The Distribution and Expression of the Nitric Oxide System During Renal Ageing and the Effect of Sex Steroid Modulation.

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-Abstract-

The protective effect of female sex in renal ageing and cardiovascular function is widely accepted, but poorly understood. Previous evidence has suggested a role for the nitric oxide and renin-angiotensin systems, though the precise mechanisms by which they elicit these effects remain elusive. Female animals and humans have increased nitric oxide bioavailability with age in comparison to males, and this effect can be negated by ovariectomy surgery, suggesting an interaction between ovarian steroids and nitric oxide. In addition, studies have shown an upregulation of the angiotensin II type 2 receptor (AT_2R) in aged females in comparison with males. Whilst incompletely understood, the AT_2R is known to mediate vasodilation, nitric oxide release, and can be modulated by oestrogen. Work in this laboratory has shown that the expression of AT_2R, renal ageing, and blood pressure may all be sensitive to the nutritional environment encountered during foetal development.

This thesis aimed to elucidate some of the mechanisms mediating this 'protective effect' of female gender in a rat model of developmentally programmed hypertension and accelerated renal ageing. It was hypothesised that ageing would result in decreased renal function and increased blood pressure. These effects would be significantly altered by sex steroid modulation, and negative effects exacerbated by exposure to a low protein diet during gestation. The mechanisms driving these effects would be, at least in part, linked in changes to renin angiotensin system-regulated nitric oxide release.

The data obtained suggested that the nitric oxide system did not significantly change with sex steroid exposure, or in response to maternal diet. Unexpectedly, ovariectomy alone did not change physiological responses as has been described previously. Instead, a significant interaction was observed between exposure to a low protein diet during gestation and ovariectomy. Offspring from mothers fed a low

protein diet had impaired responses to removal of ovarian steroids. In addition, low protein offspring had altered vascular reactivity in response to targeted agonism and antagonism of angiotensin II receptors.

In conclusion, this work has shown that the protective effect of female gender is more complex than previously described. The data did not support the hypothesis that nitric oxide mediates the beneficial effects of female sex, and targeted stimulation of the AT_2R is not an effective means of altering this. Moreover, these data suggest that foetal exposure to a low protein diet may permanently programme altered vascular function, and can significantly affect response to sex steroids.

-Acknowledgements-

I have always found the acknowledgements of a PhD thesis a little uncomfortable, and couldn't imagine expressing that level of emotion in an academic document. However, as I come to write my own, I realise that this is the easiest writing I have had to do for this thesis.

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-Publications-

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-Abbreviations-

2K1C – Two-kidney-One-Clip

ACE – Angiotensin Converting Enzyme

ACh – Acetylcholine

Ang II – Angiotensin II

ASP - Aspartate

ASS – Argininosuccinate Synthase

AT_1R – Angiotensin II Type 1 Receptor

AT_2R – Angiotensin II Type 2 Receptor

BH_4 - Tetrahydrobiopterin

BP – Blood Pressure

C21 – Compound 21

cGMP – 3', 5'-cyclic monophosphate

EDRF – Endothlin-Derived Relaxing Factor

eNOS – Endothelial Nitric Oxide Synthase

ERα – Oestrogen Receptor α

ERβ – Oestrogen Receptor β

GFR – Glomerular Filtration Rate

GPCR – G-Protein Coupled Receptor

GPER – G-Protein Coupled Oestrogen Receptor

HR – Heart Rate

HRT – Hormone Replacement Therapy

LNAME - Nω-nitro-L-arginine methyl ester

LOS - Losartan

LP – Low Protein

MAP – Mean Arterial Pressure

MAPK – Mitogen Activated Protein Kinase

MLP – Maternal Low protein

mmHg – Millimetres of Mercury

NO – Nitric Oxide

NOS – Nitric Oxide Synthase

NP – Normal Protein

OVX - Ovariectomised

PE – Phenylephrine

PKG – cGMP-Dependent Protein Kinase/Protein Kinase G

RAS – Renin-Angiotensin System

sCG – Soluble Guanylyl Cyclase

Sham – Sham-Ovariectomised

VEGF – Vascular Endothlial Growth Factor

Chapter 1 - General Introduction

1.1 - The Structure and Function of the Kidney

The kidneys are crucial organs in maintaining fluid homeostasis in the body. Comprised of as many as two million of its functional unit, the nephron, the kidney has numerous functions including maintaining fluid osmolarity, regulating plasma volume, filtering the blood of waste products and foreign compounds, and regulating arterial pressure (Sherwood, 2014; Munger et al., 2012). The kidney is supplied by a single artery, which divides into a complex vasculature within the organ (Nielsen et al., 2012). The arrangement of nephrons in the kidneys gives rise to two distinct regions of tissue, the cortex and medulla. The dark inner tissue, the medulla, is comprised of the collecting ducts and tubular components of the nephron, giving it a striated appearance. In contrast, the cortex is granular in appearance and is largely comprised of the vascular elements of the nephron (Sherwood, 2014).

The basic structure of the nephron is depicted in Figure 1.1 (Taken from Chade, 2013). It comprises a cluster of capillaries known as the glomerulus that work at high pressure to filter a protein-free plasma from the blood into the tubular component of the nephron. The glomerulus is surrounded by a thin-walled sac-like structure known as the Bowman's capsule, which is joined to the proximal tubule, a coiled and convoluted structure that passes through the cortex. This descends in a sharp U-shaped bend known as the loop of Henle, which feeds through the medulla and back into the distal tubule of the cortex. Finally, the distal tubule feeds into the collecting duct, which links to the renal pelvis, and ultimately the bladder (Sherwood, 2014; Brenner et al., 2012).

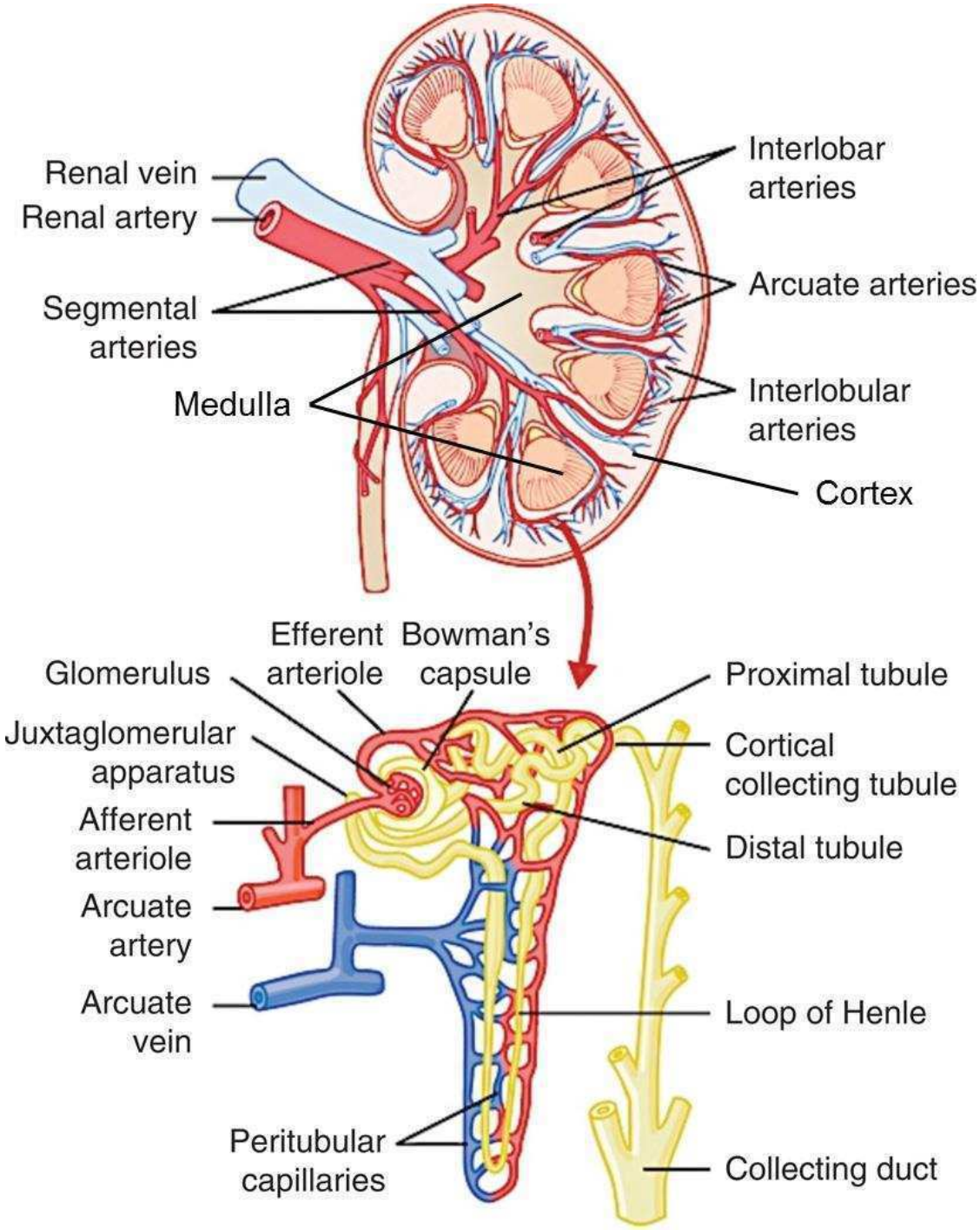


Figure 1.1: The basic structure of a bisected kidney (top), and the structure of the nephrons contained within the renal tissue (bottom). Figure from Chade (2013). The kidney is divided into two regions, the outer cortex and the inner medulla, comprising a complex vasculature and up to two million functional nephrons.

The structure of the nephron is highly specialised to perform its multiple functions. In basic terms, the actions of the nephron can be described by three processes; glomerular filtration, tubular reabsorption, and tubular secretion (Munger et al., 2012). These processes, although intrinsically linked to excretion of waste products, are ultimately the means by which the kidney regulates blood pressure, fluid balance, and osmolarity (Scott & Quaggin, 2015). As blood enters the glomerulus about 20% of the plasma flowing through it is filtered into the Bowman's capsule, the remainder passes into the peritubular capillaries surrounding the nephron (Sherwood, 2014). Glomerular filtration is indiscriminate and the filtrate may contain any constituents of the blood with the exception of the blood cells themselves. However, typically very few constituents of a molecular weight greater than 70,000 are filtered unless the golerular membrane is degraded or damaged (Lote, 2012). It is not until the filtrate passes through the tubular component of the nephron that a more selective secretion and absorption of metabolites occurs to regulate fluid balance (Munger et al., 2012).

The rate at which the kidney filters the blood can have a significant impact on the overall health of the organ, as well as major homeostatic consequences for the rest of the body. Indeed, measurement of glomerular filtration is a frequently used diagnostic test that offers insight into renal health (Stevens et al., 2006). It has been well established that aberrant fluctuations in intra-renal pressure can result in renal dysfunction, renal injury, and persistently dysregulated blood pressure (Koeners et al., 2008). As such, understanding and managing renal function is a significant area of research.

1.2 - Renal Ageing

The kidney undergoes numerous structural and functional changes over the lifespan that have been cited to be some of the most dramatic of any ageing organ system (Weinstein & Anderson, 2010; Csiszar et al., 2007). However, the rate at which kidney health deteriorates varies greatly amongst individuals. Whilst some individuals may encounter substantial declines in renal capacity, others may experience little change at all, and the consequences of these changes are variable.

1.2.1 - Structural Changes in the Kidney

Kidney size changes significantly with age, and can decrease by as much as 20% from early adulthood to the ninth decade (Brenner et al., 2012; Emamian et al., 1993). This decrease in weight is largely restricted to the cortex (Weinstein & Anderson, 2010), and is associated with an increase in glomerular sclerosis, tubular fibrosis, and tubular atrophy (Zhou et al., 2008) as well as a reduction in overall glomerular number (Hoy et al., 2003; Bolignano et al., 2014).

Evidence from both human and animal studies also suggests that the permeability of the basement membrane in glomeruli increases with age resulting in elevated excretion of proteins in the urine (Joles et al., 2010; Amakasu et al., 2011). Proteinuria is not only an indicator of changes in renal structure, but may also incite an inflammatory response in the kidneys and further exacerbate the ageing process (Gorriz & Martinez-Castelao, 2012).

An additional and important structural change that occurs during renal ageing is that of the vasculature. It has been demonstrated in multiple populations that ageing is associated with a significant increase in arterial sclerosis in the kidney (Rule et al., 2011; Kubo et al., 2003), Atherosclerotic injury, including lumen occlusion and

plaque formation, increase risk of renal ischaemic damage and subsequent kidney disease (Lerman et al., 2009; Keddis et al., 2010; Bax et al., 2003).

A key component of all of these age-related changes is inflammation (Izquierdo et al., 2012), A study by Kato et al., in 2014 suggested that ageing may not only exacerbate pathological inflammation in the kidney, but other remote organs such as the heart as well. The study showed that glomerular sclerosis and infiltration by inflammatory cells was significantly greater in older rats exposed to renal injury than in younger ones, and this elevation in the older animals led to a significant fibrotic response, suggesting an important role for this pathological relationship (Kato et al., 2014). Furthermore, Xu et al., demonstrated that the interactions between oxidative stress and inflammation play a key role in mediating the development of chronic kidney disease (Xu et al., 2015). It is clear looking at these studies, and others, that inflammation and ageing are intrinsically linked in the mediation of kidney injury, with each influencing and potentially exacerbating the other.

1.2.2 - Functional Changes in the Kidney

Associated age changes in the kidney structure have been well defined, but how these alterations translate into renal function is not as consistent (Glassock, 2011; Zhou et al., 2008). In particular, studies by Rule et al., (2010, 2011) demonstrated somewhat paradoxically that glomerular filtration rate (GFR) may increase in cases of decreased glomerular density. Additionally, Hallan et al., (2012) established that whilst GFR and urinary excretion of protein were good predictors of mortality and renal risk, they were also independent of the age of the subject. It is possible, that maintenance of GFR is linked to renal reserve. Renal reserve is the capacity of the kidney to increase basal GFR after protein overload, and was preserved in healthy older subjects when studied by Musso et al., (2013). It did, however, decrease

significantly in magnitude, suggesting compensatory mechanisms may lose efficiency with ageing.

Conversely, evidence consistently demonstrates that glomerular filtration decreases with age (Musso & Oreopoulus, 2011; Fliser, 2008; Xu et al., 2010), highlighting the importance of improving our understanding of the mechanisms by which kidney function declines. When measured correctly, glomerular filtration rate provides an accurate indication of the level of filtration occurring in the kidneys, and thus can be used to assess functional capacity of the kidneys and as a marker of renal risk (Matsushita et al., 2010).

Changes in filtration are accompanied by alterations in renal blood flow (RBF; Weinstein & Anderson, 2010). Specifically, there is a redistribution of blood flow in the kidney with renal cortical tissue vasculature becoming hyalinised, thus reducing the blood flow in the cortex (Mangoni & Jackson, 2004; Tracy et al., 2002). Weinstein and Anderson (2010) hypothesised that this is a possible cause of the decrease in renal size observed in ageing kidneys, though our understanding is still limited.

A key system altered by the ageing process is renal electrolyte handling. Generally, the elderly kidney is capable of maintaining electrolyte balance, though in instances of stress or illness fluctuations can occur. In particular, sodium handling is susceptible to disturbance and is the most common electrolyte imbalance in the ageing kidney (Schlanger et al., 2010). In aged individuals tubular reabsorption of sodium is reduced, owing to a decrease in sensitivity of the renal tubules to anti-diuretic hormone (Tareen et al., 2005). This inability to efficiently move sodium, coupled with diminished thirst leads to a significant reduction in urine concentrating capacity and subsequent hypernatremia (an excessive rise in plasma sodium; Musso & Oreopoulos, 2011). The consequences of such an electrolyte imbalance

are substantial and include a loss of the osmotic gradient in the kidney, further exacerbating the age-induced decline in function (Weinstein & Anderson, 2010).

The literature suggests that whilst we can identify multiple key markers of renal age, both structurally and functionally, our understanding of the mechanisms driving these phenomena is incomplete. Current epidemiological studies suggest that accelerated renal decline is positively associated with hypertension, smoking, atherosclerotic disease, inflammation, obesity, and gender (Weinstein and Anderson, 2010; Fox et al., 2004; Baylis, 2009; Bleyer et al., 2000; Foster et al., 2008; Vlassara et al., 2009). It is possible that the relationship between any of these states and age is more dangerous than age-related decline alone, but significantly more work needs to be done in order to understand this concept.

1.3 - Hypertension

Hypertension, or high blood pressure, is defined as a persistent elevation in blood pressure, and can be classed in multiple stages depending on severity (Table 1.1; McArdle et al., 2010).

Stage	Systolic Blood Pressure (mmHg)	Diastolic Blood Pressure (mmHg)
Average range	90-119	60-79
Pre-hypertension	120-139	80-89
Stage 1 Hypertension	140-159	90-99
Stage 2 Hypertension	160+	100+

Table 1.1: Clinical definitions of hypertension, values for systolic and diastolic blood pressure in adult humans (McArdle et al., 2010).

Regardless of its stage hypertension is associated with a number of other comorbidities (Long & Dagogo-Jack, 2011). Whilst there is no specific level of blood pressure where these complications occur, clinical definitions are crucial for patient assessment and treatment (Carretero & Oparil, 2000).

1.3.1 - Causes and Consequences of Hypertension

As there are multiple factors that can contribute to hypertension, the mechanisms that regulate it are equally variable (Hall et al., 2012). Approximately 95% of cases of hypertension are defined as 'primary hypertension', a persistent elevation in blood pressure for which there is no obvious secondary cause (such a mutation in a gene, or renovascular disease). Primary hypertension is a heterogeneous disorder, and there may be multiple, differing influences between patients that cause the disease (Hall et al., 2012). Multiple risk factors have been associated with an increased incidence of hypertension, such as obesity, high alcohol intake, insulin resistance, and sedentary lifestyle (Fuchs et al., 2001; Vaněčková et al., 2014; Soleimani, 2015; Carretero & Oparil, 2000).

The effects of persistent elevation in blood pressure have been well documented, and hypertensive patients are at considerably greater risk for a number of associated comorbidities (Long & Dagogo-Jack 2011). Numerous reviews have cited hypertension as one of the most significant risk factors for cardiovascular and cerebrovascular disease (Viazzi et al., 2013; Gorgui et al., 2014; World Health Organisation, 2013), as high blood pressure is associated with significant increases in organ damage (Schemider, 2010), increased risk of stroke (O'Donnell et al., 2010), encephalopathy and retinopathy (Sandberg & Ji, 2012), myocardial infarction (Firdaus et al., 2008), deterioration of kidney function (Bakris et al., 2009), and diabetes (Jandeleit-Dahm & Cooper, 2002).

1.3.2 - Prevalence

Hypertension places a considerable burden on the global health population. Indeed, it has been cited as the greatest risk factor globally for mortality, with elevated blood pressure believed to be a contributor to 13% of deaths worldwide (World Health Organisation, 2009). In 2012 a systematic review in the Lancet identified that of the 17 million deaths a year due to cardiovascular disease, 9.4 million of these are attributable to complications in hypertension (Lim et al., 2012). Moreover, hypertension contributes to 51% of deaths by stroke, and to 45% of deaths due to heart disease (World Health Organisation, 2013).

In the UK, circulatory conditions (including hypertension and its associated morbidities) were cited as one of the top three expenses of NHS treatments in 2011 (Harker, 2011). But hypertension is not a problem restricted to developed countries, it is also a component of the top two causes of death in in low- and middle-income countries (Kumar, 2013). With global estimates for the prevalence of hypertension increasing to 30% of the total population in 2025 (Lim et al., 2012), it is clear to see that this is a significant economic, environmental, and health burden of the populace.

1.3.3 - Links to Renal Ageing

Prevalence of hypertension increases with age (Kumar, 2013). The 2015 update on heart disease and stroke statistics from the American Heart Association highlighted that of adults over 20 years of age, as many as 36% are hypertensive and of adults over 65 years, this incidence increases to 65% of the population (Mozaffarian et al., 2015). Whilst there are multiple risk factors associated with developing hypertension as mentioned above, a key pathophysiological mechanism is associated with the

declining function of the kidney. Numerous animal models have demonstrated that kidney injury is an effective means of inducing hypertension (Hall et al., 2012). Furthermore, it has been observed that in both clinical and experimental cases of hypertension, renal pressure natriuresis is consistently disturbed, highlighting a pivotal role for this mechanism in the pathology of hypertension (McDounagh et al., 2003; Hall, 2003, Hall et al., 2012).

The percentage of the population that is elderly is increasing steadily with the continued improvements in human lifespan (Zhou et al., 2008), and as such, the prevalence of age-related disease is on the increase (Mathers, 2015). This is also true of renal-specific conditions and hypertension. Functional declines observed in renal ageing as described above (Section 1.2) are not a guarantee of developing hypertension, but there is a known relationship between the two (Brenner, 1983; Duarte et al., 2011). However, the mechanisms linking hypertension and age-related renal decline remain elusive, making future studies into the ageing kidney and hypertension imperative to our successful management of renal dysfunction and hypertension in an ageing population (Rule et al., 2010; Baylis & Corman, 1998).

1.4 - The Renin-Angiotensin System

1.4.1 - Overview

The renin-angiotensin system (RAS) is a well characterised physiological pathway responsible for the regulation of blood pressure. The major effector of the system is the peptide angiotensin II (Ang II), which is produced from an inactive precursor, angiotensinogen (Sparks et al., 2014). The classic pathway of angiotensin II production is demonstrated in Figure 1.2. Physiologically inactive, liver-secreted angiotensinogen is cleaved by kidney-derived renin to form the decapeptide

angiotensin I (Ang I), which is further cleaved by angiotensin-converting enzyme (ACE) to produce Ang II (Lavoie & Sigmund, 2003). The best-documented effects of Ang II are mediated via the angiotensin type 1 receptor (AT_1R; Section 1.4.2), and typically lead to vasoconstriction, secretion of aldosterone, cellular growth and proliferation, sodium reabsorption, and increases in blood pressure (Bosnyak et al., 2011). However, it has been demonstrated more frequently in recent years that the RAS has significant activity in numerous sites of the body and with several other receptors (Skov et al., 2014).

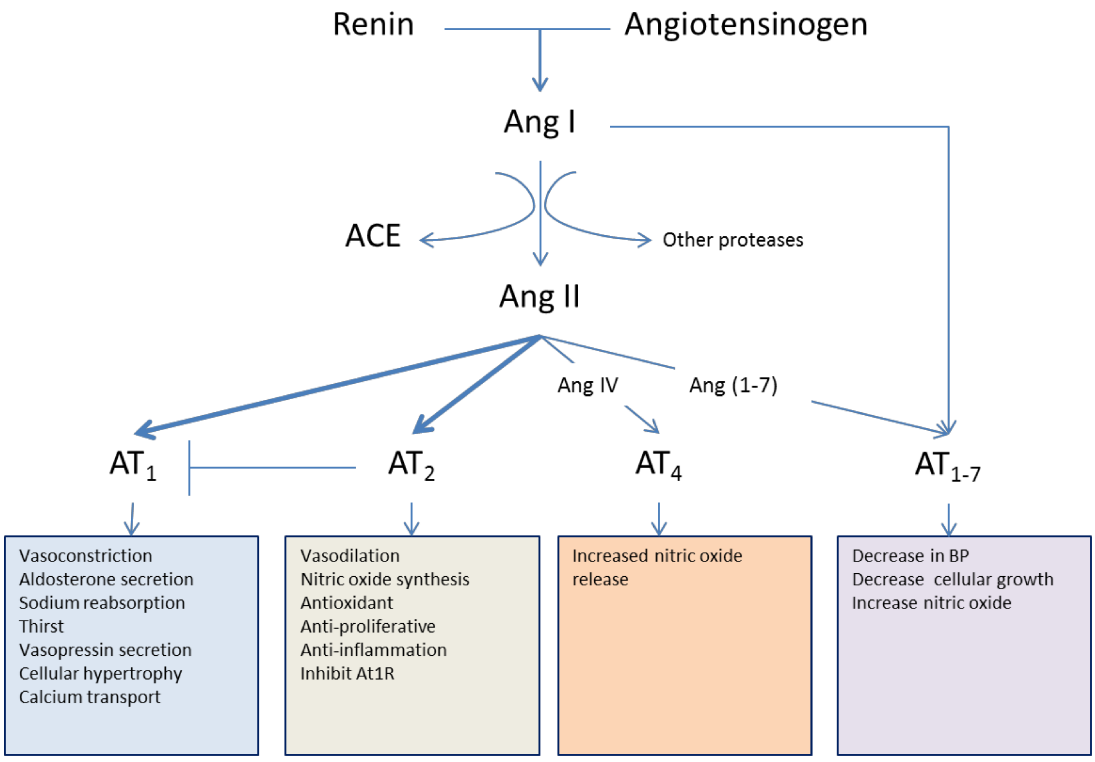


Figure 1.2: The 'classical' pathway of the Renin-Angiotensin System (RAS).

Renin, secreted by the kidney, cleaves liver-produced angiotensinogen to form angiotensin I (Ang I). Ang I is further cleaved by angiotensin-converting enzyme (ACE) and other proteases to form the physiologically active angiotensin II (Ang II). Ang II can either bind with equal affinity to angiotensin type 1 or 2 receptors to elicit physiological changes. It may also be cleaved further to produce Ang IV or Ang (1-7), which both have specific receptors.

Indeed, there are multiple sites in the body where components of the RAS are expressed including the kidney, brain, heart, vasculature, adipose tissue, and the pancreas (Lavoie & Sigmund, 2003; Bader et al., 2001). Furthermore, after its production from Ang I, Ang II may be further cleaved by angiotensin-converting enzyme type 2 (ACE2) to produce smaller physiologically active peptides such as angiotensin (1-7) (Ang (1-7); Santos et al., 2003; Iyer et al., 1998) and angiotensin IV (Ang IV; Bosnyak et al., 2012; Albiston et al., 2001). Where it has previously been described as a peptidergic endocrine system responsible largely for blood pressure regulation, increased awareness of the complexity of the RAS has given rise to a theory of 'local' or 'tissue' RAS (Paul et al., 2006). The ubiquitous expression of numerous RAS components has led to hypotheses citing Ang II, and its derivatives, as potential mediators for other physiological and pathophysiological responses. For example, Skov et al., (2014) propose that tissue RAS is a plausible link between the major predictors of metabolic disease, and as such regulating local RAS expression may prove a valuable clinical target. In addition, it is apparent that other proteases capable of cleaving Ang I to generate Ang II, such as chymase, are not involved in blood pressure regulation (Arakawa & Urata, 2012). Instead, a role has been identified for this pathway in cardiac remodelling (Wei et al., 1999; Paul et al., 2006).

The scope of this thesis focuses on the role of the RAS in blood pressure regulation. However an appreciation of the abundant expression of components of the RAS and the differing physiological actions Ang II may elicit in interacting with other receptors is essential to our deeper understanding of the RAS as a whole.

1.4.2 - Angiotensin Receptors and Their Actions

1.4.2.1 - Angiotensin Type 1 Receptor

As mentioned in Section 1.4.1, Ang II was previously believed to exert its effects through a single receptor, the angiotensin II type 1 receptor (AT_1R) in what is now frequently referred to as the 'classic' RAS pathway (Sparks et al., 2014; Paul et al., 2006). Even now our understanding of the RAS has deepened, the AT_1R is still cited as mediating the majority of physiological actions of Ang II (De Gesparo et al., 2000; Gwathmey et al., 2011; Giani et al., 2013).

The AT_1R is a member of the G-protein coupled receptor family (GPCR) and typically signals through $G_{\alpha q}$-linked pathways involving inositol triphosphate (IP_3), phospholipase C, and calcium signalling (Sparks et al., 2014). In the control of blood pressure, binding of Ang II to the AT_1R has effects on vasoconstriction, aldosterone secretion, water and electrolyte balance, thirst, cellular hypertrophy and vasopressin secretion, all of which can cause an increase in arterial pressure (De Gesparo et al., 2000).

The AT_1R is generally well-conserved among mammalian species, with the rat and human sharing approximately 95% sequence homology (Siragy, 2002). Uniquely, the rat, unlike humans, has two identified subtypes of AT_1R, $AT_{1A}R$ and $AT_{1B}R$ (McMullen et al., 2005). Past studies have demonstrated that the proteins coded for by these two distinct genes are almost identical (Dasgupta & Zhang, 2011). However, it has more recently been shown that they are differentially regulated and expressed in a tissue-specific fashion, suggesting they may differ in functionality (Elton & Martin, 2007). Despite this difference in receptor subtypes between rat and man, the $AT_{1A}R$ and $AT_{1B}R$ are known to elicit similar effects to the single human AT_1R, having been implicated in mediating pressor responses and vasoconstriction (McMullen et al., 2005).

Whilst the potentially detrimental effects of AT_1R over-activity have been well documented (Wang et al., 1997; Atlas, 2007), it has been demonstrated in numerous studies that it is equally pivotal in the maintenance of healthy blood pressure and in development. Extensive research has led to the understanding that the AT_1R is expressed in multiple tissues in the body, including the kidney (Abadir & Siragy, 2015), liver (Hirose et al., 2007), brain (Kishi et al., 2015), pregnant uterus (Yamaleyeva et al., 2013), vasculature (Nyby et al., 2007), and in multiple intracellular locations (Abadir et al., 2012). This abundant distribution of the AT_1R demonstrates its importance in multiple physiological and pathophysiological mechanisms, marking it as a significant target for research and manipulation.

1.4.2.2 - Angiotensin Type 2 Receptor

In early research of the RAS, the angiotensin II type 2 receptor (AT_2R) did not feature a great deal, being largely dismissed as a protein expressed only during early development. However, it is now understood that Ang II binds to the two major angiotensin receptor subtypes AT_1R and AT_2R with equally high affinity (Bosnyak et al., 2011; Horiuchi et al., 2012). Unlike the AT_1R, interaction of Ang II with the AT_2R elicits a vasodilatory response, which has led to speculation that it is a 'counter-regulatory' arm of the RAS (Siragy & Carey, 1997; Matavelli et al., 2011). Furthermore, AT_2R activation has been shown in some studies to antagonise the AT_1R by inhibiting its signalling pathways (Mehta & Griendling, 2006; Munzenmaier & Greene, 1996), suggesting it has a significant role to play in the regulation of RAS activity.

There is still much about the activity of the AT_2R that is unknown. Although it binds Ang II with equal affinity to the AT_1R, its expression in adult tissues is variable (Verdonk et al., 2012). In foetal life, AT_2R expression is at its highest, but this

declines shortly after birth when the AT_1R becomes the dominantly expressed subtype (Grady et al., 1991; Millan et al., 1991; Sechi et al., 1992). Studies in both humans and animals investigating the expression of the AT_2R in adult tissues have identified that it is highly expressed in the brain, liver, heart, vasculature, and kidneys (Yu et al., 2010; Booz & Baker, 1996; Tsutsumi et al., 1998; Baños et al., 2011).

Much like the AT_1R, the AT_2R binds to a G-protein coupled receptor (De Gesparo et al., 2000). However, the signalling pathway is not typical of GPCR interaction, and differs greatly from that of the AT_1R. The angiotensin II type 2 receptor has been shown to have constitutive activity (Miura et al., 2005; Li et al., 2009; Jin et al., 2002). That is, it is capable of eliciting a signalling response in the absence of a ligand binding to the receptor, which implies the level of expression of the AT_2R is associated with the level of activity even in the absence of Ang II (Funke-Kaiser et al., 2010). Activation of the receptor results in a signalling cascade involving phospotyrosine, phosphatase, and mitogen-activated protein kinase (MAPK) (Carey et al., 2000). This pathway causes the inhibition of hypertrophy and fibrosis, but the vasodilatory effects of the AT_2R are triggered by a separate signalling pathway (Li et al., 2012). Abadir et al. (2006) demonstrated effectively that the AT_2R and bradykinin B2 receptor (B2R) undergo receptor heterodimerisation to form a functional signalling unit. This receptor association leads to the activation of nitric oxide synthase via phosphorylation, and the synthesis of cyclic guanosine monophosphate (cGMP), promoting a vasodilation response (Li et al., 2012; Faria-Costa et al., 2014).

The effects of the AT_2R have become a significant topic of research. As stated above, it initiates vasodilation and decreases cell hypertrophy and fibrosis. Alongside this it has been shown to impact cardiovascular remodelling (Xu et al., 2014), reduce inflammation in response to pathological stimuli (Matavelli et al.,

2011), promote natriuresis (Carey & Padia, 2013), and inhibit the sympathetic nervous system (Faria-Costa et al., 2014). This information suggests that improving our understanding of the AT_2R, its physiological actions, and methods of manipulating it in clinical treatments for cardiovascular dysfunction should be an important RAS research target.

1.4.2.3 - Other Angiotensin Peptides and Receptors

Angiotensin II does not only exert effects through its two major receptor subtypes AT_1R and AT_2R. It may also be further cleaved by peptidases to form shorter biologically active peptides such as Ang (1-7) and Ang IV (Santos et al., 2003). These peptides may bind to the AT_2R, though they tend to do so with a lesser affinity than Ang II (Bosnyak et al., 2011), but also to their own cognate receptors (Figure 1.2; Santos et al., 2003; Kalidindi et al., 2007).

Understanding of these peptides and their receptors is limited, but it has been suggested that Ang (1-7) in particular may play a counter-regulatory role to Ang II, much as the AT_2R is proposed to do for the AT_1R (Bosnyak et al., 2012). The first demonstration of Ang (1-7) eliciting vasodilation was in 1993 by Benter et al., but these results have since been repeated in multiple human and animals models and vessel types (Iyer et al., 2000; Ueda et al., 2001; Santos et al., 2003). Additionally, Ang (1-7) has been shown to elicit beneficial effects in kidney, brain, and heart tissue, although there is still much that is not known about its actions (Ferrairo et al., 2010).

The work in this thesis largely relates to the 'classic' pathway of the RAS, and so information on these components will be limited. However, an appreciation of additional influences is essential in furthering understanding of the implications of RAS activation and potential pathways for clinical intervention.

1.4.3 - Manipulating Angiotensin Receptors – Antagonists and Agonists

Dysregulation of the RAS is well documented in cases of hypertension (Atlas, 2007; de Man et al., 2012). As such, it has become a key target in current clinical treatment. The majority of treatment strategies focus on inhibition of the pressor effects of the RAS. These can include inhibition of ACE (ACE inhibitors) or the AT_1R (angiotensin receptor blockers; ARBs), or calcium-channel blockers (CCBs). Many drugs with these capabilities are currently commercially available and are widely prescribed (Michel et al., 2013).

The 2014 evidence-based guidelines from the Eighth Joint National Committee of the American Medical Association for the management of high blood pressure recommended that: "initial antihypertensive treatment should include a thiazide-type diuretic, calcium channel blocker (CCB), angiotensin-converting enzyme inhibitor (ACEI), or angiotensin receptor blocker (ARB)" (James et al., 2014). It is evident from these guidelines, and other studies, that the treatment of hypertension is still highly variable across populations and is in need of continued research.

1.4.4 - Manipulating the AT_1R – Angiotensin Receptor Blockers

Antagonism of the AT_1R is currently one of the most common means of treating hypertension (Michel et al., 2013). Angiotensin receptor blockers (ARBs) are widely prescribed, and prevent increases in blood pressure by irreversibly binding to the AT_1R in place of Ang II (De Gasparo et al., 2000). ARBs are particularly effective in reducing blood pressure by working at the level of the receptor, they prevent AT_1R mediated actions of Ang II regardless of its site of synthesis. That is, other drugs such as ACE inhibitors for example, prevent the synthesis of Ang II, but only when it is generated by the 'classical' pathway. This means that Ang II synthesised by other

proteases may still elicit pressor effects via the AT_1R and reduce the effectiveness of blood pressure reduction using ACE inhibitors (Atlas, 2007). Moreover, AT_1R antagonists do not cause a decrease in circulating Ang II, as the peptide is still synthesised, just unable to bind its receptor. It has been suggested in some studies that this could lead to increased activation of other Ang II receptors, such as the AT_2R, and further enhance blood pressure reductions (Aramugam et al., 2015).

The first ARB developed and released for use as an antihypertensive was Losartan, though it was swiftly followed by 7 other similar compounds now referred to as the 'Sartans' (Kurtz & Kajiya, 2012; Ripley & Hirsch, 2010). There are minor differences between ARBs of the Sartan family, most notably their affinity for the receptor (Siragy, 2002). However, each has been proved to be an effective means of managing hypertension and are generally very well-tolerated medications in hypertensive cohorts (Atlas, 2007).

This thesis focusses on the actions of angiotensin receptors, and whilst AT_1R blockade has proved an effective means of reducing hypertension in many populations, it is not consistently so (Johnson, 2008). Often, administration is required in combination with an ACE inhibitor, and even still, cases of treatment resistant hypertension occur in 10-20% of the hypertensive population (Myat et al., 2012). It is apparent that there is an unmet need in the treatment of hypertension, and new pathways and strategies need consideration to combat this (Oparil & Schmieder, 2015).

1.4.5 - Manipulating the AT_2R - Compound 21

The evident need for additional means of managing hypertension, and the potential of the AT_2R for reducing blood pressure have led to significant interest in the receptor as a pharmacological target (Verdonk et al., 2012). Previously, most

studies involving the AT_2R were centred around antagonism or genetic knockdown (Faria-Costa et al., 2014). The recent development of an AT_2R agonist has allowed for more detailed consideration of the potentially beneficial effects of the AT_2R as a result of its stimulation.

Wan et al., (2004) describe the design and synthesis of Compound 21 (C21), a highly selective, non-peptide AT_2R agonist. With the full chemical name, N-Butyloxycarbonyl-3-(4-imidazol-1-ylmethylphenyl) - 5-isobutylthiophene -2- sulfonamide, it was derived from the non-selective angiotensin receptor agonist L-162,313 (Lenkei et al., 1997), and has a bioavailability of around 20-30% after oral administration in rats. Initial research suggests that the agonist binds with high affinity to the AT_2R, but not to the AT_1R when compared to the reference substances Losartan, an AT_1R antagonist and PD, 123, 319 an AT_2R antagonist (Wan et al., 2004). Details of the relative affinity of C21 for the AT1R and AT2R are presented below in table

Table 1.2 as taken from Wan et al. (2004).

Compound No.	K_i^a (nM)		AT_1/AT_2
	AT_1	AT_2	
21	>10000	0.4	>25000

Table 1.2: Relative binding affinity of Compound 21 for the AT_1R and and AT_2R.

1.4.5.1 - Current Literature

Since the development of C21, there have been multiple studies published utilising it to stimulate the AT_2R. To date, the results have been conflicting. Earlier work with C21 suggested it would be a valuable compound for use in the management of hypertension. Bosnyak at al., (2010) demonstrated effectively that C21 was capable of causing vasodilation in isolated rat vessels. This did not translate into a decrease in blood pressure *in vivo* in conscious spontaneously hypertensive rats (SHR) unless treatment was combined with concomitant AT_1R blockade. However, the decreases in blood pressure could be abolished by treatment with an AT_2R antagonist (PD123319), suggesting that the effects on blood pressure were, at least in part, being mediated by the AT_2R.

Attempts to lower blood pressure in other studies using C21 have yielded similar variable results. In 2013, Brouwers et al., reported that C21 was unable to evoke a renal vasodilatory response or lower blood pressure, even when administered simultaneously with AT_1R antagonists. They did, however, observe a nitric oxide-mediated vasodilation and increase in RBF when C21 was combined with an ACE inhibitor. This effect was only apparent in SHR, not in normotensive Wistar Kyoto (WKY) rats, suggesting that in order for the full beneficial effects of AT_2R agonism to be evident, hypertension (or potentially other pathologies) must be present.

Although the effects on blood pressure have not been dramatic, there have been consistent reports of an improved vascular response after dosing with C21. Gao et al., reported in both 2011 and 2014 that intracerebral infusion of C21 caused a decrease in norepinephrine, lower sympathetic nerve activity, and improved baroreflex sensitivity in brain tissues. Furthermore, this was associated with an increase in expression of eNOS and a decrease in expression of the AT_1R. Similar positive results have been observed in other tissues. C21 has been shown to cause an increase in renal blood flow and a decrease in renal vascular resistance (RVR) in anaesthetised rats. Interestingly, the effects in this study were limited to female animals, suggesting a potentially sex-specific mechanism. Importantly, the effects were reduced or abolished in response to an AT_2R antagonist, providing evidence that effects are being mediated by the AT_2R (Hilliard et al., 2012, 2014).

Other work has highlighted an alternative mode of action for C21. In 2012, Verdonk et al., (2012) utilised *in vitro* preparations of human, rat, and mouse vessels to observe the response to C21. They reported that there were vascular responses, but these were unlike those detailed previously. C21 was shown to induce vasoconstriction, followed by vasodilation. Treatment with an AT_1R antagonist negated the constrictor response, but antagonism of the AT_2R did not abolish the vasodilation response. These data suggested that C21 was causing constriction

through the AT_1R, and further work demonstrated that the vasodilation was linked to the blockade of calcium entry into cells.

Similar results were observed in another study considering C21 and vasodilation. SHR were treated with either C21, or C21 and losartan simultaneously for a six week period. Vascular responses were measured, and it was noted that acetylcholine-mediated (ACh) vasodilation was increased after either treatment. Administration of the NOS inhibitor LNAME (Nω-nitro-L-arginine methyl ester) blunted this effect, but only in treated groups, not controls suggesting that the effects observed were independent of the NO vasodilation pathway associated with the AT_2R (Rehman et al., 2012).

Irrespective of the conflicting data regarding vascular function, numerous studies have reported organ-protective effects of C21, including some of those listed above (Rehman et al., 2012; Hilliard et al., 2014). Kaschina et al., 2008 showed that C21 dosing improved outcomes post-myocardial infarction in rats. Ventricular function was increased in treated animals, and markers of inflammation and apoptosis were significantly decreased in response to C21. A separate study demonstrated that C21 was also capable of reducing brain damage by decreasing and preventing inflammatory cell infiltration, effects that were abolished by inhibiting the AT_2R (Gelosa et al., 2009).

It is clear from these studies that C21 has significant potential in cardiovascular and renal pathologies among others. However, there is still considerable uncertainty regarding its effects. In particular, the results presented by Verdonk et al., demonstrating the possibility of AT_1R binding C21 suggest that in order to preserve AT_2R specificity, doses of C21 need to be reconsidered and perhaps administered at much lower levels. It is clear that more research is needed into the effectiveness of C21 and its applications.

1.4.6 - The Ageing Renin-Angiotensin System

As with all other systems in the body, the RAS undergoes significant changes in expression, activity, and distribution with age (Tareen et al, 2005). Understanding these alterations may be critical to continued success in pharmacologically managing RAS-induced kidney dysfunction and hypertension (Turgot et al., 2010). It has been well-documented that elements of the RAS, in particular secretion of Ang II and activity of the AT_1R, are increased with age in numerous tissues and organs (Wang et al., 2014; Basso et al., 2005; Wirth et al., 2015). This RAS overactivity has been linked to age-dependent hypertension (Yoon et al., 2014), vascular dysfunction (Wirth et al., 2015), and the development of atherosclerosis (Weiss et al., 2001). In addition, expression of the AT_2R is at significantly lower in aged animals, minimising the counter-regulatory effects of this arm of the RAS (Tareen et al., 2005).

Typically, ageing involves accumulation of damage at the cellular and thus whole tissue level. The most commonly proposed mechanism for this deterioration is an increased generation of reactive oxygen species (ROS), which inflict damage on cellular components through reactions with the plasma membrane (Sastre et al., 2000). Numerous studies have demonstrated that the RAS is less tightly regulated with age, and Ang II production can be elevated (Herbert et al., 2008; Gilliam-Davis et al., 2007). Furthermore, angiotensin II, acting through the AT_1R, has been linked with increased generation of ROS (Conti et al., 2012). Increased Ang II release seen in aged animals results in activation of NADPH oxidase and the subsequent generation of superoxide anion, which perpetuates ROS production and cellular damage (Conti et al., 2012). This effect has been observed on multiple occasions, and can be ameliorated by dosing with AT_1R blockers such as Losartan (Gilliam-Davis et al., 2008).

1.5 - The Nitric Oxide System

1.5.1 - Overview

Nitric oxide (NO) is an important signalling molecule with a role in many aspects of physiological function and plays several critical roles in the immune, renal, and cardiovascular systems (Donald et al., 2015). NO was first described in 1980 by Furchgott & Zawadzki, though it was at the time identified as 'endothelium-derived relaxing factor' (EDRF). Whilst there was significant interest in this molecule and its physiological effects, it was not until 1988 that it was identified as nitric oxide (Furchgott, 1988).

NO is known to elicit a number of physiological effects, but is most notably a potent vasodilator (Donald et al., 2015; Moncada & Higgs, 2006; Bryan et al., 2009). The largest site of endogenous NO production is the cells of the endothelium (Buchwalow et al., 2002), and as such it can be found in multiple locations in the body. As NO has an extremely short half-life ($<2ms^{-2}$), it is usually produced directly in the locations it is required to exert its effects (Hill et al., 2010). When released, nitric oxide has a number of potential fates, but the majority of actions of NO are the result of a 3', 5'-cyclic monophosphate/protein kinase G signalling pathway (Donald et al., 2015). For example, in mediating vascular relaxation NO binds to the enzyme soluble guanylyl cyclase (sCG) which increases cGMP production. Elevated cGMP activates cyclic GMP-dependent protein kinase (PKG), the major effector enzyme for initiating muscle relaxation. PKG reduces extracellular Ca^{2+} entry, inhibits Ca^{2+} release from the sarcoplasmic reticulum, and desensitises myofilaments to Ca^{2+}, leading to a reduction in vascular tone (Gao, 2010).

Whilst the reactions with sCG and other metalloproteins are those with the highest affinity (Hill et al., 2010), NO may also react with free radicals such as superoxide to

form peroxynitrite (Donald et al., 2015). The generation of peroxynitrite is a significant pathogenic mechanism in a number of conditions such as stroke, shock, and chronic inflammation, and the simultaneous production of superoxide with NO can have deleterious consequences (Pacher et al., 2007). Under normal physiological conditions, peroxynitrite formation is at a low rate, and is believed to be responsible for increased production of prostanoid via activation of its synthetic enzyme (Gao, 2010). In pathophysiological conditions the production of peroxynitrite (and other NO derived radicals) increases significantly and can cause irreversible cellular damage. This duality of NO in its ability to mediate beneficial physiological responses as well as damaging pathological ones make understanding its actions in the body a key area of research (Förstermann & Münzel, 2006).

1.5.2 - Nitric Oxide Synthase

Nitric oxide is endogenously produced in a reaction mediated by the enzyme now referred to as nitric oxide synthase (NOS; Moncada et al., 1989). NOS appears in three isoforms in the human body, each named as a result of the location from which it was first purified:

- nNOS/NOS-I (neuronal NOS)
- iNOS/NOS-II (inducible NOS)
- eNOS/NOS-III (endothelial NOS)

(Ducsay & Myers, 2011)

However, understanding of NOS has increased substantially since its discovery, and it is now well accepted that the expression of the synthases is far more ubiquitous than was previously recorded (Michel & Feron, 1997).

1.5.2.1 - Neuronal Nitric Oxide Synthase

As the name suggests, nNOS was first identified in neuronal cells, and is constitutively expressed in the brain (Förstermann & Sessa, 2012). However, it is now understood that nNOS has the greatest expression in skeletal muscle (Tengan et al., 2012; Förstermann & Sessa, 2012), and is also found in parts of the cardiovascular system, such as cardiac myocytes and coronary artery smooth muscle cells (Zhang et al., 2014). The physiological actions of nNOS are varied; in the central nervous system it is known to regulate synaptic transmission (Izumi et al., 1992) and is involved in neurogenesis (Zhou & Zhu, 2009). In the cardiovascular system, it has been implicated in central blood pressure regulation (Sakuma et al., 1991), and may decrease sympathetic tone in the vasculature (Förstermann et al., 1994).

1.5.2.2 - Inducible Nitric Oxide Synthase

Inducible nitric oxide synthase differs slightly from the two other known isoforms of NOS in that it is not entirely constitutively expressed. Instead, it is mostly induced by cytokines, bacterial lipopolysaccharides, and other inflammatory mediators (Förstermann & Sessa, 2012). In fact, iNOS can be produced in almost any tissue providing the correct mediators are present (Förstermann et al., 1994). It is unsurprising then that iNOS functions are largely related to immune response. In a number of pathologies, such as parasitic infection and tumour, iNOS expressed in macrophages is known to mediate cytotoxic effects by producing copious quantities of NO (Nathan & Hibs Jr, 1991). Additionally, iNOS plays a substantial role in sceptic shock, which is characterised by rapid vasodilation and hypotension (Lange et al., 2009).

1.5.2.3 - Endothelial Nitric Oxide Synthase

Endothelial nitric oxide synthase is the isoform of NOS that is believed to be EDRF. It is produced, as the name suggests, predominantly in endothelial cells and is responsible for a catalogue of vascular effects (Förstermann & Sessa, 2012). Not only is eNOS involved in vasodilation, it is also responsible for preventing vascular inflammation, stimulating angiogenesis, and in the control of smooth muscle cell hypertrophy (Michel & Feron, 1997; Fukumura et al., 2000; Murohara et al., 1998). Deficiency of eNOS has been linked to a number of pathologies, including hypertension (Huang et al., 1995), aortic valve sclerosis (El Accaoui et al., 2014) diabetic nephropathy (Takahashi & Harris, 2014), and cardiac hypertrophy (Liu & Feng, 2012).

1.5.3 - The Endogenous Synthetic Pathway

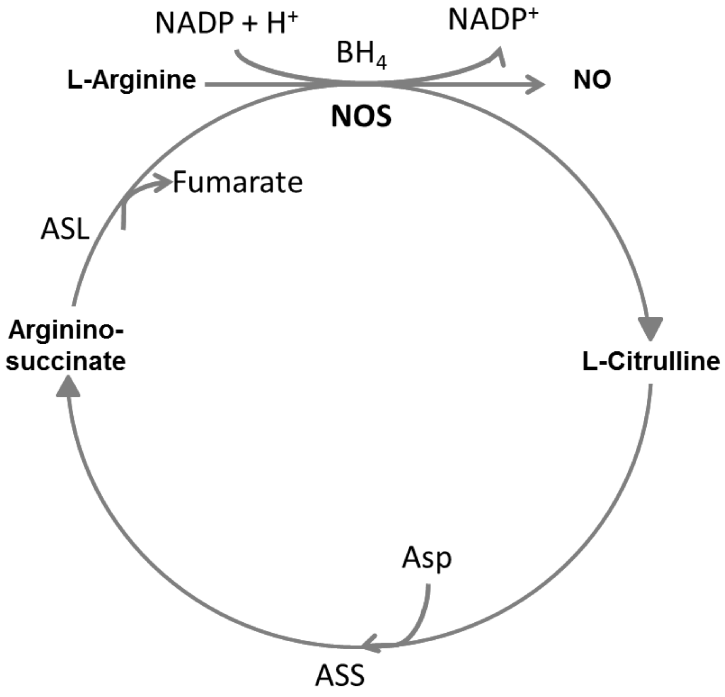


Figure 1.3: Simple representation of the endogenous nitric oxide synthesis pathway. L-arginine is oxidised by nitric oxide synthase (NOS) to form L-citrulline. The reaction requires the cofactor tetrahydrobiopterin (BH4), and the oxidation of

NADP, and causes the release of nitric oxide. L-citrulline can be used to resynthesize L-arginine in a two-step reaction mediated by the enzymes argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL), with the intermediate production of arginino-succinate. In the presence of all of the appropriate cofactors, the reaction can cycle continuously. Adapted from Wu & Morris (1998) and Li et al. (2001).

The endogenous production of nitric oxide (Figure 1.3) by NOS is a fairly complex process, but in the presence of all necessary substrates and cofactors, is a catalytically self-sufficient reaction (Bryan et al., 2009). Unlike most other enzymes, NOS requires as many as five cofactors or prosthetic groups to be bound in order for the catalysis to occur (Knowles & Moncada, 1994). In the presence of these factors, NOS may produce NO by catalysing an electron oxidation of L-arginine. Regardless of the isoform of NOS in question, this reaction occurs in two steps. In the first, L-arginine is hydroxylated to N^{ω}-hydroxy-L-arginine. Following this, the N^{ω}-hydroxy-L-arginine is oxidised by NOS to produce L-citrulline and NO (Förstermann & Sessa, 2012). This basic representation of the synthesis pathway is consistent across NOS isoforms and tissues, as depicted in Figure 1.3.

It has come to light in recent years that NO may also be formed in a second pathway, the nitrate-nitrite-NO pathway (Wobst et al., 2015). When produced in the body, NO can be rapidly oxidised to form stable metabolites nitrite and nitrate (Mian et al., 2013). It was not until 1995 that it was suggested nitrite and nitrate had any activity after their production. Zweier et al. (1995) identified NOS-independent synthesis of NO, and demonstrated that instead NO was being generated by nitrite in the ischaemic heart. Since then, it has been established that both nitrate and nitrite may be serially reduced to produce NO (Weitzberg et al., 2010), and evidence suggests that this pathway is activated in instances of limited oxygen (required for NOS-mediated production of NO) such as hypoxia (Weitzberg et al., 1998).

The capacity of the body to synthesise NO in multiple ways highlights its importance as a signalling molecule, and offers significant potential for manipulation of the NO system.

