

Protein-energy malnutrition during early gestation in sheep blunts fetal renal vascular and nephron development and compromises adult renal function

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Non-technical summary A poor diet during pregnancy has been linked to long-term health outcomes for the baby, such as an increased risk of diseases of the heart and kidney. We show in an experimental model that recreates a poor diet during pregnancy, i.e. a diet low in protein with adequate energy, that kidney development in the baby is affected in such a way as to reduce the potential for new blood vessels to form. This results in a greater number of important, functional kidney cells spontaneously dying. Later in life, these effects in the kidney manifest as permanently reduced kidney function, especially if the baby subsequently becomes overweight as an adult. The research reinforces advice to pregnant mothers about the importance of eating a nutritionally balanced diet during pregnancy.

Abstract A nutritionally poor maternal diet can reduce nephron endowment and pre-empt premature expression of markers for chronic renal disease in the offspring. A mechanistic pathway from variation in maternal diet through altered fetal renal development to compromised adult kidney structure and function with adult-onset obesity has not been described. We show that maternal protein-energy malnutrition in sheep blunts nephrogenic potential in the 0.44 gestation (65 days gestation, term ~147 days) fetus by increasing apoptosis and decreasing angiogenesis in the nephrogenic zone, effects that were more marked in male fetuses. As adults, the low-protein-exposed sheep had reduced glomerular number and microvascular rarefaction in their kidneys compensated for, respectively, by glomerular hypertrophy and increased angiogenic support. In this study, the long-term mild anatomical deficits in the kidney would have remained asymptomatic in the lean state, but when superimposed on the broad metabolic challenge that obesity represents then microalbuminuria and blunted bilateral renal function revealed a long-term physiological compromise, that is only predicted to worsen with age. In conclusion, maternal protein-energy malnutrition specifically impacts fetal kidney vascular development and prevents full functionality of the adult kidney being achieved; these residual deficits are predicted to significantly increase the expected incidence of chronic kidney disease in prenatally undernourished individuals especially when coupled with a Western obesogenic environment.

(Received 6 September 2011; accepted after revision 14 November 2011; first published online 21 November 2011)

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Abbreviations ACR, albumin:creatinine ratio; CKD, chronic kidney disease; PEM, protein-energy malnutrition; Tc^{99m}-DTPA, technetium⁹⁹ diethylenetriaminepentaacetic acid; VEGF, vascular endothelial growth factor.

Introduction

Chronic kidney disease (CKD) is a prevalent, age-related, non-communicable disease in Western societies (Winearls & Glasscock, 2009). Obesity (Ejerblad *et al.* 2006; Cignarelli & Lamacchia, 2007), hypertension (Bakris & Ritz, 2009) and type 2 diabetes (Crook, 2002) compromise renal functional reserve and increase the risk of developing CKD. In 2005, 396 million people were obese (Kelly *et al.* 2008); in 2008, 28–35% of the US and UK population were hypertensive (Egan *et al.* 2010) and with each predicted to significantly increase with time then the global burden of CKD is a major public health issue. Indeed, stage 5 CKD (i.e. end-stage renal disease) is currently increasing at 5–8% per annum, globally costing \$70–\$75 billion US dollars per annum for dialysis (Lysaght, 2002).

Obesity, hypertension and CKD are conditions virtually absent in hunter–gatherer communities whose diet is largely grain-based, alkaline, low in sodium and thus raw, natural and unrefined (i.e. a Palaeolithic diet) (Eaton & Eaton, 2000), unlike the diet commonly consumed in Westernised societies (Cordain *et al.* 2005). Dietary change (from a Western toward a Palaeolithic diet), therefore, is probably the most achievable and potentially important preventative factor that could mitigate the inexorable rise in non-communicable disease (Daar *et al.* 2007; Narayan *et al.* 2010). Increased focus on diet during a woman's (or man's; Carone *et al.* 2010; Ng *et al.* 2010) reproductive years, particularly during pregnancy and lactation, has been stimulated by the Developmental Origins of Health and Disease hypothesis (Barker & Osmond, 1986; Gluckman *et al.* 2008) where individual variability in fetal response to maternal malnutrition (reflected as disproportionate growth (Barker *et al.* 2005) and/or low birth weight (Barker & Osmond, 1988)) increases risk of those individuals developing hypertension (Huang *et al.* 2010), coronary dysfunction (Crispi *et al.* 2010), type 2 diabetes (Whincup *et al.* 2008) and kidney disease (Woods *et al.* 2004; Amann *et al.* 2006) later in life. Increased focus on the latter is particularly pertinent as nephrogenesis is complete by term in man and non-litter-bearing mammals (Wintour & Moritz, 1997) and is therefore particularly sensitive and vulnerable to maternal malnutrition. For example, a maternal low-protein, high-glucose or high-fat diet may reduce nephron endowment (Vehaskari *et al.* 2001; Nehiri *et al.* 2008; Tran *et al.* 2008). Low birth weight infants have reduced nephron number (Manalich *et al.* 2000), increased glomerular volume (Hoy *et al.* 2005), increased blood pressure (Brenner *et al.* 1988; Mackenzie & Brenner, 1995; Keller *et al.* 2003) and are prone to minimal change nephrotic syndrome (Teeninga *et al.* 2008). In a retrospective analysis of autopsies, individuals with chronic hypertension had significantly fewer nephrons than the normotensive control group (Keller *et al.* 2003).

Furthermore, more direct evidence has been collected from a cohort of Dutch individuals exposed to famine, as fetuses, during World War II when mean maternal energy intake reduced from 6.3 to 3.2 MJ day⁻¹. As middle-aged adults, those individuals exposed to famine during early gestation *vs.* those unexposed, had evidence of programmed changes to blood pressure and an increased incidence of coronary heart disease (Roseboom *et al.* 1999), but also indices of early-stage renal disease such as microalbuminuria (urinary albumin:creatinine ratio, ACR ≥ 2.5 , 12% *vs.* 7% of sampled cohort; adjusted odds ratio = 3.2, 95% confidence interval 1.4 to 7.7). These delayed developmental effects were not related to size at birth *per se* but rather to factors marking the quality of fetal growth or maternal macronutrient balance (Roseboom *et al.* 1999).

Despite small deficits (10–20%) in nephron endowment, the mammalian kidney has such functional redundancy that azotaemia only becomes evident at CKD stages 3–5, i.e. a loss of 50–70% glomeruli with glomerular filtration rate (GFR) declining to <30 ml min⁻¹ (Reilly & Perazella, 2002). Thus, the subtle effect of maternal malnutrition on nephron endowment (the 'first-hit' or Brenners' hypothesis; Brenner *et al.* 1988) is unlikely to be solely responsible for compromised adult renal function but coupled with the totality of potentially adverse environmental effects experienced within an individual's lifetime that may affect renal function, for example higher than average salt intake, increased dietary acid load and obesity (i.e. a 'double-hit'), then healthspan can be significantly reduced in those prenatally compromised individuals (Griffin *et al.* 2008). A traditional early marker for incident CKD (i.e. before overt clinical signs 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 probably initially due to effacement of the podocyte foot processes, the integrity of which is supported by a number of factors, but primarily the key nexus for angiogenesis, vascular endothelial growth factor (VEGF) (Eremina *et al.* 2008). Obesity leads to a tissue-specific increase in VEGF, to support the concomitant local hypoxia (Wood *et al.* 2009) and, in obese sheep, has been shown to be positively associated with increased intra-renal endoplasmic stress, a potential early prognostic marker for CKD (Sharkey *et al.* 2009). The significance of developmental programming in this context is that it is pleiotrophic; that is, malnutrition during fetal development may have subtle, individual effects on nephron complement (reduced), on blood pressure (increased; Law & Shiell, 1996) and on peripheral insulin sensitivity (reduced; McMillen & Robinson, 2005) but which in an obesogenic environment may all interact in a combinatorial fashion to markedly increase any underlying susceptibility to CKD (Nehiri *et al.* 2008; Simonetti *et al.* 2008).

