria after logarithmic transformation for preterm delivery (p=0.003) but not low birth weight (p=0.078). Serum albumin and proteinuria were significantly correlated with gestational age at delivery albeit with a poor co-efficient of correlation (respectively, Spearman's ρ=0.203, p=0.003; Spearman's ρ=-0.295, p<0.001; figure 2.8.3.2).

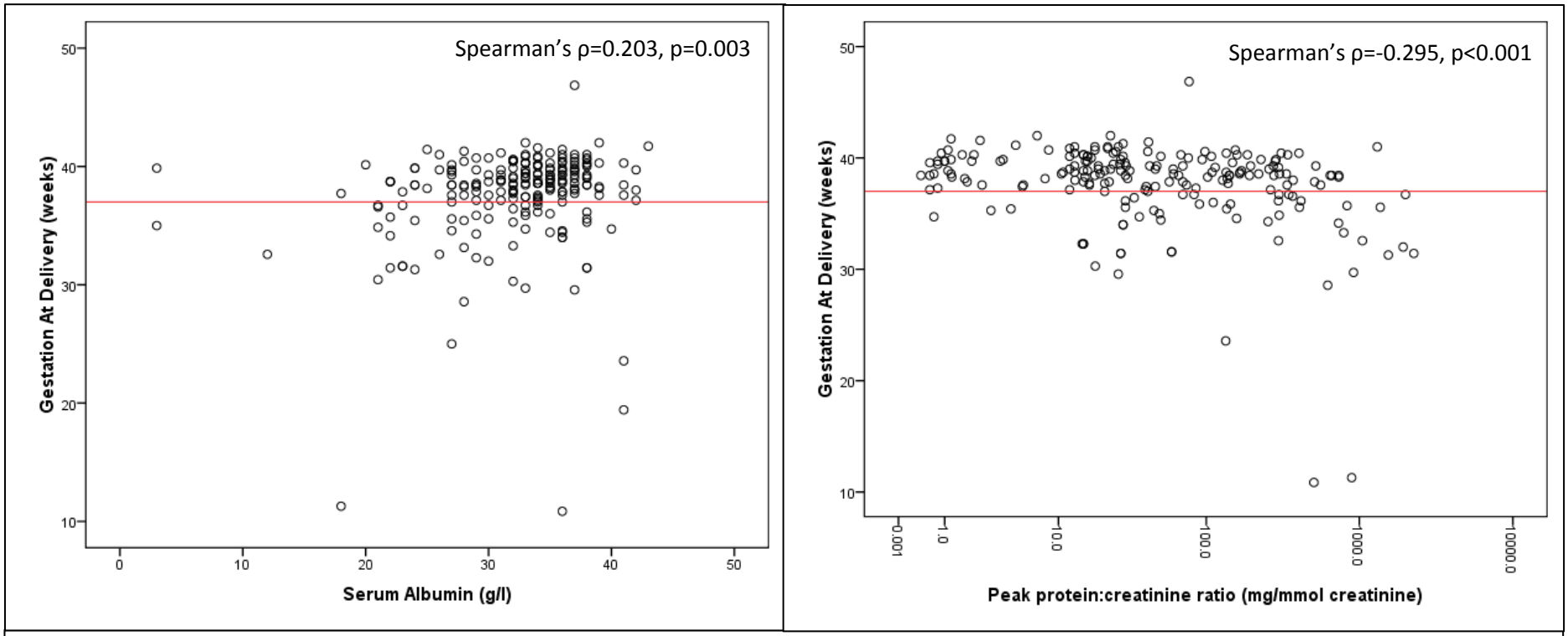


Figure 2.8.3.2. Correlation between nadir serum albumin and peak proteinuria with gestational age at delivery.

2.8.4 Discussion

In contrast to published historical series (91,92), our results, in a contemporary cohort, show that fetal outcomes are favourable in pregnancies complicated by nephrotic syndrome. The incidence of diagnosed pre-eclampsia was lower than anticipated for a cohort of patients with CKD and this may have contributed to the improved outcomes noted. Preterm delivery and low birth weight pregnancies were more common in patients with nephrotic syndrome or heavy proteinuria, however, these events were predominantly determined by baseline maternal renal function and diastolic blood pressure.

Although the development or presence of nephrotic syndrome (as defined in this study) appeared to confer no additional risk of adverse fetal outcomes over non-nephrotic pregnancies, both serum albumin and proteinuria were independently associated with preterm delivery.

It is feasible that the diagnostic criteria for nephrotic syndrome in pregnancy should be reviewed to account for the physiological changes discussed. Undoubtedly, pregnant women with heavy proteinuria are at risk of excess loss of vitamin D binding protein, antithrombin III, transferrin, immunoglobulins and intravascular fluid depletion leading to maternal malnutrition, thrombophilia and uteroplacental insufficiency (81). A proteinuric threshold beyond which maternal and fetal risks in pregnancy increase has not been identified in previous studies (86) and therefore the use of arbitrary thresholds to diagnose clinical syndromes in pregnancy in observational studies is likely to be challenging. Given that the potential risks of nephrotic syndrome remain significant in this study, it seems

prudent to continue to use established parameters to diagnose nephrotic syndrome in pregnancy.

Nevertheless, these results are reassuring for patients and clinicians faced with heavy proteinuria in pregnancy. In the absence of prognostic thresholds for serum albumin and proteinuria, close assessment of maternal nutrition, thromboembolic risk and fetal growth throughout pregnancy is required to optimise pregnancy outcomes.

2.9 Summary - Quantitative analysis of proteinuria in CKD and pregnancy

- Proteinuria can be quantified using the albumin:creatinine or protein:creatinine ratio as an alternative to a 24 hour collection In patients with CKD. Values correlate closely to 24 hour collection values. Statistically significant differences in clinical outcomes were well matched between thresholds of proteinuria identified using different methods.

- Women with CKD and abnormal quantities of proteinuria during pregnancy that failed to return to baseline postpartum had a significantly increased rate of decline in renal function from 6 months after pregnancy and were more likely to deliver low birth weight and preterm infants compared with women without proteinuria.

- Proteinuria prior to conception was not identified as a risk factor of pregnancy-associated accelerated loss of renal function. Baseline serum creatinine and blood pressure are more closely associated with adverse maternal outcomes.

- Baseline proteinuria at conception was identified to be a predictor of preterm delivery, independent of serum creatinine, blood pressure and maternal age. Although an increased rate of preterm delivery was identified in women with baseline proteinuria >100mg/mmol creatinine, ROC analysis suggested that the risk of preterm delivery increased continuously with increasing proteinuria without a clear threshold of risk.

- In contrast to historical reports, an increased risk of adverse fetal outcome was not identified in women developing nephrotic syndrome during pregnancy, independent of baseline maternal renal function and blood pressure.

3 Is there a unique urine protein fingerprint in pre-eclampsia and is this predictive of outcomes?

3.1 Introduction

3.1.1 Definition of pre-eclampsia

Pre-eclampsia (PE) is a common human-specific disease of pregnancy characterised by de-novo and progressive hypertension and proteinuria after 20 weeks gestation. Estimations of the incidence of PE are complicated by variability in definitions used (115), however, based on the International Society for the Study of Hypertension in Pregnancy (ISSHP) criteria (85), PE is reported in 2–8% of pregnancies (116). Nationally and globally, PE is a leading cause of maternal morbidity including multi-organ failure and eclampsia. Eclampsia comes from the Greek word for lightning (εκ-λαμψια) and is a life-threatening complication characterised by grand-mal seizures. Fetal complications include growth restriction, preterm delivery, stillbirth and neonatal death (18).

The diagnosis of PE is defined by international classifications (table 3.1.1.1). PE appears to be a distinct clinical entity from pregnancy-induced (or gestational) hypertension (PIH); patients developing PE have different and more severe detrimental maternal and fetal outcomes, develop specific clinical syndromes such as DIC and eclampsia (seizures), have different renal histology on biopsy and, as discussed below, develop specific patterns of serum angiogenic factor production compared to PIH. Debate remains on how best to define complicated non-proteinuric gestational hypertension and to the importance of defining "severe" PE according to gestation at onset (117,118).

Australasian Society for the Study of Hypertension in Pregnancy (ASSHP, 2000) (119)	De novo hypertension (systolic blood pressure >140mmHg, diastolic blood pressure >90mmHg) after 20 weeks' gestation plus one of the following: • Proteinuria (>300mg/d or protein:creatinine ratio >30mg/mmol) • Renal insufficiency (serum creatinine > 90µmol/l or oliguria) • Liver disease (raised transaminases or severe right upper quadrant pain) • Neurological disease (convulsions, hyperreflexia, visual disturbance) • Haematological dysfunction (thrombocytopenia, haemolysis, disseminated intravascular coagulation (DIC)) • Fetal growth restriction Followed by normalisation within 3 months
National High Blood Pressure Education Programme (NHBPEP, 2000) (30)	De novo hypertension (systolic blood pressure >140mmHg, diastolic blood pressure >90mmHg) after 20 weeks' gestation plus proteinuria (greater than 300mg/d). "In the absence of proteinuria the disease is highly suspect when increased blood pressure is accompanied by: • Headache • Blurred vision • Abdominal pain • Low platelets • Abnormal liver enzymes."
International Society for the Study of Hypertension in Pregnancy (ISSHP, 2001) (85)	A research definition was taken as that of the NHBPEP with the addition of a spot urine protein:creatinine ratio > 30mg/mmol deemed as adequate. To increase sensitivity a clinical definition based on the presence of additional factors as above should be used.
Table 3.1.1.1 Defining pre-eclampsia	

PE can occur following existing maternal disease such as chronic hypertension or CKD. Such patients can not fulfil the criteria above and yet they are at increased risk of typical complications of PE. The diagnosis of 'superimposed pre-eclampsia' is less well defined. The NHBPEP suggest that superimposed PE is highly likely with:

- New onset proteinuria (>300mg/d) in women with hypertension but no proteinuria early in pregnancy
- Sudden increase in proteinuria

- Sudden increase in previously well-controlled blood pressure
- Thrombocytopenia (<100 cells/ml)
- Or an increase in transaminases in women with hypertension and proteinuria prior to 20 weeks' gestation (120).

3.1.2 Epidemiology of pre-eclampsia

PE affects between 2 and 8% of pregnancies worldwide, equating to over 6.5 million pregnancies per year. Hypertensive disorders of pregnancy are directly implicated in an estimated 60000 maternal deaths per year. In the United Kingdom, PE complicates approximately 5% of pregnancies; this equates to about 32000 pregnancies annually. PE led to the death of 18 mothers in the United Kingdom between 2002 and 2005 (18), and 19 mothers between 2006 and 2008 (121). PE was implicated in 135 stillbirths in 2006 (122).

The incidence of PE is increased in the following groups:

- Mothers < 18 years old and >35 years old
- Obese
- Nulliparous mothers
- Personal or family history of pre-eclampsia
- Multiple pregnancies
- Chronic hypertension
- Chronic kidney disease
- Pre-existing or gestational diabetes mellitus
- Systemic lupus erythematosus or other connective tissue diseases
- Sickle cell disease
- New sexual partner

3.1.3 Pathophysiology of pre-eclampsia

3.1.3.1 Proposed aetiology of pre-eclampsia

Reports of pregnancy-related seizure disorders have been documented for over a thousand years, and hypertension and proteinuria associated with pregnancy have been recognised for over 160 years (123). Nevertheless, diagnosing PE remains descriptive and numerous theories regarding the aetiology of PE continue to be propagated (figure 3.1.3.1).

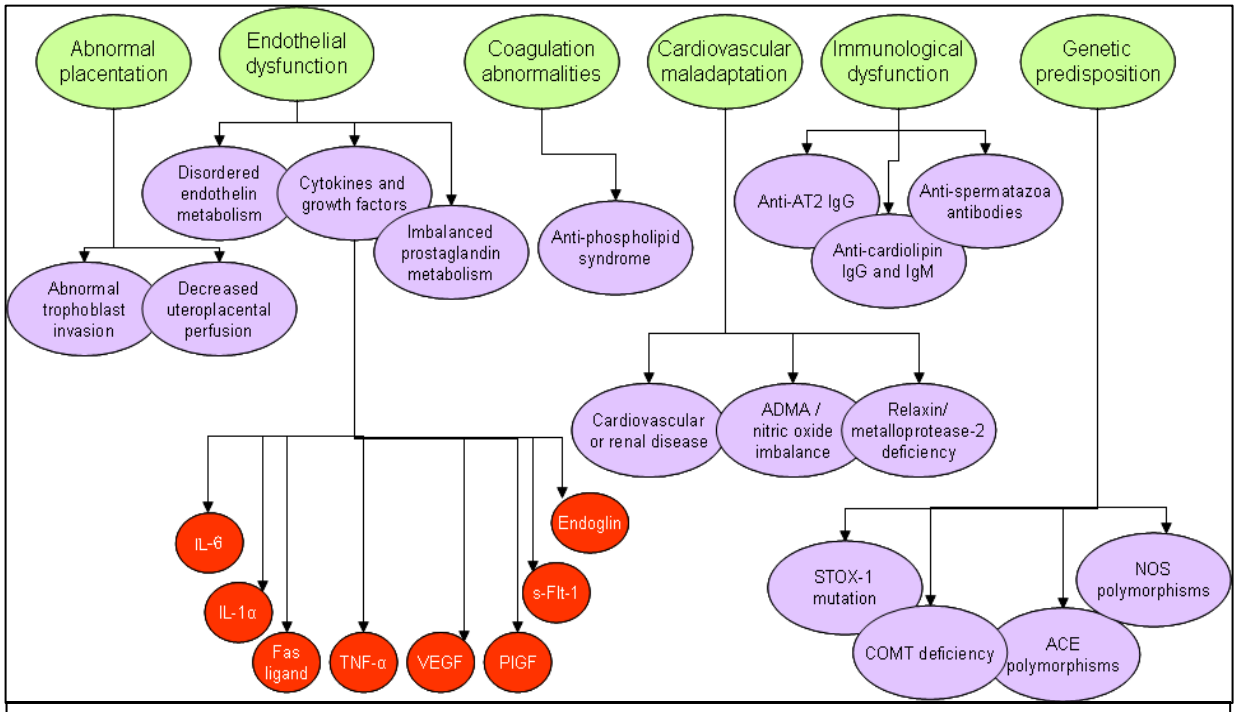


Figure 3.1.3.1. Proposed theories of the pathogenesis of pre-eclampsia.

Clinical observations support the uteroplacental unit as the root of PE (124). PE only develops in pregnancy (or molar pregnancies) and the diagnostic features of hypertension and proteinuria resolve rapidly following delivery of the infant and placenta.

A two stage pathophysiological process has been suggested. Insufficient invasion of fetal cytotrophoblasts into the maternal fetal bed leads to poor remodelling of maternal spiral arteries into low resistance vessels, a process normally complete by 18 weeks gestation (125). The resultant placental ischaemia-reperfusion incites placental oxidative and endoplasmic reticulum stress.

In the second, clinical stage, ongoing cellular stress causes release of pro-inflammatory and anti-angiogenic factors into the maternal circulation leading to maternal systemic inflammatory response, endothelial dysfunction, coagulopathy and overt clinical disease (figure 3.1.3.2) (126).

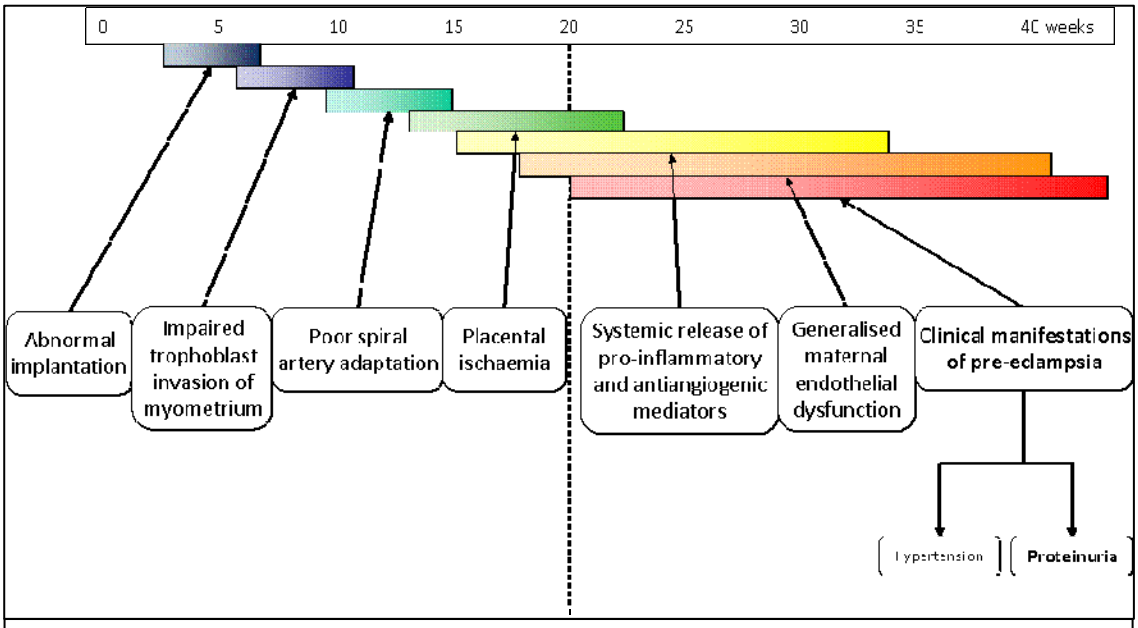


Figure 3.1.3.2 Proposed two-stage pathophysiology of pre-eclampsia. Placental ischaemia initiates maternal systemic endothelial dysfunction through the release of pro-inflammatory and antiangiogenic mediators.

3.1.3.2 Antiangiogenic factors

Since 2004, much research has been directed to the role of circulating angiogenic factors in the pathophysiology of PE. In vitro evidence from animal studies was

replicated in humans with the finding that increased levels of soluble fms-like tyrosine kinase 1 (s-Flt-1, also known as soluble vascular endothelial growth factor receptor 1, sVEGFR-1) and decreased levels of placental growth factor (PlGF) predict the subsequent development of PE (126). s-Flt-1 and PlGF are both released from the placenta and modulate the effects of vascular endothelial growth factor (VEGF) (figure 3.1.3.3).

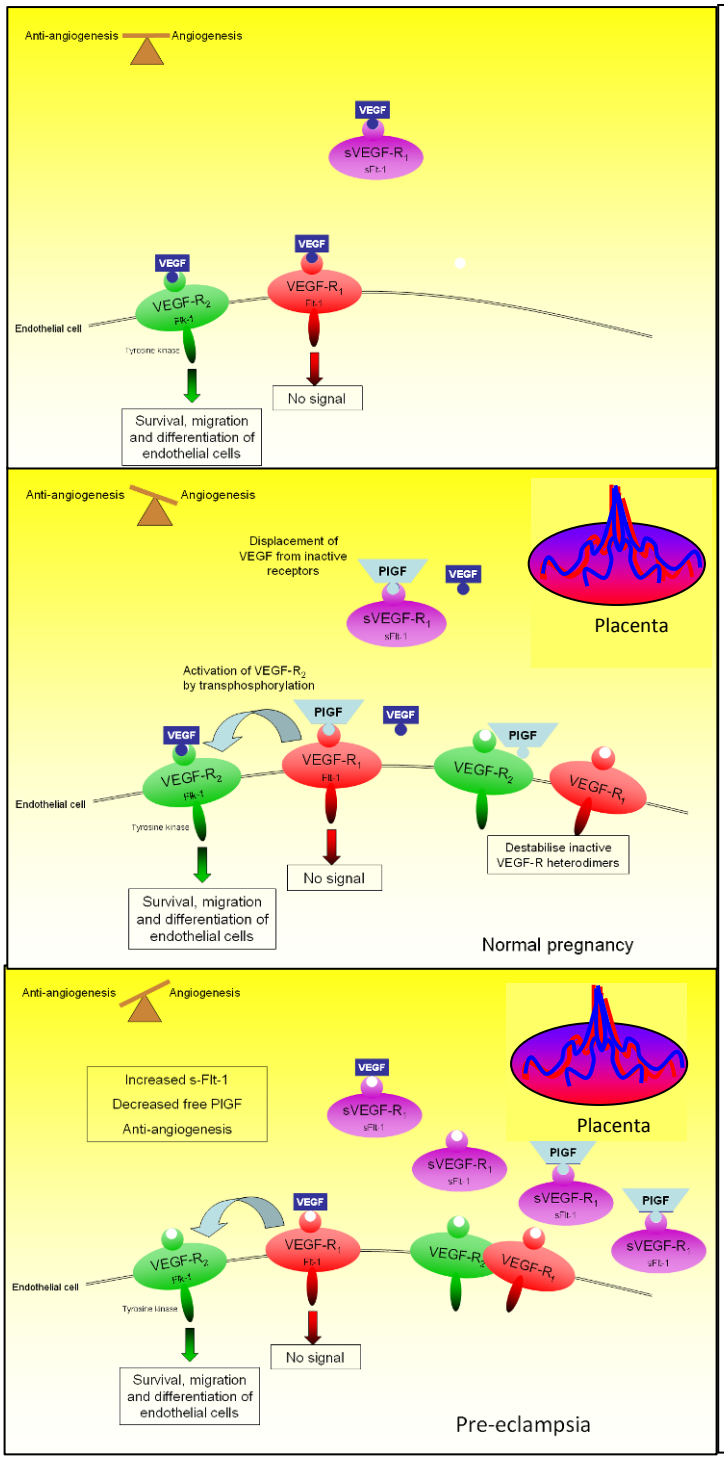


Figure 3.1.3.3. Angiogenic factors and pre-eclampsia.

In health (top), vascular endothelium requires the VEGF binding to VEGF-R_2 to maintain survival and integrity.

In normal pregnancy (middle), the placenta release placental growth factor (PlGF) which promotes angiogenesis in three ways: (a) competitive binding with VEGF on inactive receptors to increase free VEGF, (b) up-regulation of VEGF-R_2 by trans-phosphorylation and (c) destabilising inactive VEGF-R heterodimers.

In pre-eclampsia (bottom), excess placental secretion of sFlt-1, an inactive soluble VEGF receptor, reduces free PlGF levels and promotes an anti-angiogenic status.

VEGF is essential for survival during development and, on binding to VEGFR-2 (also known as Flk-1), leads to survival, migration and differentiation of endothelial cells. VEGF can bind to an inactive membrane receptor, VEGFR-1 (or Flt-1) and the soluble form as above.

Supportive evidence that angiogenic factors mediate the pathogenesis of pre-eclampsia is provided by site and time-specific VEGF-knockout mice which exhibit glomerular endotheliolsis and a PE-like illness (127). VEGF antagonists in clinical use (such as bevacizumab for malignant disease) have been associated with hypertension and proteinuria. Renal biopsies perfomed on such patients reveal changes almost identical to those seen in PE.

PlGF is essential for successful placentation and acts in 3 ways to augment the response to VEGF; (a) activation of VEGFR-2 by transphosphorylation from VEGFR-1, (b) displacement of VEGF from inactive binding sites on VEGFR-1 and s-Flt-1 and (c) destabilisation of inactive VEGFR-1/VEGFR-2 heterodimers (128). In normal pregnancy plasma PlGF concentration climb from conception to approximately 30 weeks gestation then fall. In pregnancy associated with PE there are significantly lower levels of plasma PlGF.

s-Flt-1 is an inactive soluble form of VEGFR-1 which is released in steadily increasing concentrations throughout pregnancy. It binds circulating VEGF thus damping its biological effect and has a physiological role in the avascular development of the cornea. In pregnancy associated with PE, plasma s-Flt-1 levels are significantly higher than in normal pregnancy (128). The stimulus for increased secretion of s-Flt-1 in PE is not known but, in vitro, s-Flt-1 secretion could be stimulated by hypoxia (129).

Endoglin (also known as CD105) is a functional co-receptor for transforming growth factor-β1 (TGF-β1). Normally a transmembrane protein expressed in endothelial cells and syncytiotrophoblasts, endoglin may be shed into the

circulation. Endoglin is anti-angiogenic by (a) modulating downstream signalling of TGF-β1/TGFβ-receptor binding between a pro-angiogenic ALK-1 pathway to an anti-angiogenic ALK-5 pathway and (b) circulating endoglin reduces the bioavailability of TGF-β1. In normal pregnancy, soluble endoglin levels in plasma are steady until approximately 30 weeks gestation after which they rise. In pregnancy associated with PE, this rise occurs earlier and is more exaggerated (128). The stimulus for increased shedding or secretion of endoglin in PE is not known and not induced by hypoxia (130).

Therefore, although it remains unclear what initiates abnormal placentation in PE, many clinical features can, at least in part, be explained by variations in angiogenic modulators.

3.1.3.3 Nitric oxide and pre-eclampsia

Pregnancy-induced decrease in systemic vascular resistance is a physiological response to increase the circulating volume and promote uteroplacental perfusion (see chapter 1.1.1). In addition to the morphological changes seen in (glomerular) endothelium in PE (figure 3.1.3.4) there are functional changes characterised by impaired vascular smooth muscle cell relaxation (131,132).

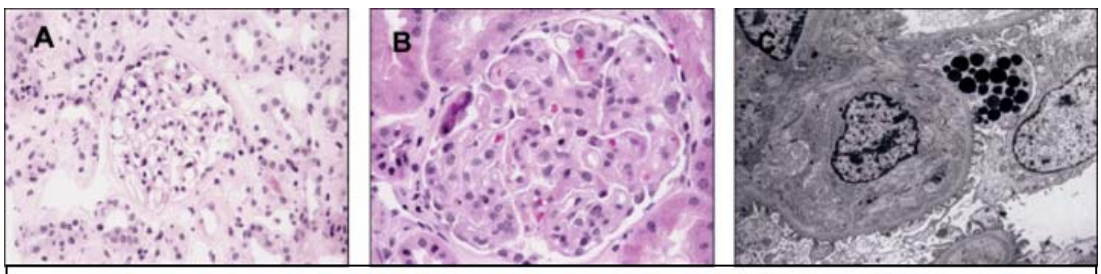


Figure 3.1.3.4. Histological changes in pre-eclampsia. A Normal glomerulus. B Glomerular endotheliosis in pre-eclampsia. C Electron micrograph of glomerular endotheliosis. Images from Karumanchi et al, 2005 (132)

Whilst endothelial-derived prostaglandins (prostacyclin) have not been shown to have a significant role in the haemodynamic adaptations to pregnancy (133), a role for nitric oxide (NO) is suggested by the observations that urinary levels of cyclic-GMP (a NO second messenger), nitrates and nitrites are increased during pregnancy in animal models.

In a rat model of uterine hypoperfusion, dietary supplementation with L-arginine, a NO donor, abrogated the associated hypertension(134). In human studies, women with PE had impaired vasodilation and raised serum asymmetric dimethylarginine (ADMA) levels, an endogenous inhibitor of endothelial NO synthase (135).

Impaired NO synthesis is also suggested as a consequence of impaired relaxin bioactivity in pregnancy associated with PE. It is proposed that in normal pregnancy, relaxin is released from ovaries in response to human chorionic gonadotrophin release from the placenta. Endothelial cells on small arteries and arterioles possess relaxin receptors which, via metalloproteinase-2 and gelatinase, activate and upregulate endothelin and endothelin-receptor ET_B. NO synthase III is promoted by ET_B leading to NO and vasodilation. How this pathway is altered in PE is unclear at present but evidence exists to suggest imbalance of metalloproteinase-2 and gelatinase activity (136)(137).

3.1.4 Management of pre-eclampsia

Definitive treatment of pre-eclampsia requires delivery of the fetus and placenta. Close monitoring by specialists during pregnancy allows the timing of delivery to be optimised for both maternal and fetal benefit. Progression of pre-eclampsia to eclampsia can be reduced with magnesium sulphate therapy (137). For women at high risk of developing pre-eclampsia numerous therapies to prevent progression have been trialled with limited success. Dietary supplementation with antioxidants, garlic or vitamin B6, prescription of diuretics, glyceryl trinitrate, L-arginine or progesterone, and increased physical exercise failed to reduce the incidence of pre-eclampsia in systematic reviews (138-143).

A systematic review of calcium supplementation during pregnancy including 12 studies and 14946 women revealed a 30% reduction in relative risk of hypertension and a 52% reduction in relative risk of pre-eclampsia. Amongst high risk patients, calcium supplementation led to a 78% reduction in the relative risk of pre-eclampsia and a 20% reduction in maternal death or serious morbidity (144). Nevertheless, further evidence suggests that calcium intake from dietary sources is adequate in most patients and calcium supplementation is only recommended when there is likely to be dietary insufficiency (145).

Low-dose aspirin has been proposed to abrogate the imbalance between prostacyclin and thromboxane observed in pregnancies complicated by pre-eclampsia. A systematic review of 59 trials including 37560 women revealed a 17% reduction in relative risk of pre-eclampsia, 8% reduction in preterm delivery

and 14% reduction in fetal or neonatal death (31). In women deemed to be at high risk of pre-eclampsia, aspirin led to a 25% relative risk reduction.

The National Institute for Health and Clinical Excellence issued guidance on the management of hypertensive disorders in pregnancy in August 2010. The key recommendations state that aspirin should be offered to all women at high risk of pre-eclampsia from 12 weeks gestation to delivery, antihypertensive treatment should be initiated to maintain blood pressure less than 150/100 mmHg, proteinuria should be assessed by the protein:creatinine ratio and that the management of hypertension and pre-eclampsia during and after pregnancy requires an integrated package of care including medical and midwifery staff in primary and secondary care (32).

3.1.5 Screening for pre-eclampsia

Routine obstetric services in the United Kingdom are focussed on the early detection of hypertensive disorders of pregnancy. Assessment of clinical features suggestive of impending pre-eclampsia (abdominal pain, headaches, visual obscuration, oedema), measurement of hypertension and detection of proteinuria are performed at each clinical interaction. Early pre-symptomatic identification of PE can expedite the timing of fetal delivery to improve outcomes and reduce the risk of maternal morbidity due to severe disease including eclampsia.

Increased frequency of monitoring is indicated in women at high risk (chapter 3.1.2), however, it is notable that 50% of cases of PE occur in women with no identified risk factor. The probability of developing PE in women at high risk can be further stratified by uterine artery Doppler at 22-24 weeks gestation.

Uterine artery Doppler scans can elucidate uteroplacental blood flow patterns during pregnancy. The resistance index (RI) is defined as the percentage reduction of end-diastolic flow versus systolic flow. A systematic review revealed that a RI > 0.58 or greater than the 90% centile gives a likelihood ratio of 2.7 for developing pre-eclampsia (31.2% versus 14.4%) (146).

The presence of absent early diastolic flow (a ‘notch’) is associated with the subsequent development of intrauterine growth retardation or pre-eclampsia (figure 3.1.5.1). The presence of any diastolic notch gave a likelihood ratio of 2.4

(28.8% versus 14.4%) and bilateral diastolic notches gave a likelihood ratio of 2.8 (32.0% versus 14.4%) of developing pre-eclampsia (146).