1.5.4 - Nitric Oxide and the RAS

The RAS is one of the major effector systems in cardiovascular function, and is closely linked with the NO system. The AT_2R, which has been identified as the 'regulatory arm' of the RAS, responsible for vasodilation (Section 1.4.2.2) is known to mediate a number of its effects through a NO signalling cascade (de Gasparo, 2002; Li et al., 2012). Studies utilising NOS inhibitor Nω-nitro-L-arginine methyl ester have been able to abolish AT_2R mediated vasodilation, confirming the pivotal role NO plays in this receptor's activity (Batenburg et al., 2004). In addition, NO release as a result of AT_2R stimulation is known to down-regulate expression of the AT_1R, the pressor arm of the RAS (Section 1.4.2.1; Ichiki et al., 1998).

At the other end of the spectrum, over activity of the AT_1R is associated with decreases in NO bioavailability, an effect that can be ameliorated by AT_1R and ACE blockade (Lemay et al., 2000; Dzau, 2001). Moreover, upregulation of the AT_1R is linked to increased superoxide production, which can lead to elevated peroxynitrite, a harmful free radical in high concentrations (de Gasparo, 2002). It is clear that NO and the RAS are closely linked, and has been suggested that the contradictory effect of differing Ang II receptors on NO production and activity are part of a coordinated regulation of kidney function (Kopkan & Cervenka, 2009). Imbalance of these systems and their components are likely fundamentally linked to a number of renal and vascular pathologies (Försetermann & Sessa, 2012).

1.5.5 - Age-Related Changes in the Nitric Oxide System

The bioavailability of NO is significantly altered with age, and this has been demonstrated on numerous occasions (Erdely et al., 2003; Baylis, 2005). This is due in part to a decrease in eNOS activity and expression (Novella et al., 2013) but it may also be linked to a shift in the ratio between eNOS and iNOS favouring iNOS. This elevated iNOS status results in an increase in the inflammatory effects of NO (Cau et al., 2012). Additionally, advancing age is linked with increases in NADPH and superoxide, both of which may increase oxidation of NO to form the harmful nitrogen radical species, peroxynitrite (Pacher et al., 2007). In some rat models, circulating asymmetric dimethyl arginine (ADMA), an endogenous NOS inhibitor, has been seen to be increased, suggesting a further pathway for the age-associated reductions observed in NO (Xiong et al., 2001).

The effects of decreases in nitric oxide have been well characterised. NO deficiency has been linked to endothelial dysfunction (Dobutović et al., 2011), increased blood pressure (Klinger et al., 2013), increased cardiovascular risk (Sverdlov et al., 2013), increased inflammation (Korish, 2009), atherosclerosis (Kawashima & Yokoyama, 2004), and many other pathologies. Moreover, nitric oxide is known to inhibit mesangial cell growth and renal vasoconstriction, two factors that are significantly involved in age-related kidney deterioration (Weinstein & Anderson, 2010; Baylis, 2005). Understanding and regulating the decline of NO and other components of the NO system in ageing may be a viable target for future pharmacological intervention.

1.6 - Sex Differences in Physiology

Male and female sex steroids have significant effects on physiological systems, aside from their normal functions as part of the reproductive system. Both ovarian steroids and androgens are capable of mediating gene expression (Piccinato et al.,

2013; Nickols & Dervan, 2012), and they have been shown to impact numerous processes including immune response (Lamason et al., 2006), brain ageing and mitochondrial function (Gaignard et al., 2015), and cancer progression (Li et al., 2012).

Whilst there are multiple physiological systems that demonstrate sex-specific differences in function, of particular interest in this thesis are those relating to cardiovascular, renal, and vascular function. As mentioned above, hypertension is a significant health burden in the global population (Lim et al., 2012), however the prevalence with which it occurs in males and females is vastly different. It has been well-documented in humans, and other mammals, that females have significantly lower blood pressure than males and the prevalence of hypertension in pre-menopause females is significantly lower than in males (Maric-Bilkan & Manigrasso, 2012). Upon reaching menopause, however, female blood pressure is very rapidly increased and often exceeds that of age-matched males (Yanes & Reckelhoff, 2011). Moreover, this is linked to an increased mortality associated with female cardiovascular incidents though the mechanisms for the progression of such cases are not understood (Miller & Best, 2011).

Likewise, renal function is significantly affected by sex as the functional capacity of the kidneys changes over the lifespan (Weinstein & Anderson, 2010; Brenner et al., 2012). Numerous studies have demonstrated that male humans and animals have a higher incidence of kidney dysfunction, and accelerated progression of renal disease (Neugarten & Golestaneh; 2013). For example, Werner et al. (2013) demonstrated that in patients where there was no overt pathology (such as diabetes), declines in renal function we significantly greater in men, and likely to result in chronic kidney disease. Work in animals has yielded similar results, it was observed in the Fischer-344 rat model of ageing that male animals experienced greater decline than female animals and higher levels of renal injury (Sasser et al.,

2012). These are just two specific examples from a well-reported field, differences between male and female renal function have been observed at different ages and in response to renal injury or disease (Kang et al., 2004; Gross et al., 2004; Hodeify et al., 2013; Short & Smyth, 2015).

The mechanisms driving these differences are still under discussion. Some studies have cited a beneficial effect of oestrogen and ovarian steroids. Satake et al. (2008), for example, demonstrated effectively that administration of oestradiol reduced ischaemic renal injury in rats, and such results have been obtained many times (Kher et al., 2005; Wang et al., 2006, Wang et al., 2009). In contrast, a number of experiments have demonstrated a lack of effect of ovarian steroids, a particularly good example of which is hormone replacement therapy (HRT). The Women's Health Initiative Studies and Heart and Estrogen/progestin Replacement Study have found that reintroducing ovarian steroids via HRT does not improve cardiovascular outcomes (Herrington, 1999; Roussouw et al., 2002; Yanes & Reckelhoff, 2011). Furthermore, other studies have identified a negative influence of androgens, marking it as a 'pro-hypertensive' hormone (Dubey et al., 2002). Studies such as those by Reckelhoff et al. (2000) reveal that castration of male rats effectively prevents or abolishes hypertension in SHR. Alongside this, Yanes et al. (2009) reported that testosterone supplementation in rats increased renal injury and blood pressure, and upregulated parts of the RAS. Current opinions on the mechanisms driving sexual dimorphism are varied, and evidence is available to implicate both families of sex steroids in mediating physiological responses. It is clear that much work still needs to be done to truly understand the effects of reproductive hormones on cardiovascular and renal health.

1.6.1 - Sex-Specific differences the RAS

The renin-angiotensin system also shows differences in function in relation to sex, and has been cited on numerous occasions as a potential mediator of some of the sex-specific effects seen in blood pressure (Maric-Bilkan & Manigrasso, 2012). Indeed, there are several examples of studies in which the RAS is shown to be differentially regulated depending on sex. A good example of this is provided by a review by Chappell et al. (2008) considering the protective effects of oestrogens in multiple studies of the mRen2.Lewis rat. The mRen2.Lewis rat is a transgenic rodent model, where a genetic variant of the renin gene causes severe hypertension. The review in question shows a clear relationship between the RAS and oestrogen, and argues that whilst oestrogen alone may be too simplistic an explanation for the complex sex-specific responses of the cardiovascular system, there is likely a regulatory role for oestrogen in the RAS (Chappell et al., 2008).

Furthermore, studies have shown there is differential expression of components of the RAS in males and females. Typically, female animals express higher quantities of the AT_2R, the angiotensin receptor associated with decreases in blood pressure and favourable renal outcomes, than male animals (Hilliard et al., 2013; Baiardi et al., 2005). Additionally, Sampson et al., (2008) demonstrated for the first time that dosing with Ang II could prompt a decrease in blood pressure in female animals, but an increase in blood pressure in males, and this was likely mediated by the AT_2R in the females. Finally, testosterone has been shown to upregulate the pressor arm of the RAS (Hilliard et al., 2013; Chen et al., 1992). It is clear that there is significant potential in the RAS as a mechanistic pathway for the sex-specific regulation of blood pressure.

1.6.2 - Sex-Specific differences in NO

A further system open to modulation by the sex steroids is the nitric oxide system. As detailed above, NO mediates a number of crucial renal and cardiovascular functions, and concentrations of NO can have significant effects on heath (Baylis, 2001). It is now well-established that NO bioavailability is significantly affected by sex, with both female humans and animals having higher concentrations than males (Loria et al., 2014; Sullivan et al., 2010). Moreover, there is clear evidence to suggest that NOS expression is significantly different between males and females, particularly in the kidney (Erdely et al., 2003; Baylis 2005). It was recently demonstrated that favourable BP outcomes are associated with increased inter medullary NOS, and this is highly influenced by sex steroids (Sasser et al., 2015).

However, much like the RAS, there is still debate as to the effects of oestrogens on the NO system. In studies considering post-menopausal women, subjects continued to present with improved kidney health and circulating NO when compared with males, despite a lack of ovarian steroids or use of HRT (Ahmed et al., 2007). This evidence suggests that it would be negligent to not consider that androgens may be mediating effects on renal health with age. The results of studies considering the effects of androgens on the NO system have been conflicting. Testosterone has been shown to mediate vasodilation as well as vasoconstriction in different parts of the vasculature (Baylis, 2012; Orshall & Khalil, 2004; Liu et al., 2003). Moreover, very recent studies by Perusquia et al. (2015) have shown a systemic NOS-dependent hypotensive response to testosterone dosing, suggesting that androgens may exert beneficial effects on vascular function. Both androgen and oestrogen receptors are well-expressed in the cardiovascular and renal systems (Baylis, 2012), and a great deal more work is required to separate the effects of both of these steroids on the endogenous synthesis and activity of NO.

1.6.3 - The 'Protective Gender' Hypothesis - Fact or Fiction?

The clear differences between male and female animals and humans have been a significant topic of research for many years now. The apparent positive outcomes in female animals have been attributed on numerous occasions to a 'protective effect of female gender' (Shaw & Protheroe, 2012; Stenvinkel et al., 2002; Claasen et al., 2012). This is the idea that up until menopause, females are, to a certain extent, protected from cardiovascular and renal injury, and this effect is the result of ovarian steroids. Certainly, a great many studies have provided evidence that supports this theory (Maric-Bilkan & Manigrasso, 2012; Chappell et al., 2008; Erdely et al., 2003). However, in recent years this central hypothesis in sexual dimorphism in physiology has been questioned. Studies such as those by Reckelhoff et al (2000), the results from the HERS trial (Herrington et al., 1999), and the Women's Health Initiative Study (Roussouw et al., 2002), have suggested that ovarian steroids are not mediating the apparently protective effect of female gender. Indeed, further investigations have cited a deleterious effect of androgens in place of a beneficial effect of ovarian steroids (Reckelhoff et al., 1994, Baltatu et al., 2002; Maranon & Reckelhoff, 2013). Where previously the argument was straightforward; female steroids are beneficial, male steroids are damaging, it now seems a more dynamic hypothesis is required. A single sex hormone argument may be too simplistic to adequately explain the sexual dimorphism in renal and cardiovascular outcomes, and there is much we do not know about differential physiological regulation in males and females.

1.7 - A Role for Animal Models

1.7.1 - Relevance

Animal models of hypertension have been frequently utilised in blood pressure research and have provided a valuable insight into the pathophysiology of the condition (Ganten et al., 2012; Campbell & Henry, 2013). Whilst there are inevitable limitations to translation of animal research into human studies, animal models of hypertension have allowed for the study of isolated factors that contribute to and cause this multifactorial and prevalent disease (Lerman et al., 2005). Indeed, the complicated nature of human hypertension creates a requirement for experimentally modifiable animal models of hypertension, as numerous factors act to confound human cohort studies (Ganten et al., 2011).

There are many different methods of inducing hypertension in an animal model, some of which are listed in Figure 1.4. The multifactorial nature of hypertension makes effectively modelling the condition challenging, however Lerman et al. (2005) identified that the ideal animal model should have “human-like” physiological responses, disease progression, and cardiovascular anatomy.

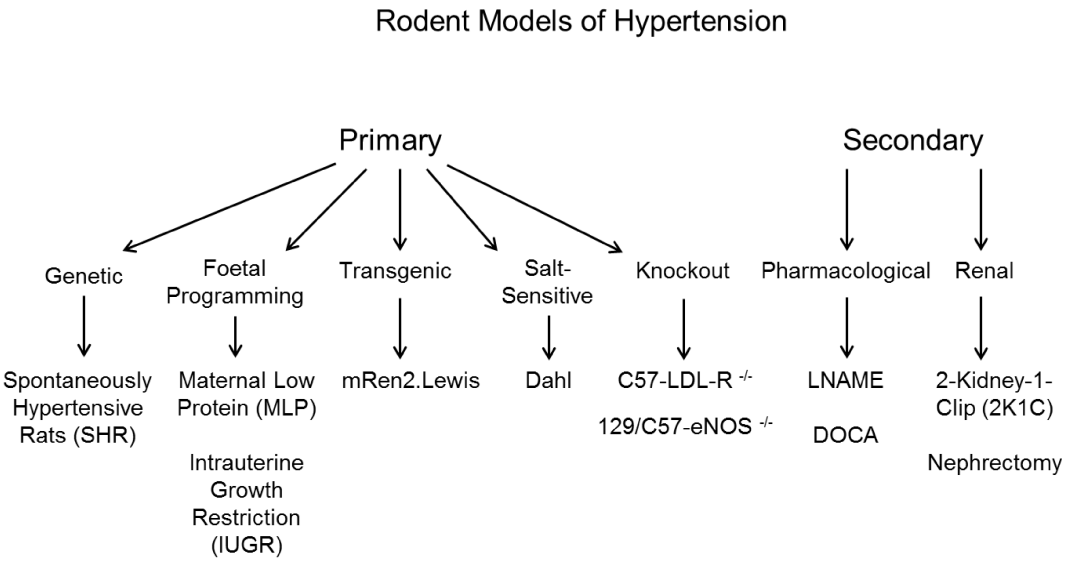


Figure 1.4: Examples of major rodent models of hypertension (Sandberg & Ji, 2012; Sun & Zhang, 2005).

1.7.2 - Foetal Programming of Hypertension

This thesis utilises a 'foetal programming' model of hypertension. Foetal programming is the idea that exposure to adverse conditions or certain stimuli during gestation can permanently 'programme' physiological outcomes in the offspring (Lucas et al., 1999). The hypothesis was first made prominent by the work of Barker et al., who demonstrated that decreased birth weight or size is associated with an increased propensity for dyslipidaemia, hypertension, and ischaemic heart disease (Barker, 1992). The programming field has expanded rapidly over the past two decades, but some of the most frequently cited examples remain the same. The offspring of the Dutch Hunger Winter are an often used cohort. In 1944, political and economic factors lead to a famine in the Western region of the Netherlands, resulting in people, including pregnant mothers, receiving as little as 400-800 calories per day. Although tragic, this has led to a full cohort of human adults exposed to a severe gestational insult, and has resulted in significant improvements in our understanding of the developmental origins of adult disease (Schultz, 2010).

This work has expanded into animal models. Rodents in particular have been well-utilised owing to their relatively short gestation period, large litter size, and short lifespan (Zhodi et al., 2014). Experiments in animals have shown that nutrient deficiency or excess, restricted growth, hormone exposure, and stress have significant negative health impacts on the phenotype of the adult offspring (de Brito Alves et al., 2015; Boubred et al., 2015; Seckl & Holmes, 2007).

1.7.2.1 - The 'Maternal Low Protein' Model

The experimental work in this thesis adopted a well-established model of foetal programming. The maternal low protein model was first developed in the 1990's by Langley & Jackson (1994), and is based on the premise that feeding a pregnant rat a moderately protein-restricted diet during gestation results in offspring with decreased renal function and hypertension. Since its inception, the model has been utilised in numerous studies as a tool to study developmental programming, renal function, and hypertension (Ashton et al., 2007; Yuasa et al., 2015; Chasaka et al., 2015; Goyal et al., 2015).

The effects of maternal low protein (LP) on offspring have been well documented, and are significant in their distribution. The earliest observations pertaining to this model were that offspring exposed to a LP diet during pregnancy presented with persistently elevated blood pressure (Langley & Jackson, 1994), and this effect was replicated on multiple occasions (Langley-Evans et al., 1999; Sahajpal & Ashton, 2003; McMullen et al; 2005). This was followed by studies demonstrating that these animals also had decreased renal function (Nwagwu et al., 2000) and reduced nephron numbers (Habib et al., 2011).

Since the first experiments using the low protein model, many studies have discovered additional effects of prenatal protein restriction on offspring. Not only do

animals have poorer renal outcomes and higher blood pressure, LP offspring have been shown to develop endothelial dysfunction (Torrens et al., 2003). This was reflected in recent work by Chisaka et al. (2015) who demonstrated that LP offspring had a significant increase in cell proliferation in the vasculature, and this was associated with increased markers of inflammation and oxidative damage. Whilst neither of the studies mentioned produced a hypertensive phenotype in the offspring, both clearly demonstrated a profound effect of LP programming on the vasculature, which could be a significant mechanism for later renal injury. In addition, such effects of the vasculature of LP offspring have been reported on multiple occasions (Brawley et al., 2003; Torrens et al., 2009).

The vasculature is not the only element of the cardiovascular system susceptible to programming by the LP model. Studies have shown that prenatal exposure to a low protein diet can result in significant effects on the heart. A number of experiments examining isolated rat hearts have shown that LP offspring are prone to poorer recovery and increased oxidative injury in the heart after myocardial ischaemia-repurfusion (Elmes et al., 2007; Elmes et al., 2008). Moreover, these differences occurred in some studies in a sex-specific manner, where LP male offspring showed programmed cardiac sympathetic activity in comparison to their female and NP counterparts (Elmes et al., 2009), and this was likely linked to a programmed alteration in adrenergic receptor expression (Ryan et al., 2012).

This is not the only instance in which a sex-specific effect of programming has been observed. McMullen et al. (2005) demonstrated that hypertension in male and female offspring exposed to a LP diet during gestation could not be ameliorated via the same anti-glucocorticoid treatment, suggesting a fundamental difference in the mechanism by which the hypertension was occurring. Furthermore, some studies have shown that females are relatively protected from programming of hypertension (Woods et al., 2005) and do not present with the same hypertensive phenotype as

the male animals. In addition, a recent study by Goyal et al. (2015) showed that female mice were relatively protected from programming of hypertension, and that this was associated with a change in ACE2 expression.

Other examples of programming effects on the RAS have been well documented. In particular, expression of the AT_2R has been influenced by LP diets. McMullen et al. (2004) showed that LP exposed offspring had significantly lower AT_2R expression than their NP counterparts at 10 weeks of age. Pijacka et al. (2015) demonstrated that this effect was reversed in older animals, LP exposed offspring at 18 months of age had significantly higher AT_2R expression than NP offspring, and this was possibly a response to an observed increase in markers of oxidative damage.

The literature base regarding the low protein model is substantial and many more examples of deleterious effects of maternal low protein could be included in this review. However, detailed above are just some of the previously observed effects of the model used within this thesis, on elements pertinent to the hypothesis of this project. It is evident that the maternal low protein model has significant potential to assess the sexually dimorphic nature of renal and cardiovascular function with age, with particular focus on the renin-angiotensin and nitric oxide systems.

1.8 - Summary and Hypotheses

Previous evidence suggests that the protective effect of female gender is mediated by an interaction of oestrogen with the renin-angiotensin and nitric oxide systems (Maric-Bilkan & Manigrasso, 2012; Chappell et al., 2008). Furthermore, it has been demonstrated that NO bioavailability decreases with age and may be associated with age-related renal injury (Erdely et al., 2003; Baylis, 2005). Females have been shown to have a higher abundance of NO and nitric oxide synthases (NOS) than males (Loria et al., 2014). Moreover, endothelial NOS is stimulated by oestrogen,

and ovariectomy can negate some of the protective effects seen in female animals. Work from this laboratory has demonstrated increased expression of the renal type 2 angiotensin receptor (AT_2R) in females, and a significant up regulation of this receptor in females but not males subsequent to the onset of developmentally programmed hypertension (McMullen & Langley-Evans, 2005). Whilst poorly understood, the AT_2R is known to be oestrogen responsive and capable of causing vasodilation. The scope of this PhD can therefore be described in a three main objectives.

Objective 1: To characterise the expression of the nitric oxide system during normal ageing, and in a model of developmentally programmed hypertension and accelerated renal ageing.

Objective 2: To explore the modulation of sex steroid exposure through gonadectomy surgery and the effect this has on nitric oxide distribution.

Objective 3: To investigate the hypothesis that the protective effect of female sex steroids is mediated by angiotensin type II receptor regulated nitric oxide activity.

The primary hypothesis of this thesis is that up-regulation of the type 2 angiotensin receptor acts to ameliorate the progression of renal injury and hypertension via the activation of nitric oxide, and that this is an oestrogen-dependent phenomenon. Using *in vivo, ex vivo*, and laboratory methods, this project will attempt to identify a mechanistic pathway for the sex-specific effects observed in renal ageing.

Chapter 2 - Materials and Methods

This thesis has been produced from data generated during three animal trials. This chapter will describe the core methods utilised, including a detailed breakdown of the animal work. Individual experimental protocols will be included for the appropriate trials.

2.1 - Animal Work

All animal experiments were performed in accordance with the 1986 Animals (Scientific Procedures) Act under Home Office licence. The study was approved by the UK Home Office and University of Nottingham Ethics Committee. All experiments were performed within the University of Nottingham Bio-Support Unit (BSU; Sutton Bonington Campus, Leicestershire). Animals were procured from Charles River Laboratories (Harlow, England).

2.1.1 - Breeding and Maintenance

Virgin female HSD/Han Wistar rats (180-200g) were mated and conception confirmed by the presence of a semen plug. Females were then randomly allocated to receive one of two isocaloric diets; normal protein (NP) 18% casein, or low protein (LP) 9% casein, for the duration of gestation as described previously (McMullen et al.,. 2005) (Section 2.1.1 -). At birth mothers were switched to a standard laboratory chow diet, the composition of which is noted in Section 2.1.2 - , pups were weighed and litters standardised to 8 offspring to minimise nutritional variation during suckling. Offspring were weaned at 3 weeks of age, maintained on a standard laboratory chow diet and weighed weekly for the remainder of the trial. Animals were microchipped at weaning for identification (AVID MUSICC Chip

Identification System, UK) and pair-housed. Holding rooms were sustained at 21°C (± 2°C) and at 55% humidity (±10%), with a 12 hour light/dark cycle.

2.1.2 - Animal Diet Composition

Table 2.1, Table 2.2, Table 2.3, and Table 2.4 show the detailed compositions of diets used in all animal trials for this PhD. For basic animal maintenance animals were provided with the 2018 Teklad Global 18% protein rodent diet, the macronutrient information for which is in Table 2.1

Macronutrient	Percent Composition
Crude Protein	18.6%
Fat (ether extract)	6.2%
Crude Fibre	3.5%
Energy Density	3.1kcal/g 13.0kJ/g
Calories from Protein	24%
Calories from Fat	18%
Calories from Carbohydrate	58%

Table 2.1: 2018 Teklad Global 18% Protein Rodent Diet (Harlan Laboratories, UK) macronutrient composition.

Pregnancy diets were prepared in house at the University of Nottingham BSU. The complete nutrient content of both the 'normal' protein and low protein diets can be found in Table 2.2.

Component	Weight (g)	
	Normal Protein (18% casein)	Low Protein (9% Casein)
Casein	180	90
Starch	425	485
Sucrose	213	243
Cellulose	50	50
Corn oil	100	100
Mineral mix (AIN-76)	20	20
Vitamin mix (AIN-76)	5	5
Choline chloride	2	2
Methionine	5	5
Total:	1000	1000

Table 2.2: Composition of complete rodent pregnancy diets. Vitamin and mineral mixes were prepared according to the American Institute of Nutrition AIN-76A formulation (AIN, 1977; Langley & Jackson, 1994).

Mineral	Weight (g)
Calcium phosphate dibasic	500.00
Sodium chloride	74.00
Potassium citrate	220.00
Sucrose	118.03
Potassium sulphate	52.00
Magnesium oxide	24.00
Ferric citrate	6.00
Manganese chloride	3.50
Zinc carbonate	1.60
Chromium potassium sulphate	0.55
Copper carbonate	0.30
Potassium iodate	0.01
Sodium selenite	0.01
Total:	1000

Table 2.3: Composition of mineral mix used in rodent pregnancy diets, AIN-76A formulation.

Vitamin	Weight (g)
Thiamine hydrochloride	0.60
Riboflavin	0.60
Pyridoxine hydrochloride	0.70
Niacin	3.00
Calcium pantothenate	1.60
Folic acid	0.20
Biotin	0.02
Vitamin B_{12} (0.1%)	1.00
Vitamin A (500000IU/g)	0.80
Vitamin D_3 (400000IU/g)	0.25
Vitamin E acetate (500IU/g)	10.0
Menadione neon bisulfite	0.08
Sucrose	981.15
Total:	1000

Table 2.4: Composition of vitamin mix used in rodent pregnancy diet, AIN-76A formulation.

2.1.3 - Gonadectomy Surgery

2.1.3.1 - Preparation and Anaesthesia

At 10 weeks of age, animals were either gonadectomised (GNX) or exposed to a sham (sham) surgical procedure (n=8 per group). Littermates were randomly allocated to different surgery groups to ensure all treatments were comprised of non-sibling animals. Anaesthetic was induced in a separate preparation room using 2.5% isofluorane gas/O_2 (2L.min^{-1}) in an induction chamber. A non-rebreathing anaesthetic circuit with an active scavenge system was used for the entirety of the procedure. The surgical area was shaved and washed with Hibiscrub and Viruscan. After preparation the animal was moved into the surgical theatre where aseptic technique was maintained for the duration of the surgery. Before the procedure was started, the animal was placed on a homoeothermic heat mat and body temperature monitored using a rectal probe.

2.1.3.2 - Surgical Protocols

For ovariectomy and sham-ovariectomy procedures, a single midline, dorsal incision was made, followed by a bilateral muscle incision of no larger than 1cm. Ovaries were fully removed, and each animal was physically examined upon dissection at cull for traces of ovarian tissue. The same incisions were adopted in the sham procedure, but ovaries were left in-tact. For castration and sham-castration procedures, a single incision was made in the scrotal sac, and a suture was placed on the main visible testicular artery to prevent bleeding. Both testicles were then removed. During the sham procedure, the same incisions were made but immediately closed. In all procedures, the same suture material was used; Coated VICRYL (Johnson & Johnson Medical Ltd, Wokingham, UK). Skin incisions were coated with a single, running, intradermal stitch to reduce recovery time and minimise risk of self-mutilation by the animals.

2.1.3.3 - Recovery

Immediately post-surgery, animals were placed in a heat box with access to food and water until fully awake, moving and consuming food and drink. For the next 7 days, all rats were monitored daily and administered analgesia as needed in the form of a semisynthetic opioid (0.005 mg/100 g Buprenorphine, Reckitt & Colman, Slough, UK), and a nonsteroidal anti-inflammatory drug (0.006 mg/100 g Metacam, Boehringer Ingelheim, Germany).

2.1.4 - Physiological Measurements across the Lifespan

2.1.4.1 - Blood Pressure Measurement via Tail Cuff

Non-invasive measurement of blood pressure can be done in three ways; photoplethysmography, piezoplethysmography, and volume pressure recording. All

three of these methods rely on the use of an occlusion cuff on the tail to obstruct the blood flow in combination with one of a variety of pressure sensors to measure it. In this experiment, a volume pressure recording sensor (VPR) was utilised. The VPR relies on a differential pressure transducer that is capable of measuring tail blood volume and can use this data to give readings for systolic, diastolic, and mean arterial blood pressure as well as heart rate. VPR blood pressure recordings have numerous advantages over photoplethysmography and piezoplethysmography, most notably that they are unaffected by skin pigmentation or ambient light, reducing error in readings (Malkoff, 2005). In a separate validation study, measurements in more than 500 animals showed that VPR readings underestimated systolic blood pressure by only 0.25mmHg on average when compared with radio-telemetry (Feng et al., 2008). Moreover, numerous hypertension and cardiovascular research studies have adopted the tail cuff methodology and it is widely regarded as a cost-effective and accurate technique (Whitesall et al., 2004).

Prior to indirect measurement of blood pressure, animals were removed from their home cages and placed in a heat box set to 30^{o}C for 30 minutes. Each animal was then individually restrained using clear animal holders with an adjustable nose cone to allow for relaxed breathing. The procedure began with a 10-minute acclimatisation period in the restraint tube to reduce the impact of stress on the readings. After acclimatisation, animals underwent 10 cycles of blood pressure measurement by non-invasively determining the tail blood volume with a volume pressure recording sensor and an occlusion tail cuff (CODA System, Kent Scientific, Torrington, CT). Of these readings, the first three were disregarded as acclimatisation cycles, and a mean was taken from the remaining seven for systolic, diastolic, and mean arterial pressure, as well as heart rate. Restraint was for no

longer than 30 minutes per animal and after measurement rats were returned to their home cage and monitored .

2.1.4.2 - Blood Pressure Measurement via Telemetry Surgery

Aortic telemetry implantation is the 'gold standard' for rodent blood pressure measurement (Kurtz et al., 2005; Morton et al., 2003). It involves implanting a catheter attached to a radio transmitter in the aorta of the rodent. Whilst an invasive procedure, the implant system offers significant advantages in its capacity for continuous monitoring of individual animals in their home environment. Once the surgical implantation has been completed the need for additional environmental stressors (such as heating or restraint) are negated, and recordings can be taken 24 hours a day. This technique was adopted in Trial III of the project.

2.1.4.2.1 - Surgical Protocol

Animals were anaesthetised and prepared as described in Section 2.1.3.1 -. A single midline, ventral incision was made in both the skin and muscle layer, roughly 5cm in length. Intestines were carefully retracted using saline-soaked gauze to allow for complete visualization of the entire length of the abdominal aorta. Clamps were placed on the aorta immediately below the renal artery and directly above the iliac bifurcation to occlude blood flow to the area. The aorta was pierced using 20G needle with a bent tip, and flexible catheter inserted ~1cm above the lower clamp with the transmitter attached to the abdominal wall (PA-C40, Data Sciences International, St Paul, MN). The aorta was sealed using a cellulose patch and vetbond surgical adhesive. Both clamps were then gently removed and the area observed for bleeding or ruptures of the aortic seal. Transmitter signal was tested using an AM radio prior to closure of the incision. After surgery, animals were allowed a 2 week recovery period before beginning baseline recordings. During the

recovery, acclimatisation and recording periods, animals were singly housed whilst 24 hour measurements of heart rate and systolic, diastolic, and mean arterial pressure were taken. During this recovery period, analgesia was administered as in Section 2.1.3.3 - above.

2.1.4.2.2 - Recording and Analysis

Telemetry data was recorded for 10 minutes at a time at 15 minute intervals. This recording period was selected for multiple reasons. First, to increase survival of the battery life in the telemetric probes, and second to reduce the volume of data requiring storage from constant telemetric recording. A study by Guild et al., (2008) investigated the benefits of constant recording in telemetric cardiovascular studies, and demonstrated that reduced recording periods could accurately reflect 24 hour values with less than 1% error. Recordings were performed using PhysioTel® receivers connected via a data exchange matrix to a desktop computer. The receivers convert frequency signals generated by the implanted transmitter into electrical impulse that can be analysed (Dataquest A.R.T.4.1 User Guide, 2007). Equipment is fitted with an ambient pressure monitor (APR-1), which measures environmental pressure fluctuations and provides corrections via a digital signal to the computer recording.

Recorded data was extracted using the Dataquest ART analysis software associated with the telemetry hardware (Data Sciences International™, USA) and hourly averages calculated and condensed for a 24 hour representation of each treatment period. Averages were also collated for hours of light and dark (08:00-20:00/20:00-08:00). Detailed statistical analysis can be found in chapter 5, with Trial III methodology.

2.1.4.3 - Cull and Tissue Collection

A week prior to culling, animals were housed in single cages for five days; 2 days adaptation, and 3 days of food consumption measurements. Following this, animals were housed in a metabolic cage for 24 hours on 2% glucose. To prevent bacterial formation in the collected urine samples, 1ml of antibiotics was added to the urine collection tube (A5955, Sigma, 1:50 diluted from stock). At the end of the 24 hour period, 3 aliquots of urine were collected. After fasting, animals were allowed a 3 day recovery period, before being returned to the metabolic cages for 24 hours, for food and water consumption measurements as well as urine collection. After this final 24 hour period, animals were culled in rising CO_2 with death confirmed by cervical dislocation. Blood was collected via cardiac puncture into heparinised tubes (sarstedt) and centrifuged at 15588g for 10 minutes at 4°C to separate plasma, which was frozen in 4 aliquots of approximately 300µL. All organs were harvested and snap frozen in liquid nitrogen, prior to storage at -80°C. Liver and Kidney samples were also fixed in 4% formalin, and gradually dehydrated with varying concentrations of ethanol following a 24 hour fixation period.

2.1.4.3.1 - Determination of Oestrus

At cull, stage of oestrus was determined in Trial II animals. Animals were restrained and vaginal smears obtained using a cotton swab. Swabs were smeared on to microscope slides and a cover slip placed immediately. Stage of oestrus was decided visually using images and descriptions in Figure 2.1 and Table 2.5, taken from The IAT Manual of Laboratory Animal Practice &Techniques.

a.

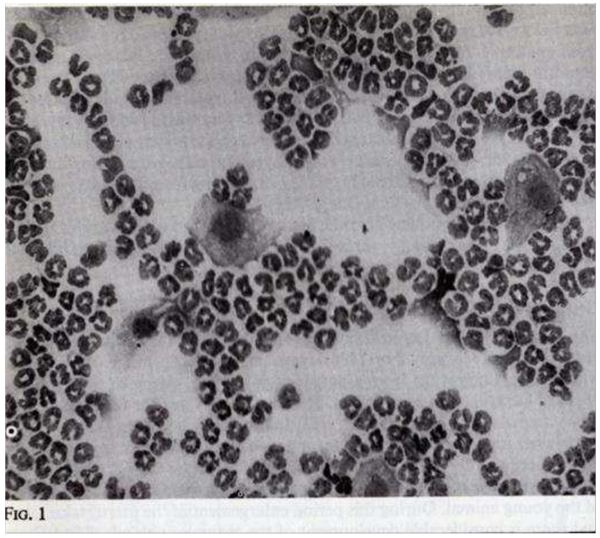


FIG. 1

b.

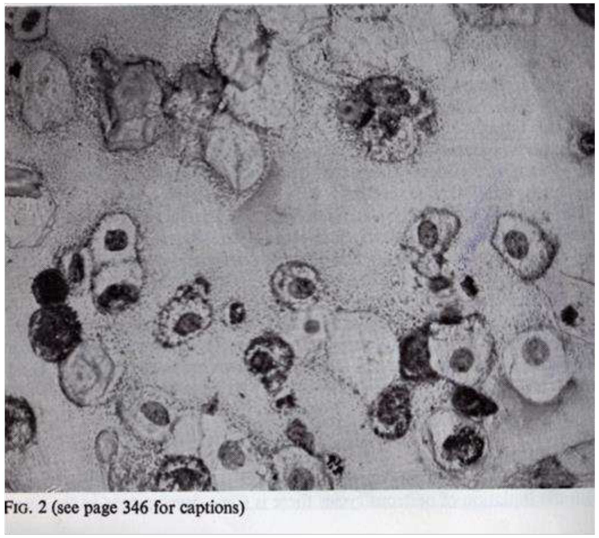


FIG. 2 (see page 346 for captions)

c.

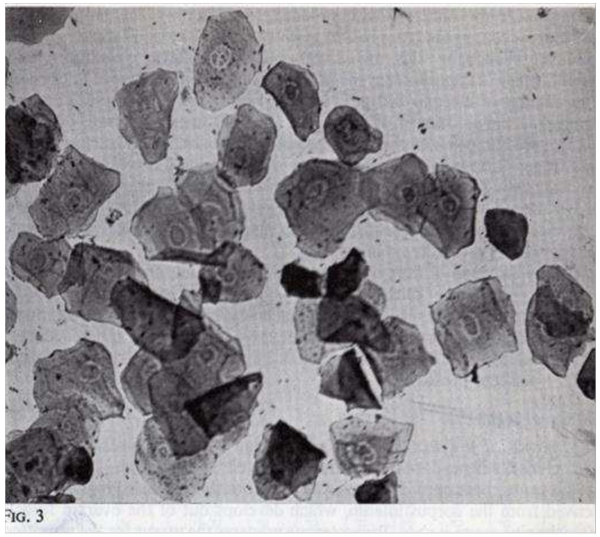


FIG. 3

d.

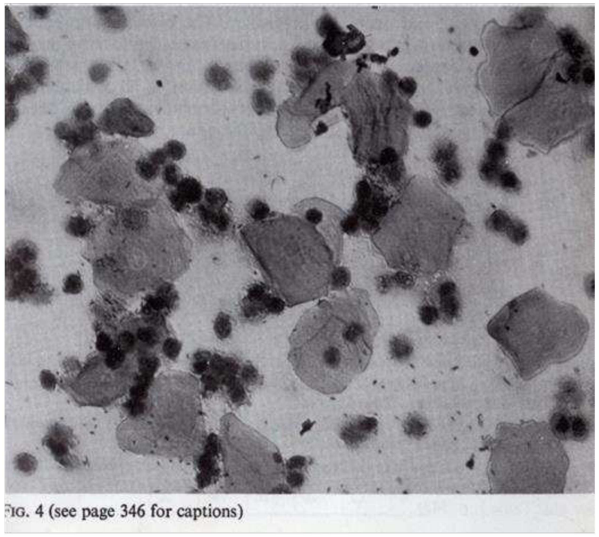


FIG. 4 (see page 346 for captions)

Figure 2.1: Reference images for stages of oestrus in rodents.

a. Dioestrus – leucocytes, present at all stages of the cycle except when the female is on heat.
b. Pro-oestrus – at the approach of oestrus, leucocytes disappear and the smear consists mainly of epithelial cells with marked nuclei.
c. Oestrus – the leucocytes have disappeared, the smear consists of cornified cells only, the female is on heat.
d. Metoestrus – the return of the leucocytes

Text taken directly from The IAT Manual of Laboratory Animal Practice & Techniques (Austin & Rowlands, 1969).

Stage	Vaginal Smear		
	Epithelial Cells	Cornified Epithelial Cells	Leucocytes
Pro-Oestrus	+++	+	+
Oestrus	-	+++	-
Met-Oestrus	+	++	++
Di-Oestrus	+	-	+++

Table 2.5: Where, - = none, + = few, ++ = many, +++ = very many.

Expected changes in cellular composition of rat vaginal smear through the stages of oestrus. Adapted from The IAT Manual of Laboratory Animal Practice &Techniques (Austin & Rowlands, 1969).

2.2 - Animal Trial Designs

2.2.1 - Trial I – Characterising the System

N.B. The majority of the animal work for trial I was performed prior to the start date of this PhD by Dr Wioletta Pijacka. The author joined the project in August 2011 as animals were reaching the final cull point.

2.2.1.1 - Trial Design

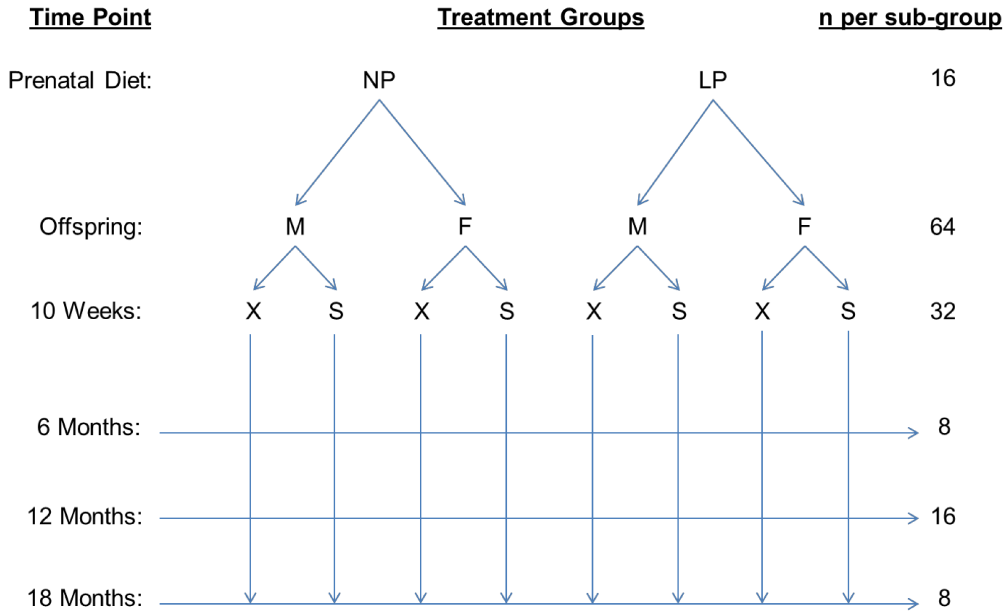


Figure 2.2: Basic protocol for animal work in Trial I, including animal *n* per group. Male (M) and female (F) offspring from dams fed a normal (NP) or low (LP) protein diet during pregnancy were either gonadectomised (X) or exposed to a sham surgical procedure (S) at 10 weeks of age. Animals were then culled at 6, 12 or 18 months of age and tissues harvested.

The largest of the three animal trials, Trial I was designed to characterise the model in use, and consider the expression of our target systems – renin-angiotensin (RAS) and nitric oxide (NO). In previous years, a significant body of work has been done (in this laboratory and others) utilising the low protein model of developmental programming, the basic protocol for which has been described in Section 2.1.2 - .

After its original development by Langley & Jackson (1994), the low protein model has been adopted widely in developmental research as both a study in its own right, and as a tool to induce hypertension (Alwasel et al., 2013). It has been documented on numerous occasions that the administration of a low protein diet during gestation 'programs' offspring towards hypertension, a shorter lifespan, decreased renal function, and increased oxidative damage, to name just some of the observed consequences (McMullen et al., 2004; Langley-Evans & Sculley, 2005; Sathishkumar et al., 2012; Sahajpal & Ashton, 2003). This PhD adopted this model in order to examine typical vs. accelerated renal ageing. The benefits of this were two-fold. First, it allowed for consideration of an 'unhealthy' system. In age, the function of most basic systems in the body is reduced (Weinstein & Anderson, 2010), however it was not logistically possible to age the animals to the length required to achieve this functional decrease. The low protein model provided an opportunity to consider animals in a condition of accelerated renal dysfunction. Furthermore, the mechanistic basis of developmental programming of disease is, in itself, a significant area of research that bears further consideration. In utilising this protocol, it was possible to focus experimental work on identifying changes in the systems of interest, renin-angiotensin and nitric oxide.

Animals were bred according to the plan in Figure 2.2. Sixteen dams were allocated either a normal (NP) or low protein (LP) diet for the duration of pregnancy and at birth, litters were culled to 8 animals to minimise nutritional variation during suckling. After weaning, offspring were maintained throughout the trial as described in Section 2.1.1 - . All offspring, from both NP and LP groups, underwent gonadectomy or sham-gonadectomy surgery at 10 weeks of age (Section 2.1.3 -). At 6, 12, and 18 months all animals underwent blood pressure measurement using an indirect tail cuff method (Section 2.1.4.1 -). In the 12 month age group, a subset of animals underwent blood pressure measurement via aortic telemetry implant

(Section 2.1.4.2 -). Culls were performed at 6, 12 and 18 months of age. Offspring were randomly allocated to these time point groups at the beginning of the trial to ensure that all groups were comprised of non-sibling animals. At these ages, urine samples, blood samples and all organs were collected for future analysis (Section 2.1.4.3 -).

2.2.2 - Trial II – Targeting the System

2.2.2.1 - Trial Design

The structure of Trial II was very similar to Trial I and can be seen in Figure 2.3.

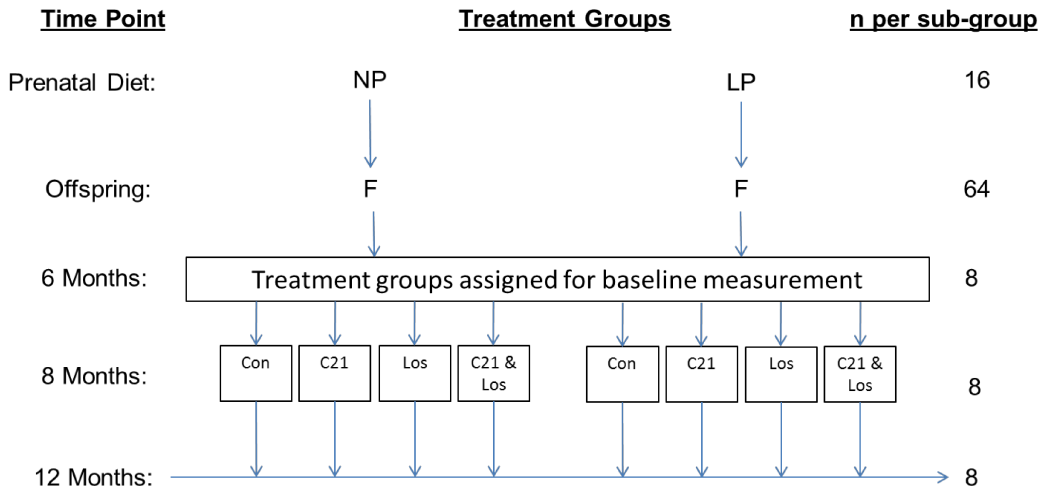


Figure 2.3 Experimental design for Trial II. Female offspring (F) from dams fed a normal (NP) or low (LP) protein diet during gestation. At 6 months of age, offspring from both low and normal protein mothers were assigned treatment groups for baseline measurement. At 8 months, a 4 month drug dosing regime was started of either control (con), C21 (Compound 21), Los (Losartan), or C21 & Los (concomitant Compound 21 and Losartan). All offspring were culled at 12 months of age and tissues collected.

Conceived as a follow on from the work characterising the renal ageing phenotype, Trial II was designed to target specific aspects of the nitric oxide and renin-angiotensin systems in an attempt to alter blood pressure. As previously discussed, numerous studies have demonstrated the beneficial effects of AT_1R blockade in eliciting a decrease in arterial pressure, and such treatments have become well established in clinical treatment of human hypertension (Michel et al., 2013). The actions of the AT_2R are less understood. Studies have demonstrated that the AT_2R may have a counter-regulatory role in the control of blood pressure, by inducing vasodilation, reducing inflammation and oxidative damage, and inhibiting tissue remodelling (Sampson et al., 2008; Berry et al., 2001; You et al., 2005). It is these factors that suggest the AT_2R might be an ideal target for pharmacological intervention to increase the effectiveness of current treatments for hypertension. Using a highly selective, non-peptide AT_2R agonist (Compound 21; C21, Section 1.4.5 -), Trial II aimed to increase nitric oxide production via enhanced activation of the AT_2R. Furthermore, additional animals were assigned to receive either an AT_1R antagonist (Losartan), or a combination of both the AT_2R agonist and AT_1R antagonist to identify whether or not the ability of the AT_2R agonist to decrease blood pressure was dependent on concomitant AT_1R blockade. In doing so, this trial was to consider how feasible manipulation of the target systems was for future work.

Virgin female Wistar rats were mated as described in Section 2.1.1 - . At birth mothers were switched to a standard laboratory chow diet, pups were weighed and litters standardised to 8 offspring to minimise nutritional variation during suckling. For this trial and Trial III, females were preferentially selected over male offspring at the point of offspring cull. One of the primary aims of this project was to consider the effects of sex steroid modulation, and to elucidate some of the mechanisms behind the ‘protective effect’ of ovarian steroids. In order to focus on the effects of ovarian

steroids, male animals were excluded from these trials. Furthermore, results from Trial I suggested that castration of males had a smaller effect on physiological outcomes than that of ovariectomy on female animals, suggesting that female animals were an ideal focal point for this study. Offspring were weaned at 3 weeks of age, maintained on a standard laboratory chow diet, and weighed weekly for the remainder of the trial. At 6 months of age, offspring from both maternal diets were randomly allocated into 1 of 4 groups: control, C21, C21 & Losartan or Losartan. Baseline blood pressure readings were obtained non-invasively by determining the tail blood volume using a volume pressure recording sensor and an occlusion tail cuff (CODA System, Kent Scientific, Torrington, CT). Measurements of food and water intake were made and urine samples collected by housing rats in metabolic cages for 24 hours. Blood pressure and metabolic measurements were carried out at both 9 and 12 months of age. At 8 months of age animals began daily drug treatments as detailed in table Table 2.6 below.

2.2.2.2 - Drug Choice and Dose Selection

Current treatments for hypertension frequently focus on inhibition or suppression of the angiotensin type I receptor, thus suppressing the pressor arm of the RAS. This trial instead focusses on the less understood angiotensin type II receptor by utilising a novel, non-peptide, orally active, AT_2R agonist, Compound 21. As a relatively new drug, C21 has a fairly small literature base, which has been discussed above. Previous experiments have focussed largely on acute, site specific dosing in short experimental windows. This experiment aimed to establish whether the potentially positive effects of C21 dosing (Gao et al., 2014; Brouwers et al., 2013; Hilliard et al., 2014) could be replicated in long-term, low dose, oral administration that is more representative of a potential clinical treatment. Compound 21 was received as a kind gift from Vicore Pharma (Uppsala, Sweden). Preliminary data in their laboratory

suggested that the minimum dose of C21 required to achieve AT_2R stimulation was 0.3mg/kg/day. Whilst other studies have utilised a far higher concentration, there has been no work to date investigating long-term, low dose administration *in vivo*. Moreover, a study by Verdonk et al. (2012) demonstrated that high dose administration of C21 causes a reduction in its selectivity for the AT_2R. As such 0.3mg/kg/day was the dose selected to proceed with (Table 2.6). Doses were administered by oral pipette, diluted with water. Current literature has utilised treatment regimes that are no longer than one week in duration. With the tremendous variation in responses seen (Chapter 1, Section 1.4.5.1) it is the author's opinion that this treatment length is insufficient to fully establish the effects of C21 dosing. In addition, publications have cited C21 as a potential treatment (or addition to treatments) for hypertension (Stecklings et al., 2012; Wan et al., 2004). In order to test this assertion, trials with significantly longer treatment periods are required. In this trial, treatments were begun at 8 months of age and continued for four months. The reasons for this were twofold; firstly, as stated above, there are no trials to date that have dosed with C21 for longer than 7 days. By increasing the dosing period to 4 months, it was hoped it would be possible to identify the effects of C21 dosing as a potential treatment in the long term for high blood pressure. Secondly, by administering the doses to animals from 8 months onwards, it would be possible to consider the effects of C21 on animals that are significantly older than those utilised in all previous C21 research.

Losartan, a well published treatment for reduction of blood pressure, was dosed at a concentration of 10mg/kg/day in accordance with previous studies (Strawn et al., 1999; Heller et al., 2005).

Maternal Diet	Group	n	Treatment	Dose
CON MLP	Control	8	No treatment	n/a
CON MLP	C21	8	Compound 21 by oral pipette	0.3mg/kg/day
CON MLP	C21 & Losartan	8	Compound 21 by oral pipette; Losartan by drinking water.	0.3mg/kg/day 10mg/kg/day
CON MLP	Losartan	8	Losartan by drinking water.	10mg/kg/day

Table 2.6: Details on groups, treatments and dosages for dosing animals between 8 and 12 months of age.

At 12 months of age, after 4 months of drug treatments, animals were culled by CO_2 asphyxiation with death confirmed by cervical dislocation. Tissues and blood were collected as before (Section 2.1.4.3 -) and stored at -80^{o}C until analysis with the exception of the mesenteric arcade, which was removed and placed in ice cold physiological saline solution for wire myography, as detailed in Section 2.3.10 -).

2.2.3 - Trial III – Modulating Sex Steroids

As with Trials I and II, Trial III utilised the low protein model of programming. The design of the trial can be seen below in Figure 2.4.

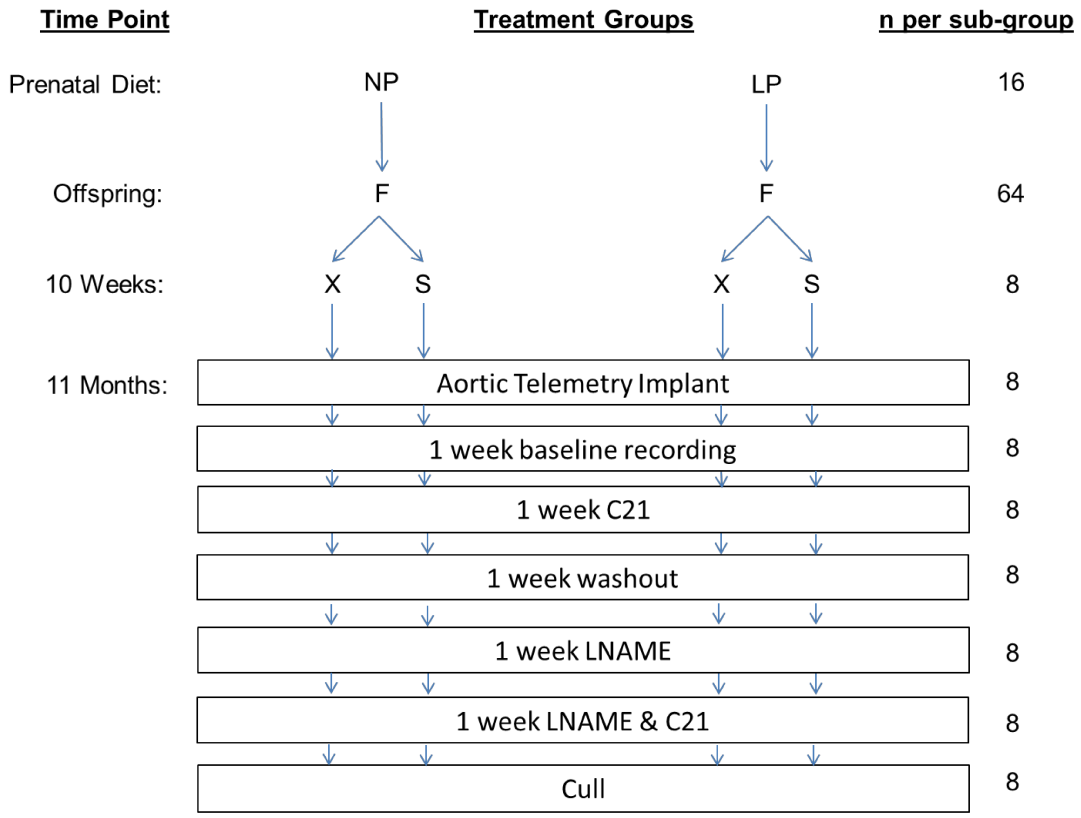


Figure 2.4: Experimental design for Trial III. Female offspring (F) from dams fed a normal (NP) or low (LP) protein diet during gestation were either gonadectomised (X) or exposed to a sham surgical procedure (S) at 10 weeks of age. At 11 months of age all animals underwent aortic telemetry implant surgery, following which, a 5 week dosing regime was employed. Animals were culled upon completion of dosing. C21 is Compound 21 administered by oral pipette, LNAME is the nitric oxide synthase inhibitor, Nω-nitro-L-arginine methyl ester, administered in drinking water.