Epidemiological studies can highlight new and potentially important aetiologies of a multi-factorial disease like CKD, but confounding factors and their often-retrospective design can mean that the results may not be as relevant for contemporary societies, in terms of current lifestyle and diet (Huxley *et al.* 2002). Thus, animal models are essential to demonstrate the biological plausibility and mechanistic detail of any potential link between early life and later health. There is a considerable body of evidence in laboratory rodents and in larger animals, such as the sheep, that early maternal diet can influence renal structure of the adult offspring (McMillen & Robinson, 2005; Ojeda *et al.* 2008; Moritz *et al.* 2009; Puddu *et al.* 2009). Sheep are particularly relevant for such studies as the start and end periods of nephrogenesis (meso/metanephros) are virtually identical to man, ending at ~ 0.90 gestation (Wintour & Moritz, 1997; Moritz & Wintour, 1999). Prenatal undernutrition of sheep increases blood pressure and decreases nephron number of the adolescent offspring (Gilbert *et al.* 2005), while a double-hit i.e. juvenile-onset obesity superimposed upon a background of fetal undernutrition has been shown by us to result in appreciable increases in systolic and diastolic blood pressure (~ 10 mmHg) with evidence of renal dysfunction (Williams *et al.* 2007).

As yet no study has elucidated a pathway from maternal protein-energy malnutrition (PEM) through alterations to fetal kidney development (the first-hit) to deficits in renal structure and function in the adult offspring, especially in the context of an adult obesogenic environment (the second-hit). This is important with regard to those individuals that may have experienced a degree of PEM *in utero* but then later in life migrate internally to more urban, industrialised and affluent areas or for those that migrate across international borders to Westernised cultures. For example, in India, 58% of women of child-bearing age are anaemic (haemoglobin < 2 SD, normal value 120 g l^{-1}), with $\sim 20\%$ children (~ 110 – 130 million individuals) suffering PEM (WHO, 2011), whilst in the Western world, obesity has increased markedly over the last 40 years and is predicted to remain high or even increase further in the next 20 years (Wang *et al.* 2011). Hence, we have tested the hypothesis that restriction of maternal protein intake during early gestation negatively impacts fetal renal development resulting in a reduced nephron endowment which, later in life when coupled with an obesogenic environment, leaves the resultant adult with a structurally and functionally compromised kidney. Furthermore, we suggest that the developing renal vasculature is particularly susceptible to maternal protein-energy malnutrition, especially in males, and may provide for the first time a potential mechanistic pathway from variation in maternal diet through sex-specific fetal responses to the programming of renal disease in the resultant adult.

Methods

Ethical approval

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 James Hutton Institute and the University of Nottingham. The authors have read, and the experiments comply with, the policies and regulations of *The Journal of Physiology* given by Drummond (2009). The study was designed to test the primary hypothesis that a maternal low protein diet would blunt fetal renal development and therefore compromise renal structure and function in the resulting adult individual. Renal functional outcomes are subject to the most measurement error, and intra-renal transit-time represents an important variable that condenses many aspects of adult renal function. In our study we had 91% power to detect a 20% effect size for renal transit-time in the adult, with at least three male and three female animals per group (3 treatment groups), significance (α) set at 0.05 and the within- and between-animal variance of 0.005 and 0.016 min, respectively, in a split-plot design.

Animals and experimental design

Thirty-five pregnant Scottish Blackface ewes carrying singleton fetuses were randomly allocated to one of three diet groups fed either a control diet (CP; $n = 13$) providing adequate protein (180 g kg^{-1} crude protein) and energy ($10.6 \text{ MJ (kg dry mass (DM))}^{-1}$; protein:energy ratio 16.9 g kg^{-1} crude protein per MJ DM) from day 0 of gestation to term (term, ~ 147 days), or a protein-restricted diet (80 g kg^{-1} crude protein, $9.2 \text{ MJ (kg DM)}^{-1}$, protein:energy ratio 8.7 g kg^{-1} crude protein per MJ DM) during either early (LPE, days 0–65 gestation; $n = 16$) or late gestation (LPL, day 66 to term; $n = 6$). On an 'as fed basis', the diets were isocaloric with the effective level of protein restriction being 8.7 vs. $17 \text{ g crude protein MJ ME (metabolic energy)}$. At day 65 gestation, chosen to coincide with peak nephrogenesis in the sheep (Wintour & Moritz, 1997), a proportion of CP ($n = 8$) and LPE ($n = 10$) ewes were killed 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 singleton offspring were delivered naturally. After weaning (10 weeks of age) the offspring were set to pasture until 1.5 years, at which point they were transferred from The James Hutton Institute to the University of Nottingham. After a brief period of acclimatisation the sheep were exposed to an obesogenic environment, specifically designed to encourage weight gain (i.e. restricted physical activity and fed to 150% ME requirements) as previously described (Rhodes *et al.* 2009). After 6–7 months (i.e. 2 years of

age) *in vivo* renography was performed on all animals as previously described (Williams *et al.* 2007). Thereafter all sheep were subsequently killed (electrocortical stunning with exsanguination) and kidneys either snap-frozen in liquid nitrogen and stored at -80°C (right kidney) or fixed in neutral buffered formalin (NBF) for 24 h, rinsed in 0.02 M PBS for 24 h and subsequently stored in 70% ethanol at room temperature (left kidney). Urine was sampled from the bladder at post mortem for a spot assessment of the albumin:creatinine ratio.

Renography

In brief, 100 MBq technetium⁻⁹⁹ diethylenetriaminepentaacetic acid ($\text{Tc}^{99\text{m}}$ -DTPA) was injected (i.v. 0.5 ml^{-1} 0.9% NaCl) in restrained sheep and from 60 frames captured over a 20 min period a dynamic renogram (adjusted for movement artefact, background and bolus deconvolution) was produced for the left and right kidney. Specific measures of interest were time to peak (minutes), upslope (counts per minute) – indicative of rate of uptake of tracer into the kidney, downslope (counts per minute) – indicative of the rate of tracer clearance and transit-time (minutes) – a composite of all previous measures. In addition, at 3, 4 and 5 h after injection, 5 ml venous blood was drawn, centrifuged and 1 ml plasma measured for gamma emission from which GFR could be accurately determined (Gleadhill *et al.* 1999).

Renal histology and nephron number

Random samples were taken from the NBF-fixed tissues, embedded in paraffin and sectioned at $5\text{ }\mu\text{m}$ (Microtome RM2255, Leica) onto polylysine slides. Slide cassettes were coded to allow for blinded analyses. Sections were stained with haematoxylin and eosin (H&E), periodic acid Schiff's reagent (PAS) or a trichrome stain as appropriate, using standard histological protocols and mounted onto coverslips with DPX mountant. Random sections from each treatment group were first examined by a consultant histopathologist to identify any obvious pathophysiological features such as monocyte infiltration, glomerular interstitial nephritis, glomerulosclerosis or basement membrane or mesangial cell thickening. Nephron number was estimated by unbiased stereology according to the random fractionator or 'Cavalieri Principle' technique, as previously described (Keller *et al.* 2003). The mean numerical density of glomeruli (N_v) was calculated as:

$$N_{v(\text{glomeruli})} = \sum Q_{-(\text{glomeruli})} / A \times d$$

where $\sum Q_{-}$ is the total number 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 stereologically estimated cortex volume, calculated as:

$$\begin{aligned} \text{Volume fraction}_{(\text{cortex or medulla})} &= \text{number of points} \\ &\quad (\text{from grid containing 200 points}[20 \times 10]) \\ &\quad \text{hitting cortex or medulla divided by} \\ &\quad \text{number of points hitting the kidney.} \end{aligned}$$

Glomerular volume density (V_v) was calculated as:

$$\begin{aligned} V_{v(\text{glomeruli})} &= \text{no of points hitting glomeruli} / \\ &\quad \text{no of points hitting the cortex} \\ \text{Total glomerular volume} &= V_{v(\text{glomeruli})} \\ &\quad \times \text{cortex volume} \\ \text{Mean glomerular volume} &= \text{total} \\ &\quad \text{glomerular volume} / N_{v(\text{glomeruli})} \end{aligned}$$

Renal immunohistochemistry

Renal sections were analysed for abundance of vascular endothelial growth factor A (VEGFA) (SC-152; Santa Cruz Biotechnology, Santa Cruz, USA) and CD34 protein (an endothelial cell marker) (AB81289; Abcam, Cambridge, UK) using an automated BOND-MAX (Leica, UK) system and primary antibodies raised against rabbit. A biotinylated ABC kit (Vector Labs, Peterborough, UK) was used to visualise VEGFA staining in fetal tissue. Appropriate negative controls were included for the primary antibody (i.e. no primary included) and for non-specific binding of the secondary antibody (i.e. using a rabbit IgG; Vector Labs, Peterborough, UK).

