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The effect of a maternal low protein diet on renal
development and function in the offspring

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Thesis submitted to the University of Nottingham for the degree of
Doctor of Philosophy

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Abstract

A poor maternal diet leads to offspring with a greater risk of developing chronic diseases later in life. This thesis considered whether a low protein diet during pregnancy in sheep affected the development of the fetal kidney, and how this impacted upon adult renal function when challenged by obesity. Pregnant ewes were fed either a control diet or a diet that was isocaloric but contained only 50% of the protein, in either early or late gestation. The effects of the diet were assessed on the ewe, day 65 fetuses (0.44 gestation), and two year old offspring which had been subjected to an obesogenic environment (*ab libitum* feed and reduced exercise). Few effects were observed on the ewe, confirming that the nutritional insult was relatively mild. Fetal renal vasculature (assessed by vascular corrosion casts) was not different between groups, although the microvasculature was significantly reduced in the early protein group, as evidenced by CD34+ staining of endothelial cells. This was accompanied by a reduction in angiogenic factors compared to control animals. Protein-energy malnutrition in the ewe led to reduced urea in maternal and fetal plasma, along with a concomitant reduction in ornithine in the fetal plasma and amniotic fluid. Other amino acids were relatively unaffected. In the adult sheep there were no effects on long-term renal function in the group fed low protein late in pregnancy, despite the lambs having lower birth weights and a period of postnatal catch up growth compared to the other groups. However, the group fed low protein during early pregnancy had reduced nephron number, microalbumuria and reduced renal function as assessed by gamma scintigraphy. There was also evidence of microvascular rarefaction which may have been exacerbated by obesity. This study did not reveal any consistent sex-specific effects of the maternal low protein diet. This study emphasises the importance of diet quality rather than quantity, and the importance of consuming a well-balanced diet during pregnancy to protect against future chronic diseases.

Publications and Presentations

Publication

Lloyd L. J., Foster T., Rhodes P., Rhind S. M. & Gardner D. S. 2012. Protein-energy malnutrition during early gestation in sheep blunts fetal renal vascular and nephron development and compromises adult renal function. *Journal of Physiology-London*, 590, 377-393.

Oral presentations

Lloyd L.J., Gardner D.S. The effect of a maternal low protein diet on renal development and function in the offspring, 15th Nephrogenesis Workshop, London, May 2011

Lloyd L.J., Rhodes P., Rhind S., Gardner D.S. *The effect of a maternal low protein diet on renal development and function in the offspring*, Society for Endocrinology BES, Birmingham, April 2011

Lloyd L.J. The effect of a maternal low protein diet on the development and function of the kidney in the offspring. Cross-Disciplinary Showcase, University of Nottingham July 2010

Poster presentations

Lloyd L.J., Sinclair K.D., Gardner D.S. *Impact of a maternal low protein diet during pregnancy on fetal amino acid availability and kidney development in sheep*. Physiological Society Meeting, Edinburgh, July 2012

Lloyd L.J., Gardner D.S. *You Are What Your Mother Eats*. SET for BRITAIN Final, Houses of Parliament, London, March 2011

Lloyd L.J. *You Are What Your Mother Eats*. Vitae Midlands Hub Regional Poster Final, Nottingham July 2010

Lloyd L.J. *You Are What Your Mother Eats*. Research Showcase 2010, University of Nottingham, May 2010

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Date

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Looking to broaden my horizons outside of the School of Biosciences, the following summer I moved about a hundred metres up the road to the Vet School to do another summer studentship, this time with Dr David Gardner. Having road tested each other, we reunited a year or so later after I finished my undergraduate degree to start my PhD. And I've been here ever since.

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Table of Contents

Abstract.....	i
Publications and Presentations	ii
Declaration.....	iii
Acknowledgements.....	iv
Table of Contents	v
List of Figures	xiv
List of Tables	xvi
Abbreviations.....	xvii
1. Introduction.....	1
1.1 Health and Disease	1
1.2 Kidney Disease	2
1.3 Health and Diet.....	5
1.3.1 Monogastric vs Ruminant Digestion	7
1.4 Mammalian kidney development	8
1.4.1 Early embryonic development.....	8
1.4.2 Nephrogenesis.....	9
1.4.3 Vascular development.....	14
1.4.4 Molecular control of development.....	15
1.5 Kidney physiology	19
1.6 Developmental Origins of Health and Disease	21

1.6.1	Epidemiological Evidence for DOHaD	22
1.6.1.1	Limitations of epidemiological studies	23
1.6.2	Animal models.....	24
1.6.2.1	Surgical models.....	27
1.6.2.2	Glucocorticoid treatment	28
1.6.2.3	Nutritional models.....	29
1.6.2.3.1	Global Nutrient Restriction	29
1.6.2.3.2	Low protein.....	31
1.6.2.3.3	Other diets	32
1.7	Mechanisms of programming	37
1.7.1	Hormones and growth factors.....	37
1.7.1.1	Angiogenic factors	37
1.7.1.2	Glucocorticoids.....	39
1.7.2	Renin-angiotensin system	40
1.7.3	Inflammatory factors.....	40
1.7.4	Apoptosis.....	41
1.7.5	Epigenetics	41
1.8	Aims and Hypotheses.....	42
1.8.1	Aims.....	42
1.8.2	Hypotheses.....	43
2.	Methods and Materials	44
2.1	Summary.....	44

2.2	Study Design	45
2.2.1	Study 1.....	46
2.2.1.1	Macauley Institute, Aberdeen: 0 – 1.5 years of age	46
2.2.1.2	University of Nottingham, Sutton Bonington: 1.5 – 2 years of age.....	47
2.2.2	Study 2.....	49
2.3	Blood and Urine Analysis	51
2.3.1	Urine Analysis	51
2.3.1.1	Albumin	51
2.3.1.2	Creatinine	51
2.3.2	Plasma Analysis: Radox Imola.....	51
2.3.2.1	Sodium.....	52
2.3.2.2	Potassium	52
2.3.2.3	Chloride	52
2.3.2.4	Glucose	52
2.3.2.5	NEFA.....	53
2.3.2.6	Triglycerides	53
2.3.2.7	Albumin	53
2.3.2.8	Total protein	53
2.3.2.9	Urea	54
2.3.2.10	Lactate	54
2.3.2.11	D-3-hydroxybutyrate	54
2.3.2.12	Quality control.....	54

2.3.3	Plasma Analysis: Amino acids	55
2.3.3.1	Amino acid extraction.....	55
2.3.3.2	Gas Chromatography – Mass Spectrometry	55
2.3.4	Amniotic Fluid: Amino Acids.....	56
2.4	Gamma Scintigraphy	56
2.5	Histology	57
2.5.1	Sample preparation	57
2.5.2	Haematoxylin and Eosin Staining	58
2.5.3	Periodic Acid Schiff’s Reagent Staining	59
2.5.4	Trichrome Staining	59
2.6	Immunohistochemistry.....	60
2.6.1	VECTASTAIN Elite ABC System	60
2.6.2	BondMax System.....	61
2.6.3	Vascular Endothelial Growth Factor A.....	62
2.6.4	CD31 and CD34.....	62
2.7	TUNEL Staining.....	63
2.8	Lectin Staining.....	64
2.8.1	Optimisation.....	65
2.9	Image Analysis	66
2.9.1	Quantification of glomerular area	66
2.9.2	Quantification of Immunohistochemical staining.....	67
2.9.3	Quantification of TUNEL positive cells.....	67

2.10	Stereology.....	68
2.10.1	Random systematic sampling.....	68
2.10.2	Cortex:medulla ratio.....	70
2.10.3	Nephron Number	70
2.11	Estimation of mammalian nephron number.....	73
2.12	Quantitative PCR.....	73
2.12.1	RNA Extraction	73
2.12.1.1	Study 1.....	73
2.12.1.2	Study 2.....	74
2.12.1.3	Quality Control	75
2.12.2	CDNA Synthesis	75
2.12.3	Primer Design	76
2.12.4	QPCR.....	77
2.12.4.1	Study 1.....	77
2.12.4.2	Study 2.....	79
2.12.5	Housekeeping genes.....	80
2.12.6	Quality Control	81
2.13	Corrosion Casting.....	81
2.13.1	Resin casting.....	81
2.13.2	Maceration	81
2.13.3	Micro CT Analysis	82
2.14	Osmolality.....	82

2.15	Statistics	83
2.15.1	Power calculations	83
2.15.2	Distribution.....	85
2.15.3	Univariate Analysis	85
2.15.4	Multivariate analysis	86
3.	Study 1: The effect of a maternal low protein diet on the ewe and her fetus	87
3.1	Introduction	87
3.2	Materials and Methods.....	90
3.2.1	Study Design.....	90
3.2.2	Renal histology	91
3.2.3	Renal immunohistochemistry.....	91
3.2.4	Detection of apoptosis	92
3.2.5	Quantitative PCR	92
3.2.6	Statistical Analysis	92
3.3	Results	93
3.3.1	Maternal weight and body condition score.....	93
3.3.2	Fetal organ weights	95
3.3.3	Fetal kidney apoptosis.....	96
3.3.4	Vascular Endothelial Growth Factor	98
3.3.5	Other genes involved in the angiogenesis pathway	98
3.4	Discussion	100
4.	Study 1: The effect of a maternal low protein diet on the offspring	107

4.1	Introduction	107
4.2	Materials and Methods.....	111
4.2.1	Study Design.....	111
4.2.2	Urine and plasma analysis	112
4.2.3	Renal function	112
4.2.4	Histology.....	112
4.2.5	Mean glomerular area.....	112
4.2.6	Stereological estimation	113
4.2.7	Estimation of mammalian nephron number	113
4.2.8	Lectin staining.....	113
4.2.9	Renal immunohistochemistry.....	113
4.2.9.1	VEGFA.....	113
4.2.9.2	CD31 and CD34.....	113
4.2.10	Quantitative PCR	114
4.2.11	Statistical Analysis	114
4.3	Results	115
4.3.1	Maternal characteristics.....	115
4.3.2	Offspring: weights and growth rates	115
4.3.3	Post mortem data.....	119
4.3.4	Urine and blood analysis	121
4.3.4.1	Sodium, potassium, chloride	121
4.3.4.2	Albumin:creatinine ratio.....	121

4.3.5	Renal function	124
4.3.6	Adult renal structure	127
4.3.7	Endoplasmic reticulum stress	129
4.3.8	Mean glomerular area	130
4.3.9	Stereology	131
4.3.10	Estimation of mammalian nephron number	133
4.3.11	Lectin staining	135
4.3.12	CD31 and CD34	138
4.3.12.1	CD31 ab32457 and ab83959 and CD34 ab63985	138
4.3.12.2	CD34 ab81289	140
4.3.13	Vascular Endothelial Growth Factor	141
4.4	Discussion	143
5.	Study 2: The effect of a maternal low protein diet on the ewe and her fetus	150
5.1	Introduction	150
5.2	Materials and Methods	152
5.2.1	Study Design	152
5.2.2	Corrosion casting	152
5.2.3	Plasma analysis: Randox Imola	153
5.2.4	Plasma analysis: Osmolality	153
5.2.5	Plasma analysis: Amino Acids	153
5.2.6	Amniotic fluid analysis: Amino acids	154
5.2.7	Renal immunohistochemistry	154

5.2.7.1	VEGFA.....	154
5.2.7.2	CD34.....	154
5.2.8	Quantitative PCR.....	154
5.2.9	Statistical Analysis.....	155
5.3	Results.....	155
5.3.1	Maternal characteristics.....	155
5.3.2	Post mortem data at day 65.....	157
5.3.3	Corrosion casts.....	159
5.3.4	Immunohistochemistry: CD34.....	161
5.3.5	Angiogenic factors.....	163
5.3.6	Metabolites and protein.....	166
5.3.7	Osmolality.....	170
5.3.8	Amino acid analysis.....	170
5.3.8.1	Maternal plasma.....	170
5.3.8.2	Fetal plasma.....	173
5.3.8.3	Amniotic fluid.....	175
5.3.8.4	Ornithine.....	175
5.4	Discussion.....	178
6.	Final Discussion.....	187
7.	Bibliography.....	193
8.	Appendix A: Lloyd <i>et al.</i> , 2012.....	225

List of Figures

Figure 1.1: Early stages of development of the embryo.....	9
Figure 1.2: Nephron development.	12
Figure 1.3: Fetal sheep kidney at day 65 gestation	13
Figure 1.4: Diagram of a renal corpuscle.....	20
Figure 2.1: Experimental design for Study 1.....	48
Figure 2.2: Experimental design for Study 2.....	50
Figure 2.3: Quantification of 2D glomerular area on Image Pro MC 6.0	66
Figure 2.4: Random, systematic sampling method	69
Figure 2.5: Stereological counting method	72
Figure 2.6: Example of a melt curve	80
Figure 2.7: Distribution and residual checking	84
Figure 3.1: Maternal weight and body condition score of ewes during pregnancy.	94
Figure 3.2: TUNEL positive staining and quantification in fetal tissue at day 65 gestation ...	96
Figure 3.3: VEGFA and receptors gene and protein expression in fetal tissue at day 65 gestation	97
Figure 3.4: Gene expression of angiogenic factors in fetal tissue at day 65 gestation	99
Figure 4.1: Schematic graphs of glomerular filtration rate (GFR) during lifetime	110
Figure 4.2: Weight of offspring at birth, weaning, 18 months and 2 years of age.	116
Figure 4.3: Weight gain of offspring during obesogenic period	117
Figure 4.4: Growth rates of offspring	118
Figure 4.5: Sodium, potassium and chloride serum concentrations	123
Figure 4.6: Gamma scintigraphy renogram results	125
Figure 4.7: Histological staining of adult kidney sections at 2 years of age	128
Figure 4.8: Mean glomerular area of two year old adult kidneys.....	130
Figure 4.9: Nephron number, total and mean glomerular volume	132

Figure 4.10: The relationship between mass and nephron number in mammals	134
Figure 4.11: Staining with Griffonia (Bandeiraea) simplifolia lectin 1 isolectin B4	137
Figure 4.12: Immunohistochemistry for endothelial cell markers	139
Figure 4.13: Immunohistochemistry for CD34 protein.....	141
Figure 4.14: VEGFA mRNA and protein expression in the adult kidney	142
Figure 5.1: Weight and body condition score of ewes prior to, and during early gestation	156
Figure 5.2: Micro CT pictures of fetal kidneys at day 65 gestation.....	159
Figure 5.3: Weight, volume and surface area of cast fetal kidneys at day 65 gestation.....	160
Figure 5.4: CD34 staining in day 65 fetal kidneys.....	162
Figure 5.5: MRNA of angiogenic factors in day 65 fetuses	164
Figure 5.6: : Vascular Endothelial Growth Factor immunohistochemical staining	165
Figure 5.7: Metabolites in maternal plasma	167
Figure 5.8: Total protein, albumin and urea in maternal plasma	168
Figure 5.9: : Urea in amniotic fluid and lactate and glucose in fetal plasma at day 65 gestation	169
Figure 5.10: Osmolality of maternal plasma and fetal amniotic fluid.....	171
Figure 5.11: Discriminant analysis of standard amino acids in fetal plasma	174
Figure 5.12: : Ornithine concentrations in maternal and fetal plasma and amniotic fluid ..	177

List of Tables

Table 1.1: US National Kidney Foundation NDOQI Stages of CKD	3
Table 1.2: Molecular control of kidney development	15
Table 1.3: Mesonephric and metanephric kidney development in several species	26
Table 1.4: Studies of nutritional models in sheep	34
Table 2.1: Energy and protein composition of the diets	46
Table 2.2: Protocol for processing tissues	58
Table 2.3: Clustal 2.1 sequence alignment for ovine and human VEGFA	62
Table 2.4: Antibody details for CD31 and CD34	63
Table 2.5: Primer details for Quantitative PCR.....	76
Table 2.6: PCR protocol for QuantiTect SYBR Green	78
Table 2.7: PCR protocol for Roche SYBR Green	79
Table 3.1: Effect of maternal low protein diet on fetal weights at 65 days gestation	95
Table 4.1: Organ weights at two years of age	120
Table 4.2: Glomerular filtration rate (GFR) at two years of age	126
Table 4.3: Histological stain details	127
Table 4.4: PCR results for endoplasmic stress markers	129
Table 4.5: Nephron number in control animals in sheep studies	133
Table 5.1: Effect of maternal low protein diet on fetal weights at 65 days gestation	158
Table 5.2: Standard amino acid concentrations in maternal plasma (μM).....	172
Table 5.3: Standard amino acid concentrations in fetal plasma (μM)	173
Table 5.4: Standard amino acids in amniotic fluid (μM).....	176

Abbreviations

A

ADH Anti-diuretic hormone

Ang1 Angiopoietin

B

BCS Body condition score

BMI Body mass index

BMP7 Bone morphogenic protein 7

C

CD31 & CD34 Cluster of differentiation 31/34

cDNA Complementary deoxyribonucleic acid

CHD Coronary heart disease

CKD Chronic kidney disease

CP Control protein group

CVD Cardiovascular disease

D, E, F

dH₂O Distilled water

EDTA Ethylenediaminetetraacetic acid

FGF Fibroblast growth factor

G

GAPDH Glyceraldehyde-3 phosphate

GC-MS Gas chromatography – mass spectrometry

GDNF Glial cell derived neurotrophic factor

GLM General linear model

GSL1_{B4} Griffonia (Bandeiraea) simplifolia lectin 1 isolectin B4

H, I, J

H & E Haematoxylin and eosin

H₂O Water

K, L, M

kg	Kilogram
LPE	Low protein early group
LPL	Low protein late group
ME	Metabolisable energy
mRNA	Messenger ribonucleic acid

N

NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBF	Neutral buffered formalin
NEFA	Non-esterified fatty acid
NS	Non significant

O, P

Pax2	Paired box 2
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor

Q, R, S

RAS	Renin angiotensin system
SEM	Standard error of the mean

T, U, V

TGF β 1	Transforming growth factor β 1
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labelling
UK	United Kingdom

W, X, Y, Z

WT-1	Wilm's tumor 1
Wnt4	Wingless-type MMTV integration site family member 4

1. Introduction

1.1 Health and Disease

Increasing life expectancy coupled with an increase in the mortality and co-morbidities associated with rising levels of obesity is leading to an additional economic burden. In the UK, total expenditure on health as a percentage of Gross Domestic Product has risen from 6.9 % in 1999 to 9.4 % in 2009 (World Health Organisation, 2012). Additionally, government expenditure per capita on health rose from \$1763 to \$3285 during this decade, an increase of over 86% (World Health Organisation, 2012).

In England and Wales in 2010 circulatory disease was the most common cause of death, accounting for 32% of all deaths (Office for National Statistics, 2008). In addition a further 2.2% of deaths had the primary cause listed as obesity, diabetes mellitus or renal disease, although these diseases often co-exist with each other and with other non-communicable 'lifestyle' diseases, such as cardiovascular disease (CVD). In Europe the mortality rates from circulatory disease are higher, with 48% of deaths due to CVD (British Heart Foundation, 2008).

However, the large impact of non-communicable disease on society is not limited to mortality alone, as the morbidity associated with these diseases is also of huge importance. The Disability Adjusted Life Year (DALY) is a measure of disease burden that combines the time lost due to disability along with the reduced life expectancy due to a disease, and one DALY is considered to be one lost year of healthy life (Murray et al., 2002). In 2004 in the UK the DALY was estimated at a total of 1.25 million, or 2,083 per 100,000 population (World Health Organisation, 2006). As more people become obese and suffer the associated co-morbidities this burden is likely to increase.

Globally the leading risks for mortality are hypertension, smoking, high blood glucose, physical inactivity, and overweight/obesity, all of which increase the risk of chronic lifestyle diseases such as CVD, type II diabetes and chronic kidney disease (World Health Organisation, 2009). These risks affect countries across all income groups, and are not limited to the wealthiest countries.

1.2 Kidney Disease

The vast majority of kidney disease falls under the umbrella term of chronic kidney disease (CKD), which is a gradual loss of kidney function over time. The two main causes of CKD are hypertension (Bakris and Ritz, 2009) and diabetes (Crook, 2002), both of which lead to nephron damage and a decline in the ability of the kidney to function. The incidence of CKD also increases greatly with age (Winearls and Glasscock, 2009) and obesity (Cignarelli and Lamacchia, 2007), so ageing populations coupled with increased prevalence of obesity mean it has become a major public health issue. Estimates of the prevalence of stage 3-5 CKD in the general population have ranged from 4.7 % in the USA (Coresh et al., 2003), 8.5% in the UK (Stevens et al., 2007) and Mexico (Amato et al., 2005), to 11.2% in Australia (Chadban et al., 2003). Stage 5 CKD (end-stage renal disease), is increasing at 5 – 8 % per annum and the global cost of providing dialysis has been estimated at \$70 - \$75 billion US dollars annually (Lysaght, 2002).

Severity of CKD is assessed according to the criteria shown in Table 1.1 and graded from stage 1 (mild) to stage 5 (end-stage renal disease, ESRD). Early stage CKD is usually asymptomatic and often identified during routine medical examinations. The mammalian kidney has such a huge functional reserve that uraemia – toxic build up of waste products in the blood - only becomes evident at CKD stages 3-5, i.e. when there has been a loss of 50-70% glomeruli with glomerular filtration rate (GFR) declining to $<30 \text{ ml} \cdot \text{min}^{-1}$ (Reilly and Perazella, 2002). However, identification at an early stage is useful because CKD increases

risk of CVD, there is the potential to try to slow the progression of the damage, and certain medications are contraindicated for those with renal disease (O'Callaghan, 2009).

Table 1.1: US National Kidney Foundation NDOQI Stages of CKD

Stage	Description	GFR (ml.min ⁻¹ .1.73 m ⁻²)
1	Kidney damage with normal or raised GFR	>90
2	Kidney damage with mildly reduced GFR	60–89
3	Moderately reduced GFR	30–59
4	Severe reduction in GFR	15–29
5	Kidney failure	<15

NDOQI – National Disease Outcomes Quality Initiative; CKD – Chronic Kidney Disease; GFR – glomerular filtration rate. Source: National Kidney Foundation (National Kidney Foundation, 2002)

The most common cause of renal failure is diabetic nephropathy and this accounts for around 22% of renal failure in the UK (The Renal Association, 2008). The UK Prospective Diabetes Study observed that a quarter of patients with type II diabetes developed microalbuminuria within ten years of diagnosis (Adler et al., 2003). Progression to macroalbuminuria was estimated at 2.8% per year, and to elevated plasma creatinine or renal replacement therapy at 2.3% per year, with those in the latter groups having an annual death rate of 19.2% (Adler et al., 2003).

Dialysis is the only viable alternative to transplantation for those with ESRD and the economic costs of providing such treatment are immense. In Europe around 2% of healthcare budgets are already taken up by dialysis costs (El Nahas and Bello, 2005), and this is likely to increase substantially if the trend of ESRD increasing at 5 – 8% per year continues (Lysaght, 2002).

The rising incidence of obesity, type II diabetes and associated co-morbidities including hypertension and kidney disease is not limited to westernised countries. It is thought that emerging countries such as India and China are likely to be the most detrimentally affected, as people from Asia tend to be particularly vulnerable to type II diabetes and suffer for a longer time from its complications (Yoon et al., 2006). Incidence of CKD increases greatly with age, so ageing populations coupled with an increase in type II diabetes are likely to lead to much higher levels of CKD (Stevens et al., 2007).

The International Diabetes Federation estimates that 285 million people worldwide had diabetes in 2010 and this is likely to be 438 million by 2030 (International Diabetes Federation, 2009). A similar pattern is envisaged in the UK, with 2.6 million people diagnosed in 2009, rising to an estimated four million by 2025 (Diabetes UK, 2009). The increasing incidence of obesity is contributing to the epidemic of type II diabetes. Around two-thirds of adults in the UK are overweight or obese, with estimated figures for 2010 of 67.8% of males and 63.8% of women having a Body Mass Index(BMI) > 25.0 (World Health Organisation Global InfoBase, 2010).

Taken together it is clear that the burden of chronic non-communicable disease is increasing globally, and it is imperative therefore to understand the aetiology/pathology and progression of disease so that interventions can be made early to prevent the onset of morbidity and ultimately mortality. However, the diseases described above are often multi-factorial (such as hypertension) and their cause can be lifestyle-related, genetic or a combination of both. In recent years the risk of succumbing to such diseases has even been shown to be influenced by prenatal growth and development.

1.3 Health and Diet

The link between diet and health has long been recognised. Back in the mid-1700s James Lind was the first to suggest that a disease (scurvy) could be cured by citrus fruits (Sutton, 2003), although the concept of vitamins was not yet known. Up until the 1950s most government public health initiatives were concerned with preventing deficiencies of nutrients or a lack of energy intake, however this changed during this decade with the recognition that over-nutrition could lead to chronic diseases (Duguid, 1954; Page et al., 1957). In recent decades dietary excess and its associated health problems have replaced concerns about dietary deficiency as the major food-related public health issue facing Westernised countries (Hill and Peters, 1998; Nestle, 1999).

However, obesity, hypertension and CKD are conditions virtually absent in hunter-gatherer communities whose diet is largely natural and unrefined (Eaton and Eaton, 2000), unlike the diet commonly consumed in Westernised societies (Cordain et al., 2005). During the Palaeolithic era (2.6 million – ~10,000 years ago) humankind evolved from early humans into the modern *Homo sapiens*, and therefore evolved to be suited to the diet available during that time (Cordain et al., 2005). A paleolithic diet was high in plant materials, such as fruit, vegetables, seeds and nuts, and also high in lean protein and/or fish (Cordain et al., 2005; Jew et al., 2009).

It was not until the advent of the agricultural revolution during the Neolithic period that carbohydrates became the staple foods for most societies (Jew et al., 2009), yet the human genome has remained largely unchanged during this period despite our diet changing greatly, particularly during the last century (O'Keefe and Cordain, 2004). In the UK in 2008/9 the percentage of energy intake from fat was 35%, from protein 18%, and from carbohydrates 47% (Food Standards Agency, 2009), whereas in the Paleolithic period these percentages were estimated to be fat 22%, protein 37%, and carbohydrates 41% (Eaton and

Konner, 1997). Other differences between the paleolithic and western diets include changes in glycaemic load, sodium-potassium ratio, and fibre content (Cordain et al., 2005).

Studies have shown that even very short-term paleolithic-style diets improve glucose tolerance (Lindeberg et al., 2007), blood pressure and lipid profiles (Frassetto et al., 2009).

Therefore dietary change (from a western toward a palaeolithic diet) is a potentially important preventative factor that could help mitigate the rise in non-communicable disease (Narayan et al., 2010; Daar et al., 2007). However, criticisms of the paleolithic-style diets are that they are difficult to follow, in particular to source meat of a similar fat:lean composition of the wild meat available in the Paleolithic era, and that the lack of non-communicable disease in hunter-gatherer communities today is confounded by other factors such as their increased activity levels, and lower life expectancy (Nestle, 1999; Milton, 2000).

Indeed considering the effect of any type of diet on disease risk is likely to be confounded by a variety of factors, partly because dietary changes tend to involve a range of changes rather than just one in isolation. For example following a low fat diet may involve changes in the types of fat ingested as well as the overall amounts, and also an increase perhaps in other food groups as the individual replaces high fat products with other foodstuffs.

Furthermore, people following certain types of diets are also likely to have other differences in lifestyle. For example vegetarians have been shown to have reduced risk of several chronic diseases, but most markedly ischaemic heart disease (Key et al., 1996), yet these results are often confounded by other factors: vegetarians tend to drink less alcohol, smoke fewer cigarettes and take more exercise than non-vegetarians (Appleby et al., 2002).

Hence, animal studies, where dietary effects can be studied in isolation from other lifestyle or environment factors, have informed much of the research about disease risk and diet.

1.3.1 Monogastric vs Ruminant Digestion

The major difference between monogastric animals and ruminants is that the latter have a four-chambered stomach, which is divided into four parts, called the rumen, reticulum, omasum and abomasum. A symbiotic relationship with microorganisms in the rumen enables the ruminant to digest cell plant wall material that is indigestible to monogastrics (Fuller, 2004).

In both monogastric animals and ruminants, food is eaten and then subjected to mechanical disruption by the teeth and to chemical degradation by salivary enzymes in the mouth which begin to break down starch (Gibney et al., 2003; Hill et al., 2008). In a monogastric species the bolus continues down the oesophagus into the stomach for further digestion by gastric juices that contain pepsin which starts protein digestion (Geissler and Powers, 2011). However, in the ruminant the bolus moves down the oesophagus into the first chamber of the stomach: the rumen (McDonald et al., 2011). The solids remaining are regurgitated and subjected to further maceration and chemical breakdown to reduce particle size (Fuller, 2004). In the rumen and reticulum bacteria, protozoa and fungi digest the fibrous plant material such as cellulose by using enzymes that break the β -glycosidic bonds (Kay, 1998). This results in the production of simple sugars, methane, carbon dioxide, and volatile free fatty acids: the latter is the main source of energy for a ruminant (Kay, 1998). Once the particle size is small enough, the digesta passes through into the omasum, where most of the water is removed, and next into the abomasum for further digestion (Fuller, 2004). The abomasum is broadly equivalent to the stomach of a monogastric animal, and further digestion occurs before the digesta (or chyme) moves into the intestines for nutrient absorption (McDonald et al., 2011). In both monogastrics and ruminants once the chyme is in the small intestines bile and enzymes from the pancreas and intestines further digest the fats and proteins, and villi aid the absorption of nutrients

(Gibney et al., 2003; Hill et al., 2008). Peristalsis transports the remaining liquid to the large intestine, where fermentation of the gut bacteria breaks down any further nutrients which are absorbed, before waste is excreted as faeces (Geissler and Powers, 2011; McDonald et al., 2011).

1.4 Mammalian kidney development

1.4.1 *Early embryonic development*

Development involves five main processes: cell division, growth, differentiation, pattern formation and morphogenesis. These processes are not linear, but overlap with each other (Twyman, 2001). In brief, the early stages of development are as follows (Wolpert et al., 2001; Twyman, 2001):

1. The egg is fertilised by the sperm and the male and female nuclei fuse together to form a diploid zygote.
2. Mitotic cell divisions occur without significant growth and therefore become progressively smaller each time. This is called cleavage.
3. After around a dozen cleavages the cells are arranged into a ball shape around a fluid-filled cavity, known as a blastula.
4. The next stage is gastrulation, during which the cells migrate and become organised into three germ layers called the ectoderm, mesoderm and endoderm.

These layers produce a specific range of differentiated cell types (Figure 1.1).

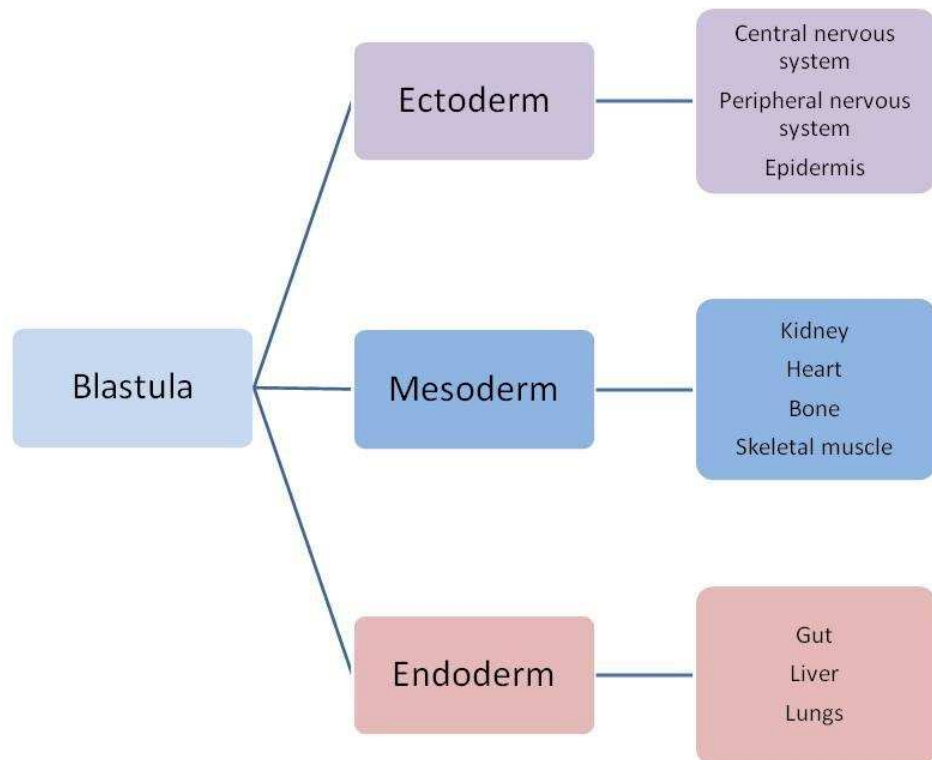


Figure 1.1: Early stages of development of the embryo

1.4.2 Nephrogenesis

The formation of the kidney has been studied to a great extent in several animal models, where the early stages of development can be carried out *in vitro* or mice can be genetically manipulated so that both the cellular and molecular basis of organogenesis can be investigated (Costantini and Kopan, 2010). There are three stages of mammalian kidney development – pronephros, mesonephros and metanephros – but only the latter develops into the mature adult kidney (Kuure et al., 2000). The nephron epithelial cells and collecting duct system originates from the intermediate mesoderm and the pronephric duct and pronephric tubules are thought to form as separate structures (Vize et al., 1997). The pronephros develops very early in gestation (20 d in humans; gestation = 268 d) and in

mammals is a non-functional kidney (Polin et al., 2004), but most of the pronephric duct regresses apart from the caudal region which becomes the Wolffian (or nephric) duct (Kuure et al., 2000).

The second of the transitional kidneys - the mesonephros - originates in the aorta-gonad-mesonephros (AGM) zone of the mesoderm, and its development is activated when the pronephric duct induces mesenchymal cells to condense (Kuure et al., 2000). In most, but not all, mammals the mesonephros is functional and acts as a transient filtration unit until the metanephros develops and takes over (Dressler, 2006). However, in other species such as fish and amphibians the mesonephros becomes the functional adult kidney (Polin et al., 2004).

The metanephros arises when the nephric duct develops an outgrowth called the ureteric bud at its caudal end, and this bud interacts with the metanephric mesenchyme (Dressler, 2006). The metanephric mesenchyme contains the progenitor cells for the nephrons but also signals to the nephric duct at the caudal end to form a ureteric bud (Costantini and Kopan, 2010). Figure 1.2 shows the process of nephron development. First the ureteric bud narrows at the tip and forms an ampulla, and the metanephric mesenchyme cluster around this and condense (Moritz and Wintour, 1999; Vainio and Lin, 2002). This condensation induces the ureteric bud to rise cranially and to branch (Wintour, 1997).

Mouse cell culture models of the developing metanephric kidney have demonstrated that the branching of the ureteric bud continues for around 10 – 15 generations (Srinivas et al., 1999; Costantini, 2006). The cells proliferate in both the tip and the trunk of the ureteric bud, however at different rates according to the phase of development, i.e whether it is during branching or collecting duct elongation (Michael and Davies, 2004). At each branch tip one side causes the mesenchyme to condense and the other branch continues onward to branch again (Wintour, 1997). The ureteric bud simultaneously induces the metanephric

mesenchyme to convert to epithelial cells, whilst other mesenchymal cells convert to interstitial stroma (Dressler, 2006). The epithelial cells form into a tube called the nephrogenic vesicle (Moritz and Wintour, 1999) which is still attached to the ureteric bud at one end (Dressler, 2006). One cleft develops in the vesicle which becomes the comma-shaped body, then a second cleft develops to produce the S-shaped body (Dressler, 2006).

Endothelial cells migrate into the s-shaped body which develops into the glomerulus and proximal and distal tubules, and the ureteric bud develops into the collecting duct system (Vainio and Lin, 2002). The base of the S-shaped body is invaded by capillaries that form the glomerulus, whilst the top fuses to the top of the ureteric bud and eventually becomes the collecting duct (Wintour and Moritz, 1997). The top half of the 'S' elongates to become the proximal and distal tubules (Wintour, 1997). The lower part of the 'S' forms the renal corpuscle, i.e. the glomerulus and Bowman's capsule (Clark and Bertram, 1999). Initially this nascent glomerulus contains primitive podocytes, a Bowman's capsule and a capillary loop (Quaggin and Kreidberg, 2008).

Basal laminae from the podocytes and the proliferating endothelial cells of the capillary fuse together to form the glomerular basement membrane (Dressler, 2006). Podocytes wrap around the capillary loops and extend projections, called foot processes, which interlink with other foot processes and form the slit diaphragm (Quaggin and Kreidberg, 2008). The renal corpuscle continues to develop, for example, by the differentiation of cells into podocytes, whilst simultaneously the tubule also differentiates into its different regions (Clark and Bertram, 1999).

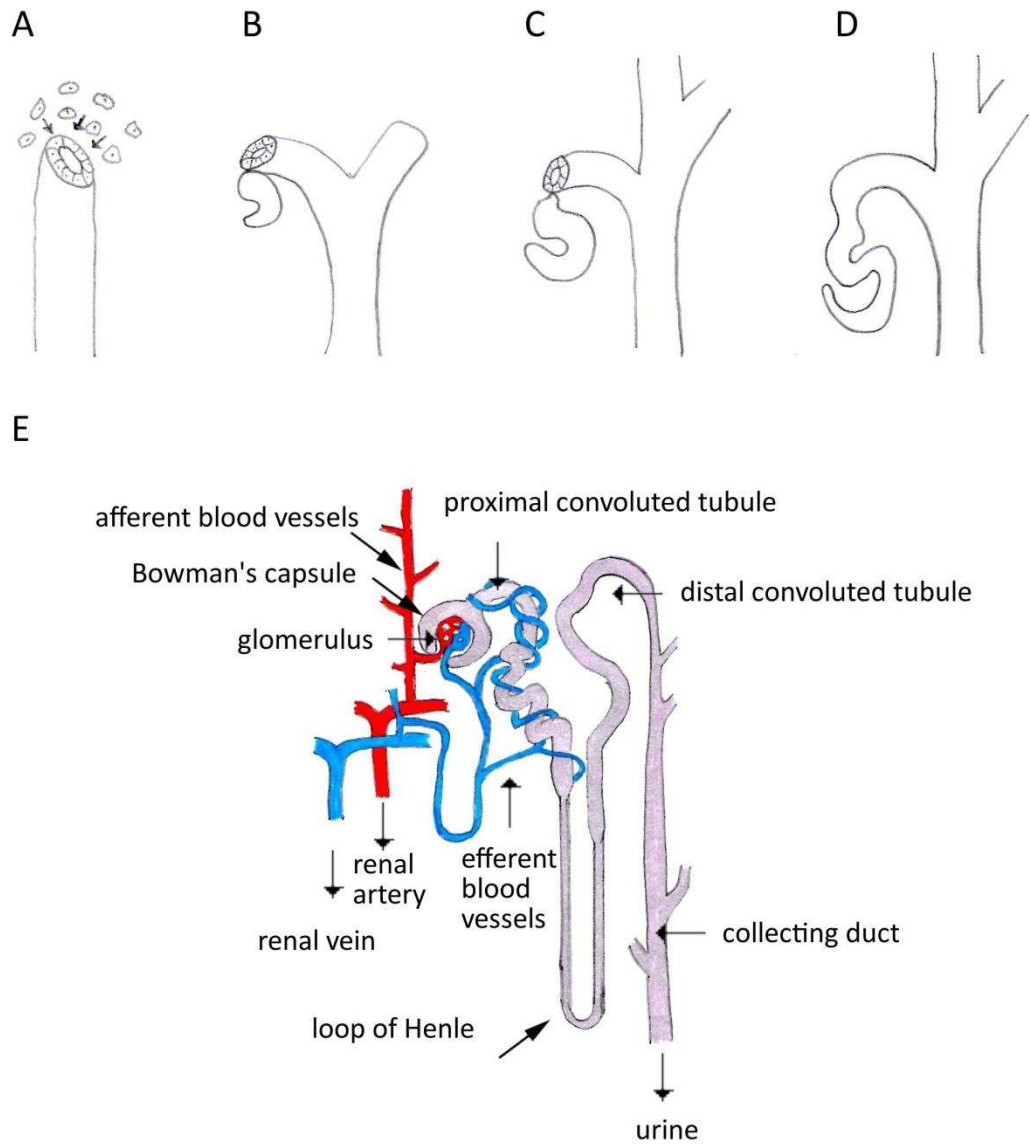


Figure 1.2: **Nephron development.**

Induction of the ureteric bud (A) is accompanied by a mesenchymal induction and condensation, branching of the ureteric bud and formation of a comma-shaped body (B). A second cleft develops and the s-shaped body (C) is formed. This fuses with the ureteric bud and a continuous tubule is formed (D), which develops into a fully functioning nephron (E). Adapted from Clark and Bertram, 1999

The ureteric bud continues to branch and generate new nephrons. This nephrogenic zone is around the edge of the cortex (Figure 1.3). As newer nephrons develop, the more mature ones move towards the medulla and therefore the first glomeruli to develop are found deepest in the cortex and the last to develop are around the edge (Dressler, 2006; Wintour, 1997). The final part of the kidney to develop is the outer medulla which contains the loops of Henle from the nephrons close to the cortex-medulla border (Wintour, 1997).

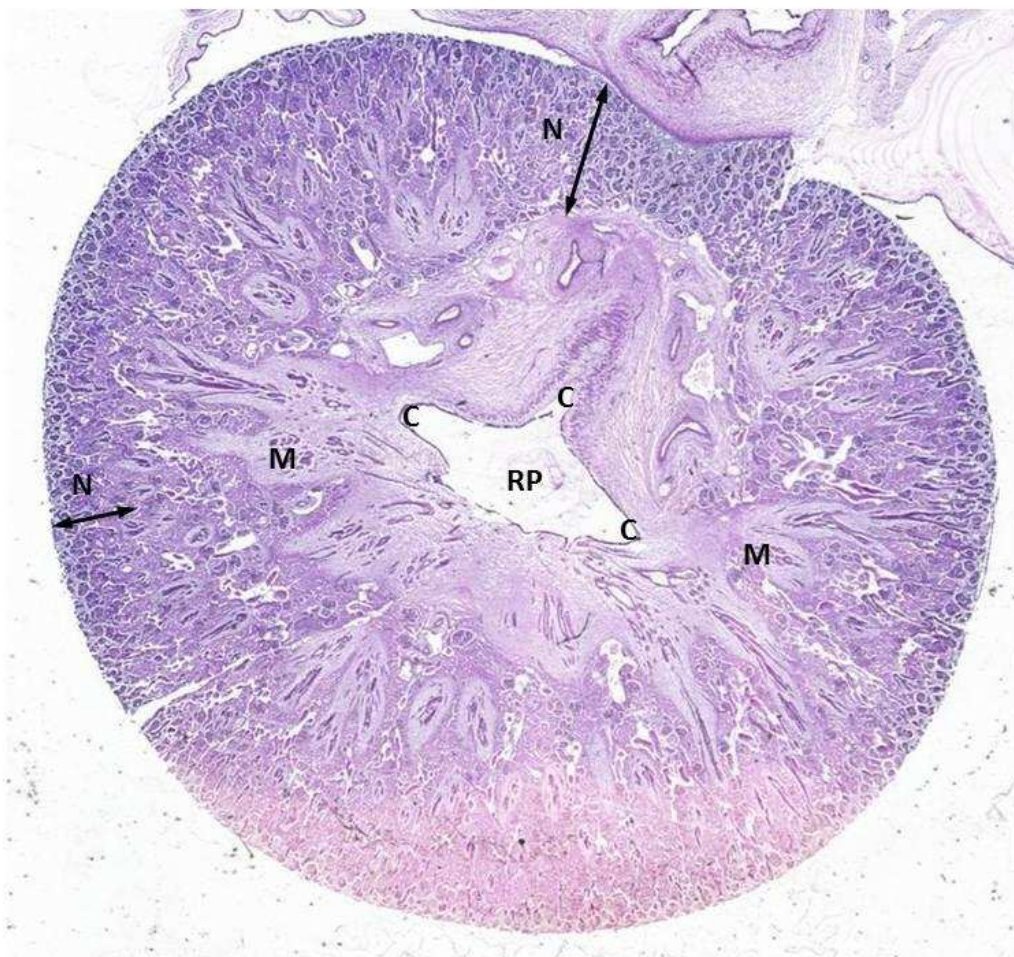


Figure 1.3: Fetal sheep kidney at day 65 gestation

Nephrogenic zone where new nephrons form (N) indicated by arrows; M, metanephric mesenchyme; developing calyces (C) and renal pelvis (RP). Author's illustration.

1.4.3 Vascular development

Angioblasts migrate to the lateral mesoderm to form 'blood islands' in the yolk sac (Flamme et al., 1997). Each blood island separates into a core of hemangioblasts (which will become the first blood cells) surrounded by endothelial cells (Polverini, 1995). These endothelial cells develop into the blood vessel endothelium, and some of the cells start to differentiate into angioblasts (Larsen, 1998). During vasculogenesis, angioblasts are assembled into the main vessels, and then angiogenesis extends these vessels with further branching eventually to form an extensive network of arterioles, venules and capillaries (Wolpert et al., 2001). The primitive venous system is made up of the cardinal system (which drains the head, neck, body wall and limbs), the vitelline veins (which drain the gut), and the umbilical veins which carry oxygenated blood from the placenta to the embryo (Larsen, 1998).

The arterial system in the embryo develops as folding takes the endocardial tubes into the thorax, and they connect to the dorsal aortae to form the first aortic arches (Larsen 1998). These five pairs of arches form the major vessels of the head, neck and thorax, the dorsal pair fuse to form a single aorta which sprouts three branches through angiogenesis (Wolpert et al, 2001). These three branches become:

- Ventral vitelline arteries, which supply the gut,
- Dorsolateral insegmental arteries which supply the head, neck, spinal column and limbs, and
- Lateral arteries, which supply the retroperitoneal organs, including the kidney (Larsen, 1998).

Glomerular capillary development starts when a capillary loop invades the cleft between the S-shaped body and the nascent podocytes (Quaggin and Kreidberg, 2008). The loop

divides into six to eight loops and the endothelial cells become fenestrated to allow filtration of the blood (Potter, 1965).

1.4.4 Molecular control of development

The molecular control of kidney development is complex and involves over 200 genes (Costantini, 2006; Dressler, 2009). Some of the main candidates are summarised in Table 1.2 below.

Table 1.2: Molecular control of kidney development

Molecular event	Genes involved
Ureteric bud branching	GDNF, Ret, angiotensin II
Mesenchymal condensation	WT-1, Pax 2, bFGF
Mesenchymal-to-epithelial conversion	Wnt-4, laminins, FGF8
Angiogenesis	VEGFA, Angiopoietins, Tie 2, bFGF, TGF β
Glomerular development	VEGFA, PDGF, Notch 1&2, cadherins

FGF, fibroblast growth factor; GDNF, glial cell-derived neurotrophic factor; Pax 2, paired box 2; PDGF, platelet derived growth factor; TGF β , transforming growth factor β ; VEGFA, vascular endothelial growth factor A; WT-1, Wilms tumour 1; WNT-4, wingless-type MMTV integration site family member 4 (Clark and Bertram, 1999; Quaggin and Kreidberg, 2008; Woolf et al., 2009; Faa et al., 2012)

Glial cell derived neurotrophic factor (GDNF) is a growth factor secreted by the metanephric mesenchyme in response to Pax 2 (Brophy et al., 2001). GDNF signals through Ret, a tyrosine kinase receptor (Shakya et al., 2005). Knockout mice for either gene usually fail to develop kidneys in the embryo as the ureteric bud fails to emerge from the nephric duct (Schuchardt et al., 1996; Sanchez et al., 1996), therefore indicating their importance at the very early stages of metanephric development as the ureteric bud begins to emerge and branch. This finding is backed up by *in vitro* studies, where explanted mouse embryo urogenital regions can be induced to form ureteric buds in the presence of GDNF (Vega et al., 1996; Michael and Davies, 2004). However in knockout mice lacking GDNF and Ret a small proportion of mice still form ureteric buds (Schuchardt et al., 1996) thus there must be some functional redundancy with other genes.

Some of this functional redundancy may be explained by the role of the fibroblast growth factor (FGF) family, members of which bind to related receptors and activate many of the same signalling pathways (Eswarakumar et al., 2005; Michos et al., 2010). FGF1 – 10 are all expressed in the developing kidney, although knockout mice for most of the FGFs do not have noticeable kidney defects, suggesting that there is overlap in function for many of these genes (Qiao et al., 2001). The exceptions are FGF7^{-/-} mice which develop smaller kidneys with fewer nephrons (Qiao et al., 1999), and FGF10^{-/-} mice which have smaller kidneys with dysplasia of the outer medulla (Ohuchi et al., 2000). However, although knockout models do not exhibit major defects, this does not mean that the FGFs do not have an important role in kidney development, rather that in their total absence, other genes are able to compensate to some extent. In fact many other studies have shown important roles for the FGF family; for example, FGF8 is needed for the survival of nephron progenitor cells and formation of the S-shaped bodies (Grieshammer et al., 2005), and FGF2 aids survival of metanephric mesenchymal cells (Perantoni et al., 1995).

Also interacting with FGF is bone morphogenic protein 7 (BMP7), which is expressed in the ureteric bud and in nascent nephrons (Godin et al., 1999). In explants of metanephric mesenchyme BMP7 has been demonstrated to promote survival by preventing apoptosis (Dudley et al., 1999). Also, knockout mice lacking BMP7 die shortly after birth due to kidney defects, which are caused a lack of differentiation of the metanephric mesenchymal cells (Luo et al., 1995); thus BMP7 has more than one role at this stage of development.

Paired box 2 (Pax2) and wingless-type MMTV integration site family member 4 (Wnt4) are also involved in the metanephric mesenchyme differentiation. Pax2 induces the metanephric mesenchyme to differentiate towards a nephron lineage (Tamimi et al., 2008), although mutant mice lacking both Pax2 and Pax8 genes are unable to form even the pronephros (Bouchard et al., 2002). Wnt4 induces the cap mesenchymal cells to undergo a

mesenchymal-epithelial transformation into the renal vesicle (Kispert et al., 1998), a finding confirmed by another study (McMahon et al., 2008) where $Wnt4^{-/-}$ mutants failed to form renal vesicles during embryonic development.

