# The nature of glomerular dysfunction in preeclampsia

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by

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**Declaration**

I, Dr Justine Norman am the author of this Thesis.

The original grant applications, protocols and infusion studies were all designed and undertaken by myself under the supervision of Professor J M Davison.

The Joint Ethics Committee for Newcastle and North Tyneside Health Authority and the Universities of Newcastle upon Tyne and Northumbria, granted ethical approval for all studies described in this thesis. Informed written consent was obtained from all volunteers prior to study.

All patient recruitment and infusion studies were performed by myself.

The in-house laboratory measures of renal haemodynamics and dextran macromolecular handling were undertaken by Mrs EA Shiells and Mrs M Kirkley. Urinary microalbumin measurement was undertaken by Mrs Fiona Harding.

The nitric oxide, cyclic GMP and amino acid work was undertaken by Professor C Baylis's laboratory in the Department of Physiology, University of West Virginia, Morgantown, USA.

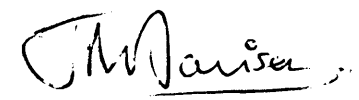
The work contained within this thesis is registered for the degree of Medical Doctorate with the University of Leicester. It has not been submitted elsewhere.

Signed:

Justine Norman

I declare the above statements are true.

Signed:



Professor JM Davison

**Publications arising from thesis**

**Oral presentations to learned societies**

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**Other publications**

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Norman JC, Davison JM and Lindheimer MD. "Renal disorders in pregnancy". Contemporary Clinical Gynecology and Obstetrics 2001;1:59-67.

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**Abbreviations**

Within the text where a term is initially used, its abbreviation is depicted and thereafter this abbreviation is used. In relation to equations and formulae there are occasions within the text where terms and abbreviations are further emphasized for the purposes of clarity.

| | |
|---|---|
| ADMA | asymmetric D-methyl-arginine |
| ANP | atrial natriuretic peptide |
| AVP | arginine vasopressin |
| BP | blood pressure |
| C | total protein concentration (g/100ml) |
| cAMP | cyclic adenosine monophosphate |
| cGMP | cyclic guanosine monophosphate |
| ECV | extracellular volume |
| EDRF | endothelial derived relaxing factor |
| EDTA | ethylenediaminetetra-acetic acid disodium salt |
| ERBF | effective renal blood flow (ml/min) |
| ERPF | effective renal plasma flow (ml/min) |
| FF | filtration fraction |
| GBF | glomerular blood flow |
| GBM | glomerular basement membrane |
| GFR | glomerular filtration rate |
| GPC | gel permeation chromatography |
| GPF | glomerular plasma flow |
| Hct | haematocrit |
| $k$ | effective hydraulic permeability (ml/min.mmHg.cm$^2$) |

| | |
|---|---|
| K | kilodalton |
| $K_f$ | ultrafiltration coefficient (ml/min/mmHg) |
| LP | late pregnancy |
| MCR | metabolic clearance rate (l/min) |
| Na | sodium (mmol/l) |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NOx | Nitrate and nitrite (i.e. no metabolites) |
| $\theta$ | fractional clearance |
| P | hydraulic pressure (mmHg) |
| PAH | paraamino hippurate |
| $P_{UF}$ | net ultrafiltration pressure (mmHg) |
| $\Delta P$ | transglomerular hydrostatic pressure difference (mmHg) |
| $\Delta P_{GC}$ | hydrostatic pressure within glomerular capillary |
| $\Delta P_{tf}$ | hydrostatic pressure within tubular fluid |
| PP | postpartum |
| $\pi$ | colloid osmotic pressure (mmHg) |
| $\Delta\pi$ | transglomerular osmotic pressure difference (mmHg) |
| $Q_E$ | efferent arteriolar plasma flow (ml/min) |
| $Q_A$ | afferent arteriolar plasma flow (ml/min) |
| RAAS | rennin-angiotensin-aldosterone system |
| RBF | renal blood flow (ml/min) |
| RIA | radioimmunoassay |
| $r_o$ | isoporous model mean pore size (nm) |
| RPF | renal plasma flow (ml/min) |
| $R_{TA}$ | total arteriolar resistance $R_A + R_E$ (dyn.s.cm$^{-5}$) |

| | |
|---|---|
| $s$ | surface area for ultrafiltration ($cm^2$) |
| S | lognormal model standard deviation (nm) |
| SEM | standard error of the mean |
| SN | single nephron e.g SNGFR (nl/min) |
| U | lognormal mean pore size (nm) |
| UAE | urinary albumin excretion (mg/24hr) |
| $V_E$ | elution volume (ml) |
| $V_{TF}$ | tubule flow rate (nl/min) |
| $\omega o$ | modelled "shunt" component |
| $X^2$ | chi-squared statistical test |

# ABSTRACT


## The nature of glomerular dysfunction in preeclampsia. Justine Norman

### Introduction

Normal pregnancy was compared to preeclamptic (PE) pregnancy with respect to glomerular ultrafiltration nitric oxide (NO) activity, derived from the metabolism of L-arginine (L-arg) to citrulline. Two biomathematical models, the "isoporous plus shunt" (mean pore size $r_o$ ,shunt component $\omega_o$) and "lognormal" (mean pore size U , standard deviation S), together with fractional dextran clearance ($\theta_D$) enabled estimation of glomerular ultrafiltration parameters ($K_f$- ultrafiltration coefficient, $\Delta P$-transglomerular pressure). NO activity was assessed from plasma and 24hr urinary NOx (nitrate and nitrite), second messenger cyclic GMP (cGMP) and NO synthase inhibitor asymmetric-D-methyl-arginine (ADMA). Late pregnant (LP) and postpartum (PP) data are presented. As NO might mediate gestational renal vasodilatation, the effects of infused L-arginine (NO precursor) compared to glycine, on ultrafiltration were examined, testing the hypothesis that NO deficiency is important in renal vasoconstriction and hypofiltration of PE.

### Methodology

16 healthy women (controls: CNTL) studied in LP and >4 months PP. 12 untreated preeclamptic (PE) primigravidas studied LP and PP. Following a 48 hour low nitrate/nitrite diet, basal measurements were undertaken: plasma NOx, cGMP, L-arg and ADMA, 24 hour urinary NOx and cGMP. Each then underwent a 4-hour neutral dextran, paraaminohippurate and inulin infusion. After 1h there were 3 x 20 min clearances to measure glomerular filtration rate (GFR), effective renal plasma flow (ERPF) and to obtain $\theta_D$ data. After 2h , each received an aminoacid (aa) infusion, either L-arginine or glycine (1ml/min). 3 x 20min collection periods were again repeated after 1h.

### Results

| Study group | | GFR ml/min | GFR during aa | ERPF ml/min | ERPF during aa | $K_f$ | $r_o$ | $\omega_o$ | U | S |
|---|---|---|---|---|---|---|---|---|---|---|
| LP CNTL | | | | | | 8.6 | 60.4 | 0.005 | 51 | 1.18 |
| | Glyc | 132+/- 9 | 144 +/-14 | 665+/-73 | 713+/-79 | | | | | |
| | L-arg | 151+/-7 | 147+/-9 | 708+/-22 | 720+/-33 | | | | | |
| PP CNTL | | | | | | 9.5 | 62.6 | 0.003 | 56 | 1.14 |
| | Glyc | 100+/-10 | 109+/-16 | 521+/-60 | 574+/-70 | | | | | |
| | L-arg | 94+/-6. | 91+/-5 | 507+/-39 | 505+/-34 | | | | | |
| LP PE | | | | | | 6.4 | 62.4 | 0.01 | 52 | 1.22 |
| | Glyc | 113+/-9 | 108+/-7.8 | 650+/-60 | 617+/-49 | | | | | |
| | L-arg | 114+/-19 | 107+/-15.3 | 541+/-71 | 542+/-74 | | | | | |
| PP PE | | | | | | 8.9 | 64 | 0.005 | 59 | 1.14 |
| | Glyc | 83+/-10 | 85+/- 9.3 | 421+/-58 | 417+/-53 | | | | | |
| | L-arg | 87+/-8 | 89+/-7 | 425+/-37 | 467+/-39 | | | | | |

| Study group | Plasma Nox (microM) | 24hrUNOx (microM) | Plasma cGMP (pmol/ml) | 24hrUcGMP (nmoles) | ADMA (uM) |
|---|---|---|---|---|---|
| LP CNTL | 14.1 +/- 2.2 | 1096 +/- 103 | 6.3 +/- 0.4 | 1728 +/- 183 | 0.47 +/- 0.07 |
| PP CNTL | 22.6 +/- 2.7 | 862 +/- 116 | 7.4 +/- 0.7 | 829 +/- 96 | 0.55 +/ 0.06 |
| LP PE | 27 +/- 5.3 | 1168 +/- 265 | 7.3 +/- 0.7 | 1255 +/- 99 | 0.58 +/- 0.1 |
| PP PE | 25.6 +/- 5.1 | 908 +/- 98 | 8 +/- 0.9 | 772 +/- 95 | 0.53 +/- 0.07 |

### Conclusions

Normal pregnancy is associated with increased GFR, ERPF, $\omega_o$ and S ($p<0.05$). PE pregnancy is associated with reduced $K_f$ but increased $r_o$ , $\omega_o$, U and S. PE values PP approach controls. From the indices that were utilised, there was no evidence of significant NO deficiency in Preeclampsia and even if there was a relative deficiency the infusion of the NO precursor L-arginine failed to augment the decreased renal haemodynamics of preeclampsia. In PE the hypofiltration therefore has both a haemodynamic and a structural basis, it recovers PP and L-arg has no ameliorating effect in LP, or PP.

# PREFACE

The work for this Thesis was completed in 1999 and 2000. The hypothesis tested by the research was formulated in 1997-1998, when funding for the work was being sought. That hypothesis was therefore devised within the context of that current literature as well as the ongoing work within the department at that time. Therefore, it has to be re-emphasised that the original literature review was researched and constructed at that time. Inevitably, the field of knowledge has considerably advanced from 2001 when the review was completed. Thus by the time of Thesis submission (in 2003-2004) and examination, the review was outdated, the hypothesis could be criticised, the frailties of study design could be exposed and overall the discussion of the derived data from 1999-2000 against this background has limitations. Nevertheless, it was decided that the discussion section of the Thesis should embrace much of the newer literature, including part of 2004.

The new literature review has been updated, even though this somewhat undermines the original hypothesis and generates obvious self- criticism in relation to study design and methodologies. There is no doubt, however, that the recruitment for and conduct of the infusion studies and subsequent laboratory determinations and analysis utilised methods and approaches whose validity was not in question. In line with generous and timely advice received, the revisions made, have been seamlessly added to the text but always bearing in mind that the work was conceived and completed 5 years ago.

This preface has been added to explain the timescale of Thesis work, preparation and submission in the light of future discussion with those concerned in the Thesis examination process. This does enhance the factual content of the Thesis but in no way should detract from the ideas and available knowledge in the late 1990's that generated the original clinical research studies.

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# 1.0 INTRODUCTION

In normal pregnancy the physiological adaptations by all systems and organs including the renal tract are truly remarkable. Glomerular filtration rate (GFR) increases by 50% and renal plasma flow (RPF) by 50-80%. Urinary protein excretion also increases but the underlying mechanisms are largely unknown. Further increments in urinary protein excretion are seen in pregnancies complicated by preeclampsia and other renal diseases reflecting in the main alterations in glomerular filtration barrier function.

Preeclampsia is an important cause of maternal and fetal morbidity and mortality complicating approximately 5% of all pregnancies. It has been described as a maternal syndrome at the extreme end of the spectrum of the gestational inflammatory disturbance that affects all vascular compartment cells, with one outcome being widespread maternal endothelial dysfunction (Redman et al, 1999). The aetiology of preeclampsia is uncertain but a failure of complete placentation with a resultant hypoxic placenta may predispose to endothelial dysfunction and preeclampsia. Whatever the aetiology of preeclampsia, the ultimate targeting of the maternal endothelium produces a multiorgan disorder with widespread enhanced vascular reactivity, activation of the coagulation cascade and disrupted volume homeostasis (Roberts et al, 1989; Redman et al, 1999; Norwitz and Repke, 2000). The exact pattern of end organ damage dictates the subsequent clinical symptoms and signs in the individual. From a clinical viewpoint there can be major diagnostic difficulties distinguishing clinically between preeclampsia, chronic hypertension, renal disease and combinations of these separate entities.

The work described in this Thesis examines glomerular dysfunction in preeclampsia and some aspects of the role of nitric oxide in the renal vasodilation of normal human pregnancy and in the renal pathophysiology of preeclampsia. The approach to these

issues involves the use of "gold standard" methods for the determination of GFR and RPF (inulin and paraaminohippurate (PAH) clearances, respectively) and the fractional clearance of neutral dextrans with all derived data analysed by theoretical biomathematical models of the glomerulus that predict functional parameters in relation to glomerular barrier function and allow assumptions to be made about transglomerular hydrostatic pressure difference.

# 2.0 BACKGROUND AND REVIEW OF THE LITERATURE

## 2.1 The kidney

### 2.1.1 Structure and function of the kidney

The kidney consists of a cortex, surrounding the central region, the medulla, which is divided into inner and outer portions, and a pelvis that connects with the ureter. The renal artery enters the kidney beside the ureter and then branches forming the interlobar arteries, the arcuate arteries, the cortical radial arteries and ultimately the afferent arterioles, which subsequently lead to the glomerular capillary network (Valtin and Schafer, 1995). The venous system with similar tributaries eventually terminates in the renal vein, also located beside the ureter. Despite only accounting for approximately 0.5% of the total body weight, the kidneys receive 25% of the cardiac output. The unique feature of the kidney is the 2 capillary networks in series i.e. the glomerular capillary network and the peritubular capillary network.

The nephron which is the functional unit of the kidney consists of the renal corpuscle (glomerulus and Bowmans capsule), proximal tubule, the loop of Henlé, the juxtaglomerular apparatus (macula densa cells and granular endothelial cells), the distal convoluted tubule and collecting duct (Valtin and Schafer, 1995). Two main types of nephron exist, juxtamedullary and superficial, which differ structurally and in location within the kidney.

The main processes involved in renal function include:

- Filtration

  The deposition of an ultrafiltrate of plasma from the glomerular capillary into the Bowmans space.

- Reabsorption

  Transport of solutes and water from tubular fluid into the peritubular capillary network.

- Secretion

  Addition of certain solutes from peritubular capillary network to tubular fluid

- Excretion

  Removal of water and solutes from the body as urine.

### 2.1.2 Structure of the glomerulus

Each kidney contains 2 million glomeruli with a combined filtration area of $1m^2$ (Stewart Cameron and Greger, 1998). The glomeruli were first described as glomerular corpuscles by Malpighi in 1666 with William Bowman in 1842 defining the relationship between glomerular corpuscles and the tubules. It was Carl Ludwig in 1843 who first considered the concept of ultrafiltration which was further developed by others who clarified what might be involved (Wearn and Richards, 1924; Marshall and Vickers, 1923).

Each glomerulus is a specialised tuft of capillaries, enclosed within Bowmans capsule and supplied by an afferent arteriole and drained by an efferent arteriole which develops deep within the centre of the tuft (Elger and Kriz, 1998). The tuft of capillaries is covered by epithelial cells (podocytes) which represent the visceral layer of Bowmans capsule. Glomeruli are roughly spherical in shape and each one is approximately 200μm in diameter.

The glomerular filtration barrier consists of 3 layers; the capillary endothelium, the glomerular basement membrane (GBM) and epithelial cells (podocytes). The GBM which consists of type IV collagen, heparin sulphate, proteoglycans and laminin (Abrahamson, 1987) was initially thought to be made up of 3 distinct laminae rarae, but this is now thought not to be the case (Williams, 1994).

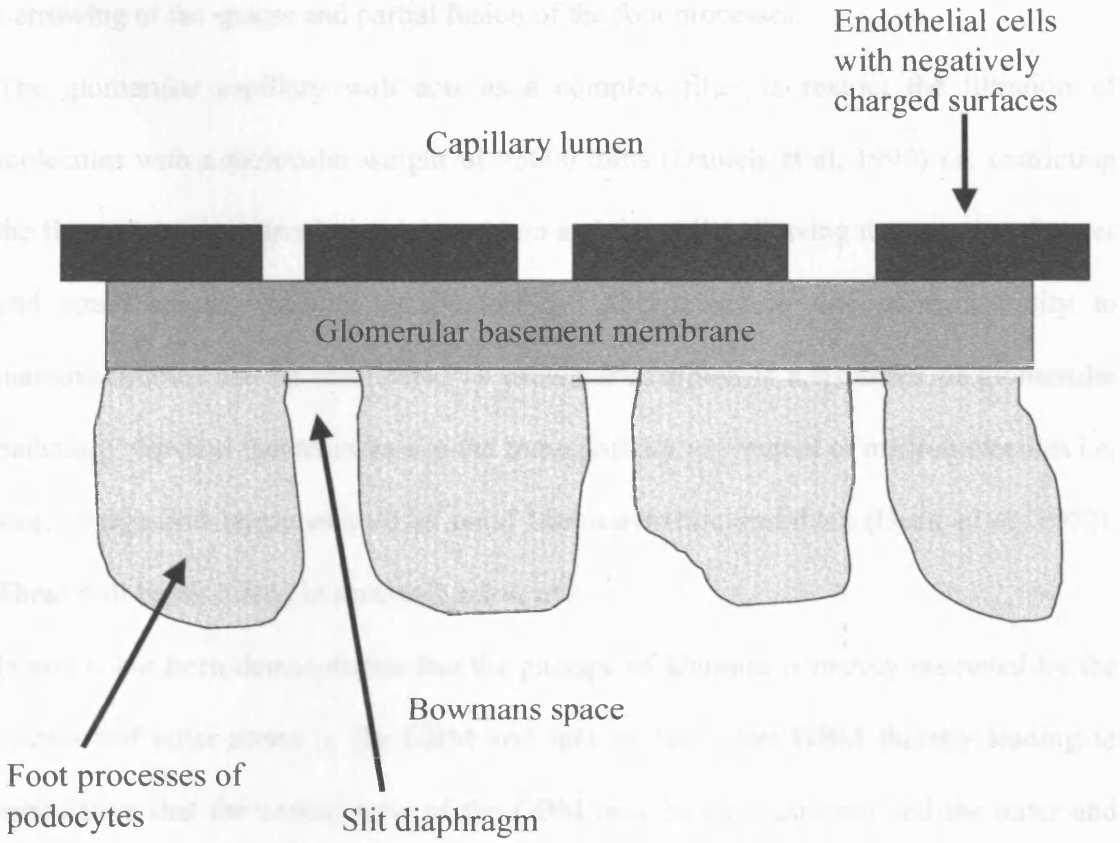



**Figure 2a: The glomerular filtration barrier**

In human glomeruli the thickness of the GBM varies between 300-370nm (Elger and Kriz, 1998). The foot processes of the podocytes regularly interdigitate leaving between them filtration slits bridged by a slit diaphragm. The capillary endothelium is fenestrated by pores 50-100nm in diameter (Larsson and Maunsbach, 1980).

Fixed negative (anionic) charge components of the glomerular capillary wall restrict the passage of circulating polyanions due to repulsion but enhance the passage of cations (Rennke et al, 1975; Ghitescu et al, 1992; Rennke and Venkatachalam, 1977). The

existence of fixed negative charges has been demonstrated morphologically in all layers of the glomerular capillary wall (Blau and Haas, 1973; Seiler et al, 1977; Latta, 1970; Caulfield and Farquhar, 1976).

The glomerular epithelial cells (podocytes) are covered with a thin layer of negatively charged glycoproteins and the epithelial slit diaphragm, GBM and the endothelial cell coat also contain negative sialoproteins. Neutralisation of the podocyte surface leads to narrowing of the spaces and partial fusion of the foot processes.

The glomerular capillary wall acts as a complex filter to restrict the filtration of molecules with a molecular weight of >5000 units (Daniels et al, 1993) i.e. restricting the flow of macromolecules such as protein and fat whilst allowing the passage of water and small solutes (Daniels et al, 1993). Aberrations in this permselectivity to macromolecules can be manifested as proteinuria, which is a hallmark of glomerular pathology. Several factors influence the transcapillary movement of macromolecules i.e. size, charge and shape as well as renal haemodynamic variables (Deen et al, 1972). These will be discussed in section 2.1.3.4, p13.

In rats it has been demonstrated that the passage of albumin is mainly restricted by the central and outer zones of the GBM and IgG by the entire GBM thereby leading to speculation that the central zone of the GBM may be size-selective and the outer and inner zones charge-selective (Fujigaki et al, 1993). In the human glomerulus, however, the degree to which each layer of the glomerular filtration barrier contributes to size and charge permselectivity is controversial (Daniels, 1993; Myers and Guasch, 1993; Remuzzi and Remuzzi, 1994). Most ultrastructural studies identify the GBM as the main filtration barrier (Brenner and Hostetter, 1978; Caulfield and Farquhar, 1974).

In addition to the above structures, mesangial cells may also influence ultrafiltration since they have contractile abilities and the GBM is the primary effector site for mesangial cell contraction (Elger and Kriz, 1998). Contraction of the mesangial cells

may lead to a reduction in the filtration surface available (Stewart Cameron and Greger, 1998) and mesangial cells also carry receptors for various hormones including prostaglandins, Angiotensin II and AVP providing a potential mechanism for hormones to influence ultrafiltration.

### 2.1.3 Ultrafiltration

Consideration of the control of glomerular ultrafiltration and its contributory factors as well as the concepts underlying mathematical modelling of this process inevitably involve equations and formulae. Measured indices (values) and theoretical parameters have abbreviations, which are well recognized. Despite having tested these earlier in the Thesis (pages **15-17**) it is thought appropriate and useful to tabulate the major indices and parameters again, prior to the ensuing paragraphs, for the purposes of clarity and consistency of approach.

The formation of urine begins with the filtration of plasma from the capillaries into Bowmans space. This process termed ultrafiltration, leaves blood cells and protein in the blood since they are unable to pass through the permselective walls of the glomerular capillaries. The mechanism of ultrafiltration has been largely investigated using animal models (Deen et al, 1972; Brenner et al, 1977) and is thought to be determined by several factors:

1. Mean transcapillary hydraulic pressure difference ($\Delta P$) (i.e the driving force for ultrafiltration.
2. Ultrafiltration coefficient ($K_f$) which depends on the characteristics of the filtration membrane
3. Glomerular haemodynamics i.e. glomerular capillary plasma flow rate ($Q_A$)
4. Characteristics of macromolecules i.e. molecular size, configuration and charge.

Mean transcapillary hydraulic pressure difference ($\Delta P$)

Glomerular filtration is promoted by the glomerular capillary hydraulic pressure difference ($\Delta P$) and is opposed by the sum of the proximal tubule hydrostatic pressure and the mean glomerular capillary plasma colloid osmotic pressure (Oken, 1989). There is some evidence to suggest that filtration rates are higher in the larger juxtaglomerular glomeruli compared to the outer cortical glomeruli.

The net ultrafiltration pressure or $P_{UF}$ is defined as (Deen et al, 1972)

*Equation 2a* $P_{UF} = \Delta P - \Delta \pi$

Where $\Delta P$: Hydrostatic pressure operating across the glomerular capillary wall

i.e. $\Delta P = \Delta P_{GC} - \Delta P_{tf}$

$\Delta P_{GC}$ = Hydrostatic pressure within the glomerular capillary

$\Delta P_{tf}$ = Hydrostatic pressure within the tubular fluid

$\Delta P$ cannot be measured directly in humans and is an assumed value.

$\Delta \pi$: Osmotic pressure of the plasma proteins

i.e. $\Delta \pi = \Delta \pi_{GC} - \Delta \pi_{tf}$

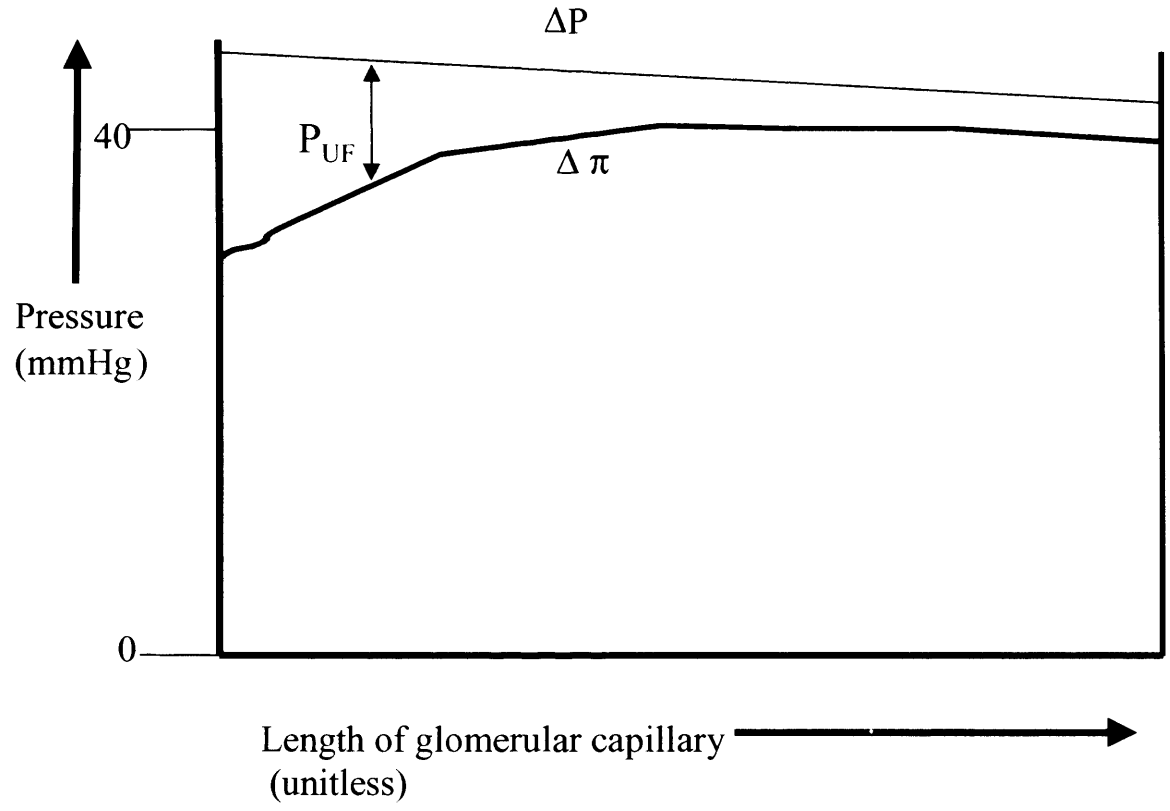
Under normal conditions $\Delta \pi_{tf} = 0$

Therefore: *Equation 2b* $P_{UF} = \Delta P - \Delta \pi_{GC}$

It has long been theoretically recognised from biomathematical modelling that the fractional clearance for a given macromolecule varies inversely with $\Delta P$ (Edwards and Deen, 1995). Since the hydrostatic pressure in the glomerular capillary ($P_{GC}$) exceeds that of tubular fluid ($P_{tf}$), $\Delta P$ will favour ultrafiltration. The reverse is true for $\Delta \pi$, however, since the filtration barrier restricts the passage of macromolecules. As filtration proceeds and filtrate is formed the colloid osmotic pressure ($\Delta \pi$) within the

glomerular capillary will increase. When $\Delta\pi$ is equal to the driving force for ultrafiltration ($\Delta P$) a filtration equilibrium is reached and no further filtration occurs (see figure 2b).

**Figure 2b: The forces involved in glomerular ultrafiltration. If $\Delta P$ is equal to $\Delta\pi$, then filtration equilibrium is reached ( modified from Valtin and Schafer, 1995)**




$P_{UF}$ is the net ultrafiltration pressure

### *2.1.3.1 Forces involved in glomerular ultrafiltration*

The net ultrafiltration pressure ($P_{UF}$) declines in glomerular capillaries mainly because plasma oncotic pressure rises rather than intracapillary hydrostatic pressure decreases. Hence, $P_{UF}$ is equivalent to the area between the $\Delta P$ and $\Delta\pi$ curves.

Studies in the rat demonstrated that filtration equilibrium is reached prior to the efferent glomerular arteriole (Deen et al, 1972) resulting in an unused filtration area. This is termed "renal reserve" and reflects the ability of the kidney to increase its level of functioning above a baseline level at times of increased GFR. This concept, however, is

controversial and has been criticised because "renal reserve" is still evident in disease states and therefore may not represent a true reserve (Thomas, 1994).

### *2.1.3.2 The ultrafiltration coefficient ($K_f$)*

The glomerular barrier freely allows the passage of water and small molecules but larger macromolecules are restricted. Filtration of larger molecules depends on the ultrafiltration coefficient ( $K_f$).

*Equation 2c* $K_f$ = Hydraulic permeability (K) x Surface area (S)

Hydraulic permeability (K) defines how easily the filtrate can pass through the membrane. Factors that influence K are the thickness and permeability of the GBM, the width and permeability of the epithelial slits, the number of fenestrae and endothelial permeability. Factors modifying the surface area for ultrafiltration (S) are glomerular volume and capillary surface density (Drummond et al, 1994). $K_f$ cannot be measured directly in humans and relies on derivation from modelling. $K_f$ is a main determinant of single nephron glomerular filtration rate (SNGFR)

*Equation 2d* $SNGFR = P_{UF} \times K_f$

### *2.1.3.3 Glomerular haemodynamics*

Transglomerular passage of macromolecules is governed by permeability and by convective and diffusive forces acting across the glomerular capillary wall which in turn is influenced by renal haemodynamics (Brenner et al, 1977). Changes in renal haemodynamics modify $\Delta P$ and $\Delta\pi$. Renal blood flow depends upon the pressure

difference across the entire kidney and the resistance to this blood flow. It is, however, only the plasma component of renal blood flow (RBF) that is subject to ultrafiltration.

*Equation 2e* Renal plasma flow (RPF) = RBF (1-haematocrit)

RPF is deduced from values for the clearance of sodium paraaminohippurate (PAH) but this only reflects the plasma which contacts the tissues resulting in the removal of PAH and therefore the subsequent flow rate determined (i.e. PAH clearance) is referred to as the effective renal plasma flow (ERPF). From ERPF values, RPF is estimated using an 85% extraction ratio (Warren et al, 1944).

Autoregulation of RBF limits the effects of systemic blood pressure changes on renal perfusion and is due to a counteracting increase in resistance in the afferent glomerular arteriole (Schnermann et al, 1984). Glomerular capillary plasma flow directly influences the flux of water into Bowmans space and the clearance of macromolecules is subject to the resultant convective and diffusive forces (Brenner et al, 1977). If the glomerular plasma flow rate ($Q_A$) increases then GFR will increase and although the absolute solute clearance will be increased ($\theta m$ x GFR) the fractional clearance of the macromolecule will be decreased ($\theta m$). This occurs because the effect of increasing $Q_A$ on the flux of restricted molecules is less than that on water and the unrestricted molecules. Increasing $Q_A$ results in net ultrafiltration for the entire length of the glomerular capillary.

Decreases in $\Delta P$ will increase the fractional clearance of macromolecules but increasing $\Delta P$ has very little effect i.e. changes in $\Delta P$ and its influence on ultrafiltration are, therefore, likely to be minimal.

### *2.1.3.4 Characteristics of macromolecules*

The 3 characteristics of macromolecules that influence ultrafiltration are molecular size, molecular charge and molecular configuration with the glomerular filter being considered to be both size and charge selective.

Molecular size

Small molecules such as inulin (effective Stokes Einstein molecular radius 1.4nm) are freely filtered but there is restriction of larger molecules e.g. albumin (radius 3.6nm) whose filtration approaches 0 (Chang et al, 1975b). This size selectivity provides the basis for the use of neutral dextrans to investigate the effect of size upon glomerular permselectivity. Neutral dextran acts as a test molecule and is a polydispersed polymer of glucopyranose (Guasch and Deen, 1993) displaying a wide range of radii whose ultrafiltration is hindered by the glomerular barrier. It is not reabsorbed or secreted and therefore its urinary clearance is equal to its glomerular filtration (Chang et al, 1975c). Comparison of the clearance of dextran with that of inulin provides fractional clearance data ($\theta m$) for dextrans of different radii:

*Equation 2f*

$$\theta m = \frac{\theta_{dextran}}{\theta_{inulin}}$$

The effective molecular radii can be estimated by standard quantitative gel chromatograph techniques (Granath and Kvist, 1967) (See Section 4.3, p79). The fractional clearance ratio will be 1 if the clearance for a particular size dextran is equal to that of inulin but will decrease, and approach 0 as the dextran radii increase above 2.1nm and filtration is progressively hindered (Chang et al, 1975b).

Molecular charge

The transglomerular passage of circulating polyanions, for example albumin is restricted and that of polycations, for example IgG, is enhanced due to electrostatic repulsion and attraction respectively by fixed negative charge components of the glomerular filtration barrier as previously described (Rennke et al, 1975, 1977; Bohrer et al, 1979; Ghitescu et al, 1992). Hence, the molecular charge of macromolecules is a determinant of their fractional clearance. The transglomerular passage of albumin is restricted to a far greater extent than size selectivity alone would predict as demonstrated by Chang et al (1975a) who studied the effect of molecular charge by comparing the sieving coefficients of neutral dextrans and the anionic polymer dextran sulphate. The fractional clearance of the latter being restricted compared to that of the former beyond a radius of 2.1nm.

In comparison studies with the cationic diethylaminoethyl (DEAE) dextran, the fractional clearance of DEAE was increased over the entire range of radii compared to that of neutral dextran and dextran sulphate hence confirming the importance of charge selectivity in ultrafiltration (Brenner et al, 1978; Rennke et al, 1975).

The abnormal filtration of anionic serum proteins e.g. albumin demonstrated in glomerulopathies is thought to be the consequence of the loss of the fixed negative charges from the glomerular filtration barrier (Caulfield and Farquhar, 1978; Blau and Haas, 1973).

Molecular configuration

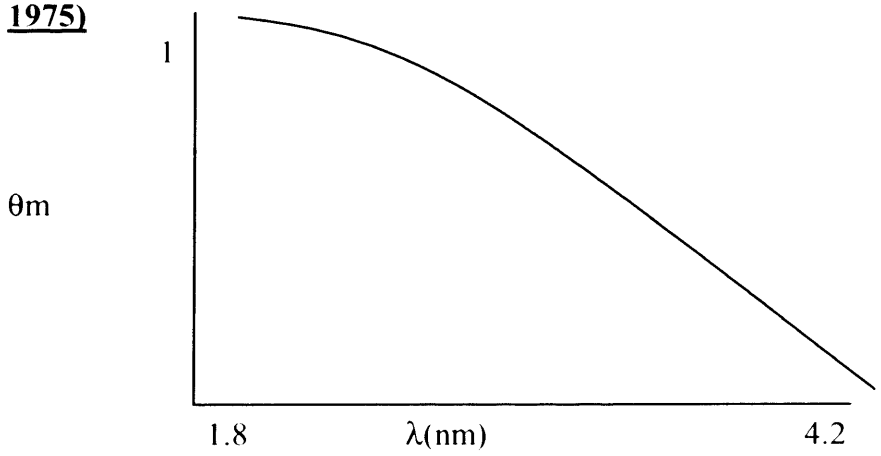
Dextran is a linear polymer and therefore the fractional clearance of dextran is not truly representative of that of the negatively charged globular plasma proteins. This obvious limitation led to the use of Ficoll, a spherical polysucrose with studies in rats demonstrating that Ficoll sieving coefficients for all radii were lower than that of neutral dextrans (Oliver et al, 1992), a finding also confirmed by Blouch and Deen (1997) in

non-pregnant healthy and nephrotic humans. Ficoll is considered to be a better marker for macromolecular filtration and although its use is limited studies have demonstrated that the restricted filtration of albumin is not just purely a function of charge-selectivity as dextran studies had previously suggested but that molecular size and molecular configuration play important roles (Remuzzi and Remuzzi, 1994).

### 2.1.4 Mathematical models for solute transport across the glomerulus

Biomathematical modelling is required to derive values for $\Delta P$, $K_f$ and $\Delta\pi$ and hence allow some description of glomerular barrier function (Roberts et al, 1996). This is achieved using measured renal haemodynamic variables together with clearance data for a test molecule e.g. neutral dextran. The clearance of the test molecule is determined in plasma and urine and individual ratios are obtained for the range of molecular radii of the molecule using gel permeation chromatography (GPC). The ratios obtained are then divided by that of the freely filtered inulin to give fractional clearance data for the macromolecule ($\theta m$). These values are then plotted against molecular size to display the glomerular sieving characteristics of the test molecule.

**Figure 2c: θm is plotted as a function of effective molecular radius (Chang et al, 1975)**



Dextran molecules with molecular radii less than 1.8nm are freely filtered but above this restriction to filtration occurs and molecules with radii above 4.2nm are virtually completely excluded from filtration.

Measured θm data is then compared with modelled clearance data for the different pore sizes and when there is a statistical fit (as determined by $\chi^2$ statistical analysis, see below) the actual pore size may be inferred.

*Equation 2g* Sum of $\chi^2 = \sum$ (measured $\theta_D$ – modelled $\theta_D$)/ modelled $\theta_D$)

These biomathematical models require the input of several variables i.e. $\Delta P$, $\pi_A$ and $K_f$ and the renal haemodynamic variables GFR and RPF. Using these values a range of possible $\theta_D$ curves are produced depending on the input value for $\Delta P$. In humans $\Delta P$ cannot be measured directly but is usually assumed to be and indeed must be between 35-45mmHg (Remuzzi, 1990).

These models are not descriptive of the structure of the glomerular filtration barrier but do provide an evaluation of the qualities that characterise the overall filtration properties of the glomerular capillary wall. Using these models with basic structural measurements does allow some predictions to be made about barrier function.

### *2.1.4.1 Theoretical Models*

The simplest model of ultrafiltration considered the glomerular capillary wall to contain uniform sized cylindrical pores, the *isoporous model*, which was successful in describing the size-selectivity properties of the glomerulus. It was not, however, sufficient to explain the proteinuria associated with certain disorders e.g. Diabetic nephropathy (Myers et al, 1982) and hence heteroporous models were developed (Myers et al, 1982; Oken, 1981; Arturson et al, 1971).

The most successful heteroporous models are the *isoporous plus shunt* and *the lognormal models*. The *isoporous plus shunt* model assumes the presence of two populations of pores, one of which is restrictive with uniform radius ($r_0$) and the other non-restrictive and large in size representing a shunt pathway ($\omega_0$) (Remuzzi and Remuzzi, 1994; Thomas, 1994).

The *lognormal* model assumes the glomerular capillary wall to be perforated by a single population of pores that have a continuous log normal distribution of radii (Remuzzi and Remuzzi, 1994; Edwards and Deen, 1995).

The data presented within this Thesis will apply the *isoporous plus shunt* and the *lognormal* models for glomerular size-selectivity.

### *2.1.4.2 The theory underlying the isoporous plus shunt model*

From work in rats Deen et al (1972) devised the *isoporous plus shunt* model of glomerular ultrafiltration. This predicted the driving force for ultrafiltration ($P_{UF}$) examining SNGFR and the effects of $Q_A$, $P_{UF}$, $\pi_A$ and K.

As previously discussed:

*Equation 2a* $$P_{UF} = \Delta P - \Delta \pi$$

$$(P_{GC} - P_{tf}) - (\pi_{GC} - \pi_{tf})$$

Where $P_{GC}$ and $P_{tf}$ are the hydrostatic pressures in the glomerular capillary and Bowmans space respectively and $\pi_{GC}$ and $\pi_{tf}$ are the corresponding colloid osmotic pressures.

The *isoporous plus shunt* model is a tool for examining the effects of K, P, Q and $\pi_A$ on SNGFR. For the purposes of modelling the glomerular capillary bed is considered to be a single tube of length l. The tube radius and hydraulic permeability of the membrane (K) are constant. The system is said to only have 2 components i.e. water and plasma

proteins since small solutes with negligible osmotic contribution are freely filtered. The tube is impermeable to protein. The concentrations and flow rates depend on x, the axial distance coordinate. As x increases (capillary distance), dQ/dX is inversely related to the length of the capillary and directly proportional to both $P_{UF}$ and $K_f$.

*Equation 2h*

$$\frac{dQ}{dX} = \frac{-SK}{L}. \; P_{UF}$$

$$\frac{dQ}{dX} = \frac{-Kf}{L}. \; P_{UF}$$

$$\frac{dQ}{dX} = \frac{-m.\,dC}{C2\,dX} \qquad C=\frac{M}{Q}$$

Where m: mass flow rate of plasma (constant)
C: plasma protein concentration and is inversely related to volume flow rate Q
If a reference pressure $P_R$ is added:

*Equation 2i*

$$\frac{dC}{dX} = \frac{K_f \Delta PR.}{Q_A}. \; C^2. \; \frac{P_{UF}}{\Delta P_R}$$

Then substituting the formula for $P_{UF}$:

*Equation 2j*

$$\frac{dC}{dX} = \frac{K_f \Delta PR.}{Q_A}. \; C^2. \; \left( \frac{\Delta P}{\Delta P_R} - \pi_1 C - \pi_2 C^2 \right)$$

Where $\pi_1$ and $\pi_2$ are osmotic pressure differences (dimensionless), directly proportional to afferent glomerular arteriole C and inversely proportional to $\Delta P_R$.

$\Delta P$ is derived from the direct measurement of $P_{UF}$ along the capillary length i.e.

*Equation 2k*

$$\Delta P = \Delta P_R \, ( 1 - \varepsilon \, (x - 1/2))$$

$\varepsilon$= a small positive constant

It is assumed that $\Delta P$ only decreases slightly along the length of the capillary.

In summary, therefore, the rate of change of protein concentration along the capillary at any point is inversely related to the initial capillary plasma flow rate and is directly proportional to $K_f$, $\Delta P$ and the protein concentration (C) at that point.

Pore theory allows an assessment of the effect of porosity on the handling of macromolecules by the glomerulus. The pore model considers transport to take place through identical pores with a radius r and length l. Solute radius is a. The solute molecule is considered to possess both diffusive properties via Brownian motion and also convective properties. This is demonstrated in Einstein's equation:

*Equation 2l* $$Dx = \frac{KT}{Fx}$$

Where:

Dx: Ficks law diffusivity of the solute

K : Boltzmann constant

T: Absolute temperature

Fx: Molecular friction coefficient

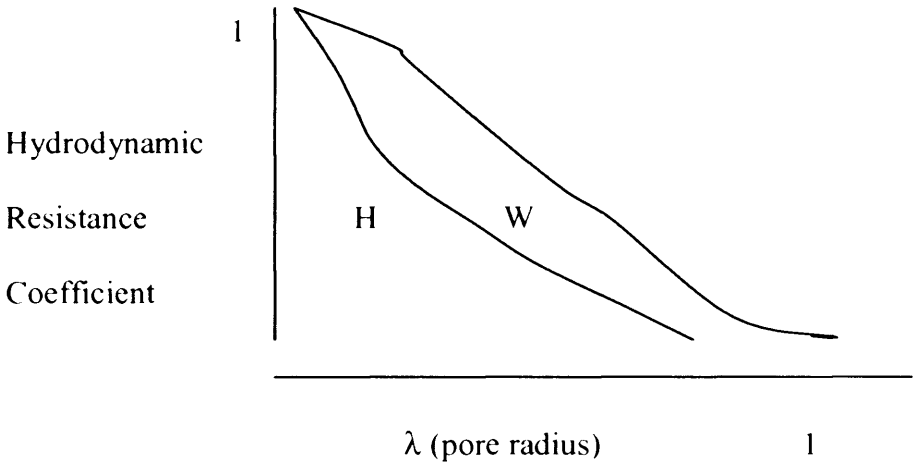
Hence, The Stokes Einstein equation relates "effective " molecular radius to diffusivity

*Equation 2m* $$Dx = \frac{KT}{6\Pi\eta a}$$

Where $\eta$= viscosity of the solvent.

Several factors are important in determining the movement of a solute, including the hydrodynamic forces between solute and solvent, the effects of the pore wall, the net axial solvent velocity and the chemical potential of the axial pore. These factors combine to give Fick's law for solute flux within the capillary and account for diffusion, the contribution from the pore wall and also the "solvent drag"( Deen et al, 1979).

**Figure 2d: The hindering effect of the pore wall is a factor both of diffusion (H) and convection (W) (Paine and Scherr, 1975).**




To summarise, this graph (figure 2d) demonstrates that as solutes increase in size and pore size reduces, the movement of a solute is restricted. At low fluid flow rates diffusion (H) governs solute transport whereas at high flow rates convection (W) is more important. Therefore, the SNGFR is an important determinant of macromolecular transport.

### *2.1.4.3 The lognormal and isoporous plus shunt models*

Both of these models require input values for GFR, RPF, $\pi_A$, and $K_f$. Each model then estimates $\pi_{GC}$, volume and solute fluxes and pore hindrance factors for either a *lognormal* distribution of pores or an *isoporous plus shunt* pore distribution. As previously mentioned, the isoporous plus shunt assumes a pore size of radius ($r_o$) and a shunt component ($\omega_o$). The *lognormal* model assumes a mean pore size, U about which there is a standard deviation, S. Fractional dextran clearance data ($\theta_D$) is then calculated from equation 2e. A range of possible $\theta_D$ curves are produced depending on input values for $\Delta P$. The curve which best fits the measured $\theta_D$ data is taken to be

representative of the $K_f$ and $\Delta P$. The "goodness of fit" is derived using equation 2f, the $\chi^2$ test.

These models are not descriptive but just provide evaluation of the parameters that can be used to characterise glomerular filtration e.g. $K_f$, $\omega o$ etc and together with structural measurements are applied to predict the glomerular barrier properties.

#### *2.1.4.4 Further models*

Several ultrastructural models using dextran have also been devised for glomerular ultrafiltration based on 3D computer fluid dynamics (Palassini and Remuzzi, 1998) and these indicate that the glomerular size selectivity of the glomerulus is determined largely by the slit diaphragms and the glomerular basement membrane and that the endothelial pores and thickness of the glomerular basement membrane are not as important. These models, however, have limitations because important factors such as the contribution of charge and molecular configuration to ultrafiltration are not totally taken into account (Edwards et al, 1999).

## 2.2 Renal function in normal pregnancy

### 2.2.1 Hormonal control of renal blood flow

Although acute variations in arterial blood pressure (BP) will cause corresponding changes in RBF and hence GFR, these are usually transient and, provided BP stays in the normal range, compensatory mechanisms come into play very quickly to return RBF and hence GFR to near normal. This is designated *autoregulation* (Persson, 2002) and it is predominantly due to changes in the caliber of the afferent arterioles, probably from a combination of two mechanisms:

(a) A myogenic reflux, with the afferent arteriolar smooth muscle wall constricting automatically when renal perfusion pressure increases.

(b) Tubuloglomerular feedback (TGF), whereby increased delivery of NaCl to the macula densa resulting from increases in BP, RBF and GFR, results in vasoconstriction of the afferent arteriole supplying that nephron's glomerulus (Thomson SC, 2002).

These mechanisms restore both RBF and ΔP (glomerular capillary pressure actually) towards normal, the initial GFR change is also reversed. The TGF negative feedback system is possible because the anatomical arrangement in the kidney is such that the macula densa region of each nephron is in close contact with its own glomerulus and afferent and efferent arterioles, a structural complex known as the juxtaglomerular apparatus (JGA). It is likely that the major mediator of TGF is adenosine, acting on adenosine $A_1$ receptors in the afferent arteriole (Thomson, 2002). The stimulus is increased by $Cl^-$ uptake by macula densa cells; this is thought to lead to ATP release into the surrounding extracellular space, which is then converted to adenosine. ATP itself may also be involved in the vasoconstrictor response (acting on afferent arteriolar P2X purinoceptors), while the sensitivity of TGF is modulated by locally produced angiotensin II, nitric oxide and certain eicosanoids (reviewed by Shirley et al, 2003).

Even though the underlying influence of autoregulation usually maintains RBF and GFR relatively constant in the mean arterial pressure range ~80-180 mmHg, a number of extrinsic factors (nervous and humoral) can bring about alterations in renal haemodynamics (see 2.2.2). Furthermore, independent and/or unequal alterations in afferent and efferent arteriolar resistance, along with Kf alterations (the latter thought to be mainly due to mesangial cell contraction – relaxation, although recent evidence also implicates contractile elements in the podocytes that line Bowman's capsule), can result in disproportionate, or even contrasting, changes in RBF and GFR. In addition, within the kidney, changes in vascular resistance in different regions of the renal cortex can alter the distribution of blood flow, e.g. diversion of blood from outer to inner cortex in

haemorrhagic shock. Some of the hormonal and other influences (factors that prevail on renal haemodynamics are listed below. It should be emphasized, however, that specific effects in isolation are unlikely to occur in vivo, where multiple factors can operate at any one time.

**The effects on glomerular haemodynamics of hormonal and other factors**

| | Afferent arteriolar resistance | Efferent arteriolar Resistance | RBF | Net ultrafiltration pressure (ΔP) | $K_f$ | GFR |
|---|---|---|---|---|---|---|
| High protein diet | ↓ | → | ↑ | ↑ | → | ↑ |
| Renal sympathetic | ↑↑ | ↑ | ↓ | ↓ | ↓ | ↓ |
| Nerves | | | | | | |
| Epinephrine | ↑ | ↑ | ↓ | → | ? | ↓ |
| Adenosine | ↑ | → | ↓ | ↓ | ? | ↓ |
| Angiotensin II | ↑ | ↑↑ | ↓ | ↑ | ↓ | ↓→ |
| Endothelin-1 | ↑ | ↑↑ | ↓ | ↑ | ↓ | ↓ |
| Glucagon | ↓ | → | ↑ | ↑ | → | ↑ |
| Nitric oxide | ↓ | ↓ | ↑ | ? | ↑ | ↑(?) |
| Atrial natriuretic | ↓ | → | ↑ | ↑ | ↑ | ↑ |
| Peptide (high dose) | | | | | | |
| Prostaglandins $E_2/I_2$ | ↓ | ↓ | ↑ | ↑ | ? | ↑ |

The overall effect on GFR will depend on RBF, net ultrafiltration pressure and $K_f$, which is controlled by mesangial cell contraction/relaxation. The effects shown are those seen when the agents are applied (or inhibited) in isolation; the actual changes that occur are dose-dependent and are modulated by other agents. (Modified from Shirley et al, 2003).