Detection of apoptosis

The presence and extent of apoptotic nuclei was determined using terminal deoxynucleotidyl transferase dUTP nick-end labelling and staining (TUNEL; TdT FragEL Calbiochem, Nottingham, UK) of $5\text{ }\mu\text{m}$ sections according to the manufacturer's protocol. In brief, dewaxed sections were rehydrated and samples permeabilised with 2 mg ml^{-1} proteinase K in 10 mM Tris pH 8 and endogenous peroxidases inactivated with 3% H_2O_2 . Samples were equilibrated with $1 \times$ TdT equilibration buffer, labelled (TdT Labelling Reaction Mix) and incubated at 37°C for 1.5 h. The reaction was terminated, samples blocked with buffer and incubated with $1 \times$ conjugate for 30 min at room temperature. Apoptotic nuclei were detected after 10 min incubation with Diaminobenzidine buffer. After rinsing (dH_2O), the samples were counterstained with methyl green, rehydrated with ethanol, cleared with xylene and mounted with DPX. Omission of primary antibody was used as a negative control.

Table 1. Primer details for quantitative PCR

Primer		Sequence 5'–3'	Annealing temp (°C)
Angiopoietin 1	F	GGGTCACACTGGGACAGCAGG	58
	R	TGGGCCACAGGCATCAAACCA	
ATF4 ¹	F	AGATGACCTGGAAACCATGC	52
	R	AGGGGGAAGAGGTTGAAAGA	
ATF6 ¹	F	AACCAGTCCTTGCTGTTGCT	52
	R	CTTCTTCTGCGGGACTGAC	
Bax ¹	F	CAGGATGCATCCACCAAGAAGC	56
	R	TTGAAAGTTGCCGTGGGAAAACATT	
Beta actin ²	F	TGTGCGTGACATCAAGGAGAA	55
	R	CGCAGTGGCCATCTCCTG	
BMP 7	F	GCCACTTCCTCACCGACGCC	58
	R	CAGCTGCAGTCACGGCCTCC	
CD68 ¹	F	GTCCTGCTACCACCACAGT	52
	R	GCTGGGAACCATTACTCCAA	
Cyclophilin	F	CATACAGGTCCTGGCATCTTGTC	56
	R	TGCCATCCAACCACTCAGTCT	
DDIT3 ¹	F	AGGACCACCAGAGGTCACAC	56
	R	TGCCACTTTCCTTCGTTTT	
bFGF	F	CTTCCCGCCCGGCACTTT	58
	R	GTCGCTCTTCTCGCGGACCC	
GAPDH ²	F	TCCGTTGTGGATCTGACCTG	55
	R	TGCTTCACCACCTTCTTGATCTC	
GRP78 ¹	F	TGAAACTGTGGGAGGTGTCA	52
	R	TCGAAAGTTCGAGAGGTG	
iNOS	F	TGATGCAGAAGGCCATGTCA	52
	R	TCTCCCTGTCTCTGTTGCAAAG	
MCP-1	F	CAAGACCATCCTGGGCAAA	52
	R	GTCCTGGACCCATTTACAGGT	
PDGF	F	CAAGACGCGCACGGAGGTGT	58
	R	GCGGTTGTTGCAGCAGCCAG	
SPARC	F	CGAGGTACCCGTGGGAGCCA	58
	R	CAAACGCGCGATGGGGGCA	
TGF β 1	F	GGAGTTGTGCGGCAGTGGCT	58
	R	AAGGGCCGGTTCATGCCGTG	
Thrombospondin	F	TGCTGTGCGGGCGGAGAAAG	58
	R	TGCCCGCCTTGCCATTGGAG	
Tie-2	F	CAGCAGACCTCGGAGGCAGGA	58
	R	TGCCCTCTCAGCTGCAGCATG	
VEGFA ³	F	GGATGTCTACCAGCGCAGC	56
	R	TCTGGGTACTCCTGGAAGATGTC	
VEGFR1 ³	F	TGGATTTCAGGTGAGCTTGGA	52
	R	TCACCGTGCAAGACAGCTTC	
VEGFR2 ³	F	CTCCAGTGGGCTGATGACC	52
	R	GCAACAAACGGCTTTTCATGT	

All primers 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; MCP-1, monocyte chemotactic protein 1; PDGF, platelet-derived growth factor; SPARC, secreted protein, acidic, cysteine-rich; TGF β -1, transforming growth factor β -1; VEGF, vascular endothelial growth factor.

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). Primers (Eurofins MWG, Ebersberg, Germany) were designed using NCBI Primer-BLAST or were as previously published (Table 1). Two micrograms of cDNA were added

to each reaction, and qPCR 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 was confirmed as a suitable housekeeping gene. mRNA quantities were normalised to cyclophilin using Roche Lightcycler 480 advanced relative quantification software.

Statistical analysis

All data 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, SED or 95% CI as indicated, to represent the measurement error. $P \leq 0.050$ was accepted as indicating statistical significance. A multivariate linear discriminant analysis was used to display multiple related outcome

measures (e.g. renography derived values for renal function). The linear discriminant analysis generates two linear vectors from all normalised data (i.e. z-scores for individual data) that capture <95% variation for all inputted variables and plots these as values on a 2D plot. The method is an objective means to effectively demonstrate statistically significant patterns in complex, non-independent datasets.

Results

Maternal and fetal body composition and growth

Maternal weight (64.0 ± 4.4 kg (mean \pm 1 SD) and body condition score (2.6 [2.58–2.68] units, median [IQR]) were similar at conception and the increase in weight with gestation and decrease in body condition score over late gestation were also similar between groups (Fig. 1A and B). At 65 days gestation, maternal (63.8 vs. 58.1 ng ml⁻¹; CP vs. LP) and fetal (64.7 vs. 66.1 ng ml⁻¹; CP vs. LP) plasma