Wilms Tumor 1 (WT1) gene encodes a transcription factor with wide-ranging effects on the expression of genes involved in kidney development (Faa et al., 2012). It may also be involved in post-transcriptional regulation (Scharnhorst et al., 2001). It is expressed in the metanephric mesenchyme where it increases as the mesenchyme condensates around the tip of the ureteric bud (Armstrong et al., 1993). Later in development the expression decreases, and in mature animals WT1 is only expressed in podocytes (Gao et al., 2005). $WT1^{-/-}$ mice are embryonically lethal due to a failure of the ureteric bud to emerge from the nephric duct (Kreidberg et al., 1993). However, WT1 is needed at several times during development, as demonstrated by one study (Davies et al., 2004) which used an siRNA-based method to switch off the WT1 at different stages of development in mouse kidney explants. The results showed that although early in development a lack of WT1 leads to a lack of growth of the ureteric bud, where WT1 was ablated later in gestation the ureteric bud remained intact but there was abnormal cell proliferation and nephron development was seriously impaired.

WT1 also regulates the expression of vascular endothelial growth factor (VEGF) in the mesenchyme (Gao et al., 2005). VEGF has multiple roles during renal development. In metanephric kidney culture it has been shown that VEGFA stimulates cell proliferation and branching morphogenesis as well as nephrogenesis (Tufro et al., 1999; Tufro, 2000). VEGFA is also expressed in early podocytes in the S-shaped bodies (Kretzler et al., 1998) and its major receptors VEGF receptors 1 and 2 (VEGFR1 and VEGFR2) are expressed in the adjacent endothelial cells (Robert et al., 1998). Signalling between VEGFA and endothelial cells is crucial for mesangial cell migration and survival (Eremina et al., 2006) and for the

induction of the development of the fenestrae in the endothelial cells of the glomerular capillaries (Eremina et al., 2003).

Given the wide variety of roles that VEGF has it is no surprise that absence of VEGF has detrimental effects. Homozygous knockout mice for either VEGFA or its receptors VEGFR1 or VEGFR2 suffer from renal agenesis and die before birth (Fong et al., 1995; Shalaby et al., 1995; Eremina et al., 2003). Heterozygous knockouts die early from renal disease due to abnormal vasculature (Eremina et al., 2003), and where VEGFA is ablated just in one cell population (podocytes) the mice do not produce mesangial cells and die early (Eremina et al., 2006).

Playing a complementary role to VEGFA and its receptors are the angiopoietins. During development angiopoietin 1 (Ang1) is thought to stabilise blood vessels by making them leak-resistant, whereas angiopoietin 2 (Ang2) is an antagonist to Ang1 and has the opposite effect (Maisonpierre et al., 1997). Ang1 is expressed from the start of metanephric development in the mesenchyme, and developing tubules and podocytes, alongside its receptor Tie2 which is found in the interstitial and glomerular capillaries (Yuan et al., 1999; Kolatsi-Joannou et al., 2001; Satchell et al., 2002). Ang1^{-/-} mutant mice are embryonically lethal due to the failure of normal vascular remodelling during development (Suri et al., 1996). Close cooperation between VEGFA and Ang1 is needed to maintain the integrity of the glomerular endothelial cell barrier (Satchell et al., 2004).

Also crucial for the development of the renal vasculature is platelet derived growth factor (PDGF). PDGF is essential during the development of the glomerular capillary loops. PDGF^{-/-} mutant mice are embryonically lethal and form only a single capillary loop (Leveen et al., 1994), whilst mice with a PDGF ablation in endothelial cells only live into adulthood but have glomerular abnormalities such as dilated capillary loops and fewer mesangial cells (Bjarnegard et al., 2004).

1.5 Kidney physiology

Mammals have two kidneys and in most mammals including humans and sheep these are bean-shaped organs located retroperitoneally. The kidney is divided into two distinct regions, with an outer cortex and an inner medulla. The cortex is surrounded by the renal capsule.

The kidney functions as a regulatory and endocrine organ as well as an excretory organ. It excretes waste products from the body which are end-products of metabolism, such as urea and creatinine, along with metabolites of hormones, and foreign substances such as drugs. The kidney has a vital role in the function of the cardiovascular system by regulating the volume of body fluid and blood pressure, and also in controlling the osmolality of body fluids to maintain normal cell volume (Koeppen and Stanton, 2007). Additionally the kidney regulates electrolyte balance by adjusting the level of excretion of electrolytes such as potassium, sodium and chloride to take account of the amount ingested, and also has a role in maintaining the correct acid-base balance for normal metabolic functions (Bray et al., 1999). In its role as an endocrine organ the kidney produces and secretes erythropoietin, renin and 1,25-dihydroxyvitamin D₃ (Koeppen and Stanton, 2007).

The functional unit of the kidney is the nephron, and each kidney in a human can contain over a million nephrons (Keller et al., 2003). The number of nephrons is generally related to the size of the animal (Kunkel, 1930). Figure 1.2E shows a diagram of a nephron, and Figure 1.4 a renal corpuscle. Blood enters the nephron through the afferent arteriole and into the glomerulus where the pressure is relatively high, and this drives water, amino acids, glucose and other small molecules such as sodium, chloride and potassium through the basement membrane into the Bowman's capsule (O'Callaghan, 2009).

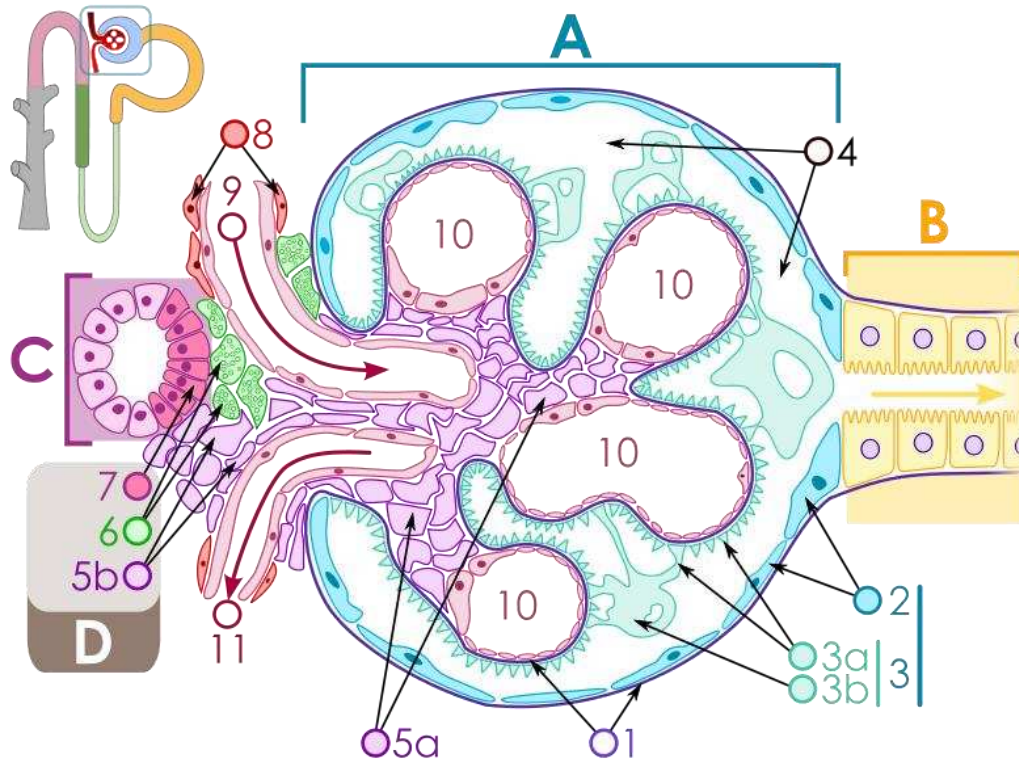


Figure 1.4: Diagram of a renal corpuscle

A – renal corpuscle; B – proximal tubule, C – distal convoluted tubule, D – juxtaglomerular apparatus; 1 – basement membrane; 2 – Bowman’s capsule, parietal layer; 3a – podocyte foot processes; 3b – podocytes; 4 – Bowman’s space; 5a – mesangium, intraglomerular cell; 5b – mesangium, extraglomerular cell; 6 – juxtaglomerular cells; 7 – macula densa; 8 – myocytes; 9 – afferent arteriole; 10 – glomerular capillaries; 11 – efferent arteriole. Copyright-free image from Wiki Commons.

Larger cells such as red blood cells and most proteins are too large to pass through and continue through the glomerulus and out into the efferent arteriole (O’Callaghan, 2009).

The filtrate that passed through into the Bowman’s capsule enters the proximal convoluted tubule where most of the water is reabsorbed, along with glucose, amino acids, and electrolytes such as sodium, chloride, potassium and phosphate through the epithelial cells and into the interstitial tissues, before being transported back into the blood in the surrounding vessels (Bray et al., 1999). The filtrate passes into the Loop of Henle, where more water and electrolytes are reabsorbed, and then into the distal convoluted tubule where sodium and chloride are actively transported through the epithelial cells before the filtrate is delivered to the collecting duct (Bray et al., 1999). The concentrated urine enters

the papilla, then through the calyces before reaching the renal pelvis and finally the ureter (Solomon et al., 2004). The amount of water reabsorbed through the collecting ducts is largely dependent on vasopressin (or anti-diuretic hormone, ADH) which is released by the posterior pituitary gland in response to high blood osmolarity, and makes the collecting duct permeable to water so it can be reabsorbed and retained in the body (Koeppen and Stanton, 2007).

1.6 Developmental Origins of Health and Disease

The Developmental Origins of Health and Disease (DOHaD) hypothesis proposes that events that occur *in utero* programme susceptibility to chronic adult diseases, such as type II diabetes (Hales and Barker, 1992) and coronary heart disease (Barker, 1995). Early in the development of the fetal origins hypothesis it was suggested that limiting nutrient supply to the fetus was an important programming factor (Barker, 1992). The term “fetal programming” refers to the concept that an insult or stimulus at a critical point during fetal development leads to a long-term effect. This ‘plasticity’ of fetal development means that developing tissues can adapt to their current circumstances by altering gene expression in response to the prenatal environment (Langley-Evans, 2006). If this occurs during critical developmental periods, the availability of nutrients could therefore have permanent effects on tissue structure and function (Barker et al., 1993).

From an evolutionary point of view a human only needs to be able to live long enough to reproduce, so fetal development should ideally provide adaptations that ensure short-medium term survival in the prevailing environment. Therefore such modifications may not be so advantageous in the long term. The ‘Thrifty Phenotype Hypothesis’ (Hales and Barker, 1992) proposes that the nutrient-restricted fetus adapts to thrifty circumstances by making adaptations that allow it to make more efficient use of limited energy substrates

available. This is a survival advantage if the postnatal environment remains poor, but a disadvantage if the postnatal environment is nutrient-rich (Hales and Barker, 1992).

1.6.1 Epidemiological Evidence for DOHaD

Epidemiological evidence supports the theory that nutritional factors during fetal development can have long-term implications for the health of the offspring. If an insult takes place during a critical period of development for the fetus then growth may be restrained, and the resulting low birth weight may be a predictor of disease later in life (Barker, 1998). In a cohort of men and women born in Hertfordshire between 1911 and 1930 death rates from coronary heart disease were twice as high for those who weighed less than 5.5 lb at birth compared with those who weighed between 8.5 and 9.5 lb (Osmond et al., 1993). Birth weight is, however, only one measure of events during intrauterine development, and responses to a sub-optimal fetal environment may lead to other permanent changes in tissue structure and function.

In addition, epidemiological studies have provided evidence that the timing of the nutritional insult is also crucial, and depending on the stage of gestation at which the insult occurs there are different long-term effects depending on the organ systems developing at that time point. For example, longitudinal evidence has been collected from a cohort of individuals exposed to famine *in utero* during the Dutch Famine at the end of World War II. At the peak of the famine, during the winter of 1944 – 1945, mean energy intake was reduced from 6.3 to 1.6 - 3.2 MJ/day (Roseboom et al., 2001). As middle-aged adults, those individuals exposed to famine during early gestation had higher LDL:HDL cholesterol ratios (Roseboom et al., 2000) and higher risk of obesity and coronary heart disease (Roseboom et al., 2006), than the control group who were not exposed to famine *in utero*. Those exposed to famine mid-gestation had evidence of programmed changes to blood pressure and an increased incidence of coronary heart disease (Roseboom et al., 1999), and also

indices of early-stage renal disease such as microalbuminuria (Painter et al., 2005).

Exposure to famine in late gestation led to poorer glucose tolerance compared to those who were not exposed (Ravelli et al., 1998). These delayed developmental effects were largely unrelated to size at birth but rather to factors marking the quality of fetal growth or maternal macronutrient balance (Roseboom et al., 1999).

Studies have shown a link between low birth weight and increased blood pressure (Barker, 1992; Mackenzie and Brenner, 1995) and this may be due to a deficit in nephron number in the kidney (Brenner et al., 1988; Manalich et al., 2000). In a retrospective analysis of autopsies of accident victims those with chronic hypertension had significantly fewer nephrons than the control group (Keller et al., 2003), and a direct linear relationship between birth weight and nephron number has also been observed with a predicted increase of 250,000 nephrons per extra kg birth weight (Hughson et al., 2003). Birth weight has also been demonstrated to be inversely correlated with mean glomerular volume (Hoy et al., 2005) as it appears that glomeruli hypertrophy to compensate for having fewer nephrons. This hypertrophy leads to hyperfiltration in the remaining nephrons, and increased pressure in the capillaries (Hershkovitz et al., 2007), and ultimately to glomerulosclerosis, proteinuria and reduced glomerular filtration rate (Brenner et al., 1996). Nephron number has also been shown to vary by sex and race, with males tending to have about 10% more nephrons than females, and Australian Aborigines having around 30% fewer than non-Aboriginal Australians (Hoy et al., 2005).

1.6.1.1 Limitations of epidemiological studies

Epidemiological studies can highlight new and potentially important aetiologies of a multi-factorial disease like chronic kidney disease. However, epidemiological studies have been criticised on a number of other fronts, including weaknesses in terms of cohort selection and failure to correct for all confounding factors. A meta-analysis of 55 studies that

reported an inverse relationship between birth weight and systolic blood pressure (typically 2 – 4 mmHg increase in systolic blood pressure per 1 kg increase in birth weight) found that the larger studies generally showed a weaker association, and many studies were not appropriately adjusted for confounding factors (Huxley et al., 2002). Once the studies were adjusted the relationship between birth weight and blood pressure was largely attenuated, and there was no conclusive evidence to support the link.

Additionally, not all epidemiological studies show evidence that maternal diet affects birth weight or the risk of chronic disease later in life. In a study of Northampton women, birth weight and infants' proportions were not affected by dietary intake during pregnancy (Langley-Evans and Langley-Evans, 2003), nor were the babies of a cohort of women from Portsmouth (Mathews et al., 1999). However, it may be that these studies both carried out in a westernised country with a high availability of food do not reflect a severe enough reduction in nutrient intake to see obvious effects on birth weight.

Another limitation of epidemiological studies in the area of DOHaD is that many are historical; the cohorts are not contemporary and may not reflect current lifestyles and diets. These limitations mean that animal studies – with their tightly controlled environments - have been crucial to demonstrating the biological plausibility of the programming effects of prenatal nutrition, and are now a valuable tool for investigating the mechanisms involved.

1.6.2 *Animal models*

Several animal models have been utilised for demonstrating the biological plausibility of the fetal programming of adult disease. The advantage of animal models when considering the effects of programming is that unlike human studies where there are many confounding

factors, the environment can be strictly controlled, so that the only difference between the control and experimental groups is the intervention.

Experimental models of intrauterine growth restriction (IUGR) can use interventions in either the mother, the placenta or the fetus. The most commonly used interventions for demonstrating programming effects are surgical models, glucocorticoid treatment or dietary interventions in the mother. These models have been used in several species, most commonly in rats and mice (around 70% of studies), but also in sheep, pigs, guinea pigs, monkeys, and chick embryos (Vuguin, 2007; Schroder, 2003). The selection of an appropriate model has several considerations. Shorter gestation and life expectancy makes rodent models attractive, and this also has financial implications for animal trials, as keeping larger animals with longer gestation and life expectancies to adulthood is time consuming and expensive.

Mammals share many common features at a biological level, for example with similar genes and biochemical pathways. However, there are also differences, which make selection of an appropriate model dependent on the variables being studied. For example, rodents are altricial which means they are born without their organs being fully developed, and a significant amount of maturation and development takes place during a short period after birth. Although human babies are also altricial, many of their internal organs are more mature, so sometimes a precocial animal model may be a more appropriate choice, depending on the organ or system being studied. Also, litter-bearing mammals, such as rodents and pigs, may have variations in the amounts of nutrient supply to different fetuses within the same litter. As humans usually have only one or two offspring, a model such as sheep may be more relevant, and indeed the sheep is a long-standing model for considering placental-fetal interactions (Fowden et al., 2006).

When considering kidney development, a sheep model is particularly appropriate.

Nephron development in the kidney is complete at birth in humans and sheep, and no new nephrons are formed postnatally. However in some other mammals, such as rats, mice and pigs nephrogenesis continues for a short period after birth (see Table 1.3).

Table 1.3: Mesonephric and metanephric kidney development in several species

Species	Gestation length (days)	Mesonephros present (% of gestation)	Metanephric development (% of gestation)
Mouse	20	0.50 – 0.65	0.55 – 1.13
Rat	21	0.57 – 0.81	0.57 – 1.38
Pig	112	0.13 – 0.21	0.18 – 1.10
Sheep	147	0.11 – 0.38	0.18 – 0.90
Human	268	0.10 – 0.40	0.12 – 0.90

Table adapted from Brace et al., 1998; Moritz and Wintour, 1999

The mesonephric and metanephric stages of kidney development are almost parallel in sheep and humans. Mesonephric development takes place between 10 to 40% of human gestation and 11 to 38% of sheep gestation (Table 1.3). This compares to a far shorter window of development in pigs (13 – 21% of gestation) and a much later developmental window in the more frequently used laboratory animals, rats (57 – 81%) and mice (50 – 65%). Similarly the period of metanephric development is analogous between human and sheep (12 – 90% gestation and 18 – 90% gestation respectively), whereas the in the pig it starts at the same time period, but extends postnatally, and in rats and mice it starts later and also extends postnatally (57 – 138% of gestation in rats, and 55 – 113% gestation in mice).

Unlike humans and most other mammals, a sheep does not appear to have a rudimentary pronephros, but instead develops a giant glomerulus at the end of what later becomes the mesonephros (Davies, 1951). However, the development of the metanephros, which

develops into the fully functioning adult kidney, is the same in both sheep and humans, thus justifying the use of sheep as an appropriate animal model with respect to renal aspects of the DOHaD paradigm.

One limitation of using sheep as a model for the human condition is that sheep are ruminants unlike humans who are monogastric, however this does not affect the functional physiology and anatomy of the kidney which are extremely similar between the species. Sheep have primarily been used in global restriction models rather than individual nutrient restriction models. However, even with a global nutrient restriction the digestive differences still apply, so it is important that any study considers this, and whether or not the results observed apply just to the sheep, or whether they may have a comparative aspect to humans or all mammals.

1.6.2.1 Surgical models

There are several models of fetal programming which are surgical in nature, and are used to generate hypoxia and intrauterine growth restriction. One commonly used method in sheep is carunclectomy, in which the majority of endometrial caruncles are removed from the uterus; this reduces the number of cotyledons that are responsible largely for the placental mass later in pregnancy (Schroder, 2003).

Studies have reported reduced gene expression of insulin-like growth factor 2 (IGF-2) and its receptors IGF-1R and IGF-2R in fetal hearts (Wang et al., 2011) and, in the liver, a reduction in IGF-1 mRNA and increased levels of hydroxysteroid dehydrogenase like 1, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha and phosphoenolpyruvate carboxykinase 2 (Gentili et al., 2009).

More commonly used as models for kidney development are sub-total maternal nephrectomy or placental embolisation models. One study (Brandon et al., 2008) showed a

reduction in mean glomerular volume in offspring whose mothers had undergone sub-total nephrectomy, although there was no change in kidney weight or nephron number. In addition the same research group (Brandon et al., 2009) reported a reduction in renal function from 6 months of age in the same experimental group.

Placental embolisation involves injecting small microspheres (15 µm in diameter) into the placenta, where they block small capillaries and thus reduce surface area for transferring oxygen and nutrients to the fetus (Morrison, 2008). Three studies which all carried out placental embolisation during the third trimester of pregnancy have shown inconsistent results, highlighting the variability inherent in the technique. In one (Zohdi et al., 2007) there was a 24% reduction in nephron number in offspring whose mothers underwent embolisation compared to the control group, although there was no change in kidney weight or renin-angiotensin system (RAS) gene expression. However, in another study (Mitchell et al., 2004) there was no change in nephron number, despite the same intervention, although there was a 34% reduction in the bodyweight of the fetuses at day 140 gestation. Furthermore, a small reduction in blood pressure (4 mmHg) was reported in young lambs (Louey et al., 2000).

1.6.2.2 Glucocorticoid treatment

Glucocorticoid treatment during pregnancy has been associated with lower birth weight and adult disease in both humans and animals (Reinisch et al., 1978; Moss et al., 2001; Newnham, 2001). In animal models artificial glucocorticoids (dexamethasone or betamethasone) have been given, as one-off doses (Dodich et al., 2001; Volk et al., 2010) or as a course over time (Malaeb et al., 2009), and various effects have been described in a range of organs and cell types in both rodent and large animals. In sheep, an increase in adult blood pressure (10 mmHg) in those offspring exposed to a glucocorticoid injection at day 27 gestation was observed (Dodich et al., 2001). In addition, in this model, nephron

number was reduced and mean glomerular volume increased (Wintour et al., 2003) when exposed to maternal dexamethasone at the same time point.

It has been suggested that changes in the relative ratio of angiotensin receptors in the kidney may explain these observations as after maternal betamethasone injection at day 80 of gestation, there was an increase in angiotensin II receptor type I (AGTR1) and a decrease in angiotensin II receptor type II (AGTR2) (Gwathmey et al., 2011).

1.6.2.3 Nutritional models

Many different dietary interventions have been studied in animal models, and they may provide useful insights into the challenges faced by pregnant women in developing countries, as well as for those women in westernised societies who do not have balanced diets or for those who suffer from *hyperemesis gravidarum*.

The main two dietary interventions used are global nutrient restriction (i.e. a reduction in caloric content of the diet, across all macronutrients) or a low protein diet which is isocaloric. Other diets have also been studied, and indeed similar programming effects are sometimes observed regardless of the intervention, which suggests that some common mechanisms may be involved. For example, hypertension has been reported in rats exposed prenatally to high fat (Khan et al., 2003), low iron (Crowe et al., 1995), and low protein diets (Langley and Jackson, 1994).

1.6.2.3.1 Global Nutrient Restriction

Many studies have utilised global nutrient restriction diets. The calorie/energy restriction may be mild, moderate or severe, and the timing of the restriction has also been varied, from pre-conception to early, mid and late gestation.

A summary of nutritional models in sheep is shown in Table 1.4. The majority of the studies utilise some form of caloric restriction. Some studies have the ewe's diet as the only intervention whereas some others have a 'double hit', with a restricted maternal diet followed by a further intervention in the lambs' diet later in life.

Several studies have considered the effect of an energy restricted diet on the hypothalamic-pituitary-adrenal (HPA) axis. One study (Long et al., 2010) which used a moderate restriction (50% of metabolisable energy, ME) during mid-gestation found little effect on the HPA axis, although fertility was reduced compared to the control group. Other studies with the same dietary restriction in the first 30 days of gestation reported a 50% increased cortisol: adrenocorticotrophic hormone (ACTH) area under the curve in females only (Poore et al., 2010) and an increase in the HPA axis in females only (Chadio et al., 2007). However, the latter study found no significant differences when the dietary restriction was later in pregnancy (day 31 – 100). A severe restriction (down to 3% ME) for 10 – 20 days at the start of the third trimester resulted in a 35% increase in (ACTH) output in response to a challenge (Bloomfield et al., 2003).

A number of studies have considered the effect of energy restriction on metabolism and energy balance. These have tended to show fairly consistent results, with a reduction in glucose tolerance in the offspring at 8 – 10 months of age, whether the intervention was prior to conception and in early gestation (Todd et al., 2009) or in mid gestation (Ford et al., 2007). In addition, insulin sensitivity was reduced whether the dietary intervention was early in gestation (Poore et al., 2007), mid-late (Rhodes et al., 2009) or confined to late gestation (Gardner et al., 2005). However, two studies have showed little to no effect on glucose metabolism and energy balance (Sebert et al., 2009; Hyatt et al., 2011).

Changes in ME (either a reduction or an increase) along with a high selenium diet have been demonstrated to reduce microvasculature in the fetal jejunum (Neville et al., 2010),

decrease mitochondrial activity and VO_{2max} (Jorgensen et al., 2009), and increase birth weight and perirenal fat at six months of age (Vonnahme et al., 2010). A 50% ME diet early in gestation resulted in increased fetal blood pressure of $3 - 8 \pm 2.5$ mmHg (Edwards et al., 2002), increased pulse pressure of 5 ± 1 mmHg at one year of age (Gardner et al., 2004), increased blood pressure (10 ± 2 mmHg) at three years (Gopalakrishnan et al., 2004), and reduced renal function at one year (Williams et al., 2007).

A milder energy restriction where ewes were fed 70% ME resulted in increased renal IGF-1 (MacLaughlin et al., 2010). However, one study (Braddick et al., 2011) showed no effect on nephron number or kidney weight for either days 1 – 31 or 104 – 127 gestation, and although a study with the period of restriction in mid-gestation (Gopalakrishnan et al., 2005) reported a 40% decrease in nephron number, this did not have a detrimental effect on blood pressure at six months of age; indeed, there was a trend for the nutrient restricted group to have lower blood pressure.

1.6.2.3.2 Low protein

Using a maternal low protein diet that is isocaloric to the control diet is a commonly used dietary intervention in rodent models. It is perhaps more relevant than the global restriction model for westernised populations, where a mismatch of nutrients is more common than a deficiency in energy intake.

There is strong evidence from animal studies that certain organs, such as the kidney, are particularly vulnerable to structural changes in response to adverse conditions *in utero*. Low protein diets in rat studies have been demonstrated to result in smaller kidneys with fewer nephrons (Langley-Evans et al., 1999), misshapen kidneys that are shorter and wider (Nwagwu et al., 2000) and fewer nephrons (Vehaskari et al., 2001) and hypertension (Langley and Jackson, 1994). Fetal programming is also associated with altered renal

function. Maternal low protein rat offspring have been found to have lower creatinine clearance, and higher blood urea and urinary albumin excretion (Nwagwu et al., 2000), reduced glomerular filtration rate (Woods et al., 2001), and increased urinary excretion of vasoactive prostaglandins (Sherman et al., 1999).

Although the low protein model is well established in rats, such dietary interventions have been rarely used in large animal studies. A low protein diet throughout gestation in the pig resulted in increased genes in lipid transport, glucose and fatty acid metabolism, and apoptosis pathways in adipose tissue (Sarr et al., 2010). Also in pigs, a low protein diet early in gestation led to lower expression of housekeeping genes with reduced histone modifications in the kidney (Denisenko et al., 2011). In cows, a low protein diet in the third trimester led to altered leptin and IGF receptors in adipose tissue (Micke et al., 2011a) and increased cross sectional areas in *longissimus dorsi* muscle (Micke et al., 2011b). However, no studies to date have currently considered the effect of a maternal low protein diet in sheep.

1.6.2.3.3 Other diets

Less commonly, other dietary interventions have been used but these tend to show similar programming effects. A high salt diet in ewes led to reduced vasopressin and increased aldosterone (Digby et al., 2010) and over nutrition (150% ME) to decreased insulin signalling to *longissimus dorsi* muscle at 22 months of age (Yan et al., 2011). A methyl deficient diet in pregnant sheep (for 56 days prior to conception to day six of gestation) also led to reduced insulin sensitivity in males only, and to an increase in blood pressure of 11 ± 2 mmHg (Sinclair et al., 2007). Ethanol administration in ewes from days 95 – 133 gestation resulted in increased cardiomyocyte and left ventricular volumes in day 133 fetuses (Goh et al., 2011) and also to an 11% reduction in nephron number, although there was no change in kidney weight, nor in renin angiotensin system (RAS) gene expression (Gray et al., 2008).

Therefore maternal malnutrition is more significant than any specific deficit, and it is the lack of ideal balance of nutrients that is more important than whether it is caused by a reduction in energy, protein, etc., or even an increase in energy, salt, or ethanol. The wide range of different maternal diets in sheep and the effects observed are shown in Table 1.4.

Table 1.4: Studies of nutritional models in sheep

Reference	Experimental diet	Timing	Results: effects on offspring
<i>Nutritional models: ewe intervention only</i>			
Yan et al, 2011	Ewe: 150% ME	-60 – 121 postnatal	↓ downstream insulin signalling in <i>longissimus dorsi</i> muscle at 22 months
Todd et al, 2009	Ewe: 10-15% weight loss	-61 to 30 days gestation	↓ glucose tolerance at 10 month
Hernandez et al, 2009	Ewe: 10-15% weight loss	-61 to 30 days gestation	altered behavioural responses
Edwards et al, 2002	Ewe: 70% ME	-60 to 7 days gestation	↑ fetal blood pressure (3 - 8 ± 2.5 mmHg)
Sinclair et al, 2007	Ewe: Methyl deficient diet	-56 to 6 days gestation	↓ insulin sensitivity (males) at 2 yr; ↑ Blood pressure (11 ± 2 mmHg)
Maclaughlin et al, 2010	Ewe:70% ME	-42 – 7 days gestation	↑ renal IGF1
Smith et al, 2010	Ewe:70% ME	-28 – 7 days gestation	No effect on adrenal function; changes in GTT response in males at 10 weeks
Neville et al, 2010	Ewe: High selenium; 40% or 140% ME	-21 – 147 days gestation 64 – 147 days gestation	↓ microvasculature in the fetal jejunum
Torrens et al, 2009	Ewe: 50% ME	-20 – 15 days gestation	↑ vasoconstrictor response in some arteries, but not in others
Chadio et al, 2007	Ewe: 50% ME	0 to 30 days gestation 31 - 100	↑ HPA axis in female vs. Male Little to no effect
Vonnahne et al, 2010	Ewe: High selenium; 40% or 140% ME	0 – 147 days gestation 50 – 147 days gestation	↑ birth weight and perirenal fat at 6 months for high selenium groups
Meyer et al, 2010	Ewe: High selenium; 40% or 140% ME	0 – 147 days gestation 50 – 147 days gestation	Little or no effect at day 21
Digby et al, 2010	Ewe: high salt	0 – 147 days gestation	↓ AVP; ↑ aldosterone

Reference	Experimental diet	Timing	Results: effects on offspring
Gardner et al, 2004	Ewe: 50% ME	1 to 30 days gestation	↑ adult pulse pressure at 1 yr (5 ± 1 mmHg)
Gardner et al, 2006	Ewe: 50% ME	1 to 30 days gestation	↓ baroreflex sensitivity
Braddick et al, 2011	Ewe: 50% ME	1 – 31 days gestation 104 – 127 days gestation	No effect on nephron number or kidney weight
Gopalakrishnan et al, 2004	Ewe: 50% ME	1 to 95 days gestation	↑ adult blood pressure at 3 yr (10 ± 2 mmHg)
Erhard et al, 2004	Ewe: 50% ME	1 to 95 days gestation	altered behavioural responses
Ford et al, 2007	Ewe: 50% ME	28 to 78 days gestation	↓ (~15%) glucose tolerance at 8 month
Long et al, 2010	Ewe: 50% ME	28 to 78 days gestation	Little effect on HPA axis; ↓ fertility
Gopalakrishnan et al, 2005	Ewe: 50% ME	28 to 80 days gestation	↓ Blood pressure (at 6 mth, 8 ± 3 mmHg). ↓ (40 ± 12 %) nephron number
Sharkey et al, 2009	Ewe: 50% ME	28 – 80 days gestation 110 -147 days gestation	Changes in adipose tissue inflammatory profile at 6 months
Hyatt et al, 2011	Ewe: 50% ME	30 – 80 days gestation	Little to no effect on glucose metabolism and energy balance
Dwyer et al, 2003	Ewe: 65% ME	28 to 147 days gestation	↓ ewe-lamb bonding behaviour at birth
Eckersall et al, 2008	Ewe: 70% ME	65 to 125 days gestation	↓ immune response to vaccination
Goh et al, 2011	Ewe: ethanol administration	95 – 133 days gestation	↑ cardiomyocyte & left ventricular volume in fetuses
Grey et al, 2008	Ewe: ethanol administration	95 – 133 days gestation	↓(11%) nephron number; no change in RAS gene expression or kidney weight
Bloomfield et al, 2003	Ewe: 3% ME	105 to 125 days gestation	↑ (35%) ACTH output to challenge

Reference	Experimental diet	Timing	Results: effects on offspring
Smith et al, 2009	Ewe: propylene glycol	110 to 147 days gestation	↑ birth weight (5%) plasma glucose and postnatal growth rates up to 12 weeks
Sebert et al, 2011	Ewe: 60% ME	110 to 147 days gestation	↑ insulin; ↓ triglycerides
Gardner et al, 2005	Ewe: 50% ME	110 to 147 days gestation	↓ (first phase) insulin sensitivity at 1 year
Nutritional models: ewe and lamb intervention			
Poore et al, 2007	Ewe: 50% ME Lamb: 85% growth	0 – 30 days gestation 3 – 6 months	↓ (30%) insulin sensitivity
Poore et al, 2010	Ewe: 50% ME Lamb: 85% growth	0 – 30 days gestation 3 – 6 months	↑ (50%) cortisol:ACTH AUC in females
Cleal et al, 2007	Ewe: 50% ME Lamb: 85% growth	0 – 30 days gestation 3 – 6 months	↑ RAAS response (5 – 8 ± 2mmHg) to furosemide at 2.5yr
Jorgensen et al, 2009	Ewe: 50% ME Lamb: high-fat	0 – 30 days gestation 0 – 6 months	↓ (40%) mitochondrial activity & VO _{2max}
Lloyd et al, 2012	Ewe: 50% protein Lamb: 1.5 ME	0 – 65 gestation 18 – 24 months	↓ renal function at 2 yr; ↓ angiogenic factors in fetus
Williams et al, 2007	Ewe: 50% ME Lamb: Ad lib	1 – 75 days gestation 3 – 12 months	↓ renal function at 1 yr
Chan et al, 2009	Ewe: 50% ME Lamb: Ad lib	1 – 75 days gestation 3 – 12 months	↑ triglyceride in left ventricle; ↓ ACC, PPAR _γ , FABP3 at 1 yr
Sebert et al, 2009	Ewe: 50% ME Lamb: Ad lib	1 – 75 days gestation 3 – 12 months	Subtle effects on energy balance
Sebert et al, 2010	Ewe: 50% ME Lamb: Ad lib	30 – 80 days gestation	FTO gene ↑ hypothalamus ↓ heart and no change in liver, kidney, pancreas at 7 days or 1yr
Rhodes et al, 2009	Ewe: 70% ME Lamb: 1.5ME	65 – 147 gestation 18 – 24 months	↑ peak insulin to GTT at 1.5yr

ACC, acetyl coenzyme A; ACTH, adrenocorticotrophic hormone; AUC, area under the curve; AVP, arginine vasopressin; FABP3, fatty acid binding protein 3; FTO, fat mass and obesity associated; GTT, glucose tolerance test; HPA, hypothalamic-pituitary axis; IGF1, insulin-like growth factor 1; ME, metabolisable energy; PPAR_γ, peroxisome proliferator-activated receptor gamma; RAS, renin-angiotensin system

1.7 Mechanisms of programming

The processes of proliferation and differentiation within a developing fetus require oxygen and nutrients, and restricting nutrient intake may lead to fewer cells within a particular tissue or changes in the balance between cell types (Gopalakrishnan et al., 2005). This can cause disruption of, or changes to, organ size and function, and metabolic and/or endocrine control (Barker, 1998). Inadequate nutrition may lead to increased demands on functional units such as nephrons and although demand may be met initially, this may be at the cost of dysfunction later in life. Altered signalling between cell types could disturb homeostatic mechanisms, and changes in gene expression could lead to changes in cell types which are unable to respond appropriately to the adult environment (Langley-Evans, 2006). Under-nutrition may also lead to permanent changes in gene expression via epigenetic mechanisms, which may programme altered endocrine or physiological function in later life (Barker, 1998; Langley-Evans, 2006).

The mechanisms and systems involved in programming are likely to be multi-factorial and some brief examples are set out in the following paragraphs.

1.7.1 Hormones and growth factors

1.7.1.1 Angiogenic factors

A traditional early marker for CKD (i.e. before overt clinical signs are present) is microalbuminuria, which can robustly predict future CKD (Fox et al., 2010). A reduced filtration barrier, allowing small amounts of albumin into the urine, is likely to be due initially to effacement of the podocyte foot processes, the integrity of which is supported by a number of factors, but primarily VEGF (Eremina et al., 2008a). VEGF is an important signalling protein that regulates endothelial cell growth and differentiation and is crucial for initiation of angiogenesis (Breier et al., 1992). For example, obesity leads to local hypoxia in

adipose tissue as the adipocytes expand and become distanced from the microvasculature and it has been shown that obesity leads to a tissue specific increase in VEGF, to support development of the new vascular network (Wood et al., 2009). In obese sheep, the increased adipose tissue mass around the kidneys has been shown to be positively associated with increased intra-renal endoplasmic stress, a potential early prognostic marker for CKD (Sharkey et al., 2009a).

The glomerular filtration barrier is comprised of endothelial cells, the basement membrane and visceral epithelial cells called podocytes, and is supported by mesangial cells (Rask-Madsen and King, 2010). The endothelial cells contain fenestrae through which plasma and small molecules can pass (Bray et al., 1999). The filtrate then passes across the glomerular basement membrane and through the slit diaphragm which is formed by interlinked foot processes from the podocytes (O'Callaghan, 2009). VEGFA is highly expressed in podocytes and plays an important role in both establishing and maintaining this barrier (Eremina et al., 2003). Mouse knockout models that are homozygous for podocyte-specific VEGFA die within a day of birth due to an absence of endothelial or mesangial cells in the mature glomeruli (Eremina et al., 2006). Heterozygous conditional knockout mice have proteinuria and ESRD by 12 weeks of age (Eremina et al., 2003). The cause of this dysfunction is multifactorial and includes a loss of endothelial cell fenestrations, a reduction in podocyte foot processes, and loss of mesangial cells (Eremina et al., 2003; Eremina et al., 2006).

Another study showed that mice treated with a VEGF receptor inhibitor for 1 – 3 weeks had significant regression of small capillaries in multiple organs, and in addition, those that remained had fewer fenestrations (Kamba et al., 2006). Within a week of stopping the administration of the inhibitor, VEGF levels increased and capillaries began to repair.

A similar finding has been observed in humans who were treated with bevacizumab, which is an anti-VEGF monoclonal antibody used to treat various types of cancer through reducing

angiogenesis (Ferrara, 2004). A study identified patients on this drug who suffered from the side effects of proteinuria, hypertension and renal failure, and through examination of renal biopsies they found thrombosis in capillaries and arterioles due to endothelial injury (Eremina et al., 2008). Taken in conjunction with the rodent studies above it would appear that VEGF is not only involved in angiogenesis and formation of the vascular structure in the developing kidney (Tufro et al., 1999), but it also is critical for maintenance and repair later in life.

Many other factors such as basic FGF and angiopoietin have complementary roles in vascular development (see 1.4.4). Other fetal nutritional studies have also demonstrated reduced VEGFA in response to a prenatal sub-optimal diet in non-human primates (Cox et al., 2006; Nijland et al., 2007); therefore angiogenic factors may have an important programming role.

1.7.1.2 Glucocorticoids

Glucocorticoids are involved in many key processes including gluconeogenesis, the regulation of gene expression and the renin-angiotensin system, and are thought to mediate some of the effects of maternal diet on the developing organs of the fetus (Edwards et al., 1993).

The glucocorticoid cortisol is about 1000 times higher in maternal circulation than in fetal circulation, despite being able to diffuse freely across the placenta (Langley-Evans, 2006). The placental enzyme 11-beta hydroxysteroid dehydrogenase (11 β -HSD) is thought to play a key role in protecting the fetus from overexposure to maternal cortisol (Bertram et al., 2001; Whorwood et al., 2001). In the rat, the effects of a maternal low protein diet led to reduced birth weight, hypertension and metabolic dysfunction and are thought to be mediated by the inhibition of placental 11 β -HSD (Langley-Evans and Nwagwu, 1998;

Bertram et al., 2001). However, these effects may be sex-specific. One study (McMullen and Langley-Evans, 2005a) observed that offspring of maternal low protein rats had higher systolic blood pressure at 4 weeks of age than the control group, however, this appeared to be dependent on glucocorticoids in males but not females.

1.7.2 Renin-angiotensin system

The RAS regulates long-term blood pressure and the volume of extracellular fluid, through the actions of renin, angiotensin I and II, and aldosterone (Bray et al., 1999). This system has been studied extensively as it plays a potential role in the mechanisms underlying the programming of hypertension in animal models. Angiotensin II is considered to be the main effector hormone of the RAS, promoting vascular smooth muscle growth and inflammation, vasoconstriction, and sodium reabsorption (Ruiz-Ortega et al., 2000). Angiotensin II works through cell surface receptors called AT₁ and AT₂, and current evidence suggests an alteration in the balance between these receptors may be involved in the programming effects of maternal diet on postnatal renal function and blood pressure control (McMullen and Langley-Evans, 2005b). In addition, the deletion of any genes involved in RAS leads to offspring with kidney abnormalities (Matsusaka et al., 2002), and in fetal sheep the RAS system is essential for the maintenance and regulation of fetal GFR (Lumbers, 1995).

1.7.3 Inflammatory factors

Whilst angiotensin II and AT₁/AT₂ receptors are well characterised regulators of blood pressure, emerging evidence suggests their involvement in the inflammatory process too. One common feature of renal disease is monocyte infiltration (Ruiz-Ortega et al., 2000). Angiotensin II has a direct role in the inflammatory response by activation of Nuclear Factor Kappa B (NF-κB) in the kidney via the AT₁ and AT₂ receptors, and increases degradation of IκB-α, which is an inhibitor of NF-κB (Ruiz-Ortega et al., 2000). AT₁ also regulates monocyte

chemoattractant protein-1 (MCP-1) via the NF- κ B pathway (Esteban et al., 2004). Some studies have demonstrated interactions between NF- κ B, I κ B- α and MCP-1. Angiotensin II activates NF κ B directly via AT₁ and AT₂ receptors and indirectly via its metabolite angiotensin IV, which results in increased MCP-1 (Esteban et al., 2005). Vascular injury in mice resulted in increased NF- κ B and MCP-1 and a decrease in I κ B- α (Gao et al., 2007), and in humans with diabetic nephropathy there was a strong positive correlation between NF κ -B and MCP-1 levels (Mezzano et al., 2004). Nutritional intervention studies have shown a reduction in the innate inflammatory response in the kidney (Sharkey et al., 2009b) and altered the adipose tissue inflammatory profile (Sharkey et al., 2009c).

1.7.4 Apoptosis

The process of programmed cell death, or apoptosis, is a normal part of tissue development and maintenance. However, evidence shows that programming may result in an abnormally increased level of apoptosis. Intrauterine growth restriction in rats resulted in altered mRNA levels for apoptosis-related proteins, increased renal apoptosis and reduced nephron number (Pham et al., 2003). A maternal low protein diet in rats reduced nephron number, increased systolic blood pressure and increased apoptosis in kidneys at eight weeks of age, despite the kidneys looking morphologically normal (Vehaskari et al., 2001). In fetal kidneys an increase in apoptosis in rat kidneys at embryonic day 13 was observed for those fed a low protein diet (Welham et al., 2002).

1.7.5 Epigenetics

Epigenetic modifications result in functional changes to the genome without any change in the underlying nucleotide sequence and are initiated by, for example, DNA methylation or histone acetylation (Ozanne and Constancia, 2007).

The methionine-homocysteine pathway and concomitant generation of 1C methyl groups is dependent on an adequate provision of amino acids, plus the vitamins B6, B12 and folic acid being provided through the diet (Geissler and Powers, 2011). Rats exposed to a MLP diet plus 1 mg/kg folic acid (to offset the dietary reduction) had lower glucocorticoid receptor and PPAR gene methylation and higher mRNA levels than the control group (normal protein plus 1 mg/kg folic acid; Lillycrop et al., 2005). A further group was subjected to the low protein diet but with 5 times the amount of folic acid, and there was no difference between this group and the controls, suggesting that the additional folic acid ameliorated the effects of the low protein diet. A further study (Sinclair et al., 2007), which restricted folate, B12 and methionine during the periconceptual period, concluded that this modest dietary restriction led to adult offspring with altered immune responses to antigenic challenge, decreased insulin sensitivity and elevated blood pressure, and that these changes were more evident in males.

1.8 Aims and Hypotheses

1.8.1 Aims

This programme of work aims to characterise the effect of a low protein maternal diet during early or late gestation on the ewe, the fetus at day 65 gestation, and the offspring at two years of age, through assessment of metabolic variables, kidney development and renal function. A further aim is to determine if any changes observed in the fetus or offspring are sex-specific.

1.8.2 Hypotheses

1. A maternal low protein diet will have little or no effect upon the nutritional status of the ewe.
2. Restriction of maternal protein intake during early or late gestation will impact upon fetal renal development resulting in a reduced nephron endowment.
3. The different timing of the low protein diet during gestation will lead to different phenotypes.
4. Later in life a nephron deficit coupled with an obesogenic environment will leave the adult offspring with a functionally compromised kidney.
5. Fetal programming of renal disease in the sheep is sex-specific.

2. Methods and Materials

2.1 Summary

All methods were carried out solely by Louise Lloyd unless acknowledged otherwise below:

- The diet was formulated by Dr Stewart Rhind, Macauley Institute, Aberdeen.
- Animal care and sample collection for the first 18 months of study 1 were carried out by Dr Rhind and his team, and for the period from 18 – 24 months by Dr David Gardner and Phillip Rhodes at the University of Nottingham. At 24 months Louise Lloyd assisted Dr Gardner with gamma scintigraphy as part of a Physiological Society summer studentship.
- Dr David Gardner was responsible for animal care and sample collection for Study 2. Professor Kevin Sinclair and Louise Lloyd assisted at post mortem.

Further details of study design and animal procedures are found at section 2.2.

- Urine and plasma metabolites and protein were measured using an autoanalyser and colorimetric assay kits (section 2.3)
- Maternal and fetal plasma amino acids were analysed by Louise Lloyd, but the amniotic fluid was measured by Dr Dongfang Li, University of Nottingham (sections 2.3.3 – 4)
- Histological stains were used to examine kidney sections for morphological differences (section 2.5)
- Manual and automated immunohistochemistry methods were used to determine protein localisation (section 2.6)
- TUNEL staining (with assistance from Tom Foster) was used to detect DNA fragmentation, as an indicator of apoptosis (section 2.7)

- Lectin staining was carried out to try to identify endothelial cells in kidney sections (section 2.8)
- Image analysis software was used to quantify glomerular area, TUNEL and immunohistochemical staining (section 2.9)
- Nephron number, total and mean glomerular volume were estimated stereologically (section 2.10), and a range of mammalian nephron numbers analysed to predict a value for sheep (section 2.11)
- Gene expression was measured using Quantitative PCR (section 2.12)
- Corrosion casts were made at post mortem of fetuses in Study 2 by Dr David Gardner and Professor Kevin Sinclair. Weights were measured by Louise Lloyd, and surface area and volume were quantified using a CT scanner operated by Craig Sturrock, University of Nottingham (section 2.13)
- Osmolality was measured using a cryoscopic osmometer (section 2.14)
- Power calculations and statistical analysis were performed using Genstat v13 (section 2.15).

2.2 Study Design

All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 and were approved by the relevant local ethical review committees of the Macaulay Research Institute and the University of Nottingham.

All chemicals, reagents and equipment were purchased from Fisher Scientific, Loughborough, UK unless stated otherwise.

2.2.1 Study 1

2.2.1.1 Macauley Institute, Aberdeen: 0 – 1.5 years of age

One month prior to mating Scottish Blackface ewes were barn housed in order to improve and align their body condition scores. Two weeks prior to mating a progesterone sponge was inserted into the vagina to induce estrus, and removed the day before mating with Scottish Blackface rams. Maternal weight and body condition score was measured 12 times; four times before mating and eight times during pregnancy. Thirty-eight pregnant Scottish Blackface ewes carrying singleton fetuses were randomly assigned to one of three diet groups fed either a control diet providing adequate protein (control protein, CP; n = 15), a low protein diet during early gestation from day 0 - 65 (low protein early, LPE; n = 17) or a low protein diet during late gestation from day 65 to term, 147 d (low protein late, LPL; n = 6). The protein and energy content of the diets are shown in Table 2.1 below.

Table 2.1: Energy and protein composition of the diets

Diet	Protein (g.kg⁻¹ crude protein)	Energy (MJ.kg⁻¹ DM)	Protein:energy ratio (g.kg.MJ DM⁻¹)
CP	180	10.6	16.9
LPE	80	9.2	8.7
LPL	80	9.2	8.7

CP, control protein; LPE, low protein early, day 0 – 65 gestation; LPL, low protein late, day 65 – term, DM, dry matter

On an as fed basis, the diets were isocaloric with the effective level of protein restriction being 8.7 vs. 17 g crude protein.MJ⁻¹ ME (metabolic energy). At day 65 gestation (chosen to coincide with the mid-point of nephrogenesis in the sheep) a randomly selected sub-group of CP (n = 9) and LPE (n = 10) ewes were euthanized (Study 1A, Figure 2.1), and fetal kidneys snap frozen in liquid nitrogen and stored at -80°C for further analysis. The remainder (CP, n = 6; LPE, n = 7; LPL, n = 6) carried to term and the offspring were delivered naturally (Study 1B, Figure 2.1).

