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# Maternal nutrition can rapidly rescue a nephron deficit in low birthweight offspring

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Low birthweight is a risk factor for hypertension and chronic kidney disease. Kidneys of low birthweight babies typically have a low nephron endowment, which is permanent. Therefore, strategies to boost or rescue nephron endowment in low birthweight offspring might be expected to decrease the prevalence of these chronic conditions. We previously reported that a high-fat diet (17% protein, 43% carbohydrate, 40% fat) fed to mice before mating and until weaning boosted nephron endowment in mice by 20%. Here, we show that offspring from dams fed a normal diet during pregnancy and switched to a high-fat diet at birth had a 14% augmented nephron endowment. Additionally, transition to a high-fat diet at birth completely rescued a 20% nephron deficit induced by feeding dams a low-protein diet (8% protein, 76% carbohydrate, 16% fat) during gestation. The augmentation and rescue of nephron endowment were associated with increased maternal caloric intake on day 1, as well as increased maternal fat and reduced carbohydrate intake during the postnatal period of rapid nephrogenesis. These findings indicate that the balance between the three macronutrients in the maternal diet, both pre- and postnatally, is crucial for nephron endowment.

Human[1–4] and animal[5–10] studies have linked low nephron number with increased risk of high blood pressure and chronic kidney disease (CKD). Low birthweight, either at term or associated with premature birth, typically results in a permanent nephron deficit[1,11–13]. Therefore, strategies to optimise growth in the foetal and/or early postnatal period may increase nephron endowment, and potentially reduce the risk of these highly prevalent chronic diseases.

Animal studies have shed light on the regulation of nephrogenesis and demonstrated the possibility to augment nephron endowment through genetic or nutritional interventions. Nephrons are derived from self-renewing nephron progenitor cells that form mesenchymal 'cap' domains around tips of the ureteric epithelium. The balance between progenitor cell self-renewal and differentiation strongly influences nephron endowment and is dictated, in part, by reciprocal signalling interactions between the ureteric tip and the mesenchymal cap (the nephrogenic niche). Briefly, tip-produced signals promote nephron progenitor self-renewal and induce nephrons to form, while nephron progenitor-derived signals promote branching of the tip for further nephron induction. Differentiation of the metanephric mesenchymal caps leads to the formation of renal vesicles, which further differentiate into comma and then S-shaped bodies before final differentiation into the mature nephron. In mice, nephron formation begins around embryonic day 13-–14 (8–9 weeks in humans) and continues until all nephron progenitor cells differentiate in a final burst of nephron formation 2–3 days after birth[14]. The exponential rate of nephron formation in mice is similar to that found in humans, with most (~60%) nephrons formed between 28-36 weeks gestation[15].

Numerous genetic and environmental perturbations result in low nephron endowment[6,7,16–19], including suboptimal nutrition[20–22]. Many studies have reported that feeding a low-protein diet to female rats or mice prior to mating and throughout pregnancy and lactation results in offspring with a 20-30% nephron deficit[7,23,24]. Studies also indicate that optimising postnatal nutrition has the potential to prevent a nephron deficit, although the outcomes are inconsistent[23,25–28]. We previously reported that feeding a high-fat diet prior to pregnancy and throughout lactation augments

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nephron endowment by 20%[5], highlighting the importance of nutrition in the establishment of nephron endowment, and the potential of nutrition to rescue a possible nephron deficit.

We previously reported that feeding mice an HFD for 6 weeks prior to conception and throughout pregnancy and lactation resulted in offspring with a 20% augmented nephron endowment[5]. Given that approximately 60% of nephron formation in mice is initiated in the first few days after birth[29–31], in the present study, we aimed to determine if maternal HFD fed during this brief postnatal period only could boost nephron endowment in normal offspring and/or rescue a programmed nephron deficit in low birthweight offspring. We found that a high-fat maternal diet post-birth augmented nephron number in normal offspring by 14%, and completely rescued a 20% nephron deficit in low birthweight offspring via lactation. While the impact of low maternal protein intake on nephron endowment has long been recognised, our findings highlight the importance of dietary fat and carbohydrate consumption during the period of rapid nephrogenesis. They indicate that maternal nutrition can have marked and rapid effects on final nephron endowment. Our findings also suggest that nutritional strategies may benefit human offspring predicted to be small for gestational age or those born early when there is a window of rapid and ongoing nephrogenesis post-birth.

## Results

Cohorts of pregnant mice were fed either an ND, HFD or LPD from conception to weaning (Fig. 1). Additional cohorts were placed on ND or LPD from mating and throughout pregnancy and then switched to an HFD from birth until weaning (N/HFD and LP/HFD; Fig. 1).

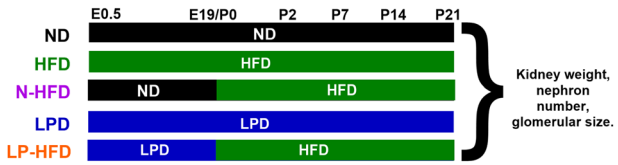


**Fig. 1 |** Schematic representation of experimental design.

### Maternal LPD programs a nephron deficit in offspring, which is present at birth

We first defined the number of PNA+ nephrons in ND, LPD and HFD offspring at birth to establish nephron number prior to the maternal diet switch. LPD offspring at birth had 10% lower body weight than ND offspring (Fig. 2a; $p = 0.0004$) and 20% fewer glomeruli (Fig. 2b, c; $p = 0.046$) but similar number of glomeruli per gram of body weight (Fig. 2d). Birthweight in HFD offspring was 5% lower than ND offspring but did not reach statistical significance ($p = 0.06$) (Fig. 2a). The number of nephrons (Fig. 1c), nephrons per gram of body weight (Fig. 2d) and nephrogenic niches (Fig. 2e, f) in HFD offspring at birth was similar to that of ND offspring.

### HFD feeding during lactation augments nephron endowment in normal offspring

Switching the maternal diet from ND to HFD at birth did not alter offspring body weight at P2 or P7 (Fig. 3a). However, body weight in N/HFD was greater than ND offspring at P14 (Fig. 3a; $p = 0.008$) and P21 (Fig. 3a; $p = 0.0005$). Kidney weight in N/HFD offspring was similar to ND offspring at P21 (Fig. 3b). Switching from ND to HFD at birth augmented nephron endowment by 14% (Fig. 3c; ND 13,100 ± 244 nephrons, N/HFD 14,890 ± 454 nephrons; $p = 0.002$). The number of nephrons per gram of body weight in N/HFD offspring was similar to ND offspring (Fig. 3d). The 1790 additional nephrons in N/HFD than ND offspring at P21 were significantly less ($p = 0.005$) than the 3586 additional nephrons in offspring from dams fed the HFD from conception to weaning. Compared to ND offspring, we observed a lower glomerular volume in HFD offspring at P21; however, this was not the case in N/HFD offspring (Fig. 3e).