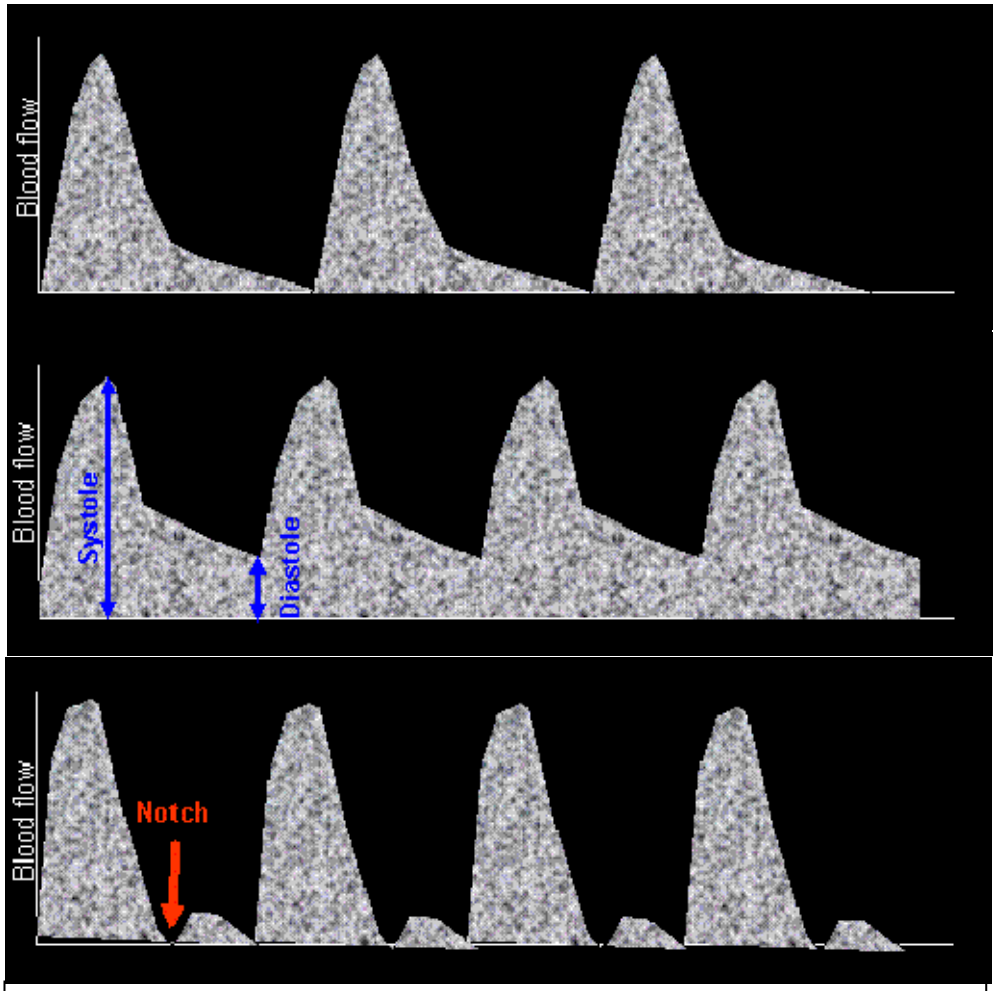


Figure 3.1.5.1. Uterine artery dopplers showing increased resistance index (upper panel), normal trace (middle panel) and diastolic notching (lower panel).

3.1.6 Biomarker studies in pre-eclampsia

Biomarkers are measurable endogenous compounds or biophysical parameters that can predict or diagnose diseases with sensitivity and specificity comparable to a gold-standard test. Using suitable biomarkers can obviate the requirement for a more invasive or expensive investigation, or for a delay in testing following an index event.

Developing disease biomarkers requires three stages:

- Identification of candidate compound/s from basic science, genomic analysis, "-omic" analysis or serendipitous discovery.
- Evaluation of the compound/s' ability to predict presence or absence of disease in a specified population with assessment of optimum 'cut-off' values.
- Evaluation of the compound/s as a diagnostic, predictive or prognostic test in the general population with assessment of the impact of false positive and negative results, safety and cost-effectiveness with respect to a "gold-standard" test.

Genomic, transcriptomic, proteomic and metabolomic analyses can be used to employed in 'hypothesis-free' studies in which biomarkers can be discovered without prior knowledge or expectation of what may be identified. Although this allows the discovery of novel biomarkers and potentially new concepts, the approach is hampered by a high risk of false positive findings (table 3.1.6.1).

Technology	Benefits	Limitations
Genomics	Stable chemistry Availability of 'normal' genome detail Availability of genome-wide arrays High volumes of samples compared easily	High volume of data, high risk of false positive findings No data on post-transcription modifications or interaction between translated protein products.
Transcriptomics	Able to assess cellular response to environment Impact of post-transcirptional modifications	No data on interaction between translated protein products. Difficult to analyse high volumes of samples Difficult to standardise collection of RNA between samples
Proteomics	Able to assess time-, cell- and environment-related responses. High volumes of samples compared easily Able to assess interaction between translated protein products.	Difficult to standardise samples for analysis – protein structure subject to processing and collection Mass/charge-based identification of putative markers subject to error
Metabolomics	Able to assess intra- or extracellular non-peptide milieu to identify biochemical activity Able to assess time-, cell- and environment-related responses.	Interpretation of relative changes in ubiquitous process difficult to interpret. Difficult to standardise sample collection Difficult to analyse high volumes of samples

Table 3.1.6.1. Overview of "-omic" technologies

Since 2000, published potential *serum* biomarkers for predicting PE are listed in table 3.1.6.2. Numerous permutations of biomarker concentration with ultrasonic

findings have been evaluated without progression to clinical practice. No single serum biomarker for PE is in clinical practice. Metabolomic analysis of serum from early pregnancy (15±1 weeks gestation) identified a panel of 14 small molecules, lipids and sterols and a combined multivariate predictive model offering ROC area under the curve of >0.9 (147). Combining panels of serum markers with uterine artery Doppler scans results in higher sensitivity and specificity but performance remains inadequate for clinical use (table 3.1.6.3) (148).

As above (chapter 3.1.3), serum s-Flt-1 and PlGF have been shown to predict PE. An increase in s-Flt-1 preceded the development of PE by approximately 5 weeks, and low levels of serum PlGF measured as early as 13 weeks gestation were associated with PE (126), however, differences in these factors are modest and do not appear to be clinically valid predictors of PE (149). Serum endoglin elevations during pregnancy are exaggerated in pregnancies complicated by PE (128).

Endoglin
Serum fms-like tyrosine kinase 1 (s-Flt-1)
Placental growth factor
Adipocyte fatty acid binding protein
Adrenomedullin
Ischaemia-modified albumin
ADAM12
Adiponectin
Complement fragment Bb
Selectin-E and –P
Proangiogenic CYR61 and NOV
Insulin-like growth factor-1 and IGF-binding protein-1
Asymmetric dimethylarginine
Pregnancy-associated plasma protein A (PAPP-A)
Soluble CD30
Soluble CD40-ligand
Serum PP13
Activin A
Cell-free fetal DNA
SERPINA-3
Endostatin
Vascular endothelial growth factor
Granulysin
Inhibin A
Leptin
Serum heat-shock protein 70
PP5/TFPI-2
Vascular cell-adhesion molecule-1
Tumour necrosis factor-receptor
Calprotectin
Transforming growth factor-1β
Plasminogen activator inhibitor type 2
5-hydroxytryptophan
Monosaccharide
Decanoylcartinine
Dodecanoylcartinine
Methylglutaric acid
Oleic acid
Docosahexenoic acid
γ-butyrolactone
2-oxovaleric acid
Acetoacetic acid
Hexadecenoyleicosatetraenoyl-sn-glycerol
Di-(octadecandienoyl)-sn-glycerol
Sphingosine 1-phosphate
Sphinganine 1-phosphate
Vitamin D3
Table 3.1.6.2. Proposed serum biomarkers of pre-eclampsia published since 2000

Biomarkers studied	Time of testing	Sensitivity	Specificity
High risk populations			
PP-13 and PI (cutoffs unspecified) (150)	First trimester	90%	90%
sFlt-1>614pg/ml and PI>1.45 ± bilateral uterine artery notches (151)	Second trimester	96%	87%
AFP>2.5 MoM + hCG >2.5 MoM) PI > 95% centile±bilateral uterine artery notches (152)	20 to 24 weeks	64%	97%
Low risk populations			
PP-13 + PAPP-A + PI (cutoff unspecified) (153)	First trimester (serum), second trimester (ultrasound)	74%	80%
Activin A > 6.58 MoM + uterine artery notch (154)	Second trimester	79%	100%
PlGF + PAPP-A, PI + mean arterial pressure + multiple maternal demographic factors (155)	First trimester	93%*	95%*

Table 3.1.6.3 Summary of selected studies combining biochemical and ultrasonographic markers in predicting pre-eclampsia. Adapted from Giguère et al (148) PP-13, placental protein-13; PI, pulsatility index; sFlt-1, soluble fms-like tyrosine kinase; AFP, alphafetoprotein; hCG, human chorionic gonadotrophin; PAPP-A, pregnancy-associated plasma protein A; MoM, multiples of median. *Values for prediction of early (<34 weeks) pre-eclampsia.

No predictive *urine* biomarkers for PE are in clinical practice. Published studies of urine biomarkers for PE are summarised in table 3.1.6.4. It is notable that s-Flt-1 and PlGF can be found in the urine of patients with PE at the time of diagnosis (156) but urine concentrations have not been found to be predictive of the subsequent development of PE. Podocyturia has been reported to have particurlarly robust capabilities of diagnosing and predicting the onset of pre-eclampsia, albeit in small trials and with methodology too complex to enter clinical practice (157). Podocyte-specific mRNA has been used as a simpler method to identify podocyturia with impressive results in a preliminary study (158). Buhimsci et al identified SERPINA-1 and non-random fragments of albumin

from urine proteomic profiles to be predictive of the subsequent development of pre-eclampsia up to 25 weeks before clinical features, albeit in a cohort of 19 patients of whom 3 developed pre-eclampsia (159). In a further study, proteomic analysis identified small molecule urinary biomarkers predictive of pre-eclampsia at 12 to 16 weeks gestation. A model of 50 peptide markers - predominantly including fragments of collagen, fibrinogen and uromodulin - was able to predict the subsequent development of pre-eclampsia with reasonable confidence in an initial cohort. However, outcomes were not replicated when the derived model was applied to a novel cohort of patients (160).

Paper	Biomarker	Patient group	Timing	Comments
Kronborg 2007 (161)	Urine and plasma orosomucoid	Prospective longitudinal study of healthy and PE pregnancies	From 18/40 gestation	Urine Orosomucoid:Cr higher at 20 weeks in PE patients and preceded rise in A:CR
Garovic 2007 (157)	Podocyturia	44 PE and 23 normotensive controls	Within 24h of delivery	PPV of podocyturia better than serum s-Flt-1, PIGF and endoglin, but difficult to measure
Williams 2007 (162)	Inositol phosphoglycan P-type (IPP)	27 PE women and 47 healthy pregnant controls	?	Urine IPP 30x higher in PE and increase occurred 7+ weeks before clinical diagnosis
Hamar 2006 (163)	Serum and urine inhibin A	75 women (30 severe PE, 11 mild PE, 9 chronic HTN, 16 pregnant controls, 9 nonpregnant control)	On admission with HTN or for delivery	Urine inhibin A >45pg/ml predicted severe PE (sensitivity 96.8%, specificity 87.5%)
Aggarwal, 2006 (164)	Urine PlGF	69 women (35 PE, 34 normotensive control)	?	Urine PlGF and PlGF/Cr lower in PE
Roes, 2005 (165,165)	Urine Glutathione S-transferase-A1-1 (GSTA1-1) and –P1-1	22 with severe PE, 30 non-pregnant with PMH PE, 18 normal pregnancy and 30 non-pregnant controls	?	Urine GSTA1-1 increased in PE but also normal pregnancy
Heyl, 2005 (166)	Urine VCAM-1	10 PE and 10 normal pregnancies	?	Although serum VCAM-1 elevated in pregnancy, urine VCAM-1 shows a circadian rhythm
Buhimschi 2005 (156)	Urine s-Flt-1, VEGF and PlGF	14 non-preg, 16 healthy preg, 21 hypertensive not severe PE, 17 with severe PE	Time of clinical manifestation	Increased sFlt-1 and decreased PlGF associated with severe PE. Log[sFlt-1/PlGF] > 2.1 gives 88.2% sens and 100% spec for severe PE.
Levine 2005 (167)	Urine PlGF	240 women; 120 with PE, 120 normotensive	Variable	Decreased urine PlGF mid-trimester (25 – 28 weeks) strongly associated with subsequent early development of PE.
Paternoster 1999 (168)	NAG, α1-microglobulin, albumin, uric acid	23 normal preg, 54 PE, 34 PIH	On admission	U-NAG increased in PE and PIH Urine alpha-1-microglobulin increased in PE
Mills 1999 (169)	Urinary metabolites of PGI_2 and thromboxane A2	134 PE, 139 controls	<22/40 26-29/40 and 36/40	Lower PGI2 metabolites as early as 13/40 in PE pregnancies.
Bahado-Singh 1998 (170)	Urine beta-core fragment of hCG	347 white non-smokers undergoing amniocentesis	Mid-trimester	Presymptomatic elevation of urine beta-hCG predicts subsequent PE: >2x median -> 2.07 x risk; >4x median -> 5.17 x risk
Leaños-Miranda 2008 (171)	Urine prolactin and fragments	207 healthy pregnancies, 124 with gestational HTN, 48 mild PE, 167 severe PE	After 20 weeks	Urine PRL significantly higher in PE. Antiangiogenic PRL fragments (14-16kDa) only found in severe PET (but only in 21.6%).
Buhimsci 2008 (159)	SERPINA 1 and albumin fragments	215 pregnant women and 10 non pregnant women with a variety of clinical and laboratory based diagnoses	Longitudinal study of 19 women. Cross-sectional study of 206 women.	Proteomic analysis of urine. SERPINA1 and non-random fragments of albumn associated with the development of pre-eclampsia,

Table 3.1.6.4. Urinary biomarkers studies in pre-eclampsia. PE, pre-eclampsia; Cr, creatinine; A:Cr, albumin-to-creatinine ratio; PPV, positive predictive value; HTN, hypertension; PMH, past medical history; VCAM-1, vascular cell adhesion molecule-1; PIH, pregnancy induced hypertension; PGI_2, prostacyclin; PlGF, placental growth factor; s-Flt-1, soluble fms-like tyrosine kinase 1; hCG, human chorionic gonadotrophin; NAG,N-acetyl glucosaminidase.

3.1.7 Pre-eclampsia and the kidney

Given that proteinuria is a ubiquitous and diagnostic marker of pre-eclampsia it is not surprising that histological examination of the kidney reveals significant pathology. Within the kidney marked abnormalities in endothelial cells can be observed in biopsies with capillary endothelial oedema, vasospasm and microthrombi, collectively termed glomerular endotheliosis (see figure 3.1.3.2) (172). Consequently, the glomerular filtration barrier is disrupted and proteinuria develops. The impairment to capillary blood flow leads to a reduction in glomerular filtration rate. Serum creatinine concentrations are higher in pregnancies complicated by PE (51).

Acute kidney injury affects 1% - 2% of cases of pre-eclampsia. Requirement for renal replacement therapy is rare and more often a result of hypovalaemia following haemorrhagic complications than direct renal injury. Progression to chronic kidney disease and end stage renal disease is less common still, although failure to recover is more likely if the patient had pre-existing hypertension or kidney disease (173). Longitudinal population studies have identified pre-eclampsia as an independent risk factor for the subsequent development of end stage renal disease over a 17 year follow-up (relative risk 4.7), although the incidence remains low (87).

The changes seen on light microscopy resolve by 40 days post-partum and, although some glomerular basement membrane thickening can persist on ultrastructural examination, excretory dysfunction and proteinuria almost always returns to normal (174).

3.1.8 Urine proteomics in pre-eclampsia

For this thesis I aimed to perform proteomic analysis of a large number of urine samples to identify putative urinary biomarkers predictive of the subsequent development of pre-eclampsia. I questioned whether the morphological changes identified on renal histology and the systemic endothelial changes characteristic of PE would alter the nature of filtered protein encountered in urine.

As above (chapter 1.5), urine contains micromolar concentrations of over a thousand peptides in health (107). The constitution of this milieu of peptides – the urinary proteome – is dependent on:

- the concentration of circulating peptides
- the integrity of the glomerular basement membrane
- efficiency of tubular protein scavenging systems
- intra-tubular and intracellular peptide proteolysis
- secretion of peptides from the tubular and urothelial membrane

To date, there are no international guidelines on standardisation of urine collection and storage for proteomic analysis although this has been highlighted as an area of need by the human kidney and urine proteome project (HKUPP), the human proteome organisation (HuPO) and the European Proteomics Association. These guidelines will be of significance since, unlike genomic analysis where the source material is stable, proteomic profiles can undergo marked changes

dependent on the collection, processing and storage of the urine (see chapter 3.1.11).

Urine proteomic profiles are obtained by separation of the protein constituents by gel electrophoresis, liquid chromatography or mass spectrometry (table 3.1.8.1).

Technique	For	Against
1D gel electrophoresis	Cheap Simple Protein bands extractable for identification	Poor mass resolution Unable to identify low abundance peptides Challenging to compare multiple complex samples
2D gel electrophoresis	Relatively cheap Protein spots extractable for identification Improved mass resolution	Time consuming Unable to identify low abundance peptides.
High performance liquid chromatography	High mass resolution for simple mixtures Amenable to tandem mass spectrometry for peptide identification Portion of proteome selected according to physicochemical properties.	Time consuming Unsuitable for large sample numbers
Matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI)	High mass resolution for complex mixtures. Amenable to tandem mass spectrometry for peptide identification. Portion of proteome selected according to physicochemical properties	Expensive Time consuming.
Surface-enhanced laser desorption ionisation time of flight mass spectrometry (SELDI)	Simple Rapid preparation and analysis Portion of proteome selected according to physicochemical properties	Reasonable mass resolution for complex mixtures Challenging to identify protein peaks from spectrometry data Large coefficient of variance of signal intensity between samples
Ion-trap/Fourier transform cyclotron analysers	Very high mass resolution Relatively simple to identify protein peaks Rapid results	Very expensive Difficult to compare large numbers of samples

Table 3.1.8.1 Comparison of proteomic analysis techniques

3.1.9 Surface-Enhanced Laser Desorption Ionisation Time-Of-Flight Mass Spectometry

Surface-Enhanced Laser Desorption Ionisation Time-of-Flight Mass Spectrometry (SELDI) was developed in the Baylor College of Medicine, Texas by T. William Hutchens in 1993.

To improve the resolution of a signal obtained from a complex mixture of peptides, a proportion of peptides are selected for analysis according to their physicochemical properties. A 'spot' or 'chip' is supplied coated with an appropriate compound to select out the proteome portion for study. The study mixture is spotted on to the surface prepared with a specified buffer and then washed off.

The bound peptides are allowed to crystalise with an energy absorbing matrix (EAM). Once crystalised, the bound peptides are ionised by a laser pulse and enter a gaseous phase. The ionised gaseous peptides are accelerated across an electric potential and the time of flight from 'spot' to sensor is recorded.

By calibrating the system to peptides of known mass and charge the time of flight can be correlated to the mass/charge of the bound peptides (figure 3.1.9.1).

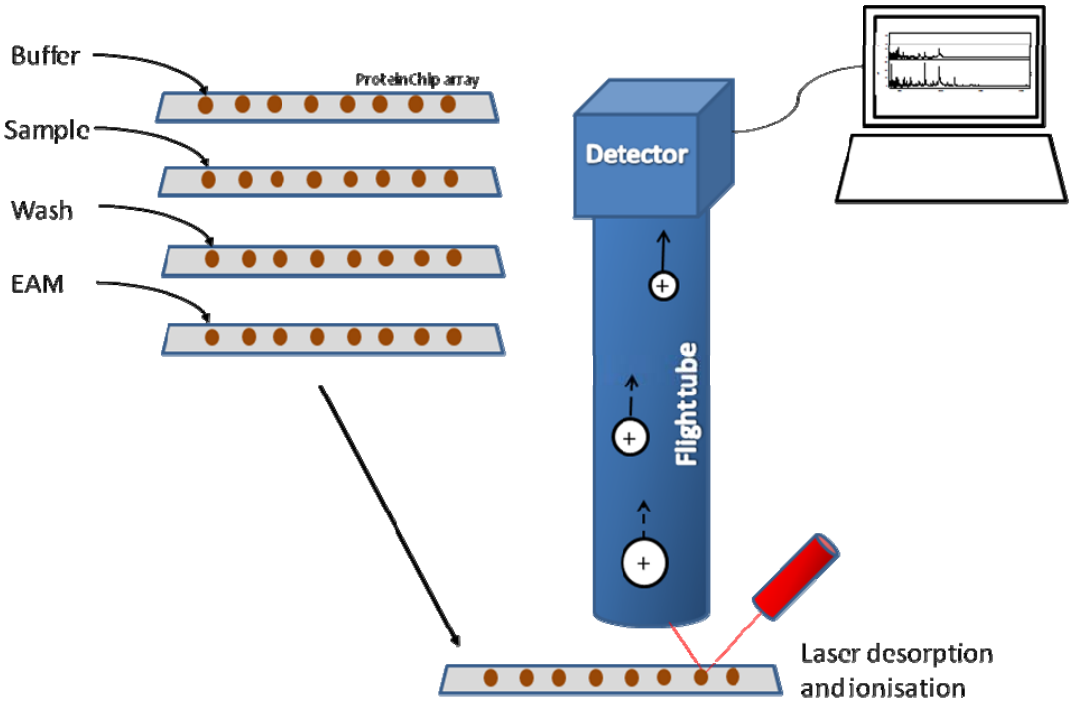


Figure 3.1.9.1 Schematic of SELDI. EAM, energy absorbing matrix.

A fluid can be analysed using multiple 'chips' with different surfaces to maximise the proteome studied, however, because SELDI can produce large amounts of data relatively quickly, it is most suited to screening large numbers of samples for putative biomarkers. The choice of chip surface can be made based on the fluid type studied, a predicted biomarker or biomarkers, or by preliminary investigations across all surface types (table 3.1.9.1).

ProteinChip® array	Surface chemistry	Peptide selectivity	Washing and binding buffer	EAM
Q10	Cationic quaternary ammonium ions	Negative surface charged proteins	Tris 0.1M pH 7.0 to 9.0	Sinapinic acid or CHCA
CM10	Anionic carboxylate groups	Positive surface charged proteins	Sodium acetate 0.1M pH 4.0 to 7.0	
IMAC30	Nitrilotriacetic acid chelated to iron, copper, zinc or gallium	Metal-binding protein domains or phosphorylated proteins	Sodium phosphate 0.1M, sodium chloride 0.5M pH7.0	
H50	Methylene chains	Hydrophobic proteins	10% acetonitrile, 0.1% trifluoroacetic acid	
NP20	Silicon dioxide	Hydrophilic proteins	Sodium acetate 0.1M pH 4.0 to 7.0	

Table 3.1.9.1 ProteinChip® arrays and their binding properties. EAM, energy absorbing matrix.

The specificity of peptide binding to a particular chip type can be further manipulated by altering the chemical properties of the washing and binding buffer used in the chip preparation. For example, using a strongly alkaline washing and binding buffer (Tris 0.1M pH 9.0) with a Q10 array maximises negative charging of soluble peptides. This will increase the stringency of binding to the chip surface, whereas a more selective proteome will bind using a neutral buffer (Tris 0.1M pH 7.0).

Finally, the resolution of signal obtained can be further optimised by choice of energy absorbing matrix (EAM) applied. Commonly utilised matrices are sinapinic acid (3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid) for larger peptides or CHCA (alpha-cyano-4-hydroxy cinnamic acid) for smaller molecules

Compared to alternative proteomic methods, SELDI benefits from allowing a high throughput of samples with rapid acquisition of data (175). This makes it ideal for searching complex fluids for putative biomarkers, albeit at the expense of decreased signal resolution and increased inter-assay coefficient of variance of signal intensity (176). Although these limitations have generally limited the use of SELDI as a clinical tool (177), it allows a hypothesis-free approach to novel biomarker discovery and subsequent evaluation. This approach has been used to identify proteomic patterns to differentiate diabetic from non-diabetic nephropathy (178,179), early acute kidney injury (180-182), acute renal transplant rejection (183,184), lupus nephritis (185,186) and urogenital malignancy (187-189).

SELDI was chosen as the most appropriate method of analysis for this project since the rapid acquisition of data would allow for same-day analysis of samples, minimising possible interference from collection and storage variations.

3.2 Sample collection and storage in urine proteomics

3.2.1 Introduction

By definition, the proteome is “the entire complement of proteins, including the modifications made to a particular set of proteins, produced by an organism or system….this will vary with time and distinct requirements, or stresses, that a cell or organism undergoes” (106). Consequently, alterations in the method of sample collection, modifications and storage will affect the proteome analysed. The concentration of urine will greatly affect the quantity of excreted protein and is addressed in chapter 3.2.6.

There are no published standardised methods for the collection, preparation and storage of urine although this is being addressed by the human kidney and urine proteome project (HKUPP), the human proteome organisation (HuPO) and the European Proteomics Association. Key areas of importance for consideration in urinary proteomics were summarised by Thongboonkerd in 2007 (190), namely:

- removal of cells and cellular debris
- addition of protease inhibitors
- addition of inhibitors of bacterial growth
- addition of denaturing buffer
- sample storage and the effect of freeze-thaw cycles
- methods for concentrating or isolating urinary proteins

- removal of high abundance proteins (such as albumin) prior to analysis

Controversy over these issues exists since any processing of the urine sample may introduce proteome signal artefact. In our study we aimed to find biomarkers that may be able to be identified using a urine dipstick as a point-of-care test. I therefore developed a protocol with minimal interventions to mimic bedside conditions as closely as possible.

A series of pilot studies were performed to derive optimum methodology for the subsequent clinical study (chapter 3.2 to 3.4).

3.2.2 Removal of cells and cellular debris

3.2.2.1 Introduction

Normal healthy urine has less than 5 cells/high power field under light microscopic examination. This is increased in patients with intrinsic renal disease, urothelial malignancy, urinary tract infection or urothelial inflammation. Cellular degradation *ex vivo* leads to release of intracellular proteins and organelles contaminating the urinary proteome and, at room temperature, occurs from 30 minutes post-micturition. Centrifugation has been shown to adequately separate cells and cellular debris from urine prior to proteome analysis (191).

3.2.2.2 Methods

Urine was separated into 500µl aliquots and chilled on ice for 10 minutes. Centrifugation was performed at 13400rpm for 0 to 2 minutes. Urine samples were examined by light microscopy for cells and cellular debris. Whole urine or supernatant was mounted under a cover slip and examined at 100x magnification using a GX-optical GXD-30 inverting microscope. Images were captured using a Deltopix DP200 digital camera.

3.2.2.3 Results

Cells and cellular debris were seen in whole healthy urine. These were absent or much reduced in samples undergoing centrifugation at 13400 rpm for 1 minute or 2 minutes. Representative field images are shown in figure 3.2.2.1.

Figure 3.2.2.1 Removal of cells and cellular debris from urine by centrifugation at 13400 rpm for 0, 1 or 2 minutes (100x magnification)

3.2.2.4 Conclusion

Cells and cellular debris were removed from urine by centrifugation of healthy urine at 13400 rpm for 2 minutes. In the clinical study, we opted to perform centrifugation for 2 minutes.

3.2.3 Addition of protease inhibitors

Protease inhibitors are not recommended in urine proteomics (190). Their importance is greater in serum proteomic analysis given the higher concentrations of endogenous proteases present and the richer depth of proteome present, whereas in urine there is less endogenous protease activity (192). Furthermore, peptide protease inhibitors may contaminate the proteome studied by interacting with or competing with endogenous peptides (eg, aprotinin (193)) and small molecule protease inhibitors may affect the physicochemical properties of endogenous peptides (eg, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (194)).

We did not further investigate the effect of protease inhibitors on the urinary proteome, however, we aimed to minimise the possibility of proteolysis by performing proteomic analysis of samples within 24 hours of collection.

3.2.4 Addition of inhibitors of bacterial growth

Bacterial urinary tract infection or bacterial contamination of collected samples will dramatically affect the urinary proteome as a result of active cellular protein secretion and cell degradation. There is less evidence that inhibitors of bacterial overgrowth (eg, sodium azide or boric acid) have a significant effect on the urinary proteome as compared to protease inhibitors, however, there is a theoretical risk that the alteration in acid-base resulting from their addition may affect peptide charge.

The effect of bacterial contamination on the urine proteome has been shown to be minimised by centrifugation shortly after collection, storage at 4°C prior to freezing and freezing within 48 hours of collection (195).

We did not study the effect of inhibitors of bacterial growth on the urinary proteome but aimed to minimise the effect of bacteruria or bacterial contamination on the urinary proteome by (a) discarding data obtained from bacteruric samples, (b) centrifugation of urine within 30 minutes of collection and (c) storage on ice prior to proteomic analysis within 24 hours. Inhibitors of bacterial growth were not added to samples.

3.2.5 Addition of denaturing buffer and urine storage

3.2.5.1 Introduction

Denaturation of proteins leads to loss of their tertiary and secondary structure. In proteomics, this may increase the depth of peptides to be identified, particularly with gel-based techniques where the morphology of secondary and tertiary structures has an effect on electrophoretic mobility independent of molecular mass. Previous studies have shown that protein denaturation prior to SELDI analysis or urine can increase the number of peptide peaks obtained (192), however, there is a theoretical risk that the alteration in acid-base status of the fluid can affect binding of peptides to the ProteinChip® array surface.

Left at room temperature or 4°C, protein degradation leads to considerable changes in the urinary proteome within 3 days, with the production of multiple low molecular weight peptide fragments, presumably as a result of enzymatic and non-enzymatic proteolysis (196). Conversely, promptly frozen urine supernatant shows little proteomic degradation over 4 to 5 freeze-thaw cycles (190,192) albeit at the expense of signal intensity (196).

A pilot study was performed to identify the effect of denaturing buffer and storage conditions over 24 hours prior to proteomic analysis. The results were used in design of the clinical study.

3.2.5.1 Methods

Midstream urine from 4 healthy volunteers (2 male, 2 female) was separated into 500µl aliquots and chilled on ice for 10 minutes. Urinary tract infection was excluded by dipstick reagent analysis for leucocyte esterase and nitrites. Cells and cellular debris were removed by centrifugation at 13400 rpm for 1 minute. Supernatant was separated into 150µl aliquots in 12 Eppendorf tubes and 2 cryotubes. Samples were not standardised to creatinine or protein content as intrasubject comparisons were performed.

Denaturing buffer (urea 9M, CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) 4%, TRIS (2-Amino-2-hydroxymethyl-propane-1,3-diol) pH 9.0 40mM) was added to alternate urine supernatant samples (56µl per 150µl sample) from each volunteer.

Prior to proteomic analysis, samples were stored at:

- 4°C for less than 2 hours
- 4°C for 16 hours
- -20°C for 16 hours
- -80°C for 16 hours
- or frozen in liquid nitrogen (-196°C) for 16 hours.

Proteomic analysis by SELDI was performed on CM10 ProteinArray® chips using the manufacturer's Bioprocessor® unit. The array was washed with 200µl washing and binding buffer (sodium acetate 0.1M pH 4.0) per well, incubated with shaking

for 5 minutes twice and the buffer removed. Urine supernatant samples (thawed on ice where necessary) were added to each spot; 150µl for samples with denaturing buffer and 109µl for samples without denaturing buffer to standardise the protein content per sample applied. The samples was incubated for 30 minutes with shaking then removed and the spots washed with washing and binding buffer for 5 minutes with shaking three times. Spots were washed with 200µl deionised ultrapure water twice and allowed to dry. One microlitre of energy absorbing matrix (sinapinic acid 20µg/µl, acetonitrile 50%, trifluoroacetic acid 0.5%, deionised ultrapure water 49.5%) was applied per spot and allowed to dry twice.

Mass spectrometry was performed by SELDI-TOF MS using a Ciphergen ProteinChip System Series 4000 (Ciphergen Biosystems, California, USA), with focus mass of 9000Da, range 0 to 100000Da, warming shot energy 4400nJ (1 shot), data shot energy 4000nJ (10 shots), sampling rate 400Hz, matrix attenuation 2500Da and a 25kV source of positive ions.

Peak analysis was performed with Ciphergen Express Client 3.0 software defining a peak as a signal-to-noise ratio greater than 6. Total numbers of peaks per spectrum were counted and compared by one-way analysis of variance (ANOVA).

3.2.5.2 Results

There was no significant difference in the number of peaks recorded following any storage method and a single freeze-thaw cycle (table 3.2.5.1, figure 3.2.5.1).

Storage	Number of peaks				
	V1	V2	V3	V4	Average
Immediate analysis	22	25	24	29	25
4°C	21	22	24	21	22
-20°C	16	16	27	21	20
-80°C	21	23	32	19	23.75
Liquid nitrogen	19	21	19	23	20.5
Average	19.8	21.4	25.2	22.6	

Table 3.2.5.1 Identified peaks between healthy volunteers and storage methods. ANOVA of between storage method; F=1.2, p=0.35.