After birth, ewes were offered 1.5 kg of feed and unlimited hay, to ameliorate the effects of the diet during gestation on milk quality during lactation. All male lambs were castrated. At two weeks of age the lambs were vaccinated and then lambs and ewes were put to pasture. Ewes were fed 1 kg.day⁻¹ feed pellets (North Eastern Farmers, Aberdeen, UK) for 4 weeks, and then 0.6 kg.day⁻¹ until weaning at ten weeks of age. Every three weeks, weights and body condition scores were recorded for both ewe and lamb, and a blood sample taken from the jugular vein of the lambs. The sheep were at pasture with the feed supplement until euthanised (ewes; a lethal dose of sodium pentobarbital followed by exsanguination) or transferred to the Sutton Bonington Campus of the University of Nottingham at 1.5 years of age (offspring).

2.2.1.2 University of Nottingham, Sutton Bonington: 1.5 – 2 years of age

On arrival at the University of Nottingham, the offspring were weighed and had their body condition score assessed. After a brief period of acclimatisation of four weeks the sheep were exposed to an obesogenic environment, specifically designed to encourage weight gain, being fed to 150% ME requirements, and barn housed to restrict physical activity, and reduce energy expenditure needed for thermogenesis. All animals were weighed weekly to monitor the weight gain, and the endpoint was an approximately 50% weight gain, as this represented a theoretical gain in body mass index (BMI) from 22.5 to 32.5 kg.m⁻², i.e. from a normal to an obese BMI (World Health Organisation, 2000). After six months (at two years of age) *in vivo* renography was performed on all animals, with the sheep being subsequently euthanized (one week later) by electrocortical stunning with exsanguination. Urine was sampled from the bladder at *post mortem*. The right kidney was snap frozen in liquid nitrogen and stored at -80°C and the left fixed in neutral buffered formalin (NBF), rinsed in 0.02 M phosphate buffered saline (PBS) for 24 h and then stored in 70% ethanol at room temperature.

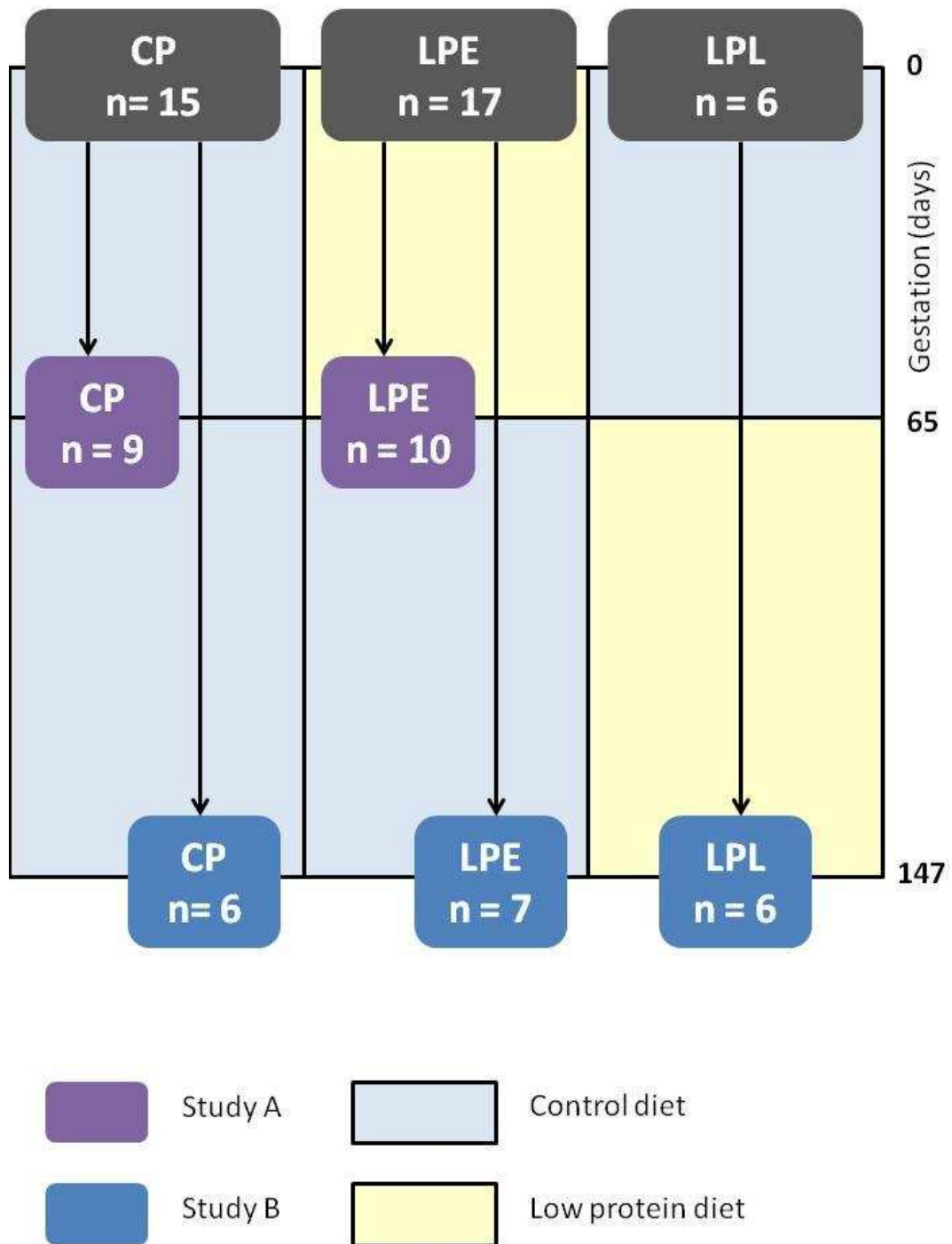


Figure 2.1: Experimental design for Study 1

CP, control protein; LPE, low protein early; LPL, low protein late.

2.2.2 Study 2

Fifty-six Scottish Blackface ewes were weighed, body condition scored (1 = very thin to 5 = obese) and gradually acclimatised to the environment at Sutton Bonington over a one-month period, prior to a baseline blood sample being taken by venepuncture. Over this period, sheep were fed 600 g concentrate with unlimited hay being available. Ewes were oestrus synchronised using progesterone sponges and after 14 days were artificially inseminated by laparoscopy with semen from a Scottish Blackface ram. This was designated as day 0 of pregnancy and at this time, an equal number of ewes were randomly allocated to a control or low protein diet. The sheep were fed these diets (900 g pellets with 100 g hay) until day 65 gestation. On two occasions (days 17 and 18) pregnancy was confirmed by measurement of progesterone (Ridgeway Science, UK) and non-pregnant ewes were returned to stock. Ewe weights, body condition score and blood samples were collected on four further occasions to day 65.

From 56 ewes, seven were found to be non-pregnant, and upon completion of the study and retrieval of the products of conception it was found that 18 ewes carried either triplet or singleton pregnancies. Only twin-bearing ewes ($n = 31$) were entered into the study for analysis. Hence, thirty-one pregnant Scottish Blackface ewes carrying twin fetuses ($n = 62$ fetuses) were randomly assigned to one of two diet groups fed either a control diet providing adequate protein (control protein, CP; $n = 15$), or a low protein diet during early gestation from day 0 - 65 (low protein early, LPE; $n = 16$). The study design is summarised in Figure 2.2. The protein and energy content of the diets are shown in Table 2.1 above.

Blood samples were taken from the ewe prior to mating, and at days 28, 42 and 65 of pregnancy. The blood was withdrawn into K^+ -EDTA tubes, centrifuged at 3000 rpm (800 g) for 10 min, and the resulting plasma taken and stored at -20°C . At day 65 of gestation the sheep were euthanized by barbiturate overdose and the twin fetuses retrieved. Fetal blood

samples were withdrawn from the umbilical artery into K⁺-EDTA tubes, centrifuged and the plasma removed and stored at -20°C. Amniotic fluid was sampled from each fetus and stored at -20° C. One twin from each pair was corrosion cast (CP, n = 6 males and n = 8 females; LPE, n = 8 males and n = 7 females), and from the other twin (CP, n = 8 males and n = 8 females; LPE, n = 9 males and n = 8 females) the right kidney was snap frozen in liquid nitrogen and stored at -80°C and the left fixed in 4% w/v paraformaldehyde, rinsed in 0.02 M PBS for 24 h and then stored in 70% ethanol at room temperature.

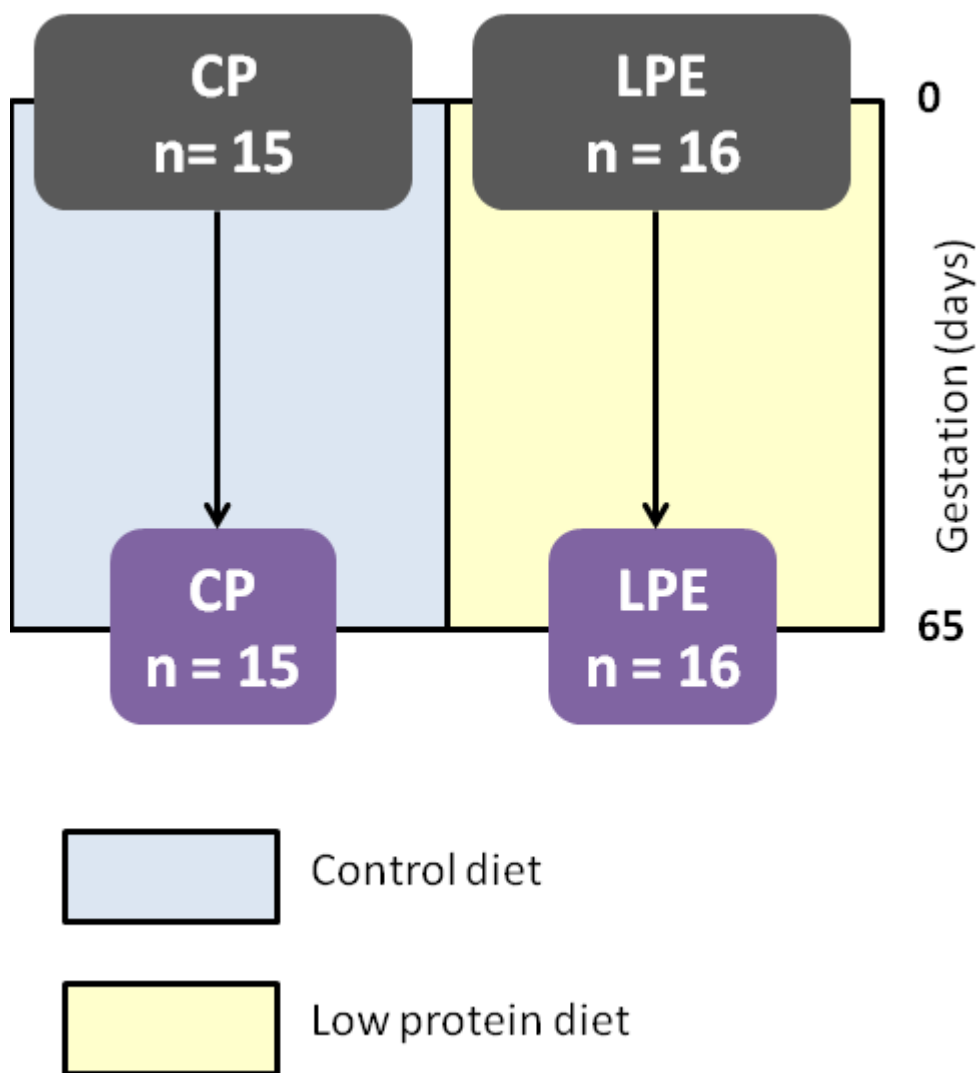


Figure 2.2: Experimental design for Study 2

CP, control protein; LPE, low protein early

2.3 Blood and Urine Analysis

2.3.1 Urine Analysis

Urine from adult offspring at two years of age was collected from the bladder at post-mortem and albumin (g.l^{-1}) and creatinine ($\mu\text{mol.l}^{-1}$) were measured using a Randox Rx Imola autoanalyser and quantitative colorimetric assay kits (Randox Laboratories, Co Antrim, UK).

2.3.1.1 Albumin

Albumin levels were determined using a Randox albumin kit. Albumin has the ability to bind to bromocresol green in a quantitative manner. The albumin-bromocresol green complex can then be detected as it absorbs maximally at 578 nm.

2.3.1.2 Creatinine

Creatinine levels were determined enzymatically using a Randox creatinine kit. Creatinine deaminase converts creatinine and H_2O to N-Methylhydantoin and ammonia. Glutamate dehydrogenase catalyses ammonia and α -ketoglutarate in the presence of NADPH to form glutamate and NAD^+ . The absorbance decrease (at 340 nm) due to NADPH reduction is proportional to the concentration of creatinine.

2.3.2 Plasma Analysis: Randox Imola

All two-year old offspring plasma samples from Study 1 were defrosted and analysed for sodium, potassium and chloride using a Randox Rx Imola autoanalyser and quantitative colorimetric assay kits (Randox Laboratories, Co Antrim, UK). All maternal plasma samples from Study 2 were defrosted and analysed for glucose, non-esterified fatty acids (NEFA), triglycerides, albumin, total protein, urea, lactate and D-3-hydroxybutarate. Fetal plasma

samples from Study 2 were defrosted and analysed for glucose and lactate only. All concentrations were measured in mmol.l^{-1} unless stated otherwise.

2.3.2.1 *Sodium*

Sodium concentrations were determined enzymatically using a Randox sodium kit. O-nitrophenol- β -D-galactopyranoside (OPNG) is added to the plasma. B-galactosidase hydrolyses sodium + OPNG to form o-nitrophenol, which is yellow. The absorbance of the yellow product is measured at 405 nm.

2.3.2.2 *Potassium*

Potassium was determined enzymatically using a Randox potassium kit. Pyruvate kinase reacts with phosphoenolpyruvate to yield pyruvate. The pyruvate reacts with NADH in the presence of lactate dehydrogenase to form lactate and NAD. This causes a decrease in absorbance at 340 nm which is proportional to the concentration of potassium.

2.3.2.3 *Chloride*

Chloride concentrations were determined using a Randox chloride kit, using a thiocyanate method. Mercurous thiocyanate reacts with the chloride ions to form mercury perchlorate and thiocyanate. The thiocyanate forms a red complex with ferric chloride and the absorbance of the red dye is measured at 500 nm.

2.3.2.4 *Glucose*

Glucose concentrations were determined using a Randox glucose kit, which uses the hexokinase method. The action of the hexokinase enzyme results in D-glucose being phosphorylated with an ATP molecule to form glucose-6-phosphate. Glucose-6-phosphate

dehydrogenase catalyses G6P in the presence of NADP, to form 6-phosphogluconate and NADPH. The absorbance of NADPH is measured at 340 nm.

2.3.2.5 NEFA

NEFA concentrations were determined using a Randox NEFA kit. The enzyme acyl-CoA synthetase is added to plasma and allows NEFA to form thiol esters of coenzyme A (acyl-CoA) in the presence of adenosine triphosphate (ATP), magnesium cations and CoA. Hydrogen peroxide is produced when Acyl-CoA is oxidised by acyl-CoA oxidase. Peroxidase enzymes then catalyse 3-methylN-(hydroxyethyl)aniline with 4-aminoantipyrine in the presence of hydrogen peroxide to form a purple coloured adduct which is maximally absorbed at 550 nm.

2.3.2.6 Triglycerides

Triglyceride concentrations were determined using a Randox triglyceride kit, which utilises the GPO-PAP method. The triglycerides undergo enzymatic hydrolysis using lipases. Peroxidase then catalyses hydrogen peroxide, 4-aminophenazone and 4-chlorophenol to produce quinoneimine, which is maximally absorbed at 540 nm.

2.3.2.7 Albumin

Albumin levels were determined using a Randox albumin kit, see 2.3.1.1.

2.3.2.8 Total protein

Total protein concentration was determined using a Randox total protein kit, and was measured in g.l^{-1} . The kit uses the Biuret method: under alkali conditions peptides form a violet-coloured complex with copper ions. The intensity of the colour, and therefore the absorption at 540 nm, is directly proportional to the concentration of protein.

2.3.2.9 Urea

Urea concentration was determined using a Randox urea kit, utilising a kinetic method. The urea is hydrolysed in the presence of water and the enzyme urease to form ammonia and CO₂. Glutamate dehydrogenase catalyses the ammonia to react with α-oxoglutaric acid and NADH₂, to form glutamic acid and NAD⁺. The absorbance decrease (at 340 nm) due to NADH₂ reduction is proportional to the concentration of urea.

2.3.2.10 Lactate

Lactate concentration was determined using a Randox L-Lactate kit. The kit uses an enzymatic method, where lactate oxidase catalyses the oxidation of L-lactate to form pyruvate and H₂O₂. The H₂O₂ is catalysed with a peroxidase to form a purple dye. The intensity of the dye (absorbed at 550 nm) is proportional to the lactate content.

2.3.2.11 D-3-hydroxybutyrate

D-3-hydroxybutyrate concentration was determined using a Randox ranbut (hydroxybutyrate) kit. This is a kinetic enzymatic method. The enzyme 3-Hydroxybutyrate dehydrogenase oxidises D-3-hydroxybutyrate to form acetoacetate. Concurrently NAD⁺ is reduced to NADH and the associated change of absorbance at 340 nm can be directly correlated with the D-3-hydroxybutyrate concentration.

2.3.2.12 Quality control

Experimental samples were run in duplicate, and each run contained the same two quality control (QC) samples. Inter-assay variation was calculated from the variance from the QC samples, and intra-assay variation from the variance of the duplicate experimental samples. Intra-assay variance ranged from 0.35 – 3.52, and inter-assay from 3.23 – 7.07.

2.3.3 Plasma Analysis: Amino acids

Amino acids were isolated from maternal and fetal plasma and derivatised using the EZ:faast™ Amino Acid Kit (Phenomenex, Macclesfield, UK) according to the manufacturer's protocol.

2.3.3.1 Amino acid extraction

Fifty µl of plasma and 100 µl of internal standard solution (20 nmol norvaline) was pipetted into a preparation vial, and then passed very slowly through a tip containing EZ:faast™ solid phase extraction sorbent using a syringe. Next, 200 µl of washing reagent (sodium carbonate) was passed through the sorbent tip, before 200 µl eluting medium (propranol and sodium hydroxide) was drawn up into the tip and then expelled along with the sorbent containing the amino acids into a clean vial. Fifty µl of chloroform and isooctane were sequentially added to derivatise the amino acids and the resulting organic layer was recovered. The organic layer containing the amino acids was dried down using nitrogen, and the sample redissolved in 100 µl iso-octane:chloroform (4:1 v/v). Where necessary the amino acids were stored at -20°C for up to 12 hours whilst other samples were prepared.

Amino acid standards were prepared using the same method, with 50 µl of EZ:faast standard solutions 1 and 2 added to 100 µl internal standard. A standard was run every 15 samples, to check for consistency.

2.3.3.2 Gas Chromatography – Mass Spectrometry

One µl of each amino acid sample was injected in splitless mode (split closed for 10s) using an AS3000 autosampler (Thermo, Manchester, UK). The injector of the Trace GC Ultra gas chromatograph (Thermo) was kept at 250°C, with a starting oven temperature of 90°C which was increased to 320°C at the rate of 20°C min⁻¹ (transfer line from the oven to mass

spectrometer was 300°C). Helium (8 psi) was used as the carrier gas to elute the amino acids from the ZB-AAA column (10 m x 0.25 mm ID).

The DSQ II mass spectrometer (Thermo) was operated in selected ion mode recording ions 84, 101, 114, 116, 130, 144, 146, 155, 156, 158, 172, 180, 184, 243 and 244 with a dwell time of 0.03s. To calibrate the data the peak areas for the amino acids in the standards and samples were adjusted for variation in the peak area of the internal standard.

2.3.4 Amniotic Fluid: Amino Acids

Dr Dongfang Li carried out this analysis on behalf of the study. In brief, 980 µl of the plasma sample and 20 µl internal standard norleucine were added to a conical centrifuge tube containing 60 mg of solid 5-sulphosalicylic acid (SSA), immediately mixed and allowed to stand for 1 hour at 4°C. Next the mixture was centrifuged for 15 minutes at 4°C in a high speed centrifuge to spin down the precipitate, and the supernatant was removed and filtered through a 0.2 µm filter. Standard solutions of amino acids were prepared in the same way. Finally, 20 µl of treated standards or sample were injected to a Biochrom 20 amino acid analyser (Pharmacia LKB, Biochrom Ltd, Cambridge, UK) and peak integration was performed using EZChrom Elite software. This method successfully measured 19 out of the 20 amino acids.

2.4 Gamma Scintigraphy

This work was carried out during a Physiological Society summer studentship in 2007.

Adult sheep were restrained and stood underneath a gamma camera, and a radioactive nuclide 100 megabecquerels (MBq) technetium⁻⁹⁹ diethylenetriaminepentaacetic acid (Tc^{99m}-DTPA) was injected (*i.v.* 0.5 ml⁻¹ 0.9% NaCl) into the jugular vein. Sixty frames were captured over a 20 min period and a dynamic renogram (adjusted for movement artefact,

background and bolus deconvolution) was produced for the left and right kidney. Specific measurements of interest were:

- time to peak (min), i.e. how quickly the maximum amount of Tc^{99m} -DTPA is detected in the kidney
- upslope (counts per minute, cpm): the slope upwards as the radioactive nuclide increases towards its peak
- downslope (cpm): the downward slope as the nuclide leaves the kidney
- transit-time (min): a composite of all previous measurements, which measures the time the Tc^{99m} -DTPA takes to pass through the kidney.

The time to peak and upslope are indicative of the rate of uptake of tracer into the kidney, whereas the downslope is indicative of the rate of tracer clearance.

In addition, at 3, 4 and 5 h after injection, 5 ml venous blood was drawn from the contralateral jugular cannula, centrifuged in heparinised tubes, and plasma collected. This was used to determine the glomerular filtration rate (GFR) using the method described by (Gleadhill et al., 1999). In brief, duplicate 1 ml plasma aliquots, along with timed control samples were measured on a gamma counter and the GFR determined by the decline in radioactivity over time.

2.5 Histology

2.5.1 *Sample preparation*

Random samples were taken from the NBF or paraformaldehyde-fixed tissues and processed (Tissue processor TP1020, Leica, Germany) by increasing concentrations of ethanol to dehydrate the tissue, followed by clearing with HistoClear, and impregnating with paraffin wax (Table 2.2).

Sections were embedded at random orientations (Paraffin Embedding Center EG1160, Leica) in cassettes containing paraffin wax. All cassettes were given a code unrelated to the animal number so all analyses were blinded. Sections were cut using a microtome (Microtome RM2255, Leica) at 5 μm thick and mounted onto Menzel polysine slides (Fisher Scientific, Loughborough, UK).

Table 2.2: Protocol for processing tissues

Step	Chemical	Time
1	70% ethanol	60 min
2	80% ethanol	60 min
3	95% ethanol	60 min
4	100% ethanol	30 min
5	100% ethanol	30 min
6	100% ethanol	30 min
7	100% ethanol	30 min
8	Histoclear	60 min
9	Histoclear	60 min
10	Histoclear	60 min
11	Paraffin wax	60 min
12	Paraffin wax	90 min

2.5.2 Haematoxylin and Eosin Staining

Sections were stained with Haematoxylin and Eosin (H&E), using a standard protocol. In brief, the sections were dewaxed in histoclear (2 x 5 min), then rehydrated through a series of ethanol baths (100%, 95% and 70% ethanol) for 2 min each, and then rinsed in tap water. They were stained in Harris haematoxylin for 2 min (adult kidney tissue) and 1 min (fetal tissue). After differentiation in 1% alcohol (2 dips) and bluing in ammoniated water (2 dips) the sections were counterstained in eosin (VWR, Lutterworth, UK) for 4 min (adult tissue) or 3 min (fetal), before being dehydrated back through the series of ethanol baths, and cleared in xylene (2 x 5 min). The sections were mounted with glass coverslips using DPX mountant (Sigma-Aldrich, Dorset, UK).

2.5.3 Periodic Acid Schiff's Reagent Staining

Adult kidney sections were dewaxed in histoclear (2 x 5 min), then rehydrated through a series of ethanol baths (100%, 95% and 70% ethanol) for 2 min each, and then rinsed in tap water. They were oxidised in 0.5% periodic acid solution (VWR, Lutterworth, UK) for 5 min. Next the sections were rinsed in water before being stained with Schiff's reagent (VWR, Lutterworth, UK) for 15 min, and then rinsed in tap water for 8 min. The sections were counterstained in haematoxylin for 1 min. After differentiation in 1% alcohol (2 dips) and bluing in ammoniated water (2 dips) the sections were dehydrated back through the series of ethanol baths, and cleared in xylene (2 x 5 min). The sections were mounted with glass coverslips using DPX mountant.

2.5.4 Trichrome Staining

Adult kidney sections were dewaxed in histoclear (2 x 5 min), rehydrated through a series of ethanol baths (100%, 95% and 70% ethanol) for 2 min each and then rinsed in tap water. They were stained in Harris' haematoxylin for 15 min. After differentiation in 1% alcohol (2 dips) and bluing in ammoniated water (2 dips) the sections were placed in phosphomolybdic acid for 2.5 min, then into Orange G for 5 min, before being rinsed in tap water. The sections were put in 1% acetic acid for 2 min, and then in Fast Green for 5 min before being returned to the 1% acetic acid for a further 3 min. The sections were rinsed in 95% ethanol before being dehydrated back through 95% and 100% ethanol baths (3 min each), and cleared in xylene (2 x 5 min). The sections were mounted with glass coverslips using DPX mountant.

2.6 Immunohistochemistry

2.6.1 VECTASTAIN Elite ABC System

Immunohistochemistry (IHC) was carried out using The VECTASTAIN Elite ABC system (Vector Labs, Peterborough, UK). All steps were carried out at room temperature unless stated otherwise. Antibody dilutions are given in the individual experimental chapters.

Random samples were taken from NBF (Study 1) or paraformaldehyde (Study 2) fixed tissues, embedded in paraffin and sectioned at 5 μm (Microtome RM2255, Leica) onto Menzel polysine slides. Slides were coded to allow for blinded analysis.

Slides were de-waxed in xylene (2 x 5 min) and rehydrated through a series of ethanol baths (100%, 95%, 70%) and into distilled water for 5 min each. Next the slides were equilibrated in PBS (Invitrogen Life Technologies, Paisley, UK) for 2 x 5 min. The slide surrounding the sample was carefully dried (taking care to keep the sample hydrated), the sample was circled with a hydrophobic barrier pen (ImmEdge pen, Vector Labs, Peterborough, UK) and the slides were placed onto a shelf in a humidity chamber. Endogenous peroxidases were inactivated using 3% H_2O_2 for 15 min, then rinsed in PBS for 2 x 5 min. An avidin-biotin blocking kit (Vector Labs, Peterborough, UK) was used to block endogenous biotin, with avidin applied for 15 min, rinsed in PBS, then blocked with biotin for 15 min, before being rinsed in PBS again. Non-specific binding was prevented by applying a block of 20% normal goat serum (diluted in PBS) for 30 min. Primary antibodies were diluted in PBS + 0.05% Tween 20 (Sigma Aldrich, Gillingham, UK) and incubated in a fridge overnight at 4°C. The next day unbound antibody was removed by rinsing slides in PBS for 2 x 5 min, before incubating with the secondary antibody (Biotinylated Goat anti-rabbit IgG, Vector Labs) diluted 1:200 in PBS + 0.05% Tween for 30 min. This was followed by 2 x 5 min washes in PBS to remove the unbound secondary antibody. Next the samples were incubated with

Vectastain® Elite ABC Reagent (Vector Labs) for 30 min, before rinsing in PBS (2 x 5 min).

The staining was visualised using a 3,3' diaminobenzidine (DAB+) substrate kit (Vector Labs). To 2.5 ml distilled water, one drop of buffer, two drops of DAB substrate, and 1 drop of H₂O₂ were added. This was applied to each tissue section and developed for 2 min (fetal tissue) and 4 min (adult tissue). When the appropriate intensity of stain was achieved, slides were immersed in running tap water and rinsed for 5 min. The samples were then counterstained using haematoxylin and mounted as described previously (see 2.5.2).

Negative controls were omission of primary antibody, and rabbit IgG. Positive controls for each experiment are listed in the experimental chapters.

2.6.2 BondMax System

Random samples were taken from NBF-fixed tissues, embedded in paraffin and sectioned at 5 µm (Microtome RM2255, Leica) onto Menzel polylysine slides. Slides were coded to allow for blinded analysis.

Samples were stained using the Leica BOND-MAX™ automated system, using Ready-to-use Bond™ reagents (Leica, Wetzlar, Germany). Sections were dewaxed and then antigen retrieval performed for 10 min using EDTA pH9. Protocol X was selected, with a peroxidase block, followed by incubation with primary antibodies diluted in Bond Primary Antibody Diluent at room temperature for 30 min. The secondary antibody, polymer and DAB were applied sequentially for 10 min each. The specimens were counterstained with haematoxylin, then rehydrated with ethanol and cleared with Xylene before being mounted using DPX mountant.

Negative controls were omission of primary antibody, and rabbit IgG. Positive controls are listed in the relevant experimental chapters.

2.6.3 Vascular Endothelial Growth Factor A

The ovine VEGFA protein sequence was checked against the sequences of other large mammals using the CLUSTAL 2.1 multiple sequence alignment tool (European Bioinformatics Institute, 2012) and had high homology (99% with bovine, 96% porcine, 95% equine and 93% human). The VEGFA antibody is a rabbit polyclonal IgG with epitope mapping at the N-terminus of human VEGFA which is identical to ovine VEGFA in that region (see Table 2.3).

Table 2.3: Clustal 2.1 sequence alignment for ovine and human VEGFA

Accession Number	Species	Amino acids
gi 3228693 gi 3719221	Ovis aries Homo sapiens	MNFLLSVWHWSLALLLYLHHAKWSQAAPMAEGG-QKPHEVMKFMVDVYQRS MNFLLSVWHWSLALLLYLHHAKWSQAAPMAEGGGQNHHEVVKFMVDVYQRS ***** *: ***:*****
gi 3228693 gi 3719221	Ovis aries Homo sapiens	FCRPIETLVDFIQEYYPDEIEFIFKPCVPLMRCGGCCNDESLECVPTTEF YCHPIETLVDFIQEYYPDEIEYIFKPCVPLMRCGGCCNDEGLECVPTTEES :*.*****:*****.***.*****
gi 3228693 gi 3719221	Ovis aries Homo sapiens	NITMQIMRIKPHQSQHIGEMSFLQHNKCECRPKKDKARQENPCGPCSERR NITMQIMRIKPHQSQHIGEMSFLQHNKCECRPKKDRARQENPCGPCSERR *****.*****.*****
gi 3228693 gi 3719221	Ovis aries Homo sapiens	KHLFVQDPQTCKCCKNTDSRCKARQLELNERTCRCDKPRR KHLFVQDPQTCKCCKNTHSRCKARQLELNERTCRCDKPRR *****.*****

2.6.4 CD31 and CD34

CD31 and CD34 were selected for their properties as markers of endothelial cells. However, the ovine CD31 and CD34 protein sequences are not available. In such cases it is conventional to use bovine sequences as they tend to have greater homology to ovine. Using the CLUSTAL 2.1 multiple sequence alignment tool (European Bioinformatics Institute, 2012) the homology between bovine CD31 and CD34 and that of other mammals was 100% (human, mouse, pig, horse), therefore it is likely that these proteins are highly conserved between species. As there were no published papers using CD31 or CD34 antibodies in sheep at this time, four antibodies were trialled, for which details are given in Table 2.4.

Table 2.4: Antibody details for CD31 and CD34

Antibody	Abcam ref	Polyclonal or monoclonal	Epitope mapping (amino acid number)	Dilutions
CD31	ab32457	Polyclonal	600 – 700	1:100, 200, 500, 1000, 2000
CD31	ab83959	Polyclonal	700 – C-terminus	1:50, 100, 250, 500, 1000
CD34	ab63985	Polyclonal	1 - 100	1:50, 100, 250, 500, 1000
CD34	ab81289	Monoclonal	C-terminus	1:25, 50, 100, 250, 500, 1000

2.7 TUNEL Staining

Fetal kidney sections were sectioned at 5 µm thick, and the presence of apoptotic nuclei was determined using a *terminal* deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) kit (TdT FragEL™ DNA fragmentation detection kit; Calbiochem, Nottingham, UK) according to the manufacturer's protocol. All steps were undertaken at room temperature, and slides were rinsed between stages with 1x TBS (20 mM Tris pH 7.6, 140 mM NaCl) unless stated otherwise.

Sections were deparaffinised in xylene, and then rehydrated through a series of 100%, 90%, 80% and 70% ethanol baths. The slides were rinsed briefly in 1x TBS, and then the samples circled with a paraffin pen (ImmEdge hydrophobic barrier pen; Vector Labs, Peterborough, UK). Next the samples were permeabilised with 2 mg·ml⁻¹ Proteinase K diluted 1:100 in 10 mM Tris pH8 for 20 min, before endogenous peroxidases were inactivated with 3% H₂O₂ for 5 min.

Samples were equilibrated with 1x TdT Equilibration buffer for 10 min, before the excess was blotted, and then incubated with TdT enzyme diluted 1:20 with TdT Labeling Reaction Mixture at 37°C for 1.5 hours in a humidified chamber. The reaction was terminated using Stop Solution for 5 min. Next, blocking buffer was applied for 10 min, then the excess was blotted and samples incubated with 1x conjugate for 30 min in a humidified chamber.

Apoptotic nuclei were detected after 10 min incubation with 3,3' diaminobenzidine (DAB) solution. After rinsing in dH₂O, the samples were counterstained with methyl green for 3 min, rehydrated through a series of ethanol baths, cleared with Xylene and mounted with DPX.

Omission of primary antibody was used as a negative control. A mixture of HL60 cells incubated with 0.5 µg/ml actinomycin D for 19 hours to induce apoptosis was used as a positive control.

2.8 Lectin Staining

Surface carbohydrates on vascular endothelial cells act as ligands for mammalian lectins (Rhodes and Milton, 1998). *Griffonia simplicifolia* lectin 1 isolectin B4 (GSL1_{B4}) was selected as this binds to murine endothelial cells and most species apart from humans (Laitinen, 1987).

Sections were deparaffinised in xylene and then rehydrated through a series of 100%, 95%, and 70% ethanol baths. The slides were placed in distilled H₂O for 5 min and then in PBS for 5 min. The samples were circled with a paraffin pen, and endogenous peroxidases were inactivated using 3% H₂O₂ for 5 min before being rinsed in PBS (2 x 5 min).

An avidin-biotin blocking kit (Vector Labs, Peterborough, UK) was used to block endogenous biotin (15 min with avidin, rinsed with PBS, then 15 min with biotin) and then the samples were covered with Carbo-free blocking solution (Vector Labs, Peterborough, UK) for 30 min to prevent non-specific binding.

The excess solution was tipped off, then the samples were incubated with biotinylated *Griffonia (Bandeiraea) simplicifolia* lectin 1 isolectin B4 (Vector Labs, Peterborough, UK).

Next the samples were incubated with Vectastain® Elite ABC Reagent (Vector Labs) for 30 min, before rinsing in PBS (2 x 5 min). The staining was visualised using a 3,3'-diaminobenzidine (DAB+) substrate kit (Vector Labs). To 2.5 ml distilled water, one drop of buffer, two drops of DAB substrate, and 1 drop of H₂O₂ were added. This was applied to each tissue section and developed for 5 min. When the appropriate intensity of stain was achieved, slides were immersed in running tap water and rinsed for 5 min. The samples were then counterstained using haematoxylin and mounted as described previously (see 2.4.2).

Omission of primary antibody and 500 mM galactose (a competing sugar) were used as negative controls. Bovine ovary was used as a positive control.

2.8.1 Optimisation

GSL1_{B4} was diluted at 5, 10, 20 and 40 µl.ml⁻¹, and incubated for either 2 hours at room temperature, or overnight at 4°C. These variations were tried both with and without antigen retrieval. Antigen retrieval was carried out after the sections were rehydrated through xylene and a series of ethanol baths. A pressure cooker containing 1x citrate buffer was used, with the samples immersed in the pressure cooker for 2 min 30 sec, before the steam was released and the samples left to cool for 30 min. Next the samples were rinsed in PBS twice for 5 min, before carrying on with the peroxidase blocking step shown in 2.8.

Other optimisation steps included increasing blocking steps to try to remove non-specific staining, and decreasing the incubation time with the DAB substrate.

2.9 Image Analysis

Image Analysis was carried out using ImagePro MC 6.0 software, to quantify 2D glomerular area, immunohistochemical staining, and TUNEL staining. Macros were written to automate the processes.

2.9.1 Quantification of glomerular area

Sections stained with H&E were used to assess mean glomerular area (μm^3) of the adult kidneys. Using a light microscope (Leica, Germany) and x200 magnification, twenty glomeruli were randomly selected per adult animal and photographed. The glomerular area for each glomeruli was calculated using Image Pro MC 6.0 software. Figure 2.3 shows the process, whereby each glomerulus is identified (A), then circled (B), before the circled is filled and the black area measured. Each measurement was automatically exported to an Excel spreadsheet, and the mean from 20 glomeruli per animal calculated.

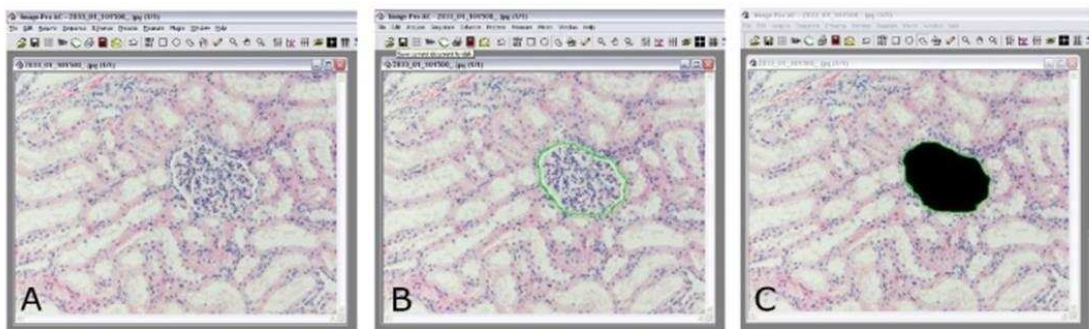


Figure 2.3: Quantification of 2D glomerular area on Image Pro MC 6.0

Microphotographs were taken (A) and glomeruli outlined in green (B). The region outlined was filled in (C) and the software calculated the area.

2.9.2 Quantification of Immunohistochemical staining

Sections stained as per the methods described in 2.6.1 or 2.6.2 were used to assess the amount of antibody staining. Using a light microscope (Leica, Germany) and x200 magnification, twenty images of random areas were photographed. The amount of staining was calculated using Image Pro MC 6.0 software. For each field of view, brown DAB staining was picked up by the software and its percentage area relative to the whole picture calculated. Each measurement was automatically exported to an Excel spreadsheet, and the mean from 20 fields of view per animal calculated. For quality control purposes three 100 x 100 µm sections were counted by eye and compared with the automated results. Variation was <5% between the methods.

2.9.3 Quantification of TUNEL positive cells

Sections stained as per the method described in 2.7 were used to assess the amount of TUNEL positive cells. Using a light microscope (Leica, Germany) and x200 magnification, twenty images of random areas were photographed. The number of TUNEL positive cells was calculated using Image Pro MC 6.0 software. For each field of view the number of cells stained brown with DAB was counted by the software, along with the number of cell nuclei stained blue with haematoxylin. Each measurement was automatically exported to an Excel spreadsheet, and percentage of TUNEL positive cells was calculated using the formula:

$$\text{Number of TUNEL positive cells} \div \text{Total number of cells} \times 100$$

The mean from 20 fields of view per animal was calculated. For quality control purposes three 100 x 100 µm sections were counted by eye and compared with the automated results. Variation was <5% between the methods.

2.10 Stereology

Sheep kidneys were previously halved and snap frozen, then defrosted and fixed in NBF.

The volume of the total kidney was calculated using water displacement.

2.10.1 *Random systematic sampling*

Figure 2.4 details the random systematic sampling method. First, each half kidney was cut into 3 mm slices (Figure 2.4A). Depending on the size of the kidney this meant a range of 8-12 slices. Next a dice was rolled to select a random starting point (in this case number 3), and the dice rolled again (again, number 3); hence, every third slice was taken for sampling, starting with the third section (Figure 2.4B).

Each randomly selected slice was then cut vertically into 5 mm strips, and horizontally every 10 mm (range 6 -18 segments). A dice was rolled again (randomly selecting number 5), hence every 5th piece was taken (from the random start point of 3 previously selected) counting from left to right, top to bottom then moving to the next slice until 6 samples were obtained from each animal (Figure 2.4C). The six samples were processed (see 2.5.1), and then embedded (Paraffin Embedding Center EG1160, Leica) at random orientation in cassettes containing paraffin wax. All cassettes were given a code unrelated to the animal number so all analysis was carried out blind.

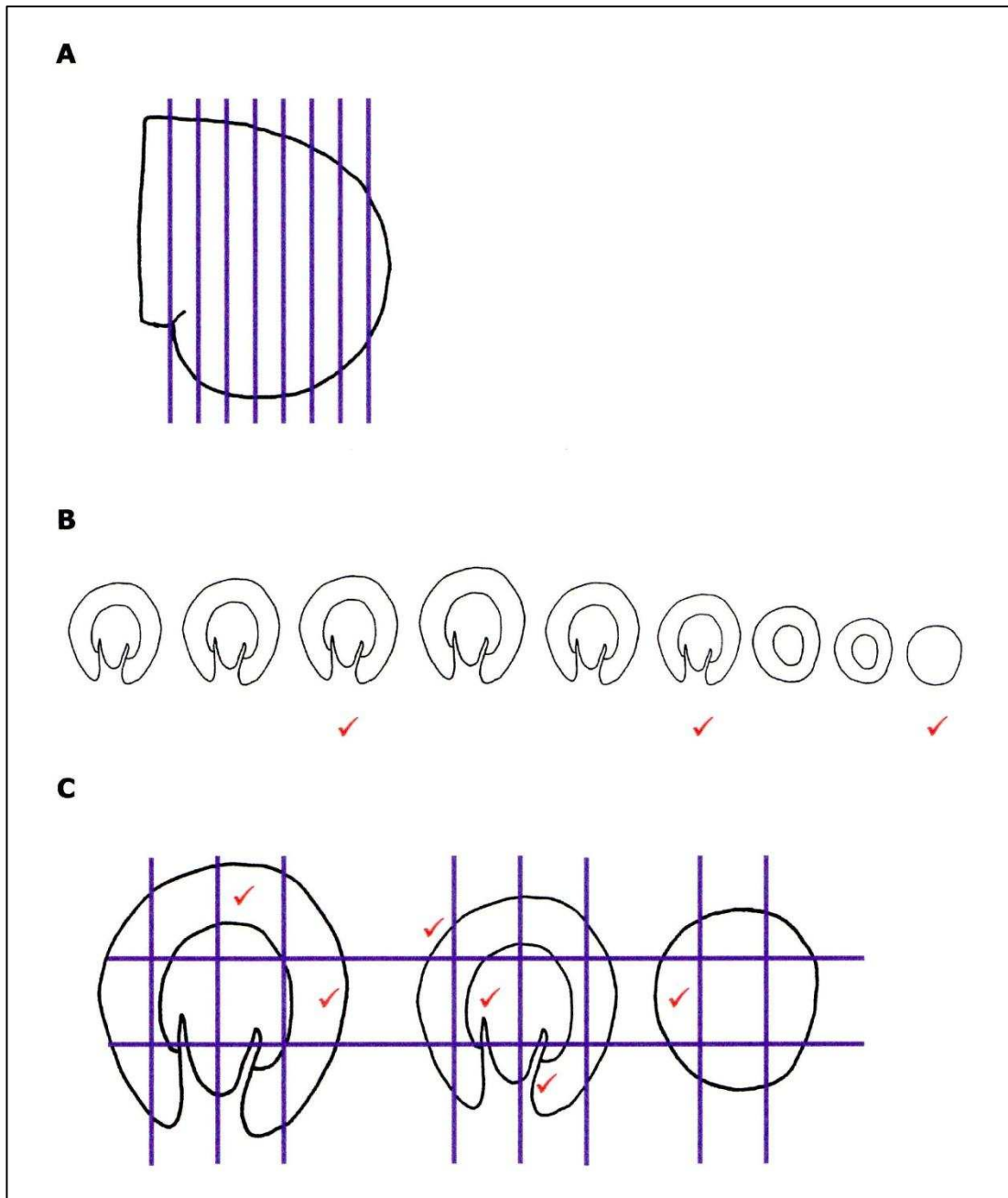


Figure 2.4: Random, systematic sampling method

A, the kidney is cut into 3 mm slices; B, every third slice is selected; C, every fifth segment is selected, until six in total are collected.

2.10.2 Cortex:medulla ratio

Sections were cut using a microtome (Microtome RM2255, Leica) at 5 μm thick and mounted onto polysine slides and stained using H&E (see 2.5.2).

Using a slide box (PathScan Enabler IV, Electron Microscopy Sciences) the whole of each section was photographed at high resolution and was used to estimate the ratio of the cortex to medulla, using the Cavalieri Principle which gives an unbiased volume estimate based upon reference areas on sections. First a point-counting method was applied which is considered to be the most efficient method for estimating the area of cut surfaces of tissue (Gundersen, 1986). A point counting grid containing 200 points (20 x 10) was used (Figure 2.5A), and the volumes of cortex and medulla were worked out using the formula:

Volume fraction_(cortex) = no of points hitting cortex/no of points hitting the kidney

Volume fraction_(medulla) = no of points hitting medulla/no of points hitting the kidney

The volume fraction of the cortex and medulla were multiplied by the volume of the kidney to give the volumes of the cortex and medulla.

2.10.3 Nephron Number

Two die were rolled and the total was used to select a random starting point – 7 – and every 7th (the “reference”) and 8th pair (the “look-up”) with a distance of 30 μm between were selected for the physical dissector, and were mounted adjacent on slides. The block was serially sectioned until five dissector pairs had been acquired. Six blocks were cut per animal, so a total of 30 dissector pairs per animal were obtained. Next, the slides were stained with H&E (2.5.2) so the glomeruli could be easily identified.

Photographs were taken using the slidebox scanner (PathScan Enabler IV, Electron Microscopy Sciences) and an unbiased counting frame measuring 2 mm x 2 mm was placed on the reference and look-up sections in the same position. Glomeruli were counted if they appeared in the reference section but not in the look-up section and were not hit by the solid line (glomeruli were counted if they hit the dotted line). In the example illustrated in Figure 2.5D only the glomeruli indicated by the black arrows were counted. To double the efficiency of this process the same pictures were counted in the reverse direction, i.e. counting glomeruli that appeared in the look-up section but not in the reference section (white arrows, Figure 2.5D). To ensure consistency, where the cross was not fully within the glomeruli, it was counted whenever the top right intersection (Figure 2.5C) was on the glomeruli. The mean numerical density of glomeruli (N/V) was calculated:

$$N/V_{(\text{glomeruli})} = \Sigma Q_{-(\text{glomeruli})} / A \times d$$

Where ΣQ_{-} is the total no of glomeruli counted in the reference section, d is the distance between the planes and A is the area of the unbiased counting frame. The results were multiplied by the cortex volume to give a nephron number per kidney.

Glomerular volume density (V_v) was calculated using a point counting grid containing 200 points (20 x 10) was used (Figure 2.5B), and the volume of glomeruli was calculated using the formulae:

$$V_v_{(\text{glomeruli})} = \text{no of points hitting glomeruli} / \text{no of points hitting the cortex}$$

$$\text{Total glomerular volume} = V_v_{(\text{glomeruli})} \times \text{cortex volume}$$

$$\text{Mean glomerular volume} = \text{total glomerular volume} / N_{v_{(\text{glomeruli})}}$$

For quality control purposes, three animals had their nephrons recounted on a different day. The difference between results was < 5%.

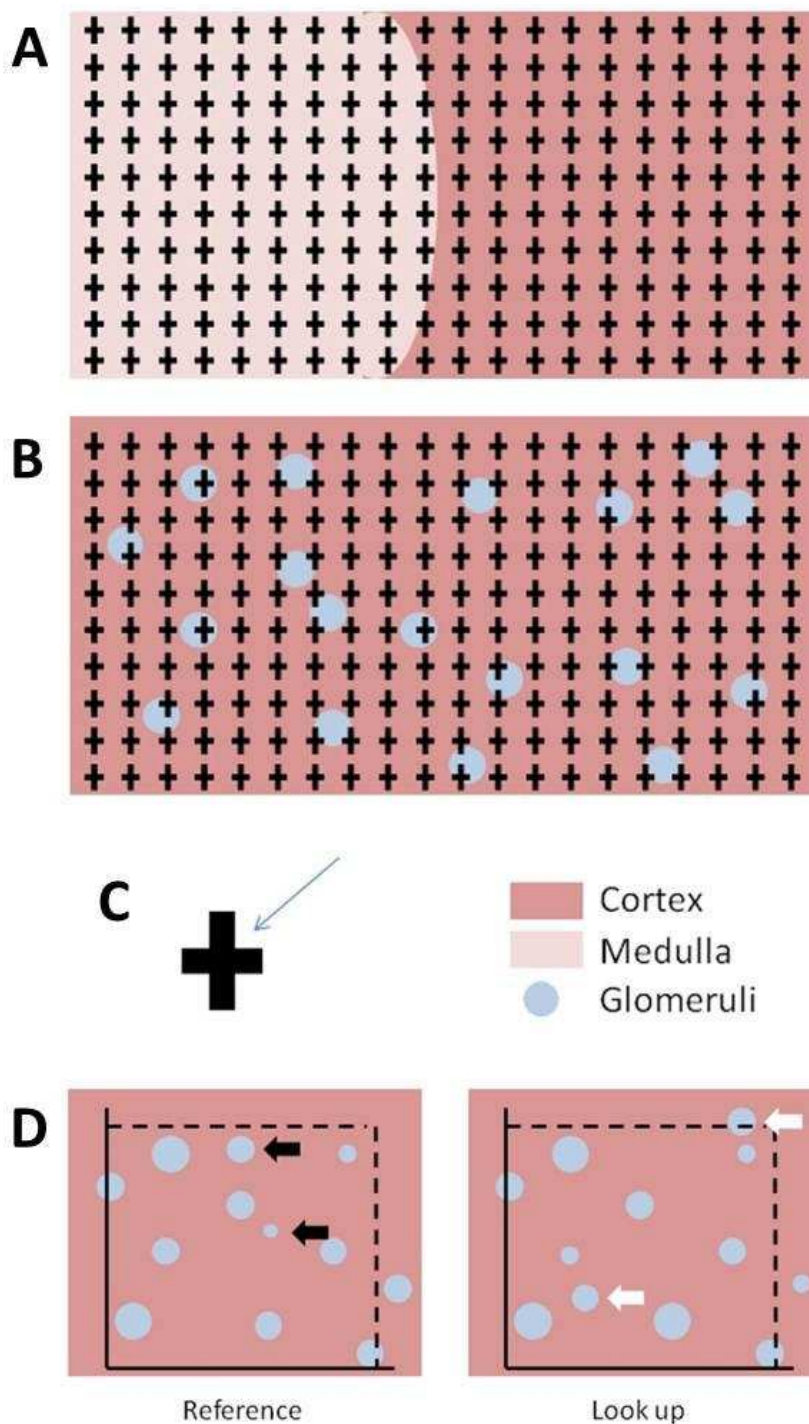


Figure 2.5: Stereological counting method

A point counting grid was used to estimate cortex:medulla ratio (A) and glomeruli:cortex ratio (B), whereby crosses hitting the item of interest were counted in relation to the total number of crosses. As crosses sometimes are only partly on an item of interest, it was counted wherever the top right hand corner of the cross lay (C). Glomeruli were counted if they appeared in the reference section but not in the look-up section and were not hit by the solid line (D). To double the efficiency of this process the same pictures were counted in the reverse direction, (white arrows)

2.11 Estimation of mammalian nephron number

Relevant studies were identified using computerised searches of the online electronic databases PubMed (MEDLINE) and ISI Web of Science from inception up to 30 June 2009. Data for as many different mammalian species as possible were included, where body weight and nephron number had been reported. These data were added to the data provided in an earlier study (Kunkel, 1930) and using \log_{10} data for published values of nephron number from a mouse weighing 20 g to an elephant weighing 3700 kg generated an equation of $y = 0.613x + 3.116$. This was used to predict the nephron number for sheep.