### Switching from LPD to HFD at birth rapidly normalises body weight and completely rescues the programmed nephron deficit in low birthweight offspring

LPD offspring were growth restricted at birth and throughout lactation, weighing 10% less than ND offspring at birth (Fig. 3a; $p = 0.0004$), 15% less at P2, 33% less at P7, 37% less at P14, and 35% less at P21 (Fig. 3a; $p < 0.0001$ at P2, 7, 14, and 21). As expected, LPD offspring had smaller kidneys than ND offspring at P21 (Fig. 3b; $p < 0.0001$), and these kidneys contained 28%

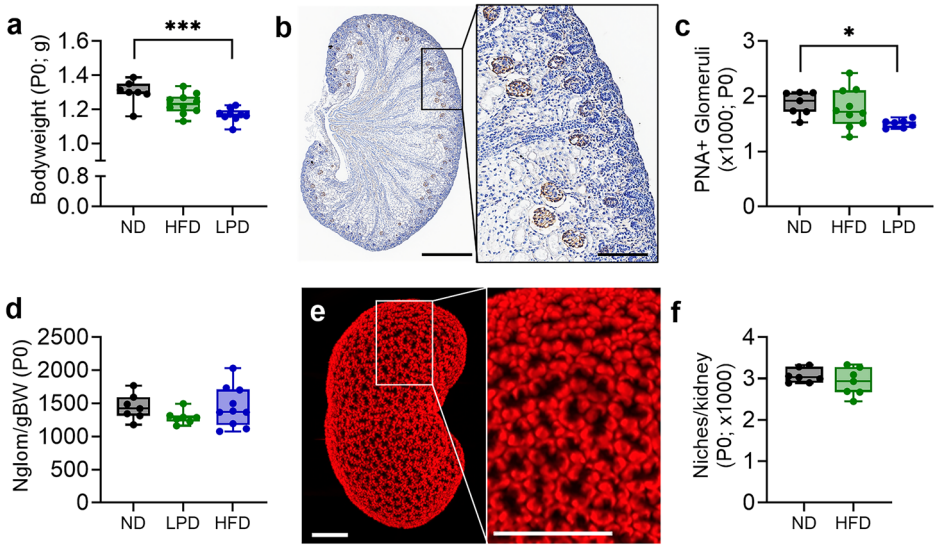


**Fig. 2 | Maternal HFD programs normal nephron and niche number in offspring at birth, while maternal LPD programs an offspring nephron deficit at birth.** Body weight at P0 in ND ($n = 3$ females and 4 males from each of 4 litters), LPD ($n = 4$ females and 5 males from 5 litters) and HFD ($n = 5$ females and 5 males from 5 litters) offspring; each dot represents a litter; $p = 0.0004$ (**a**); Representative PNA-labelled histological section of a P0 kidney marking the plasma membranes of podocytes for glomerular counting (brown); scale bar: left = 0.5 mm, right = 150 µm (**b**); number of PNA+ glomeruli per kidney at P0 in ND ($n = 3$ females and 4 males from 4 litters), LPD ($n = 4$ females and 5 males from 5 litters) and HFD ($n = 5$ females and 5 males from 5 litters) offspring; $p = 0.047$ (**c**); number of PNA+ glomeruli per kidney per gram of body weight at P0 in ND ($n = 3$ females and 4 males from 4 litters), LPD ($n = 4$ females and 5 males from 5 litters) and HFD ($n = 5$ females and 5 males from 5 litters) offspring (**d**); representative whole-mount P0 kidney immunofluorescently labelled with Six2 to mark nephrogenic niches (caps) in the whole kidney; scale bars = 500 µm (**e**); number of nephrogenic niches per kidney at P0 in ND and HFD ($n = 3$ females and 4 males from 4 litters per group) (**f**).

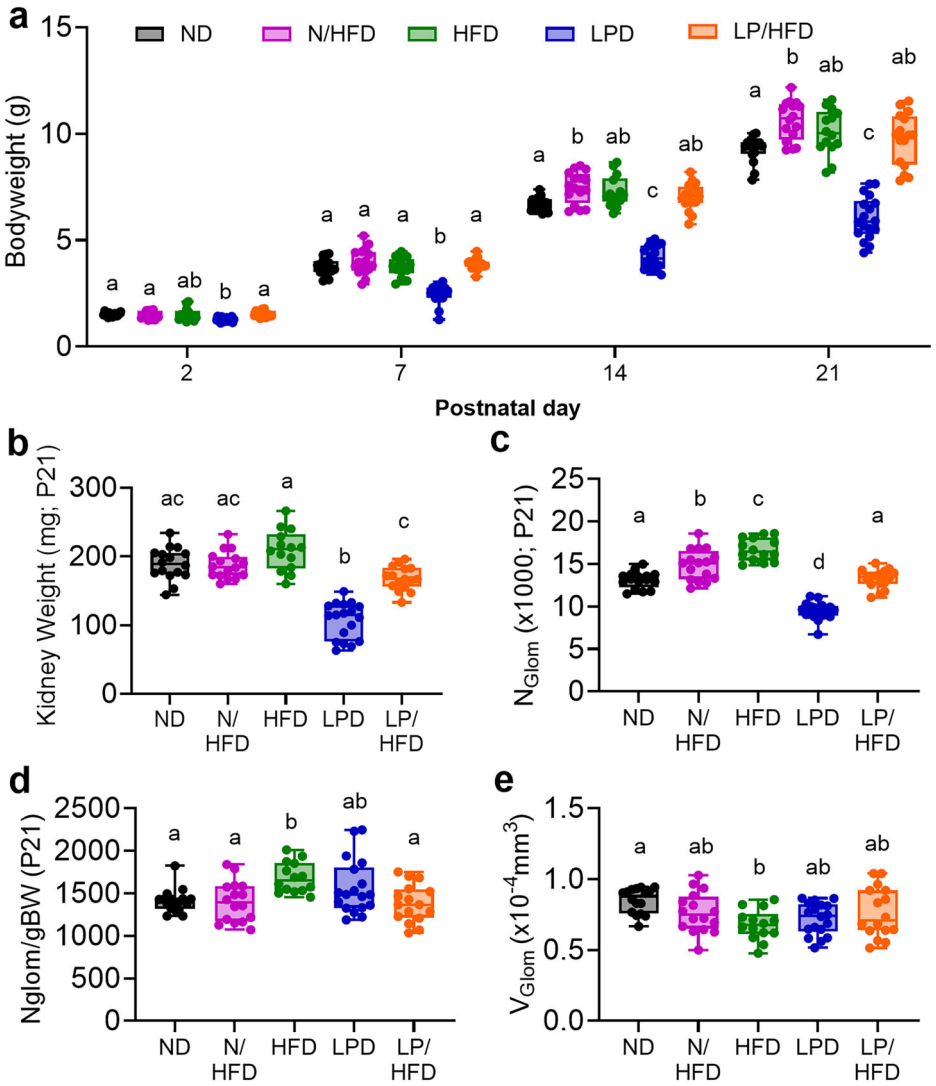


**Fig. 3 | Feeding HFD during lactation augments nephron number during the final days of nephrogenesis and can rescue a programmed nephron deficit.** Body weight in ND ($n = 8$ litters), N/HFD ($n = 8$ litters), HFD ($n = 7$ litters), LPD ($n = 9$ litters) and LP/HFD ($n = 8$ litters) at P2, P7, P14 and P21 (**a**). Each point in **a** represents the average body weight for each litter (sexes combined following no detectable sex difference), with whiskers extending to the smallest and largest value, and the box extending from the 25th to 75th percentiles, with a solid line indicating the median. Kidney weight (**b**), total number of glomeruli per kidney ($N_{glom}$) (**c**), total number of glomeruli per kidney per gram of body weight (**d**) and glomerular volume (**e**) in ND, N/HFD, HFD, LPD and LP/HFD at P21. Each point in the box and whiskers plot in **k–m** represents an individual male or female from a single litter, with whiskers extending to the smallest and largest value, and the box extending from the 25th to 75th percentiles, with a solid line indicating the median where $n = 7–9$ litters (HFD: $n = 14$ from 7 litters, ND, N/HFD and LP/HFD: $n = 16$ from 8 litters, LPD: $n = 18$ from 9 litters. Different letters indicate statistically significant differences ($p < 0.05$).

fewer nephrons (Fig. 3c; $p < 0.0001$). Nephron number per gram of body weight in LPD offspring was similar to ND offspring at P21 (Fig. 3d).