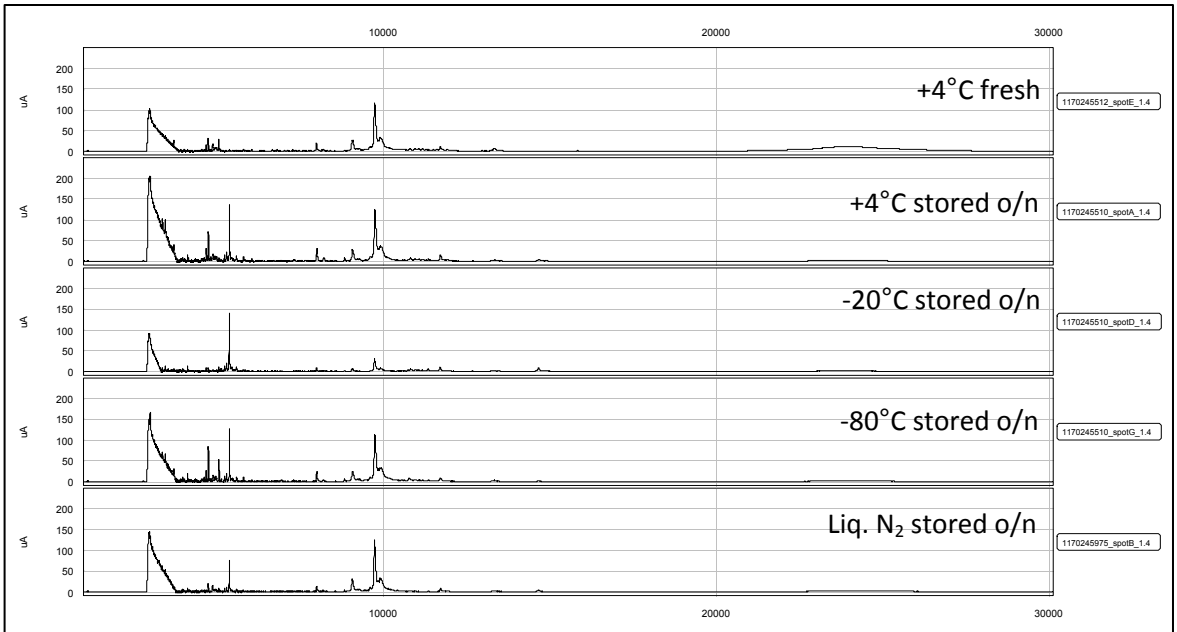


Figure 3.2.5.1. SELDI-TOF MS profiles of a single representative sample stored in 5 different conditions. o/n, overnight.

No beneficial effect on preserving or optimising peptide peaks were found in samples treated with denaturing buffer. Use of denaturing buffer caused loss of

peaks at mass/charge values of 8018, 9091 and 9764Da with no gained peaks (figure 3.2.5.2).

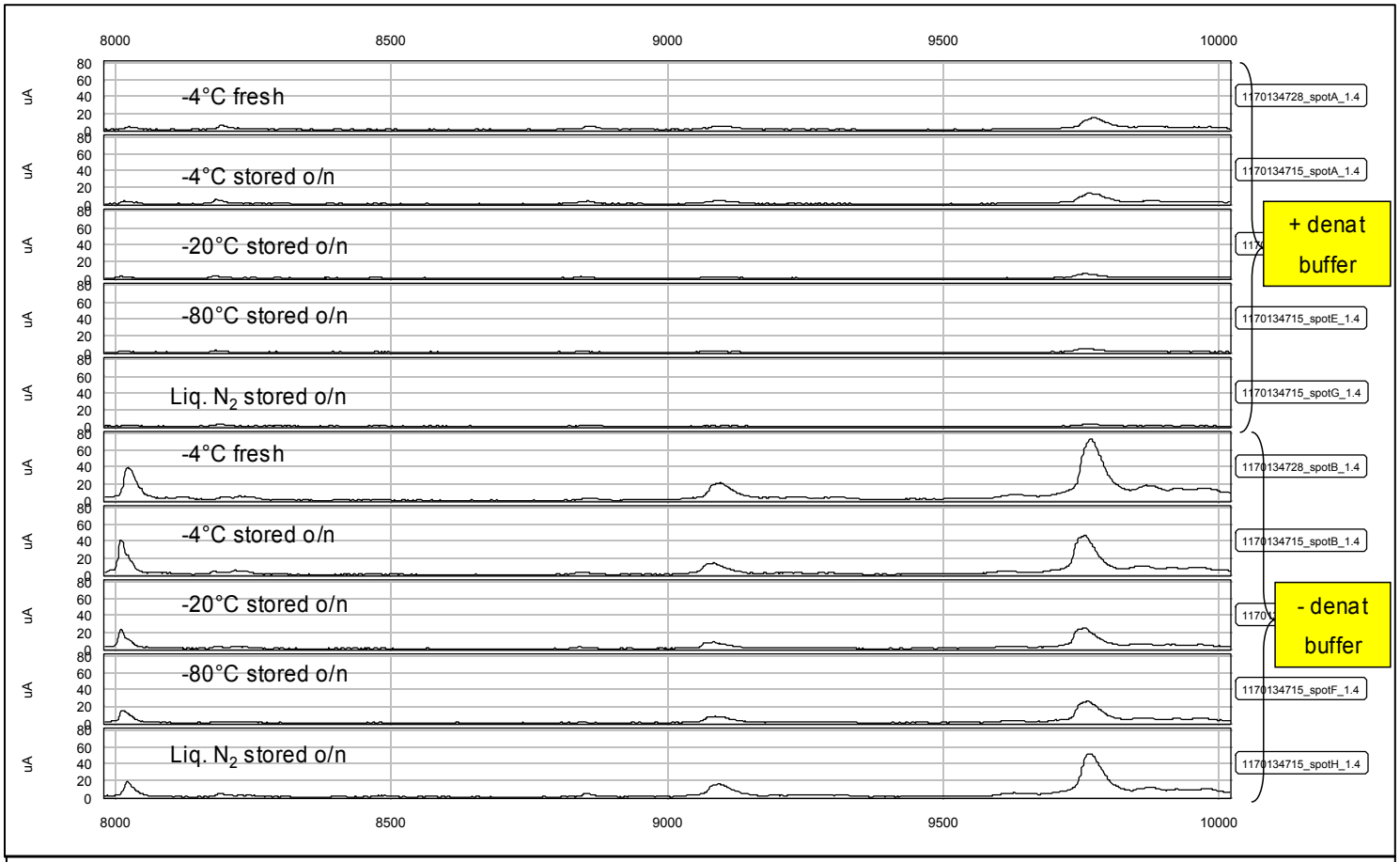


Figure 3.2.5.2. Effect of denaturing buffer in reducing sensitivity of CM10 ProteinChip® array analysis of healthy urine. Addition of denaturing buffer reduced signal intensity at peaks from 8000 to 10000 Da irrespective of storage method. No additional peaks were identified with the addition of denaturing buffer. Liq. N_2, liquid nitrogen; o/n, overnight.

3.2.5.3 Discussion

Little difference was found in the proteome of urine stored overnight at 4°C compared with urine analysed within 2 hours or stored overnight at -196°C. Subsequently samples were analysed on the day of collection or stored overnight at 4°C.

We found denaturing buffer to have no beneficial effect and possible detrimental effect on the depth of urinary proteome in healthy urine. Denaturing buffer was not used in subsequent experiments.

3.2.6 Creatinine assay

3.2.6.1 Introduction

The concentration of protein in urine is dependent on the quantity of protein filtered, reabsorbed and secreted in urine; and its relative degree of water dilution. Urine concentration is predominantly determined by salt reabsorption in the proximal convoluted tubule, loop of Henlé and distal convoluted tubule under the influence of tubuloglomerular feedback and aldosterone, and the permeability of the collecting ducts to water through aquaporins under the influence of antidiuretic hormone. Through these mechanisms, urine osmolarity may vary from 200 to 1000mOsm/l.

Prior to analysing the urine proteome, this variation in water content must be addressed. It is appropriate to normalise samples according to their total protein content when it is anticipated that there will be little variation in protein content between samples. In our study, where it is anticipated that protein content will rise in patients with evolving pre-eclampsia, it is more appropriate to normalise samples according to the creatinine content. This assumes that creatinine is being produced, filtered and secreted at a steady rate.

Creatinine is an amino- and methylated imidazole ring formed as a breakdown product of muscle metabolism. It can be measured by colorimetric reaction (197,198), enzymatic reactions (199,200), high performance liquid chromatography (201,202) or isotope dilution mass spectrometry (203) (table 3.2.3.1).

We compared the performance of the colorimetric alkaline picrate method of creatinine measurement with published results.

Method	Advantages	Disadvantages
Colorimetric alkaline picrate (Jaffe reaction)	Cheap No specialist equipment needed Widely available in routine laboratories	Subject to interference by glucose, acetoacetate, proteins and drugs. Moderate coefficients of variance
Enzymatic methods	Modest improvement in coefficients of variance compared with colorimetric method	More cumbersome and expensive than colorimetric methods Remain subject to interference from endogenous and exogenous compounds
High performance liquid chromatography	Very low interference from non-creatinine compounds	Cumbersome and time-consuming No standardised methodology
Isotope dilution mass spectrometry	The 'gold standard' for accurate measurement of creatinine concentration. Excellent specificity and bias <0.3%.	Multistage analytical procedure requiring specialised equipment.
Table 3.2.3.1 Summary of analytical methods for measuring creatinine		

3.2.6.2 Methods

Creatinine solution (250mg/dl) was diluted 1 in 10 in phosphate buffered saline (PBS) then serially diluted to create standards ranging from [creatinine]=25mg/dl to 0.0976mg/dl. On a 96 well plate, 25µl of the diluted standards were placed in quadruplicate with a final standard containing PBS alone. Urine sample supernatants were diluted 1 in 12.5 in PBS. 25µl aliquots were placed in quadruplicate on a 96 well plate.

Creatinine concentrations were obtained by colorimetric analysis of the Jaffe reaction. The Jaffe reaction produces an orange-yellow colour when creatinine reacts with picric acid (2,4,6-trinitrophenol) and alkali (sodium hydroxide) (198) (figure 3.2.6.1).

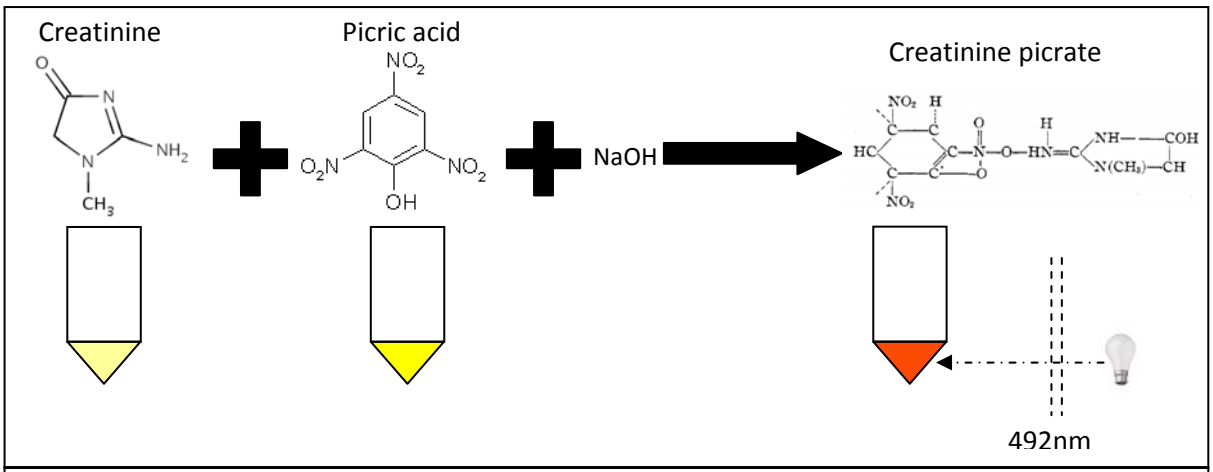


Figure 3.2.6.1 Colorimetric alkaline picrate creatinine assay

Solutions of detergent buffer (anhydrous Na_2HPO_4 61.56mM, $Na_2B_4O_7.10H_20$ 62.5mM, SDS 4% and Triton X-100 0.0625%), alkali (NaOH 468.5mM) and a saturated solution of picric acid (56mM) were mixed in a ratio of 2:2:1. 100µl of this working reagent were added to each sample and the plate was swirled to mix.

After 30 minutes incubation, absorbance was measured at 492nm with a Titertek Multiscan Plus. Mean results for the quadruplicate samples were used.

3.2.6.3 Results

Intra-assay co-efficient of variance (CV) was calculated from 105 sets of quadruplicate data derived from standard calibration curves and urine samples. The mean (SD) intra-assay CV was 2.5% (2.1%).

Inter-assay CV was calculated from 5 urine samples each analysed 7 times using different standards. The mean (SD) inter-assay CV was 11.2% (4.3%).

Creatinine concentration was quantified in 449 urine specimens obtained in the study, analysed with 68 assays. Linear regression analysis of absorbance against creatinine concentration for the standard curves revealed excellent correlation (r^2>0.99) for all assays.

Measured creatinine concentration ranged from 5.03mg/dl to 543mg/dl (0.04mmol/l to 4.79mmol/l) with a mean of 120.7mg/dl (1.07mmol/l) and 95% confidence interval 111.9mg/dl to 129.5mg/dl (0.99mmol/l to 1.14mmol/l) (figure 3.2.6.2).

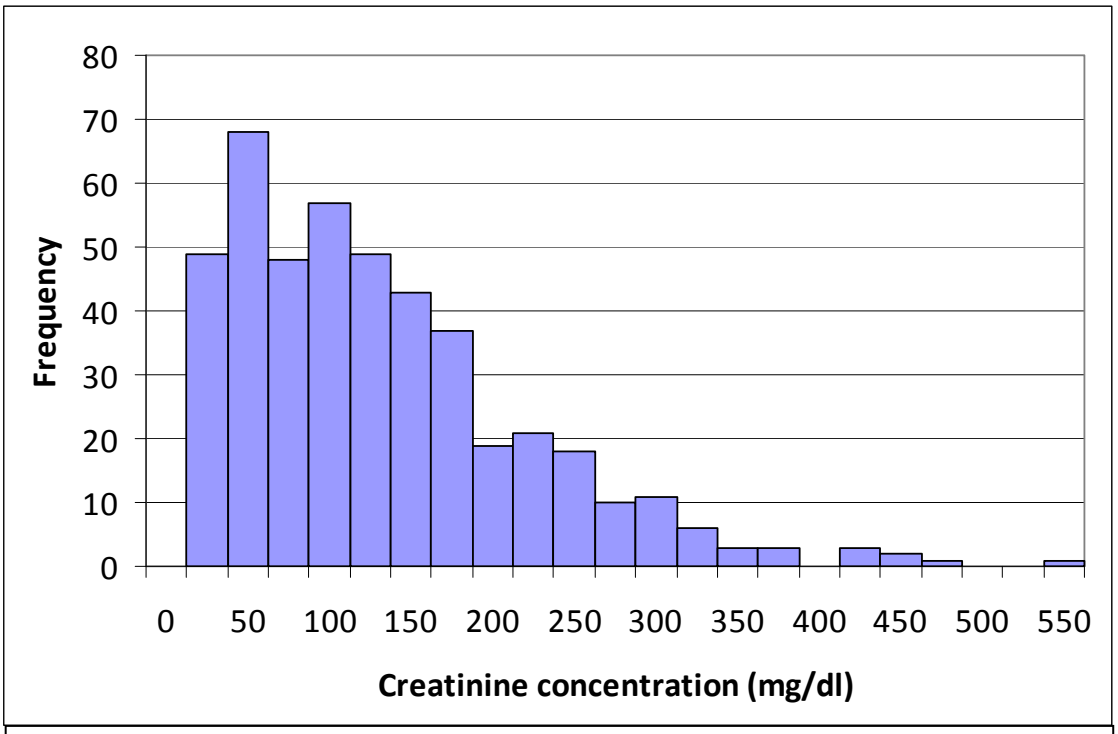


Figure 3.2.6.2. Histogram of measured urine creatinine concentration

3.2.6.4 Discussion

The urine creatinine concentrations found in our study are comparable with values obtained from 11635 women in the NHANES III cohort where the reported mean was 113.5mg/dl (95%CI, 110.7 to 116.3 mg/dl) (204).

The intra-assay CV (2.5%) was comparable with values obtained from commercially available colorimetric assay kits (2.7%) (Cayman Chemical Company, Ann Arbor, Michigan), enzymatic methods (2.2%) (205) and high performance liquid chromatography (HPLC) (5.2%) (206). The inter-assay CV (11.2%) was greater than that quoted for commercially available colorimetric assay kits (3.0%) and HPLC (5.2%) but comparable with the inter-assay CV encountered in a study of multiple assay types in routine clinical practice (14.2%) (207).

The Laboratory Working group of the National Kidney Disease Education Program studied the impact of different techniques of measuring creatinine in serum and identified that inaccuracy from biological variability and analytical interference outweighed the inaccuracies introduced by more or less accurate laboratory methods (208).

We used the colorimetric alkaline picrate method for analysing urine creatinine concentrations for our study given its simplicity and low cost and results comparable to other methods and other laboratories.

For the clinical study, urine was diluted with ultrapure deionised water to a standardised creatinine. Based on our preliminary results from and the results of the NHANES III study (204), all urine samples were diluted to a concentration of

20mg/dl (1.78mmol/l) creatinine. This threshold was chosen to prevent over-dilution of the proteome whilst ensuring that the number of samples encountered more dilute than this was kept to a minimum. Retrospective analysis of the samples obtained in our study shows that 418 (93%) urine specimens had a creatinine concentration greater than 20mg/dl. Sample with creatinine concentration less than 20mg/dl were neither concentrated nor diluted.

3.3 SELDI protocol optimisation

3.3.1 Introduction

Mass spectrometry-based biomarker discovery allows a hypothesis-free or "bottom-up" approach to study design; that is, the proteome can be examined for discriminating differences between samples without prior expectation of putative biomarkers. Novel compounds or pathophysiological theories can evolve from this approach. It is generally accepted that putative biomarkers identified by mass spectrometry should be validated in a novel cohort with an alternative methodology (177).

As above (see chapter 3.1.9), compared to alternative proteomic methods, SELDI-TOF MS benefits from allowing a high throughput of samples with rapid acquisition of data (175). Selective binding of portions of the proteome to the ProteinChip® surface according to the peptide's physicochemical properties enables increased mass/charge resolution in the resultant spectrum, albeit at the expense of discarding non-binding peptides.

It was financially and logistically impractical to perform our clinical study on multiple ProteinChip® arrays. We therefore developed our array preparation prior to the study to optimise capture of putative biomarkers.

3.3.2 Methods

3.3.2.1 Sample collection and preparation

To compare different ProteinChip® arrays, urine specimens from healthy non-pregnant volunteers and patients at various stages of pregnancy without clinical evidence of pre-eclampsia underwent centrifugation to remove cells and cellular debris as above (chapter 3.2.2). To compare the effect of different washing and binding buffers on the identified urine proteome, urine specimens collected as part of a separate study on the effect of spironolactone in cardiovascular disease were analysed. Samples were standardised according to serum creatinine in ultrapure deionised water. Serum creatinine was measured using the method described above (chapter 3.2.6). Denaturing buffer, inhibitors of bacterial growth or protease inhibitors were not added to the samples.

3.3.2.2 Comparing ProteinChip® arrays

Different ProteinChip® arrays were prepared according to the manufacturer's instructions using the least selective recommended washing and binding buffer. The following ProteinChip® arrays and buffers were used (see table 3.1.9.1):

- CM10 array, sodium acetate 0.1M pH 4.0
- Q10 array, TRIS 0.1M pH 9.0
- NP20 array, sodium acetate 0.1M pH 4.0
- H50 array, prewashing with 50µl acetonitrile, washing and binding with 10% acetonitrile, 0.1% trifluoroacetic acid

- IMAC30 array with copper, iron or gallium loading, sodium phosphate 0.1M/sodium chloride 0.5M.

Sinapinic acid was used as energy absorbing matrix in all experiments.

Thawed standardised urine samples were added to the ProteinChip® arrays as per the manufacturer's instructions using a Bioprocessor® unit and agitation for 30 minutes. Proteomic profiles were obtained by SELDI using a Ciphergen ProteinChip System Series 4000 (Ciphergen Biosystems, California, USA). Each sample analysis was performed with focus mass of 9000Da, range 0 to 100000Da, warming shot energy 4400nJ, 10 data shots energy 4000nJ, sampling rate 400Hz, matrix attenuation 2500Da and a 25kV source of positive ions.

Peaks were identified as a signal to noise ratio greater than 5.0 using Ciphergen Expresss Client 3.0 software (Ciphergen Biosystems, California, USA) and counts compared using one-way ANOVA with Tukey post-hoc analysis.

3.3.2.3 Comparing washing and binding buffers

To investigate the effect of different washing and binding buffers on signal preservation and resolution, SELDI was performed as above on CM10 and IMAC30 ProteinChip® arrays using high specificity (HEPES 50mM, pH 7.0 and sodium phosphate 0.1M/sodium chloride 0.5M/imidazole 5mM respectively) or low specificity (sodium acetate 0.1M pH 4.0 and sodium phosphate 0.1M/sodium chloride 0.5M) buffer.

3.3.2.4 Comparing SELDI protocols

The intensity and number of laser pulses used to ionise bound peptides from the ProteinChip® array, the mass focus and degree of attenuation of signal caused by the matrix can be varied per SELDI protocol. More intense laser pulses can increase the ionisation and of bound peptide and increase signal intensity at the expense of risk of protein fragmentation. The mass focus improves resolution within a proportion of the proteome and needs to be optimised according to the specimen studied.

Based on the size of peptides identified in healthy urine, previously proposed urinary biomarker characteristics and manufacturer's recommendations for protocol settings, three protocols were studied:

- matrix attenuation 2500 Da, focus mass 9000 Da, mass range 0 to 100000 Da, sampling rate 400MHz, warming shot 1 x 4400 nJ, data shots 10 x 4000 nJ (protocol A)

- matrix attenuation 10000 Da, focus mass 30000 Da, mass range 0 to 250000 Da, sampling rate 400MHz, warming shot 1 x 6600 nJ, data shots 10 x 6000 nJ (protocol B)

- matrix attenuation 1000 Da, focus mass 5000 Da, mass range 0 to 100000 Da, sampling rate 400MHz, warming shot 1 x 4400 nJ, data shots 10 x 4000 nJ. (protocol C)

3.3.3 Results

The mean number of peaks from each array type is shown in table 3.3.3.1. In volunteer and pregnant patient groups, the CM10 and IMAC30 copper preparations produced the most identified peaks (figure 3.3.3.1).

Array	Mean	Std. Deviation	p value
CM10	26.88	7.302	
IMAC Cu^{2+}	25.94	8.177	0.999
Q10	24.31	7.499	0.899
NP20	23.37	3.384	0.673
H50	17.88	5.414	0.001
IMAC Ga^{2+}	16.38	4.241	<0.001
IMAC Fe^{3+}	15.50	5.254	<0.001

Table 3.3.3.1. Number of peaks per ProteinChip® array type. N=16. ANOVA F=9.619, p<0.001. p values compare array peak counts against CM10 chip by Tukey post-hoc analysis.

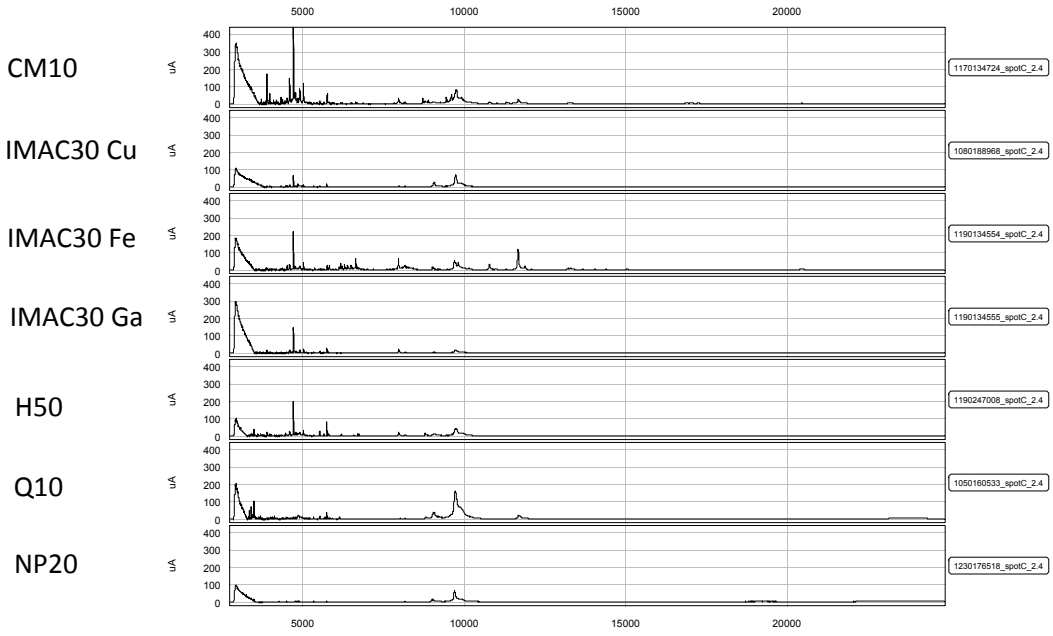


Figure 3.3.3.1. Representative spectra from a single urine sample analysed on different ProteinChip® arrays

Washing and binding buffers with increased specificity reduced the binding of higher mass/charge peptides but preserved smaller molecules. In known proteinuric samples, increased specificity buffers (imidazole 5mM added) reduced albumin binding (figures 3.3.3.2 and 3.3.3.3).

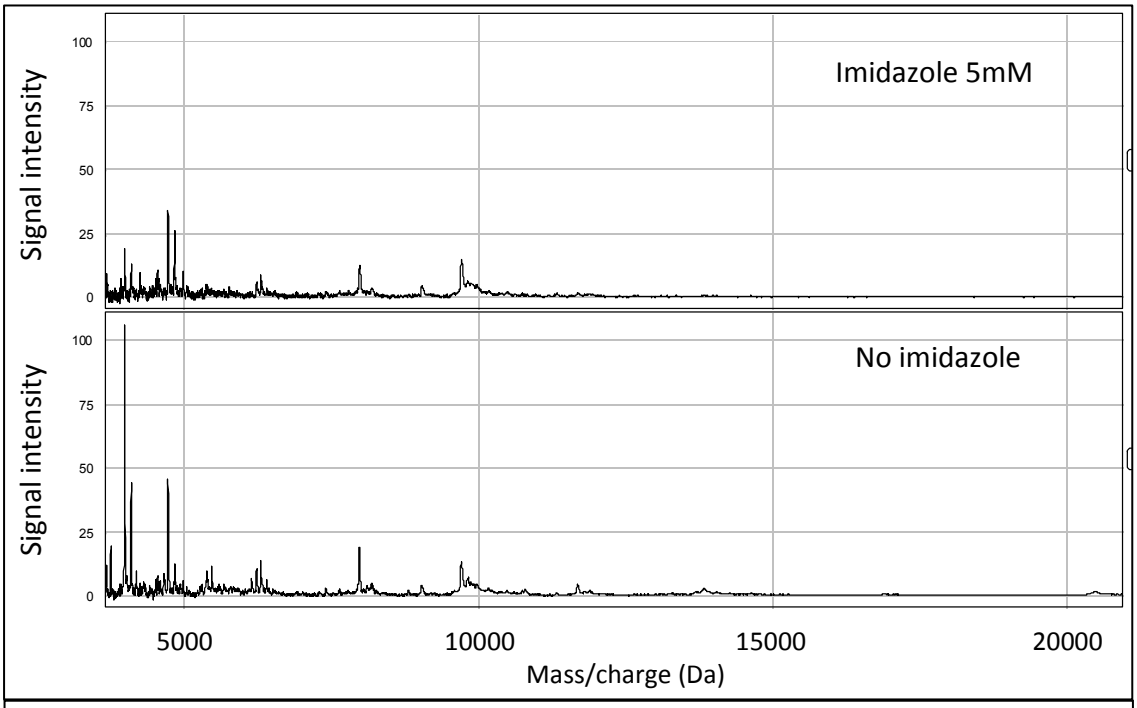


Figure 3.3.3.2. Representative spectra of urine SELDI analysis using an IMAC30 ProteinChip® array and copper loading; the effect of imidazole on peptide binding sensitivity. Imidazole reduced the sensitivity of peptide binding.

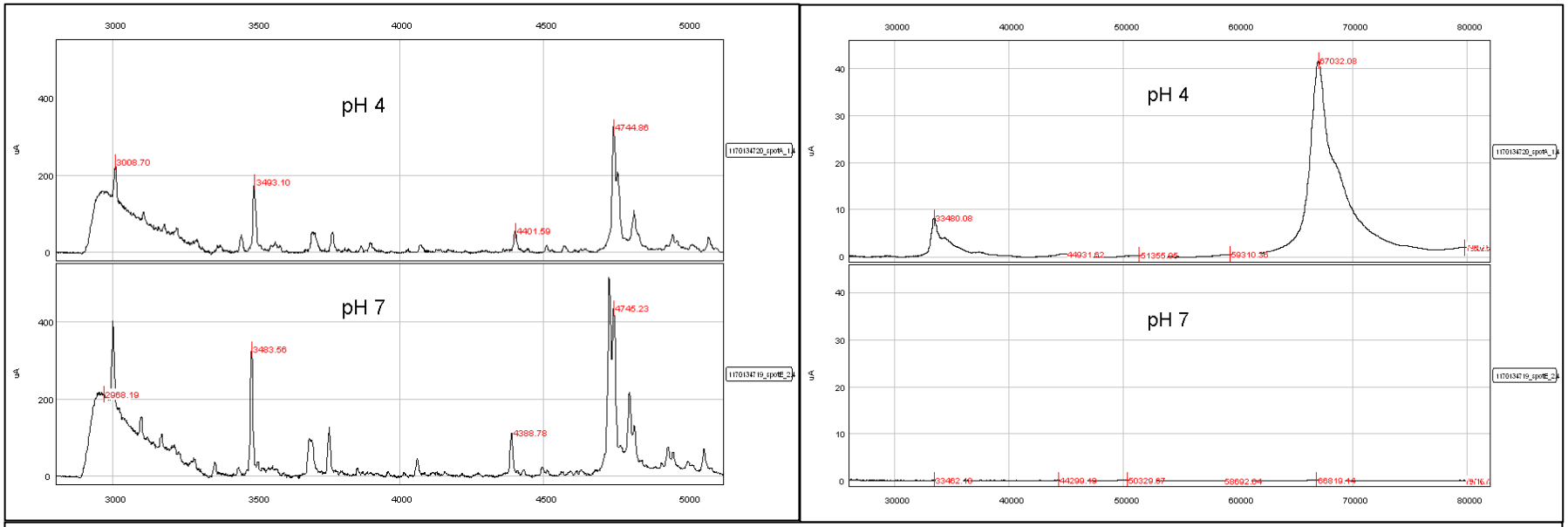


Figure 3.3.3.3. Representative spectra of low specificity washing and binding buffer (sodium acetate 0.1M pH 4.0) and high specificity washing and binding buffer (HEPES 50mM pH 7.0) with CM10 ProteinChip® array. Small peptide binding (left panel) is preserved but albumin binding is reduced (right panel).

Variation in the SELDI acquisition protocol improved the resolution of signal at the selected focus mass as expected (figure 3.3.3.4). Decreasing the matrix attenuation increased the number of small peptides revealed but at the expense of increased interference from the matrix (figure 3.3.3.5).