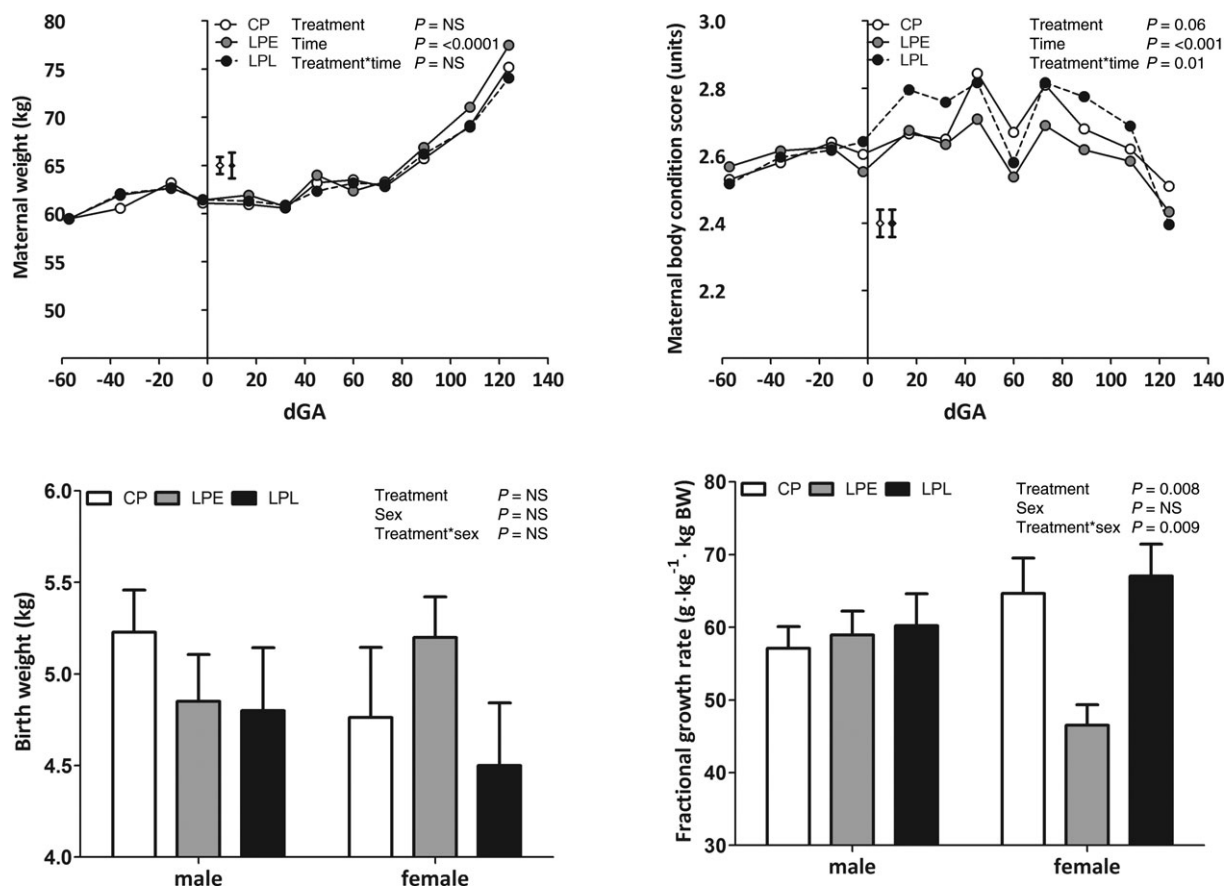


Figure 1. The effect of a maternal low protein diet on maternal and neonatal characteristics

Data are means \pm SEM: for ewes; control protein (CP, $n = 8$), low protein early (LPE, days 0–65 gestation, $n = 10$) and low protein late (LPL, days 65 to term, $n = 10$) and for offspring; CP, 4 males and 4 females; LPE, 4 males and 6 females; LPL and 5 males and 5 females. Data were analysed by General Linear Model for the fixed effects of treatment and time (maternal data) plus gender (offspring data) with testing for suitable interactions (Genstat v13, VSNi, UK). Maternal weight and body condition were recorded at intervals before and during gestation and birth weight within 12 h of birth. BW, birth weight; dGA, days gestation. In A and B, errors bars on the two symbols indicate variation associated with time (open) and treatment (filled), respectively.

Table 2. Effect of maternal low protein diet on maternal and fetal weights at 65 days gestation

		Sex		P value		
	Treatment	Male	Female	Treatment	Sex	Treat*Sex
Maternal						
Liver (g)	CP	–	730 ± 25	NS	NS	NS
	LP	–	730 ± 35			
Fetal						
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	0.04	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	0.09	NS
	LP	1.66 ± 0.16	1.50 ± 0.07			

Data are estimated marginal means ± SEM 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 one-way ANOVA 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 $P < 0.05$, but $P = 0.06$ – 0.10 taken to indicate an effect falling close to the arbitrary significance boundary. NS, not significant. CP, control protein diet; LP, low protein diet.

cortisol were not different and after post mortem only sex of the fetus, not maternal diet, had an effect on fetal body and organ weights (male > female; Table 2). Birth weights and neonatal fractional growth rate of singleton male and female lambs were not different between diet groups, with the exception of female lambs exposed to maternal low protein during early gestation (LPE), who grew at a lower daily rate (Fig. 1C and D).

Fetal kidney angiogenesis and apoptosis at 0.44 gestation

The quantity of immunopositive cells undergoing apoptosis was significantly higher (Fig. 2A and B) and positively stained for VEGFA protein significantly ($P = 0.02$) lower in the nephrogenic zone of the LPE fetal kidney vs. controls (Fig. 2C and D). In addition, at this time transcript expression for angiogenic markers including VEGFA, angiopoietin 1 (ANG1), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factor beta 1 (TGFβ1) and the Tie-2 receptor were all significantly decreased in male LPE fetuses only ($P < 0.01$, treatment × sex interaction for all cases; Fig. 2E–J). In addition, transcript expression of VEGFR1 showed a strong trend ($P = 0.052$) towards reduced expression in the LPE group in both sexes, relative to controls (17.8 ± 1.2 vs. $22.3 \pm 1.36 \times 10^{-3}$ units).

Adult renal function

At 1.5 years of age and at a similar weight (47.1 ± 0.7 kg, all animals) the offspring were reared in an obesogenic environment to facilitate weight gain and fat deposition to a level considered obese i.e. an 8–10 unit increase in theoretical BMI (weight (kg)/height (m²)) from 22 to 32 (Fig. 3A). Offspring gained similar amounts of weight in each treatment group, but females gained less than males (Fig. 3B). Upon stabilisation of weight (weeks 25–33), all offspring underwent bilateral *in vivo* renography. In contrast to fetal life, we observed few treatment × sex interactions (i.e. an effect specifically in male LPE offspring) in the adult offspring, but rather revealed with obesity a number of important effects specific to both the male and female offspring exposed to a maternal low protein diet during early gestation (group LPE): relative to controls, the upslope of the renogram in LPE was unaffected (Fig. 4A) but all other indices suggested a subtle reduction in flow-based renal function; for example, the downslope (indicating clearance of tracer) tended to be decreased in LPE (Fig. 4B), the time to peak (indicating renal uptake of tracer) tended to be increased (Fig. 4C) with renal transit-time, a composite parameter of whole organ function, being significantly increased (~20%) in LPE offspring (Fig. 4D). Measurement of glomerular filtration rate (GFR), however, indicated preservation of GFR in LPE vs. other groups in the longer term

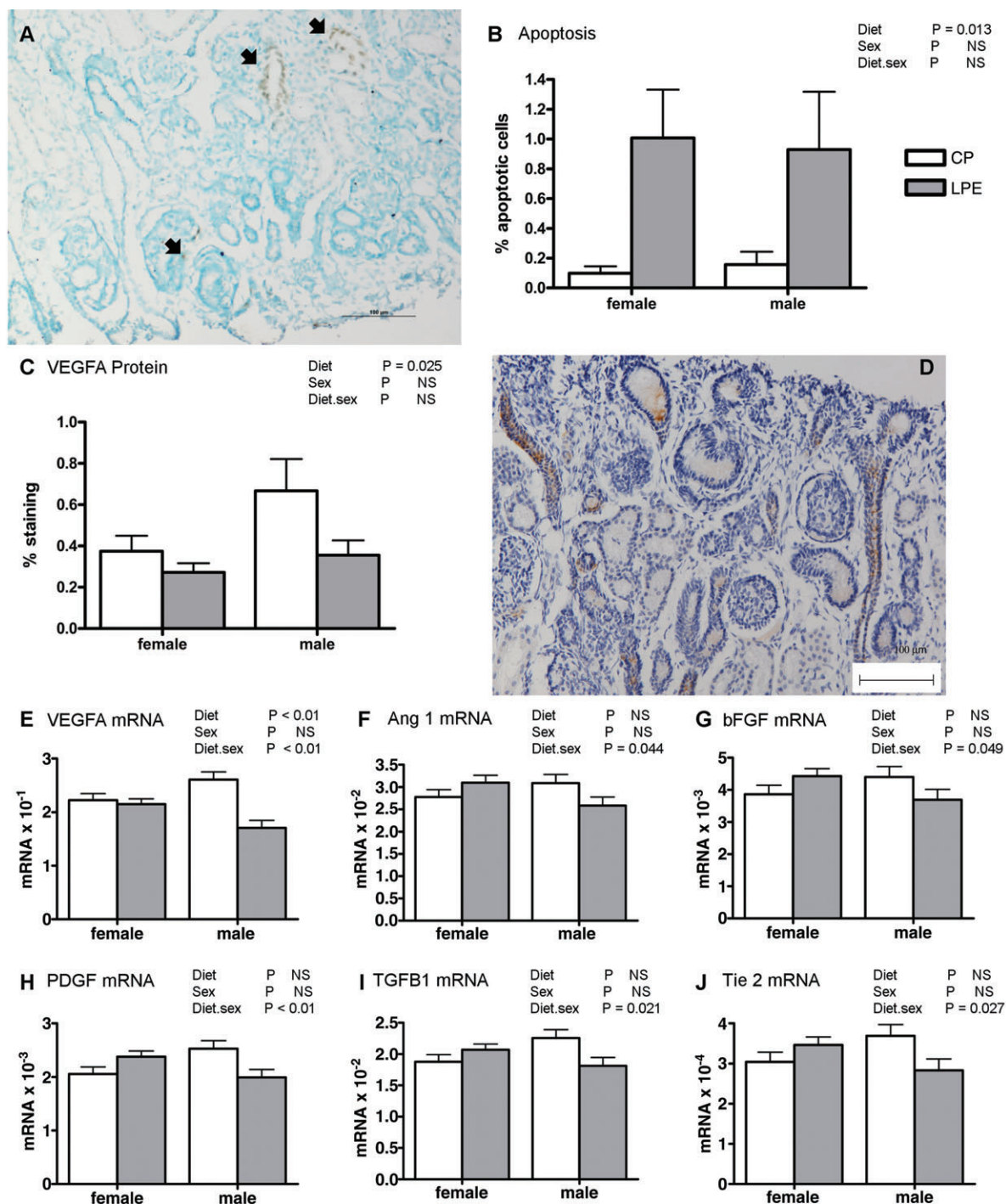


Figure 2. The effect of a low protein maternal diet on markers of angiogenesis and apoptosis in the fetal kidney