Switching the diet from LPD to HFD at birth increased offspring growth rate so that by P2 LP/HFD offspring were 11% heavier than LPD offspring ($p < 0.0001$) and had body weights similar to ND offspring (Fig. 3a). Body weight in LP/HFD offspring remained similar to ND offspring throughout lactation (Fig. 3a). Compared to LPD, switching the maternal LPD to HFD at birth increased kidney weight by 57% (Fig. 3b; $p < 0.0001$) and nephron endowment by 40% (LPD 9433 ± 240 nephrons, LP/HFD 13,240 ± 278 nephrons; $p < 0.0001$); Fig. 3c), so that both kidney weight and nephron number at P21 were similar to ND offspring. Nephron number per gram of body weight in LP/HFD offspring was similar to ND offspring (Fig. 3d). Glomerular volume in both LPD and LP/HFD offspring was similar to ND offspring at P21 (Fig. 3e).

### Augmented nephron endowment in offspring of dams fed HFD throughout pregnancy and lactation was associated with altered macronutrient intake, not increased caloric intake

Given the higher caloric density of the HFD compared with the NPD and the LPD, and the altered macronutrient ratio, we next assessed whether the augmentation and rescue of nephron endowment observed was associated with increased total caloric intake or alterations to individual macronutrient intake. We measured maternal food intake (Fig. 4a) every 2–3 days for 26 days post-conception (19 days of gestation and the first 7 lactational days) with particular interest in days 11–19 (the *in utero* nephrogenic period) and lactational days 1–3 (the lactational nephrogenic period). Caloric and macronutrient intakes were calculated using digestible energy densities (Supplementary Table 1).

During the first 10 days of gestation (prior to commencement of metanephric kidney development), the greater caloric density of the HFD resulted in approximately 30% higher caloric intake (Fig. 4b) in HFD than ND fed dams. Caloric intake in HFD dams during gestational days 11-13 was similar to ND dams. Although caloric intake was on average 17% higher in HFD dams than ND dams between 14- and 17-days gestation, this increase was not statistically significant. As a consequence, HFD dams maintained normal caloric intake throughout the period of *in utero* nephron induction (Fig. 4b).

HFD fed dams consumed a similar number of calories from protein as ND dams throughout gestation and the nephrogenic lactational period with the exception of reduced protein intake on gestational day 11 and lactational day 2 (Fig. 4c). HFD dams consumed on average 25% fewer calories from carbohydrate than ND dams between gestational days 6 to 19 (birth), and on average 35% fewer calories from carbohydrate throughout the lactational nephrogenic period (Fig. 4d). Caloric intake from fat was on average 200% higher in HFD than ND dams throughout the gestational period, and on average 140% higher throughout early lactation (Fig. 4e).