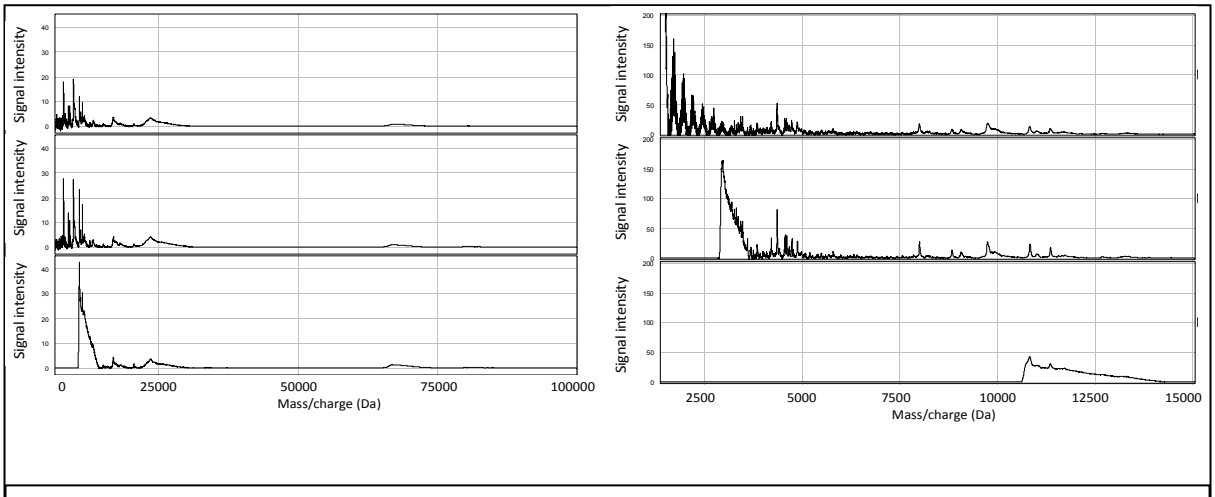


Figure 3.3.3.4 Representative spectra using CM10 ProteinChip® array and three protocols. Left panels, proteome from 3000 to 100000 Da; right panels, magnification of proteome from 2000 to 15000 Da. Top panels, protocol A; middle panels, protocol B; lower panels, protocol C (see text)

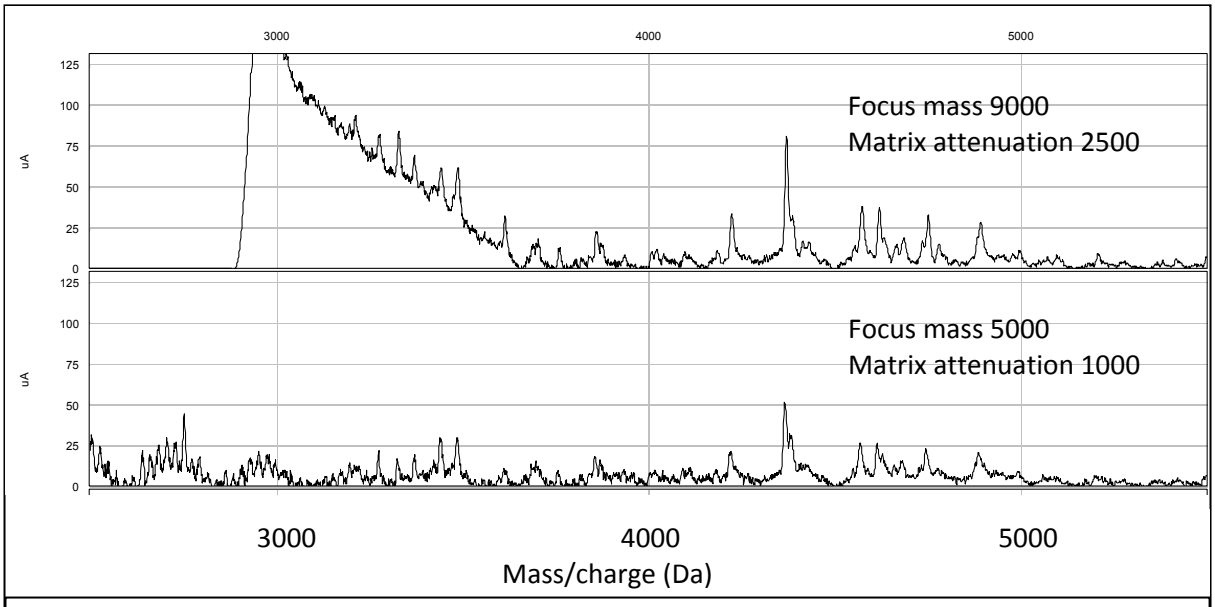


Figure 3.3.3.5. Representative spectra of CM10 ProteinChip® array SELDI analysis of urine. Decreasing the matrix attenuation reveals small peptide peaks (mass/charge 3000 to 4000 Da) at the expense of increased matrix interference (mass/charge <3000 Da).

High abundance proteins such as albumin can reduce the sensitivity of proteomic analysis by obscuring the peaks of lower abundance proteins, particularly in overtly albuminuric samples. Furthermore, fragments of proteins like albumin can be identified over a wide range of molecular weights (209). Removal of albumin by immunoprecipitation will increase the ability to examine low abundance proteins.

Unfortunately, albumin binds to many other peptides in urine and removal of the albumin will also remove these potential biomarkers. The techniques involved can be expensive and cumbersome and, although removal of albumin may be crucial in overtly albuminuric samples, in 'normal' urine the amount of albumin is likely to be tolerable. Data from this study support this, in that a peak at about 68000Da (likely to represent albumin) is very small or absent in all non-proteinuric patient samples compared to other peaks (figure 3.3.3.6).

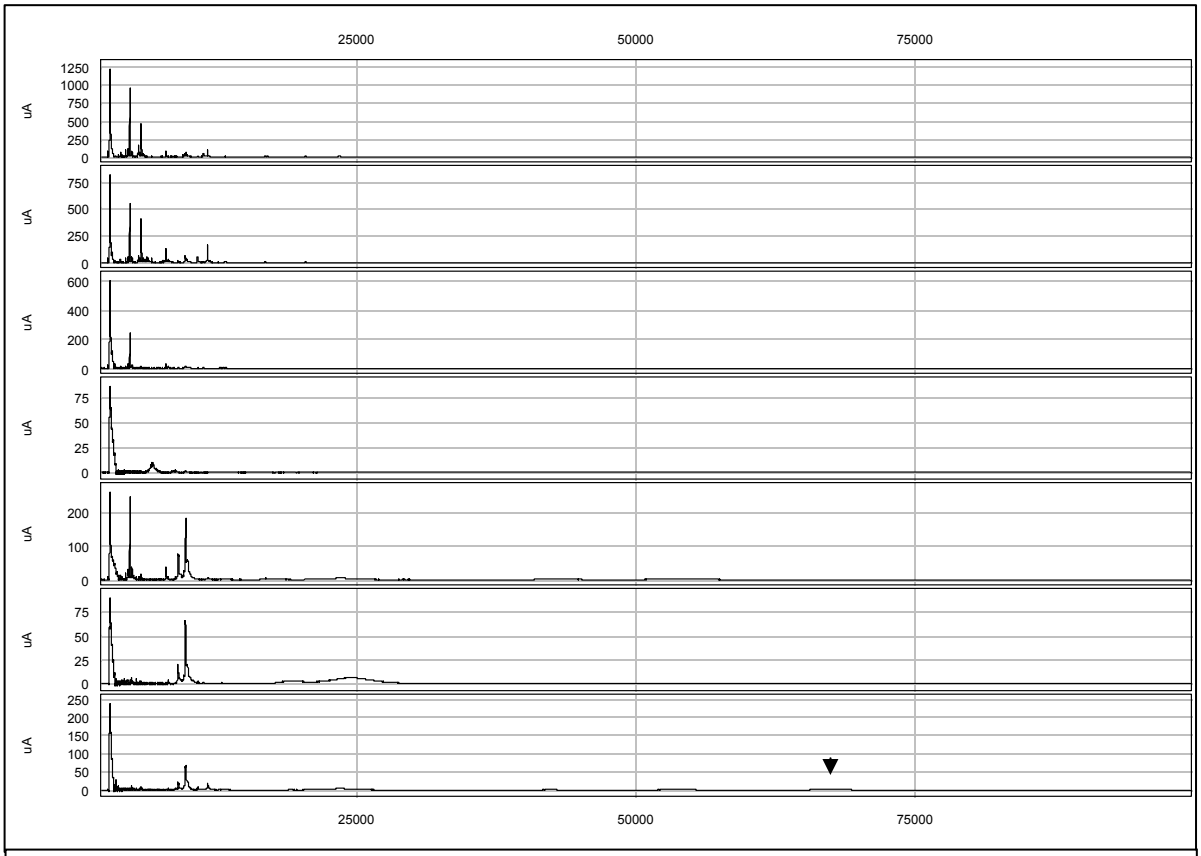


Figure 3.3.3.6. CM10 ProteinChip® SELDI profiles of healthy urine samples. A peak at about 68000Da (representing albumin, arrowhead) is absent or minimal in all spectra.

3.3.4 Discussion

Our results show that the most peaks were obtained with a CM10 ProteinChip® array which binds hydrophilic peptides. Given that the urinary proteome will predominantly contain soluble peptides this is to be expected. Hydrophobic peptides are found in urinary exosomes which can be extracted using established methodology (210), however, these would not be amenable to identification by a point-of-care test and were not extracted. The resolution of signal obtained using the least specific washing and binding buffer (sodium acetate 0.1M pH 4.0) appears to be sufficient for use in a comparative study. Removal of albumin was not performed in the study.

Although no urinary biomarkers predictive of pre-eclampsia are established in clinical practice, it was prudent to ensure that previously published putative predictive peptides would be identified with our protocol (114-126). Based on their physicochemical characteristics of previously described biomarkers and the binding properties of different ProteinChip® arrays stated by the manufacturer, almost all the peptides previously described would be expected to bind to a CM10 ProteinChip® array with sodium acetate 0.1M pH 4.0 washing and binding buffer (table 3.3.4.1). Subsequently this combination was selected for the clinical study.

Chip Array	pH	β-hCG	PlGF	α-1-μglobulin	Orosomucoid	Inhibin A	NAG	Albumin	s-Flt-1	PRL	SP-1 frag 1	SP-1 frag 2
CM10	4	1	1	1	1	1	1	1	1	1	0	1
	5	1	1	1	0	1	0	0	1	1	0	1
	6	1	1	0	0	1	0	0	1	0	0	1
	7	1	1	0	0	1	0	0	1	0	0	1
Q10	7.5	0	0	1	1	0	1	1	0	1	1	0
	9	0	0	1	1	0	1	1	0	1	1	0

Table 3.3.4.1. Predicted binding of proposed urinary biomarkers of pre-eclampsia with different washing and binding buffers. Key: 1, binding; 0, no binding.

Similarly, based on the molecular weight of the above biomarkers and accepting the findings of our optimisation study, a protocol with focus mass of 9000Da and matrix attenuation 2500 Da was chosen for the clinical study (protocol B).

3.4 Analysing proteomic data

Digital mass spectrometry generates large amounts of complex data. Maximising the utility of the information generated has led to an evolution in statistical methods paralleling the developments in data acquisition (211). The main challenges posed by such datasets are how to minimise false positive results as a result of the depth of data obtained. The benefits and limitations of techniques to address this are now discussed.

3.4.1 Univariate parametric or non-parametric continuous data comparison

The simplest method of identifying discriminatory peaks in mass spectrometry data is to quantify the signal intensity of each identified peptide peak and compare the average intensity between groups of spectra (pre-eclamptic versus normal pregnancy for example). Spectral data calculated in this way are seldom normally distributed and therefore median signal intensities can be compared using the Mann-Whitney *U* test (or Kruskal-Wallis test for multiple comparisons).

These tests are simple and rapid to perform, however, there is a high risk of identifying false positive results. There is also no consideration for potential interactions between peaks.

3.4.2 Principal component analysis

An alternative attempt to optimise data analysis is to reduce the complexity of the data. Instead of comparing the signal intensity of (for example) 300 peaks between two conditions, the data is collated into a reduced number of dimensions, each representing the most discriminatory features. In this way, data from 300 peaks might be summarised into as little as 10 “principle components” of which only 4 or 5 account for the vast majority of the differences seen between conditions. This limited number of components can then be compared statistically in more depth.

Although this is a useful statistical method to simplify the dataset, it is challenging to then “reverse engineer” the principle component to its constituent primary experimental variables. In mass spectrometry data this means that although a difference in proteome between samples might be identified, it is not possible to identify which peptide peaks are contributing to that difference.

3.4.3 Hierarchical clustering

A high number of data dimensions can be reduced whilst minimising loss of potentially important peaks by clustering variables (signal intensity of peptide peaks) together according to how close to each other they behave in discriminating groups. The importance of individual variables in defining a descriptive model can then be modified according their proximity in a dendogram (such as a heat map).

Hierarchical clustering of mass spectrometry data is challenging. The depth of complexity of data produced leads to inadequate clustering of variables and therefore limits the desired reduction of dimensionality.

3.4.4 Support vector machines

Support vector machines are supervised learning algorithms that identify discriminatory variables by combining them into multidimensional functions. These are developed by repeated iteration to obtain the optimum descriptive function of a binary classification (pre-eclamptic versus normal pregnancy, for example). A sample of unknown classification is then classified according to the derived function on which it comes to lie.

The use of support vector machines in mass spectrometry data is limited predominantly by the computational processing power required to derive the function. Similarly, allocating samples of unknown classification to the model in the test phase becomes slow to the point of being impractical.

3.4.5 Decision trees and random forest classifiers

An alternative method to utilise the depth of the mass spectrometry data obtained is to rank each variable and identify an optimum cut-off value for each variable according to its discriminatory potential. A binary logical model can then be derived to allocate each path through a decision tree to an outcome, and this model applied to data from samples of unknown classification in a test or utilisation phase (figure 3.4.5.1).

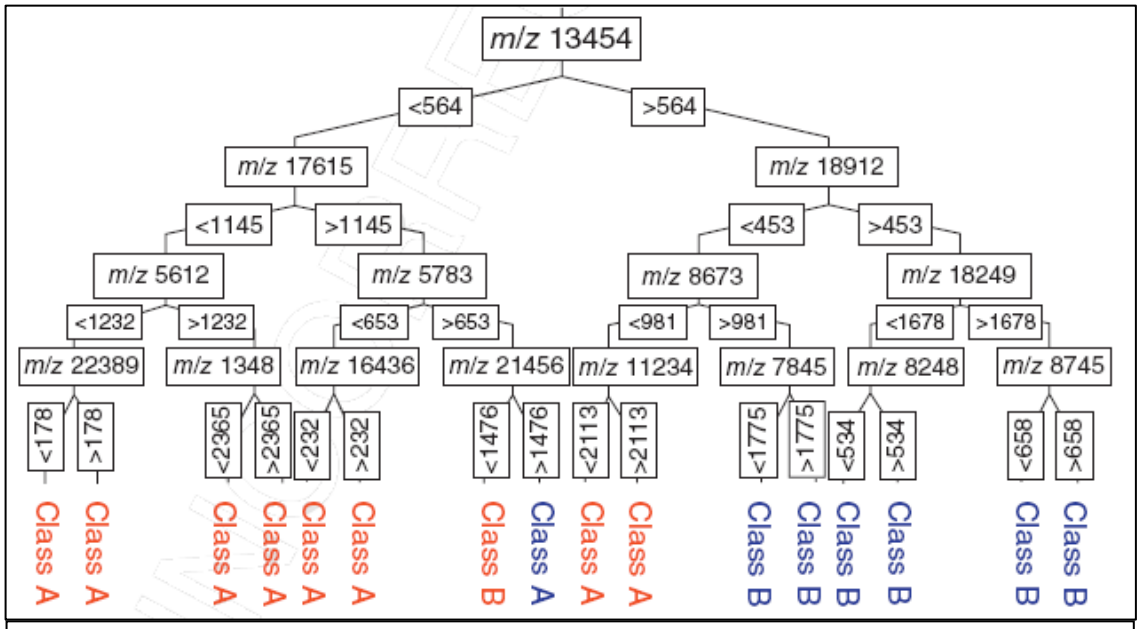


Figure 3.4.5.1 Example of a decision tree in a proteomic study. Peptide peaks are ranked according to their discriminatory power and cut-offs of signal intensity allocated to each. A logical model is derived to define each path through the tree to an outcome. Figure obtained from Ball and Al-Shahib (2010) (156)

In this example, a peak intensity of 700 at m/z 13454, 300 at m/z 18912, 1200 at m/z 8673 and 800 at m/z 11234 would result in the sample being identified as Class A. To improve the performance of decision trees, multiple trees can be derived from random subsets of variables. This “forest” of trees is linked such that

the output from data from a sample of unknown classification is defined by the most common output of all of the trees.

3.4.6 Artificial neural networks

Artificial neural networks (ANN) formulate a function to model data to an output. The function is derived through repeated iterations whereby the magnitude of change to the subsequent model is proportional to the error in performance of the preceding model. Multiple variables are input to each model through a variable number of hidden 'nodes' that interact within a network. The repeated iterations enable the model to learn the optimum function. Once the model has been 'learned' it can be applied to a training set to quantify its performance. Multiple models can be integrated in a final model.

ANNs are unsupervised learning algorithms and therefore it is difficult to evaluate how a function came to be derived from its constituent variables. Nevertheless, they are capable of reducing the excess dimensionality of proteomic data into simplified functions that can be used to classify novel data with high levels of accuracy.

3.5 Clinical study of urine proteomics in predicting pre-eclampsia

3.5.1 Aims

Given that proteinuria is a near-ubiquitous finding in PE, we hypothesised that the development of overt PE may be preceded by subtle alterations in urine protein excretion that would previously have been undetectable by standard methods of analysis. We therefore chose to use surface-enhanced laser desorption and ionization combined with time-of-flight mass spectrometry (SELDI) to screen urine early in pregnancy for biomarkers predictive of the development of PE.

In this study we aimed to find biomarkers that may be able to be identified using a urine dipstick as a point-of-care test. We therefore developed a protocol to minimise the chance of proteome degradation or contamination to mimic bedside conditions as closely as possible.

Early diagnosis of PE will permit focussed monitoring of maternal and fetal progress during pregnancy to optimise timing of delivery and successful outcomes. Similarly, exclusion of PE in early pregnancy in women at known risk of the condition will obviate the anxiety associated with frequent hospitalisation and reduce the demand on medical resources.

To our knowledge there has been only one other mass spectrometry based biomarker discovery study to follow women throughout pregnancy to identify urine biomarkers predictive of future PE prior to 20 weeks gestation (160).

3.5.2 Methods

3.5.2.1 Participants and recruitment

Participants were recruited from the high risk pregnancy clinic at the Leicester Royal Infirmary between March 2008 and July 2009. Patients were referred to the clinic from community midwives, primary care physicians or general antenatal clinics based on a past or family history of hypertensive disorders of pregnancy, chronic hypertension or obesity (figure 3.5.2.1). Consecutive patients less than 20 weeks gestation were invited to participate and informed consent obtained by the investigator prior to submission of urine samples at subsequent visits. Patients with known chronic kidney disease (CKD stage 1-5) or diabetes mellitus were excluded.

Demographic and clinical data were obtained from hospital records and patient self-reporting.

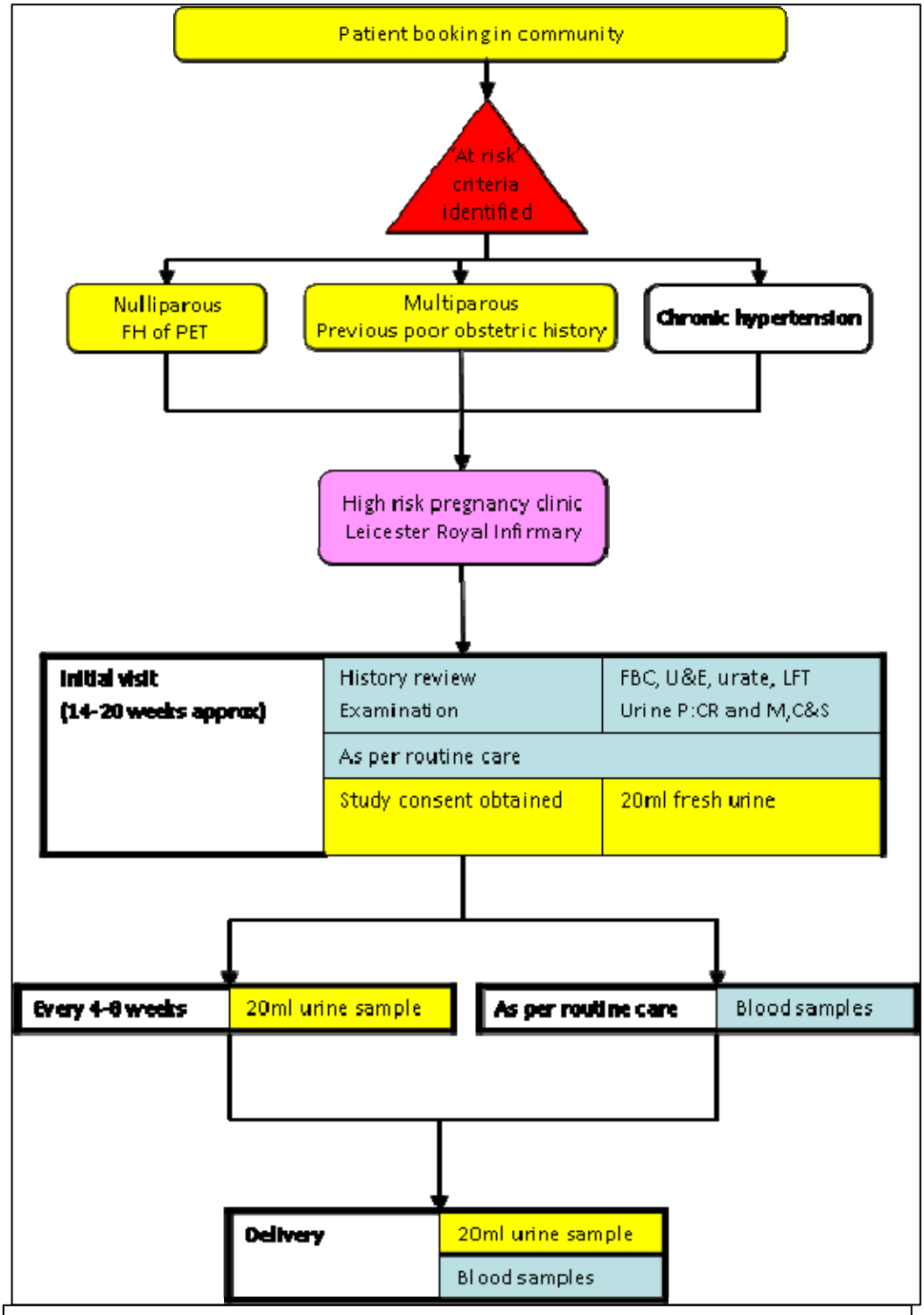


Figure 3.5.2.1. Recruitment and study protocol

Blood pressure, medication history and a 20ml mid-stream clean catch urine specimen were obtained after consent and at each subsequent outpatient clinic attendance for the duration of the pregnancy. Standard clinical care including blood tests, medication prescription, radiological investigation, timing and method of delivery were directed by an independent team of clinicians.

Ethical approval for the study was granted by the Leicestershire, Northamptonshire and Rutland Research Ethics Committee.

3.5.2.2 Classification of Pregnancy Outcomes

Following delivery pregnancies were characterised into five categories; normal, pre-eclampsia, superimposed pre-eclampsia, pregnancy-induced (gestational) hypertension or chronic hypertension. Samples were grouped according to gestational age at time of collection; less than 20 weeks, 20 to 25 weeks, 26 to 31 weeks, 32 to 37 weeks or after 37 weeks

Pre-eclampsia was defined according to the research definition of the International Society for the Study of Hypertension in Pregnancy (85); namely, *de novo* hypertension (>140/90 mmHg) and proteinuria (urinary protein excretion >300mg/d or urine protein:creatinine ratio >30mg/mmol creatinine) after 20 weeks gestation that resolved postpartum. Superimposed pre-eclampsia was defined according to the criteria of the National High Blood Pressure Education Programme; namely, new onset proteinuria (>300mg/d or protein:creatinine ratio >30mg/mmol creatinine) in women with hypertension but no proteinuria early in pregnancy, sudden increase in proteinuria, sudden increase in previously well-controlled blood pressure, thrombocytopenia (<100 cells/ml) or increase in

transaminases in women with hypertension and proteinuria prior to 20 weeks' gestation (120). Pregnancy-induced (gestational) hypertension was defined as *de novo* hypertension (>140/90 mmHg) during pregnancy with or without the need for antihypertensive medication that resolved postpartum. Chronic hypertension was defined as a blood pressure greater than 140/90mmHg on more than one occasion prior to conception or less than 20 weeks gestation, or the requirement for antihypertensive medication prior to conception.

Preterm delivery was defined as delivery before 37 weeks gestation. Low birth weight was defined as less than 2.5kg and very low birth weight as less than 1.5kg. Corrected birth weight percentiles were calculated from the data of Gardosi et al. (17). Small for gestational age infants were defined as those below the 10% centile at birth.

3.5.2.3 Urine analysis and preparation

Clean catch urine samples were immediately placed on ice and underwent centrifugation within 30 minutes of micturition (13400g x 2 minutes) to remove cellular debris. Protease inhibitors and inhibitors of bacterial growth were not added to samples. Supernatants were kept on ice until SELDI TOF MS analysis later that day. Simultaneous urine dipstick analysis was performed on all collected urine samples, and in those testing positive for protein the proteinuria was quantified by measuring urine protein:creatinine ratio as per routine care. Samples positive for leucocyte esterase or nitrites underwent microscopy and culture. Treatment of symptomatic and asymptomatic bacteruria was as per local

clinical guidelines. Urine samples in which bacteruria was later confirmed were excluded from all spectral analyses.

To account for variations in hydration status, normalisation of urine biomarker excretion between women was achieved by correcting all samples to the same creatinine concentration (20mg/dl) before SELDI (212). Urine creatinine concentrations were measured using a colorimetric assay based on the Jaffe reaction (chapter 3.2.6) (198).

3.5.2.4 Proteomic analysis by SELDI

Mass spectra were obtained from normalised urine specimens on the same day as collection by SELDI. Urine did not undergo denaturation. We selected the cation-selective peptide array (CM10 ProteinChip®, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) for the entire study because of the large number of mass/charge features observed and its reproducibility in detecting protein species in urine (see chapter 3.3.2).

CM10 ProteinChip® arrays were prepared in a Bioprocessor® unit according to an optimised variant of the manufacturer's instructions using 0.1M sodium acetate pH 4.0 as a low-specificity binding and washing buffer. Each array spot was washed with 200µl of buffer for 5 minutes with agitation twice. 150µl of the normalised urine sample supernatants were applied to the array spot for 30 minutes with agitation and then removed. Following three further 5 minute washes with buffer, spots were rinsed twice with 200µl deionised purified water. Sinapinic acid (3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid) was used as an energy absorbing matrix and 1µl of a saturated solution in 0.5% (v/v)

trifluoroacetic acid and 50% (v/v) acetonitrile was applied to each array spot twice and allowed to air dry.

ProteinChip® arrays were analysed using a Ciphergen ProteinChip System Series 4000. Each sample analysis was performed with focus mass of 9000Da, range 0 to 100000Da, warming shot energy 4400nJ, data shot energy 4000nJ, sampling rate 400Hz, matrix attenuation 2500Da and a 25kV source of positive ions. The mass spectra of the samples were generated using an average of 10 laser shots. Upon acquisition of all the data, spectra were normalised according to total ion content. The mass-charge ratio (m/z) of each of the proteins/peptides captured on the array surface was determined according to the externally calibrated standards (Protein MW Standards (Bio-Rad Laboratories Ltd, Hemel Helpstead, UK): Arg8-vasopressin (1084.25 Da), somatostatin (1637.9 Da), dynorphin (2147.5 Da), bovine insulin β-chain (3495.94 Da), human insulin (5807.65 Da), bovine ubiquitin (8564.8 Da), and bovine cytochrome C (12230.9 Da)) and the system recalibrated according to the manufacturer's instructions.

Due to the prospective nature of the study and the immediate SELDI analysis of all specimens, urine samples were, by default, analysed in random order on consecutive ProteinChip® array spots with the investigator blinded to clinical outcome.

3.5.2.5 Statistical analysis

Baseline characteristics and pregnancy outcomes were compared between normal and pre-eclamptic pregnancies using 2-sided independent sample t tests for continuous parametric variables, Mann Whitney *U* test for continuous non-

parametric variables and Fisher's Exact test or Chi squared test for categorical variables.

Given the hypothesis-free nature of the study, power calculations to identify required patient recruitment were not valid. In the absence of published evidence or guidelines, we used results from studies using similar technology to estimate the required samples size. In previous exploratory clinical trials, 5 to 50 samples per group have been used (213,214). From the high risk pregnancy clinic at Leicester Royal Infirmary, an incidence rate of pre-eclampsia of 10% to 15% was estimated, requiring approximately 150 patients to be recruited to yield about 15 patients with pre-eclampsia .

Spectral analysis was based on artificial neural network modelling (ANN) using the predictive performance of multiple non linear ANN based models to identify the most predictive (and therefore most biologically relevant) ion species from the SELDI TOF MS spectra. Extensive Monte Carlo cross validation was incorporated preventing over-fitting whilst ensuring the ions found had relevance for the broader population. This approach has been shown to determine a parsimonious subset of ions across a range of diseases and sample types (213,215-217). In brief, spectra obtained from specimens collected at <20 weeks gestation categorised as normal pregnancy and pre-eclampsia were included and then aligned into equal mass/charge bins and peaks identified based on median intensity to noise ratio. The 2000 most intense peaks with mass/charge between 4000 and 20000 Da were input to an artificial neural network algorithm to identify peaks discriminating normal and pre-eclamptic pregnancies (Compandia Ltd.,

Nottingham) (213,216). Spectra obtained from urine samples in pregnancies progressing to superimposed pre-eclampsia, pregnancy-induced (gestational) hypertension or in patients with chronic hypertension were not included in comparative analysis. Random sample cross-validation with 50 models was performed and single features ranked based on their classification performance for blind data. Receiver operating curve (ROC) analysis for each random sample model was performed and the mean area under the curve calculated to identify the most predictive peaks. Stepwise addition of these peaks created a final predictive model based on a panel of putative biomarkers which was applied to the original spectra to assess performance.

The same procedure was performed independently on spectra from sample obtained between 20 and 25 weeks gestation.

A p value ≤ 0.05 was considered significant.

3.5.3 Results

3.5.3.1 Patient demographics and clinical details

Of 155 women invited to participate, 145 consented to the study and 142 contributed at least one urine specimen. Forty patients were excluded from the spectral analysis; 12 with chronic hypertension, 4 with superimposed pre-eclampsia, 22 who developed gestation (pregnancy-induced) hypertension, 2 who had a normal pregnancy but failed to provide a urine sample at <25 weeks gestation and 2 who were lost to follow up (figure 3.5.3.1).

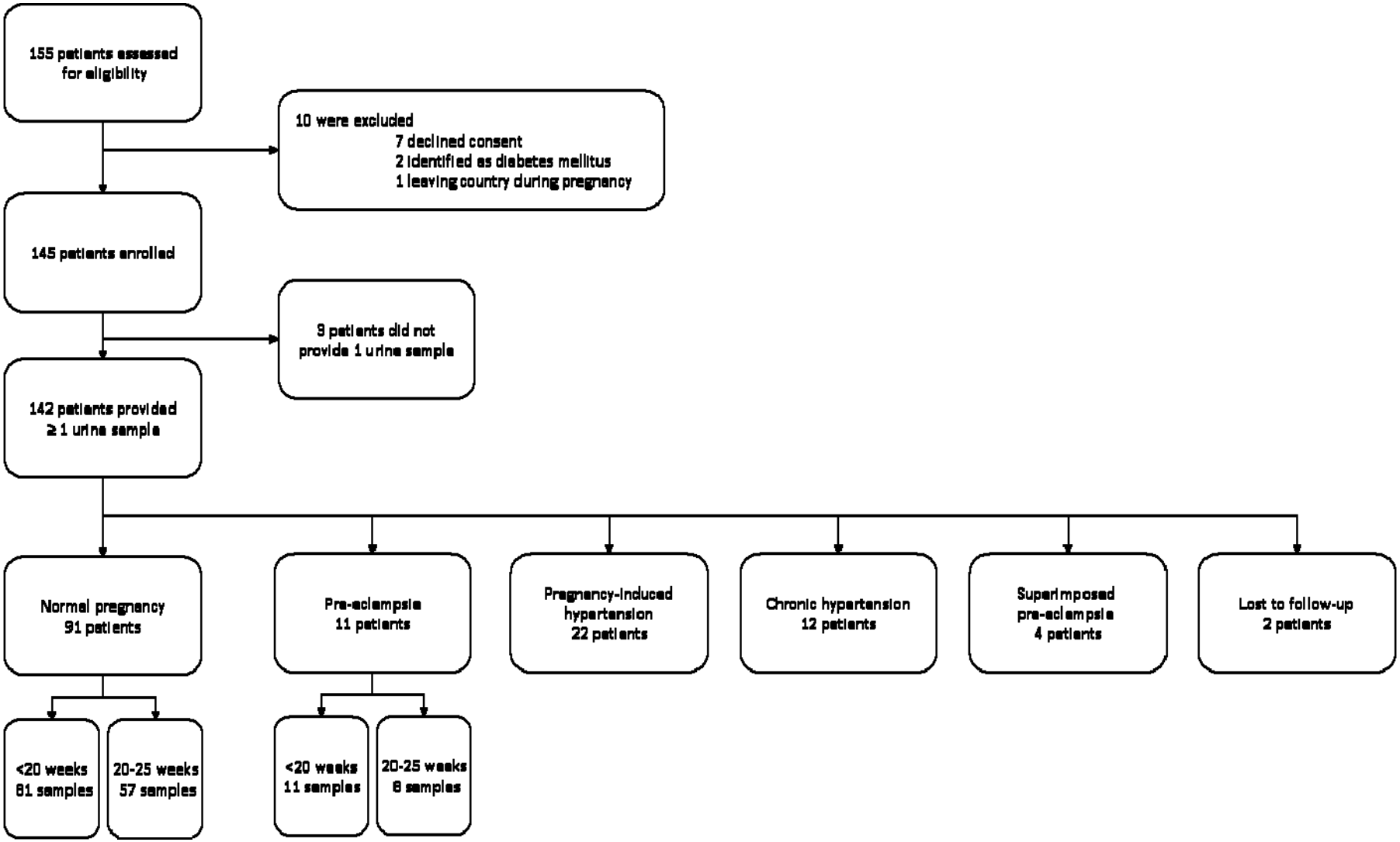


Figure 3.5.3.1. Participant enrolment and outcomes

The remaining recruited patient cohort was typical of a population at high risk of pre-eclampsia (218). There were no significant differences in any demographic features at enrolment between those high risk women who developed pre-eclampsia and those who had a normal pregnancy (table 3.5.3.1).