Above all else it must be emphasized that most regulatory mechanisms within the kidney are focused ultimately on the control of "effective circulating volume", an undeterminable "volume", poorly defined, that somehow reflects the degree of "vasculature fullness", so essential for any organ with a vascular network. The extracellular fluid volume (ECFV) is what actually governs the effective circulating volume (ECV) (except in some pathological clinical conditions where "triggering

mechanisms" become dysfunctional) and in turn renal control of this is achieved by regulating the sodium content of the body. Said otherwise, control of extracellular sodium ensures that ECV is kept under scrutiny and control too. ECV is perceived by intravascular receptors in the aorta and carotid arteries (baroreceptors), the renal arterioles and cardiac atria. Thence, effector mechanisms act in unison influencing both GFR and tubular resorption. The important 'players' (as seen in the table) are the renal sympathetic nerves, the rennin-angiotensin-aldosterone system, atrial natriuretic peptide (ANP), the endothelins, the purines, the eicosanoids as well as renal interstitial hydrostatic pressure (Kohan, 1997; Welsh and Wilcox, 2002). Essentailly the balance between GFR and tubular reabsorption ensures strict control over sodium excretion and thus control of ECV. Many of the factors involved do not just modulate day to day sodium balance but have other effects elsewhere within the kidney and throughout the cardiovascular system.

### 2.2.2 Renal haemodynamics

As intimated in the previous section haemodynamic alterations are widespread in normal human pregnancy. Cardiac output increases by 33% by the end of the 1st trimester and is maintained throughout pregnancy (Robson et al, 1989). In addition, total peripheral resistance falls resulting in a reduced diastolic blood pressure of approximately 15mmHg in the second trimester which slowly returns to non-pregnant levels by term (Williams and DeSwiet, 1997). The extracellular compartment accumulates 6-8 litres of extra fluid by the creation of a salt conserving state and plasma volume increases by more than 50% (Gallery and Brown, 1987). The physiological haemodilution of normal pregnancy is associated with a decrease in blood viscosity which may be beneficial for intervillous perfusion (Whittaker and Lind, 1993).

Renal haemodynamics also undergo dramatic changes in normal pregnancy with effective renal plasma flow (ERPF) increasing early in pregnancy (from week 5) to

reach levels 80% higher than non-pregnant ERPF. This is maintained throughout the 2nd trimester but gradually reduces as term approaches to levels approximately 60% greater than non-pregnant values (Dunlop, 1981; Sturgiss et al, 1994). The reduction throughout the 3rd trimester is not entirely understood but may be due to the effects of posture and/or venocaval compression from the gravid uterus (Chesley and Sloan, 1964). There is, however, good evidence to show that decreases still occur when these factors are controlled and/or negated (Ezimokhai et al, 1981).

Glomerular filtration rate (GFR) also increases early in pregnancy (from week 4) and remains elevated at a level 50% above non-pregnant values. Hence, filtration fraction (FF= GFR/ERPF) is significantly reduced in early gestation but increases during the 3rd trimester (Dunlop, 1981; Davison et al, 1981; Milne et al, 2002). GFR is determined by the glomerular plasma flow, glomerular permeability and the hydrostatic and oncotic pressure gradients across the glomerular membrane (Duvekot and Peeters, 1994). The rise in GFR in normal pregnancy is due to an increase in renal plasma flow (RPF) without a concomitant rise in glomerular capillary blood pressure (Baylis, 1994). Studies in rats demonstrated that gestational increments in GFR were not associated with differences in $\Delta P$, $K_f$ or $\pi_A$ (Baylis, 1980, 1982). Human studies, using neutral dextran sieving data and biomathematical modelling, have demonstrated that the hyperfiltration is primarily due to increments in RPF, with a small contribution due to decreased glomerular oncotic pressure, without evidence of increased $\Delta P$ (Roberts et al, 1996).

Glomerular blood flow is controlled by changes in the resistance of the afferent and efferent glomerular arterioles. If the resistance of the afferent arteriole is decreased then glomerular blood flow would increase but unless the efferent resistance also changes the glomerular pressure would increase. Therefore, the ratio of the afferent to efferent resistance determines glomerular pressure and hence $\Delta P$. Animal model investigations

(Baylis and Reckelhoff, 1991) endorse the view that resistances in both the afferent and efferent arterioles decrease during rat pregnancy, resulting in increases in GFR and glomerular plasma flow and no change in $\Delta P$.

The mechanisms that mediate the gestational changes in renal haemodynamics are not fully understood as previously discussed (section 2.2.1). Obviously, a maternal factor must be involved since glomerular filtration is increased prior to the development of the fetoplacental unit (Davison and Noble, 1981) and both ERPF and GFR are increased in studies of pseudopregnant rats (Baylis, 1994) and even in pseudopregnant rats with uteri removed soon after mating (Slangen et al, 2000). Several potential renal vasodilators as mentioned earlier, have been suggested including prostaglandins, Atrial natriuretic peptide (ANP), nitric oxide (NO) and Relaxin. Studies in pregnant rats have demonstrated that vasodilatory prostaglandins do not appear to mediate renal vasodilation since acute inhibition of cyclooxygenase with indomethacin did not reverse the increases in GFR and ERPF or affect the attenuated renal response to an infusion of Angiotensin II (AII) (Conrad and Colpoys, 1986; Baylis, 1987). Also, measurement of the renal production of prostaglandins in pregnant animals has failed to demonstrate any increase with gestation (Conrad and Dunn, 1987; Brown and Venuto, 1990). Indomethacin administration to pregnant women increases total peripheral resistance by only 5% which is far less than the overall reduction in total peripheral resistance seen during pregnancy (Sorensen et al, 1992). Therefore, all this evidence casts doubt on a significant role for prostaglandins in the mediation of gestational renal vasodilation.

Other studies have suggested that endothelial derived factors e.g. NO, acting via cyclic GMP may play a crucial role in mediating gestation renal vasodilation and this will be discussed further in section 2.46. More recently a possible role for relaxin in mediating gestational renal vasodilation has been implied since the renal effects of chronic relaxin administration in rats resemble those of normal pregnancy i.e. plasma osmolality was

reduced, ERPF and GFR were increased and there was attenuation of the renal circulatory response to the vasoconstrictor AII (Danielson et al, 1999).

### 2.2.3 Renal handling of protein and albumin.

Urinary excretion of protein increases progressively throughout pregnancy with a normal limit of 300mg/24hour at term (Sturgiss et al, 1994; Brown et al, 2001). The contribution of albumin and other proteins is still in doubt as are the mechanisms involved. The cause of increased protein excretion in pregnancy is likely to be multifactorial. Possible theories for increased excretion include altered tubular handling of proteins, alterations in permeability of the glomerular barrier and changes in GFR. The increased excretion of retinol binding protein (RBP) and N acetyl β glycosaminadase in pregnancy, both proteins usually absorbed in the tubules, (Beetham et al, 1988; Skrha et al, 1989), might be explained by the increased GFR leading to an increased tubular load of protein which is above the tubular reabsorptive capacity. The relative amounts of these proteins within the urine are small (Bernard et al, 1992).

When considering urinary albumin excretion, it is still debatable as to whether or not this is increased in normal pregnancy. Some studies have reported an increased albumin excretion in pregnancy (Konstantin-Hansen et al, 1992; Taylor and Davison, 1997) whereas other studies have failed to demonstrate an increase (Misiani et al, 1991; Brown et al, 1994). Despite this, microalbuminuria of up to 20mg/24hr is considered normal (Higby et al, 1994) and as albumin only accounts for a small percentage of total protein excretion in pregnancy, this is good evidence for an intact glomerular filtration barrier resistant to the passage of macromolecules in pregnancy. Fractional clearance data using neutral dextrans in normal pregnancy have demonstrated that the clearance of smaller radii dextrans was reduced in normal gestation without increases in the clearance of larger radii dextrans (Roberts et al, 1996).

## 2.3 Preeclampsia

### 2.3.1 Diagnosis of preeclampsia and classification of hypertension in pregnancy and their importance for management

Bearing in mind the necessity to recruit "subjects with preeclampsia", strict criteria had to be met (see pages 69-71), taking into account internationally defined and acceptable definitions for diagnosis.

Because "hypertensive disorders" are the most common medical disorders in pregnancy, the diagnosis of preeclampsia as a subgroup, needs attention to detail (Broughton-Pipkin, 1995). Hypertension actually complicates 8-9% of pregnancies, with approximately 6-7% being hypertension which occurs during pregnancy and 2% being pre-existing hypertension. Preeclampsia is the most serious hypertensive complication of pregnancy occurring primarily in first pregnancies, usually after 20 weeks gestation and most often near term. From a clinical point of view, the original commonly used definition of preeclampsia emphasized 3 features – oedema, hypertension and proteinuria – not because they were important but because, historically, these were the first to be defined and were easily clinically accessible. Nowadays, our better understanding of the pathophysiology of preeclampsia has led to the modern approach to classification and diagnosis. In addition, cognisence of the blood pressure (BP) changes of normal pregnancy underpins the chosen criteria establishing a dividing line between normal and high BP. Also acceptable now is that (i) oedema is commonplace in normal pregnancy and its assessment is subjective and (ii) proteinuria invariably occurs as a late sign of preeclampsia and may not be a necessary component (Roberts et al, 2003).

Thus for the purposes of definition the significance given to oedema, hypertension and proteinuria has changed considerably and although national and international

organizations have come to recognize this, the literature relating to classification of the hypertensive disorders in pregnancy and the diagnostic definitions of each category can still be confusing.

There is an assortment of terms and schemes, some quite complex, and on occasion the same term is used to include different disorders by various authorities. This lack of consensus on classification and diagnosis was the main reason for controversies in a variety of areas including counseling, management and research and in documenting immediate and remote prognoses. Since the proposals from the American College of Obstetrics and Gynecology (ACOG) in 1972 many other organizations have re-examined classification, especially in the last 5-10 years, including the World Health Organisation (WHO), the US National High Blood Pressure Education Program (NHBPEP), the Australasian Society for the Study of Hypertension in Pregnancy (ASSHP), the Canadian Hypertension Society (CHS) and the International Society for the Study of Hypertension in Pregnancy (ISSHP). (Helewa et al, 1997; National High BP Program working Group, 2000; Brown et al, 2000 and 2001; Roberts et al, 2003).

All organisations now accept that the classical triad concept on which definitions were based is outdated and the term 'toxaemia of pregnancy' is not now recommended. There is general agreement on the following points:

1. Definition of hypertension involves BP ≥140 mmHg and/or a diastolic BP ≥90 mmHg (using Korotkoff sound 5 (K5)), respectively (Brown et al, 2001). To date there has not been much emphasis placed upon differentiating between mild and severe in any of the categories but some do consider that this should be reconsidered, with severe being systolic BP ≥160 mmHg and/or diastolic BP ≥110 mmHg.

2. Classification recognizes 4 categories: preeclampsia, gestational hypertension, chronic hypertension and preeclampsia superimposed on chronic hypertension.
3. As preeclampsia can be a "silent" disorder, it is better to overdiagnose it when differentiating between it and the more benign gestational hypertension, then follow the clinical course as if the patient had the more potentially serious disease. This, however, has the disadvantage of undermining the specificity of diagnostic criteria but recognizes what is needed by clinicians and thus for the purposes of research it has been emphasised that preeclampsia should have the more restricted definition.

In terms of clinical diagnosis and usefulness the following summarises the current international consensus:

1. Preeclampsia. *De novo* hypertension after 20 weeks gestation plus proteinuria. The latter, defined as protein excretion >300mg/24h may correlate with 30 mg/dl in a spot urine but given the problems with dipstick tests, many still prefer a 24h or some timed quantitative determination. Use of spot urine protein/creatinine ratios ≥30 mg/μmol is, however, an alternative.

   Normalisation of BP within 3m postpartum is also considered by many to be another requirement. For the purposes of research most would now agree that to recruit true preeclamptics into clinical studies, subjects should be restricted to having new-onset hypertension after 20 weeks gestation with properly documented proteinuria. Although hypertension and proteinuria remain the only criteria in determination of preeclampsia, further guidance is necessary because in the absence of proteinuria the disease is still highly suspect when there is increased BP accompanied by headache, blurred

vision, abdominal pain or with abnormal laboratory tests, specifically thrombocytopenia and/or abnormal liver enzymes. Interestingly, NHBPEP, ASSHP and ISSHP no longer recognise BP increases of 15 mmHg and 30 mmHg diastolic and systolic levels, respectively, emphasizing absolute values for the diagnosis of hypertension. That is not to say, however, that rises in systolic and/or diastolic BP alluded to above, do not warrant close observation, especially if accompanied by proteinuria and other abnormalities in either clinical or laboratory assessments (see Brown et al, 2001).

2. Gestational hypertension. *De novo* hypertension alone, appearing after 20 weeks gestation (NHBPEP classification states that this label can be changed to transient hypertension when BP normalizes postpartum).
3. Chronic hypertension. The presence or history of hypertension before pregnancy or in the first half of pregnancy. It is considered to be essential hypertension if there is no obvious underlying cause and to be secondary if associated with another definitive pathology/aetiology. (In NHBPEP classification (2000), essential and secondary hypertension are not recognized with emphasis on *de novo* hypertension alone which fails to resolve postpartum, although no length of time is attached to this (Roberts et al, 2003)).
4. Preeclampsia superimposed on chronic hypertension. Development of new signs and/or symptoms associated with preeclampsia after 20 weeks gestation (as above) in women with chronic hypertension. Important issues include sudden increase in the magnitude of the hypertension, the appearance of thrombocytopaenia and/or abnormal liver function tests and/or

a sudden increase in proteinuria if the woman has had proteinuria since early pregnancy.

Therefore it can be stated that the major consensus statements from national and international organizations reveal the real movement that there has recently been towards agreed diagnostic criteria and terminology for the hypertensive disorders of pregnancy, with emphasis not only on exact definitions of hypertension and timing of appearance, with or without proteinuria, but also on the correct measurement of BP and proper quantification of the proteinuria. Inevitably, precise definitions can no longer depend only on traditional clinically accessible signs which, although readily detectable, are just secondary features of the primary trophoblastic pathology which exists within the uterus. The multisystem sequelae can be thought of as secondary maternal adaptations with the large variations and clinical presentations reflecting the variable susceptibility of maternal target organs, including the kidney. Only now are the complexities of the pathogenesis of the hypertensive disorders of pregnancy being exposed, hence the need to keep under review terminology, definitions and classification.

Lastly, it should be emphasized that preeclampsia is a major cause of poor outcome in pregnancy (Greer, 2005). Preeclamptic conditions represent 1 in 3 cases of severe obstetric morbidity, hypertension and/or proteinuria is the leading single identifiable risk factor in pregnancy associated with stillbirth and preeclampsia is strongly associated with fetal growth restriction, low birth weight, preterm delivery, respiratory distress syndrome and admission to neonatal intensive care (Duckitt and Harrington, 2005). Furthermore, in 46% of maternal deaths (Confidential Enquiries in Maternal Deaths) and 65% of fetal deaths (Enquiry into Stillbirths and Deaths in Infancy), due to preeclampsia, different management would have been expected on balance to affect the outcome. As there was failure in many cases to identify and act on known risk factors

at booking and to recognize and respond to symptoms and signs from 20 weeks gestation onwards, guidelines have been proposed for screening and early detection of preeclampsia in the community. This should ensure uniformity in referral thresholds and assessment strategies. The preeclampsia community guideline (PRECOG), is evidence based, supported by many national UK bodies and provides a framework by which women with preeclampsia are offered specialist care if/as needed to ensure optimal outcome for mother and baby (Milne et al, 2005).

### 2.3.2 Background

Pregnancy induced hypertension is defined as a blood pressure of 140/90mmHg recorded on two or more occasions, four hours apart, after the 20$^{th}$ week of pregnancy. If this hypertension is accompanied by the presence of proteinuria (300mg/24hour) then the condition is described as preeclampsia (Davey and MacGillivray, 1988: Brown et al, 2001). A diastolic increment of 25mmHg can also be used when defining the condition.

### 2.3.3 Pathophysiology

Preeclampsia is an important cause of maternal and fetal morbidity and mortality complicating approximately 5% of all pregnancies (Broughton-Pipkin, 1995). It is characterised by hypertension, proteinuria and oedema. It has been described as a maternal syndrome at the extreme end of the spectrum of the gestational inflammatory disturbance that affects all vascular compartment cells, with one outcome being widespread maternal endothelial dysfunction (Redman et al, 1999). In preeclampsia the mother can die following a multiorgan illness that can include convulsions, acute renal failure, hepatic compromise with maternal death usually being attributed to intracerebral haemorrhage or adult respiratory distress syndrome (ARDS).

Considered as a continuum, the considerable overlap between normal pregnancy and preeclampsia is explainable, as is the lack of an ideal screening test and/or prophylactic

measure. Whatever the aetiology of preeclampsia, the ultimate targeting of the maternal endothelium produces a multiorgan disorder with widespread enhanced vascular reactivity, activation of the coagulation cascade and disrupted volume homeostasis (Roberts et al, 1989; Redman et al, 1999; Carbillon et al, 2000).

There is a familial tendency to preeclampsia since Chesley et al (1968) discovered that there was a 26% incidence of preeclampsia in the daughters of mothers with a history of preeclampsia but only an 8% incidence in the daughter-in laws of mothers with a history of preeclampsia. Certain conditions predispose to the development of preeclampsia including preexisting hypertension, diabetes mellitus, multiple pregnancy, hydatidiform mole and collagen vascular diseases (Roberts and Redman, 1993). Several theories exist for the aetiology of preeclampsia including placental ischaemia, immune maladaptation, genetic imprinting and very low density lipoprotein (VLDL) versus toxicity preventing activity (Dekker and Sibai, 1998).

Poor placental perfusion due to inadequate placentation is the unique feature of all pregnancies predisposing to preeclampsia (Redman et al, 1999). The vascular endothelium provides a target for the factors released by a poorly perfused placenta that could explain the diverse pathophysiological changes of preeclampsia (Roberts et al, 1989). With activation or injury the endothelial cells produce vasoconstrictor substances and procoagulants which ultimately result in the clinical syndrome of preeclampsia (Roberts et al, 1989).

At present the factors released by the placenta resulting in endothelial cell injury in preeclampsia are largely unknown but speculation exists that they could possibly be lipid peroxides, lipoproteins, free fatty acids, anti-endothelial antibodies or platelet derived growth factor (Roberts et al, 1989; Hubel et al, 1989; Endresen et al, 1994). Fibronectin, factor VIII mitogen and tissue plasminogen activator (TPA) are increased in the blood of preeclamptics and are known to be factors released from injured

endothelial cells (Roberts et al, 1989; Friedman et al, 1995). In addition, endothelin-1 which is also released by endothelial cells has been demonstrated to be elevated in women with preeclampsia and especially in women with HELLP (haemolysis, elevated liver enzymes and low platelets) (Nova and Sibai, 1991). It is the most potent vasoconstrictor with the kidney being very sensitive to its vasoactive effects and has therefore been implicated in the pathogenesis of preeclampsia (Pouta and Vuolteenaho, 1998). Other factors which may prove to be clinically useful markers in preeclampsia are Activin A, Inhibin A, Cytokeratin-18 and neurokinin B, all of which are increased in preeclamptic women compared to normal pregnant controls (Silver et al, 1999; Aquilina et al, 1999; Hefler et al, 2001; Page et al, 2000).

As for the prediction and prevention of preeclampsia, abnormal uterine and umbilical artery doppler flow between 12-16 weeks has been used to predict women at high risk for preeclampsia. Sonographic evidence of diastolic notching in the arcuate vessels of the uterus at 16-20 weeks has a positive predictive value for preeclampsia of 42-70% in a high-risk population (Norwitz and Repke, 2000). In addition, several preventative measures have been studied but there is no conclusive evidence to suggest that supplementation with magnesium, calcium, fish oils or low dose aspirin can prevent the onset of preeclampsia within a low risk population (Sibai, 1998; Levine et al, 1997). Women at high risk for preeclampsia may, however, benefit from low dose aspirin early in gestation (CLASP, 1994; Caritis et al, 1998; Heyborne, 2000; Duley et al, 2001).

In summary, the aetiology of preeclampsia may be multifactorial and it is therefore unlikely that any one single gene will predispose to preeclampsia or that any single intervention will be effective in its prevention.

### 2.3.4 Renal lesion in preeclampsia and glomerular dysfunction

Electron microscopy of renal biopsy specimens from women with preeclampsia demonstrates that the primary pathological change is within the glomerular capillary cells which are increased in size and contain electron dense cytoplasmic inclusions which may lead to occlusion of the capillary lumen (Spargo et al, 1976). These glomerular changes referred to as "glomerular capillary endotheliosis" were first described by Spargo et al in 1959. The glomerulus appears bloodless and swollen under the light microscope and there is mesangial and endothelial cell hypertrophy, which encroaches on the capillary lumen. The endothelial fenestrae may be difficult to discern with subendothelial granular deposits of either immune complexes or fibrin related products (Gaber et al, 1994).

This preeclamptic nephropathy lesion is not now thought to be pathognomonic of preeclampsia but it is present in more than 70% of primiparous women with preeclampsia (Roberts et al, 1989) and starts to resolve within 48 hours of delivery. Limited information with reported renal biopsies postpartum suggest that the glomerular deposits of various haemostatic factors disappear quite quickly but that the subendothelial enlargement and endothelial swelling may persist for a variable length of time (Suzuki and Gejyo, 1997). Some studies have demonstrated persistent structural changes up to 6 months postpartum (Furukawa et al, 1983).

Naicker et al (1997) examined renal biopsies two weeks postpartum in women with early onset preeclampsia. There were various glomerular structural changes and more specifically the GBM had a reduced number of anionic sites together with foot process fusion, GBM thickening and mesangial cell proliferation implying loss of cell surface charge. Overall this suggests the possibility of a loss of charge selectivity in preeclampsia.

Lafayette et al (1999) speculated from morphometric analysis and PAH and inulin infusion clearance data (but not dextran sieving) that their mathematical derivation of a reduction in $K_f$ suggests that hypofiltration of preeclampsia is a consequence of glomerular structural changes which alter glomerular ultrafiltration capacity without evidence of haemodynamic changes. This study was, however, undertaken in patients immediately postpartum, following caesarean section under regional anaesthetic blockade who were volume loaded and therefore has its limitations.

Strevens et al (2003 a & b) performed renal biopsy examinations on 36 hypertensive and 12 normotensive pregnant women (a discussion of the ethics of the study can be found in a letter exchange). By noting glomerular endotheliosis in 5 of the normal pregnant volunteers, these investigators were questioning the previous literature in which the diagnosis of preeclampsia was established by the presence or absence of that lesion. However, their results revealed that under light microscopy the intensity of the lesion in normotensive gravidas, on a scale of 0 to 3, was 1+ in 4 of 5 speicmens (often a subjective call between something and nothing for the pathologist), and 2+ in the other specimen. Similarly, electron microscopy showed a slight degree of endotheliosis in 5 specimens and mesangial cell interposition and electron dense deposits were not seen. Lesions in the preeclamptic specimens, however, were considerably more robust and, as would be expected, glomerular volume was increased significantly in those presenting with hypertension and proteinuria. Thus, these studies, in reality, confirm our experience that the lesion in its fully developed form is quite distinctive, and the term most investigators have used, *characteristic*, not *pathognomonic*, still is quite valid.

Barrier function in normal pregnancy has been assessed by using infusion of neutral dextran-40, which, similar to inulin, is neither secreted nor reabsorbed by the renal tubule, resulting in a urinary clearance equal to its glomerular filtration (Chang et al, 1975 a & b). Results suggest that the normal gestational hyperfiltration is caused by

increments in ERPF without a change in intraglomerular pressure ($\Delta P$), $K_f$ increases, and that a significant nondiscriminating shunt (derived by mathematic modelling of fractional clearance data) appears concomitantly with increasing proteinuria. All changes resolve sequentially (the shunt component being the last) by 4 to 5 months after delivery.

Obviously $\Delta P$ cannot be measured in humans so this precludes precise estimation of $K_f$. However studies (Moran et al, 2003) show a substantial decrease in $K_f$ in preeclamptic women (50%) studied prepartum, compared to their own postpartum values as well as to data from normotensive third trimester controls. The magnitude of this change leaves little doubt that the attenuated $K_f$ compromises preeclamptic glomerular ultrafiltration in association with decreased ERPF.

It appears paradoxic that structural changes that hinder the ultrafiltration of water and might reasonably be expected to retard the passage of proteins, do not. One possible explanation is that broadened podocyte foot processes capable of reducing k may, in a small proportion, completely disrupt the integrity of the slit diaphragm. This then would allow for high loss of protein in small pockets of the glomerular wall while only contributing to a small increase in the net flux of water.

The transglomerular filtration of the negatively charged albumin is restricted to a much greater extent than would be expected from its size alone. Indirect evidence from renal biopsy studies have raised the possibility that preeclamptic morphometric changes might lead to or result from a loss of charge selectivity (Naicker and Randeree, 1997). In specimens obtained 2 weeks postpartum from 15 African women with early onset preeclampsia the glomeruli were enlarged in 50%, with fusion of podocyte foot processes (80%) and the glomerular basement membrane was thickened in two thirds of these women. These ultrastructural changes were seen in combination with a significant decrease in the amount of anionic heparin sulphate in the glomerular basement

membrane. The loss of negative charge on the podocyte surface was implied because the repelling action of adjacent cell membranes is thought to b e necessary to maintain the numerous narrow slits characteristic of the podocyte epithelial cell layer. However, these biopsy examinations were performed 2 weeks postpartum and may represent an atypical patient population.

Postpartum preeclamptic renal biopsy examinations also have been assessed by Lafayette et al (1998) in an attempt to derive filtration parameters for the glomerular barrier, using morphometric analysis and theoretical modelling of 13 women at 48 hours after caesarean section. The validity of this has been questioned for the following reasons: the heterogeneous postpartum population; failing to control for differences in glomerular haemodynamics; use of pharmacologic manipulations; and contrary to most other studies in the literature, ERPF in their preeclamptic patients was similar to the normotensive postpartum controls, thus making this one of the rare studies that failed to show some haemodynamic contribution to hypofiltration in preeclampsia. Nevertheless, the investigators' modelling results suggested a decrease in $K_f$ of approximately 40%. Subendothelial deposits, endothelial and mesangial cell hypertrophy, and swollen endothelial segments without fenestrae reduced the effective filtration surface area by a third. This was offset by the increased overall glomerular volume, resulting in a net reduction of only 10%. Morphometric analysis led to the conclusion that hypofiltration was caused by a decrease in $K_f$ secondary to decreases in both porosity and available filtration surface area. Structural bases for preeclamptic renal changes must still be sought but new approaches are needed.

### 2.3.5 Renal handling of protein and albumin

Emphasis is placed on the appearance and progression of proteinuria in preeclampsia with most classification systems including proteinuria (>300mg/24hr) for the clinical

diagnosis of preeclampsia (Brown et al, 2001). The proteinuria consists of a number of different proteins including albumin, the alpha and beta globulins and $\alpha_2$ macoroglobulin (Kelly and McEwan, 1973; McEwan, 1969; Katz and Berlyne, 1974). The most accepted way of assessing proteinuria is by a 24 hour urine collection (Taylor and Davison, 1997) but other controversial methods exist including dipstick analysis, automated analysis and protein:creatinine ratio, with dipstick analysis demonstrating observer error (Halligan et al, 1999). More recently, however, the clinical significance of proteinuria with respect to maternal and perinatal outcome has been questioned and termination of pregnancy on the degree of proteinuria alone if other indicators of fetal and maternal wellbeing are favourable is no longer advocated (Schiff, 1996). Therefore, in view of this, newer classification systems for preeclampsia have focused more on the presence of other systemic abnormalities (deSwiet and Brown, 1999) and also gestational blood pressure changes (Redman and Jefferies, 1988).

Proteinuric preeclampsia may lead to persistent renal changes later in life. A study comparing women with a history of preeclampsia with women with normal pregnancy histories demonstrated a higher rate of microalbuminuria in the preeclamptic group both at 2-4 months and 3-5 years postpartum (Bar et al, 1999). This may reflect residual glomerular damage or the presence of covert renal disease. Therefore, the presence of persistent microalbuminuria may be a useful marker for the development of hypertension or glomerular disease in women who have had a pregnancy complicated by preeclampsia (Nisell and Lintu, 1995).

### 2.3.6 Glomerular barrier function

The integrity of the filtering glomerular barrier is altered in preeclampsia allowing increased passage of protein. There are conflicting opinions as to the contributions of glomerular and tubular factors to proteinuria. Maclean et al (1972) claimed the

proteinuria to be of glomerular origin because the protein selectivity resembled that of freely filtered neutral dextrans whereas, Kelly and McEwan (1973) suggested a tubular origin since they demonstrated a relative increase in the clearance of IgA immunoglobulin compared to IgG. There is agreement, however, that most of the proteinuria in preeclampsia is due to an increase in excretion of albumin. The increase in total protein excretion and albuminuria is thought to be secondary to the vasoconstriction of preeclampsia together with alterations in the electrostatic properties of the glomerulus and the reabsorptive capacity of the proximal tubule.

Proteinuria is described as being selective or non-selective. Selective proteinuria suggests that the glomerular barrier restricts the passage of larger molecules but that this property is lost in non-selective proteinuria. The proteinuria of preeclampsia is thought to be non-selective and has been described as "intermediate range proteinuria" (Maclean et al 1972; Wood et al, 1976). Maclean et al (1972) used intermediate sized neutral dextrans in preeclamptic women demonstrating a loss of selectivity compared with non-pregnant controls suggesting therefore that proteinuria in preeclampsia is due to a size selective deficit in the filtration barrier. In most cases the proteinuria of preeclampsia resolves by 4-6 months postpartum.

## 2.4 Endothelial activation and nitric oxide

### 2.4.1 Background

Endothelial cells produce several paracrine vasoactive substances, which regulate the tone of the underlying smooth muscle cells. These hyperpolarising factors include prostacyclin, which is produced from arachidonic acid by the cyclooxygenase pathway, endothelial derived hyperpolarising factor (EDHF) which requires further specific identification and endothelial derived relaxing factor (EDRF), which is derived from L-arginine by the action of nitric oxide synthase (NOS) (see below) (Moncada et al, 1976;

Feletou and Vanhoutte, 1999). The existence of EDRF was first described in 1980 by Furchgott and Zawadski who demonstrated that the endothelium was necessary for acetylcholine induced vascular relaxation, suggestive of the existence of a labile substance, namely EDRF.

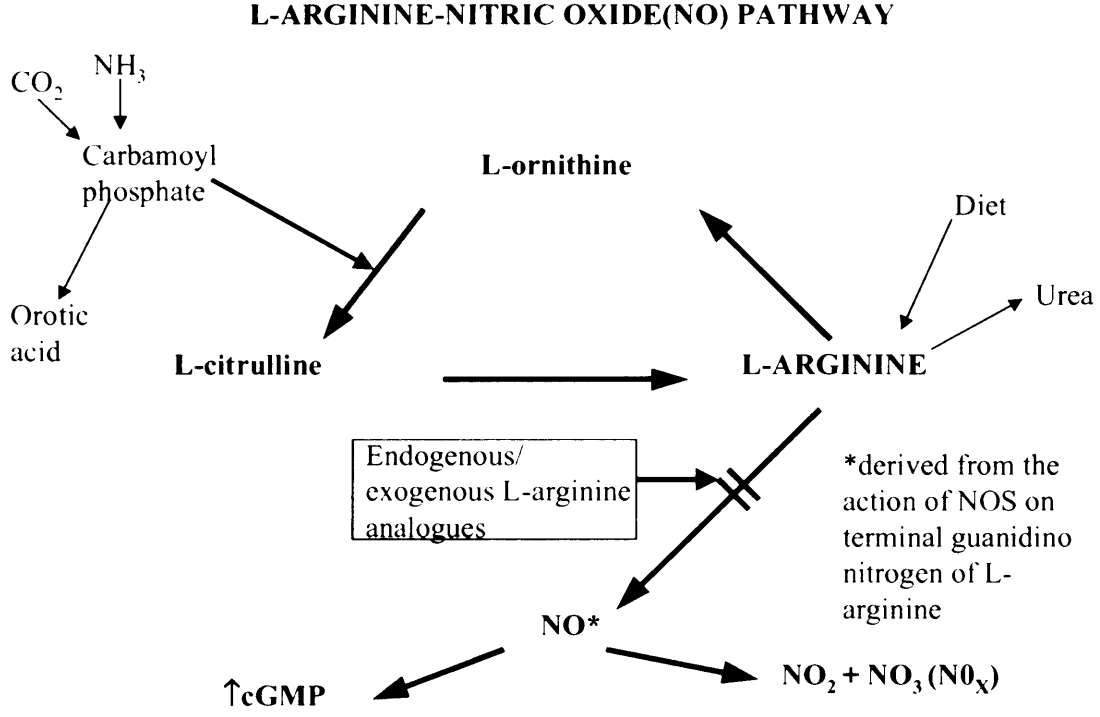
EDRF is known to be a substance with a half life of only a few seconds (Cocks et al, 1985) and its release has been observed under basal conditions as well as after stimulation with acetylcholine (Martin, 1985). The effects of EDRF are mediated by the stimulation of guanylate cyclase and the consequent production of cyclic guanosine monophosphate (cGMP), being inhibited by a number of agents including methylene blue and haemoglobin (Rapoport and Murad, 1983; Martin, 1985). Later it was suggested that EDRF may be nitric oxide (NO) (Ignarro et al, 1987) as half lives of EDRF and NO were very similar, both inhibited platelet aggregation and acted on vascular smooth muscle (Radomski et al, 1987; Kukovetz, 1979).

In 1988 it was demonstrated that L-arginine but not D-arginine was the precursor for the production of NO by endothelial cells, (Palmer et al, 1988; Schmidt et al, 1988) with the calcium dependent nitric oxide synthase (NOS) being the responsible enzyme (Moncada et al, 1991). Certain analogues of L-arginine were subsequently shown to inhibit the synthesis of nitric oxide and these included $N^{G}$-mono-methyl-L-arginine (L-NMMA), $N^{G}$ nitro-L-arginine methyl ester (L-NAME) and Nω-nitro-L-arginine (Rees et al, 1990; Gross et al, 1990; Palmer et al, 1988). Concomitantly with these discoveries it became apparent that endothelial dependent relaxation occurred in response to hypoxia, shear stress, electrical stimulation and to various substances including acetylcholine, adenine, thrombin, substance P and bradykinin (Furchgott and Vanhoutte, 1989, Furchgott, 1999).

### 2.4.2 Production of NO

The free radical NO is synthesised from the amino acid L-arginine (Palmer et al, 1988). This is an oxidative reaction involving a family of enzymes, nitric oxide synthases (NOS) which act on the terminal guanidino nitrogen of l-arginine to produce nitric oxide (NO) (Moncada et al, 1991; Myers et al, 1991; Miller et al, 1999). Citrulline, the byproduct of this reaction can be recycled back to L-arginine (Hecker et al, 1993). The production of NO from L-arginine requires molecular oxygen together with several cofactors including tetrahydrobiopterin, nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide and flavin adenine dinucleotide (Hevel and Marletta, 1994; Neri and DiRenzo, 1995).

**Figure 2e: Production of nitric oxide**




Orotic acid is an intermediate metabolite in pyrimidine synthesis. It is a sensitive marker for L-arginine deficiency

### *2.4.2.1 Nitric oxide synthase (NOS)*

NOS exists as 3 isoforms; neuronal NOS (type 1), endothelial NOS (type 2), and inducible NOS (type 3) (Forsterman et al, 1991). These 3 isoforms are encoded by distinct genes and display approximately 50% amino acid sequence homology (Rutherford et al, 1995). They are present in many cell types and their expression is regulated at the transcription level (Massmann et al, 1999). There is considerable overlap in the distribution of these NOS isoforms (Peters et al, 1999). Both nNOS and iNOS are cytosolic proteins but eNOS, which is associated with cell membranes, is myristoylated (Busse and Mulsch, 1990; Pollock et al, 1991; Stuehr et al, 1991). eNOS and nNOS are said to be constitutively expressed and dependent on the cofactor calmodulin. Elevated calcium levels result in NOS and calmodulin binding with subsequent activation of NOS (Klatt et al, 1992). Hence, eNOS and nNOS are designated calcium dependent (Busse and Mulsch, 1990; Bredt and Snyder, 1990) whereas, iNOS, once thought to be independent of calcium, is now known to be twice as active in the presence of calcium than in its absence (Kone and Baylis, 1997).

iNOS is expressed after transcriptional induction in virtually all nucleated cells (Forsterman et al, 1991; Knowles and Moncada, 1994). Bacterial toxins, IFN and IL-1 are known to stimulate gene expression of iNOS (Neri and DiRenzo, 1995). NO is produced in large amounts by the activity of iNOS and this NO is used for cytoactive purposes and may play a role in the hypotension and vascular changes that occur in various clinical shock syndromes (Moncada and Higgs, 1993; Kone and Baylis, 1997).The activation of iNOS is associated with a sustained need for large amounts of NO (Nathan, 1992).

The two constitutive NOS isoforms, eNOS and nNOS have limited tissue expression and are regulated by various stimuli. Stimuli which trigger the production of NO

include adenosine diphosphate (ADP), acetylcholine, bradykinin, amino acids, platelet derived substances and shear stress (Neri and DiRenzo, 1995). Both eNOS and nNOS produce NO in a tonic fashion for signaling purposes. NO produced by nNOS is important as a neurotransmitter and may have a role in functions such as learning and memory (Moncada, 1997; Kone, 1997).

The distribution of these isoforms within the kidney has been studied in the rat where iNOS mRNA is mainly localised to the outer medulla (Morrissey et al, 1994; Kone and Baylis, 1997), nNOSmRNA to the inner medullary collecting duct and the macula densa (Terada et al, 1992; Mundel et al, 1992; Bachman and Mundel, 1994) and eNOS principally localised to the endothelium of the glomerular capillaries, intrarenal arteries, medullary vasa recta and the afferent and efferent arterioles (Bachman and Mundel, 1994). nNOS within the kidney is involved in glomerular haemodynamics, renin release, BP control and other kidney functions via both central and peripheral neural activity (Reyes et al, 1994; Cabrera and Bohr, 1995; Peters and Noble, 1996).

#### *2.4.2.2 L-arginine*

L-arginine is a non-essential amino acid in humans (Visek, 1986). This naturally occurring L-isomer of arginine is the substrate for the production of NO, whereas the D-isomer of arginine is not (Palmer et al, 1988). It has been demonstrated that L-arginine treatment is associated with increases in plasma nitrate and citrulline, a byproduct of NO production, both in pregnant and non-pregnant women and that L-arginine has hypotensive properties in pregnant women (Neri et al, 1996; Smulders et al, 1997; Facchinetti et al, 1999).

Maintenance of plasma L-arginine levels is mainly dependent on its dietary intake and synthesis by the kidney (Reyes et al, 1994). The normal dietary intake of L-arginine is approximately 3-6g/day (Reyes et al, 1994; Tangphao et al, 1999). Whilst endogenous

synthesis of L-arginine occurs mainly in the kidney smaller amounts are produced in the liver by the conversion of citrulline to arginine (Reyes et al, 1994). The normal plasma concentration of L-arginine in both rats and humans is 100 micromoles per litre (Peters et al, 1999). An increased urinary excretion of orotic acid is suggestive of relative L-arginine deficiency within the body since when arginine levels are suboptimal pyrimidine biosynthesis is increased with ammonia utilisation and hence increased levels of orotic acid (Reyes et al, 1994; Baylis et al, 1996) (See figure 2e).

The precise relationship between plasma L-arginine and the production of NO is not entirely understood but it has been demonstrated that in the non-pregnant healthy individual exogenous L-arginine plays a very limited role as a precursor of NO and therefore does not appear to be a rate limiting factor in its production (Tangphao et al, 1999). In humans only approximately 1% of the daily dietary L-arginine intake is metabolised in the L-arginine-NO-pathway (Castillo et al, 1995) and it has been shown that a high L-arginine diet in young men that has no effect on plasma NO metabolites or excretion in the urine compared to a normal L-arginine diet (Beaumier et al, 1995).

The production of NO can be inhibited by a number of $N^G$ substituted L-arginine analogues eg $N^G$ nitro-L-arginine methyl ester (L-NAME). These inhibitors are competitive and act on the nitric oxide synthases to inhibit their activity. Endogenous inhibitors of NO production include asymmetric dimethylarginine (ADMA) and $N^G$-mono-methyl-L-arginine (L-NMMA), both of which are found within human plasma and urine. ADMA is, however, found in far higher concentrations within the body than L-NMMA (Holden and Fickling, 1998).

### 2.4.3 Properties of NO

NO is a labile, soluble colourless gas with a half-life of seconds. In both water and plasma NO is metabolized to the stable end-product nitrite ($NO_2$) (Bachman and Mundel, 1994). Hence, urinary and plasma $NO_2$ concentration are used extensively as

an indirect measure of NO activity within the body (Facchinetti et al, 1999). NO exerts a wide variety of biological effects throughout the body many of which are mediated by guanylate cyclase, which is activated to increase the production of the second messenger cGMP (Bachman and Mundel, 1994), in turn activating protein kinases, resulting in dephosphorylation of myosin light chains and subsequent smooth muscle relaxation (Murad, 1986).

NO has many functions other than vasodilatation; it acts synergistically with prostacyclin to inhibit platelet adhesion and it inhibits platelet aggregation (Radomski et al, 1987; Moncada et al, 1991; Moncada and Higgs, 1993). Within both the central and peripheral nervous system NO acts as a neurotransmitter and blood vessels are innervated by nonadrenergic, noncholinergic nerves that release NO as their neurotransmitter. NO is also produced in large quantities during immunolgic reactions and may have a role in non specific immunity (Moncada and Higgs, 1993). It may also play an important role in the regulation of cardiovascular function and the sympathetic nervous system (Fujisawa et al, 1999) and it has been postulated that NO may be of importance in the mid-term quiescence of the uterus in pregnancy (Sladek et al, 1997) with a reduction in responsiveness to NO at term possibly mediating the initiation of labour (Buhimschi et al, 1995b).

NO may also be important in both acute and chronic inflammation as well as conditions such as liver cirrhosis and septic shock (Moncada and Higgs, 1993). In support of this inhibitors of NO synthesis have been shown to decrease inflammation and administration of L-arginine has increased inflammation (Neri et al, 1996). The likely sources of NO here are neutrophils, endothelium and macrophages since NO derived from macrophages has been demonstrated to have a non specific cytotoxic effect (Bachman and Mundel, 1994). NO reacts with oxygen to yield both nitrogen dioxide

and peroxynitrites both of which are potent oxidants with cytotoxic potential, they can both mediate massive oxidative injury and inflammation (Peters et al, 1999).

NO is important in the regulation of blood pressure as it exerts effects on vascular smooth muscle, sympathetic outflow and on sodium balance within the kidney in response to alterations in arterial pressure and extracellular fluid volume (Umans, 1997). Also, eNOS produces NO, which blunts the responsiveness to vasoconstrictors and also has direct vasodilatory actions, which control vascular tone and blood pressure (Moncada et al, 1991; Kone and Baylis, 1997). Also, as previously mentioned nNOS may also influence both blood pressure and kidney functions, as can iNOS under certain circumstances (Kone and Baylis, 1997).

### 2.4.4 Measurement of NO metabolites

NO metabolites (nitrite and nitrate, NOx) within body fluids originate from endogenous production of NO, dietary intake of nitrates ($NO_3$) and nitrites ($NO_2$) and a small amount is derived from the inhalation of polluted air (Baylis and Vallance, 1998). The plasma level of NOx will be influenced both by the production as well as the elimination from the body of these metabolites. Foodstuffs high in nitrate and nitrite include green vegetables and processed foods and ideally should be avoided in advance of assessing baseline NO status (Baylis and Beinder, 1998). Approximately 50% of ingested $NO_3$ and $NO_2$ is excreted within the urine and the rest is metabolised and lost in the faeces (Green et al, 1981). Other factors affecting the measurement of plasma and urinary NOx include vitamin C, exercise, alcohol and atmospheric pollution (Baylis and Vallance, 1998).

NO is produced by the kidney in the proximal tubule and this can contribute to plasma NOx levels (Bouby et al, 1993). Furthermore, urinary NOx levels do not reliably reflect

NO production since they may reflect changes in renal epithelial handling of NO and/or alterations in tubular reabsorption (Baylis et al, 1996).

Therefore, for a qualitative measure of total NO production the ideal sample is a bacteria free 24 hour urine collection from subjects who have strictly followed a pre-investigation low nitrate/nitrite diet (Baylis and Vallance, 1998). Even then it is crucial to realise that $NO_3$, $NO_2$ and NOx excretions just reflect total production and do not represent measures of biologically active NO (Baylis and Vallance, 1998). The measurement of plasma and urinary cGMP may also be informative with respect to NO production but since ANP also uses cGMP as a second messenger then cGMP levels can ultimately be misleading (Baylis et al, 1996).

### 2.4.5 NO and the kidney

NO affects many aspects of renal function. In rats it has been shown that administration of L-arginine causes a large renal vasodilatation together with a marked natriuretic, diuretic and kaliuretic responses (Suto and Losonczy, 1995). Thus, it must contribute to the regulation of water excretion, sodium excretion and renal blood flow (Broere et al, 1998). Within the kidney NO produced by the endothelium is thought to counteract the vasoconstrictor effects of angiotensin II (A II) as well as reduce A II driven hypertrophy and matrix production (Peters and Noble, 1996; Peters et al, 1999). The proximal tubule is also a major site of L-arginine synthesis (Bouby et al, 1993). As mentioned previously (page 33) the 3 isoforms of NOS are distributed throughout the kidney but the renal medulla has a greater capacity to produce NO than the renal cortex.

Renal functional reserve or renal reserve represents the capacity of the kidney to increase its renal haemodynamics under certain demands. It has been demonstrated that patients with a renal reserve will exhibit a vasodilatory response to oral protein loading or intravenous administration of amino acid mixtures, including L-arginine (Bosch et al, 1983, 1986; Reyes et al, 1994; Sturgiss et al, 1996; Milne et al, 2002). Renal reserve is

evident from increases in both GFR and RBF. Some patients have a reduced or absent renal reserve (i.e. some patients with renal disease) and do not exhibit any response to protein loading or amino acid challenge. In pregnant rats and humans, despite the already chronic renal vasodilatation of pregnancy, administration of amino acids can produce a further augmentation of renal haemodynamics, demonstrating the presence of a renal reserve in pregnancy (Baylis, 1988; Sturgiss et al, 1996; Milne et al, 2002).

Infusion of amino acids also causes a significant decrease in renal vascular resistance (Krishna and Newell, 1988) and it has been suggested that elevated hormone levels of insulin, growth hormone, glucagon or the renin-angiotensin system may be responsible for the renal response to hyperaminoacidaemia (Castellino and Giordano, 1988; Woods, 1993). Furthermore, experiments with isolated kidneys suggest that the amino acids may have some direct renal vasodilatory actions and that intact renal innervation is not necessary for the protein induced renal haemodynamic augmentation (Woods, 1993). It has also been suggested that normal proximal tubular function is necessary for the protein induced effects and that prostaglandins may also be involved (Krishna and Newell, 1988; Woods, 1993). Only the naturally occurring L-isomers of amino acids, not the D-isomers, cause increases in GFR and RBF (Premen and Dobbins, 1990).

A proposed model for protein induced changes involves hyperaminoacidaemic stimulated proximal tubular sodium reabsorption and in response to the consequent reduction in tubular sodium concentration nitric oxide and prostaglandins are released which cause afferent arteriolar vasodilatation and an increase in both RBF and GFR (Woods, 1993).

In rats it has been shown that infusion of L-NAME, an inhibitor of NO production, produces more vasoconstriction of the efferent glomerular arteriole than the afferent which causes relative preservation of the GFR despite a decrease in ERPF (Broere et al, 1998). At low levels of inhibition diuresis and natriuresis are decreased without changes

in renal haemodynamics but greater inhibition is required to reduce RBF and increase SBP (Lahera et al, 1991). It has been demonstrated that NO can influence tubular transport directly and regulate tuboglomerular feedback (Baylis et al, 1990; Mundel et al, 1992; Roczniak and Burnsk, 1996; Garcia et al, 1996; Bachman and Mundel, 1994). Chronic inhibition of NO synthesis in rats has been shown to produce proteinuria and glomerular injury compared with controls and also elevations in glomerular capillary blood pressure ($P_{GC}$) and reduced glomerular capillary ultrafiltration coefficient ($K_f$) (Baylis et al, 1992).

### 2.4.6 NO and normal pregnancy

As previously discussed, normal human pregnancy is associated with major haemodynamic changes including increases in circulating blood volume and in cardiac output with decreased total peripheral resistance, this latter contributes significantly to the reduction in blood pressure from early gestation (Curtis et al, 1995; Baylis and Beinder, 1998).

Gestational augmentation of renal haemodynamics, with a significant increment in GFR sustained throughout pregnancy ensures a correspondent reduction in plasma creatinine (Davison and Noble, 1981; Conrad et al, 1999). This hyperfiltration is secondary to elevated RPF consequent on vasodilatation. It is known from rat studies that the increase in GFR is entirely due to an increase in the RPF without concomitant increases in the glomerular blood pressure ($\Delta P$ or $P_{GC}$), since there is equal relaxation of both the preglomerular and postglomerular arterioles (Baylis and Beinder, 1998).

The cause of the systemic vasodilatation of pregnancy remains to be fully elucidated but there is strong evidence to suggest that factors derived from the endothelium play key roles (Molnar et al, 1994). Despite the increase in prostaglandins in pregnancy, however, studies suggest that they do not directly mediate the fall in blood pressure or

changes in renal haemodynamics associated with normal pregnancy (Conrad and Colpoys, 1986). NO has also been implicated as a key factor in this systemic vasodilatation of pregnancy as oestrogen is known to increase NOS activity (Weiner and Knowles, 1994).