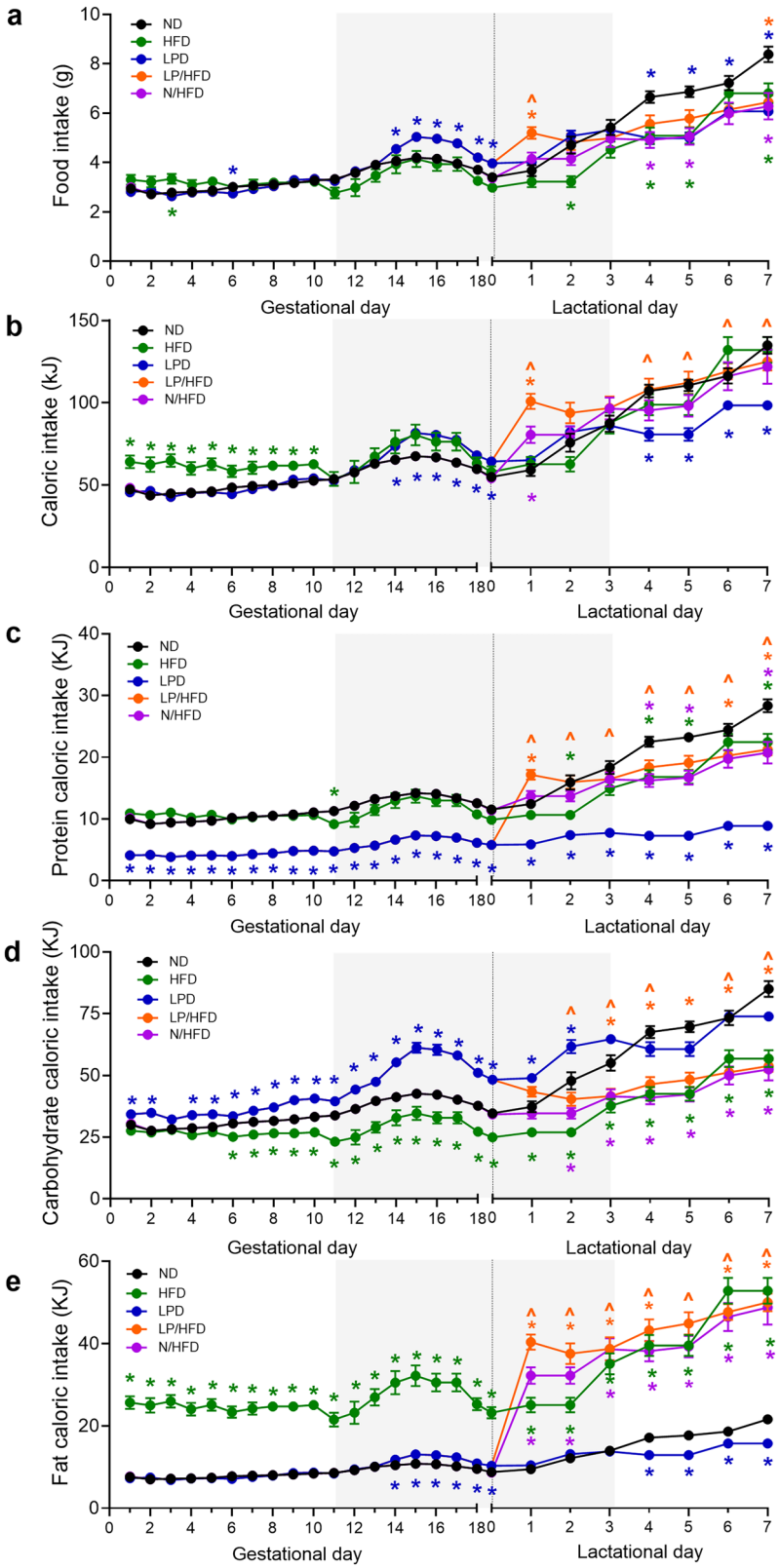


**Fig. 4 | Daily food and energy intake across pregnancy and the first 7 days of lactation.** Food intake (**a**); total caloric intake (**b**); protein caloric intake (**c**); carbohydrate caloric intake (**d**); fat caloric intake (**e**) in ND ($n = 21$), LPD ($n = 14$) and HFD ($n = 12$) dams throughout pregnancy, as well as in ND (n = 11), LPD ($n = 6$), HFD ($n = 12$), N/HFD ($n = 10$) and LP/HFD ($n = 8$) dams during the first 7 days of lactation. **Green** asterisks denote significant difference between HFD and ND; **blue** asterisks denote significant difference between LPD and ND; **purple** asterisks denote significant difference between N/HFD and ND; **orange** asterisks denote significant difference between LP/HFD and ND; **orange** ^ denote significant difference between LP/HFD and LPD. Differences between other dietary groups were included in analysis but are not shown.

### Dams switched from ND to HFD at birth, consumed fewer calories from carbohydrate and more calories from fat in early lactation

Offspring of dams fed ND throughout gestation and switched to HFD at birth (N/HFD) had augmented nephron endowment at the end of nephrogenesis. Assessment of maternal nutritional intake during the lactational period showed that N/HFD dams consumed 36% more total calories than ND dams on lactational day 1 (Fig. 4b; $p = 0.022$). However, acclimatisation to the HFD normalised caloric intake in N/HFD dams to that of ND dams from day 2 onwards (Fig. 4b). While calories from protein in N/HFD dams was similar to that of ND dams throughout lactational days 1-3, calories from carbohydrate were approximately 25% lower on

lactational day 2 ($p = 0.038$) and 3 ($p = 0.034$; Fig. 4d), and calories from fat were 240% greater on day 1, and approximately 170% greater on days 2 and 3 of lactation (Fig. 4e; $p < 0.0001$ at each timepoint).

### LPD dams showed 'protein leverage' during late pregnancy but not in the first few postnatal days

We analysed maternal nutritional intakes associated with a nephron deficit in offspring of dams fed LPD. Food intake and total caloric intake in LPD dams were similar to ND dams during days 1-13 of gestation (Fig. 4a and b). However, the composition of the LPD lead to consumption of, on average, 57% less protein and 20% more carbohydrate calories during the first 13 days of gestation (Fig. 4c–e). Fat intake in LPD and ND was similar during this period.

In contrast to the first 13 days of gestation, total food (Fig. 4a) and caloric (Fig. 4b) intake were both greater in LPD than ND dams during gestational days 14-19, which likely reflects the need for LPD dams to consume more protein ('protein leverage') during this period. However, this increased food intake did not normalise macronutrient intake, with LPD dams consuming on average 50% fewer protein calories (Fig. 4c), 40% more carbohydrate calories (Fig. 4d) and 18% more fat calories than ND dams during this period (Fig. 4e).

In contrast to findings during late pregnancy, total food and caloric intake in LPD dams were similar to ND dams during the first three days of postnatal life (Fig. 4a, b). Given the low protein and high carbohydrate composition of the diet, LPD dams consumed on average 55% fewer protein calories (Fig. 4c), 26% more carbohydrate calories (Fig. 4d) but a similar amount of fat calories as ND dams during this period (Fig. 4e). Total food and caloric intake in LPD dams were lower than ND dams during lactational days 4-7. Lower food intake (Fig. 4a) together with composition of the LPD diet resulted in LPD dams consuming substantially less protein and fat (Fig. 4c, e) across lactational days 4-7.

### Switching from LPD to HFD at birth increased caloric intake, normalised protein and carbohydrate intake and provided additional fat calories in the early lactational period

Dams fed LPD throughout pregnancy and switched to HFD at birth (LP/HFD) significantly increased their food intake (Fig. 4a; $p = 0.01$) on lactational day 1. Given the greater food intake and the higher caloric density of the HFD, LP/HFD dams consumed 55% more calories than LPD dams ($p = 0.0003$) and 70% more calories than ND dams on lactational day 1 ($p < 0.0001$) (Fig. 4b). However, during lactational days 2 and 3, the final stages of nephrogenesis, total caloric intake in LP/HFD dams was no longer greater than LPD or ND dams (Fig. 4b).

The diet switch from LPD to HFD combined with higher food intake on lactational day 1 resulted in higher protein intake than ND dams ($p = 0.004$). However, on lactational days 2 and 3, protein intake in LP/HFD was comparable to ND dams (Fig. 4c). Similarly, the diet switch from LPD to HFD lowered carbohydrate intake from day 2, and by day 3 carbohydrate intake was significantly lower than in ND dams (Fig. 4d; $p = 0.047$). LP/HFD dams consumed significantly more fat than during the lactational nephrogenic period than both LPD (P1: $p < 0.0001$, P2: $p = 0.0001$ and P3 $p = 0.0003$) and ND dams (P1 and P2: $p < 0.0001$, P3 $p = 0.0002$) (Fig. 4e).

### Prolonged nephron induction likely contributes to augmented nephron endowment

Given that HFD can both boost and rescue nephron endowment via lactation, we next investigated whether prolongation of nephrogenesis might be responsible for these alterations in nephron endowment. We assessed the number of self-renewing and differentiating SIX2+ structures per tip (Fig. 5a; Supplementary Table 5) at P2 and P3. The number of SIX2+ self-renewing structures was similar in ND, HFD, LPD and LP/HFD offspring at P2 (Fig. 5b). By P3, the reduction in the number of self-renewing SIX2+ structures in ND and LPD offspring indicated nephrogenesis was almost complete. In contrast, HFD offspring showed significantly more SIX2+ self-renewing structures than ND offspring at P3 (Fig. 5b), indicating that compared with ND offspring, NPC are maintained for an additional day in HFD offspring. The number of differentiating SIX2+ structures was similar in all groups at P2 (Fig. 5c). At P3 there were a greater number of differentiating structures in ND compared to LP-HFD ($p < 0.05$) and a trend for a similar finding in HFD offspring ($p = 0.06$; Fig. 5c).