This final animal trial was designed with the intention of integrating the concepts considered in Trials I and II. A key area of focus in this thesis is the sex-specific differences seen in physiological systems with age, in humans and animals alike. In particular, the so called 'protective effect' of oestrogen and other female sex

steroids is not fully understood. To attempt to isolate the effects of the sex hormones on these systems, gonadectomy surgery was adopted to provide a 'knock-out' of oestrogen to observe how the system responded to pharmacological intervention in the presence and absence of this sex steroid.

Female rats were mated as described previously (Section 2.1.1 -), and female offspring selected as with Trial II. Offspring were assigned to receive either a sham- or ovariectomy procedure at 10 weeks of age as before (Section 2.1.3 -). Blood pressure measurements were taken by tail cuff at 6 and 9 months of age (Section 2.1.4.1 -). At 11 months of age, all animals underwent aortic telemetry implant surgery (Section 2.1.4.2 -) for continuous blood pressure measurement.

2.3 - Laboratory Work

2.3.1 - Determination of Creatinine Clearance

Creatinine is a stable compound produced by creatinine phosphate during muscle contraction. In the blood, creatinine is freely filtered through the kidney and secreted in urine. In a healthy individual, levels of plasma creatinine are maintained fairly consistently (Sherwood, 2014). As such, by measuring both plasma and urinary levels of creatinine, a reasonably accurate estimation of glomerular filtration can be made.

The level of creatinine in both plasma and urine was determined in samples from animals at multiple time points using an adaptation of the Jaffé alkaline picrate method (Lustgarten and Wenk, 1972; Greenwald, 1930). The process is dependent on the formation of a coloured complex between picrate and creatinine proportional to creatinine concentration, and can be measured spectrophotometrically.

2.3.1.1 - Urinary Creatinine

Urine samples were diluted 20-fold using HPLC grade water prior to assay. A range of creatinine standards (0-20mg/dl) was prepared by dilution of a stock creatinine solution (100mg/dl) with HPLC grade water. For the assay, 50µl of sample or standard was pipetted into individual wells of a 96-well plate in triplicate. 100µl of working reagent (comprising 2.5ml 1M NaOH and 12.5ml 0.13% picric acid for one plate) was then added, and the plate incubated at room temperature on a shaker for 30 minutes. Absorbance was then read at 492nm (A1), and following this 30µl of 30% acetic acid was added to each well before incubating for a further 5 minutes. The absorbance was read once more at 492nm (A2). Creatinine was then determined by subtracting A2 values from A1 values to correct for background, and then, by using the standard curve, values for creatinine in mg/dl were obtained.

2.3.1.2 - Reliability Criteria

In order to assess the sensitivity of the urinary creatinine assay, a composite standard curves was generated and calculations performed to determine intra- and inter-assay coefficients of variation.

2.3.1.3 - Standard Curve

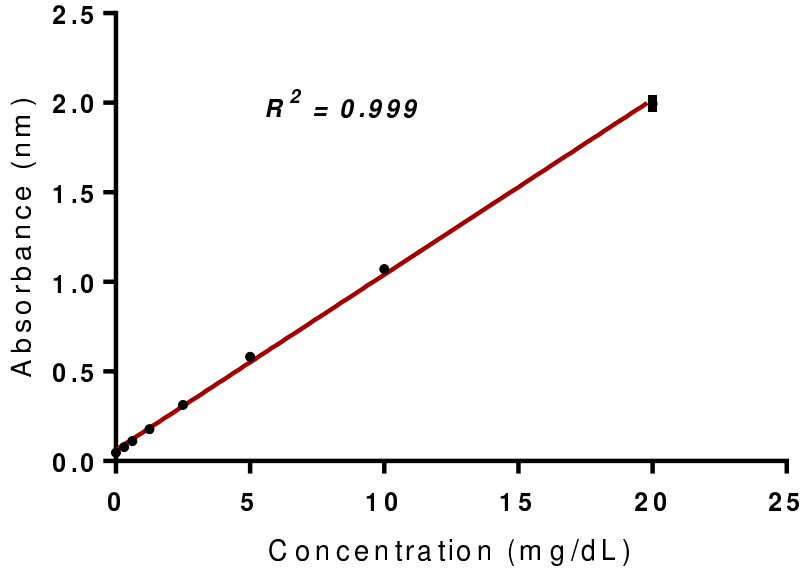


Figure 2.5: Composite standard curve for urinary creatinine data. Values presented are mean ± SEM compiled from 9 creatinine standard curves.

Creatinine assays were prepared using conventional units (mg/dL). All data are presented in Système International (SI) units. Creatinine values were converted from conventional to SI units by multiplying with the accepted conversion factor of 88.4 (Fowler & Mikota, 2006).

2.3.1.4 - Sensitivity

Assay sensitivity was determined by subtracting two standard deviations from the mean creatinine concentration of the zero standards. The value generated was taken to be the lower limit of sensitivity. Using this definition, the limit of detection of the creatinine assays was calculated to be 0.17mg/dL or 14.6µmol/l.

2.3.1.5 - Precision

The inter-assay coefficient of variation (%CV) was calculated for each assay by running a quality control sample on each assay plate which had creatinine concentrations that lay within the middle of the standard curve. The coefficient of variation could then be calculated using the following equation ((SD/mean) x 100). The inter-assay %CV for urinary creatinine was 4.3% across 9 assays.

The intra-assay coefficient of variation was determined using 10 random sets of samples from each plate. CV values were calculated between replicates on each plate in the assay and then an average taken of all 9 plates to give the overall value for intra-assay variation. Using this method, the intra-assay %CV for urinary creatinine was 2.26%.

2.3.1.6 - Plasma Creatinine

Unlike the urine samples, Trial I plasma samples were measured using the Quantichrom Creatinine Assay Kit (DICT-500, BioAssay Systems, Hayward, CA). In short, a 2mg/dl standard was prepared by diluting a 50mg/dl standard with pure water, and 30uL of this standard was pipetted in duplicate, twice on each 96-well assay plate. Alongside this, 30ul of each plasma sample (undiluted) and a pure water blank were pipetted in duplicate. Working reagent was prepared as per the manufacturer's instructions and 200ul added to each well. Optical density was read

immediately (OD0) at 492nm (Tecan, Sunrise) and again after 5 minutes of incubation (OD5). The creatinine concentration of the sample was determined using the following equation:

$$\frac{(\text{SampleOD5-SampleOD0})}{(\text{StandardOD5-Standard OD0})} \times [\text{STD}]$$

Where 'Standard' and 'Sample' refer to plasma samples and the prepared standard control at reading times 0 and 5 minutes respectively. [STD] is 2mg/dl.

Assay sensitivity information was obtained from the manufacturer, with the lower limit of detection for plasma creatinine cited as 0.10mg/dL.

2.3.1.7 - Creatinine Clearance

Using the concentrations as determined above, and the values for urine volume (ml/24hr), creatinine clearance was calculated in ml/min per 100g of bodyweight. This was done using the following formula:

$$\frac{\left[\frac{(\text{UCr} \times \text{UV24})}{(\text{PCr} \times 24 \times 60)}\right]}{\left(\frac{\text{BW}}{100}\right)}$$

Whereby:

UCr = Urinary Creatinine (µmol/l)

UV24 = 24 hour urine volume (ml)

PCr = Plasma Creatinine (umol/l)

BW = Bodyweight (g)

2.3.2 - Determination of Urea

Urinary urea concentration was determined in urine samples using the QuantiChrom Urea Assay Kit (DIUR-500, BioAssay Systems, Hayward, CA). Prior to assaying, urine samples were diluted 50-fold using HPLC grade water. To perform the assay, 5µl of a 50mg/dl standard was pipetted in duplicate on to a 96-well assay plate. Following this, 5µl of each diluted urine sample and a pure water blank were pipetted in duplicate. Working reagent was prepared as per the manufacturer's instructions and 200µl added to each well within 20 minutes of mixing. The plate was left at room temperature on a shaker for 20 minutes to incubate. At the end of the incubation period, optical density was read at 520nm (Tecan, Sunrise). The urea concentration of the sample was determined using the following equation:

$$\frac{(\text{SampleOD} - \text{BlankOD})}{(\text{StandardOD5} - \text{Blank OD0})} \times n \times [\text{STD}]$$

Where 'Sample' refers to urine samples, 'blank' is the pure water control, and the prepared standard concentration is 50mg/dl. Data for assay sensitivity was obtained from the manufacturer, with the lower limit of detection defined as 0.006mg/dL.

2.3.3 - Determination of Protein Concentration

Protein assays were utilised in numerous samples from all trials, in each case the same experimental protocol was employed. The Bio-Rad DC Protein Assay is based on the principles of the Lowry assay (Lowry et al., 1951) and allows for the colorimetric determination of protein concentration in biological samples. In brief, urine and tissue homogenate samples were diluted 10-fold by adding 20µL sample to 180µL pure water prior to assay. A range of protein standards was made up (concentrations 0.2mg/ml – 1.5mg/ml) using bovine serum albumin and pure water (or homogenisation buffer for tissue samples). 5µL of standard, sample, and pure

water blank were pipetted in triplicate on to a 96-well microtitre plate. Then, 25µL of Bio-Rad Reagent A (an alkaline copper tartrate solution, Bio-Rad Laboratories Inc) was added to each well. This was followed immediately by 200µL Bio-Rad Reagent B (a dilute Folin Reagent) and the plate incubated for 15 minutes at room temperature. Absorbance was read at 750nM (BioRad 680XR, Bio-Rad laboratories Inc.) and the protein concentration determined using the standard curve generated.

2.3.3.1 - Reliability Criteria

Reliability of the protein assay was determined in the same manner as described in Section 2.3.1.2 -.

2.3.3.2 - Standard Curve

A composite standard curve was created from six protein assay plates performed.

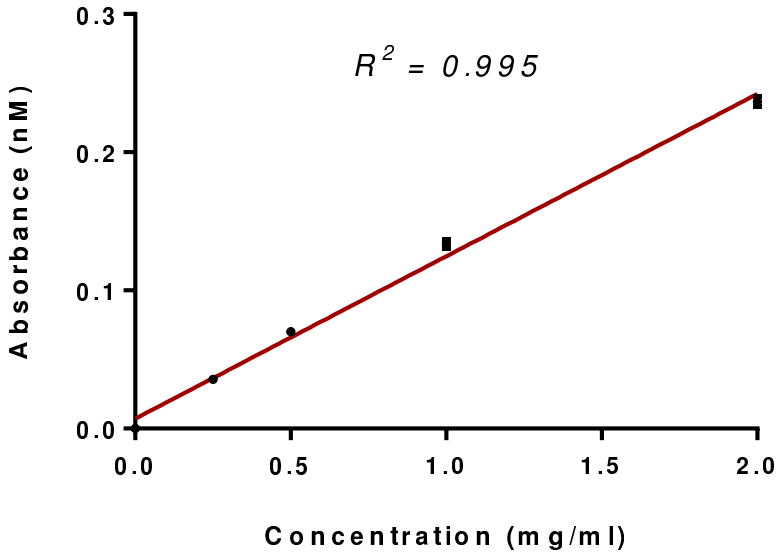


Figure 2.6: Composite standard curve from six protein assays. Data presented are mean ± SEM.

2.3.3.3 - Sensitivity

The sensitivity of this assay was determined using the mean value obtained for zero concentration standards as described in Section 2.3.1.4 -. Using this method, the lower limit of detection was found to be 0.04mg/mL.

2.3.3.4 - Precision

Coefficients of variation (%CV) were determined as noted in Section 2.3.1.5 -. Using these definitions, inter-assay %CV was calculated as 1.90% and intra-assay %CV was calculated as 6.1%.

2.3.4 - Determination of Protein Carbonyls

The breakdown of protein in tissue due to free radical damage results in the formation of stable products known as protein carbonyls (Dalle-Donne et al., 2003). When incubated with 2, 4-Dinitrophenylhydrazine (DNP) carbonyls react specifically to produce a coloured compound that can be analysed spectrophotometrically. Protein carbonyls were determined using the method described by Langley-Evans and Sculley (2005). Crushed, frozen kidneys were homogenised in 50mM potassium phosphate buffer with 5mM EDTA, pH 7.4. Samples were assayed for protein content (mg/ml) as per the Bio-Rad DC Protein Assay protocol (Section 2.3.3 -). All samples were then diluted to an equal concentration of 1mg/ml. Per sample, four aliquots of 100µl were removed to perform the assay in duplicate. Protein was precipitated from the samples by incubation with 500µl trichloroacteic acid for 15 minutes at 4°C. Samples were then centrifuged at 15,588g for 5 minutes, and the resultant pellet re-suspended in either 2M hydrochloric acid (blank), or 2M hydrochloric acid containing 0.1% DNP. After incubating for an hour, protein was re-precipitated using TCA as before, centrifuged, and the pellet washed 3 times with

ethanol:ethyl acetate solvent to remove excess DNP. The final pellet was suspended in 800µl of 6M guanidine hydrochloride and absorbance measured as 370nm. The extinction coefficient of 21000$M^{-1}cm^{-1}$ was then used to calculate the concentration of protein carbonyls in nM/mg protein.

2.3.5 - Determination of Triglycerides and Cholesterol

2.3.5.1 - Circulating Lipids

Total plasma triglycerides and total cholesterol were determined using the Randox RX Imola clinical chemistry analyser (Randox Laboratories LTD). Experiments were performed with the assistance of Dr Nigel Kendall (University of Nottingham, School of Veterinary Medicine and Science). Randox cholesterol (Cat. No. CH3810), LDL (Cat No. CH3841), HDL (Cat. No. CH3811) and triglyceride (Cat. No. TR3823) reagents were used as per the manufacturer's protocol. Sensitivity and precision data were obtained from the manufacturer. Cholesterol intra-assay coefficient of variation (%CV) is noted as 1.8%, inter-assay %CV is 2.81%. The lower limit of detection is listed as 0.189mmol/l. For triglycerides, intra-assay %CV is 1.47%, inter-assay %CV is 2.5%. The lower limit of detection was found to be 0.189mmol/l.

2.3.5.2 - Triglyceride Deposition in the Liver

Total liver triglyceride levels were determined using an Infinity™ triglyceride assay reagent. Prior to assay, lipids were extracted from the liver samples. Per sample, 200mg of crushed, frozen liver was homogenised in 1.6ml of sodium sulphate and the homogenate decanted into 5.4ml of hexane:isopropanol (3:2, v/v), and a further 2ml of sodium sulphate added. Samples were centrifuged at 830g for 15 minutes at room temperature. After centrifugation, the top layer of supernatant was removed into fresh tubes and the samples dried down under nitrogen. The resultant solid was

re-suspended in 1ml of hexane and 100µl aliquots made for each sample. The aliquots were dried down under nitrogen once more and re-suspended in 100µl of isopropanol to be used for assaying. A standard curve ranging 0-2mg/ml was created using Infinity™ Triglyceride stock solution and assayed alongside the samples. Exactly 10µl of standard or sample was pipette on to a 96-well microplate and 200µl of Infinity™ Triglycerides reagent added. The plate was incubated for 15 minutes at 37°C. Absorbance was read at 550nm with a reference wavelength of 655nm (BioRad 680XR, Bio-Rad laboratories Inc.) and triglyceride concentrations calculated in mg/ml using the standard curve produced.

2.3.5.3 - Reliability Criteria

Assay reliability was determined through creation of a composite standard curve and by performing calculations for coefficients of variation, as described in Section 2.3.1.2 -for urinary creatinine assay.

2.3.5.4 - Standard Curve

The assay was performed over two plates, the standard curves for which were compiled in the figure below.

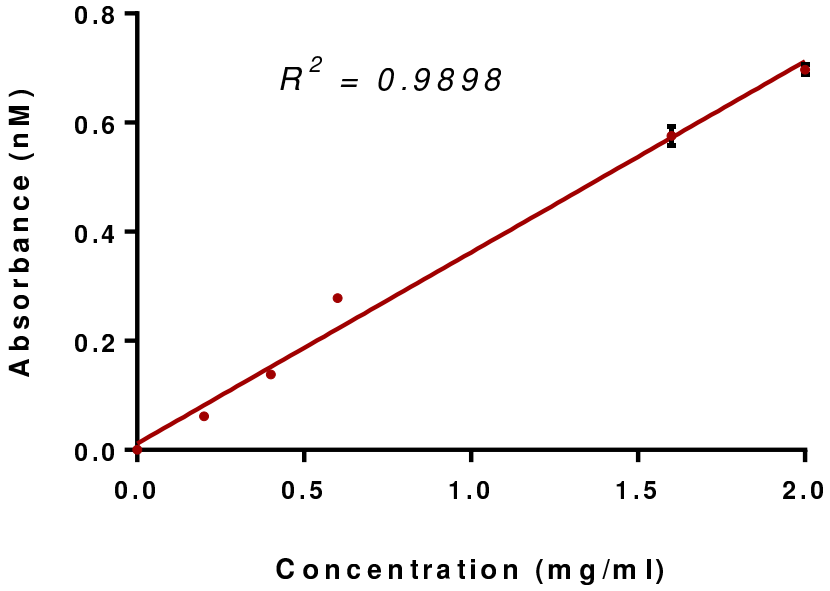


Figure 2.7: Composite standard curve for liver triglycerides. Data presented are mean ± SEM and collated from two standard curves.

2.3.5.5 - Sensitivity

Sensitivity of the assay was determined by averaging the values acquired for blank standards, and subtracting two standard deviations from this value. Using this definition the limit of detection was found to be 0.125mg/ml.

2.3.5.6 - Precision

The inter-assay coefficient of variation was determined using a control sample in each assay with a triglyceride concentration in the middle of the standard curve. Using the equation ((SD/mean)*100), %CV for two triglyceride assay plates was calculated to be 15%.

Intra-assay variation was determined by comparing replicates on each assay plate. The coefficient of variation was calculated for each and an average taken for the

entire plate. Using this method, intra-assay coefficient of variation was determined to be 22% over two assays.

2.3.6 - Determination of Nephron Number

Nephrons are the functional unit of the kidney, and the number expected in a given species is usually characteristic of that species, having been established during gestation (Amri et al., 1999). Decreases in the functional number of nephrons can have significant implications for overall kidney function throughout the lifespan. Nephron number was determined in trial I kidneys using the following protocol, performed by an undergraduate student, Shair-Li Hoh, under supervision of the author, using the method described by Harrison & Langley-Evans (2009).

Portions of formalin fixed kidney weighing 0.2g were prepared and placed in a 12.5mL tube with sufficient 0.1M hydrochloric acid to cover the entire piece of tissue. The kidneys were incubated in the acid for 30 minutes at 37°C after which the acid was removed and replaced with 5mL of phosphate buffered saline solution. Each kidney was thoroughly homogenised and three 20µL drops of the homogenate pipetted separately on to a microscope slide and secured with a cover slip. The 'droplets' were examined using a microscope and nephrons counted by hand. An average was taken from the 3 droplets for each kidney and this number used to determine overall nephron number in the entire kidney with the following calculation:

$$\text{Average count} \times 250 \times \frac{\text{Weight of kidney}}{\text{Weight of cut piece}}$$

2.3.7 - Determination of Urinary & Plasma Osmolality

Urinary and plasma osmolality were measured as an indicator of the concentrating capacity of the kidneys. Measurements were made on undiluted samples using the Fiske® Model 210 Osmometer (Advanced Instruments, Inc.). A 20µL sample was inserted into the machine and a reading in milliosmoles per kilogram of water (mOsm/kg H_2O) taken. All samples were performed in duplicate and an average reading noted.

2.3.8 - Determination of Urinary Nitrites

Urine samples were analysed for nitrite concentration using the Sievers Nitric Oxide Analyser (NOA 280, GE Water & Process Technologies, Trevose, PA, USA). In biological systems, in the absence of superoxide anion or oxyhaemaoglobin, nitrite (NO_2^-) is the primary oxidation product of NO. High concentrations of circulating nitric oxide will result in increased concentration of nitrites in both urine and plasma. As such, measurement of nitrites can give an estimation of the NO levels of certain samples. The Sievers NOA works as a highly sensitive detector by using a gas-phase chemiluminescent reaction between nitric oxide and ozone:

$$NO + O_3 \rightarrow NO_2^* + O_2$$

$$NO_2^* \rightarrow NO_2 + hv$$

(GE Water & Process Technologies, 2010).

In brief, nitric oxide and ozone are combined in a reaction chamber within the analyser. The reaction emits a chemiluminescent light that is detected by a red-filtered photomultiplier tube. In liquid sampling, an inert gas is bubbled through reducing agents and samples are injected into this mix one by one. In the reaction

purge vessel, nitrites are converted back to nitric oxide which can then be carried back to the analyser for detection as noted above.

Urine samples were collected as detailed in Section 2.1.4.3 - and stored protected from light. The NOA was set-up according to manufacturer's instructions for liquid sampling (GE Water & Process Technologies, 2010). A series of nitrite standards of known concentration ranging from 10mM – 10nM were prepared via serial dilutions of a 0.1M nitrite stock solution with deionised water. Standards were injected one by one into the NOA purge vessel containing sodium iodide and glacial acetic acid. Readings obtained in mV were plotted in excel alongside the overall concentrations to generate a standard curve with a value for slope and intercept. Urine samples were injected into the purge vessel one by one and mV readings obtained for each. These were then converted to concentrations using the standard curve generated and the following equation:

$$\text{Concentration (nM)} = \left[\frac{(\text{Peak area} - \text{Intercept})}{\text{Slope}}\right]$$

All samples were performed in duplicate, and an average taken for concentration values.

2.3.8.1 - Standard Curve

A single standard curve was generated for sample analysis as described above. All standards were performed in triplicate and an average value generated for analysis.

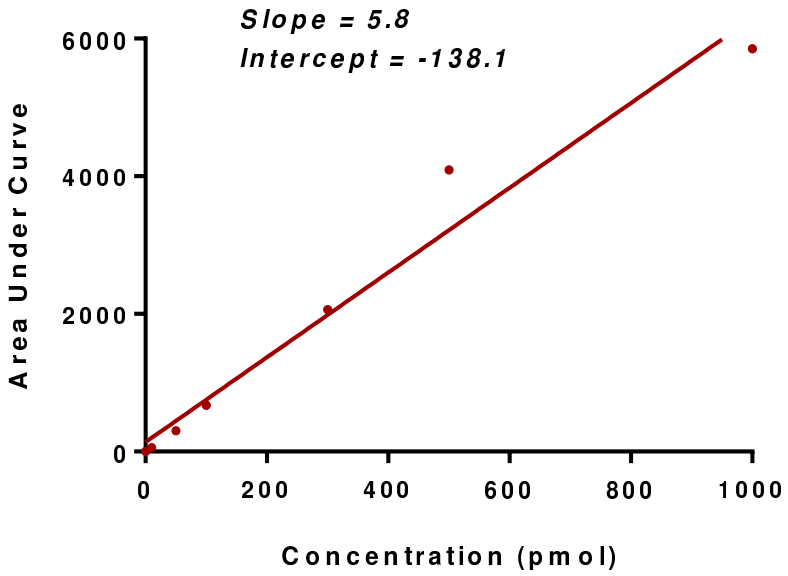


Figure 2.8: Standard curve for nitrite analysis on the Sievers Nitric Oxide Analyser. Data are mean ± SEM. Slope and intercept were calculated using NOA software built-in functions (GE Water & Process Technologies, 2010).

2.3.9 - Measuring Gene Expression

2.3.9.1 - Extraction and Preparation of Ribonucleic Acid (RNA)

Prior to extraction, all tubes, pipette tips, and glassware were cleaned and autoclaved to ensure sterility. All work was performed using molecular grade, RNase and DNase-free water (Sigma-Aldrich Co. LLC). Extraction of RNA was performed using Roche High Pure Tissue kits (Roche Diagnostics Ltd); crushed snap-frozen kidney samples, weighing approximately 20mg, were added to 400µL lysis buffer in a clean Eppendorf tube. Samples were 'lysed' by passing the buffer and tissue through a 21g needle and syringe a minimum of ten times. The resultant lysate was centrifuged for two minutes at 15,588g and the supernatant removed into a fresh sterile tube with 200µL pure ethanol. Roche high filter tubes were combined with collection tubes, and the entire sample/ethanol mixture pipetted to the upper chamber of the collection assembly. Samples were centrifuged at 15,588g for 30

seconds and the flow-through liquid discarded, keeping both the high filter and collection tubes. Per sample, 90µL DNase incubation buffer and 10µL DNase were mixed together, and 100µL of this solution added to each filter tube and these incubated at room temperature for 15 minutes to remove contaminating genomic DNA. Wash buffers I and II were passed through the filter tubes in sequence, which were then removed from the collection tubes and placed into a new, clean Eppendorf. Elution buffer was added to each filter tube reservoir (50µL) and the entire assembly centrifuged for a minute at 5903g. The resultant liquid in the Eppendorf contained the RNA for each sample.

2.3.9.2 - Testing RNA Concentration and Quality

Following RNA extraction, every sample was tested for RNA concentration (ng/µL) using a Nanodrop 2000 (Thermo Scientific). Samples were measured, blanked against pure water, at both 260nm (for nucleic acids) and 280nm (for proteins) and a ratio was calculated for these two readings. Samples with a concentration of RNA greater than 100ng/µL and a ratio value between 1.8 and 2.0 were deemed suitable for further analysis. Those failing to achieve this were discarded and RNA extraction repeated as above.

2.3.9.3 - Agarose Gel Electrophoresis

All RNA samples were resolved on a horizontal agarose gel. Gels were prepared in-house at a concentration of 1% agarose by mixing 1.5g agarose with 150ml 1 x TAE buffer (TAE buffer diluted from 50x, comprising 2.0M Tris Acetate + 100mM NA_2 EDTA, Flowgen Bioscience, Sittingbourne UK). The liquid was microwaved until clear, cooled under running water, and poured immediately into a gel tank with a 20-well comb in place. Gels were allowed to set completely before being submerged in 1xTAE. Samples were loaded in 5µL volumes with 2µL loading dye (0.25% (w/v)

bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% Ficoll (Type 400), in water). Gels were electrophoresed at 100v for approximately 20 minutes, or until the bands were roughly 2cm from the bottom of the gel. The entire gel was stained in ethidium bromide for 30 minutes before being visualised with a UV light source (Gel Doc 2000, Bio-Rad Laboratories Inc). All samples presenting with two clear bands (18S and 28S) were deemed acceptable for use, those with unclear, distorted, or absent bands were discarded and RNA extraction performed again as in (Section 2.3.9.1 -).

2.3.9.4 - Synthesis of Complimentary DNA (cDNA)

Accepted RNA samples were synthesised into first strand complimentary DNA using a RevertAid™ reverse transcriptase enzyme reaction and random primers (RevertAid RT Kit, Thermo Fisher Scientific Inc.). Reactions were prepared according to manufacturer's instructions in the following manner (Table 2.7):

Component	**Volume**
Total RNA (50ng/µL)	10µL
Random Hexamer Primer	1µL
Water (Nuclease Free)	1µL
Total Volume	**12µL**

Table 2.7: Components of RevertAid-RT reaction first step.

Reagents were pipetted into a fresh 96-well plate and incubated at 65°C for 5 minutes in a GeneAmp PCR System (9700; Applied Biosystems). Once completed, the plate was removed and immediately placed on ice to halt the reaction. Following this, the reagents in Table 2.8 were added to each reaction and the plate incubated once more for 5 minutes at 25°C and for 60 minutes at 42°C. The reaction was

terminated by heating the entire plate to 70°C for a further 5 minutes. Prior to storage, each cDNA well was made up to 100µL with RNase-free water and mixed gently.

Component	Volume
5x Reaction Buffer	4µL
Ribolock RNase Inhibitor	1µL
10mM dNTP Mix	2µL
Revertaid RT	1µL
Total Volume	**20µL**

Table 2.8: Components of RevertAid cDNA synthesis second step and volumes required.

The cDNA produced was used to generate a standard curve for every subsequent reaction. This was achieved by combining equal aliquots from each cDNA sample to create a 'pool' of the samples within that trial. Prior to inclusion in the pool, each cDNA sample was quantified independently by running it with the primer pair of interest. This was performed using the run and reagents detailed in Table 2.9. An average value was taken from the entire dataset, and an individual sample that fell more than 2 standard deviations from the mean of the group was excluded from the cDNA pool. This was done to prevent outliers skewing the dataset and to ensure any samples that may contain contaminants were not being amplified.

2.3.9.5 - Design and Testing of Primer Pairs

Oligonucleotide primers were designed for each experiment using online databases and Primer Express software. The gene sequence was selected in NCBI (National Center for Biotechnology Information), and the exon-intron boundaries identified

using Ensembl Genome Browser. This sequence information was inserted into Primer Express in FASTA format (Software Version 3.0; Applied Biosystems) and primers generated that cross an exon-intron junction to increase specificity. Newly designed primer pairs were checked using an online Basic Local Alignment Search Tool (BLAST) for alignment with the sequence of interest and to ensure no other sequences were detected by the primers. Selected primers were ordered via Sigma-Aldrich Custom Oligos.

2.3.9.6 - Primer Sequences

Below in Figure 2.9 and Figure 2.10 is the sequence information for endothelial nitric oxide synthase (eNOS) and Rab4 used in the design of primers for PCR. Gene and primer sequences were identified as detailed above (Section 2.3.9.5 -). Primer sequences are highlighted in green (forward) and yellow (reverse). Exon-Intron boundaries in the sequence are marked in changes of colour from black to blue.

ATGGGCAACTTGAAGAGTGTGGGCCAGGAGCCTGGGCCACCCTGTGGCCTAGGGCTCGGGCTGGGCCTAGG
GCTATGCGGCAAGCAGGGCCCAGCCTCACCGGCACCAGAGCCTAGCCAGGCACCAGTACCCCCGTCCCCAA
CCCGACCAGCACCAGACCACAGCCCCCCGTTAACCCGGCCCCCAGACGGACCCAAGTTTCCTCGAGTAAAGA
ACTGGGAAGTGGGCAGCATCACCTACGATACCCTCAGTGCACAGGCTCAGCAGGATGGGCCCTGTACCCCAA
GACGCTGCTTGGGATCCCTGGTATTTCCAAGGAAGTTACAGAGCCGGCCCACCCAGGGCCCTTCACCCACTG
AGCAGCTATTGGGTCAAGCCCGGGACTTCATCAATCAGTACTATAACTCGATCAAAAGGAGTGGTTCCCAGGC
TCATGAGCAGCGGCTTCAGGAAGTGGAAGCTGAGGTGGTGGCCACGGGCACCTACCAGCTCCGGGAGAGTG
AGCTGGTGTTTGGGGCCAAACAGGCCTGGCGCAACGCTCCCCGCTGTGTGGGGCGGATCCAGTGGGGGAAA
CTGCAGGTATTTGATGCTCGGGACTGCAGGACAGCACAGGAAATGTTCACCTACATCTGTAACCACATTAAGTA
TGCAACAAACCGAGGCAATCTTCGTTCAGCCATCACGGTGTTCCCCCAGCGCTACGCTGGCCGGGGAGACTT
CCGGATCTGGAACAGCCAGCTGGTGCGCTACGCGGGCTATAGGCAGCAGGACGGCTCTGTGCGAGGGGACC
CTGCCAACGTGGAGATCACTGAGCTCTGTATTCAACATGGCTGGACCCCAGGAAATGGCCGCTTTGATGTGCT
GCCCCTGCTACTCCAGGCTCCCGATGAGCCCCCAGAACTCTTCACTCTGCCCCCAGAGCTGGTCCTCGAGGT
GCCTCTGGAGCACCCCACGCTAGAGTGGTTTGCTGCCCTTGGCCTGCGCTGGTATGCCCTCCCAGCTGTGTC
CAATATGCTGCTAGAAATCGGGGGCCTGGAGTTTCCGGCTGCCCCTTTCAGCGGCTGGTACATGAGTTCAGAG
ATTGGCATGAGGGACCTGTGTGACCCTCACCGATACAACATACTTGAGGATGTGGCTGTCTGCATGGATCTAG
ACACCCGGACAACCTCATCACTGTGGAAAGACAAGGCAGCAGTGGAAATTAACGTGGCTGTGCTGTACAGTTA
CCAGCTGGCCAAAGTGACCATTGTGGACCACCATGCCGCCACAGCCTCCTTCATGAAGCACTTGGAAAATGAG
CAGAAGGCCAGAGGGGGCTGCCCTGCTGACTGGGCCTGGATCGTGCCCCCCATCTCAGGCAGCCTCACCCC
TGTCTTCCATCAAGAGATGGTCAACTATTTCCTGTCCCCTGCCTTCCGCTACCAGCCTGACCCCTGGAAAGGAA
GTGCAGCAAAAGGCACAGGCATCACCAGGAAGAAGACTTTTAAGGAAGTAGCCAATGCAGTGAAGATCTCTGC
CTCACTCATGGGCACGGTGATGGCGAAGCGTGTGAAGGCGACTATCCTGTATGGCTCTGAGACTGGCCGCGC
CCAGAGCTACGCACAGCAGCTGGGGAGGCTCTTTCGGAAGGCGTTTGACCCCCGGGTCCTGTGCATGGATGA
ATACGATGTGGTATCCCTAGAGCATGAGGCCTTGGTATTGGTGGTGACCAGCACATTTGGCAATGGGGATCCC
CCGGAGAATGGAGAGAGCTTTGCAGCAGCGCTGATGGAAATGTCGGGCCCCTACAACAGCTCCCCTCGGCC
TGAGCAGCACAAGAGTTACAAAATCCGATTCAACAGTGTCTCCTGCTCGGACCCACTGGTATCCTCTTGGCGG
CGCAAAAGGAAGGAATCCAGTAACACAGACAGTGCAGGGGCCCTGGGCACCCTCAGGTTCTGTGTGTTTGGG
CTGGGCTCCAGAGCATACCCGCACTTCTGTGCCTTTGCTCGAGCGGTGGACACAAGGCTGGAGGAGCTGGGC
GGGGAGCGACTGTTGCAGCTGGGCCAGGGTGATGAGCTCTGCGGCCAGGAGGAGGCTTTCCGAGGCTGGGC
CCAGGCAGCCTTCCAGGCTGCCTGTGAAACTTTCTGTGTGGGAGAAGATGCCAAGGCTGCTGCCCGAGATAT
CTTCAGTCCCAAGCGCAGCTGGAAACGCCAGAGGTACCGGCTGAGTACCCAAGCTGAGAGCCTGCAATTACT
GCCAGGGCTGACTCACGTGCACAGACGGAAGATGTTCCAGGCTACAATTCTTTCTGTGGAGAACCTACAGAGC

AGCAAATCCACCCGAGCCACAATCCTGGTGCGTCTGGACACTGGAAGCCAGGAGGGACTGCAGTACCAGCCA
GGGGACCACATAGGTGTGTGCCCACCCAACCGGCCTGGCCTAGTGGAGGCGCTGCTGAGCCGAGTGGAGGA
CCCTCCGCCATCCACAGAGCCTGTGGCCGTGGAACAACTGGAAAAAGGCAGCCCTGGTGGCCCTCCCCCCG
GCTGGGTACGGGACCCCCGGCTTCCCCCATGTACGCTGCGCCAGGCTCTCACTTACTTCCTGGACATCACTTC
CCCGCCCAGCCCTCGCCTTCTTCGACTGCTGAGCACCCTGGCAGAGGAGTCCAGCGAACAGCAGGAGCTAGA
GGCTCTCAGCCAGGACCCCCGGCGCTACGAAGAATGGAAGTGGTTCCGCTGCCCCACACTGCTAGAGGTGCT
GGAACAATTTCCATCCGTGGCACTGCCTGCCCCGCTGATCCTCACCCAGCTGCCCCTGCTCCAGCCCCGGTA
CTACTCTGTCAGCTCAGCACCCAGCGCCCACCCAGGAGAGATCCACCTCACTGTAGCTGTGCTGGCATACAGA
ACCCAGGATGGGCTGGGCCCTCTGCACTATGGGGTCTGTTCCACATGGATGAGCCAACTCAAGGCAGGAGAC
CCGGTGCCCTGCTTCATCAGGGGGGCTCCCTCCTTCCGGCTGCCACCTGATCCTAACTTGCCTTGCATCCTGG
TGGGCCCAGGGACTGGTATTGCACCCTTCCGGGGATTCTGGCAAGACCGATTACACGACATTGAGATCAAAG
GACTGCAGCCTGCCCCCATGACTTTGGTGTTTGGCTGCCGATGCTCCCAACTGGACCATCTCTACCGGGACGA
GGTACTGGACGCCCAGCAGCGTGGAGTGTTTGGACAAGTCCTCACCGCCTTTTCCAGGGATCCTGGCAGCCC
TAAGACCTATGTGCAAGACCTCCTGAGGACAGAGCTGGCCGCGGAGGTTCACCGCGTGCTGTGCCTCGAGCA
AGGACACATGTTTGTCTGCGGTGATGTCACTATGGCAACCAGCGTCCTGCAAACCGTGCAGCGAATTCTGGCA
ACAGAGGGCAGCATGGAGCTGGATGAAGCCGGTGACGTCATCGGCGTGCTGCGGGATCAGCAACGCTACCA
CGAGGACATTTTCGGACTCACATTGCGCACCCAGGAGGTGACGAGCCGCATCCGCACCCAGAGTTTTTCTTTG
CAGGAGCGACAGCTGAGGGGTGCAGTGCCCTGGTCCTTTGACCCGCCTACCCAAGAAACACCTGGTT

Figure 2.9: Gene sequence for endothelial nitric oxide synthase (eNOS) from the rat. Exon-Intron boundaries are marked by a change in colour from black to blue. Primer sequences are highlighted in green (forward) and yellow (reverse).

ATGGCGCAGACCGCCATGTCCGAGACTTACGATTTCTTGTTTAAGTTCTTGGTCATTGGAAATGCGGGAACTG
GCAAATCCTGCTTGCTCCATCAGTTCATTGAGAAGAAATTCAAAGATGACTCAAATCATACCATAGGAGTGGAA
TTCGGCTCAAAGATAATAAATGTTGGTGGTAAATATGTGAAGTTACAGATATGGGACACGGCTGGACAGGAGC
GGTTCAGGTCTGTGACGAGAAGCTACTACAGAGGTGCGGCTGGGGCACTCCTCGTCTATGACATCACCAGCC
GAGAAACCTACAATGCGCTTACTAATTGGTTAACAGATGCCAGAATGCTGGCGAGCCAGAACATCGTCATCATT
CTCTGCGGGAACAAGAAGGACCTGGATGCCGACCGGGAAGTCACCTTCCTTGAAGCCTCCAGGTTCGCACAA
GAGAATGAGCTCATGTTCCTGGAAACCAGTGCACTGACTGGCGAGAACGTCGAAGAGGCTTTCATGCAGTGC
GCAAGGAAGATACTTAACAAAATTGAATCAGGTGAGCTGGACCCCGAGAGGATGGGCTCTGGTATCCAGTATG
GAGACGCCGCCTTGAGACAGCTACGGTCACCCCGACGTACACAGGCTCCAAGTGCACAGGAGTGTGGCTGCT
AG

Figure 2.10: Gene sequence for Rab4 from the rat. Exon-Intron boundaries are marked by a change in colour from black to blue. Primer sequences are highlighted in green (forward) and yellow (reverse).

2.3.9.7 - The Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) can be performed in different ways to measure gene expression, though the basic principle is the same in each instance. The reaction comprises heating single stranded cDNA samples with a specific primer pair designed to bind and amplify a target sequence (i.e. the gene of interest). This amplification can be detected by fluorescent dyes capable of binding only double stranded products.

The process involves a number of amplification cycles, in which each cDNA sample is first denatured before primers can anneal and amplify the target sequence. The quantity of amplification is relative to the amount of starting sequence, thus giving an indication of the overall abundance of that gene. However, it is important to note that the point of measurement is a critical concept in utilising PCR. In the early cycles of the reaction, amplification is characterised by an exponential phase in which the target sequence is doubled; this is reflective of the starting abundance of the target sequence. As the reaction progresses, and individual components (such as primers) decrease in quantity, the amplification can be limited and thus not entirely representative of the starting quantities. As a consequence, it is important to ensure quantification of expression and any analysis performed is based upon data generated in the exponential phase of the PCR reaction as demonstrated in Figure 2.11.

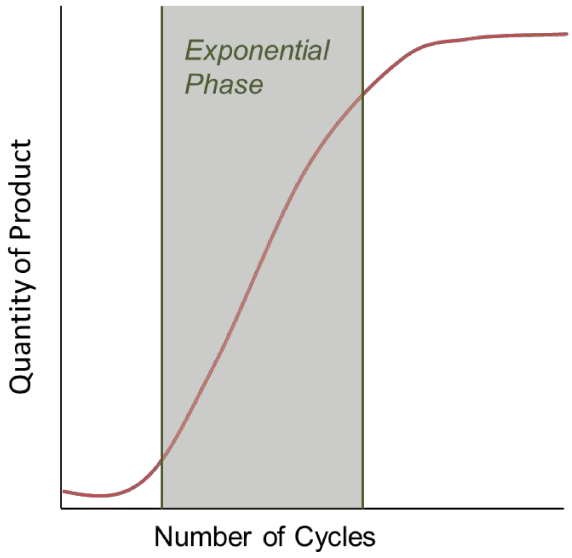


Figure 2.11: A hypothetical PCR reaction in which products are amplified over a number of reaction 'cycles'. A plateau is reached when reaction components decrease in quantity.

2.3.9.8 - Reverse Transcriptase Quantitative PCR (RT-qPCR)

This project relied on a specific method of measuring gene expression, reverse transcriptase quantitative PCR (RT-qPCR). In this experimental set-up the quantity of product can be measured at the end of every individual cycle; this is in contrast to endpoint PCR, which only allows for measurement of products upon completion of the reaction, potentially outside of the exponential phase.

In brief, RNA and cDNA samples were prepared as in Sections 2.3.9.1 - and 2.3.9.4 -. For the PCR reaction, a 'master mix' incorporating SYBR green fluorescent dye (Roche Diagnostics), forward and reverse primers for the target gene (Sigma-Aldrich) and RNase free water was created. Exactly 10µL of this prepared master mix was pipetted into each well to be used on a 384-well lightcycler plate, and 5µL of cDNA sample added to the reaction. Plates were covered with lightcycler foils and set to run on a Light Cycler machine (LC480; Roche Diagnostics, Table 2.9).

Phase of Reaction	Number of Cycles	Settings per Cyle
Pre-Incubation	1	95°C, 5 minutes
Amplification	45	95°C, 10 seconds 60°C, 15 seconds 72°C 15 seconds
Melting Curve	1	95°C, 5 seconds 60°C, 1 second 97°C cont.
Cooling	1	40°C

Table 2.9: Phases of the PCR SYBR Green run used in the experimental protocol. Measurements were performed in 'real-time' at the end of each amplification cycle to obtain a more accurate reflection of gene expression. Melt curve cycles were performed at the end of amplification to ensure a single product of the appropriate size was being produced.

In order to obtain quantitative data, samples were run in triplicate alongside a standard curve of serially diluted cDNA pool ranging from 1:2 to 1:128 in dilution. All experiments were normalised using Quant-IT™ Oligreen® ssDNA reagent. Oligreen is a dye reagent that binds only single stranded sequences and emits a fluorescent signal proportional to the level of binding (Gray & Wickstrom, 1997). As such it can be used as a marker of overall cDNA concentration and account for any 'background' interference that may skew gene expression data. Further details on the genes measured and primers used may be found within respective results chapters.

2.3.10 - Assessment of Vascular Function Via Wire Myography

2.3.10.1 - Preparation of Vessels

Wire myography was used to determine vascular reactivity to vasoconstrictors and vasodilators. The entire mesentery was removed from animals at cull and placed in ice cold physiological saline solution (composition mM: NaCl 119, KCl 4.7, $CaCl_2$ 2, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.026 and $C_6H_{12}O_6$ 5). Mesenteric resistance arteries (of approximate internal diameter 300μm) were dissected and cleaned of all connective tissue under a dissecting microscope. Sections of roughly 2mm in length were cut and gently threaded, with care to maintain the integrity of the endothelium, onto two, parallel stainless steel wires (diameter 40µm). Once secured in place, the vessels were bathed in PSS at 37°C and gassed continuously with 95% O2/5% CO_2.

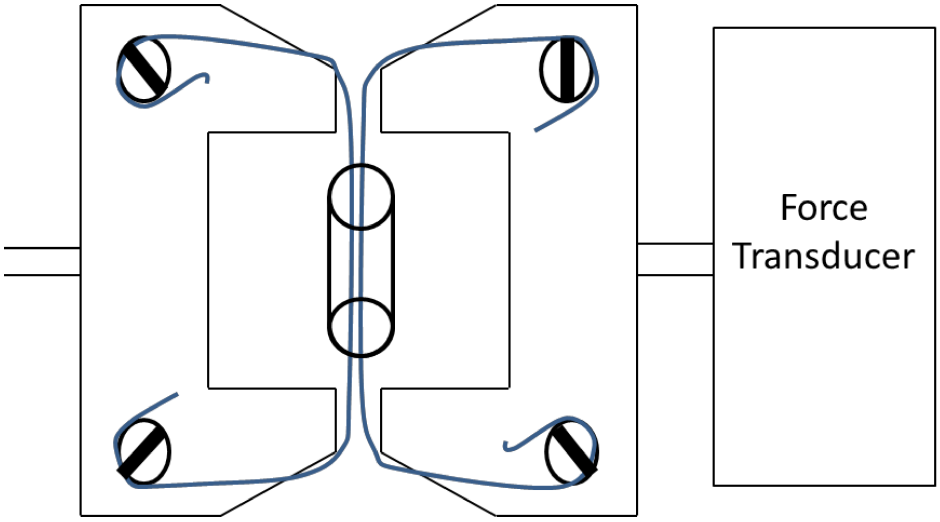


Figure 2.12: Schematic of a vessel mounted in a wire myography chamber. Two stainless steel wires run at parallel through the lumen of the mesenteric artery. Wires are secured at each end using screws. Jaws are connected to a force transducer.

2.3.10.2 - Testing Vessel Quality

Using the Laplace relationship (Letic, 2012) to calculate, vessels were incrementally stretched to a circumference equivalent to a transmural pressure of 100mmHg and the diameter of the vessels set at a value equivalent to 90% of this for the remainder of the experiment. Prior to producing dose-response curves, vessels were subjected to assessment of functional integrity using a 125mM KPSS solution (PSS solution with an equimolar substitution of KCl for NaCl), and any vessel that failed to produce an active tension of 13.3kPa (or 100mmHg) was excluded from the study and replaced. Cumulative dose-response curves were performed in response to phenylephrine (1nM - 100µM). Following this, vessels were administered a dose of the thromboxane mimetic 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α at a concentration capable of eliciting an 80% constriction (approximately 10nM), this was used as a 'pre-constriction' to perform a dose response curve to acetylcholine (1nM-100µM).

Reactivity values were recorded in mV using PowerLab® and extracted using LabChat software (ADInstruments, Oxford, UK). Values for constriction were taken as percentages of a value for 'maximal' contraction, which was generated by a single dose with noradrenaline (10µM) and KPSS (125mM), Values for relaxation were taken as a percentage decrease of a pre-constriction.

Chapter 3 - Characterising the Animal Model

3.1 - Introduction

Sexual dimorphism in renal ageing is well documented (Baylis & Corman, 1998; Neugarten et al., 2000; Baylis, 2009). Over the lifespan, many changes occur in functional renal capacity with significant decreases in glomerular filtration, renal blood flow, arteriolar resistance, and renal mass. These reductions are associated with alterations to glomerular haemodynamics and increased basement membrane permeability, which leads to elevated protein leakage (and thus, proteinuria) (Weinstein & Anderson, 2010; Bolton & Sturgil, 1980; McLachlan et al., 1970). Evidence shows that whilst age-related changes can be fairly well predicted, these effects are often more significant and more deleterious in males. For example, a study by Xu et al., (2010) highlights that the progression to end-stage chronic kidney disease in men is significantly faster than that seen in females (Xu et al., 2010). However, post-menopause renal decline and cardiovascular risk in women not only increases to reach a level similar to that in males, but in some instances exceeds it (Cutler et al.,. 2008; Reckelhoff, 2001). The mechanisms for this have yet to be elucidated, though particular emphasis has been placed on the so-called 'protective effect' of oestrogen in females (Farhat et al., 1996; Mendelsohn & Karas, 1999).

In attempting to isolate a mechanistic pathway by which oestrogen may impact blood pressure regulation, much focus has been placed on the nitric oxide system. Nitric oxide (NO) is a potent vasodilator, that can promote natriuresis and diuresis, and modulate salt and water absorption along the nephron (Herrera et al., 2006). It is produced endogenously by three different isoforms of 'nitric oxide synthase' (NOS), each named for the location in which they were first observed (Ducsay & Myers 2011). Of interest in the present study is the third isoform, endothelial NOS

(eNOS), as it is largely responsible for the production of nitric oxide in the vasculature that contributes to vasodilation and pressure regulation. Nitric oxide and endothelial nitric oxide synthase deficiency are common features of sustained hypertension. Studies in both humans and animals have demonstrated that levels of eNOS and NO production decline with age, and that this decline is more rapid in males when compared with pre-menopausal females (Ahmed et al., 2007; Erdely et al., 2003; Sverdlov et a, 2014). Furthermore, it has been observed that oestrogen is capable of modulating eNOS production (Duckles & Miller, 2010; Stirone et al., 2005), and whilst symptoms of renal decline and cardiovascular disease increase post-menopause with oestrogen and NO decline, they can be reduced by supplementation with oestrogenic hormones (Bush and Barrett-Connor, 1985; Bush et al., 1987; Ernster et al., 1988).

It is not only female sex steroids that are believed to impact renal and cardiovascular function. Androgens have also been implicated in disease progression (Reckelhoff & Granger, 1999; Hayward et al., 2001). Experiments in rats demonstrated that antagonism or mutation of androgen receptors improved hypertension-induced end-organ damage, and reduced levels of circulating renin (Baltatu et al., 2002). Moreover, studies involving castration of young male rats have successfully attenuated the onset of hypertension in spontaneously hypertensive animals (Reckelhoff et al., 1999). Such data has led to speculation that not only is there a beneficial effect of circulating oestrogen, but there may be a deleterious effect of androgens on hypertension and renal function. However, in recent years it has been observed that cardiovascular and mortality risk is significantly higher in men with free testosterone levels in the lowest quartile (Maranon & Reckelhoff, 2013), and recent research by Perusqia et al., (2015) has demonstrated significant and neuronal nitric oxide synthase-dependent (nNOS) hypotensive effects of testosterone in Sprague-Dawley rats. Whilst interactions of androgens with

elements of the nitric oxide system have been less frequently documented, it is clear that their role in renal ageing requires further consideration.

These apparent effects of sex steroids are evident in models of developmental programming. Numerous studies have demonstrated that not only does exposure to a low protein diet during gestation result in elevated blood pressure and poorer renal function in offspring, it can do so in a sex-specific manner (Ozaki et al., 2001; McMullen et al., 2005; Sathishkumar et al., 2012). Prenatal protein restriction in the rat has successfully induced hypertension in male offspring, but not in females (Woods et al., 2005; Woods & Weeks, 2005). These differences in response between males and females seen in the five studies listed above (and others), suggest that the sex steroids may play a significant role in modulating response to a nutritional challenge during foetal life.

It is clear that sex steroids have an important role to play in the development and progression of renal decline, cardiovascular risk and hypertension. However, the mechanisms by which this is occurring are far from clear. Thus, the aim of the following study was to fully characterise the sex-specific processes of normal and accelerated renal ageing in a model of developmentally programmed hypertension.

3.2 - Objectives

The experiments described in the following chapter were utilised to characterise the differences in male and female animals in terms of renal function and distribution of elements of the nitric oxide system in kidney samples, with the following specific objectives:

1) To investigate the impact of sex steroids and the removal of oestrogen/testosterone on the processes of normal and accelerated renal ageing across the life-course.
2) To characterise the expression and activity of elements of the nitric oxide system during these processes and monitor changes in such across the life-course.

3.3 - Methods

Data in the following chapter pertains to animals in Trials I and III, details of which can be found in Chapter Two. In brief, offspring of mothers on low or normal protein diets during gestation were exposed to a gonadectomy or sham-gonadectomy surgical procedure at 10 weeks of age. Offspring were aged until either 6, 12 or 18 months of age when tissue, blood, and urine samples were collected. Analyses for renal function, gene expression, and dyslipidaemia were performed to characterise the physiological profile of animals exposed to a mild gestational nutritional insult over the life course, and the effect of sex steroid modulation.