Several animal studies have supported the role of NO in the vasodilatation of pregnancy. Studies in rats and rabbits demonstrated an increase in plasma and urinary cGMP, the second messenger of NO, in pregnancy compared to non-pregnant controls (Conrad and Vernier, 1989; Losonczy and Mucha, 1996). Also, in pregnant sheep and rats increased plasma and urinary nitrates have been demonstrated together with increased NOS activity within the uterine artery endothelium (Magness et al, 1996). It has been suggested that in rats the endothelial NO system is crucial for the adaptations required in the circulation in response to the extracellular volume expansion of pregnancy (Baylis et al, 1996; Fiol and Machado, 1998). In pregnant guinea pigs the increased sensitivity to acetylcholine and relaxation is secondary to a pregnancy-mediated increase in NO activity either by increased release or increased vessel responsiveness (Weiner et al, 1989).

Administration of $N^{w}$nitro L-arginine, a potent competitive inhibitor of NO production, to fetal lambs produced a significant reduction in umbilical blood flow thus suggesting an important role for NO in the maintenance of umbilical-placental perfusion (Chang and Roman, 1992). The potential implications of this might be that impairment of NO production could ultimately lead to fetal growth restriction. Also, it has been demonstrated that pregnant rats are more responsive to the effects of NOS inhibitors than non-pregnant controls with a consequent greater reduction in GFR, RPF and a greater increase in vascular resistance (Danielson and Conrad, 1995).

Further studies in rats using selective and non selective NOS blockade in late pregnancy have demonstrated that although endothelial derived NO is important in blunting the

vascular response to vasoconstrictors in pregnancy, some other mechanisms must be responsible for the vasodilatation associated with late pregnancy (Lubarsky and Ahokas, 1997). Also, despite chronic inhibition of NO production in late pregnancy in rats, maternal systemic vasodilatation still occurs suggesting that other compensatory mechanisms are up-regulated during a period of chronic NOS inhibition (Ahokas and Lubarsky, 1998). Therefore, in general, whilst animal studies suggest that NO contributes to the reduction in vascular resistance in some vascular beds in pregnancy including the uteroplacental and renal circulations (Raij & Baylis, 1995), it is likely that other mechanisms may also be involved (Conrad et al, 1993a, 1993b; Danielson and Conrad, 1995).

In healthy pregnant women plasma and urinary cGMP are increased compared to non-pregnant controls and remain elevated throughout gestation (Kopp et al, 1977; Boccardo and Soregaroli, 1996). As cGMP is also the second messenger of atrial natriuretic peptide (ANP) it was speculated that altered ANP activity could be implicated in this increase in cGMP. ANP, however, is known not to be increased in normal pregnancy and therefore the increased cGMP might merely reflect NO (Fournier et al, 1991).

Urinary $NO_3$ excretion has been demonstrated to be higher in pregnant women than non-pregnant with a greater difference in the third trimester compared to early pregnancy (Seligman et al, 1994; Conrad et al, 1999). As mentioned earlier, however, there are difficulties in measuring NO metabolites and the subsequent interpretation of results because levels are diet dependent (Baylis and Vallance, 1998).

Increased NOS distribution and activity have been shown within the placenta, particularly in villous trophoblast cells where maternal NO production and eNOS expression also appear to be increased. This may be of importance in the increased uteroplacental blood flow that occurs in pregnancy (Conrad et al, 1993a; Myatt et al, 1993; Morris et al, 1996; Ramsay et al, 1996). It is possible to increase uterine artery

blood flow by the administration of a NO donor e.g.GTN (Ramsay et al, 1996). Although it has not been possible to detect iNOSmRNA and nNOSmRNA in the placentae of healthy women, (Myatt et al, 1993; Schonfelder et al, 1996) placental NOS activity has been shown to be inhibited by a calmodulin antagonist, supporting the notion that calcium dependent NOS predominates (Garvey et al, 1994).

During pregnancy plasma arginine levels are markedly reduced possibly reflecting an increased production of NO as well as fetal requirements and urea production (Raij, 1994; Baylis and Beinder, 1998). Also, endogenous inhibitors of NO production such as ADMA have been shown to be reduced in normal pregnancy possibly indirectly augmenting the effects of NO (Fickling et al, 1993). This reduction is evident by the end of the first trimester and may reflect an important method of regulating NO production in pregnancy. There is correlation between blood pressure at all stages of gestation and plasma concentrations of ADMA (Holden et al, 1998).

### 2.4.7 NO and preeclampsia

As previously discussed preeclampsia is a common complication of human pregnancy with multisystem involvement resulting in proteinuria, hypertension, oedema, increased intravascular coagulability, decreased renal haemodynamics, hyperuricaemia, hepatic dysfunction, increased responsiveness to vasoconstrictors, generalised organ hypoperfusion and fetal growth restriction (Cooper et al, 1993). In preeclampsia the normal vascular adaptations to pregnancy are compromised with vasoconstriction and platelet aggregation as important features of the disorder (Cotton et al, 1988; Roberts and Redman, 1993).

The precise aetiology of preeclampsia is unknown but it is thought that abnormal trophoblast migration resulting in reduced placental blood flow is important and that the subsequent widespread and differential endothelial cell dysfunction mediates the generalised vasospasm (Brosens et al, 1972; Roberts et al, 1991; Roberts and Redman,

1993). Thus it seems that it is poor perfusion of the fetoplacental unit in preeclampsia that results in the release of factors hostile to endothelium which theoretically could lead to a reduction in endothelial NO release (Zeeman et al, 1992). Therefore, it has been hypothesised that a derangement of the L-arginine-NO pathway may be important in the pathophysiology of preeclampsia and it is certainly true that NO can inhibit vasospasm, platelet activation and activation of leucocytes (Roberts et al, 1991). Doppler studies in pregnant women with preeclampsia have confirmed that both the uteroplacental and fetoplacental circulations are compromised which can ultimately lead to fetal growth restriction (Trudinger and Cook, 1990).

In support of this hypothesis several animal studies have demonstrated that administration of NOS inhibitors to pregnant rats does result in an increase in blood pressure, the appearance of proteinuria and fetal growth restriction (Baylis and Engels, 1992; Yallampalli and Garfield, 1993; Diket et al, 1994; Molnar et al, 1994; Edwards et al, 1996; Baylis et al, 2002) and abolition of the refractoriness to vasoconstrictors such as angiotensin II, norepinephrine and/or arginine vasopressin (AVP) (Molnar and Hertelendy, 1992). These effects were also demonstrated to be partially reversible by administration of L-arginine, but not D-arginine (Buhimschi et al, 1995a; Helmbrecht et al, 1996).

It has also been noted that GFR, normally elevated in pregnant rats, was significantly decreased after administration of L-NAME, a NOS inhibitor (Molnar et al, 1994), often also associated with severe glomerular structural abnormalities. Interestingly, the chronic blockade of NO production in both pregnant and non-pregnant rats results in significant reduction in plasma NOx concentration (Ahokas and Lubarsky, 1998).

Human studies comparing preeclamptic women with healthy pregnant women, with measurement of urinary NOx concentrations in maternal, uteroplacental and fetoplacental circulations, have yielded inconsistent results. Some studies have

demonstrated higher levels of NOx in preeclamptics as compared to normal pregnant controls, (Lyall et al, 1995; Smarason and Allman, 1997; Ranta et al, 1999; Norris et al, 1999) whilst others have demonstrated lower levels (Seligman et al, 1994; Davidge and Stranko, 1996). Some found no significant difference between the two groups (Cameron et al, 1993; Wang et al, 1994; Curtis et al, 1995; Brown et al, 1995; Conrad and Mosher, 1995; Egerman et al, 1999) (see table 1). These inconsistencies in results are also apparent in studies comparing plasma and urinary cGMP levels in preeclamptics versus normal pregnant controls. Some studies showed an increase in cGMP in preeclampsia (Schneider et al, 1996), others no difference (Barton et al, 1992; Boccardo and Soregaroli, 1996) or a decrease (Begum et al, 1996; Lopez-Jaramillo et al, 1996) (see table 2).

As already mentioned, however, dietary intake significantly influences NOx within the body fluids as well as NOx elimination, therefore where studies were not controlled for diet the interpretation of data is difficult. Also, as previously mentioned, the measurement of NOx may not be an accurate measurement of the haemodynamically active NO within the body (Baylis and Vallance, 1998).

In two human studies ADMA concentrations were found to be significantly higher in women with preeclampsia compared to normal pregnant women (Fickling et al, 1993; Holden and Fickling, 1998). This was, however, contested by another study where ADMA, L-arginine and ANP levels were no different between the two groups nor indeed differed from women who were not pregnant (Lopez-Jaramillo et al, 1996).

The argument against the hypothesis that reduced NO production by dysfunctional endothelial cells is responsible for the vasoconstriction of preeclampsia is supported by the studies previously mentioned which revealed elevated plasma NOx in women with preeclampsia compared to normal pregnant controls (Nobunaga et al, 1996; Smarason and Allman, 1997; Ranta et al, 1999) encouraging speculation that increased production

of NO could be compensatory for the vasoconstriction and hypertension of preeclampsia. Reduced renal clearance in preeclampsia was also cited as a possible cause but Ranta et al (1999) found renal clearance to be identical in both the normal pregnant group and the preeclamptic group. A further study demonstrated increased $NO_2$ concentrations within the fetal circulation of women with preeclampsia but not within the maternal circulation (Lyall et al, 1995). They suggested that this compensatory increase may improve blood flow within the fetoplacental circulation which is compromised in preeclampsia secondary to the defective placentation. Again, however, there was no dietary control of nitrites and nitrates during these studies.

One study speculated that although no clear role for NO in preeclampsia could be demonstrated, endogenous sources of NO may have resulted in difficulties with data interpretation (Curtis et al, 1995). A further study which demonstrated no difference in plasma NOx between the two groups did, however, show reductions in the 24 hour urinary NOx, thought to be an overall better measure of NO activity, in preeclampsia compared to normal pregnancy (Conrad and Mosher, 1995).

Another study suggested a compensatory increase in NO production (Davidge et al, 1995), where plasma from women with preeclampsia was shown to cause an upregulation in constitutive NOS and hence an increase in NO production from healthy endothelium. Moreover, they also speculated that it was calcium dependent iNOS isoforms that were responsible for the increase.

When compared to healthy pregnancies NOS activity has been shown to be decreased in placentae from pregnancies with pregnancy induced hypertension, fetal growth restriction and abnormalities in umbilical flow velocity as demonstrated on Doppler studies, again which may reflect the placental dysfunction (Giles et al, 1997; Beinder et al, 1999). In contrast, another study demonstrated increased levels of NOS in placentae

from women with preeclampsia but a decrease in NOS in the umbilical artery endothelium of these same women (Rutherford et al, 1995).

The argument for the explanation that NO deficiency is important in the pathogenesis of preeclampsia is supported by the finding of significantly lower serum $NO_2$ levels in women with preeclampsia compared with normotensive pregnancies (Seligman et al, 1994) and decreased urinary NOx levels in preeclampsia but without an associated reduction in plasma NOx (Davidge and Stranko, 1996; Begum et al, 1996). When L-arginine has been administered to pregnant women hypotension and increased NO production ensues suggesting that the L-arginine-NO-cGMP pathway may be involved in preeclampsia and that perhaps L-arginine supplementation may be beneficial in reducing blood pressure in these women (Facchinetti et al, 1999).

With respect to cGMP, increased in normal pregnancy, the results of studies are inconsistent. Where studies have shown an increase in cGMP in preeclampsia compared to normotensive controls it must be noted that ANP, which has cGMP as a second messenger, is also increased in preeclampsia and therefore, any increase in cGMP secondary to an increase in ANP may in fact mask any reduction in cGMP secondary to NO deficiency (Fournier et al, 1991).

Overall it remains unclear as to whether NO deficiency is important in the pathogenesis of preeclampsia. Some of the confusion originates from the difficulty in assessing activity of NO by urinary and plasma measurements and the need for fastidious pre-study dietary control of nitrates and nitrites.

**Table 1: NOx in preeclampsia and normal pregnancy**

| Author | late pregnancy vs non pregnant | preeclampsia vs normal pregnancy |
|---|---|---|
| Cameron et al 1993 | | no difference in spot urine NOx |
| Seligman et al 1994 | ↑plasma NOx | ↓plasma NOx |
| Curtis et al 1995 | no difference in plasma NOx | no difference |
| Brown et al 1995 | no difference in 24 hr urine NOx | no difference |
| Conrad et al 1995 | no difference in plasma NOx or 24hr urine NOx | no difference in plasma NOx<br>↓ in 24hr urine NOx |
| Lyall et al 1995 | | no difference in plasma NOx |
| Silver et al 1996 | | no difference in plasma NOx |
| Nobunaga et al 1996 | ↑plasma NOx | ↑plasma NOx |
| Begum et al 1996 | ↑ spot urine NOx | ↓ spot urine NOx |
| Davidge et al 1996 | | no difference in plasma NOx<br>↓ spot urine NOx |
| Smarason et al 1996 | no difference in plasma NOx | ↑plasma NOx |
| Egerman et al 1999 | | No difference in plasma nitrite |

**Table 2: cGMP in pregnancy and preeclampsia**

| Author | late pregnancy vs non pregnant | preeclampsia vs normal pregnancy |
|---|---|---|
| Kopp et al 1977 | ↑ 24 hr urine cGMP | – |
| Barton et al 1992 | | ↓ spot urine cGMP but not significant |
| Schneider et al 1996 | ↑plasma cGMP | ↑plasma cGMP |
| Boccardo et al 1996 | ↑plasma cGMP | no difference in plasma cGMP |
| Lopez-Jaramillo et al 1996 | ↑plasma and spot urine cGMP | ↓plasma and spot urine cGMP |
| Begum et al 1996 | ↑spot urine cGMP | ↓spot urine cGMP |

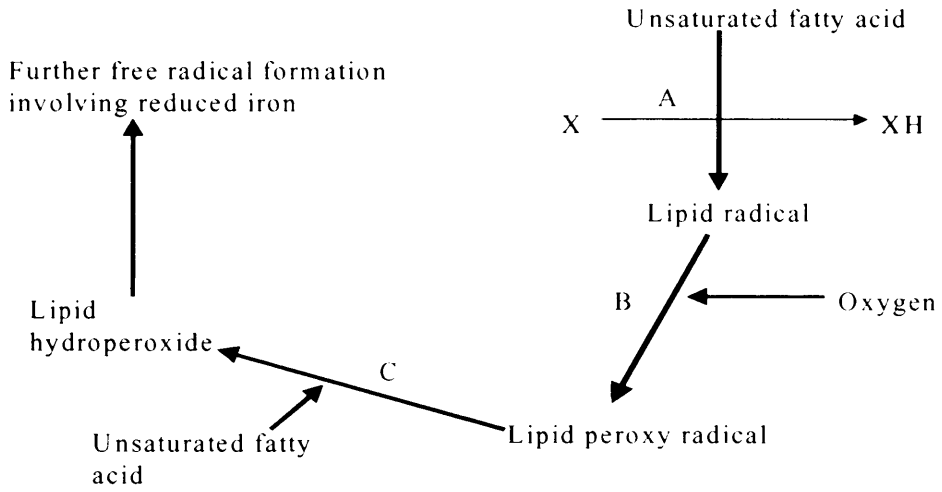
## 2.5 Lipid peroxidation

### 2.5.1 Background

Several workers are currently investigating the possible role of lipid peroxidation in the pathogenesis of preeclampsia (Chappell et al, 1999; Hubel, 1999). Lipid peroxides form when polyunsaturated fatty acids interact with free radicals (see figure 2f). Both free oxygen radicals and lipid peroxides stimulate peroxidation reactions that are toxic to cells and cell membranes resulting in endothelial cell injury and dysfunction amongst other effects (Walsh, 1994). Antioxidants, whether endogenous or derived from the diet, oppose the toxic actions of lipid peroxides and free oxygen radicals. Endogenous antioxidants include superoxide dismutase, glutathione peroxidase and catalase and dietary antioxidants include vitamins C and E (Walsh, 1994).

The balance between the peroxide forming mechanism and the peroxide removing antioxidant system may control the rate of prostaglandin generation and the balance between prostacyclin, a vasodilator and thromboxane A2, a vasoconstrictor (Broughton-Pipkin, 1995). Peroxides inhibit prostacyclin synthase but do not influence thromboxane synthase thereby favouring vasoconstriction (Hubel et al, 1989, 1999). Malondialdehyde (MDA) is frequently analysed as one of the end products of the lipid peroxidation process (Uotila et al, 1993). Oxidative stress is aetiologically important in many diseases and vitamin E supplementation has been shown to be of some benefit in the prevention of cardiovascular disease (Stephens et al, 1996).

**Figure 2f: Simplified diagram of the free radical mechanism of lipid peroxidation**




Step A: a free radical of proper oxidative power(X) removes a hydrogen from the fatty acid to form a lipid radical

Step B: Combination with oxygen to form a lipid peroxy radical

Step C: This then attacks further unsaturated fatty acids, propagating the cycle

### 2.5.2 Pregnancy and preeclampsia

In normal pregnancy lipid peroxides increase but antioxidants also increase to offset their toxic activity. In preeclampsia there is an increase in lipid peroxides but their antioxidant activity is not sufficient to counteract their toxic activity. This is thought to be due to a reduced placental production of the antioxidant glutathione peroxidase (Walsh, 1994). In addition, reduced vitamin levels have been demonstrated in preeclamptics compared with normal pregnancy (Tsukatani, 1983) and riboflavin deficiency has also been suggested as a possible risk factor for preeclampsia (Wacker et al, 2000). Rat studies have shown that the effects of lipid peroxides on blood vessels and uteroplacental perfusion resemble the pathophysiological changes of human preeclampsia (Iwasa et al, 1990). It has been suggested that the increase in uric acid in

preeclampsia is an oxidative response and that this compensatory rise in antioxidants is a result of the increased peroxide load and may reflect disease severity (Uotila et al, 1993).

In a study of antioxidant supplementation in women with severe preeclampsia, no effects were demonstrated on malondialdehyde levels but serum uric acid levels were lower in the antioxidant group compared to the placebo group. There was also a tendency for prolongation of pregnancy in the antioxidant group but the results did not suggest routine use of antioxidants in the treatment of preeclampsia (Gulmezoglu et al, 1997).

In contrast, a study of dietary supplementation with vitamins C and E in women considered to be at high risk for preeclampsia demonstrated that supplementation at 16-22 weeks may be beneficial for the prevention of preeclampsia in a high risk group. The associated improvement in clinical outcome also strongly suggested a role for oxidative stress in the pathophysiology of preeclampsia (Chappell et al, 1999). In order to adequately assess the benefits of vitamin supplementation for the prevention of preeclampsia, however, proper adequately controlled large scale trials must be undertaken on adequate numbers of women (Roberts and Hubel, 1999).

## 2.6 Animal model studies in preeclampsia

There have been many attempts to generate animal models of preeclampsia, a lot of which have not been successful. This may be due to the "subtleties" underlying maternal adaptations to pregnancy and how these conflict with or are opposed by the very procedure utilized to prepare a specific animal model. For instance, although essential hypertension is a risk factor for development of preeclampsia in women, pregnancy is powerfully antihypertensive when superimposed on a variety of models of established hypertension in rats, whether genetic or experimentally induced (Baylis,

1994). In rats with various forms of underlying renal disease, pregnancy often improves renal function and only in one situation (passive Heymann nephritis; a model of membranous glomerulonephritis) is renal function acutely worsened by the pregnancy. Sot it is essential that such issues be borne in mind when assessing animal models of preeclampsia, in terms of strengths and weaknesses (Podjarny et al, 2004).

Workers in this field have tended to focus on a specific pathogenic mechanism believed to have a role in the human clinical condition. These mechanisms include uterine ischaemia, impairments of the nitric oxide (NO) system, induction of renal disease, insulin resistance, over-activity of the autonomic nervous system and/or rennin-angiotensin system, activation of a systemic inflammatory response and the activation of circulating proteins that interfere with angiogenesis.

### 2.6.1 Uteroplacental ischaemia model

The concept behind this model is from the defect believed to trigger preeclampsia in women. Uterine blood increases considerably during normal pregnancy, taking up to 20-25% of cardiac output by term (Longo, 1983). Dilation of the spiral arteries during the first trimester reduces uteroplacental vascular resistance and increases uteroplacental blood flow. In women destined to develop preeclampsia, uteroplacental blood flow is reduced by 50-70%, so-called uteroplacental ischaemia (Brosens, 1964). Several authors have investigated the effects of aortic clamping or uterine artery banding, procedures which do lead to hypertension in a variety of species (see Podjarny et al, 2004). Overall, whilst it does appear that such techniques lead to hypertension but aortic occlusion may have many other haemodynamic consequences in addition to uteroplacental ischaemia. Thus, clear proof of significant hypertension being induced is still lacking and not always will there be changes in other systems and organs that can be seen as relevant to the human disease (Alexander et al, 2001; Crews et al, 2000).

### 2.6.2 Chronic NO synthesis inhibition

A novel approach is to concentrate on what happens at endothelial level in preeclampsia rather than try to emulate the entire clinical condition. Focus has inevitably been on NO because of the evidence that vascular tone is tonically controlled by shear regulated, locally produced, endothelial NO. Chronic nitric oxide synthase (NOS) inhibition produced dose-dependent sustained hypertension in non-pregnant animals, together with renal vasoconstriction, increased glomerular blood pressure, proteinuria and glomerular injury (Zatz and Baylis, 1998). Whether NO plays a role in the gestational vasodilation is less certain. Most animal and clinical evidence argues against a role for NO as the exclusive peripheral vasodilator and the endothelial NOS (eNOS) knock-out mouse exhibited a decrease in BP during pregnancy, to the wild-type value (Shesely et al, 2001), although others reported further increases of BP in pregnancy in the eNOS knock-out model.

Although increased NO may not be responsib le for the entire peripheral vasodilation of normal pregnancy, it probably does play a role in some vascular beds, most notably the kidney (Baylis et al, 1998). In particular, there is good evidence for a primary role of NO-mediated renal vasodilation that is ignaled by the ovarian hormone relaxin and requires activation of the endothelin type B receptor. This model may be of further use but it must be remembered that it involves manipulation of an end point whose resemblance to preeclampsia may be fortuitous.

### 2.6.3 Adriamycin nephropathy (AN)

A single dose of adriamycin given to the nonpregnant rat produces an experimental model of nephropathy with proteinuria, hypertension and, eventually, chronic renal failure (O'Donnell et al, 1985). Micropuncture studies showed that glomerular blood

pressure remained normal (Fogo et al, 1988), indicating that glomerular hypertension was not required for the development of glomerular injury.

Impaired NOS may be implicated in the hypertension because total NO production can be reduced in late pregnancy in rats that have AN and L-arginine administration, the NO synthase substrate, may normalize BP in these rats too. Furthermore, acute NOS inhibition has no effect on BP in late pregnancy AN rats whereas a pressor response was evident in normal pregnant rats as in AN virgin rats.

The AN model resembles preeclampsia in women in that the maternal symptoms disappear soon after delivery, suggesting that all the systemic and renal derangements are functional in origin (Pomeranz et al, 1995). However, the situation is different after 2 pregnancies. At the end of the second pregnancy, BP again was increased and proteinuria also was evident and these remained higher after 7 weeks compared with virgin AN rats. Histologic examination of the kidneys in AN rats at the end of the second pregnancy and 5 weeks thereafter revealed a significant and irreversible worsening of the renal disease, characterized by a marked mesangial expansion (Vasquez et al, 1997).

### 2.6.4 Insulin resistance

Insulin resistance and hyperinsulinaemia are characteristic of normal pregnancy and are maximal in the third trimester. Marked hyperinsulinaemia has been described in women with preeclampsia and/or gestational hypertension (Aruso et al, 1999) with a strong association between hyperinsulinaemia and preeclampsia and even 2 months after delivery, preeclamptic women remain insulin resistant andd hyperinsulinaemic; a finding that may be related to the increased incidence of hypertension in women with a previous history of preeclampsia. The available clinical data also suggest that hyperinsulinaemia, insulin resistance, and/or hyperglycaemia in early or mid-pregnancy

may be prognostic of later gestational hypertension and/or preeclampsia (Innes et al, 2001).

The pathogenic role of insulin resistance/hyperinsulinaemia has been evaluated in pregnant rats with chronic hyperinsulinaemia induced with sustained-release insulin pellet subcutaneously. BP increases close to term and pregnant hyperinsulinaemic rats also have mildly reduced blood serum glucose levels, hypertriglyceridaemia, and a reduced fractional excretion of sodium although urinary protein excretion remains unchanged (Burztyn et al, 2003). The normal increase in GFR (creatinine clearance) seen in pregnant rats was blunted in hyperinsulinaemic pregnant rats (Burztyn et al, 2003). Total NO production was diminished and systolic BP correlated directly with serum insulin levels and inversely with total NO production. Treatment with L-arginine normalized BP and increased total NO production and also increased the renal eNOS protein levels.

### 2.6.5 Inflammation

Normal pregnancy can be thought of, and indeed has been documented as, an inflammatory condition or state, activated by placental debris, with preeclampsia being the extreme of the spectrum of inflammation (Redman et al, 1999). Cytokines are in fact more abundant in preeclampsia than normal pregnancy, and are produced by the placenta. Moreover, markers of oxidative stress are increased in the placenta of women with preeclampsia and nitrotyrosine staining (a marker peroxynitrite, a highly reactive toxic molecule), has been found in the preeclamptic placenta (Many et al, 2000). In normal placenta, there is an adequate concentration of L-arginine, which permits normal eNOS activity to form NO. In preeclampsia, a lower than normal L-arginine concentration is caused by arginase II overexpression (Li et al, 2001). This, together with the high oxidant state induces eNOS uncoupling with increased generation of

reactive oxygen species, more peroxynitrite synthesis, and further oxidative stress (Norris et al, 2004). In pregnant mice, there is a switch from anti-inflammatory to pro-inflammatory cytokines in response to lipopolysaccharide challenge. In the rat, injection of an ultra low dose of lipopolysaccharide caused increased BP, proteinuria, low platelet count, and glomerular thrombosis only in pregnancy (Faas et al, 1994). Furthermore, pregnant rats given lipopolysaccharide developed glomerular thrombosis secondary to NO substrate limitation, indicated by plasma arginine levels decreasing to near zero. In contrast, the same dose of lipopolysaccharide in nonpregnant rats produced only approximately 50% decrements in plasma L-arginine, greater NO generation and no glomerular thrombosis, thus pregnant females are particularly susceptible to development of L-arginine deficiency (Raij, 1994; Raij and Baylis, 1995). Direct administration of tumour necrosis factor α results in a preeclamptic-like response in rats in association with a reduction in NOS abundance as well as amplified vascular contraction and blunted NO vascular relaxation in isolated vessels. Circulating tumour necrosis factor α levels are correlated tightly to indices of insulin resistance in pregnant women.

### 2.6.6 Angiogenesis antagonism

Vascular endothelial growth factor (VEGF) plays an important role in angiogenesis and defective angiogenesis may underly defective placentation, leading to preeclampsia. Alternative splicing of the messenger RNA for one of the VEGF receptors (FLT1) produces a soluble form of FLT1 (Sflt1) that binds with, and antagonises, both VEGF and placental growth factor (PlGF). Amniotic fluid levels and placental sFLT1 expression are increased in preeclamptic women and increased circulating levels of sFLT1 are seen in preeclamptic patients (Maynard et al, 2003). Furthermore, these increased levels of sFLT1 lead to decreases in the plasma levels of free VEGF and PlGF

(Levine et al, 2004), and these changes precede, and predict, both the occurrence and severity of preeclamsia and may be additive with other risk factors, such as insulin resistance.

In addition to these clinical observations a "new" rat model of preeclampsia has been created by administration of sFLT1-expressing adenovirus that causes high circulating levels of sFLT1. The adenovirus alone (the control) had no effect in pregnant or nonpregnant rats on BP or urinary protein excretion, and histological assessment of glomeruli was normal. Administration of Sflt1-expressing adenovirus (days 8-9 of gestation) produced a dose-dependent hypertension, proteinuria, and glomerular endotheliosis in 16- to 17-day pregnant rats; whch is impressive because the pregnant rat is refractory to many huypertensive interventions and rarely exhibits glomerular endotheliosis. The low-dose sFLT1 adenovirus produced levels of circulating sFLT1 seen in preeclamptic women. Further, sFLT1 also produced similar symptoms in nonpregnant rats and administered excess VEGF rescued the sFLT1-infused rat. In addition to their angiogenic actions, both VEGF and PlGF caused relaxation when superfused on precontracted rat renal arterioles in vitro, and this vasodilation was blocked by sFLT1 (Maynard et al, 2003), perhaps via inhibition of an NO-dependent pathway. This new model has been developed based on sound predictions from clinical observations. The fact that the hypertension, proteinuria, and glomerular endotheliosis are all reproduced by VEGF/PlGF antagonism suggest that we may be close to actually identifying a primary cause for the maternal preeclamptic syndrome (further discussed by Podjarny et al, 2004).

### 2.6.7 Renin angiotensin system (RAS)

The RAS is greatly modified in normal pregnancy, with increased levels of pro-renin, renin, angiotensinogen and angiotensin II (ANGII) in the circulation, along with marked

blunting of the pressor actions of ANGII. In preeclamptic patients, the levels of prorenin, rennin, angiotensinogen, and ANGII are all lower thaninnormal pregnancy and the vascular responsiveness to ANGII is restored (Baylis et al, 1998). Some (but not all) groups have reported that certain polymorphisms in the angiotensinogen gene increase the risk for preeclampsia and a recent study reported the presence of agonistic autoantibodies to ANGII in all women with preecclampsia (Wallukat et al, 2003).

Overexpression of angiotensinogen led to higher baseline and pregnancy BP that was amplified in the presence of a double mutant, eNOS knock-out and angiotensinogen over-expression model. A transgenic mouse model has been created with females expressing human angiotensinogen that developed hypertension, proteinuria and glomerular enlargement only during pregnancy and only when mated with males expressing the human rennin. A similar model has been developed in the rat with similar results (Takimoto et al, 1996; Bohlender et al, 2000).

These models are of interest, but they may not be relevant to humans because in the main the hypertension and other pathologies were associated with increased rennin and angiotensin levels, whereas in preeclamptic women the various components of the renin angiotensin system are down-regulated versus normal pregnancy.

### 2.6.8 Conclusions about animal models

The VEGF antagonist model is perhaps the most exciting and itenhances and dovetails with the clinical data which stresses the importance of high free circulating levels of VEGF and PIGF in the maternal responses to pregnancy. In fact, of all the animal models of preeclampsia, the sFLT1 shows the greatest promise because, in addition to the hypertension and proteinuria, it is the only animal model with clearly shown glomerular endotheliosis. So in conclusion, whilst models are definitely of use in

preeclampsia research, the fact that this disease only occurs spontaneously in primates dictates that the definitive studies on preeclampsia will, of necessity, be clinical.

# 3 Clinical studies

## 3.1 Aims and objectives of the study

## 3.2 Study design and subjects

### 3.2.1 Ethical considerations

### 3.2.2 Funding

### 3.2.3 Study groups, timetable and recruitment

### 3.2.4 Informed consent

## 3.3 Infusion protocol

### 3.3.1 Basal measurements

### 3.3.2 Infusion methodology

## 3.4 Further studies

### 3.4.1 High dose L-arginine study

### 3.4.2 Vitamin study

## 3.0 Clinical Studies

### 3.1 Aims and Objectives of the study

The basic aims were:

1. To quantify the renal haemodynamic response (renal reserve) in normal and preeclamptic pregnancy to an intravenous infusion of L-arginine or glycine.
2. To elucidate factors contributing to renal reserve in normal and preeclamptic pregnancy and to the relatively depressed GFR and proteinuria in preeclampsia in terms of alterations in RPF, $\Delta P$, $K_f$, $\omega_o$ and other glomerular barrier function parameters.
3. To investigate the contribution of reduced availability of NO substrate (L-arginine) to the renal vasoconstriction and hypofiltration of preeclampsia.
4. To quantify the effects of intravenous vitamin C and oral vitamin E on the renal haemodynamic response (renal reserve) and the NO system in normal pregnancy. A subsidiary pilot study to assess the potential for a definitive future investigation.

The specific questions relating to the kidney in preeclampsia were:

1. Is depression of GFR and the magnitude of UAE related to haemodynamic factors and/or altered glomerular barrier function?
2. Is there a renal reserve and is it associated with increments in ERPF and/or $\Delta P$?
3. Is the magnitude and/or absence of renal reserve dependent on the particular amino acid infused?
4. Is the time course of post delivery renal recovery after preeclampsia related to its severity, extent of gestational glomerular dysfunction, renal reserve and/or duration of expectant management?

## 3.2 Study design and subjects

### 3.2.1 Ethical considerations

All protocols used in this study were approved by the Joint Ethics Committee of the Universities of Newcastle upon Tyne and Northumbria at Newcastle and the North Tyneside and Newcastle Health Authority.

### 3.2.2 Funding

Candidate's salary funded by and the costs of all consumables were a grant from Wellbeing (2 years from 01/02/99 to 31/01/01) awarded to Professor JM Davison (see acknowledgements).

### 3.2.3 Study groups, timetable and recruitment

This was a longitudinal study of 16 healthy pregnant women and 12 preeclamptic women studied both in late pregnancy (LP) (mean gestations=35+5 & 37+5 weeks, respectively) and then reevaluated at either 3 or 6 months postpartum (PP) (mean18+5 and 17+6 weeks respectively), thus acting as their own non-pregnant controls. The study groups can be defined as follows:

**Healthy controls**

16 healthy primigravid pregnant controls with singleton pregnancies were recruited from the antenatal clinic of the Royal Victoria Infirmary, Newcastle upon Tyne and from community antenatal clinics attended by myself after discussion with the General Practitioners responsible for the supervision of the clinic. All 16 were studied during late pregnancy (LP) (mean gestation=35+5 weeks) and postpartum (PP) (mean =18+5 weeks) (see Appendix A1). All had uneventful pregnancies and successful obstetric outcomes. Exclusion criteria are listed below:

No history of hypertension, renal disease, diabetes mellitus or preeclampsia.

Current pregnancy:

Drug treatment (any)

Significant proteinuria (24 hour excretion > 300mg prior to the infusion study)

Urinary tract infection

By strict adherence to these criteria the 16 women were comparable to one another and the group itself was "tightly" characterized. *Each woman was her own non-pregnant control.*

**Table 3: Characteristics of the Healthy controls**

| Initials | Amino acid | Gestation LP (weeks) | Post delivery PP (weeks) | 24 hr urinary protein LP(g/24h) | 24 hr urinary protein PP(g/24h) | Basal MAP LP | Basal MAP PP |
|---|---|---|---|---|---|---|---|
| NB | L-arg | 33 | 14+4 | 0.11 | 0.1 | 83 | 87 |
| SB | L-arg | 32+6 | 15+3 | 0.07 | 0.1 | 88 | 86 |
| HM | L-arg | 33+1 | 14+4 | 0.06 | 0.1 | 83 | 83 |
| HC | L-arg | 35+2 | 12 | 0.15 | 0.1 | 83 | 92 |
| RSB | L-arg | 34+2 | 14 | 0.1 | 0.1 | 67 | 80 |
| CN | L-arg | 36+2 | 16&24 | 0.11 | 0.06 | 89 | 86 |
| EW | L-arg | 33 | DNR | 0.19 | DNR | 88 | DNR |
| KF | L-arg | 36+3 | 23 | 0.1 | 0 | 85 | 95 |
| MW | glyc | 36 | 23 | 0.1 | 0.1 | 79 | 83 |
| VH | glyc | 39 | 16 | 0.08 | 0.1 | 99 | 95 |
| JM | glyc | 38 | 27 | 0.11 | 0.1 | 103 | 98 |
| EW | glyc | 37 | 14 | 0.22 | 0.05 | 83 | 77 |
| SC | glyc | 35+2 | 25+4 | 0.16 | 0.1 | 89 | 88 |
| MG | glyc | 37 | 28 | 0.14 | 0.1 | 77 | 82 |
| VP | glyc | 35+4 | 14 | 0.1 | 0.1 | 93 | 85 |
| SM | glyc | 37+3 | 24 | 0.11 | 0.1 | 86 | 93 |
| | | | | | | | |
| **Mean** | | 35+5 | 18+5 | 0.124 | 0.087 | 85.9 | 87.3 |
| **S.e.m** | | 0.48 | 1.445 | 0.01 | 0.0075 | 2 | 1.6 |

**Preeclamptic women**

12 preeclamptic women were recruited from the antenatal ward of the Royal Victoria Infirmary, Newcastle upon Tyne. In order to obtain a "pure" preeclamptic study group, patient recruitment was strictly confined to *primigravidae* with significant proteinuria

(see below) and *de novo* hypertension without hypertensive medication(s) at the time of the study. All subjects conformed to the International Society for the Study of Hypertension in Pregnancy (ISSHP) definitions of gestational proteinuric hypertension (Brown et al, 2001) (see 2.3.1). Blood pressure was measured using a Datascope Accutorr (Datascope, new Jersey, USA) which is a automated measuring device fully validated for pregnancy and the non-pregnant. The inclusion criteria are listed below:

Past medical history:

No drug treatment

No history of renal disease, hypertension or diabetes mellitus

Current pregnancy:

No urinary tract infection

First pregnancy (singleton)

Proteinuria > 300mg/24 hour

Hypertension with BP> 140/90mmHg sustained over 30 minutes

No hypertensive medication(s)

By strict adherence to all these criteria (and recommendations), the 12 preeclamptic women were equivalent to one another and as a group were "very tightly" characterized. *Each woman acted as her own non-pregnant control.*

All 12 women were studied in LP (mean gestation= 37+5 weeks) and PP (mean= 17+6 weeks) (see Appendix A2). At the time of the LP study none of them had been given antihypertensive medication(s). Of the 12 participating patients only 11 returned for follow up as one patient was uncontactable PP.

The basic patient characteristics and pregnancy outcomes for both the healthy controls and preeclamptics are given in the Appendix tables.

**Table 4: Characteristics of the Preeclamptic group**

| Initials | Amino acid | Gestation LP (weeks) | Post delivery PP (weeks) | 24 hr urinary protein LP(g/24h) | 24 hr urinary protein PP(g/24h) | Basal MAP LP | Basal MAP PP |
|---|---|---|---|---|---|---|---|
| DM | L-arg | 36+3 | 16+4 | 0.57 | 0.18 | 98 | 94 |
| KT | L-arg | 37+1 | 13+4 | 2.7 | 1.5 | 96 | 104 |
| DJ | L-arg | 36+6 | 24 | 0.56 | 0.1 | 103 | 96 |
| RL | L-arg | 40+3 | 28+5 | 0.6 | 0.1 | 109 | 87 |
| BG | L-arg | 40+1 | 16+6 | 0.6 | 0.1 | 95 | 85 |
| EB | L-arg | 35+6 | 14+6 | 0.63 | 0.1 | 118 | 99 |
| MG | glyc | 39 | 16+4 | 2.2 | 0.17 | 117 | 100 |
| YM | glyc | 39 | 14 | 0.58 | 0 | 105 | 97 |
| JO | glyc | 37+3 | 12+4 | 1.3 | 0.1 | 104 | 98 |
| KM | glyc | 36+6 | DNR | 4.1 | DNR | 117 | DNR |
| AB | glyc | 37+1 | 24 | 8.4 | 0.1 | 95 | 83 |
| PG | glyc | 35+6 | 15+5 | 0.9 | 0.1 | 91 | 85 |
| | | | | | | | |
| **Mean** | | 37+5 | 17+6 | 1.93 | 0.23 | 104 | 93.5 |
| **S.e.m** | | 0.451 | 1.57 | 0.67 | 0.128 | 3 | 2 |

### 3.2.4 Informed consent

Written, informed, witnessed consent was obtained from all volunteers (see Appendix A13)

## 3.3 Infusion protocol

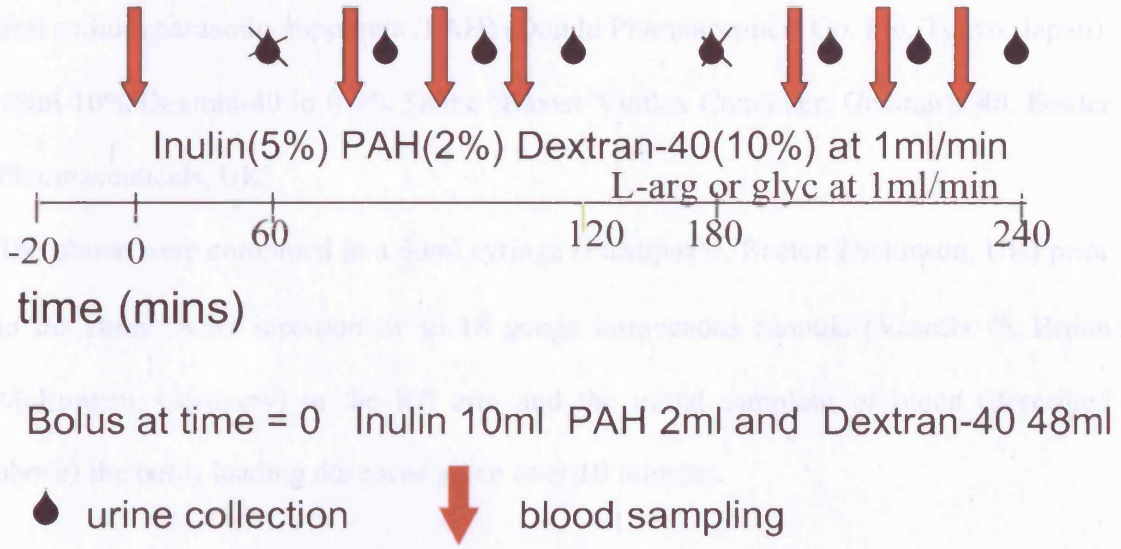
For 48 hours prior to the infusion the patient was requested to modify her diet by eliminating processed foods and green vegetables. Advice regarding diet was obtained from Professor Christine Baylis of the University of West Virginia, USA whose laboratory collaborated with us on this study (Baylis and Vallance, 1998) and diet sheets were completed and distributed to all patients (see Appendix A14). Each patient undertook a 24-hour urine collection the day before the infusion.

All infusions were performed in a purpose built secluded room with the temperature controlled at 21-24°C. All subjects had a light breakfast prior to attending for the study. During the infusion the patients were requested to drink water to encourage voiding and

were seated for the duration of the study only rising to use the commode. Blood pressure was measured throughout the study, using a Datascope Accutorr (Datascope, New Jersey, USA).

The infusion protocol is summarised below:

**Figure 3a: Infusion protocol regimen**




### 3.3.1 Basal Measurements

After insertion of an 18 gauge venous cannula blood was taken for:

- Urea & electrolytes and to accompany the 24hour urine collection– for creatinine clearance, performed by the Department of Clinical Chemistry at the Royal Victoria Infirmary, Newcastle upon Tyne.
- 10ml Lithium heparin sample for osmolality, electrolytes, PAH and Inulin blanks
- 4ml Haematocrit- performed by the Haematology laboratory at the Freeman Hospital, Newcastle upon Tyne.

- 5ml lithium heparin sample for the measurement of nitric oxide surrogates (see page 103).
- 4ml EDTA sample for the measurement of cGMP and amino acids (see page 101).

### 3.3.2 Infusion methodology

Loading dose:

10ml Inulin (Inutest® Fresenius, Austria)

2ml sodium paraaminohippurate (PAH) (Daiidu Pharmaceutical Co. Ltd, Tokyo, Japan)

48ml 10% Dextran-40 in 0.9% Saline (Baxter Viaflex Container, Gentran® 40, Baxter Pharmaceuticals, UK)

The above were combined in a 60ml syringe (Plastipak®, Becton Dickinson, UK) prior to the study. After insertion of an 18 gauge intravenous cannula (Vasofix ®, Braun Melsungen, Germany) in the left arm and the initial sampling of blood (described above) the bolus loading dose was given over 10 minutes.

Maintenance infusion:

The bolus was followed by a maintenance infusion of 1ml/min to provide steady state conditions after one hour using a Baxter Flogard® 6201 Volumetric infusion pump (Roberts, 1995, Roberts et al, 1996; Milne et al, 2002 and Moran et al, 2003).

75ml Inulin (Inutest®)

36ml PAH

264ml 10% Dextran-40 in 0.9% Saline.

After the hour for equilibration, the bladder was emptied by spontaneous voiding. This was followed by 3 consecutive urine collection periods of 20 minutes. Blood samples were taken in the midpoint of each 20-minute collection period from another 18-gauge cannula in the antecubital fossa of the right arm of the patient. L-arginine (Arginine

Hydrochloride 12.5%) or glycine (2g/10ml) were then infused via a syringe driver (P1000 Syringe pump, Welmed) at 6g/hour for the next 2 hours in addition to the maintenance infusion previously described. In total 12g of either amino acid was given. Prior to commencement of the study each woman was randomised to receive either L-arginine or glycine for her two test occasions. After the initial equilibration hour of the amino acid, the 3 clearances were repeated. Variations from baseline of blood pressure, heart rate, osmolality, creatinine, electrolytes and haematocrit are measured using standard laboratory techniques (see methodology section 4.1). We collaborated with Professor C Baylis of the University of West Virginia, USA for the laboratory investigations relating to the activity of the NO system. The methods for the measurement of urine and plasma amino acids, cGMP, ADMA and NOx are described in section 5.0.

**Table 5: Samples per infusion- NO system**

| Sample type | NOx | cGMP | ADMA* | L-arginine |
|---|---|---|---|---|
| **24hour urine** | Yes | Yes | - | - |
| **Basal blood** | Yes | Yes | Yes | Yes |
| **Clearance urine (3)** | Yes | - | - | - |
| **Clearance blood (3)** | Yes | - | - | Yes |
| **Clearance urine (6)** | Yes | - | - | - |
| **Clearance blood (6)** | Yes | - | - | Yes |

*endogenous Asymmetric dimethylarginine(ADMA)

Samples were sent to the laboratory of Professor Christine Baylis at the University of West Virginia, Morgantown, USA using Federal Express rapid shipment. The samples were sent in dry ice. There was no formal assessment undertaken of the impact of storage prior to shipment or during shipment on the samples. One batch of samples was seriously damaged during transit but further duplicate samples were sent at a later date.

It was hoped that more information regarding the NO system during the infusion protocol would be forthcoming but not all assays were undertaken on all the specimens dispatched, resulting in an incomplete data set. As the assays could not be undertaken in-house, this was beyond our control and hence impacted on the results and subsequent discussion relating to the NO system presented in this Thesis.

The metabolic roles of L-arginine, which are secondary to stimulation of hormone release, probably do not significantly influence glomerular function but this amino acid was utilised because of its role as a NO donor (Umans, 1997). The role and safety of the optically different isomer D-arginine are controversial and in any case it was unavailable in a form suitable for our study (Umans, 1997). Therefore, glycine was chosen as an alternative amino acid. As the main focus of this study was glomerular haemodynamics in preeclampsia, the investigation of a control group of normal primigravidae and the use of glycine infusion in both preeclamptic and healthy women were therefore crucial.

## 3.4 Further studies

### 3.4.1 High dose L-arginine study

This group of women consisted of 3 healthy pregnant women with singleton pregnancies in LP and a non-pregnant woman (see appendix A5). Each woman underwent the same infusion regime and sampling as previously described but received 24g IV L-arginine over the latter 2 hours instead of the 12g described. This small pilot study was to assess whether we were giving enough L-arginine to produce a significant effect on renal haemodynamics and the NO system. These women were studied on only one occasion.

### 3.4.2 Vitamin study

As a brief study (n=4), which may lead on to further work and investigation 4 healthy pregnant women fulfilling the exclusion criteria as previously discussed were recruited to attend for a vitamin study. They were studied both in LP and PP (see appendix A4). The infusion regime was identical to that described previously including the dietary modification but instead of receiving an amino acid for the second 2 hours of the infusion, they received 250mg of intravenous vitamin C (Ascorbic acid Injection, 500mg in 5ml, South Devon Healthcare) and 400mg of oral Vitamin E (200mg capsules, Pharmacy, Royal Victoria Infirmary). The standard measurements were the same as the main infusion study.

# 4 Methods

## 4.1 Basic laboratory measurements

- 4.1.1 Sodium
- 4.1.2 Creatinine
- 4.1.3 Chloride
- 4.1.4 Total serum protein (C)
- 4.1.5 Total urinary protein
- 4.1.6 Albumin
- 4.1.7 Estimation of systemic oncotic pressure ($\pi_A$)

## 4.2 Renal haemodynamics

- 4.2.1 Preparation of samples
- 4.2.2 Measurement of glomerular filtration rate (GFR)
- 4.2.3 Measurement of effective renal plasma flow (ERPF)

## 4.3 Gel permeation chromatography (GPC) for dextran clearance analysis

- 4.3.1 Background
- 4.3.2 GPC equipment
- 4.3.3 Preparation of samples for GPC
- 4.3.4 Dextran analyses

## 4.4 Modelling of dextran fractional clearance data ($\theta_D$)

- 4.4.1 *Isoporous plus shunt* model
- 4.4.2 *Lognormal* model

## 4.5 Statistical analysis

# 4.0 Methods

## 4.1 Basic laboratory measurements

### 4.1.1 Sodium

Sodium is measured by the process of Flame Photometry, which takes 40 seconds per sample (Corning 480 Flame Photometer, Corning Ltd, Essex, UK). A plasma sample of 75µl is used to analyse sodium covering the range 0-200mmol/l. The process involves the excitation of individual sodium atoms by a low temperature flame. As the atoms cool light radiation is emitted which is detected by a sensor, then isolated, amplified and compared with a reference signal to produce a final value.

The Corning 480 flame photometer utilises compressed air (3.15kg/cm2 at 10l/min) and instrument grade propane (pressure= 1.75-2.1kg/cm2).

Assay reproducibility has already been calculated previously in our Department with intra-assay and inter-assay coefficients of variation of 1% and 1.3% respectively (Moran, 2000).

### 4.1.2 Creatinine

Creatinine in urine and plasma is quantified using the reaction between creatinine and picric acid (picrate). The reagents used are 45ml of 0.05mol/l picric acid and 180ml of 0.188M sodium hydroxide acting as an alkaline buffer. In the presence of the hydroxyl ions the reaction between creatinine and picrate produces a red colour which is detected by a Beckman Creatinine Analyser 2 (Beckman Instruments Inc, California, USA). The formation of the red colour is directly proportional to the amount of creatinine in the sample. A 1ml-sample size is pipetted into the machine, of this 25µl is then analysed at an absorbance wavelength of 520nm. The machine is designed to have a delay time of

25.6 seconds before recording in order to allow any colour changes not attributable to creatinine to make their contribution without affecting the final result.