## Discussion

Hypertension and chronic kidney disease are leading causes of morbidity and mortality worldwide[32]. Compromised kidney development before birth, as well as premature birth, can result in low nephron endowment, which has been associated with increased risk of developing these diseases. Given that even healthy individuals lose almost half their nephrons across their lifetime, reducing kidney function with age[33], it is clear that many low birthweight babies with their low nephron endowment begin postnatal life with a heightened risk of kidney and cardiovascular disease in later life. Therefore, strategies to optimise kidney development and nephron endowment have the potential to reduce rates of these highly prevalent chronic conditions. We previously reported that an HFD fed to pregnant mice boosted nephron endowment by 20%[5]. In the present study, we found that the HFD fed to ND offspring only during lactation (the final stages of nephrogenesis) rapidly boosted nephron endowment. In addition, while many have shown that LPD induces a nephron deficit, we show that this deficit is present at birth and is further amplified by LPD feeding in the lactational period. Notably, we show that the LPD-induced nephron deficit at birth can be completely rescued within 72 hours by altering postnatal maternal nutrition to HFD. This rescue of nephron endowment was associated with strikingly increased food and caloric intake on lactational day 1, accelerated early neonatal growth, as well as increased protein and fat and decreased carbohydrate intake. These results are discussed below.

Lactational high-fat feeding augmented nephron number and rescued a programmed nephron deficit. Many studies have examined the effects of prenatal or combined prenatal/postnatal maternal nutrition on nephron endowment[5–7,17–19,34,35], with most studies focusing on LPD and reporting reduced endowment. Here, we show that switching ND to HFD at birth in mice can augment offspring nephron endowment by 14%. Only a few previous studies have examined the impact of postnatal nutrition on nephron number in normal birthweight offspring. In rats, postnatal food restriction (via cross-fostering additional pups during suckling) reduced nephron endowment[36] and postnatal hyper-nutrition (via litter size reduction) of appropriate birthweight pups resulted in pups with 20% more nephrons[20]. Similar to the Boubred et al. study, the augmentation in nephron endowment in N/HFD offspring in the present study was associated with excess weight gain, so that by 2 weeks of age, N/HFD offspring were significantly heavier than ND offspring.

To determine whether a diet switch at birth could rescue low nephron endowment in low birthweight offspring, we utilised a maternal LPD model, which we and others have previously shown gives rise to offspring with a nephron deficit[6,7,18,23,24,34]. Importantly, we show here that this model results not only in low birthweight but also reduced neonatal growth, which can stem from a wide range of factors, including, but not limited to, poor lactational nutrition and/or supply during maternal LPD feeding, offspring malabsorption as well as increased offspring energy expenditure on thermogenesis or due to lung immaturity. In the present study, we showed for the first time that a nephron deficit induced by maternal low-protein and high carbohydrate feeding is present at birth (20% deficit) and that the deficit increases to approximately 30% by weaning, indicating that LPD impairs nephrogenesis in both the prenatal and postnatal periods. At birth and weaning, the nephron deficit in LPD offspring is coupled to the lower body weight in these animals; however, our previous studies on LPD offspring indicate that LPD offspring undergo post-weaning catch-up growth and are susceptible to adult disease[24].

Switching the LPD to HFD at birth rapidly improved pup growth and restored nephron endowment to normal levels, rescuing a 20% deficit and preventing a potential 30% nephron deficit by the completion of nephrogenesis (postnatal day 3). This is a dramatic finding, both in terms of the

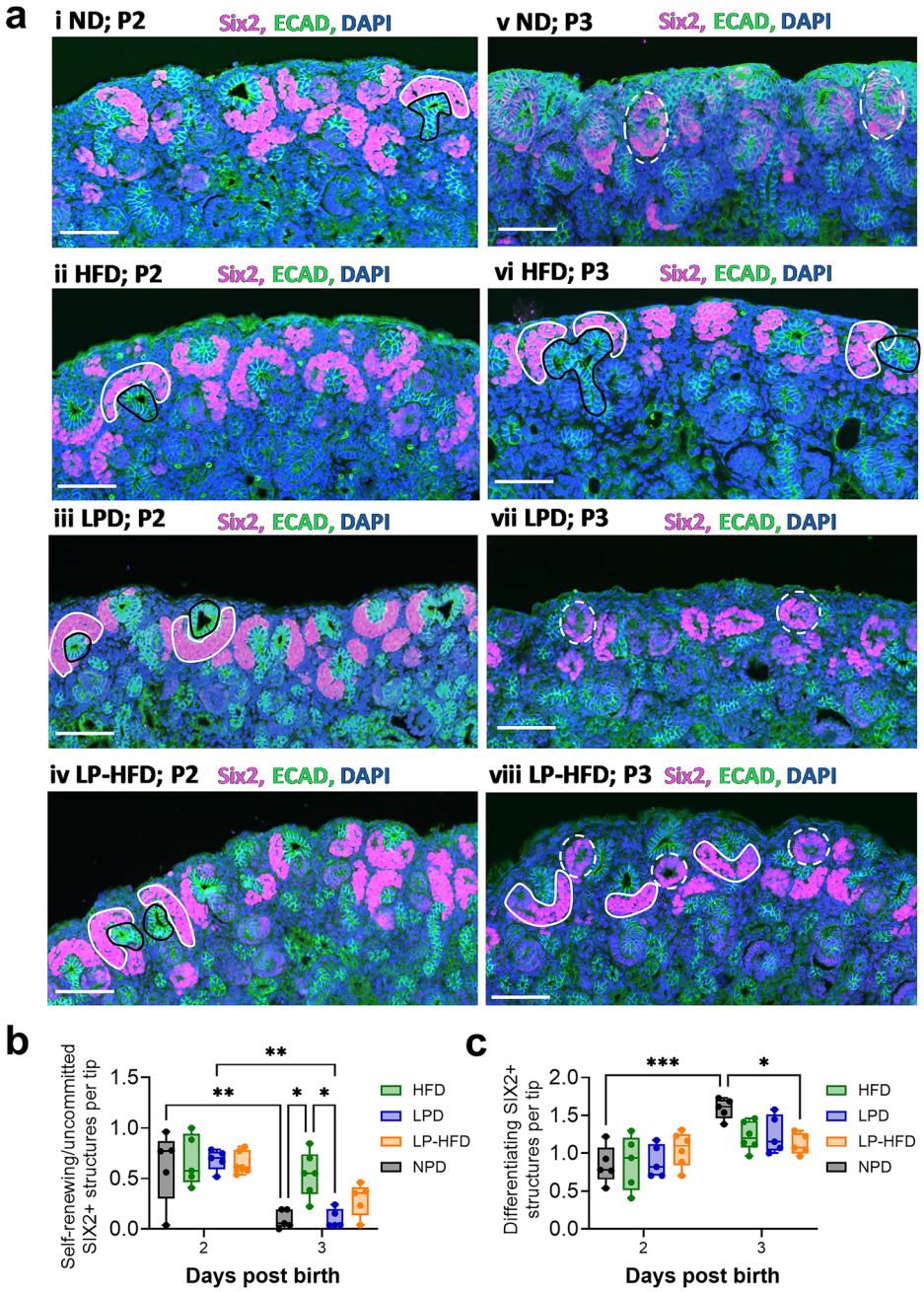


**Fig. 5 | Prolonged nephron induction contributes to augmented nephron endowment.** **a** Representative images of the nephrogenic zone in ND (i), HFD (ii), LPD (iii) and LP-HFD (iv) offspring at P2, and ND (v), HFD (vi), LPD (vii) and LP-HFD (viii) at P3. Scale bars = 50 µm. Representative self-renewing SIX2+ (pink) cells outlined in white, ECAD+ (green) ureteric tips outlined in black. Representative committed SIX2+ structures are indicated with a dashed outline. **b** The number of self-renewing SIX2+ structures per tip in ND, HFD, LPD and LP-HFD offspring at P2 and P3 (**c**). The number of differentiated SIX2+ structures per tip in ND, HFD, LPD and LP-HFD offspring at P2 and P3. Points in (**b**) and (**c**) represent average values from >25 tips for one sample, from 5-6 litters per diet per timepoint. $^{*}p < 0.05$, $^{**}p < 0.01$. Only significant differences with each time point and within each diet across time are shown.