3.4 - Statistical Analysis

All data are presented as mean values ± standard error of the mean unless otherwise stated. Data from both Trial I and Trial III were analysed using the Statistical Package for Social Science, (vers. 22, SPSS Inc, Chicago Il, USA). Datasets were subjected to a test for normality in the form of a histogram. A three-way ANOVA was performed considering the effects of age, diet, and surgery on dependent variables with a Bonferroni post-hoc test where appropriate. Sex was not included as a separate factor as the ANOVA, as this comparison was encompassed by the control groups in the four surgery categories. A p value of <0.05 was taken to be statistically significant. All graphs were generated in GraphPad Prism 6

(GraphPAD software Inc., San Diego, USA). Significant effects are denoted on the graph, significant interactions are noted in the figure legend. In each group, starting *n* was 8 animals, outliers for each experiment were determined using a box and whisker plot. Samples that fell more than two standard deviations outside the mean of the group were taken to be outliers and excluded from statistical analysis. Any group with a variable *n* is indicated within the figure legend.

3.5 - Results

3.5.1 - Metabolic Measurements

Body weight and organ weights were measured at each cull point at 6, 12, and 18 months of age. Data for body weight and kidney weight are presented in Table 3.1. There was no significant effect of maternal diet on body weight in grams ($p=0.120$) or on kidney weight expressed as a percentage of body weight ($p = 0.433$). There were, however, significant effects of surgery and age on both of these parameters. Intact males had the highest bodyweight, as would be expected, and intact females had the lowest bodyweight ($p<0.01$). Ovariectomy of females resulted in a significant increase in weight at all ages ($p<0.01$), though final bodyweight in these animals was still significantly lower than all male animals irrespective of surgery. Bodyweight increased with age, regardless of surgical group ($p<0.01$).

		6 months				12 months				18 months			
	DIET	IF	OF	IM	CM	IF	OF	IM	CM	IF	OF	IM	CM
Body Weight (g)	NP	265 ± 8.09^{a†}	299 ± 12.71^{b†}	452 ± 17.09^{c†}	398 ± 11.71^{d†}	296 ± 19.74^{e†}	351 ± 20.84^{f†}	504 ± 22.03^{g†}	427 ± 14.73^{h†}	372 ± 18.00^{i†}	422 ± 14.14^{j†}	612 ± 18.50^{k†}	568 ± 34.76^{l†}
	LP	273 ± 8.66^{a†}	330 ± 7.52^{b†}	456 ± 16.41^{c†}	418 ± 12.84^{d†}	291 ± 6.09^{e†}	371 ± 10.40^{f†}	515 ± 18.23^{g†}	472 ± 14.65^{h†}	361 ± 10.48^{i†}	430± 10.80^{j†}	645 ± 25.52^{k†}	525 ± 13.08^{l†}
Kidney Weight (% BW)	NP	0.276 ± 0.012^{a†}	0.241 ± 0.014^{b†}	0.297 ± 0.008^{c†}	0.246 ± 0.009^{b†}	0.242 ± 0.005^{a†}	0.261 ± 0.016^{b†}	0.301 ± 0.011^{c†}	0.250 ± 0.011^{b†}	0.329 ± 0.017^{d†}	0.232 ± 0.009^{e†}	0.331 ± 0.019^{f†}	0.208 ± 0.007^{e†}
	LP	0.276 ± 0.014^{a†}	0.251 ± 0.020^{b†}	0.314 ± 0.021^{c†}	0.236 ± 0.013^{b†}	0.350 ± 0.011^{a†}	0.228 ± 0.015^{b†}	0.293 ± 0.009^{c†}	0.247 ± 0.009^{b†}	0.289 ± 0.012^{d†}	0.229 ± 0.006^{e†}	0.251 ± 0.009^{f†}	0.215 ± 0.005^{e†}

Table 3.1: Body weight (g) and kidney weight (expressed as a percentage of bodyweight) in offspring at 6, 12, and 18 months of age. All animals were offspring of dams exposed to a normal protein (NP) or low protein (LP) diet during gestation. At 10 weeks of age, offspring were ovariectomised (OF), castrated (CM), or exposed to a 'sham' surgical procedure (sham-ovariectomy, IF; sham-castration IM).Bodyweight was significantly different depending on surgery group and age of the animals. Kidney weight was also significantly affected by both surgery and age. There were no effects of maternal diet on either parameter measured. Significant differences between groups are denoted by differing superscript letters ($p<0.05$). † denotes significance at $p < 0.01$.

Food intake, water consumption, and urinary excretion rate were measured in all animals at each timepoint. Data is presented in Table 3.2 below. As with body weight data, there were no significant effects of maternal diet on any dataset. Food intake, water consumption, and urinary output were all significantly affected by surgery and age. Food intake was highest in 6 month old animals, with values decreasing as the animals aged ($p<0.01$). Intake levels were greater in intact females in comparison with all other surgery groups ($p<0.01$). Male animals consumed less than female animals, irrespective of castration surgery ($p<0.01$).

Water consumption followed similar trends; 18 month old animals consumed significantly less water than 6 and 12 month old animals ($p<0.01$). Intact female animals consumed the greatest quantities of water per 100g of bodyweight, and this was significantly greater than all other groups. Intact males consumed the lowest portion of water per day per 100g of bodyweight in comparison with all other groups. Interestingly, there were no significant differences between castrated and ovariectomised females although both differed significantly from their intact counterparts ($p<0.01$).

Urinary excretion (measured as a 24 hourly rate per 100g bodyweight) was lowest in older animals with 18 month animals excreting significantly less than both 12 and 6 month animals ($p<0.01$). This change is likely a reflection of the changes in water consumption noted above. Surgery also significantly altered excretion rates ($p<0.01$), with intact females once again presenting with greater values than all other groups ($p<0.01$). Whilst ovariectomised females differed significantly from intact females, there were no significant differences between castrated and intact males.

		6 months				12 months				18 months			
	DIET	IF	OF	IM	CM	IF	OF	IM	CM	IF	OF	IM	CM
Water Intake (mL/24hr)	NP	5.13 ± 1.23^{a†}	6.93 ± 1.13^{b†}	5.02 ± 0.42^{c†}	5.20 ± 0.29^{b†}	3.80 ± 0.33^{a†}	8.70 ± 2.60^{b†}	5.58 ± 0.28^{c†}	5.02 ± 0.499^{b†}	7.73 ± 0.95^{d†}	4.40 ± 0.48^{e†}	10.53 ± 1.32^{f†}	4.25 ± 0.46^{e†}
	LP	6.54 ± 0.53^{a†}	7.89 ± 0.60^{b†}	10.15 ± 1.16^{c†}	5.70 ± 0.90^{b†}	10.99 ± 1.13^{a†}	7.01 ± 1.69^{b†}	4.69 ± 0.37^{c†}	7.07 ± 2.18^{b†}	7.85 ± 0.74^{d†}	5.74 ± 0.44^{e†}	4.03 ± 0.25^{f†}	4.51 ± 0.41^{e†}
Urine Volume (mL/24hr)	NP	1.68 ± 0.11^{a†}	3.97 ± 0.72^{b†}	2.44 ± 0.17^{c†}	2.45 ± 0.16^{b†}	1.70 ± 0.23^{d†}	3.10 ± 0.26^{e†}	2.24 ± 0.11^{f†}	2.49 ± 0.32^{e†}	3.65 ± 0.69^{d†}	1.88 ± 0.16^{e†}	5.05 ± 0.90^{f†}	2.63 ± 0.42^{e†}
	LP	2.75 ± 0.16^{a†}	4.40 ± 0.24^{b†}	4.73 ± 0.70^{c†}	3.15 ± 0.56^{b†}	4.83 ± 0.72^{d†}	2.48 ± 0.27^{e†}	2.01 ± 0.18^{f†}	1.98 ± 0.21^{e†}	4.04 ± 0.44^{d†}	2.92 ± 0.30^{e†}	1.69 ± 0.20^{f†}	2.13 ± 0.28^{e†}
Food Intake (g/24hr)	NP	4.82 ± 0.74^{a†}	6.60 ± 0.35^{b†}	6.03 ± 0.61^{c†}	5.06 ± 0.21^{d†}	3.74 ± 0.23^{d†}	5.93 ± 0.40^{e†}	5.66 ± 0.33^{f†}	4.94 ± 0.31^{g†}	6.44 ± 0.46^{h†}	3.58 ± 0.30^{i†}	8.45 ± 0.70^{j†}	3.78 ± 0.29^{k†}
	LP	5.43 ± 0.40^{a†}	6.88 ± 0.47^{b†}	7.56 ± 0.52^{c†}	5.11 ± 0.41^{d†}	7.43 ± 0.42^{d†}	5.61 ± 0.27^{e†}	4.67 ± 0.33^{f†}	4.64 ± 0.25^{g†}	5.41 ± 0.32^{h†}	4.58 ± 0.19^{i†}	4.02 ± 0.24^{j†}	3.72 ± 0.17^{k†}

Table 3.2: Data for food and water intake and urinary excretion, and urinary osmolality in offspring of mothers fed a normal (NP) or low protein (LP) diet during pregnancy. At 10 weeks of age, offspring were ovariectomised (OF), castrated (CM), or exposed to a 'sham' surgical procedure (sham-ovariectomy, IF; sham-castration IM). Values are mean ± SEM of measures taken at 6, 9, and 12 months of age. N is 8 per group. Results largely followed similar trends. There were no effects of maternal diet on food intake, water consumption, or urinary output. However, surgery group and age had a significant effect on all three paramaters ($p<0.01$ in all instances). Significaance is denoted by differing superscript letters ($p<0.05$). † denotes significance at ($p<0.01$).

3.5.2 - Expression of Endothelial Nitric Oxide Synthase (eNOS)

mRNA expression of endothelial nitric oxide synthase was determined in the kidneys of all animals from Trial I, in an attempt to observe the effects of age and surgery on expression, and whether or not this affected the production of nitric oxide.

Figure 3.1 shows data from Trial I animals in all groups. There was no significant effect of age ($p=0.959$), surgery ($p=0.122$) or maternal diet ($p=0.105$) on gene expression of eNOS.

These data suggest that any changes apparent in other markers of the nitric oxide system are unrelated to changes in the inherent expression of endothelial nitric oxide synthase in the kidney.

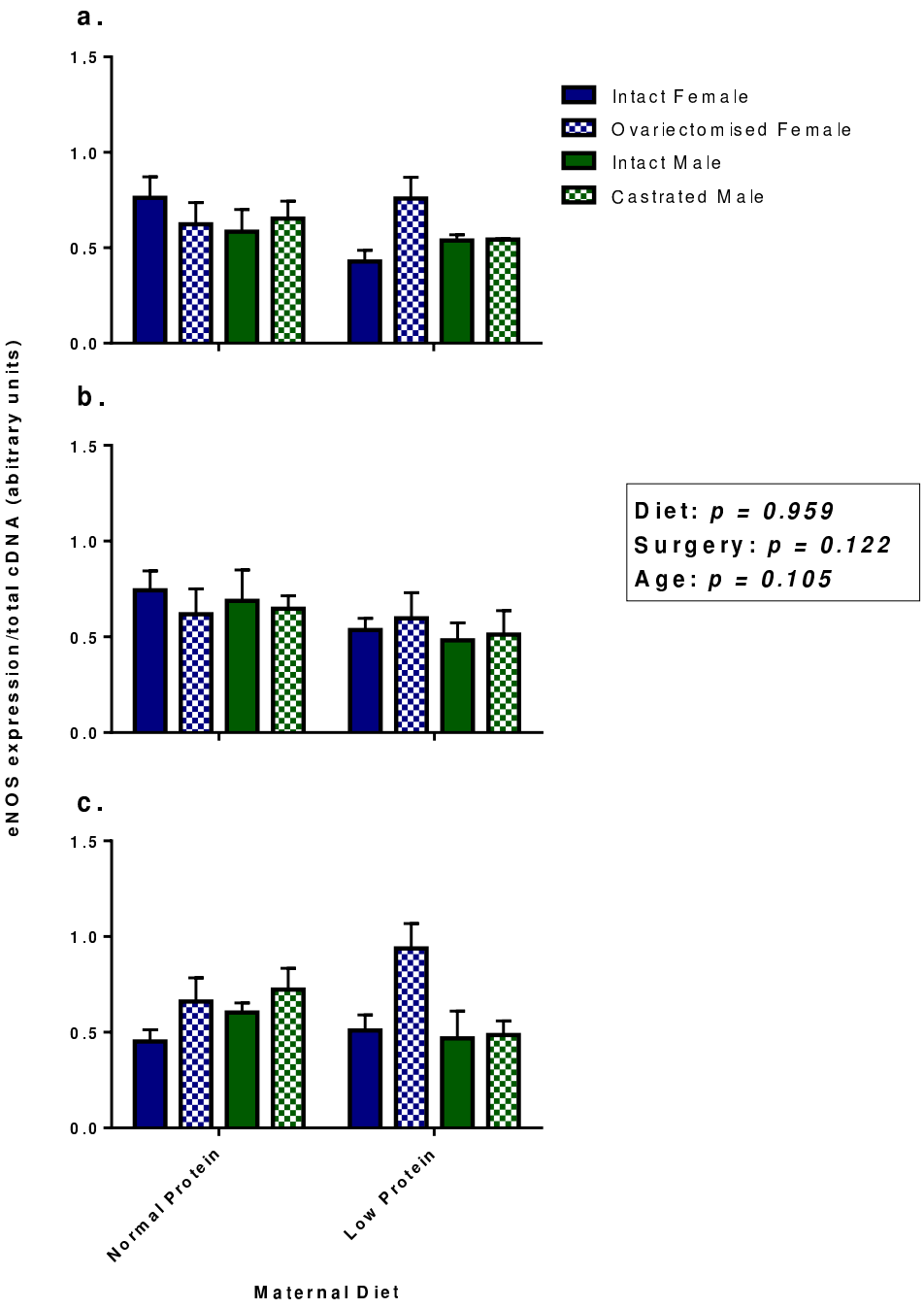


Figure 3.1: Gene expression of endothelial nitric oxide synthase was determined in kidneys from a) 6 month, b) 12 month, and c) 18 month old offspring from normal- and low protein prenatal diet groups. Offspring were either castrated, ovariectomised, or exposed to a sham surgical procedure. All expression was normalised to the total concentration of cDNA. In each group *n* is between 5 and 8. No significant differences were seen between groups.

3.5.3 - Expression of Rab4

Expression of Rab4 mRNA was determined alongside endothelial nitric oxide synthase. Rab4 is a member of a family of renin-angiotensin system GTPases responsible for intracellular protein trafficking (Seachrist & Ferguson, 2003). Recent evidence suggests that Rab4 may be implicated in the resensitization of the AT_1R in the presence of angiotensin II (Esseltine et al., 2011). Increased AT_1R sensitivity and activity are common features of hypertension (Kobori et al., 2007), and as such inappropriate sensitization of the AT_1R via Rab4 may play a role in perpetuating the renal ageing phenotype considered within this thesis. Data was generated from kidneys of Trial I animals at 6, 12 and 18 months of age after exposure to a normal or low protein prenatal diet and either a gonadectomy or sham-gonadectomy procedure.

Data are shown in Figure 3.2. There was no significant effect of age, surgery or diet individually. There was however a significant interaction between age and diet ($p<0.05$). In normal protein offspring, expression decreased with age. Expression of Rab4 at 18 months in normal protein animals was approximately 20% less than that seen at 6 months. In low protein offspring, there was a 15% increase in Rab4 expression between 6 and 18 months. It should be noted that this significance associated with low protein offspring is likely driven by a dramatic increase in low protein ovariectomised female Rab4 expression at the 18 month time point. These results suggest that there are different mechanisms active in the low protein offspring when compared to their normal-protein counterparts.

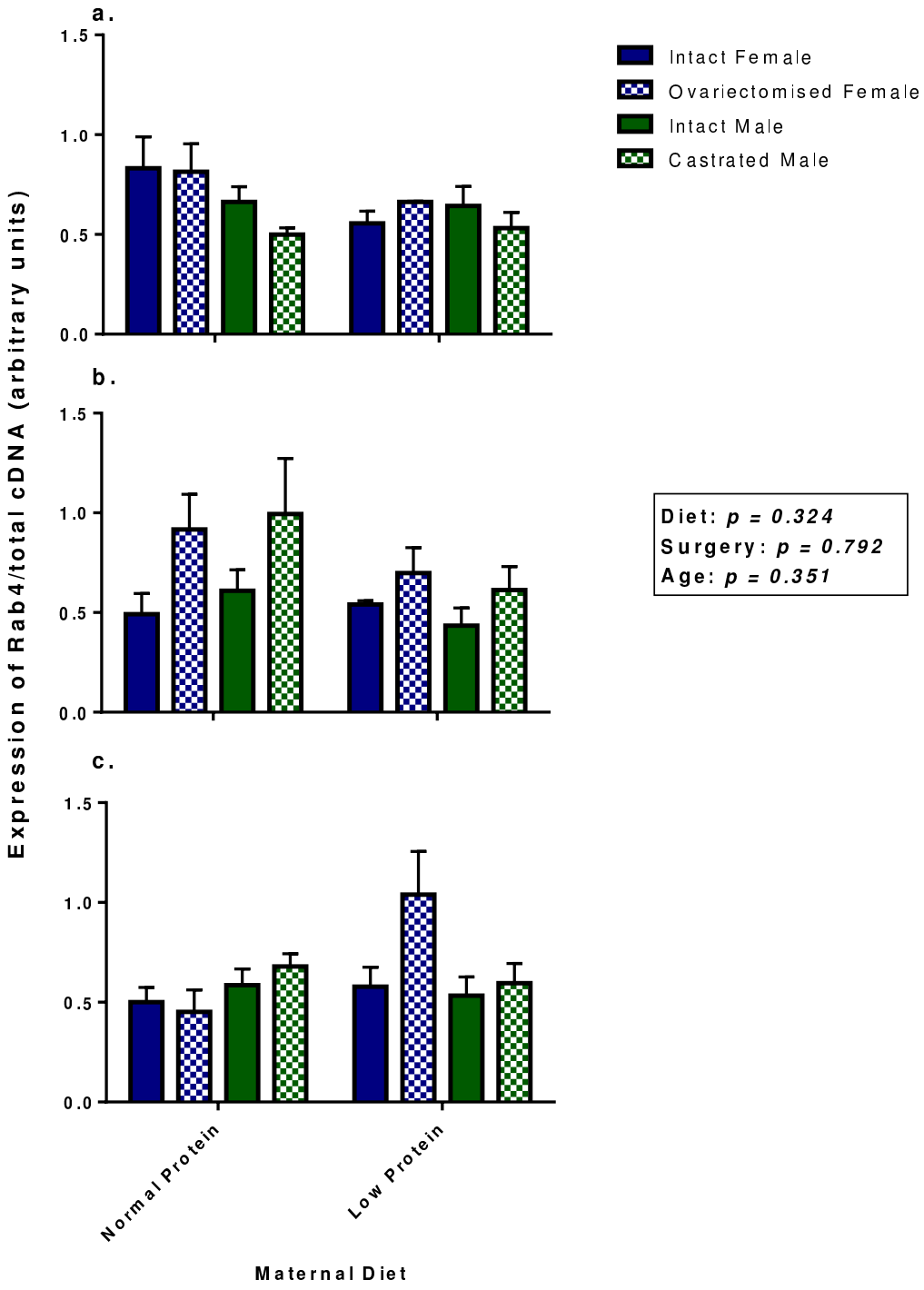


Figure 3.2: Gene expression of Rab4, normalised to total cDNA concentration, in kidneys of normal and low protein offspring from Trial I. Samples are from animals at a) 6 months, b) 12 months, and c) 18 months. A significant interaction was observed between age and diet ($p<0.05$). N per group = 5-8.

3.5.4 - Urinary Nitrite Excretion

Concentration of nitrites was measured in urine samples of animals from all animals in trial I as an indicator of nitric oxide concentrations.

In Trial I (Figure 3.3) a number of significant effects were observed. Urinary nitrite excretion was significantly altered by age ($p<0.001$), with 18 month old animals excreting significanrtly less nitrites than both 6 and 12 month old animals. In addition, 12 month old animals excreted significantly more nitrites than 6 month old animals as well ($p<0.01$).

There were also significant differences between surgical groups. Intact females had the highest values for urinary nitrite excretion when compared to all other groups ($p<0.01$). This was accompanied by a significant interaction between age and surgery ($p<0.01$). This is likely driven by a drastic increase in nitrite excretion in 12 month old intact females of both normal and low protein fed mothers. There was no significant effect of maternal diet alone or in conjunction with another factor.

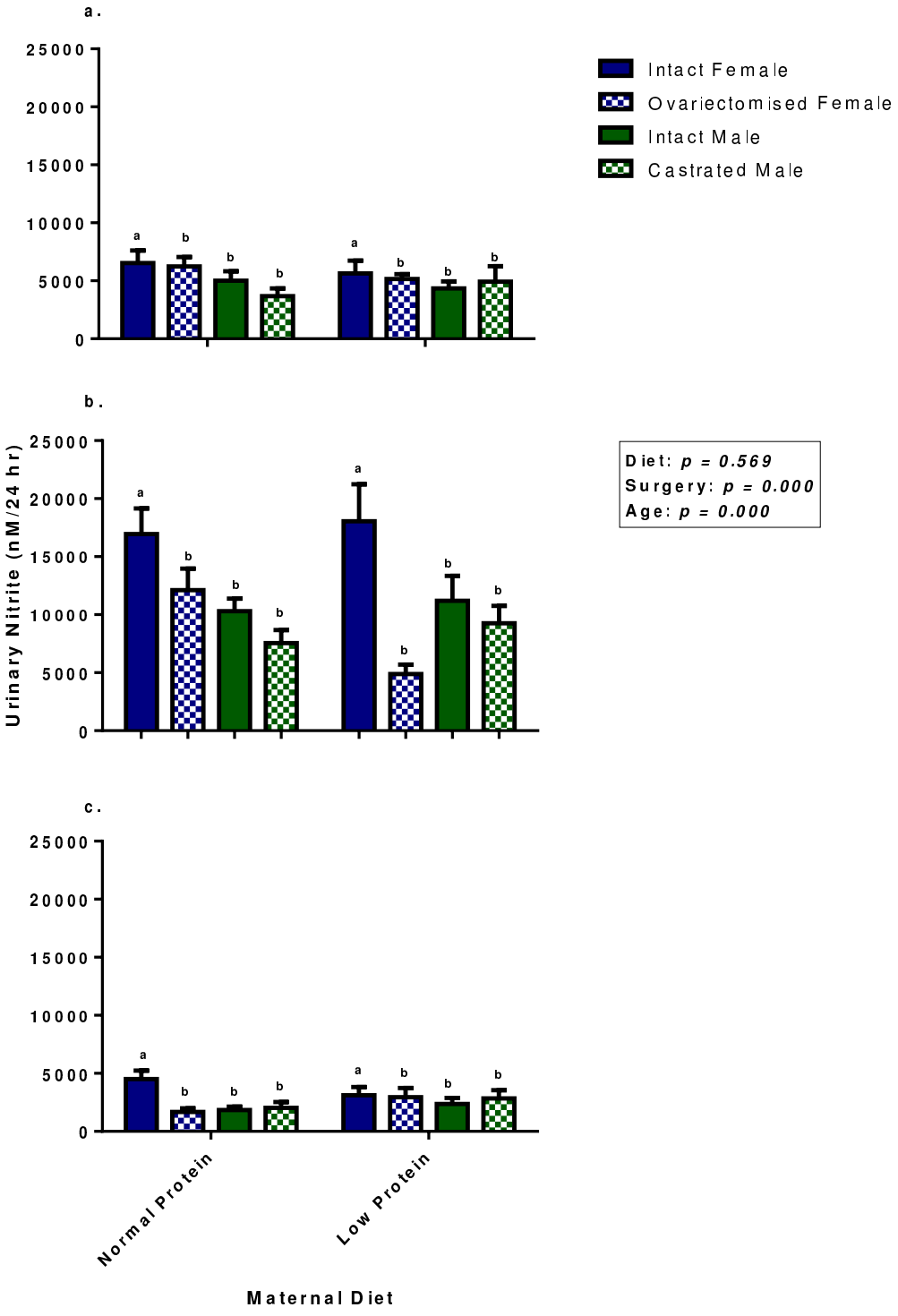


Figure 3.3: Concentration of urinary nitrites (nM/24hr) in samples from a) 6 month, b) 12 month, and c) 18 month old animals. Animals were from mothers on either a normal or low protein diet during pregnancy, and were exposed to either gonadectomy or sham-gonadectomy surgery. Nitrite concentration was significantly affected by age ($p<0.001$) and surgery ($p<0.001$), and there were significant interactions of age with with surgery ($p<0.05$). N is 8 per group.

3.5.5 - Markers of Renal Function

3.5.5.1 - Nephron Number

Total number of nephrons was determined in fixed kidneys from 12 and 18 month animals exposed to either a normal- or low protein prenatal diet, from sham- or gonadectomy surgery groups.

Figure 3.4 illustrates the total number of nephrons per kidney. As has been demonstrated in previous studies, nephron number was significantly decreased in offspring exposed to a low protein prenatal diet when compared to offspring of the normal protein group ($p<0.001$) (McMullen et al., 2004; Langley-Evans et al., 1998). Significant differences were seen across surgical groups, with intact females having significantly higher nephron number per kidney than both ovariectomised females and intact males ($p<0.05$). Castration of males conferred some preservation of nephron number, but this was not statistically significant ($p = 0.068$). There was no decline in nephron number apparent with age.

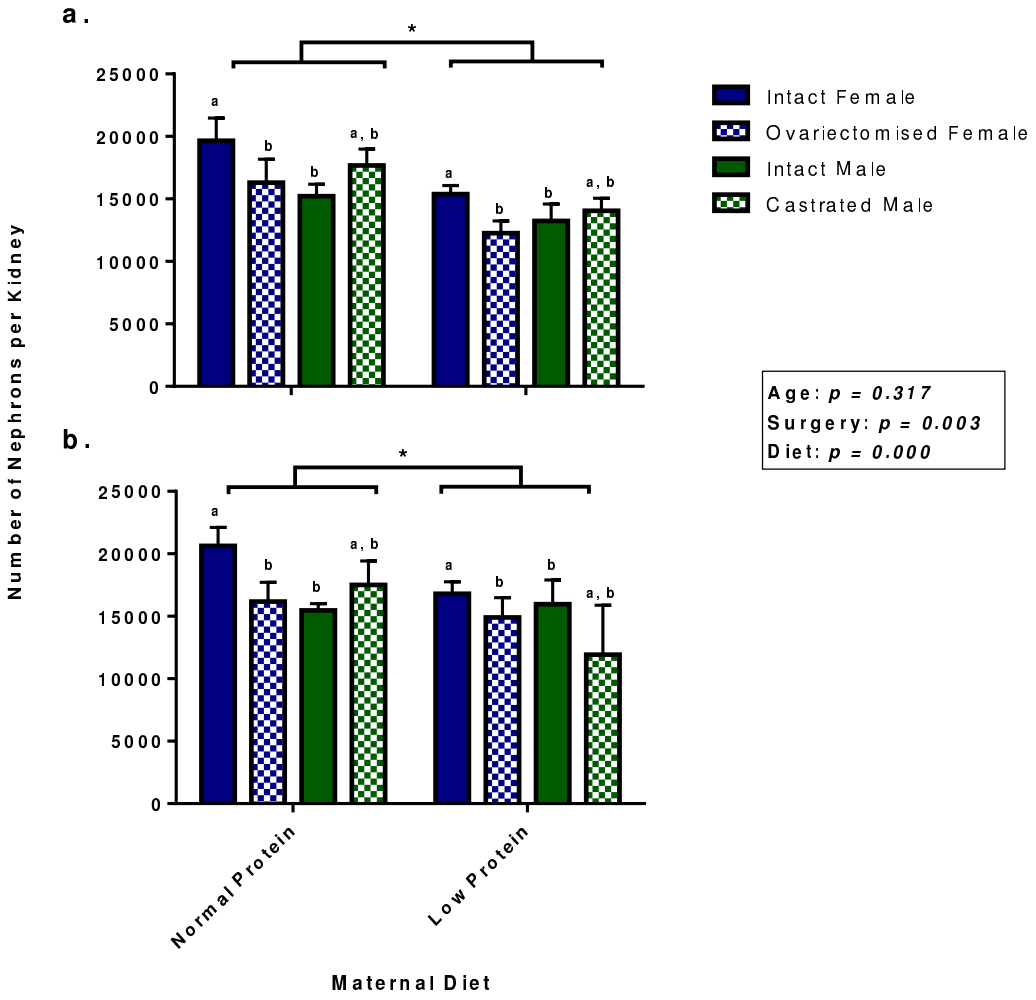


Figure 3.4: Total kidney nephrons in a) 12 month and b) 18 month old offspring of dams exposed to a normal protein (NP) or low protein (LP) diet during gestation. Data from animals that were ovariectomised, castrated, or exposed to a 'sham' surgical procedure. Maternal diet significantly affected nephron number; NP>LP $p<0.001$. Nephron number was also affected by surgical group, intact female > intact male and ovariectomised females ($p<0.05$). N is 8 per group. Significant differences between surgical groups are denoted by superscript letters. Asterisks denote significant differences between maternal diet groups.

3.5.5.2 - Creatinine Clearance

Creatinine clearance was determined at 6, 12, and 18 months in offspring from normal- and low protein mothers after exposure to gonadectomy or sham-gonadectomy surgery as a proxy measurement for glomerular filtration rate (GFR), as is demonstrated by Figure 3.5. As creatinine undergoes tubular secretion and can therefore overestimate glomerular filtration (Nguyen et al., 2009), there has been speculation as to the precision of creatinine clearance as a measure of GFR. Still, it is generally accepted to be the most expedient and practical measurement of glomerular filtration widely available as creatinine is freely filtered, not metabolised by the kidney, and physiologically inert (Perrone et al., 1992).

In accordance with the literature, clearance rate was significantly diminished with age (Musso & Oreopoulos, 2011; Weinstein & Anderson 2010), with 6 months animals presenting with significantly higher clearance values than both 12 and 18 month old offspring ($p<0.001$). A significant effect of surgery was also observed and intact females demonstrated higher creatinine clearance than all other groups ($p<0.001$). This is reflective of the 'protective gender' hypothesis (Silbiger and Neugarten, 1995; Kummer et al., 2012), with the beneficial effects of oestrogen on GFR being negated by ovariectomy surgery. Male offspring had significantly lower creatinine clearance than intact females ($p<0.001$), and this was unaffected by castration. Regardless of surgery or age, there was no effect of maternal diet on creatinine clearance.

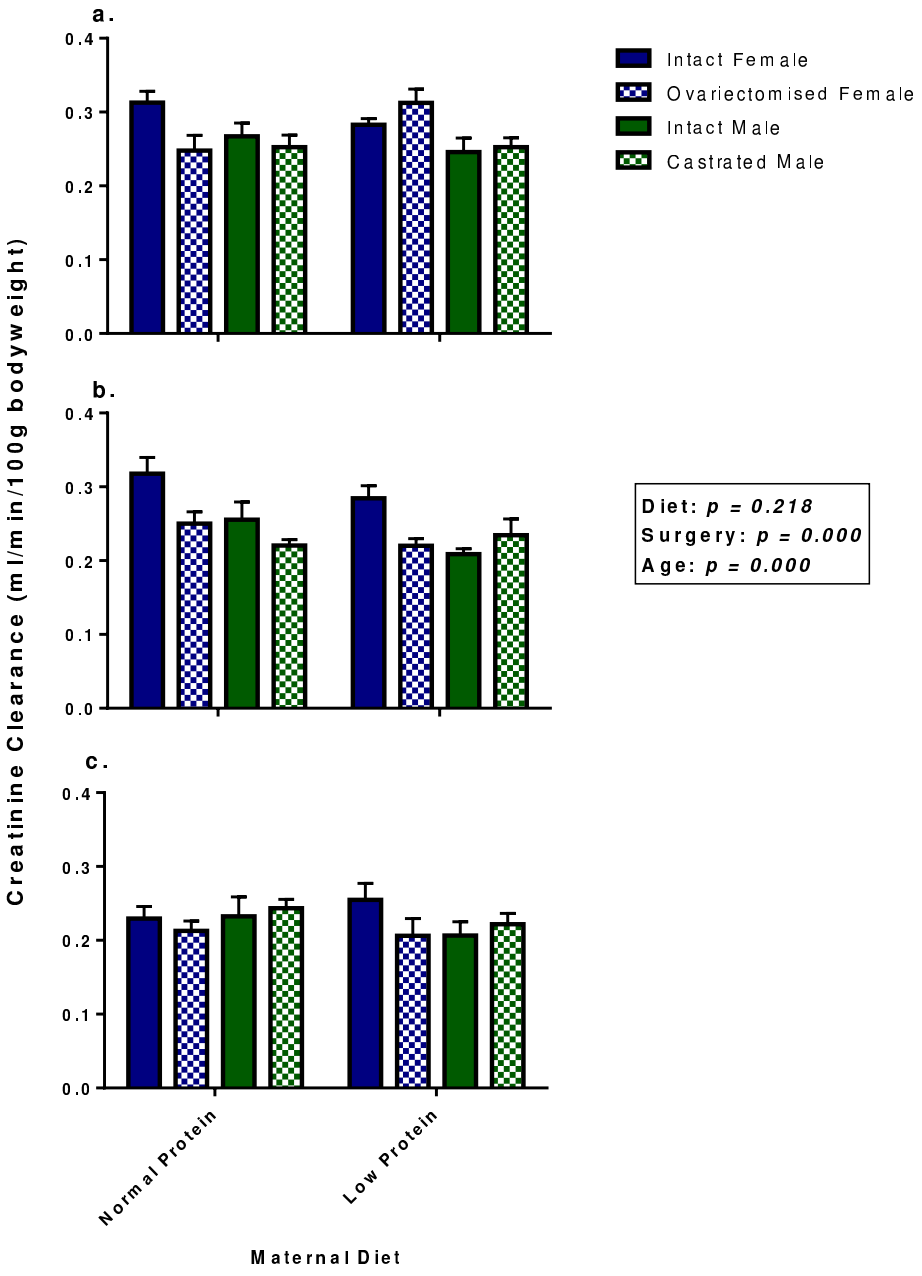


Figure 3.5: Creatinine clearance (ml/min/100g bodyweight) in offspring at a) 6 months of age, b) 12 months of age, and c) 18 months of age. All animals were offspring of dams exposed to a normal protein (NP) or low protein (LP) diet during gestation. At 10 weeks of age, offspring were ovariectomised, castrated, or exposed to a 'sham' surgical procedure. Animals in the 6 month group had significantly higher clearance rates than those in the 12 and 18 month groups ($p<0.001$). N is 8 per group. Significant differences between surgical groups are denoted by superscript letters. Asterisks denote significant differences between maternal diet groups.

3.5.5.3 - Urinary Creatinine Excretion and Plasma Creatinine

In order to determine a rate of creatinine clearance, urinary and plasma creatinine were measured in all Trial I animals, the data for which can be seen in Figure 3.6 and Figure 3.7.

As would be expected, urinary creatinine values closely reflect the patterns seen in creatinine clearance. Urinary creatinine was significantly altered with age ($p<0.001$), with 6 month animals excreting more creatinine than both 12 and 18 month animals ($p<0.05$), and 12 month animals also excreting significantly more than 18 month old animals ($p<0.02$). There was a significant effect of surgery on urinary creatinine excretion, with intact females having higher values than all other surgery groups ($p<0.001$).

The concentration of plasma creatinine is kept fairly constant in the body (Sherwood, 2014), and as such minimal differences were seen in the data generated. There was no significant effect of maternal diet or surgery group on the results. There was however, a significant effect of age. Animals in the 6 month group had significantly lower plasma creatinine concentrations than animals of 12 and 18 months of age ($p<0.02$). However, whilst statistically significant, the differences in plasma creatinine are numerically very small. As such, these differences should be considered with caution. In a large trial, with a good number of animals per group, small physiologically insignificant differences can be identified by analysis, and the relevance of this with regards to renal function cannot be overstated.

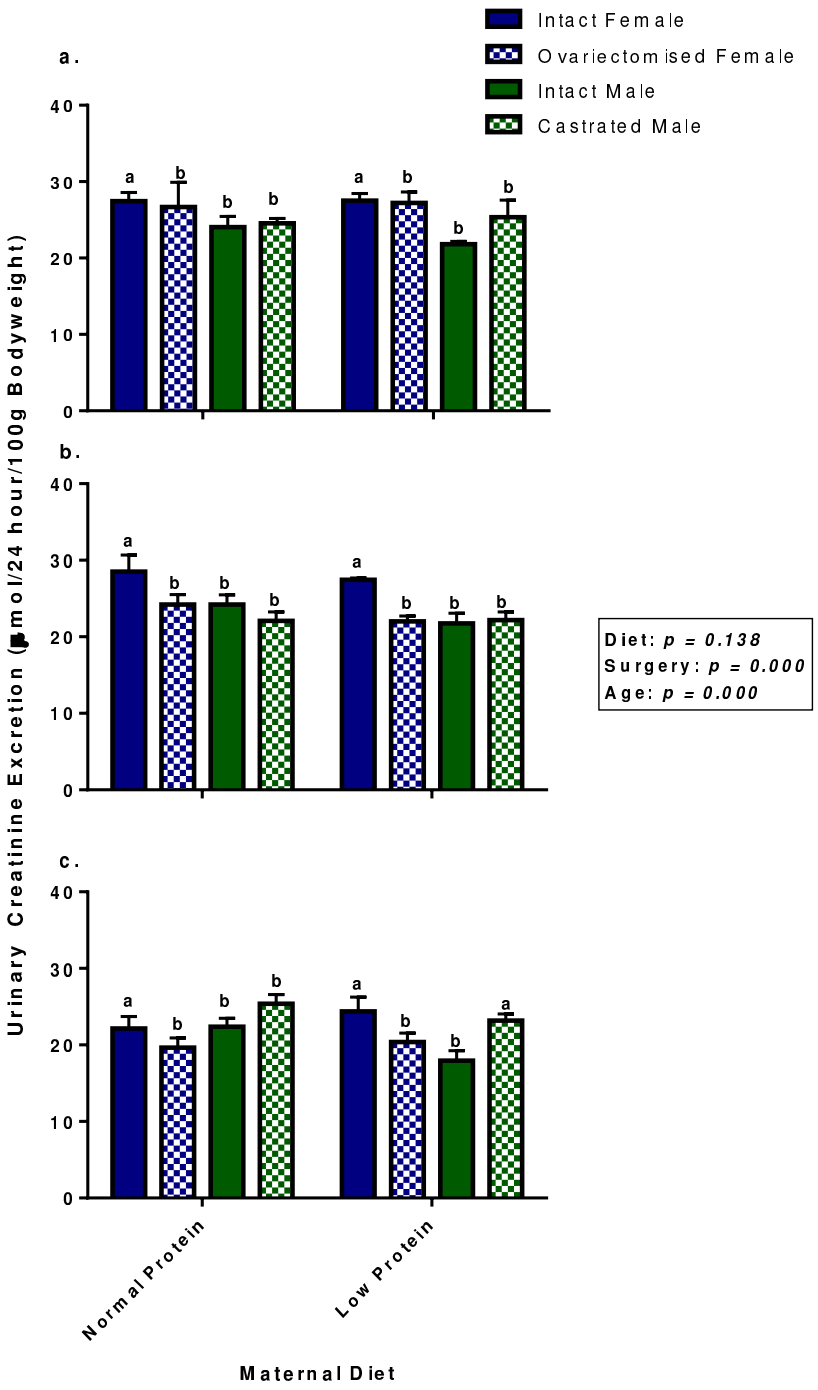


Figure 3.6: Urinary creatinine excretion over 24 hours in all offspring from trial I at a) 6 months, b) 12 months, and c) 18 months of age. A significant effect of age was observed; creatinine excretion decreased with age, with 6 month values greater than 12 months, and 6 and 12 month values greater than 18 months ($p<0.001$). N is 8 per group. Significant differences between surgical groups are denoted by superscript letters.

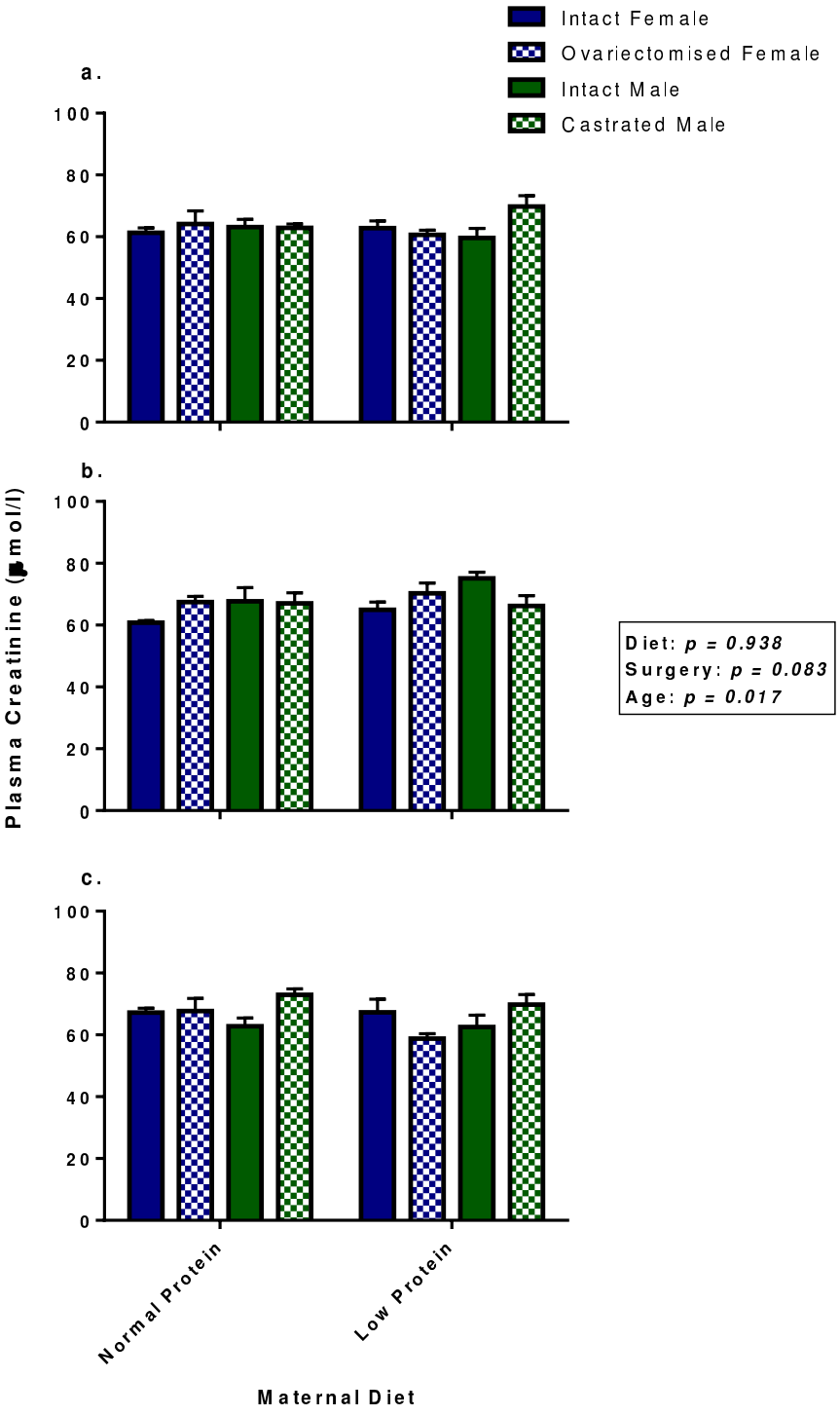


Figure 3.7: Plasma creatinine concentration (µmol/l) in Trial I animals at a) 6 months, b) 12 months, and c) 18 months of age. Only a significant effect of age was observed, with 6 month plasma creatinine lower than both 12 and 18 month values ($p<0.02$). N is 8 per group

3.5.5.4 - Plasma Osmolality

Plasma osmolality was measured in all Trial I animals to add to the portfolio of renal markers. Whilst often used as an indicator of hydration, osmolality can offer insight into the concentrating capacity of the kidneys, and has been demonstrated to be influenced by sex hormones (Wenner & Stachenfeld, 2012). Constant plasma osmolality plays a significant role in maintaining stable blood pressure, and as such large fluctuations can be key indicators of overall renal function (Henrich et al., 1980).

Plasma osmolality (mOsm /kg H_2O) was unchanged with age, but it was significantly affected by both maternal diet ($p<0.01$), and by surgery ($p<0.01$) as can be seen in Figure 3.8. On average, normal protein offspring had significantly higher plasma osmolality that low protein offspring, and this was true at all ages. Both intact and ovariectomised females had significantly lower plasma osmolality than intact and castrated males ($p<0.01$), but were not different from one another. There were no significant differences between intact and castrated males.

There was a significant interaction between maternal diet and surgery ($p<0.05$). This is less clear in its import, as the trends observed between surgery groups are the same between maternal diet groups. It is possible that the high statistical power of the experiment is highlighting very small, physiologically insignificant differences between groups.

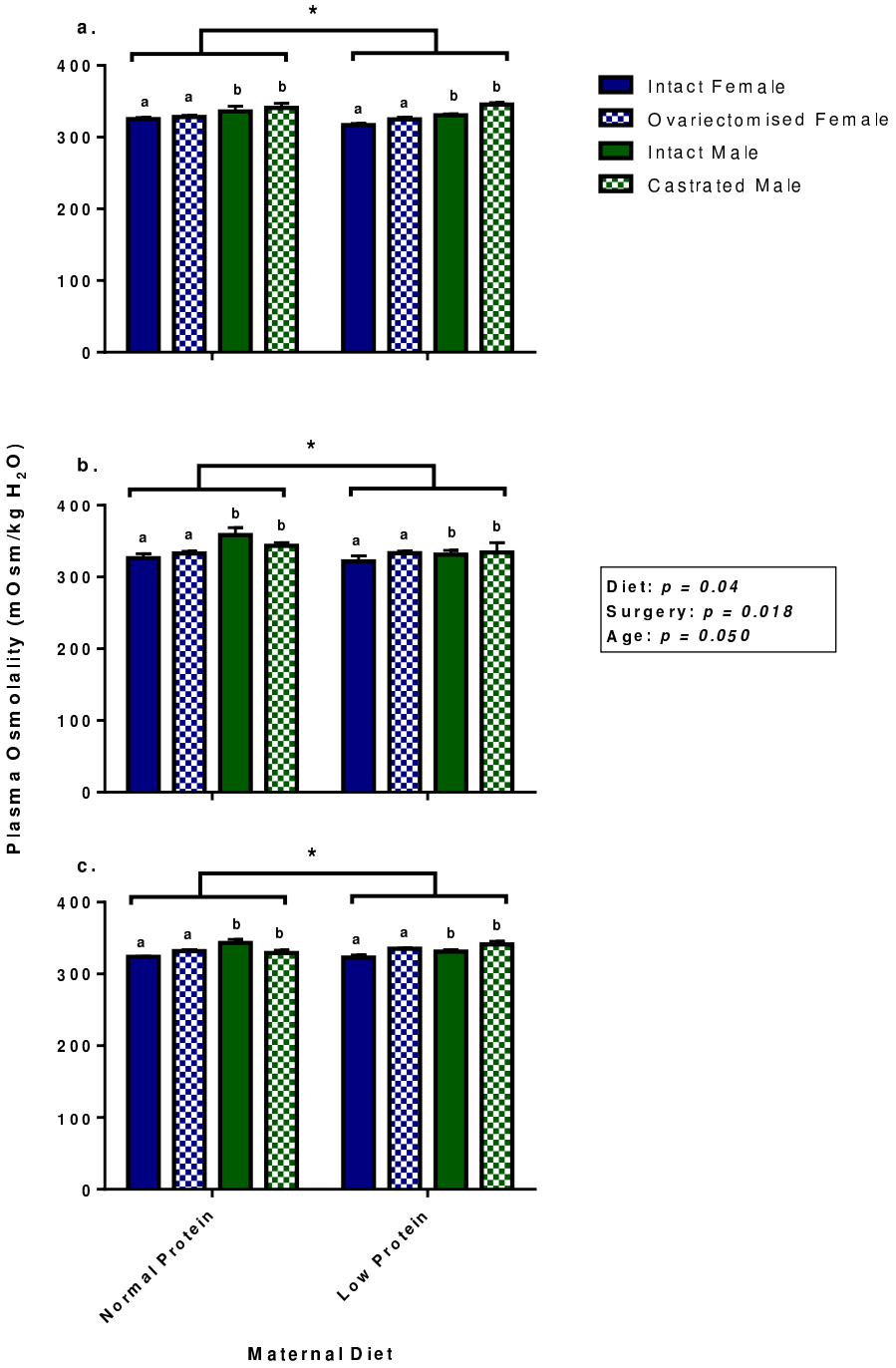


Figure 3.8: Plasma osmolality (mOsm/kg H_2O) measured in undiluted plasma samples from all offspring in trial I at a) 6 months, b) 12 months, and c) 18 months of age. Univariate ANOVA showed a significant interaction between diet and surgery ($p<0.05$), and significant effects of diet and surgery alone ($p<0.01$ in both instances). In each group, *n* is between 5 and 8 animals. Significant differences between surgical groups are denoted by superscript letters. Asterisks denote significant differences between maternal diet groups.

3.5.5.5 - Urinary Urea Excretion

Data for urinary urea excretion are presented in Figure 3.9. Measurements were taken in animals from 6, 12 and 18 month time points in all groups of Trial I. Urea excretion was not affected by age ($p = 0.176$), but did vary with surgery group ($p<0.001$) and in response to maternal diet ($p<0.05$). On average, offspring from mothers fed a control diet excreted significantly less urea than offspring of low protein fed mothers. As with other datasets, intact female animals differed the most from other groups, excreting significantly less urea than male animals irrespective of castration. Ovariectomy of female animals had no effect on outcomes.

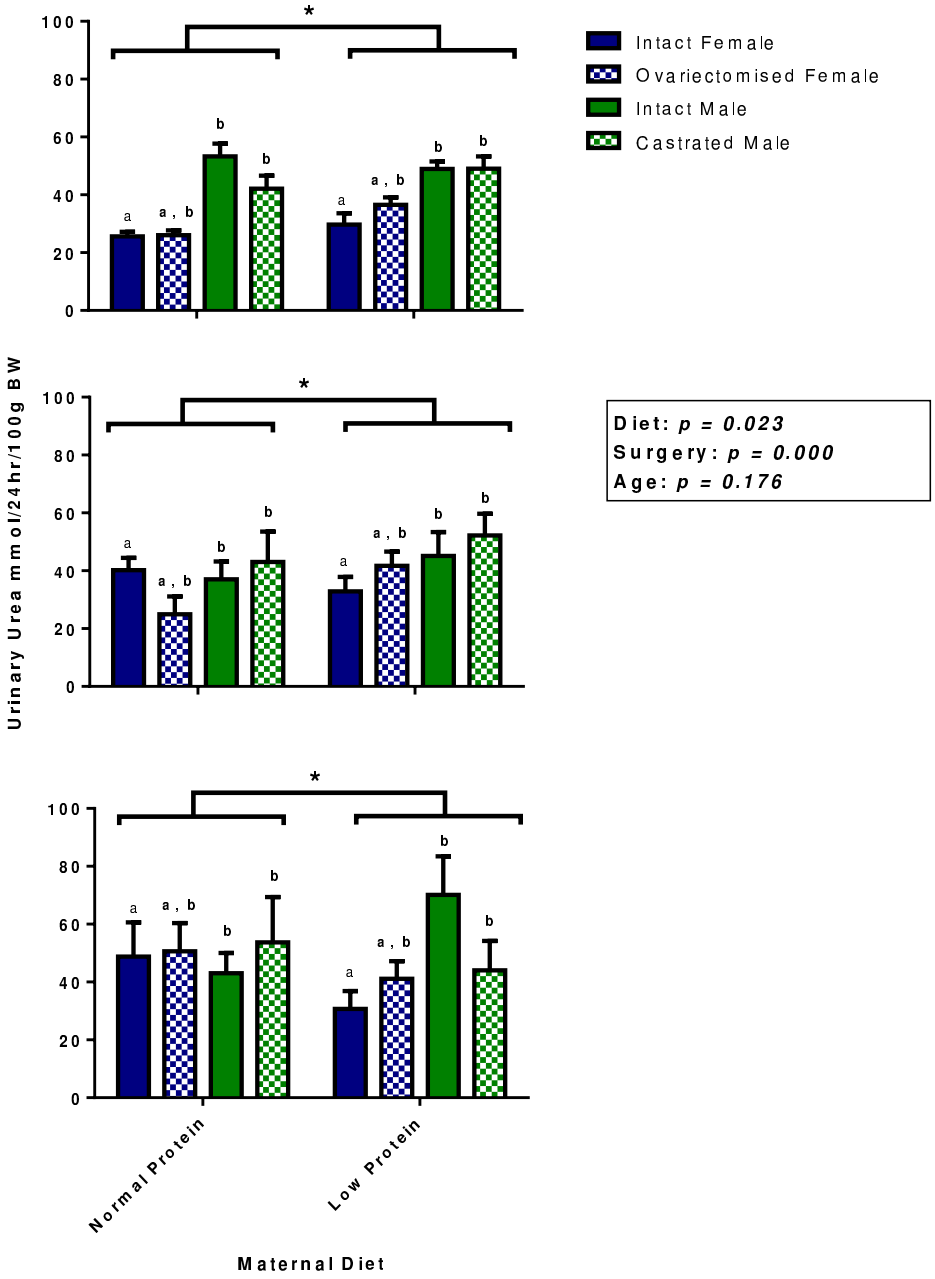


Figure 3.9: Urinary urea excretion (mmol/l/ 100g bodyweight) in animals at a) 6 months, b) 12 months and c) 18 months of age. Animals are offspring from mothers on normal- and low protein diets, and have undergone gonadectomy or sham-gonadectomy surgery at 10 weeks of age. A significant effect of age was observed; 18 month old animals excreted significantly less urea than both 6 and 12 month old animals ($p<0.001$). N = 8 per group.