2.12 Quantitative PCR

All procedures carried out used RNase-free water, tubes and pipette tips. All steps were carried out at room temperature unless otherwise stated.

2.12.1 RNA Extraction

2.12.1.1 Study 1

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Crawley, UK), in accordance with the manufacturer's instructions. Kidney samples were ground to a fine powder under liquid nitrogen in an autoclaved pestle and mortar, then ~20 μg of the powder was added to lysis buffer RLT + 1% 2-mercaptoethanol in a cooled Eppendorf tube and homogenised by passing through a 19-gauge needle ten times. The homogenate was centrifuged at 12,000 $\times g$ for 3 min, and the supernatant transferred to a new RNase-free tube. One volume of 70% ethanol was added to each tube and mixed by pipetting. A maximum of 700 μl of the supernatant was added to the spin column, and centrifuged at 8,000 $\times g$ for 15 sec at room temperature. The flow-through collected in the collection tube underneath was discarded and the spin column reinserted in the same collection tube.

Buffer RW1 was added to the spin column and centrifuged at $8,000 \times g$ for 15 sec to wash the spin column. The spin column was placed into a new Collection Tube, buffer RPE added, and centrifuged at $8,000 \times g$ for 15 sec. Buffer RPE was added a second time, and then centrifuged at $8,000 \times g$ for 2 min to dry the membrane, then the spin column was placed into a collection tube. Thirty μl of RNase-Free Water was added to the centre of base of the spin column and centrifuged for 1 min at $8,000 \times g$. This step was repeated using the eluted RNA to put it through the centre of the spin column again. The purified RNA was stored in a -80°C freezer.

2.12.1.2 Study 2

Total RNA was extracted using PureLink™ Mini Kit (Invitrogen, Paisley, UK), in accordance with the manufacturer's instructions. Kidney samples were ground to a fine powder under liquid nitrogen in an autoclaved pestle and mortar, then $\sim 20 \mu\text{g}$ of the powder was added to lysis buffer + 1% 2-mercaptoethanol in a cooled eppendorf and homogenised by passing through a 19-gauge needle ten times. The homogenate was centrifuged at $12,000 \times g$ for 2 minutes, and the supernatant transferred to a new RNase-free tube. One volume of 70% ethanol was added to each tube and vortexed to disperse any visible precipitate. A maximum of 700 μl of the supernatant was added to the spin cartridge, and centrifuged at $12,000 \times g$ for 15 seconds at room temperature. The flow-through was discarded and the spin cartridge reinserted in the same collection tube.

Wash Buffer I was added to the spin cartridge and centrifuged at $12,000 \times g$ for 15 seconds. The spin cartridge was placed into a new collection tube, Wash Buffer II added, and centrifuged at $12,000 \times g$ for 15 seconds. This step was repeated.

Next the spin cartridge was centrifuged at $12,000 \times g$ for 1 min to dry the membrane and then the spin cartridge was placed into a recovery tube. Fifty μl of RNase-Free Water was

added to the centre of the base of the spin cartridge and incubated at room temperature for 1 min. This was centrifuged for 2 min at $\geq 12,000 \times g$. This step was repeated using the eluted RNA to put it through the centre of the spin cartridge again. The purified RNA was stored in a -80°C freezer.

2.12.1.3 Quality Control

The quality of the RNA was checked using a Nanodrop (Fisher Scientific, Loughborough, UK). The parameters set were that RNA yield was $\geq 100 \text{ ng}\cdot\mu\text{l}^{-1}$, and the 260/280 absorbance ratio was between 1.90 and 2.10.

2.12.2 CDNA Synthesis

CDNA was synthesised using an Omniscript reverse transcriptase kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. RNA was thawed and kept on ice, and all other kit components were thawed at room temperature and then kept on ice during the protocol. First the RNA was treated by DNase to remove any DNA contamination prior to cDNA synthesis, using a RQ1 RNase-free DNase kit (Promega, Southampton, UK). Two μl of RNA was added to an Eppendorf tube containing RNase-free DNase, buffer and water and incubated in a water bath at 37°C for 30 min. Next RQ1 DNase stop was added to terminate the reaction and the solution denatured at 65°C in a heat block for 10 min to inactivate the DNase.

A mastermix was prepared containing 10x RT buffer, random primers, Dntp, RNase inhibitor, Omniscript and RNase-free water. This was added to each tube and incubated in a water bath at 37°C for 2 h. Finally, the samples were diluted in 130 μl RNase-free water, and stored in the -20°C freezer until used for qPCR.

2.12.3 Primer Design

Primers were designed using NCBI Primer-BLAST (Table 2.5), and purchased from Eurofins MWG (Ebersberg, Germany). Stock primers (100 pmol.µl⁻¹) were diluted to 5 pmol.µl⁻¹ in RNase-free H₂O.

Table 2.5: Primer details for Quantitative PCR

Primer		Sequence 5' – 3'	Annealing temp (°C)
Angiopoietin 1	F	GGGTCACACTGGGACAGCAGG	58
	R	TGGGCCACAGGCATCAAACCA	
ATF4	F	AGATGACCTGGAAACCATGC	52
	R	AGGGGGAAGAGGTTGAAAGA	
ATF6	F	AACCAGTCCTTGCTGTTGCT	52
	R	CTTCTTCTGCGGGACTGAC	
Bax	F	CAGGATGCATCCACCAAGAAGC	56
	R	TTGAAGTTGCCGTGGAAAACATT	
Beta actin	F	TGTGCGTGACATCAAGGAGAA	55
	R	CGCAGTGGCCATCTCCTG	
BMP 7	F	GCCACTTCTCACCGACGCC	58
	R	CAGCTGCAGTCACGGCCTCC	
CD68	F	GTCCTGCTACCACCACAGT	52
	R	GCTGGGAACCATTACTCAA	
Cyclophilin	F	CATACAGGTCTGGCATCTTGTC	56
	R	TGCCATCCAACCACTCAGTCT	
DDIT3	F	AGGACCACCAGAGGTCACAC	56
	R	TGCCACTTTCCTTCGTTTT	
bFGF	F	CTTTCCCGCCCGGCACTTT	58
	R	GTCGCTCTTCTCGGGACCC	
GAPDH	F	TCCGTTGTGGATCTGACCTG	55
	R	TGCTTACCACCTTCTTGATCTC	
GRP78	F	TGAAACTGTGGGAGGTGTCA	52
	R	TCGAAAGTCCAGAAAGGTG	
iNOS	F	TGATGCAGAAGGCCATGTCA	52
	R	TCTCCCTGTCTCTGTTGCAAAG	
MCP-1	F	CAAGACCATCTGGGCAAA	52
	R	GTCCTGGACCCATTTCAGGTT	
PDGF	F	CAAGACGCGCACGGAGGTGT	58
	R	GCGGTTGTTGCAGCAGCCAG	

Primer		Sequence 5' – 3'	Annealing temp (°C)
SPARC	F	CGAGGTACCCGTGGGAGCCA	58
	R	CAAACCTCGCCGATGGGGGCA	
TGFβ1	F	GGAGTTGTGCGGCAGTGGCT	58
	R	AAGGGCCGGTTCATGCCGTG	
Thrombospondin	F	TGCTGTGCGGGCGGAGAAAG	58
	R	TGCCCGCCTTGCCATTGGAG	
Tie-2	F	CAGCAGACCTCGGAGGCAGGA	58
	R	TGCCCTCTTCAGCTGCAGCATG	
VEGFA	F	GGATGTCTACCAGCGCAGC	56
	R	TCTGGGTACTCTGGAAGATGTC	
VEGFR1	F	TGGATTTAGGTGAGCTTGGA	52
	R	TCACCGTGCAAGACAGCTTC	
VEGFR2	F	CTTCCAGTGGGCTGATGACC	52
	R	GCAACAAACGGCTTTTCATGT	

All primers were designed using NCBI Primer-BLAST; ATF, Activating Transcription Factor; BMP 7, Bone Morphogenic Protein 7; DDIT3, DNA Damage Inducible Transcript 3; bFGF, basic Fibroblast Growth Factor; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; iNOS, inducible Nitric Oxide Synthase; GRP78, 78 kDa Glucose-regulated Protein ; MCP1, Monocyte Chemotactic Protein 1; PDGF, Platelet-derived Growth Factor; SPARC, Secreted Protein, Acidic, Cysteine-rich; TGFβ-1, Transforming Growth Factor β-1; VEGF, Vascular Endothelial Growth Factor.

2.12.4 QPCR

2.12.4.1 Study 1

QPCR was performed using QuantiTect SYBR Green RT-PCR Kit (Qiagen, Crawley, UK) on a Roche Lightcycler® 480 (Roche Diagnostics, Burgess Hill, UK).

For each reaction, 2 µl of diluted cDNA was added to 10 µl SYBR Green, 1.25 µl each of forward and reverse primers and 5.5 µl RNase-free water, making 20 µl volume per well on a 96-well plate (Roche Diagnostics, Burgess Hill, UK). The protocol for the Roche Lightcycler is shown in Table 2.6.

Table 2.6: PCR protocol for QuantiTect SYBR Green

Program name		Cycles	Analysis mode		
Pre-incubation		1	None		
Amplification		40 – 45*	Quantification		
Melting curve		1	Melting curves		
Cooling		1	None		
Pre-incubation					
Target °C	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)	
95	None	00:15:00	4.4		
Amplification					
Target °C	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)	
95	None	00:00:20	4.4		
55*	None	00:00:20	2.2		
72	Single	00:00:20	4.4		
Melting curve					
Target °C	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)	
95	None	00:00:05	4.4		
72	None	00:01:00	2.2		
95	Continuous		0.11	5	
Cooling					
Target °C	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)	
40	None	00:00:30	2.2		

*primer dependent

Melting curves were used to confirm reaction specificity (example in Figure 2.7). MRNA quantities were normalised to three housekeeping genes (2.12.5) using Roche Lightcycler® 480 advanced relative quantification software.

2.12.4.2 Study 2

QPCR was performed using a Roche SYBR Green kit (Roche Diagnostics, Burgess Hill, UK) on a Roche Lightcycler® 480. For each reaction, 5 µl of diluted cDNA was added to 10 µl SYBR Green, 1 µl each of forward and reverse primers and 3 µl RNase-free water, making 20 µl volume per well on a 96-well plate (Roche Diagnostics). The protocol for the Roche Lightcycler is shown in Table 2.7.

Table 2.7: PCR protocol for Roche SYBR Green

Program name		Cycles	Analysis mode		
Pre-incubation		1	None		
Amplification		40 – 45*	Quantification		
Melting curve		1	Melting curves		
Cooling		1	None		

Pre-incubation				
Target °C	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)
95	None	00:10:00	4.4	

Amplification				
Target °C	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)
95	None	00:00:10	4.4	
55*	None	00:00:10	2.2	
72	Single	00:00:10	4.4	

Melting curve				
Target °C	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)
95	None	00:00:05	4.4	
65	None	00:01:00	2.2	
97	Continuous		0.11	5

Cooling				
Target °C	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)
40	None	00:00:10	1.5	

*primer dependent

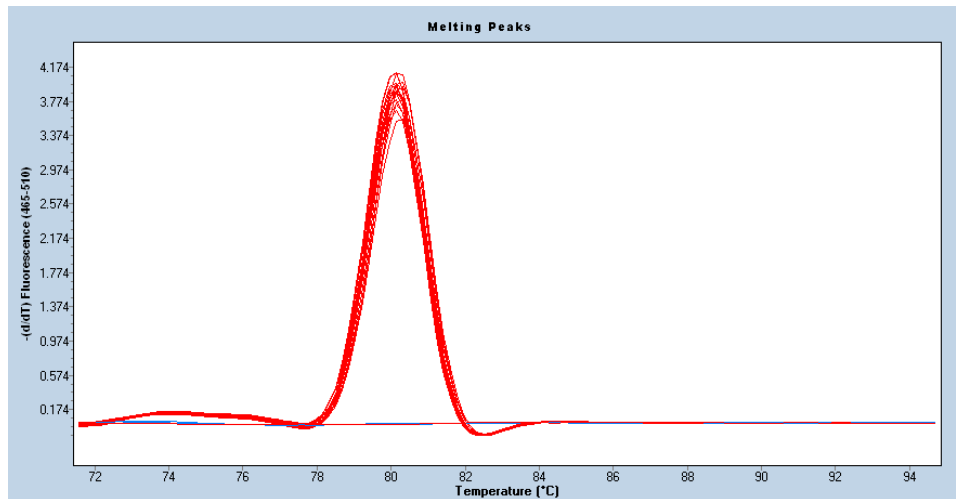


Figure 2.6: Example of a melt curve

Melt curve demonstrating the primer's specificity with all samples having the same melt curve and the blanks flat along the bottom of the graph

Melt curves were used to confirm reaction specificity (example in Figure 2.6). MRNA quantities were normalised to housekeeping genes (2.12.5) using Roche Lightcycler® 480 advanced relative quantification software.

2.12.5 Housekeeping genes

Beta-actin, cyclophilin and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) were selected as housekeeping genes. Crossing points for the CP, LPE and LPL groups were compared to confirm that the housekeeping genes were not significantly different between groups, and therefore suitable to be used in this context. Results for all genes of interest were normalised relative to all three housekeeping genes and all significant results stood whichever housekeeping gene was used. The results for beta actin are shown in this thesis, as this was the housekeeping gene expressed at levels closest to the genes of interest.

2.12.6 Quality Control

All standards were done in triplicate and samples in duplicate. Intra-assay variation was set at 5%, although most duplicates and triplicates had less than 1% variation between them. Inter-assay agreement was also validated, for example, VEGFA run on two different plates on different days had an R^2 value of 99.1.

2.13 Corrosion Casting

Corrosion casting was carried out using Batson's No. 17 Plastic Replica and Corrosion Kit (Polysciences Inc; Eppelheim, Germany) in accordance with the manufacturer's protocol, to visualise and quantify the gross vasculature of the fetus.

2.13.1 Resin casting

First 2 - 4 ml of the catalyst was added to 10 ml of the base solution A, and left to stand at room temperature. To a further 10 ml of base solution A, 2 - 4 drops of promoter C were added, and the solution mixed slowly. After combining the two preparations, 7 ml of the resulting solution was injected slowly into the umbilical artery of the fetus using a fine-gauge (21G) needle. The perfusion-fixed fetus was stored on ice for 2 - 3 h whilst the resin set, to aid the polymerisation process, and to prevent expansion and distortion of the specimen.

2.13.2 Maceration

Once the specimen was set, it was immersed in maceration solution (20% potassium hydroxide) for 12 - 18 h at 50°C to remove the surrounding soft tissue. After all the tissue was dissolved, the remaining vascular cast was rinsed in water, dried gently and stored at room temperature in a protective plastic container.

2.13.3 Micro CT Analysis

The surface area and volume of the resin casts of each kidney were quantified using a Nanotom® high resolution computed tomography (CT) scanner (GE Sensing and Inspection Technologies, Wunstorf, Germany) operated by Craig Sturrock, University of Nottingham.

X-ray slices were taken every 40 µm throughout the cast, averaging 2000 slices per fetus.

Using the Nanotom® software, these images were stacked and reconstructed to provide quantitative volumetric data and to calculate the surface area of each cast.

2.14 Osmolality

Osmolality of plasma samples was measured using an OSMOMAT 030 automatic cryoscopic osmometer (Gonotec, Berlin, Germany).

Fifteen µl of each sample was pipetted into a measuring vessel and then pushed onto the measuring vessel holder. The holder was moved down onto the lower cooling system and the osmolality was measured automatically.

The system was calibrated by performing measurements with distilled H₂O and a calibration standard of 300 mOsmol.kg⁻¹ (Gonotec, Berlin, Germany). For quality control purposes all samples were measured in duplicate and all results presented had < 5% coefficient of variation.

2.15 Statistics

2.15.1 Power calculations

For Study 1, renal functional outcomes were considered to be subject to the most measurement error, based upon a previous study (Williams et al., 2007). Intra-renal transit-time represents an important variable that condenses many aspects of adult renal function. Genstat v13 (VSNi, UK) was used to perform the power calculations, using a split-plot design with diet as the whole plot and sex as the sub plot. Significance was set at 0.05, and within animal variance (residual mean square, SD^2) was 0.005 min and between animal variance 0.016 min. For this study it was estimated that there was 91% power to detect a 20% effect size for renal transit-time in the adult, with at least 3 male and 3 female animals per group (three groups; CP, LPE and LPL; n = 19 total).

For Study 2, amino acid concentrations were considered to be the main outcome of interest. For example, the study had 86% power to detect a change of 10% in ornithine concentrations. Genstat v13 was used to perform the split-plot design with diet as the whole plot and sex as the sub plot. Significance was set at 0.05 and diet variance was 166 μmol and sex variance 25 μmol , with at least 4 male and female animals per group (two groups; CP and LPE; n = 31 total).

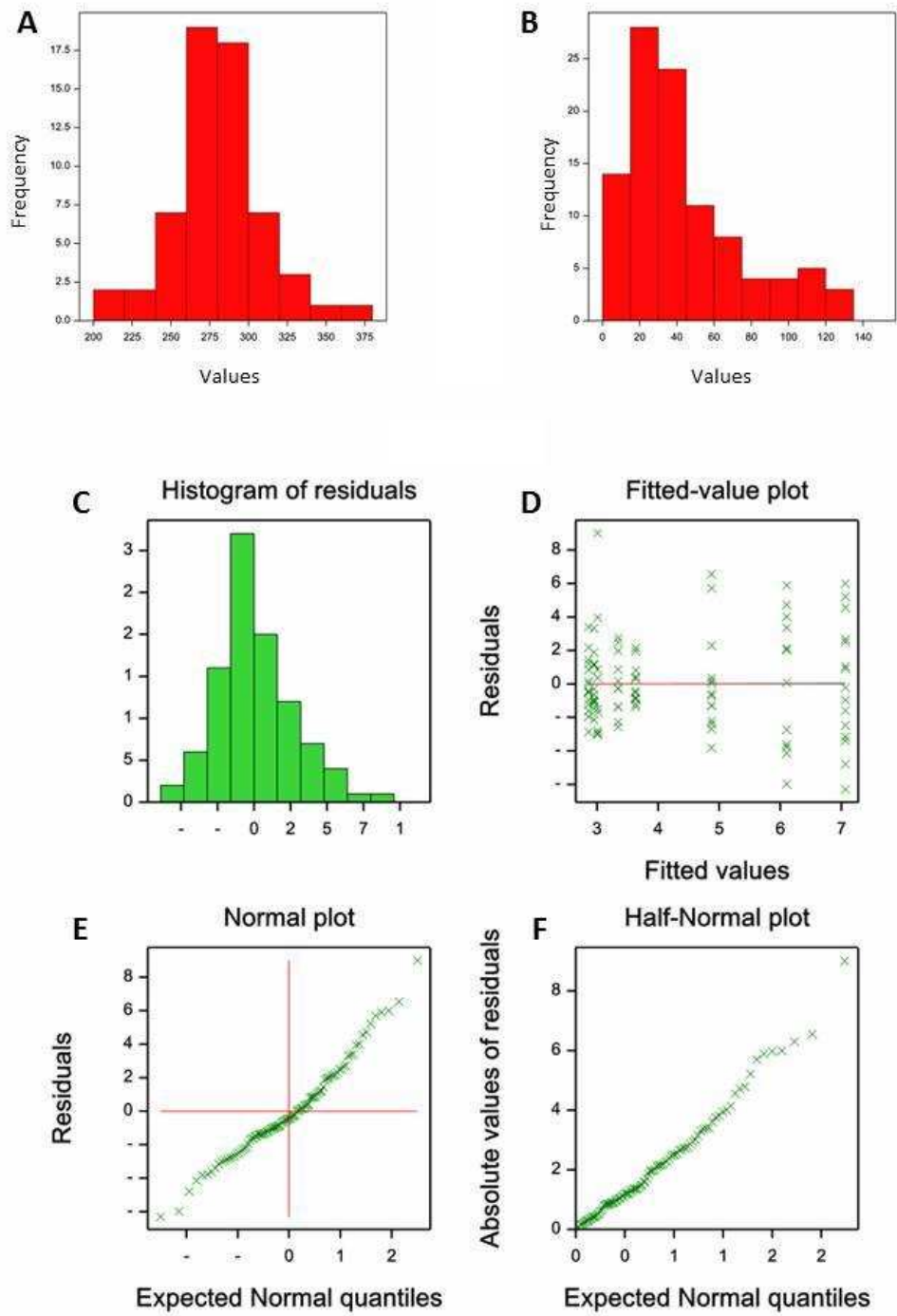


Figure 2.7: Distribution and residual checking

Examples shown of A, normal distribution; B, gamma distribution; C, normal distribution of residuals; D, fitted-value plot; E, normal plot; F, half-normal plot

2.15.2 Distribution

The distributions of the data were checked for normality using a Shapiro-Wilks test (Genstat v13). Figure 2.7 shows examples of variables that were either normally distributed (A) or had a skewed distribution (B). Where there was a skewed distribution, the data were log-transformed. The models were checked to ensure the residuals had a normal distribution (C), and the fitted-value plot (D) shows no tendency of the residuals to increase or decrease with the fitted values. This ensures that the constant variance assumption in the model is satisfied (Petrie and Sabin, 2009). The normal and half-normal plots (E and F) should show a linear relationship between the residuals and expected values.

2.15.3 Univariate Analysis

The data (unless stated below) were analysed using General Linear Models (GLM; Genstat v13).

All data were first checked for normality (or otherwise) of the distribution and appropriate GLM statistical models used with or without data transformation (log-link functions). Fixed effects were diet, sex and a diet.sex interaction. For the fetal experiments where data was included from both twins, the ewe was added as a random effect, and therefore a residual maximum likelihood (REML) model was used as this is capable of taking into account both fixed and random effects (Galwey, 2006).

All data are presented as predicted means \pm SEM. Data with a gamma distribution was log transformed for statistical analysis and the means and SEM back transformed for presentation purposes. Whilst $P < 0.05$ was accepted as indicating statistical significance, values of P from 0.05 - 0.09 are also presented to indicate effects falling close to the arbitrary significance boundaries.

The albumin:creatinine ratio data did not fit usual distribution patterns, therefore was analysed by the Wilcoxon rank sum test, a non-parametric test that does not depend upon assumptions about distribution (Petrie and Sabin, 2009).

The data for the developmental stage of the fetal kidneys was assessed using a Chi-squared test, as this is appropriate for categorical data (Petrie and Sabin, 2009).

Correlations were performed using Pearson's correlation coefficients.

2.15.4 Multivariate analysis

Amino acid concentrations are not all independent of each other, therefore GLM analysis is not necessarily the best statistical method, and a multivariate discriminant analysis was also carried out (Genstat v13) in addition to GLM analysis of each individual amino acid. This is a type of linear discriminant analysis whereby one or two numerical values (linear descriptors called canonical variate 1 and canonical variate 2) capture the majority of variation for all non-independent variables (Altman, 1991). Furthermore, combining related variables in this way improves the power and precision of the analysis and offers a more objective interpretation of the physiology. Data are presented as canonical variates plot with group means enclosed by circles representing 95% confidence of the mean.

3. Study 1: The effect of a maternal low protein diet on the ewe and her fetus

3.1 Introduction

The incidence of non-communicable diseases such as CVD, type II diabetes and CKD rises with both age and degree of obesity (Wilson et al., 1998; Calle et al., 1999; Wahba and Mak, 2007; Danaei et al., 2009). Whilst some risk factors are unavoidable (age, sex), others are lifestyle-related (e.g. obesity, smoking) and can be modified to reduce the risk of such chronic diseases. In 2005, 396 million people worldwide were estimated to be obese (Kelly et al., 2008); in 2008, 28-35% of the US and UK population were hypertensive (Egan et al., 2010) and in 2010, 285 million people were estimated to have type II diabetes (Diabetes UK, 2009). With each disease predicted to increase significantly in frequency over the next few decades, the global burden of these 'lifestyle' diseases is a major public health issue.

However, the risk of these diseases is not dependent on one factor alone, but rather on a combination of genetics, diet, lifestyle and other environmental influences. Unlike genetics and some environmental influences, an individual living in a country where food is freely available usually has control over their diet. A 16-year long study of female nurses in the USA (Hu et al., 2001) identified that a diet high in fibre and polyunsaturated fat and low in trans-fats and glycaemic load leads to the lowest risk of type II diabetes, even after adjustment for BMI. Another study of the same cohort showed similar results for coronary heart disease (CHD), with the least risk of death or non-fatal infarctions in those who ate a diet high in fibre, n-3 fatty acids and folate, and low in trans fats and glycaemic load (Stampfer et al., 2000). In addition, other studies of men and women have shown an inverse relationship between dietary folate intake and risk of stroke and CVD (Bazzano et al., 2002), and a Mediterranean diet (high in monosaturates, fruit, vegetables, fish and unrefined cereals) led to a decreased risk of CVD (Knoops et al., 2004). Furthermore, a

huge increase in CHD mortality in Beijing between 1984 and 1999 was attributed to rises in total cholesterol as a result of an increasingly 'westernised' type of diet (Critchley et al., 2004). Changes in diet are, therefore, a potentially easily achievable way of reducing some of the risk of non-communicable disease in the population.

Increased focus on diet during a woman's reproductive years, particularly during pregnancy and lactation, has been stimulated by the Developmental Origins of Health and Disease hypothesis (Barker and Osmond, 1986; Gluckman et al., 2008). The response of the fetus to maternal diet has been demonstrated to increase the risk of those offspring developing hypertension (Huang et al., 2010; Stein et al., 2006), coronary dysfunction (Crispi et al., 2010), type II diabetes (Whincup et al., 2008) and kidney disease (Amann et al., 2006; Woods et al., 2004) later in life. Increased focus on kidney disease is particularly pertinent as nephrogenesis is complete by term in humans and non-litter bearing mammals (Wintour and Moritz, 1997) and is therefore particularly sensitive and vulnerable to maternal malnutrition. For example, nephron endowment has been shown to be reduced by maternal diets low in protein (Langley-Evans et al., 1999), low in iron (Crowe et al., 1995), high in glucose (Nehiri et al., 2008) or high in fat (Khan et al., 2003).

The inclusion of pregnant women in experimental trials has major ethical and legal considerations, with the potential to cause harm to both the woman and the developing fetus. In addition, the need to follow the offspring through to middle or old age makes a trial extremely time-consuming and expensive; hence most such studies have tended to be retrospective rather than prospective. Other major limitations of human dietary studies are a lack of accurate recall regarding diet, and many confounding factors. Taken together, these difficulties mean that animal studies - with their tightly controlled environments and short life-spans - provide an ideal opportunity to study the effect of maternal diet during pregnancy on the mother, the developing fetus and the offspring later in life.

Research into the nutrition of sheep has been ongoing for more than 60 years. Initially this was focused on discovering the optimal nutrition for ewes during pregnancy in order to maximise offspring weight, reduce lamb mortality and increase the number of live twin births (Blaxter, 1957; Louca et al., 1974; Quirke et al., 1978). However, in the last two decades sheep have increasingly been used as a model for the effect of diet during pregnancy on fetal development due to similarities between human and ovine development *in utero*.

Most fetal programming studies in sheep have focused on a global restriction model in which total energy (and all other macro/micronutrients) is restricted. These studies have shown a range of metabolic and cardiovascular effects (see Table 1.4). However, in Westernised countries a lack of energy intake is rarely a problem for pregnant women, due to the high availability of energy dense foodstuffs. More common is an imbalance of nutrients such that a greater proportion of energy is consumed relative to, for example, the proportion of protein leading to a mismatch of food groups. In addition, the timing of any nutritional insult is important, as particular organs develop at different rates and periods during gestation. Organogenesis proceeds first by a priority of cell differentiation, then proliferation and finally by maturation of these cells. Therefore differing effects of undernutrition will be observed depending on the relative priority of cellular processes being undertaken at the time of a given nutritional perturbation.

Hence, this study has considered two periods of nutrient restriction, one up to day 65 of gestation (chosen to coincide with the peak period of nephrogenesis) and one from day 65 to full term (~147 days) and has specifically considered the effect of a low protein (but adequate energy) diet compared with a control ruminant diet (i.e. one providing 9% protein vs. 18% protein). The protein content of 18% more than adequately meets the protein requirements of a pregnant ewe, and indeed such an animal has no additional

requirements compared with a non-pregnant ewe during early to mid-gestation (Robinson and Forbes, 1967). Although similar percentages of protein have also been used in programming studies of rats (Langley-Evans et al., 1999; Brawley et al., 2003), these figures were set independently as 18% is a normal protein intake for a sheep at pasture, whereas 9% is considered to be equivalent to that available to ewes reared on a heather-dominant pasture. The latter situation may occur in many hill areas in the UK where increased consumption of tannins (for example in heather) limit amino acid availability to the ewe.

To summarise, this chapter considers the effect of a low protein diet both on the ewe throughout gestation and on the fetus at day 65 (or 0.44 gestation). The investigation characterises the effect of a low protein maternal diet on maternal gestational weight gain and body condition score. In addition it investigates the effect on the developing fetus in terms of renal development, at both a morphological and molecular level.

3.2 Materials and Methods

3.2.1 Study Design

Full details are given in Chapter 2. A brief summary of the experimental design is as follows:

All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 and were approved by the relevant local ethical review committees of the Macauley Institute and the University of Nottingham. Thirty-seven pregnant Scottish Blackface ewes carrying singleton fetuses were randomly allocated to one of three diet groups fed either a control diet (CP; n = 15) providing adequate protein from day 0 of gestation to term (term ~147 days), or a protein-restricted diet during either early (LPE, days 0 – 65 gestation; n = 16) or late gestation (LPL, day 66 – term; n = 6). On an 'as fed basis', the diets were isocaloric with the effective level of protein restriction being 8.7 vs. 17

g crude protein. MJ ME (metabolic energy). At day 65 gestation, chosen to coincide with peak nephrogenesis in the sheep (Wintour and Moritz, 1997), a proportion of CP (n = 9) and LPE (n = 9) ewes were euthanised and the fetal kidneys snap frozen in liquid nitrogen and stored at -80°C for further analysis. The remaining ewes carried to term and are reported in Chapter 4.

Maternal weight and body condition score was measured prior to and up to day 65 in the group that were euthanised at this time point, and to term in the other group, and data for the whole pregnancy is reported in this chapter. All fetal data presented in this chapter relates to the group that were euthanised at day 65 gestation.

3.2.2 Renal histology

One kidney from each fetus was sectioned transversely, stained with periodic acid Schiff's reagent (section 2.5.3) and assessed for morphological differences between the diet groups.

3.2.3 Renal immunohistochemistry

Renal sections were analysed for abundance of VEGFA (SC-152; Santa Cruz Biotechnology, Santa Cruz, USA) using a biotinylated ABC kit (Vector Labs, Peterborough, UK). The full method is given in section 2.6.1.

The primary and secondary antibodies were both diluted 1:200. Appropriate negative controls were included for the primary antibody (i.e. omission of primary antibody) and for non-specific binding of the secondary antibody (i.e. using a rabbit IgG; Vector Labs, Peterborough, UK). Adult sheep kidney was used as a positive control.

3.2.4 Detection of apoptosis

The presence and extent of apoptotic nuclei was determined using a TUNEL kit (Calbiochem, Nottingham, UK). The full method is described in section 2.7. Omission of TdT enzyme and reaction mixture was used as a negative control, and a mixture of HL60 cells incubated with 0.5 µg/ml actinomycin D for 19 hours to induce apoptosis (provided by the manufacturer) was used as a positive control.

3.2.5 Quantitative PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Crawley, UK) and cDNA was synthesised using an Omniscript reverse transcriptase kit (Qiagen, Crawley, UK). QPCR was performed using QuantiTect SYBR Green RT-PCR Kit (Qiagen, Crawley, UK) on a Roche Lightcycler® 480. Melt curves were used to confirm reaction specificity and cyclophilin, beta actin and GAPDH were used as housekeeping genes. Negative controls were H₂O, and samples with Omniscript omitted. mRNA quantities were normalised to housekeeping genes using Roche Lightcycler® 480 advanced relative quantification software. Full details of the method are described at sections 2.12.1.1, 2.12.2, 2.12.3, 2.12.4.1 and 2.12.5.

3.2.6 Statistical Analysis

All data apart from the qualitative scoring of kidney maturity were analyzed using a General Linear Model (GLM) or general linear mixed model approach where appropriate (Genstat v13, VSNi, UK) after checking for normality (or otherwise) of the error distribution. Data with skewed errors were log transformed before analysis. Estimated marginal means are presented with SEM, or s.e.d. as indicated, to represent the measurement error. $P < 0.05$ was accepted as indicating statistical significance. For the qualitative scoring of kidney maturation, scores for each treatment group were assessed by χ^2 .

3.3 Results

3.3.1 Maternal weight and body condition score

Maternal weight was not significantly different between diet groups neither before pregnancy, at conception, nor during gestation. As would be expected, weight increased during the course of gestation in all groups, from 61.3 ± 0.94 kg at conception up to 75.6 ± 0.94 kg at day 124 of gestation (average for all groups; data for individual groups shown in Figure 3.2). However, there was no diet*sex interaction and the effects of time on weight gain were seen equally in all groups.

Body condition score (BCS) was similar at conception, increased slightly during early pregnancy and declined toward the end of gestation (Figure 3.1). The LPE group tended to have lower BCS (2.60 ± 0.04 LPE vs 2.65 ± 0.05 CP and 2.66 ± 0.04 LPL; $P = 0.06$), however the changes observed were very small, with LPE having only a 1.8% reduction compared with controls.

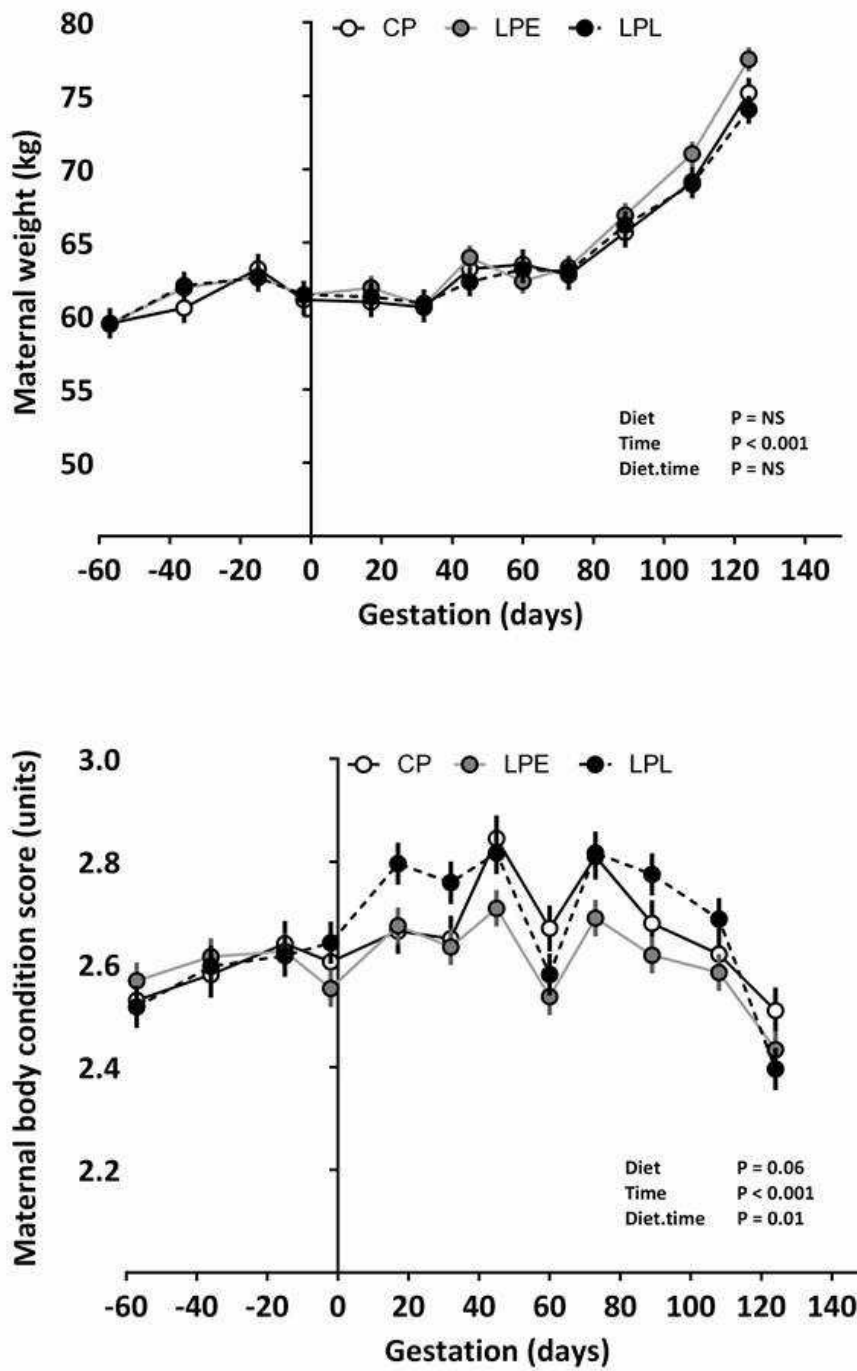


Figure 3.1: Maternal weight and body condition score of ewes during pregnancy.

3.3.2 Fetal organ weights

At *post mortem* only sex of the fetus, but not maternal diet, had a significant effect on fetal body weight, liver, and heart weights which were all significantly higher in males. Kidney weights tended to be higher in males although this did not reach statistical significance ($P = 0.09$) and brain and lung weights were not different between the sexes (Table 3.1).

Table 3.1: Effect of maternal low protein diet on fetal weights at 65 days gestation

	Diet	Sex		P value		
		male	female	Diet	Sex	Diet*Sex
Body weight (g)	CP	111 ± 4	89 ± 5	NS	0.004	NS
	LP	110 ± 9	95 ± 3			
Brain weight (g)	CP	3.46 ± 0.08	3.32 ± 0.15	NS	NS	NS
	LP	3.55 ± 0.15	3.52 ± 0.04			
Liver weight (g)	CP	8.42 ± 0.39	6.92 ± 0.51	NS	0.007	NS
	LP	9.02 ± 0.37	7.30 ± 0.52			
Heart weight (g)	CP	0.97 ± 0.08	0.90 ± 0.09	NS	0.040	NS
	LP	1.17 ± 0.06	0.90 ± 0.07			
Lung weight (g)	CP	5.32 ± 0.45	4.79 ± 0.79	NS	NS	NS
	LP	5.88 ± 0.99	4.76 ± 0.42			
Kidney weight (g)	CP	1.77 ± 0.22	1.43 ± 0.11	NS	NS	NS
	LP	1.66 ± 0.16	1.50 ± 0.07			

Data are predicted means ± S.E.M. for ewes (CP, n = 8; LP, n = 10) fed a control protein diet (CP fetuses, male, n = 4; female, n = 4) or a low protein diet to day 65 gestation (LP fetuses, male, n = 4; female, n = 6). Data were analysed by general linear model for the fixed effects of treatment (control vs. low protein), sex (male vs. female) or their interaction using Genstat v13 (VSNi, UK). Statistical significance was accepted at $P < 0.05$, NS, not significant.

3.3.3 Fetal kidney apoptosis

The percentage of cells that were TUNEL positive (indicating DNA fragmentation resulting from apoptosis) was significantly higher in both the nephrogenic zone around the outer edge of the cortex, and throughout the whole kidney (Figure 3.2). In the whole kidney there was a two-fold increase in the number of TUNEL positive cells in the low protein group compared with controls, however in the nephrogenic zone the difference was more pronounced, with an eight-fold increase in the LPE group.

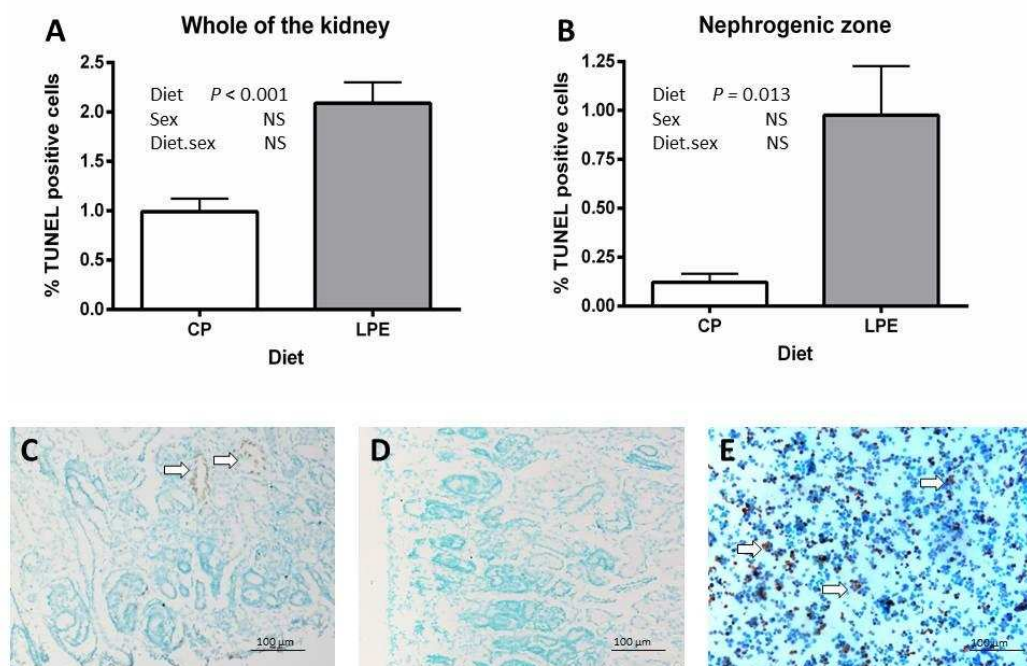


Figure 3.2: TUNEL positive staining and quantification in fetal tissue at day 65 gestation

A – the percentage of TUNEL positive cells in the whole of the fetal kidney section; B – the percentage of TUNEL positive cells in the nephrogenic region; C – a representative micrograph of the TUNEL staining (brown) with methyl green counterstain and arrows indicating TUNEL positive cells; D – negative control; E- positive control (cells incubated with 0.5 $\mu\text{g}/\text{ml}$ actinomycin D for 19 hours to induce apoptosis). Data in graphs are predicted means \pm S.E.M. for ewes (CP, $n = 8$; LP, $n = 10$) fed a control protein diet (CP fetuses, male, $n = 4$; female, $n = 4$) or a low protein diet to day 65 gestation (LP fetuses, male, $n = 4$; female, $n = 6$). Data were analysed by general linear model for the fixed effects of treatment (control vs. low protein), sex (male vs. female) or their interaction using Genstat v13 (VSNi, UK). Statistical significance was accepted at $P < 0.05$, NS, not significant.

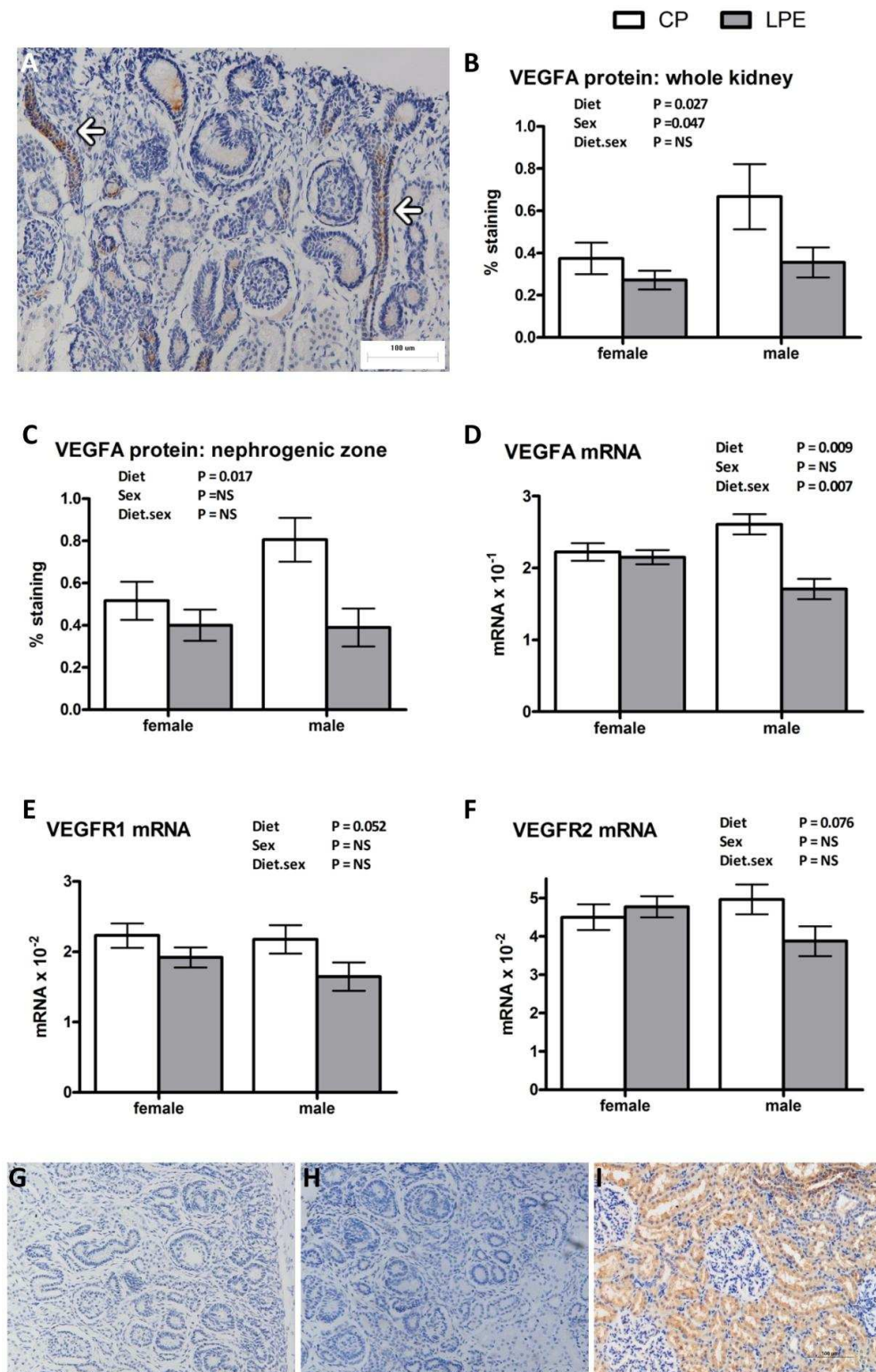


Figure 3.3: VEGFA and receptors gene and protein expression in fetal tissue at day 65 gestation

A – representative micrograph of VEGFA staining (brown) with arrows indicating staining in collecting ducts; B & C quantification of staining in the whole sample and nephrogenic zone; D – F, mRNA for VEGFA and its receptors VEGFR1 & 2; negative controls were G (omission of antibody) and H (IgG control); I, positive control, adult kidney. Data in graphs are predicted means \pm S.E.M. for ewes (CP, n = 8; LP, n = 10) fed a control protein diet (CP fetuses, male, n = 4; female, n = 4) or a low protein diet to day 65 gestation (LP fetuses, male, n = 4; female, n = 6). Data were analysed by general linear model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance was accepted at $P < 0.05$, NS, not significant.

3.3.4 Vascular Endothelial Growth Factor

VEGFA protein as measured by immunohistochemical staining was significantly lower in the LPE group compared to controls, in both the whole of the fetal kidney and the nephrogenic zone around the outer edge of the cortex (Figure 3.3 A-C). In addition, for the whole kidney, males had around 50% more staining than females ($P = 0.047$). However although the numerical mean for males was higher than for females in the nephrogenic zone, this was not statistically significant. Furthermore, the difference between control and low protein VEGFA levels was more pronounced in males than females, which was also mirrored by the levels of mRNA for VEGFA (Figure 3.3 D) where there was a significant diet*sex interaction ($P < 0.01$). VEGFA mRNA was also reduced in the LPE group compared to controls (0.237 ± 0.009 vs 0.199 ± 0.008 ; $P < 0.01$). The mRNA for the VEGF receptor VEGFR1 showed a strong trend ($P = 0.052$) towards reduced expression in the LPE group in both sexes, relative to controls (1.78 ± 0.12 vs. $2.23 \pm 0.14 \times 10^{-2}$ units). VEGFR2 also tended to be lower in the LPE group compared to controls, but this was not statistically significant (Figure 3.3 E - F). However, as for the other variables, greater differences between treatment groups were observed in males.