	Total	Normal pregnancy	Pregnancy complicated by pre-eclampsia	p value
n	102	91	11	
Age at conception (years)	28.8±5.8	28.8±6.0	29.2±4.3	0.83
Ethnicity European South Asian African-Caribbean Other	 89 (86%) 6 (6%) 7 (7%) 1 (1%)	 78 (86%) 6 (7%) 7 (8%) 0(0%)	 10 (91%) 0 (0%) 0 (0%) 1 (9%)	0.06
Current smoker	7 (10%)*	5 (8%)*	2 (18%)*	0.13
Gravida (median (range))	2 (1-11)	2 (1-11)	2 (1-8)	0.65
Primigravida (n (%))	26 (26%)	22 (24%)	4 (36%)	0.46
Multiple pregnancy (n (%))	0 (0%)	0 (0%)	0 (0%)	NA
Past history of pre-eclampsia (n (%))	35 (46%)**	31 (45%)**	4 (57%)**	1.00
Family history of pre-eclampsia (n (%))	15 (15%)	12 (13%)	3 (27%)	0.20
BMI at booking (kg/m2)	30.8±8.4	30.5±8.1	33.4±10.2	0.30
Systolic blood pressure at booking (mmHg)	123±12	123±12	124±13	0.67
Diastolic blood pressure at booking (mmHg)	77±11	77±11	79±9	0.54
Prescription of aspirin prophylaxis (n (%))	15 (15%)	14 (15%)	1 (9%)	1.00

Table 3.5.3.1. Demographic characteristics of participants included in urine proteomic spectral analysis. *Missing data for 28 patients **Excluding primigravida

Pre-eclampsia was diagnosed at 27 to 41 weeks (median 38 weeks, IQR 36.8 to 38.2 weeks) and, as expected, was associated with an increased frequency of pre-term delivery, caesarean section and low birth weight (table 3.5.3.2). No episodes of pre-eclampsia were severe accordingly to ISSHP criteria (table 3.5.3.3).

	Normal pregnancy (n=91)	Pre-eclampsia (n=11)	p
Caesarean section	28 (31%)	7 (63.6%)	0.04
Stillbirth	0 (0%)	0 (0%)	NA
Neonatal death	0 (0%)	0 (0%)	NA
Survival with impairment	0 (0%)	2 (18.2%)	0.01
Gestational age at delivery (weeks)	39.8±1.5	37.7±3.3	<0.001
Preterm delivery	6 (6.6%)	2 (18.2%)	0.21
Birth weight (kg)	3.51±0.5	3.21±1.08	<0.001
Low birth weight	2 (2.2%)	3 (27.3%)	0.008
Very low birth weight	0 (0%)	1 (9.1%)	0.11
Small for gestational age	9 (10%)	2 (18%)	0.33

Table 3.5.3.2 Pregnancy outcomes in those patients who had a normal pregnancy and those who developed pre-eclampsia. Values mean±SD or n (%).

Patient	Maternal age at conception (years)	Gravida	Gestational age (weeks)		Maximum blood pressure (mmHg)	Maximum proteinuria (P:CR, mg/mmol creatinine)	Delivery Method	Birth Weight (kg)	Birth weight centile (%)	Fetal Outcome
			at diagnosis of pre-eclampsia	at delivery						
1	25.4	2	27.1	29.6	159/97	37	Elective Caesarean	0.75	<1	Alive with impairment
2	31.0	8	30.9	33.6	145/99	351	Elective Caesarean	1.96	10.5	Alive with impairment
3	34.7	3	37.1	37.4	149/91	194	Normal Vaginal	2.98	31.9	Alive and no impairment
4	25.0	5	36.4	37.9	162/98	78	Emergency Caesarean	3.94	96.9	Alive and no impairment
5	28.2	2	37.1	37.9	158/108	82	Elective Caesarean	2.48	5.9	Alive and no impairment
6	24.1	5	38.0	39.0	165/116	48	Normal Vaginal	3.62	47.3	Alive and no impairment
7	24.4	6	38.6	39.0	157/92	39	Normal Vaginal	3.96	81.5	Alive and no impairment
8	37.2	1	38.0	39.6	158/95	48	Emergency Caesarean	3.9	94.1	Alive and no impairment
9	29.7	1	38.0	39.6	170/104	210	Emergency Caesarean	4.24	76.5	Alive and no impairment
10	31.7	1	39.9	40.1	164/93	50	Emergency Caesarean	3.82	60.2	Alive and no impairment
11	29.5	1	41.0	41.1	160/110	86	Normal Vaginal	3.65	46.4	Alive and no impairment

Table 3.5.3.3. Clinical characteristics of pre-eclamptic pregnancies. P:CR, protein:creatinine ratio

Urine specimens included in the predictive models were collected prior to the development of clinical features of pre-eclampsia (figure 3.5.3.2). In the model derived from samples obtained <20 weeks gestation samples were obtained at a minimum of 12.7 weeks (median 21 weeks, interquartile range (IQR) 20 to 22 weeks) before a clinical diagnosis of pre-eclampsia was made in affected cases.

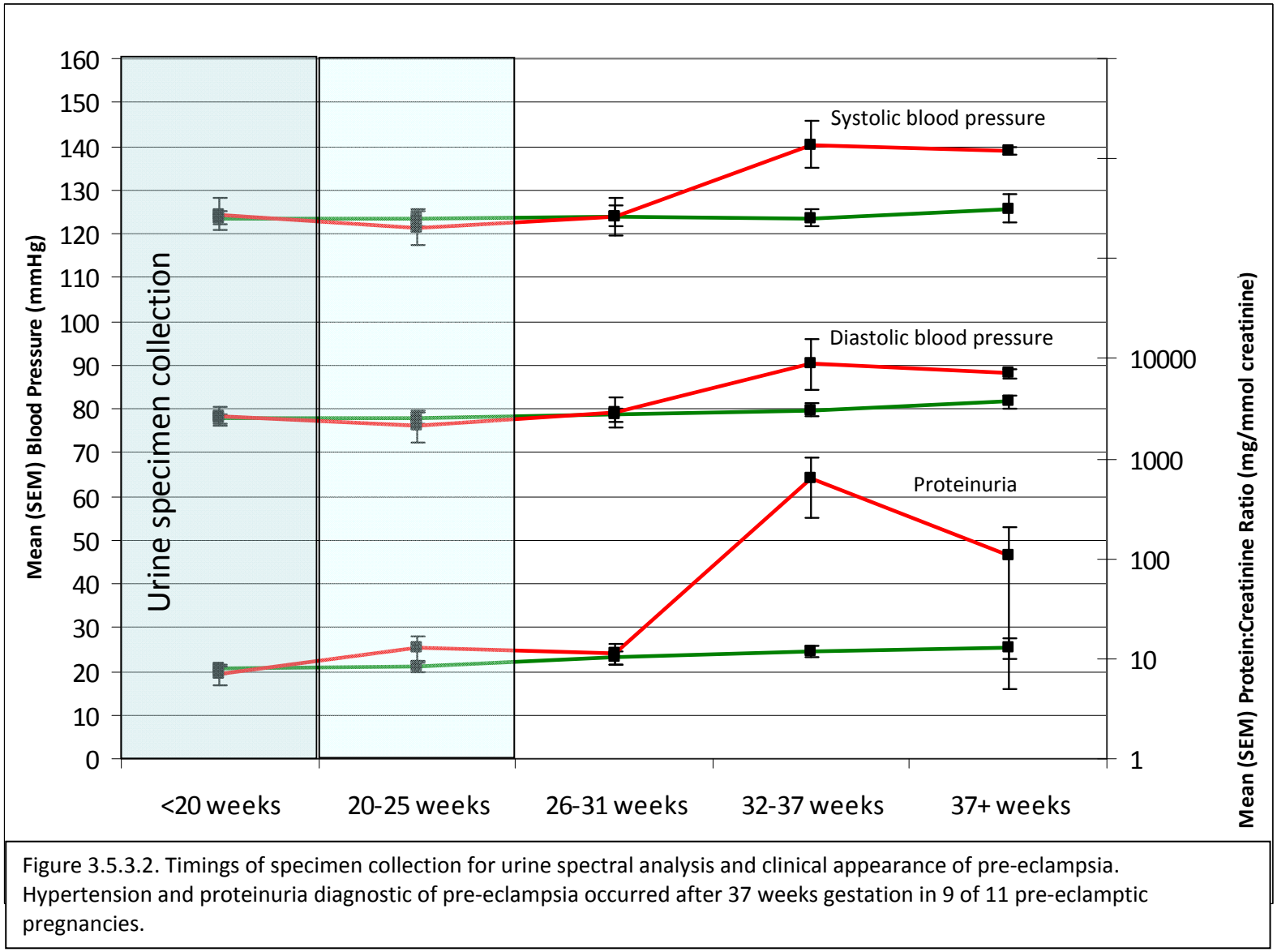


Figure 3.5.3.2. Timings of specimen collection for urine spectral analysis and clinical appearance of pre-eclampsia. Hypertension and proteinuria diagnostic of pre-eclampsia occurred after 37 weeks gestation in 9 of 11 pre-eclamptic pregnancies.

3.5.3.2 Development of diagnostic models from SELDI TOF MS analysis: <20 weeks gestation

In the training set, which included spectra from all pre-eclamptic pregnancies and a subset from normal pregnancies, 793 peaks were found to be differentially expressed in urine samples collected <20 weeks gestation between those women who had a normal pregnancy and those who went on to develop pre-eclampsia. ANN modelling using two hidden nodes and approximately 3000 iterations was performed to identify which peaks described the outcome data in the most parsimonious manner.

ANN modelling identified 5 protein peaks that provided the greatest discrimination between normal and pre-eclamptic pregnancies. Inclusion of further discriminating peaks did not improve the overall performance of the model. In decreasing order of contribution to the final model, mass/charge of the included peaks were 9080 Da, 8020 Da, 4648 Da, 4813 Da and 11320 Da. Representative spectra are shown in figure 3.5.3.3.A.

For each included peak, a non-linear regression function was derived relating the signal intensity of each identified peak to the probability of developing pre-eclampsia (figure 3.5.3.3.B). Peak intensity was positively correlated to probability of pre-eclampsia for 9080 Da and 11320 Da, negatively correlated for 8020 Da and 4813 Da and a parabolic relationship identified for peak 4648 Da.

The model was applied to 50 cross validation training sets, each of 40 spectra. Spectra from pre-eclamptic pregnancies used to derive the model were also used in the training sets, with random spectra from normal pregnancies not included in

derivation of the model. Spectra from women with chronic hypertension and superimposed pre-eclampsia were not included in these evaluations.

The output correctly classified 100% of women who would go on to have a normal pregnancy and 92% of women who would develop pre-eclampsia (mean ROC area under curve 0.88 , range 0.5–0.97; figure 3.5.3.3.C. A representative model output for one of the 50 training sets is shown in figure 3.5.3.4. In this example, a probability cut-off of 0.50 correctly identified 28 of 29 (97%) normal pregnancies and 7 of 11 (63%) pre-eclamptic pregnacies. One of two early onset pre-eclamptic pregnancies was correctly identified.

Of the five protein peaks identified, peak 1 (m/z 9080.29 Da) contributed 80% of the model's predictive power (figure 3.5.3.5); the remaining 4 peaks <10% each.

Application of this derived 5 peak model to all urine specimens collected at <20 weeks gestation, yielded a sensitivity of 87% and specificity of 82% for the detection of pre-eclampsia.

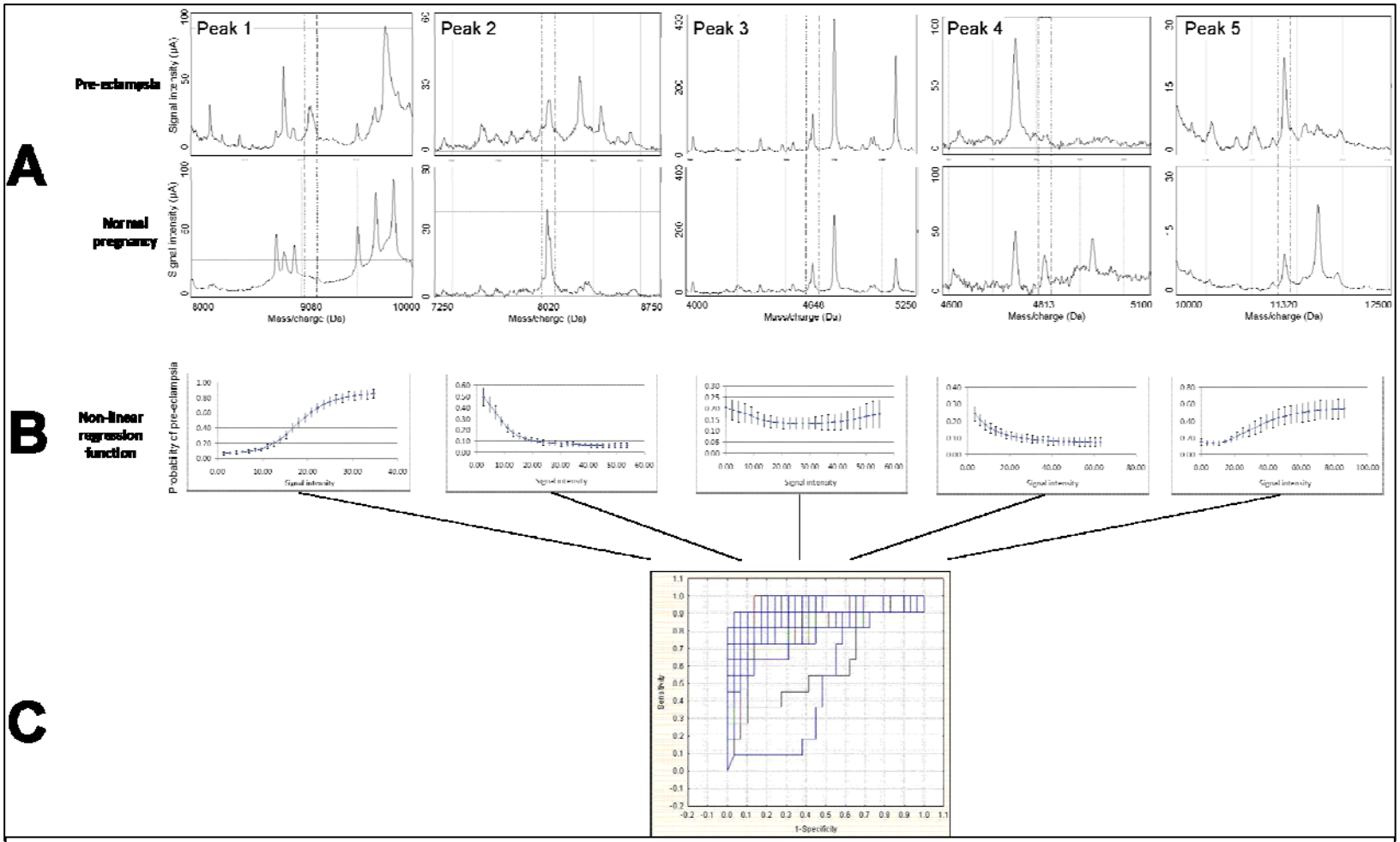


Figure 3.5.3.3. Development of diagnostic models from SELDI TOF MS analysis. A: Representative SELDI TOF MS spectra of five peptide peaks included in an artificial neural network model predictive of subsequent pre-eclampsia, obtained from urine collected <20 weeks gestation in pre-eclamptic and normal pregnancy. B: Non-linear regression functions of individual protein peaks in predicting pre-eclampsia. C: Receiver operator characteristic (ROC) curves for the performance of the multivariate 5 protein panel in discriminating women at <20 weeks gestation who develop pre-eclampsia from those who have a normal pregnancy. Multiple curves represent results from 50 cross validated models (mean area under curve 0.88).

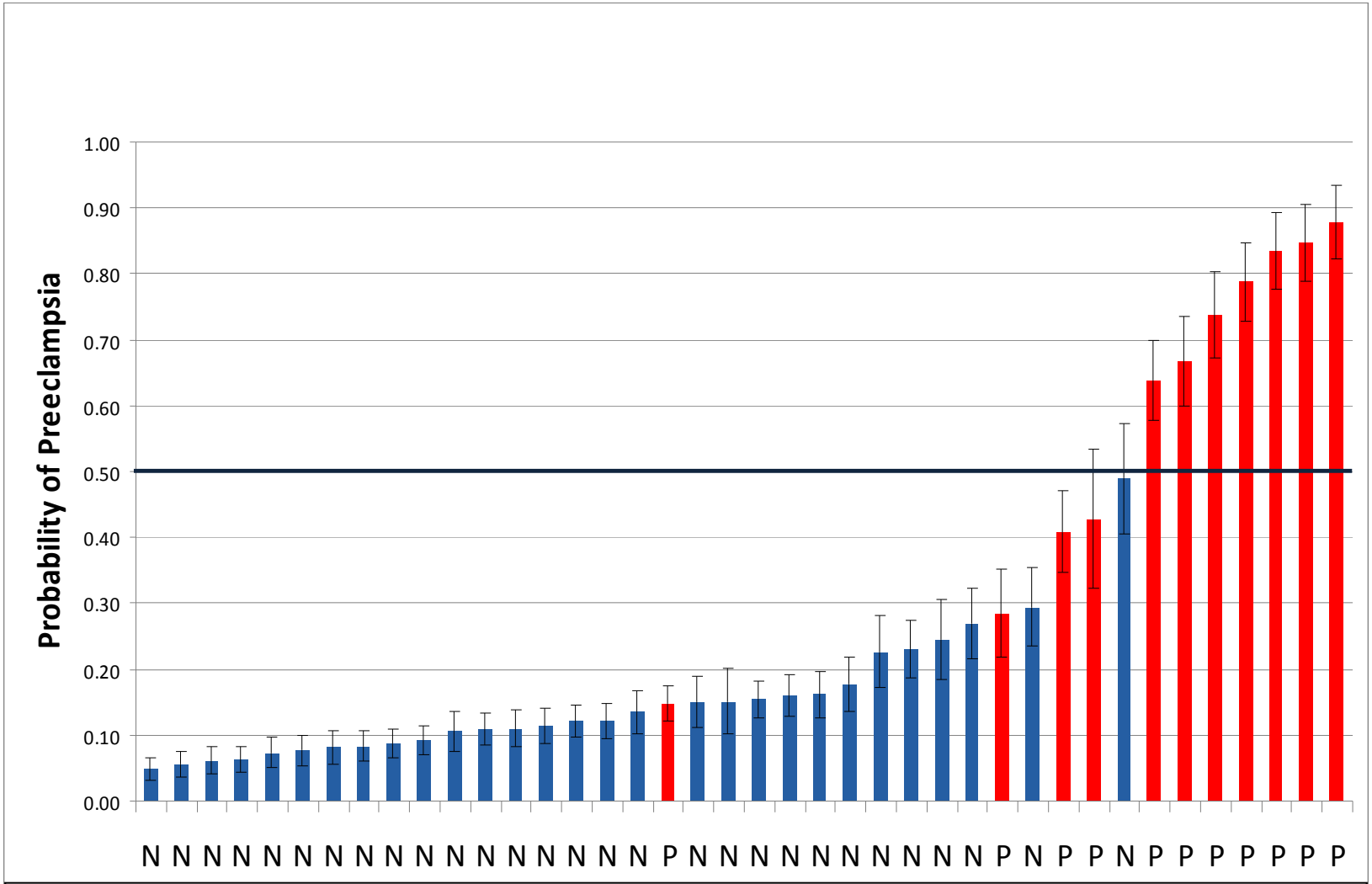


Figure 3.5.3.4. Development of diagnostic models from SELDI TOF MS analysis: <20 weeks gestation. Performance data for a test set. Columns represent individual participants; red bars (P) represent cases of pre-eclampsia, blue bars (N) represent normal pregnancies. Using a 50% probability cutoff, the model misclassified one normal pregnancy and 4 pre-eclamptic pregnancies.

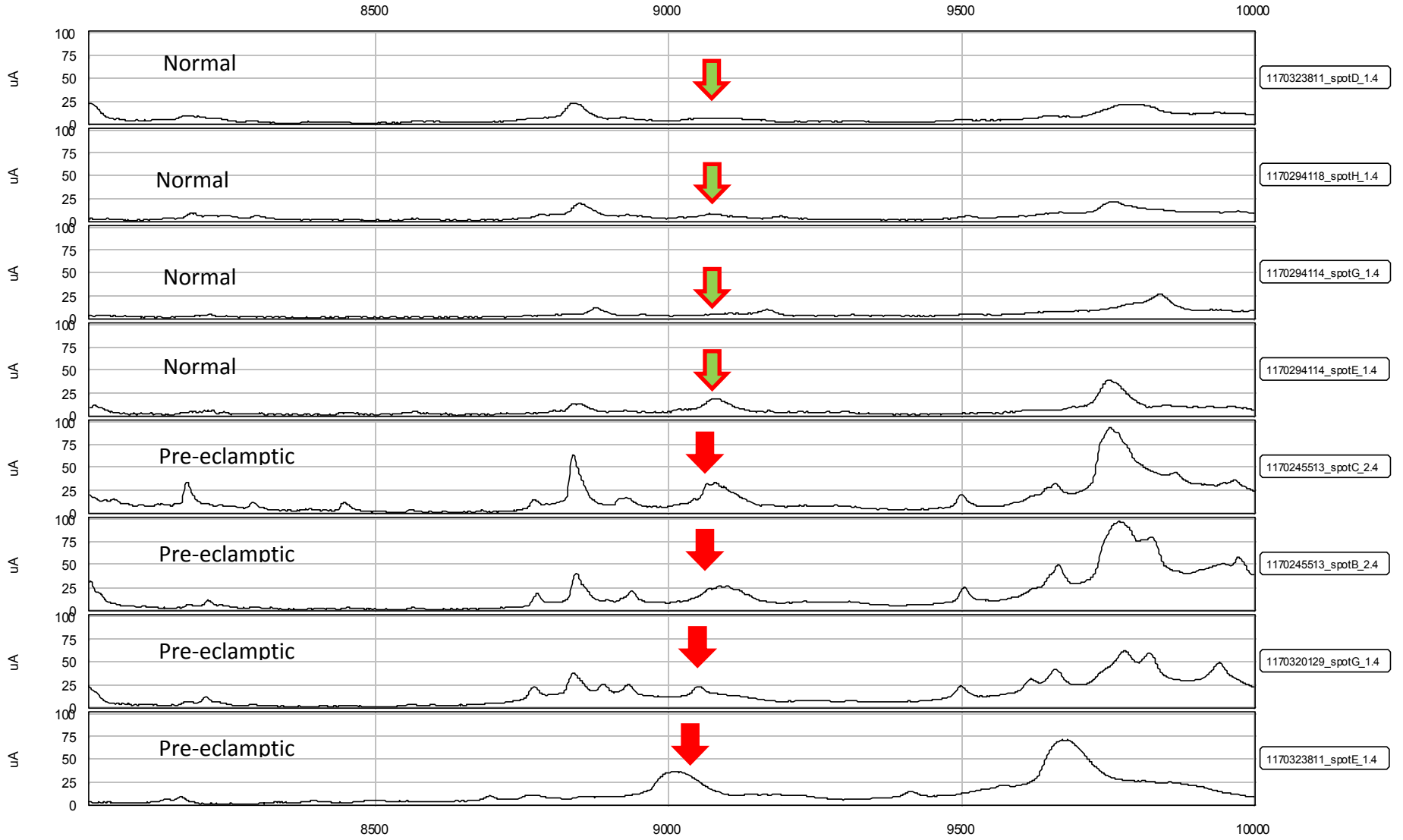


Figure 3.5.3.5. Representative SELDI spectra from urine samples obtained less than 20 weeks gestation in normal and pre-eclamptic pregnancies. Signal intensity of a peak at 9080 Da (arrows) is increased in pre-eclamptic pregnancies.

3.5.3.3 Development of diagnostic models from SELDI TOF MS analysis: 20 to 25 weeks gestation

ANN modelling of 6756 peaks in spectra from samples collected at 20 to 25 weeks gestation identified a single peak (m/z 4254.4 Da) predictive of subsequent pre-eclampsia (figures 3.5.3.6. and 3.5.3.7). The addition of further discriminating peaks did not improve the model performance further.

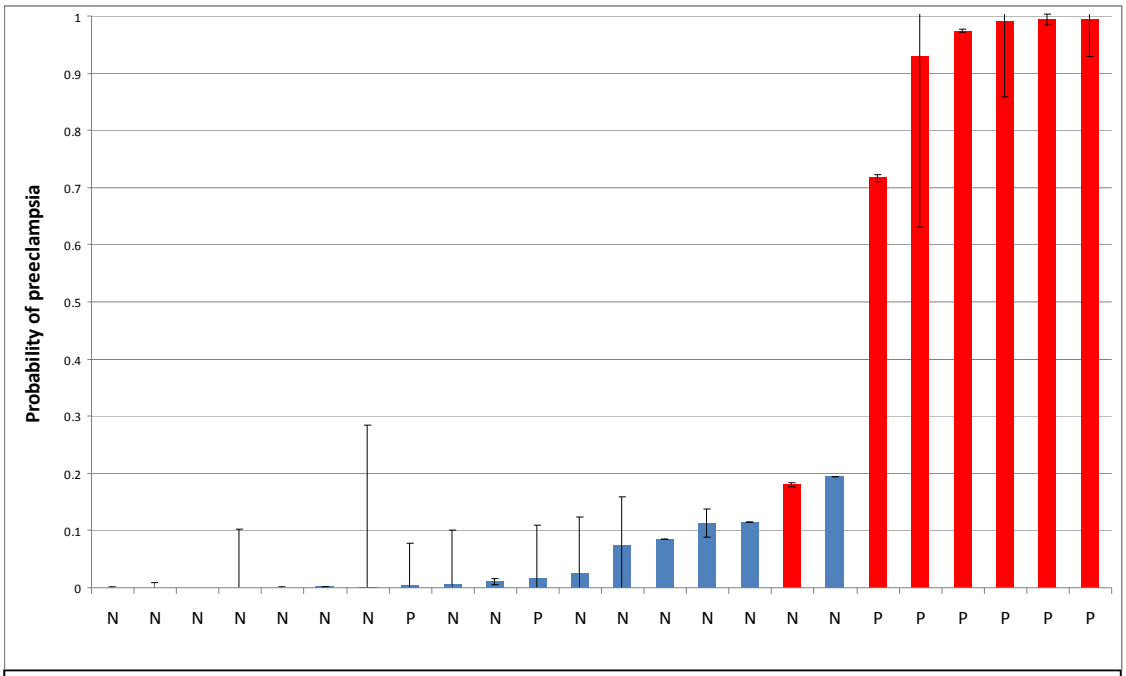


Figure 3.5.3.6. Development of diagnostic models from SELDI TOF MS analysis: 20 - 25 weeks gestation. Performance data for a test set. Columns represent individual participants; red bars (P) represent cases of pre-eclampsia, blue bars (N) represent normal pregnancies. Using a 50% probability cutoff, the model misclassified no normal pregnancy and 2 pre-eclamptic pregnancies.

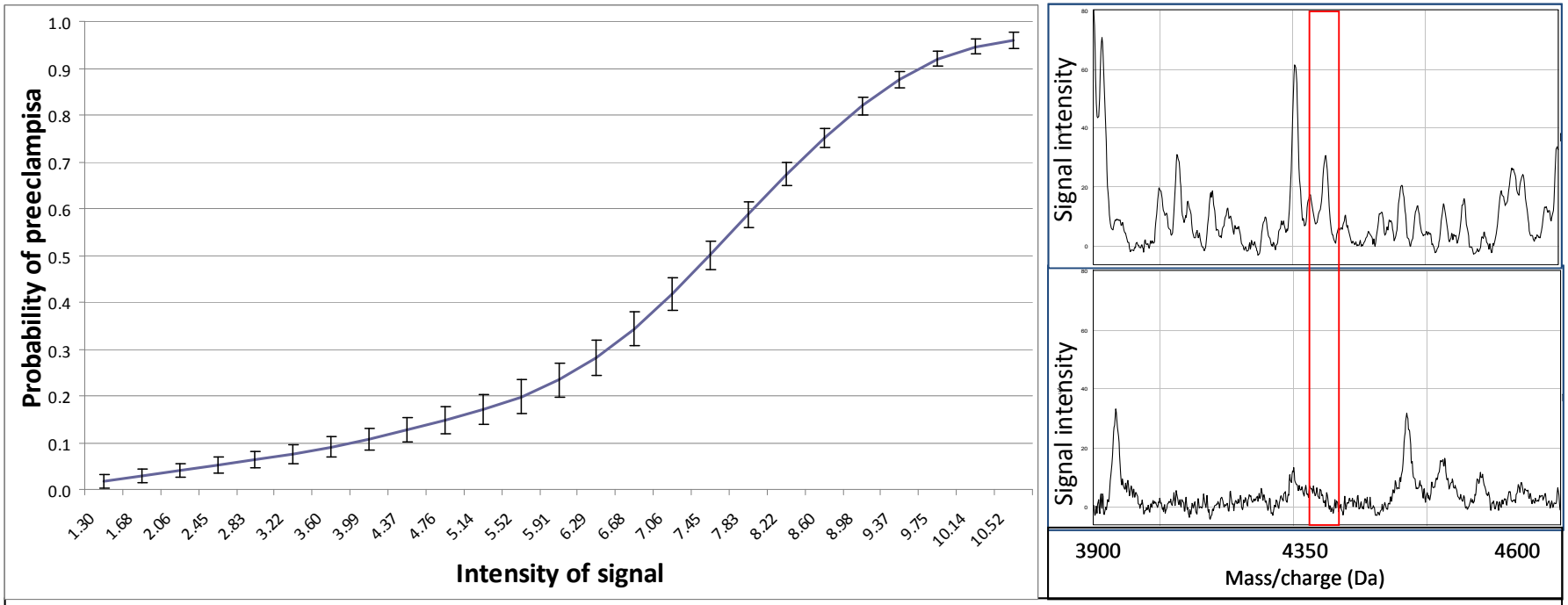


Figure 3.5.3.7. Development of diagnostic models from SELDI analysis: 20 to 25 weeks gestation. *Left.* ANN non-linear regression function of probability of pre-eclampsia against signal intensity of peak at 4254.4 Da. *Right.* Representative spectra from pre-eclamptic (top) and normal pregnancy (bottom).