3.5.6 - Protein Carbonyl Concentrations

Protein carbonyls were measured in homogenised frozen kidney samples from animals at 6 and 18 months of age, to offer a comparison of the 'young' and 'old' ends of the ageing model. All surgery groups and both diet groups were measured in these two time points.

There were no significant univariate effects of surgery, age, or maternal diet groups (Figure 3.10) There were, however, significant interactions between surgery and age ($p<0.02$), and between surgery, age, and diet ($p<0.05$). In both instances these interactions are likely driven by the dramatic age-related increase in carbonyl concentration seen in low protein females in comparison with any other group. Indeed, it can be argued that effects of surgery are likely an effect of sex as there is no significant difference between sham- and gonadectomy animals when compared. Effects of diet appear related to the direction of change of carbonyl concentrations with ageing. In normal protein offspring carbonyls tended to decrease with age, whilst in the low protein offspring, the opposite was true.

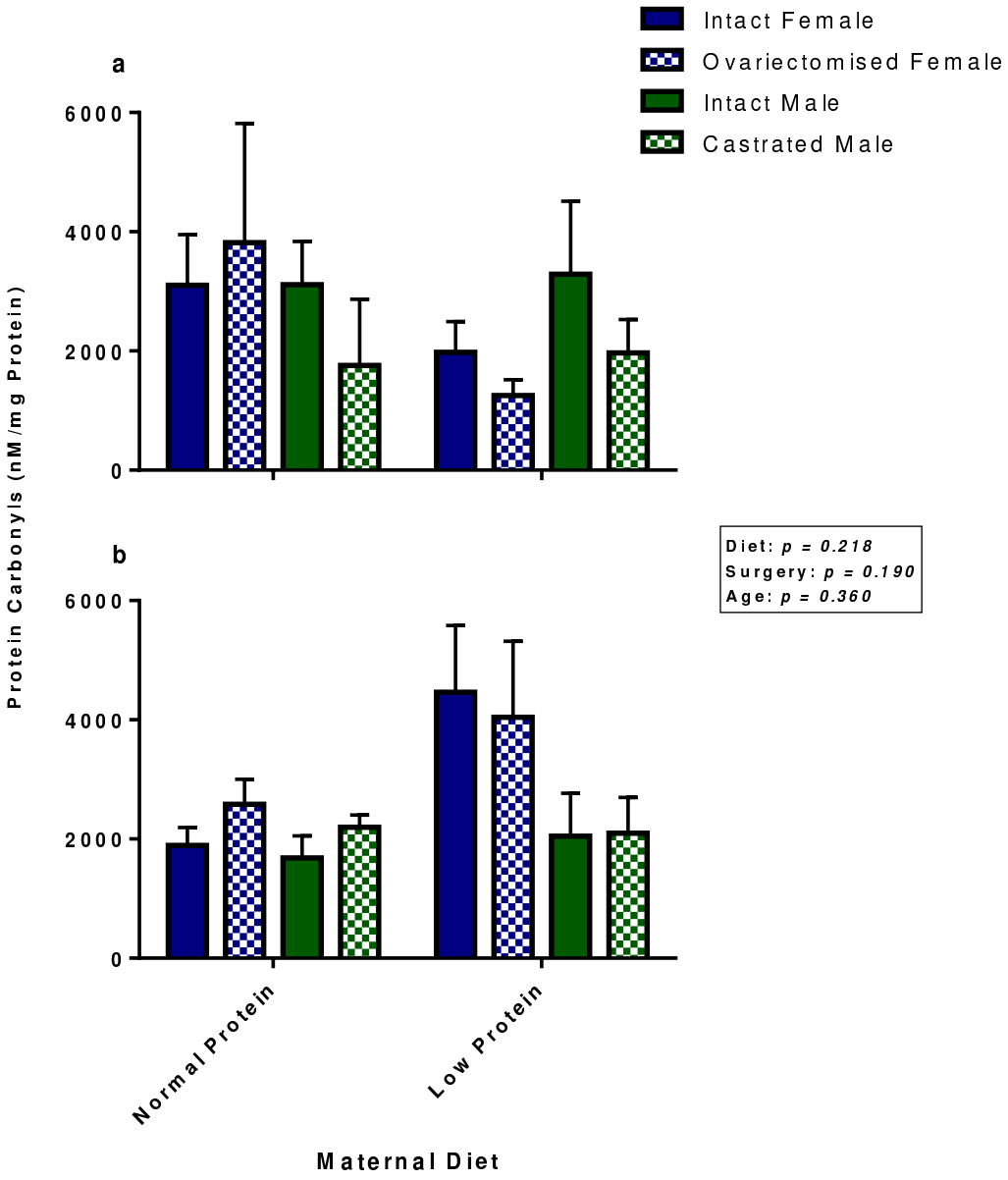


Figure 3.10: Protein carbonyls measured per microgram of protein in a) 6 month and b) 18 month animals. Data are from offspring of mothers on normal and low protein diets during pregnancy that have undergone a gonadectomy or sham-gonadectomy procedure. Significant interactions were observed with surgery*age $p<0.02$, and surgery*age*diet $p<0.05$. N is 5-8 per group.

3.5.7 - Circulating Lipid Concentrations

Lipid profiles are commonly used as an indicator of overall health. Increased circulating and hepatic lipid concentrations are typically associated with increases in cardiovascular risk (Miller et al., 2011; Sarwar et al., 2007). Furthermore, Peinado et al., (2007) argue that there is a strong correlation between certain serological markers, including triglycerides and cholesterol, and nitric oxide concentrations. Whilst this correlation is contrary to the hypotheses of this project, suggesting higher levels of NO in males are associated with increased cardiovascular risk (Peinado et al., 2007), it is evident that circulating lipids bear further scrutiny in the investigation of nitric oxide and renal ageing. In addition, it has been demonstrated that animals exposed to a low protein diet *in utero* are predisposed to aberrant lipid regulation with age (Erhuma et al., 2007; Burdge et al., 2004). Given this, circulating lipids were measured as an indicator of cardiovascular risk and as a biomarker of the low protein model.

3.5.7.1 - Plasma Cholesterol

Plasma cholesterol was measured in Trial I plasma samples in animals from all surgery, diet and age groups (Figure 3.11).

Plasma cholesterol was unaffected by maternal diet, and there was no effect of surgical group alone. Cholesterol concentrations were significantly higher at 12 months of age ($p<0.02$) when compared with samples at 6 and 18 months. A statistically significant interaction between age and surgery was observed ($p<0.05$). This is likely linked to the magnitude of change in concentration observed in female animals (both ovariectomised and intact), compared to males (both castrated and intact) across the 6, 12, and 18 month time points. Whilst plasma cholesterol increased at 12 months in male animals as it did in the female animals, this increase

was smaller. Furthermore, the decrease in concentration at 18 months was smaller in male animals when compared with female animals.

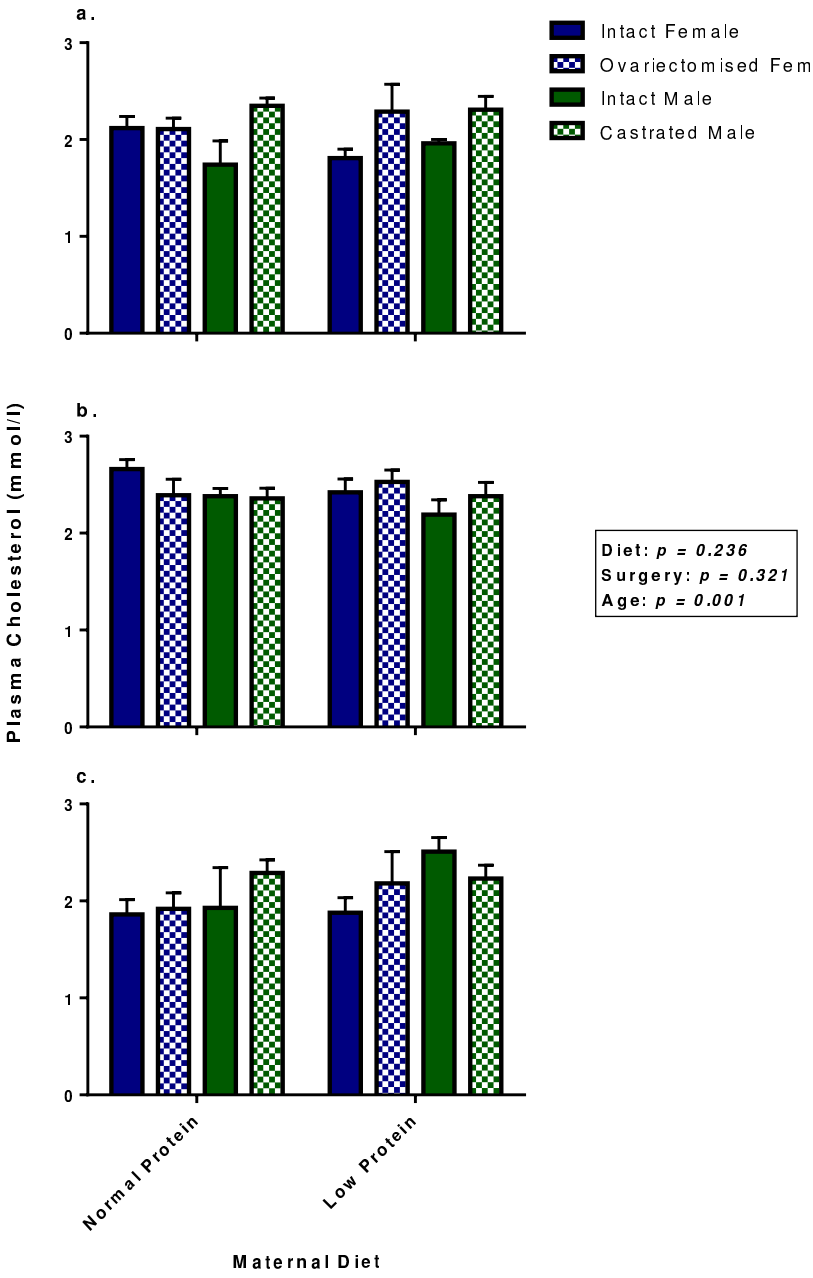


Figure 3.11: Plasma cholesterol (mmol/l) in animals from trial I at a) 6 months, b) 12 months, and c) 18 months of age. Offspring exposed to a normal or low protein diet during gestation and exposed to a sham- or gonadectomy procedure were sampled. Age of the animals had a significant effect on concentration with 12 month animals demonstrating significantly higher total cholesterol than both 6 and 18 month old animals ($p<0.02$). There was also a statistically significant interaction between age and surgery ($p<0.05$). N is 8 per group.

3.5.7.2 - Plasma Triglycerides

As with plasma cholesterol, triglycerides were measured in all animals from trial I and are presented in Figure 3.13.

There was a significant effect of age on triglyceride concentrations. 6 month old animals in all groups had lower plasma triglycerides than both 12 and 18 month old animals ($p<0.001$).

This was accompanied by a significant effect of surgery; intact male animals had significantly higher circulating triglyceride concentrations than all other groups ($p<0.001$). Whilst this was not reflected in plasma cholesterol, liver triglyceride deposition at 18 months of age was significantly higher in intact males animals than all other groups (Section 3.5.7.3 -, Figure 3.12, Figure 3.13), and may be linked to circulating triglycerides.

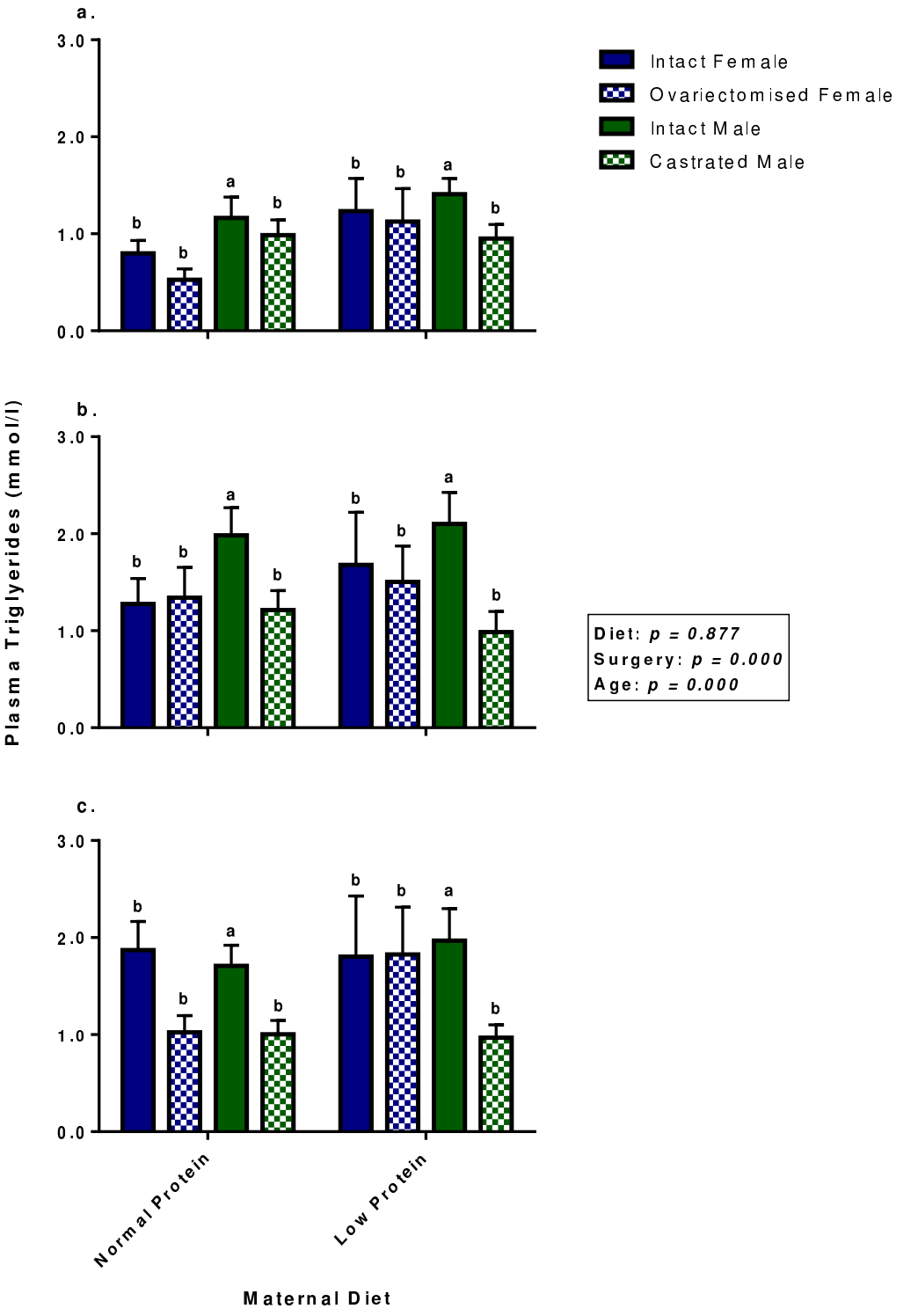


Figure 3.12: Plasma triglycerides (mmol/l) in animals at a) 6, b) 12, and c) 18 months of age from trial I. Triglycerides increased significantly with age ($p<0.001$), and a significant interaction was observed between surgery and age ($p<0.05$). N is 8 per group. Significant differences between surgical groups are denoted by superscript letters.

3.5.7.3 - Liver Triglyceride Deposition

Total triglyceride concentration in crushed liver tissue was measured in 18 month old animals as a marker of age-related adiposity. Tissues were taken from both normal and low protein offspring, and from animals in all surgery groups.

There was a highly significant effect of surgery on liver triglyceride deposition (Figure 3.13). Intact and ovariectomised females had the lowest concentrations of liver triglycerides ($p<0.01$). Intact males had the highest concentration, particularly when compared with females ($p<0.01$), and this elevated liver triglyceride concentration was somewhat ameliorated by castration ($p<0.05$).

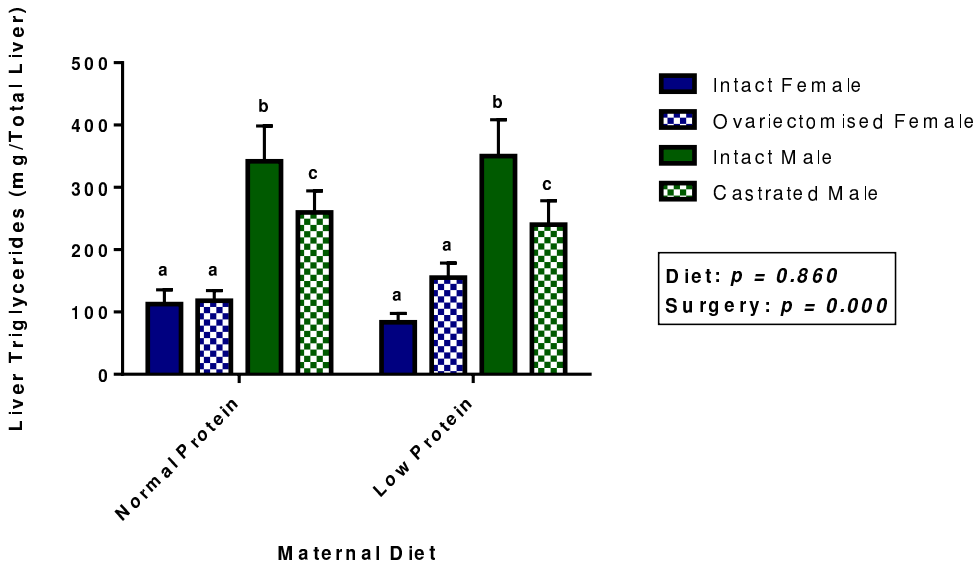


Figure 3.13: Total triglyceride deposition in livers of 18 month old offspring from mothers fed a normal or low protein diet during pregnancy. Offspring were castrated, ovariectomised, or exposed to a sham surgical procedure. A highly significant effect of surgery was observed ($p<0.001$); both intact and castrated males had higher triglyceride levels than female animals from either surgery group.

3.6 - Discussion

The aim of the experiments within this chapter was two-fold. The primary aim was to characterise programmed differences across the life-course in offspring exposed to a low protein diet during gestation. Secondly, the studies were designed to consider changes in expression and activity of the nitric oxide system during the ageing process. We hypothesised that renal function in rats would be diminished with age, particularly in male animals. Furthermore, it was hypothesised that male animals would be more susceptible to accelerated renal ageing in response to a gestational nutritional insult. We also anticipated that these changes in renal function would be closely linked with changes in the nitric oxide system. A summary table of the results obtained within this chapter can be found at the end of the discussion for reference (Table 3.3).

Markers of renal function largely followed expected trends. Rates of creatinine clearance were significantly decreased with age, suggesting a reduction in glomerular filtration rate (GFR). Intact females had the highest creatinine clearance rates, and this was reduced by ovariectomy. In contrast, whilst intact males had lower clearance than female animals, this difference was not diminished by castration. The role of sex steroids has not been conclusively determined in mediating age-related changes in renal filtration. The current results suggest that the effect may largely be mediated by ovarian steroids, as removal of testosterone conferred no protective effect. In an attempt to increase the accuracy of GFR estimations, urinary urea was also measured. Urea, like creatinine, is filtered through the kidney. In contrast to creatinine, urea undergoes tubular reabsorption and so often underestimates excretion (Brenner et al., 2012). In this experiment, urinary urea did not directly reflect results seen in urinary creatinine, with no significant decrease in excretion with age. Whilst there are limitations to its

application, these results suggest that either the level of filtration is decreasing with age, or the level of tubular reabsorption is increasing.

Another factor that must be considered in analysis of these data is the method by which they were collected. Urine and plasma samples were collected from animals using a metabolic cage, which has, on multiple occasions, been shown to cause stress to the animals within. Rodents in metabolic cages have shown unfavourable responses to separation from cage mates, restricted floor area in the cages, and the metal grid flooring typical of these types of cage (Tarland, 2007). As a consequence, housing in metabolic cages has been shown to have a significant effect on rodent cardiac response (Hoppe et al., 2009), water consumption, neurohormonal excretion (Eriksson et al., 2004), and urinary metabolites (Tarland, 2007). Many of the conclusions drawn within this study rely heavily on the results obtained from plasma and urine samples that were collected in metabolic cages. Given the proven impact of such housing, it must be considered that the results generated within this thesis may have differed were samples collected in another manner. Whilst this is a significant limitation, and must be considered in analyses, data collected suggest that the animals were reasonably well-adjusted to the application of these cages. For example, there was no statistically significant weight loss associated with metabolic cage housing, as animals were weighed at the start and end of the metabolic cage period. Whilst it is not possible to compare the food and water intake of these animals with the intake in standard housing conditions due to group housing, the lack of weight loss is a promising indication that the animals were eating and drinking, at least to a certain extent, as they normally would. Furthermore, average urinary output for an adult rat is in the region of 2.3 - 3.3ml per 100g bodyweight, per 24 hours (Johns Hopkins University, 2015), and values for the animals within this study were in this range. Although this is a

potential source of variation in the data generated, evidence collected suggests that the effect was minimal.

The data presented for creatinine clearance was somewhat reflected by the nephron numbers determined by maceration. Intact females had the highest numbers of nephrons, and these numbers decreased in animals exposed to ovariectomy surgery. This may contribute to the differences observed in creatinine clearance as previous studies have identified a relationship between decreased nephron number and glomerular filtration (Luycyk & Brenner, 2005; Celsi et al., 1998). In addition to this, a significant difference was observed between offspring of rats fed normal or low protein diets. Low protein exposed animals had significantly decreased nephron number in comparison to their normal protein counterparts. This is a relationship that has been observed in many previous studies (McMullen et al., 2004; Langley-Evans et al., 1998; Habib et al., 2011). However it is interesting to note that maternal diet did not affect creatinine clearance in Trial I animals. Whilst maternal diet is in this instance had a clear effect on nephron number, this did not translate into an effect on GFR. This discrepancy suggests that the differences in clearance and excretion between animals of different surgery groups may be mediated by differences other than changes in the number of functional kidney units. Conversely, the kidneys of the LP offspring may be maintaining their functional capacity by utilising a compensatory mechanism. Although no measurements were made to confirm such a hypothesis, work such as that by MacKenzie and Brenner (1995) would offer a reasonable explanation for these results. Decreased nephron endowment at birth may, instead of resulting in decreased kidney filtration, result in localised glomerular hypertension. This response would in itself have negative effects on renal function over time, but would offer an immediate compensation for the inherent deficit in functional units seen in LP offspring by maintaining filtration rate.

Somewhat surprisingly, there were no changes in the expression of endothelial nitric oxide synthase, suggesting that any changes in concentrations of nitric oxide were not linked to enhanced eNOS expression. This is contrary to the hypothesis of this experiment, and to a substantial proportion of the literature (Sverdlov et al., 2014; Sasser et al., 2015; Khalil, 2005). It has been shown in previous studies that eNOS is susceptible to dynamic regulation of mRNA expression in response to external influences such as vascular shear stress (Sase & Michel, 1997; Uematsu et al., 1995) and ovarian steroid concentrations (Grazul-Bilska et al., 2006; Searles, 2006). Furthermore, it has been demonstrated that regulation of vascular tone and vasodilatory responses is decreased in ageing blood vessels, which is in part mediated by a decrease in eNOS mRNA (Tanabe et al., 2003). In an ageing model of prenatally programmed renal dysfunction, with removal of sex steroids, it was anticipated that one or all of these factors would likely influence eNOS mRNA expression. The data generated suggests that eNOS mRNA expression is not playing the dominant regulatory role in these states. Instead, additional protein-level or post-translational modifications may be the key mediators. Moreover, these results were repeated in intact females of Trial III suggesting this may be a pathway for further study.

One example of such regulation may be found in looking at vascular endothelial growth factor (VEGF). VEGF is an endothelium-specific protein that stimulates angiogenesis, microvascular hyperpermeability, and vasodilation (Hood et al., 1998). It has been shown to mediate vasodilatory effects through activation of eNOS via phosphorylation. VEGF signalling can cause the phosphorylation of eNOS at numerous serine sites, thereby stimulating nitric oxide release and vascular relaxation (Aramoto et al., 2004; Rafikov et al., 2011). Activation of this

mechanism would result in changes in levels of nitric oxide without changes in overall expression of eNOS.

A study by Kang et al., (2004) demonstrated that 17β-estradiol can mediate changes in expression of VEGF, which may improve renal vasculature function and thus confer reno-protective effects. Although only speculation at this point, it is possible that the intact females of Trial I, are experiencing a protective effect of maintained circulating ovarian steroids when compared with other surgery groups. Such ovarian hormones could result in activation of VEGF and cause subsequent eNOS phosphorylation in the kidneys. This would, in theory, impact the NO system without affecting mRNA expression. Whilst no conclusions can be drawn in this direction at this time, this may be a mechanism for further study.

If changes in eNOS activation were being elicited as a result of VEGF activity, it would stand to reason that changes would be seen in nitric oxide concentration as a result of altered production. Measurement of stable nitric oxide oxidation products has become a common tool for assessment of nitric oxide levels (Schmidt & Baylis, 2008). Upon release in the body, NO undergoes rapid sequential oxidation to form the stable metabolite nitrite (NO_{2^-}), which is excreted via the kidneys in urine (Mian et al., 2013). Typically, urinary nitrite excretion is taken to represent systemic NO production (Goggins et al., 2001; van den Berg et al., 2013), with the understanding that this will also incorporate any production from gut bacteria, or from the diet (Mian et al., 2013). As a measure of nitric oxide concentration, urinary nitrites alone have limitations. Whilst regarded as a reasonable means of observing changes in the nitric oxide system, urinary nitrite concentrations are heavily influenced by diet (Baylis & Vallance, 1998) and changes in environmental conditions, such as storage and handling, of samples (Moshage et al., 1998). As a consequence, it is the author's opinion that whilst every care was taken to ensure consistency in urine

sample handling, considerably more work would be required to conclusively determine the impact of nitric oxide concentrations on the physiological outcomes described in this thesis. The data presented regarding nitrite concentration offers an insight into the NO system and is intended to aid discussion, but would require supplementary data to draw solid conclusions.

The results for urinary nitrite were not as clear cut as those presented for renal clearance or eNOS expression. However, a significant effect of age was evident, as 12 month animals demonstrated drastically elevated urinary nitrite in comparison to both 6 and 18 month old animals. This was true of every group with the exception of intact female normal-protein offspring, where nitrite concentration remained constant regardless of age. This statistically significant interaction between age, diet and surgery is suggestive of a more complex relationship between the NO system and renal function than has been previously understood. It was hypothesised at the start of this study that favourable renal outcomes would be associated with increases in NO system activity or concentration. The results thus far have indicated that this is not necessarily the case. Indeed, the group demonstrating the best maintained renal function, the intact female normal protein offspring, have showed limited changes in nitrite concentration. This data suggests that it may be aberrant fluctuations in NO activity, or inconsistent NO production that may elicit decreases in renal function, rather than a direct concentration-dependent effect.

As detailed in Chapter 1, the RAS is susceptible to programming, with multiple studies identifying changes in angiotensin receptor expression as a result of prenatal low protein exposure (McMullen et al., 2004; Goyal et al., 2015; Pijacka et al., 2015). Rab4, is a membrane trafficking protein in the renin-angiotensin system partially responsible for the resensitization of the angiotensin II type 1 receptor (AT_1R) (Esseltine et al., 2011). Binding of angiotensin II to the type 1 receptor

activates the classic pressor pathway of the RAS, and has been widely cited for its involvement in promoting hypertension (Crowley et al., 2006; Chen & Coffman, 2015). Ligand receptor binding also results in a decrease in receptor sensitivity, thus blunting the response of the AT_1R for a period of time (Li et al., 2008). Chronic over-activity of the AT_1R has been generally acknowledged as a key factor in hypertension and renal dysfunction. Given its capacity for AT_1R resensitization, excessive activity or increased expression of Rab4 could result in increased AT_1R binding with angiotensin II, and thus lead to an increase in the pressor effects of the RAS. The results in this experiment did not definitively support such a hypothesis, however they provided an interesting insight into the effects of differential Rab4 expression on renal outcomes. Whilst the statistical tests demonstrate on average a significant increase with age of Rab4 expression, it is clear looking at the figures that this change is largely apparent in the 18 month cohort. Data for Rab4 expression in 6 and 12 month old animals is relatively consistent, and according to the statistical analysis entirely unaffected by surgical or dietary group. Normal protein offspring experience a steady decrease in Rab4 expression across groups at 18 months. This is in contrast with the low protein offspring, where change in expression is variable depending on surgical group. In particular, LP ovariectomised females present with a drastic upregulation of Rab4, in contrast with any other group.

Expression of Rab4 mRNA did not correlate with changes in renal function or eNOS expression. It does, however, correspond well to changes in protein carbonyls concentration. In particular, low protein females presented with a significant increase in carbonyl concentration at 18 months of age, in a similar fashion to the change in Rab4 expression. Rincon et al., (2015) demonstrated that AT_1R elicits NO system-dependent increases in blood pressure via overexpression of inflammatory mediators and increased oxidative stress. Whilst only speculation at this early stage,

it seems reasonable to hypothesise that renal function and blood pressure regulation are modulated by complex interactions between regulatory proteins of the RAS and inflammatory mediators, a situation that is exacerbated by prenatal programming.

The impact of increased protein carbonyl concentrations (a measure of oxidative injury) is not, in this instance, particularly clear. The data in the present study showed no association between concentrations of protein carbonyls and GFR. A study by Oberg et al., (2004) demonstrated clearly that GFR in patients with CKD did not significantly correlate with markers of oxidative damage, despite such inflammatory markers being significantly increased in such patients. Our results appear to support this observation, suggesting that whilst inflammation may have an impact on renal capacity, the mechanisms behind it are seemingly more complex than previously anticipated.

Lipids are particularly susceptible to oxidative damage within cells, owing to their double bond rich molecular structure (Ho et al., 2013) and have been seen not only to contribute to declines in cardiovascular health across the lifespan (Hausman et al., 2011), but may also impact on renal health (Zhou et al., 2008). A number of studies have identified a relationship between increased oxidation of low density lipoproteins (LDL) and activation of the AT_1R (Mehta & Li, 2001; Papademetriou, 2002). Furthermore, previous studies have demonstrated a significant effect of prenatal programming on lipid profiles of offspring and their predisposal for developing the metabolic syndrome (Lee et al., 2013; Erhuma et al., 2007). These multiple factors serve to highlight the importance of the regulation of lipid metabolism and transport, and the potential role dyslipidaemia may play in the development of metabolic and reno-vascular phenotypes following foetal exposure

to a maternal low protein diet. Cholesterol and triglyceride levels were measured in offspring to aid in the understanding of an overall metabolic profile of these animals.

Contrary to previous research, there was no effect of maternal diet on any lipid profile. Significant changes to offspring cholesterol have been noted in previous studies considering the low-protein model. In particular, Sohi et al. (2011) demonstrated effectively that a low-protein diet during gestation results in long-term augmentation of cholesterol in offspring as a consequence of epigenetic modification. Moreover, maternal low-protein has been linked with an increase in atherosclerotic lesions, likely as a result of changes to members of the LDL receptor family or SREBP1c (sterol regulatory element binding protein) and subsequent cholesterol regulation (Yates et al, 2008). It is evident from such studies that changes to the maternal environment can have significant effects on cholesterol production and concentration in offspring. Despite this, no effects at all were seen as a consequence of maternal diet in this study, casting further questions on the success of the programming model adopted.

There were, however, significant effects of age. In this study, animals at 12 months of age had significantly elevated circulating cholesterol in comparison with their 6 month and 18 month old siblings. This sharp increase occurred in tandem with a significant increase in urinary nitrite excretion (a marker of nitric oxide production). Data generated within this thesis suggested that any changes observed in NO metabolites were not associated with changes in the activity of endothelial nitric oxide synthase (eNOS). However, no consideration was given to the activity of iNOS (inducible NOS), which has been shown to play a role in cholesterol metabolism by altering cholesterol efflux capacity and has even been cited as proatherogenic in excess (Zhao et al., 2014). It may be that increases in cholesterol seen in this age group were linked to an increase in inducible nitric oxide synthase

activity and a subsequent alteration of cholesterol accumulation, which may answer for the simultaneous increase seen in nitrites. Unfortunately, these may only be considered speculations. No measures were made that may offer additional insight into this link, and the relationship may be association rather than causal.

It is well established that elevated circulating triglycerides and increased fat deposition in the liver are associated with the development of metabolic syndrome (Eckel et al., 2005; Vasan, 2006). Moreover, it has been shown that there is an increased risk of aberrant lipid regulation and developing the metabolic syndrome in offspring of mothers fed a low protein diet during gestation (Jahan-Mihan et al., 2015). With this in mind, it is unusual that there was once again no effect of maternal diet on any of the parameters measured. Whilst some differences were seen in the triglyceride levels, these were in no way related to maternal diet group, and the differences observed were still of small magnitude. Plasma and liver triglycerides were both significantly affected by sex and surgical groups, with levels higher in intact males than all other groups, and this effect was somewhat ameliorated in the liver by castration. This was not unexpected as males typically have higher lipid concentrations than pre-menopausal females (Williams, 2004). The reductive effect of castration suggests that in this instance, androgens are mediating the elevations in lipids instead of a protective effect of ovarian steroids in the female animals. However, the elevations in lipids observed in males were not in substantial excess of expectation for aged animals, and there appeared to be no significant relationship between elevated lipids and other measured parameters (such as oxidative damage). The data suggests that whilst lipid profile remains an important risk factor in predicting cardiovascular health, the interactions between lipid concentration and the renal NO system observed in this study may be an association rather than causal or mechanistically linked.

The results from Trial I did not support the hypotheses made at the beginning of the work. It was expected that ageing would result in decreases in renal function, the severity of which would be significantly altered by differing exposure to sex steroids and prenatal protein restriction. As discussed above, there is a substantial body of evidence suggesting that male animals and humans are more prone to poor renal function and associated morbidities with age, and it was predicted that these effects would be mediated by changes in the nitric oxide system. Whilst some of the data suggested a protective effect of female sex steroids on renal function, there was no association between these factors and measured elements of the nitric oxide system. Instead, possible interactions were seen between indirectly associated factors such as Rab4 and oxidative damage, and these were significantly affected by maternal diet. Considerably more work would be required to truly elucidate the mechanisms behind some of the sex-specific effects of renal ageing, but it is possible to conclude that it is not a simple concentration-dependent effect of nitric oxide, but more likely a complex interaction between multiple factors that can cause aberrant fluctuations in elements of the nitric oxide system.

Measurement	Baseline Reading		Change in value as a result of:		
			Increased Age	Maternal Diet	Gonadectomy Surgery
Creatinine Clearance (ml/min/100g BW)	Female	0.31 ± 0.016	↓	-	↓
	Male	0.27 ± 0.018	↓	-	-
Plasma Osmolality (mOsm/kg H_2O)	Female	325 ± 2.23	-	↓	-
	Male	3.35 ± 7.50	-	↓	-
Protein Carbonyls (nM/Mg protein)	Female	3100 ± 852	-	-	-
	Male	3110 ± 729	-	-	-
Urinary Urea (mmol/24hr/100g BW)	Female	25.6 ± 1.63	-	↑	-
	Male	53.3 ± 4.41	-	↑	-
Plasma Cholesterol (mmol/l)	Female	2.12 ± 0.12	↑	-	-
	Male	1.74 ± 0.25	↑	-	-
Plasma Triglycerides (mmol/l)	Female	0.78 ± 0.13	↑	-	-
	Male	1.16 ± 0.22	↑	-	↓
eNOS Expression	Female	0.76 ± 0.11	-	-	-
	Male	0.58 ± 0.12	-	-	-
Rab4 Expression	Female	0.83 ± 0.16	-	-	-
	Male	0.66 ± 0.08	-	-	-
Total Kidney Nephrons	Female	19664 ±1809	-	↓	↓
	Male	15223 ± 956	-	↓	-
Urinary Nitrites (nM/24hr)	Female	6520 ± 1101	↑	-	↓
	Male	5011 ± 808	-	-	-

Table 3.3: Summary of changes from control in male and female animals as a consequence of increased age, differing maternal diets, or gonadectomy surgery. Increased results denoted by '↑', decreased results denoted by '↓', where there was no change '–' is used.

Chapter 4 - Targeting the Renin-Angiotensin System

4.1 - Introduction

The renin-angiotensin system (RAS) is a major target for the treatment of hypertension (Michel et al., 2013). It is known to mediate a number of effects including vasoconstriction, water and sodium retention, sympathetic activation and inflammation (Atlas, 2007; Schriffin, 2002; Section 1.4 - . The actions of angiotensin II are mediated via three major receptor subtypes; $AT_{1a}R$, $AT_{1b}R$ and AT_2R. The two type I receptor subtypes are understood to regulate the majority of pressor effects. Numerous studies have demonstrated the beneficial effects of AT_1R blockade in eliciting a decrease in arterial pressure (Ferrario, 2006) and such treatments have become well established in clinical treatment of human hypertension (Chappell, 2015). The AT_2R, however, is less understood. Previous evidence indicates that activation of the type II receptor subtype results in effects opposite to those of the type I receptor including a decrease in arterial pressure, reduced inflammation, and inhibition of tissue remodelling, cell growth and proliferation (Stoll et al., 1995; Dimitripoulou et al., 2001; Batenburg et al., 2004). These effects occur through a number of mechanisms, including activation of the nitric oxide system (Siragy and Carey, 1997).

It is these observations that suggest the AT_2R might be an ideal target for pharmacological intervention to increase the effectiveness of current treatments for hypertension. A study by Widdop et al., (2002) demonstrates effectively that AT_2R stimulation may result in vasodilation and this phenomena is evident after long-term AT_1R blockade. Such data offers 'proof of principle' that stimulation of the AT_2R may well contribute to the anti-hypertensive effects of AT_1R blockade and, furthermore,

may be a useful target for development of further pharmacological interventions (Widdop et al., 2002).

The recent development of a highly selective AT_2R agonist, Compound 21 (C21), offers the opportunity to consider the actions of the AT_2R in mediating changes in blood pressure more carefully. Derived from the non-selective angiotensin receptor agonist L-162, 313, C21 is an orally active means of selectively targeting AT_2R (Wan et al., 2004). Current data on the effects of C21 administration is varied. Whilst there is evidence to suggest that C21 confers significant organ-protective effects after dosing through reductions in inflammation and structural changes to tissues (Gelosa et al., 2009; Kaschina et al., 2008), its effects on reducing blood pressure are seemingly more complex. It is evident that C21 is capable of eliciting a vasorelaxation response in isolated vessels as demonstrated by Bosnyak et al., (2010). However, this effect was not replicated *in vivo* as spontaneously hypertensive rats showed no decrease in systolic blood pressure unless C21 was administered concomitantly with the AT_1R antagonist candesartan. Furthermore, recent *in vitro* studies by Verdonk et al., (2012) demonstrated that C21-induced vasodilation is not necessarily mediated via the AT_2R. Additionally, in some instances, C21 doses resulted in vasoconstrictor responses, further complicating our understanding of the potential benefits of AT_2R stimulation. Against this backdrop of conflicting evidence from acute studies, relatively little work has been completed to consider the effects of long-term administration of C21. In order to assess the capacity of C21 *in vivo* as a method of regulating blood pressure, further work involving administration of AT2R agonists over a longer time period is needed.

The present study aimed to address the following questions:

1. Does long-term, low-dose administration of C21 infer a reduction in systolic blood pressure?
2. Is this response dependent on concomitant dosing with AT_1R antagonists?
3. Does long-term administration of C21 infer alternative protective effects, for example, increased vascular reactivity?

Utilising the low protein model of cardiovascular programming (Section 1.7.2.1 -) the experiment described in this chapter investigated whether stimulation of the AT_2R was capable of eliciting a long-term reduction in blood pressure using the novel, highly-specific AT_2R agonist, C21. To date, there has been no work considering the impact of prenatal programming on response to C21, making this a novel study design.

4.2 - Hypothesis

Though current data on the effects of C21 on blood pressure is conflicting, the potential for the beneficial effects of AT_2R stimulation remains. We hypothesize that long-term, low-dose administration of C21 would result in significant decreases in systolic blood pressure that may be linked with organ-protective effects. Furthermore, this effect would be expected to be enhanced by concomitant AT_1R blockade.

4.3 - Materials and Methods

All data discussed in this Chapter relates to animals from Trial II (Section 2.2.2 -), detailed methodology can be found in Chapter Two. As with Trial I, females were mated and assigned a normal (NP) or low protein (LP) diet for the duration of pregnancy. Eight female offspring from each mother were kept, fed on standard laboratory chow and maintained until 12 months old. Measurements for blood

pressure, heart rate, food intake, and urinary excretion were made at 6, 9, and 12 months of age (Section 2.1.4 - At 8 months of age, animals were assigned to receive one of four treatments; control, Compound 21, Losartan (a selective AT_1R antagonist), or Compound 21 and Losartan simultaneously. Treatments were administered daily until the end of trial at 12 months of age. At cull, all major organs were collected and snap frozen in liquid nitrogen. Samples of liver and kidney were also preserved in 4% formalin. The mesenteric arcade was removed and placed in ice cold physiological saline solution (PSS composition mM: NaCl 119, KCl 4.7, CaCl2 2, MgSO4 1.17, NaHCO3 25, KH2PO4 1.18, EDTA 0.026 and $C_6H_{12}O_6$ 5) for wire myography (Section 2.3.10 -).

4.3.1 - Statistical Analysis

All values are presented as means ± SEM unless otherwise stated. Constriction values are presented as a percentage of constriction in response to 125mM KPSS (KPSS composition is as with PSS bar an equimolar substitution of NaCl with KCl). Relaxation values are expressed as a percentage reduction of tone induced by pre-constriction with the thromboxane mimetic 9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α. Data for blood pressure, weight, and metabolic measurements were analysed using the Statistical Package for Social Science, (vers. 19, SPSS Inc, Chicago Il, USA) using ANOVA with a Bonferroni post-hoc correction where applicable. Data groups showing significant differences in main factor effects (age, maternal diet, or treatment) from the overall output were subject to secondary analysis within that factor group. Myography data were first log transformed using Graphpad Prism and the equation x=Log[X]. Initial analysis was performed in the form of a non-linear regression plot using GraphPad Prism 6 (GraphPAD software Inc., San Diego, USA) and values for LogEC50, Hill slope and

maximal response were generated by this package. A four-parameter variable slope fit was used. The model in question utilises the following equation:

$$Y=Bottom + (Top-Bottom)/(1+10^{((LogEC50-X)*HillSlope)})$$

Whereby the 'top' and 'bottom' of the graph are determined by the dose response and starting point of the vessel.

The values generated by the regression analysis were then assessed in the same manner as the other data, using ANOVA in SPSS with a Bonferroni post-hoc correction where applicable. A *p* value of <0.05 was taken to be statistically significant.

4.3.2 - Power Calculations

Power calculations were used to determine the numbers of animals required for blood pressure analysis, based on standard deviations from previous studies (McMullen & Langley-Evans, 2005; Ozaki et al.., 2001). The probability of a type I error (α) was set at 0.05 and of a type II error (β) set at 0.10, to give a power (β-1) of 0.90. Offspring within a litter cannot be regarded as independent, as variation within a litter may differ from variation between litters. Therefore, the sample size calculated dictated the number of litters, rather than offspring, required.

The following calculation was performed to determine the number of animals required to observe statistical significance:

$$n = ((9^2+9^2)*(1.96+1.28)^2)/15^2 = 7.56 \text{ litters.}$$

Based on these calculations, 8 litters per group was deemed an appropriate number to generate sufficient offspring for adequately powered analysis for each group.

4.4 - Results

4.4.1 - Systolic Blood Pressure

Blood pressure was measured in all animals from Trial II at 6, 9, and 12 months of age using the tail cuff method, and the entire dataset is presented in Figure 4.1 below. On analysis of the entire dataset a significant effect of age was observed. Animals at 12 months of age had significantly lower systolic blood pressure than animals at 6 and 9 months of age ($p<0.05$). Whilst an effect of maternal diet was evident, this was contrary to previous literature with control offspring demonstrating higher systolic blood pressure than maternal low protein offspring ($p <0.05$). It is important to note that the power for these main factor effects (age, maternal diet, treatment group) was quite high, allowing for relatively small differences to be picked up as statistically significant. Furthermore, the values for systolic blood pressure in control animals across all age groups lay between 115-130mmHg, which would be considered within normal range and are reasonably similar to the overall range of the maternal low protein animals (110-129mmHg). Further analysis of the interacting factors highlights that the values for systolic blood pressure at 12 months are where the greatest differences lay. Maternal low protein diet exposed offspring presented with systolic pressures between 110-116mmHg at 12 months of age compared to the 116-130mmHg of the 12 month controls. These values in the MLP offspring would be considered verging on hypotensive, particularly in aged animals.

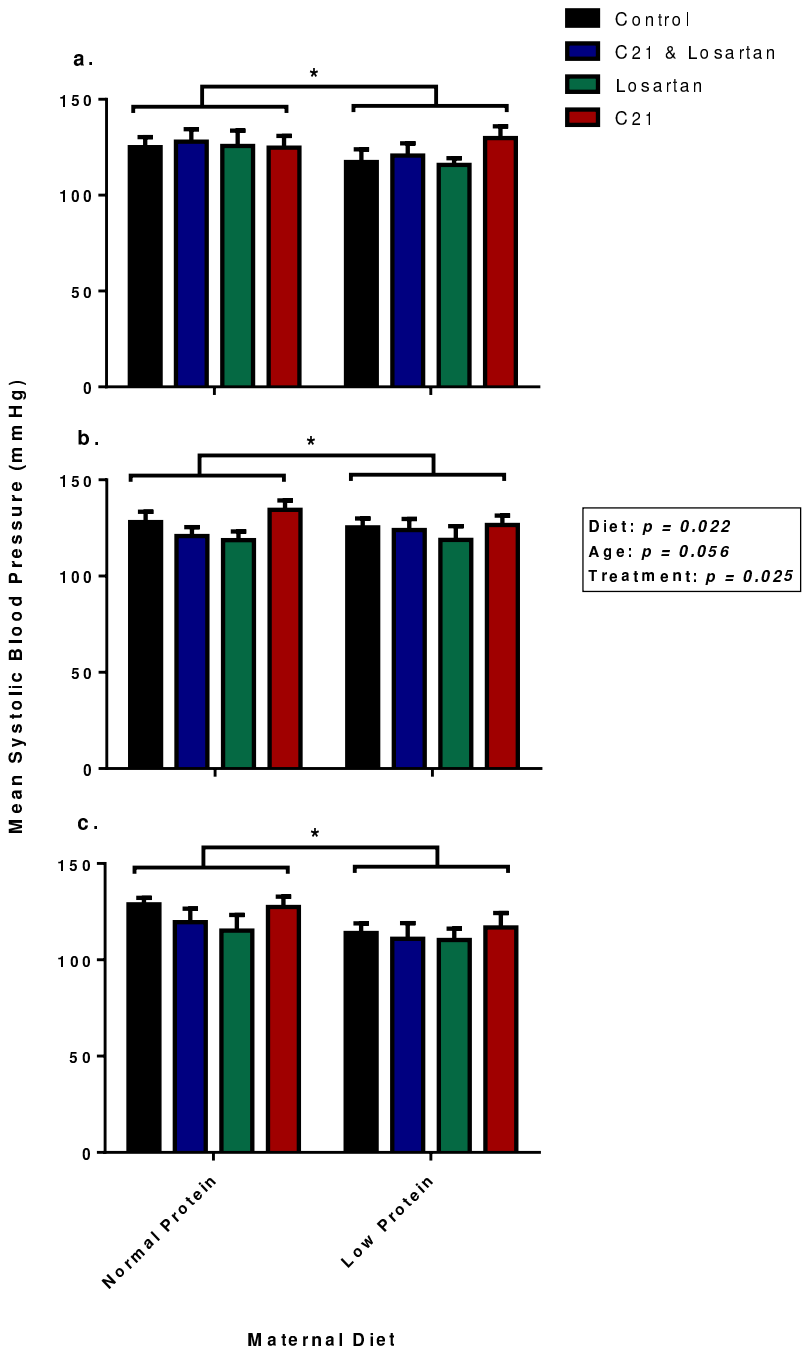


Figure 4.1: Systolic blood pressure (SBP) in offspring from mothers fed a normal or low protein diet during gestation at a) 6 months, b) 9 months, and c) 12 months of age from control, C21, Losartan, or C21 and Losartan treatment groups. Animals at 12 months of age had significantly lower blood pressure than both 6 and 9 month animals ($p<0.05$). Low protein offspring had significantly lower blood pressure than their control counterparts ($p<0.02$). N is 8 per group. Superscript letters denote significant differences between drug treatment groups. Asterisks denote significant differences between maternal diet groups.

Changes in systolic blood pressure with age were also analysed in all animals as shown in Figure 4.2. There were no significant differences between treatment groups, however, the change in pressure with age is well demonstrated by the graphs.

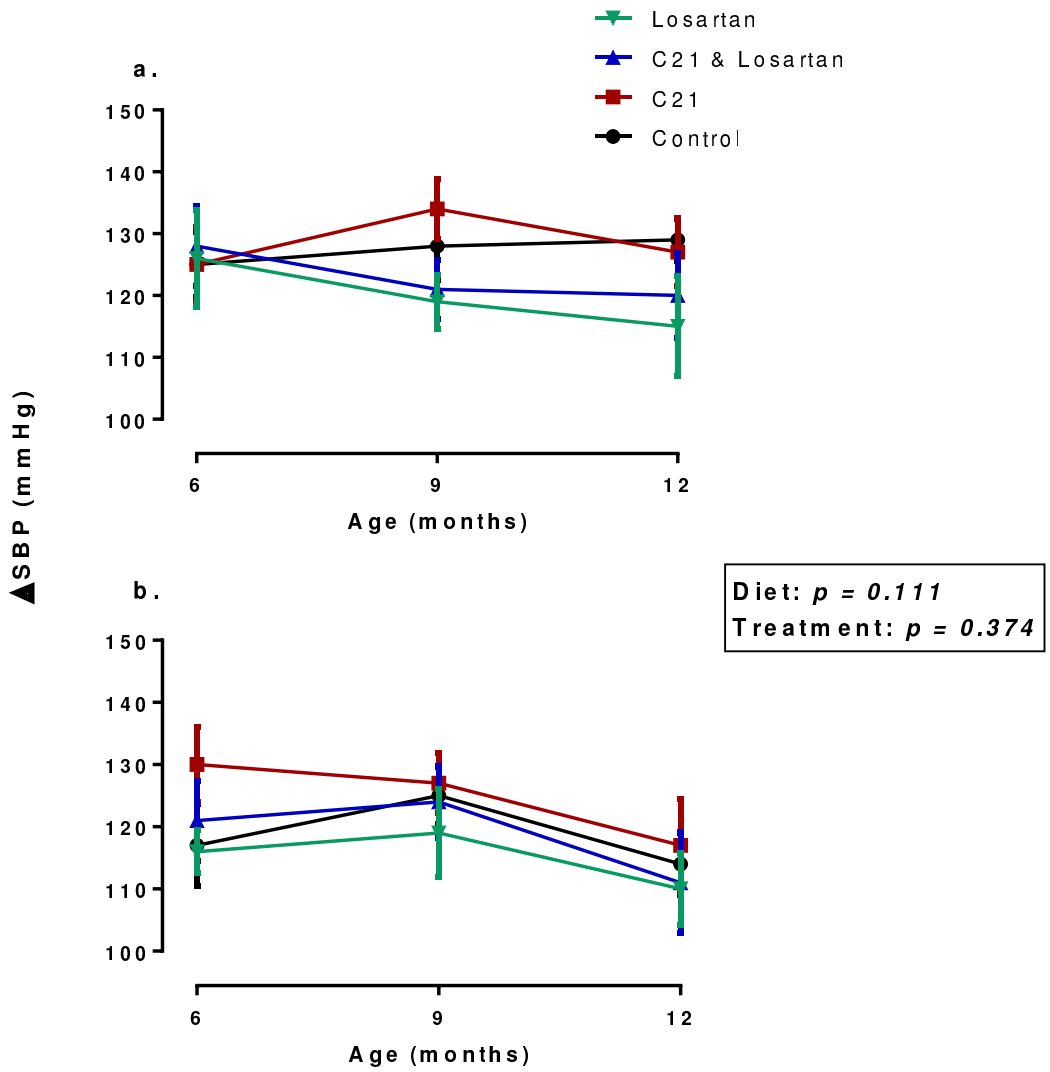


Figure 4.2: Change in systolic blood pressure from 6 to 12 months of age in offspring of a) normal and b) low protein fed mothers from control, C21, Losartan and C21/Losartan treatment groups. No significant differences were observed. N is 8 per group.

The effects of treatment were not as clear cut as anticipated. At 6 months of age, as would be expected for baseline readings, there was no significant effect of drug treatment on blood pressure response, nor was there any significant effect of maternal diet alone. At both 9 and 12 months of age, and when the dataset was analysed as a whole, there was a single effect of treatment. Losartan treated animals had significantly lower systolic blood pressure than C21 treated animals ($p<0.05$) as demonstrated in Figure 4.3 below. There were no other differences between treatment groups. The loss of effect of maternal diet in the pooled dataset supports the suggestion that the significant differences observed in maternal diet groups are driven by the differences at 12 months of age discussed above.