3.3.5 Other genes involved in the angiogenesis pathway

There was no effect of diet or sex on transcript expression for angiogenesis promoters including angiopoietin 1 (Ang1), the Tie 2 receptor, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and transforming growth factor beta 1 (TGF β 1). However, for Ang1, Tie2, bFGF, PDGF and TGF β 1 there was a significant diet.sex interaction, with lower mRNA levels in male LPE fetuses only (Figure 3.4).

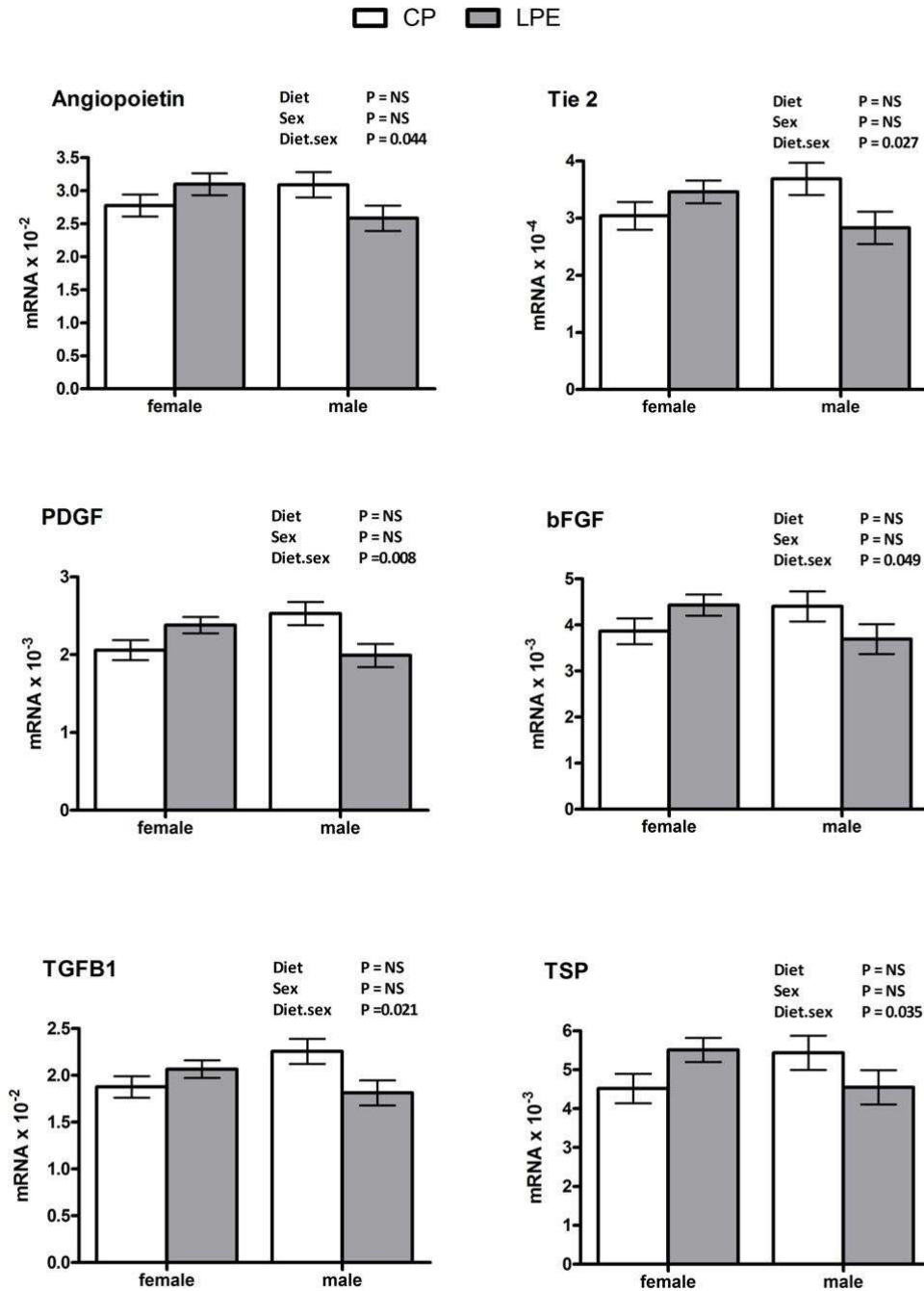


Figure 3.4: Gene expression of angiogenic factors in fetal tissue at day 65 gestation

Data are predicted means \pm S.E.M. for ewes (CP, n = 8; LP, n = 10) fed a control protein diet (CP fetuses, male, n = 4; female, n = 4) or a low protein diet to day 65 gestation (LP fetuses, male, n = 4; female, n = 6). Data were analysed by general linear model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance was accepted at $P < 0.05$; NS, not significant; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; TGF β 1, transforming growth factor beta-1; TSP, thrombospondin.

Bone morphogenetic protein 7 (BMP7) was not different between groups ($5.68 \pm 0.34 \times 10^{-3}$ CP, vs $6.13 \pm 0.30 \times 10^{-3}$ LPE), and neither was secreted protein, acidic, cysteine-rich (SPARC; $7.83 \pm 0.49 \times 10^{-2}$ CP, vs $7.47 \pm 0.46 \times 10^{-2}$ LPE) an angiogenesis inhibitor. However, one inhibitor of angiogenesis, thrombospondin (TSP) had a significant diet.sex interaction, with gene expression increased in female LPE fetuses but decreased in male LPE fetuses compared to controls (Figure 3.4).

3.4 Discussion

Protein-energy malnutrition (PEM) is highly prevalent in developing or emerging countries with up to 20% and 8% children in India and China, respectively - which equates to over 130 million individuals in these two countries alone - being affected (WHO, 2011). Whilst PEM is less prevalent in developed Westernised societies e.g. (0.8% children in the USA) this still equates to ~2 million children (WHO, 2011). In addition, the high incidence of pregnancy-induced nausea and vomiting (75-90% of women) and in its extreme version *hyperemesis gravidarum* (1% of women) during early pregnancy means that many women (and therefore their fetuses) may experience some degree of macro/micronutrient deficiency at this time (Fejzo et al., 2009).

In this study protein restriction early or late in gestation had no effect on the ewe's weight gain during pregnancy, which is likely to reflect the isocaloric nature of the low protein and control diets. Body condition score – a subjective marker of overall fat mass - was slightly lower in the LPE group by the end of gestation but only by 2%. These results indicate that the nutritional insult is mild, as the ewe is not detrimentally affected during gestation, with no clear indications of maternal malnutrition. In contrast global nutrient restriction has been shown to affect maternal weight gain when given in mid gestation (Vonnahme et al.,

2003) or late gestation (Chadio et al., 2007). However, most studies fail to report the effects on the ewe and concentrate solely on the effects on the offspring.

Fetal body weight and organ weights were not different between dietary groups, although there was an effect of sex, with males having higher total body and organ weights.

However, the greater organ weights were a reflection of the higher overall body weight, and once adjusted for body weight the effects did not persist. Interestingly, the difference in fetal weight for males and females at such an early stage in gestation (day 65 out of term 147 days), is at a time when there is no exposure to secondary sex hormones, suggesting that differences in size between males and females are not entirely dependent on these hormones. In contrast, another programming study of sheep (MacLaughlin et al., 2005) found no differences in fetal weight at d 53 – 56 gestation. It may be that differences are only observed at particular time points during gestation, as fetal growth does not follow a linear course, but instead is a pulsatile process, and differences in fetal growth rates in humans have been shown to be sex-specific (Lampl and Jeanty, 2003).

A further consideration is that larger animals like sheep and humans have a greater metabolic reserve capacity compared to smaller mammals and are therefore better able to cope with temporary nutritional deficits in protein intake. However, because sheep are ruminants and the rumen contains microbes which are able to synthesise amino acids, a dietary deficit may not always lead to a deficit for the fetus. This is considered further in Chapter 5 through experiments measuring the levels of amino acids in maternal and fetal plasma, and fetal amniotic fluid.

The development of the kidney depends on the balance between survival factors promoting growth and factors promoting cell death. The process of apoptosis, or programmed cell death, is a normal part of cellular development and maintenance and tight control of this process is crucial. Dysregulation of apoptotic pathways is present in

many renal disease states. An increase in apoptosis has been observed in diabetic nephropathies in mice (Susztak et al., 2006), rats (Zhang et al., 1997) and humans (Kumar et al., 2004). Furthermore, higher levels of apoptosis have been found at different stages of disease progression in a rat model of experimental glomerulonephritis (Shimizu et al., 1996) and in human polycystic and dysplastic kidneys both pre- and post-natally (Woo, 1995; Winyard et al., 1996). In addition, evidence shows that fetal programming may also result in an abnormally increased level of apoptosis. This current study found that apoptosis was increased in the LPE group at the midpoint of nephrogenesis, both in the whole of the kidney but also, crucially, in the nephrogenic zone, and this finding is replicated in other programming studies. Intrauterine growth restriction in rats resulted in altered mRNA levels for apoptosis-related proteins, increased renal apoptosis and reduced nephron number (Pham et al., 2003). Also a maternal low protein diet in rats reduced nephron number, increased systolic blood pressure and increased apoptosis in kidneys at 8 weeks of age, despite the kidneys looking morphologically normal (Vehaskari et al., 2001). In rat fetal kidneys an increase in apoptosis in kidney condensing mesenchymal cells (i.e. nephron progenitors) and in loose mesenchymal cells (precursors of interstitial cells) at embryonic day 13 was observed for those fed a low protein diet (Welham et al., 2002).

This study has also demonstrated decreased expression of important mediators of vasculogenesis (*de novo* vessel formation) and angiogenesis (sprouting of new vessels from existing vessels), which indicates potential for reduced renal vascular growth during fetal development. Vascular development involves overlapping processes of formation, stabilisation, branching, remodelling and pruning, and specialisation (Jain, 2003). VEGFA is a signalling protein that regulates endothelial cell growth and differentiation and is essential for vasculogenesis and angiogenesis in all organs (Breier et al., 1992; Tufro et al., 1999). VEGFA has many important biological roles which vary according to the stage of development and physiological function in the organ in which it is expressed (Breen, 2007).

Initially VEGF was called vascular permeability factor (VPF), following its discovery as a protein in tumour ascites fluids that increased microvascular permeability (Senger et al., 1983). Independently of this work VEGF was identified as a potent mitogen for vascular endothelial cells from both small and large vessels (Leung et al., 1989), before it was realised that VEGF and VPF were in fact the same protein which had a variety of roles (Keck et al., 1989). Transgenic mouse gene knockout models have been utilised to further prove the importance of VEGFA. Homozygous deletions lead to perinatal lethality (Eremina et al., 2003) and heterozygous deletions result in renal disease at a young age (Eremina et al., 2003) due to abnormal formation of blood vessels (Carmeliet et al., 1996). VEGFA is highly expressed in podocytes and plays an important role in both establishing and maintaining the glomerular barrier (Eremina et al., 2003). Podocyte-specific heterozygous VEGFA knockouts also die early from renal failure and do not produce differentiated mesangial cells, thus showing VEGFA's role in mesangial cell development (Eremina et al., 2006). In the present study, VEGFA mRNA was reduced in the LPE group, although the effect was much more pronounced in males. However, both sexes showed a more substantial reduction at the protein level, both in the whole of the kidney and – critically – in the nephrogenic zone, suggesting that VEGFA is the key angiogenic factor affected by the nutritional deficit of maternal dietary protein intake. This has also been verified in other fetal studies involving a large mammal model, where reduced VEGFA in response to a prenatal sub-optimal diet in non-human primates has been demonstrated (Cox et al., 2006; Nijland et al., 2007).

In this study VEGF staining was primarily found in the developing collecting ducts (see Figure 3.3), and very little was observed in the mature glomeruli, despite podocytes being a main source of VEGF expression (Rask-Madsen and King, 2010). However, this pattern of staining has also been observed in other studies, which tend to find minimal staining in glomeruli when paraffin sections are used (Burt et al., 2007), and demonstrate evidence of

VEGF staining in the collecting ducts (Cooper et al., 1999; Gealekman et al., 2004). In later fetal life and mature kidneys VEGF is also expressed by other parts of the tubules (Tufro et al., 1999; Kang et al., 2001).

In the fetal kidney peritubular capillaries have been shown using immunohistochemistry techniques to be positive for VEGF receptor proteins (Simon et al., 1998), therefore the VEGF in the collecting ducts may act through paracrine signalling via these nearby receptors. In addition, VEGF receptors have been identified in non-endothelial cells and tissues, including the tubular cells of both fetal and adult kidneys, and it is hypothesised that VEGF may have both autocrine as well as paracrine effects. For example, one study found a rat renal tubular epithelial cell line expressed VEGFR1 and VEGFR2 mRNA and protein, a finding that was replicated *in vivo* with sections of normal rat kidney (Kanellis et al., 2000). In addition, when the cell line was exposed to VEGF it significantly stimulated their proliferation via VEGFR2 and the Akt signalling pathway, as well as protecting the cells from hydrogen peroxide-induced apoptosis. Furthermore, another study revealed both autocrine and paracrine functions of VEGF (Villegas et al., 2005). Using three different cell lines (rat and mouse proximal tubular cells, and normal rat kidney cells) it was confirmed that all three secreted VEGF and this stimulated proliferation and survival of tubular epithelial cells via the VEGFR2 receptor in a paracrine manner. In addition there was a paracrine effect through the VEGF acting to chemoattract and recruit endothelial cells when they were co-cultured.

Further studies have shown the importance of the VEGF receptors, VEGFR1 and VEGFR2. Homozygous VEGFR1-deficient knockout mice form endothelial cells but these are arranged into abnormal vascular channels and the embryos die *in utero* (Fong et al., 1995). Homozygous VEGFR2-deficient mice fail to develop blood islands in the yolk sac and have no apparent organised blood vessels, and this is also embryonically lethal (Shalaby et al.,

1995). Therefore both of these receptors are critical for normal vascular development.

This study showed a trend for decreasing VEGFR1 and VEGFR2 mRNA in the LPE fetuses, but this did not reach statistical significance (VEGFR1, $P = 0.052$; VEGFR2, $P = 0.076$). Hence VEGFR1, having only just missed statistical significance was re-tested in a further fetal study (see Chapter 5).

In addition this study considered the effect of a low protein diet on key angiogenic factors by measuring gene expression of both promoters and inhibitors of angiogenesis. Previous research has shown that whilst VEGFA is the main initiator of vasculogenesis and angiogenesis, other factors also have essential roles as the vasculature forms. The primitive vessels initially consist of a tube of endothelial cells and as these mature they become stabilised through recruitment of mural cells (pericytes and vascular smooth muscle cells) and extracellular matrix (ECM); this varies according to whether they are specialising into a capillary, artery or vein (Jain, 2003). At this stage of stabilisation and specialisation PDGF is secreted by endothelial cells to recruit mural cells, and through its receptor PDGFR- β (which is expressed on mural cells) it causes proliferation and migration of these cells (Hellstrom et al., 2001).

TGF β 1 also has a role at this stage of vascular development, as it induces differentiation of mesenchymal cells to mural cells (Chambers et al., 2003), and Ang1/Tie 2 receptor assists in the stabilisation of the nascent vessels by making them leak-proof (Thurston et al., 1999). Overexpression of Ang1 in transgenic mice has been shown to lead to a higher number of blood vessels that are considerably larger and more highly branched (Suri et al., 1998), and at high doses TGF β 1 appears to inhibit endothelial cell differentiation (Yancopoulos et al., 2000), thus, like VEGFA, a tight control of these factors is needed in order to ensure normal development.

The present study showed that all the measured promoters of angiogenesis were decreased in the LPE group at 0.44 gestation, but only in males, suggesting that females may have some protective mechanisms. This indicates reduced potential for intrarenal microvascular growth, which is explored further by measuring CD34, an endothelial cell marker, in the adult kidney (Chapter 4). This study is the first to show sex-specific effects in a large animal model, although it is not uncommon in rodent programming studies to find that males are more adversely affected than females (Kwong et al., 2000; Ozaki et al., 2001; Woods et al., 2005). Furthermore, taken in conjunction with the increased proportion of apoptotic nuclei within the nephrogenic zone of the developing kidney it would appear that the dietary protein deficit in the maternal environment has an impact on the fetus, and specifically on those organs such as the kidney undergoing hyperplastic growth at that time. Although gross differences in the fetus such as a reduction in kidney weight were not observed between dietary groups, it appears that more subtle changes have occurred.

Chapter 4 will consider the effects of the low protein diet on fetuses at full term and on their postnatal growth trajectories, alongside possible changes in the structure and function of the adult kidneys at two years of age, to see whether the subtle effects observed in the fetus have longer term effects.

4. Study 1: The effect of a maternal low protein diet on the offspring

4.1 Introduction

In the previous chapter, subtle yet significant changes were identified in the developing kidneys of the fetus, where diet was lower in protein during the first 65 days of gestation. Although there was no evidence of disturbance to the gross morphology of the kidneys at day 65, there were changes in the expression and translation of genes that mediate angiogenesis, including VEGFA, alongside increased apoptosis in the nephrogenic zone.

The changes observed in the fetus are interesting *per se*, however the strength of this study lay in the opportunity to follow a cohort of sheep subject to the same experimental conditions (i.e. a maternal low protein diet over the first 65 days of gestation) to adulthood, and therefore to observe whether programming of the fetus translated into altered renal function in the adult. In addition, there was the opportunity to consider whether the timing of a nutritional insult was important in the programming of renal function, as a further group were subjected to a low protein diet from day 65 through to the end of gestation (full term = ~147 days).

The effect of a low protein maternal diet on nephron endowment is likely to be subtle. It is unlikely to be solely responsible for compromised adult renal function but is considered to be a 'first-hit' (or Brenner's hypothesis; Brenner et al., 1988). Fewer nephrons, coupled with a 'second hit' such as obesity, type II diabetes, or hypertension, may affect renal function, and 'healthspan' can be significantly reduced in those prenatally compromised individuals (Griffin *et al.*, 2008).

The prevalence of obesity and its associated co-morbidities is rising rapidly. In the UK in 2010 65.8% of the population aged between 15 and 100 were overweight or obese with a

BMI $\geq 25.0 \text{ kg.m}^{-2}$ (World-Health-Organisation-Global-InfoBase, 2010). WHO statistics also show that in the USA the situation is even more pronounced, with 78.6% classified as overweight or obese. The emerging nations such as India and China are also experiencing a rapid rise in the levels of obesity, with 22.5% and 38.5% respectively being overweight or obese in 2010. As these two countries are the most populous in the world (India, 1.18 billion; China, 1.33 billion; (United Nations Statistics Division, 2012) this equates to 266 million and 514 million individuals respectively with a BMI $\geq 25 \text{ kg.m}^{-2}$.

Overweight and obesity are associated with increased risk of mortality from kidney disease (Flegal et al., 2007). The relative risk for end stage renal disease rises with each category of overweight, even after adjustment for other major risk factors such as smoking, hypertension and type II diabetes. Compared with lean controls, the relative risk for overweight was 1.87 [95% CI 1.64 – 2.14]; for class I obesity, 3.57 [3.05 – 4.18]; for class II obesity, 6.12 [4.97 – 7.54]; and for class III obesity, 7.07 [5.37 – 9.31] (Hsu et al., 2006). Furthermore, men and women with a BMI $\geq 25 \text{ kg.m}^{-2}$ at age 20, 40 and 60 had a 3-fold increased risk of CKD compared with those with a normal BMI (Ejerblad et al., 2006).

The mechanisms through which obesity may cause renal disease are still poorly understood, although it is thought that inflammation, lipotoxicity and haemodynamic factors may play a role (for reviews, see Hall, 2003; Wahba and Mak, 2007).

A traditional early marker for CKD (i.e. before there are any overt clinical signs) is microalbuminuria, which can reliably predict future CKD (Fox *et al.*, 2010). A reduced filtration barrier in the glomerulus allows small amounts of albumin to pass through into the urine, and is likely initially to be due to effacement of the podocyte foot processes. The integrity of the foot processes is supported by a number of factors, but primarily by VEGFA (Eremina et al., 2008a). Obesity leads to a tissue specific increase in VEGFA, to support the concomitant local hypoxia (Wood *et al.*, 2009) and in obese sheep it has been shown to be

positively associated with increased intra-renal endoplasmic stress, a potential early prognostic marker for CKD (Sharkey et al., 2009a). Maternal under-nutrition during fetal development may have subtle effects on nephron number, increased blood pressure and other metabolic variables; however, it is obesity later in life alongside the natural process of ageing that intensifies and exacerbates these effects and may lead to CKD.

An important indicator of renal function is glomerular filtration rate (GFR) which is the rate of flow of filtered fluid through the kidney (O'Callaghan, 2009). In humans it is most common to estimate GFR (eGFR), so as to avoid exposure to the radioactive nuclides that are needed to provide an exact measurement. There are several methods of eGFR estimation, although most laboratories in the UK use the Modification of Diet in Renal Disease (MDRD) formula, which uses serum creatinine levels along with information on age, race and gender (Levey et al., 1999). A criticism of this method is that it tends to underestimate GFR by around 6% in patients with CKD, but by around 29% in healthy individuals (Rule et al., 2004) when compared to GFR calculated by urinary clearance of I-125-iothalamate. However, it is more accurate than another estimation method called the Cockcroft-Gault equation (Levey et al., 2006). Using an animal model and applying the radioactive isotope method means that greater accuracy can be ensured.

A decline in GFR is an inevitable part of ageing, as nephrons cease to function. Peak GFR is reached around the age of 18-20 years, and after that point there is a slow decline. The rate of decline starts to increase after the age of 45, at an annual rate of around $8 \text{ ml} \cdot \text{min}^{-1} \cdot 1.73 \text{ m}^{-2}$ (Poggio et al., 2009). Reduced nephron number leads to compensatory mechanisms so that, for example, a reduction of 50% of the nephrons due to a nephrectomy only leads to around 20-30% reduction in function (Addis et al., 1924; Krohn et al., 1966; Fehrman-Ekholm et al., 2001).

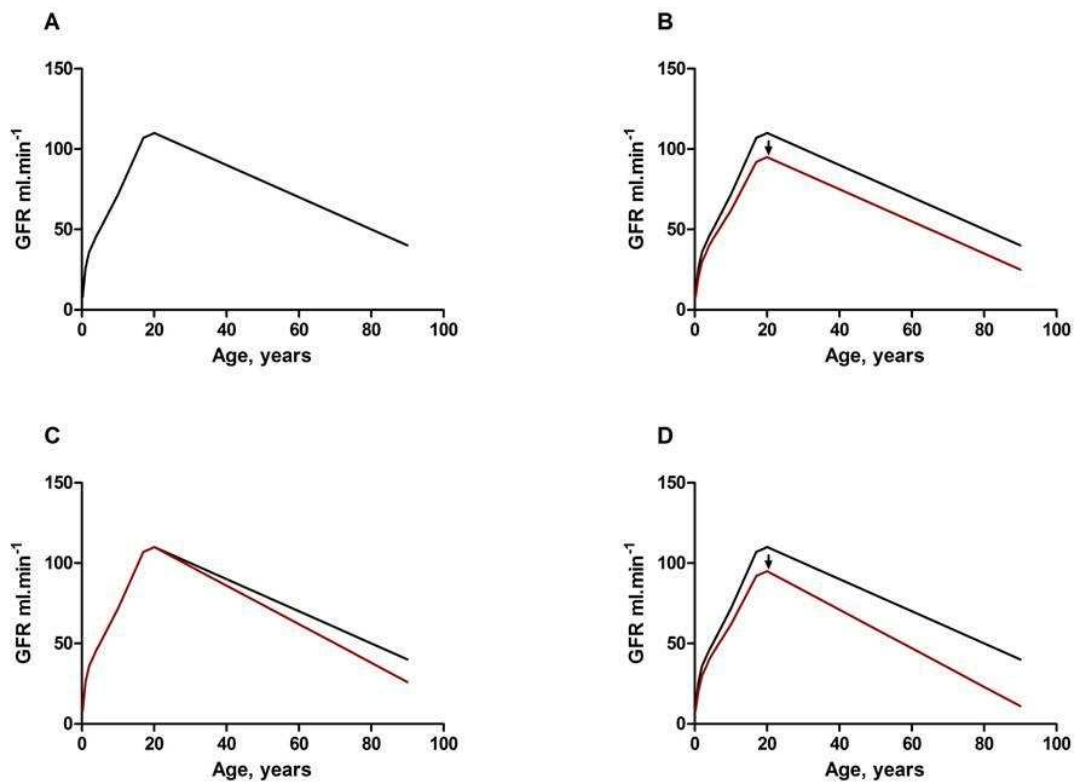


Figure 4.1: Schematic graphs of glomerular filtration rate (GFR) during lifetime

A: normal GFR reaches peak during early adulthood, before declining with age; B: a shift in the line downwards (red line) means a failure to reach the peak and GFR falls below $60 \text{ ml}\cdot\text{min}^{-1}\cdot 1.73\text{m}^{-2}$ (the cut off point for stage I chronic kidney disease) in late middle age, rather than in old age. C: The peak GFR is still reached, but there is a faster rate of decline (red line) and GFR declines below $60 \text{ ml}\cdot\text{min}^{-1}\cdot 1.73\text{m}^{-2}$ around a decade earlier. D: a combination of B and C means that the peak is not reached, and also function declines at a faster rate, leading to CKD in early middle age.

Figure 4.1A shows a schematic diagram of GFR with increasing age. Although absolute GFR does not reach its peak until early adulthood, this is largely a reflection of body surface area. When adjusted for this, the peak is reached much earlier (around 2-3 years of age; Wahl et al., 2003). In Figure 1.4B, the red line shows a scenario where the peak reached is lower, perhaps due to a reduction in the nephron number at birth. This means that the natural decline observed later in life may lead to chronic kidney disease once the GFR falls below $60 \text{ ml}\cdot\text{min}^{-1}\cdot 1.73\text{m}^{-2}$. Alternatively, there may be a full nephron complement, but a failure to maintain functionality caused by either changes *in utero*, or through lifestyle as an

adult. This would lead to the same peak but a faster rate of decline, as shown by the red line in Figure 4.1C. A combination of both scenarios might mean a lower peak and an increased rate of decline, as shown by the red line in Figure 4.1D.

In Chapter 3, the fetal kidney exposed to a maternal low protein diet was clearly influenced by the dietary pattern. The investigation in this chapter considers whether there is evidence for any of the scenarios in Figure 4.1, by looking at renal function, nephron complement, and other changes within the adult kidney at the molecular level.

4.2 Materials and Methods

4.2.1 Study Design

Full details are given in Chapter 2. A brief summary of the experimental design follows.

All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 and were approved by the relevant local ethical review committees of the Macauley Institute and the University of Nottingham.

Thirty-seven pregnant Scottish Blackface ewes carrying singleton fetuses were randomly allocated to one of three diet groups fed either a control diet (CP; n = 15) providing adequate protein from day 0 of gestation to term (term ~147 days), or a protein-restricted diet during either early (LPE, days 0 – 65 gestation; n = 16) or late gestation (LPL, day 66 – term; n = 6). On an 'as fed basis', the diets were isocaloric with the effective level of protein restriction being 8.7 vs. 17 g crude protein·MJ ME (metabolic energy). Eighteen fetuses were killed on day 65 gestation (Chapter 3) and the remainder carried to term with the offspring delivered naturally. All male lambs were castrated. Lambs were vaccinated and put to pasture at two weeks of age and then weaned at 10 weeks. Lamb weight and body condition score were measured every three weeks.

The offspring were transferred to the University of Nottingham at 18 months of age and barn housed to create an obesogenic environment. At 24 months, *in vivo* renography was performed and then the sheep were euthanised, urine was sampled and the kidneys collected.

4.2.2 Urine and plasma analysis

Urine and plasma samples were analysed for a spot measurement of the urinary albumin:creatinine ratio (urine from the bladder) and plasma electrolytes (Na⁺, K⁺ and Cl⁻) as described in sections 2.3.2.1, 2.3.2.2 and 2.3.2.3.

4.2.3 Renal function

Renal function was assessed using gamma scintigraphy. Full details are given in section 2.4.

4.2.4 Histology

Random paraffin-embedded sections from each treatment group were stained with (H&E), Periodic-Acid Schiff Reagent (PAS) or a trichrome stain (sections 2.5.2, 2.5.3 and 2.5.4). These were examined to identify any obvious pathophysiological features such as monocyte infiltration, glomerular interstitial nephritis, glomerulosclerosis or basement membrane or mesangial cell thickening, the findings being confirmed by a consultant histopathologist.

4.2.5 Mean glomerular area

Mean glomerular area (μm^3) was assessed on 20 randomly selected glomeruli per animal using ImagePro MC 6.0 (section 2.9.1).

4.2.6 Stereological estimation

Kidney volume was measured using water displacement. Cortex:medulla ratio, nephron number, total glomerular volume and mean glomerular volume were estimated by unbiased stereology. The full method is explained in section 2.10.

4.2.7 Estimation of mammalian nephron number

A review of papers reporting estimates of nephron number in different mammalian species was carried out and the data used to predict nephron number in sheep as described in 2.11.

4.2.8 Lectin staining

Kidney sections were stained for lectin as a marker of endothelial cells. The lectin staining method is described in 2.8. Negative controls were omission of GSL1_{B4}, and as a further negative control galactose (a competing sugar) was added to GSL1_{B4}. Bovine ovary was used as a positive control.

4.2.9 Renal immunohistochemistry

4.2.9.1 VEGFA

Renal sections were analysed for abundance of VEGFA using the Leica BOND-MAX™ automated system (full method is given in 2.6.2, and further details of the VEGFA protein sequence and antibody selection are given in 3.2.3. Negative controls were omission of primary antibody and rabbit IgG, and mouse kidney was used as a positive control.

4.2.9.2 CD31 and CD34

CD31 and CD34 antibodies were tested on renal sections, using both the ABC system (section 2.6.1), and the BondMax automated system with and without antigen retrieval (section 2.6.2).

Negative controls were omission of primary antibody and rabbit IgG, and a multi-species microarray was used as a positive control.

4.2.10 Quantitative PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Crawley, UK) and cDNA was synthesised using an Omniscript reverse transcriptase kit (Qiagen, Crawley, UK). QPCR was performed using QuantiTect SYBR Green RT-PCR Kit (Qiagen, Crawley, UK) on a Roche Lightcycler® 480. Melt curves were used to confirm reaction specificity and cyclophilin, beta actin and GAPDH were used as housekeeping genes. Negative controls were H₂O and samples with Omniscript omitted. mRNA quantities were normalised to housekeeping genes using Roche Lightcycler® 480 advanced relative quantification software. Full details of the method are described at sections 2.12.1.1, 2.12.2, 2.12.4.1 and 2.12.5.

4.2.11 Statistical Analysis

All data apart from the albumin:creatinine ratio (ACR) results were analysed using a General Linear Model (GLM) or general linear mixed model approach where appropriate (Genstat v13, VSNi, UK) after checking for normality (or otherwise) of the error distribution. Data with skewed errors were log transformed before analysis. Estimated marginal means are presented with SEM. ACR was analysed using the Wilcoxon's rank sum test and results are presented at median and interquartile range. $P < 0.05$ was accepted as indicating statistical significance.

4.3 Results

4.3.1 *Maternal characteristics*

Maternal weight gain and body condition score is described at 3.3.1. In brief, there was no significant difference in weight gain during pregnancy between dietary groups, although there was a small (1.8%) tendency for reduction in body condition score in the LPE group during gestation.

4.3.2 *Offspring: weights and growth rates*

Birth weight was significantly reduced in the LPL, but not LPE, group compared with controls (Figure 4.2 A), however there was no effect of sex, nor a diet.sex interaction. The LPL group experienced a period of catch-up growth as there were no differences between dietary groups by the time of weaning at 12 weeks, or for weight at 18 months or 2 years of age (Figure 4.2 B-D), although by 2 years males tended to be heavier than females (male, 77.6 ± 2.67 ; female 70.7 ± 2.53 kg; $P = 0.076$).

At 1.5 years of age and at a similar weight (47.1 ± 0.7 kg, all groups) the offspring were reared in an obesogenic environment (barn housed to restrict exercise and decrease thermogenic loss, and fed *ad libitum*) for the following 35 weeks.

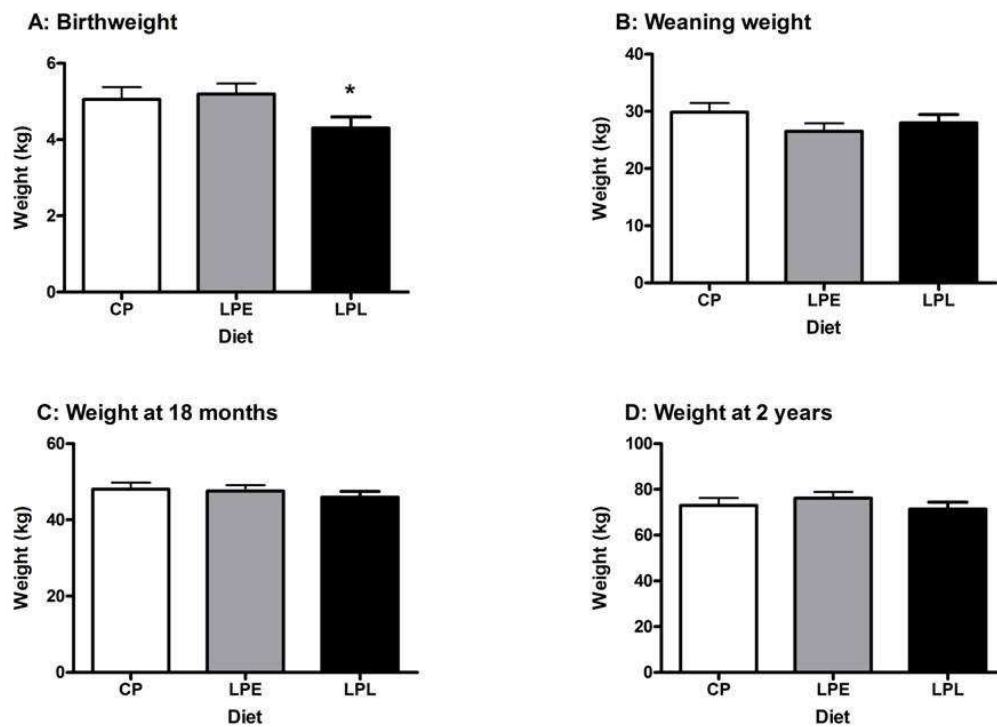


Figure 4.2: Weight of offspring at birth, weaning, 18 months and 2 years of age.

Data are Means \pm S.E.M. for offspring of ewes fed a control protein diet (CP, n=6), or a low protein diet during early gestation (days 0-65; LPE, n=7) or a low protein diet during late gestation (days 66-term; LPL, n=6). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. Data were analysed by General Linear Model for the fixed effects of treatment, sex and their interaction with (Genstat v13, VSNi, UK). CP, control protein diet; LPE, low protein early diet; LPL, low protein late diet; * = $P < 0.05$.

The purpose was to increase weight and body fat deposition to a point that corresponded to a theoretical gain in body mass index (BMI) from 22.5 to 32 $\text{kg}\cdot\text{m}^{-2}$, i.e. a change from a normal BMI to an obese one. Figure 4.3 shows the weight gain during this period. All groups gained weight at a similar rate, regardless of prenatal diet, although there was an effect of time because weight increased during the obesogenic period ($P < 0.001$) before stabilising at around 30 weeks of duration.

Growth in obesogenic environment

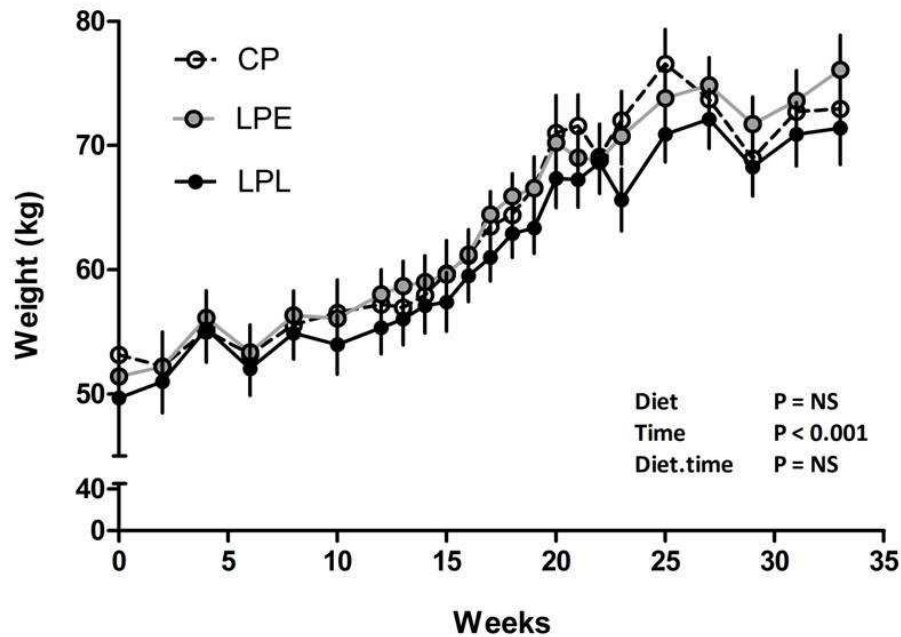


Figure 4.3: Weight gain of offspring during obesogenic period

Sheep were barn housed and fed *ad libitum* to promote weight gain from age 18 months. Data are Means \pm S.E.M. for offspring of ewes fed a control protein diet (CP, n=6), or a low protein diet during early gestation (days 0-65; LPE, n=7) or a low protein diet during late gestation (days 66-term; LPL, n=6). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. Data were analysed by General Linear Model for the fixed effects of treatment, sex and their interaction with (Genstat v13, VSNi, UK). CP, control protein diet; LPE, low protein early diet; LPL, low protein late diet; NS, non-significant.

There was no effect of diet or sex on the growth rate between birth and weaning at 12 weeks of age (CP, 272 ± 16.2 ; LPE, 235 ± 13.9 ; LPL 260 ± 14.6 g.day⁻¹; Figure 4.4 A) nor between weaning and 18 months (CP, 35.9 ± 3.12 ; LPE, 41.5 ± 2.68 ; LPL, 35.4 ± 2.82 g.day⁻¹; Figure 4.4 B). Between 18 months and 2 years of age, the rate of growth increased compared to that observed between weaning and 18 months, although there were no statistically significant differences between dietary groups (CP, 84.77 ± 8.87 ; LPE, 96.96 ± 7.61 ; LPL, 86.65 ± 8.00 g.day⁻¹; Figure 4.4 C). However, males tended to grow faster than females (males, 100.61 ± 7.28 vs females, 82.03 ± 6.15 g.day⁻¹; $P = 0.075$).

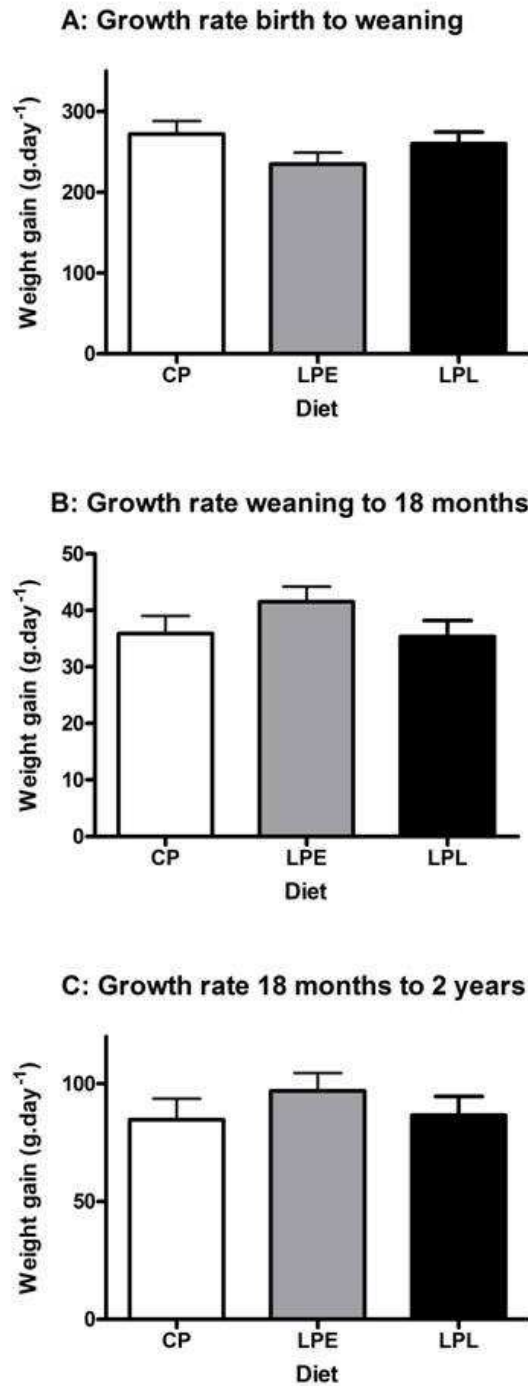


Figure 4.4: Growth rates of offspring

Growth rates of offspring, from birth to weaning (A), weaning to 18 months (B) and in an obesogenic environment from 18 months to 2 years of age (C). Data are Means \pm S.E.M. for offspring of ewes fed a control protein diet (CP, n = 6), or a low protein diet during early gestation (days 0-65; LPE, n = 7) or a low protein diet during late gestation (days 66-term; LPL, n = 6). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. Data were analysed by General Linear Model for the fixed effects of treatment, sex and their interaction with (Genstat v13, VSNi, UK). CP, control protein diet; LPE, low protein early diet; LPL, low protein late diet;

4.3.3 Post mortem data

Diet had no effect on the weights at post mortem of brain, heart, liver, kidneys, lungs or liver (Table 4.1). Brain, liver and total kidney weight was significantly higher in males than females; however this was attenuated once adjusted for total body weight. Other organs measured – spleen, adrenal and pituitary glands – were not significantly different in weight between groups.

The heart left ventricular thickness was higher in the LPE groups compared to the LPL and control groups (CP, 15.39 ± 0.80 ; LPE, 17.86 ± 0.69 ; LPL 15.37 ± 0.72 cm).

Various adipose tissue depots were also measured: total visceral fat, perirenal fat and omental fat were not different between groups. However, the amount of pericardial fat was significantly higher in the LPE group (CP, 5.29 ± 0.10 ; LPE, 5.53 ± 0.09 ; LPL, 5.15 ± 0.09 g.kg⁻¹ body weight).

Table 4.1: Organ weights at two years of age

<i>Organ</i>	<i>Diet</i>	<i>Sex</i>		<i>P value</i>		
		<i>male</i>	<i>female</i>	<i>Diet</i>	<i>Sex</i>	<i>Diet*Sex</i>
Brain (g)	CP	103 ± 3.63	91.1 ± 7.26			
	LPE	110 ± 5.14	91.0 ± 3.25	NS	<0.01	NS
	LPL	108 ± 7.26	91.1 ± 3.63			
Heart (g)	CP	299 ± 33.7	271 ± 43.1			
	LPE	313 ± 49.8	322 ± 32.4	NS	NS	NS
	LPL	303 ± 48.3	264 ± 29.7			
Liver (g)	CP	781 ± 47.3	640 ± 66.9			
	LPE	813 ± 66.9	684 ± 42.3	NS	0.018	NS
	LPL	774 ± 66.9	663 ± 47.3			
Lungs (g)	CP	660 ± 153	537 ± 176			
	LPE	682 ± 224	498 ± 103	NS	NS	NS
	LPL	610 ± 200	376 ± 87			
Total kidney (g)	CP	162 ± 10.3	128 ± 14.6			
	LPE	156 ± 14.6	117 ± 9.20	NS	0.010	NS
	LPL	150 ± 14.6	131 ± 10.3			
Kidney (g.kg⁻¹ weight)	CP	2.15 ± 0.15	1.83 ± 0.22			
	LPE	1.90 ± 0.22	1.63 ± 0.14	NS	NS	NS
	LPL	2.05 ± 0.22	1.86 ± 0.15			
Perirenal fat (kg)	CP	2.31 ± 0.35	2.60 ± 0.50			
	LPE	2.57 ± 0.50	2.41 ± 0.31	NS	NS	NS
	LPL	1.37 ± 0.50	2.57 ± 0.35			

Data are means ± S.E.M. for offspring of ewes fed a control protein diet (CP, n=6), or a low protein diet during early gestation (days 0-65; LPE, n=7) or a low protein diet during late gestation (days 66-term; LPL, n=6). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. Data were analysed by General Linear Model for the fixed effects of treatment, sex and their interaction with (Genstat v13, VSNi, UK). CP, control protein diet; LPE, low protein early diet; LPL, low protein late diet; NS, non-significant.

4.3.4 Urine and blood analysis

4.3.4.1 Sodium, potassium, chloride

Plasma sodium, potassium and chloride concentrations are shown in Figure 4.5. There was no significant effect of diet, sex or a diet.sex interaction on plasma sodium concentration (CP, 131.7 ± 4.95 ; LPE, 133.6 ± 4.24 ; LPL, 138.1 ± 4.46 mmol.L⁻¹), although the concentrations observed were slightly below sheep reference values 142 – 160 mmol.L⁻¹ (Aitken, 2007). Neither were any differences observed for chloride (CP, 93.6 ± 2.93 ; LPE, 92.5 ± 2.51 ; LPL, 96.1 ± 2.64 mmol.L⁻¹), which fell in the lower end of sheep reference values (Radostits et al., 2000).

Potassium was not significantly different between diets (CP, 7.98 ± 0.39 ; LPE, 7.64 ± 0.33 ; LPL, 7.90 ± 0.35 mmol.L⁻¹), but was reduced in males compared to females (7.23 ± 0.32 vs 8.27 ± 0.27 mmol.L⁻¹, $P = 0.029$). These values were higher than the published reference range of 3.9 – 5.4 mmol.L⁻¹ (Aitken, 2007) although it was not possible to check breed-specific values. However, haemolysis of plasma samples may lead to unreliable results with an incorrectly high level of potassium (Dimeski et al., 2005; Jeffery et al., 2009). Although precautions were taken to minimise the risk of haemolysis, i.e. minimal agitation of collection tubes and the immediate separation of plasma (Ismail et al., 2005), a visual inspection for haemolysis can be unreliable (Hawkins, 2002). In future studies, if potassium levels were as high as measured in this study a haemolyser index on an autoanalyser could be used to check definitively for haemolysis.

4.3.4.2 Albumin:creatinine ratio

The urinary albumin:creatinine ratio (ACR) was significantly increased in the LPE group compared with the controls and the LPL group (CP, 1.73 [1.71 – 2.67]; LPE, 8.88 [7.9-24.7];

LPL, 2.49 [1.63-2.98] mg. μmol^{-1} ; $P = 0.03$, median [IQR]) and there was no effect of sex, nor a diet.sex interaction.

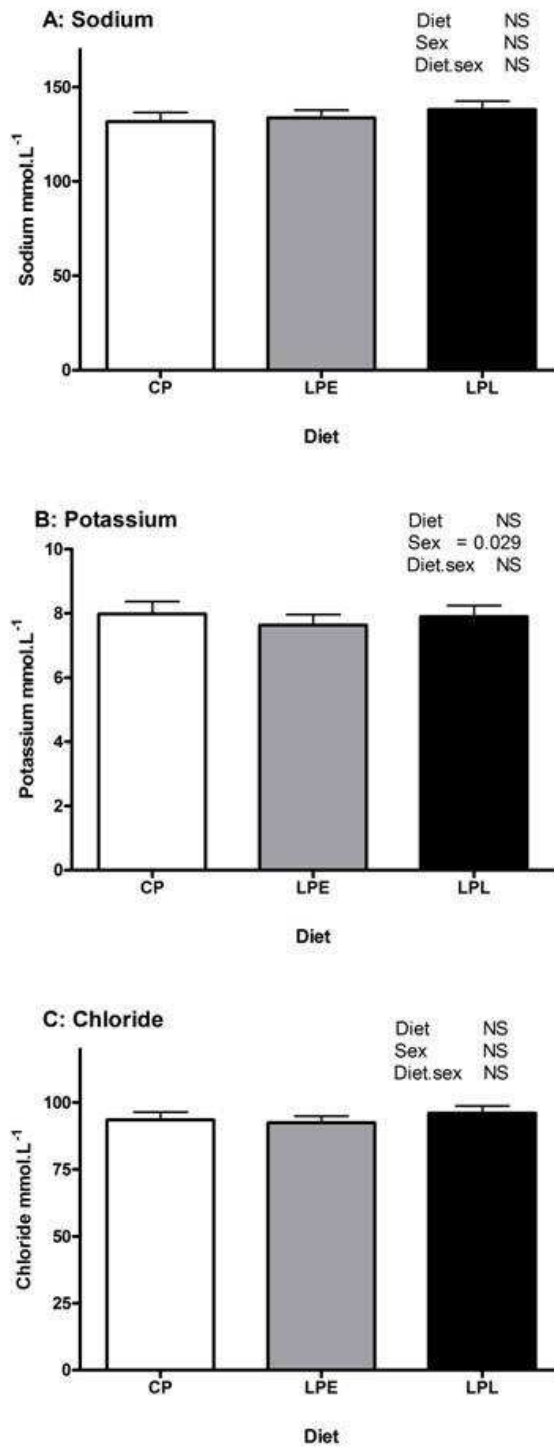


Figure 4.5: Sodium, potassium and chloride serum concentrations

Data are Means \pm S.E.M. for offspring of ewes fed a control protein diet (CP, n=6), or a low protein diet during early gestation (days 0-65; LPE, n=7) or a low protein diet during late gestation (days 66-term; LPL, n=6). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. Data were analysed by General Linear Model for the fixed effects of treatment, sex and their interaction with (Genstat v13, VSNi, UK). CP, control protein diet; LPE, low protein early diet; LPL, low protein late diet; NS, non-significant

4.3.5 Renal function

The upslope of the renogram (indicating the rate of uptake of the radioactive nuclide) was not significantly different between diets or between sexes (CP, 44.8 ± 11.40 ; LPE 37.4 ± 6.76 ; LPL, 50.0 ± 6.76 cpm $^{99\text{M}}\text{Tc}$ -DPTA; Figure 4.6A). The downslope (indicating the rate of clearance of the radioactive nuclide) was also not different between groups (CP, 10.30 ± 3.04 ; LPE 6.08 ± 1.05 ; LPL, 8.18 ± 1.48 cpm $^{99\text{M}}\text{Tc}$ -DPTA; Figure 4.6B). The time taken for the peak to be reached from injection to the peak concentration in the kidney was not different between males and females, however there was an effect of diet (CP, 2.98 ± 0.111 ; LPE 3.13 ± 0.071 ; LPL, 2.88 ± 0.065 min; $P = 0.046$; Figure 4.6C). Transit time through the kidney (a composite measure of the other parameters and an indication of the whole kidney function) was greater in the LPE group compared to controls and the LPL group (CP, 1.07 ± 0.076 ; LPE 1.27 ± 0.054 ; LPL, 0.97 ± 0.041 min; $P < 0.001$; Figure 4.6D). Males had a slower transit time than females (1.028 min ± 0.0471 vs 1.164 ± 0.0407).