rapidity and the extent of nephron endowment rescue achieved. In addition, the rescue occurred completely via lactation, so our findings highlight the importance of post-natal nutrition on nephron endowment following preterm birth. Following human preterm birth, kidney development and maturation coincide with various factors that impair nephrogenesis, including an altered redox status promoting oxidative stress, and often growth restriction, exposure to interventional nephrotoxic drugs, inflammation and altered postnatal nutrition[37,38]. The outcome is often accelerated nephron maturation[39,40] and although ongoing for up to 40 days post preterm birth[41], early termination of nephrogenesis results in low nephron endowment. Therefore, strategies to enhance postnatal nephrogenesis have the potential to provide a therapeutic option to babies born preterm.

In contrast to the augmentation of nephron endowment in HFD offspring, the rescue of nephron endowment in LP/HFD offspring was associated with accelerated neonatal somatic growth, which was first observed just 2 days after birth. Interestingly, this rapid weight gain resulted in normal but not excess body weight compared to ND offspring at 2 days, and this normal body weight was maintained throughout the remainder of the lactational period. Our findings suggest this accelerated growth may be associated with higher caloric intake during lactational day 1, which returns to normal at day 2.

The impact of postnatal nutrition on offspring body weight has been reported in several studies. When Boubred et al (2007, 2009) applied their litter size reduction/overfeeding model to growth-restricted rat pups (induced also by maternal LPD), the overfeeding normalised offspring body weight by 15 days of age, but failed to normalise nephron number[25]. Wlodek et al showed that cross-fostering growth-restricted rat pups (induced by placental restriction, which impairs lactation) to control dams with a normal lactational environment improved body weight and prevented the nephron deficit and hypertension observed in restricted offspring[21]. While low birthweight/growth restriction is a key factor in the development of impaired adult renal function[13], accelerated postnatal 'catch-up' growth in low birthweight infants has been associated with cardiovascular disease[42] and these findings have been replicated in rodent models of intrauterine

growth restriction[21,43]. The rescue of nephron endowment in LP/HFD offspring is coupled to the correction in body weight; whether the rescue of nephrogenesis in this LP/HFD model is beneficial alongside the rapid postnatal growth observed remains to be determined. Regardless, the impact of the nutritional change at birth on the rapid rescue and normalisation of nephron endowment is clear, and can be anticipated to motivate more studies on nutritional prevention of a nephron deficit and resultant kidney and cardiovascular health.

The 27% higher nephron endowment in HFD offspring than ND offspring in the present study is similar to what we observed previously[5]. Given that total caloric intake in HFD dams was similar to ND dams throughout the nephron induction period, we suspect that the augmentation in nephron number is associated with differences in levels of specific macronutrients and/or micronutrients, and/or differences in nutrient balance. While many studies have assessed the effect of maternal diet on nephron endowment, like the present study, they did not consider the balance and interactions between macronutrients (i.e, a higher percentage of fat requires a reduction in one or both of the other macronutrients). While the current study did not determine the multidimensional impact of maternal diet on nephrogenesis, our findings do show that both HFD and N/HFD dams consumed substantially more fat and less carbohydrate during the nephrogenic period than ND dams.

During the in-utero nephron induction period (gestational days 11–19), LPD dams significantly increased their food and caloric intake. This finding is indicative of protein leverage, whereby compensatory feeding for protein results in over-consumption of fat and carbohydrate in diets low in percent protein (and vice versa)[44]. Protein leverage is incomplete in mice (Simpson et al. 2025), hence the increased food intake in LPD dams was not sufficient to normalise macronutrient intake and as a result LPD dams consumed less protein, more carbohydrate and more fat than ND dams during the in-utero nephron induction period. While fat intake was normal in the first postnatal days in LPD dams, the high carbohydrate intake and low protein intake was maintained through the early postnatal period when the rate of nephron induction normally peaks, suggesting a role for not only adequate dietary protein but also reduced carbohydrate intake in the establishment of nephron endowment.

In early lactation, when LPD dams were switched to HFD, the altered composition of the diet raised and then normalised protein intake, lowered carbohydrate intake and dramatically increased caloric intake from fat. In this study, we did not assess the impact of LPD switched to NPD at birth to observe the impact of normalisation of both protein and carbohydrate intake in the early postnatal period. However, while Boubred et al.[25] and Woods et al[23,28] have both shown in rats that gestational low protein feeding followed by postnatal normal protein control diet results in offspring with a nephron deficit, other reports have shown that cross fostering rat pups from prenatal low protein to postnatal standard chow can prevent a nephron deficit[26,27] supporting the concept that the multidimensional nature of nutrition is complex and difficult to disentangle.

The present study measured for the first time maternal caloric intake across the entire nephrogenic period. Similar maternal caloric intake profiles were observed in HFD, N/HFD and LP/HFD dams with offspring with augmented or rescued nephron endowment, that being greater fat and reduced carbohydrate across the *in utero* nephrogenic or lactational nephrogenic period.

Maternal macronutrient intake significantly influences milk composition, with fat content being the most variable and the level of dietary fat and variation in specific fatty acids present in the mother's diet being readily reflected in the milk[45]. We expect that the proportion of individual fatty acids in mouse milk samples varies in response to the maternal diet and likely reflects the source of dietary fat. Additional studies are required to understand how maternal diet impacts milk composition, macronutrient ratio and milk production volume, in order to further delineate the impact of maternal diet on nephron endowment.

Taken together, our findings, together with findings from the studies cited above, indicate that in addition to the long-known impact of protein, levels of fat and carbohydrate may also be instrumental in determining nephron endowment. Our findings suggest that a specific balance of all three macronutrients is likely crucial for optimal nephron endowment – namely, adequate protein, reduced carbohydrate and higher fat. Explicitly exploring this prediction will require further studies in which the ratios and amounts of macronutrients are systematically explored[46].

Augmentation of nephron endowment was associated with an extension to the period of nephron induction. At birth, HFD pups had similar numbers of nephrogenic niches and maturing nephrons as ND offspring, indicating that the augmentation of nephron number in HFD offspring occurred postnatally during the final stage of nephrogenesis. This finding is in contrast to our previous finding that nephron augmentation in HFD offspring was evident at embryonic day 18[5]. These contrasting findings may be associated with the differing compositions of the control diets used in the two studies, or the length of diet exposure (from 6 weeks pre-gestation in Hokke et al.[5] vs from conception in the present study). Regardless, high-fat feeding throughout pregnancy and lactation induced a 27% augmentation in nephron endowment, and when fed only during lactation augmented nephron endowment by 14%.