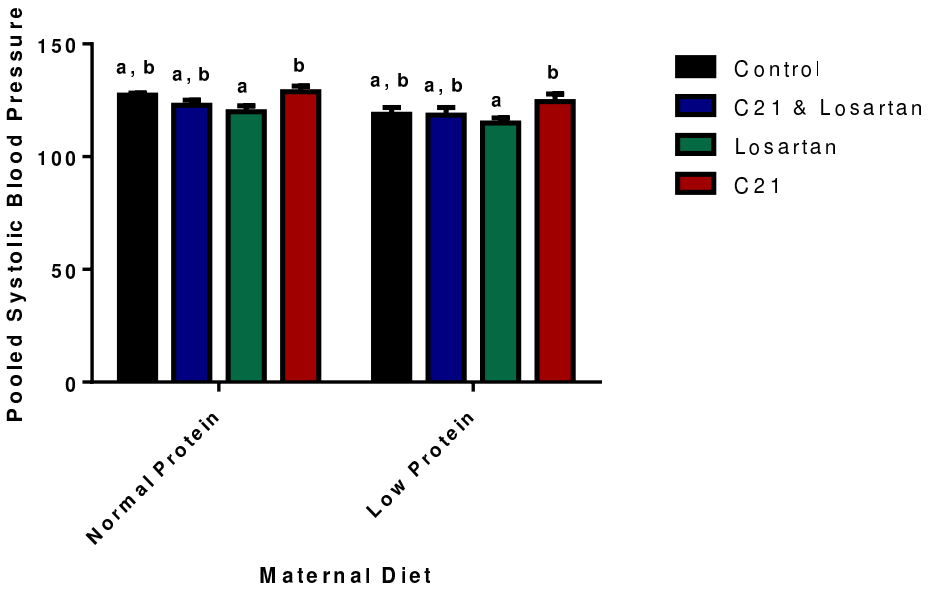


Figure 4.3: Pooled systolic blood pressure (mmHg) for Trial II offspring of mothers fed a normal or low protein diet during gestation. Animals are from control, C21, Losartan, or C21 and Losartan treatment groups at both 9 and 12 months of age. An effect of treatment was observed in Losartan treated animals, who had lower systolic blood pressure than C21 treated animals ($p<0.05$). No other effects were observed.

4.4.2 - Diastolic and Mean Arterial Pressure by Tail Cuff

Both diastolic and mean arterial pressures were measured in Trial II offspring in tandem with systolic blood pressure measurements, as shown in Figure 4.4 and Figure 4.6 respectively.

Diastolic blood pressure readings followed the same patterns as observed with systolic data. Offspring from low protein mothers had significantly lower diastolic blood pressure than offspring of normal protein mothers ($p<0.05$), and this effect was enhanced by age with 12 month old low protein offspring having the lowest diastolic blood pressure values of the entire cohort ($p<0.05$). These results are demonstrated in Figure 4.4.

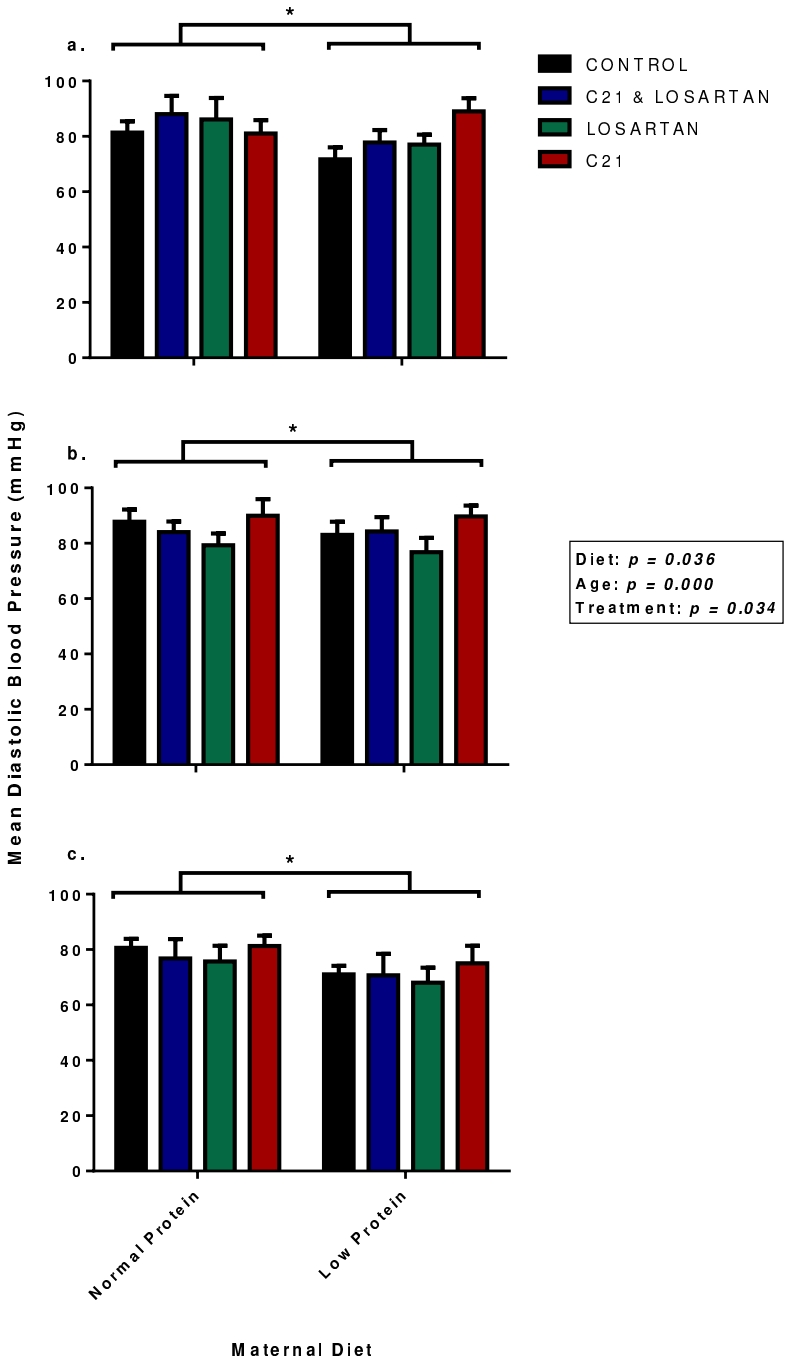


Figure 4.4: Diastolic blood pressure (mmHg) measured by tail cuff in Trial II offspring at a) 6, b) 9, and c) 12 months of age. Losartan treated animals had lower diastolic pressure than C21 treated animals ($p<0.05$). Offspring from mothers fed a low protein diet during pregnancy had significantly lower diastolic pressure than offspring of normal protein mothers ($p<0.05$). Animals at 12 months of age had significantly lower diastolic pressure than both 6 and 9 month old animals ($p<0.05$). In each group, n = 8. Asterisks denote significant differences between maternal diet groups.

When data was pooled for analysis of treatment effect (Figure 4.5), Losartan treated animals once more had lower diastolic blood pressure than their C21 treated counterparts ($p<0.05$), but there were no other significant effects of drug treatment. Pooled treatment data is presented below in Figure 4.5, as averaged for animals at 9 and 12 months of age.

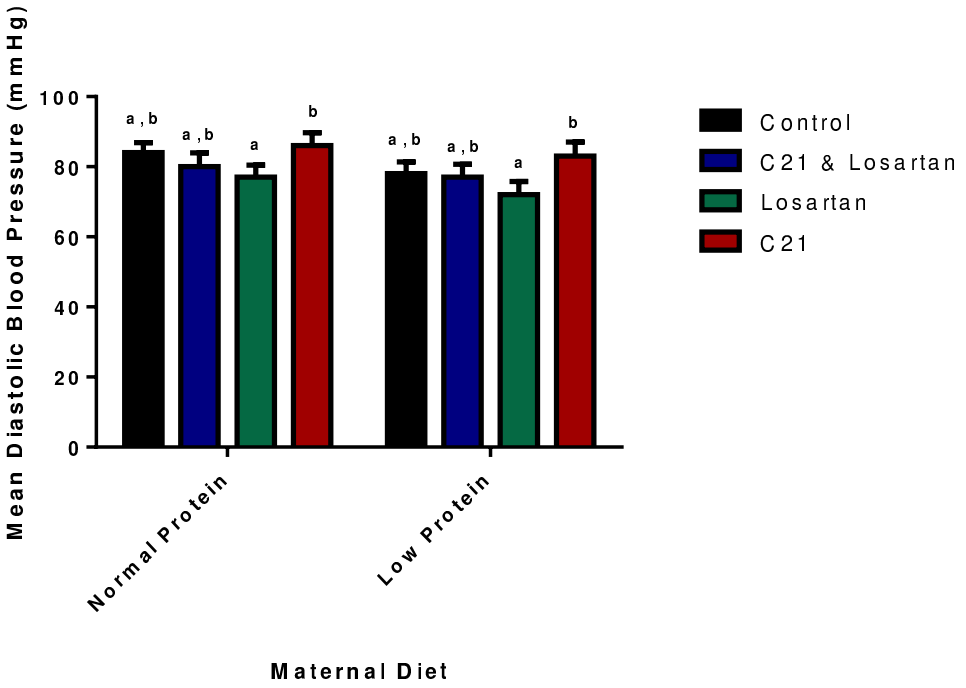


Figure 4.5: Pooled diastolic blood pressure readings for animals at 9 and 12 months of age. Animals are from control, C21, Losartan, or C21 and Losartan treatment groups. Losartan treated animals had significantly lower blood pressure readings than C21 treated animals ($p<0.05$). Significance denoted by differing superscript letters.

Mean arterial pressures (Figure 4.66) did not follow the same patterns of variation as were noted for systolic and diastolic blood pressures. Losartan treated animals no longer presented with significantly lower blood pressure than C21 treated animals, suggesting the result seen in systolic and diastolic analyses may be a factor of the statistical power of the experiment. However, there was once more a significant difference between prenatal diet groups, with low protein offspring having lower mean arterial blood pressure than normal protein offspring ($p<0.05$). Furthermore, 12 month old animals still had significantly lower mean arterial pressure than 6 and 9 month animals ($p<0.02$).

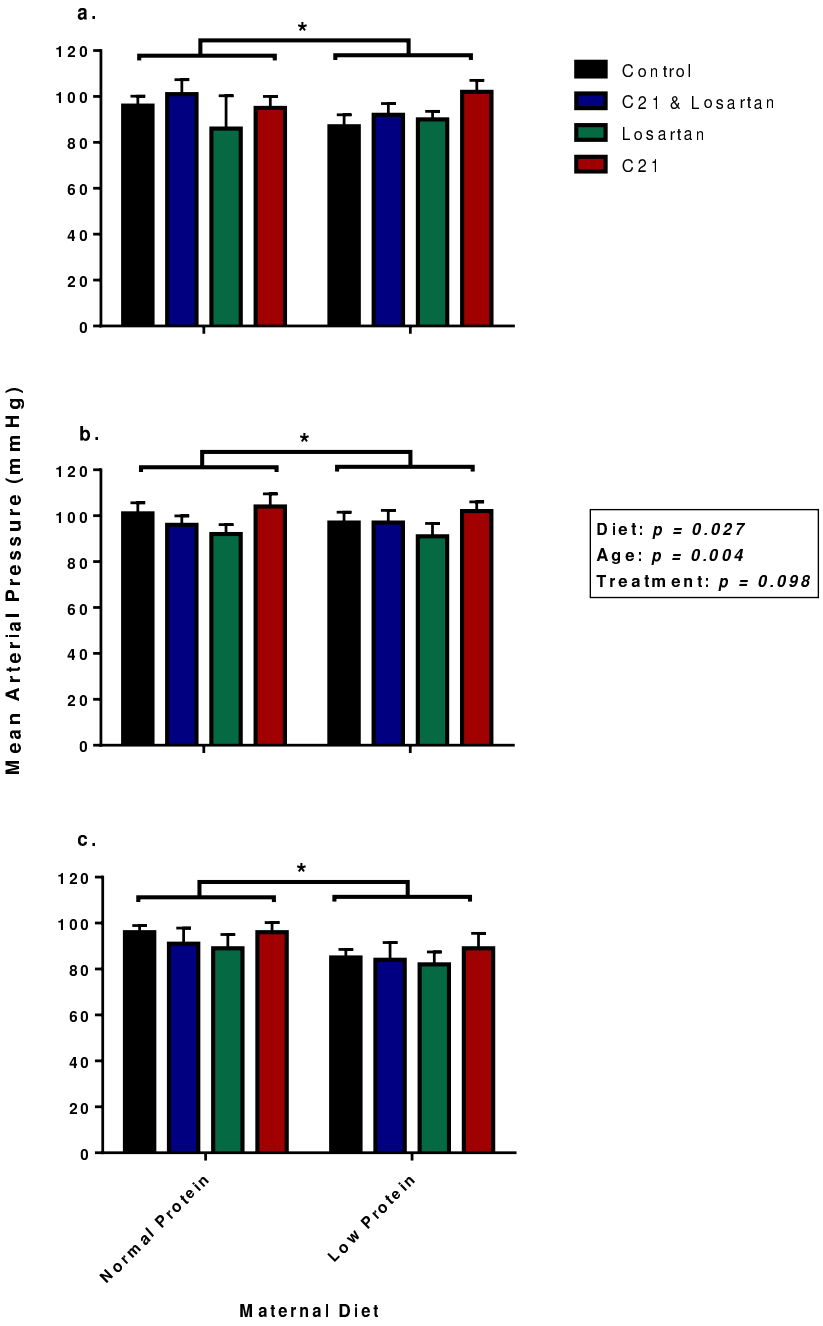


Figure 4.6: Mean arterial pressure (mmHg) measured in Trial II offspring by tail cuff at a) 6, b) 9, and c) 12 months of age. Offspring of low protein fed mothers had significantly lower blood pressure than offspring of normal protein fed mothers ($p<0.05$). Age had a significant effect on results, 12 month animals had lower mean arterial pressure than 6 and 9 month old animals ($p<0.02$). N is 8 per group. Asterisks denote significant differences between maternal diet groups.

4.4.3 - Heart Rate

As with systolic, diastolic and mean arterial pressure, heart rate was measured by tail cuff in animals at 6, 9 and 12 months of age.

There was a significant effect of age with 6 month old animals having significantly higher heart rates than 9 month animals ($p<0.05$). There was also a highly significant interactive effect between maternal diet and treatment group ($p<0.01$). The apparent effect of drug treatment on mean heart rate was almost entirely opposite across maternal diet groups, with C21 and Losartan treatments tending to increase heart rate in rats exposed to normal protein in foetal life, whilst decreasing heart rate in the low protein group (Figure 4.7).

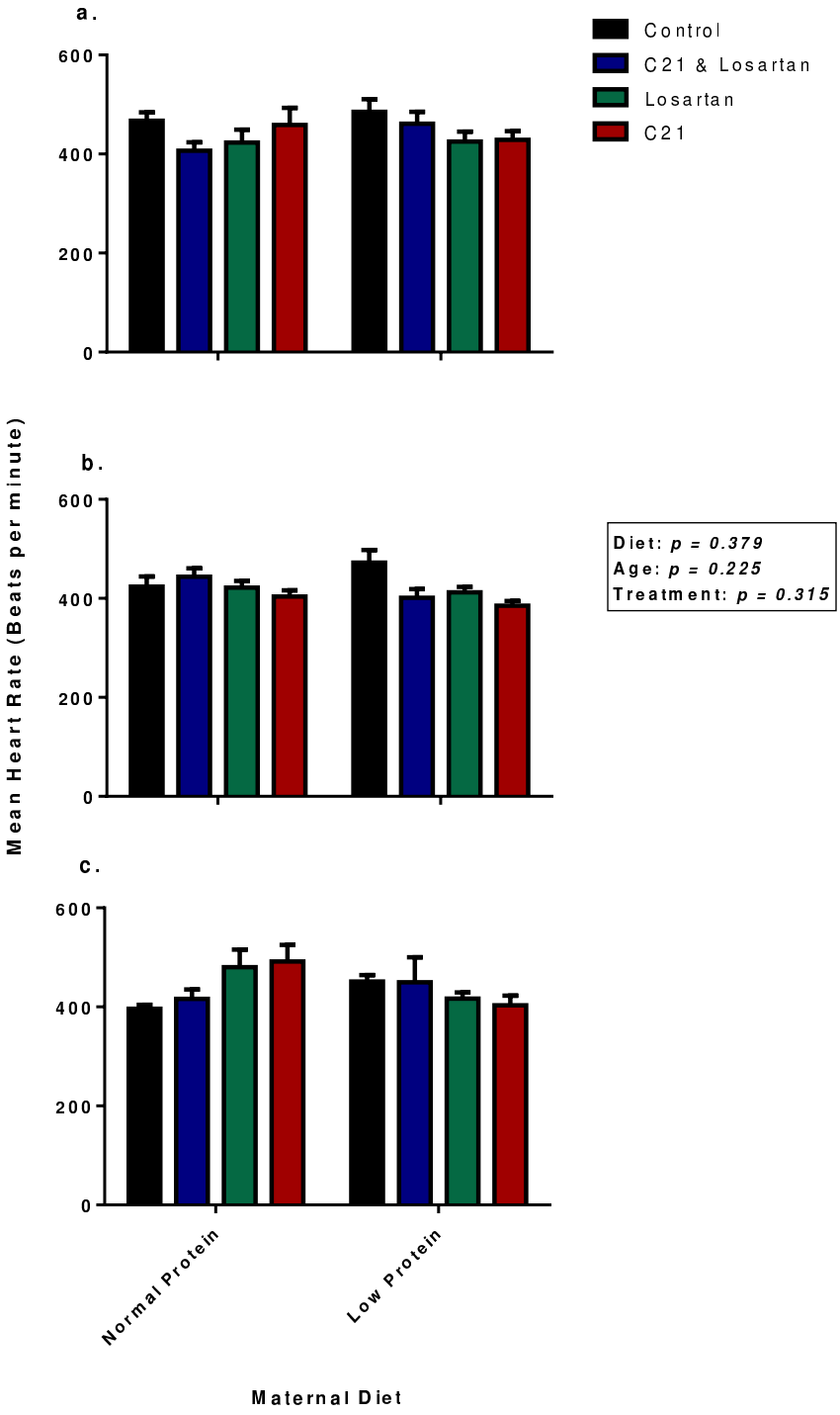


Figure 4.7: Mean heart rate (beats per minute) in Trial II offspring at a) 6, b) 9, and c) 12 months of age. Heart rate was significantly affected by age, 6 month animals had higher heart rates than 9 month animals ($p<0.05$). A significant interaction was observed between maternal diet and treatment ($p<0.01$), but there were no main factor effects of either diet or treatment alone. N = 8 per group.

4.4.4 - Weight and Growth Parameters

No significant differences in weight at cull were observed between groups at 12 months of age, nor were any differences seen in growth rates across the lifespan (data not shown). There were, however, significant differences evident between treatment groups with regards to kidney size. Animals from both Losartan and C21 + Losartan treatment groups presented with significantly larger kidneys than control and C21 treated animals, expressed as a percentage of total bodyweight ($p<0.01$; Table 4.1). Aside from this, there were no other significant differences between organ weights regardless of whether weights were expressed as percentages of bodyweight or as true mass in grams.

Treatment Group:	Control		C21		C21 & Losartan		Losartan	
Maternal Diet:	**NP**	**LP**	**NP**	**LP**	**NP**	**LP**	**NP**	**LP**
Bodyweight (g)	312.0 ± 9.9	317.5 ± 12.2	303.5 ± 6.8	303.6 ± 12.5	284.5 ± 3.5	292.0 ± 9.1	295.2 ± 15.8	306.6 ± 12.9
Kidney (g)	0.936 ± 0.038	0.950 ± 0.036	0.935 ± 0.017	0.930 ± 0.027	0.906 ± 0.032	0.930 ± 0.030	0.949 ± 0.039	0.950 ± 0.029
Kidney (%BW)	0.302 ± 0.018[a]	0.300 ± 0.008[a]	0.309 ± 0.007[a]	0.309 ± 0.013[a]	0.318 ± 0.011[b]	0.319 ± 0.008[b]	0.324 ± 0.010[b]	0.312 ± 0.010[b]
Liver (g)	10.772 ± 0.331	10.673 ± 0.475	10.818 ± 0.407	10.209 ± 0.401	10.051 ± 0.282	10.739 ± 0.492	10.077 ± 0.540	10.991 ± 0.469
Liver (% BW)	3.478 ± 0.188	3.368 ± 0.113	3.573 ± 0.147	3.378 ± 0.118	3.531 ± 0.079	3.678 ± 0.113	3.434 ± 0.151	3.597 ± 0.110
Heart (g)	1.046 ± 0.032	1.080 ± 0.036	1.056 ± 0.032	1.100 ± 0.038	1.007 ± 0.023	1.042 ± 0.035	1.017 ± 0.026	0.984 ± 0.039
Heart (% BW)	0.337 ± 0.014	0.349 ± 0.016	0.306 ± 0.044	0.360 ± 0.019	0.355 ± 0.010	0.358 ± 0.009	0.350 ± 0.017	0.324 ± 0.016

Table 4.1: Mean body and organ weights at cull for control and low protein offspring from all treatment groups. Data are presented as mean ± SEM. Kidneys in animals treated with either C21 & Losartan, or Losartan alone were larger than kidneys from animals in the control treated group and the C21 treated group, irrespective of maternal diet group. Different superscript letters denote a significant difference at $p<0.05$ level.

4.4.5 - Metabolic Measurements

Food and water intake, urine excretion, and urinary osmolality were measured at 6, 9, and 12 months of age using metabolic cages for collection and an osmometer for analysis (Table 4.2).

There were no significant differences between food and water intake across treatment groups, regardless of age or maternal diet.

There were significant differences in both urine volume (mL/24hr/100g BW) and urinary osmolality (mOsm/g water). Urine volume excreted was significantly affected by maternal diet, as normal protein offspring excreted more than low protein offspring ($p<0.001$). This was also affected by age, as the volume excreted increased between 6 and 12 months ($p<0.05$). These data were reflected in urinary osmolality, the solute concentration in the urine of low protein offspring was significantly higher than that in the urine of normal protein offspring ($p<0.001$). Given the decrease in urine excretion observed in the low protein offspring, it is not unsurprising that there would be increased osmolality. As these changes were not coupled with changes in water consumption, it is possible that there are differences in the kidney concentrating capacity of normal and low protein offspring.

		6 months				9 months				12 months			
	DIET	**CON**	**C21 & LOS**	**LOS**	**C21**	**CON**	**C21 & LOS**	**LOS**	**C21**	**CON**	**C21 & LOS**	**LOS**	**C21**
Water Intake (mL/24hr/100g BW)	**NP**	11.82 ± 1.59	11.50 ± 0.57	13.18 ± 1.53	13.69 ± 0.87	9.68 ± 1.74	11.14 ± 0.54	12.84 ± 1.19	10.70 ± 1.28	9.94 ± 1.13	11.12 ± 0.73	12.85 ± 1.59	11.56 ± 0.93
	LP	9.18 ± 1.22	10.56 ± 0.78	11.39 ± 1.28	10.28 ± 0.87	15.28 ± 5.30	11.84 ± 1.59	10.82 ± 0.95	14.87 ± 4.86	11.27 ± 2.08	9.88 ± 0.87	11.59 ± 1.03	10.95 ± 0.94
Urine Volume (mL/24hr/100g BW)	**NP**	4.39 ± 1.32	4.61 ± 0.31	4.54 ± 0.71	5.62 ± 0.62	5.05 ± 0.78	4.82 ± 0.35	5.23 ± 0.69	6.44 ± 0.53	5.17 ± 0.65	5.27 ± 0.88	5.48 ± 0.69	5.39 ± 0.69
	LP	3.51 ± 0.57	4.12 ± 0.55	4.31 ± 0.64	3.18 ± 0.50	3.43 ± 0.84	3.96 ± 0.39	4.07 ± 0.64	3.75 ± 0.71	3.18 ± 0.17	4.29 ± 0.65	5.27 ± 0.75	4.39 ± 0.56
Food Intake (g/24hr/100g BW)	**NP**	7.88 ± 0.47^{a†}	8.98 ± 0.65^{a†}	8.39 ± 0.50^{a†}	8.63 ± 0.36^{a†}	6.87 ± 0.58^{a†}	8.10 ± 0.56^{a†}	8.19 ± 0.43^{a†}	8.29 ± 0.58^{a†}	6.77 ± 0.45^{a†}	7.86 ± 0.43^{a†}	6.88 ± 0.44^{a†}	7.74 ± 0.74^{a†}
	LP	6.84 ± 0.46^{b†}	7.66 ± 0.37^{b†}	8.07 ± 0.78^{b†}	8.44 ± 0.49^{b†}	6.58 ± 0.53^{b†}	8.37 ± 0.58^{b†}	7.72 ± 0.42^{b†}	7.28 ± 0.62^{b†}	7.06 ± 0.38^{b†}	9.05 ± 0.81^{b†}	8.23 ± 0.66^{b†}	7.42 ± 0.57^{b†}
Urine Osmolality (mOsm/g H_2O)	**NP**	141.6 ± 50.68^{c†}	98.8 ± 10.45^{c†}	91.0 ± 22.03^{c†}	97.1 ± 9.22^{c†}	65.4 ± 9.77^{c†}	105.9 ± 14.41^{c†}	101.9 ± 19.95^{c†}	63.1 ± 13.63^{c†}	94.7 ± 17.49^{c†}	87.9 ± 11.20^{c†}	111.1 ± 27.33^{c†}	93.2 ± 13.89^{c†}
	LP	197.5 ± 41.99^{d†}	156.1 ± 22.47^{d†}	123.7 ± 22.15^{d†}	140.3 ± 13.32^{d†}	147.1 ± 29.92^{d†}	132.0 ± 29.05^{d†}	157.6 ± 36.84^{d†}	196.0 ± 43.21^{d†}	157.3 ± 22.62^{d†}	149.5 ± 28.14^{d†}	111.5 ± 21.46^{d†}	108.3 ± 13.35^{d†}

Table 4.2: Data for food and water intake, urinary excretion, and urinary osmolality in control treated (CON), C21 treated (C21), C21 and Losartan treated (C21 & LOS), or Losartan treated (LOS) offspring of mothers fed a normal (NP) or low protein (LP) diet during pregnancy. Values are mean ± SEM of measures taken at 6, 9, and 12 months of age. N is 8 per group. There was a significant effect of maternal diet on food intake, on average NP>LP ($p<0.001$), and on urinary osmolality LP>NP ($p<0.01$). Significance ($p<0.05$) denoted by differing superscript letters, † = significant at $p<0.01$.

4.4.6 - Urinary Protein Excretion

Persistent elevation in urinary protein can be indicative of glomerular damage (Gorriz & Martinez-Castelao, 2012), and is marked as part of an ageing rat phenotype (Joles et al., 2010). As such, urinary protein concentrations can be used as a marker of renal function. Urinary protein was measured in all Trial II offspring at 6, 9, and 12 months of age. Studies have demonstrated a nephroprotective effect of C21 (Rehman et al., 2012; Hilliard, 2014), and it was proposed in this study that long term dosing with C21 would have a beneficial impact on renal function, and this would be reflected in urinary protein.

There was no significant difference between treatment or diet groups in concentrations of urinary protein excreted (mg/24 hour; Figure 4.8). Urinary protein was significantly affected by age with an increase in urinary protein observed in animals at 12 months old when compared with those at 9 months ($p<0.05$). There was no significant effect of treatment group for offspring exposed to either maternal diet, and there were no interactions between any of the factors.

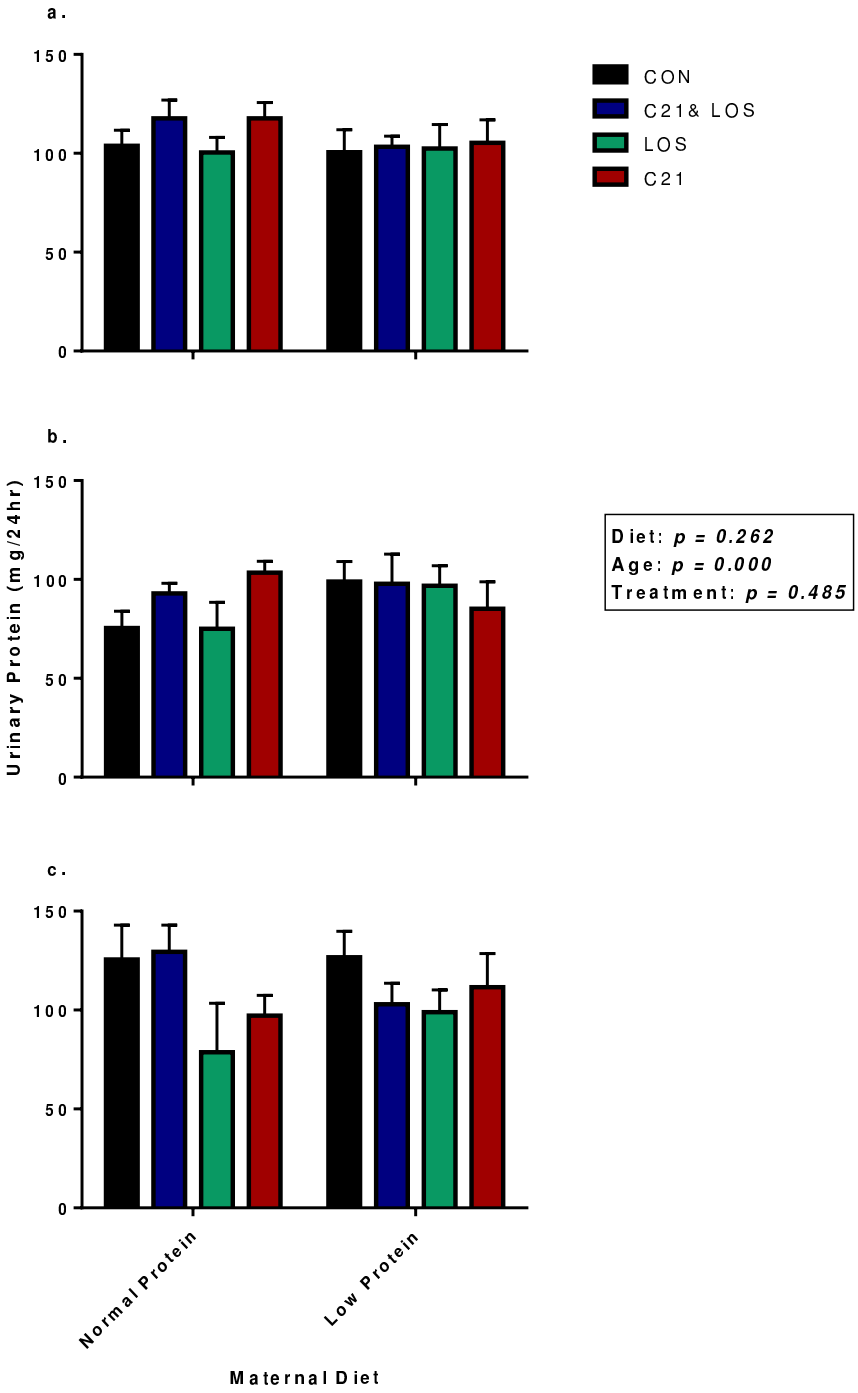


Figure 4.8: Urinary protein excretion in offspring of mothers fed a normal or low protein diet during pregnancy from control (CON), C21, C21 & Losartan (C21 & LOS), or Losartan (LOS) treatment groups. Data taken from animals at a) 6 months, b) 9 months and c) 12 months of age. A significant effect of age was observed; urinary protein significantly increased between 9 and 12 months of age *($p<0.05$)*. N is 8 per group.

4.4.7 - Assessment of Vascular Function

Vascular function was measured using a wire myograph (Section 2.3.10 -). At the point of cull, 5 animals from each treatment group were selected at random and tissues removed for myograph analysis. In all data pertaining to vascular function, n = 5 per group unless otherwise stated.

4.4.7.1 - Vasoconstrictor Responses

Vasoconstriction was measured in two ways. First the response to exposure to a single dose of 125mM KPSS was determined, and then the response to a cumulative dose range of the α_1-adrenoceptor agonist, phenylephrine was recorded. Doses selected were based upon previously published experiments (Torrens et al., 2009a; 2009b; Rodford et al., 2008).

4.4.7.2 - Maximal Constriction with KPSS

There were significant differences noted in the response to application of KPSS (Figure 4.9). Doses were administered three times (with washes in between) and an average value taken for the replicates. Control animals had greater constriction that Losartan treated animals ($p<0.05$). There was also a significant interaction effect between maternal diet and treatment ($p<0.05$). The magnitude of difference in response between drug treatment groups was far greater in normal protein offspring. These data suggest there was a significantly different effect of treatment between normal and low protein offspring.

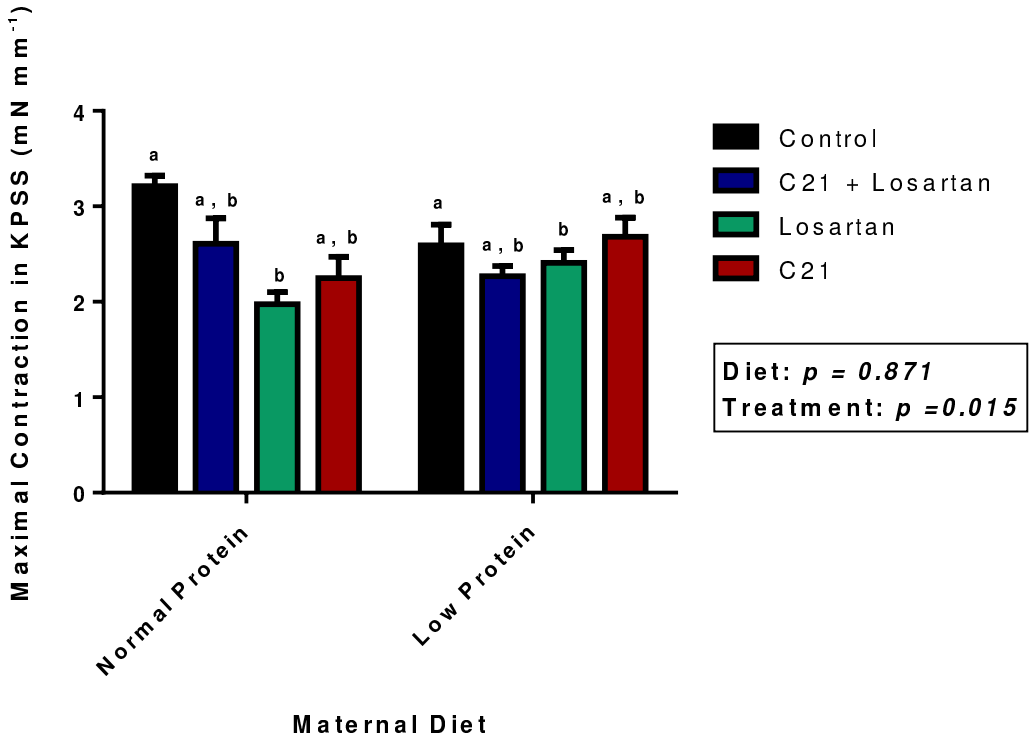


Figure 4.9: Maximal contraction in response to application of 125mM KPSS (mN mm^{-1}) in normal and low protein offspring from treatment groups control, C21 & Losartan, Losartan or C21. Control treated animals had significantly greater responses than Losartan treated animals ($p<0.05$), and this difference was greater in normal protein offspring ($p<0.05$). Differing subscript letters denote significance between drug treatment groups ($p<0.05$).

4.4.7.3 - Lumen Diameter of Vessels

Lumen diameter was recorded for each artery collected (Figure 4.10). There were no significant differences between animals of any treatment or maternal diet group. This is valuable as it serves to demonstrate that differences observed in vascular response were not an artefact of vessel size.

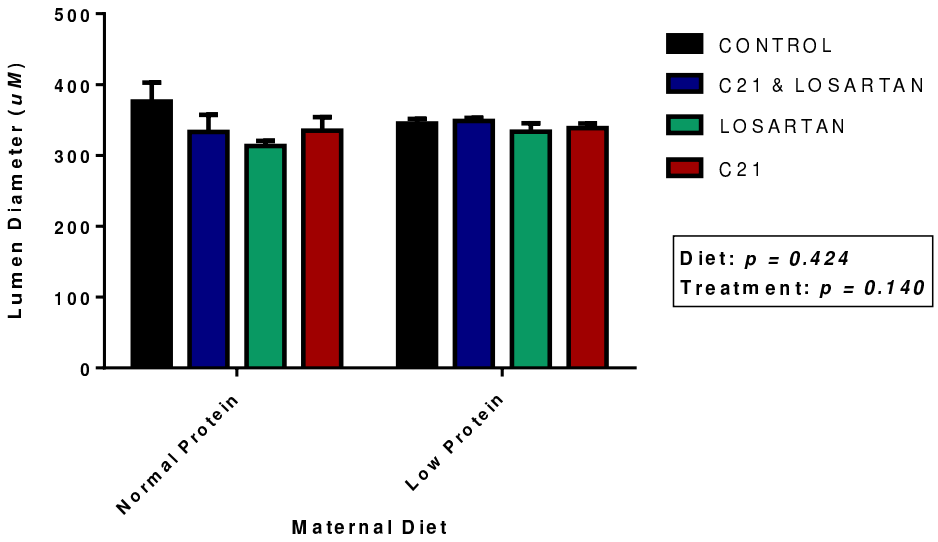


Figure 4.10: Lumen Diameter (µM) of vessels used in the wire myography experiments. Animals from mothers fed a normal or low protein diet during gestation were exposed to a 4 month drug treatment period in one of the following treatment groups: control, C21 & Losartan, Losartan, and C21.

In Table 4.3 below, is a summary of the vascular data generated in this experiment as a complement to Figure 4.11, Figure 4.12, Figure 4.13, and Figure 4.14. Response to KPSS was significantly different between control and Losartan treated animals ($p<0.05$), and this effect was more notable in normal protein offspring ($p<0.05$). Response to phenylephrine was significantly higher in low protein offspring when compared to normal protein offspring ($p<0.05$), but there was no effect of treatment. Maximal relaxation in response to acetylcholine was significantly different in treatment groups depending on maternal diet. Normal protein offspring treated with C21, Losartan, or C21 & Losartan had decreased maximal relaxation responses when compared to low protein offspring.

	Normal Protein Offspring				Low Protein Offspring			
	Control	**C21**	**C21 + Losartan**	**Losartan**	**Control**	**C21**	**C21 + Losartan**	**Losartan**
Lumen Diameter (μM)	375.94 ±26.92	334.86 ± 19.21	333.24 ± 24.09	313.38 ± 7.28	345.00 ± 26.9	338.73 ± 6.31	348.76 ± 4.14	333.46 ± 12.05
Maximal Constriction								
KPSS (mN mm^{-1})	3.84 ± 0.53^{a}	2.25 ± 0.23$^{a, b}$	2.61 ± 0.12$^{a, b}$	2.89 ± 0.27^{b}	2.59 ± 0.22^{a}	2.68 ± 0.20$^{a, b}$	2.27 ± 0.10$^{a, b}$	2.41 ± 0.13^{b}
PE (% KPSS)	87.69 ± 1.66^{c}	86.62 ± 2.71^{c}	84.68 ± 4.45^{c}	84.42 ± 2.85^{c}	89.10 ± 1.20^{d}	87.74 ± 1.99^{d}	82.29 ± 0.48^{d}	89.08 ± 0.41^{d}
% Maximal Relaxation								
Acetylcholine	94.58 ± 2.83^{e†}	61.91 ± 14.78^{f†}	63.88 ± 16.35^{f†}	72.60 ± 15.58^{f†}	95.12 ± 3.62^{e†}	97.83 ± 0.57^{f†}	97.76 ± 1.58^{f†}	100.69 ± 2.08^{f†}
pEC50 (-log M)								
Phenylehphrine	5.734 ± 0.009	5.958 ± 0.018	5.688 ± 0.013	5.965 ± 0.054	5.912 ± 0.019	5.929 ± 0.021	5.824 ± 0.009	5.79 ± 0.011
Acetylcholine	8.125 ± 0.072	8.779 ± 0.542	8.173 ± 0.249	7.837 ± 0.040	8.058 ± 0.061	8.315 ± 0.081	8.174 ± 0.056	8.166 ± 0.027

Table 4.3: Summary of vascular data for offspring of normal and low protein mothers. All values presented are mean ± SEM. Differing superscript letters denote significant differences at $p<0.05$. † = statistically significant at $p<0.01$.

4.4.7.4 - Response to Phenylephrine

Cumulative dose-response curves ranging from 1nM to 100µM were performed using the α_1-adrenoceptor agonist phenylephrine (PE). Isolated vessels were treated with doses of PE at two minute intervals and readings for constriction recorded in mV. Data are presented as a percentage of maximal constriction to KPSS, calculated based on these readings.

The EC50 (-logM) after dosing with the α_1-adrenoceptor agonist phenylephrine did not differ amongst treatment groups from either maternal diet cohort (Figure 4.11). There was, however, a significant main factor effect of maternal diet on maximal response to phenylephrine; LP offspring had a significantly greater maximal response than normal protein offspring ($p < 0.05$, Figure 4.12). This effect was only evident in animals from the C21, C21 & Losartan, and Losartan groups. Control treated animals were not significantly different between normal and low protein offspring.

These observations reflected the data demonstrated in response to KPSS dosing, suggesting that the effect of the treatment regime is different in normal and low protein offspring, possibly as a result of programmed physiological differences.

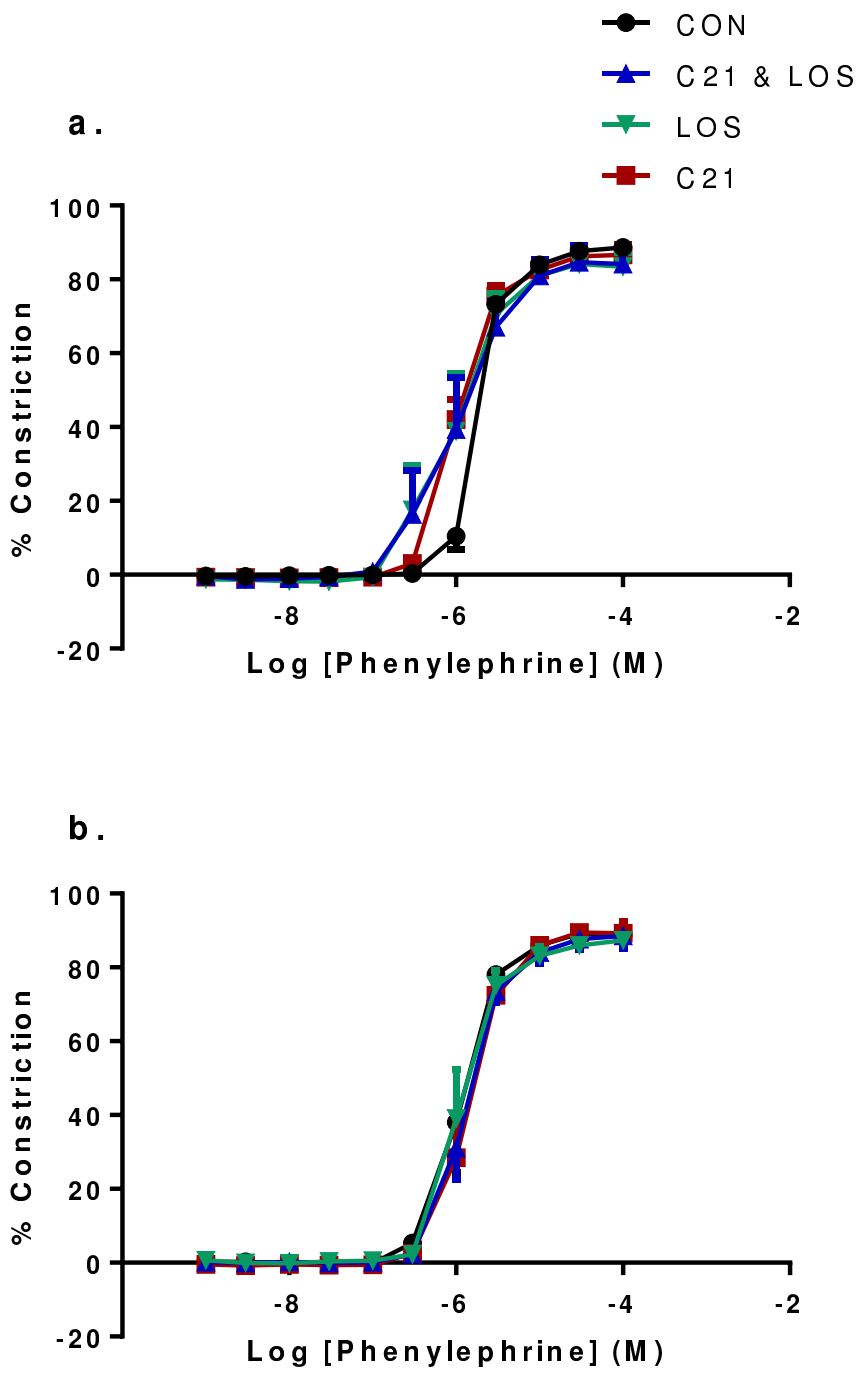


Figure 4.11: Cumulative dose-response curves for phenylephrine (1nM-100µM) in a) normal protein and b) low protein offspring, from control (CON), C21, C21 & Losartan (C21 & LOS), or Losartan (LOS) treatment groups. Data are presented as a percentage of maximal constriction to 125mM KPSS.

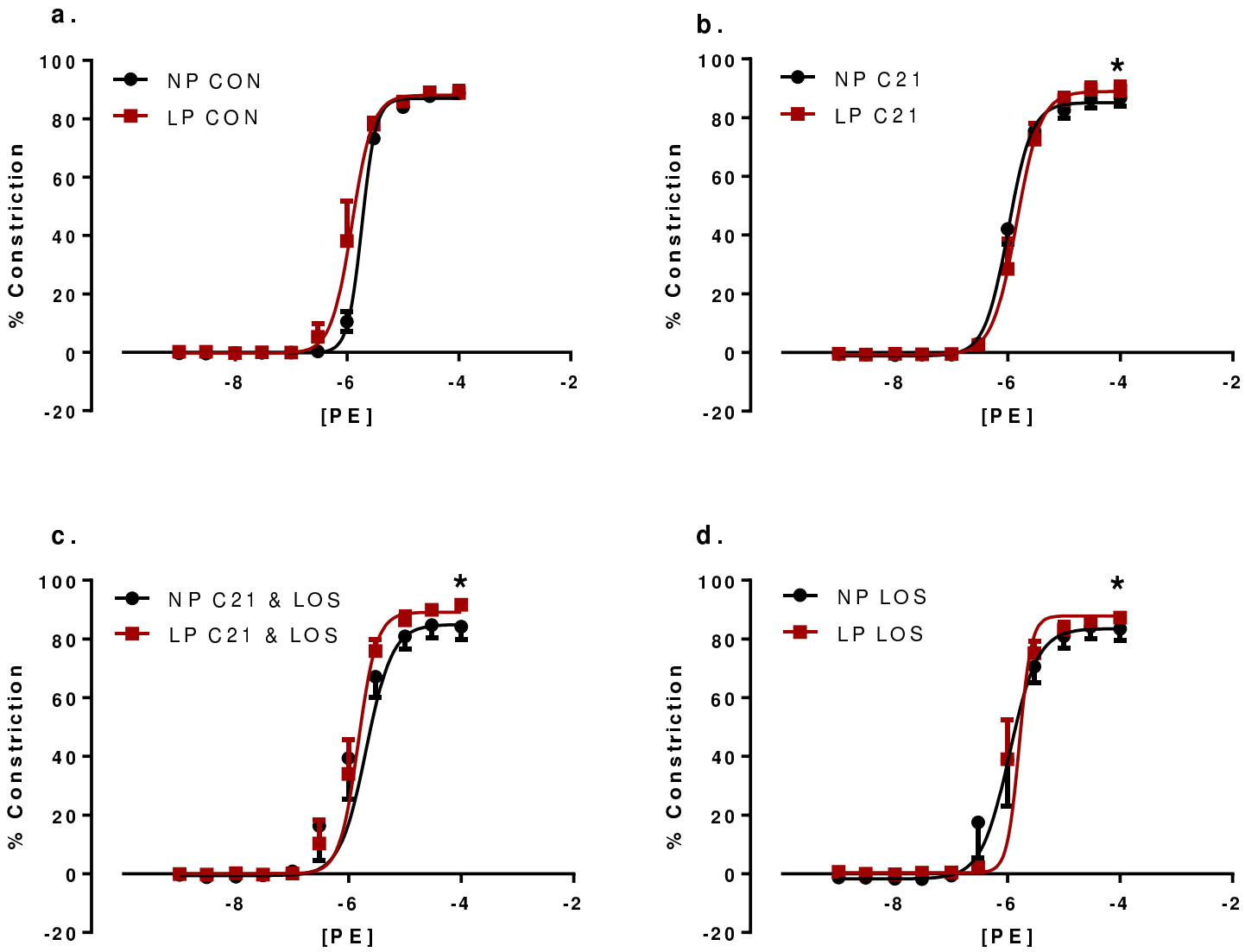


Figure 4.12: Vasoconstriction in mesenteric arteries.

Response to doses of phenylephrine in a) control treated animals from both diet groups, b) C21 treated animals from both diet groups, c) C21 + Losartan treated animals from both diet groups and d) Losartan treated animals from both diet groups. * Overall effect of diet, significant at $p<0.05$.

4.4.7.5 - Response to Acetylcholine

Vasodilation responses were measured in vessels pre-constricted with a single dose of thromboxane mimetic, 9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α (TBXM). Values are presented as a percentage relaxation of this pre-constriction. Cumulative dose-response curves were generated with doses of acetylcholine ranging from 1nM to 100μM.

There was no significant differences observed between groups in the EC50 (-log M) of the regression curves. However, there were significant differences in the maximal response to acetylcholine across groups. Firstly, there was a main factor effect of treatment; C21 treated animals had significantly decreased maximal response to acetylcholine in comparison with control treated animals ($p<0.05$, Figure 4.13). Arguably, this effect was being driven by difference in control and C21 animals in the normal protein offspring, as when considered alone there is no significant difference between animals in the low protein cohort (Figure 4.13; Table 4.3).

There was also a significant effect of maternal diet. Normal protein offspring had significantly diminished maximal responses to acetylcholine in comparison with MLP offspring ($p<0.01$, Figure 4.14). This difference was only significant in C21, C21 + Losartan and Losartan treated animals but not controls, suggesting that there was a difference in response to treatment between control and low protein offspring.

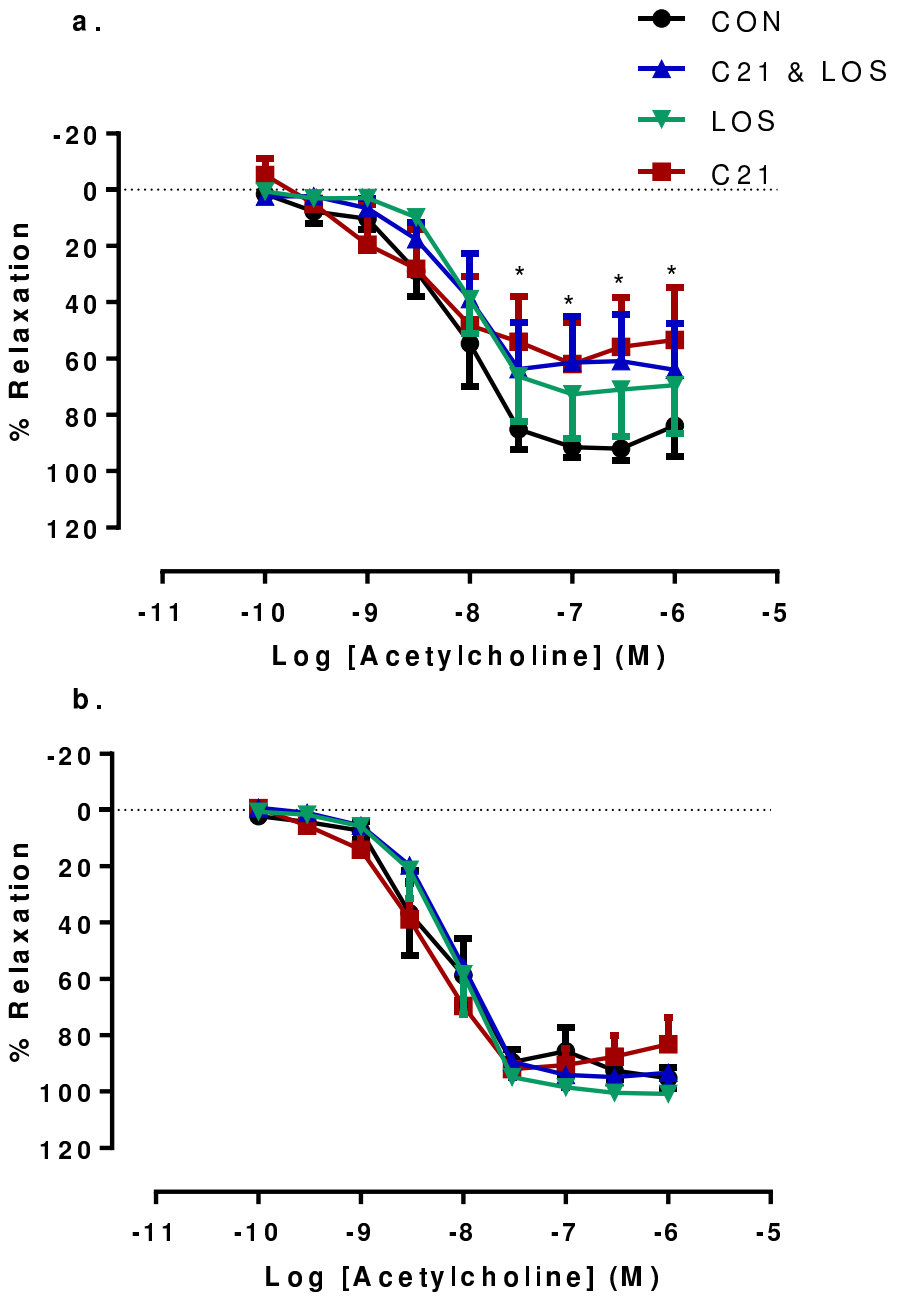


Figure 4.13: Vasorelaxation in mesenteric arteries in response to acetylcholine (ACh) after pre-constriction with TBXM in a) normal protein offspring from all treatment groups, b) low protein offspring from control (CON), C21, C21 & Losartan (C21 & LOS), or Losartan (LOS) treatment groups. Data are presented as a percentage decrease of pre-constriction.