Assay reproducibility for creatinine measurement has been previously calculated in our Department with intra-assay and inter-assay coefficients of variation of 1.2% and 1.3% respectively (Moran, 2000).

### 4.1.3 Chloride

Chloride is measured using a Corning 920 Chloride meter (Corning, Essex, UK) which produces silver ions from two silver electrodes. These silver ions then combine with the chloride ions to produce silver chloride, which precipitates. Once all of the available chloride ions have combined with silver ions, free silver ions are then able to pass between the electrodes altering the conductivity of the solution. This change in conductivity is detected by the chloride meter and expressed as milliequivalents of chloride per litre (range 10-350mEqCl/l). Reproducibility is quoted as +/- 1mEqCl/l at 135 mEqCl/l with a resolution of 1mEqCl/l (data from Corning, Essex,UK).

The procedure involves placing the electrodes into a "combined acid buffer". Once prepared, a standard is added (100µl of 100mEqCl/l ) and a reading is taken. This is repeated until the measurement obtained for the standard is accurate at 100mEqCl/l. Once the standard has been read, 100µl of sample is added and a reading taken. Results for a 100µl sample are expressed as mEqCl/L +/- 1mEqCl/l.

### 4.1.4 Total serum protein (C)

The Biuret method is a reliable method for determining total serum protein (Dawnay et al, 1991).

Reagents:

Copper (II) sulphate (alkalinised using sodium hydroxide)

Sodium potassium tartrate (prevents the copper from precipitating)

Standards:

Bovine serum albumin (BDH Chemicals Ltd, Dorset, UK)

Biuret reagent (used as blank)

The principle behind this assay is that protein will react with the copper ions in the alkaline solution to form a violet-coloured complex. Any compound containing carbamyl groups linked through carbon or nitrogen (i.e. peptides) will show a positive biuret reaction. The intensity of the colour change is proportional to the amount of complex formed, which in turn depends on the number of peptide linkages present. Different proteins will give different absorption spectra, but absorbance properties at the optimum wavelength (540nm) are the most accurate for the number of peptide linkages. The development of colour is both temperature and time dependent, therefore, both of these parameters are standardised. An automated analyser is used which initially dilutes the serum sample to obtain a protein concentration within the linear range of colour change. The analyser then automatically calculates the total serum protein concentration (C) using:

*Equation 4a*

C= (OD sample/ OD standard) x plasma dilution x concentration nearest sample x 100

(Where: OD =optical density)

Results are expressed as protein g/100ml plasma.

### 4.1.5 Total urinary protein

Total urinary protein is measured using a turbidimetric method (Iwata and Nishikaze, 1979; McElderry et al, 1982). In alkaline solution, protein reacts with benzethonium

chloride to produce a temperature stable turbidity. EDTA is first added to the solution to remove any non-protein components, which could cause erroneous turbidity. The degree of turbidity is read using spectrophotometry and is linear for sample concentrations of 40-1600mg/l.

Procedure:

Sodium hydroxide (0.05mmol/l) and benzethonium chloride (4g/l) are added to standard solutions (125-1000mg/l "Quantimetrix"), urine samples, and saline blank and vortex mixed. After 40 minutes the absorbance at 600nm is compared with the blank using an automated analyser. The calculation of total urinary protein is as for serum protein, using equation 4a.

### 4.1.6 Albumin

The concentration of urinary albumin was measured by the Diabetic Research Group, Department of Medicine, University of Newcastle upon Tyne using an in-house radioimmunoassay (Christensen and Orfkov, 1984). An anti-human albumin antibody was used as a template for competitive binding of a radiolabelled albumin ($^{125}$I-albumin) and the unknown urinary albumin. The larger the proportion of unlabelled bound albumin the higher the unknown urinary albumin concentration. 30ml of calf bovine serum (DCBS, ICN Biomedicals Ltd, Oxford, UK) which precipitates the antibody that albumin is bound to is used to separate bound albumin from unbound albumin. The radioactivity count recorded is inversely proportional to the amount of unlabelled albumin present in the sample.

Reagents:

Assay buffer- the agents below are combined with deionised water to a volume of 100ml and the pH is adjusted to 8.0

Sodium dihydrogen orthophosphate (0.624g)

Bovine serum albumin (0.2g)

EDTA (1.1g)

Thiomersal (0.0242g)

Labelled albumin:

$^{125}$I-albumin (200KBq/100µl)- diluted to 1:30 with buffer (prepared in-house)

Antibody:

Rabbit anti-human albumin serum (code no A001, Dako, UK)-diluted (1:320 – 1:64,000) with buffer to achieve optimum binding of approximately 70%

Precipitation reagents:

Donor calf bovine serum (DCBS, ICN Biomedicals Ltd, UK)

Polyethylene glycol 6000 (PEG) prepared with 75g-assay buffer

Standards:

10 working standards (Protein concentrations 0.1µg/ml- 10µg/ml) (Kabi Diagnostica, Sweden)

Procedure:

All assays were performed in triplicate. 5µl of assay buffer is added to all tubes (i.e. blanks, excess antibody, zero standard, standards and unknown). Then 100µl radiolabelled albumin is added and mixed to all tubes. 100µl of assay buffer is added to blanks, 100µl of 1:320 antibody to excess tubes and 100µl of diluted antibody to standards, unknowns and interassay control. Precipitation agents are then added after overnight incubation at 4°C and the tubes are centrifuged for 40 minutes at 3000rpm. Following decanting and drainage for 1 hour, each tube is counted for 1 minute.

Analysis:

The radioactivity count is inversely proportional to the unknown urinary albumin concentration. Albumin concentration (mg/l) is calculated from:

*Equation 4b*

Albumin concentration (S/U) x urine dilution x nearest standard concentration

Where: S = nearest standard (counts/min)

U= unknown sample (counts/min)

The sensitivity of the assay is 0.1mg/100ml. Assay reproducibility has previously been determined at albumin concentrations above and below 10μg/ml (Moran, 2000).

### 4.1.7 Estimation of systemic oncotic pressure ($\pi_A$)

The heteroporous membrane models used in this study (*isoporous plus shunt* and *lognormal*) require input values for afferent glomerular arteriole oncotic pressure ($\pi_A$). Systemic plasma oncotic pressure ($\pi_{plasma}$) is equivalent to the afferent glomerular arteriole oncotic pressure ($\pi_A$).

Oncotic pressure is derived from the Landis and Pappenheimer (1963) equation which utilises C (total serum protein):

*Equation 4c* $\pi_{plasma} = 2.1C + 0.16C^2 + 0.009C^3$

This equation is based upon albumin representing approximately 50% of total serum protein in the study groups.

NB. The above equation can also be used to calculate the efferent glomerular oncotic pressure ($\pi_E$), when C is replaced by $C_E$ (efferent arteriole protein concentration)

$C_E$ is derived from:

*Equation 4d* $C_E = C/(1 - FF)$

Where: FF is the filtration fraction:

*Equation 4e* FF= GFR/ERPF

## 4.2 Renal haemodynamics

### 4.2.1 Preparation of samples

Blood samples

The blood samples for the measurement of glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) using inulin and sodium aminohippurate (PAH), respectively, are collected into 10ml lithium heparin bottles. Once collected the samples are centrifuged at 3000rpm for 10 minutes at 25°C. The plasma is then separated from the sample and 1ml aliquots are diluted with 5ml distilled water. 4ml of 20% trichloroacetic acid is then added to precipitate plasma proteins and then the samples are centrifuged again for 10 minutes to obtain a clear supernatant which is then used for the inulin and PAH assays. All samples from the test occasion are batched together.

Urine samples

The urine samples obtained during the study occasion are diluted with distilled water to obtain inulin and PAH concentrations within the workable range of the assays (see table 4). Deproteinisation is not necessary since the amount of protein in the diluted urine samples is negligible.

**Table 6: Urine dilutions for inulin and PAH assays**

| Urine sample volume(ml) | Dilution of urine sample |
|---|---|
| 0-50 | 1:1000 |
| 50-100 | 1:500 |
| 100-200 | 1:250 |
| 200-300 | 1:100 |
| 300-400 | 1:50 |
| 400-600 | 1:25 |
| >600 | 1:10 |

### 4.2.2 Measurement of glomerular filtration rate (GFR)

A modification of the Heyrovsky method (1956) was used for the determination of plasma and urine inulin. Inulin, a fructose polymer, is not secreted, reabsorbed or metabolised by the kidney and freely passes through the glomerular membrane due to its small size and lack of charge and therefore is an ideal substance for estimating GFR. The procedure involves adding an alcoholic solution of purified 4-indol-3yl-acetic acid to the samples, which then produces a blue-purple colour in the presence of fructose. This colour change, which is proportional to the amount of fructose present, is then measured using spectrophotometry. The interference from glucose is less than 1%.

Reagents:

Hydrochloric acid (specific gravity 1.18)

5% 4-indol-3yl-acetic acid in 96% ethanol

Blanks:

Distilled water was used as the reagent blank for the standards and urine samples for calibration purposes. A pre-infusion plasma sample was used as the blank for the plasma samples.

Standards:

Standard aqueous solutions of inulin (Inutest®, Fresenius, Austria) were prepared in concentrations of 1,2,3,4,5 and 7mg/100ml and were analysed in the same batch as the samples of urine and plasma.

Procedure:

0.2ml of 4-indol-3yl-acetic acid is added to all samples (plasma supernatant, blanks, urines and standards). After mixing, hydrochloric acid (8ml) is then added and the samples are incubated at 37°C for 75minutes in a water bath, followed by a cooling period of 30 minutes. The samples are analysed using a spectrophotometer (Pye Unicam SP6 200) set at a wavelength of 530nm. The plasma sample is read against the plasma blank and the urine samples and standards against the reagent blank of distilled water.

Results:

Analysis of the range of inulin standards produces a straight-line curve of inulin concentration versus optical density (OD). Plasma inulin ($P_{in}$) and urine inulin ($U_{in}$) concentration are then calculated using the following formulae:

*Equation 4f*

$P_{in}$ = (OD sample/ OD standard) x plasma dilution x concentration of nearest standard

*Equation 4g*

$U_{in}$ = (OD sample/ OD standard) x urine dilution x concentration of nearest standard

Where: OD= optical density

Inulin assay characteristics

The reproducibility of the in-house assay has been calculated as follows. Known amounts of inulin were added to plasma and urine stock to achieve the concentration range found in the infusion study samples (plasma: 20mg/100ml and urine: 200mg/100ml). 10 samples were prepared and a 1ml aliquot of each sample analysed as described above.

Intra-assay (within batch) variation

This was calculated for plasma and urine using 10 samples (see table 5). Previous in-house laboratory data using the Heyrovsky method suggests that inter-assay variability between samples over the diluted standard range (1-7mg inulin/100ml) is less than 3% (Dunlop, 1981; Davison and Dunlop, 1984). This is minimised by the study samples being analysed in a single batch. The mean recovery rate (the amount measured/amount added to the stock) for urine and plasma was 102.5%, this reflects small errors introduced by the mixing and aliquoting of small volumes and includes intra-assay variation.

**Table 7: Intra-assay coefficient of variation for inulin (CV%)**

| | Number of samples | Mean +/- S.D | CV% |
|---|---|---|---|
| **Urine** | 10 | 194.1 +/- 2.7 | 1.4 |
| **Plasma** | 10 | 21.7 +/- 0.32 | 1.5 |

Calculation of GFR

GFR is calculated from the following standard formula:

*Equation 4h*

$$GFR(ml/min) = \frac{\text{urinary concentration of inulin x urine volume(ml/min)}}{\text{Plasma concentration of inulin}}$$

### 4.2.3 Measurement of effective renal plasma flow (ERPF)

Effective renal plasma flow (ERPF) is determined using sodium paraaminohippurate (PAH). PAH is a suitable substance for this since as well as being filtered by the kidney it is also actively secreted into the renal tubular system. Therefore, PAH is almost completely removed from the circulation in just one pass through the kidney. However, not all renal plasma flow (RPF) passes through the glomerulus or is in direct contact with the tubule epithelium and since PAH clearance will only measure the RPF which does come into contact with the tubular epithelium that effectively removes it from the circulation, what PAH clearance actually measures is called the effective renal plasma flow (ERPF). Hence, when plasma concentrations of PAH are low, the concentration of PAH in the renal vein is approximately 8% and not zero as might be expected i.e. the renal extraction of PAH is 92% (Warren et al, 1944). This study was performed in non-pregnant subjects but a later study suggested this also to be true within a pregnant population (Bucht et al, 1951).

An in-house modification of the Bratton and Marshall (1939) method for sulphanilamide determination was used to estimate PAH concentration in plasma and urine. PAH contains a free amino group ($-NH_2$) which can be diazotised with sodium nitrite. Excess sodium nitrite is then removed with ammonium sulphamate, and the remaining diazo complex formed is coupled with an indicator to produce a pink colour, which is detected by spectrophotometry. The amount of pink colour produced is proportional to the amount of PAH present.

Reagents:

0.1% aqueous sodium nitrite

1.2M hydrochloric acid (HCL)

0.5% aqueous ammonium sulphamate

0.1% N-1-napthyl ethylenediamine dihydrochloride in alcohol (indicator)

Standards:

Standard aqueous solutions of PAH (Merck, Sharp & Dohme, USA and Daiidu Pharmaceutical Co. Ltd, Tokyo, Japan) were prepared in concentrations of 0.5, 1, 1.5 and 3mg/100ml.

Blanks:

As for inulin, 5ml distilled water blanks were used for the standard solutions and urine samples and a pre-infusion plasma sample was used as the blank for the plasma samples.

Procedure:

1ml aliquots of diluted urine and standards were further diluted with distilled water to 5ml. 1ml of HCL (1.2M) was added to all tubes followed by sodium nitrite (0.1%). After 5 minutes 0.5ml of ammonium sulphamate (0.5%) was added, mixed and left for 3 minutes before the indicator was added (0.1% N-1-naphthyl ethylenediamide). 10 minutes was allowed for the pink colour change to occur which was then detected using a spectrophotometer (Pye Unicam SP6 200) set at a wavelength of 520nm. Standards and urines were read against the distilled water blank and the plasma samples against the plasma blank.

Results:

The results are calculated in the same way as inulin (see above) using the curve attained from the standards. Results are expressed as PAH mg/100ml plasma or urine.

PAH assay characteristics

Stock urine and plasma samples with known added amounts of PAH are used to calculate the intra-assay variation. The PAH concentration ranges (2mg/100ml plasma

and 100mg /100ml urine) found in plasma and urine during the infusion studies were attained. Sample preparation was repeated 10 times for both plasma and urine and is the same as for inulin (see above).

Intra-assay (within batch) variation

This was calculated for plasma and urine using 10 samples (see table 6). Since measurements for each infusion were performed within the same batch, calculation of the clearance ratio would cancel the effect of inter-assay variation. As with the inulin assay previous in-house data estimates inter-assay variation to be less than 3% over the range of diluted PAH standards (Dunlop, 1981; Davison and Dunlop, 1984). The mean recovery rate (amount measured/amount added to the stock) for urine and plasma was 100.9%.

**Table 8: Intra-assay coefficient of variation for PAH (CV%)**

| | Number of samples | Mean +/- S.D | CV % |
|---|---|---|---|
| **Urine** | 10 | 97.4 +/- 1.1 | 1.1 |
| **Plasma** | 10 | 2.09 +/- 0.02 | 1.1 |

Calculation of ERPF

ERPF is calculated using the following standard formula:

*Equation 4i*

$$\text{ERPF (ml/min)} = \frac{\text{(urinary concentration of PAH x urine volume (ml/min))}}{\text{Plasma concentration of PAH}}$$

Throughout this thesis ERPF is used, however, RPF which can be estimated from ERPF is required for biomathematical modelling of glomerular ultrafiltration. Renal blood

flow (RBF) is not required for modelling purposes but can theoretically be derived from:

*Equation 2e* (see section 2.1)

RPF (ml/min) = RBF (1-haematocrit)

The filtration fraction (FF) is the fraction of plasma flowing through the kidneys that is filtered in the Bowmans space. Throughout this thesis, the FF has been calculated using:

*Equation 4e* FF= GFR/ ERPF

## 4.3 Gel permeation chromatography (GPC) for dextran clearance analysis

### 4.3.1 Background

GPC is the method whereby different sized dextran molecules are separated from plasma and urine samples in order to provide fractional dextran clearance ($\theta_D$) data. The fractional clearance data can then be compared to calculated clearance data derived from biomathematical models (i.e. *isoporous plus shunt* and *lognormal*) (Roberts et al, 1996; Milne et al, 2002).

The different sized dextran molecules were separated using GPC, which is a form of size exclusion chromatography that uses an aqueous system for the separation of water-soluble samples. The principle of the procedure is based on the differential permeation of various sized molecules into matrices of defined porosity i.e. dextrans are separated by the speed at which they percolate down a column containing porous beads. Large molecules, which cannot pass through the pores, are the first to leave the columns and smaller molecules enter the bead matrix and will leave the column later. Elution order is a function of molecular size with the largest dextrans eluting first and the smallest last. Since only dextran is being analysed, separation can be expressed in terms of weight or molecular size, and therefore the molecular weight of an eluted fraction can be equated with elution volume ($V_E$).

### 4.3.2 GPC equipment

The GPC equipment consists of 3 columns (2 active columns and 1 guard column) (Anachem, Luton, UK) containing hydrophilic beads of pore size 20nm-50nm made up of a stable co-polymer of ethylene glycol and methacrylate (TosoHaas, Philadelphia, PA, USA). The 20nm pore size is suitable for measuring dextran molecules up to a molecular weight of 60,000 Daltons and the larger pore size is suitable for dextrans in the range 1,000-700,000 Daltons.

The first column is a guard column, which protects the size exclusion columns, following in descending order of pore size. The columns are housed in a "Spectraphysic" column oven (ThermoFinnigan, Stoke on Trent, Staffs, UK) which is switched on the day before use in order to ensure a stable temperature of 35°C. A "Constametric 3200" pump (ThermoFinnigan, Stoke on Trent, Staffs, UK) infuses the system with the mobile phase phosphate buffer pH 6.5 at a flow rate of 0.75ml/min. This pump is independent of any increase in backpressure. The samples are injected through a port in the side. The sample volumes are small which produces a narrow chromatogram and minimises the risk of overloading the column.

A "Shodex RI74" refractive index meter (ThermoFinnigan, Stoke on Trent, Staffs, UK) analyses the eluted fractions in terms of their refractive index and the resultant chromatogram plots the change in refractive index ($\Delta$RI) against the retention volume ($V_E$).

**Table 9: The GPC columns used**

| Column | Column size | Particle size (μm) | Average pore size (nm) | Dextran separation range (mw) |
|---|---|---|---|---|
| Guard | 7.5mm x 7.5cm | N/A | N/A | N/A |
| 3000PW | 7.5mm x 30cm | 10, 17, 20 | 20nm | Up to 60,000 |
| 4000PW | 7.5mm x 30cm | 17, 22 | 50nm | 1,000-700,000 |

N/A Not applicable

### 4.3.3 Preparation of samples for GPC

Both the urine and plasma samples need to be deproteinised since large proteins would block the columns and smaller proteins may interfere with the elution volumes.

Reagents:

Zinc Sulphate

Sodium Hydroxide

Phosphate Buffer (pH 6.5)

Procedure:

1.2ml of plasma or 1.5ml of urine is mixed with the zinc sulphate and sodium hydroxide to precipitate proteins. The tubes are centrifuged at 3000rpm for 10 minutes and the supernatant decanted and then filtered (2μ pore size) into pre-weighed tubes. These tubes are then re-weighed to calculate the precise starting volume. The samples are then frozen to –80°C and vacuum evaporated leaving a stable residue, which is then stored at –70°C until analysis. This enables all samples from the same patient to be processed together on completion of the study.

Prior to GPC analysis, the sample are reconstituted with phosphate buffer to their original sample volume and then finally re-filtered prior to injection into the GPC column.

Dextran is a stable molecule and storage of plasma and urine samples at –70°C has been shown not to affect the distribution of dextran molecular sizes (Roberts et al, 1996).

### 4.3.4 Dextran analysis

Dextran separation by GPC

The dextran used in this study is Gentran 40 (10% Dextran in 0.9% saline, Baxter Pharmaceuticals, UK) which has a broad molecular size distribution (3.5K-71K MW).

Analysis of the fractional concentration and size distribution of the dextran is achieved using GPC and the change in refractive index is measured using the elution volume.

The samples are prepared as previously discussed in section 4.33. An aqueous solution of phosphate buffer is used as a mobile phase to reduce the disturbance of pH and temperature on column performance and also to obtain a steady chromatogram (i.e. the refractive index remains stable). This refractive index is then set as the baseline before the samples are injected. The columns are calibrated before each batch of samples using known molecular weight dextran standards (78.8, 42.7, 27, 11.7 and 6.7 kiloDaltons). In order to reduce clearance variation, each plasma sample is analysed after the corresponding urine sample for each clearance period. All samples for an individual were analysed in one run (i.e. antenatal and postnatal). A 42K standard is run at the start of each day to assess day to day performance. The area under the curve in the chromatogram is integrated to determine dextran concentrations.

Estimation of fractional dextran concentration

The density of a solution can be measured by refractive index ($\Delta$RI) and is related to solute concentration. Since the molecular size distribution of dextran is not discrete but continuous, when $\Delta$RI is plotted against elution volume the result is a broad peak over the entire dextran range. Fractional dextran concentration was equated with $\Delta$RI by integrating the chromatogram.

Chromatogram analysis

The peaks in the chromatogram are formed by dextran molecules with the larger molecules forming the early peaks, since they freely pass through the columns and the smallest molecules forming the later peaks. Intermediate sized molecules (i.e. not the largest or smallest) will simply record as peaks between these 2 extremes. A calibration curve is derived by plotting the retention volumes at maximum peak ($V_E$) of the standards against the corresponding molecular weight (logarithmic scale). The

regression line drawn between the 5 standard points is calculated using a fourth polynomial curvilinear regression (PCGPC software, Thermo Separation Products, Staffs, UK).

Equation 4j $$\text{Molecular weight} = \sum_{0}^{5} a(n).x^2$$

Where: a= the regression coefficient specific to the calibration curve

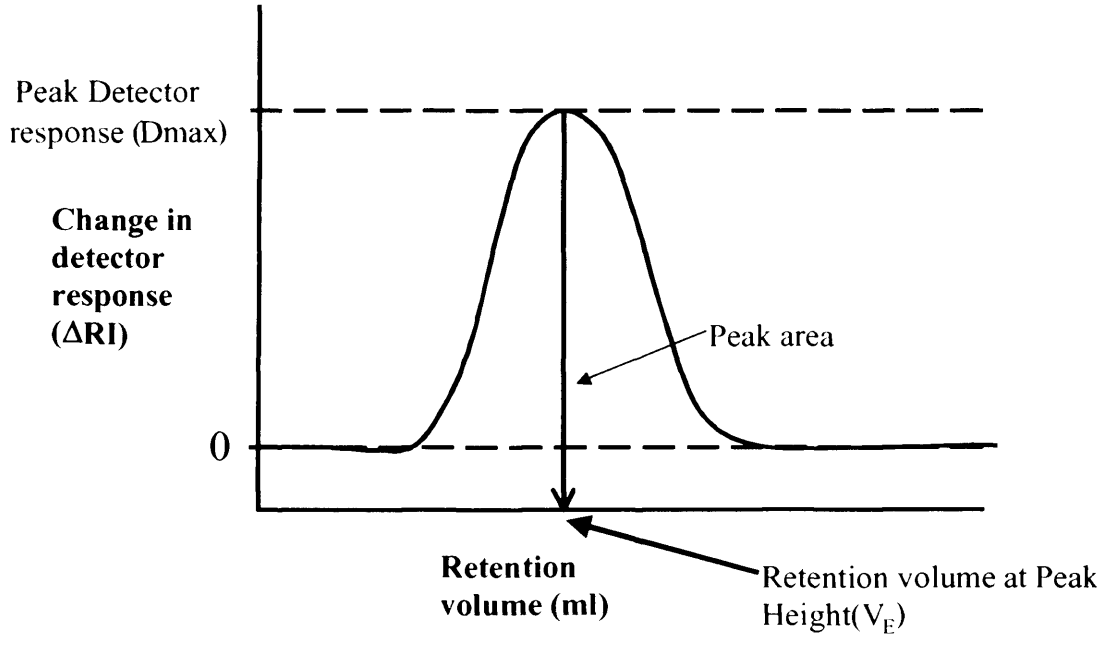
n=0-4

$x=V_E$

Subsequently, when a plasma or urine sample is then analysed, the molecular weights are read directly from this standard calibration curve.

The chromatogram for each test dextran of specific molecular weight is a bell shaped curve.

**Figure 4a: Chromatogram of a 42K standard**



$V_E$ is the retention volume at peak height of the chromatogram and is representative of the molecular weight of the dextran it contains.

$V_E$ can be calculated using:

*Equation 4k* $V_E = V_o + K_D . V_i$

Where:

$K_D$: is the distribution coefficient (governs the volume of solvent that is accessible within the porous beads to the solute)

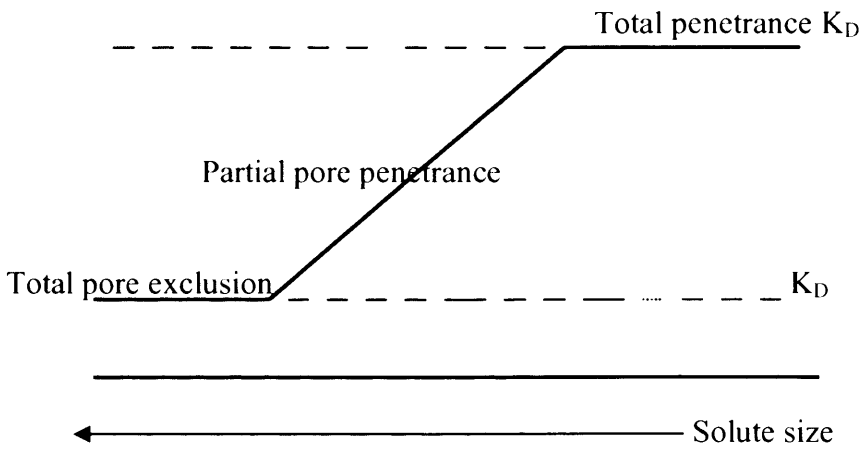
$V_o$: the void volume of the column (mobile phase) i.e. the unhindered molecules in the first timed elution fractions. This is the interstitial volume available to all molecules.

$V_i$: the eluent volume within the co-polymer beads (stationery phase)

When separations are performed at slow flow rates equilibrium is achieved between the mobile and stationary phases.

For large molecules, which cannot enter the pores $K_D$=0 but for very small molecules with free access $K_D$=1. This relationship is shown in figure 4c.

**Figure 4b: Curve showing the relationship of solute size to $K_D$**



The plateau range corresponds to the useful separation range for dextran molecular size discrimination.

The dextran radius ($r_s$) is calculated from the following:

*Equation 4l* $$r_s = 0.33 \times (\text{molecular weight})^{0.463}$$

Each curve is integrated at 38 integrations per ml elution volume (retention volume) which is equivalent to 86 integrations. This encompasses dextrans of molecular size 3.0nm to 6.5nm. Retention volumes for each dextran size is then compared with the detector response ($\Delta$RI), which is proportional to the amount of dextran present.

From here, fractional clearance data for dextrans ($\theta_D$) can be calculated using urine and plasma concentrations of both dextran and inulin:

*Equation 4m* $$\theta_D = \frac{\text{Dextran u / Dextran p}}{\text{Inulin u / Inulin p}}$$

Where: Dextran u is the concentration of dextran in urine

Dextran p is the concentration of dextran in plasma

Inulin u is the concentration of inulin in urine

Inulin p is the concentration of inulin in plasma

$\theta_D$ data are collected in this way for dextran radii 3-6.5nm. This data are then compared with the $\theta_D$ data calculated from the biomathematical models (*isoporous plus shunt* and *lognormal*).

Refinements

Prior to the start of each run, the 5 standards were used to produce a calibration curve. Runs lasted no longer than 3 weeks and at the start of each week a 42K standard was

used and compared with the calibration curve to achieve accuracy between results. In addition, a single dextran standard (42.7K) was analysed 12 times and was taken to be representative of the Dextran radii of interest (3.0-6.5nm). In this way, GPC reproducibility was assessed regularly_by means of chromatogram ability to produce a peak for a known sized Dextran molecule. The reproducibility is assessed in terms of peak area (total solute concentration), the detector response and the peak height ($P_{max}$) retention volume. Widening of the chromatogram peak and a loss of the symmetry around $P_{max}$ may be indicative of column inefficiency.

## 4.4 Modelling of dextran fractional clearance data ($\theta_D$)

### 4.4.1 *Isoporous plus shunt* model

This model assumes that the glomerulus is made up of a number of restrictive pores of size $r_o$ and a small number of pores, which do not restrict the flow of molecules. The smaller number of non-restrictive pores is referred to as the "shunt" component or $\omega_o$. The model requires the input of values for GFR, RPF, $\pi_A$, calculated $\pi_E$ and an assumed value for $\Delta P$. The assumed input values for $\Delta P$ are 37,40 and 43mmHg. From these input values the model will calculate $K_f$ and $\theta_D$ data for pore radii 3-6.5nm. Different $K_f$ and $\theta_D$ values are obtained for each of the 3 assumed $\Delta P$ values. The assumed data are then compared with the measured $\theta_D$ data to see if they agree. The Chi-squared test ($\chi^2$ test) is used to compare measured and modelled data at each dextran radius, with the lowest $\chi^2$ value giving the "closest fit" or "best fit" and an estimate of the $\Delta P$ value (Deen et al, 1972; Roberts et al, 1996; Milne et al, 2002; Moran et al, 2003).

### 4.4.2 *Lognormal* model

The process for this model is identical as for the *isoporous plus shunt* model. The same input values are required and it calculates $K_f$ and $\theta_D$ data. However, the *lognormal*

model is different in that it assumes that the glomerulus is made up of a continuous pore distribution with radii U and standard deviation, S. The model expresses glomerular pore sizes in these terms, providing different $\theta_D$ data for the dextran range 3-6.5nm. The assumed values for $\Delta P$ are the same (37,40 and 43mmHg) and the $\chi^2$ test is again used to assess the degree of concordance between measured and modelled $\theta_D$ data (Deen et al, 1972; Roberts et al, 1996; Milne et al, 2002; Moran et al, 2003).

Once measured $\theta_D$ are obtained, these are plotted against dextran radius to give dextran fractional clearance curves. These dextran fractional clearance curves give an illustration of the sieving characteristics of the glomerulus and may be analysed statistically since theoretical $\Delta P$ values have not yet been used to provide modelled $\theta_D$ data.

### 4.5 Statistical analysis

Statistical advice was provided by Mr T Butler, Department of Mathematics and Statistics, University of Newcastle upon Tyne.

Results are presented as mean or median ± standard deviation (S.D.). When results are derived from the mean of several measurements, the standard error of the mean (S.E.M.) replaces the S.D. The coefficient of variation (C.V.) is the intra-assay S.D. divided by the mean, expressed as a percentage (Swinscow, 1996), as in the formula:

$$CV = \frac{\text{intra-assay S.D.}}{\text{mean}}$$

The C.V. is independent of measurement and its use has been restricted to intra-subject variability.

Significance testing using Student's t-distribution was used entirely appropriately for parametric data (paired or unpaired) with $P<0.05$ considered significant.

The Chi-squared test ($\chi^2$) was used to compare observed with expected results. Measured $\theta_D$ data (observed) was compared with $\theta_D$ modelled (expected) data using the standard $\chi^2$ formula (Campbell and Machin, 1993) as follows:

$$\chi^2 = \Sigma \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$

It further follows that the sum of the $\chi^2$ for Dextran radii 3.0 to 6.5 nm is given by the equation:

$$\text{Sum of } \chi^2 = \sum_{3.0nm}^{6.5nm} \frac{(\text{measured } \theta_D - \text{modelled } \theta_D)^2}{\text{modelled } \theta_D}$$

Differences between fractional Dextran sieving curves were performed by comparing individual fractions with paired Student's t-tests in line with recommendations of the inventors of curve analysis and their interpretation (Deen et al, 1972, 1979 and 1985) Because the theoretical analysis utilised assumed $\Delta P$ values, it was not possible to apply true significance testing to the results of the log-normal or isoporous + shunt modelled data. All analysis of the predicted biomathematical results was therefore restricted to descriptive interpretations.

By definition preeclamptics will have a urinary albumin excretion (UAE) greater than 300mg/24hr by an unpredictable and variable amount, therefore the non-parametric Wilcoxon rank sum test was used to analyse differences between control and preeclamptic UAE.

Values for gestational changes in glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) in normal and preeclamptic pregnancies and for the effects of a mixed amino acid infusion in normal pregnancies (renal reserve) were taken from the publications of Dunlop (1981), Davison & Dunlop (1984), Sturgiss et al (1996), Moran et al (2003) and Milne et al (2002) and from the literature reviewed by Conrad and Lindheimer (1999). These values were used to assist in determining the number of women to be studied.

Using a standard power calculation with a significance level of 5% (0.05) and power of 90% (probability of detecting a significant difference) the number of women required to identify significant differences in

1. GFR and RPF between normal pregnancy and preeclamptic pregnancy would be 6 and

2. GFR and RPF in response to a single amino acid infusion (renal reserve) would be 5.

In fact, the overall size of the preeclamptic cohort was limited by the actual availability of women with that specific condition given the strict selection criteria for inclusion in this cohort. Mainly they were women booking at the Royal Victoria Infirmary with a small number referred during the course of pregnancy from outlying hospitals. The recruited groups taken from a normal population, were entirely adequate and appropriate as assessed against the power calculations. The biological and demographic characteristics of the women conformed to a normal distribution as did the derived data, with mean and median coinciding.

On the basis of the Department's previous 30 years clinical research experience it was predicted that "sufficient" preeclamptic women would be recruited during the two year time span of the definitive study.

# 5 Methods for measuring nitric oxide (NO) activity

## 5.1 Background

## 5.2 Preparation of samples

5.2.1 Samples for the measurement of L-arginine and cGMP

5.2.2 Samples for the measurement of NOx and ADMA

## 5.3 Measurement of cGMP

## 5.4 Measurement of NOx

## 5.5 Measurement of L-arginine

## 5.6 Measurement of ADMA

## 5.0 Methods for measuring nitric oxide (NO) activity

### 5.1 Background

As previously described (section 2.4) $NO_2$ and $NO_3$, otherwise known as NOx, are the stable oxidation products of NO. It is widely accepted that NOx and cGMP, the second messenger of NO, are indices of in vivo NO production. The amount of NOx produced by the body, when the dietary intake of NOx is strictly controlled, should provide an index of NO production, which can be measured in the plasma and urine. Ideally a 24-hour urine collection is used since acute changes in plasma and urinary NOx may not reflect the true systemic NO production (Baylis and Vallance, 1998).

Apart from NOx and cGMP, other indices that are useful in defining the activity of the NO system are the amino acids arginine and citrulline and the endogenous NOS inhibitors asymmetric dimethylarginine (ADMA), symmetric dimethylarginine ( SDMA ) and n-monomethyl-L-arginine(L-NMA) (Holden and Fickling, 1998).

In our investigations we measured NOx, cGMP, L-arginine and ADMA as part of our infusion study (see page 76) in order to thoroughly analyse the activity of NO in normal pregnancy and preeclampsia. The preparation of samples for analysis was undertaken by us in our laboratory and the samples were stored in a freezer at –20°C until later rapid shipment in "dry ice" to the laboratory of Professor C Baylis at the University of West Virginia, who collaborated with us in this study. The methodology below for the measurement of NOx, cGMP, amino acids and endogenous inhibitor is from Professor Baylis' laboratory where these methods have been developed and fully validated (Suto et al, 1995).

## 5.2 Preparation of samples

### 5.2.1 Samples for the measurement of L-arginine and cGMP

A 4ml-blood sample in an EDTA tube (stored on ice) is required for the measurement of arginine and cGMP. The EDTA within the tube acts as an anticoagulant. Once blood is taken the tube is centrifuged at 2300G for 17 minutes (4°C) and the plasma is then aliquoted into 2 x 1ml samples. For amino acid analysis the 1ml sample is frozen and stored at -20°C. For cGMP analysis, however, 50µl of IBMX (10mM 3-isobutyl-1-methylxanthine) is added to the 1ml sample and this is then frozen and stored at -20°C. IBMX is a phosphodiesterase inhibitor, which prevents the breakdown of cGMP in vitro. The urine samples for the measurement of cGMP (24hour collection and clearance periods 3 and 6) are aliquoted into 3x1ml samples (each) and then frozen at -20°C. Once prepared, the samples are stored until shipment to the laboratory in the USA.

### 5.2.2 Samples for the measurement of NOx and ADMA

A 5ml-blood sample in a heparinised tube (stored on ice) is required for the measurement of NOx and ADMA. Once collected the tube is centrifuged at 2300G for 17 minutes (4°C). Once centrifuged, an equal volume of 5-sulphosalicylic acid is then added, mixed and then left for 60 minutes at 4°C. Following this, the sample is further centrifuged at 2300G for 22 minutes (4°C) and the deproteinised plasma is then filtered, frozen and stored at -80°C. 3 x 1ml samples of the 24-hour urine collection are aliquoted and frozen at -20°C for the measurement of NOx. Again, these samples are stored (-20°C) until rapid shipment to the USA.

### 5.3 Measurement of cGMP

Urinary and plasma cGMP is measured using radioimmunoassay, which is a competitive enzyme immunoassay for the determination of cGMP in biological fluids (Cayman Chemical Co, Ann Arbor, MI, USA). The principle is that antibodies to cGMP competitively bind to cGMP within the sample or cGMP covalently attached to an alkaline phosphatase molecule. Post incubation and washing, the reaction is stopped and the yellow colour generated read on a microplate reader. The intensity of the yellow colour produced is inversely proportional to the amount of cGMP present.

Reagents:

cGMP alkaline phosphatase conjugate, 6ml

Polyclonal rabbit antibody to cGMP, 6ml

Assay buffer 2, 30ml (sodium acetate)

Wash buffer concentrate, 15ml

pNpp substrate, 20ml

sodium hydroxide, 5ml(2N)

Well plate:

One 96 Well plate (coated with goat antibody specific to rabbit IgG)

Standards:

cGMP standards (500,100,20,4,0.8,0.16 pmol/l)- these are made up with cGMP 0.5ml (5000pmol/l) and assay buffer.

Procedure:

Assay buffer, standards, samples, blue conjugate solution and yellow antibody solutions are pipetted into the wells of the well plate as laid out in the instructions. The plate is then incubated on a plate shaker at room temperature for 120minutes at 500rpm.The contents of the wells is then washed out 3 times with wash buffer. The blue conjugate

solution and substrate solution are then added to the wells as per instructions before another period of incubation at room temperature of 45minutes (without shaking). Sodium hydroxide is then added to each well which stops then reaction and then the optical density of each well can then be read at 405nm. All samples and standards are run in duplicate. The measured optical density is used to calculate the cGMP concentration in the sample.

Reproducibility of the cGMP assay

The sensitivity of the cGMP assay is 0.367pmol/l. The cross reactivity for a number of other related compounds to cGMP is <0.001%. The intra and inter-assay coefficients of variation for low, medium and high cGMP concentrations are as follows:

**Table 10: Intra and inter-assay coefficients of variation (CV%)**

| | Low cGMP concentration | Medium cGMP concentration | High cGMP concentration |
|---|---|---|---|
| Intra-assay CV % | 5.2 | 4 | 7.6 |
| Inter-assay CV% | 13.7 | 3.5 | 5 |
| | | | |

### 5.4 Measurement of NOx

Following preparation of samples as previously described in section 5.22, the concentration of NOx ($NO_2$ and $NO_3$) in plasma and urine is measured using the Griess assay after conversion of $NO_3$ to $NO_2$ with a nitrate reductase enzyme (Suto et al, 1995).

Reagents:

Nitrate reductase enzyme- produced from Ecoli (ATCC 25922), grown in an anaerobic incubator in a nitrate reductase medium for 14 hours.

Buffer (1.0M HEPES, 2,4mM ammonium formate, pH 7.2)

Griess reagent

Standards:

$NO_2$ standards (5-500mM)

$NO_3$ standard (100mM)

Procedure:

125µl samples (diluted 3-4x) are incubated with 100µl of buffer and 25µl of nitrate reductase enzyme (100mg/ml) in a shaking water bath at 37°C for 60 minutes. Following this the tubes are then centrifuged for 10 minutes at 2000G. Then, 100µl of supernatant is mixed with 150µl Griess reagent in 96 well plates. These plates are then read in an ELISA plate reader at 543nm for the estimation of NOx. Standards are run in addition to the samples to test for complete reduction by the nitrate reductase enzyme.

**5.5 Measurement of L-arginine**

Plasma samples for the measurement of L-arginine are prepared as described in section 5.21. Once prepared, L-arginine is measured using reverse phase high-pressure liquid chromatography (HPLC) with fluorescent detection using a modification of the Acc Q Tag system for amino acid analysis (Waters, Milford MA, USA).

Reagents:

Acc Q fluor borate buffer

Acc Q fluor reagent 2A

Buffers:

Buffer A: 1/10 dilution of AccQ fluor reagent made up to pH 4.7 with HPLC grade phosphoric acid 85% (Fisher A260-500)

Buffer B: 100% Acetonitrile (Fisher a998-4)

Buffer C: 100% HPLC grade water

Standards:

Amino acid standards made up in Acc Q fluor borate buffer (6.25, 12.5, 25, 50, 100,200mM)

Procedure:

On the day of analysis, the samples and standards are allowed to thaw at room temperature. Once thawed, 10µl plasma samples and standards are mixed with 70µl Acc Q fluor borate buffer and vortexed. Then, 20µl of Acc Q fluor reagent is added and vortexed again for a few seconds. 60 seconds after mixing the samples and standards are placed into a 55°C oven for 10 minutes and then placed in an autosampler (Waters 717 plus, Waters, USA). 10µl samples are then injected onto a C18 reversed phase column at 41 °C (Nova-Pak, 4µm, 39 x 150mm silica-based column, Waters Acc Q Tag, Waters, USA). The eluting products are then measured using a scanning fluorescence detector (Waters 474) at excitation and emission wavelengths of 395nm and 250nm (gain 100), respectively. A flow rate of 1ml/min was attained using a Waters's 600s controller and 616-pump system using a gradient elution table in which buffers are mixed in the following percentages:

**Table 11: Buffer percentages (%)**

| **Time** | **0** | **0.5** | **18** | **19** | **29.5** | **33** | **36** | **65** |
|---|---|---|---|---|---|---|---|---|
| **% A** | 100 | 99 | 95 | 91 | 83 | 0 | 100 | 0 |
| **% B** | 0 | 1 | 5 | 9 | 17 | 60 | 0 | 60 |
| **% C** | 0 | 0 | 0 | 0 | 0 | 40 | 0 | 40 |

The concentration of L-arginine is calculated using Millennium chromatography manager (version 2.10) software to do integrations and calculations based on the established curves for each run.

**5.6 Measurement of ADMA**

ADMA is measured using a slightly modified Acc Q tag method of reverse phase HPLC (Anderstam et al, 1997). Prior to analysis the samples are prepared as previously described in Section 5.22.

Reagents:

Acc Q fluor borate buffer

Acc Q fluor reagent 2A

Standards:

Equal amount of ADMA is made up with Acc Q fluor borate buffer to achieve concentrations of 0.625, 1.25, 2.5, 5 and 10μM.

Buffers:

A Sodium acetate (19.04g, Sigma s-7670), Triethylamine (1940μl, Sigma t-0886), 1L HPLC grade water (Fisher w5-4). All of the above combined with phosphoric acid 85% to give pH 5.8. This is then filtered through a 0.22μm filter disc (Millipore GVW PO4700).

B 100% acetonitrile (Fisher a998-4)

C 100% HPLC grade water

Procedure:

On the day of analysis samples and standards are thawed at room temperature. Once thawed, 20μl of deproteinised plasma is added to 60μl Acc Q fluor borate buffer and vortexed. Then 20μl of Acc Q fluor reagent is added and vortexed again for a few

seconds. In addition, 20µl of a stock deproteinised control plasma is added to 40µm of Acc Q fluor borate buffer, 20µl of standard and 20µl of Acc Q fluor reagent. 60 seconds after mixing, the samples and standards are placed for 10 minutes in a 55°C oven and then into an autosampler cooled to 8-10°C (Waters 717 plus, Waters, Milford MA, USA). 30µl of samples and standards are then injected, using a column heater (Waters) and control module, into a C18 reversed phase column at 37 °C (Nova-Pak, Waters Acc Q Tag, 3.9 x150mm, 4µm, silica based.).

Fluorescence is then measured at 250nm emission and 395nm excitation (gain 100) using a scanning fluorescence detector (Waters 474, Waters, Milford MA, USA). A flow rate of 1ml/min is attained using a Waters's 600s controller and 616-pump system using a gradient elution table in which the buffers are mixed in the following percentages:

**Table 12: Buffer percentages (%)**

| Time (min) | 1 | 16 | 25 | 35 | 40 |
|---|---|---|---|---|---|
| %A | 99 | 97 | 94 | 92 | 86 |
| %B | 1 | 3 | 6 | 8 | 14 |
| %C | 0 | 0 | 0 | 0 | 0 |

NB. Before each subsequent injection there is 6 minutes of flushing with 60% buffer B and 40% buffer C and then 9 minutes of flushing with 100% buffer A.

Concentration of ADMA is calculated using Millennium chromatography manager (version 2.10) software which undertakes integrations and calculations based on established standard curves for each run.

Reproducibility

Information from Professor Baylis' laboratory regarding the reproducibility of the ADMA assay show a recovery of 98+/- 4% (on plasma spiked with 2.5mM, n=4) and replicate measurements (n=5) on a sample of 4mM give a coefficient of variation of 5.7%. The interassay variability for plasma with 2.5mM was 9.2% (n= 10 runs).

# 6 Results for normal controls in late pregnancy (LP)/ postpartum (PP)

## 6.1 Effect of glycine and L-arginine on renal haemodynamics

6.1.1 Glomerular filtration rate (GFR)
6.1.2 Effective renal plasma flow (ERPF)
6.1.3 Filtration fraction (FF)

## 6.2 Effect of glycine and L-arginine on haematocrit and electrolyte excretion

6.2.1 Haematocrit
6.2.2 Electrolyte excretion

## 6.3 Effect of glycine and L-arginine on the renal handling of macromolecules with biomathematical modelling

6.3.1 Fractional dextran clearance curves for controls in LP and PP
6.3.2 Summary of effect of glycine and L-arginine on fractional clearance curves in LP and PP
6.3.3 Biomathematical modelling
6.3.4 The effect of glycine and L-arginine on urinary albumin excretion

## 6.4 Effect of glycine and L-arginine on the NO system

6.4.1 L-arginine
6.4.2 cGMP
6.4.3 NOx
6.4.4 ADMA

## 6.0 Results for normal controls in late pregnancy (LP)/ postpartum (PP)

### 6.1 The effect of glycine and L-arginine on renal haemodynamics

Within this section the results are presented for glomerular filtration rate (GFR) and effective renal plasma flow (ERPF). Both the effects of glycine and L-arginine are presented. It is important to note that during the infusion studies neither L-arginine nor glycine produced any significant alteration in systemic blood pressure or mean arterial pressure (MAP). Those data are demonstrated within the appendix.

#### 6.1.1 Glomerular filtration rate (GFR)

GFR was, as expected, significantly increased by 46% in late pregnancy compared to postpartum ($p<0.0001$). The infusion of glycine increased GFR by 9.6% in late pregnancy and by 8.8% postpartum. Neither increment attained statistical significance. The infusion of L-arginine both in LP and PP did not produce any significant effect on GFR.

**Figure 6a: The effect of glycine infusion on GFR (mean +s.e.m. n=8)**

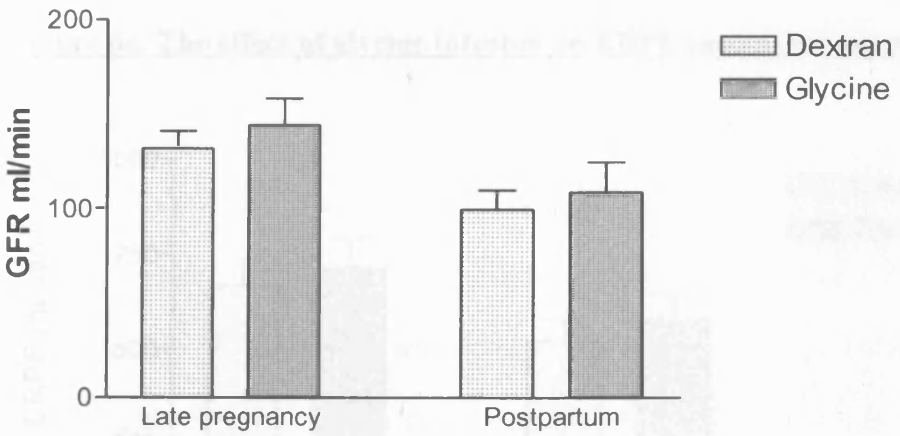
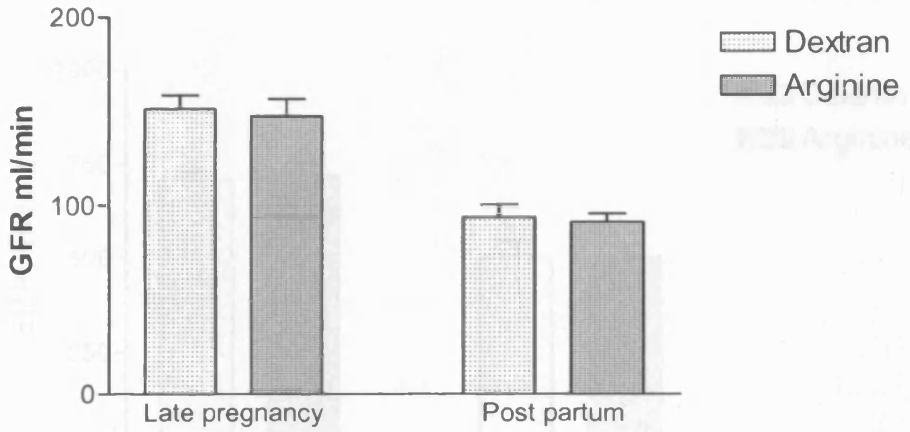


**Figure 6b: The effect of L-arginine infusion on GFR (mean +s.e.m. n=8)**




### 6.1.2 Effective Renal plasma flow (ERPF)

ERPF was, as expected, significantly increased by 31% in LP compared to PP (p=0.0009). Glycine infusion increased ERPF by 7.2% in LP and by 10.3% PP. Neither increment attained statistical significance. L-arginine did not produce any significant change in ERPF in LP or PP.