The cessation of nephrogenesis is marked by loss or reduced expression of nephron progenitor self-renewal markers such as SIX2[14,47]. In mice, this normally occurs by postnatal day 3[14,47]. In the present study, we identified that nephron augmentation in HFD offspring was associated with a 1-day extension of nephrogenesis. This is one of only a handful of studies to show that extension of nephrogenesis can increase nephron endowment. *Lin28a* and *Lin28b* encode RNA-binding proteins that regulate stem and progenitor cell state and metabolism, functioning in part by inhibiting the *let-7* family of microRNAs. Knockout of *Lin28b* led to a 50% reduction in progenitor cell niche number at P0 in mice, while suppression of *let-7* resulted in a 60% increase in niche number at P0, and extended nephrogenesis by 1 day[48]. In addition, mice expressing abnormally high levels of Glial cell line-derived neurotrophic factor (GDNF) maintain uncommitted NPCs for an additional day but suffer developmental abnormalities that compromise nephron endowment[49]. One example of extended nephrogenesis without apparent pathology involves heterozygous knockout of *Tsc1*, a regulator of cell growth, metabolism and proliferation. Loss of a single copy of *Tsc1* from NPCs resulted in a 25% increase in nephron endowment, with some uncommitted NPCs evident for an additional day compared to control mice. In contrast to these models with extended nephrogenesis, heterozygous loss of the transcriptional regulator *Six2*, led to an 18% increase in niche number at P0 and nephron number at P21, but no change in the timing of cessation was observed in this model. Rather, an increase in NPC proliferation drove the boost in nephron number[50]. Our finding that the rescue of nephron endowment in LP/HFD offspring was not associated with a 1-day extension of nephrogenesis (although there may have been a shorter extension to the nephrogenic period) supports the concept that multiple cellular mechanisms are involved in the regulation of final nephron endowment.

How HFD and other dietary interventions alter NPC regulation remains unclear. However, cellular metabolism is emerging as an important regulator of stem and progenitor cell state in health and disease[51–53]. Targeted studies in mice point to a role for high glycolytic flux in NPC maintenance and inhibition of glycolysis as a trigger for differentiation[54,55]. These findings are supported by metabolic analyses in the developing human kidney with NPC renewal relying on glycolysis, and differentiation shifting towards oxidative phosphorylation[51]. NPC state has a substantial impact on cell proliferation, with self-renewing NPCs taking twice as long to progress through the cell cycle as partially committed NPCs[31]. Moreover, the *Six2* heterozygous model and recent studies on NPC plasticity demonstrate a capacity to shift nephron progenitor identity towards a partially committed state with increased proliferative capacity, without compromising self-renewal[50,56,57]. As such, diets such as HFD that are less favourable for glycolysis may push NPCs into a partially committed state with enhanced proliferation, thereby extending and augmenting nephrogenesis.

While the present findings demonstrate the significant potential of nutrition to boost and rescue nephron endowment, the structure and function of the resultant kidneys in adulthood, and under stressful conditions, were not analysed. Such studies may reveal renal susceptibility to advanced aging and/or disease. However, we previously reported that offspring of female mice fed HFD from 6 weeks prior to pregnancy and up to weaning had normal body composition, glomerular filtration rate, protein excretion and renal histology at 6 and 9 months of age[5]. Although we would expect similar findings, long-term studies to investigate these outcomes in LP/HFD offspring are warranted.

Interventions to optimise prenatal and postnatal health and development, and thereby reduce the prevalence of chronic diseases, have huge potential economic, societal and individual benefit[58]. Nutritional interventions appear to have considerable promise in this regard. While the current study cannot delineate the impact of each macronutrient nor define the impact of the altered ratio of these macronutrients on nephron endowment, future studies to disentangle the impact of the three macronutrients on nephrogenesis and determine optimal nutritional strategies to protect renal development in at-risk infants are warranted.

## Methods

### Experimental design

Animal experiments were conducted in accordance with guidelines provided by the Monash University Animal Research Platform (ethics approval numbers: MARP 21115, 24666, 30142) and performed in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals. Virgin female C57BL/6J mice aged 6-10 weeks of age were housed under SPF conditions with a 12-hour light/dark cycle and randomly allocated to experimental groups, and fed either a normal diet (ND; AIN93G; Specialty Feeds, Western Australia), a high fat diet (HFD; SF00-219, Specialty Feeds, Western Australia) or a low protein diet (LPD; SF01-026, Specialty Feeds, Western Australia) from conception and throughout pregnancy and lactation (Fig. 1). Additional cohorts were fed a ND or LPD from conception to birth and then fed the HFD throughout lactation (N/HFD, LP/HFD; Fig. 1). At all times, mice had *ad libitum* access to food and water. Diet compositions are provided in Supplementary Table 1. Females were mated overnight and the presence of a vaginal plug the following morning identified post-conceptional day 0.5 (P0.5). Gestational length and litter size were not affected by diet (Supplementary Tables 2, 3), but to control for these factors, only litters with 4-9 pups and a 19-day gestation were included in the study.

### Estimating total nephron number at birth

One cohort of offspring was collected at birth (ND: $n = 4$; HFD: $n = 5$ and LPD: $n = 5$ litters) to estimate the total number of nephrons prior to the diet switch. The total number of nephrons (lectin peanut agglutinin PNA + S-shaped body stage to capillary loop stage glomeruli) per kidney at P0 in was estimated using the physical disector/fractionator combination as previously described[59]. In brief, pups were weighed and sexed and kidneys were dissected in phosphate buffered saline (PBS) and fixed in methacarn (bioWORLD; 21730013). One kidney from a single male and female per litter (ND: $n = 4$ litters; HFD: $n = 5$ and LPD: $n = 5$ litters) was randomly chosen, processed to paraffin and exhaustively sectioned at 4 µm. Ten evenly-spaced section pairs were systematically sampled and stained with PNA (Sigma-Aldrich, Castle Hill, NSW, Australia; L3165). Section pairs were projected using a light microscope and all PNA+ structures (Fig. 2b) counted using the physical disector counting principle[59].

### Nephrogenic niche analysis at birth

P0 kidneys from ND and HFD pups were collected at P0 for quantitation of the total number of NPC niches (marked by Six2+ mesenchymal caps) per kidney. Kidneys were dissected in PBS and fixed in 4% paraformaldehyde for 20 minutes, washed 3x for 5 minutes in ice-cold PBS and stored for up to a week in PBS. One kidney from a single male and female per litter ($n = 4$ litters/group) was randomly chosen and immunofluorescently labelled for Six2 (marker of NPCs). Kidneys were washed using PBS/0.1% Triton for 2 hours and then incubated in 5% Donkey serum, 0.1% Triton X-100 in PBS overnight at 4 °C followed by incubation in 5% Donkey serum, 0.1% Triton X-100 in PBS containing the rabbit anti-Six2 antibody (11562–1-AP; Proteintech 1:400, diluted in Agilent antibody diluent S3022) for 48 hours at 4 °C. After washing, kidneys were incubated with Alexa Fluor donkey anti-rabbit 568 (Invitrogen A10042; 1:750, diluted in Agilent antibody diluent S3022) for 24 hours at 4 °C followed by an overnight wash in PBS. and dehydrated in methanol, cleared using Benzyl Alcohol/Benzyl Benzoate (BABB) and embedded in 2% low melting point agarose as previously described[60]. Whole kidneys were scanned by optical projection tomography (OPT) with images reconstructed in Imaris (Bitplane AG) (Fig. 2e). The total number of Six2+ caps in whole kidneys was counted using the measurement points function as previously reported[61].