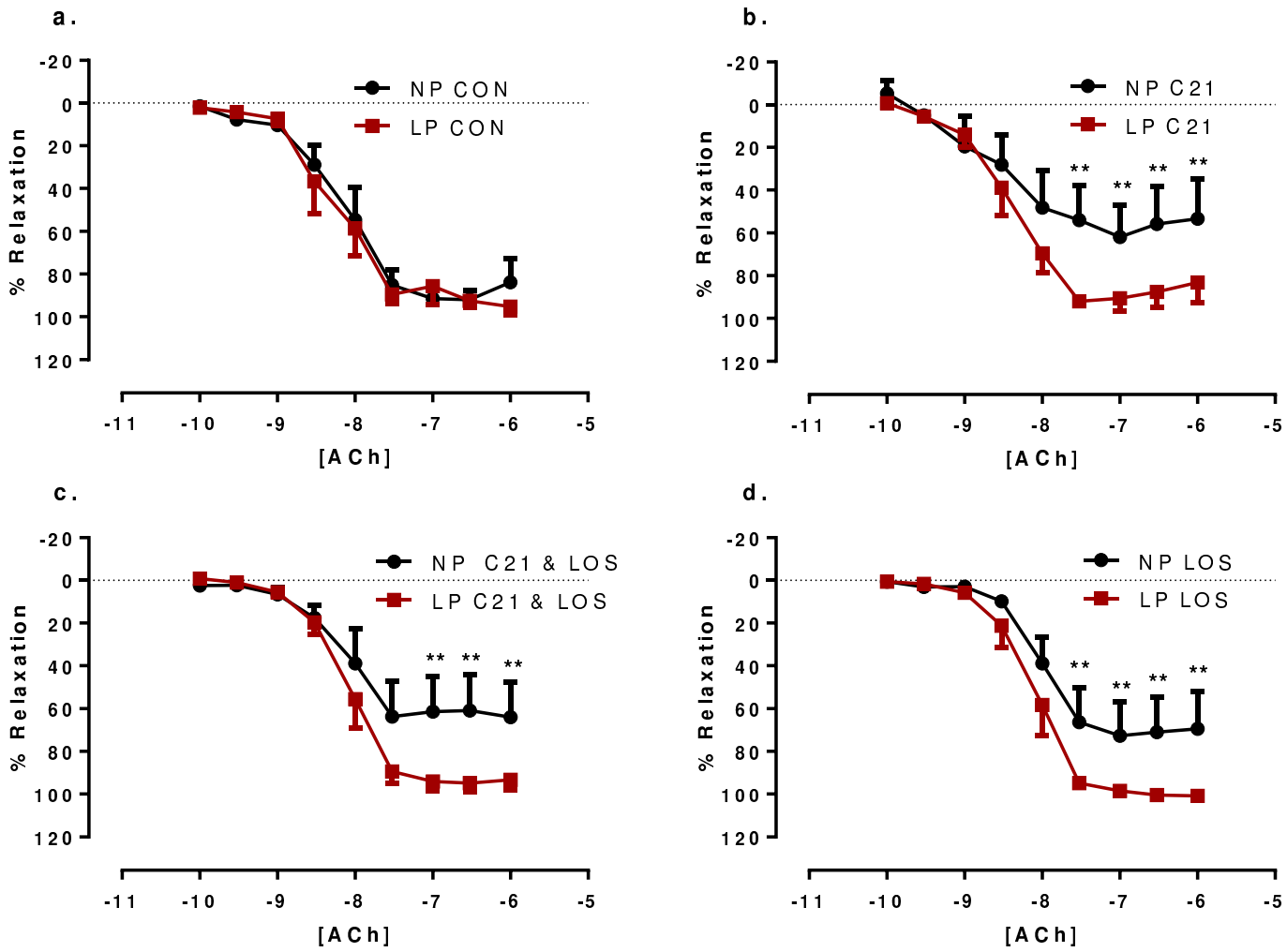


Figure 4.14: Vasorelaxation (as a percentage decrease of a pre-constriction) in mesenteric arteries in response to acetylcholine (Ach) in a) control (CON) treated animals from both diet groups, b) C21 treated animals from both diet groups, c) C21 + Losartan (C21 & LOS) treated animals from both diet groups and d) Losartan (LOS) treated animals from both diet groups.

**effect of maternal diet, *p<0.01.*

4.5 - Discussion

The aim of this study was to explore the potential benefits of long-term administration of the selective AT_2R agonist C21, both alone and administered concomitantly with AT_1R antagonist, Losartan. The study design incorporated the use of a well-established method of programming hypertension and renal injury; the maternal low protein diet model, with the aim of providing a comparison between typical and accelerated ageing in the kidney.

In contrast to previous research (Langley & Jackson, 1994; McMullen & Langley-Evans, 2005; Nwagwu et al., 2000), no elevation in blood pressure was observed in low protein offspring. Instead, normal protein (NP) offspring presented with significantly higher blood pressure than the low protein (LP) group. However, it is important to note that the absolute values for systolic blood pressure in the NP animals were within normal range for rats of that age. The average blood pressure of a Wistar rat is around 125-130mmHg and with this in mind we should consider that the readings for LP offspring at 12 months of age may even be considered mildly hypotensive. Ranging between 110-116mmHg, the LP 12 month systolic blood pressure readings are unusually low not only for animals from a low protein mother, but also for normal animals of that age group. It has been suggested that differences in blood pressure may be the result of an increase in stress associated with the tail cuff procedure used for measurement. In order to obtain a reading animals must be removed from their home cage, warmed (to cause vasodilation in the tail), restrained for extended periods of time, and have an occlusion cuff and sensor attached to their tails. Whilst this could be a source of variation in many instances, it seems unlikely to be the source of the discrepancy in this experiment. A study by Swali et al., (2010) demonstrated effectively through use of simultaneous tail cuff and telemetry (gold standard) blood pressure measurement, that responses to stressors involved in the tail cuff measurement are the same across control and

LP offspring. This supports the hypothesis that is unlikely that differences between the NP and LP animals in this study were due to differing stress responses. Alterations in blood pressure evident as a result of the measurement procedure would likely have been the same across all groups and therefore the magnitude of difference between control and low protein animals the same. Moreover, the greatest differences between diet groups were observed at 12 months of age, at which point the animals had experienced the tail cuff procedure twice before (6 and 9 month readings) and would be more acclimatised to the methods involved. Finally, if LP animals are analysed independently from the NP animals, the decrease in systolic blood pressure at 12 months of age compared to 6 and 9 months of age is statistically significant. In NP animals this is not the case. From these details we can conclude that the differences observed between control and LP offspring may be a differing response to the treatments administered, possibly as a result of a programmed physiological difference between the groups.

It has been frequently reported that exposure to a low protein diet during gestation results in elevation in blood pressure in the offspring throughout the lifespan. In this study there was no significant difference in blood pressure in LP and control offspring in contrast with the literature. In analysing this, we must consider the age of the animals used in the study. Firstly, animals were sacrificed at 12 months of age. Whilst there was no effect on blood pressure up until this point, it is possible that higher blood pressure could have been seen at a later stage. Previously, studies ageing animals to 18 months have been sufficient for inducing age-related disease. A review by the author compared survival rates between three cohorts of laboratory rats aged to 18 months (two historical, one current). In both historical cohorts survival to 18 months of age was poor, with odds ratios (likelihood of death before 18 months) of 28 and 32%. In contrast, survival to 18 months of age in the recent cohort was 100%, suggesting animal health was vastly improved in the more

recently bred laboratory animals (Clifford et al., 2013) With these data in mind, it may be possible that the length of the trial performed for this report was insufficient for induction of increases in blood pressure associated with ageing.

Alternatively, there are limited studies considering the effect of maternal diet on blood pressure in aged offspring. The majority of programming studies have demonstrated an elevation in blood pressure in offspring at relatively young ages, with the maximum age for a reported difference in SBP being around 20 weeks (Bai et al., 2012). It is possible that the BP programming effect of the LP diet is negated or compensated for in the long-term, though the mechanism for this remains unclear. Increased localised glomerular hypertension would certainly be able to counteract increases in BP globally (Brenner & Mackenzie, 1995). However, it would be expected that this would cause greater renal damage in the long term and present in other forms of renal dysfunction aside from blood pressure.

As in this particular animal cohort there was no hypertensive effect of exposure to the LP diet during gestation at any time point (Pijacka et al., 2015), it is not unreasonable to assume that the programming phenomenon is not a consistent or strong model of renal ageing. Certainly, this data does not contain any of the hallmark effects of low protein programming. Moreover, limited negative effects of the LP diet were seen on any outcome reported throughout this thesis. Other studies have reported inconsistencies with this particular model in rats (Woods, 2004 & 2005), and work in humans has suggested that LP during gestation does not alter blood pressure in infants (Huh et al., 2005). These data present a strong argument for the use of an alternative model.

Compound 21, administered chronically, appeared to exert no effect on systolic blood pressure when compared to controls in either normal or low protein offspring in agreement with a number of other studies (Gelosa et al., 2009; Matavelli et al.,

2011; Kaschina et al., 2008). This was true even when administered in combination with a well-known AT_1R antagonist. Moreover, blood pressure was significantly reduced by administration of the AT_1R antagonist Losartan alone, suggesting that Compound 21 and AT_2R stimulation are potentially eliciting a reduction in response to AT_1R antagonism. Whilst a great deal of the literature suggests AT_2R stimulation may result in vasodilation as previously mentioned, evidence is also available to suggest that it is equally possible for AT_2R stimulation to invoke a constrictor response (Verdonk et al., 2012). Our data agrees with current literature suggesting that AT_2R agonism and C21 do not cause a reduction in blood pressure. It must be considered that the initial study design incorporated the low protein model to provide a hypertensive cohort for comparison of the effects of C21 in normotensive and hypertensive animals. At 6 months of age there was no elevation is systolic blood pressure observed in low protein offspring. Thus, additional research in hypertensive animals, for example spontaneously hypertensive rats, may prove to be interesting as the present study refers only to animals considered normotensive at the start of treatment.

In addition to the decrease in blood pressure that was observed in Losartan-treated animals, kidney size was significantly increased. This was also apparent in combined C21 and Losartan treated animals (but not C21 alone), suggesting that Losartan is mediating the effects on kidney size. This was, however, the only apparent effect of drug treatment on renal measurements. There were no effects of treatment on urine volume, osmolality, or protein excretion. In contrast, there were significant effects of maternal diet on these parameters, demonstrating a clear difference in physiological response in offspring exposed to a nutritional insult during gestation. The impact of foetal programming on nephron number has been well documented (McMullen et al., 2004; Benz & Amman, 2010), although measurements were not performed in this study that can verify this, it is a

reasonable explanation for the differences seen. Additionally, it would have been beneficial in this project to include a more comprehensive selection of tests considering the renal function of these animals. Measurement of inulin clearance, albuminuria (and albumin:creatinine ratio), and pro-inflammatory markers would have vastly enhanced the understanding of the effects of long term dosing on overall renal health. Indeed, a study by Koulis et al. (2015) showed that compound 21 was an effective means of ameliorating diabetes mellitus-induced elevations in cystatin c, albuminuria, and glomerulosclerosis. It is possible that the improved BP response in the low protein offspring is an increased susceptibility to the effects of treatment – in other words, Compound 21 is more effective at improving renal function and filtration in offspring predisposed to renal injury, and thus presents with significant improvements in renal outcome. Regrettably, this is mere speculation at this point and would require a substantial body of additional labwork to fully understand.

Responses to the vasodilator acetylcholine showed a dramatic difference between maternal diet groups. NP offspring presented with significantly diminished relaxation responses in comparison with LP offspring. Whilst relaxation reaches a maximum of 94% in control treated NP offspring (within 1% of the maximum response achieved in low protein offspring), it was reduced to 73% in Losartan-treated animals, 64% in combined C21 and Losartan-treated animals, and 62% in C21 treated animals. The lack of this effect in untreated animals from both diet groups suggests a difference in response to treatment between the maternal diet groups. This is in contrast to a great deal of the literature concerning the low protein diet and vascular function. Previous studies have demonstrated on numerous occasions that LP offspring are prone to decreased relaxation responses in isolated arteries and that this is linked to a change in blood pressure (Torrens et al., 2009; Brawley et al., 2004; Watkins et al., 2010). The discrepancy between the results in this experiment and the

published literature is perhaps unsurprising, as the data for blood pressure does not follow the expected pattern either. With LP blood pressure readings significantly lower than their NP controls, it follows that LP vascular responses would not be poorer than the NP offspring. However, the reasons for this are not immediately obvious.

It is well accepted that decreases in vascular reactivity can be linked with increases in systolic blood pressure, and this is evident in the data presented. The differences between the response to acetylcholine in control and LP offspring were highly significant. The *ex vivo* nature of the myography procedure removes the vessels from immediate stimulation by angiotensin II. For this reason it is possible that the vascular effects that were observed are the result of a long-term physiological or structural change to the vessels that has occurred as a result of exposure to maternal dietary insult. Swali et al., (2010) noted disparities between tail cuff blood pressure (measuring peripheral blood pressure) and central blood pressure (measured by radiotelemetry from the descending aorta) specifically in the offspring of mothers fed a low protein diet during pregnancy. Their explanation for this was that there may be programmed differences in the structure of the peripheral vasculature, which may be consistent with the assertion made above.

In order to understand this in depth, we must consider pathways in these animals that may normally respond to both Ang II and acetylcholine. G-protein coupled receptors (GPCRs) are instrumental in the signalling pathways of a number of vascular contractile and relaxation responses. Their complex nature allows for their activation to result in both constriction and vasodilation in various tissues (Brinks and Eckhart, 2010). The GPCR, $G\alpha_{q/11}$, can be activated by both acetylcholine and angiotensin II and is therefore a potential site of variation as a result of programming in the low protein model. A study by Xing et al., (2002) demonstrated that exposing cultured vascular smooth muscle cells to angiotensin II for varying time periods

altered $G\alpha_{q/11}$ expression in the tissue. Ryan et al., (2012) examined expression of G proteins associated with the cardiac adrenergic receptors and found no effect of maternal diet on expression of G*i* or G*s*. Finally, previous studies have demonstrated the maternal low protein diet can alter the levels of $AT_{1/2}R$ expression in rat kidney tissue (McMullen et al., 2004; Sahajpal and Ashton, 2003). A combination of altered $G\alpha_{q/11}$ and angiotensin receptor expression due to programming could dramatically alter the response to the treatments administered in this trial. Whilst the evidence in this area is currently limited, it provides a solid focal point for continuation of this work. It is clear that response to angiotensin receptor antagonism and agonism differs in control and low protein offspring and further work must be done to elucidate the mechanism by which this is occurring.

Differences in the GPCR between diet groups may also go some way to explaining differences in constrictor responses. Previous literature would suggest that increased contractile responses are linked with increased systolic blood pressure. In these data, this is not the case; MLP offspring demonstrate greater contraction in response to pheyleprhine, but have lower systolic blood pressure than controls. However, phenylephrine also activates $G\alpha_{q/11}$ (Brinks and Eckhart, 2010) and if fundamental changes to the GPCR have been made as is hypothesised above, the responses to constrictors may be equally altered and thus not reflective of a typical hypertensive state.

4.6 - Conclusions

The results from this study do not support the original hypothesis under investigation. Long-term, low-dose treatment with Compound 21 does not reduce systolic blood pressure in offspring from either control- or low protein fed mothers, and this was unaffected by concomitant dosing with AT_1R antagonist Losartan.

However, we can conclude that the maternal diet during gestation significantly alters the response to treatment with C21 and Losartan alike. Whilst the mechanism for this is currently unclear, our data suggests a potential role for the G-protein coupled receptor $G\alpha_{q/11}$ and long-term physiological or structural changes to the vasculature.

Chapter 5 - The Effect of Sex Steroid Modulation

5.1 - Introduction

There is a significant literature base concerning the effects of sex steroids on blood pressure. In particular, it has been well documented that oestrogen confers a 'protective effect', and high levels of oestrogen are associated with favourable physiological outcomes (Sandberg & Ji, 2012; Farhat et al., 1996; Mendelsohn & Karas, 1999). Evidence suggests that prior to menopause the prevalence of hypertension is greater in males than in females (Kearney et al., 2005). However, post-menopause hypertension and cardiovascular risk in women not only increases to reach a level similar to that in males, but in some instances exceeds it (Cutler et al.,. 2008). In addition, work in this laboratory demonstrated that blood pressure in normal protein, intact female offspring is significantly lower than their low protein counterparts, and this effect is negated by ovariectomy surgery (Pijacka et al., 2015). Although these improved renal outcomes in aged females in comparison with males are well-documented (McMullen & Langley-Evans, 2005; Saez et al., 2014; Reverte et al., 2011), there is still debate as to the precise nature of the nephroprotective effects of oestrogen (Vitolo et al., 2014).

Chapter 3 of this thesis considered the hypothesis that nitric oxide concentrations would be altered with age and elicit subsequent effects on renal function, and that this would happen in a sex-specific manner. The experiments conducted found this to be untrue; whilst a protective effect of female sex steroids was observed, this did not appear to be mediated via moderations in the nitric oxide system. Data suggested that although no direct concentration effect of nitric oxide (or associated compounds) was observed, there may be interactions between alternative elements of the renin-angiotensin system that are playing a role in mediating these sex-

specific differences. Additionally, changes in levels of nitrites (measured as a proxy for nitric oxide concentration) were statistically significant leading to the hypothesis that aberrant fluctuations in NO activity may be physiologically important, rather than a proportional concentration relationship.

There is a large evidence base linking the angiotensin II type 2 receptor with healthier blood pressure, increased vascular reactivity, and reduced inflammation, potentially through increased activity of nitric oxide (Siragy & Carey, 1997; Sampson et al., 2008; Dimitropoulou et al., 2001; Padia & Carey, 2013). Furthermore, differential expression and activity of the AT_2R has been observed in males and females, and cited as a potential mechanistic pathway for sex-specific differences in blood pressure (Hilliard et al., 2011; Hilliard et al., 2013). As such, the development of a highly selective AT_2R agonist, Compound 21 (C21; Wan, 2004; Hilliard et al., 2012) was a promising means of increasing NO and potentially mimicking some of the protective effects associated with female gender. A trial considering the long-term administration of C21 in this lab was unable to elicit a decrease in blood pressure in rats (Chapter 4). Other, more acute studies have successfully utilised C21 to stimulate desirable responses in the renin-angiotensin and nitric oxide systems (Verdonk et al., 2012; Bosnyak et al., 2010). It is for this reason, that the following study incorporated shorter periods of treatment with C21 to investigate whether or not it is capable of causing a short-term alteration in blood pressure, in the hopes of identifying potential pathways for future study and intervention.

The work described in this chapter aimed to examine potential mechanistic pathways that may be responsible for sexual dimorphism in blood pressure regulation. Through utilisation of a well-established model of normal vs. accelerated renal ageing and hypertension (Langley & Jackson 1994; McMullen et al., 2005) and ovariectomy surgery, the study addressed the following questions:

1. Does the removal of oestrogen result in sustained elevation in blood pressure, and is this effect exacerbated in a model of accelerated renal ageing?
2. Does the removal of oestrogen result in a differential response to drug treatment targeting the renin-angiotensin system?
3. Can these results offer a target pathway for future understanding of the 'protective effect' of oestrogen?

It was hypothesised that ovariectomy would result in elevation of blood pressure, and this increase would be sustained with age as a result of altered nitric oxide synthesis and activity. This effect may be ameliorated by pharmacologically stimulating nitric oxide production through treatment with the novel, highly-specific angiotensin type II receptor agonist, Compound 21. As the mechanisms by which C21 has beneficial physiological effects have not been fully elucidated, an additional nitric oxide synthase inhibition challenge was incorporated to establish whether or not C21 is acting on the NO system.

5.2 - Methods

Data in this chapter pertains to Trial III (Section 2.2.3 -), detailed methodology can be found in Chapter Two. In brief, virgin female Wistar rats were mated as described for Trials I and II. At birth, litters were culled to 8 offspring with preferential selection of females. At 10-weeks of age, all females were either ovariectomised or exposed to a sham surgical procedure (Section 2.1.3 -). Following recovery, animals were housed in pairs and weighed weekly for the remainder of the trial. At 11 months of age, all animals underwent aortic telemetry implantation for continuous monitoring of blood pressure responses (Section 2.1.4.2 -). For the final 5 weeks of Trial, at 12 months of age, animals underwent a series of week-long drug interventions with simultaneous measurement of blood pressure and heart rate, full details of which can be found in Chapter Two (Section 2.2.3 -). Animals were culled upon completion of dosing and blood, urine, and kidneys collected for measurement of renal function and nitric oxide concentration.

5.3 - Statistical Analysis

All data are presented as mean ± SEM unless stated otherwise. Starting n per group was 8, losses during surgery or signal dropout during telemetry recording mean that n is variable for blood pressure data. Changes in n are noted in the appropriate figure legends. Analyses were performed with the Statistical Package for Social Science (SPSS, vers. 22, SPSS Inc, Chicago Il, USA). Tail cuff blood pressure data were analysed using a three-way ANOVA, with a Bonferroni post-hoc correction where applicable. Telemetry blood pressure data were analysed using a mixed between-within ANOVA with time and treatment as repeated measures. Where Mauchly's test for sphericity was failed, significance was calculated using the Greenhouse-Geisser correction. Separate three-way ANOVA was performed on 24 hour averages, as well as 'day' and 'night' averages. Telemetry data are presented

in two ways, first comparing surgical groups, and second considering the response to treatment within surgical groups. All graphs were drawn using GraphPad Prism (GraphPAD software Inc., San Diego, USA). A p value of less than 0.05 was taken to be statistically significant. Sample size was determined using the same power analyses performed in section 4.3.2.

5.4 - Results

5.4.1 - Measurements by Tail Cuff

5.4.1.1 - Blood Pressure and Heart Rate

Systolic, diastolic, and mean arterial blood pressure and heart rate measurements were made at six and nine months of age using the tail cuff method (Section 2.1.4.1 -).

No significant differences were observed in systolic, diastolic (Figure 5.1), or mean arterial blood pressure (Figure 5.2) regardless of maternal diet, or surgery. This was also true of heart rate (Figure 5.2), where neither maternal diet nor surgery had a significant effect on the data. This did not change with age and no significant differences were observed between six and nine month old animals.

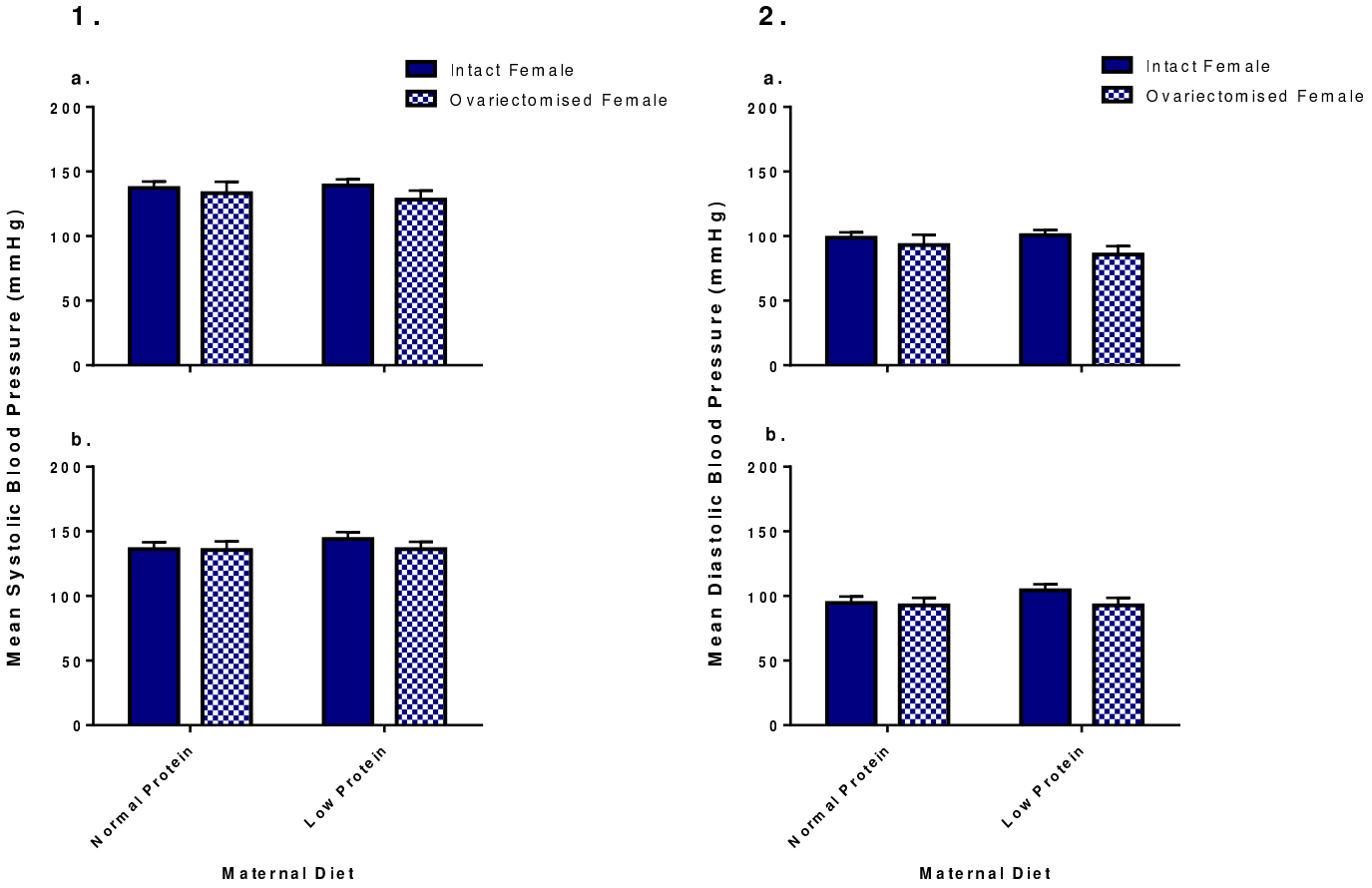


Figure 5.1: Panel 1: Mean systolic blood pressure. Panel 2: Mean diastolic blood pressure. Data was collected by tail cuff in ovariectomised or sham-ovariectomised female offspring at a) 6 months of age and b) 9 months of age. In all groups, n = 8. No significant differences were observed between animals of any group at either age.

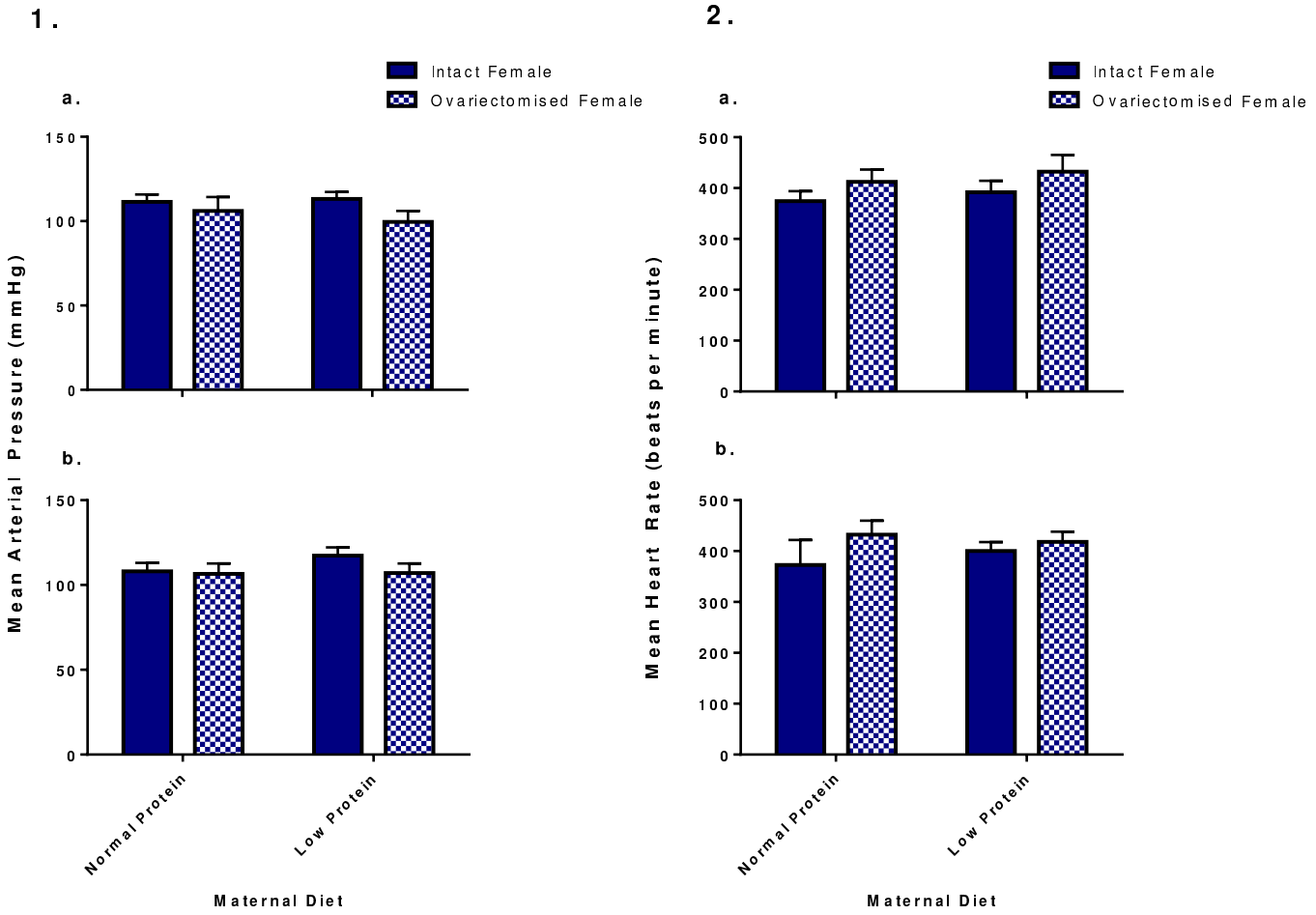


Figure 5.2: Panel 1: Mean arterial pressure (mmHg). Panel 2: Average heart rate (beats per minute). Data was collected by tail cuff in ovariectomised or sham-ovariectomised female offspring at a) 6 months of age and b) 9 months of age. In all groups, n = 8. No significant differences were observed between animals of any group at either age.

5.4.2 - Endothelial Nitric Oxide Synthase Expression

PCR was repeated in animals from Trial III; offspring of mothers on a low- or normal-protein diet were exposed to sham- or ovariectomy surgery, and expression was measured in kidneys at 12 months of age. Unlike Trial I, Trial III did not incorporate culls at time points prior to the final time point, and as such measurements in tissues were only made at 12 months of age. As with Trial I, there was no significant effect of diet (*p=0.237*) or surgery (*p=0.*327) on eNOS expression, as demonstrated in Figure 5.3.

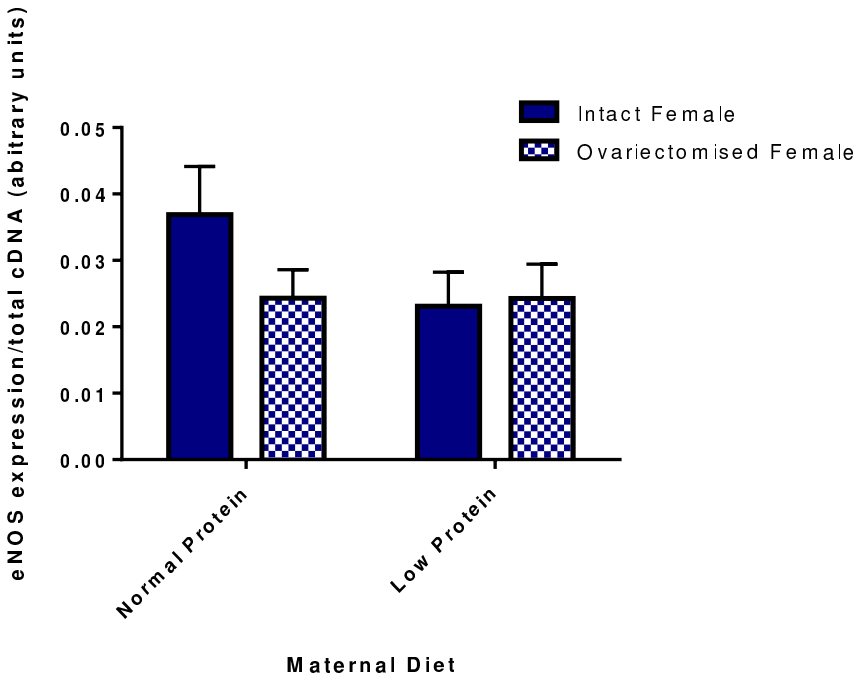


Figure 5.3: Gene expression of endothelial nitric oxide synthase in the kidney of 12 month old animals from trial III. Female offspring from mothers on normal- and low protein diets during gestation were exposed to ovariectomy (ovariectomised) or sham-ovariectomy (intact) surgery at 10 weeks of age. Kidneys were harvested at cull and expression determined using RT-qPCR, all expression data are normalised to the total concentration of cDNA in the samples. No significant differences were seen between groups. Normal protein intact *n = 5*, normal protein ovariectomised *n = 8*, low protein intact *n = 7*, low protein ovariectomised *n = 6*.

5.4.3 - Markers of Renal Function

Alongside gene expression, urinary creatinine was measured in Trial III animals, to see if differences with age and surgery were repeated in a second trial. Offspring of low- and normal protein mothers were either ovariectomised or exposed to a sham surgical procedure, and repeat samples taken from the same animals at 6, 9 and 12 months of age (Figure 5.4).

As with the data from Trial I, there was a significant effect of age, with 12 month animals having significantly lower urinary creatinine excretion than animals at both 6 and 9 months of age ($p<0.001$). There was also a statistically significant effect of surgery. Intact females had higher excretion rates than ovariectomised females across all age groups. Excretion rate was unaffected by maternal diet.

As animals were not culled at the 6 and 9 month time points, acquisition of blood samples for determination of plasma creatinine (and thus clearance), was not feasible in these age groups. However, as was demonstrated in Trial I, plasma creatinine is kept at a relatively constant concentration (Sherwood, 2014), and as such significant effects in urinary excretion may offer us insight into the effects we could expect in creatinine clearance.

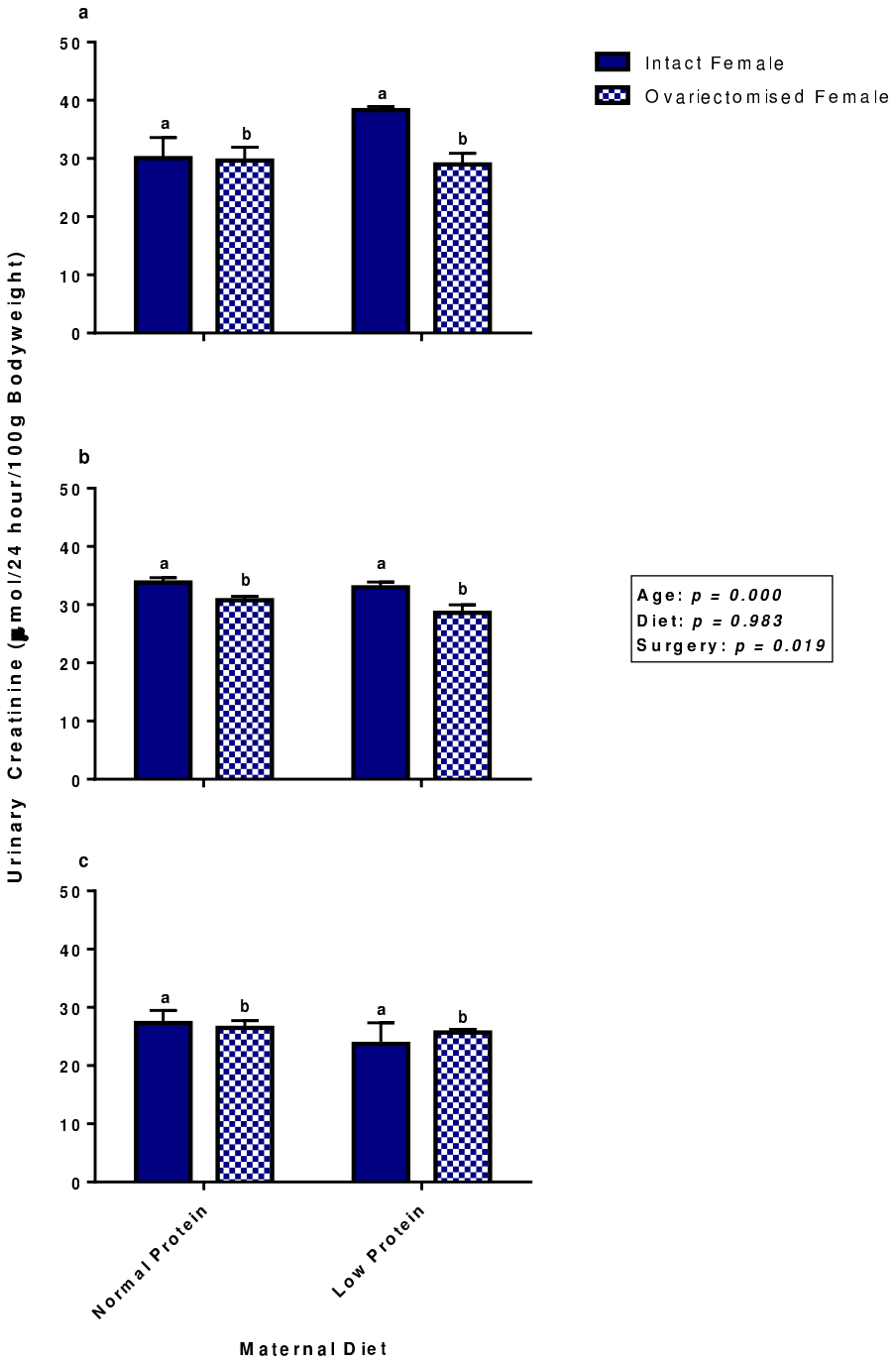


Figure 5.4: Urinary creatinine excretion in a 24 hour period in offspring from normal- and low protein mothers after exposure to sham- or gonadectomy surgery. Samples were taken from the same animals at a) 6 months, b) 9 months, and c) 12 months of age. Animals at 12 months of age had significantly lower excretion than animals at both 6 and 9 months of age ($p<0.001$). Normal protein intact $n = 5$, normal protein ovariectomised $n = 8$, low protein intact $n = 7$, low protein ovariectomised $n = 6$. Significant differences between surgical groups are denoted by superscript letters.

5.4.4 - Food and Water Intake

At six, nine, and twelve months of age, data for food and water intake and urine excretion was collected by housing the animals in metabolic cages (Table 5.1).

Food intake (g/24hr/100g bodyweight) was significantly affected by age ($p<0.01$), with animals at 12 months old consuming significantly less than at 6 and 9 months of age. This was accompanied by significant effects of both surgery and maternal diet. Across all time points, sham-ovariectomised animals consumed more food per 100g bodyweight than ovariectomised animals ($p<0.01$). Similarly, across all groups, offspring of mothers fed a normal protein diet during gestation ate more than their low protein counterparts ($p<0.02$).

Water intake (g/24hr/100g bodyweight) was also significantly affected by maternal diet, surgery, and age. As age increased in the animals, water intake decreased and this was significant at each time point ($p<0.01$). As with the data for food intake, normal protein offspring consumed more than low protein offspring ($p<0.01$), and sham-ovariectomised animals consumed more than their ovariectomised counterparts ($p<0.01$).

Urine excretion (g/24hr/100g bodyweight) was not affected by maternal diet ($p=0.514$) or by age ($p=0.921$). There was however, a significant effect of surgical group. sham-ovariectomised animals excreted more than ovariectomised animals ($p<0.01$).

		6 months		9 months		12 months	
	Diet	Sham	Ovex	Sham	Ovex	Sham	Ovex
Food Intake	NP	$7.25 \pm 0.61^{a\dagger}$	$5.77 \pm 0.46^{b\dagger}$	$9.20 \pm 0.39^{c\dagger}$	$6.51 \pm 0.68^{d\dagger}$	$5.16 \pm 1.09^{e\dagger}$	$5.44 \pm 0.65^{f\dagger}$
(g/24hr/100g BW)	LP	$6.91 \pm 0.24^{g\dagger}$	$5.32 \pm 0.38^{h\dagger}$	$6.56 \pm 0.78^{i\dagger}$	$4.94 \pm 0.33^{j\dagger}$	$5.07 \pm 0.75^{k\dagger}$	$5.36 \pm 0.16^{l\dagger}$
Water Intake	NP	$10.15 \pm 0.94^{a\dagger}$	$9.49 \pm 1.67^{b\dagger}$	$12.65 \pm 0.93^{c\dagger}$	$8.44 \pm 1.06^{d\dagger}$	$9.83 \pm 1.43^{e\dagger}$	$7.11 \pm 0.68^{f\dagger}$
(mL/24hr/100g BW)	LP	$11.55 \pm 0.87^{g\dagger}$	$8.16 \pm 0.89^{h\dagger}$	$9.16 \pm 0.90^{i\dagger}$	$5.81 \pm 0.48^{j\dagger}$	$5.60 \pm 1.57^{k\dagger}$	$7.00 \pm 0.39^{l\dagger}$
Urine Volume	NP	$4.35 \pm 0.97^{a\dagger}$	$3.74 \pm 0.50^{b\dagger}$	$5.75 \pm 1.34^{a\dagger}$	$4.47 \pm 0.41^{b\dagger}$	$4.94 \pm 1.30^{a\dagger}$	$3.83 \pm 0.40^{b\dagger}$
(mL/24hr/100g BW)	LP	$5.45 \pm 0.76^{a\dagger}$	$3.70 \pm 0.55^{b\dagger}$	$4.73 \pm 0.49^{a\dagger}$	$3.04 \pm 0.18^{b\dagger}$	$5.43 \pm 0.89^{a\dagger}$	$3.16 \pm 0.34^{b\dagger}$

Table 5.1: Food intake, water intake, and urine excreted in sham-operated (Sham) and ovariectomised (Ovex) offspring of mothers fed a normal (NP) or low protein (LP) diet during pregnancy. Data presented is from 6, 9, and 12 months of age. Food and water intake were both significantly affected by maternal diet, surgery group, and age ($p<0.05$). Urine excretion was significantly affected by surgery ($p<0.05$). NP Sham $n = 5$, NP Ovex $n = 8$, LP Sham $n = 7$, LP Ovex $n = 6$. Significance denoted by differing superscript letters ($p<0.05$), † = significant at $p<0.01$.

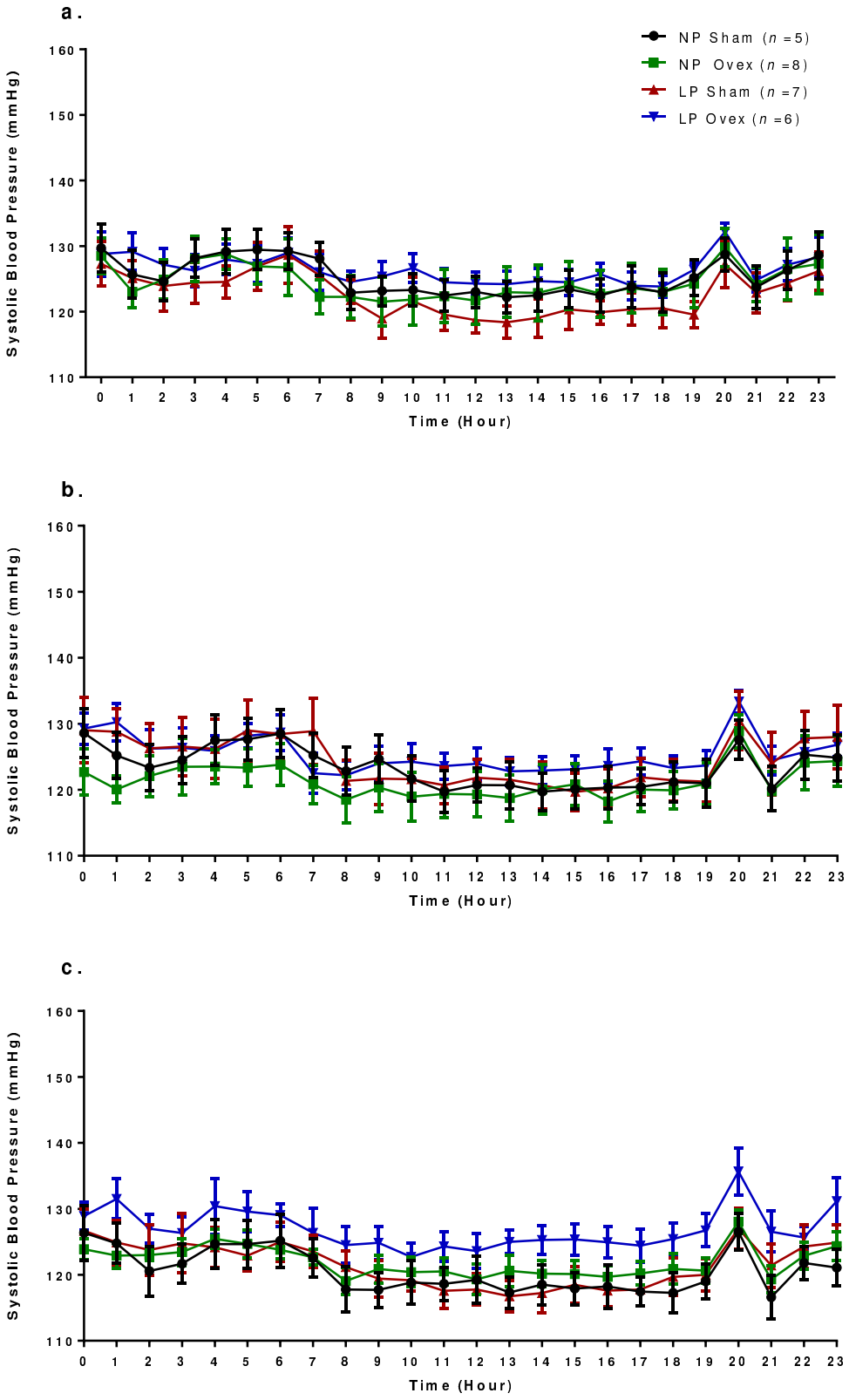
5.4.5 - Blood Pressure – Measurements by Radio Telemetry

Blood pressure measurements at 12 months of age were performed using the gold standard radio telemetry technique.

Systolic blood pressure (SBP), determined by telemetry (Figure 5.5), did not differ between maternal diet and surgery groups at baseline, when animals were treated with C21, or during the 'washout' period.

However, a consistent interaction was observed between diet and surgery groups during the LNAME treatment phase ($p<0.02$). Whilst LNAME increased blood pressure in all groups, the magnitude of the response was related to diet and surgery group. In normal protein offspring, sham operated animals had a larger increase in systolic blood pressure in response to LNAME when compared with ovariectomised animals. In low protein offspring, ovariectomised animals had a larger increase in SBP in response to LNAME when compared with their sham operated counterparts. This was accompanied by a significant interaction between time and surgery ($p<0.01$)

The increased response to LNAME observed in ovariectomised low protein offspring may be indicative of an increased sensitivity to ovarian steroids. These data suggest that animals predisposed to accelerated renal ageing may experience more significant physiological responses to the removal or fluctuation of oestrogen.


a.
NP Sham (n =5)
NP Ovex (n =8)
LP Sham (n =7)
LP Ovex (n =6)
Systolic Blood Pressure (mmHg)
Time (Hour)
b.
Systolic Blood Pressure (mmHg)
Time (Hour)
c.
Systolic Blood Pressure (mmHg)
Time (Hour)

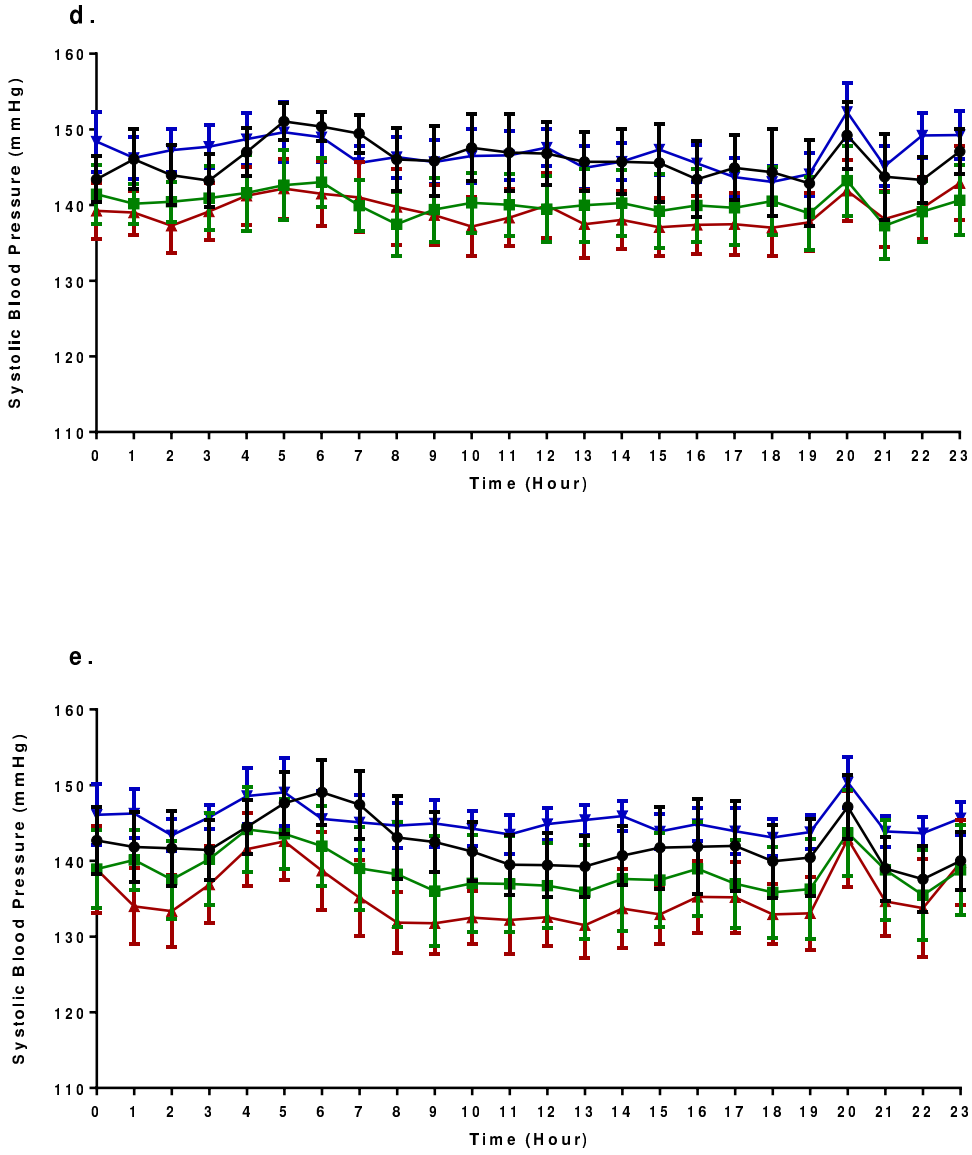


Figure 5.5: (Figure contained on pages 202 & 203) Average systolic blood pressure readings measured by radio telemetry in sham-operated (sham) and ovariectomised (ovex) normal (NP) and low protein (LP) offspring in the following one week treatment periods: a) Baseline, b) C21, c) Washout, d) LNAME, and e) LNAME and C21. Treatments were administered for 7 days and readings averaged for each hour in a 24 hour period. Details of treatment regime can be found in section 2.2.3). There was a significant interaction between maternal diet and surgery in response to LNAME treatment (panel d) ($p<0.02$)

Drug treatment had a significant effect on blood pressure when considered within surgical and diet groups.

There were no changes from baseline after treatment with C21 alone, and no changes during the 'washout' period that was employed to ensure any variations in blood pressure had been normalised. Treatment with LNAME induced a significant increase in blood pressure ($p<0.01$), in accordance with previous studies (Boe et al., 2013; Sung et al., 2013), and this occurred in all diet and surgical groups (Figure 5.6 & Figure 5.7). C21 treatment was not capable of ameliorating the effects of LNAME-induced hypertension in any treatment group, with no significant differences observed between the LNAME and the LNAME/C21 treatment phases.