**Figure 6c: The effect of glycine infusion on ERPF (mean +s.e.m. n=8)**

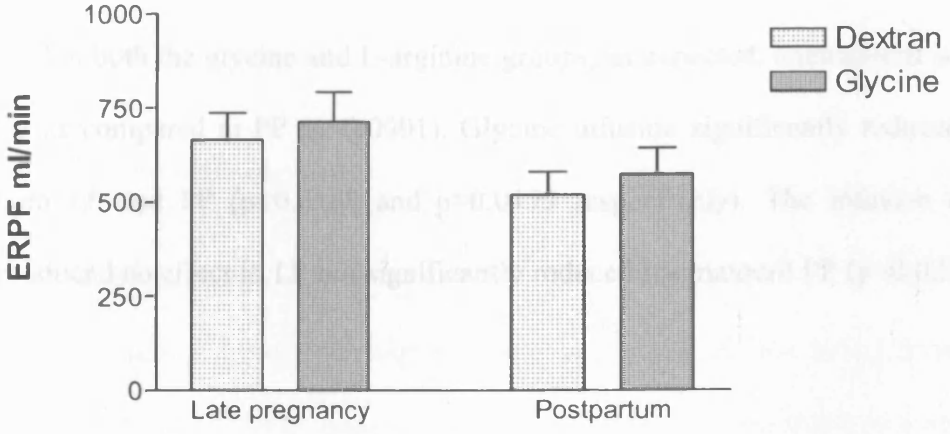
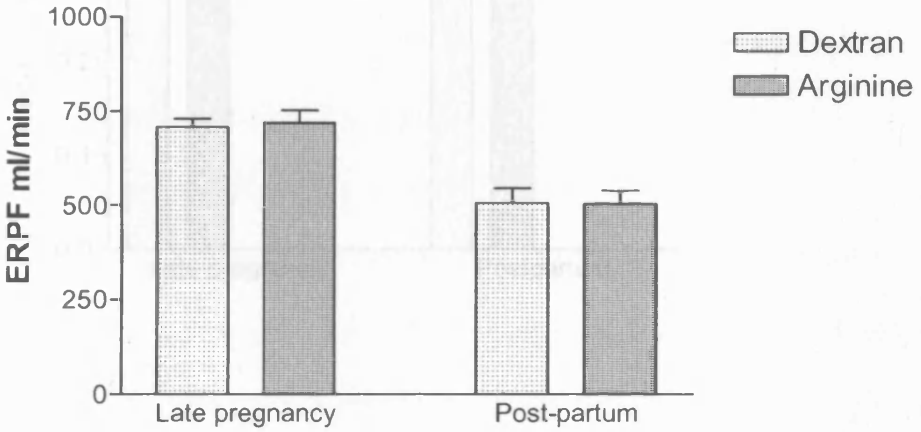


**Figure 6d: The effect of L-arginine infusion on ERPF (mean +s.e.m. n=8)**




### 6.1.3 Filtration fraction (FF)

Filtration fraction is derived from both GFR and ERPF i.e. GFR/ERPF.

Neither glycine nor L-arginine produced any significant change in FF either LP or PP.

## 6.2 Effect of glycine and L-arginine on haematocrit and electrolyte excretion

### 6.2.1 Haematocrit

Within both the glycine and L-arginine groups, as expected, haematocrit was reduced in LP as compared to PP ($p<0.0001$). Glycine infusion significantly reduced haematocrit both LP and PP ($p=0.0168$ and $p=0.0179$ respectively). The infusion of L-arginine produced no effect in LP but significantly reduced haematocrit PP ($p=0.0258$).

**Figure 6e: The effect of glycine infusion on haematocrit (mean +s.e.m. n=8)**

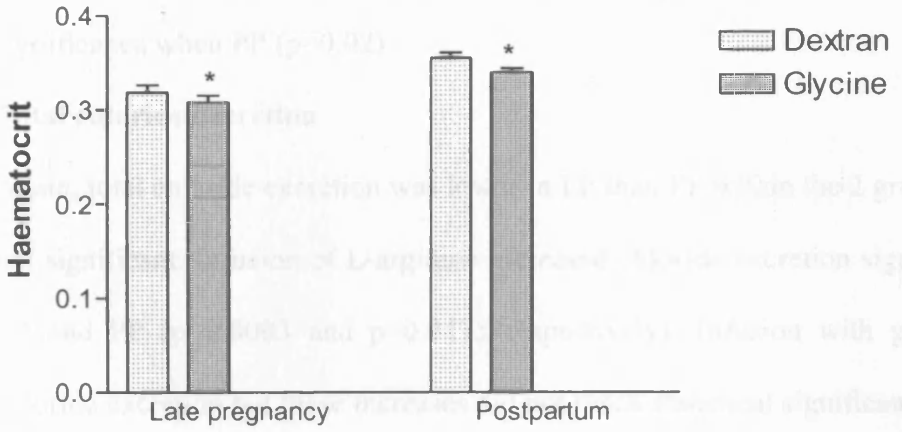
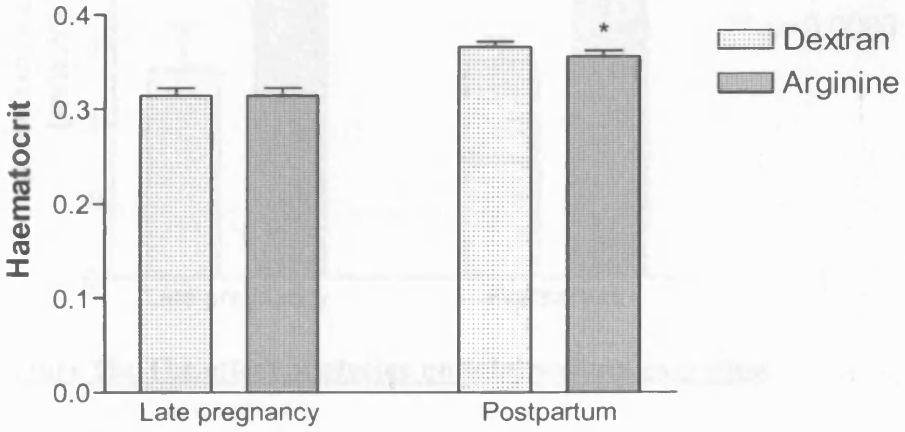



**Figure 6f: The effect of L-arginine infusion on haematocrit (mean +s.e.m. n=8)**




### 6.2.2 Electrolyte excretion

**Total sodium excretion**

Total sodium excretion was slightly lower in LP than PP but this difference was non-significant within the 2 groups. Infusion of L-arginine increased sodium excretion in

both LP and PP but the effect only reached statistical significance in LP (p=0.0093). Infusion with glycine also increased sodium excretion but this only reached statistical significance when PP (p=0.02)

**Total chloride excretion**

Again, total chloride excretion was lower in LP than PP within the 2 groups but this was not significant. Infusion of L-arginine increased chloride excretion significantly both in LP and PP (p=0.0003 and p=0.0475 respectively). Infusion with glycine increased chloride excretion but these increases did not reach statistical significance.

Hence, in summary although both L-arginine and glycine infusions increased electrolyte excretion the effect of L-arginine was greater than glycine.

**Figure 6g: The effect of L-arginine on total sodium excretion**

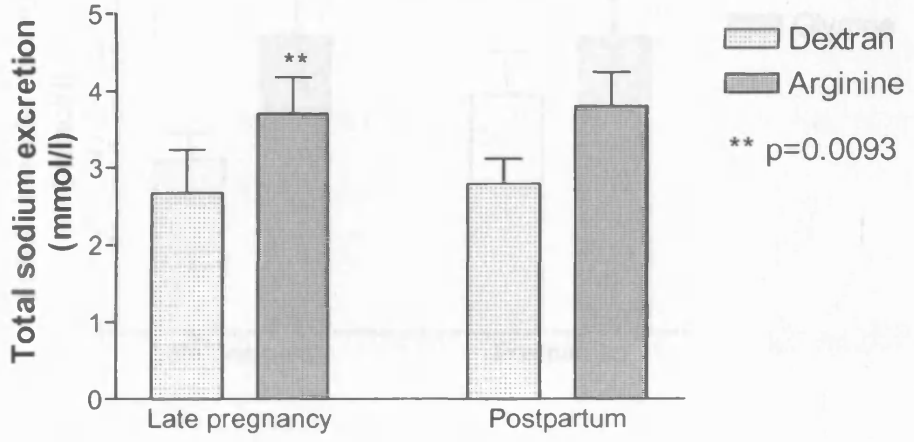



**Figure 6h: The effect of glycine on total sodium excretion**

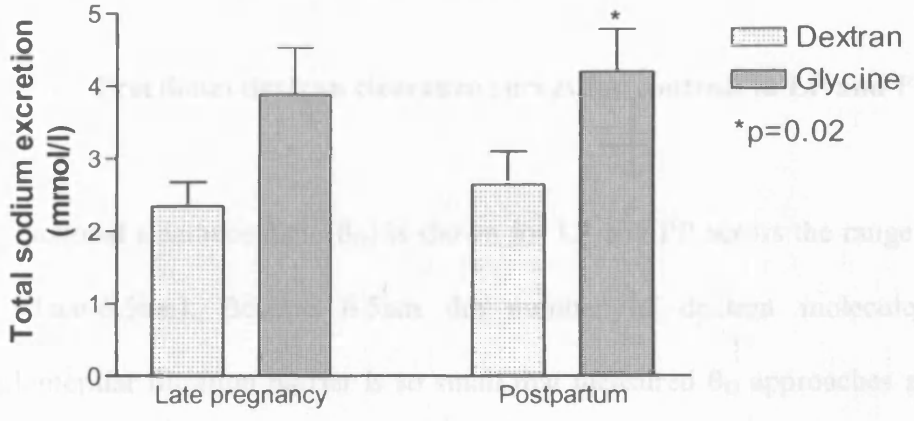


**Figure 6i: The effect of L-arginine on total chloride excretion**

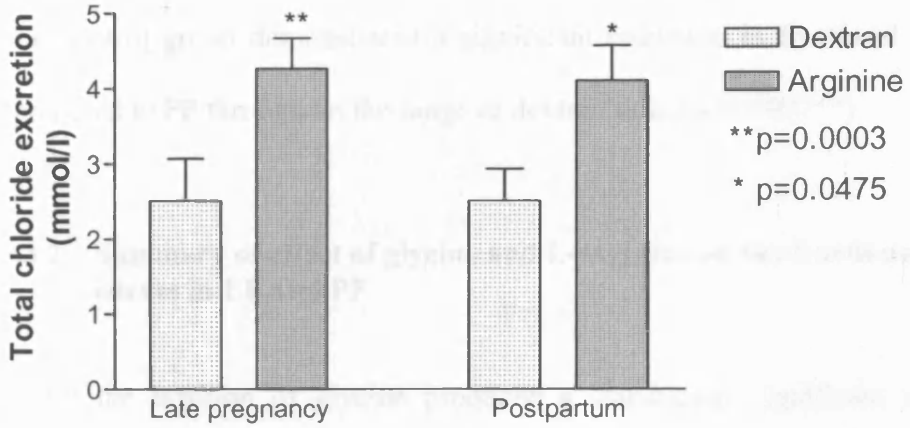
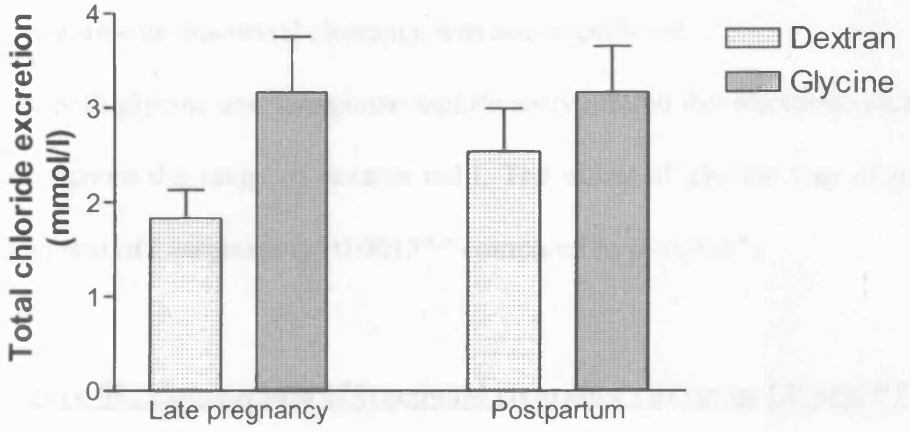



**Figure 6j: The effect of glycine on total chloride excretion**




## 6.3 Effect of glycine and L-arginine on the renal handling of macromolecules with biomathematical modelling

### 6.3.1 Fractional dextran clearance curves for controls in LP and PP

Fractional clearance data ($\theta_D$) is shown for LP and PP across the range of dextran radii (3.1nm-6.5nm). Beyond 6.5nm the number of dextran molecules crossing the glomerular filtration barrier is so small that measured $\theta_D$ approaches zero. The curves

are shown for infusion with dextran alone and then with an infusion of either glycine or L-arginine.

The control group demonstrated a significant reduction in fractional clearance in LP compared to PP throughout the range of dextran radii (p=0.0097**)

### 6.3.2 Summary of effect of glycine and L-arginine on the fractional clearance curves in LP and PP

In LP the infusion of glycine produced a statistically significant reduction in the fractional clearance of dextran throughout the entire range of dextran radii (p=0.0086**). L-arginine, however, only produced a significant reduction in fractional clearance between the radii 5.3-6.3nm (p<0.0001*** to p=0.02*). The overall effect of L-arginine on fractional clearance was non-significant.

PP, both glycine and L-arginine significantly altered the fractional clearance of dextran throughout the range of dextran radii. The effect of glycine was of greater magnitude than that of L-arginine (p=0.0015** compared to p=0.016*).

**Figure 6k: Comparison of fractional clearance curves in LP and PP in healthy controls (n=16)**

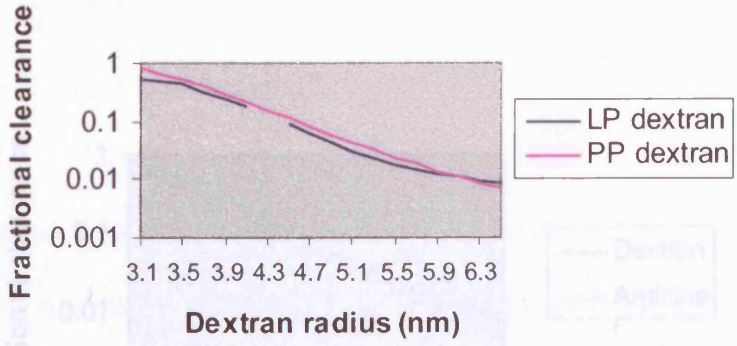
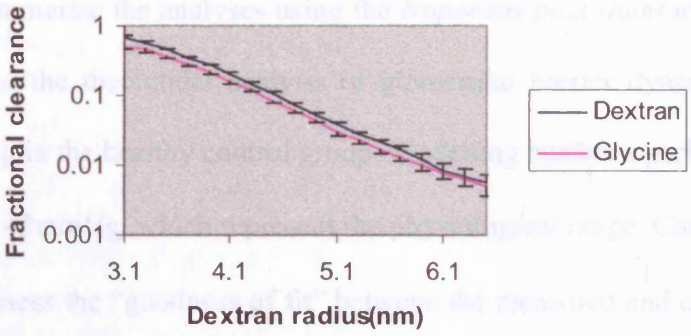
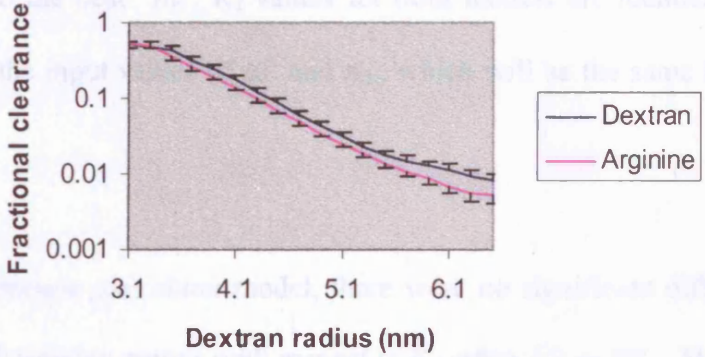


**Figure 6l: Effect of amino acids on fractional clearance in healthy controls**

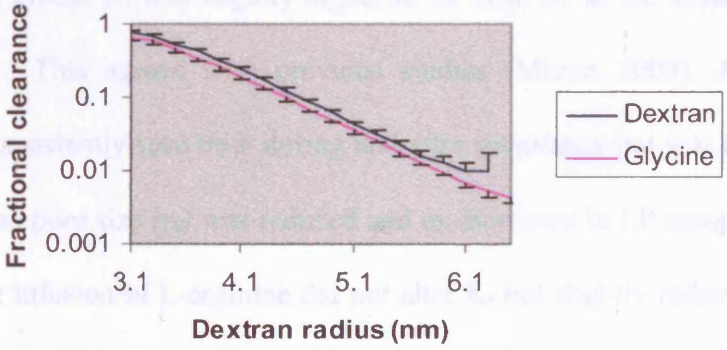
Late pregnancy-
dextran vs glycine
(p=0.0086)



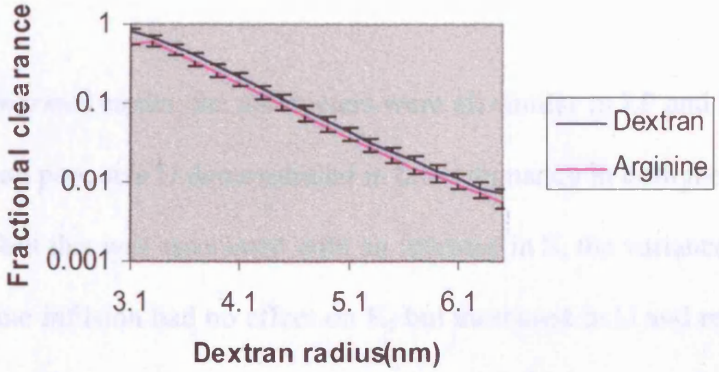

Late pregnancy-
dextran vs L-arginine
(p=ns)




Postpartum-
dextran vs glycine
(p=0.0015)




Postpartum-
dextran vs L-arginine
(p=0.016)



### 6.3.3 Biomathematical modelling

The following tables summarise the analyses using the *isoporous plus shunt* model and the *lognormal* model for the theoretical analysis of glomerular barrier dynamics and macromolecular handling in the healthy control group. Modelling has been performed at the $\Delta P$ range 37, 40 and 43mmHg, which represent the physiological range. Chi-squared ($X^2$) testing is used to assess the "goodness of fit" between the measured and calculated fractional clearance of dextran ($\theta_D$) As can be seen from the tables below, the $\Delta P$ of 37mmHg corresponds to the best "fit". $K_f$ values for both models are identical as $K_f$ determination relies on the input values of $\Delta P$ and $\pi_{gc}$, which will be the same for both models.

With respect to the *Isoporous plus shunt* model, there were no significant differences between the glycine and arginine groups with respect to $K_f$ either LP or PP. However, when taken as a whole group, $K_f$ was slightly higher in LP than PP at the assumed $\Delta P$ values of 40 and 43. This agreed with previous studies (Moran 2003). A shunt component ($\omega_o$) was consistently seen both during and after pregnancy but was reduced PP in both groups. Mean pore size ($r_o$) was reduced and $\omega_o$ increased in LP compared to PP in both groups. The infusion of L-arginine did not alter $K_f$ but slightly reduced $r_o$ in LP and PP and reduced $\omega_o$ in LP. As for the effect of glycine infusion, this produced no change in $K_f$ or $\omega_o$ but did reduce $r_o$ both LP and PP.

With respect to the *lognormal* model, the parameters were all similar in LP and PP apart from a reduction in mean pore size U demonstrated in late pregnancy in both the glycine and L-arginine groups but this was associated with an increase in S, the variance around the mean. The L-arginine infusion had no effect on $K_f$ but increased in U and reduced S

in LP and reduced U and increased S when PP. The glycine infusion had no effect on $K_f$ but reduced U and increased S both LP and PP. Overall, individual chi-squared values were small but did not represent as close a fit as those obtained with the *isoporous plus shunt* model.

Therefore, in summary, an increase in the clearance of larger dextran molecules in pregnancy is reflected by a higher shunt component ($\omega_o$) of the *isoporous plus shunt* model in LP compared to PP and an increase in S for a given value of U (mean pore size) in the *lognormal* model. As the chi-squared values were consistently low in the *isoporous plus shunt* model as compared to the *lognormal* model, this therefore provides the closest overall fit to the measured fractional clearance data ($\theta_D$). With respect to the glycine infusion, the glycine reduced $r_o$ and U both in LP and PP, the 2 models being in close agreement. The L-arginine infusion, however, significantly reduced the shunt component in LP but had different effects on mean pore size U in LP and PP.

**Table 13: The *Isoporous plus shunt* model in the healthy controls**

**Arginine (n=8 LP, n=7 PP)**

**Arginine infusion →**

| LP/PP | ΔP | Kf | $r_o$ | ωo | $\chi^2$ | Kf | $r_o$ | $\varpi_o$ | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| **LP** | **37** | 9.7 | 59 | 0.006 | 0.7 | 9.9 | 58.5 | 0.003 | 1.1 |
| | **40** | 7.9 | 59 | 0.006 | 0.8 | 8.0 | 58.6 | 0.004 | 1.2 |
| | **43** | 6.7 | 59 | 0.007 | 0.8 | 6.7 | 58.6 | 0.004 | 1.2 |
| | | | | | | | | | |
| **PP** | **37** | 9.7 | 62.6 | 0.002 | 1.1 | 8.8 | 60.9 | 0.003 | 0.7 |
| | **40** | 6.9 | 62.3 | 0.003 | 1.0 | 8.0 | 61.2 | 0.003 | 1.1 |
| | **43** | 5.5 | 63 | 0.003 | 1.7 | 5.8 | 61 | 0.004 | 0.8 |
| | | | | | | | | | |

**ΔP- transglomerular pressure (mmHg), $r_o$- pore size (nm)**
**Kf- ultrafiltration coefficient (ml/min/mmHg), ωo- shunt component**
**$\chi^2$- chisquared.**

**Glycine (n=8 LP/PP)**

**Glycine infusion →**

| LP/PP | ΔP | Kf | $r_o$ | $\varpi_o$ | $\chi^2$ | Kf | $r_o$ | $\varpi_o$ | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| **LP** | **37** | 7.5 | 61.7 | 0.004 | 0.8 | 10.3 | 60.2 | 0.004 | 0.8 |
| | **40** | 6.1 | 61.8 | 0.004 | 0.9 | 8.5 | 60.2 | 0.004 | 0.8 |
| | **43** | 5.2 | 61.8 | 0.004 | 0.97 | 7.3 | 60.4 | 0.004 | 1.3 |
| | | | | | | | | | |
| **PP** | **37** | 9.2 | 62.5 | 0.003 | 0.8 | 8.7 | 61.3 | 0.002 | 0.9 |
| | **40** | 6.6 | 62.6 | 0.003 | 0.95 | 6.7 | 61.4 | 0.003 | 0.96 |
| | **43** | 5.3 | 62.6 | 0.004 | 1.1 | 5.4 | 61.4 | 0.003 | 1.0 |
| | | | | | | | | | |

**Table 14: The *Lognormal* model in the healthy controls**

**Arginine** **Arginine** →

| LP/PP | ΔP | Kf | U | S | $\chi^2$ | Kf | U | S | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| **LP** | **37** | 9.7 | 47 | 1.22 | 1.3 | 9.9 | 47.8 | 1.19 | 0.9 |
| | **40** | 7.9 | 47.1 | 1.22 | 1.4 | 8 | 48 | 1.19 | 1.0 |
| | **43** | 6.7 | 47.2 | 1.22 | 1.4 | 6.8 | 48.1 | 1.19 | 1.0 |
| | | | | | | | | | |
| **PP** | **37** | 9.7 | 55.6 | 1.15 | 0.9 | 8.7 | 51.9 | 1.18 | 0.7 |
| | **40** | 6.9 | 56 | 1.15 | 1.1 | 7.95 | 52 | 1.18 | 0.8 |
| | **43** | 5.5 | 56.1 | 1.15 | 1.2 | 5.8 | 52.3 | 1.17 | 0.9 |
| | | | | | | | | | |

**U**- mean pore size (nm)
**S**- standard deviation about the mean

**Glycine** **Glycine** →

| LP/PP | ΔP | Kf | U | S | $\chi^2$ | Kf | U | S | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| **LP** | **37** | 7.5 | 55.1 | 1.14 | 1.0 | 10.3 | 51.4 | 1.17 | 0.8 |
| | **40** | 6.1 | 55.2 | 1.14 | 1.1 | 8.5 | 51.5 | 1.17 | 0.9 |
| | **43** | 5.2 | 55.2 | 1.14 | 1.1 | 7.3 | 51.6 | 1.17 | 0.9 |
| | | | | | | | | | |
| **PP** | **37** | 9.2 | 56.8 | 1.13 | 1.0 | 8.7 | 54.1 | 1.15 | 0.7 |
| | **40** | 6.6 | 57.1 | 1.13 | 1.2 | 6.7 | 54.9 | 1.14 | 0.9 |
| | **43** | 5.3 | 57.2 | 1.13 | 1.3 | 5.4 | 55 | 1.14 | 1.0 |
| | | | | | | | | | |

### 6.3.4 Effect of glycine and L-arginine on urinary albumin excretion

Within the control group the microalbumin excretion (μg/min) was higher PP than LP although this was not statistically significant. Both L-arginine and glycine did not have any significant effects on microalbumin excretion but glycine tended to reduce excretion in LP and PP whereas L-arginine reduced excretion LP but increased excretion PP. In summary, L-arginine and glycine had very little effect on microalbumin excretion within the control group.

**Table 15: Microalbumin excretion (μg/min)- LP vs PP (mean+/- s.e.m)**

| | Late pregnancy | Postpartum |
|---|---|---|
| **Mean** | 5.335 | 5.99 |
| **S.e.m** | 0.61 | 1.83 |

**Table 16: Microalbumin excretion (μg/min)- summary of results in controls (mean+/- s.e.m)**

| | Dextran | L-arginine | Dextran | Glycine |
|---|---|---|---|---|
| **Late pregnancy** | 5.63 +/- 0.99 | 4.75 +/- 0.4 | 5.0 +/- 0.7 | 4.38 +/- 0.8 |
| **Postpartum** | 5.51 +/- 0.98 | 6.6 +/- 1.5 | 6.4 +/- 3.4 | 7.94 +/- 3.7 |

## 6.4 The effect of glycine and L-arginine on the NO system

### 6.4.1 L-arginine

The measurement of L-Arg concentration (μmol/l) on a basal sample was taken both in LP and PP, these measurements were repeated following the 2 hour infusion of either L-arginine or glycine. Basal measurements revealed that L-Arg concentration was significantly lower in LP than PP (p=0.0031) within both control groups.

The infusion of glycine did not produce any alteration in the L-Arg concentration either LP or PP. The L-arginine infusion, however, significantly increased plasma L-Arg concentrations both in LP and PP (p=0.014 and p=0.0067 respectively.

**Figure 6m : L-Arg concentration (µmol/l) - LP vs PP (mean +s.e.m, n=16)**

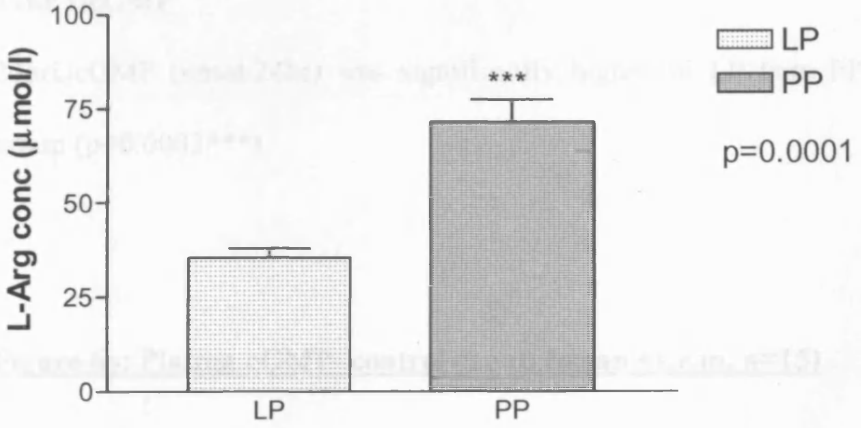
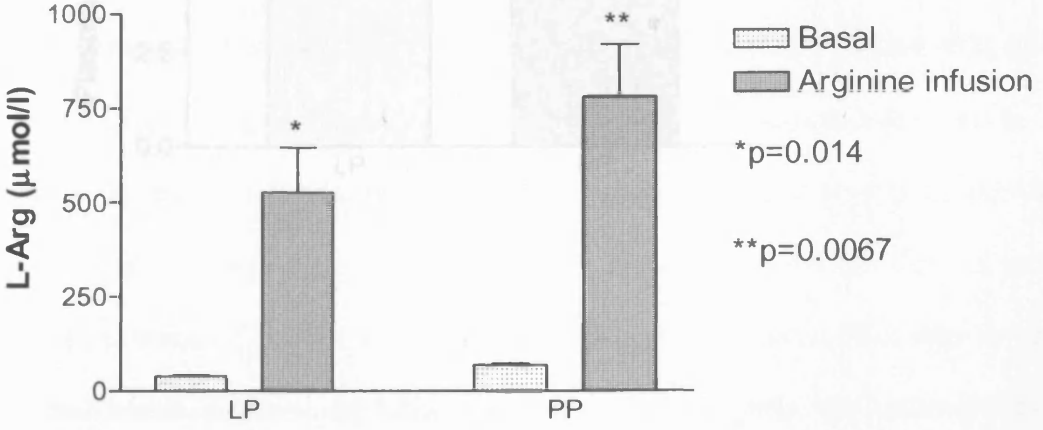



**Figure 6n: L-Arg concentration (µmol/l)- the effect of L-arginine infusion (mean+s.e.m, n=8)**



### 6.4.2 cGMP

The measurement of plasma cGMP (nmol/l) on a basal blood sample was taken both in LP and PP together with a measurement of 24 hour urinary cGMP (24hrUcGMP) (nmol/24hr).

**Plasma cGMP**

Within the control group, basal plasma cGMP (nmol/l) was lower in LP than PP (p=ns).

**24hr UcGMP**

24hrUcGMP (nmol/24hr) was significantly higher in LP than PP within the control group (p=0.0003***).

**Figure 6o: Plasma cGMP- control group (mean +s.e.m, n=15)**

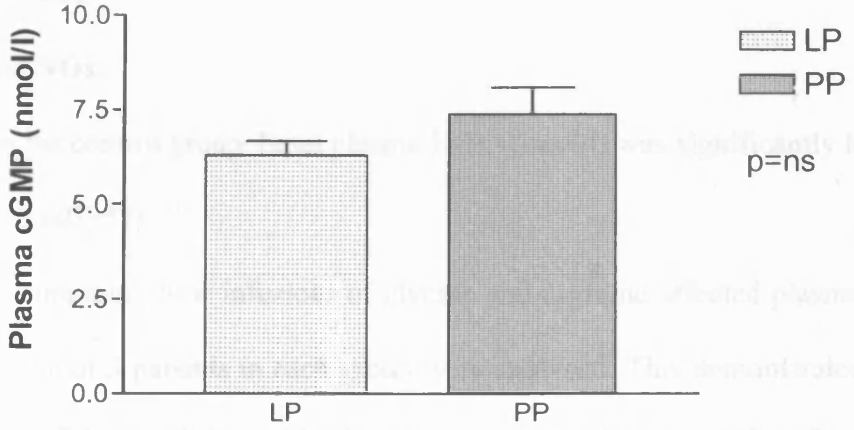
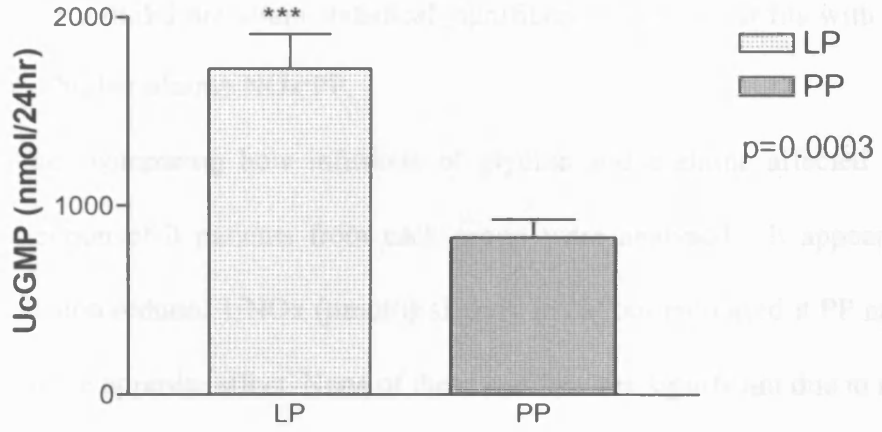


**Figure 6p: 24hrUcGMP- control group (mean +s.e.m, n=15)**




### 6.4.3 NOx

The measurement of plasma NOx (µmol/l) on a basal blood sample was taken both in LP and PP together with a measurement of 24 hour urinary NOx (24hr UNOx) (µmol/24hr).

**Plasma NOx**

Within the control group, basal plasma NOx (µmol/l) was significantly lower in LP than PP (p=0.0023**).

When comparing how infusions of glycine and arginine affected plasma NOx (µmol/l), a selection of 3 patients in each group were analysed. This demonstrated that in LP the infusion of dextran followed by L-arginine increased plasma NOx after the dextran and was then maintained at that level during the L-arginine infusion but this did not occur PP. The infusion of dextran followed by glycine reduced plasma NOx after the dextran and then was further lowered following the infusion of glycine, this occurred both in LP and PP. Due to the small numbers, these results are not statistically significant.

**24 hr UNOx**

24 hr UNOx (µmol/24hr) was higher in LP than PP within the control group. However, this result did not attain statistical significance. This result fits with the previous result of a higher plasma NOx PP.

When comparing how infusions of glycine and arginine affected UNOx (µmol/l), a selection of 3 patients from each group were analysed. It appeared that L-arginine infusion reduced UNOx (µmol/l) slightly in LP but increased it PP and glycine infusion had the opposite effect. None of these results were significant due to small numbers.

**Figure 6q: Plasma NOx –control group (mean +s.e.m, n=15)**

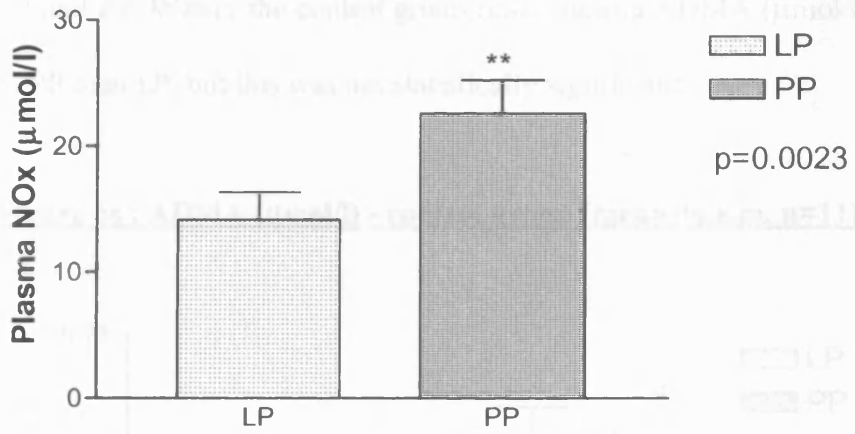
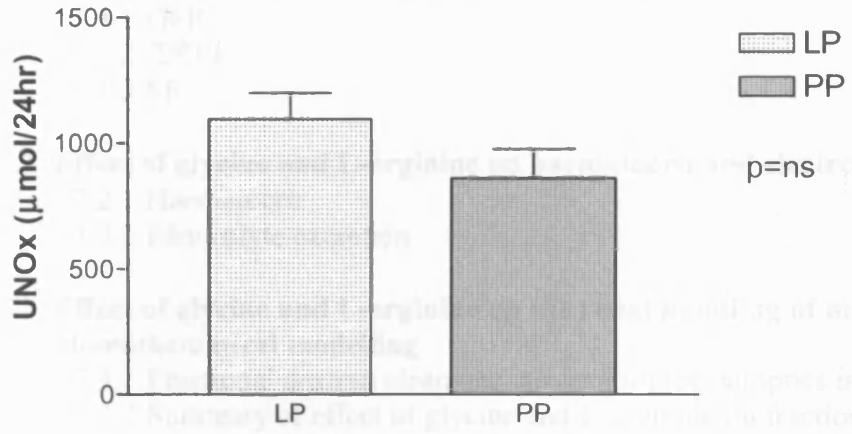



**Table 17 : Plasma NOx (µmol/l) during amino acid infusion (n=3 in each group)**

| | LP | | | PP | | |
|---|---|---|---|---|---|---|
| | **P0** | **P3** | **P6** | **P0** | **P3** | **P6** |
| Arginine | 12.7 | 17.3 | 17 | 17 | 18.3 | 17 |
| Glycine | 20.5 | 19.5 | 16.5 | 28.5 | 23.5 | 20.5 |

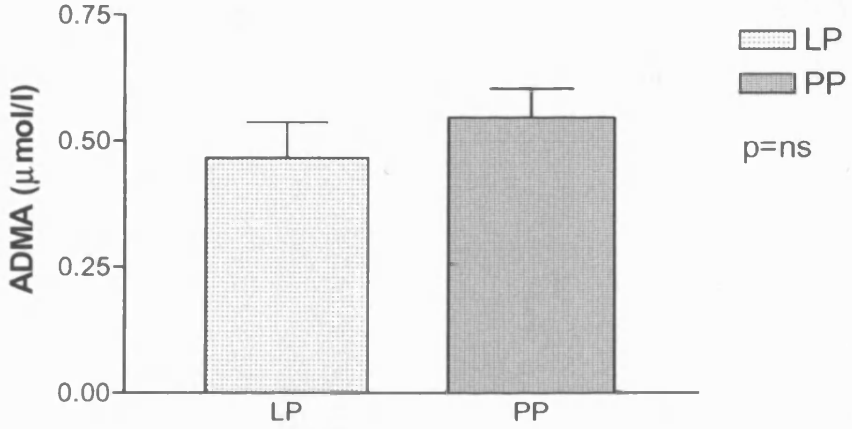
**Figure 6r: 24hr UNOx- control group (mean +s.e.m, n=15)**




### 6.4.4 ADMA

The measurement of plasma ADMA (µmol/l) on a basal blood sample was taken both in LP and PP. Within the control group basal plasma ADMA (µmol/l) was slightly higher in PP than LP, but this was not statistically significant.

**Figure 6s : ADMA (µmol/l) - control group (mean +s.e.m, n=11)**



# 7 Results for preeclamptics in late pregnancy (LP)/ postpartum (PP)

## 7.1 Effect of glycine and L-arginine on renal haemodynamics

7.1.1 GFR
7.1.2 ERPF
7.1.3 FF

## 7.2 Effect of glycine and L-arginine on haematocrit and electrolyte excretion

7.2.1 Haematocrit
7.2.2 Electrolyte excretion

## 7.3 Effect of glycine and L-arginine on the renal handling of macromolecules with biomathematical modelling

7.3.1 Fractional dextran clearance curves for preeclamptics in LP and PP
7.3.2 Summary of effect of glycine and L-arginine on fractional clearance curves in LP and PP
7.3.3 Biomathematical modelling
7.3.4 Effect of glycine and L-arginine on urinary albumin excretion

## 7.4 Effect of glycine and L-arginine on the NO system

7.4.1 L-arginine
7.4.2 cGMP
7.4.3 NOx
7.4.4 ADMA

## 7.0 Results for preeclamptics in late pregnancy (LP)/ postpartum (PP)

### 7.1 Effect of glycine and L-arginine on renal haemodynamics

#### 7.1.1 GFR

GFR was demonstrated to be significantly increased by 33% in LP compared to PP ($p=0.0082$) . Neither the glycine or L-arginine infusion produced any effect on GFR either in LP or PP.

**Figure 7a: The effect of glycine infusion on GFR (mean+s.e.m. n=6)**

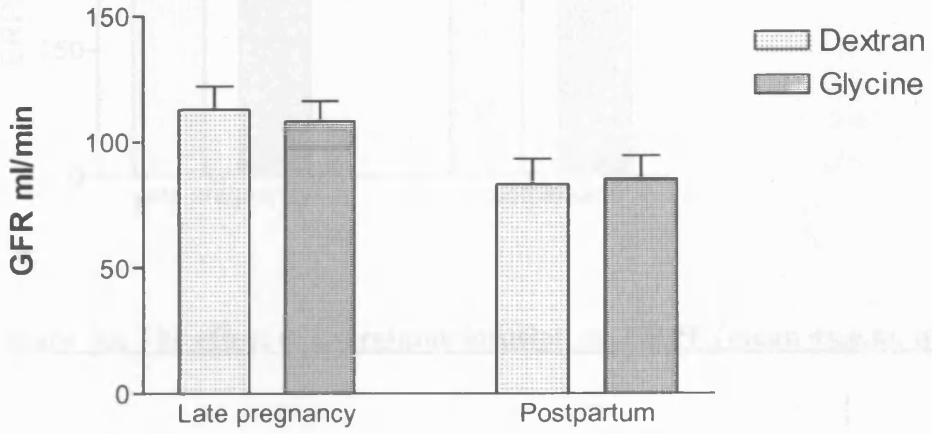
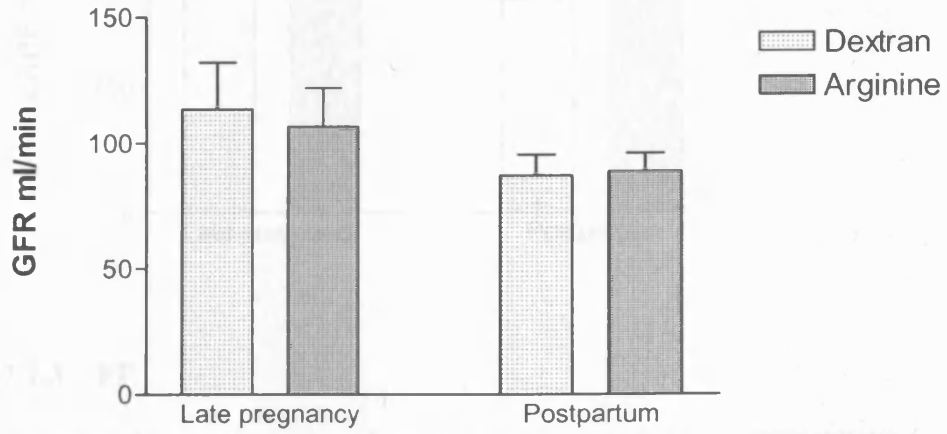



**Figure 7b: The effect of L-arginine infusion on GFR (mean+s.e.m. n=6)**



### 7.1.2 ERPF

ERPF was demonstrated to be significantly higher by 41% in LP than PP within this group (p=0.01). Neither the glycine or L-arginine infusion produced any effect on ERPF either in LP or PP.

**Figure 7c: The effect of glycine infusion on ERPF (mean +s.e.m, n=6)**

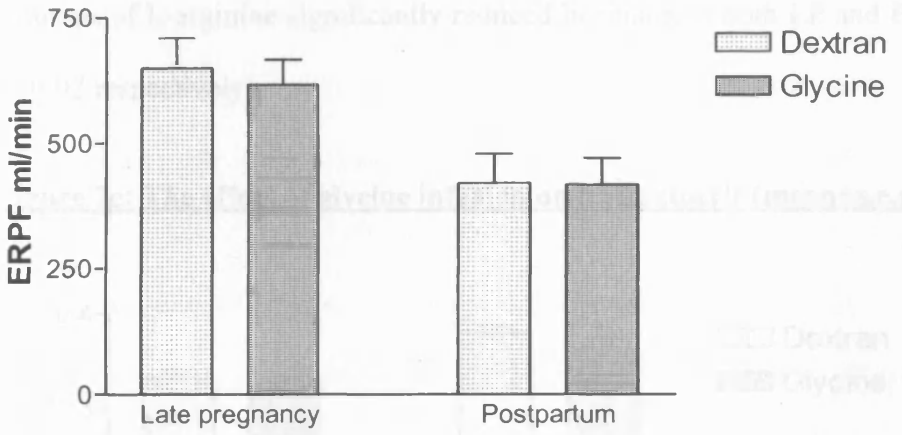
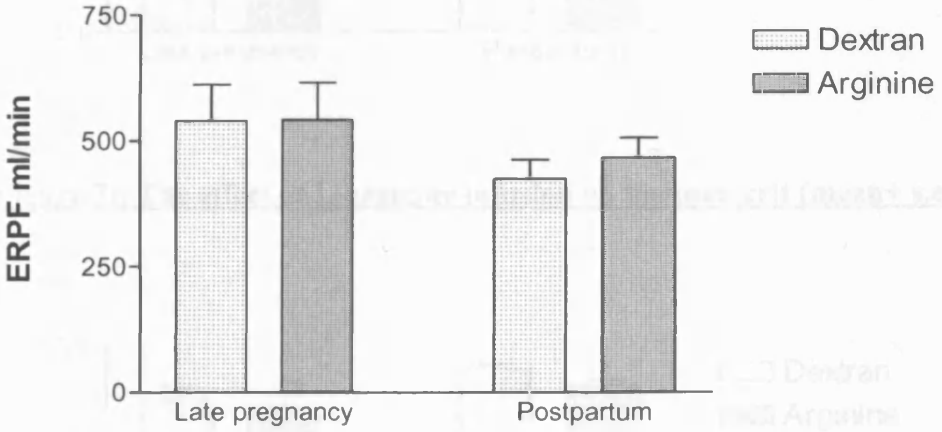



**Figure 7d: The effect of L-arginine infusion on ERPF (mean +s.e.m, n=6)**




### 7.1.3 FF

Filtration fraction is derived from both GFR and ERPF i.e GFR/ERPF

Neither glycine nor L-arginine produced any significant change in FF either LP or PP.

## 7.2 Effect of glycine and L-arginine on haematocrit and electrolyte excretion

### 7.2.1 Haematocrit

As expected, haematocrit was significantly lower in LP than PP in both the glycine and L-arginine groups (p=0.0087 and p=0.04 respectively).

Glycine infusion did not produce any significant effect on haematocrit in LP but did significantly reduce haematocrit PP (p=0.035).

Infusion of L-arginine significantly reduced haematocrit both LP and PP (p=0.0085 and p=0.02 respectively).

**Figure 7e: The effect of glycine infusion on haematocrit (mean+s.e.m, n=6)**

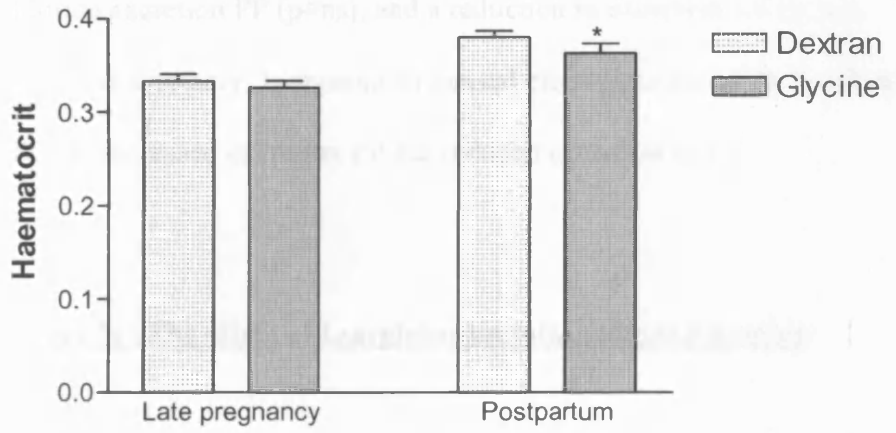
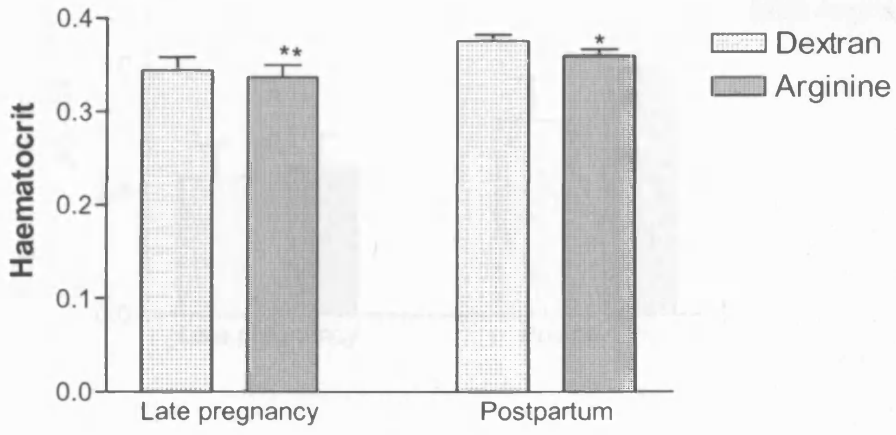



**Figure 7f: The effect of L-arginine infusion on haematocrit (mean+ s.e.m, n=6)**



### 7.2.2 Electrolyte excretion

**Total sodium excretion**

Total sodium excretion was lower in LP than PP in both groups (p=ns). Infusion of L-arginine increased sodium excretion in LP and PP but this did not reach statistical significance. Infusion with glycine resulted in a slight increase in sodium excretion when PP (p=ns), and a reduction in sodium excretion in LP (p=ns).