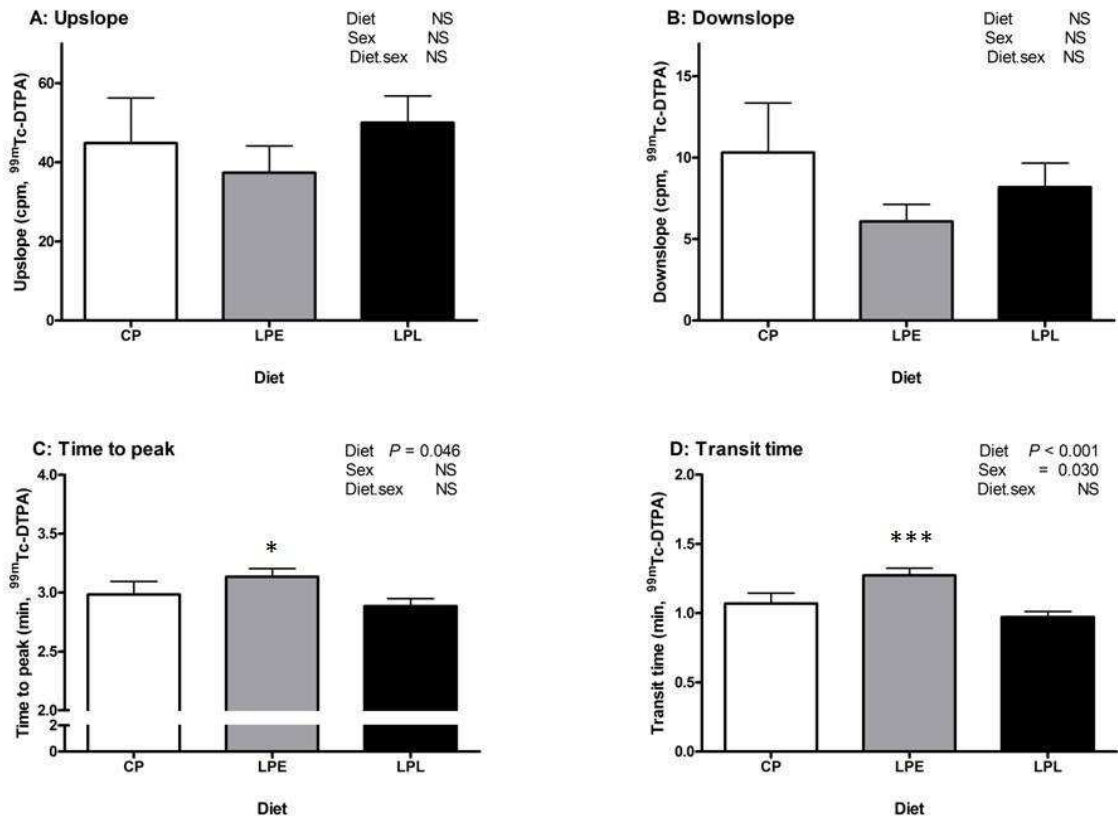


Figure 4.6: Gamma scintigraphy renogram results

Renal function was assessed by renography using Tc99m-DTPA. The upslope (A) is defined as the positive slope of the renogram occurring before the peak and computed using the X and Y values of the renogram at 5 and 90% of the peak Y; Time to peak (C) is from bolus injection to peak counts in the right kidney, the downslope is defined as the negative slope of the renogram occurring after the peak and computed using the X and Y values of the renogram at the peak and half-peak value on the downslope of the curve (B); transit-time is the mean time taken for tracer to pass through the kidney (D). Data are Means \pm S.E.M. for offspring of ewes fed a control protein diet (CP, n=6), or a low protein diet during early gestation (days 0-65; LPE, n=7) or a low protein diet during late gestation (days 66-term; LPL, n=6). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. Data were analysed by General Linear Model for the fixed effects of treatment, sex and their interaction with (Genstat v13, VSNi, UK). CP, control protein diet; LPE, low protein early diet; LPL, low protein late diet; NS, non-significant, * $P < 0.05$, *** $P < 0.001$

Measurement of glomerular filtration rate (GFR) indicated preservation of GFR in LPE animals in the longer-term (Table 4.2), as there were no differences between diet or sex, nor evidence of a diet.sex interaction.

Table 4.2: Glomerular filtration rate (GFR) at two years of age

	<i>GFR</i>	Diet	Sex		<i>P</i> value		
			male	female	Diet	Sex	Diet*Sex
<i>ml.ml⁻¹</i>	CP		146 ± 16	147 ± 23			
	LPE		114 ± 23	150 ± 14	NS	NS	NS
	LPL		133 ± 23	139 ± 16			
<i>ml.ml⁻¹.g⁻¹ kidney</i>	CP		0.97 ± 0.14	1.12 ± 0.22			
	LPE		0.89 ± 0.21	1.32 ± 0.13	NS	NS	NS
	LPL		1.02 ± 0.21	0.98 ± 0.14			
<i>ml.ml⁻¹.kg⁻¹ lean mass</i>	CP		3.21 ± 0.37	3.68 ± 0.53			
	LPE		3.27 ± 0.53	3.78 ± 0.33	NS	NS	NS
	LPL		2.84 ± 0.53	3.63 ± 0.37			

Glomerular filtration rate was assessed through decay of Tc99m-DTPA in plasma samples collected after 3, 4 and 5h post 100MBq I.V. bolus dose. Ewes were euthanized as per standard operating procedures and the wet weight of organs recorded. Data are Means ± S.E.M. for offspring of ewes fed a control protein diet (CP, n=6), or a low protein diet during early gestation (days 0-65; LPE, n=7) or a low protein diet during late gestation (days 66-term; LPL, n=6). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. Data were analysed by General Linear Model for the fixed effects of treatment, sex and their interaction with (Genstat v13, VSNi, UK). NS, not significant; CP, control protein diet; LPE, low protein early diet; LPL, low protein late diet; GFR, glomerular filtration rate.

4.3.6 Adult renal structure

The general morphology of the kidneys was assessed by staining with a range of histological stains, details of which are shown in Table 4.3.

Table 4.3: Histological stain details

Stain	Stain component	Colour	Structures stained
H & E	Haematoxylin	Dark blue	Cell nuclei
	Eosin	Pink	Cytoplasm (pink)
		Red	red blood cells (red)
PAS	Periodic acid-Schiff	Magenta	Basement membranes, tubule brush borders
	Haematoxylin	Dark blue	Cell nuclei
Trichrome	Fast green	Green	Collagen
	Orange G	Orange	Red blood cells
	Haematoxylin	Dark blue	Cell nuclei

Adapted from Young et al., 2006

Histological examination of the kidney cortex indicated no obvious morphological or structural abnormality in either the glomeruli or distal and proximal convoluted tubules in any of the diet groups (Figure 4.7 A, C-F). In addition there was neither evidence of consistent monocyte/lymphocyte infiltration (example shown in Figure 4.7 B) nor any indication of glomerulosclerosis.

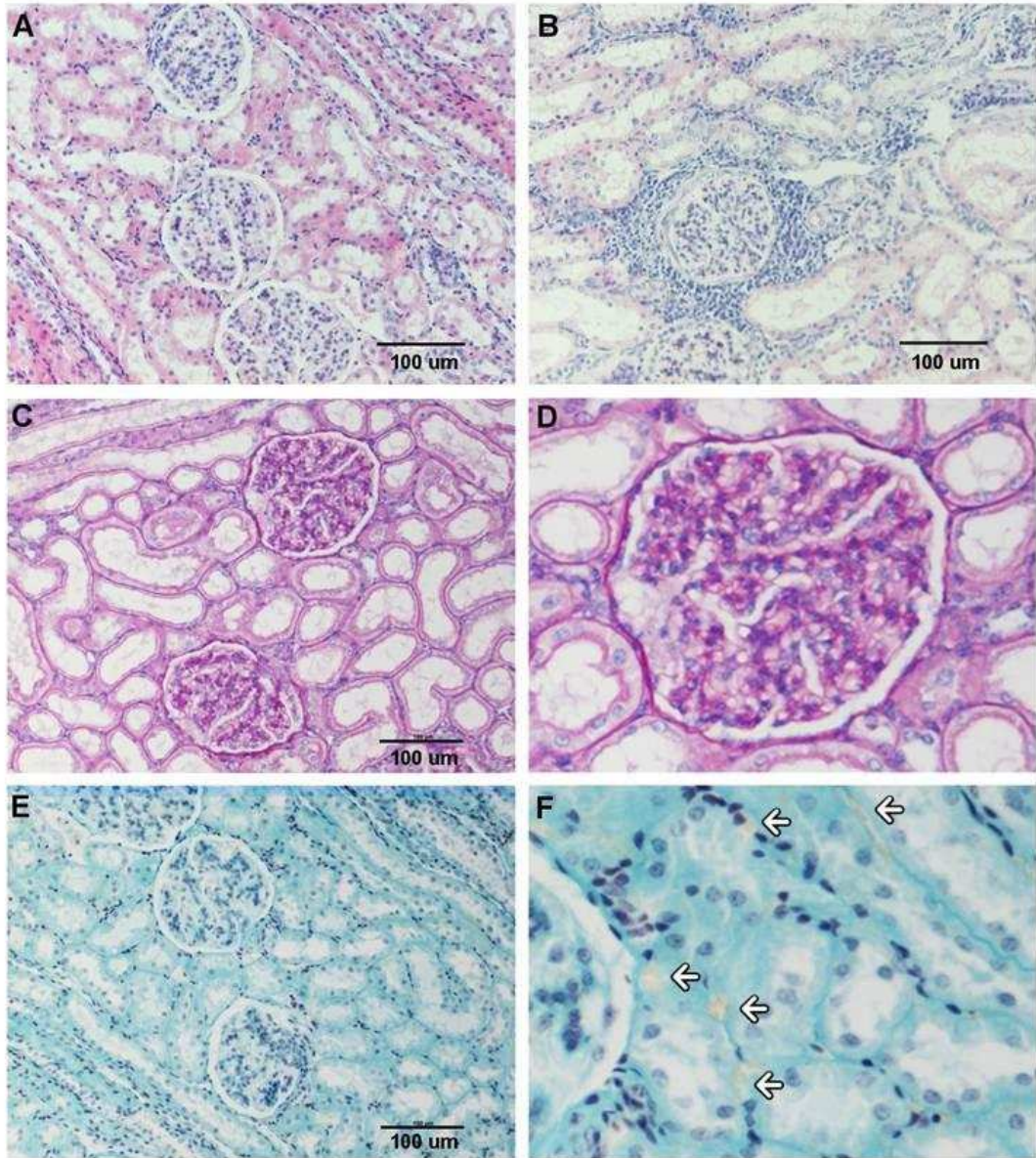


Figure 4.7: Histological staining of adult kidney sections at 2 years of age

Representative section from the LPE group stained with haematoxylin and eosin, showing normal kidney structure (A, magnification x 200); representative section from the control group showing monocyte infiltration around the glomeruli (B, magnification x 200); representative histological sections from the LPE group (C, magnification x200; D, magnification x400) stained with Periodic Acid Schiffs reagent shows normal kidney structure; representative histological section (x200) from the LPE group stained with trichrome (E) and showing red blood cell staining (orange) as indicated by white arrows (F).

Table 4.4: PCR results for endoplasmic stress markers

	Treatment	Sex		P value		
		Male	Female	Diet	Sex	Diet.sex
ATF4	CP	5.85 ± 0.80	4.95 ± 1.13			
	LPE	5.00 ± 1.13	5.29 ± 0.72	NS	NS	NS
	LPL	4.51 ± 1.13	6.33 ± 0.80			
ATF6	CP	5.36 ± 0.98	5.86 ± 1.39			
	LPE	5.90 ± 1.39	4.73 ± 0.88	NS	NS	NS
	LPL	4.88 ± 1.39	4.83 ± 0.98			
Bax	CP	8.37 ± 0.98	9.23 ± 1.38			
	LPE	8.39 ± 1.38	8.51 ± 0.87	NS	NS	NS
	LPL	10.40 ± 1.38	8.48 ± 0.98			
CD68	CP	0.09 ± 0.03	0.11 ± 0.04			
	LPE	0.11 ± 0.04	0.18 ± 0.03	NS	NS	NS
	LPL	0.22 ± 0.04	0.17 ± 0.03			
DDIT3	CP	0.68 ± 0.16	0.46 ± 0.15			
	LPE	0.81 ± 0.26	1.17 ± 0.24	0.02	NS	NS
	LPL	0.62 ± 0.20	1.08 ± 0.25			
HSPA5	CP	2.80 ± 0.47	2.28 ± 0.54			
	LPE	2.10 ± 0.50	2.84 ± 0.43	NS	NS	NS
	LPL	2.06 ± 0.49	2.76 ± 0.47			
MCP-1	CP	1.26 ± 0.30	0.69 ± 0.43			
	LPE	1.45 ± 0.43	1.40 ± 0.27	NS	NS	NS
	LPL	1.80 ± 0.43	1.27 ± 0.30			

Advanced relative quantification of mRNA normalised to cyclophilin using Roche Lightcycler® 480 software. Data are Means ± S.E.M. for offspring of ewes fed a control protein diet (CP, n=6), or a low protein diet during early gestation (days 0-65; LPE, n=7) or a low protein diet during late gestation (days 66-term; LPL, n=6). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. Data were analysed by General Linear Model for the fixed effects of treatment, sex and their interaction (Genstat v13, VSNi, UK). NS, not significant; CP, control protein diet; LPE, low protein early diet; LPL, low protein late diet. Gene symbols according to HUGO nomenclature (<http://www.genenames.org/>); ATF, Activating Transcription Factor; DDIT3, DNA Damage Inducible Transcript 3; HSPA5, Heat Shock 70 kDa Protein 5; MCP1, Monocyte Chemotactic Protein 1.

4.3.7 Endoplasmic reticulum stress

A previous study (Sharkey et al., 2009a) indicated that juvenile obesity led to increased intra-renal endoplasmic reticulum (ER) stress. This study tested many of the same markers

of ER stress, but only one (DDIT3) was found to be significantly different, with increased mRNA expression in the LPE group compared to the control and LPL groups (Table 4.4).

4.3.8 Mean glomerular area

Mean glomerular area was assessed using 2-D images taken from 20 randomly selected areas of kidney cortex per animal. There were no significant differences between diet groups (Figure 4.8), however, there was an effect of gender, with females having larger glomerular area than males ($16,448 \pm 590$ vs $14,249 \pm 699 \mu\text{m}^2$; $P= 0.034$). However, 2-D analysis has been criticised on a number of fronts (see discussion), and therefore the gold standard method of stereological analysis was used instead to calculate mean glomerular volume.

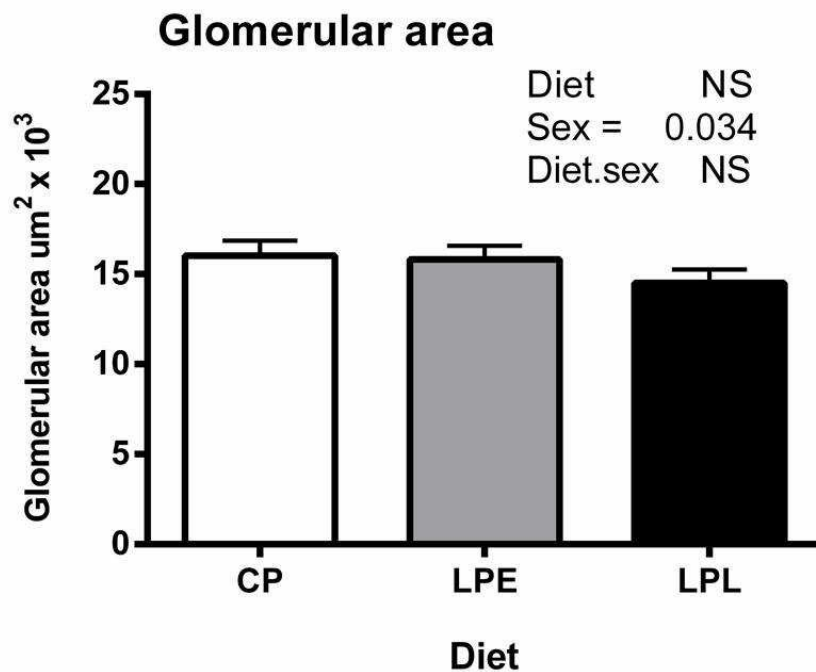


Figure 4.8: Mean glomerular area of two year old adult kidneys

Data are Means \pm S.E.M. for offspring of ewes fed a control protein diet (CP, n=6), or a low protein diet during early gestation (days 0-65; LPE, n=7) or a low protein diet during late gestation (days 66-term; LPL, n=6). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. Data were analysed by General Linear Model for the fixed effects of treatment, sex and their interaction with (Genstat v13, VSNi, UK). CP, control protein diet; LPE, low protein early diet; LPL, low protein late diet; NS, non-significant.

4.3.9 Stereology

As assessed by stereology, there was a strong trend for reduced nephron number (~16% fewer) in the LPE group compared to controls and the LPL group (CP, 1,042,981 ± 83,165; LPE, 876,694 ± 55,682; LPL, 1,018,677 ± 58,297; $P = 0.054$; Figure 4.9 A), although there was no effect of sex. Total glomerular volume was not different between dietary groups (Figure 4.9 B), but there was a significant effect of sex, with males having higher total glomerular volume than females (males, 2.14 ± 0.13; females, 1.65 ± 0.11 cm³), which probably reflects the higher kidney volume in male animals. Conversely the mean glomerular volume was not affected by sex but was significantly higher in the LPE group (CP, 1.94 ± 0.15; LPE, 2.29 ± 0.13; LPL, 1.66 ± 0.14 mm²; $P = 0.022$; Figure 4.9 C).

However, although nephron number and mean glomerular volume were altered in the LPE group, the overall size and structure of the kidney were not different. As expected, kidney volume was highly correlated with kidney weight (Pearson's correlation coefficient of $r^2 = 0.91$; $P < 0.001$). Kidney volume was not different between dietary groups (CP, 72.6 ± 5.15; LPE, 65.4 ± 4.42; LPL, 71.6 ± 4.65 cm³), however there was a weak trend for a difference between males and females (males, 75.4 ± 4.43; females, 65.4 ± 3.58 cm³; $P = 0.097$). In addition there were no effects of diet or sex observed on the volume of the renal cortex (CP, 46.3 ± 3.68; LPE, 44.8 ± 3.15; LPL, 48.7 ± 3.32 cm³). Furthermore there were no diet.sex interactions for any of the stereological variables.

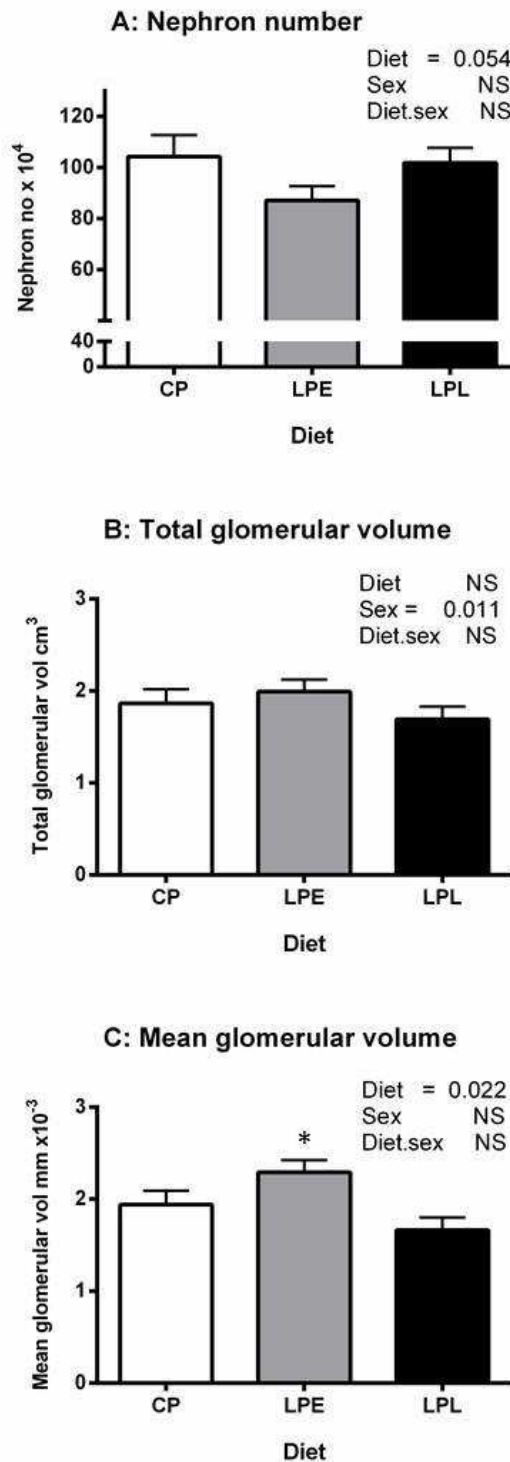


Figure 4.9: Nephron number, total and mean glomerular volume

Variables were assessed using unbiased stereology. Data are Means \pm S.E.M. for offspring of ewes fed a control protein diet (CP, n=6), or a low protein diet during early gestation (days 0-65; LPE, n=7) or a low protein diet during late gestation (days 66-term; LPL, n=6). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. Data were analysed by General Linear Model for the fixed effects of treatment, sex and their interaction with (Genstat v13, VSNi, UK). CP, control protein diet; LPE, low protein early diet; LPL, low protein late diet; NS, non-significant, * $P < 0.05$

4.3.10 Estimation of mammalian nephron number

This study reported a nephron number in sheep that was higher than other reported estimates from control animals, as shown in Table 4.5. Therefore the possibility of an error in the stereological method was considered. The method was checked by Professor Antonio Ribeiro, Professor of Stereology at University of Sao Paulo who confirmed it to be correct. Given the substantial variation in the reported nephron numbers, next it was considered whether it was possible to predict the nephron number of any given mammal, based upon analysis of nephron number and body weight from other studies.

Table 4.5: Nephron number in control animals in sheep studies

Paper	Nephron number	N
Bains et al., 1996	289,439 ± 36,263	6
Douglas-Denton et al., 2002	365,672 ± 36,016	7
Wintour et al., 2003	402,787 ± 30,458	7
Mitchell et al., 2004	559,000 ± 19,800	6
O'Connell et al., 2006	425,406 ± 27,560	5
Zohdi et al., 2007	371, 219 ^a	7
Brandon et al., 2008	423, 520 ± 22,194 ^b	9
Gray et al., 2008	423,177 ± 17,178	5
Muhle et al., 2010	681,000 ± 46,000	9
Zhang et al., 2010	896,814 ± 45,772	11
Galinsky et al., 2011	600,000 ^c	4

Data are means ± s.e.m. ^a s.e.m. or s.d. not reported; ^b one animal was excluded as an outlier for having a nephron number of 701, 450; ^c numbers not given in the paper, this is an estimate from a graph.

A paper published in 1930 (Kunkel, 1930) was the first to consider this in detail, and reported that animals such as dogs (which vary hugely in size according to breed) varied in nephron number and size according to their weight. This paper also reported details of nephron number in a variety of other mammals. By updating these data for nephron estimates from studies using modern stereological methods wherever possible, and adding

other species reported more recently, it was possible to observe a strong correlation between body weight and nephron number ($R^2 = 0.98$; $P < 0.0001$). Using \log_{10} data for published values of nephron number from a mouse weighing 20 g to an elephant weighing 3700 kg (Figure 4.10) generates an equation of $y = 0.613x + 3.116$, therefore accurately predicting an adult sheep (50 - 70 kg) to have between 994,050 – 1,221,839 nephrons per kidney.

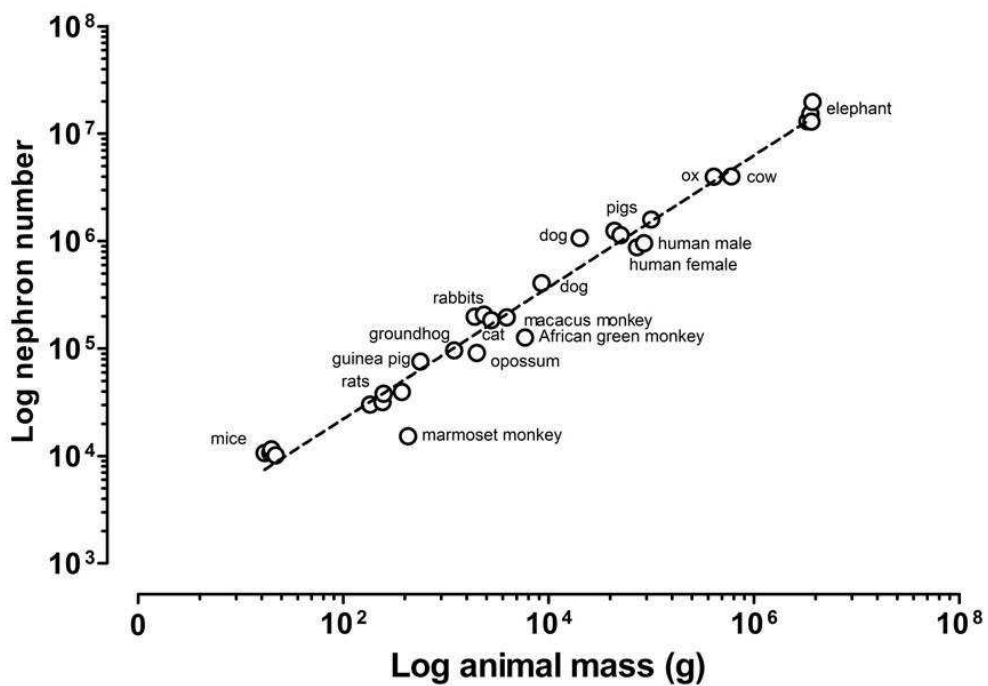


Figure 4.10: The relationship between mass and nephron number in mammals

Data are \log_{10} of mass (g) and nephron number per kidney. $R^2 = 0.98$; $P < 0.0001$.

4.3.11 Lectin staining

As the reduction in nephron number was only around 16% it is unlikely that this alone would cause a reduction in renal function during young adulthood, so other structural changes in the kidney were considered, in particular the vascular system.

Lectins are non-immune proteins that have specificity for certain carbohydrate residues (Rhodes and Milton, 1998), and these are exploited for use in histochemical methods.

Mammalian vascular endothelial cells act as ligands for lectins, and *Griffonia simplicifolia* lectin and the isolectin GSL B4 bind to the endothelial cells of most species, excluding humans (Rhodes and Milton, 1998).

Initially, concentrations of lectin at 5, 10 and 20 $\mu\text{g}\cdot\text{ml}^{-1}$ were trialled, along with bovine ovary as a positive control as this has been previously shown to stain with this lectin when incubated at room temperature for 2 hours. Although the positive control worked (Figure 4.11 G), there was no staining on the sheep kidney sections (Figure 4.11 A), therefore the lectin was applied at the original concentrations, plus a higher one (40 $\mu\text{g}\cdot\text{ml}^{-1}$) and incubated overnight at 4°C. At 5 $\mu\text{g}\cdot\text{ml}^{-1}$ there was no staining (not shown) and at 10 and 20 $\mu\text{g}\cdot\text{ml}^{-1}$ there was evidence of non-specific staining (Figure 4.11 B and C respectively) but no staining of endothelial cells. At 40 $\mu\text{g}\cdot\text{ml}^{-1}$ there was clear staining of endothelial cells (white arrows, Figure 4.11 D) although the non-specific staining in the proximal tubules was stronger (Figure 4.11 E).

To try to counteract the non-specific staining, various strategies were used, including increasing the time of the blocking steps during the protocol. A citrate buffer antigen retrieval method was also used to see whether this improved the specificity of the staining, but this made the staining in the proximal tubules even more pronounced, even at a lower

concentration of lectin (Figure 4.11 F), and also resulted in staining in the negative control that omitted lectin.

Without antigen retrieval the omission of lectin resulted in a section without positive staining (Figure 4.11 H), but the other negative control of a competing sugar, galactose, stained just as much as the lectin itself did. Therefore although the lectin did seem to stain endothelial cells, it was impossible to be sure this was specific. There was also a large amount of non-endothelial staining in the proximal tubules. A similar finding has also been found in pigs (Kirkeby and Mikkelsen, 2008), where isolectin GSL B4 was shown to bind to both the basement membrane and brush borders of proximal convoluted tubules.

Thus it would appear that the lectin is not suitable as an endothelial cell marker for ovine kidney.

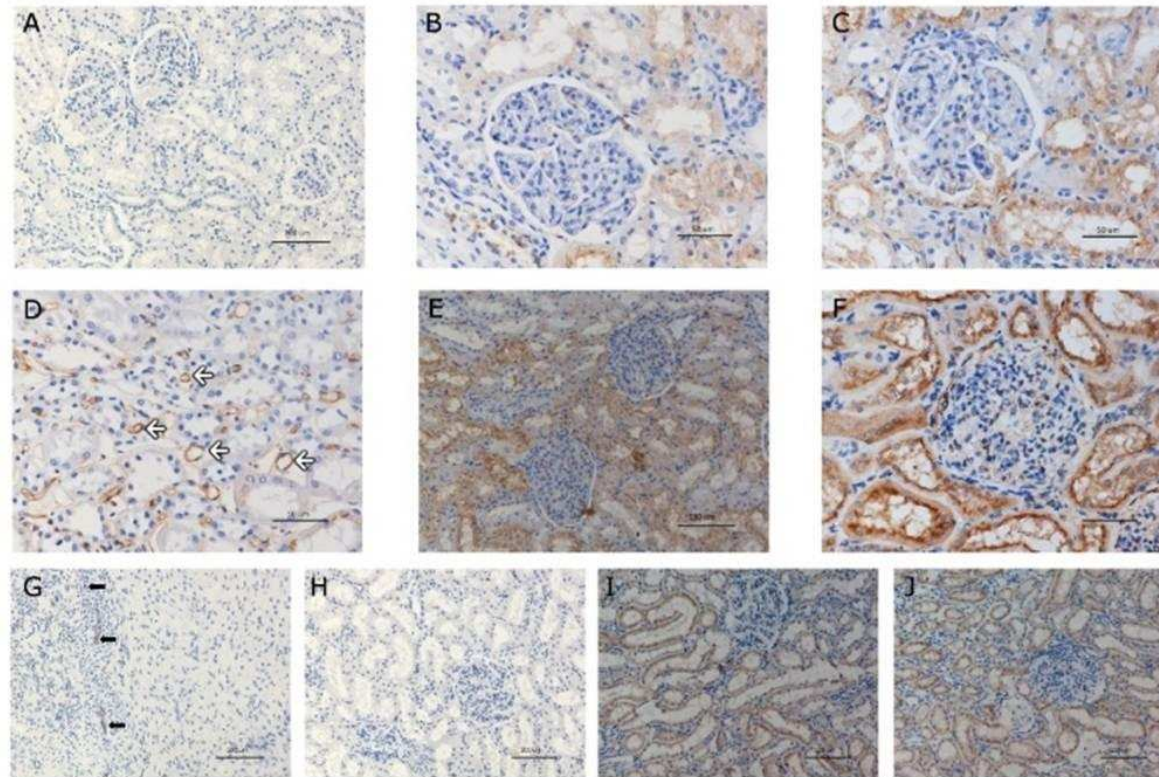


Figure 4.11: Staining with Griffonia (*Bandeiraea*) simplifolia lectin 1 isolectin B4

Representative micrographs show absence of lectin staining with 2h room temp incubation at $20 \mu\text{g}\cdot\text{ml}^{-1}$ (A; magnification x200); non-specific staining with overnight incubation at 4°C at $10 \mu\text{g}\cdot\text{ml}^{-1}$ (B; x400) and $20 \mu\text{g}\cdot\text{ml}^{-1}$ (C; x400); specific staining at $40 \mu\text{g}\cdot\text{ml}^{-1}$ in endothelial cells indicated by white arrows (D; x400) but intense non-specific staining in proximal tubules (E; x200), antigen retrieval amplified non-specific staining even at $5 \mu\text{g}\cdot\text{ml}^{-1}$ (F, x400). Positive control was bovine ovary (G, x200), negative control (omission of lectin) with and without antigen retrieval (J & H respectively, x200) and stained for competing sugar, galactose (I, x200).

4.3.12 CD31 and CD34

As the lectin staining was not successful due to non-specific staining, other endothelial cell markers were needed. Due to a lack of availability of antibodies successfully reported for use in sheep, four antibodies were trialled for two other endothelial cell markers CD31 (also known as platelet/endothelial cell adhesion molecule 1, PECAM) and CD34.

4.3.12.1 CD31 ab32457 and ab83959 and CD34 ab63985

All three of these antibodies were tried using both the Vectastain Elite ABC system (section 2.6.1) and the automated Bondmax system with and without antigen retrieval (section 2.6.2).

The CD31 ab32457 antibody was tried at five different concentrations ranging from 1:100 to 1:2000. However, there was no evidence of specific staining of endothelial cells.

Representative micrographs of control sections as shown in Figure 4.12 demonstrate that at higher concentrations (1:250 and above) there was evidence of non-specific staining however this starts to be diluted out by 1:500 and by 1:1000 there is no staining at all.

The CD31 ab83959 antibody was tried at concentrations ranging from 1:50 to 1:1000, and a similar pattern was observed to the other CD31 antibody, with the highest concentration (1:50) having non-specific staining, which started to fade by 1:100, and had disappeared by 1:250 (Figure 4.12).

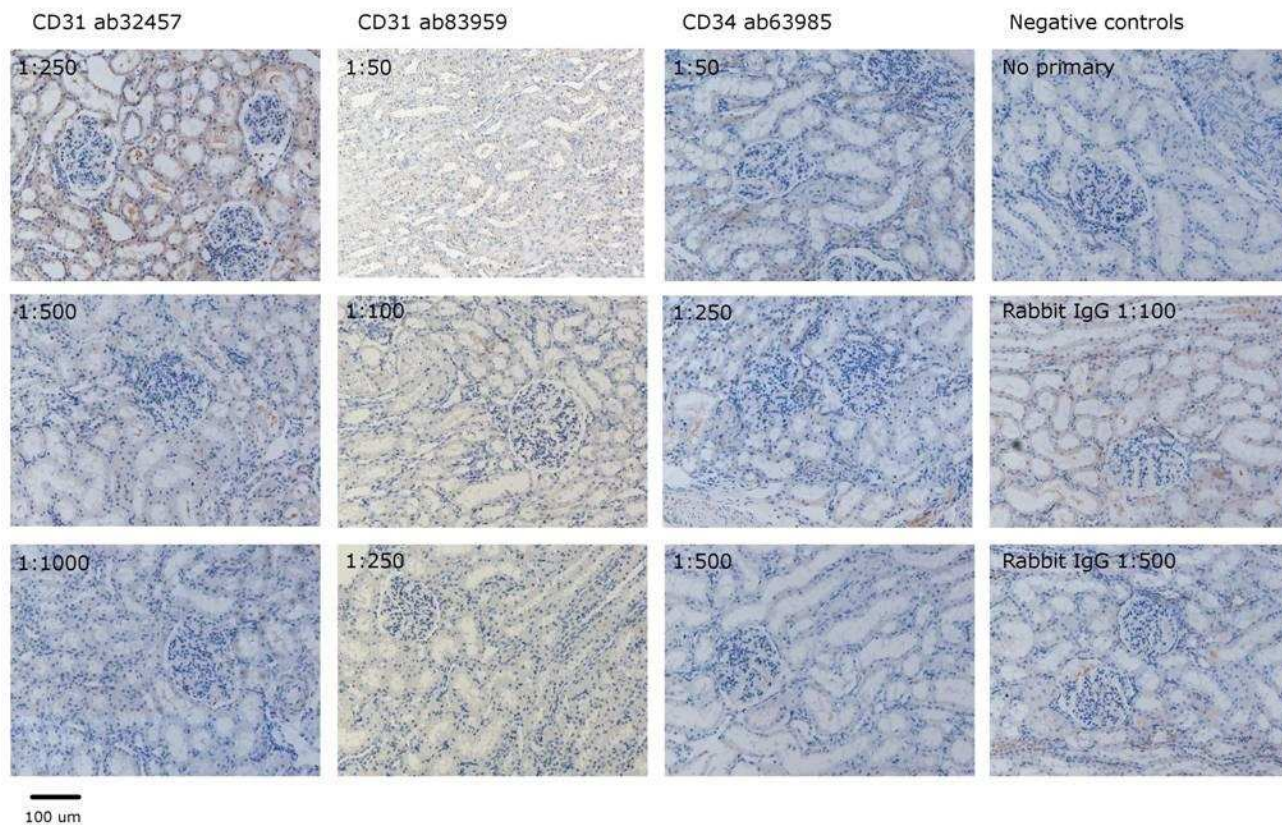


Figure 4.12: Immunohistochemistry for endothelial cell markers

Representative micrographs at magnification x200 show non-specific staining for CD31 ab32457 at 1:250 and 1:500, which disappears by 1:1000; non-specific staining for CD31 ab83959 at 1:50, which has largely disappeared by 1:100 and totally disappeared by 1:250; non-specific staining for CD34 ab63895 at 1:50, which has largely disappeared by 1:250 and totally disappeared by 1:500. Negative controls were omission of antibody and rabbit IgG control which evidenced the non-specific staining at a higher concentration of 1:50, and a smaller amount at 1:500.

The CD34 ab63895 showed exactly the same pattern as the CD31 ab83959 at the same concentrations (Figure 4.12), with no evidence of specific staining of endothelial cells.

The negative controls where the primary antibody was omitted were all negative regardless of method or antigen retrieval status. However the rabbit IgG control stained the tissue when it was diluted to match the higher primary antibody concentrations. Once the IgG control was diluted low enough to remove this non-specific staining, the primary antibodies were diluted such that all staining from them also disappeared. However, the final antibody (CD34 ab81289) was successful.

4.3.12.2 CD34 ab81289

CD34 ab81289 was tried at concentrations ranging from 1:25 to 1:500 initially. At the higher concentrations there was an abundance of non-specific staining, which was also seen in the rabbit IgG control at the same concentrations; however by 1:500 dilution the IgG control had very little staining and genuine specific staining could be seen on the sample albeit alongside some additional staining in the proximal tubules. This was counteracted by diluting further to 1:1000, where the staining could be seen in endothelial cells alone (indicated by white arrows in Figure 4.13 A), and staining was entirely absent in both negative controls (omission of primary antibody, Figure 4.13 C; Rabbit IgG Figure 4.13 D).

There was a significant decrease in the abundance of CD34 protein in the LPE group compared to CP and LPL groups (Figure 4.13 B, $P < 0.001$) which suggests a reduction in the microvascular structures in the kidney.

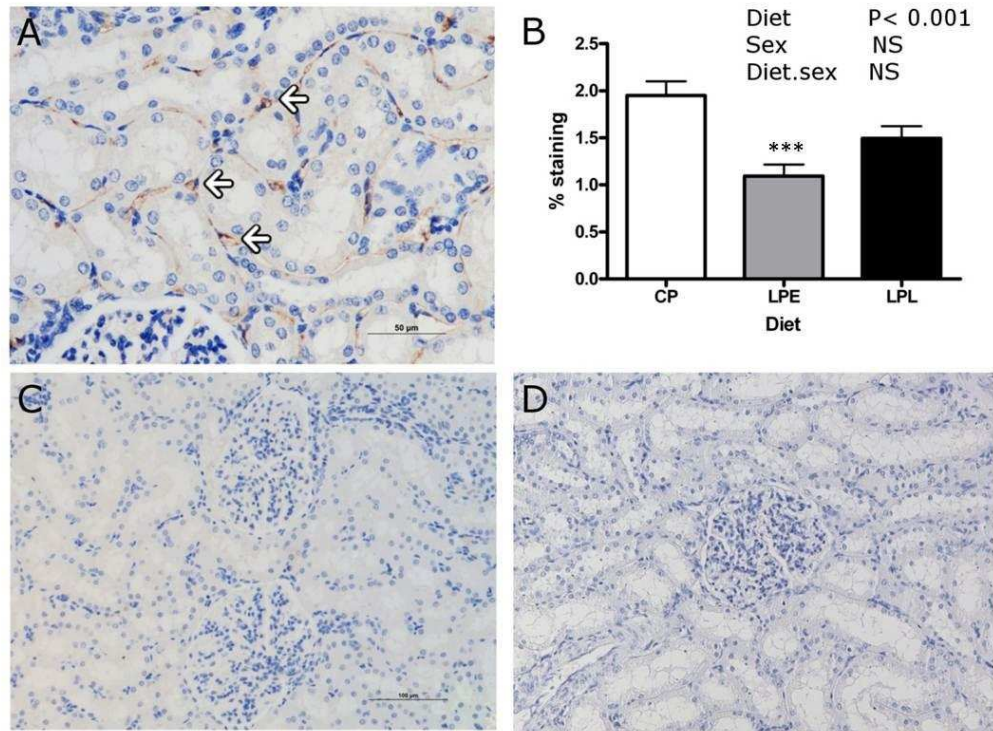


Figure 4.13: Immunohistochemistry for CD34 protein

A representative micrograph of a control kidney with staining for CD34 indicated by the white arrows (A, magnification x 400); negative controls were omission of antibody (C, x200) and rabbit IgG (D, x200). The graph (B) shows means \pm S.E.M. for offspring of ewes fed a control protein diet (CP, n=6), or a low protein diet during early gestation (days 0-65; LPE, n=7) or a low protein diet during late gestation (days 66-term; LPL, n=6). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. Data were analysed by General Linear Model for the fixed effects of treatment, sex and their interaction with (Genstat v13, VSNi, UK). CP, control protein diet; LPE, low protein early diet; LPL, low protein late diet; NS, non-significant, *** $P < 0.001$

4.3.13 Vascular Endothelial Growth Factor

To explore whether the effects of VEGFA seen in the fetus (section 3.2.3) were also observed in the adult tissue, qPCR and immunohistochemistry were carried out. In direct contrast to the fetal results where VEGFA was significantly lower in LPE animals, the mRNA of VEGFA tended to be increased in the LPE group compared to controls and LPL animals, whereas the mRNA for the receptors tended to be increased in both LPE and LPL groups compared to controls (Figure 4.14 A-C; VEGFA, $P = 0.07$; VEGFR1, $P = 0.05$; VEGFR2, $P = 0.09$). At the protein level, VEGFA was significantly increased in both LPE and LPL groups compared to controls (Figure 4.14 D – F).

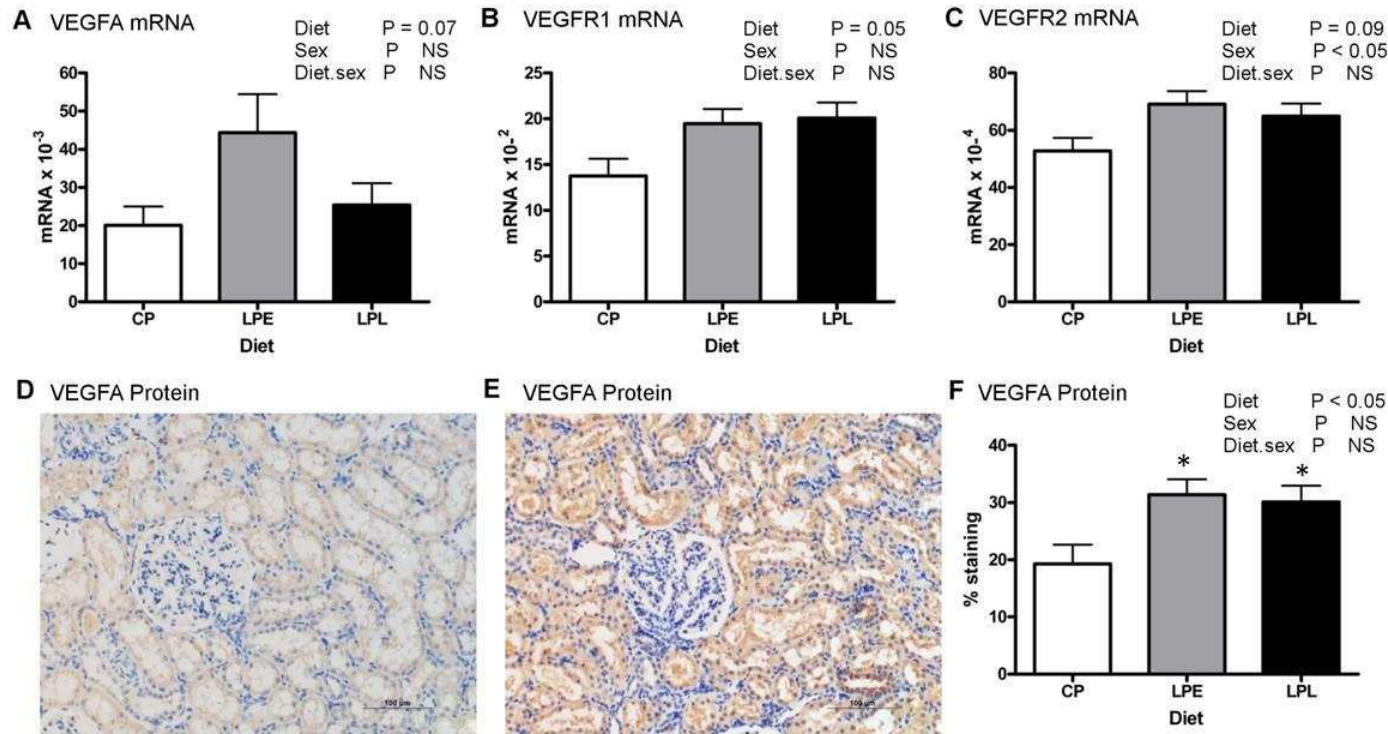


Figure 4.14: VEGFA mRNA and protein expression in the adult kidney

Quantification of mRNA expression of VEGFA (A), VEGFR1 (B) and VEGFR2 (C); representative micrographs (magnification x200) of immunostaining for VEGFA for the control group (D) and LPE group (E); and its quantification (F). Negative controls were omission of primary antibody (G) and rabbit IgG (H); positive control was mouse kidney (I). Data are Means \pm S.E.M. for control protein (CP, n=6), low protein early (days 0-65 gestation; LPE, n=7) and low protein late (days 66-term; LPL, n=6). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. Data were analysed by General Linear Model for the fixed effects of treatment and sex with their interaction (Genstat v13, VSNI, UK). NS, non significant; VEGF, vascular endothelial growth factor, * $P < 0.05$

4.4 Discussion

In this study of maternal protein-energy malnutrition, despite a 50% reduction in protein availability, the diet had few effects on maternal body weight or body condition score, which indicates that the nutrient restriction was relatively mild. However, there were effects on the fetus that were determined by the timing of the nutritional insult, as the group fed low protein from day 65 – term (LPL group) had a significantly lower birth weight than the controls (CP) or those fed the maternal low protein diet from day 0 -65 gestation (LPE). In addition, the LPL group exhibited a period of early catch-up growth such that by weaning there were no differences in body weight between groups. Both low birth weight and early catch-up growth are considered developmental markers for later adult disease (Barker et al., 1989; Osmond et al., 1993). This study illustrates, however, that the quality of nutrition early in development is of more importance with respect to the programming of later adult dysfunction and potentially disease. Although the LPL animals had lower birth weight and a period of catch-up growth early in life, there was no evidence of a detrimental effect on kidney function at two years of age. In contrast, the LPE group, which had normal birth weights and early growth patterns, had reduced kidney function in adulthood.

There were few effects of diet on fetal organ weight (Chapter 3) or on adult organ weights at 2 years of age. This contrasts with laboratory rodent studies in which a similar degree of protein restriction has often resulted in disproportionate growth (Langley-Evans et al., 1999), low birthweight (Levy and Jackson, 1993; Desai et al., 1996), smaller kidneys (Langley-Evans et al., 1999) and misshapen kidneys (Nwagwu et al., 2000). One possible explanation for the difference between rodent studies and this study is that the ewes in this study were monocotous (i.e. the uterus was supporting a singleton pregnancy) and thus the growth of the utero-placental unit has a lower anabolic demand than on polycotous species (where the uterus supports multiple pregnancies) such as rodents (McCance and

Widowson, 1986). Indeed, other nutritional programming studies of sheep have also shown no effect on kidney weight (Gray et al., 2008; Braddick et al., 2011), and in another monocotous species (marmoset monkeys) kidney weight was also unaffected by maternal diet (Bramlage et al., 2009). These findings suggest that the effects on smaller mammals are not necessarily replicated in larger mammals. Thus, as is often the case, laboratory rodents are likely to present a more severe response to a dietary regimen that has much less effect on larger animals.