Data are mean \pm SEM where appropriate for: A, immunohistochemical staining of TUNEL +ve cells (representative micrograph from LPE group, black arrows indicate apoptotic cells); B, quantification of apoptotic data (CP, $n = 8$ (4 males, 4 females); LP, $n = 10$ (4 males, 6 females)); C, quantification of VEGFA protein abundance (CP, $n = 8$ (4 males, 4 females); LP, $n = 10$ (4 males, 6 females)); D, immunohistochemical staining of VEGFA protein abundance (representative micrograph from nephrogenic zone of LPE group, arrows indicate +ve staining); E–J, quantification of transcript expression (CP, $n = 8$ (4 males, 4 females); LP, $n = 10$ (4 males, 6 females)). Data were analysed using General Linear Model without or with \log_{10} transformation (% apoptotic cells) for the fixed effects of treatment, gender and their interaction (Genstat v13, VSNi, UK). Log transformed data are presented as back-transformed means. Scale bars in A and D, 100 μm .

(i.e. 3–5 h, Table 3). Indeed, plasma Na^+ (pooled estimate, 132 [128–143] mmol l^{-1} ; median [IQR]), plasma K^+ (pooled estimate, 8.02 [7.12–8.54] mmol l^{-1}) and plasma Cl^- (pooled estimate, 108 [103–113] mmol l^{-1}) were not different between treatment groups or sexes. However, the urinary albumin:creatinine ratio (ACR; $\text{mg } \mu\text{mol}^{-1}$) was significantly ($P = 0.03$, Wilcoxon's Rank sum test) increased in the LPE group, with no effect of gender

(LPE, 8.88 [7.9–24.7] vs. CP, 1.73 [1.71–2.67]; LPL, 2.49 [1.63–2.98] median [IQR]), suggesting reduced glomerular barrier function. Stereological analysis of renal anatomy indicated significantly reduced ($F = 3.7$, $P = 0.05$) nephron number in the adult LPE kidney *per se* (LPE, $8.52 [8.04–9.28] \times 10^6$ vs. CP, $10.6 [10.5–10.9] \times 10^6$; LPL, $10.4 [8.19–11.9] \times 10^6$; Fig. 4E). Hence, using a multivariate linear discriminant analysis that predicted <99% of the variance for all renal outcome measures for all animals (e.g. upslope, time to peak, downslope, transit-time and nephron number) within just two eigenvectors (i.e. two linear orthogonal) indicates the separation of treatment groups with respect to *in vivo* adult renal function after exposure to a maternal low protein diet (Fig. 4F).

Adult renal structure

As assessed by stereology, reduced nephron number and microalbuminuria in LPE vs. controls was accompanied by increased mean glomerular volume in this group (Table 4), without effect on overall kidney mass, volume or relative volume of the renal cortex (Table 4). Histological examination of glomeruli and proximal tubules in all treatment groups indicated no obvious morphological or structural abnormality, no consistent lymphocyte infiltration or any evidence of glomerulosclerosis in LPE or LPL vs. CP (Fig. 5A–D). However, through immunohistochemistry, we found significantly decreased ($P < 0.001$) abundance of CD34 protein (an endothelial cell marker) in LPE vs. controls (quantification in Fig. 5H, micrograph in Fig. 5I), suggesting microvascular rarefaction in this group with increased ($P = 0.02$) abundance of VEGFA (quantification in Fig. 5G, micrograph in Fig. 5E). Further analysis of transcripts for angiogenic markers revealed a consistent tendency for increased expression in LPE vs. other groups (VEGFA, $P = 0.07$; VEGFR1, $P = 0.05$; VEGFR2, $P = 0.09$) without any consistent indication of increased intra-renal endoplasmic reticulum stress (Table 5).

Discussion

In this study we have traced a pathway from mild, maternal protein-energy malnutrition (PEM) acting during early gestation to blunt the microvascular development of the fetal kidney in a sex-specific manner to a structurally- and functionally-compromised adult kidney with evidence of microvascular rarefaction in the face of an obesogenic environment. The potential implications of the work are relevant for those individuals born to mothers with varying degrees of PEM that may retain residual, asymptomatic effects of the maternal nutritional imbalance within their kidneys that manifest later in life as an earlier than expected onset of CKD, especially

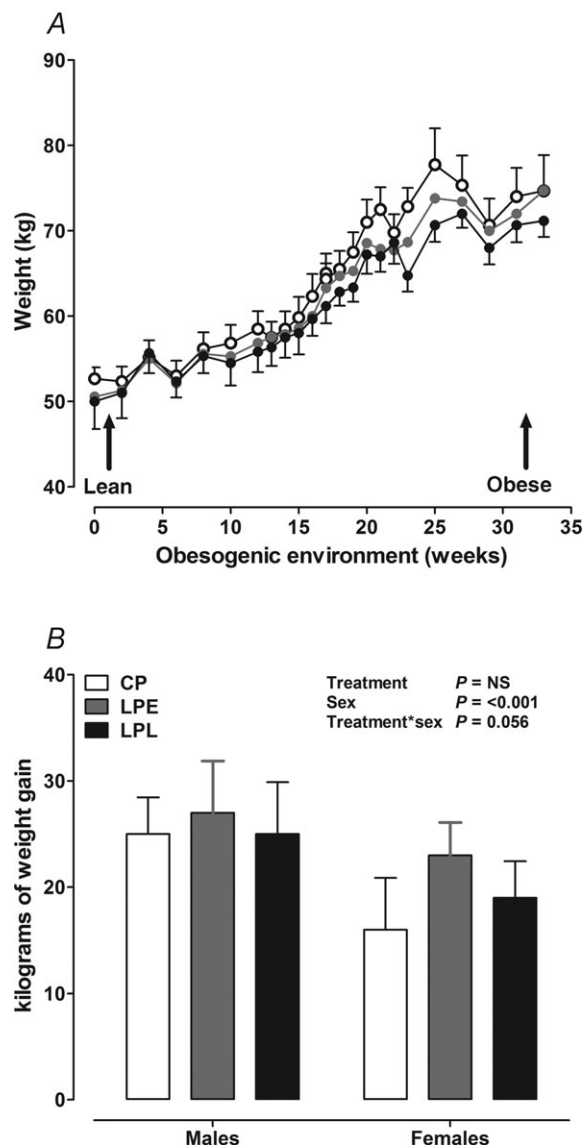


Figure 3. Weight gain during exposure to an obesogenic environment in adult sheep

Data are means \pm SEM for control protein (CP, $n = 6$), low protein early (LPE, days 0–65 gestation, $n = 7$) and low protein late (LPL, days 66 to term, $n = 6$). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. A, absolute body weight gain; B, kgs of weight gain after exposure to the obesogenic diet. Data were analysed by General Linear Model for the fixed effects of treatment, gender and time with testing for suitable interactions (Genstat v13, VSNi, UK). NS, non-significant.