**Total chloride excretion**

Again, total chloride excretion was lower in LP than PP in both groups (p=ns). Infusion of L-arginine increased chloride excretion in both LP and PP, this was only statistically significant when PP (p=0.001). Infusion with glycine resulted in a slight increase in chloride excretion PP (p=ns), and a reduction in excretion LP (p=ns).

Hence, in summary, L-arginine increased electrolyte excretion in LP and PP and glycine slightly increased excretion PP but reduced excretion in LP.

**Figure 7g: The effect of L-arginine on total sodium excretion**

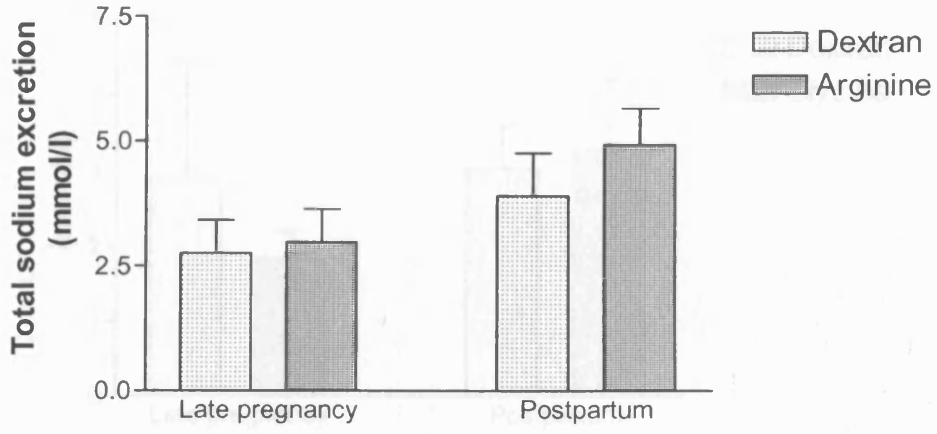


**Figure 7h: The effect of glycine on total sodium excretion**

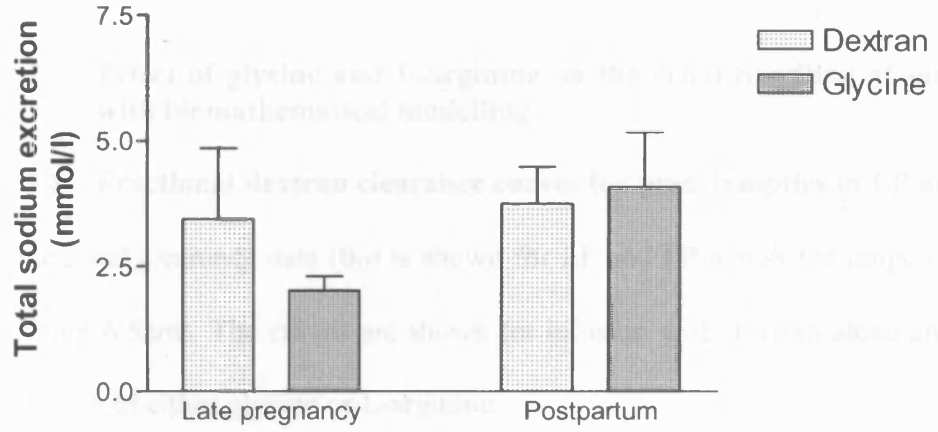



**Figure 7i: The effect of L-arginine infusion on total chloride excretion**

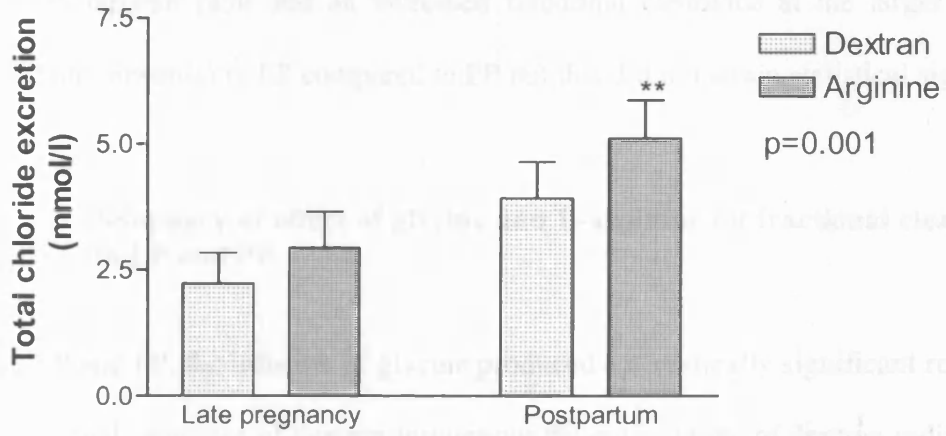
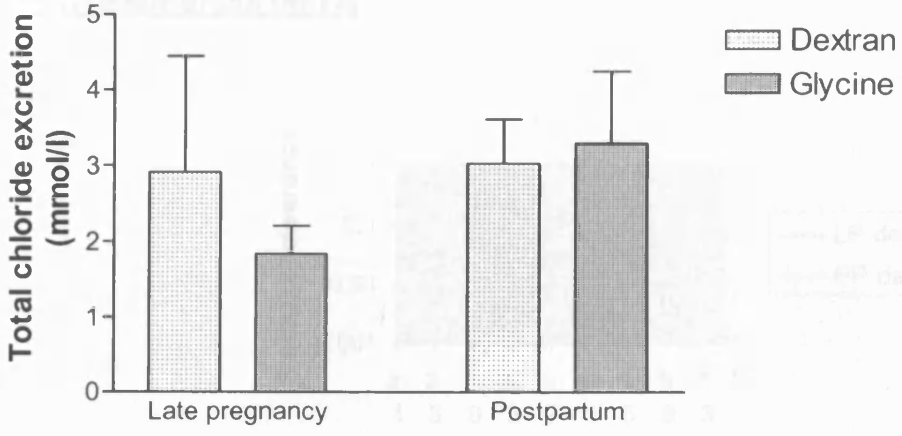



**Figure 7j: The effect of glycine infusion on total chloride excretion**



## 7.3 Effect of glycine and L-arginine on the renal handling of macromolecules with biomathematical modelling

### 7.3.1 Fractional dextran clearance curves for preeclamptics in LP and PP

Fractional clearance data ($\theta_D$) is shown for LP and PP across the range of dextran radii (3.1nm-6.5nm). The curves are shown for infusion with dextran alone and then with an infusion of either glycine or L-arginine.

The preeclamptic group demonstrated a slight reduction in fractional clearance at the lower dextran radii and an increased fractional clearance at the larger dextran radii (5.5nm onwards) in LP compared to PP but this did not attain statistical significance.

### 7.3.2 Summary of effect of glycine and L-arginine on fractional clearance curves in LP and PP

In LP and PP, the infusion of glycine produced a statistically significant reduction in the fractional clearance of dextran throughout the entire range of dextran radii (p=0.0051** and 0.002** respectively). The infusion of L-arginine did significantly reduce fractional clearance in LP (p=0.0001***) but not PP.

**Figure 7k: Comparison of fractional clearance curves in LP and PP in the preeclamptic group (n=12)**

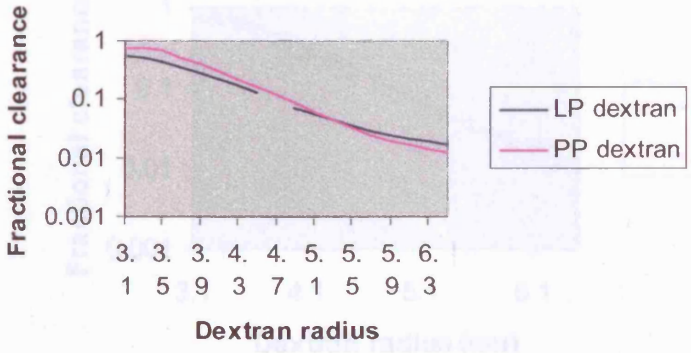
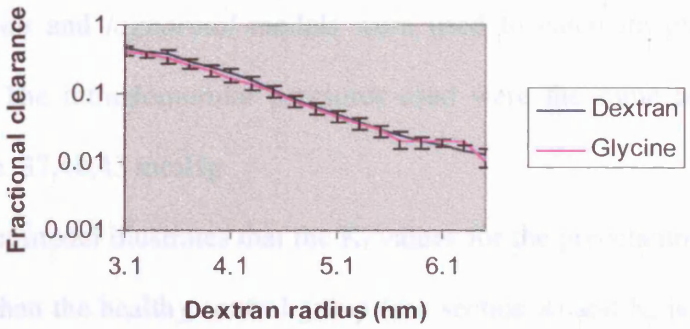
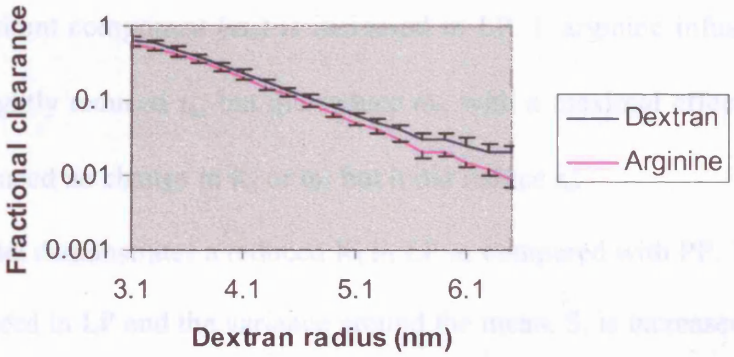


**Figure 7l: Effect of amino acids on fractional clearance in the preclamptic group**

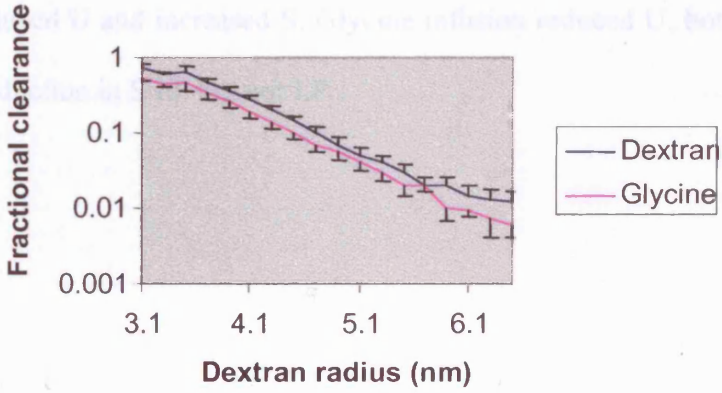
Late pregnancy-dextran vs glycine (p=0.0051)



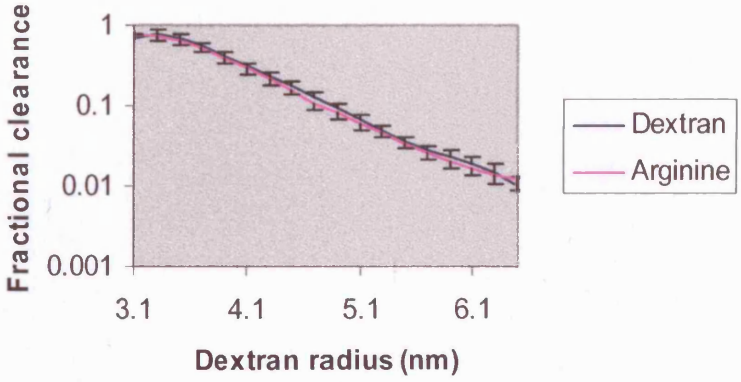

Late pregnancy-dextran vs L-arginine (p=0.0001)




Postpartum-dextran vs glycine (p=0.002)




Postpartum-dextran vs L-arginine (p=ns)



### 7.3.3 Biomathematical modelling

The *isoporous plus shunt* and *lognormal* models were used to calculate glomerular sieving characteristics. The intraglomerular pressures used were the same as for the healthy control group i.e. 37,40,43 mmHg.

The *isoporous plus shunt* model illustrates that the $K_f$ values for the preeclamptic group are significantly lower than the healthy control group (see section 8) and $K_f$ is lower in LP compared to PP within the PE group. Mean pore size ($r_o$) is reduced in LP compared to PP and the shunt component ($\omega_o$) is increased in LP. L-arginine infusion had little effect on $K_f$, slightly reduced $r_o$, but did reduce $\omega_o$, with a maximal effect in LP. Glycine infusion produced no change in $K_f$ or $\omega_o$ but it did reduce $r_o$.

Again, the *lognormal* model demonstrates a reduced $K_f$ in LP as compared with PP. The mean pore size, U, is reduced in LP and the variance around the mean, S, is increased in LP compared to PP. L-arginine infusion increased U in LP and reduced S but PP it had the opposite effect and reduced U and increased S. Glycine infusion reduced U, both in LP and PP, with a small reduction in S PP but not LP.

**Table 18 :The *Isoporous plus shunt* model in the preeclamptic group**

**Arginine ( n=6 LP, PP)** **Arginine** →

| LP/PP | ΔP | Kf | $r_o$ | $\varpi_o$ | $\chi^2$ | Kf | $r_o$ | $\varpi_o$ | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| **LP** | **37** | 6.8 | 63 | 0.01 | 0.5 | 6.7 | 62.9 | 0.007 | 0.4 |
| | **40** | 5.6 | 63.2 | 0.01 | 0.5 | 5.6 | 63 | 0.007 | 0.4 |
| | **43** | 4.7 | 63 | 0.01 | 0.6 | 4.8 | 63 | 0.008 | 0.5 |
| | | | | | | | | | |
| **PP** | **37** | 8.5 | 65 | 0.006 | 0.7 | 6.5 | 64.8 | 0.005 | 0.9 |
| | **40** | 6.6 | 65.1 | 0.006 | 0.9 | 5.0 | 64.4 | 0.005 | 0.9 |
| | **43** | 5.0 | 65.2 | 0.006 | 1.0 | 4.2 | 64.4 | 0.007 | 0.9 |
| | | | | | | | | | |

**Glycine (n=6 LP, n=5 PP)** **Glycine** →

| LP/PP | ΔP | $K_f$ | $r_o$ | $\omega_o$ | $\chi^2$ | Kf | $r_o$ | $\varpi_o$ | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| **LP** | **37** | 6.0 | 61.7 | 0.01 | 0.3 | 5.6 | 60.7 | 0.009 | 0.7 |
| | **40** | 5.1 | 61.7 | 0.01 | 0.3 | 3.7 | 60.8 | 0.01 | 0.7 |
| | **43** | 4.5 | 61.7 | 0.01 | 0.35 | 4.0 | 60.8 | 0.01 | 0.6 |
| | | | | | | | | | |
| **PP** | **37** | 9.3 | 63.1 | 0.004 | 0.8 | 8.5 | 61.4 | 0.003 | 0.8 |
| | **40** | 6.7 | 63 | 0.005 | 0.9 | 6.3 | 61.5 | 0.003 | 0.8 |
| | **43** | 5.3 | 63.1 | 0.006 | 1.0 | 5.1 | 61.6 | 0.004 | 0.9 |
| | | | | | | | | | |

**Table 19 :The *Lognormal* model in the preeclamptic group**

**Arginine** **Arginine** →

| LP/PP | ΔP | Kf | U | S | $\chi^2$ | Kf | U | S | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| **LP** | **37** | 6.8 | 53 | 1.22 | 0.7 | 6.7 | 54.7 | 1.18 | 0.4 |
| | **40** | 5.6 | 53.4 | 1.21 | 0.75 | 5.6 | 55 | 1.18 | 0.5 |
| | **43** | 4.7 | 53.6 | 1.22 | 0.8 | 4.8 | 55.1 | 1.15 | 0.5 |
| | | | | | | | | | |
| **PP** | **37** | 8.5 | 61.1 | 1.12 | 1.0 | 6.5 | 59 | 1.14 | 1.0 |
| | **40** | 6.6 | 61.3 | 1.12 | 1.2 | 5.0 | 59.3 | 1.14 | 1.1 |
| | **43** | 5.0 | 61.4 | 1.12 | 1.3 | 4.2 | 59.3 | 1.14 | 1.2 |
| | | | | | | | | | |

**Glycine** **Glycine** →

| LP/PP | ΔP | Kf | U | S | $\chi^2$ | Kf | U | S | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | |
| **LP** | **37** | 6 | 51.2 | 1.21 | 0.4 | 5.6 | 49.8 | 1.2 | 0.4 |
| | **40** | 5.1 | 51.4 | 1.21 | 0.6 | 4.7 | 50.1 | 1.2 | 0.4 |
| | **43** | 4.5 | 51.5 | 1.21 | 0.65 | 4.0 | 50.3 | 1.21 | 0.45 |
| | | | | | | | | | |
| **PP** | **37** | 9.3 | 57.1 | 1.15 | 1.1 | 8.5 | 53.4 | 1.16 | 0.5 |
| | **40** | 6.7 | 57.4 | 1.15 | 1.3 | 6.3 | 53.8 | 1.16 | 0.6 |
| | **43** | 5.3 | 57.5 | 1.15 | 1.4 | 5.0 | 53.9 | 1.16 | 0.7 |
| | | | | | | | | | |

### 7.3.4 Effect of glycine and L-arginine on urinary albumin excretion

Microalbumin excretion (μg/min) was higher in LP than PP within the preeclamptic group as you would expect but this did not achieve statistical significance. The infusion of L-arginine slightly reduced excretion in LP but slightly increased excretion PP (p=ns). Glycine infusion slightly reduced excretion in both LP and PP but again these results were not significant. In summary, L-arginine and glycine had very little effect on microalbumin excretion.

**Table 20 : Microalbumin excretion (μg/min)- LP vs PP (mean +/- s.e.m.)**

| | Late pregnancy | Postpartum |
|---|---|---|
| **Mean** | 1146 | 70.97 |
| **S.e.m.** | 499.8 | 57.3 |

**Table 21: Microalbumin excretion (μg/min)- summary of results in preeclamptics (mean +/- s.e.m)**

| | Dextran | L-arginine | Dextran | Glycine |
|---|---|---|---|---|
| **Late pregnancy** | 484.2 +/- 312.6 | 271.4 +/- 176 | 1807 +/- 909.2 | 1746 +/- 892.8 |
| **Postpartum** | 115.1 +/- 105.7 | 137.2 +/- 129 | 18.07 +/- 2.79 | 11.99 +/- 2.84 |

## 7.4 Effect of glycine and L-arginine on the NO system

### 7.4.1 L-arginine

The measurement of L-Arg concentration (μmol/l) on a basal sample was taken both in LP and PP, these measurements were repeated following the 2 hour infusion of either L-arginine or glycine. Basal measurements revealed that L-Arg concentration was significantly lower in LP than PP (p=0.0013) within both preeclamptic groups.

The infusion of glycine did not produce any alteration in the L-Arg concentration either LP or PP. The L-arginine infusion, however, significantly increased plasma L-Arg concentrations both in LP and PP (p=0.01 and p=0.0009 respectively).

**Figure 7m: L-Arg concentration (μmol/l) - LP vs PP ( mean + s.e.m.)**

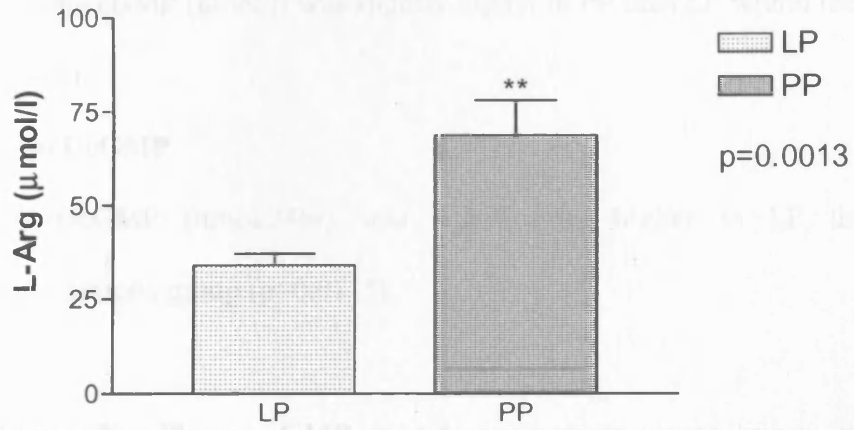
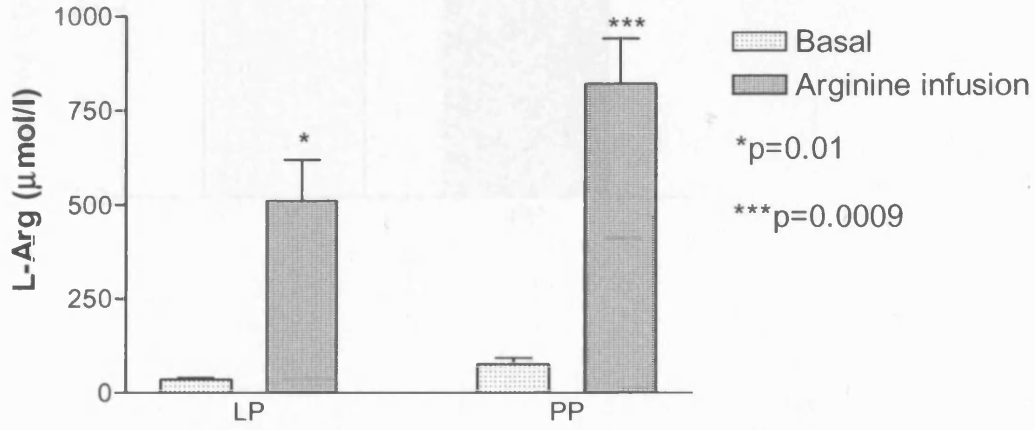



**Figure 7n: L-Arg concentration(μmol/l) - the effect of L-arginine infusion (mean+s.e.m)**



### 7.4.2 cGMP

The measurement of plasma cGMP (nmol/l) on a basal blood sample was taken both in LP and PP together with a measurement of 24-hour urinary cGMP (24hrUcGMP) (nmol/24hr).

**Plasma cGMP**

Plasma cGMP (nmol/l) was slightly higher in PP than LP within the preeclamptic group (p=ns).

**24hrUcGMP**

24hrUcGMP (nmol/24hr) was significantly higher in LP than PP within the preeclamptic group (p=0.011*).

**Figure 7o : Plasma cGMP- preeclamptic group (mean +s.e.m., n=11)**

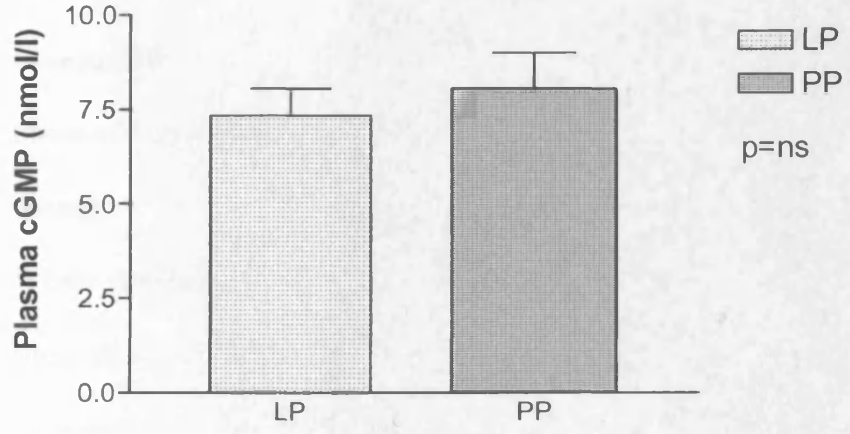
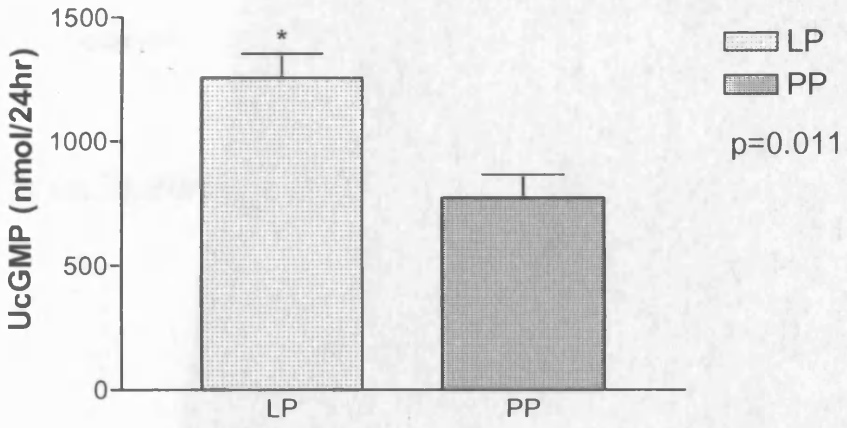


**Figure 7p : 24hrUcGMP- preeclamptic group (mean + s.e.m., n=11)**




### 7.4.3 NOx

The measurement of plasma NOx (µmol/l) on a basal blood sample was taken both in LP and PP together with a measurement of 24 hour urinary NOx (24hrUNOx) (µmol/24hr).

**Plasma NOx**

Plasma NOx (µmol/l) was slightly higher in LP than PP within the preeclamptic group (p=ns).

When comparing how infusions of glycine and L-arginine affected Plasma NOx (µmol/l) a selection of 3 patients in each group were analysed. In both groups the combination of dextran followed by either amino acid resulted in a lowering of plasma NOx, these results are not considered statistically significant due to the small numbers looked at.

**24hr UNOx**

24hr UNOx (µmol/24hr) was slightly higher in LP than PP within the preeclamptic group (p=ns)

When comparing how infusions of L-arginine and glycine affected UNOx (µmol/l), a selection of 3 patients from each group were analysed. This demonstrated that infusion

of L-arginine had no effect on UNOx in LP but increased it slightly PP, whereby glycine reduced UNOx in LP and increased it PP. These results are not considered significant due to the small numbers involved.

**Figure 7q: Plasma NOx- preeclamptic group (mean +s.e.m., n=11)**

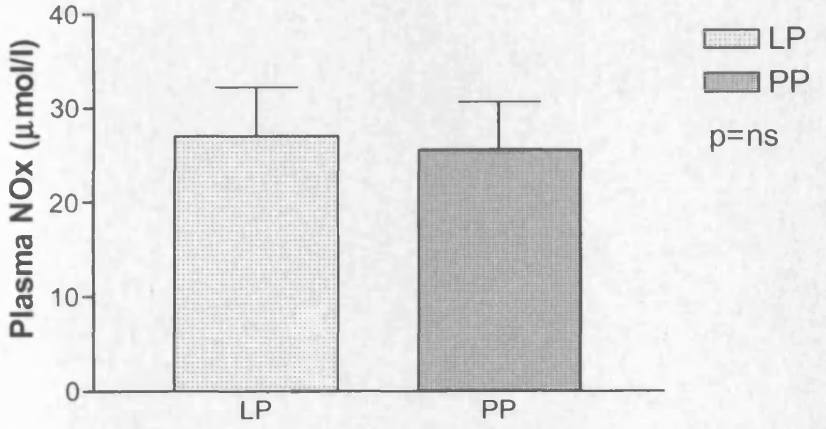
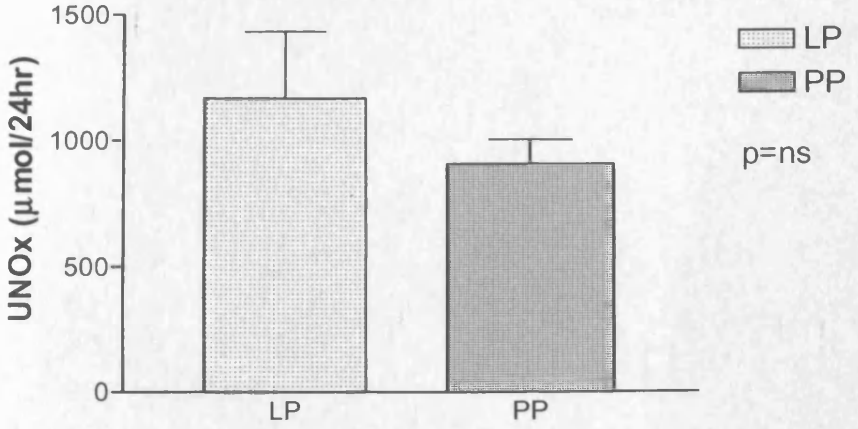



**Table 22: Plasma NOx (µmol/l) during amino acid infusion (n=3 in each group)**

| | LP | | | PP | | |
|---|---|---|---|---|---|---|
| | **P0** | **P3** | **P6** | **P0** | **P3** | **P6** |
| Arginine | 20.6 | 20.7 | 19 | 25.7 | 23.7 | 22 |
| Glycine | 44.7 | 40 | 34.3 | 26.7 | 22 | 17.3 |

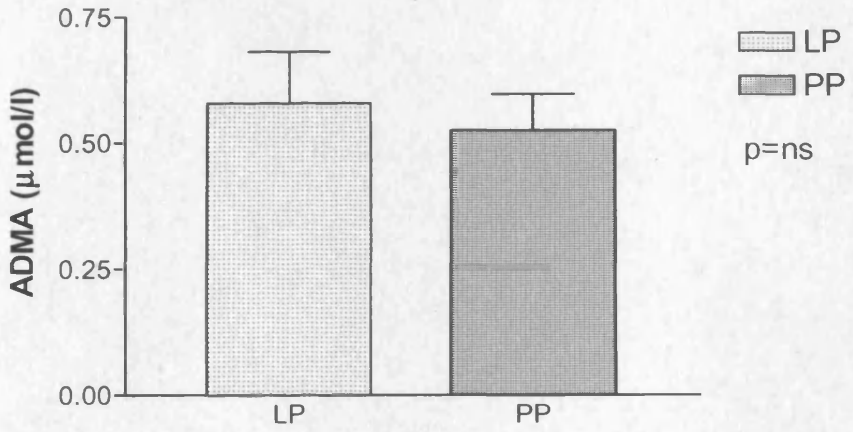
**Figure 7r: 24hr UNOx- preeclamptics (mean +s.e.m., n=11)**



### 7.4.4 ADMA

The measurement of plasma ADMA (µmol/l) on a basal blood sample was taken both in LP and PP. Within the preeclamptic group, basal plasma ADMA (µmol/l) was slightly higher in LP than PP, but this was not statistically significant.

**Figure 7s: ADMA (µmol/l) - preeclamptic group (mean +s.e.m., n=11)**



# 8 Comparison of healthy controls vs preeclamptics

## 8.1 Renal haemodynamics

8.1.1 GFR
8.1.2 ERPF
8.1.3 FF

## 8.2 Haematocrit and electrolyte excretion

## 8.3 Renal handling of macromolecules with biomathematical modelling

8.3.1 The ultrafiltration coefficient ($K_f$)
8.3.2 Comparing fractional clearance curves for controls and preeclamptics
8.3.3 Biomathematical modelling
8.3.4 Urinary albumin excretion

## 8.4 The NO system

8.4.1 Amino acids
8.4.2 cGMP
8.4.3 NOx
8.4.4 ADMA

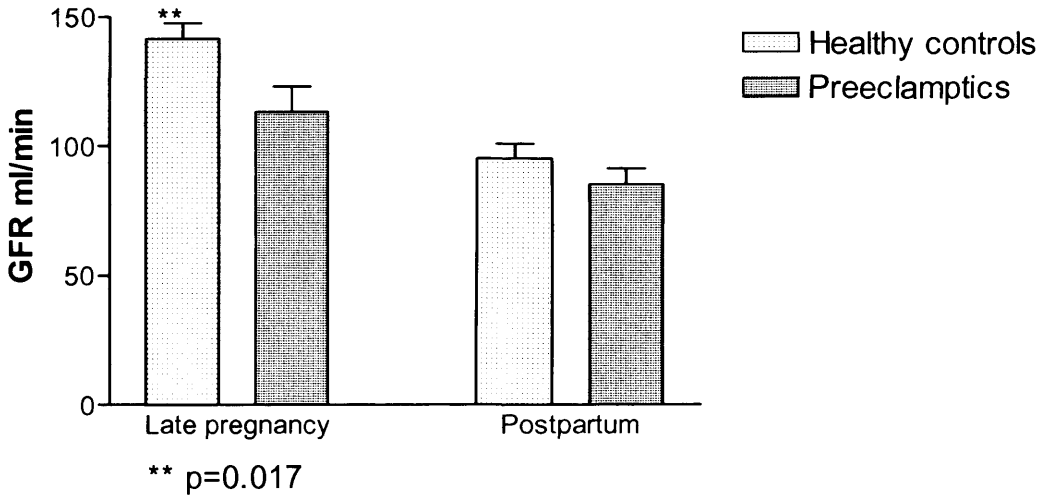
# 8.0 Comparison of healthy controls vs preeclamptics

## 8.1 Renal Haemodynamics

### 8.1.1 GFR

In LP there was a 25% increase in GFR (p=0.017) in the control group compared to the preeclamptic group. There was no significant difference between the two groups when PP.

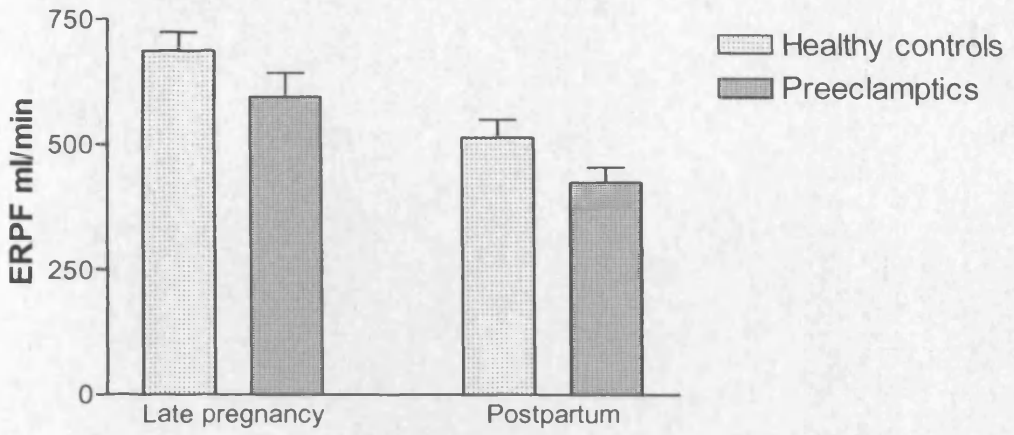
**Figure 8a: Comparison of GFR in controls vs preeclamptics (mean + s.e.m)**




### 8.1.2 ERPF

Although there was a 15% increase in the LP ERPF in controls compared to the preeclamptic group this did not reach statistical significance. There was a significant 21% increase in the PP ERPF in controls compared to the preeclamptic group (p=0.0173).

**Figure 8b: Comparison of ERPF in controls vs preclamptics (mean + s.e.m.)**




### 8.1.3 FF

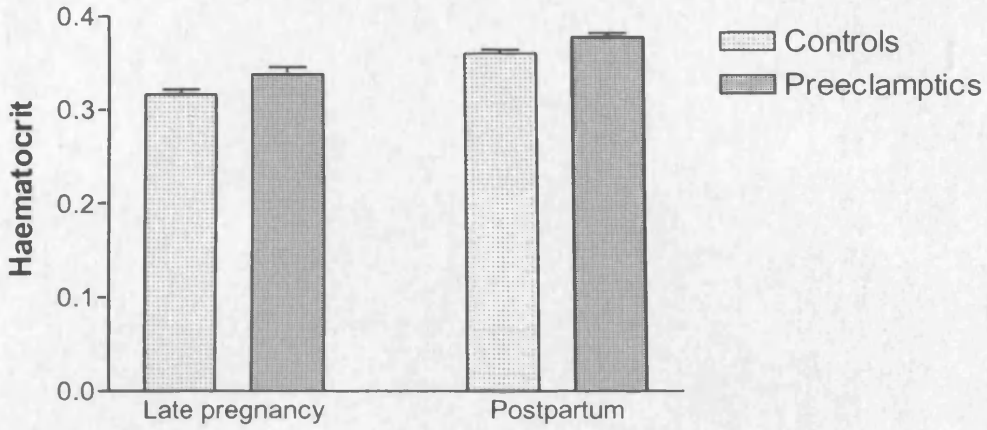
Filtration fraction was increased by 8.3% in the control group as compared to the preeclamptic group LP but this did not achieve statistical significance. There was no difference between the 2 groups PP.

## 8.2 Haematocrit and electrolyte excretion

### Haematocrit

Although the preeclamptic group demonstrated higher haematocrit levels than the control group in both LP and PP, these results were not statistically significant.

**Figure 8c: Comparison of haematocrit in controls vs preeclamptics (mean +s.e.m)**




**Electrolyte excretion**

In LP, the preeclamptic group demonstrated higher total sodium and chloride excretion than the control group but these results did not attain statistical significance. PP, again the preeclamptic group demonstrated higher total sodium and chloride excretion but only the sodium excretion reached statistical significance (p=0.0097).

**Table 23 :Total sodium and chloride excretion (mmol/l)- controls vs preeclamptics (mean +/-s.e.m)**

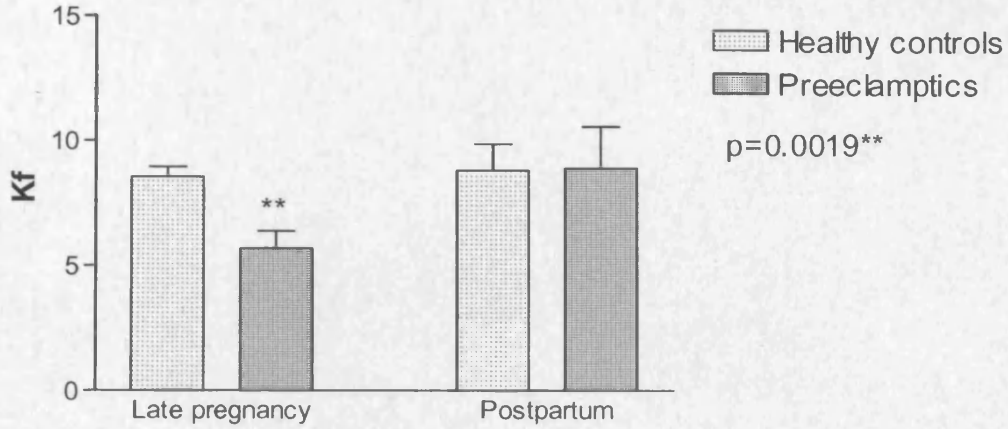
| | Controls | Preeclamptics |
|---|---|---|
| LP sodium | 2.51 +/-0.17 | 3.1 +/- 0.7 |
| **LP chloride** | 2.17 +/- 0.33 | 2.57 +/- 0.74 |
| **PP sodium** | 2.72 +/- 0.08 | 3.82 +/- 0.55 |
| **PP chloride** | 2.53 +/- 0.3 | 3.48 +/- 0.47 |

## 8.3 Renal handling of macromolecules with biomathematical modelling

### 8.3.1 The ultrafiltration coefficient ($K_f$)

The preeclamptic group had a significantly reduced $K_f$ as compared to the controls in LP (p=0.0019). However, there was no significant difference when PP.

**Figure 8d: Comparison of $K_f$ in controls vs preeclamptics**

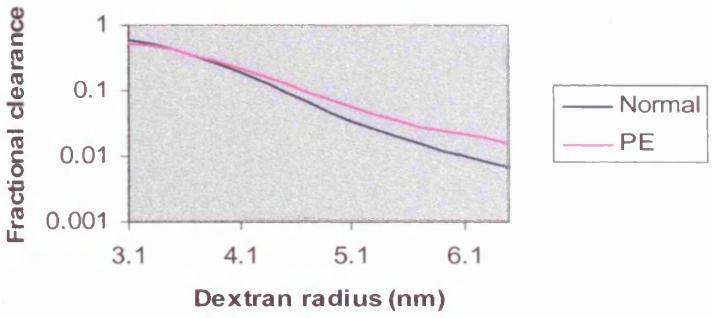



### 8.3.2 Comparing fractional clearance curves for controls and preeclamptics

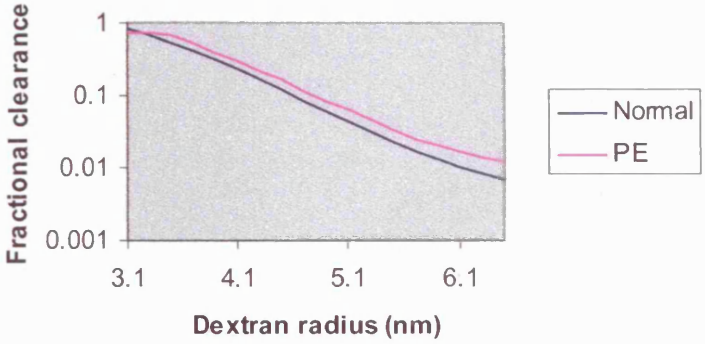
Dextran fractional clearance curves are plotted for the preeclamptic group and the control group over the range of dextran radii 3.1-6.5nm. From the curves it is evident that in the preeclamptic group in LP fractional clearance of dextran is increased compared to the control group, but only attaining statistical significance for the dextran radii 5.7-6.5nm (p=0.02*). In PP, the preeclamptic group also demonstrated increased fractional clearance but this was only significant at the radii 6.3-6.5nm (p=0.04*). The comparison curves between the control and preeclamptic groups are demonstrated in the figures below followed by graphs for the glycine and L-arginine subgroups.

**Figure 8e: Comparison of fractional clearance curves- controls vs preeclamptics**

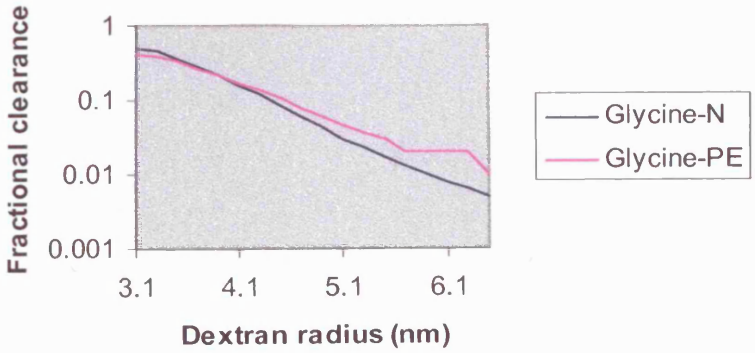
Late pregnancy



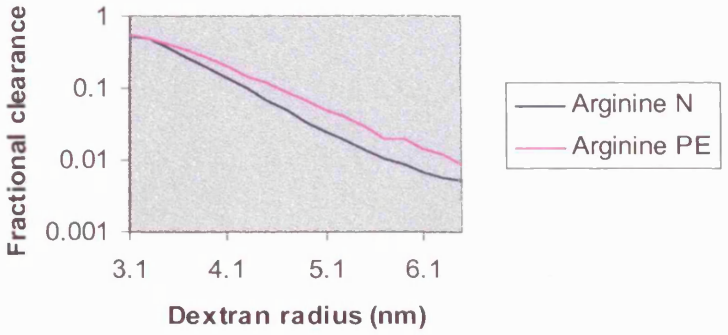

Postpartum



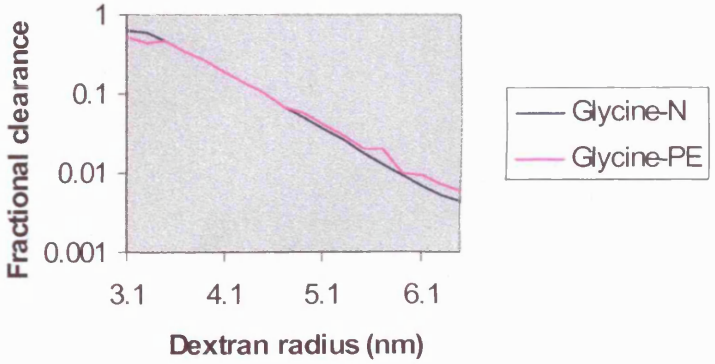

Late pregnancy-glycine



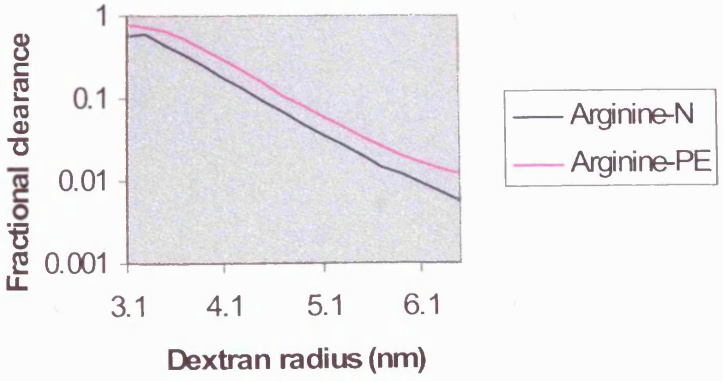
Late pregnancy-
L-arginine




Postpartum-
Glycine




Postpartum-L-arginine



### 8.3.3 Biomathematical modelling

The comparison of $K_f$ between the 2 groups has already been discussed in section 8. With respect to the other modelled parameters, the *isoporous plus shunt* model predicted significant increases in mean pore size ($r_o$) and shunt component ($\omega_o$) in preeclamptic pregnancy, changes that were also seen when comparing the groups PP but magnitudes were not as great. Infusion with L-arginine and glycine had similar effects within the 2 groups LP and PP.

With respect to the *lognormal* model there was very little difference between the 2 groups in U or S in LP. For the preeclamptic group PP, there were higher values for U. If the groups were further split to account for glycine and L-arginine infusions, then in LP the L-arginine preeclamptic group had higher values for U, but the glycine group had lower values for U. The preeclamptic glycine group also had higher values for S in LP when compared to their control group. In general, the effects of L-arginine and glycine infusion were similar between the 2 groups studied.

**Table 24 :*Isoporous plus shunt*- comparison of controls vs preeclamptics**

ΔP=37mmHg

Arginine →

| LP/PP | $K_f$ | $r_o$ | $\omega_o$ | $\chi^2$ | $K_f$ | $r_o$ | $\omega_o$ | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| **LP-control** | 9.7 | 59 | 0.006 | 0.7 | 9.9 | 58.5 | 0.003 | 1.1 |
| **LP-PE** | 6.8 | 63 | 0.01 | 0.5 | 6.7 | 62.9 | 0.007 | 0.4 |
| | | | | | | | | |
| **PP-control** | 9.7 | 62.6 | 0.002 | 1.1 | 8.8 | 60.9 | 0.003 | 0.7 |
| **PP-PE** | 8.5 | 65 | 0.006 | 0.7 | 6.5 | 64.8 | 0.005 | 0.9 |
| | | | | | | | | |

Glycine →

| LP/PP | $K_f$ | $r_o$ | $\omega_o$ | $\chi^2$ | $K_f$ | $r_o$ | $\omega_o$ | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| **LP-control** | 7.5 | 61.7 | 0.004 | 0.8 | 10.3 | 60.2 | 0.004 | 0.8 |
| **LP-PE** | 6.0 | 61.7 | 0.01 | 0.3 | 5.6 | 60.7 | 0.009 | 0.7 |
| | | | | | | | | |
| **PP-control** | 9.2 | 62.5 | 0.003 | 0.8 | 8.7 | 61.3 | 0.002 | 0.9 |
| **PP-PE** | 9.3 | 63.1 | 0.004 | 0.8 | 8.5 | 61.4 | 0.003 | 0.8 |
| | | | | | | | | |

**Table 25 : *Lognormal*- comparisons of controls vs preeclamptics**

Arginine →

| LP/PP | $K_f$ | U | S | $\chi^2$ | $K_f$ | U | S | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| **LP-control** | 9.7 | 47 | 1.22 | 1.3 | 9.9 | 47.8 | 1.19 | 0.9 |
| **LP-PE** | 6.8 | 53 | 1.22 | 0.7 | 6.7 | 54.7 | 1.18 | 0.4 |
| | | | | | | | | |
| **PP-control** | 9.7 | 55.6 | 1.15 | 0.9 | 8.7 | 51.9 | 1.18 | 0.7 |
| **PP-PE** | 8.5 | 61.1 | 1.12 | 1.0 | 6.5 | 59 | 1.14 | 1.0 |
| | | | | | | | | |

Glycine →

| LP/PP | $K_f$ | U | S | $\chi^2$ | $K_f$ | U | S | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| **LP-control** | 7.5 | 55.1 | 1.14 | 1.0 | 10.3 | 51.4 | 1.17 | 0.8 |
| **LP-PE** | 6 | 51.2 | 1.21 | 0.4 | 5.6 | 49.8 | 1.2 | 0.4 |
| | | | | | | | | |
| **PP-control** | 9.2 | 56.8 | 1.13 | 1.0 | 8.7 | 54.1 | 1.15 | 0.7 |
| **PP-PE** | 9.3 | 57.1 | 1.15 | 1.1 | 8.5 | 53.4 | 1.16 | 0.5 |
| | | | | | | | | |

### 8.3.4 Urinary albumin excretion

The preeclamptic group demonstrated higher microalbumin excretion than the control group in both LP and PP. These results did not attain statistical significance.

**Table 26: Microalbumin excretion (μg/min)- summary of comparison between controls and preeclamptics (mean +/-s.e.m))**

| | Controls | Preeclamptics |
|---|---|---|
| **Late pregnancy** | 5.32 +/- 0.32 | 1146 +/- 661.4 |
| **Postpartum** | 34.8 +/- 8 | 66.6 +/- 48.5 |

## 8.4 The NO system

### 8.4.1 Amino acids

As previously described in sections 6.41 and 7.41 basal plasma L-Arg concentrations (Um) were measured both in LP and PP and L-arginine infusion significantly increased L-Arg concentration in both the controls and preeclamptics. However, there was no significant difference between the basal measurements for L-Arg when comparing the controls with the preeclamptics either in LP or PP.

### 8.4.2 CGMP

**Plasma cGMP**

The preeclamptic group demonstrated higher levels of plasma cGMP in both LP and PP compared to the control group. These results were not statistically significant.

**24hrUcGMP**

The preeclamptic group demonstrated lower levels of 24hrUcGMP in LP and PP compared to the control group. Again, these results were not statistically significant.