Additionally a number of small animal studies have suggested that reduced maternal protein intake, regardless of when the intervention occurs during gestation, can have quite remarkable effects on anatomical development of the fetal kidney with reports of a 20-50% deficit in nephron number (Vehaskari et al., 2001; McMullen et al., 2004; Hoppe et al., 2007). Here, as in larger animals (Brennan et al., 2005; Braddick et al., 2011), few gross effects on kidney size or development are observed, but on closer examination there are some subtle renal effects. In the adult animals exposed to maternal protein restriction during early gestation only, a small reduction (~15%) in nephron number was evident, together with an increase in mean glomerular volume (determined stereologically). This study is the first to demonstrate a renal phenotype after changes to the macronutrient composition – rather than energy content - of the maternal diet and suggests that the protein-energy ratio of the maternal diet is more important in terms of developmental programming than a balanced reduction in nutrient intake (Brennan et al., 2005). In humans, there is much descriptive evidence to suggest that an optimal fetal environment is a prerequisite for adequate development of the kidney and for good blood pressure control later in life; low birth weight babies have fewer nephrons (Manalich et al., 2000; Hughson et al., 2003) and tend to develop higher blood pressure later in life (Brenner et al., 1988; Mackenzie and Brenner, 1995b; Keller et al., 2003). In addition, individuals with a history of

hypertension have significantly fewer nephrons than normotensive controls (Keller et al., 2003).

Furthermore our estimate of nephron number in control sheep (~1,042,981) is of academic value and interest for although it was determined stereologically, it contrasts with other estimates for sheep, e.g. from 289,000 to 420,000 (Table 4.5). Nephron number may vary subtly within a species (Zimanyi et al., 2009; McNamara et al., 2010), but this is usually due to size and lean mass as the number of glomeruli is highly correlated with both birth weight (Manalich et al., 2000) and weight-adjusted metabolic mass between species (Kunkel, 1930). Using \log_{10} data for nephron number from a range of species predicts a sheep (weighing between 50 and 70kg) to have between 994,050 – 1,221,839 nephrons per kidney; the value found in this study (1,042,981) falls within this range. In a study (Holt and Rhode, 1976) that considered the similarity of various renal glomerular dynamics between mammals, a log relationship between nephron number and bodyweight was observed that applied to mammals ranging from mice to whales. This relationship would also predict a nephron number of ~1.2 million in sheep.

It may be that there are breed differences between sheep, however no other data are reported for the Scottish Blackface breed used in this study, and due to the sparsity of similar sheep studies it is not possible to draw meaningful conclusions. However, for the three studies which have used Merino: Border-Leicester crosses (Mitchell et al., 2004; Zohdi et al., 2007; Gray et al., 2008) it is interesting to note that whilst they provide substantially lower values than the predicted nephron number reported here, they still vary considerably (from 371,000 to 559,000). This may suggest that the effects of prenatal programming on nephron number are best considered relative to their own control groups, rather than to species specific values.

Stereology is defined as: “a body of mathematical methods relating three-dimensional parameters defining the structure to two-dimensional measurements obtainable on sections of the structure” (Weibel, 1979) and is currently considered to be the gold standard for estimating nephron number. It has largely superseded the acid maceration method previously used, which exploited the capacity of the Bowman’s capsule to resist acid digestion, thus leaving the glomeruli intact whilst the rest of the kidney is dissolved. The acid maceration method had several limitations including the assumption that the acid maceration affects all the glomeruli equally, whereas damaged glomeruli may actually have differing levels of acid resistance (Schreuder and Nauta, 2007). Alternative methods using histological sections were developed, however, one limitation of estimating numbers of 3-D particles such as glomeruli from 2-D sections is that the likelihood of them appearing on a 2-D section is not equal to their frequency in 3-D, but rather to their size (Bertram, 2001). Indeed in this study there were contrasting results with a 2-D analysis of mean glomerular area showing no effect of diet, whereas the stereological analysis of mean glomerular volume revealed glomerular hypertrophy in the LPE group.

The effect of renal dysfunction in the LPE animals is significant, but fairly subtle. This may reflect the age of the animals (young adults, around 2 years out of a 10 – 12 year life expectancy), and had it been possible to keep the animals into old age then more marked effects may have been observed. Although GFR is the most useful measure of renal function in a clinical setting, the use of gamma scintigraphy in this study meant the possibility of measuring additional renal functional parameters on a real-time basis, such as the upslope and downslope of the renogram, the time to peak from injection in the carotid artery to the peak level of radioactive nuclide in the kidney, and the transit time through the kidney.

Nevertheless, microalbuminuria is an excellent early predictive marker of future progressive renal disease; thus, it would appear that exposure to a defined period of malnutrition *in utero* may put the offspring at greater risk of progressive renal failure. In the adult, the kidney has marked functional redundancy and the subtle changes to renal development observed here are unlikely to result in symptoms of clinical disease. However, in a study from the Dutch Famine cohort, young adults protein restricted during early gestation displayed microalbuminuria, one of the first signs of early-stage renal disease (Painter et al., 2005a), when metabolically challenged with obesity as adults. Those individuals exposed to famine conditions as early gestation fetuses were at higher risk of developing obesity (Ravelli et al., 1976) and microalbuminuria in middle age (Painter et al., 2005a). This finding is also replicated in the present study. Taken together with the impaired renal function and mild glomerular hypertrophy observed, this suggests that the young kidneys in these obese sheep have been permanently adversely affected by the protein-energy malnutrition *in utero*. With age, the mild hyperfiltration of the remaining nephrons and increased pressure in the capillaries (Hershkovitz et al., 2007b) will ultimately lead to an earlier onset of CKD. At this early stage, however, the lack of any morphological change, apparent monocyte infiltration, or glomerulosclerosis suggests the existence of Minimal Change Disease in the young adult LPE group.

In addition, although previous research in sheep (Sharkey et al., 2009a) demonstrated increased gene expression of endoplasmic reticulum stressors in response to juvenile obesity, this was not the case in the present study. This may be due to differences between the studies in terms of timing of the obesity, as the obesogenic environment was from weaning to one year of age in the former study, but from 18 months onwards in this current study.

The microalbuminuria observed in the LPE animals is suggestive of reduced glomerular barrier function, and this is likely to be due in part to single-nephron hyperfiltration, as this can be a consequence of obesity (Williams et al., 2007). However, in this study the combination of other factors including reduced nephron number, and microvascular rarefaction (evidenced by reduced levels intra-renal endothelial CD34) and increased intra-renal pressure (indicated by reduced TC-^{99m} DTPA time to peak and transit time) are likely to exacerbate this. Furthermore additional evidence of a compromised glomerular filtration barrier is provided by the increased levels of VEGFA in the LPE group, as over-expression of VEGFA is implicated in renal disease. In a model of induced diabetic nephropathy in rats, VEGFA gene expression increased (Cooper et al., 1999). Over-expression of VEGF is also linked to renal cell carcinoma (Jacobsen et al., 2000) and, in the cortex only, to ageing (Kang et al., 2001). VEGF receptors also have important roles in the maintenance of normal renal function and in progression of kidney disease. Diabetic nephropathy led to increased VEGFR2 gene expression (Cooper et al., 1999), and inhibitors of VEGF receptors ameliorated the albuminuria associated with the disease (Sung et al., 2006). Also, where VEGFA was increased in response to hypertension, an inhibitor of VEGFR2 led to severe glomerulosclerosis (Advani et al., 2007). Interestingly, although an increase in VEGFA is indicated in some diseases of the kidney, under-expression also has deleterious effects. Under-expression of VEGFA has been linked to thrombotic microangiopathy in the kidney (Eremina et al., 2008b), and focal segmental glomerulosclerosis (Shulman et al., 1996). Thus the levels of VEGFA are strictly regulated for normal kidney function.

The disparity between VEGFA gene and protein levels at different ages – decreased in the fetal LPE group, yet increased in the same group in the adults – probably reflects the differing roles of VEGFA throughout the lifespan. In fetal kidneys its primary role is in the regulation of angiogenesis (Carmeliet et al., 1996; Tufro et al., 1999), whereas in the adult

kidneys a significant part of its role is in the maintenance and repair of the glomerular barrier (Eremina et al., 2007).

To summarise, this study has identified a renal phenotype in adult LPE (but not LPL) sheep, with reduced nephron number and a decline in renal function despite evidence of some compensatory glomerular hypertrophy. Although the general morphological structure of the kidneys was not affected by the diets there is evidence for microvascular rarefaction.

In the final experimental chapter the macro- and micro-vascular structure of the fetal kidney is considered in more detail. In addition, mechanistic links between diet and kidney development are explored further by measuring metabolites and amino acids in both the maternal and fetal environments.

5. Study 2: The effect of a maternal low protein diet on the ewe and her fetus

5.1 Introduction

In the previous chapters it was demonstrated that a low protein diet during early, but not late, gestation led to reduced nephron number and poorer kidney function during early adulthood in the offspring. In addition, at a molecular level, changes in gene and protein expression of VEGFA and its receptors were observed. At this relatively young age glomerular filtration rate was preserved and there were no gross morphological differences between the kidneys of the adult animals subjected to a low protein diet *in utero* and the control group. Nevertheless, a reduction in CD34 (a marker of endothelial cells) in their kidneys suggested blunted microvascular development and function in these adult animals.

Examination of fetal kidneys at day 65 (0.44 gestation in sheep) identified increased apoptosis and decreased angiogenic factors in the low protein exposed fetal kidneys relative to controls, suggesting a mechanism explaining the adult phenotype of reduced nephron number and microvascular rarefaction. However, the mechanistic pathway from maternal low protein through to inadequate fetal kidney development could not be ascertained. Hence, in the present study, the low protein diet paradigm was again employed in order to study more closely the relationship between maternal nutrition, fetal nutrition and fetal kidney development.

Several studies of sheep have shown that reduced nutrition during early or mid-gestation does not affect fetal growth or body weight at birth (Trahair et al., 1997; McCrabb et al., 1991). Furthermore nutritional restriction during sheep pregnancy is associated with increased placental weight (Faichney and White, 1987; McCrabb et al., 1991), which may have compensatory effects for protecting the fetus from the lack of nutrients. Thus this study aimed to measure metabolites in both the maternal and fetal environments to assess

the precise effects of the maternal low protein diet. The same amino acids are essential to sheep and humans. However, one key difference is that whilst humans must get these through their diet, for ruminants there is an additional source of amino acids through the breakdown of proteins synthesised by microbes in the rumen (McDonald et al., 2011). Thus dietary amino acid intake in a sheep may not entirely correlate with the amino acid levels in the maternal plasma, and therefore during this second study it was considered whether the amino acid profile in ewes was indeed different between the dietary groups, and whether an effect could be seen in the fetal environment.

In addition this study considered the vascular structure of the developing kidney. To visualise and analyse the macrovascular structure of the kidney a corrosion casting method was used to create a resin structure that replicates the vascular architecture of the fetal kidney. Using a micro-computed tomography (micro-CT) scanner a 3-D model can be constructed from a series of 2-D slices (Mondy et al., 2009), and this can be analysed using software to provide accurate estimations of vessel surface area and volume, including small vessels of less than 20 μm in diameter (Marxen et al., 2004). Corrosion casting has been used to visualise the vascular structure of a variety of organs including the ovaries of cattle (Jiang et al., 2003) and rabbits (Macchiarelli, 2000), sheep lungs (Schraufnagel et al., 1995), the kidneys of pigs (Xu et al., 1994) or mice (Wagner et al., 2011), and also cancerous tumours in mice (Konerding et al., 1999). However, it is most commonly used in placental studies, where a number of species have been studied, including horse (Abd-Elnaeim et al., 2006), cow (Pfarrer et al., 2001), donkey (Saber et al., 2008), and goat (Leiser et al., 1997). In sheep the maternal and fetal microvasculature of the placenta at different time points throughout gestation has been studied using resin casts (Hafez et al., 2010), and another study examined the anatomy of the renal arteries in the sheep (Aksoy et al., 2004). However, no study has so far considered the renal vascular structure in the fetal ovine kidney in the context of nutritional programming.

To summarise, this chapter builds upon Chapter 3 and considers further the effect of a low protein diet on the ewe during the early part of pregnancy and the fetus at day 65 (or 0.44 gestation). The chapter characterises the effect of a low protein maternal diet on maternal and fetal nutritional status, and examines the vascular development in the fetus.

5.2 Materials and Methods

5.2.1 Study Design

Full details of general methods are given in Chapter 2. A brief summary of further methodological approaches used specifically in Chapter 5 are:

All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986 and were approved by the relevant local ethical review committees of the Macauley Institute and the University of Nottingham.

Thirty-one pregnant Scottish Blackface ewes carrying twins were randomly allocated to groups fed either a control diet (CP; n = 15) providing adequate protein from day 0 to day 65 gestation (term ~147 days), or a protein-restricted diet from day 0 – 65 gestation (LPE, days 0 – 65 gestation; n = 16). On an 'as fed basis', the diets were isocaloric with the effective level of protein restriction being 8.7 vs. 17 g crude protein·MJ ME (metabolisable energy). At day 65 gestation, ewes were euthanised. Amniotic fluid and fetal plasma were collected. One fetus was corrosion cast, and from the other fetus organs were weighed, one kidney snap frozen in liquid nitrogen and stored at -80°C and the other fixed in 4% paraformaldehyde.

5.2.2 Corrosion casting

One fetus from each ewe was corrosion cast using Batson's No. 17 Plastic Replica and Corrosion Kit in accordance with the manufacturer's protocol, to visualise and quantify the

gross vasculature of the fetus. Full details are given at section 2.13. The casts were weighed, and then scanned using a micro CT scanner, from which volume and surface area were calculated.

5.2.3 Plasma analysis: Radox Imola

Maternal plasma was sampled prior to pregnancy, and then at days 13, 28, 42 and 65 of gestation, and stored at -20°C. All maternal plasma samples were defrosted, and analysed for glucose, NEFA, triglycerides, albumin, total protein, urea, lactate and D-3-hydroxybutarate using a Radox Rx Imola autoanalyser and quantitative enzymatic colorimetric assay kits, as described in section 2.3.2. Plasma samples from day 65 fetuses were defrosted and analysed for glucose and lactate only.

5.2.4 Plasma analysis: Osmolality

Osmolality of maternal plasma samples prior to pregnancy and at days 13, 42 and 65 of gestation was determined using an automatic cryoscopic osmometer (Osmomat 030; Gonotec, Berlin, Germany), see section 2.14.

5.2.5 Plasma analysis: Amino Acids

Amino acids were isolated from maternal and fetal plasma and derivatised using the EZ:faast™ Amino Acid Kit according to the manufacturer's protocol. After derivatisation the samples were analysed on a gas chromatography mass spectrometer. Full details are given at section 2.3.3.

5.2.6 Amniotic fluid analysis: Amino acids

The EZ:Faast kit described above was not able to detect two, and gave poor results for a further three amino acids in amniotic fluid. Thus, a further analysis was conducted using an ion exchange chromatography system as described in section 2.3.4.

5.2.7 Renal immunohistochemistry

5.2.7.1 VEGFA

Renal sections were analysed for abundance of VEGFA (SC-152; Santa Cruz Biotechnology, Santa Cruz, USA) using a biotinylated ABC kit (Vector Labs, Peterborough, UK). The full method is given in section 2.6.1, and further details of the VEGFA protein sequence and antibody selection are given in section 2.6.3. Negative controls were omission of primary antibody and rabbit IgG, and mouse kidney was used as a positive control.

5.2.7.2 CD34

Renal sections were analysed for abundance of CD34 (ab81289; Abcam, Cambridge, UK) using the Leica BOND-MAX™ automated system. The full method is given in section 2.6.2, and further details of the CD34 protein sequence and antibody selection are given in section 2.6.4. Negative controls were omission of primary antibody and rabbit IgG, and adult ovine kidney was used as a positive control.

5.2.8 Quantitative PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Crawley, UK), and cDNA synthesised using an Omniscript reverse transcriptase kit (Qiagen, Crawley, UK). QPCR performed using a Roche SYBR Green kit (Roche Diagnostics, Burgess Hill, UK) on a Roche Lightcycler® 480. Melt curves were used to confirm reaction specificity and cyclophilin,

beta actin and GAPDH were used as housekeeping genes. Negative controls were H₂O and samples with Omniscript omitted. mRNA quantities were normalised to housekeeping genes using Roche Lightcycler® 480 advanced relative quantification software. Full details of the method are described at sections 2.12.1.2, 2.12.2, 2.12.4.2 and 2.12.5.

5.2.9 Statistical Analysis

All data were analyzed using a General Linear Model (GLM) or general linear mixed model approach where appropriate (Genstat v13, VSNi, UK) after checking for normality (or otherwise) of the error distribution. Data with skewed errors were log transformed before analysis. Predicted means are presented with SEM to represent the measurement error. Correlations between variables were tested using Pearson's correlation tests. $P < 0.05$ was accepted as indicating statistical significance. Full details are described at section 2.15.

5.3 Results

5.3.1 Maternal characteristics

There was no difference in weight gain during pregnancy up to day 65 gestation between dietary groups, although weight increased with time (Figure 5.1A). In addition there were no differences in body condition score between the CP and LPE groups, and although there was an improvement with time this was during the pre-pregnancy period, rather than during gestation (Figure 5.1B). There was also statistical evidence of a diet.time interaction for both variables.

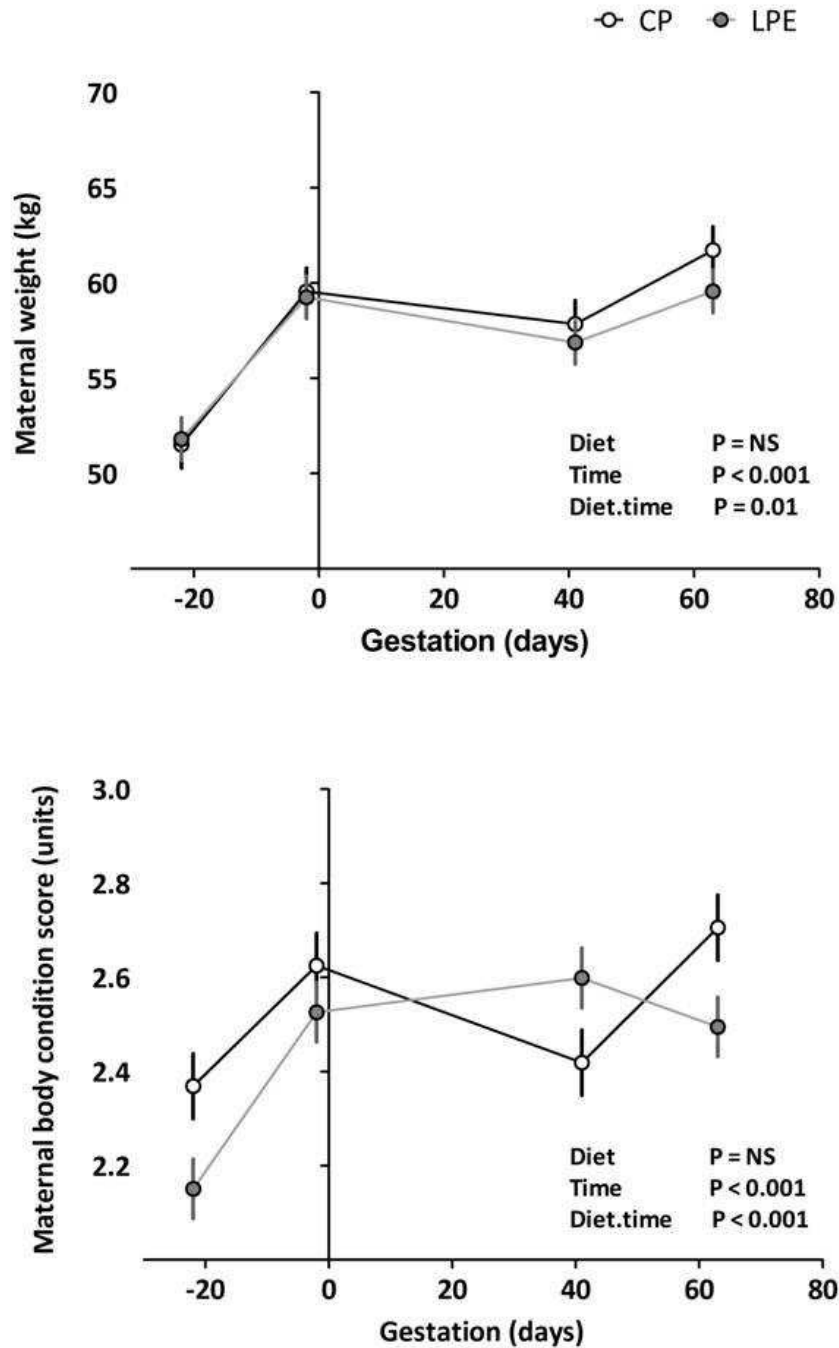


Figure 5.1: Weight and body condition score of ewes prior to, and during early gestation

Data are Means \pm S.E.M. for ewes fed a control protein diet (CP, n = 15), or a low protein diet during early gestation (days 0-65; LPE, n = 16). There were 8 males and 7 females in CP and 8 males and 8 females in LPE. Data were analysed by General Linear Model for the fixed effects of diet, time and their interaction with (Genstat v13, VSNi, UK). CP, control protein diet; LPE, low protein early diet; NS, non-significant; significance indicated by P < 0.05.

5.3.2 *Post mortem data at day 65*

Post mortem data is presented in Table 5.1. The ewe weight at *post mortem* was significantly lower in the LPE group compared to controls (CP, 60.5 ± 0.72 ; LPE, 58.4 ± 0.68 kg), however the percentage difference was small, i.e. a reduction of ~3.5%. In addition a significant effect of diet on gravid uterus weight was observed, with the LPE group weighing ~8% less (CP, 3.00 ± 0.08 ; LPE, 2.77 ± 0.07 kg). However, the effects on the ewe and uterus were not translated to the fetus itself. Diet and sex had no effect on the fetal weights at *post mortem* of kidney, heart, or liver, nor on total body weight or crown rump length (Table 5.1). Adjusting organ weight for total body weight had no effect on the findings. Total gonad weight did not vary according to diet, but testes were significantly larger than ovaries (male, $4.80 \pm 0.45 \times 10^{-2}$; female, $2.29 \pm 0.49 \times 10^{-2}$ g).

When compared to study 1, fetuses at day 65 gestation weighed more in study 2 (study 1, 101 ± 4.13 ; study 2, 125 ± 3.71 g; $P < 0.001$) and had larger hearts (study 1, 0.99 ± 0.05 ; study 2, 1.16 ± 0.03 g; $P < 0.001$), however, the other organs measured in both studies (kidney, liver) were not significantly different.

Table 5.1: Effect of maternal low protein diet on fetal weights at 65 days gestation

	Diet	Sex		P value		
		Male	Female	Diet	Sex	Diet*Sex
Body weight (g)	CP	130 ± 6.54	131 ± 6.18	NS	NS	NS
	LPE	128 ± 5.87	118 ± 5.77			
Total Kidney weight (g)	CP	1.79 ± 0.12	1.68 ± 0.13	NS	NS	NS
	LPE	1.74 ± 0.12	1.73 ± 0.12			
Liver weight (g)	CP	8.68 ± 0.55	9.03 ± 0.58	NS	NS	NS
	LPE	8.40 ± 0.55	8.26 ± 0.54			
Heart weight (g)	CP	1.21 ± 0.07	1.14 ± 0.08	NS	NS	NS
	LPE	1.11 ± 0.07	1.17 ± 0.07			
Crown rump length (mm)	CP	166 ± 4.08	173 ± 4.36	NS	NS	NS
	LPE	175 ± 4.36	167 ± 4.36			
Total gonads weight (g x 10⁻²)	CP	4.80 ± 0.45	2.29 ± 0.49	NS	<0.001	NS
	LPE	5.02 ± 0.52	2.01 ± 0.45			

Data are predicted means ± S.E.M. for ewes (CP, n = 15; LPE, n =16) fed a control protein diet (CP fetuses, male, n=8; female, n=7) or a low protein diet to day 65 gestation (LPE fetuses, male, n=8; female, n=8). Data were analysed by General Linear Model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance was accepted at P<0.05, NS, not significant; CP, control protein diet; LPE, low protein early diet.

5.3.3 Corrosion casts

Figure 5.2 shows examples of micro CT pictures of the kidneys of a male control fetus (147M) and a male low protein fetus (127M). Weight was highly correlated with both volume ($R^2 = 0.93$; $P < 0.001$) and surface area ($R^2 = 0.88$; $P < 0.01$). The micro CT results of volume and surface area were also strongly correlated ($R^2 = 0.97$; $P < 0.001$). There was a large variation in the cast weights, volumes, and surface areas, precluding any significant difference between diet groups, or between sexes (Figure 5.3). As there was no clear indicator of an effect of diet on the macrovasculature, the microvasculature was considered instead, using CD34 as a marker of endothelial cells.

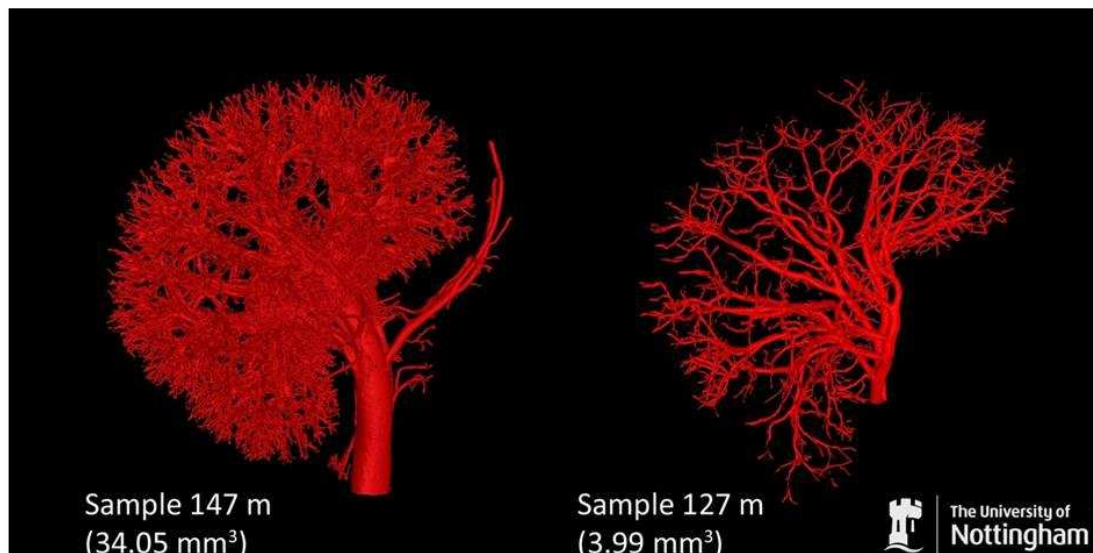


Figure 5.2: Micro CT pictures of fetal kidneys at day 65 gestation

Sample 147M is a male control fetus and sample 127M is a low protein male fetus, and volumes are shown in mm^3 . Corrosion casting was carried out using Batson's No. 17 Plastic Replica and Corrosion Kit (Polysciences Inc; Eppelheim, Germany). The volume of the resin casts was quantified using a Nanotom[®] high resolution computed tomography (CT) scanner (GE Sensing and Inspection Technologies, Wunstorf, Germany).

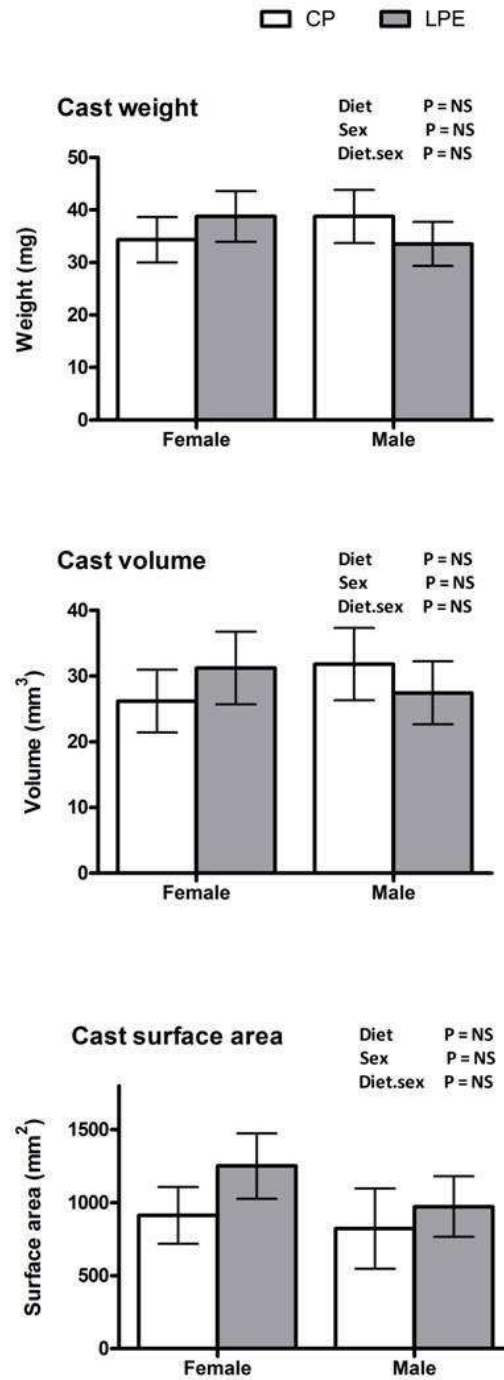


Figure 5.3: Weight, volume and surface area of cast fetal kidneys at day 65 gestation

Corrosion casting using Batson's No. 17 Plastic Replica & Corrosion Kit. Surface area and volume of resin casts were quantified using a Nanotom[®] high resolution computed tomography scanner. Data are predicted means \pm S.E.M. for ewes fed a control protein diet (CP fetuses, male, n = 8; female, n = 7) or a low protein diet to day 65 gestation (LPE fetuses, male, n = 8; female, n = 8). Data were analysed by General Linear Model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance was accepted at $P < 0.05$, NS, not significant.

5.3.4 Immunohistochemistry: CD34

The percentage of the tissue section that stained for CD34 was reduced in LPE compared to CP fetuses in both the whole of the kidney and in the nephrogenic zone around the edge of the developing cortex (Figure 5.4). In the whole of the kidney, LPE fetuses had 25% less staining than controls (CP, 3.60 ± 0.28 %; LPE 2.83 ± 0.26 %; $P = 0.054$), and the same proportional reduction was also found in the nephrogenic region (CP, 4.41 ± 0.35 %; LPE 3.29 ± 0.33 %; $P = 0.029$).

Compared to the adult kidney, the fetal kidney microvascular network was proportionally more of the total area of the kidney (adult, 1 - 2 %; fetal, 2 - 5 %). In addition, although still significant, the effect of the maternal diet on the LPE group was less striking in the fetuses compared to the adults (adult, ~50 % reduction in the LPE group; fetal ~25% reduction).

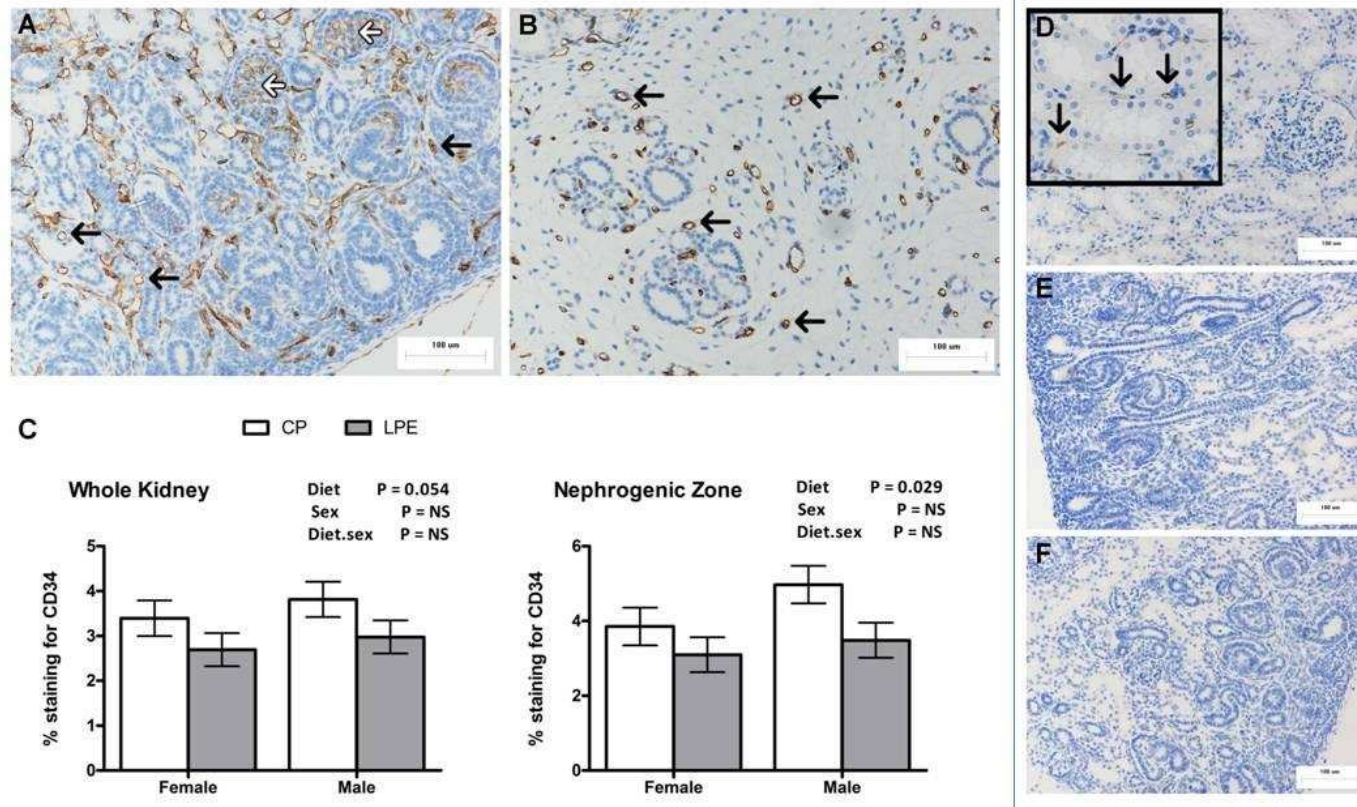


Figure 5.4: CD34 staining in day 65 fetal kidneys

Representative micrographs of the nephrogenic zone (A) and developing medulla (B); CD34 staining is brown, black arrows indicate endothelial cells, white arrows show staining of glomerular capillaries. D, positive control, adult ovine kidney; negative controls were omission of primary antibody (E) and rabbit IgG (F) All pictures are x200 magnification. Data in graphs are predicted means \pm S.E.M. for fetuses of ewes fed a control protein diet (CP; male, n=8; females n=7) or a low protein diet to day 65 gestation (LPE; male, n=8; female, n=8). Data were analysed by General Linear Model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance $P < 0.05$, NS, not significant.

5.3.5 Angiogenic factors

To check for reproducibility of the angiogenic factor results seen in the fetuses in Chapter 3, qPCR and immunohistochemistry were carried out. In Study 1 for a range of angiogenic promoters, LPE males (but not females) had reduced mRNA expression compared to controls. In the current study, two of these factors (angiopoietin and Tie 2 receptor) were measured, and although there was a strong trend for the LPE fetuses to have lower mRNA expression, this was true for both sexes, and not just males (Figure 5.5). In Study 1 VEGFA was significantly reduced in the LPE group compared to controls, which was replicated in this present study with the exact same pattern of gene expression as was found in the earlier study, with LPE fetuses having less mRNA than controls (CP, $1.37 \pm 0.06 \times 10^{-1}$; LPE, $1.14 \pm 0.06 \times 10^{-1}$ units). VEGFR1 mRNA showed the same pattern of results in both studies. However, in the current study the results were more uniform across both sexes, whereas in the first study it was more pronounced in males than females (see Figure 3.4) in VEGFA, angiopoietin and Tie 2.

At the protein level, VEGFA results in Study 1 were replicated in this current study (Figure 5.6), with a significant reduction of VEGFA staining in the nephrogenic zone in the LPE fetuses. As before, the staining was primarily evident in the ureteric buds and the developing collecting ducts.

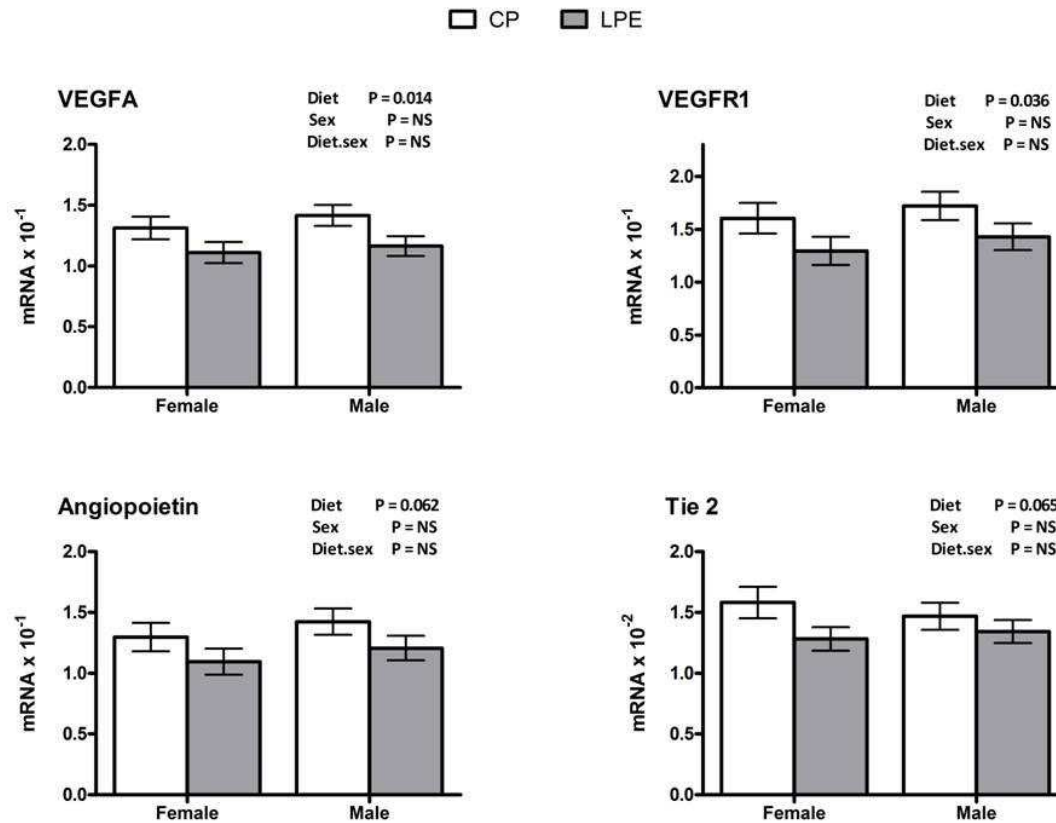


Figure 5.5: MRNA of angiogenic factors in day 65 fetuses

Data are predicted means \pm S.E.M. for fetuses of ewes fed a control protein diet (CP; male, n = 8; females n = 7) or a low protein diet to day 65 gestation (LPE; male, n = 8; female, n = 8). Data were analysed by General Linear Model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance $P < 0.05$, NS, not significant; VEGF, vascular endothelial growth factor.

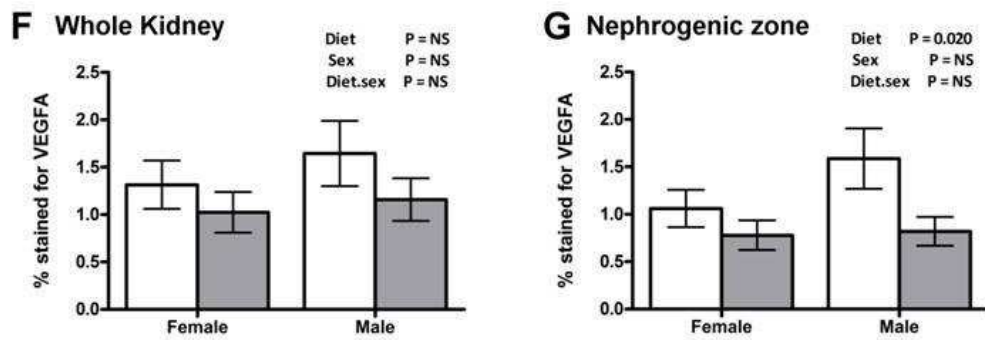
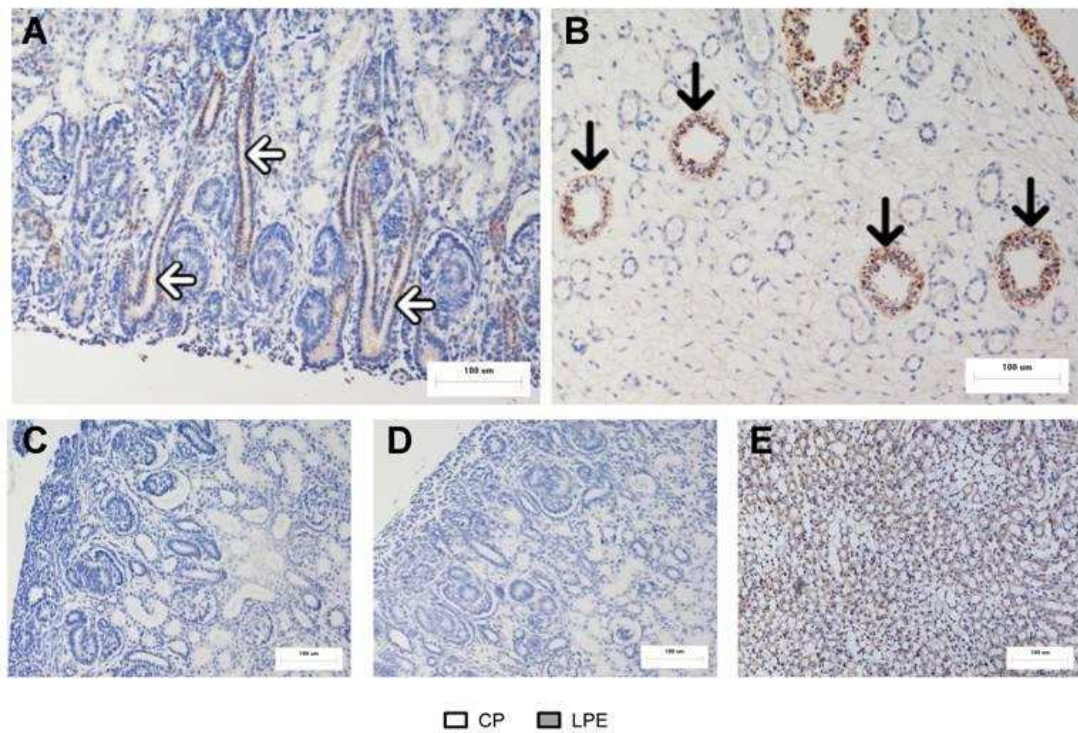


Figure 5.6: : Vascular Endothelial Growth Factor immunohistochemical staining

Representative micrographs of the nephrogenic zone (A) and developing medulla (B); VEGFA staining is brown; white arrows indicate ureteric buds; black arrows indicate collecting ducts. Negative controls were omission of primary antibody (C) and rabbit IgG isotype control (D). Positive control (E) was mouse kidney. All micrographs taken at x200 magnification. Data in graphs are predicted means \pm S.E.M. for fetuses of ewes fed a control protein diet (CP; male, n = 8; females n = 7) or a low protein diet to day 65 gestation (LPE; male, n = 8; female, n = 8). Data were analysed by General Linear Model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance $P < 0.05$, NS, not significant.

5.3.6 Metabolites and protein

To consider the maternal and fetal nutritional environments, various metabolites and protein levels were measured in the plasma of the ewes at 10 days prior to pregnancy and at days 28, 42 and 65 of gestation, and in the fetuses at *post mortem*.

In the ewes prior to pregnancy no plasma metabolite was different between groups, with the exception of non-esterified fatty acids (NEFA) which was lower in the LPE group compared to controls. Over the course of gestation the metabolites (NEFA, triglycerides, glucose and lactate) became significantly higher in the LPE ewes compared to controls (Figure 5.7), and there was also a significant effect of time. In addition, total protein and albumin were also significantly higher in the LPE group compared to the CP group (Figure 5.8), which was unexpected given the lower protein content of the diet. D-3-hydroxybutyrate was not significantly different between groups or over time. Urea was significantly lower in the LPE ewes compared to controls (Figure 5.8), with a reduction of around 20% (average of all time points, CP, 5.52 ± 0.26 ; LPE, 4.51 ± 0.25 mmol.l⁻¹).

To investigate if the findings translated into the fetal environment, lactate, glucose and urea were measured in fetal plasma (Figure 5.9). Glucose was not significantly different between groups. Lactate and urea results reflected the maternal environment, with lactate being significantly higher in LPE fetuses compared to controls (CP, 4.22 ± 0.14 ; LPE 4.79 ± 0.14 mmol.l⁻¹), and urea being significantly reduced (CP, 6919 ± 351 ; LPE, 3511 ± 340 mmol.l⁻¹). There were no effects of sex.

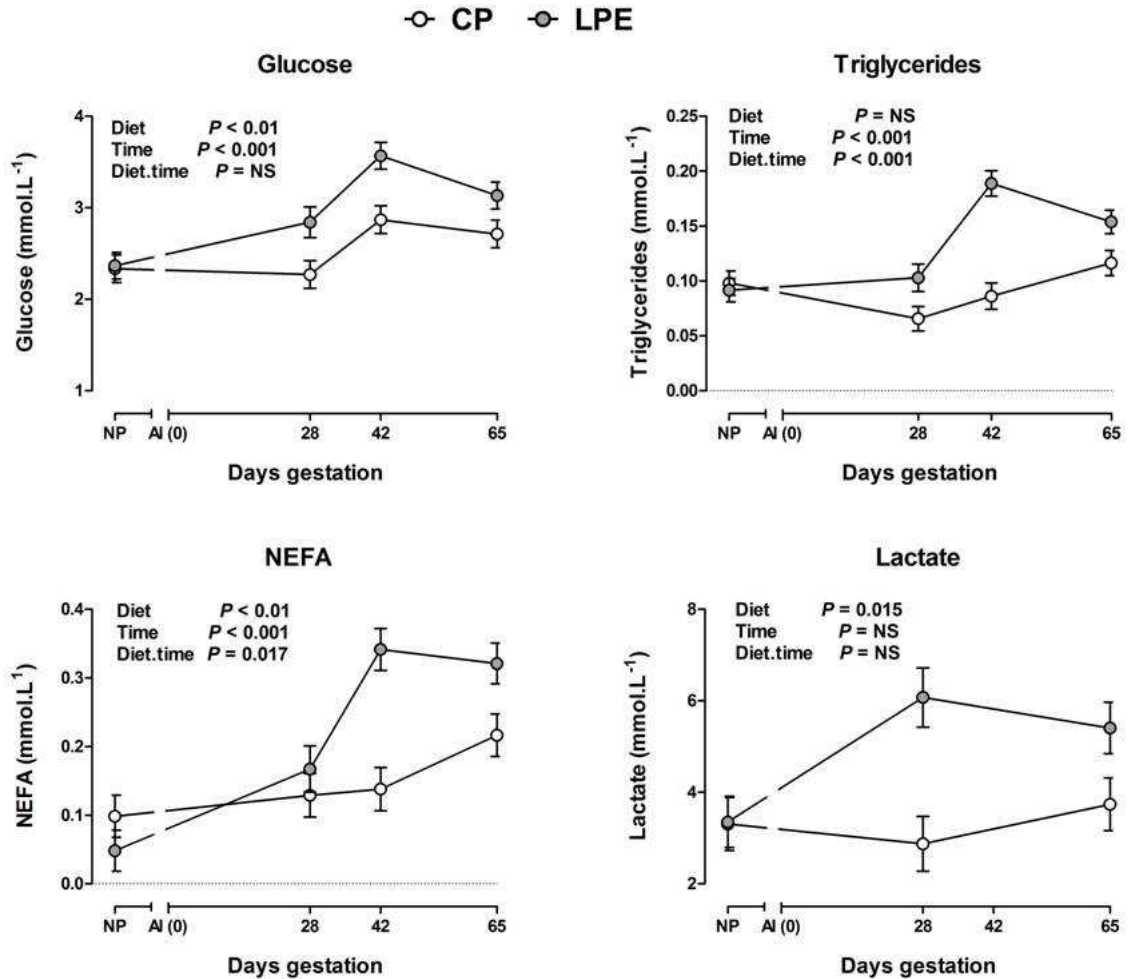


Figure 5.7: Metabolites in maternal plasma

Metabolites were measured prior to pregnancy, and at days 28, 42 and 65 of gestation using a Randox Imola autoanalyser. Data in graphs are predicted means \pm S.E.M. for fetuses of ewes fed a control protein diet (CP; male, n=8; females n=7) or a low protein diet to day 65 gestation (LPE; male, n=8; female, n=8). Data were analysed by General Linear Model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance $P < 0.05$, AI, artificial insemination at day 0; NP, non-pregnant at ten days prior to AI; NS, non significant.

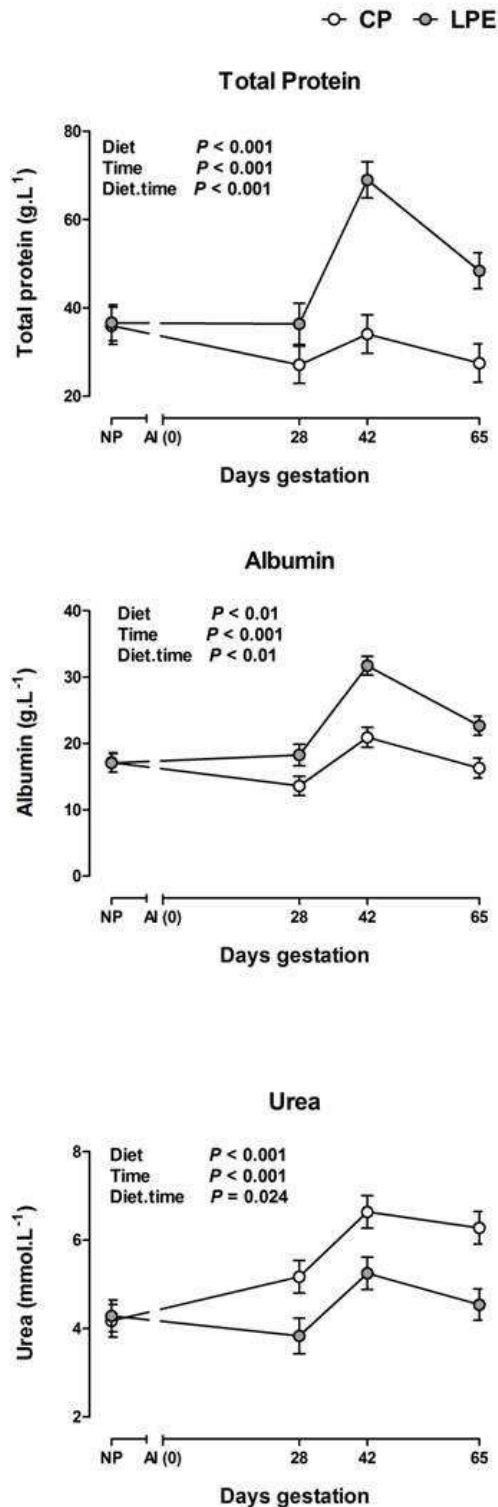


Figure 5.8: Total protein, albumin and urea in maternal plasma

Variables were measured prior to pregnancy, and at days 28, 42 and 65 of gestation using a Randox Imola autoanalyser. Data in graphs are predicted means \pm S.E.M. for fetuses of ewes fed a control protein diet (CP; male, $n=8$; females $n=7$) or a low protein diet to day 65 gestation (LPE; male, $n=8$; female, $n=8$). Data were analysed by General Linear Model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance $P < 0.05$, AI, artificial insemination at day 0; NP, non-pregnant ewes at ten days prior to AI; NS, non significant.