3.5.3.4. Temporal changes in SELDI spectra during pre-eclamptic and normal pregnancy

Having identified peaks predictive of pre-eclampsia from ANN models, retrospective comparison of individual peak intensity at different gestational ages was performed to identify variation with gestation and the predictive potential of individual model constituents. Mean signal intensities of the 5 peaks in the model from samples obtained < 20 weeks gestation and the 1 peak in the model from samples obtained 20 to 25 weeks gestation are shown in figure 3.5.3.8 for normal and pre-eclamptic pregnancies.

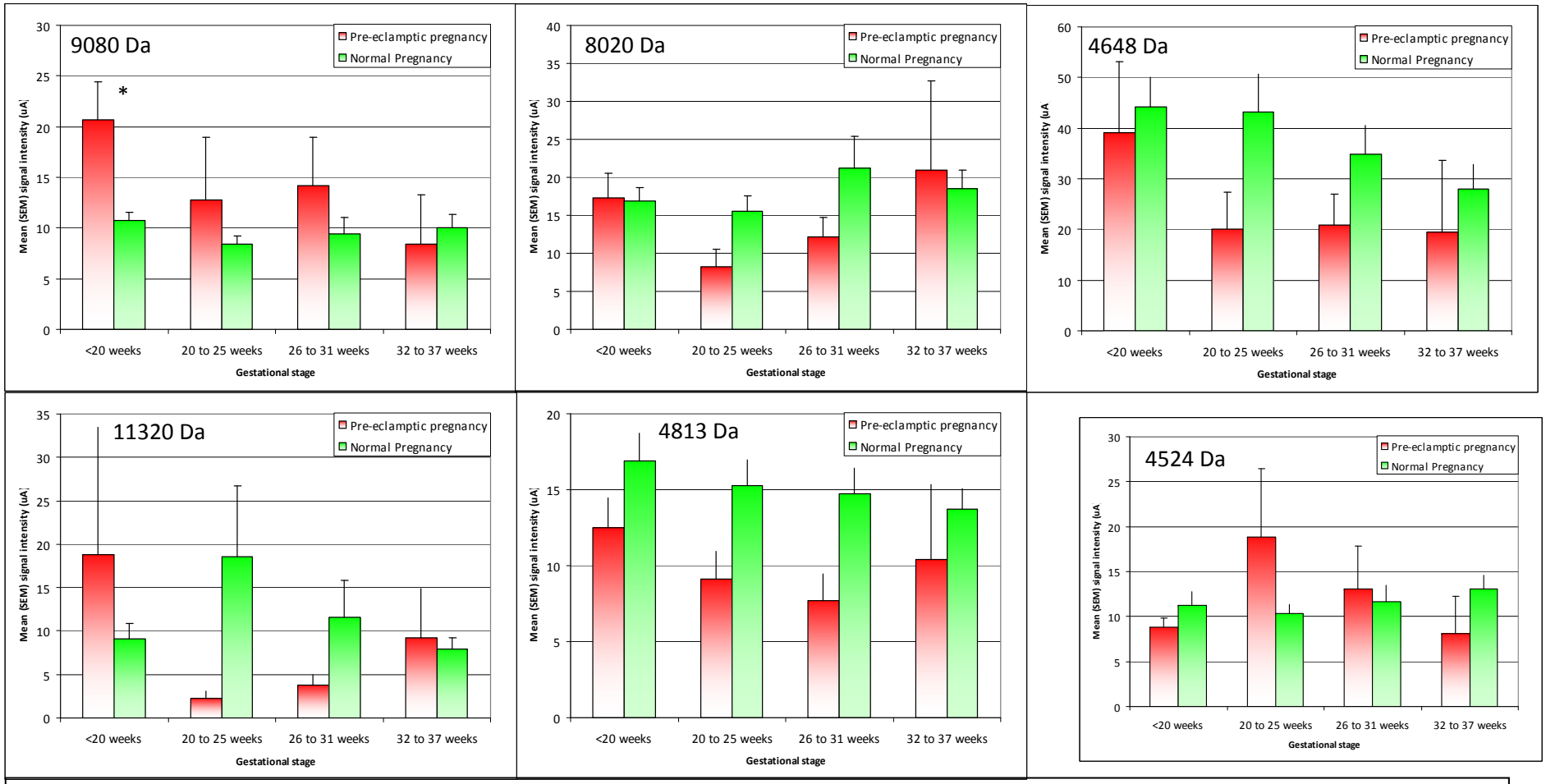


Figure 3.5.3.8. Mean (SEM) signal intensity of peptide peaks identified in predictive models of pre-eclampsia by gestational stage. First five panels: peaks in model from samples obtained < 20 weeks gestation. Lower right panel: peak in model from samples obtained 20 to 25 weeks gestation. *Signal intensity was significantly higher for the peak at 9080 Da in pre-eclamptic pregnancies in samples taken <20 weeks gestation (<0.05).

Peak 1 (9080 Da) contributed 80% to the ANN model derived from samples obtained less than 20 weeks gestation and, as above, intensity was greater in pregnancies destined to end in pre-eclampsia (figure 3.5.3.8). Further analysis by week of gestation shows that a difference in signal intensity existed from week 17 to 21 only (figure 3.5.3.9).

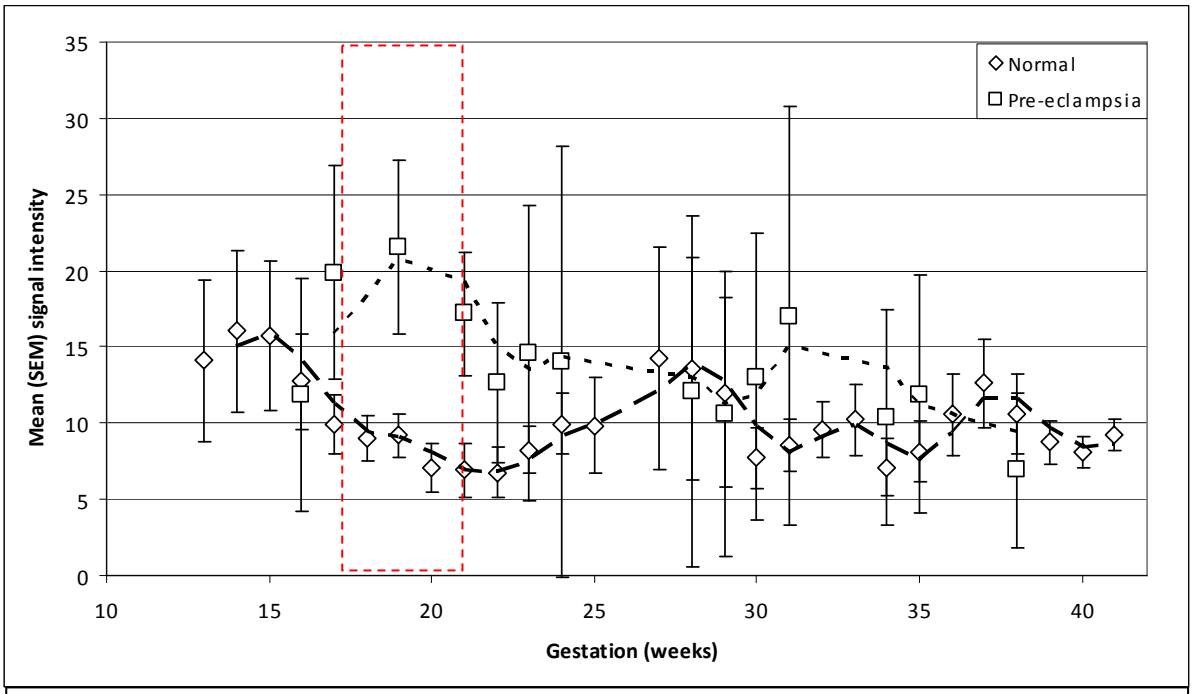


Figure 3.5.3.9. Signal intensity of peak at 9080 Da identified by SELDI in normal and pre-eclamptic pregnancies. Signal intensity was significantly higher in pregnancies ending in pre-eclampsia in samples collected from week 17 to 21 (red box)

3.5.4 Discussion

We identified a panel of five proteins from specimens collected prior to 20 weeks gestation that accurately predict the subsequent development of pre-eclampsia up to 23 weeks before clinical manifestation in women at high risk of developing pre-eclampsia. A further peptide peak identified in samples collected between 20 and 25 weeks gestation independently predicted the subsequent development of pre-eclampsia.

Identifying an effective predictive test of the risk of pre-eclampsia has proved challenging despite an explosion in exploratory studies over the last five years. Although studies of single markers from serum, urine, biophysical parameters or demography have identified statistical differences between subsequent normal or pre-eclamptic pregnancies, translation to clinical practice is hampered by poor sensitivity and specificity or overly cumbersome laboratory methods (146). The most effective predictive models for pre-eclampsia have combined serum biomarker analysis with uterine artery waveform analysis and maternal demographic features (148). Sensitivities and specificities greater than 90% have been reported from retrospective analysis of serum levels of placental growth factor, pregnancy associated peptide A or placental protein 13 from samples collected in the first trimester with uterine artery Doppler and demographic data (148,150,155) but are yet to be validated in prospective cohorts.

The concept that healthy urine is free from protein has been overridden by evidence from increasingly sensitive proteomic techniques identifying over 1500 proteins albeit in low quantity (107). Exploration of the urinary proteome in

health and disease has been hampered by a lack of standardisation in urine collection and preparation methods which can have a significant impact on the reproducibility of results (190,192,219). Compared to alternative proteomic methods, SELDI benefits from allowing a high throughput of samples with rapid acquisition of data (175). This makes it ideal for searching complex fluids for putative biomarkers, albeit at the expense of decreased signal resolution and increased inter-assay coefficient of variance of signal intensity (176). Although these limitations have generally limited the use of SELDI as a clinical tool (177), it allows a hypothesis-free approach to novel biomarker discovery and subsequent evaluation. This approach has been used to identify proteomic patterns to differentiate diabetic from non-diabetic nephropathy (178,179), early acute kidney injury (180-182), acute renal transplant rejection (183,184), lupus nephritis (185,186) and urogenital malignancy (187-189). Validation of these findings have led to the identification of candidate biomarkers for clinical utilitisation such as EN-2 in prostate cancer (220) or a panel of urine markers in lupus nephritis (221).

Proteomic analysis using liquid chromatography mass spectrometry identified a panel of 50 small molecule urinary peptide biomarkers - predominantly fragments of collagen, fibrinogen and uromodulin - predictive of pre-eclampsia at 12 to 16 weeks gestation however outcomes were not replicated in a separate cohort of patients (160). A small longitudinal proteomic study of urinary biomarkers predictive of pre-eclampsia identified SERPINA1 and non-random fragments of albumin as predicitive albeit in samples obtained late in the second trimester (159).

Our results suggest that accurate prediction of pre-eclampsia can be derived from a single urine specimen in early pregnancy. A simple urinary test to identify patients at high risk of pre-eclampsia would facilitate prioritisation of women in need of more intensive monitoring during pregnancy, particularly in developing countries where access to maternity resources and medical input is limited and maternal and fetal outcomes from hypertensive disorders of pregnancy are poor.

As a longitudinal study we were able to follow the signal intensities of peaks identified in the ANN model through normal and pre-eclamptic pregnancies. Univariate analysis of these peaks did not show marked differences between normal and pre-eclamptic pregnancies except for the most discriminatory peak (9080 Da) in the ANN model from samples obtained prior to 20 weeks gestation. A difference in mean intensity did not persist after 20 weeks gestation and is therefore unlikely to have been identified in previous studies of urine protein biomarkers where samples have usually been obtained at the time of evaluation of hypertension in pregnancy.

The mass/charge ratios of peaks identified in this study do not correlate with the molecular masses of putative predictive biomarkers previously proposed. Correlation of mass/charge data obtained from SELDI traces with peptide identification from the known urine proteome is hampered by (a) possible alteration in protein fragmentation in disease, (b) variation in protein charge and (c) the presence of disease-specific peptides in urine. Tandem mass spectroscopy with peptide fragmentation is used to assist with peptide identification, however, coupling SELDI mass spectrometry to such technology is not widely available.

Given that the peak identified at 9080 Da in our study contributed 80% to the ANN model from samples obtained less than 20 weeks gestation, it is likely that this may represent a possible protein biomarker of disease. Using the same technology, a peak at 9080 Da was identified by Katz-Jaffe and colleagues in the secreted proteome of human blastocysts (unpublished data, patent application) although this peptide has not been identified to date. In the published human urine proteome of Adachi and colleagues (107), neither peptides with a mass of 9060 to 9100 Da (NEDD8 precursor, small breast epithelial mucin and liver-expressed antimicrobial peptide 2 precursor) nor those with mass multiples of 9080 to account for multiple charges (thy-1 membrane glycoprotein precursor, 18151 Da; secreted and transmembrane protein 1 precursor, 27307 Da; apoprotein E precursor, 36246 Da; 2,4-dienoyl-CoA reductase, mitochondrial precursor, 36330 Da; gamma-glutamyl hydrolase precursor, 36340 Da; ADP-ribosyl cyclase 2 precursor, 36342 Da; tumour-associated calcium signal transducer 2 precursor, 36371 Da) have been associated with pre-eclampsia in previous studies. A predicted urine protein with mass 27241 Da similar to an oxidoreductase is notable given the hypothesised pathophysiological role of nitric oxide synthetase polymorphisms in pre-eclampsia (222).

Alternatively, D-Erythro-7,8-dihydroneopterin triphosphate synthetase is a peptide of mass 9080 Da involved in neopterin and folate metabolism. Neopterin is a marker of cellular inflammation and serum levels are elevated in pre-eclampsia as compared to normal pregnancy (223). D-Erythro-7,8-

dihydroneopterin triphosphate synthetase has not been identified as a urinary protein however.

In summary we identified a urinary proteomic “fingerprint” in early pregnancy capable of predicting the subsequent development of pre-eclampsia with high accuracy. Validation of these findings in a novel cohort and characterisation of the identified biomarkers will allow development of simple and accurate tests to identify patients at high risk of pre-eclampsia.

3.6 Future development

The findings in this study are clinically relevant since this is the first time that accurate prediction of pre-eclampsia in early pregnancy has been achieved from a single test. Moreover, given that obtaining urine specimens is non-invasive, painless and does not require specialist equipment or personnel, there is great potential for a urinary biomarker test for pre-eclampsia to be used in the developing world where access to medical care is more limited.

Translating these results to clinical practice will require two future steps. Firstly, the presence of the protein fingerprint identified in our study should be validated in a novel prospective clinical cohort. This cohort can be extended to low-risk as well as high-risk pregnancies to evaluate its utility as a routine screening test. This is important as 50% of cases of pre-eclampsia occur in patients without identified clinical or demographic risk factors for the condition.

Secondly, but contemporaneously, the peptide peaks identified by the ANN models should be characterised. To characterise peptides identified by SELDI, investigators have used a quadrupole time of flight tandem mass spectrometer equipped with a ProteinChip® interface to identify peptides directly from the array without offline purification of the samples (159). Alternatively, sodium dodecyl sulfate polyacrylamide gel electrophoresis of the collected specimen can be used to separate the constituent peptides (224). The bands corresponding to the peptide peaks identified on SELDI can be cut out if identified and characterised using more established techniques such as liquid chromatography tandem mass spectrometry coupled to matrix-assisted laser desorption time of

flight mass spectrometry (MALDI TOF MS/LC MS/MS), or if the putative biomarker is known, Western blotting. Characterised peptides from the ANN model peaks can then be assayed in the stored samples and novel clinical cohort by enzyme-linked immunosorbent assay (ELISA).

It is envisaged that validated urine biomarkers of pre-eclampsia from this study can be utilised in clinical practice by SELDI, ELISA or a semi-quantitative urine dipstick similar to current urine pregnancy tests to evaluate an individual patient's risk of pre-eclampsia. Characterisation of the peptides identified from the ANN models will improve knowledge of the pathophysiology of pre-eclampsia and may lead to therapeutic interventions.

3.7 Summary - Qualitative analysis of proteinuria in predicting pre-eclampsia

- A proteomic-based approach to urine biomarker discovery was used to predict pre-eclampsia from a prospective cohort of patients at increased risk of the disease
- A panel of five peptides was identified in samples obtained less than 20 weeks gestation that correctly predicted pre-eclampsia with 92% accuracy.
- Validation of these findings in a novel cohort and characterisation of the peptides in the panel will allow development of a simple and accurate point-of-care test to identify women at greatest risk of developing the disease.

Appendix

A. UK CORD dataset

A.1 Maternal data

Patient ID
Last Name
First Name
Date of Birth
Hospital Number
Ethnicity – according to 2001 UK census groups
Diagnosis – according to EDTA codes (when known)
Timing when diagnosis known – before, during or following pregnancy
Provisional diagnosis – proteinuria, haematuria, structural abnormalities
Center - Hospital

A.2 Pregnancy data

Patient ID	
Pregnancy ID	
Gravidity	
Para a	
Para b	
Number of fetuses	
Last menstrual period	
Estimated date of delivery	
Date of delivery	
Delivery method	
Preterm labour?	
Mid term scan?	
Indication for delivery	
Past medical history	Acute renal failure Dialysis Transplant Recurrent UTI Treated hypertension Diabetes Thyrotoxicosis Venous thromboembolism CVA Thrombophilia
Past obstetric history	Eclampsia Pre-eclampsia Gestational hypertension HELLP IUGR Preterm labour Miscarriage Stillbirth Early neonatal death Late neonatal death Proteinuria in pregnancy
Preconception serum creatinine	Date of test
Preconception urine albumin:creatinine ratio	Date of test
Preconception systolic blood pressure	Date of measurement
Preconception diastolic blood pressure	Date of measurement

A.3 Child data

Child ID
Patient ID
Weight
Gestation at delivery
Weight centile
Gender
Apgar Score at 1 minute
Apgar Score at 5 minutes
Apgar Score at 10 minutes
Arterial pH and base excess
Venous pH and base excess
Outcomes – alive and well, alive with impairment, termination, miscarriage, stillbirth, NND
Congenital anomaly

A.4 Visit data

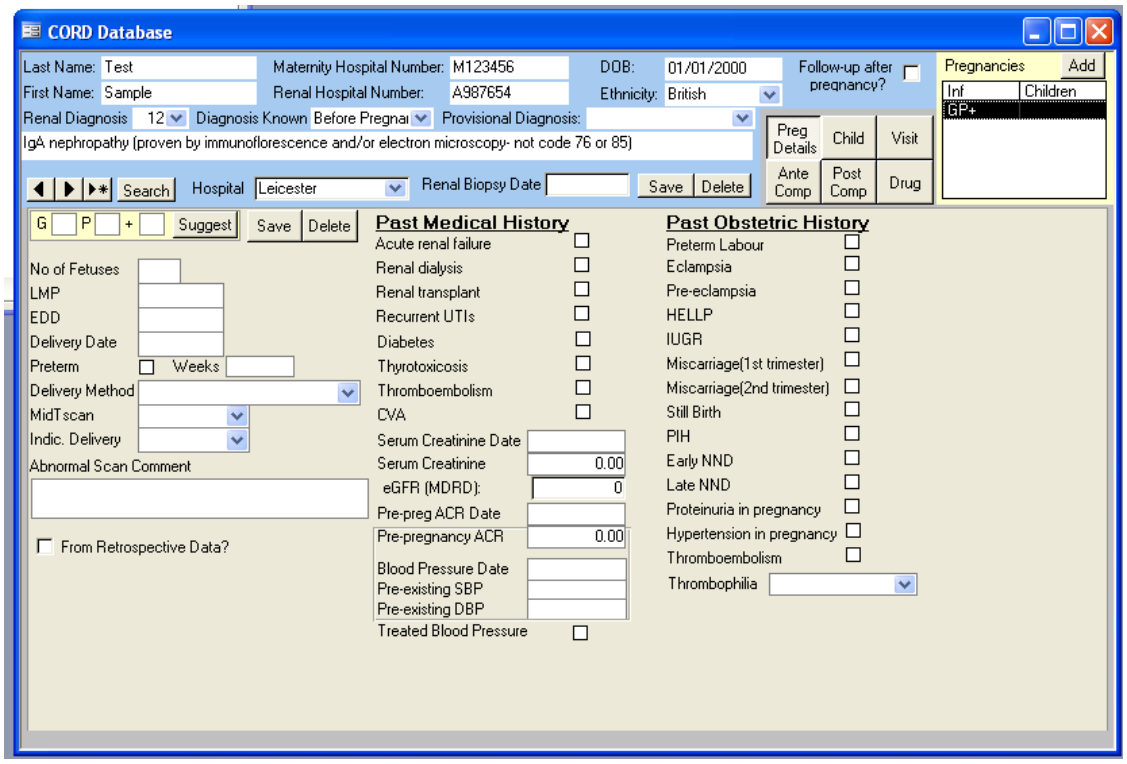
Visit ID	
Pregnancy ID	
Visit number	
Date of visit	
Gestation/Postnatal at visit (weeks)	
Weight	
Height	
Systolic blood pressure	
Diastolic blood pressure	
Mean 24 hour systolic and diastolic blood pressure	
Mean day time systolic and diastolic blood pressure	
Mean night time systolic and diastolic blood pressure	
Urine dip blood	
Urine dip protein	
Urine protein:creatinine ratio	
Urine albumin:creatinine ratio	
24 hour urine volume	
24 hour urine creatinine concentration	
24 hour urine protein concentration	
24 hour urine protein excretion	
Urinary tract infection?	
Serum tests	Creatinine Albumin Sodium Potassium Urate ALT AST Bile acids Haemoglobin Platelets APPTR INR Glycated haemoglobin Ciclosporin level C-reactive protein
Immunology	ANA ENA dsDNA (critihidia and ELISA) pANCA cANCA MPO PR3 C3 C4 C3d IgA

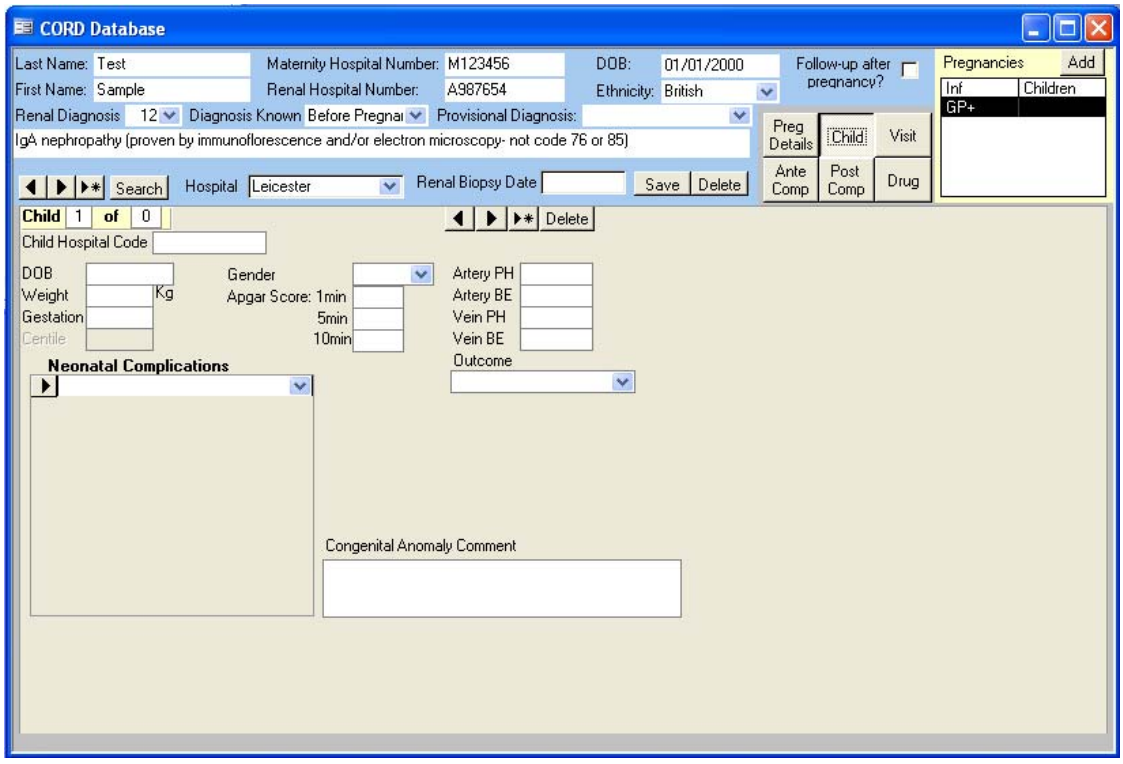
	IgM IgG Anti-cardiolipin IgG and IgM Lupus anticoagulant
Renal ultrasound	Renal size – left and right Cortical scarring? – left or right Duplex system? – left or right
Fetal ultrasound	Head circumference Abdominal circumference Umbilical artery Doppler result Liquor volume Uterine artery notching?

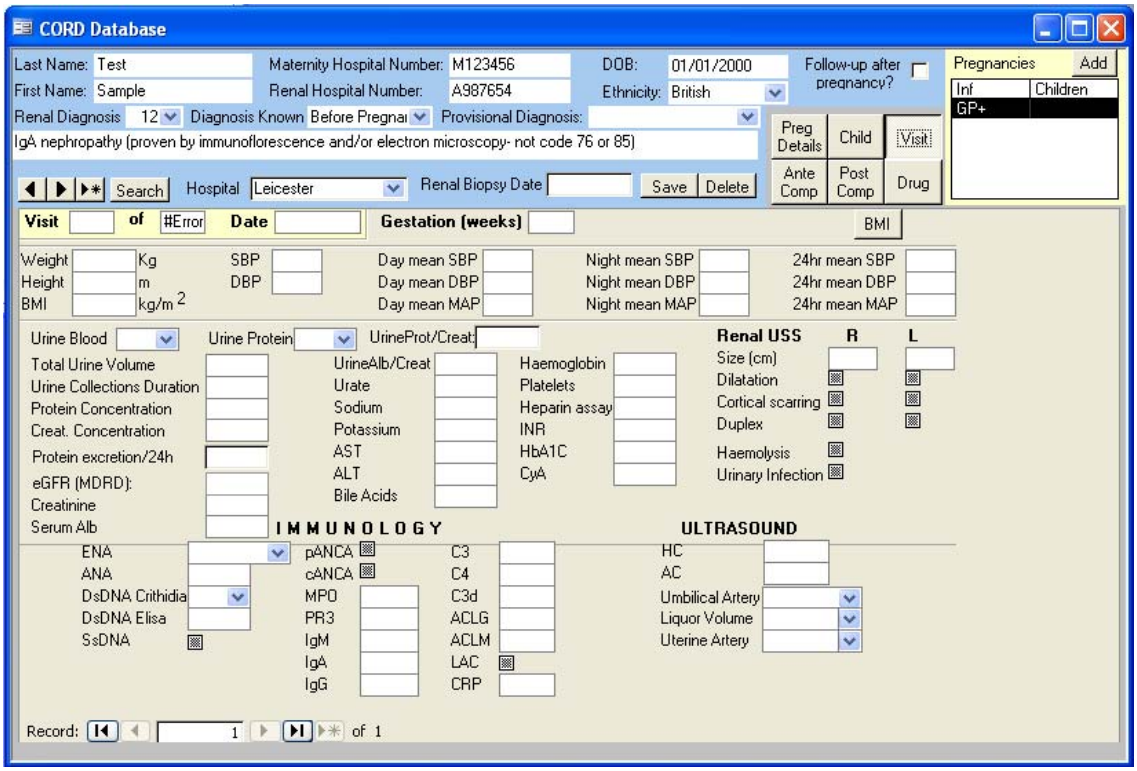
A.5 Common drugs

Visit ID
Low molecular weight heparin?
Aspirin?
Prophylactic antibiotics
Labetalol dose
Methyldopa dose
Nifedipine dose
Hydralazine dose

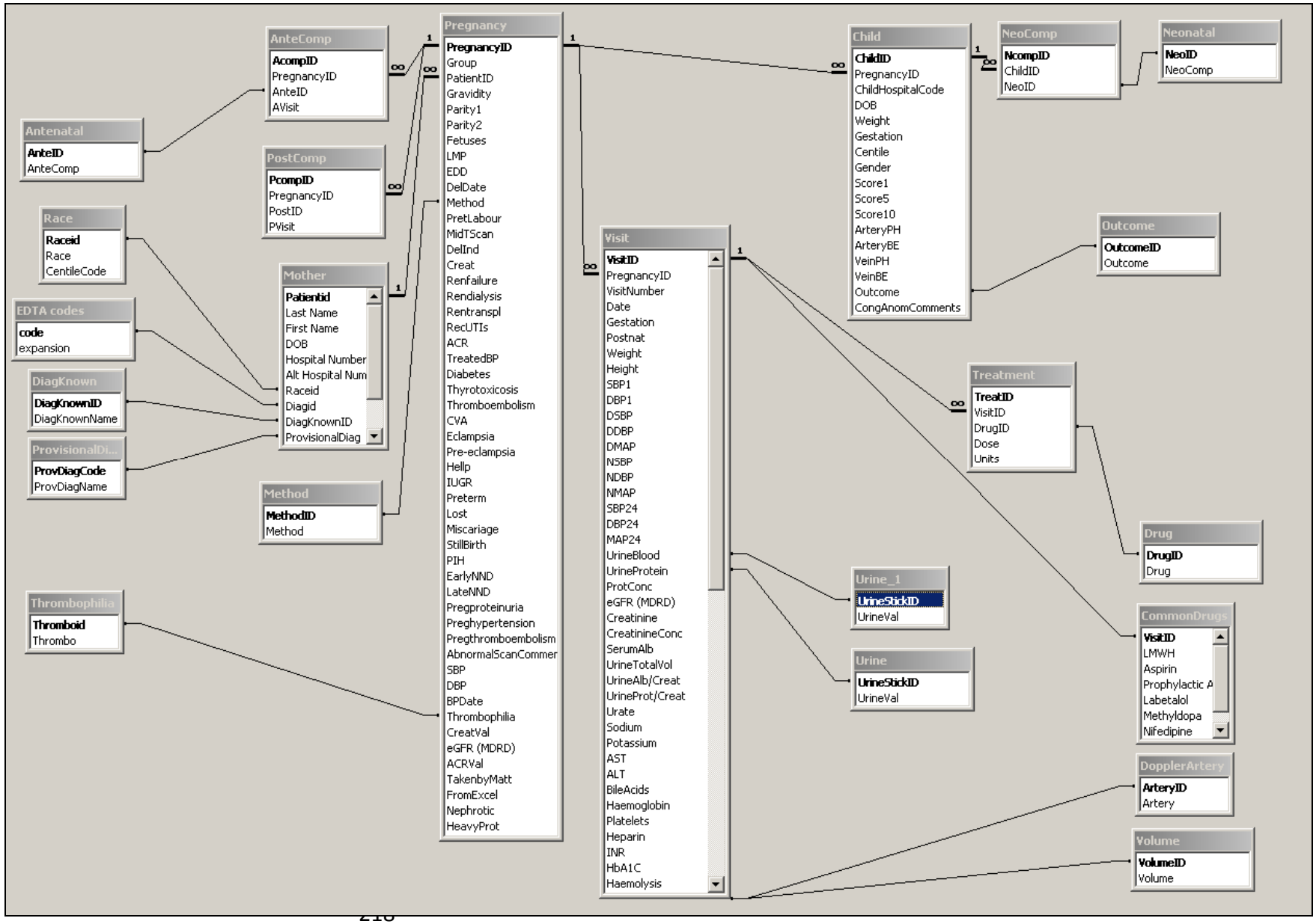
A.6 Database screenshots






CORD Database
Last Name: Test
First Name: Sample
Maternity Hospital Number: M123456
Renal Hospital Number: A987654
DOB: 01/01/2000
Ethnicity: British
Follow-up after pregnancy?
Pregnancies
Add
Inf
Children
GP+
Renal Diagnosis 12
Diagnosis Known Before Pregnar
Provisional Diagnosis:
IgA nephropathy (proven by immunoflorescence and/or electron microscopy- not code 76 or 85)
Preg Details
Child
Visit
Ante Comp
Post Comp
Drug
Search
Hospital Leicester
Renal Biopsy Date
Save
Delete
Visit
of #Error
Date
Gestation (weeks)
BMI
Weight Kg
Height m
BMI kg/m 2
SBP
DBP
Day mean SBP
Day mean DBP
Day mean MAP
Night mean SBP
Night mean DBP
Night mean MAP
24hr mean SBP
24hr mean DBP
24hr mean MAP
Urine Blood
Urine Protein
UrineProt/Creat:
Total Urine Volume
Urine Collections Duration
Protein Concentration
Creat. Concentration
Protein excretion/24h
eGFR (MDRD):
Creatinine
Serum Alb
UrineAlb/Creat
Urate
Sodium
Potassium
AST
ALT
Bile Acids
Haemoglobin
Platelets
Heparin assay
INR
HbA1C
CyA
Renal USS
R
L
Size (cm)
Dilatation
Cortical scarring
Duplex
Haemolysis
Urinary Infection
IMMUNOLOGY
ENA
ANA
DsDNA Crithidia
DsDNA Elisa
SsDNA
pANCA
cANCA
MPO
PR3
IgM
IgA
IgG
C3
C4
C3d
ACLG
ACLM
LAC
CRP
ULTRASOUND
HC
AC
Umbilical Artery
Liquor Volume
Uterine Artery
Record: 1 of 1