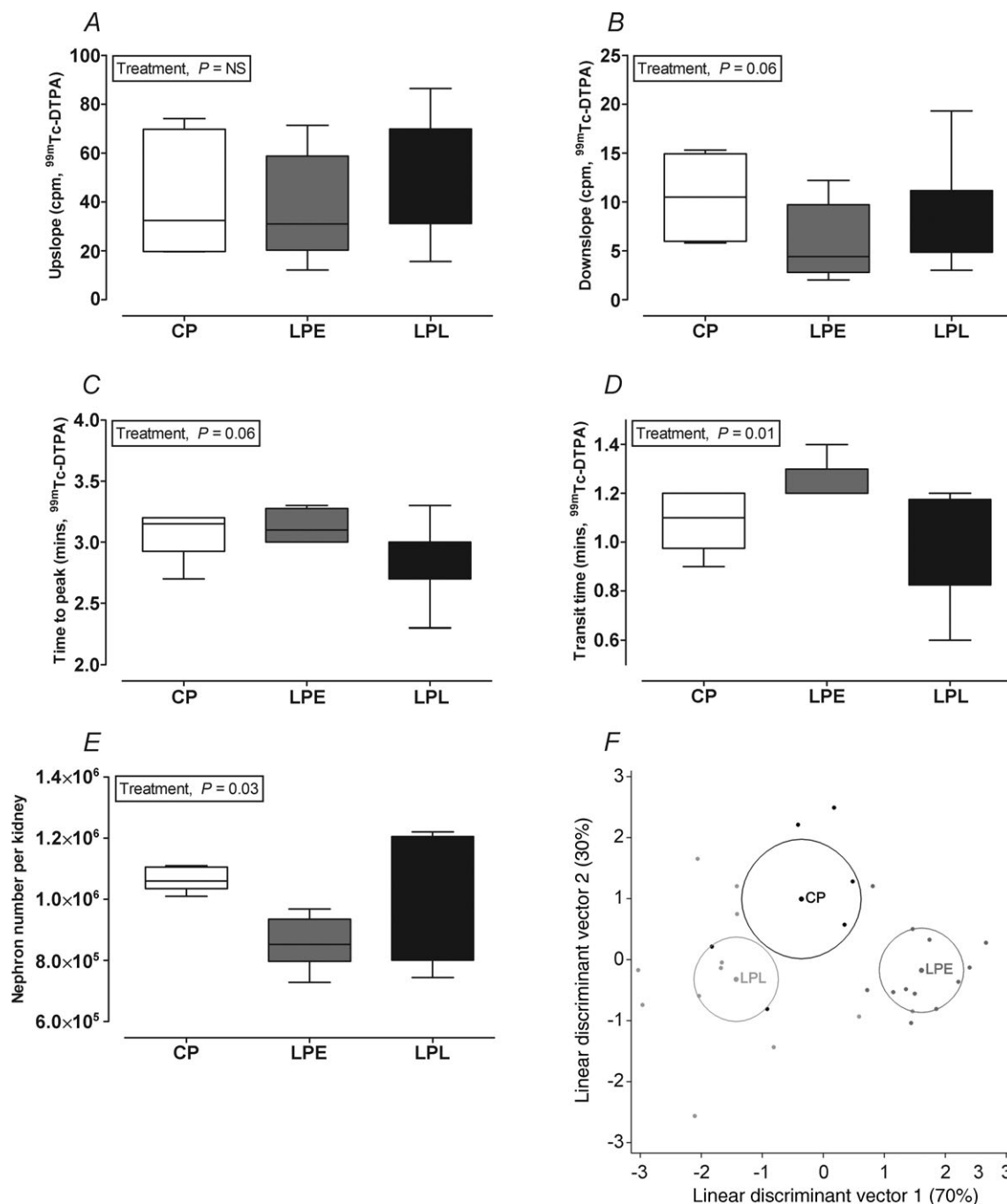


Figure 4. The effect of a low protein maternal diet on adult offspring renal function as assessed by renography

Boxplots show median (IQR with whiskers representing 5–95th centile) for control protein (CP, $n = 6$), low protein early (LPE, days 0–65 gestation, $n = 7$) and low protein late (LPL, days 66 to term, $n = 6$). There were 3 males and 3 females in CP and LPL and 3 males and 4 females in LPE. Data were analysed by Restricted Maximum Likelihood (REML) for the fixed effects of treatment and gender with interaction (Genstat v13, VSNi, UK). Renal function was assessed by renography using $\text{Tc}^{99\text{m}}$ -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 (B) 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 (C); transit time is the mean time taken for tracer to pass through the kidney (D); nephron number was estimated by unbiased stereology (E) and F shows the mean for each treatment group (circles representing 95% confidence interval of the mean) after linear discriminant analysis of normalised (i.e. z-scores) data for upslope, time to peak, downslope, transit time, nephron number and mean glomerular volume.

Table 3. Effect of maternal low protein diet on offspring glomerular filtration rate

	Treatment	Sex		P value		
		Male	Female	Treatment	Sex	Treat*Sex
GFR (ml ml ⁻¹)	CP	146 ± 16	147 ± 23	NS	NS	NS
	LPE	114 ± 23	150 ± 14			
	LPL	133 ± 23	139 ± 16			
GFR (ml ml ⁻¹ (g kidney) ⁻¹)	CP	0.97 ± 0.14	1.12 ± 0.21	NS	NS	NS
	LPE	0.89 ± 0.21	1.32 ± 0.13			
	LPL	1.02 ± 0.21	0.98 ± 0.14			
GFR (ml ml ⁻¹ (kg lean mass) ⁻¹)	CP	3.21 ± 0.37	3.68 ± 0.53	NS	NS	NS
	LPE	3.27 ± 0.53	3.78 ± 0.33			
	LPL	2.84 ± 0.53	3.63 ± 0.37			

Glomerular filtration rate was assessed through decay of Tc^{99m}-DTPA in plasma samples collected after 3, 4 and 5 h post 100 MBq i.v. bolus dose. Ewes were killed as per standard operating procedures and the wet weight of organs recorded. Data are means ± SEM for offspring of ewes fed a control protein diet (CP, *n* = 6), or a low protein diet during early gestation (LPE, days 0–65, *n* = 7) or a low protein diet during late gestation (LPL, days 66 to term, *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; GFR, glomerular filtration rate.

Table 4. Effect of a maternal low protein diet on offspring kidney weight and stereology

	Treatment	Sex		P value		
		Male	Female	Treatment	Sex	Treat*sex
Kidney weight (g (kg BW) ⁻¹)	CP	2.63 ± 0.20	2.66 ± 0.28	NS	NS	NS
	LPE	2.72 ± 0.29	2.13 ± 0.18			
	LPL	2.44 ± 0.29	2.61 ± 0.20			
Kidney volume (cm ³)	CP	79.67 ± 5.99	67.50 ± 7.91	NS	NS	NS
	LPE	73.85 ± 7.92	59.20 ± 5.01			
	LPL	73.00 ± 7.92	70.50 ± 5.60			
Per cent cortex volume (%)	CP	61.17 ± 3.37	67.62 ± 5.26	NS	NS	NS
	LPE	70.01 ± 5.45	67.14 ± 3.31			
	LPL	68.60 ± 5.34	67.14 ± 3.70			
Cortex volume (cm ³)	CP	48.29 ± 4.00	44.82 ± 5.65	NS	NS	NS
	LPE	51.55 ± 5.65	39.82 ± 3.57			
	LPL	50.49 ± 5.65	47.32 ± 3.99			
Total glomerular volume (cm ³)	CP	1.96 ± 0.17	1.79 ± 0.23	NS	0.011	NS
	LPE	2.39 ± 0.23	1.70 ± 0.15			
	LPL	2.04 ± 0.23	1.44 ± 0.17			
Mean glomerular volume (mm ³ × 10 ⁻³)	CP	1.81 ± 0.17	2.03 ± 0.24	0.022	NS	NS
	LPE	2.61 ± 0.24	2.06 ± 0.15			
	LPL	1.93 ± 0.24	1.47 ± 0.17			

Data are means ± SEM for offspring of ewes fed a control protein diet (CP, *n* = 6), or a low protein diet during early gestation (LPE, days 0–65, *n* = 7) or a low protein diet during late gestation (LPL, days 66 to term, *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.

when coupled with the ever-increasing likelihood of having to metabolically compensate to an obesogenic adult environment.