### Estimation of total glomerular number (nephron endowment) and glomerular volume at P21

For estimation of total glomerular number at P21, one male and one female offspring per litter (ND: $n = 8$ litters, HFD: $n = 7$ litters; LPD: $n = 9$ litters; N/HFD: $n = 8$ litters and LP/HFD: $n = 8$ litters) was anesthetised with isoflurane and euthanised by exsanguination via perfusion of PBS via the left ventricle, followed by perfusion fixation with 10% formalin. Both kidneys were removed and immersion-fixed in 10% formalin. Whole left kidneys were weighed and embedded in paraffin for glomerular counting. Total glomerular number ($N_{glom}$) per kidney was estimated using the physical disector/fractionator combination, as previously described[59]. Briefly, whole left kidneys embedded in paraffin were exhaustively sectioned at 5µm. Ten evenly-spaced section pairs were systematically sampled and stained with PNA and counterstained with haematoxylin. Section pairs were projected using a light microscope and all PNA+ glomeruli were counted using the disector counting principle[62]. Tissue sections were sampled for glomerular volume estimation, for each field of view $P_{glom}$ (grid points on glomerular tuft) and $P_{kid}$ (grid points overlaying kidney tissue) were counted[62]. Mean glomerular volume ($V_{glom}$) was estimated at P21 using the formula:

$$V_{glom} = \frac{P_{glom}/P_{kid}}{N_{glom}/V_{kid}}$$

### Food intake throughout pregnancy and early lactation

Additional cohorts were utilised to assess maternal food intake and thereby calculate maternal caloric intake in ND ($n = 21$), LPD ($n = 14$) and HFD ($n = 12$) dams throughout pregnancy, as well as in ND ($n = 11$), LPD ($n = 6$), HFD ($n = 12$), N/HFD ($n = 10$) and LP/HFD ($n = 8$) throughout lactation. Food intake in grams was recorded every 2 or 3 days from conception to P7 (completion of nephrogenesis) and daily intakes calculated. Food intake was not measured daily in order to minimise stress to the dams and thereby minimise cannibalism of pups. Food spillage was accounted for by sifting the bedding at each food intake measurement. Caloric intake was calculated using grams consumed and digestible energy density. Caloric intake of protein, fat and carbohydrate was calculated using the percentage of digestible energy of each macronutrient (Supplementary Table 1).

### Timing of the cessation of nephron induction

Separate cohorts of pups were collected at P2.5 ($n = 5$ ND, HFD, LPD, $n = 6$ LP/HFD litters), and P3.5 ($n = 5$ ND, HFD, LPD and LP-HFD litters) for analysis of nephron induction. Kidneys were dissected, fixed in methacarn, processed and embedded in paraffin. One kidney from each pup in the litter was sectioned at 4 µm and antigen retrieval was performed using an automated Dako PT Link (PT10126) system. Non-specific binding of antibodies was blocked by incubating sections in 4% BSA solution for 30 minutes followed by incubation with the primary antibodies against Six2 (1/400; 11562–1-AP; Proteintech) and e-cad (ureteric epithelium marker; 1:400; AF748, Bio-techne) overnight at 4 °C. Tissue was washed and incubated with Alexa Fluor donkey anti-rabbit 647 (1:800; A32795, Invitrogen), Alexa Fluor chicken anti-goat 488 (1:800; A21467, Invitrogen) and DAPI (1:1000)

for 1 hour at room temperature. Sections were mounted with ProLong Diamond Anti-Fade Mountant (ThermoFisher) and analysed by fluorescent confocal microscopy using an Olympus VS200 slide scanner. Immunofluorescence images were converted from .vsi to .ome.tiff format using QuPath[63] and subsequently imported to ImageJ[64]. The Cell Counter plugin in ImageJ (https://github.com/fiji/Cell_Counter) was used to annotate tips, areas of self-renewing/uncommitted SIX2+ nephron progenitor cells and areas of differentiating nephron progenitors or early nephrons based on morphology and expression of SIX2 and ECAD as follows (Supplementary Fig. 1): Uncommitted regions were identified by higher intensity SIX2 staining in cells packing closely around the 'top' or 'shoulder' of the tip and the absence of a lumen. differentiating nephron progenitors and early nephron structures were defined by the presence of a lumen (differentiating) or morphology reminiscent of a comma-shaped body (early nephron). Regions where groups of SIX2+ cells formed structures with uncertain morphology or with "cap"-like structures not located on the 'top' or 'shoulder' of the tip were ignored, as well as any SIX2 staining where tip morphology was not observed. At least 26 tips per sample were quantified by an individual unaware of the sample stage or experimental group. Regions of SIX2+ self-renewing/uncommitted and differentiating/committed nephron progenitor cells are reported as number/tip.

### Statistical analysis

Datasets were tested and passed the Shapiro-Wilk normality test. Body weight, nephron number and nephron progenitor cell niche number at P0 were analysed by two-way ANOVA (maternal diet and sex). Body weight at P2, 7, 14 and 21 was initially analysed by a three-way linear mixed-effects model (maternal diet, sex, and time) using R package lmerTest. This model revealed that sex was not a significant predictor of body weight, either as a main effect or in any interaction (Supplementary Table 4). Consequently, we proceeded with a simplified two-way repeated-measures ANOVA model (maternal diet x time) for the final analysis and post-hoc comparisons. Kidney parameters at P21 in ND, LPD, HFD, N/HFD and LP/HFD were analysed by two-way ANOVA (maternal diet and sex). No sex difference was identified in any parameter (Supplementary Table 4), so the data presented have been consolidated and *P*-values have been adjusted for the Tukey multiple comparisons test for differences between diets. Food intakes were analysed by two-way ANOVA with repeated measures and *P*-values presented have been adjusted for the Tukey multiple comparisons test. The number of self-renewing/uncommitted and differentiating SIX2+ structures per tip was analysed by two-way ANOVA (maternal diet and time). Statistical analyses were performed utilising GraphPad Prism 10. Data in tables are mean ± SEM; *n* refers to the number of litters in all experiments, and dams in reference to food and caloric intakes. Statistical significance was defined as $P < 0.05$.

## Data availability

The authors declare that the data supporting the findings of this study are available within this manuscript and the accompanying supplementary information files.

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## Author contributions

All authors participated in the review of the manuscript and the data shown and have read and approved the final version of the manuscript. Specifically: Conception and design (L.A.C.-M., A.J.R., S.JS, SS-B, AN.C, J.FB); data acquisition (L.A.C.-M., S.EG, G.C, J.E, NdZ, JL.M, Y.W, R.H); data analysis (LAC-M, SE.G, YM); data interpretation (L.A.C.-M., SJ.S, JF.B), preparation of manuscript (LAC-M, S.JS, A.NC, JF.B).

## Competing interests
The authors declare no competing interests.

## Additional information
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