**Figure 8f: Comparison of plasma cGMP- controls vs preeclamptics (mean +s.e.m)**

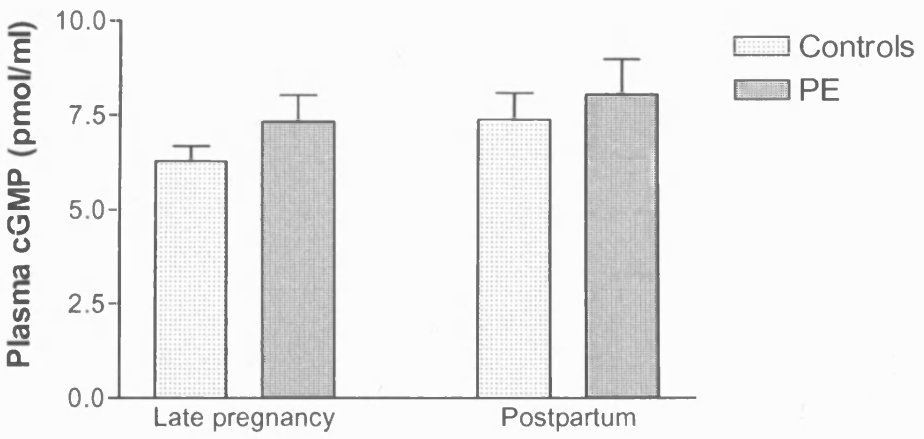
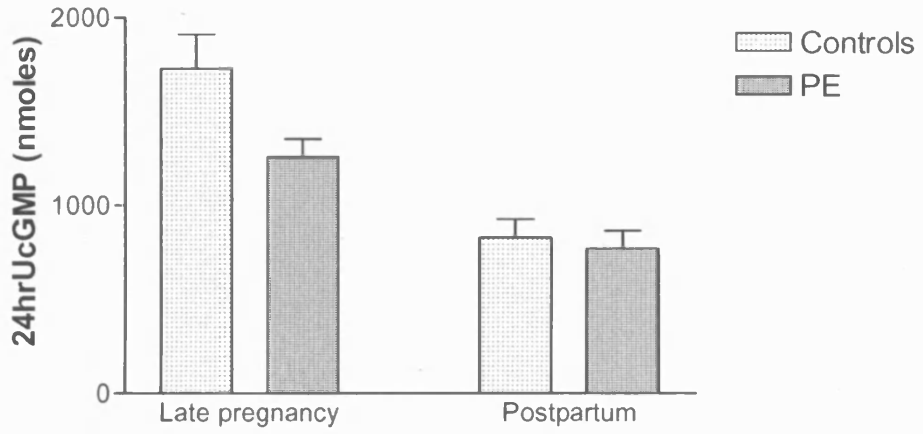


**Figure 8g : Comparison of 24hrUcGMP- controls vs preeclamptics (mean +s.e.m)**




### 8.4.3 NOx

**Plasma NOx**

The preeclamptic group demonstrated higher levels of plasma NOx in both LP and PP compared to the control group. This finding was only statistically significant in LP ($p=0.02$).

**24hr UNOx**

Again, the preeclamptic group demonstrated higher levels of 24hr UNOx in LP and PP compared to the control group. These results were not statistically significant.

**Figure 8h: Comparison of plasma NOx- controls vs preeclamptics (mean +s.e.m)**

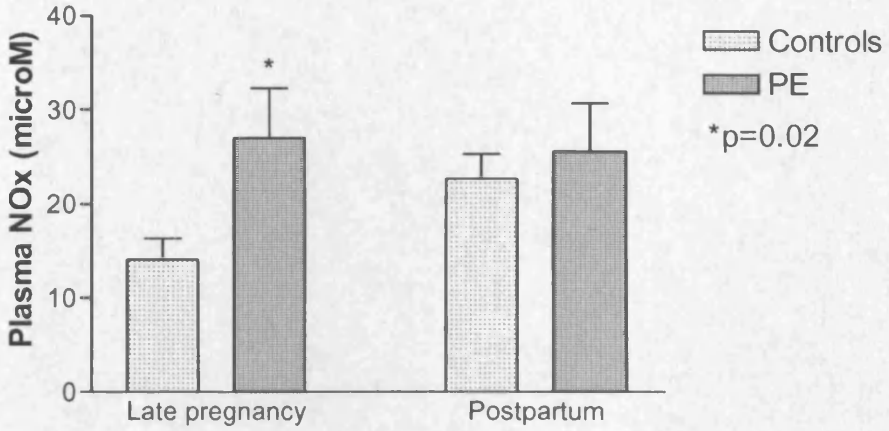
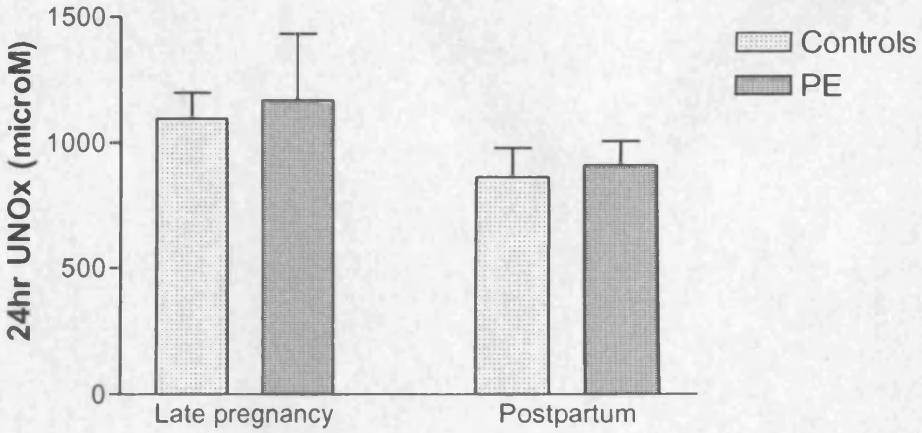


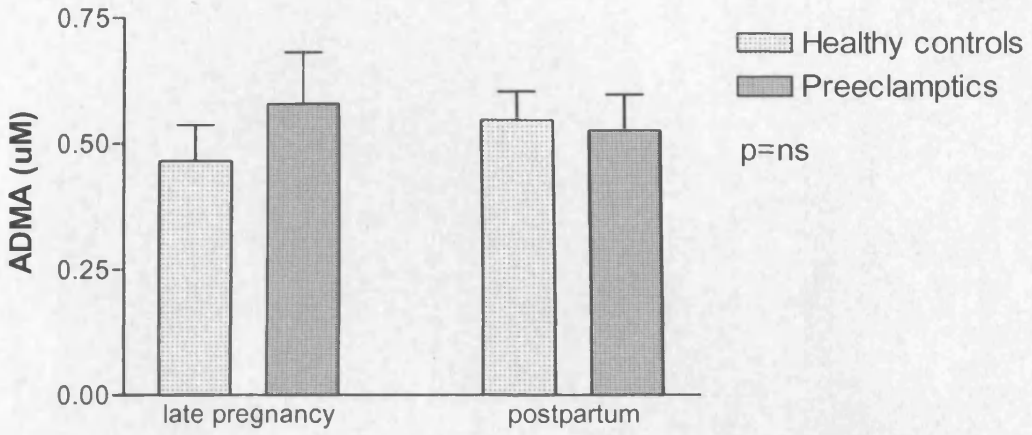

**Figure 8i: Comparison of 24hr UNOx- controls vs preeclamptics (mean +s.e.m)**




### 8.4.4 ADMA

The preeclamptic group demonstrated slightly higher levels of plasma ADMA (uM) in LP and slightly lower levels in PP when compared to the control group. These results were not statistically significant.

**Figure 8j: Comparison of plasma ADMA- controls vs preeclamptics (mean +s.e.m)**



# 9 Further studies

## 9.1 Pilot study: The effect of high dose L-arginine on renal haemodynamics and biomathematical modelling in pregnant and non-pregnant subjects

9.1.1 Renal haemodynamics, haematocrit and electrolyte excretion
9.1.2 Fractional clearance curves
9.1.3 Biomathematical modelling
9.1.4 Urinary albumin excretion

## 9.2 Pilot vitamin study: The effect of vitamins C and E in pregnant and non-pregnant healthy controls

9.2.1 Renal haemodynamics, haematocrit and electrolyte excretion
9.2.2 Fractional clearance curves
9.2.3 Biomathematical modelling
9.2.4 Urinary albumin excretion

# 9 Further studies

## 9.1 Pilot study: the effect of high dose L-arginine on renal haemodynamics and biomathematical modelling in pregnant and non-pregnant subjects

This study had the same design and infusion protocol as the main study. However, in order to see whether the dose of L-arginine we had been using in the main study was too small to induce any effect on renal parameters, we doubled the dose of L-arginine given i.e. 24g over a 2-hour period instead of 12g and studied the same effects. 3 of the subjects were pregnant at the time of study and one was not pregnant.

### 9.1.1 Renal haemodynamics, haematocrit and electrolyte excretion

This table demonstrates the renal haemodynamic changes observed with high dose L-arginine infusion. The changes are minimal and were not statistically significant.

**Table 27: The effect of high dose L-arginine on GFR, ERPF, FF, haematocrit (mean +/- s.e.m, n=4)**

| | GFR | ERPF | FF | Haematocrit |
|---|---|---|---|---|
| Dextran | 127.3 +/- 24.1 | 616.4 +/- 96.8 | 20.5 +/- 1.4 | 0.341 +/- 0.01 |
| High dose L-arginine | 123.5 +/- 14.6 | 632.7 +/- 37 | 19.3 +/- 1.1 | 0.327 +/- 0.01 |

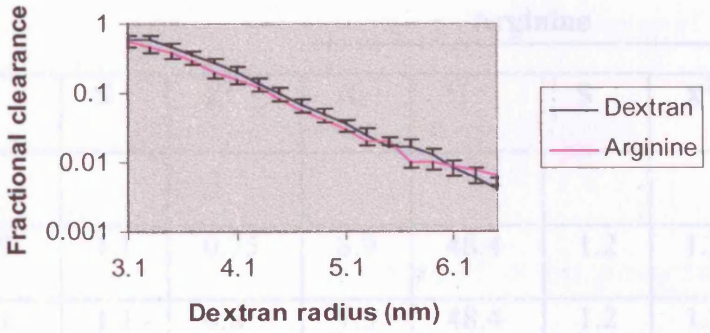
**Electrolyte excretion**

The total excretion of sodium and chloride were increased with the infusion of high dose L-arginine. Total sodium excretion increased by 3.1 mmol/l to 6.2 mmol/l which was not statistically significant but total chloride excretion increased from 2.84mmol/l to 9mmol/l which did reach statistical significance (p=0.0088**).

### 9.1.2 Fractional clearance curves

Dextran fractional clearance curves were plotted for the dextran radii 3.1-6.5nm. The high dose L-arginine significantly reduced the fractional clearance of dextran across the entire range studied (p=0.0056).

**Figure 9a: Fractional clearance curves**




This finding differed from the control group who received the standard dose of L-arginine. Within this group, reduced fractional clearance in LP was only demonstrated to be significant in the radii range 5.3-6.5nm.

### 9.1.3 Biomathematical modelling

The tables below illustrate the effect of high dose L-arginine on the modelling parameters. With respect to the *lognormal* model, it is predicted that high dose L-arginine will reduce mean pore size U but increase the variance about the mean, S. The *isoporous plus shunt* model predicts that high dose L-arginine will increase the shunt component ($\omega_o$) but decrease the pore size ($r_o$). Both models predict no alteration in $K_f$. The predictions for this strength of L-arginine are slightly different to those seen in the control group described previously, here the changes were minimal but included predictions of reduced $r_o$ and $\omega_o$ both LP and PP, increased U and reduced S when LP but reduced U and increased S when PP.

**Table 28 : *Lognormal***

| | | | | | Arginine → | | | |
|---|---|---|---|---|---|---|---|---|
| **ΔP** | **$K_f$** | **U** | **S** | **$X^2$** | **$K_f$** | **U** | **S** | **$X^2$** |
| | | | | | | | | |
| **37** | 8.6 | 56.9 | 1.1 | 0.75 | 8.9 | 48.4 | 1.2 | 1.1 |
| **40** | 7.2 | 57.1 | 1.1 | 0.8 | 7.5 | 48.4 | 1.2 | 1.2 |
| **43** | 6.4 | 57 | 1.1 | 0.9 | 6.7 | 48.4 | 1.2 | 1.3 |

**Table 29: *Isoporous plus shunt***

| | | | | | Arginine → | | | |
|---|---|---|---|---|---|---|---|---|
| **ΔP** | **$K_f$** | **$r_o$** | **$\omega_o$** | **$X^2$** | **$K_f$** | **$r_o$** | **$\omega_o$** | **$X^2$** |
| | | | | | | | | |
| **37** | 8.6 | 61.2 | 0.002 | 1.1 | 8.9 | 59.2 | 0.005 | 0.7 |
| **40** | 7.2 | 61.3 | 0.002 | 1.2 | 7.4 | 59.2 | 0.005 | 0.8 |
| **43** | 6.4 | 61.2 | 0.002 | 1.3 | 6.7 | 59.2 | 0.005 | 0.8 |

### 9.1.4 Urinary albumin excretion

Infusion of high dose L-arginine produced a slight, non-significant reduction in microalbumin excretion (4.35 to 3.085μg/min)

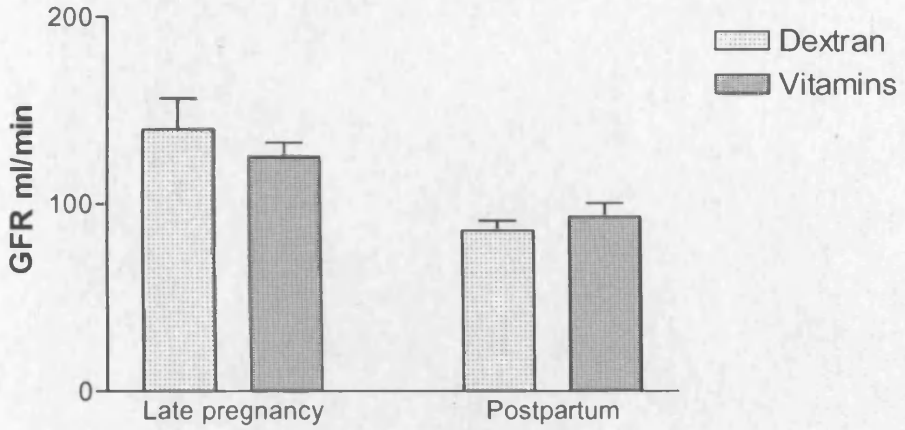
## 9.2 Pilot vitamin study: The effect of vitamins C and E in pregnant and non-pregnant healthy controls

### 9.2.1 Renal haemodynamics, haematocrit and electrolyte excretion

Glomerular filtration rate (GFR)

Although GFR was increased by 63% in LP compared to PP in this group, this was not statistically significant possibly due to the small numbers in this study (n=4). The infusion of vitamin C and oral vitamin E did not produce any alteration in GFR.

**Figure 9b: The effect of vitamins on GFR (mean + s.e.m. n=4)**

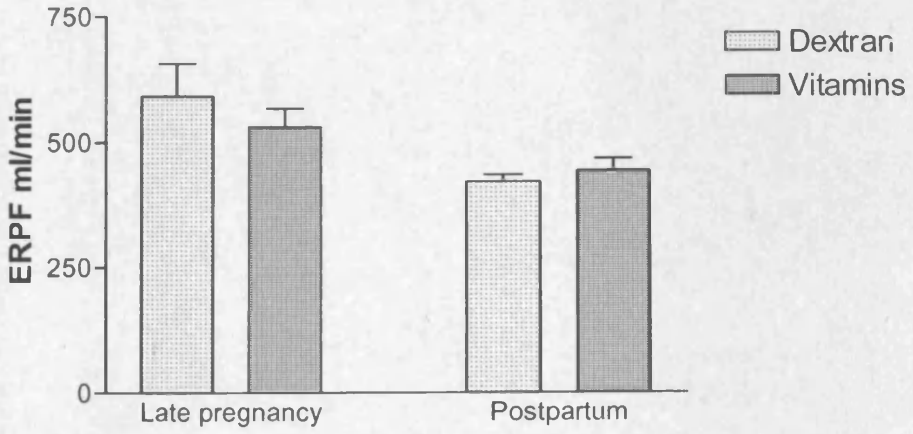



Effective renal plasma flow (ERPF)

Again, ERPF was higher in LP than PP (40%), but this was not statistically significant.

The vitamins did not produce any effect on ERPF.

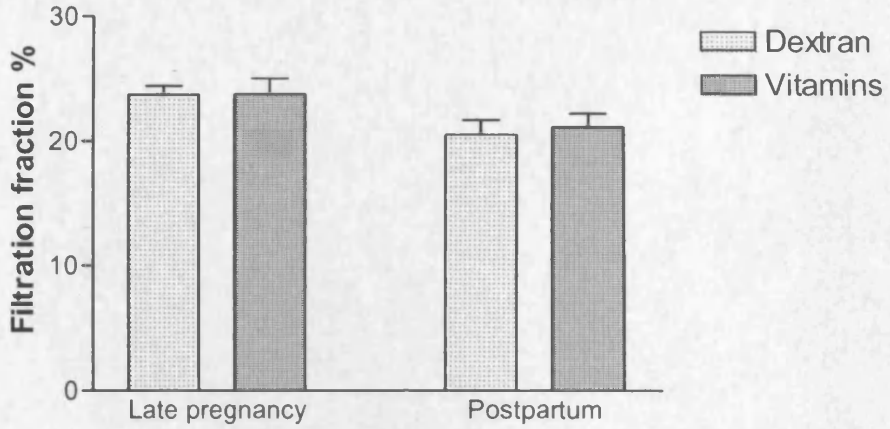
**Figure 9c: The effect of vitamins on ERPF (mean + s.e.m. n=4)**




Filtration fraction (FF)

The filtration fraction was increased in LP compared to PP (p=ns), but vitamins C and E had no effect on FF.

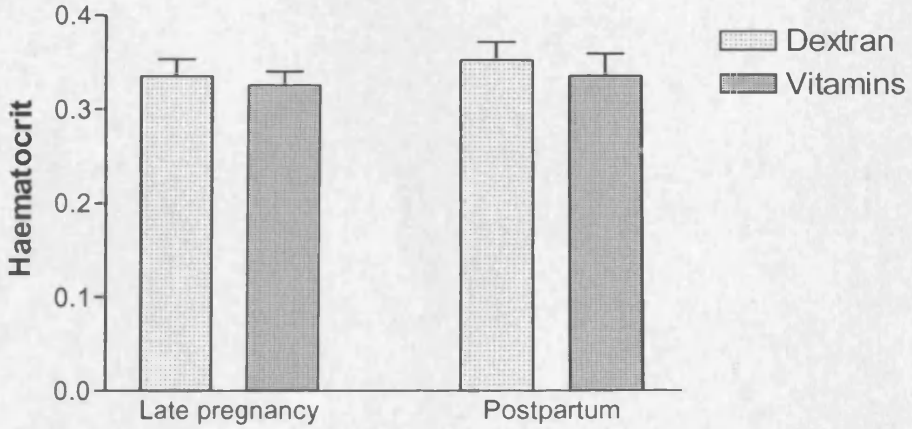
**Figure 9d: The effect of vitamins on FF (mean +s.e.m. n=4)**




Haematocrit

Vitamins C and E did not produce any significant effect on the haematocrit

**Figure 9e: The effect of vitamins on haematocrit (mean +s.e.m. n=4)**




Electrolyte excretion

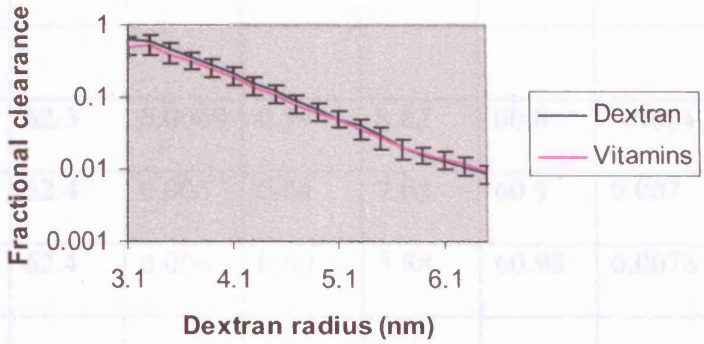
Vitamins C and E did not produce any alteration in the total excretion of sodium or chloride (mmol/l) either in LP or PP.

### 9.2.2 Fractional clearance curves

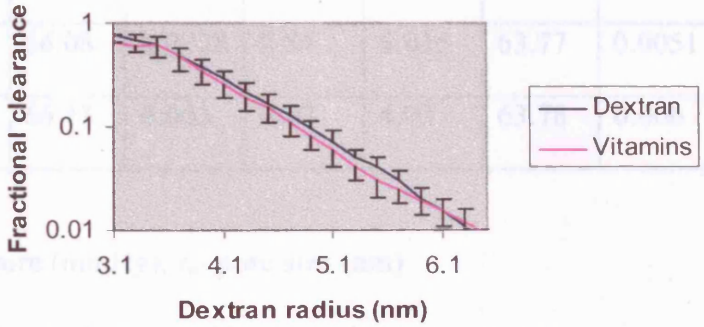
The effect of vitamins C and E was to reduce fractional clearance throughout the entire range of dextran radii (3.1-6.5nm) both in LP and when PP. Both reductions were statistically significant (p=0.0143 and p=0.0164 respectively).

**Figure 9f: Fractional clearance curves**

Late pregnancy




Postpartum




### 9.2.3 Biomathematical modelling

The following tables summarise the analyses using the *isoporous plus shunt* and *lognormal* models as described previously. It is evident that the transglomerular pressure (ΔP) of 37mmHg provides the "best fit".

**Table 30 : *Isoporous plus shunt*- vitamin study (LP n=4, PP n=3)**

| | | | | | | Vitamins → | | | |
|---|---|---|---|---|---|---|---|---|---|
| **LP/PP** | **ΔP** | **$K_f$** | **$r_o$** | **$\omega_o$** | **$\chi^2$** | **$K_f$** | **$r_o$** | **$\omega_o$** | **$\chi^2$** |
| | | | | | | | | | |
| **LP** | **37** | 10.78 | 62.3 | 0.0049 | 0.59 | 8.82 | 60.8 | 0.0064 | 0.86 |
| | **40** | 8.58 | 62.4 | 0.006 | 0.64 | 7.03 | 60.9 | 0.007 | 0.84 |
| | **43** | 7.16 | 62.4 | 0.006 | 0.69 | 5.88 | 60.98 | 0.0076 | 0.84 |
| | | | | | | | | | |
| **PP** | **37** | 7.07 | 66.07 | 0.002 | 0.51 | 7.72 | 63.7 | 0.0043 | 0.775 |
| | **40** | 5.45 | 66.08 | 0.0028 | 0.84 | 6.015 | 63.77 | 0.0051 | 0.854 |
| | **43** | 4.44 | 66.13 | 0.003 | 0.92 | 4.93 | 63.78 | 0.006 | 0.91 |

ΔP- transglomerular pressure (mmHg), $r_o$- pore size (nm)

$K_f$- ultrafiltration coefficient (ml/min/mmHg), $\omega_o$ - shunt component

$\chi^2$- chi squared.

$K_f$ is slightly higher in LP than PP and is slightly decreased by the administration of vitamins in LP and increased when PP. The mean pore size ($r_o$) is lower in LP than PP and is decreased by the vitamins, both LP and PP. The shunt component ($\omega_o$) is higher LP than PP and is subsequently increased by the vitamins in both LP and PP.

**Table 31 : *Lognormal***

| | | Vitamins → | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **LP/PP** | **ΔP** | **$K_f$** | **U** | **S** | **$\chi^2$** | **$K_f$** | **U** | **S** | **$\chi^2$** |
| | | | | | | | | | |
| **LP** | **37** | 10.78 | 54.96 | 1.16 | 0.75 | 8.82 | 50.4 | 1.21 | 0.52 |
| | **40** | 8.58 | 55.13 | 1.16 | 0.85 | 7.03 | 50.8 | 1.2 | 0.59 |
| | **43** | 7.16 | 55.24 | 1.16 | 0.9 | 5.88 | 51.04 | 1.2 | 0.64 |
| | | | | | | | | | |
| **PP** | **37** | 7.07 | 63.28 | 1.09 | 0.64 | 7.72 | 57.24 | 1.15 | 0.83 |
| | **40** | 5.45 | 63.34 | 1.09 | 0.78 | 6.015 | 57.4 | 1.15 | 0.93 |
| | **43** | 4.44 | 63.5 | 1.09 | 0.87 | 4.93 | 57.5 | 1.15 | 1.0 |

U- mean pore size (nm)

S- standard deviation about the mean

Mean pore size (U) is lower in LP than PP and is reduced by the vitamins in both LP and PP. S is higher in LP than PP and is increased by the vitamins in both LP and PP. In summary, the 2 models predict similar effects of vitamins C and E within this small group of women.

### 9.2.4 Urinary albumin excretion

The administration of vitamins C and E only slightly reduced microalbumin excretion in LP and PP (p=ns).

**Table 32 : Microalbumin excretion (µg/min)- summary of vitamin effect (mean +/- s.e.m)**

| | Dextran | **Vitamins** |
|---|---|---|
| Late pregnancy | 5.44 +/- 1.8 | 5.09 +/- 1.3 |
| **Postpartum** | 11.13 +/- 8.6 | 8.58 +/- 5.2 |

# 10 Discussion

## 10.1 Basal renal haemodynamics

## 10.2 Control of glomerular permeability

## 10.3 Effect of infused amino acids

## 10.4 Fractional dextran clearance curves and modelled data

10.4.1 Control group

10.4.2 Preeclamptic group

## 10.5 Effect of infused amino acids on modelled data

## 10.6 Nitric Oxide activity

10.6.1 Amino acids

10.6.2 Cyclic GMP and NO activity

## 10.7 Conclusions specific to aims and objectives of this study

## Some conclusions and future directions

## 10.0 Discussion

Preeclampsia, a pregnancy specific disease, is a multisystem disorder whose renal manifestations include functional loss and proteinuria. The cause of preeclampsia remains obscure, and several of the leading theories are discussed in the literature review as well as elsewhere in the literature (Conrad and Lindheimer, 1999; Davison et al, 2004; Agatisa et al, 2004) and will be reiterated in this discussion where relevant. Whatever the proximate cause of preeclampsia there is widespread endothelial dysfunction, and in this respect renal function with its reliance upon adequate glomerular blood flow and barrier integrity is particularly susceptible to these endothelial changes. Indeed, it is the kidney that has provided a unique window through which to view the disease process. In addition, measurement of circulating markers of GFR (i.e. serum creatinine), and, to some extent, the degree of proteinuria, aid in managing the disorder. This Thesis has focused on the compromised glomerular haemodynamics and disordered glomerular barrier function in preeclampsia, discussing how these pathologic changes effect clinical markers such as GFR and protein excretion and how the changes might or might not be ameliorated by infusion of specific amino acids.

Almost 50 years ago Homer Smith wrote "a pregnant woman is a very interesting phenomenon: I do not know of any other way to increase filtration rate by fifty per cent or more for prolonged periods" (Smith 1956). Since then, every piece of human or animal model renal research in pregnancy has approached this central important question from many different angles and the overall aim of the current work was to innovatively further interrogate this gestational sustained hyperfiltration and any accompanying renal reserve in terms of its underlying glomerular dynamics, using neutral dextran sieving and biomathematical modelling in pregnant women in health

and disease, namely preeclampsia. Once again, it has been clearly shown by these studies that in health renal heamodynamic increments are often in excess of 50% above non-pregnant values whilst clearly compromised in preeclampsia but nevertheless, may remain above the non-pregnant range.

In discussing the results obtained by these studies, inevitably these have to be placed in the context of characteristic renal structural changes seen in preeclampsia as well as concentrating on the disordered renal haemodynamics overall and the other determinants of ultrafiltration and proteinuria. Where necessary, the link to generalised endothelial dysfunction will be mentioned as well as identification of the many circulating factors postulated to mediate the changes. Obviously, the hope that amino acid infusion, and specifically L-arginine would have a beneficial effect in attenuating the disease process, as in some animal studies, has not been fulfilled. The reason for this and the frailties of the study and perhaps the naivety of the approach will be addressed.

## 10.1 **Basal Renal Haemodynamics**

The renal haemodynamic response to pregnancy was evident in both the control (healthy women) and preeclamptic groups. In LP the control group demonstrated a 46% increment in GFR and a 34% increment in ERPF compared to the preeclamptic group with a 27% increment in GFR and 41% increment in ERPF. Overall, however, the increments in the controls were larger than in the preeclamptics for both GFR and ERPF, by 25% and 15% respectively. The ultrafiltration coefficient $K_f$ was significantly reduced in the PE group compared to the control group and may therefore, together with reduced renal blood flow, explain the relative gestational reduction in GFR within this group. Filtration fraction (FF=GFR/ERPF) was 8.3% greater in the control group in LP. Postpartum values for GFR and ERPF for both groups were similar. These data endorse strongly a recent review where renal haemodynamic data from 23 studies (many of

variable quality) of preeclamptic women were compared in terms of GFR and ERPF (mainly measured by renal clearances of inulin and para-aminohippurate, respectively) (Conrad and Lindheimer, 1999). All 23 studies were characterised by decrease in GFR, and all but one, a decrease in ERPF in preeclampsia, compared with matched late pregnancy controls. Nine of the ten studies had post-partum data, revealing that the preeclamptic GFR and ERPF values could be lower than non-pregnant values.

It has been postulated that high renal vascular resistance due primarily to increased afferent arteriole sphincter tone is probably responsible for the reduction in ERPF consistently seen in preeclampsia. In this respect Conrad and Lindheimer (1999) estimated total renal vascular, afferent arterial and venular resistances, respectively, in 97 normotensive and 65 preeclamptic or eclamptic women from 5 studies. The almost three-fold increase in total resistance was due to a more than four-fold increase in afferent-, a small increment in venular-, but little change in efferent resistance, respectively. This may serve a protective role in preeclampsia, autoregulating intra-glomerular pressure, maintaining it within narrow limits despite systemic hypertension.

## 10.2 Control of glomerular permeability

In order to put into context the modeling data and their implications for glomerular barrier function some consideration should be given to the stability and mechanisms of barrier function itself. Concomitantly, it must be emphasized that modeling is a concept not necessarily in line with minute anatomy and that values derived from modeling are not necessarily "applicable" in vivo. This area has also been covered extensively in the literature review.

The barrier function of the glomerular capillary wall for macromolecules is selective for size, shape and charge (Kriz et al, 1998). The charge selectivity of the barrier results from the dense accumulation of negatively charged molecules throughout the entire

depth of the filtration barrier, including the surface coat of endothelial cells, and the high content of negatively charged heparan sulfate proteoglycans in the GBM. Polyanionic macromolecules, such as plasma proteins, are repelled by the "electronegative shield" originating from these dense assemblies of negative charges (Deen et al, 1979)

The size selectivity of the filtration barrier is in part established by the desne network of the GBM. The most restrictive structure, however, appears to be the slit diaphragm (Drummond et al, 1994). Uncharged macromolecules up to an effective radius of 1.8nm pass freely through the filter. Larger components are more and more restricted (indicated by their fractional clearances, which progressively decrease) and are totally restricted at effective radius > 4.0nm. Plasma albumin has an effective radius of 3.6nm; without the repulsion from the negative charge, plasma albumin would pass through the filter in considerable amounts (Brenner and Hostetter, 1978)

It follows, therefore, that the principle challenge for the glomerular capillaries is to combine "leakiness" with stability. The walls of capillaries do not appear to be capable of resisting high transmural pressure gradients. Several structures/mechanisms are involved in counteracting the distending forces to which the capillary wall is constantly exposed. The locus of action of all these forces is the GBM (Deen et al, 1972)

Two systems appear to be responsible for the development of stabilizing forces. Firstly, there is a basic system that consists of the GBM and the mesangium. Cylinders of the GBM, in fact, largely define the shape of glomerular capillaries. These cylinders, however, do not completely encircle the capillary tube; they are open towards the mesangium. Mechanically, they are completed by contractile mesangial cell processes that bridge the gaps of the GBM by interconnecting the opposing mesangial angles.

Podocytes act as the second structure-stabilising system. Two mechanisms appear to be involved. First, podocytes stabilize the folding pattern of glomerular capillaries by

fixing the turning points of the GBM between neighbouring capillaries. Second, podocytes may contribute to structural stability of glomerular capillaries by a mechanism similar to that of pericytes elsewhere in the body. Podocytes are attached to the GBM by foot processes that cover almost entirely the outer aspect of the GBM. The foot processes possess a well-developed contractile system connected to the GBM. Since the foot processes are attached in various angles on the GBM, they may function as numerous small, stabilizing patches on the GBM, counteracting locally the elastic distension of the GBM (Kriz et al, 1998)

### 10.3 Effect of infused amino acids

The infusion of glycine and L-arginine did not produce any statistically significant changes in GFR, ERPF or FF in the control or preeclamptic groups, LP or PP. The only increment observed, albeit non-significant, was the increase in GFR (9.6% LP, 8.8% PP) and ERPF (7.2%LP, 10.3% PP) with glycine infusion in the control group only.

Previous studies infusing an amino acid mixture (Vamin-9) demonstrated significant increases in GFR and ERPF in both LP and PP (Sturgiss et al, 1996; Milne et al, 2002). This current study demonstrated a non-significant increase in the controls with glycine infusion, with L-arginine having no effect. The ultrafiltration coefficient $K_f$ was unchanged by either L-arginine or glycine, implying that the glycine-induced increment in GFR, albeit non-significant, is ERPF driven, agreeing with the previous studies.

Our finding of no change in renal haemodynamics with L-arginine and only a non-significant increment with glycine could be interpreted as demonstrating that the amino acid effect demonstrated by previous studies (Sturgiss et al, 1996; Milne et al, 2002) may simply have been a result of amino acid load and not necessarily related to a specific amino acid or amino acids. As described in sections 6.41 and 7.41, the L-arginine infusion significantly increased plasma L-arginine concentrations both in LP

and PP within both groups and making it unlikely that the lack of effect was due to inadequate dosing. Moreover, high dose L-arginine (24g compared with 12g) also had no effect on renal haemodynamics.

In retrospect, it may have been naïve to expect that major changes would have been seen and/or that an intravenous infusion of a single amino acid, even L-arginine as a NO donor, under a short-term infusion protocol would have had any effect. Modulation of ERPF is possible but with a kidney already working at the "top of the range" no further augmentation might have been possible. And paradoxically it was the glycine that unmasked the renal haemodynamic reserve optimally.

It is difficult to know in an acute classical physiological whole body experiment whether or not a plasma estimation of the amino acid, which was certainly increased, has any relevance to what is happening within the kidney both in terms of concentration achieved *in-situ* and the adequacy and/or efficacy of the duration of exposure. What constitutes adequate dosing? It was thought to have been achieved but renal effects obviously do not correlate with results from renal function studies. Interestingly, there is some evidence of specific endothelial defects in women with preeclampsia (Haller et al, 1997) and furthermore, supportive evidence for NO deficiency in preeclamptics has been obtained from the reduced uterine perfusion pressure rat model (Haller et al, 1998). These workers did demonstrate that supplementation with L-arginine decreased BP by 19mmHg in pregnant rats with reduced uterine perfusion pressure (untreated vs treated) compared to a 12mmHg decrement in pregnant (untreated vs treated) rats. These results are suggestive that L-arginine supplementation may be beneficial in attenuating the hypertension in preeclampsia but this is animal model evidence and of course, renal haemodynamics were not specifically investigated.

## 10.4 Fractional dextran clearance curves and modelled data

It is the large radii dextran $\theta_D$ values that are particularly important for glomerular modelling i.e > 5.5nm, because they have a significant influence on the modelling for S and $\omega_o$ values, which represent the range of or biggest pore sizes in the *lognormal* and *isoporous plus shunt* models, respectively (Oken et al, 1981; Myers et al, 1982a; Deen et al, 1985) and correlate with proteinuria.

Modelling for both the control and preeclamptic groups was performed at values for $\Delta P$ of 37, 40 and 43mm Hg, values that are representative of the physiological range. Two theoretical models were used to analyse glomerular barrier dynamics and macromolecular handling, namely the *isoporous plus shunt* and *lognormal* models. Both models were in close agreement (tables 22 and 23). Each predicted a gestational decrease in $r_o$ (*isoporous plus shunt*) or U (*lognormal* ) together with an increased variance about the pore size in ωo ( the shunt component of *isoporous plus shunt* ) or S (*lognormal* ), which endorsed the previous studies of Milne et al (2002) and Moran et al (2003).

### 10.4.1 Control group

The control group demonstrated a significant reduction in fractional clearance in LP compared to PP (p=0.0097) The graph also shows a non-significant increase in fractional clearance from dextran radius 6.3nm upwards. Total protein excretion was non-significantly higher in LP than PP within the control group but no difference in UAE was observed. It is important to state that urinary microalbumin was measured from 20 minute collection periods and hence may not be as reliable as a 24 hour urine collection.

The overall reduction in fractional clearance may be explained by an increased glomerular plasma flow rate ($Q_A$), reflected in an increased GFR and may imply altered

glomerular size selectivity of pregnancy. The reduction in fractional clearance is reflected in the gestational decrease in mean pore size $r_o$ (*isoporous plus shunt*) and U (*lognormal*) of both biomathematical models. The non-significant increase in fractional clearance above 6.3nm is reflected in LP by increases in the non-restrictive $\omega_o$ (*isoporous plus shunt*) and S (*lognormal*) and may account for the slight non-significant increase in TPE in LP.

Our study agrees with previous dextran studies of Roberts et al (1996) and Milne et al (2002) which also demonstrated gestational decreases in fractional clearance. Another dextran study of Moran (2000) did not show any pregnancy alteration in fractional clearance and utilised the same collection period ($3^{rd}$ out of 3 collection periods) as the current study.

In the previous study by Milne et al (2002) an amino acid load (using Vamin-9 which contains 18 aminoacids, including glycine and L-arginine) produced a further reduction in fractional clearance at all dextran radii in LP, attaining significance for radii 4-4.9nm (LP) and 4-5.9nm (PP). In our study the infusion of both L-arginine and glycine further reduced fractional clearance but the effect of glycine was more consistent.

Glycine reduced fractional clearance significantly throughout the range of dextran radii both in LP and PP. This was reflected in both models by a reduction in $r_o$ and U in both LP and PP. Glycine had a greater enhancing effect on increasing GRF and ERPF (although not statistically significant) both in LP and PP when compared to L-arginine. The rise in GFR during glycine infusion may be due to increased glomerular plasma flow rate ($Q_A$), as this would increase the flux of water and unhindered solutes through the glomerular barrier greater than restricted macromolecules like dextran, hence reducing the fractional clearance of dextran. This may be a possible explanation for the effect of glycine on fractional clearance.

The reduction in fractional clearance by L-arginine was significant throughout the range of dextran radii in PP but in LP was only statistically significant for the dextran radii 5.3-6.3nm. Both models were in close agreement in PP with a reduction in $r_o$ and U, but the models disagreed in LP with the *isoporous plus shunt* model providing the best explanation with a reduction in $r_o$ and $\omega_o$. L-arginine had very little effect on renal haemodynamics in LP and PP and therefore any effect on fractional clearance must be independent of this.

There is no doubt that inulin and PAH clearances are "gold standard" measurements of GFR and ERPF, respectively, and the data from this study are entirely acceptable. The question has to be asked, however, is how relevant are neutral dextran sieving curves and biomathematical modelling? This approach, well grounded in (non-pregnant) nephrology has only been used in human pregnancy with confidence for 10 years and mainly by our Department, as referred to earlier. Strictly refereed publications attend to the acceptability and credibility of that work but the modelling approach is essentially a theoretical interpretation of a biological phenomenon and is based on certain assumptions. Crucially, $\Delta P$ is assumed to take a value between 37mmHg and 43mmHg, based on micropuncture studies in rats but plausible in humans, given that $\Delta P$ must be greater than intraglomerular oncotic pressure ($\pi_{GC}$) but substantially less than systemic arterial pressure. The modelling also assumes that the primary determinant of basement membrane permeability is its structural configuration, which is able to respond "quickly" to altered physiological states and/or challenges. Other membrane characteristics may have a role, however, for example membrane charge, which may be critically important in the preeclamptic glomerulus. Said otherwise, of the critical deteminants of GFR only one, ERPF, is truly determined, the rest being modelled, indirectly calculated or inferred. These are the ultrafiltration coefficient ($K_f$) and the Starling forces acting across the glomerular barrier- the net oncotic pressure and $\Delta P$.

Whilst it is not unreasonable to expect that a condition of fairly longstanding nature or duration like established preeclampsia with a structurally definable renal lesion might yield modelling data different from healthy pregnant women, is it naïve to postulate that by comparison, a very short amino acid infusion could ameliorate this lesion let alone produce a measurable change/effect in dextran sieving curves? Admittedly, GFR and ERPF might change in response to a physiological challenge by a pharmacological agent but structural changes and their sequelae take time. This will be further discussed when the preeclamptic results are reviewed. Bearing in mind that modelling is only a theoretical concept this further adds to the uncertainty that it would be sensitive enough to detect minor changes emerging during a short infusion.

### 10.4.2 Preeclamptic group

In the preeclamptic group, fractional clearance was non-significantly reduced in LP compared to PP up to dextran radius 5.5nm, after which it increased (again non-significant). As to be expected microalbumin excretion (μg/min) was greater in LP than PP within the preeclamptic group, but this was non-significant. This gestational reduction in fractional clearance in the PE group below 5.5nm is reflected in the modelling with a reduction in $r_o$ and an increase in the shunt component $\omega_o$ (*isoporous plus shunt*) and a reduction in U and an increase in S (*lognormal*). The increase in $\omega_o$ and S correlate with the clearance of the larger dextran molecules and hence would explain the increased clearance above 5.5nm. GFR was relatively increased in the PE group in LP compared to PP and this may imply an increase in $Q_A$ which may also contribute to a gestational reduction in fractional clearance.

When comparing the control group with the preeclamptic group in LP, the latter demonstrated significantly increased fractional clearance at the larger dextran radii (5.7-6.5nm) and higher microalbumin excretion (but not significantly) at this time. This

could explain some of the increased total protein excretion in the PE group. Microalbumin excretion, however, should correspond with $\theta_D$ at 3.6nm (close equivalent to albumin radius), thus it has to be postulated that for increased microalbumin excretion in the presence of reduced GFR and unchanged $\theta_D$, a loss of glomerular barrier charge selectivity might be involved. Indeed, even in normal pregnancy, the transglomerular filtration of the negatively charged albumin is restricted to a much greater extent than would be expected from its size alone. Loss of glomerular barrier charge is implicated in the development of several nephropathies usually coupled with a size selective defect. Unfortunately, charged dextrans are not suitable for use in human pregnancy and alternative techniques need to be sought and developed.

Those studies of other proteinuric states included diabetic nephropathy and glomerulonephritis (Myers et al, 1982ab), where there was loss of charge selectivity together with disordered size selectivity. Guasch et al (1993) were able to account for the proteinuria of minimal change and membranous nephropathy using anionic dextran sulphate as a test molecule. The loss of charge selectivity, and not size selectivity explained the higher proportion of smaller to larger proteins excreted. There are a number of drawbacks with dextran sulphate, it binds to plasma proteins and this binding is dependent on molecular size and therefore plasma size chromatography is necessary in addition to urine in order to establish true fractional clearance (Comper and Glasgow, 1995).

As mentioned earlier, the major issue from our point of view is that dextran sulphate is not licensed for use in human pregnancy, thus altered glomerular charge selectivity can only be inferred indirectly. Nevertheless, the indirect evidence is convincing. In fact it is the indirect evidence from renal biopsy specimens that has raised the possibility that preeclamptic morphometric changes might lead to or result from, a loss of charge selectivity (Naicker et al, 1997). In specimens from 15 African women obtained 2

weeks postpartum the glomeruli were enlarged in 50% with fusion of podocyte foot processes (80%) and the glomerular basement membrane (GBM) was thickened in two thirds. These ultrastructural changes were seen in combination with a significant decrease in the amount of anionic heparin sulphate in the GBM. The loss of negative charge on the podocyte surface was implied, as the repelling action of adjacent cell membranes is thought to be necessary to maintain the numerous narrow slits characterisic of the podocytes epithelial cell layer. It must be borne in mind, however, that these biopsies were performed two weeks post partum, and may represent an atypical patient population. In the absence of dextran sieving data, Lafayette et al (1998) have assessed postpartum preeclamptic renal biopsies in an attempt to derive filtration parameters for the glomerular barrier, using morphometric analysis and theoretical calculations in 13 women at 48h post caesarean section. The validity of this has been questioned for the following reasons; the heterogeneous postpartum population, failing to control for differences in glomerular haemodynamics, use of pharmacological manipulations, and contrary to most other studies in the literature ERPF in the preeclamptic patients was similar to the normotensive postpartum controls, thus making this, one of the rare studies that failed to demonstrate some haemodynamic contribution to hypofiltration in preeclampsia. Still, the authors anatomical modelling results (not from dextran sieving as earlier emphasised) suggested a reduction in $K_f$ of 40%. Subendothelial deposits, endothelial and mesangial cell hypertrophy and swollen endothelial segments without fenestrae, reduced the effective filtration surface area by a third. This was offset by the increased overall glomerular volume resulting in a net reduction of only 10%. Morphometric analysis led to the conclusion that hypofiltration was due to a reduction in $K_f$ secondary to reductions in both porosity and available filtration surface area.

Of interest, in the women studied for this investigation, the preeclamptic group postpartum still demonstrated statistically significant increased fractional clearance at dextran radii 6.3-6.5nm with increased micoalbumin excretion (non-significant) and total protein excretion (non-significant), possibly reflecting that at the time of the PP study (average 4 months post delivery), glomerular integrity had not completely recovered.

This finding compliments the reports of increased urinary albumin excretion (>14mg/24h) up to 3-5 years after preeclampsia, which may reflect residual glomerular damage from gestational protein trafficking (Abbate et al, 1999) and/or covert renal anomalies (Bar et al, 1999). Indeed, there is now interesting literature accumulating on the delayed/subsequent cardiovascular risks of preeclampsia (Smith et al, 2001; Irgens et al, 2001). Asking about preeclampsia, low birthweight infants or any "adverse" obstetric outcome gives valuable information for assessing cardiovascular risk in women (Sattar and Greer, 2002).

As mentioned, the renal changes in preeclampsia, persistent or otherwise, are, as already alluded to, supported by the histological features (Spargo et al, 1959; Sheehan 1980) in the older studies as well as evident in biopsy specimens taken in early onset preeclampsia by Naicker et al (1997) where a reduction in glomerular anionic sites correlated both with proteinuria and histological changes such as thickening of the glomerular basement membrane and fusion of foot processes.

These results have quite logically been discussed against the background of what is known about the structural changes in the kidney in preeclampsia. Indeed, the presence of endothelial cell swelling in glomeruli form women dying of eclampsia was noted as early as 1924 (Mayer, 1924) and more recently, histopathological and morphometric examination of renal biopsy and autopsy material, has aided in understanding of preeclampsia-induced "end-organ pathology".

As mentioned earlier, it was Spargo et al (1959) who coined the phrase "glomerular endotheliosis" and initially claimed the lesion was pathognomonic for preeclampsia. In contrast, others have described such lesions in nongravid women with a variety of glomerular lesions, non-preeclamptic hypertensive pregnant women and in abruption. Furthermore, very recently, it has been described in pregnant women with uncomplicated pregnancy. This latter claim deserves some comment bearing in mind the thrust and rationale of the earlier discussion. Strevens and colleagues (2003) performed renal biopsies on 36 hypertensive and 12 normotensive pregnant women. (A discussion of the ethical issues can be found in later ensuing correspondence: British Journal of Obstetrics and Gynaecology, 111:191-195, 2004). By noting "glomerular endotheliosis" in 5 of the normal pregnant volunteers has put under some doubt the previous literature where the diagnosis of preeclampsia was established by the presence or absence of that lesion. However, the results reveal that under light microscopy the intensity of the lesion in normotensive gravidas, on a scale 0-3, was 1+ in 4/5 specimens (often a subjective call between something and nothing for the pathologist), and 2+ in the other. Similarly, electron microscopy showed a "slight degree of endotheliosis" in 5 and mesangial cell interposition and electron dense deposits were not seen. Lesions in the preeclamptics, however, were considerably more robust, and, as would be expected, glomerular volume was significantly increased in those presenting with hypertension and proteinuria. Thus, these studies, in reality, confirm the consensus that the lesion in its fully developed form is quite distinctive, and the term "characteristic" (not "pathognomonic") is still highly relevant.

As discussed earlier, glomerular endotheliosis usually reverses fairly rapidly after delivery, the glomeruli resuming normal appearance within 2-3 weeks, but some have attributed abnormalities present years after the putative disease to residua of the preeclamptic lesion. Whether pure, as opposed to superimposed preeclampsia, leaves

permanent histological residua, however, is controversial but the long-term cardiovascular sequelae of preeclampsia are now recognised.

### 10.5 Effect of infused amino acids on modelled data

The effect of the amino acids in the PE group was similar to the control group. Glycine consistently reduced fractional dextran clearance throughout the range of dextran radii in LP and PP but the effect of L-arginine was only to reduce fractional clearance in LP and not PP. Neither amino acid had any effect on GFR or ERPF in the PE group and hence the small effect on fractional dextran clearance must be independent of any renal haemodynamic effect. Glycine reduced $K_f$, $r_o$, $\omega_o$ and U with no change in S in LP and PP reflecting the reduced clearance. L-arginine did not produce consistent results with respect to the modelling but the *isoporous plus shunt* model was the closest in agreement.

With respect to modelling parameters, $K_f$ was markedly reduced in LP preeclamptic women compared to controls but there was no significant difference PP between these groups. Mean pore size ($r_o$) and the shunt component ($\omega_o$) and U and S were greater in the preeclamptic group compared to controls in LP and PP, with the LP increases being of greater magnitude, thus possibly explaining the increased fractional clearance within the preeclamptic group and the proteinuria of preeclampsia.

Both the *lognormal* and *isoporous plus shunt* models were in close agreement, although the *isoporous plus shunt* when analysed using "goodness of fit" most closely predicted the measured $\theta_D$ data. The significant reduction in $K_f$ in preeclampsia in LP when compared to the control group was the most notable feature of the models. However, despite this significant reduction in $K_f$ in the PE group fractional clearance was still higher than the control group at the higher dextran radii implying that other factors must

be more important. Postpartum, the differences between the groups were much smaller reflecting the resolution of disordered preeclamptic glomerular barrier function.