In this study of PEM in sheep, despite a 50% decrease in the availability of protein (8.7 vs. 16.9 g kg⁻¹ MJ) fed to pregnant ewes we observed few effects of the diet on

maternal weight or her body condition score (a subjective marker of overall fat mass) or on fetal body or organ weights at 0.44 gestation or at term (e.g. Fig. 1, Table 1), suggesting the overall dietary regimen to be modest. This contrasts with studies in laboratory rodents in which a similar degree of protein restriction often results

in disproportionate fetal growth (Langley-Evans *et al.* 1996), low birth weight (Zeman, 1967; Levy & Jackson, 1993; Desai *et al.* 1996) and a reported 20–50% deficit in nephron endowment in the permanent kidney (Vehaskari *et al.* 2001; McMullen *et al.* 2004; Hoppe *et al.* 2006). In this study, we observe only a small, ~15% reduction in nephron number, but a large (~45%) compensatory increase in mean glomerular volume in LPE. The likely explanation is first due to allometry; larger animals like sheep and man have greater metabolic reserve capacity (and lower surface area:volume ratio) compared to smaller

mammals and are thus more able to metabolically adjust to nutritional deficit. Second, being monotocous, the anabolic demands required to support the uteroplacental unit in sheep are much lower relative to the polytocous rodent (McCance & Widdowson, 1986). Hence, nutritional protocols in laboratory rodents probably present a more severe phenotype, due to the relatively greater impact of malnutrition. Nevertheless, in this study, mild maternal protein:energy malnutrition translated to the fetal environment with significant impact upon an organ undergoing hyperplastic growth at that time e.g. the fetal

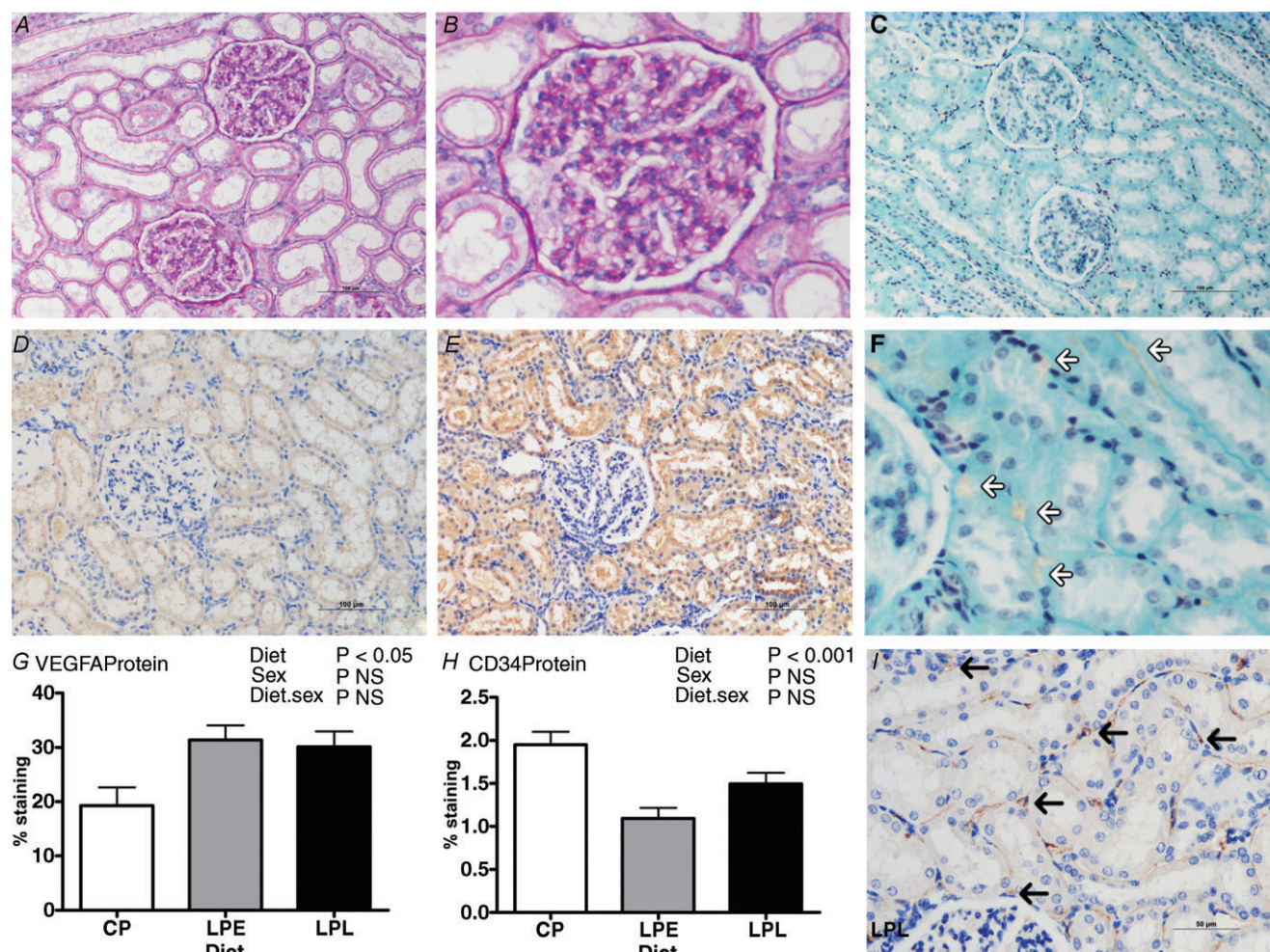


Figure 5. The effect of a low protein maternal diet on adult offspring renal structure and vascularisation as assessed by expression and abundance of markers for renal endothelial cells

Data are means \pm SEM for control protein (CP, $n = 6$), low protein early (LPE, days 0–65 gestation, $n = 7$) and low protein late (LPL, days 66 to term, $n = 6$). 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). *A* and *B*, representative histological sections from the LPE group (*A*, magnification $\times 200$; *B*, magnification $\times 400$) stained with periodic acid Schiffs reagent shows normal kidney structure; *C* and *F*, representative histological sections from the LPE group stained with trichrome; *D* and *E*, representative histological sections with immunohistochemical staining for vascular endothelial growth factor A (VEGFA) in CP (*D*) vs. LPE (*E*), and *G* shows quantification of VEGFA staining (using ImagePro MC); *H*, quantification of CD34 stained sections (using ImagePro MC) and *I*, a representative section from CP, immunostained for CD34 (black arrows indicate endothelial cells). Scale bars in *A*, *C*, *D* and *E*, 100 μm ; in *I*, 50 μm .

Table 5. Effect of a maternal low protein diet on renal endoplasmic reticulum stress genes

		Sex		P value		
	Treatment	Male	Female	Treatment	Sex	Treat*sex
ATF4	CP	5.85 ± 0.80	4.95 ± 1.13	NS	NS	NS
	LPE	5.00 ± 1.13	5.29 ± 0.72			
	LPL	4.51 ± 1.13	6.33 ± 0.80			
ATF6	CP	5.36 ± 0.98	5.86 ± 1.39	NS	NS	NS
	LPE	5.90 ± 1.39	4.73 ± 0.88			
	LPL	4.88 ± 1.39	4.83 ± 0.98			
Bax	CP	8.37 ± 0.98	9.23 ± 1.38	NS	NS	NS
	LPE	8.39 ± 1.38	8.51 ± 0.87			
	LPL	10.40 ± 1.38	8.48 ± 0.98			
CD68	CP	0.09 ± 0.03	0.11 ± 0.04	NS	NS	NS
	LPE	0.11 ± 0.04	0.18 ± 0.03			
	LPL	0.22 ± 0.04	0.17 ± 0.03			
DDIT3	CP	0.68 ± 0.16	0.46 ± 0.15	0.02	NS	NS
	LPE	0.81 ± 0.26	1.17 ± 0.24			
	LPL	0.62 ± 0.20	1.08 ± 0.25			
HSPA5	CP	2.80 ± 0.47	2.28 ± 0.54	NS	NS	NS
	LPE	2.10 ± 0.50	2.84 ± 0.43			
	LPL	2.06 ± 0.49	2.76 ± 0.47			
MCP-1	CP	1.26 ± 0.30	0.69 ± 0.43	NS	NS	NS
	LPE	1.45 ± 0.43	1.40 ± 0.27			
	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 ± SEM for offspring of ewes fed a control protein diet (CP, $n = 6$), or a low protein diet during early gestation (LPE, days 0–65, $n = 7$) or a low protein diet during late gestation (LPL, days 66 to term, $n = 6$). 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. 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; MCP-1, monocyte chemotactic protein 1.

kidney. This is emphasised by the observation that, for the LPL group, which experienced a longer period of maternal protein restriction, spanning almost the entire period of hypertrophic growth of the fetus (weights at 65 days gestation are only ~3–4% weight at term), there was no consistent renal phenotype. To our knowledge, this study is the first to demonstrate a renal phenotype in large animals after changes to the macronutrient composition of the maternal diet and 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 *per se* (i.e. *diet quantity*) (Brennan *et al.* 2005). This clearly has relevance when considered in context to the marked changes in nutritional quality that have occurred over the last 50 years, in the UK (Prynne *et al.* 1999) but also elsewhere.