Repeated measures ANOVA highlighted a significant interaction between time and treatment ($p<0.01$).

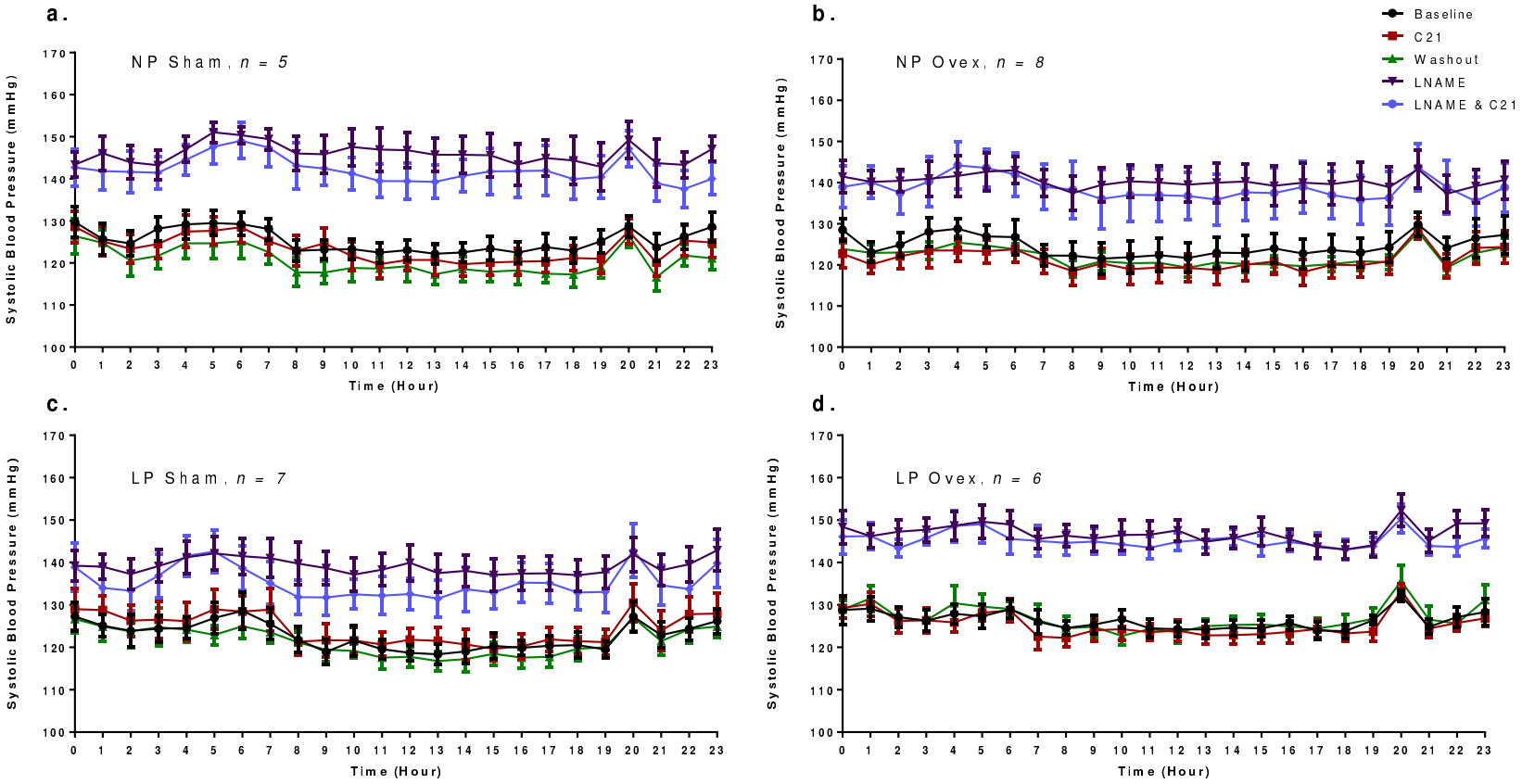


Figure 5.6: Change in systolic blood pressure in response to treatment in a) sham-operated normal protein offspring, b) ovariectomised normal protein offspring, c) sham-operated low protein offspring, and d) ovariectomised low protein offspring.

Systolic blood pressure values were averaged for hours of daylight (Day, 08:00-20:00) and darkness (Night, 20:00-08:00). On average during baseline, C21, and washout periods, systolic blood pressure was significantly increased in the night ($p<0.01$), as would be expected for nocturnal animals. This difference was unaffected by surgery or drug treatment (Figure 5.7).

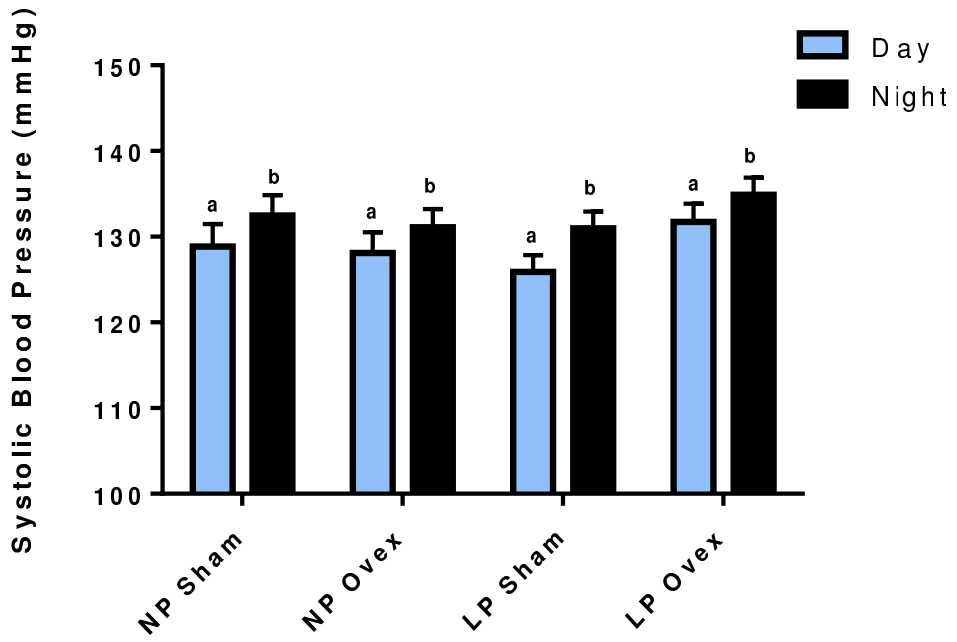


Figure 5.7: Average systolic blood pressure in sham-operated normal protein offspring (NP sham, *n = 5*), ovariectomised normal protein offspring (NP Ovex, *n = 8*), sham-operated low protein offspring (LP Sham, *n = 7*), and ovariectomised low protein offspring (LP Ovex, *n = 6*) in the hours of daylight (08:00-20:00) and darkness (20:00-08:00). Values are mean ± SEM. Date presented is from the baseline treatment period, no significant differences were observed between baseline, C21 and washout treatment phases. Average values for night time blood pressure were significantly higher than those for hours of daylight across groups ($p<0.05$). Different superscript letters denote a significant difference between day and night at $p<0.05$.

However, during the LNAME and LNAME/C21 combined treatment phases, diurnal blood pressure changes were lost. In these periods there were no significant differences between day and night averages for blood pressure (Figure 5.8) and this was unaffected by maternal diet or surgery. There were no significant differences between the LNAME and LNAME/C21 treatment phases.

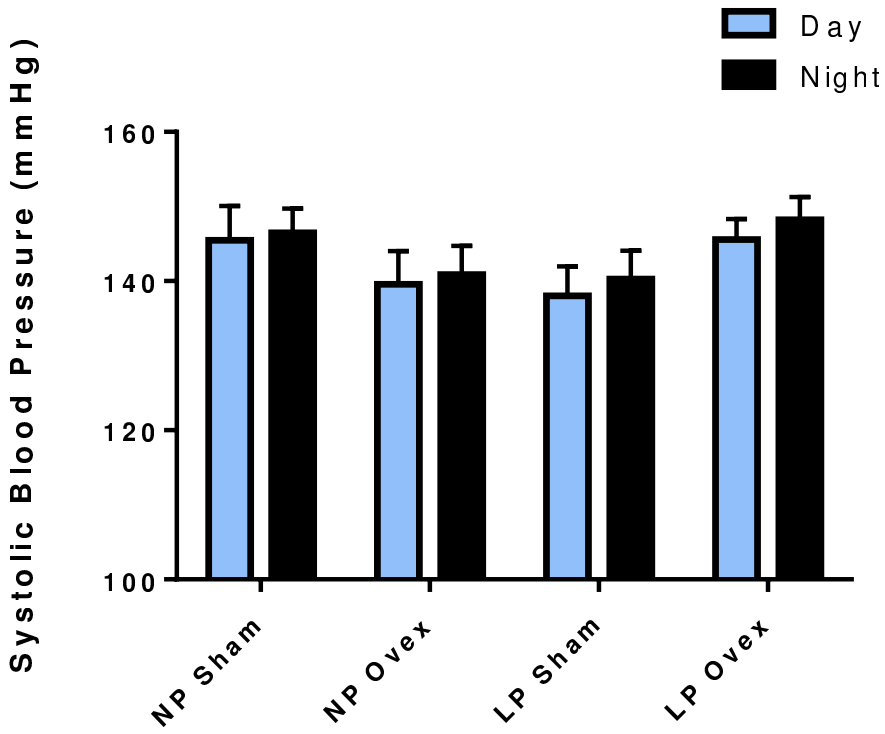


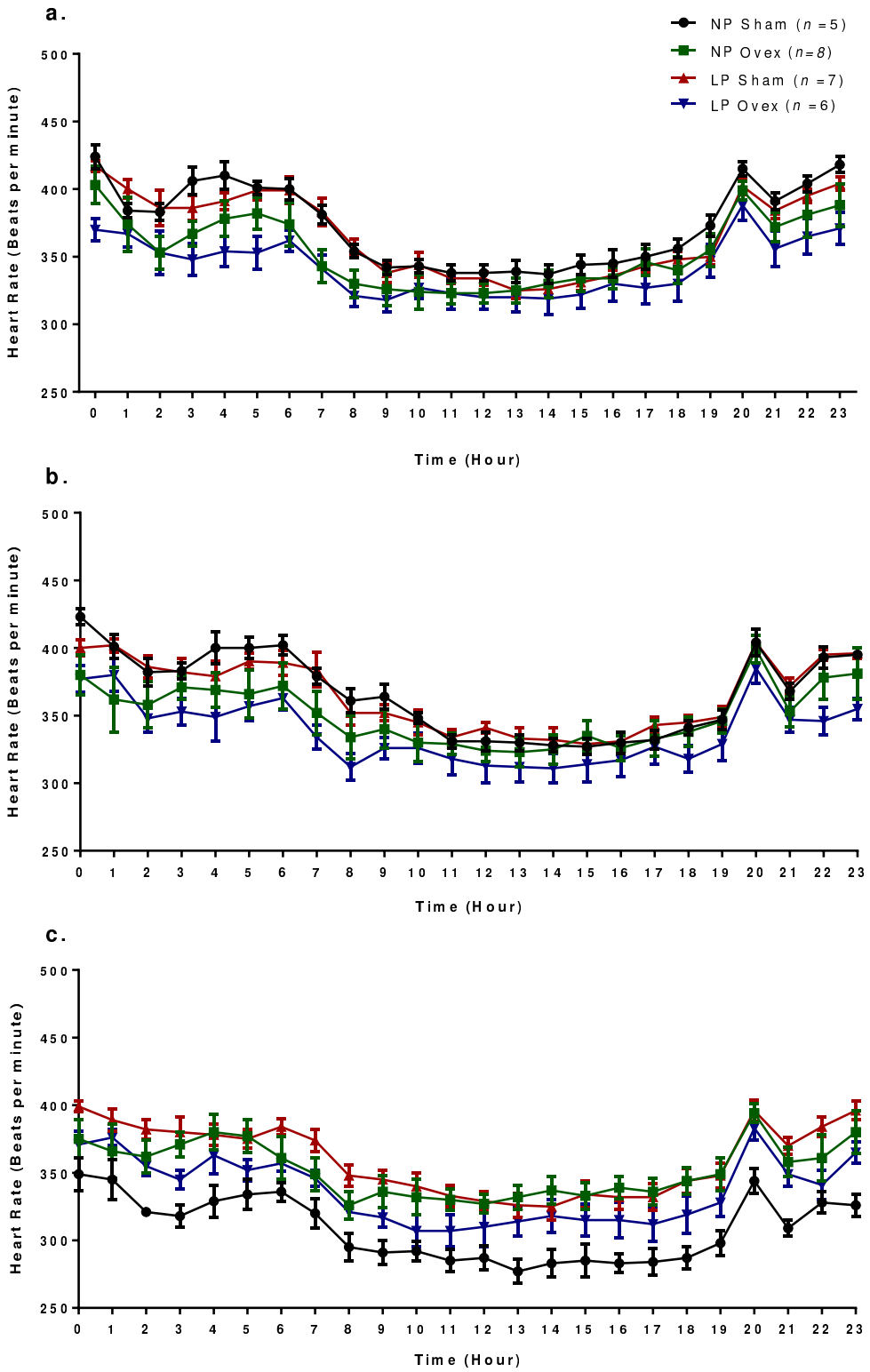
Figure 5.8: Average systolic blood pressure in sham-operated normal protein offspring (NP sham, *n = 5*), ovariectomised normal protein offspring (NP Ovex, *n = 8*), sham-operated low protein offspring (LP Sham, *n = 7*), and ovariectomised low protein offspring (LP Ovex, *n = 6*) in the hours of daylight (08:00-20:00) and darkness (20:00-08:00). Values are mean ± SEM. Data presented are from the LNAME treatment phase.

5.4.6 - Heart Rate – Measurements by Radio Telemetry

Heart rate was measured alongside blood pressure in animals at 12 months of age using radio telemetry. Typically, heart rate changes in proportion to fluctuations in blood pressure.

In this experiment, there was a significant effect of surgery on heart rate ($p<0.01$; Figure 5.9), whereby sham-operated animals had a significantly higher heart rate on average than ovariectomised animals, regardless of maternal diet or treatment. This was accompanied by a significant interaction between surgery and time ($p<0.01$). Differences were greatest between surgical groups in hours of darkness, when the animals were awake and active (Figure 5.10 & Figure 5.11).

There was no significant effect of treatment on heart rate irrespective of surgery or maternal diet.


a.
NP Sham (n =5)
NP Ovex (n=8)
LP Sham (n =7)
LP Ovex (n =6)
Heart Rate (Beats per minute)
500
450
400
350
300
250
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (Hour)
b.
Heart Rate (Beats per minute)
500
450
400
350
300
250
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (Hour)
c.
Heart Rate (Beats per minute)
500
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0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (Hour)

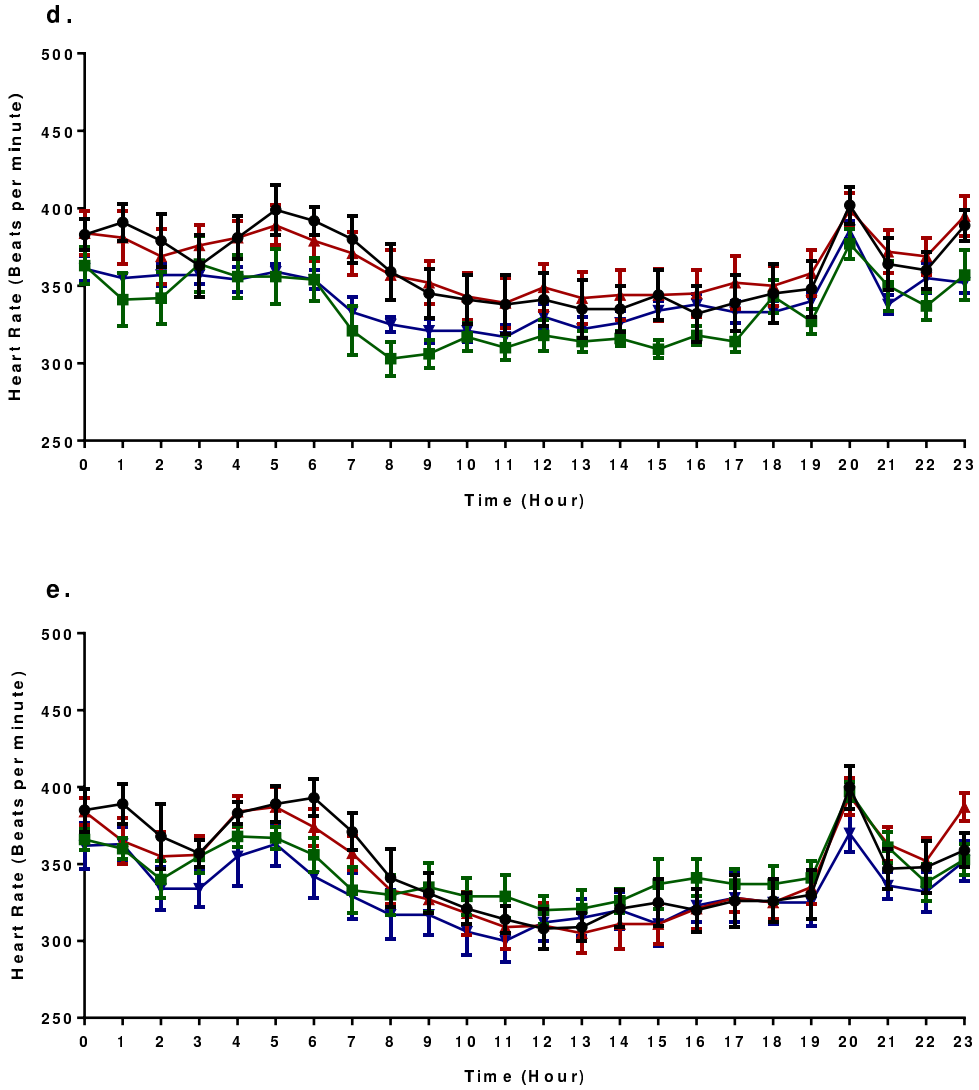

Figure 5.9: (Figure contained on pages 209 & 210) Average heart rate in beats per minute measured by radiotelemetry in sham-operated (sham) and ovariectomised (ovex) normal (NP) and low protein (LP) offspring in the following one week treatment periods: a) Baseline, b) C21, c) Washout, d) LNAME, and e) LNAME and C21. Treatments were administered for 7 days and readings averaged for each hour in a 24 hour period. Details of treatment regime can be found in Section 2.2.3 - .

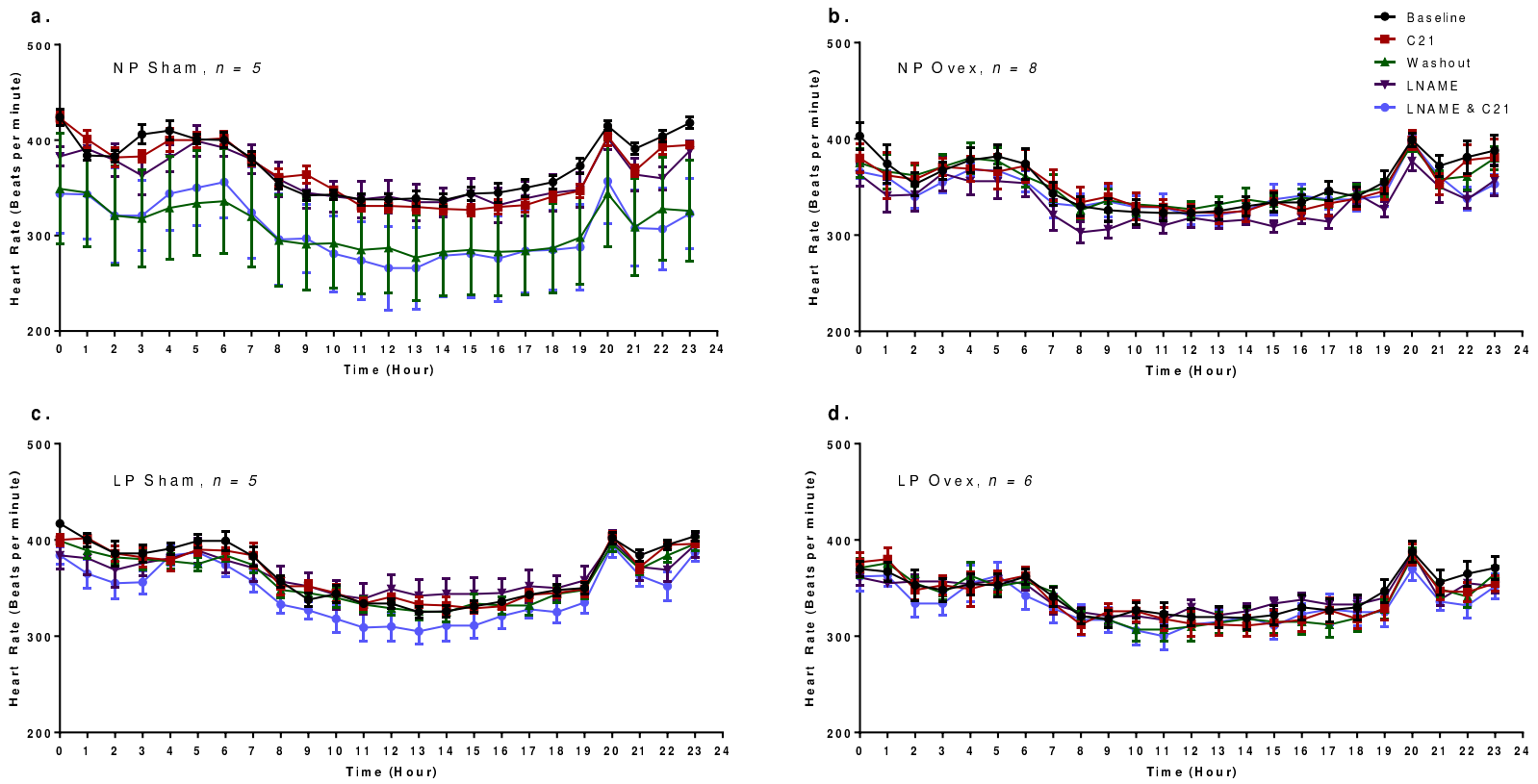

Figure 5.10: Change in heart rate in response to treatment in a) sham-operated normal protein offspring, b) ovariectomised normal protein offspring, c) sham-operated low protein offspring, and d) ovariectomised low protein offspring.

Values for heart rate were averaged for hours of daylight (Day, 08:00-20:00) and darkness (Night, 20:00-08:00) as demonstrated in Figure 5.11. On average, heart rate was significantly increased in the night ($p<0.01$), when the animals would have been awake and at their most active. This was completely unaffected by maternal diet or surgery.

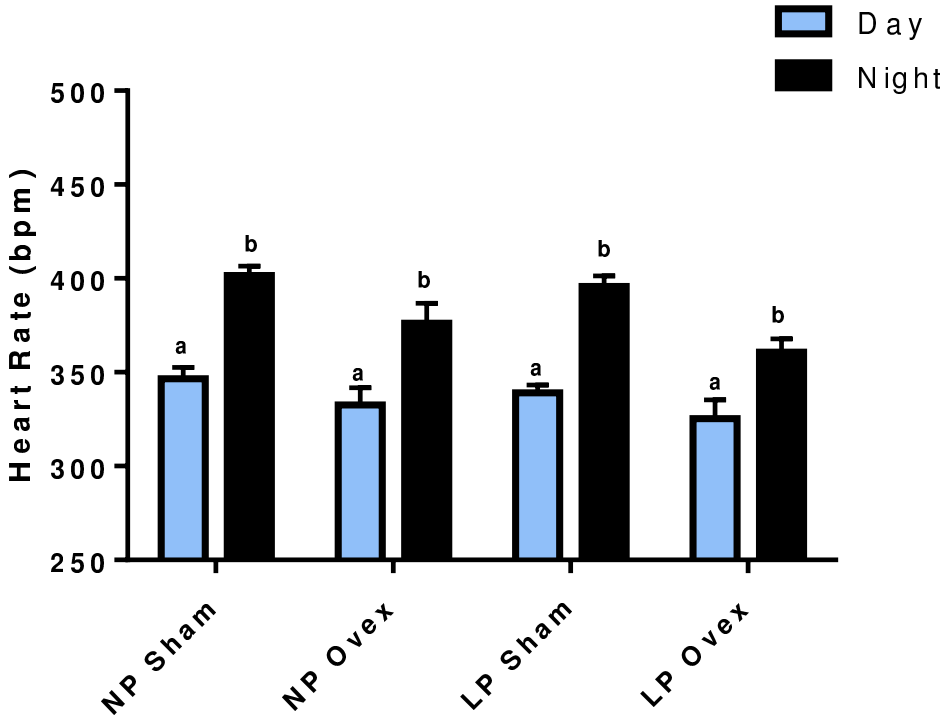

Figure 5.11: Average heart rate (beats per minute) in hours of daylight and darkness from sham-operated normal protein offspring (NP sham, $n = 5$), ovariectomised normal protein offspring (NP Ovex, $n = 8$), sham-operated low protein offspring (LP Sham, $n = 7$), and ovariectomised low protein offspring (LP Ovex, $n = 6$). Heart rate was significantly higher in hours of darkness ($p<0.01$) in all diet and surgery groups. Differing superscript letters denote significant differences between groups.

5.4.7 - Body Weight and Organ Data

Body and organ weights at cull are shown in Table 5.2 below. Body weight was significantly increased ($p<0.01$) in ovarectomised animals from both normal and low protein mothers, and this was likely due to a significant increase in peri-renal fat (g) ($p<0.01$). Neither maternal diet nor surgery had a significant effect on kidney, heart, liver, or gonadal fat depot weight. Increased food intake was observed in ovariectomised animals when compared with their sham-operated counterparts, which may contribute to the increased accumulation of fat.

	Normal Protein						Maternal Low Protein					
	Sham			Ovex			Sham			Ovex		
Body Weight (g)	303.68	±	16.86^{a†}	363.73	±	12.02^{b†}	310.00	±	6.26^{a†}	364.20	±	18.77^{b†}
Gonadal Fat (g)	7.50	±	1.50	10.64	±	0.02	7.65	±	0.05	8.69	±	0.04
Peri-Renal Fat (g)	7.07	±	1.67^{c†}	9.40	±	0.37^{d†}	5.34	±	0.38^{c†}	11.22	±	0.47^{d†}
Kidney (g)	0.93	±	0.04	0.95	±	0.04	1.01	±	0.06	0.91	±	0.05
Liver (g)	9.28	±	0.49	10.08	±	1.34	10.67	±	0.42	9.73	±	0.57
Heart (g)	1.04	±	0.07	1.15	±	1.60	1.19	±	0.28	1.11	±	2.25

Table 5.2: Body weight, fat depot weight, kidney, liver, and heart weights (g) at cull in sham-operated (sham) and ovariectomised (Ovex) offspring from mothers fed a normal (NP) or low protein (LP) diet during pregnancy. Body weight was significantly increased in Ovex animals, regardless of maternal diet ($p<0.01$). This was accompanied by a significant increase in peri-renal fat depot ($p<0.01$). Significance at $p<0.05$ denoted by differing superscript letters, † = $p<0.01$.

5.5 - Discussion

The lack of effect of both surgery and maternal diet on blood pressure responses in this trial is unexpected. In particular, the effect of removal of oestrogen has been well documented (Reckelhoff 2001) alongside its effectiveness at ameliorating increases in blood pressure following ovariectomy surgery (Mercier et al., 2002). It was hypothesised in starting this experiment that ovariectomy surgery would have a pronounced effect on systolic blood pressure, and this increase would persist for the duration of the trial. However, no overall effect of ovariectomy on blood pressure was observed. Instead, a significant interaction between maternal diet and surgery was observed. As detailed in the results section, responses to LNAME were differentially affected by surgery in normal and low protein exposed animals. Ovariectomy negatively affected blood pressure only in the low protein offspring, suggesting that there may be a programming effect of maternal diet on sensitivity of the offspring to a decrease in oestrogen.

This difference between control and low protein animals may be the result of multiple factors. The most basic potential explanation is that oestrogen levels have not been reduced, thus blunting the usual increase in blood pressure seen with ovariectomy. Although oestrogen was not measured in the animals due to technical difficulties, it is most unlikely that the surgery did not result in complete removal of ovarian tissue in some animals. During surgery the completed removal of intact ovaries was confirmed for each animal, and all rats were physically examined upon dissection for traces of ovary with none reported. This would also not explain the differential response in control and low protein animals.

It is also possible that an alternative site of steroid production is compensating for the long term reduction in oestrogen levels. A significant increase in peri-renal fat

depot size was observed in ovariectomised offspring from both control and low protein mothers. This increased volume of adipose tissue is unsurprising. It has been previously reported that reduction of oestrogen can result in an increase in visceral fat deposition and is a strong indicator that the ovariectomy surgery was successful (Wajchenberg, 2000). However, when we consider that adipose tissue is known to be a site of production for steroid hormones, oestrogen included (Siiteri, 1987; Simpson, 2003), it may be possible that this depot is acting as an oestrogen producing organ in the absence of the ovaries. There are limitations to this theory, it has been demonstrated in humans that post-menopausal synthesis of oestrogens in adipose tissue is significantly lower than production by the pre-menopausal ovary (Simpson et al., 2005). Furthermore, studies have shown that circulating oestrogens are minimal in post-menopausal women, and that ovarian steroids produced appear to act in a local, paracrine fashion (Labrie et al., 2003). In this respect, it is unlikely that the adipose tissue in these ovariectomised rats could produce enough oestrogen to mimic that of an intact animal.

Interestingly, these data suggest that oestrogen (or another ovarian steroid) is capable of reducing the impact of nitric oxide synthase inhibition, but this only occurs in animals exposed to a low protein diet in utero. The presence of ovarian steroids in offspring of NP animals appeared to increase the impact of NOS inhibition, which implies that there is regulation of eNOS by ovarian steroids. A review by Searles (2006) highlighted oestrogen as a transcription factor for eNOS, offering a potential mechanism by which the presence of oestrogen (or absence) may influence this system. However, data generated for gene expression of eNOS (Chapter 3, Section 0) in the animals in this trial does not support this theory. Indeed, a complete lack of effect was observed, suggesting that there was no effect of gonadectomy surgery on eNOS, or oestrogen specific regulation of eNOS in the mediation of blood pressure responses.

The maternal low protein model has been well-documented in previous years, and is known to have deleterious effects on offspring health and sensitivity to environmental insult (Sayer et al., 2001; Swali, McMullen, & Langley-Evans, 2010). It has been shown that the glucocorticoid receptor (GR), a steroid receptor, is susceptible to programming by maternal diet (Bertram et al., 2001). Such programming typically involves epigenetic modification of the receptor gene, and has significant influences on subsequent function and distribution of the GR (Xiong & Zhang, 2013). To the author's knowledge there is little research considering the programming of other steroid receptors, such as the oestrogen receptor, after exposure to a maternal low protein diet. However, other studies have demonstrated that the oestrogen receptor is liable to foetal programming via other gestational insults (Matsuda, 2014; Kundakovic et al., 2013). Moreover, sexual dimorphism in oestrogen receptor expression is programmed during development, and persists into adulthood (Champagne & Curley, 2008).

In Chapter Four, we observed a significant difference in the vascular responses of NP and LP offspring. One of the proposed mechanisms for this was programmed differences in G-protein coupled receptors and their signalling pathways. In this study, it is possible that a similar effect is being observed. The differential response to ovariectomy in NP and LP offspring could be the result of altered receptor expression. Oestrogens have two types of receptors in the mammalian body, the oestrogen receptors α and β (ERα and ERβ), which are member of the ligand-regulated transcription factor receptor family, and GPER, a G-protein coupled oestrogen receptor (Cheng et al., 2014). ERα and ERβ exert effects on the body via direct binding with specific DNA sequences and acting as transcription factors, whereas GPER activates numerous signalling pathways including the MAPK pathway (Pupo et al., 2016). Interestingly, the GPER has been cited as a mediator of some reno-protective effects (Cheng et al., 2014) and as one of the main

effectors of ovarian steroid non-genomic, vasodilatory actions in the vasculature (Gros et al., 2011). This has potential as an explanation for the lack of ovariectomy effect on blood pressure, and for the unusual interaction between maternal diet and surgery observed in response to LNAME treatment. Exposure to the low protein diet during gestation may result in offspring with altered basal GPER expression. This would, in theory, result in altered sensitivity to the steroid hormones in intact animals, and could also result in significantly different responses to gonadectomy surgery.

Whilst it seems a strong possibility that there is a programming effect in the LP offspring in their response to ovariectomy, it is unusual that no consistent effect has been seen in blood pressure. As discussed in Chapter 4, this has been a consistent result throughout this thesis. The low protein model has failed to programme hypertension as it has in previous studies (Langley & Jackson, 2004; McMullen et al., 2004). The possible reasons for this have been discussed in Chapter 4, however it is important to note that despite the lack of a hypertensive phenotype, a significant effect of programming is still evident.

Other studies have investigated response to LNAME with use of the MLP model. In 2002, Itoh et al. demonstrated that the effects of LNAME-induced hypertension differed between NP and LP offspring. Hypertensive response to LNAME was greater in NP offspring, and this was accompanied by a more significant inhibition of ACh-mediated relaxation in mesenteric arteries in the NP offspring. The data led the authors to conclude that there was an inherent difference in the endothelium of offspring exposed to the LP diet during gestation, and that LP offspring responses to nitric oxide were significantly poorer to those of NP offspring. A similar effect was reported by Sathishkumar et al., (2009) who also showed NP offspring to show a greater hypertensive response to LNAME. This study did not test the NO-mediated

response in vessels of the offspring, and so we cannot conclude that it was affected by maternal diet in this instance. However, this in combination with the proposed differences in oestrogen receptor activity, may offer a potential explanation of the interaction observed between maternal diet and ovariectomy surgery in these animals. Further work would be required to study this mechanism properly.

The final question laid out in the objectives of this trial was whether the nitric oxide system could be manipulated through use of Compound 21 to positively affect blood pressure in the presence of accelerated renal ageing and ovariectomy. The data suggested that this is not the case. Blood pressure was not affected by dosing with Compound 21 in both normotensive and LNAME-induced hypertensive animals. Compound 21 is a highly selective angiotensin II type 2 receptor agonist that is believed to have beneficial effects on blood pressure (Wan et al., 2004), and may provide an alternative/addition to current treatments. Data regarding the effectiveness of Compound 21 is conflicting. Studies have shown it is capable of eliciting a vasorelaxation response *in vitro* (Bosnyak et al., 2010), but *in vivo* results have been variable. Work in this laboratory, as described in Chapter Four, established that long-term, low dose administration of C21 was not effective at reducing blood pressure. Previous C21 work has utilised fairly short-term treatment both *in vivo* and *in vitro* to observe effects (Hilliard et al.. 2012; Verdonk et al., 2012; Hilliard et al., 2014; Bosnyak et al., 2010), and so an acute treatment period was included in this study to see if any of the results were replicated. As expressed in the results, Compound 21 did not effectively reduce blood pressure, nor was there any tendency for this. Without a hypertensive phenotype, it is difficult to speculate on the ability of C21 to reduce basal blood pressure. The initial treatment period may have elicited different results if LP offspring had had elevated blood pressure, as in previous studies. Compound 21 was also unable to bring about a reduction in blood pressure after induction of hypertension using LNAME. Additional work is

required to establish whether or not this is due to an inability of C21 to elevate nitric oxide levels, or due to an inability of nitric oxide to mediate a reduction in BP in light of a severe hypertensive challenge.

5.6 - Conclusions

Whilst work to date on this project cannot offer a complete picture, it is clear from the current evidence that the interaction between oestrogen and blood pressure is a complex one, and cannot be explained by considering one system alone. Data collected suggests that Compound 21 is not an effective means of modulating blood pressure, though the detailed mechanisms in action and the reasons behind this need further work. Moreover, a maternal low protein diet during gestation adds a further layer of complexity. It may be that the MLP model induces permanent changes in the vasculature of offspring that result in decreased NO-mediated vascular relaxation, and altered oestrogen receptor expression. If and how these two factors work together is unclear, further work is needed to fully elucidate the mechanisms behind this complex interaction.

Chapter 6 - Discussion

6.1 - Introduction

It has been well established that pre-menopausal females are relatively protected from renal injury and cardiovascular disease when compared with age-matched males (Sandberg & Ji, 2012). The mechanisms behind this facet of renal ageing are not clearly defined. Evidence has shown that males and females have significantly different nitric oxide bioavailability, and this fluctuates greatly with age (Erdely et al., 2003; Baylis, 2005). Moreover, endothelial nitric oxide synthase, the enzyme responsible for the endogenous production of NO, is regulated by oestrogen (Duckles & Miller, 2010), making it a viable candidate for involvement in regulating the protective effect of female sex.

A significant body of research has been published detailing sex-specific expression of the renin-angiotensin system. It has been shown that the angiotensin II type 2 receptor is upregulated in female animals when compared to their male counterparts (McMullen et al., 2004; Okumura at al., 2005). In addition, oestrogen down regulates the angiotensin type 1 receptor that is responsible for the majority of presser effects of angiotensin and is widely acknowledged to play a role in mediating renal injury and hypertension when overexpressed (Fischer et al., 2002).

In an ageing population, improving our understanding of the mechanisms by which kidney function deteriorates over the lifecourse may be essential to successfully managing renal health (Zhou et al., 2008).

6.2 - Summary of Findings

The work in this thesis was compiled to establish some of the mechanisms underpinning sexual dimorphism in renal ageing and whether or not age-related decline in function was associated with the nitric oxide and renin-angiotensin systems.

At the start of the work, we hypothesised that ageing would result in declining renal function and increased blood pressure. These changes would be significantly affected by the presence or absence of sex hormones, and negative effects would be exacerbated by exposure to a low protein diet during gestation. We suggested that these effects would be mediated, at least in part, by RAS-regulated NO release.

6.2.1 - The Effects of Sex Steroids on Renal Function with Age

Previous studies have argued clear cut roles for both oestrogens and androgens in mediating or protecting against renal decline and associated health problems (Sasser et al., 2015; Sainz et al., 2003; Maranon & Reckelhoff, 2013). This study hypothesised that protective effects on renal function would be linked to the presence of oestrogen, and that deleterious effects would be ameliorated by the removal of androgens. The results generated suggest a mildly protective effect of ovarian steroids, with intact females preserving renal function better than their ovariectomised counterparts and male animals. Contrary to other literature, there was no significant protection conferred by castration in males (Reckelhoff et al., 1999; Hayward et al., 2001). To some extent this shows consistency with cohort studies in humans that have shown that males with the lowest testosterone have the highest cardiovascular and renal risk (Liu et al., 2003). Whilst it was not possible to measure androgen concentrations in this study, that androgens have a role to play in this phenotype is a theory we should consider.

6.2.2 - Manipulating the AT_2R – A Potential Therapeutic?

The AT_2R has received significant research attention in the past decade due to its potential to downregulate and counteract the pressor effects of the AT_1R (Savoia & Volpe, 2014). This project utilised the recently developed AT_2R agonist, C21 to attempt to increase production of NO. To our knowledge, there are very few studies considering the effects of long-term dosing with C21, making the experiments in Chapter Four particularly novel. These experiments demonstrated that long-term, low dose administration was not effective at changing blood pressure in normotensive rats. We also showed that C21 was not able to reduce LNAME-induced hypertension. This is in agreement with more recent work regarding C21 that has also shown it does not consistently reduce blood pressure *in vivo* despite demonstrating vasodilatory effects *in vitro* (Danyel et al., 2014). The majority of work with C21 to date has been performed using direct vascular infusions of the drug doses (Brouwers et al., 2015), and whilst these have been effective at producing beneficial effects in an experimental setting, they are not representative of a real world application of C21.

Some recent studies have shown that C21 can prevent increases in blood pressure *in vivo*. For example, Ali et al., (2015) found that oral C21 administration prevented BP increases in animals fed a high salt diet. The dose in this study was significantly lower than the dose published in this experiment, which may be the reason for the discrepancy. Alternatively, it may be that the effects of C21 on blood pressure are not evident in the absence of a strongly hypertensive phenotype.

Importantly, whilst the AT_2R remains a potentially beneficial target of the RAS for therapeutic intervention, the work in this thesis suggests that it will be best used in

conjunction with other treatments for blood pressure rather than as a standalone measure.

6.2.3 - Exposure to Low Protein During Gestation has Long Lasting Effects on the Vasculature of Offspring.

This thesis utilised the well documented 'maternal low protein' model of foetal programming. Unlike previous work, the model did not induce hypertension in the offspring of mothers fed a low protein diet during pregnancy (Langley & Jackson, 1994; McMullen et al., 2004). However, there were other significant effects of programming. In particular, Chapter Four demonstrated a significant difference between NP and LP offspring in their response to treatment with C21, Losartan, or a mixture of the two with regards to vasodilatory responses to acetylcholine. Vascular reactivity was substantially reduced in NP offspring in contrast to the LP offspring, who maintained full responses throughout. Similarly, in Chapter Five, whilst no overall difference in blood pressure was observed in NP and LP offspring, response to treatment with the NOS inhibitor LNAME and ovariectomy surgery were significantly different between NP and LP offspring.

In both instances, these effects could be explained by programmed differences in vascular receptors. Past studies have shown differing responses in NP and LP endothelial function, and have concluded that the mechanisms by which these animals regulate vasodilation are significantly different (Itoh et al., 2002; Sathishkumar et al., 2009). The work generated in this thesis supports this argument. Moreover, we propose that this difference may be specifically mediated by programmed differences in vascular receptors. The MLP model has been shown to result in altered receptor expression before (McMullen et al., 2005; Bertram et al., 2001; Xiong & Zhang, 2013). In this study we cannot identify a single receptor

responsible for these effects, though there are potential candidates. It is the author's opinion that GPCRs, specifically GPER and the GPCR subunit, Gα, are worthy candidates for future research. GPER is an oestrogen receptor that has been shown to activate MAPK signalling (Pupo et al., 2016). It has also been shown to bind aldosterone, a key hormone in activation of the renin-angiotensin system (Gros et al., 2011). Both of these features would offer some explanation for the ovarian steroid-specific changes in response observed in LP offspring in their response to inhibition of NOS. Increased binding of aldosterone to GPER in the absence of oestrogens may result in increases in BP, and this effect may be more pronounced in the LP offspring depending on the receptor expression and distribution. The GPCR subunit Gα is activated by both angiotensin II and acetylcholine, and is capable of eliciting both vasoconstrictor and vasodilator responses (Brinks & Eckhart, 2010). Exposure to maternal undernutrition may result in either programmed differences in receptor distribution, or possibly differences in receptor activation. The scope of this project did not cover such work, but it would be an interesting future direction.

6.2.4 - Changes in Nitric Oxide Expression and Distribution

This project hypothesised that the protective effects of female gender were being mediated by the nitric oxide system. Numerous studies have demonstrated that female animals and humans have higher NO activity and bioavailability than males (Baylis, 2005; Baylis, 2012; Erdely et al., 2003). Furthermore, eNOS, an enzyme responsible for the endogenous synthesis of NO, can be transcriptionally and post-transcriptionally regulated by oestrogen, making it a potential mediator for sex-specific effects.

The hypotheses were not supported by the data obtained. No changes were observed in eNOS expression, and changes in NO metabolites were not linked to markers of renal function. The data in Chapter Three suggested instead that the animals with the best preserved renal function demonstrated the least change in NO metabolites. These results infer that aberrant fluctuations in the nitric system are more likely contributors to renal decline than a direct effect of NO concentration.

This is particularly pertinent when we consider the phenomena of blood pressure variability. On a day-to-day basis, blood pressure is constantly in flux (Mancia et al., 1983). Typically, the body deals with these BP fluctuations by utilising parasympathetic and sympathetic signalling to cause changes in heart rate and vascular tone (Stauss et al., 2000). Studies have demonstrated that excessive daily variability in blood pressure is an important risk factor in cardiovascular disease (Floras, 2013). Stauss et al. (2000) showed that NO may be involved in the short term stabilising of blood pressure, to act alongside or in place of the normal baroreceptor reflex. This would add credence to the suggestion that NO fluctuations may be more significant than NO concentration alone, but further work would be required to fully understand this.

6.3 - Limitations of the Study

As with any scientific experiment, there were limitations to this study. The original hypotheses set out to measure elements of the nitric oxide system. Of the intended measures, only two were completed, mRNA expression of endothelial nitric oxide synthase, and urinary nitrites. Attempts were made to measure protein expression of NOS, using western blotting and immunofluorescence. However, results obtained were inconsistent, unrepeatable, and often did not work at all, casting doubt upon the reliability of the measure as a whole. Whilst mRNA expression was successfully

measured, this does not give us an indication of the levels of protein being successfully translated.

Additionally, attempts were made to measure plasma nitrite. In this case, the level of each of these metabolites was too low to measure, and thus readings could not be obtained. It has been established that nitrites are unstable in whole blood (Nagababu & Rifkind, 2010). When samples were collected for these trials they were held on ice until centrifuging, rather than immediately processed. This short delay between blood collection and separation of plasma may have been a contributing factor to these readings too low to measure.

Facilities were not available to measure nitrates or nitrothiosols in plasma and urine, and this may also be a significant limitation of this element of the study. In attempting to study nitric oxide in biological fluids, the most accurate method is to measure nitrites, nitrates, and nitrothiosols (Baylis & Corman, 2005). Without these additional measures, the data generated regarding the nitric oxide system must be analysed with caution. Additionally, other techniques are available to measure components of the nitric oxide system. For example, Luminex assays are a comprehensive system for measuring changes in numerous metabolites simultaneously. Unlike other assays, Luminex relies on the use of pre-coated antibody plates (much like ELISA) and so offers increased specificity in the procedure. Inclusion of such data would have greatly enhanced any conclusions made regarding circulating NO.

In addition, the two other nitric oxide synthases, neuronal NOS and inducible NOS, have both been implicated in the function of the cardiovascular system in both healthy and unhealthy adults (Forstermann & Sessa, 2012; Zhang et al., 2014; Zhao et al., 2014). Neglecting to measure these components of the NO system may have left gaps in our understanding of the actions of nitric oxide and its distribution in

renal ageing. This is particularly important as no changes at all were seen in endothelial nitric oxide synthase, refuting the hypothesis made at the start of this project that eNOS and oestrogen are closely linked in the regulation of renal ageing. Many datasets in both humans and animals demonstrate declines in the NO system associated with high blood pressure and age (Erderly et al., 2003; Baylis, 2005). It is possible that the measurements employed within this PhD were simply not comprehensive enough to fully elucidate some of the mechanisms involved in this phenomenon.

An unusual finding in this thesis was the lack of a hypertensive phenotype in offspring of low protein mothers, in contrast to the majority of literature regarding this model (Habib et al., 2011; Satishkumar et al., 2012; McMullen et al, 2004). Whilst other programming effects were still evident, suggesting the model was working, the lack of a hypertensive phenotype in each of the animal trials did limit conclusions somewhat. For example, studying the AT_2R agonist C21 and its potential as a therapeutic for hypertension was inherently restricted by the absence of elevated blood pressure. Whilst we were still able to observe the effects of C21, and to conclude it does not reduce blood pressure in a normotensive animal, confirmation that this was also the case in hypertensive animals would have been preferable. Utilisation of an alternative model such as the spontaneously hypertensive rat (SHR) may have proven beneficial in acquiring this hypertensive phenotype, however caution should be applied in stating the relevance of genetic models such as this. For example, Loria et al. (2014) demonstrated that SHR present with altered vascular responses to other models of rodent hypertension. In selecting an alternative means of inducing hypertension it would be essential to compare against a more physiologically representative control.

Aside from this, previous studies performed in this laboratory that reported a 'low protein phenotype' were performed in an alternative animal facility, with differing tail

cuff devices, and utilising conventional open top cages. Since the relocation from those premises, the upgrade of the blood pressure measuring equipment, and change of caging to enclosed, air-filtered habitats, four studies (including the three presented within this thesis and Swali et al., 2010) have failed to progamme blood pressure changes in offspring. It is possible that previous observations were influenced by the facilities utilised, and that some of the differences were an artefact of the equipment.

Furthermore, the animals used in this study were the first in our laboratory to be housed in enclosed, air-filtered caging for a maternal low protein study. It is possible that changing from an open-top cage system to an enclosed unit may have resulted in significant changes in the microbiome of the animals in the study. Indeed, research argues that the environment of an animal can override some of the genetically determined elements of the microbiota (Lees et al., 2014). Evidence is emerging that suggests a significant role for the microbiota in the gut in the modulation of numerous physiological processes (Sommer & Bäckhed, 2013). Of particular interest is the work by Heijtz et al. (2011) and Manco et al. (2012), whose studies effectively demonstrate that the microbiome of the pregnant mother can significantly impact development of the foetal brain. Whilst there is no evidence to date considering the detailed effects of the microbiome on other developmental pathways, it is possible that a change such as that in the environmental conditions observed here may have drastically altered the phenotypic response seen in this model. This possibility creates additional concern regarding the strength of the animal model adopted, as should the effects of programming not be robust enough to withstand such environmental modulation, their application in humans is inherently limited. However, this hypothesis would need to be thoroughly tested before any conclusions regarding the effects of the maternal microbiome on offspring outcomes could be made.

There are always limitations associated with animal work, and this thesis is no exception. The most frequently highlighted problem with rats as a model for human disease is the intrinsic physiological differences between rodents and man (Lerman et al., 2005). Many animal models of accelerated renal ageing are reliant on significant physical injury such as the two kidney one clip (2K1C). Often, such models induce hypertension rapidly and in a fashion dissimilar to the development of high blood pressure in humans (Grossman, 2010). In contrast the MLP model is a mild, physiological challenge that progressively worsens with time, which may provide a closer simulation of human hypertension. Whilst this work could not be taken as anything other than a 'proof of principle' study, the model adopted was very appropriate in principle.

One of the main aims of this project was to understand some of the nuances of renal ageing. Whilst some methods were adopted to measure renal filtration and injury, there are a host of other tests that may have been employed to fully characterise the stage of ageing the animals within these trials were at. For example, measurement of inulin clearance, glomerular sclerosis, albuminuria, cystatin c, renal flow rate, and comparison of plasma and urinary osmolality could have been highly informative. This is particularly pertinent owing to the lack of overt programming and ageing phenotypes observed inother parameters.

In considering ageing, it is also important to note that these animals were, at their oldest, only close to middle age. The lifespan of a Wistar rat is 2-3 years, and thus age-related changes may have been more significant had these animals been allowed to age to natural death. Previous studies with aged animals have demonstrated significantly decreased survival rates to 18 months than presented in this bosy of work (Clifford et al., 2013) suggesting that this was a healthy cohort of animals that may not have presented with overt ageing responses until significantly later in life. Moreover, previous studies have documented numerous secondary

observations at cull such as tumours, enlarged spleens, and liver sclerosis. No such observations were made in the culls of these animals, further adding to the suggestion that they were a healthy cohort that required a longer ageing challenge.

Finally, a large portion of the study revolved around the effects of sex steroids on our chosen outcomes. Attempts were made to measure oestrogen, oestradiol, and testosterone via enzyme-linked immunosorbent assay (ELISA). Despite numerous attempts, no kits could be optimised to work in the plasma samples available. In each instance, the sensitivity of the ELISA was not adequate, and could not detect sex steroids in the samples. This is a significant drawback in the analysis of the effects of sex steroids in this study. However, all other measures relating to the success of the gonadectomy surgery suggested that the procedures had been successful. In all cases, rats were inspected both during surgery and at cull for traces of gonadal tissue, and none were found. Moreover, gonadectomised animals (both male and female) presented with significantly increased body weight as a result of an increase in adipose tissue, an indicator that the surgery was successful at reducing circulating sex steroids (Wachenberg, 2000).

6.4 - Further Work

This thesis has raised many questions surrounding the sexual dimorphism observed in renal function and cardiovascular disease. Where previously it was hypothesised that the nitric oxide system would be a key regulator in these processes, the data obtained does not support this. Whilst the NO system may play a role in accelerated renal age and cardiovascular problems, it is not likely working alone.

Despite the inconclusive findings regarding a mechanism for sex-specific differences in physiological response, it is clear that this phenomenon still requires significant research attention. It is suggested that in order to truly identify

mechanistic drivers behind sexual dimorphism in renal ageing, an approach starting with clarifying gene and protein expression in age will offer a powerful means of identifying potential targets for whole body physiology studies such as those contained within this thesis. Ageing is a complex process, and as such characterising the profile of renal age would offer a means of managing kidney function in an older population more effectively.

Studies such as that by Amelina & Cristobal (2009) demonstrate effectively that measurement of the proteome can identify sex-specific, age-related changes in protein expression and associated modifications in the kidney. An experiment encompassing whole proteome analysis, coupled with whole transcriptome analysis could offer a profile of renal ageing that could then be subjected to further manipulation. Moreover, a complex study design, such as that used within this thesis, would benefit from a streamlined approach to analysis. Labelling a 'typical' protein and gene profile with age would allow for observations of change in said profile with sex steroid modulation.

6.5 - Concluding Remarks

Renal ageing, hypertension, and vascular dysfunction are significant health concerns. The distinct sexual dimorphism observed in the progression and presentation of these conditions is not fully understood, but has a strong impact on our future treatment and management of them in an ageing population. Data in this thesis suggests that the sex-specific effects on the renal and cardiovascular systems are mediated by complex interactions of ovarian steroids and receptors in the vasculature. However, these effects are not mediated by concentrations of nitric oxide alone, instead the results suggest that such conditions are an artefact of abnormal fluctuations in the nitric oxide system. Moreover, there is a clear effect of

foetal programming on the vasculature, which may go some way to explaining the tremendous variations observed in vascular function with age.

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