The only published values for $K_f$ measured during preeclamptic pregnancy (as opposed to post-caesarean section (Lafayette et al, 1999)) are those of Moran et al (2003) who indeed demonstrated a 50% reduction in $K_f$ in preeclampsia. These data agree with that study. It is unlikely that reductions in $K_f$ solely account for the glomerular hypofiltration of preeclampsia, reflected in the gestational reduction in GFR but not a reduction in fractional clearance within our study, because also to be taken into account is the renal blood flow depression consistently reported in preeclampsia (Chapman, 1998 ; Conrad and Lindheimer, 1999), and endorsed by this current study.

Again, it has to be emphasised that the models could only provide a theoretical interpretation of what is altered intraglomerular morphology in preeclampsia. Whilst it may be possible to define changes in modelled parameters in the established basal preclamptic state, compared to normal pregnancy, this mathematical approach may not be sensitive enough or indeed appropriate to identify the changes during a short term amino acid infusion, NO-donor or not, if indeed there are alterations in morphology in this time span.

## 10.6 Nitric oxide activity

### 10.6.1 Amino acids

The plasma concentration of L-arginine in both the control and preeclamptic groups was lower in LP than PP, possibly reflecting the increased plasma volume of pregnancy and/or gestational increment in GFR, although the former would be blunted in preeclampsia. If NO is important in gestational vasodilatation it might be expected that L-arginine levels would be lower in pregnancy (LP control) as demonstrated here, whereas plasma NOx in pregnancy (LP control) would be increased, which it was not.

In the relatively volume depleted preeclamptic group with reduced GFR it might be expected that L-arginine could be higher than the control group but L-arginine concentration was the same in both controls and preeclamptics, both in LP and PP. Plasma NOx was, however, demonstrated to be higher within the PE group compared to the control group in both LP and PP but only significant in LP. As L-arginine is the substrate for the formation of NO, increased production of NO could theoretically lead to a reduction in L-arginine levels to equal that of the control group.

Infusion of L-arginine in both controls and preeclamptics significantly increased L-arginine concentrations, endorsing the validity of the dosage calculation. Glycine infusion did not alter L-arginine concentrations in either group, LP or PP. The infusion of L-arginine and of glycine in both groups in LP and PP had no effect on systemic blood pressure, disagreeing with Facchinetti et al (1999) who demonstrated reductions in blood pressure with L-arginine administration but protocol details are scanty in their publication

### 10.6.2 Cyclic GMP and NO activity

Both plasma cGMP (nmol/l) and 24 hour UcGMP (nmol/24hr) were measured as indices of NO activity since cGMP is the second messenger of NO. In the control group in LP 24 hour UcGMP was increased above PP levels agreeing with Kopp et al (1977). Plasma cGMP was, however, slightly reduced in LP compared to PP (non-significant) in contrast to other studies (Schneider et al, 1996; Boccardo et al, 1996; Lopez-Jaramillo et al, 1996) where increments in plasma cGMP were evident in normal pregnancy. Similarly, the preeclamptics, akin to controls, also demonstrated reduced plasma cGMP and increased 24hrUcGMP in LP compared to PP. The reductions in LP may purely be a function of an increase in urinary excretion reflected in the 24-hour urine results because careful control of diet had been maintained. Comparing the

preeclamptic group to controls, plasma cGMP was increased (non-significant) and 24hUcGMP was decreased (non-significant) in the preeclamptics. The best studies in the literature indicate that plasma concentration, urinary excretion and metabolic production of cGMP are increased in normal pregnancy (Sala et al, 1995; Conrad et al, 1999)

If NO is important in the vasodilatation of pregnancy it might be anticipated that there would be a gestational increase in plasma cGMP and by the same argument, if NO deficiency is implicated in preeclampsia then a decrease might have been expected within this group. In fact, within the Preeclamptic group, the plasma cGMP was higher (not significant) than the control group in LP and PP. This may reflect the increase in plasma NOx seen in our study in preeclampsia or may be explained by a reduction in urinary excretion as compared to the control group. Interestingly, when the cGMP/ creatinine ratio was examined there were no differences in plasma cGMP between the controls or preeclamptics in LP or PP.

As cGMP acts a second messenger to other substances, importantly ANP, as well as NO and as ANP is increased in preeclampsia (Moran, 2003) it is difficult to ascertain whether any changes in cGMP are truly reflective of NO activity or other mechanisms.

Relatively crude assessments were obtained of NO activity from plasma NOx (micromol/l) and 24hrUNOx (micromol/24hr). In both LP and PP the preeclamptic group demonstrated higher values for plasma NOx and 24hrUNOx compared to the controls, in agreement with data from Nobunaga et al (1996) and Smarason et al (1996). Also, although both groups demonstrated increased 24hrUNOx in LP compared to PP, the preeclamptic group only demonstrated increased plasma NOx in LP compared to PP thus disagreeing with most studies comparing LP with the non pregnant (table1, page 63). When the haemoconcentrating effect of decreased renal clearance is factored out by

calculating the plasma NOx/ creatinine ratio the results are the same but the reduction in plasma NOx in the LP control group becomes statistically significant.

As described in the methodology and results sections (pages 128,150) there were only a limited number of plasma samples available to assess the effects of L-arginine and glycine infusion on plasma NOx. The results were inconsistent and the statistical advice was that these were inappropriate to undertake any meaningful analyses.

ADMA (an endogenous endothelial NO synthase inhibitor) was the only inhibitor of the NO pathway that was measured (micromol/l) in this work. All results were non-significant. Plasma ADMA was elevated in preeclampsia compared to the control group in LP but reduced in preeclampsia PP, compared to controls. The preeclamptic group demonstrated higher levels of ADMA in LP than PP (non-significant), which was in contrast to the control group which demonstrated reduced ADMA in LP compared to PP. Holden and Fickling (1998) also demonstrated increased levels of ADMA in preeclampsia compared to healthy pregnant controls.

So data from the work do "implicate " ADMA because of the trend to higher levels of ADMA in preeclampsia, compared to LP in controls and PP in both. Obviously, dietary control is important when studying the role of ADMA but also assays can be variable, so that normal ranges may be difficult to agree on. Non-specific interference in the assay is always a worry. Sample collecting and storage are other frailties in this area of study. Our samples were collected meticulously and dispatched to a reliable laboratory taking all necessary precautions to avoid delay or losses.

Having said this, the work in the literature post 2002 have addressed most of the above criticisms, but at the time of our work the potential problems were more prominent.

In 2003, Savvidou and colleagues tested the hypothesis that ADMA contributes to the development of preeclampsia. The authors measured ADMA (and its symmetrical analogue) and forearm ischaemia-reperfusion as a marker of endothelial function and

also monitored uterine blood flow by Doppler flow techniques. Women with preeclampsia had significantly lower flow-mediated vasodilation than women with normal uterine perfusion. In the preeclamptics there was a tight correlation between ADMA levels and flow-mediated vasodilation prompting the concept that endothelial dysfunction develops before clinical preeclampsia and all the perinatal sequelae and ADMA may be a potentially contributing factor in these women.

When reviewing the current data it is evident that when preeclamptic LP is compared to control LP that plasma cGMP, plasma NOx, 24hr UNOx and ADMA are increased with 24hrUcGMP reduced. The only significant difference, however, was in plasma NOx. This may infact reflect increased activity of the NO system in preeclampsia which does not endorse the initial hypothesis which predicted a reduction in endothelial NO release due to poor fetoplacental perfusion in preeclampsia. It must be remembered that cGMP is also the second messenger for other substances e.g. ANP and hence, results relating to cGMP may not be purely a reflection of NO activity. It is possible that the increased production of NO demonstrated here may be a compensatory mechanism in response to the vasoconstriction and hypertension of preeclampsia in order to attenuate platelet adhesion and aggregation, which are features of preeclampsia. Also, increased NO activity in preeclampsia could theoretically reduce the effects of increased endothelin and inflammatory cytokines described in preeclampsia.

Another argument could be that increased NO levels may be implicated in the pathogenesis of preeclampsia because increased NO may increase peroxynitrites which in turn could impair vascular function and stimulate production of lipid peroxides, implicated in the endothelial dysfunction of preeclampsia. (Myatt et al, 1996; Davidge et al, 1998) ADMA may be important in regulating the production of NO and may be increased in preeclampsia in response to increased NO activity. Within the control group, reduced ADMA could be an attempt to augment NO production but within this

group this is not reflected in the plasma NOx results. Interestingly when ADMA was compared to L-arginine, i.e. the ADMA/L-arg ratio which is thought to be a better predictor of NOS inhibitory activity, the results are the same, except for increased ADMA in the control group in LP. This could, therefore, be a further explanation for the reduced plasma NOx within the control group in LP.

This interrogation of the NO system in these investigations is the most disappointing aspect of the research. This study certainly had a big advantage over the *majority* of previous studies assessing the NO system in pregnancy in that we strictly controlled diet (appendix A14) with each woman following a low nitrate/nitrite diet prior to the study period. Despite this fastidious attention to detail it has to be accepted that measurement of plasma and urinary NOx is almost certainly not an accurate measure of haemodynamically active NO within the body (Baylis and Vallance, 1998). In fact, the animal model work presents a more convincing argument for the potential role of NO in gestational renal vasodilatation and hyperfiltration. Urinary NOx and cGMP excretion is elevated in gravid rats even when on a diet low in nitrate and nitrite (Conrad et al, 1999). Furthermore, NO metabolites are increased in the plasma of pregnant rats and NO-haemoglobin in erythrocytes is detected in pregnant but not in non-pregnant rats (Conrad et al, 1999)

Overall, therefore, it has been shown that the kidney, with its reliance on glomerular blood flow and glomerular barrier integrity provides a unique window to view the preeclamptic process. Both ERPF and GFR decrease in preeclampsia although absolute values may remain above the non-pregnant range. A reduction in the ultrafiltration coefficient ($K_f$) in the order of 50%, either alone or in combination with reduced RBF is presented as the most likely mechanisms for the reduction in GFR. Proteinuria develops, at least in part secondary to impaired glomerular barrier integrity with a loss of size selectivity revealed by fractional dextran clearance studies and it is proposed

though yet to be proven, that this is accompanied by a loss of glomerular barrier charge selectivity.

There has been no convincing demonstration of reduced activity of the NO system nor that any said reduction could be ameliorated by a NO-donor such as L-arginine. The predominant results relate to glomerular haemodynamics and barrier function with the NO activity data providing little support for the original hypothesis.

### 10.7 Conclusions specific to aims and objectives of this study

Bearing in mind the specific questions that were posed (page 90) relating to the kidney in preeclampsia, it is possible to deal with the answers in a "closing statement", with answers to the 4 questions:

1. ***Is depression of GFR and magnitude of UAE related to haemodynamic factors and/or altered barrier function?***

   Both RBF and GFR decrease in preeclampsia, although absolute values may remain above the non-pregnant range. A decrease in the ultrafiltration coefficient ($K_f$) of around 50%, either alone or in tandem with reduced RBF is perhaps the most likely mechanism for the GFR decrease. Proteinuria develops, at least partly, secondary to impaired glomerular barrier integrity with a loss of size/selectivity revealed by $\phi_D$ studies and it is proposed, but not yet proven, that this is accompanied by a loss of glomerular barrier charge selectivity.

2. ***Is there a renal reserve and is it associated with increments in RBF and/or $\Delta P$?***

   The infusion of glycine and L-arginine (postulated to unmask renal reserve) did not produce any statistically significant changes in GFR, RBF or FF in the control or preeclamptic groups, either in LP or PP. The only increases recorded (albeit non-significant in each case) was that in GFR (9.6% LP & 8.8% PP) and ERPF (7.2% LP & 10.3% PP), with glycine *in the controls only.* L-arginine had no effect at all.

$K_f$ was not changed by either glycine or L-arginine, implying that the glycine-induced increments in GFR were RBF-dependent. In all, therefore, there was no renal reserve in the preeclamptic women, despite two different amino acid challenges. This also prevailed at the study time chosen for the repeat tests PP.

3. ***Is the magnitude and/or absence of renal reserve dependent on a particular amino acid?***

As mentioned, surprisingly, L-arginine had no augmentation effect at all. Glycine was effective, however, but not in the preeclamptic women.

4. ***Is the time course of post-delivery renal recovery after preeclampsia related to its severity, extent of glomerular dysfunction, renal reserve and/or duration of expectant management?***

It was not possible to individualise recovery phases between patients, based on the severity of their preeclampsia and/or expectant management that had been utilised. In the controls 24hr total protein excretion (TPE) had "normalized" by the time of the PP study (18±(sem) 1.44 wks) compared to LP (LP 0.124±(sem) 0.01g vs PP 0.087±0.0075g) but in the PP preeclamptics "return to normal" was not achieved in that time (17±1.57 wks) and there was no way of assessing when indeed it would nromalise (LP 1.93±0.67g vs PP 0.23±0.128). There was, however, no significant difference between TPE in controls and (previous) preeclamptics PP. In the controls all modelled parameters were "normalised" by PP, as was also the case in the preeclamptics, but specifically $\omega_o$ and U.

Lastly, if this study could be replanned and undertaken again what changes would be needed? In discussing these changes there should be no undermining of the considerably effort put into what has been compiled in this Thesis, but an honest reappraisal shows scientific integrity. Also these remarks have to be taken alongside those in the last section "Some general conclusions and future directions".

At the outset it must be admitted that the oral supplementation studies were at best naïve and at worst ill-designed and irrelevant in their construction and execution. The infusion studies, however, used techniques that were fully validated and that had tackled and yielded important data in relation to many crucial aspects of maternal adaptations in pregnancy. Having said this, a short-term infusion of an amino acid in the presence of established structural preeclamptic pathology may have been insufficient to elicit change. A series of experiments to assess the effect of duration of infusion would be valuable, especially if such an approach had any hope of being translated onto a therapeutic footing. Other amino acids could have been used and as mentioned earlier, the ideal "control" for L-arginine would have been D-arginine, but it was unavailable. In addition, it should be mentioned that glycine, as used here, could have a specific effect *per se* on NO activity, which would obviously make difficult interpretation of "NO markers" data, although the undouobted renal augmentation effects in these studies could not be doubted. Precise plasma measurements of the infused amino acids could have been helpful, if only retrospectively to assess dose-finding and patient-to-patient variation, in the search for the "best" infusion regimen. The surrogates of NO activity were thought to be "the best" available and the most relevant at the time of the work (1999-2000) but this area would need considerable re-examination now. Furthermore, even though sample collection and subsequent pre-assay preparation were meticulous, the assays were "offsite" and factors involved with shipment and lack of control in the analysing laboratory meant these data had to be regarded as less robust than those derived "in-house".

**Some conclusions and future directions**

Preeclampsia remains a major cause of maternal and fetal morbidity and mortality but in the last decade progess has been logarithmic in understanding its pathogenesis, predicting its occurrence and managing the disorder once it has appeared. Generalised endothelial dysfunction is still the favoured "disorder" responsible for all of the clinical aspects of the maternal syndrome. New forays into the deranged cell biology of the placenta, the role of antiangiogenic proteins and their inhibitors, autoantibodies, cytokines , growth factors and oxidative stress as well as the status of the systemic inflammatory response have all altered our thinking about preeclampsia. These far ranging fields cannot all be reviewed or covered. Exciting new examples of progress, however, can be briefly mentioned. For instance the increased placental expression and secretion of soluble fms-like tyrosine kinase (sflt-1), a naturally occurring circulating VEGF antagonist in preeclamptic patients. (Maynard et al, 2003; Levine et al, 2004)

The work of this Thesis, however, has focused on changes in one of the important organs, the kidney, testing a hypothesis which ultimately was "not proven". So where do we go form here in relation to kidney research that is relevant to the clinical problems in humans? Is human pregnancy "whole body" research still a useful proposition? Are there any new developments since the inception and execution of the work in this Thesis?

Perhaps the most relevant current work is that concerning relaxin and its effect on the circulation and on matrix metalloproteinases (MMP). Relaxin is secreted by the corpus of the ovary during rodent and human pregnancy. It is a potent vasodilator when chronically administered to conscious, non-pregnant or pregnant or ovariectomised female and to male rats (Danielson et al, 2000; Novak et al, 2002). Four important issues appear (1) Relaxin effect(s) appear not to be gender specific and (2) Neutralising endogenous circulating relaxin with specific antibodies or eliminating relaxin by

ovariectomy completely abrogates the renal circulatory and osmoregulatory changes of pregnancy (3) An important mechanism for pregnancy (relaxin)-induced renal vasodilation and hyperfiltration involves up-regulation of vascular matrix metalloproteinase-2 activity (MMP-2) (which has a pivotal role in the relaxin-gelatinase pathway), which in turn, processes big endothelin (ET) to ET $_{1\text{-}32}$ thereby activating the endothelial $ET_B$ receptor and NO. (4)Because increased vascular gelatinase activity by relaxin is a proximal step in the vasodilatory pathway(s) of pregnancy, inappropriate MMP-2 activity may contribute to reduced renal function in preeclampsia (Hsu et al, 2002).

Much work is needed now to examine the role of relaxin in human pregnancy and clinical investigations involving "whole body" studies are still essential. It is important to pursue an understanding of the pathways bringing about gestational renal adaptation as only then is there any hope of helping women when these pathways start to fail, as in preeclampsia. The vasodilatory pregnancy factor, be it relaxin or not, must trigger the renal adaptive response. If it is relaxin then the recent availability of human recombinant relaxin (rhRLX) that will allow the animal studies described to be undertaken in humans and may go somewhat towards solving Homer Smith's curiosity as well as establishing whether or not amelioration of renal problems in pregnancy is possible, let alone the role of RLX in renal medicine generally.

It is intriguing to theorise about *impossible* investigations. Certainly, undertaking micropuncture studies in healthy women and women with preeclampsia would provide some of the answers (sought by other indirect methods over decades) to questions about the mechanisms underlying renal haemodynamic dysfunction. Overall, it seems from work presented in this Thesis that during pregnancy the presence of renal compromise is detrimental to maintaining renal haemodynamic augmentation. A micropuncture approach in humans *can never be*, so we are left with animal model highly invasive

procedures to provide data which must then only cautiously be extrapolated to humans. Regarding primate studies, to date there has only been one small study of renal haemodynamics and renal reserve reporting the presence of renal reserve similar to that in non-pregnant humans (Easterling et al, 1998).

Practically, as well as ethically, since the human kidney is not directly accessible to investigation and in light of the limitations that pregnancy imposes on the materials and methods used for investigating renal function, the collection of physiological data in humans will always require the utilisation of fully validated protocols for the use of indirect techniques such as the application of biomathematical modelling of glomerular dynamics.

Infusion studies such as those described in this Thesis provide for the safe acquisition of "whole body" data in humans. Measurement of fractional dextran clearances ($\theta_D$) with the subsequent biomathematical modelling allows analysis of glomerular ultrafiltration parameters (transglomerular hydrostatic pressure, $\Delta P$, ultrafiltration coefficient, $K_f$, and predicted barrier parameters). Whilst this highly theoretical concept has its own pitfalls, the data obtained using such techniques bear striking similarities to those directly assessed in animal studies validating there use in humans. The future for this approach therefore must be positive.

Human infusion studies are laborious and rely on consistent and honest recruitment strategies and the generous nature of the volunteers as well as rigorous adherence to methodological protocols and laboratory techniques. It is only by undertaking further studies in a statistically appropriate number of women that natural inherent idiosyncrasies related to inter- and intra- subject variability can be overcome. This would allow determination of whether differences between health and disease states are in fact real.

We will always keep returning to that statement by Homer Smith because despite considerable efforts, the pathways responsible for triggering the kidney changes in pregnancy are not fully determined yet so elucidating the aberrations in such pathways will take some further time. Meanwhile, preeclampsia remains a multisystem disorder of unknown aetiology and without a totally unifying hypothesis. The endothelium is targeted at an early stage resulting in disordered renal vascular sensitivity and glomerular dysfunction. The kidney, therefore continues to provide a unique opportunity to study the pathogenesis of preeclampsia, remains central to its diagnosis and management, and may allow assessment of subtle and remote effects of the disorder as well.

## Appendix A

**A1** Gestation and follow up of healthy controls (n=16)

| Initials | Amino acid | Gestation -LP (weeks) | PP (weeks) |
|---|---|---|---|
| NB | L-arg | 33 | 14+4 |
| SB | L-arg | 32+6 | 15+3 |
| HM | L-arg | 33+1 | 14+4 |
| HC | L-arg | 35+2 | 12 |
| RSB | L-arg | 34+2 | 14 |
| CN | L-arg | 36+2 | 16 & 24 |
| EW | L-arg | 33 | Did not return |
| KF | L-arg | 36+3 | 23 |
| MW | Glyc | 36 | 23 |
| VH | Glyc | 39 | 16 |
| JM | Glyc | 38 | 27 |
| EW | Glyc | 37 | 14 |
| SC | Glyc | 35+2 | 25+4 |
| MG | Glyc | 37 | 28 |
| VP | Glyc | 35+4 | 14 |
| SM | Glyc | 37+3 | 24 |
| | **Mean** | **35+5** | **18+5** |
| | **Standard error(se)** | **0.48** | **1.445** |

**A2** Gestation and follow up of preeclamptics (n=12)

| Initials | Amino acid | Gestation- LP (weeks) | PP (weeks) |
|---|---|---|---|
| DM | L-arg | 36+3 | 16+4 |
| KT | L-arg | 37+1 | 13+4 |
| DJ | L-arg | 36+6 | 24 |
| RL | L-arg | 40+3 | 28+5 |
| BG | L-arg | 40+1 | 16+6 |
| EB | L-arg | 35+6 | 14+6 |
| MG | Glyc | 39 | 16+4 |
| YM | Glyc | 39 | 14 |
| JO | Glyc | 37+3 | 12+4 |
| KM | Glyc | 36+6 | Did not return |
| AB | Glyc | 37+1 | 24 |
| PG | Glyc | 35+6 | 15+5 |
| | **Mean** | **37+5** | **17+6** |
| | **se** | **0.451** | **1.57** |

**A3** Gestation and follow up of validation study (n=11)

| Initials | Diagnosis- preeclamptic(PE) or normal (N) | Gestation- LP (weeks) | PP (weeks) |
|---|---|---|---|
| LW | PE | 36+6 | 14+4 |
| PP | PE | 37 | 14+2 |
| AM | PE | 35 | Did not return |
| SQ | PE | 33+1 | 13+1 |
| DC | PE | 39+6 | 12+6 |
| VT | PE | 37+5 | 15+4 |
| YK | PE | 32+1 | 13 |
| DH | N | 36+1 | 15 |
| CJ | N | 34+3 | 13+4 |
| JT | N | 36+2 | Did not return |
| SM | N | 39+6 | 13 |
| | **Mean** | **36+2** | **13+6** |
| | **se** | **0.75** | **0.33** |

**A4** Gestation and follow up of Vitamin study (n=4)

| Initials | Gestation-LP (weeks) | PP (weeks) |
|---|---|---|
| HH | 36+5 | 13+4 |
| DR | 38+2 | Did not return |
| JH | 35+1 | 14 |
| SH | 34+4 | 16 |
| **Mean** | **36+2** | **14+4** |
| **se** | **0.83** | **0.74** |

**A5** Gestation and follow up of high dose arginine study (n=4)

| Initials | Gestation (weeks) / non pregnant (NP) |
|---|---|
| LB | 37+3 |
| MT | 38+5 |
| NW | 37+1 |
| HO | NP |
| **Mean** | **37+6** |
| **se** | **0.48** |

## A6 Haemodynamic data for healthy controls

Mean arterial blood pressure (MAP), mmHg.
LP= late pregnancy
PP= postpartum

| Initials | LP Basal | LP Dextran | LP Dextran + amino acid | PP basal | PP Dextran | PP Dextran + amino acid |
|---|---|---|---|---|---|---|
| CN | 89 | 82 | 81 | 86 | 91 | 90 |
| VH | 99 | 83 | 85 | 95 | 97 | 99 |
| MW | 79 | 88 | 78 | 83 | 85 | 89 |
| EWO | 83 | 90 | 77 | 77 | 82 | 85 |
| RSB | 67 | 79 | 82 | 80 | 81 | 88 |
| NB | 83 | 84 | 79 | 87 | 86 | 82 |
| SB | 88 | 81 | 80 | 86 | 80 | 80 |
| SC | 89 | 88 | 80 | 88 | 79 | 73 |
| HM | 83 | 84 | 81 | 83 | 89 | 88 |
| HC | 83 | 85 | 82 | 92 | 95 | 95 |
| JM | 103 | 83 | 83 | 98 | 86 | 87 |
| MG | 77 | 81 | 81 | 82 | 79 | 85 |
| EW | 88 | 86 | 88 | DNR | DNR | DNR |
| VP | 93 | 83 | 86 | 85 | 85 | 75 |
| KF | 85 | 82 | 76 | 95 | 85 | 86 |
| SM | 86 | 98 | 92 | 93 | 95 | 98 |
| | | | | | | |
| Mean | 85.9 | 84.8 | 81.9 | 87.3 | 86.3 | 86.7 |
| **Sem +/-** | 2 | 1 | 1 | 1.6 | 1.5 | 2 |

DNR= did not return

## A7 Haemodynamic data for vitamin study

| Initials | LP Basal | LP Dextran | LP Dextran + amino acid | PP basal | PP Dextran | PP Dextran + amino acid |
|---|---|---|---|---|---|---|
| HH | 91 | 93 | 88 | 94 | 99 | 93 |
| DR | 87 | 90 | 89 | DNR | DNR | DNR |
| JH | 86 | 81 | 81 | 85 | 84 | 82 |
| SH | 82 | 103 | 85 | 88 | 90 | 95 |
| | | | | | | |
| Mean | 86.5 | 91.2 | 85.8 | 89 | 91 | 90 |
| **Sem +/-** | 2 | 4.5 | 2 | 2.6 | 4 | 4 |

**A8** Haemodynamic data for preeclamptic group

Mean arterial blood pressure (MAP), mmHg.
LP= late pregnancy
PP= postpartum

| **Initials** | **LP** **Basal** | **LP** **Dextran** | **LP** **Dextran + amino acid** | **PP** **basal** | **PP** **Dextran** | **PP** **Dextran + amino acid** |
|---|---|---|---|---|---|---|
| DM | 98 | 110 | 110 | 94 | 93 | 96 |
| MG | 117 | 111 | 99 | 100 | 87 | 88 |
| DJ | 103 | 106 | 109 | 96 | 99 | 99 |
| YM | 105 | 100 | 99 | 97 | 99 | 101 |
| KT | 96 | 102 | 103 | 104 | 106 | 100 |
| JO | 104 | 105 | 106 | 98 | 101 | 101 |
| KM | 117 | 107 | 113 | DNR | DNR | DNR |
| EB | 118 | 113 | 100 | 99 | 94 | 96 |
| RL | 109 | 102 | 104 | 87 | 97 | 87 |
| AB | 95 | 106 | 98 | 83 | 84 | 86 |
| PG | 91 | 93 | 101 | 85 | 89 | 88 |
| BG | 95 | 101 | 102 | 85 | 90 | 88 |
| | | | | | | |
| Mean | 104 | 105 | 104 | 93.5 | 94.5 | 94 |
| **Sem +/-** | 3 | 2 | 1 | 2 | 2 | 2 |

**A9** Pregnancy outcomes for healthy controls

VD= vaginal delivery
LSCS= lower segment caesarean section

| **Initials** | **Fetal sex** | **Fetal birth weight (g)** | **Gestational age at delivery** | **Mode of delivery** |
|---|---|---|---|---|
| CN | Male | 3240 | 40+3 | VD |
| VH | Male | 3360 | 41+2 | VD |
| MW | Male | 3400 | 40+4 | LSCS |
| EWO | Female | 3005 | 40+5 | VD |
| RSB | Male | 3285 | 38 | VD |
| NB | Male | 3400 | 40+3 | VD |
| SB | Male | 3510 | 38+3 | VD |
| SC | Male | 4030 | 41+5 | VD |
| HM | Male | 3600 | 40+6 | VD |
| HC | Male | 3685 | 42+3 | VD |
| JM | Male | 2780 | 40+3 | VD |
| MG | Male | 3010 | 38 | LSCS |
| EW | Male | 3540 | 39+6 | VD |
| KF | Male | 3550 | 40+5 | VD |
| SM | Female | 3260 | 40+1 | VD |
| VP | Female | 3125 | 41+1 | VD |
| | | | | |
| Mean | | 3361 | | |
| **Sd +/-** | | 303 | | |

**A10** Pregnancy outcomes for vitamin study

| Initials | Fetal sex | **Fetal birth weight (g)** | **Gestational age at delivery** | **Mode of delivery** |
|---|---|---|---|---|
| HH | Female | 3330 | 39+6 | VD |
| DR | Female | 4165 | 40+5 | VD |
| JH | Male | 3990 | 42 | VD |
| SH | Female | 2710 | 42 | VD |
| | | | | |
| Mean | | 3549 | | |
| **Sd +/-** | | 665 | | |

**A11** Pregnancy outcomes for preeclamptic group

| Initials | Fetal sex | Fetal birth weight(g) | Gestational age at delivery | Mode of delivery |
|---|---|---|---|---|
| DM | Female | 2450 | 37+1 | VD |
| MG | Female | 2600 | 39+4 | LSCS |
| DJ | Male | 2570 | 38 | VD |
| YM | Female | 2680 | 38+6 | VD |
| KT | Female | 3055 | 38 | VD |
| JO | Female | 3325 | 38+2 | VD |
| KM | Female | 2920 | 37+1 | VD |
| EB | Male | 4130 | 37+5 | LSCS |
| RL | Male | 4160 | 40+5 | LSCS |
| AB | Male | 2490 | 37+3 | VD |
| PG | Male | 2670 | 36+1 | LSCS |
| BG | Male | 3570 | 40+6 | VD |
| | | | | |
| Mean | | 3052 | | |
| **Sd +/-** | | 614 | | |

**A12** Pregnancy outcomes for validation study

| Initials | Fetal sex | Fetal birth weight(g) | Gestational age at delivery | Mode of delivery |
|---|---|---|---|---|
| CJ | Female | 2500 | 35+2 | LSCS |
| JT | Female | 3660 | 39+2 | VD |
| SM | Male | 3640 | 39+6 | VD |
| DH | Male | 2870 | 37+6 | LSCS |
| LW | Male | 2975 | 37+4 | LSCS |
| PP | Male | 3340 | 37+1 | VD |
| AM | Male | 2325 | 37+1 | VD |
| SQ | Female | 2195 | 34+2 | VD |
| DC | Male | 3200 | 40+3 | VD |
| VT | Female | 2940 | 38 | VD |
| YK | Male | 1360 | 32+1 | LSCS |
| | | | | |
| Mean | | 2819 | | |
| **Sd** +/- | | 686 | | |

**A13**

## CONSENT FORM

I agree to participate in the study, which examines the role of Nitric oxide in pregnancy. Dr Norman has explained the study to me and I have read the information leaflet provided.

I agree to have blood samples taken whilst an infusion of Amino acid and Dextran 40 is running.

I understand that this study will not affect my care in pregnancy and that I may, at any time, withdraw from the study and that I do not need to give reasons for this.

Signed:

Date:

Witnessed:

Date:

**A14**

## DIET ADVICE SHEET
## (AVOIDING GREEN VEGETABLES AND PROCESSED FOOD [TINNED FOOD])

TIME: FOR 2 DAYS BEFORE THE STUDY

List of ideas to combine at mealtimes:

| FOOD | AMOUNT |
|---|---|
| <u>BREAKFAST</u> | |
| Banana | 1 |
| Low fat milk | 1 carton |
| Hot cereal | 1 portion |
| Margarine | 1tsp |
| Bread | 2 slices |
| Honey | 2 tsp |
| Orange juice | ½ glass |
| <u>LUNCH</u> | |
| Turkey, roasted | 2oz |
| Swiss cheese | ½ oz |
| Mayonnaise | 1 tsp |
| Tomato | ¼ medium size |
| Bread | 2 slices |
| Tuna salad | 1 portion |
| Orange | 1 medium |
| Cod, baked | 3 oz |
| Corn, boiled | 2 oz |
| Apple | 1 |
| <u>AFTERNOON SNACK</u> | |
| Crackers | 6 |
| Cheddar cheese | 1 / 2 oz |
| Sugar Cookies | 2 |
| <u>DINNER</u> | |
| Chicken, skinless breast roasted | 4oz |

| | |
|---|---|
| Macaroni | 2oz |
| Corn, boiled | 2oz |
| Bread | 1 slice |
| Margarine | 1 tsp |
| Orange | 1 |
| Roasted beef | 4oz |
| Noodles | 2oz |
| Peas | 2oz |
| Pear | 1 small |
| Rice | 2oz |

EVENING SNACK

| | |
|---|---|
| Crackers | 3 |
| Low fat milk | 1 carton |
| Angel food cake | 1 slice |

## Appendix B Data for the healthy control group

B1 Healthy control GFR (ml/min)- during dextran and dextran plus amino acid infusion

| Patient | Amino acid (aa) | LP Dextran | LP Dextran +aa | PP Dextran | PP Dextran +aa |
|---|---|---|---|---|---|
| NB | L-arg | 170.3 | 141.8 | 95.8 | 83 |
| HC | L-arg | 134.9 | 148.7 | 113 | 112.5 |
| KF | L-arg | 124.6 | 121 | 88.1 | 89.8 |
| HM | L-arg | 126.9 | 118.2 | 73.3 | 82 |
| CN | L-arg | 152 | 141.6 | 72.7 | 76.3 |
| RSB | L-arg | 162.4 | 141 | 98.3 | 95 |
| EW | L-arg | 163 | 164.2 | | |
| SB | L-arg | 176.2 | 201.3 | 115.4 | 99.1 |
| SC | Glyc | 145.2 | 156.9 | 130.5 | 131.6 |
| MG | Glyc | 121.7 | 133 | 81 | 52.2 |
| JM | Glyc | 148.4 | 204.1 | 104.1 | 156.5 |
| SM | Glyc | 148.3 | 162.5 | 109.6 | 118.2 |
| EW | Glyc | 111.4 | 111.2 | 66.2 | 62 |
| VH | Glyc | 119.9 | 118.9 | 73.9 | 83.3 |
| VP | Glyc | 170.7 | 180.6 | 133 | 155.9 |
| MW | Glyc | 88.2 | 86.8 | 72.6 | 77.7 |

B2 Healthy control ERPF (ml/min)- during dextran and dextran plus amino acid infusion

| Patient | Amino acid (aa) | LP Dextran | LP Dextran +aa | PP Dextran | PP Dextran +aa |
|---|---|---|---|---|---|
| NB | L-arg | 795.6 | 736.5 | 518.7 | 486.9 |
| HC | L-arg | 688.3 | 726 | 644.4 | 628.4 |
| KF | L-arg | 617.6 | 626.6 | 550.7 | 592.8 |
| HM | L-arg | 647.3 | 578.3 | 323.2 | 362.3 |
| CN | L-arg | 688.3 | 676.9 | 423.1 | 438.2 |
| RSB | L-arg | 788.1 | 736.5 | 531.7 | 538.4 |
| EW | L-arg | 744.5 | 832.5 | | |
| SB | L-arg | 697.6 | 846 | 555.4 | 485.6 |
| SC | Glyc | 742.7 | 763.3 | 667 | 676.5 |
| MG | Glyc | 652.1 | 717.7 | 462 | 401.8 |
| JM | Glyc | 645.3 | 856.3 | 459.4 | 766.7 |
| SM | Glyc | 681.1 | 754.4 | 558 | 624.5 |
| EW | Glyc | 543.8 | 519.4 | 450.9 | 437.8 |
| VH | Glyc | 549 | 594.5 | 345.4 | 381 |
| VP | Glyc | 1103.5 | 1113.7 | 855.7 | 905.8 |
| MW | Glyc | 398.8 | 381.7 | 366.6 | 401.1 |

B3 Healthy control FF (GFR/ERPF)- during dextran and dextran plus amino acid infusion

| Patient | Amino acid (aa) | LP Dextran | LP Dextran +aa | PP Dextran | PP Dextran +aa |
|---|---|---|---|---|---|
| NB | L-arg | 21 | 19 | 18.5 | 17 |
| HC | L-arg | 19.6 | 20.5 | 17.5 | 17.9 |
| KF | L-arg | 20.2 | 19.3 | 16 | 15.2 |
| HM | L-arg | 19.6 | 20.4 | 22.7 | 22.6 |
| CN | L-arg | 22.1 | 20.9 | 17.2 | 17.4 |
| RSB | L-arg | 20.6 | 19.1 | 18.5 | 17.6 |
| EW | L-arg | 21.9 | 19.7 | | |
| SB | L-arg | 25.3 | 23.8 | 20.8 | 20.4 |
| SC | Glyc | 19.6 | 20.6 | 19.6 | 19.5 |
| MG | Glyc | 18.7 | 18.5 | 17.5 | 13 |
| JM | Glyc | 23 | 23.8 | 22.7 | 20.4 |
| SM | Glyc | 21.8 | 21.5 | 19.6 | 18.9 |
| EW | Glyc | 20.5 | 21.4 | 14.7 | 14.2 |
| VH | Glyc | 21.6 | 20 | 21.4 | 21.9 |
| VP | Glyc | 15.5 | 16.2 | 15.5 | 17.2 |
| MW | Glyc | 22.1 | 22.7 | 20 | 19.4 |

B4 Healthy control- 24 hour urinary protein (g/24h)- in LP and PP

| **Patient** | **LP** | **PP** |
|---|---|---|
| NB | 0.11 | 0.1 |
| HC | 0.15 | 0.1 |
| KF | 0.1 | 0.0 |
| HM | 0.06 | 0.1 |
| CN | 0.11 | 0.06 |
| RSB | 0.1 | 0.1 |
| EW | 0.19 | |
| SB | 0.07 | 0.1 |
| SC | 0.16 | 0.1 |
| MG | 0.14 | 0.1 |
| JM | 0.11 | 0.1 |
| SM | 0.11 | 0.1 |
| EW | 0.22 | 0.05 |
| VH | 0.08 | 0.1 |
| VP | 0.1 | 0.1 |
| MW | 0.1 | 0.1 |
| Mean | 0.124 | 0.087 |
| s.e.m | 0.01 | 0.0075 |

B5 Healthy control- Plasma NOx (microM)- in LP and PP

| Patient | **Amino acid (aa)** | **LP** | **PP** |
|---|---|---|---|
| NB | L-arg | 4 | 12 |
| HC | L-arg | 9 | 37 |
| KF | L-arg | 16 | 15 |
| HM | L-arg | 8 | 17 |
| CN | L-arg | 14 | 19 |
| RSB | L-arg | 21 | 26 |
| EW | L-arg | | |
| SB | L-arg | 6 | 27 |
| SC | Glyc | 8 | 16 |
| MG | Glyc | 13 | 21 |
| JM | Glyc | 7 | 10 |
| SM | Glyc | 8 | 7 |
| EW | Glyc | 23 | 26 |
| VH | Glyc | 28 | 28 |
| VP | Glyc | 14 | 37 |
| MW | Glyc | 33 | 41 |

B6 Healthy control- 24hrUNOx (microM)- in LP and PP

| Patient | **Amino acid (aa)** | **LP** | **PP** |
|---|---|---|---|
| NB | L-arg | 1473 | 914 |
| HC | L-arg | 1035 | 1368 |
| KF | L-arg | 1156 | 947 |
| HM | L-arg | 699 | 1037 |
| CN | L-arg | 984 | 680 |
| RSB | L-arg | 1128 | 608 |
| EW | L-arg | | |
| SB | L-arg | 1088 | 1088 |
| SC | Glyc | 844 | 1238 |
| MG | Glyc | 1675 | 301 |
| JM | Glyc | 728 | 516 |
| SM | Glyc | 1010 | 486 |
| EW | Glyc | 432 | 692 |
| VH | Glyc | 1954 | 986 |
| VP | Glyc | 1424 | 1918 |
| MW | Glyc | 817 | 145 |

B7 Healthy control- plasma cGMP (pmol/ml)- in LP and PP

| Patient | **Amino acid (aa)** | **LP** | **PP** |
|---|---|---|---|
| NB | L-arg | 5.66 | 5.74 |
| HC | L-arg | 6.14 | 12.04 |
| KF | L-arg | 5.5 | 4.9 |
| HM | L-arg | 6.93 | 7.82 |
| CN | L-arg | 4.53 | 5.08 |
| RSB | L-arg | 8.85 | 7.89 |
| EW | L-arg | | |
| SB | L-arg | 7 | 14 |
| SC | Glyc | 6.06 | 5.94 |
| MG | Glyc | 5.5 | 6.1 |
| JM | Glyc | 8.1 | 8.6 |
| SM | Glyc | 4.9 | 5 |
| EW | Glyc | 9 | 6.85 |
| VH | Glyc | 5.2 | 5.4 |
| VP | Glyc | 4 | 6 |
| MW | Glyc | 7 | 9.5 |

B8 Healthy control- 24hrUcGMP (nmoles)- in LP and PP

| Patient | **Amino acid (aa)** | **LP** | **PP** |
|---|---|---|---|
| NB | L-arg | 1757 | 843 |
| HC | L-arg | 1706 | 1062 |
| KF | L-arg | 860 | 500 |
| HM | L-arg | 954 | 910 |
| CN | L-arg | 704 | 585 |
| RSB | L-arg | 2572 | 685 |
| EW | L-arg | | |
| SB | L-arg | 2688 | 1731 |
| SC | Glyc | 1249 | 701 |
| MG | Glyc | 1418 | 429 |
| JM | Glyc | 1536 | 1394 |
| SM | Glyc | 1665 | 488 |
| EW | Glyc | 3227 | 355 |
| VH | Glyc | 2176 | 1039 |
| VP | Glyc | 1983 | 799 |
| MW | Glyc | 1432 | 916 |

B9 Healthy control-ADMA (uM)- in LP and PP

| Patient | **Amino acid (aa)** | LP | PP |
|---|---|---|---|
| NB | L-arg | 0.394 | 0.619 |
| HM | L-arg | 1.03 | 0.917 |
| CN | L-arg | 0.128 | 0.38 |
| RSB | L-arg | 0.354 | 0.749 |
| SC | Glyc | 0.35 | 0.362 |
| JM | Glyc | 0.566 | 0.563 |
| EW | Glyc | 0.253 | 0.38 |
| MW | Glyc | 0.477 | 0.731 |

## Appendix C Data for the preeclamptic group

C 1 Preeclamptic GFR (ml/min)- during dextran and dextran plus amino acid infusion

| Patient | Amino acid (aa) | LP Dextran | LP Dextran +aa | PP Dextran | PP Dextran +aa |
|---|---|---|---|---|---|
| DM | L-arg | 72.5 | 76.7 | 69 | 90.4 |
| DJ | L-arg | 51.3 | 49.7 | 62.5 | 58.1 |
| KT | L-arg | 169.8 | 137.2 | 102.9 | 106.2 |
| EB | L-arg | 112.6 | 103.4 | 76.6 | 79.4 |
| RL | L-arg | 122.6 | 127.9 | 107 | 100.3 |
| BG | L-arg | 152.7 | 144.7 | 104.7 | 97.7 |
| MG | Glyc | 97.5 | 89.3 | 88.4 | 91.2 |
| YM | Glyc | 88.4 | 102.1 | 54.9 | 59.2 |
| JO | Glyc | 148.3 | 143.8 | 117.2 | 115.3 |
| KM | Glyc | 102.8 | 113.5 | | |
| AB | Glyc | 132.6 | 93.5 | 83 | 85 |
| PG | Glyc | 107.5 | 106.7 | 71.7 | 75.2 |

C2 Preeclamptic ERPF (ml/min)- during dextran and dextran plus amino acid infusion

| Patient | Amino acid (aa) | LP Dextran | LP Dextran +aa | PP Dextran | PP Dextran +aa |
|---|---|---|---|---|---|
| DM | L-arg | 316.8 | 364.3 | 316.5 | 521.6 |
| DJ | L-arg | 374.6 | 310.9 | 326.7 | 293.7 |
| KT | L-arg | 785.7 | 628.1 | 538.2 | 554.3 |
| EB | L-arg | 527.9 | 544 | 405.5 | 465.8 |
| RL | L-arg | 658.5 | 801 | 495.7 | 527.3 |
| BG | L-arg | 579.7 | 605.7 | 469.7 | 439 |
| MG | Glyc | 585.4 | 562.7 | 430.7 | 435.6 |
| YM | Glyc | 396.3 | 437.7 | 243.3 | 258.1 |
| JO | Glyc | 744.3 | 780 | 607.8 | 586.7 |
| KM | Glyc | 673.4 | 715.8 | | |
| AB | Glyc | 819.9 | 577.1 | 433.2 | 431.8 |
| PG | Glyc | 680.7 | 629.4 | 388.3 | 373.9 |

C3 Preeclamptic FF (GFR/ERPF)- during dextran and dextran plus amino acid infusion

| Patient | Amino acid (aa) | LP Dextran | LP Dextran +aa | PP Dextran | PP Dextran +aa |
|---|---|---|---|---|---|
| DM | L-arg | 22.9 | 21 | 21.8 | 17.3 |
| DJ | L-arg | 13.7 | 16 | 19 | 19.8 |
| KT | L-arg | 21.6 | 21.8 | 19 | 19 |
| EB | L-arg | 21.3 | 19 | 19 | 17 |
| RL | L-arg | 18.6 | 16 | 21.6 | 19 |
| BG | L-arg | 26.4 | 23.9 | 22.2 | 21.9 |
| MG | Glyc | 16.7 | 15.9 | 20.5 | 21 |
| YM | Glyc | 22.3 | 23.3 | 22.6 | 23 |
| JO | Glyc | 20 | 18.4 | 19.3 | 19.7 |
| KM | Glyc | 15.3 | 16 | | |
| AB | Glyc | 16.2 | 16.2 | 19.2 | 19.7 |
| PG | Glyc | 15.8 | 17 | 18.5 | 20 |

C4 Preeclamptic- 24 hour urinary protein (g/24h) – in LP and PP

| Patient | LP | PP |
|---|---|---|
| DM | 0.57 | 0.18 |
| DJ | 0.56 | 0.1 |
| KT | 2.7 | 1.5 |
| EB | 0.63 | 0.1 |
| RL | 0.6 | 0.1 |
| BG | 0.6 | 0.1 |
| MG | 2.2 | 0.17 |
| YM | 0.58 | 0.0 |
| JO | 1.3 | 0.1 |
| KM | 4.1 | |
| AB | 8.4 | 0.1 |
| PG | 0.9 | 0.1 |
| Mean | 1.93 | 0.23 |
| s.e.m | 0.67 | 0.128 |

C5 Preeclamptic- Plasma NOx (microM)- in LP and PP

| Patient | **Amino acid (aa)** | **LP** | **PP** |
|---|---|---|---|
| DM | L-arg | 24 | 31 |
| DJ | L-arg | 21 | 20 |
| KT | L-arg | 14 | 5 |
| EB | L-arg | 23 | 30 |
| RL | L-arg | 27 | 15 |
| BG | L-arg | 17 | 26 |
| MG | Glyc | 77 | 16 |
| YM | Glyc | 16 | 8 |
| JO | Glyc | 23 | 40 |
| KM | Glyc | | |
| AB | Glyc | 34 | 24 |
| PG | Glyc | 21 | 66 |

C6 Preeclamptic- 24hrUNOx (microM)- in LP and PP

| Patient | **Amino acid (aa)** | **LP** | **PP** |
|---|---|---|---|
| DM | L-arg | 663 | 1019 |
| DJ | L-arg | 610 | 839 |
| KT | L-arg | 1612 | 1173 |
| EB | L-arg | 811 | 926 |
| RL | L-arg | 1478 | 43 |
| BG | L-arg | 973 | 1009 |
| MG | Glyc | 3390 | 986 |
| YM | Glyc | 1643 | 1073 |
| JO | Glyc | 877 | 1115 |
| KM | Glyc | | |
| AB | Glyc | 37 | 641 |
| PG | Glyc | 756 | 1165 |

C7 Preeclamptic- plasma cGMP (pmol/ml)- in LP and PP

| Patient | **Amino acid (aa)** | **LP** | **PP** |
|---|---|---|---|
| DM | L-arg | 5.2 | 6.4 |
| DJ | L-arg | 9 | 5.5 |
| KT | L-arg | 4.9 | 7.2 |
| EB | L-arg | 11.8 | 11.9 |
| RL | L-arg | 6.1 | 8.7 |
| BG | L-arg | 10.1 | 8.9 |
| MG | Glyc | 7.5 | 4.6 |
| YM | Glyc | 5.7 | 9 |
| JO | Glyc | 9 | 6.2 |
| KM | Glyc | | |
| AB | Glyc | 4.8 | 5.4 |
| PG | Glyc | 6.7 | 15 |

C8 Preeclamptic- 24hrUcGMP (nmoles)- in LP and PP

| Patient | **Amino acid (aa)** | **LP** | **PP** |
|---|---|---|---|
| DM | L-arg | 1689 | 457 |
| DJ | L-arg | 641 | 656 |
| KT | L-arg | 1557 | 478 |
| EB | L-arg | 1052 | 1533 |
| RL | L-arg | 908 | 528 |
| BG | L-arg | 1446 | 609 |
| MG | Glyc | 1329 | 950 |
| YM | Glyc | 1493 | 871 |
| JO | Glyc | 996 | 1013 |
| KM | Glyc | | |
| AB | Glyc | 1553 | 755 |
| PG | Glyc | 1140 | 637 |

C9 Preeclamptic ADMA (uM)- in LP and PP

| Patient | **Amino acid (aa)** | LP | PP |
|---|---|---|---|
| DM | L-arg | 0.453 | 0.582 |
| DJ | L-arg | 0.422 | 0.479 |
| KT | L-arg | 1.384 | 0.677 |
| EB | L-arg | 0.507 | 0.667 |
| RL | L-arg | No peak | 0.351 |
| BG | L-arg | 0.782 | 0.679 |
| MG | Glyc | 0.215 | 0.145 |
| YM | Glyc | 0.528 | 0.466 |
| JO | Glyc | 0.414 | 0.176 |
| KM | Glyc | | |
| AB | Glyc | 0.414 | 0.594 |
| PG | Glyc | 0.683 | 0.969 |

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