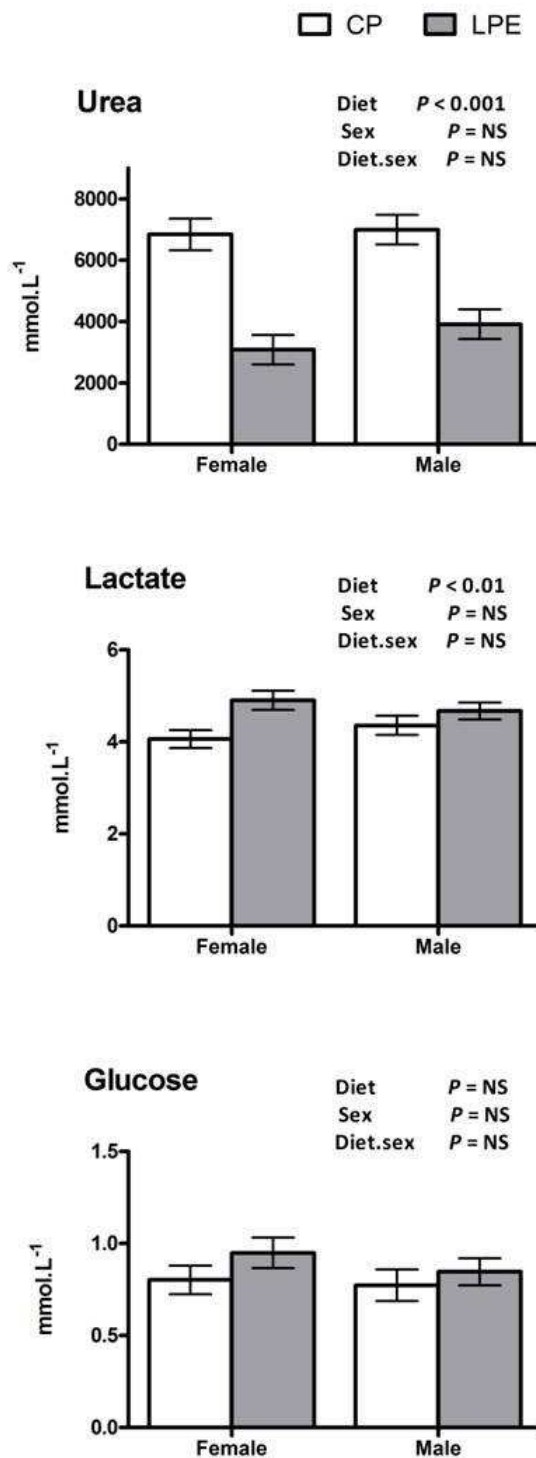


Figure 5.9: : Urea in amniotic fluid and lactate and glucose in fetal plasma at day 65 gestation

Variables were measured using a Randox Imola autoanalyser. Data in graphs are predicted means \pm S.E.M. for fetuses of ewes fed a control protein diet (CP; male, n=8; females n=7) or a low protein diet to day 65 gestation (LPE; male, n=8; female, n=8). Data were analysed by General Linear Model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance $P < 0.05$, NS, non significant.

5.3.7 Osmolality

Maternal plasma and fetal amniotic osmolality results are shown in Figure 5.10. Maternal plasma osmolality was not different between the LPE and CP groups in non-pregnant ewes, however, during gestation the LPE group had significantly higher osmolality compared to the control ewes (for all time points, CP, 303 ± 3.29 ; LPE, 314 ± 3.28 mOsm), and there was also an effect of time. However, the opposite effect was observed in the fetal amniotic fluid, with LPE fetuses having significantly lower osmolality than CP fetuses (CP, 288 ± 4.10 ; LPE 275 ± 3.80 mOsm, $P = 0.030$). There was no effect of sex on fetal amniotic osmolality, nor was there a diet.sex interaction.

5.3.8 Amino acid analysis

5.3.8.1 Maternal plasma

In maternal plasma, most standard amino acids were not significantly different between dietary groups but changed as gestation progressed (Table 5.2). Four amino acids were different in the LPE group compared to controls: asparagine concentration was reduced in the LPE ewes compared to CP ewes (average for all time points: CP, 25.8 ± 1.79 ; LPE, 21.5 ± 2.26 μM), as was the concentration of leucine (average for all time points: CP, 122 ± 5.90 ; LPE, 103 ± 6.10 μM). Conversely, lysine concentration was higher in the LPE group compared to controls (average for all time points: CP, 162 ± 31.1 ; LPE, 196 ± 40.3 μM), and also tryptophan concentration (average for all time points: CP, 41.7 ± 7.55 ; LPE, 58.9 ± 9.54 μM). Linear discriminant analysis showed no particular overall effects of an interaction between diet groups and timing of gestation.

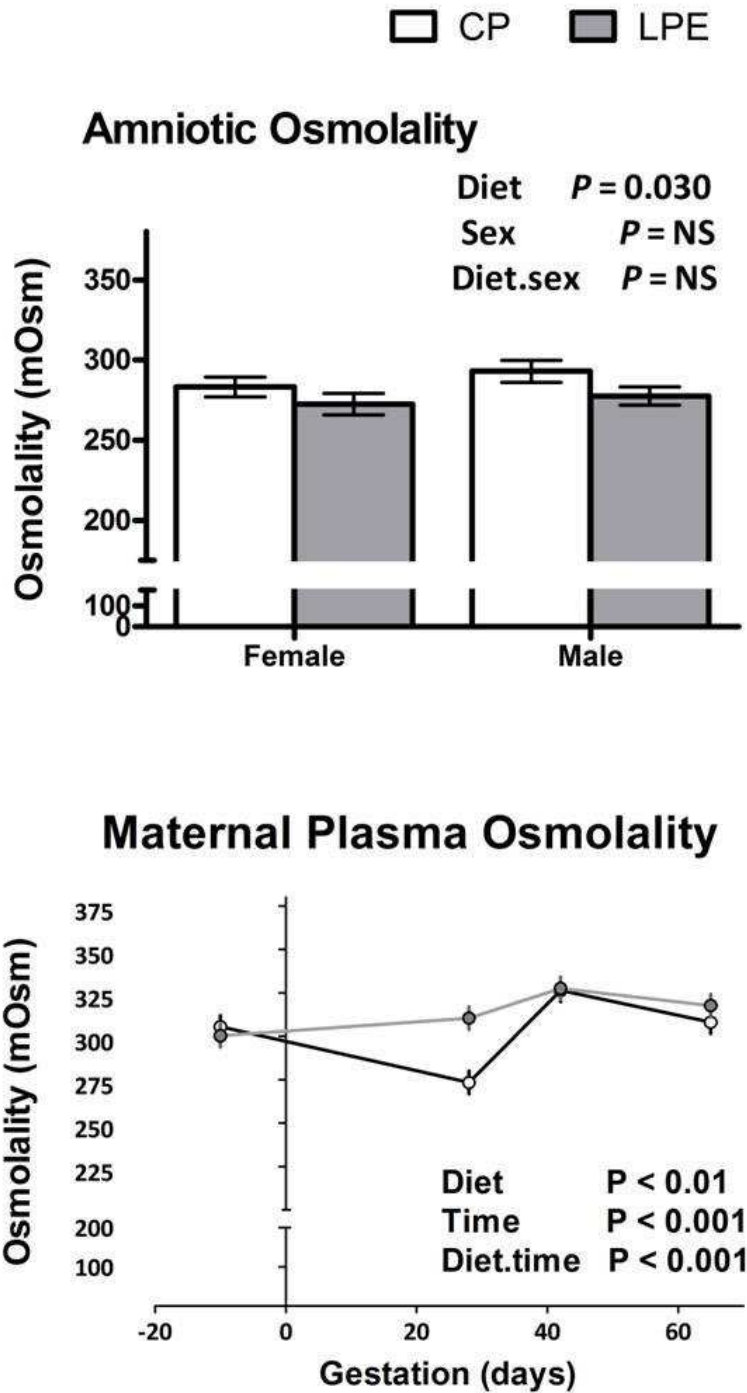


Figure 5.10: Osmolality of maternal plasma and fetal amniotic fluid

Variables were measured prior to pregnancy, and at days 13, 42 and 65 of gestation using a Gonotec Osmometer. Data in graphs are predicted means \pm S.E.M. for fetuses of ewes fed a control protein diet (CP; male, n=8; females n=7) or a low protein diet to day 65 gestation (LPE; male, n=8; female, n=8). Data were analysed by General Linear Model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance $P < 0.05$, NS, non significant.

Table 5.2: Standard amino acid concentrations in maternal plasma (μM)

Amino acid	Non pregnant		Day 28		Day 42		Day 65		Diet	P-value Time	Diet.time
	CP	LPE	CP	LPE	CP	LPE	CP	LPE			
Alanine	389 \pm 22.3	368 \pm 21.6	345 \pm 76.5	516 \pm 85.4	331 \pm 35.1	364 \pm 32.8	300 \pm 30.2	302 \pm 30.0	NS	0.041	NS
Asparagine	35.9 \pm 4.17	33.9 \pm 4.93	32.0 \pm 5.04	20.6 \pm 7.10	21.2 \pm 1.85	15.0 \pm 1.90	14.3 \pm 2.78	16.5 \pm 2.48	0.031	<0.001	NS
Aspartic acid	6.65 \pm 1.72	4.88 \pm 1.91	4.63 \pm 1.69	7.33 \pm 2.03	7.20 \pm 2.31	5.25 \pm 2.50	10.0 \pm 3.52	8.18 \pm 3.39	NS	NS	NS
Glutamic acid	103 \pm 12.1	85.4 \pm 15.2	162 \pm 27.7	124 \pm 40.8	45.6 \pm 4.36	50.0 \pm 4.46	49.9 \pm 10.8	48.1 \pm 8.15	NS	<0.001	NS
Glycine	622 \pm 49.5	582 \pm 49.3	919 \pm 121	1077 \pm 149	563 \pm 58.4	811 \pm 54.7	823 \pm 95.1	974 \pm 91.3	NS	<0.001	NS
Isoleucine	116 \pm 9.81	119 \pm 9.50	102 \pm 12.4	74.4 \pm 16.0	85.3 \pm 6.51	71.0 \pm 6.06	66.0 \pm 5.57	72.3 \pm 5.57	NS	<0.001	NS
Leucine	143 \pm 10.4	147 \pm 10.0	133 \pm 15.2	96.6 \pm 16.9	113 \pm 7.60	83.4 \pm 7.10	100 \pm 9.70	83.9 \pm 9.40	<0.01	<0.001	NS
Lysine	171 \pm 32.9	257 \pm 41.1	253 \pm 85.1	223 \pm 132	127 \pm 24.8	155 \pm 21.1	95.6 \pm 22.8	149 \pm 21.1	<0.01	<0.01	0.047
Phenylalanine	80.1 \pm 7.33	74.8 \pm 7.29	67.6 \pm 9.64	54.1 \pm 11.8	49.1 \pm 4.02	51.0 \pm 3.77	60.2 \pm 7.63	47.8 \pm 7.35	NS	<0.001	NS
Proline	84.6 \pm 6.45	84.7 \pm 6.45	85.1 \pm 9.86	68.3 \pm 13.5	66.1 \pm 4.29	48.8 \pm 4.31	49.3 \pm 5.47	55.7 \pm 5.06	NS	<0.001	NS
Threonine	68.8 \pm 13.4	81.9 \pm 16.6	64.0 \pm 15.4	74.4 \pm 23.9	40.1 \pm 7.84	42.6 \pm 8.66	27.6 \pm 11.7	45.6 \pm 9.31	NS	0.047	NS
Tryptophan	61.3 \pm 10.8	78.1 \pm 14.1	55.0 \pm 20.4	81.7 \pm 30.0	20.4 \pm 6.54	44.0 \pm 7.18	30.1 \pm 11.8	31.8 \pm 9.25	0.032	<0.01	NS
Tyrosine	70.6 \pm 9.92	61.0 \pm 10.9	48.8 \pm 9.17	30.1 \pm 11.1	36.3 \pm 4.43	33.4 \pm 4.42	29.5 \pm 4.72	28.6 \pm 4.24	NS	<0.01	NS
Valine	497 \pm 43.8	563 \pm 42.4	434 \pm 61.8	356 \pm 74.3	358 \pm 28.5	263 \pm 26.8	315 \pm 27.4	259 \pm 27.4	NS	<0.001	NS
Total	2956 \pm 213	2527 \pm 159	2736 \pm 464	2988 \pm 485	1949 \pm 61	1969 \pm 101	1738 \pm 253	2115 \pm 77	NS	<0.001	NS

Data are predicted means \pm S.E.M. for ewes fed a control protein diet (CP; n=15) or a low protein diet to day 65 gestation (LPE; n=16). Data were analysed by General Linear Model for the fixed effects of diet, time or their interaction using Genstat v13 (VSNi, UK). Statistical significance $P < 0.05$, NS, non significant.

5.3.8.2 Fetal plasma

In fetal plasma the only standard amino acid that was significantly different between groups was glycine, which had concentrations ~25% higher in the LPE group compared to the CP group (Table 5.3). In addition, glycine had a diet.sex interaction, with the increase in the LPE group compared to controls being much more marked in female fetuses.

Furthermore, asparagine (female, 49.5 ± 2.71 ; male, 59.6 ± 2.80 μM) and also proline (female, 108 ± 5.18 ; male, 128 ± 6.35 μM) were lower in female compared to male fetuses.

Table 5.3: Standard amino acid concentrations in fetal plasma (μM)

Amino acid	CP	LPE	P-value		
			Diet	Sex	Diet.sex
Alanine	285 ± 14.5	266 ± 14.5	NS	NS	NS
Asparagine	54.6 ± 2.87	54.1 ± 2.73	NS	0.012	NS
Aspartic acid	9.66 ± 0.66	9.16 ± 0.60	NS	NS	NS
Glutamic acid	218 ± 16.8	224 ± 15.9	NS	NS	NS
Glycine	397 ± 22.8	479 ± 26.2	0.016	NS	0.018
Isoleucine	78.9 ± 4.44	76.1 ± 4.11	NS	NS	NS
Leucine	140 ± 6.06	127 ± 5.93	NS	NS	NS
Lysine	702 ± 65.1	540 ± 61.8	NS	NS	NS
Phenylalanine	175 ± 8.97	174 ± 8.75	NS	NS	NS
Proline	119 ± 5.99	116 ± 5.57	NS	0.019	NS
Serine	700 ± 49.1	691 ± 48.1	NS	NS	NS
Threonine	448 ± 35.3	463 ± 32.9	NS	NS	NS
Tryptophan	87.2 ± 12.7	88.6 ± 12.7	NS	NS	NS
Tyrosine	277 ± 27.7	231 ± 22.9	NS	NS	NS
Valine	212 ± 12.3	199 ± 11.3	NS	NS	NS
Total	3821 ± 179	3580 ± 170	NS	NS	NS

Data are predicted means \pm S.E.M. for fetuses of ewes fed a control protein diet (CP; male, n=8; females n=7) or a low protein diet to day 65 gestation (LPE; male, n=8; female, n=8). Data were analysed by General Linear Model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance $P < 0.05$, NS, non significant.

Discriminant analysis (Figure 5.11) however did reveal differences between the experimental groups and sexes.

There were few correlations between fetal and maternal plasma, and those that existed were generally weak. The strongest correlation between maternal and fetal plasma was for glycine (Pearson's correlation, $R^2 = 0.31$, $P = 0.028$).

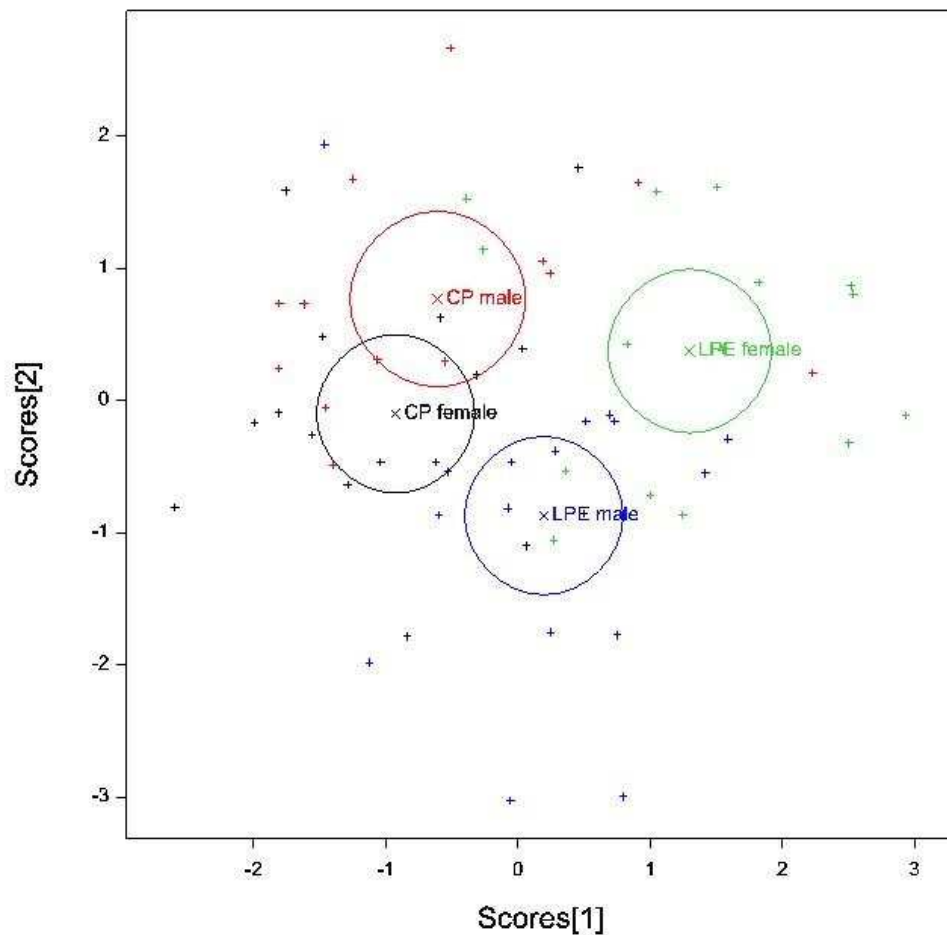


Figure 5.11: Discriminant analysis of standard amino acids in fetal plasma

X marks the mean and the circles represent 95% confidence intervals after linear discriminant analysis (Genstat v13, VSNi, UK) of normalised data (i.e. Z-scores) for fetal plasma amino acid concentrations.

5.3.8.3 Amniotic fluid

In amniotic fluid, there were no significant differences between dietary groups, nor any effects of sex or a diet.sex interaction (Table 5.4). Linear discriminant analysis did not reveal any further effects of diet or sex.

There was a moderate correlation between fetal plasma and amniotic fluid (for all standard amino acids, Pearson's correlation, $R^2 = 0.42$, $P < 0.001$), however there were no significant correlations between the variables in maternal plasma and amniotic fluid.

5.3.8.4 Ornithine

As well as measuring the standard amino acids, the plasma and amniotic fluid concentration of ornithine was measured. Although ornithine was not different between LPE and control ewes (Figure 5.12), it was significantly lower in both amniotic fluid (CP, 109 ± 11.0 ; LPE, 74.3 ± 7.21 μM) and in fetal plasma (CP, 432 ± 31.1 ; LPE, 264 ± 30.0 μM).

Table 5.4: Standard amino acids in amniotic fluid (μM)

Amino acid	CP	LPE	P-value		
			Diet	Sex	Diet.sex
Alanine	178 \pm 31.1	192 \pm 30.1	NS	NS	NS
Arginine	39.4 \pm 7.88	37.6 \pm 7.31	NS	NS	NS
Asparagine	30.8 \pm 4.50	33.6 \pm 4.35	NS	NS	NS
Cysteine	6.65 \pm 0.72	6.75 \pm 0.70	NS	NS	NS
Glutamic acid	46.8 \pm 43.4	43.4 \pm 8.39	NS	NS	NS
Glutamine	124 \pm 18.6	152 \pm 21.9	NS	NS	NS
Glycine	117 \pm 19.2	160 \pm 18.6	NS	NS	NS
Histidine	9.98 \pm 1.76	12.1 \pm 2.22	NS	NS	NS
Isoleucine	11.7 \pm 2.18	13.1 \pm 2.41	NS	NS	NS
Leucine	24.1 \pm 5.05	25.1 \pm 5.16	NS	NS	NS
Lysine	98.9 \pm 11.6	98.5 \pm 11.3	NS	NS	NS
Methionine	9.87 \pm 1.98	11.7 \pm 2.18	NS	NS	NS
Phenylalanine	10.8 \pm 1.96	12.3 \pm 2.05	NS	NS	NS
Proline	66.8 \pm 6.18	57.7 \pm 5.20	NS	NS	NS
Serine	80.3 \pm 11.2	98.8 \pm 10.8	NS	NS	NS
Threonine	61.7 \pm 21.1	84.2 \pm 12.1	NS	NS	NS
Tryptophan	1.28 \pm 0.37	1.61 \pm 0.34	NS	NS	NS
Tyrosine	14.0 \pm 2.58	16.0 \pm 2.75	NS	NS	NS
Valine	30.6 \pm 5.63	36.3 \pm 6.67	NS	NS	NS
Total	1325 \pm 122	1329 \pm 102	NS	NS	0.037

Data are predicted means \pm S.E.M. for fetuses of ewes fed a control protein diet (CP; male, n = 8; females n = 7) or a low protein diet to day 65 gestation (LPE; male, n = 8; female, n = 8). Data were analysed by General Linear Model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance $P < 0.05$, NS, non significant.

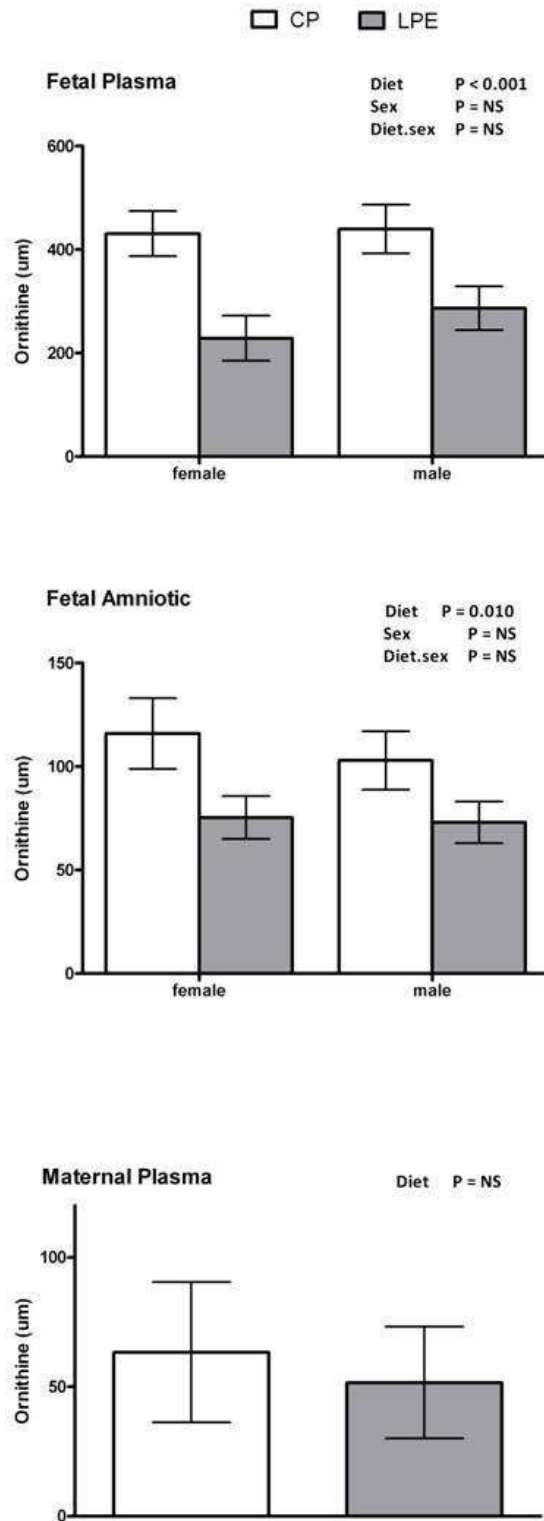


Figure 5.12: : Ornithine concentrations in maternal and fetal plasma and amniotic fluid

Data are predicted means \pm S.E.M. for fetuses of ewes fed a control protein diet (CP; male, n = 8; females n = 7) or a low protein diet to day 65 gestation (LPE; male, n = 8; female, n = 8). Data were analysed by General Linear Model for the fixed effects of diet, sex or their interaction using Genstat v13 (VSNi, UK). Statistical significance $P < 0.05$, NS, non significant.

5.4 Discussion

The previous chapters have revealed an adult phenotype with reduced nephron number and kidney function that may be as a consequence of changes in angiogenesis and apoptosis in the fetal kidney during its early development.

This second study repeated the experimental conditions of Study 1, but involved a change of location and environment which may introduce random error into the experiment.

Hence, it was critical to ensure consistency between the two studies so that any conclusions about the effect of a low protein diet on fetal development are valid, comparable and repeatable. Maternal body condition score and weight gain up to day 65 of pregnancy were not different between the dietary groups in Study 1, and this was replicated in Study 2, showing that at this stage of pregnancy the effect of diet on the ewe is not pronounced, which is likely due to the isocaloric nature of the diet, despite a reduction in protein content.

However, the second study did show a weight gain over time that was not reflected in the earlier study up to day 65 gestation. However, most of the gain in the variables was in the two weeks prior to pregnancy, which was the acclimatisation period; therefore these changes were due to improvements in living environment and diet during this time, rather than as a consequence of the study.

Fetal body and organ weights were also not significantly different between the dietary groups in either study, although in the first study there was an effect of sex, with body, liver and heart weight all being slightly higher in the male fetuses. This finding was not replicated in the current study, nor in two studies of fetal sheep at day 53 – 58 gestation (MacLaughlin et al., 2005; MacLaughlin et al., 2010), both of which had around double the number of fetuses compared to Study 1. Therefore it seems likely that the observation

regarding sex differences shown in this previous study (section 3.3.2) maybe have been a type I error.

However, the fetal body weight was higher in the second study compared with the first study. The larger fetal size may be due to small differences in the dietary intake, as in both studies the food intake varied from 600 – 1200 g.d⁻¹ depending upon the stage of gestation, but this was not exactly the same between the studies. However, the percentage protein intake remained exactly the same between both studies, so there were no overall differences in the proportions of the diet consumed.

The macrovascular structure of the fetal kidneys was determined using corrosion casting. Batson's methylmethacrylate corrosion casting method has been demonstrated to be a superior method for accurate high resolution imaging of microvascular structures when compared to other resins (Mondy et al., 2009). Micro-CT allows imaging of the larger blood vessels in the kidney, however in this study it was not possible to see the individual glomeruli or peritubular capillaries, as to observe these tiny structures either nanotomography or laser scanning confocal microscopy is needed (Wagner et al., 2006; Wagner et al., 2011). The corrosion casts showed no significant differences in macrovascular structures in the kidney between dietary groups, however it was clear that some kidneys were far less developed than others, and these tended to be in the males of the LPE group. However, the large variation in results meant that this did not reach statistical significance, and indeed both the highest and lowest surface areas and volumes were observed in the same group (LPE males). This taken in conjunction with the fact that there were not obvious morphological differences in the kidneys in adulthood (Chapter 4) suggests that the renal macrovascular development may potentially only be delayed, rather than halted by the dietary regime. Unfortunately it was not possible to check this possibility in the current study as all ewes were killed at the same time in gestation.

However, at a microvascular level, the reductions observed in the angiogenic factors (VEGFA, VEGFR1, Ang1 and Tie 2) in both Study 1 and Study 2 in the LPE group would suggest that there are likely to be differences in blood vessel (particularly endothelial cell) development. Immunohistochemical staining for endothelial cells using a CD34 antibody as a marker, showed a clear reduction in the amount of staining in the LPE group in the peritubular and glomerular capillaries, thus showing an effect of the LPE diet on the microvascular development in the fetus at this stage of gestation. As observed in a previous study (Ferraz et al., 2008), CD34+ staining was found only in the peritubular capillaries and mature nephrons, and not in the primitive nephrons. However, the difference between the LPE and CP groups was more marked in adult animals (Chapter 4) than at day 65 in the fetus. The lower level of reduction in the fetus suggests that either vascular network development continues to be detrimentally affected later in gestation, or that obesity or another factor in the adult environment damages the smaller blood vessels in adulthood.

To investigate further the effect of the nutritional environment on the mother and fetus, metabolites and protein were measured in maternal and fetal plasma. Plasma metabolite concentrations in ruminants may vary according to the diet, age, breed and reproductive status (Alonso et al., 1997; Lindsay et al., 2005; Forbes et al., 1998). Most metabolites measured in this study fell within the published reference ranges for sheep (Aitken, 2007), or reported values during pregnancy (Redmer et al., 2012; Moallem et al., 2012; Dubreuil et al., 2005). The exceptions were triglycerides which were slightly below reference values, and total protein and albumin which were below the reference range in the control group, although just within the lower end of the range for LPE animals.

Maternal glucose in the control ewes (varying between 2.33 and 2.87 mmol.l⁻¹, depending on the stage of gestation) was similar to reference values in non-pregnant sheep (Aitken,

2007) and values reported in pregnant sheep (Redmer et al., 2012; Tanaka et al., 2008; Sharma et al., 2012). In fetal plasma, the glucose concentration was far lower than maternal levels, as also observed by other studies (Redmer et al., 2012; Bazer et al., 2012) and in the latter study where glucose concentrations were measured at 14 time points during gestation, the concentrations varied from 0.11 – 0.70 mmol.l⁻¹, and were not significantly different over the period of gestation.

Fewer studies have reported values for other metabolites in pregnant ewes' plasma. This study found triglyceride levels slightly lower than either reference values (Aitken, 2007) or for ewes at day 140 gestation (Tanaka et al., 2008). The latter study showed no effect of a global restriction diet on glucose, triglycerides, or total protein, although lactate was significantly lower in the group fed around 50% than an ad libitum group. Lactate concentrations in this current study (3.3 – 6.09 mmol.l⁻¹) were above reference values (1.0 – 3.0; Aitken, 2007), but not significantly different between groups until day 65 where they were higher in LPE ewes compared with CP ewes. In a previous study comparing *ad libitum* feeding to a diet 50% less in calorie intake, lactate concentrations were reduced in the lower calorie intake group (2.0 ± 0.4 vs. 3.2 ± 0.4 mmol.l⁻¹; Tanaka et al., 2008).

Conflicting results between studies make the identification of what are normal concentrations during pregnancy somewhat difficult. For example, one study reported no difference in glucose concentrations between pregnant and non-pregnant ewes (Alonso et al., 1997) whereas another reported that glucose concentrations were significantly lower in pregnant ewes (Lindsay et al., 2005). However, in nutritional studies where ewe weight during pregnancy may be affected by reductions in nutrient intake, the blood metabolites such as glucose, triglycerides, lactate, and total proteins remain largely unaffected (McMullen et al., 2005; Tanaka et al., 2008; Sosa et al., 2009). In a study of adolescent sheep which had previously been over-nourished during early pregnancy (Redmer et al.,

2012) subsequent under-nutrition during late gestation had no significant effect on metabolites, with the exception of NEFAs, which were raised in the low dietary intake group, a finding that was replicated in a study considering a global nutrient restriction in mid-gestation (McMullen et al., 2005), and in this current study.

Surprisingly, the total protein content in maternal plasma was higher in the ewes fed less protein in their diets. In addition, the levels of total protein and albumin in the ewes in this study were far lower than those reported in other studies (Dubreuil et al., 2005; Tanaka et al., 2008; Khatun et al., 2011), although the latter two reported values far in excess of the reference range for sheep (Aitken, 2007) and should therefore be viewed with some caution. One study of young Merino sheep (Lepherd et al., 2009) calculated a lower reference range for total protein (51 – 64 vs 60 – 79 g.l⁻¹; Aitken, 2007), suggesting that there may be species or age variation; however most of the values in this study still fell below this range. Blood analyses are highly dependent upon the conditions during specimen collection (Braun et al., 2010), although these samples were collected in optimal circumstances and there is no reason to doubt their validity.

Physiological adaptations occur during pregnancy to allow the cardiovascular system of the mother to adapt to increased metabolic needs to ensure delivery of oxygenated blood to the fetus and peripheral tissues (Silversides and Colman, 2007). A rise in cardiac output occurs, alongside a decrease in mean arterial pressure and a rise in plasma volume expansion (Longo, 1983; Chapman et al., 1998). The latter occurs primarily in response to oestrogen which in turn stimulates the renin-angiotensin system (Longo, 1983). In humans, plasma volume increases from around 6 weeks (0.15) of gestation (Chapman et al., 1998; Bernstein et al., 2001) up to 34 weeks (0.85 gestation), where it plateaus out (Pirani and Campbell, 1973). These changes are also observed in other mammals, including rats (Atherton et al., 1982) sheep (Magness et al., 1993), and baboons (Phippard et al., 1986).

Although red blood cell mass also increases during gestation (Taylor and Lind, 1979), this is in a lesser proportion to the plasma volume expansion; hence haemodilution occurs (Silversides and Colman, 2007). Plasma volume expansion in human pregnancies has been shown to correlate with birth weight (Pirani and Campbell, 1973), and this expansion is reduced in women with pre-eclampsia or fetal growth restriction (Salas et al., 1993; Salas et al., 2006), suggesting that plasma volume expansion is needed to provide an optimum uterine environment for the fetus. In this study total plasma volume was not measured, however, the concentration of plasma metabolites in LPE together with increased osmolality suggests a lack of appropriate plasma volume expansion in the LPE group. In rats, a low protein diet has been demonstrated to result in significantly reduced plasma volume expansion compared to control animals (Rosso and Streeter, 1979).

In the fetal environment, the changes observed in the ewe were not translated in all cases; fetal plasma glucose concentration was similar between diet groups. The increase in lactate observed in the LPE ewes (36% higher) was blunted in the fetuses (a 13% increase), suggesting that the placenta has a primary role in modifying the nutritional provision to the developing fetus and further reiterating that maternal nutrition does not necessarily equate to fetal nutrition.

Glucose, lactate and amino acids are the primary sources of energy for fetal oxidative metabolism and growth (Harding, 2001; Bell and Ehrhardt, 2002). Fetal amino acids are generally in higher concentration than those in maternal plasma, although the ratios between the two vary both according to the amino acid and the timing of gestation (Kwon et al., 2003; Jobgen et al., 2008). Fetal plasma amino acid concentrations in other published studies of sheep (Kwon et al., 2003; Kwon et al., 2004) show some variability between the concentrations reported and those found in this study. Nevertheless, the same patterns are observed, with the most abundant amino acids being glycine, serine and

threonine, and the least abundant being aspartic acid. However, in this study lysine concentrations were far higher in the control group than in the previous two studies (this study, 702 μM ; Kwon *et al.*, 2003, 200 μM ; Kwon *et al.*, 2004, 272 μM). In the maternal plasma, the pattern of amino acids is replicated in this study and others (Kwon *et al.*, 2003; Kwon *et al.*, 2004; Ashworth *et al.*, 2011) with the highest concentrations for alanine, glycine and valine and lowest for aspartic acid, although the amounts reported also varied between studies. The only study with published values for amino acid concentrations in ovine amniotic fluid (Kwon *et al.*, 2003) showed similar patterns to those in the current study with alanine having the highest concentration and tryptophan having the lowest.

Studies investigating the effects of maternal under-nutrition on maternal and fetal amino acid concentrations have shown differing results. In a sheep model of a maternal 50% global nutrient restriction from day 28 – 135 gestation most amino acids were reduced in both ewes and fetuses in the restricted group (Kwon *et al.*, 2004). However in sheep fed the same diet from day 28 – 78 only nine of the amino acids were lower in the ewes and none were significantly different between the control and nutrient restricted fetuses (Jobgen *et al.*, 2008). This may reflect differences between breeds of sheep, as one of the studies used Colombian/Suffolk crosses (Kwon *et al.*, 2004) and the other used Baggs ewes (Jobgen *et al.*, 2008); or it may be that the longer duration of nutrient restriction in the Kwon study had more effect. However, a further study looking at the effect of a maternal diet with a 25% reduction in energy requirements in two different breeds of sheep found that the ewe breed had a greater effect on amino acid concentrations than the restricted diet (Ashworth *et al.*, 2011).

Taken together, the metabolite and amino acid data suggest that there is no consistent pattern of change in nutritional substrate that may explain the differences in microvascular development of the fetal kidney. However, the concentration of ornithine was lower in

both fetal plasma and amniotic fluid and may well reflect the significant reduction in maternal and fetal plasma urea, since ornithine is derived from urea metabolism and urea can freely cross the feto-placental unit. Nitrogen intake is highly correlated with plasma urea concentration in sheep (Nolan and Leng, 1970), and a low protein diet in sheep has been shown to lead to lower plasma urea in ewes (Rabinowitz et al., 1973). In all mammals nitrogenous waste is excreted in order to prevent accumulation and toxicity (Solomon et al., 2004). During metabolism of amino acids the amino group is deaminated then converted to ammonia, and the liver converts the ammonia to urea, which is less toxic and easily excreted in the urine (Solomon et al., 2004). Urea is synthesised by the liver in larger amounts than is excreted in the urine, as some of the urea enters the digestive tract and is reabsorbed or utilised by microbes as a source of nitrogen to enable the synthesis of protein (Sarraseca et al., 1998). This occurs in all mammals including monogastric species such as humans (Jackson, 1995), pigs (Torrallardona et al., 1994) and rats (Torrallardona et al., 1996); however in ruminants, the large number of microbes in the forestomachs enhances this effect (Sarraseca et al., 1998).

Other nutritional studies in humans (Meakins and Jackson, 1996) and sheep (Whitelaw et al., 1990) have shown that a reduction in protein intake leads to urea-nitrogen production exceeding nitrogen intake and this is effected through mobilisation of protein stores and a negative nitrogen retention. In a study of sheep fed either a control diet or low protein diet, although nitrogen intake was nearly 60% lower in the low protein group, the effect on microbial protein synthesis was negligible (Tebot et al., 2002), suggesting that urea is recycled to meet the needs of the animal rather than being excreted. During pregnancy there is an increased need for protein to meet the needs of the placenta and fetus, however, this can be met from the mother's own nutrient reserves in lean and fat tissue in addition to that provided by the diet (Duggleby and Jackson, 2002), therefore it is to be

expected that a reduction in dietary protein may lead to greater utilisation of these reserves as a compensatory mechanism.

To conclude, this second study has confirmed the findings from study 1, i.e. a maternal low protein diet leads to a reduction in angiogenic factors, such as VEGFA. This has an impact on blood vessel development, but at the micro- rather than macro-vascular level, as evidenced by CD34+ staining of endothelial cells in the peritubular and glomerular capillaries. In addition, despite few changes in amino acid concentrations, a pathway from lower urea to reduced ornithine concentrations in the LPE group compared with controls has been identified.

6. Final Discussion

This thesis has described the effect of mild PEM on the ewe and her fetus, and how the fetus is particularly sensitive to PEM during early but not late gestation. In addition it has described how PEM blunts microvascular, but not macrovascular development of the fetal kidney and how the effects of PEM carry through to a structurally and functionally compromised adult kidney when challenged by obesity.

The potential implications of the work are relevant for those individuals born to mothers with varying degrees of PEM which may retain residual, asymptomatic effects of the maternal nutritional imbalance within their kidneys. These effects may manifest later in life as an early onset of CKD, especially when coupled with the ever-increasing likelihood of having to adjust metabolically to an obesogenic adult environment.

In this study of PEM in sheep, despite a 50% decrease in the availability of protein (8.7 vs. 16.9g·kg⁻¹·MJ) fed to pregnant ewes, there were few effects of the diet on maternal weight, her body condition score or on fetal body or organ weights at 0.44 gestation or at term, suggesting the overall dietary regimen to be modest. As described in chapter 4 this differs from rodent studies where a similar degree of protein restriction has resulted in disproportionate fetal growth (Langley-Evans et al., 1996), low birth weight (Zeman, 1967, Desai et al., 1996, Levy and Jackson, 1993) and a 20-50% deficit in nephron endowment in the kidney (Hoppe et al., 2006, McMullen et al., 2004, Vehaskari et al., 2001). In this study only a small (~15%) reduction in nephron number was observed in LPE animals, although this was coupled with a larger (~45%) compensatory increase in mean glomerular volume. The likely explanation is first due to allometry: larger mammals like sheep and humans have a lower surface area:volume ratio and a greater metabolic reserve capacity compared to smaller mammals and are therefore more able to adjust metabolically to nutritional deficit. Secondly, as sheep usually carry singletons or twins the anabolic demands required to

support the utero-placental unit are much lower relative to rodents with large litters (McCance and Widdowson, 1986). Hence, nutritional paradigms in laboratory animals are likely to present a more severe phenotype, due to the relatively greater impact of malnutrition.

Although few consistent effects on metabolites or amino acid concentrations were observed in this study, one key metabolic cycle appeared to be affected: the urea cycle. Previous studies have shown a link between protein intake and urea production in sheep (Payne and Morris, 1969, Rabinowitz et al., 1973) and that is also reflected in this current study. In the pregnant ewes in this study, urea was decreased in both the maternal and fetal compartments in the LPE group compared with controls, probably due to the ingested protein being utilised to meet the needs of the developing fetus as well as those of the ewe. In addition, another important intermediate the urea cycle, ornithine, was also reduced in the LPE fetuses compared with controls, in both amniotic fluid and fetal plasma. As ornithine is a precursor of polyamines, which are involved in the key processes of proliferation and differentiation during fetal development (Heby, 1981, Thomas and Thomas, 2001, Childs et al., 2003), future work will investigate this more deeply.

Despite relatively few differences between the groups in terms of nutritional variables, the mild maternal PEM did have an effect on the fetal environment, with a significant impact upon the fetal kidney, an organ that is undergoing hyperplastic growth during that time. This is emphasised by the observation that for Group LPL which experienced a longer period of maternal protein restriction (day 65 – term) spanning almost the entire period of hypertrophic growth of the fetus, there was no consistent renal phenotype. This study is the first to demonstrate a renal phenotype in large animals after changes to the macronutrient composition of the maternal diet and it suggests that the protein-energy ratio of the maternal diet (*i.e. diet quality*) is more important in terms of developmental

programming than a balanced reduction in nutrient intake. The lack of effect of a global nutrient restriction on kidney development is also reported in other species (Brennan et al., 2005, Braddick et al., 2011). This clearly has in the context of the marked changes in nutritional quality and macronutrient balance that have occurred over the last 50 years, in the UK (Prynne et al., 1999) but also elsewhere.

In a rodent study (Welham et al., 2002), a potential mechanistic pathway to explain loss of nephrons with maternal protein restriction was suggested: increased apoptosis of mesenchymal cells (either nephron progenitor or interstitial supportive cells). However, the determining factor remained elusive. Here, these findings are for the first time replicated in a large animal model and demonstrate a potential mechanism in which fetal kidneys exposed to a maternal low protein diet had increased apoptosis and decreased angiogenesis in the nephrogenic zone. Whilst previous work has shown how one or other of these pathways may be influenced by maternal nutrition (Welham et al., 2002, Cox et al., 2006) this study shows a consistent effect on both. Taken together, the data suggest that maternal PEM specifically limits fetal intra-renal vascularity, resulting in blunted nephrogenesis and restricted nephron endowment of the permanent kidney. These effects are asymptomatic until revealed later in life when the adult sheep is exposed to an obesogenic environment and renal dysfunction becomes apparent.

The extent of the renal dysfunction in LPE, demonstrated through microalbuminuria and blunted intra-renal transit of a radionuclide, is subtle but, considering the age of the animals (young adults), is significant. Microalbuminuria is a good early predictor of later renal disease (Viberti et al., 1982, Mogensen et al., 1995) and the likelihood is that this programmed phenotype would deteriorate with age as GFR declines with age, even in a normal population (Coresh et al., 2003). However, without aged sheep this is only an assumption, although a programming study of humans exposed to famine in the early

stages of pregnancy also revealed microalbuminuria in middle age (Painter et al., 2005). In this current study, microalbuminuria in LPE animals was an indicator of reduced renal barrier function. This is partly due to single-nephron hyperfiltration which occurs with obesity (Williams et al., 2007) but may also have been exacerbated in this study by reduced nephron number, increased intra-renal pressure, and microvascular rarefaction. Microvascular rarefaction in LPE animals compared with controls occurred during adulthood, and to a lesser extent during fetal development, suggesting that although microvascular development is blunted by the LPE diet during development, it is exacerbated by other factors in the adult such as obesity (Frisbee, 2005, Pasarica et al., 2009). With no clear difference in gross renal morphology, inflammatory infiltration or indices of intra-renal stress, the functional deficits in LPE (both males and females) suggest Minimal Change Disease, perhaps due to podocyte effacement (Haraldsson et al., 2008, Veron et al., 2010). However, lack of appropriately fixed tissue for transmission electron microscopy precludes this possibility being explored. Nevertheless, the molecular and immunohistochemical data for the adult animals (increased VEGFA) suggest greater support for a compromised renal barrier: VEGFA is highly expressed in podocytes, plays an important role in establishing and maintaining the glomerular barrier (Eremina *et al.*, 2008) and when overexpressed, is associated with glomerular disease in mice (Veron et al., 2010) and impaired angiogenesis in rats (Kang et al., 2001).

Finally, in the first fetal study, many aspects of the adverse renal phenotype described appeared to be sex-specific, that is the effect was greater in males than females (e.g. the reduction in angiogenic factors in the male LPE fetal kidney). A sex effect in the experimental endpoints of studies examining relationships between maternal diet and offspring phenotype (males being adversely affected more often than females) is not uncommon (Grigore et al., 2008). These effects are unlikely to be due to differences in the plasma concentration of sex-hormones (very low in the fetus, and the male adult offspring

were castrates) but could be related to growth since male fetuses and offspring grow at a faster rate than females (seen at day 65 gestation and in fractional growth rate postnatally). Faster growing animals may be more susceptible to deficits and/or changes in the pattern of substrate supply, in particular of amino acids. Alternatively, the effect may be a product of nutritionally-induced sex-specific epigenetic programming – a paradigm recently described in bovine blastocysts (Bermejo-Alvarez et al., 2010) but also in adult sheep (Sinclair et al., 2007).

However, in this current study at two years of age, the sex-specific programming of the kidney was less evident. The study was powered to reveal sex-specific differences in renal function (e.g. transit-time) as the primary outcome. Thus, if any clear sex-specific differences in renal function and molecular end-points were to exist they would have been revealed with the study design. The second fetal study indicated there were no sex-specific effects, and this taken in conjunction with the adult data would seem to suggest that the sex-specific effects revealed in the first fetal study were the result of a type I error.

Nevertheless, an adult renal phenotype has been described that indicates a specific effect of maternal low protein acting during early development of the fetal kidney. A suggestion for a potential mechanism at this time is that it is mediated through the key nexus for control of angiogenesis, VEGF.

Future work will aim to determine the nature of the relationship between maternal PEM, the fetal nutritional environment and organ growth by studying other organs, such as the liver, to see whether the effects seen in the kidney are common to other developing organs. In addition, as ornithine was identified as the key amino acid reduced by the low protein diet, this warrants further investigation. Aside from its role in the urea cycle, ornithine is also a precursor for the synthesis of the polyamines putrescine, spermine and spermidine (Wu and Morris, 1998). Polyamines have a variety of roles, including the

regulation of cell proliferation (Porter and Bergeron, 1983) and differentiation (Heby, 1981), and therefore if levels of polyamines were reduced in LPE animals compared to controls this may be another mechanism which affects fetal development. Thus levels of polyamines in amniotic fluid and fetal liver will be measured.

To conclude, PEM is highly prevalent in developing countries (WHO, 2011), and also to some degree in women in westernised countries who suffer from pregnancy-induced nausea (75-90% of women) and who may as a result experience some degree of macro/micronutrient deficiency (Fejzo et al., 2009). This current study has shown that PEM at this time may specifically affect the developing fetal kidney by limiting renal vascularisation and nephrogenesis. This may have functional consequences later in life, exacerbating the age-related decline in renal function.

In the scientific literature there are no reports of a new nephron forming in its entirety in a mammalian adult kidney. As the adult kidney has such limited opportunity for repair compared with other organs, it is vital to understand those processes which adversely affect kidney development. With the prevalence of renal morbidity increasing by ~5% per annum, largely through the increased prevalence of obesity and type II diabetes (Lysaght, 2002), a clinical focus on a good, balanced and high quality maternal diet during pregnancy as a potentially modifiable risk factor may help to mitigate a proportion of the expected cases of CKD in the future.

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8. Appendix A: Lloyd *et al.*, 2012

Lloyd L. J., Foster T., Rhodes P., Rhind S. M. & Gardner D. S. 2012. Protein-energy malnutrition during early gestation in sheep blunts fetal renal vascular and nephron development and compromises adult renal function. *Journal of Physiology-London*, 590, 377-393.