The small reduction in the full complement of nephrons in LPE illustrates an important consequence of maternal protein-energy deprivation but the reported

absolute numbers are also of academic value and interest and deserve comment. Epidemiological studies have illustrated how an optimal fetal environment is a prerequisite for adequate kidney development and for good blood pressure control later in life (Manalich *et al.* 2000; Hughson *et al.* 2003; Keller *et al.* 2003). Our stereologically derived estimate of nephron number in control sheep (~1,064,611) was determined according to the methodology of Keller (Keller *et al.* 2003), but contrasts markedly with other stereologically derived estimates for sheep (e.g. from 289,000 to 420,000; Bains *et al.* 1996; Gray *et al.* 2008). Nephron number may subtly vary 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 are highly correlated with both birth weight (up to 3 kg, 250,000 glomeruli (kg BW^{-1}); $r = 0.870$, $P < 0.0001$) (Manalich *et al.* 2000) and weight-adjusted metabolic mass between species (Kunkel, 1930). Using \log_{10} data for published values of

nephron number from a mouse (20 g) to an elephant (3700 kg) generates an equation of $y = 0.613x + 3.116$, therefore accurately predicting an adult sheep (50–70 kg) to have between 994,050 and 1,221,839 nephrons per kidney. This estimate encompasses the reported values in this manuscript, but not others (Bains *et al.* 1996; Gray *et al.* 2008) and emphasises, perhaps, that prenatal effects on nephron number are best considered relative to appropriate controls rather than to species-specific values.

In rodents, Welham *et al.* (2002) were the first to suggest a potential mechanistic pathway to explain loss of nephrons. They just with maternal protein restriction – increased loss (apoptotic) of mesenchymal (either nephron progenitor or interstitial supportive) cells, but the determining factor remained elusive. Here, we replicate these findings for the first time in a large animal model and demonstrate a potential mechanism: fetal hyperplastic kidneys exposed to maternal low protein had increased apoptosis and decreased angiogenesis in the nephrogenic zone. While 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) we show a coherent effect on both. Taken together, the data suggest that maternal protein-energy malnutrition specifically limits fetal intrarenal vascularity, resulting in blunted nephrogenesis and restricted nephron endowment of the permanent kidney, effects that are asymptomatic until superimposed on an adult obesogenic environment when renal dysfunction is revealed.

The extent of the renal dysfunction in LPE (microalbuminuria and blunted intra-renal transit of radio-nuclide) is subtle, but considering the age of the animals (1.5–2 years of age of an expected ~10–12 year lifespan) is significant. Microalbuminuria is a good early predictor of later renal disease (Painter *et al.* 2005) but without aged animals, one can only make the assumption that the programmed phenotype would deteriorate with age. From epidemiological studies, those human individuals exposed to famine during early (but not late) gestation were at higher risk of developing obesity (Ravelli *et al.* 1976) with concomitant microalbuminuria in middle age (Painter *et al.* 2005). In our study, microalbuminuria in LPE, marking reduced renal barrier function, is probably due to single-nephron hyperfiltration as this occurs with obesity *per se*, as shown by us in a previous study (Williams *et al.* 2007) but also may be exacerbated in this study by (1) reduced nephron number, (2) microvascular rarefaction (marked by reduced intra-renal endothelial CD34⁺ abundance) and (3) increased intra-renal pressure (indicated by reduced Tc-^{99m} DTPA time to peak and intra-renal transit-time). With no clear difference in gross renal morphology, inflammatory infiltration or indices of intra-renal stress then the functional deficits in LPE (both males and females) suggests 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 (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 over-expressed, is associated with glomerular disease in mice (Veron *et al.* 2010).

Finally, in fetal life, many aspects of the adverse renal phenotype described were sex-specifically altered, that is, the effect was greater in males *vs.* females (witness the reduction in angiogenic factors in the male LPE fetal kidney as an example). A sex effect in the experimental endpoints of studies examining relationships between maternal diet and offspring phenotype (males adversely affected more often than females) is not uncommon (Grigore *et al.* 2008). These effects are unlikely due to differences in the plasma concentration of sex hormones (very low in the fetus, male adult offspring were castrates) but could be related to growth since males (fetuses and offspring) grow at a faster rate than females (reflected at day 65 gestation here, and in fractional growth rate post-natally). 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 phenomenon recently described in bovine blastocysts (Bermejo-Alvarez *et al.* 2010) but also in adult sheep (Sinclair *et al.* 2007). However, in this study at 2 years of age, the sex-specific programming of the kidney was less evident. Our study was powered to reveal sex-specific differences in renal function (e.g. transit time) as our primary outcome. Thus, we are confident that if any clear sex-specific differences in renal function (and molecular end-points) were to exist we would have been able to reveal them with our study design. However, for alternative outcomes in which a treatment \times sex interaction appears likely (e.g. for mean glomerular volume the effect size was +27%, male LPE *vs.* controls) but for which the measurement error increases, then we acknowledge a potential Type II error (in this example we have 85% power to detect a 27% difference at the treatment level, but only 56% at the treatment \times sex level). 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. We have described a potential mechanism, at this time, that is mediated through the key nexus for control of angiogenesis, VEGF, and that the blunting of renal development appears more marked in male fetuses. Further, mechanistic studies aimed at determining the nature of the relationship between maternal protein-energy malnutrition, the fetal

nutritional environment and renal growth (micro-vascular, nephrogenesis) in male and female fetuses is warranted.

To conclude, protein-energy malnutrition (PEM) is highly prevalent in developing countries with up to 20%, 8% and 0.8% children in India, China and the USA, respectively, being affected (equating to ~130, 18 and 1.1 million individuals; WHO, 2011). Whilst it is less prevalent in developed, Westernised societies the incidence of pregnancy-induced nausea (75–90% women) and, in extremis, hyperemesis gravidarum (1% women) during early pregnancy means that many women (and their fetuses) may experience some degree of macro/micronutrient deficiency at this time (Fejzo *et al.* 2009). Here we show that PEM at this time may specifically impact the developing, hyperplastic fetal kidney to limit renal vascularisation and nephrogenesis with functional consequences later in life that are predicted to exacerbate the age-related decline in renal function. With the prevalence of renal morbidity increasing by ~5% per annum largely through the increased prevalence of obesity and T2D then a clinical focus on a good, balanced and high-quality maternal diet 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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Author contributions

The experiments were conducted at the School of Veterinary Medicine and Science, Sutton Bonington Campus, University of Nottingham. D.S.G., P.R. and S.M.R. conceived and designed the experiments; L.J.L., D.S.G., P.R., T.F. and S.M.R. conducted research; L.J.L. and D.S.G. analysed and interpreted research and L.J.L. and D.S.G. wrote the paper. D.S.G. has primary responsibility for its final content and all authors have approved the final version of the manuscript.

Acknowledgements

The authors would like to acknowledge the contributions of: Dr Ali Mobasheri (University of Nottingham) and Dr Cindy Wong (Abcam) for the gift of the CD34 antibody, Dr Tom McCulloch (Consultant Pathologist, Nottingham University Hospitals Trust) for advice concerning renal histopathology and Dr Jim Craighan for statistical advice on the manuscript. Mrs. Louise Lloyd was supported by a British Heart Foundation PhD studentship (FS/09/011/26562).