B Database table relationships


Pregnancy
PregnancyID
Group
PatientID
Gravidity
Parity1
Parity2
Fetuses
LMP
EDD
DelDate
Method
PretLabour
MidTScan
DelInd
Creat
Renfailure
Rendialysis
Rentranspl
RecUTIs
ACR
TreatedBP
Diabetes
Thyrotoxicosis
Thromboembolism
CVA
Eclampsia
Pre-eclampsia
Hellp
IUGR
Preterm
Lost
Miscariage
StillBirth
PIH
EarlyNND
LateNND
Pregproteinuria
Preghypertension
Pregthromboembolism
AbnormalScanCommer
SBP
DBP
BPDate
Thrombophilia
CreatVal
eGFR (MDRD)
ACRVal
TakenbyMatt
FromExcel
Nephrotic
HeavyProt
AnteComp
AcompID
PregnancyID
AnteID
AVisit
Antenatal
AnteID
AnteComp
PostComp
PcompID
PregnancyID
PostID
PVisit
Race
Raceid
Race
CentileCode
EDTA codes
code
expansion
DiagKnown
DiagKnownID
DiagKnownName
ProvisionalDi...
ProvDiagCode
ProvDiagName
Mother
Patientid
Last Name
First Name
DOB
Hospital Number
Alt Hospital Num
Raceid
Diagid
DiagKnownID
ProvisionalDiag
Method
MethodID
Method
Thrombophilia
Thromboid
Thrombo
Visit
VisitID
PregnancyID
VisitNumber
Date
Gestation
Postnat
Weight
Height
SBP1
DBP1
DSBP
DDBP
DMAP
NSBP
NDBP
NMAP
SBP24
DBP24
MAP24
UrineBlood
UrineProtein
ProtConc
eGFR (MDRD)
Creatinine
CreatinineConc
SerumAlb
UrineTotalVol
UrineAlb/Creat
UrineProt/Creat
Urate
Sodium
Potassium
AST
ALT
BileAcids
Haemoglobin
Platelets
Heparin
INR
HbA1C
Haemolysis
Child
ChildID
PregnancyID
ChildHospitalCode
DOB
Weight
Gestation
Centile
Gender
Score1
Score5
Score10
ArteryPH
ArteryBE
VeinPH
VeinBE
Outcome
CongAnomComments
NeoComp
NcompID
ChildID
NeoID
Neonatal
NeoID
NeoComp
Outcome
OutcomeID
Outcome
Treatment
TreatID
VisitID
DrugID
Dose
Units
Drug
DrugID
Drug
Urine_1
UrineStickID
UrineVal
Urine
UrineStickID
UrineVal
CommonDrugs
VisitID
LMWH
Aspirin
Prophylactic A
Labetalol
Methyldopa
Nifedipine
DopplerArtery
ArteryID
Artery
Volume
VolumeID
Volume

C Predicting pre-eclampsia GCP documents

C.1 Consent form

Patient name, address, DOB (or ID label)

Centre Number: _____
Study Number: _____
Patient Identification Number for this trial: _____

CONSENT FORM

Title of Project: **Finding a urinary protein fingerprint in Pre-Eclampsia**

Name of Researcher / Principal Investigator: **Prof Nigel Brunskill**

Please initial bo

1. I confirm that I have read and understand the information sheet dated 20th May 2007 version 2 for the above study and have had the opportunity to ask questions. ☐
2. I understand that I may withdraw my consent to my tissue being used at any time without justifying my decision and without affecting my normal care and medical management. ☐
3. I agree to donate the tissue samples as detailed below and allow their use in medical research as described in the Patient Information Leaflet. ☐
4. I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue. ☐
5. I understand that tissue samples will not be used to undertake any genetic tests whose results may have adverse consequences on my or my families insurance or employment. ☐
6. I understand that sections of any of my medical notes may be looked at by responsible individuals from the research team or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
7. I understand that blood and/or urine samples and associated clinical data may be transferred to commercial / non-commercial research partners of the University Hospitals of Leicester NHS Trust, but that the information will be anonymised prior to transfer. ☐

1. The samples which I hereby consent to donate are: **(please type if possible)** ☐
……

Blood

Urine

2. I agree to take part in the above study. ☐

_____	_____	_____
Name of Participant	Date	Signature

_____	_____	_____
Researcher	Date	Signature

Original for researcher/site file/CRF
copy for patient, copy for hospital notes

C.2 Patient information leaflet

Patient Information Leaflet
(Normal Pregnancy)

Title of Study: Finding a urinary protein fingerprint in Pre-Eclampsia

Principal Investigator: Prof Nigel Brunskill

You may contact: Prof Nigel Brunskill on 0116 258 8043

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. This research will contribute towards a research doctorate for the principal investigator.

Thank you for reading this.

1. What is the purpose of the study?

Pre-eclampsia is a disorder that occurs only during pregnancy and can affect both the mother and the unborn baby. 1 in every 20 pregnancies is affected.
It usually becomes apparent 20 weeks or more into the pregnancy.
It is a condition that causes protein in the mother's urine and high blood pressure. If undiagnosed Pre-eclampsia and similar disorders are the leading global causes of maternal and infant illness in pregnancy.
Currently there are no tests to diagnose pre-eclampsia or even predict which women may develop it.
The only clue a doctor or midwife may get is a rise in a patient's blood pressure or detection of protein in her urine. Unfortunately this can occur in many other conditions making the diagnosis very difficult.
If Pre-eclampsia is detected early there is every chance that with treatment the pregnancy will progress normally and without complication, however late diagnosis can lead to illness for both the mother and the unborn baby.

2. Why have I been chosen?

You have been chosen because you have a normal pregnancy. It is important to have samples of urine from women with normal pregnancies so we can compare them with urine from women with Pre-eclampsia to find what it is that makes them different.

3. Do I have to take part?

You may be approached at your next clinic visit to discuss whether you would like to join the study. We aim to give you at least 7 days after receiving this information sheet to consider your decision. You may take as long as you like to think about it. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

4. What will happen to me if I take part?

If you agree to take part we will ask to keep a proportion of the blood left over from any blood sample you will have as part of your routine care. You need do nothing to achieve this as we will take the sample after all the routine testing has been done.
At your clinic appointments when you have blood and urine tests, the unused blood and urine can be recycled into this study making up most of the study samples.
We may then ask you to collect further urine samples at intervals, no more frequently than every 28 days. This will continue until two months after you deliver your baby, making a maximum possible number of times we approach you to be 9 (although it is likely to be far less than this).
You will continue to have you usual antenatal care and we do not expect this study to cause you any inconvenience at any time however, in the unlikely event of the research team requesting a urine sample at a time that you are not attending a clinic we will reimburse any travelling expenses that you incur in getting the sample to the Hospital.
You may refuse any of these requests for samples and still remain in the study, however we will be able to gain more information the more samples you agree to give.

5. What do I have to do?

As detailed above most of the study blood and urine tests you need do nothing other than sign the consent form and attend you usual antenatal care.
Any extra urine tests are best collected by you first thing in the morning and then either taken or sent to the hospital.

.

6. Will I receive payment for the tissue that I donate for this research study?

You will not receive any payment for the blood or urine. The samples are gifts - neither you nor your relatives will benefit from any inventions that result from the use of these samples. Any reasonable travelling expense you incur in taking part in the study will be covered by the principal investigators team.

7. What are the possible disadvantages and risks of taking part?

Beyond the inconvenience collecting the samples there will be no disadvantage or risk to either you or your babies care.

8. What are the possible benefits of taking part?

While there will be no direct clinical benefit for you taking part in the study the work may help in the future management of patients with Pre-eclampsia.

9. What if something goes wrong?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms would be available to you.
These can be accessed through the Patient Advice & Liaison Service (PALS) which is a free and confidential service that helps patients.

PALS Telephone **0116 258 3100.**

PALS e-mail: **pals@uhl-tr.nhs.uk**

PALS address: PALS Office
Glenfield Hospital
Groby Road
Leicester, LE3 9QP

10. Will my taking part in this study be kept confidential?

Access to your samples will be only available to members of the research team.
You will not be identifiable from your samples. All your samples will be given a unique code and only the NHS professionals responsible for your care will have access to your medical details. The handling of your blood and urine will be treated with the usual degree of confidentiality you expect within the NHS.

11. What will happen to the results of the research study?

The results of this study will be published in peer reviewed scientific journals. You will not be identifiable in these publications.
If you wish to be notified of the results at the end of the study (estimated summer 2012) please send an e-mail to *preeclampsiastudy@btinternet.com*

12. Who is organising and funding the research?

This work is being performed by doctors working in the John Walls Renal Unit and the Department of Infection, Immunity & Inflammation, University of Leicester. Funding has been obtained by application for a research grant from charitable funds
.

13. Who has reviewed the study?

All research that involves NHS patients or staff, information from NHS medical records or uses NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be approved by an NHS Research Ethics Committee before it goes ahead. Approval does not guarantee that you will not come to any harm if you take part. However, approval means that the Committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits and that you have been given sufficient information on which to make an informed decision to take part or not.

14. Contact for Further Information

If you wish to withdraw your consent and remove any stored blood or urine samples or if you require any further information please contact Prof Nigel Brunskill (0116) 258 8043.

Thank you for reading this information leaflet.
You will receive a copy of the information sheet and a signed consent form to keep.

C.3 Invitation letter

University Hospitals of Leicester NHS
NHS Trust

John Walls Renal Unit
Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW

Re: “Finding a urinary protein fingerprint in Pre-Eclampsia” Research.

Congratulations on your pregnancy.

I am writing to you as part of a group of doctors investigating diseases in pregnancy. We are currently recruiting participants into a trial investigating the causes of Pre-Eclampsia.

In order to perform this study we want pregnant women like you to allow us to perform analyses on their urine samples. We would also like to study left over blood taken as part of the routine sampling taken during pregnancy.
The enclosed Information sheet give more specific information on how this may affect you should you choose to help us out.

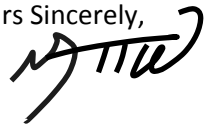
At your next clinic you will have the opportunity to discuss this in more detail with your doctor. If you are then keen to join our study you would need to sign a consent form. The study tests are predominantly performed on Left over samples that would be discarded after routine analysis. These left over samples would be sent to the renal research lab at the Leicester General hospital and analyzed for the study.

Signing consent does not bind you to giving us all your samples as you are free to withdraw your consent at any time. You may even choose to give us some samples and not others, it is up to you.

Please read the enclosed leaflet and feel free to discuss it with you doctor.

For those women whose first language is not English we recommend booking a translator for your next clinic visit. Do this by phoning the booking center in advance, you will find this number on your appointment notification. Alternatively a family member may act as a translator if you want.

Thank you for your attention.
Yours Sincerely,



Dr Matthew Williams
Specialist Registrar in Renal Medicine
John Walls Renal Unit
Leicester General Hospital

D. Publications and presentations arising from this work

2011 Youssouf S, Hall M, Lipkin G, Lightstone L, Brunskill N, Carr S.
Pregnancy in women with CKD stage 3 to 5: maternal outcomes.
Oral presentation, American Society of Nephrology Renal Week, Philadelphia

2011 Hall M.
Renal Disease and Pregnancy.
Oral presentation, SpR Club, Belfast

2010 Hall M, Brunskill N.
Glomerulonephritis and the Nephrotic Syndrome in Pregnancy.
Fetal and Maternal Medicine Review 2010; 21(2):163-184.

2010 Hall M, Brunskill N.
Renal Disease in Pregnancy.
Obstet. Gynae. Repro. Med. 2010; 10(5):131-137

2010 Hall M, Ball G, Bosio P, Carr S, Brunskill N.
An Early Urinary Proteomic Fingerprint Accurately Predicts Later Pre-Eclampsia.
Poster presentation, RA/BRS Annual Conference, Manchester. (Best Abstract award)
Oral presentation, British Maternal and Fetal Medical Conference, Gateshead
Oral presentation, American Society of Nephrology Renal Week, Denver

2009 Hall M, Lightstone L, Lipkin G, Day C, Carr S, Brunskill N.
Nephrotic Range Proteinuria in Pregnancy: Fetal Outcomes.
Poster presentation**,** American Society of Nephrology Renal Week, San Diego

2009 Hall M, Lightstone L, Lipkin G, Day C, Brunskill N, Carr S.
Favourable pregnancy outcomes in a prospective contemporary cohort of women with CKD 3 to 5.
Poster presentation, American Society of Nephrology Renal Week, San Diego

2009 Hall M, Al-Jayyousi R, Brunskill N, Carr S.
Hypertension and baseline creatinine predict pregnancy-related accelerated loss of renal function.
Poster presentation, RA/BTS Annual Conference, Liverpool
Poster presentation, WCN/ERA-EDTA conference, Milan

2009 Hall M, Al-Jayyousi R, Ferraro A, Lightstone L, Lipkin G, Mihaescu A, Noguiera E, Sinnamon K, Tullett K, Brunkill N, Carr S.
Validation of the protein:creatinine ratio in pregnant patients with chronic kidney disease.
Poster presentation, RA/BTS Annual Conference, Liverpool
Poster presentation, WCN/ERA-EDTA conference, Milan (Top 20% abstract)

2008 Hall M, Al-Jayyousi R, Brunskill N, Carr S.
Kidney disease in pregnancy: Effect of proteinuria on outcomes.
Poster presentation, RA/BRS Annual Conference, Glasgow

References

(1) HAMILTON HF. The cardiac output in normal pregnancy; as determined by the Cournand right catheterization technique. J Obstet Gynaecol Br Emp 1949 Aug;56(4):548-552.

(2) SPETZ S. Peripheral Circulation during Normal Pregnancy. Acta Obstet Gynecol Scand 1964;43:309-329.

(3) Ogueh O, Clough A, Hancock M, Johnson MR. A longitudinal study of the control of renal and uterine hemodynamic changes of pregnancy. Hypertens Pregnancy 2011;30(3):243-259.

(4) Brenner WE, Hendricks CH. Interdependence of blood pressure, weight gain, and fetal weight during normal human pregnancy. Health Serv Rep 1972 Mar;87(3):236-246.

(5) Larsson A, Palm M, Hansson LO, Axelsson O. Reference values for clinical chemistry tests during normal pregnancy. BJOG 2008 Jun;115(7):874-881.

(6) Evans KN, Bulmer JN, Kilby MD, Hewison M. Vitamin D and placental-decidual function. J Soc Gynecol Investig 2004 Jul;11(5):263-271.

(7) Jensen D, Duffin J, Lam YM, Webb KA, Simpson JA, Davies GA, et al. Physiological mechanisms of hyperventilation during human pregnancy. Respir Physiol Neurobiol 2008 Mar 20;161(1):76-86.

(8) Risberg A, Olsson K, Lyrenas S, Sjoquist M. Plasma vasopressin, oxytocin, estradiol, and progesterone related to water and sodium excretion in normal pregnancy and gestational hypertension. Acta Obstet Gynecol Scand 2009;88(6):639-646.

(9) Higby K, Suiter CR, Phelps JY. Normal values of urinary albumin and total protein excretion during pregnancy. Am J Obstet Gynecol 1994;171(4):984-989.

(10) Kawakami H, Murakami T, Kajii T. Normal values for 24-h urinary protein excretion: total and low molecular weight proteins with a sex-related difference. Clin Nephrol 1990 May;33(5):232-236.

(11) NHS Maternity Statistics, 2009-10. 2010; Available at: http://www.hesonline.nhs.uk/Ease/servlet/AttachmentRetriever?site_id=1937&file_name=d:\efmfiles\1937\Accessing\DataTables\Annual maternity release 2010\NHS Maternity Statistics, England 2009-10.xls&short_name=NHS Maternity Statistics, England 2009-10.xls&u_id=8943. Accessed December 3rd, 2010.

(12) Everett C. Incidence and outcome of bleeding before the 20th week of pregnancy: prospective study from general practice. BMJ 1997 Jul 5;315(7099):32-34.

(13) Clifford K, Rai R, Regan L. Future pregnancy outcome in unexplained recurrent first trimester miscarriage. Hum Reprod 1997 Feb;12(2):387-389.

(14) Van Lerberghe W, Manuel A, Matthews Z, Wolfheim C. Great expectations: making pregnancy safer. In: Van Lerberghe W, Phumaphi J, Evans T, editors. The World Health Report 2005 - make every mother and child count Switzerland: World Health Organization; 2005. p. 40-58.

(15) Live births, stillbirths and infant deaths: babies born in 2007, in England and Wales. 2010; Available at: http://www.statistics.gov.uk/pdfdir/bircoh1010.pdf. Accessed December 3rd, 2010.

(16) Preterm Births 2005. 2005; Available at: http://www.statistics.gov.uk/downloads/theme_population/pretermbirths_2005.xls.

(17) Gardosi J, Francis A. Software program for the calculation of customised birth weight percentiles. 2000-2009;6.4.

(18) Lewis.G editor. The Confidential Enquiry into Maternal and Child Health (CEMACH). Saving Mothers' Lives: Reviewing maternal deaths to make motherhood safer - 2003-2005. The Seventh Report on Confidential Enquiries into Maternal Deaths in the United Kingdom. London: CEMACH; 2007.

(19) Hogan MC, Foreman KJ, Naghavi M, Ahn SY, Wang M, Makela SM, et al. Maternal mortality for 181 countries, 1980-2008: a systematic analysis of progress towards Millennium Development Goal 5. Lancet 2010 May 8;375(9726):1609-1623.

(20) Pregnancy and renal disease. Lancet 1975(2):801-802.

(21) Mackay EV. Pregnancy and renal disease: a ten year survey. Aust N Z J Obstet Gynecol 1963;3:21.

(22) Hou S. Historical perspective of pregnancy in chronic kidney disease. Adv Chronic Kidney Dis 2007 Apr;14(2):116-118.

(23) Williams D, Davison J. Chronic kidney disease in pregnancy. BMJ 2008 Jan 26;336(7637):211-215.

(24) Imbasciati E, Gregorini G, Cabiddu G, Gammaro L, Ambroso G, Del Giudice A, et al. Pregnancy in CKD stages 3 to 5: fetal and maternal outcomes. Am J Kidney Dis 2007 Jun;49(6):753-762.

(25) Piccoli GB, Attini R, Vasario E, Conijn A, Biolcati M, D'Amico F, et al. Pregnancy and chronic kidney disease: a challenge in all CKD stages. Clin J Am Soc Nephrol 2010 May;5(5):844-855.

(26) Okundaye I, Abrinko P, Hou S. Registry of pregnancy in dialysis patients. Am J Kidney Dis 1998 May;31(5):766-773.

(27) Steenkamp R, Castledine C, Feest T, Fogarty D. UK Renal Registry 13th Annual Report (December 2010): Chapter 2: UK RRT prevalence in 2009: national and centre-specific analyses. Nephron Clin Pract 2011;119 Suppl 2:c27-52.

(28) Fahy BG, Gouzd VA, Atallah JN. Pregnancy tests with end-stage renal disease. J Clin Anesth 2008 Dec;20(8):609-613.

(29) Hou S. Pregnancy in End Stage Renal Disease. American Society of Nephrology Renal Week; November 19th 2010; ; 2010.

(30) Zamorski MA, Green LA. NHBPEP Report on High Blood Pressure in Pregnancy: A Summary for Family Physicians. Am Fam Physician 2001;64:263-270.

(31) Duley L, Henderson-Smart DJ, Meher S, King JF. Antiplatelet agents for preventing pre-eclampsia and its complications. Cochrane Database Syst Rev 2007 Apr 18;(2)(2):CD004659.

(32) Visintin C, Mugglestone MA, Almerie MQ, Nherera LM, James D, Walkinshaw S, et al. Management of hypertensive disorders during pregnancy: summary of NICE guidance. BMJ 2010 Aug 25;341:c2207.

(33) Burrows RF, Burrows EA. Assessing the teratogenic potential of angiotensin-converting enzyme inhibitors in pregnancy. Aust N Z J Obstet Gynaecol 1998 Aug;38(3):306-311.

(34) Cooper WO, Hernandez-Diaz S, Arbogast PG, Dudley JA, Dyer S, Gideon PS, et al. Major congenital malformations after first-trimester exposure to ACE inhibitors. N Engl J Med 2006 Jun 8;354(23):2443-2451.

(35) Brown M. CKD in pregnancy: Patterns of care and general principles of management. In: Davison JM, Nelson-Piercy C, Kehoe S, Baker P, editors. Renal disease in pregnancy. 1st ed. London: RCOG; 2008. p. 31-44.

(36) Magee LA, von Dadelszen P, Chan S, Gafni A, Gruslin A, Helewa M, et al. The Control of Hypertension In Pregnancy Study pilot trial. BJOG 2007 Jun;114(6):770, e13-20.

(37) Holley JL, Reddy SS. Pregnancy in dialysis patients: a review of outcomes, complications, and management. Semin Dial 2003 Sep-Oct;16(5):384-388.

(38) Reddy SS, Holley JL. The importance of increased dialysis and anemia management for infant survival in pregnant women on hemodialysis. Kidney Int 2009 Jun;75(11):1133-1134.

(39) Barua M, Hladunewich M, Keunen J, Pierratos A, McFarlane P, Sood M, et al. Successful pregnancies on nocturnal home hemodialysis. Clin J Am Soc Nephrol 2008 Mar;3(2):392-396.

(40) Jefferys A, Wyburn K, Chow J, Cleland B, Hennessy A. Peritoneal dialysis in pregnancy: a case series. Nephrology (Carlton) 2008 Oct;13(5):380-383.

(41) Chou CY, Ting IW, Lin TH, Lee CN. Pregnancy in patients on chronic dialysis: a single center experience and combined analysis of reported results. Eur J Obstet Gynecol Reprod Biol 2008 Feb;136(2):165-170.

(42) Hou S. Conception and pregnancy in peritoneal dialysis patients. Perit Dial Int 2001;21 Suppl 3:S290-4.

(43) Asamiya Y, Otsubo S, Matsuda Y, Kimata N, Kikuchi K, Miwa N, et al. The importance of low blood urea nitrogen levels in pregnant patients undergoing hemodialysis to optimize birth weight and gestational age. Kidney Int 2009 Jun;75(11):1217-1222.

(44) Diaz JM, Canal C, Gimenez I, Guirado L, Facundo C, Sola R, et al. Pregnancy in recipients of kidney transplantation: effects on the mother and the child. Nefrologia 2008;28(2):174-177.

(45) Fuchs KM, Wu D, Ebcioglu Z. Pregnancy in renal transplant recipients. Semin Perinatol 2007 Dec;31(6):339-347.

(46) Josephson MA, McKay DB. Considerations in the medical management of pregnancy in transplant recipients. Adv Chronic Kidney Dis 2007 Apr;14(2):156-167.

(47) Watnick S. Pregnancy and contraceptive counseling of women with chronic kidney disease and kidney transplants. Adv Chronic Kidney Dis 2007 Apr;14(2):126-131.

(48) Kim HW, Seok HJ, Kim TH, Han DJ, Yang WS, Park SK. The experience of pregnancy after renal transplantation: pregnancies even within postoperative 1 year may be tolerable. Transplantation 2008 May 27;85(10):1412-1419.

(49) Kashanizadeh N, Nemati E, Sharifi-Bonab M, Moghani-Lankarani M, Ghazizadeh S, Einollahi B, et al. Impact of pregnancy on the outcome of kidney transplantation. Transplant Proc 2007 May;39(4):1136-1138.

(50) Thompson BC, Kingdon EJ, Tuck SM, Fernando ON, Sweny P. Pregnancy in renal transplant recipients: the Royal Free Hospital experience. QJM 2003 Nov;96(11):837-844.

(51) Sibai BM, Lindheimer M, Hauth J, Caritis S, VanDorsten P, Klebanoff M, et al. Risk factors for preeclampsia, abruptio placentae, and adverse neonatal outcomes among women with chronic hypertension. National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. N Engl J Med 1998 Sep 3;339(10):667-671.

(52) Jungers P, Chauveau D, Choukroun G, Moynot A, Skhiri H, Houillier P, et al. Pregnancy in women with impaired renal function. Clin Nephrol 1997 May;47(5):281-288.

(53) Ferraro AJ, Somerset DA, Lipkin G, Al-Jayyousi RA, Carr S, Brunskill NJ, et al. Pregnancy in women with pre-existing renal disease: maternal and fetal outcomes. J Obstet Gynecol 2005;25:S13.

(54) Abe S. Pregnancy in IgA nephropathy. Kidney Int 1991 Dec;40(6):1098-1102.

(55) Hansson L, Zanchetti A, Carruthers SG, Dahlof B, Elmfeldt D, Julius S, et al. Effects of intensive blood-pressure lowering and low-dose aspirin in patients with hypertension: principal results of the Hypertension Optimal Treatment (HOT) randomised trial. HOT Study Group. Lancet 1998 Jun 13;351(9118):1755-1762.

(56) Tight blood pressure control and risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 38. UK Prospective Diabetes Study Group. BMJ 1998 Sep 12;317(7160):703-713.

(57) Bar J, Ben-Rafael Z, Padoa A, Orvieto R, Boner G, Hod M. Prediction of pregnancy outcome in subgroups of women with renal disease. Clin Nephrol 2000 Jun;53(6):437-444.

(58) Cunningham FG, Cox SM, Harstad TW, Mason RA, Pritchard JA. Chronic renal disease and pregnancy outcome. Am J Obstet Gynecol 1990 Aug;163(2):453-459.

(59) Holley JL, Bernardini J, Quadri KH, Greenberg A, Laifer SA. Pregnancy outcomes in a prospective matched control study of pregnancy and renal disease. Clin Nephrol 1996 Feb;45(2):77-82.

(60) Jones DC, Hayslett JP. Outcome of pregnancy in women with moderate or severe renal insufficiency. N Engl J Med 1996 Jul 25;335(4):226-232.

(61) McKay DB, Josephson MA. Pregnancy in recipients of solid organs--effects on mother and child. N Engl J Med 2006 Mar 23;354(12):1281-1293.

(62) Strauch BS, Hayslett JP. Kidney disease and pregnancy. Br Med J 1974 Dec 7;4(5944):578-582.

(63) Surian M, Imbasciati E, Cosci P, Banfi G, Barbiano di Belgiojoso G, Brancaccio D, et al. Glomerular disease and pregnancy. A study of 123 pregnancies in patients with primary and secondary glomerular diseases. Nephron 1984;36(2):101-105.

(64) Imbasciati E, Ponticelli C. Pregnancy and renal disease: predictors for fetal and maternal outcome. Am J Nephrol 1991;11(5):353-362.

(65) Jungers P, Dougados M, Pelissier C, Kuttenn F, Tron F, Lesavre P, et al. Lupus nephropathy and pregnancy. Report of 104 cases in 36 patients. Arch Intern Med 1982 Apr;142(4):771-776.

(66) Hayslett JP, Lynn RI. Effect of pregnancy in patients with lupus nephropathy. Kidney Int 1980 Aug;18(2):207-220.

(67) Moroni G, Quaglini S, Banfi G, Caloni M, Finazzi S, Ambroso G, et al. Pregnancy in lupus nephritis. Am J Kidney Dis 2002 Oct;40(4):713-720.

(68) Venning M, Patel M. Lupus and connective tissue disease in pregnancy. In: Davison JM, Nelson-Piercy C, Kehoe S, Baker P, editors. Renal Disease in Pregnancy. 1st ed. London: Royal College of Obstetricians and Gynaecologists; 2008. p. 95-109.

(69) Jungers P, Houillier P, Forget D. Reflux nephropathy and pregnancy. Baillieres Clin Obstet Gynaecol 1987 Dec;1(4):955-969.

(70) Jungers P, Houillier P, Chauveau D, Choukroun G, Moynot A, Skhiri H, et al. Pregnancy in women with reflux nephropathy. Kidney Int 1996 Aug;50(2):593-599.

(71) Macejko AM, Schaeffer AJ. Asymptomatic bacteriuria and symptomatic urinary tract infections during pregnancy. Urol Clin North Am 2007 Feb;34(1):35-42.

(72) Smaill F. Antibiotics for asymptomatic bacteriuria in pregnancy. Cochrane Database Syst Rev 2000;(2)(2):CD000490.

(73) Chen YK, Chen SF, Li HC, Lin HC. No increased risk of adverse pregnancy outcomes in women with urinary tract infections: a nationwide population-based study. Acta Obstet Gynecol Scand 2010 Jul;89(7):882-888.

(74) Sharma P, Thapa L. Acute pyelonephritis in pregnancy: a retrospective study. Aust N Z J Obstet Gynaecol 2007 Aug;47(4):313-315.

(75) Iseki K, Kinjo K, Iseki C, Takishita S. Relationship between predicted creatinine clearance and proteinuria and the risk of developing ESRD in Okinawa, Japan. Am J Kidney Dis 2004 Nov;44(5):806-814.

(76) Kent DM, Jafar TH, Hayward RA, Tighiouart H, Landa M, de Jong P, et al. Progression risk, urinary protein excretion, and treatment effects of angiotensin-converting enzyme inhibitors in nondiabetic kidney disease. J Am Soc Nephrol 2007 Jun;18(6):1959-1965.

(77) Katz AI, Davison JM, Hayslett JP, Singson E, Lindheimer MD. Pregnancy in women with kidney disease. Kidney Int 1980 Aug;18(2):192-206.

(78) Stettler RW, Cunningham FG. Natural history of chronic proteinuria complicating pregnancy. Am J Obstet Gynecol 1992 Nov;167(5):1219-1224.

(79) Franceschini N, Savitz DA, Kaufman JS, Thorp JM. Maternal urine albumin excretion and pregnancy outcome. Am J Kidney Dis 2005 Jun;45(6):1010-1018.

(80) Hemmelder MH, de Zeeuw D, Fidler V, de Jong PE. Proteinuria: a risk factor for pregnancy-related renal function decline in primary glomerular disease? Am J Kidney Dis 1995 Jul;26(1):187-192.

(81) Basgul A, Kavak ZN, Sezen D, Basgul A, Gokaslan H, Cakalagaoglu F. A rare case of early onset nephrotic syndrome in pregnancy. Clin Exp Obstet Gynecol 2006;33(2):127-128.

(82) Fisher KA, Ahuja S, Luger A, Spargo BH, Lindheimer MD. Nephrotic proteinuria with pre-eclampsia. Am J Obstet Gynecol 1977 Nov 15;129(6):643-646.

(83) First MR, Ooi BS, Jao W, Pollak VE. Pre-eclampsia with the nephrotic syndrome. Kidney Int 1978 Feb;13(2):166-177.

(84) Imasawa T, Nishiwaki T, Nishimura M, Shikama N, Matsumura R, Nagai M, et al. A case of "pure" preeclampsia with nephrotic syndrome before 15 weeks of gestation in a patient whose renal biopsy showed glomerular capillary endotheliosis. Am J Kidney Dis 2006 Sep;48(3):495-501.

(85) Brown MA, Lindheimer MD, de Swiet M, Van Assche A, Moutquin JM. 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 2001;20(1):IX-XIV.

(86) Chan P, Brown M, Simpson JM, Davis G. Proteinuria in pre-eclampsia: how much matters? BJOG 2005 Mar;112(3):280-285.

(87) Vikse BE, Irgens LM, Leivestad T, Skjaerven R, Iversen BM. Preeclampsia and the risk of end-stage renal disease. N Engl J Med 2008 Aug 21;359(8):800-809.

(88) Klumb EM, de Andrade MC, Jesus NR, Campani C, Campos CF, Levy RA, et al. Primary antiphospholipid nephropathy beginning during pregnancy. Rheumatol Int 2007 Jan;27(3):289-293.

(89) Pandya BK, Gibson SP, Robertson IG. Nephrotic syndrome in early pregnancy--is renal biopsy always necessary? Nephrol Dial Transplant 2002 Apr;17(4):672-674.

(90) Weinstock RS, Kopecky RT, Jones DB, Sunderji S. Rapid development of nephrotic syndrome, hypertension, and hemolytic anemia early in pregnancy in patients with IDDM. Diabetes Care 1988 May;11(5):416-421.

(91) McLigeyo SO, Otieno LS, Kinuthia DM, Mwongera FK, Wairagu SG. Outcome of pregnancy in nephrotic syndrome: a report on five cases. East Afr Med J 1991 Jun;68(6):477-483.

(92) Yao T, Yao H, Wang H. Diagnosis and treatment of nephrotic syndrome during pregnancy. Chin Med J (Engl) 1996 Jun;109(6):471-473.

(93) Kayali F, Najjar R, Aswad F, Matta F, Stein PD. Venous thromboembolism in patients hospitalized with nephrotic syndrome. Am J Med 2008 Mar;121(3):226-230.

(94) Lindqvist PG, Kublikas M, Dahlback B. Individual risk assessment of thrombosis in pregnancy. Acta Obstet Gynecol Scand 2002 May;81(5):412-416.

(95) Heit JA, Kobbervig CE, James AH, Petterson TM, Bailey KR, Melton LJ,3rd. Trends in the incidence of venous thromboembolism during pregnancy or postpartum: a 30-year population-based study. Ann Intern Med 2005 Nov 15;143(10):697-706.

(96) Retnakaran R, Cull CA, Thorne KI, Adler AI, Holman RR, UKPDS Study Group. Risk factors for renal dysfunction in type 2 diabetes: U.K. Prospective Diabetes Study 74. Diabetes 2006 Jun;55(6):1832-1839.

(97) Russo LM, Sandoval RM, McKee M, Osicka TM, Collins AB, Brown D, et al. The normal kidney filters nephrotic levels of albumin retrieved by proximal tubule cells: retrieval is disrupted in nephrotic states. Kidney Int 2007 Mar;71(6):504-513.

(98) Peti-Peterdi J. Independent two-photon measurements of albumin GSC give low values. Am J Physiol Renal Physiol 2009 Jun;296(6):F1255-7.

(99) Milne JE, Lindheimer MD, Davison JM. Glomerular heteroporous membrane modeling in third trimester and postpartum before and during amino acid infusion. Am J Physiol Renal Physiol 2002 Jan;282(1):F170-5.

(100) Cote AM, Firoz T, Mattman A, Lam EM, von Dadelszen P, Magee LA. The 24-hour urine collection: gold standard or historical practice? Am J Obstet Gynecol 2008 Dec;199(6):625.e1-625.e6.

(101) Lambers Heerspink HJ, Gansevoort RT, Brenner BM, Cooper ME, Parving HH, Shahinfar S, et al. Comparison of different measures of urinary protein excretion for prediction of renal events. J Am Soc Nephrol 2010 Aug;21(8):1355-1360.

(102) Leanos-Miranda A, Marquez-Acosta J, Romero-Arauz F, Cardenas-Mondragon GM, Rivera-Leanos R, Isordia-Salas I, et al. Protein:creatinine ratio in random urine samples is a reliable marker of increased 24-hour protein excretion in hospitalized women with hypertensive disorders of pregnancy. Clin Chem 2007 Sep;53(9):1623-1628.

(103) Ruggenenti P, Gaspari F, Perna A, Remuzzi G. Cross sectional longitudinal study of spot morning urine protein:creatinine ratio, 24 hour urine protein excretion rate, glomerular filtration rate, and end stage renal failure in chronic renal disease in patients without diabetes. BMJ 1998 Feb 14;316(7130):504-509.

(104) Home P, Mant J, Diaz J, Turner C, Guideline Development Group. Management of type 2 diabetes: summary of updated NICE guidance. BMJ 2008 Jun 7;336(7656):1306-1308.

(105) Department of Health. National Service Framework for Renal Services - Part Two: Chronic kidney disease, acute renal failure and end of life care. February 3rd 2005; Available at: http://www.dh.gov.uk/dr_consum_dh/groups/dh_digitalassets/@dh/@en/documents/digitalasset/dh_4102680.pdf. Accessed February 16th, 2010.

(106) Wasinger VC, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, et al. Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. Electrophoresis 1995 Jul;16(7):1090-1094.

(107) Adachi J, Kumar C, Zhang Y, Olsen JV, Mann M. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. Genome Biol 2006;7(9):R80.

(108) Fischer MJ. Chronic kidney disease and pregnancy: maternal and fetal outcomes. Adv Chronic Kidney Dis 2007 Apr;14(2):132-145.

(109) McNeil BJ, Hanley JA. Statistical approaches to the analysis of receiver operating characteristic (ROC) curves. Med Decis Making 1984;4(2):137-150.

(110) Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 1982 Apr;143(1):29-36.

(111) Guy M, Borzomato JK, Newall RG, Kalra PA, Price CP. Protein and albumin-to-creatinine ratios in random urines accurately predict 24 h protein and albumin loss in patients with kidney disease. Ann Clin Biochem 2009 Nov;46(Pt 6):468-476.

(112) Cote AM, Brown MA, Lam E, von Dadelszen P, Firoz T, Liston RM, et al. Diagnostic accuracy of urinary spot protein:creatinine ratio for proteinuria in hypertensive pregnant women: systematic review. BMJ 2008 May 3;336(7651):1003-1006.

(113) Keane WF, Brenner BM, de Zeeuw D, Grunfeld JP, McGill J, Mitch WE, et al. The risk of developing end-stage renal disease in patients with type 2 diabetes and nephropathy: the RENAAL study. Kidney Int 2003 Apr;63(4):1499-1507.

(114) Davison JM, Nelson-Piercy C, Kehoe S, Baker P. Renal Disease in Pregnancy. 1st ed. London: Royal College of Obstetricians and Gynaecologists; 2008.

(115) Chappell L, Poulton L, Halligan A, Shennan AH. Lack of consistency in research papers over the definition of pre-eclampsia. Br J Obstet Gynaecol 1999 Sep;106(9):983-985.

(116) Geographic variation in the incidence of hypertension in pregnancy. World Health Organization International Collaborative Study of Hypertensive Disorders of Pregnancy. Am J Obstet Gynecol 1988 Jan;158(1):80-83.

(117) Lowe SA, Brown MA, Dekker GA, Gatt S, McLintock CK, McMahon LP, et al. Guidelines for the management of hypertensive disorders of pregnancy 2008. Aust N Z J Obstet Gynaecol 2009 Jun;49(3):242-246.

(118) Lindheimer MD, Taler SJ, Cunningham FG. Hypertension in pregnancy. J Am Soc Hypertens 2008 Nov-Dec;2(6):484-494.

(119) Brown MA, Hague WM, Higgins J, Lowe S, McCowan L, Oats J, et al. The detection, investigation and management of hypertension in pregnancy: executive summary. Aust N Z J Obstet Gynaecol 2000 May;40(2):133-138.

(120) Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. American Journal of Obstetrics and Gynecology 2000;183:S1-S22.

(121) Cantwell R, Clutton-Brock T, Cooper G, Dawson A, Drife J, Garrod D, et al. Saving Mothers' Lives: Reviewing maternal deaths to make motherhood safer: 2006-2008. The Eighth Report of the Confidential Enquiries into Maternal Deaths in the United Kingdom. BJOG 2011 Mar;118 Suppl 1:1-203.

(122) Acolet D editor. Confidential Enquiry into Maternal and Child Health (CEMACH) Perinatal Mortality 2006: England, Wales and Northern Ireland. London: CEMACH; 2008.

(123) Airoldi J, Weinstein L. Clinical significance of proteinuria in pregnancy. Obstet Gynecol Survey 2007;62(2):117-123.

(124) Cudihy D, Lee RV. The pathophysiology of pre-eclampsia: current clinical concepts. J Obstet Gynaecol 2009 Oct;29(7):576-582.

(125) Redman CW, Sargent IL. Latest advances in understanding preeclampsia. Science 2005 Jun 10;308(5728):1592-1594.

(126) Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, et al. Circulating angiogenic factors and the risk of preeclampsia. N Engl J Maed 2004;350(7):672-683.

(127) Eremina V, Sood M, Haigh J, Nagy A, Lajoie G, Ferrara N, et al. Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. J Clin Invest 2003 Mar;111(5):707-716.

(128) Romero R, Nien JK, Espinoza J, Todem D, Fu W, Chung H, et al. A longitudinal study of angiogenic (placental growth factor) and anti-angiogenic (soluble endoglin and soluble vascular endothelial growth factor receptor-1) factors in normal pregnancy and patients destined to develop preeclampsia and deliver a small for gestational age neonate. J Matern Fetal Neonatal Med 2008 Jan;21(1):9-23.

(129) Munaut C, Lorquet S, Pequeux C, Blacher S, Berndt S, Frankenne F, et al. Hypoxia is responsible for soluble vascular endothelial growth factor receptor-1 (VEGFR-1) but not for soluble endoglin induction in villous trophoblast. Hum Reprod 2008 Jun;23(6):1407-1415.

(130) Baumann MU, Bersinger NA, Mohaupt MG, Raio L, Gerber S, Surbek DV. First-trimester serum levels of soluble endoglin and soluble fms-like tyrosine kinase-1 as first-trimester markers for late-onset preeclampsia. Am J Obstet Gynecol 2008 Sep;199(3):266.e1-266.e6.

(131) Haller H, Ziegler EM, Homuth V, Drab M, Eichhorn J, Nagy Z, et al. Endothelial adhesion molecules and leukocyte integrins in preeclamptic patients. Hypertension 1997 Jan;29(1 Pt 2):291-296.

(132) Karumanchi SA, Maynard SE, Stillman IE, Epstein FH, Sukhatme VK. Preeclampsia: a renal perspective. Kidney Int 2005;67(6):2101-2113.

(133) Sorensen TK, Easterling TR, Carlson KL, Brateng DA, Benedetti TJ. The maternal hemodynamic effect of indomethacin in normal pregnancy. Obstet Gynecol 1992 May;79(5 (Pt 1)):661-663.

(134) Davison JM, Homuth V, Jeyabalan A, Conrad KP, Karumanchi SA, Quaggin S, et al. New aspects in the pathophysiology of preeclampsia. J Am Soc Nephrol 2004;15:2440-2448.

(135) Savvidou MD, Hingorani AD, Tsikas D, Frolich JC, Vallance P, Nicolaides KH. Endothelial dysfunction and raised plasma concentrations of asymmetric dimethylarginine in pregnant women who subsequently develop pre-eclampsia. Lancet 2003 May 3;361(9368):1511-1517.

(136) Jeyabalan A, Novak J, Danielson LA, Kerchner LJ, Opett SL, Conrad KP. Essential role for vascular gelatinase activity in relaxin-induced renal vasodilation, hyperfiltration, and reduced myogenic reactivity of small arteries. Circ Res 2003 Dec 12;93(12):1249-1257.

(137) Altman D, Carroli G, Duley L, Farrell B, Moodley J, Neilson J, et al. Do women with pre-eclampsia, and their babies, benefit from magnesium sulphate? The Magpie Trial: a randomised placebo-controlled trial. Lancet 2002 Jun 1;359(9321):1877-1890.

(138) Rumbold A, Duley L, Crowther CA, Haslam RR. Antioxidants for preventing pre-eclampsia. Cochrane Database Syst Rev 2008 Jan 23;(1)(1):CD004227.

(139) Meher S, Duley L. Garlic for preventing pre-eclampsia and its complications. Cochrane Database Syst Rev 2006 Jul 19;3:CD006065.

(140) Meher S, Duley L. Nitric oxide for preventing pre-eclampsia and its complications. Cochrane Database Syst Rev 2007 Apr 18;(2)(2):CD006490.

(141) Meher S, Duley L. Progesterone for preventing pre-eclampsia and its complications. Cochrane Database Syst Rev 2006 Oct 18;(4)(4):CD006175.

(142) Meher S, Duley L. Exercise or other physical activity for preventing pre-eclampsia and its complications. Cochrane Database Syst Rev 2006 Apr 19;(2)(2):CD005942.

(143) Churchill D, Beevers GD, Meher S, Rhodes C. Diuretics for preventing pre-eclampsia. Cochrane Database Syst Rev 2007 Jan 24;(1)(1):CD004451.

(144) Hofmeyr GJ, Atallah AN, Duley L. Calcium supplementation during pregnancy for preventing hypertensive disorders and related problems. Cochrane Database Syst Rev 2006 Jul 19;3:CD001059.

(145) Hofmeyr GJ, Duley L, Atallah A. Dietary calcium supplementation for prevention of pre-eclampsia and related problems: a systematic review and commentary. BJOG 2007 Aug;114(8):933-943.

(146) Conde-Agudelo A, Villar J, Lindheimer MD. World Health Organization Systematic Review of Screening Tests for Preeclampsia. Am J Obstet Gynecol 2004;104(4):1367-1391.

(147) Kenny LC, Broadhurst DI, Dunn W, Brown M, North RA, McCowan L, et al. Robust early pregnancy prediction of later preeclampsia using metabolomic biomarkers. Hypertension 2010 Oct;56(4):741-749.

(148) Giguere Y, Charland M, Bujold E, Bernard N, Grenier S, Rousseau F, et al. Combining biochemical and ultrasonographic markers in predicting preeclampsia: a systematic review. Clin Chem 2010 Mar;56(3):361-375.

(149) Powers RW, Jeyabalan A, Clifton RG, Van Dorsten P, Hauth JC, Klebanoff MA, et al. Soluble fms-Like tyrosine kinase 1 (sFlt1), endoglin and placental growth factor (PlGF) in preeclampsia among high risk pregnancies. PLoS One 2010 Oct 11;5(10):e13263.

(150) Nicolaides KH, Bindra R, Turan OM, Chefetz I, Sammar M, Meiri H, et al. A novel approach to first-trimester screening for early pre-eclampsia combining serum PP-13 and Doppler ultrasound. Ultrasound Obstet Gynecol 2006 Jan;27(1):13-17.

(151) Diab AE, El-Behery MM, Ebrahiem MA, Shehata AE. Angiogenic factors for the prediction of pre-eclampsia in women with abnormal midtrimester uterine artery Doppler velocimetry. Int J Gynaecol Obstet 2008 Aug;102(2):146-151.

(152) Jauniaux E, Gulbis B, Tunkel S, Ramsay B, Campbell S, Meuris S. Maternal serum testing for alpha-fetoprotein and human chorionic gonadotropin in high-risk pregnancies. Prenat Diagn 1996 Dec;16(12):1129-1135.

(153) Spencer K, Cowans NJ, Chefetz I, Tal J, Meiri H. First-trimester maternal serum PP-13, PAPP-A and second-trimester uterine artery Doppler pulsatility index as markers of pre-eclampsia. Ultrasound Obstet Gynecol 2007 Feb;29(2):128-134.

(154) Ay E, Kavak ZN, Elter K, Gokaslan H, Pekin T. Screening for pre-eclampsia by using maternal serum inhibin A, activin A, human chorionic gonadotropin, unconjugated estriol, and alpha-fetoprotein levels and uterine artery Doppler in the second trimester of pregnancy. Aust N Z J Obstet Gynaecol 2005 Aug;45(4):283-288.

(155) Poon LC, Kametas NA, Maiz N, Akolekar R, Nicolaides KH. First-trimester prediction of hypertensive disorders in pregnancy. Hypertension 2009 May;53(5):812-818.

(156) Buhimschi CS, Norwitz ER, Funai E, Richman S, Guller S, Lockwood CJ, et al. Urinary angiogenic factors cluster hypertensive disorders and identify women with severe preeclampsia. Am J Obstet Gynecol 2005 Mar;192(3):734-741.

(157) Garovic VD, Wagner SJ, Turner ST, Rosenthal DW, Watson WJ, Brost BC, et al. Urinary podocyte excretion as a marker for preeclampsia. Am J Obstet Gynecol 2007 Apr;196(4):320.e1-320.e7.

(158) Hans JB, Kelder TP, Penning ME, Scherjon S, Bruijn JA. Podocyte Specific mRNA Levels Measured in Urine of Patients with Preeclampsia Are Increased Compared to Healthy Pregnant Controls. American Society of Nephrology Renal Week; November 18th - 21st 2010; Denver, CO. USA; 2010.

(159) Buhimschi IA, Zhao G, Funai EF, Harris N, Sasson IE, Bernstein IM, et al. Proteomic profiling of urine identifies specific fragments of SERPINA1 and albumin as biomarkers of preeclampsia. Am J Obstet Gynecol 2008 Nov;199(5):551.e1-551.16.

(160) Carty DM, Siwy J, Brennand JE, Zurbig P, Mullen W, Franke J, et al. Urinary proteomics for prediction of preeclampsia. Hypertension 2011 Mar;57(3):561-569.

(161) Kronborg CS, Allen J, Vittinghus E, Knudsen UB. Pre-symptomatic increase in urine-orosomucoid excretion in pre-eclamptic women. Acta Obstet Gynecol Scand 2007;86(8):930-937.

(162) Williams PJ, Gumaa K, Scioscia M, Redman CW, Rademacher TW. Inositol phosphoglycan P-type in preeclampsia: a novel marker? Hypertension 2007 Jan;49(1):84-89.

(163) Hamar BD, Buhimschi IA, Sfakianaki AK, Pettker CM, Magloire LK, Funai EF, et al. Serum and urine inhibin A but not free activin A are endocrine biomarkers of severe pre-eclampsia. Am J Obstet Gynecol 2006 Dec;195(6):1636-1645.

(164) Aggarwal PK, Jain V, Sakhuja V, Karumanchi SA, Jha V. Low urinary placental growth factor is a marker of pre-eclampsia. Kidney Int 2006 Feb;69(3):621-624.

(165) Roes EM, Raijmakers MT, Roelofs HM, Peters WH, Steegers EA. Urinary GSTP1-1 excretion is markedly increased in normotensive pregnancy as well as in preeclampsia. J Nephrol 2005 Jul-Aug;18(4):405-408.

(166) Heyl W, Heintz B, Reister F, Harwig S, Witte K, Lemmer B, et al. Increased soluble VCAM-1 serum levels in preeclampsia are not correlated to urinary excretion or circadian blood pressure rhythm. J Perinat Med 2005;33(2):144-148.

(167) Levine RJ, Thadhani R, Qian C, Lam C, Lim KH, Yu KF, et al. Urinary placental growth factor and risk of preeclampsia. JAMA 2005 Jan 5;293(1):77-85.

(168) Paternoster DM, Stella A, Babbo GL, Pignataro R, Mussap M, Plebani M. Markers of tubular damage in pre-eclampsia. Minerva Ginecol 1999 Oct;51(10):373-377.

(169) Mills JL, DerSimonian R, Raymond E, Morrow JD, Roberts LJ,2nd, Clemens JD, et al. Prostacyclin and thromboxane changes predating clinical onset of preeclampsia: a multicenter prospective study. JAMA 1999 Jul 28;282(4):356-362.

(170) Bahado-Singh RO, Oz U, Isozaki T, Seli E, Kovanci E, Hsu CD, et al. Midtrimester urine human chorionic gonadotropin beta-subunit core fragment levels and the subsequent development of pre-eclampsia. Am J Obstet Gynecol 1998 Sep;179(3 Pt 1):738-741.

(171) Leanos-Miranda A, Marquez-Acosta J, Cardenas-Mondragon GM, Chinolla-Arellano ZL, Rivera-Leanos R, Bermejo-Huerta S, et al. Urinary prolactin as a reliable marker for preeclampsia, its severity, and the occurrence of adverse pregnancy outcomes. J Clin Endocrinol Metab 2008 Jul;93(7):2492-2499.

(172) Altchek A, Albright NL, Sommers SC. The renal pathology of toxemia of pregnancy. Obstet Gynecol 1968 May;31(5):595-607.

(173) Gammill HS, Jeyabalan A. Acute renal failure in pregnancy. Crit Care Med 2005 Oct;33(10 Suppl):S372-84.

(174) Morris RH, Vassalli P, McCluskey RT. Immunofluorescent studies on renal biopsies in pregnancy. Clin Obstet Gynecol 1968 Jun;11(2):522-532.

(175) Roelofsen H, Alvarez-Llamas G, Schepers M, Landman K, Vonk RJ. Proteomics profiling of urine with surface enhanced laser desorption/ionization time of flight mass spectrometry. Proteome Sci 2007 Jan 15;5:2.

(176) Rai AJ, Stemmer PM, Zhang Z, Adam BL, Morgan WT, Caffrey RE, et al. Analysis of Human Proteome Organization Plasma Proteome Project (HUPO PPP) reference specimens using surface enhanced laser desorption/ionization-time of flight (SELDI-TOF) mass spectrometry: multi-institution correlation of spectra and identification of biomarkers. Proteomics 2005 Aug;5(13):3467-3474.

(177) Check E. Proteomics and cancer: running before we can walk? Nature 2004 Jun 3;429(6991):496-497.

(178) Papale M, Di Paolo S, Magistroni R, Lamacchia O, Di Palma AM, De Mattia A, et al. Urine proteome analysis may allow noninvasive differential diagnosis of diabetic nephropathy. Diabetes Care 2010 Nov;33(11):2409-2415.

(179) Dihazi H, Muller GA, Lindner S, Meyer M, Asif AR, Oellerich M, et al. Characterization of diabetic nephropathy by urinary proteomic analysis: identification of a processed ubiquitin form as a differentially excreted protein in diabetic nephropathy patients. Clin Chem 2007 Sep;53(9):1636-1645.

(180) Ho J, Lucy M, Krokhin O, Hayglass K, Pascoe E, Darroch G, et al. Mass spectrometry-based proteomic analysis of urine in acute kidney injury following cardiopulmonary bypass: a nested case-control study. Am J Kidney Dis 2009 Apr;53(4):584-595.

(181) Bennett MR, Ravipati N, Ross G, Nguyen MT, Hirsch R, Beekman RH, et al. Using proteomics to identify preprocedural risk factors for contrast induced nephropathy. Proteomics Clin Appl 2008;2(7-8):1058-1064.

(182) Vanhoutte KJ, Laarakkers C, Marchiori E, Pickkers P, Wetzels JF, Willems JL, et al. Biomarker discovery with SELDI-TOF MS in human urine associated with early renal injury: evaluation with computational analytical tools. Nephrol Dial Transplant 2007 Oct;22(10):2932-2943.

(183) Liang SL, Clarke W. Urine proteomic profiling for biomarkers of acute renal transplant rejection. Methods Mol Biol 2010;641:185-191.

(184) O'Riordan E, Orlova TN, Podust VN, Chander PN, Yanagi S, Nakazato M, et al. Characterization of urinary peptide biomarkers of acute rejection in renal allografts. Am J Transplant 2007 Apr;7(4):930-940.

(185) Zhang X, Jin M, Wu H, Nadasdy T, Nadasdy G, Harris N, et al. Biomarkers of lupus nephritis determined by serial urine proteomics. Kidney Int 2008 Sep;74(6):799-807.

(186) Suzuki M, Ross GF, Wiers K, Nelson S, Bennett M, Passo MH, et al. Identification of a urinary proteomic signature for lupus nephritis in children. Pediatr Nephrol 2007 Dec;22(12):2047-2057.

(187) Okamoto A, Yamamoto H, Imai A, Hatakeyama S, Iwabuchi I, Yoneyama T, et al. Protein profiling of post-prostatic massage urine specimens by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry to discriminate between prostate cancer and benign lesions. Oncol Rep 2009 Jan;21(1):73-79.

(188) Wu DL, Zhang YF, Guan M, Liu WW, Xu YM, Jin SB, et al. Tree analysis pattern of mass spectral urine profiles in differential diagnosis of bladder transitional cell carcinoma. Zhonghua Zhong Liu Za Zhi 2007 Apr;29(4):274-277.

(189) Wu DL, Zhang WH, Wang WJ, Jing SB, Xu YM. Proteomic evaluation of urine from renal cell carcinoma using SELDI-TOF-MS and tree analysis pattern. Technol Cancer Res Treat 2008 Jun;7(3):155-160.

(190) Thongboonkerd V. Practical points in urinary proteomics. J Proteome Res 2007 Oct;6(10):3881-3890.

(191) Saetun P, Semangoen T, Thongboonkerd V. Characterizations of urinary sediments precipitated after freezing and their effects on urinary protein and chemical analyses. Am J Physiol Renal Physiol 2009 Jun;296(6):F1346-54.

(192) Papale M, Pedicillo MC, Thatcher BJ, Di Paolo S, Lo Muzio L, Bufo P, et al. Urine profiling by SELDI-TOF/MS: monitoring of the critical steps in sample collection, handling and analysis. J Chromatogr B Analyt Technol Biomed Life Sci 2007 Sep 1;856(1-2):205-213.

(193) Marshall J, Kupchak P, Zhu W, Yantha J, Vrees T, Furesz S, et al. Processing of serum proteins underlies the mass spectral fingerprinting of myocardial infarction. J Proteome Res 2003 Jul-Aug;2(4):361-372.

(194) Rai AJ, Gelfand CA, Haywood BC, Warunek DJ, Yi J, Schuchard MD, et al. HUPO Plasma Proteome Project specimen collection and handling: towards the standardization of parameters for plasma proteome samples. Proteomics 2005 Aug;5(13):3262-3277.

(195) Thongboonkerd V, Saetun P. Bacterial overgrowth affects urinary proteome analysis: recommendation for centrifugation, temperature, duration, and the use of preservatives during sample collection. J Proteome Res 2007 Nov;6(11):4173-4181.

(196) Schaub S, Wilkins J, Weiler T, Sangster K, Rush D, Nickerson P. Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. Kidney Int 2004 Jan;65(1):323-332.

(197) Jaffe M. Ueber den Niederschlag, welchen Pikrinäurein normalem Harn erzeugt und über eine neue Reaktion des Kreatinins. Z Physiol Chem 1886;10:391-400.

(198) Greenwald I. The Chemistry of Jaffe's reaction for creatinine. V. The isolation of the red compound. J Biol Chem 1928;80:103-105.

(199) Fossati P, Prencipe L, Berti G. Enzymic creatinine assay: a new colorimetric method based on hydrogen peroxide measurement. Clin Chem 1983 Aug;29(8):1494-1496.

(200) Munz E, Bernt E, Wahlefeld AW. An evaluation of a fully enzymatic method for creatinine determination. Z Klin Chem Klin Biochem 1974 May;12(5):259.

(201) Brown ND, Sing HC, Neeley WE, Koetitz SE. Determination of "true" serum creatinine by high-performance liquid chromatography combined with a continuous-flow microanalyzer. Clin Chem 1977 Jul;23(7):1281-1283.

(202) Lim CK, Richmond W, Robinson DP, Brown SS. Towards a definitive assay of creatinine in serum and in urine: separation by high-performance liquid chromatography. J Chromatogr 1978 Jan 1;145(1):41-49.

(203) Welch MJ, Cohen A, Hertz HS, Ng KJ, Schaffer R, Van der Lijn P, et al. Determination of serum creatinine by isotope dilution mass spectrometry as a candidate definitive method. Anal Chem 1986 Jul;58(8):1681-1685.

(204) Barr DB, Wilder LC, Caudill SP, Gonzales AJ, Needham LL, Pirkle JL. Urinary creatinine concentrations in the U.S. Population: Implications for Urinary Biologic Monitoring Measurements. Environ Health Perpect 2005;113:119.

(205) Tidman M, Sjostrom P, Jones I. A Comparison of GFR estimating formulae based upon s-cystatin C and s-creatinine and a combination of the two. Nephrol Dial Transplant 2008 Jan;23(1):154-160.

(206) Catlin DH, Starcevic B. HPLC Method for the Assay of Creatinine in Urine. J Liq Chromatography & Rel Technol 1991;14(12):2399-2408.

(207) Seronie-Vivien S, Galteau MM, Carlier MC, Hadj-Aissa A, Hanser AM, Hym B, et al. Impact of standardized calibration on the inter-assay variation of 14 automated assays for the measurement of creatinine in human serum. Clin Chem Lab Med 2005;43(11):1227-1233.

(208) Myers GL, Miller WG, Coresh J, Fleming J, Greenberg N, Greene T, et al. Recommendations for improving serum creatinine measurement: a report from the Laboratory Working Group of the National Kidney Disease Education Program. Clin Chem 2006 Jan;52(1):5-18.

(209) Candiano G, Musante L, Bruschi M, Petretto A, Santucci L, Del Boccio P, et al. Repetitive fragmentation products of albumin and alpha1-antitrypsin in glomerular diseases associated with nephrotic syndrome. J Am Soc Nephrol 2006 Nov;17(11):3139-3148.

(210) Gonzales PA, Zhou H, Pisitkun T, Wang NS, Star RA, Knepper MA, et al. Isolation and purification of exosomes in urine. Methods Mol Biol 2010;641:89-99.

(211) Ball G, Al-Shahib A. Data Mining for Predictive Proteomics. In: Shah HN, Gharbia SE, editors. Mass Spectrometry for Microbial Proteomics London: John Wiley & Sons. Ltd; 2010. p. 411-422.

(212) Zhou H, Yuen PS, Pisitkun T, Gonzales PA, Yasuda H, Dear JW, et al. Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney Int 2006 Apr;69(8):1471-1476.

(213) Ball G, Mian S, Holding F, Allibone RO, Lowe J, Ali S, et al. An integrated approach utilizing artificial neural networks and SELDI mass spectrometry for the classification of human tumours and rapid identification of potential biomarkers. Bioinformatics 2002 Mar;18(3):395-404.

(214) Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, et al. Use of proteomic patterns in serum to identify ovarian cancer. Lancet 2002 Feb 16;359(9306):572-577.

(215) Rajakaruna L, Hallas G, Molenaar L, Dare D, Sutton H, Encheva V, et al. High throughput identification of clinical isolates of Staphylococcus aureus using MALDI-TOF-MS of intact cells. Infect Genet Evol 2009 Jul;9(4):507-513.

(216) Matharoo-Ball B, Ball G, Rees R. Clinical proteomics: discovery of cancer biomarkers using mass spectrometry and bioinformatics approaches--a prostate cancer perspective. Vaccine 2007 Sep 27;25 Suppl 2:B110-21.

(217) Matharoo-Ball B, Miles AK, Creaser CS, Ball G, Rees R. Serum biomarker profiling in cancer studies: a question of standardisation? Vet Comp Oncol 2008 Dec;6(4):224-247.

(218) Poston L, Briley AL, Seed PT, Kelly FJ, Shennan AH, Vitamins in Pre-eclampsia (VIP) Trial Consortium. Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebo-controlled trial. Lancet 2006 Apr 8;367(9517):1145-1154.

(219) Yamamoto T. The 4th Human Kidney and Urine Proteome Project (HKUPP) workshop. 26 September 2009, Toronto, Canada. Proteomics 2010 Jun;10(11):2069-2070.

(220) Morgan R, Boxall A, Bhatt A, Bailey M, Hindley R, Langley S, et al. Engrailed-2 (EN2): A Tumor Specific Urinary Biomarker for the Early Diagnosis of Prostate Cancer. Clin Cancer Res 2011 Mar 1.

(221) Wu T, Fu Y, Brekken D, Yan M, Zhou XJ, Vanarsa K, et al. Urine proteome scans uncover total urinary protease, prostaglandin D synthase, serum amyloid P, and superoxide dismutase as potential markers of lupus nephritis. J Immunol 2010 Feb 15;184(4):2183-2193.

(222) Sandrim VC, Palei AC, Sertorio JT, Cavalli RC, Duarte G, Tanus-Santos JE. Effects of eNOS polymorphisms on nitric oxide formation in healthy pregnancy and in pre-eclampsia. Mol Hum Reprod 2010 Jul;16(7):506-510.

(223) von Versen-Hoeynck FM, Hubel CA, Gallaher MJ, Gammill HS, Powers RW. Plasma levels of inflammatory markers neopterin, sialic acid, and C-reactive protein in pregnancy and preeclampsia. Am J Hypertens 2009 Jun;22(6):687-692.

(224) Devarajan P, Krawczeski CD, Nguyen MT, Kathman T, Wang Z, Parikh CR. Proteomic identification of early biomarkers of acute kidney injury after cardiac surgery in children. Am J Kidney Dis 2010 Oct;56(4):632-642.