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RESEARCH ARTICLE

Podocyte endowment and the impact of adult body size on kidney health

Luise A. Cullen-McEwen,[1] James van der Wolde,[1] Kotaro Haruhara,[1,2] Leon Tribolet,[1,3] John P. Dowling,[4] Michael G. Bertram,[5,6] Robert de Matteo,[1] Fabian Haas,[7] Jan Czogalla,[7] Yusuke Okabayashi,[2,7] James A. Armitage,[8] M. Jane Black,[1] Wendy E. Hoy,[9] Victor G. Puelles,[1,7] and John F. Bertram[1]

[1]*Stem Cells and Development Program, Monash Biomedicine Discovery Institute and Department of Anatomy and Developmental Biology, Monash University, Melbourne, Victoria, Australia;* [2]*Division of Nephrology and Hypertension, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo, Japan;* [3]*Health and Biosecurity, CSIRO, Geelong, Victoria, Australia;* [4]*Department of Anatomical Pathology, Monash Medical Centre, Clayton, Victoria, Australia;* [5]*Department of Wildlife, Fish, and Environmental Studies, Swedish University of Agricultural Sciences, Umea, Sweden;* [6]*School of Biological Sciences, Monash University, Melbourne, Victoria, Australia;* [7]*III. Department of Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany;* [8]*School of Medicine (Optometry) and Institute for Mental and Physical Health and Clinical Translation, Deakin University, Waurn Ponds, Victoria, Australia; and* [9]*Centre for Chronic Disease, University of Queensland, Royal Brisbane and Women's Hospital, Herston, Queensland, Australia*

Abstract

Low birth weight is a risk factor for chronic kidney disease, whereas adult podocyte depletion is a key event in the pathogenesis of glomerulosclerosis. However, whether low birth weight due to poor maternal nutrition is associated with low podocyte endowment and glomerulosclerosis in later life is not known. Female Sprague–Dawley rats were fed a normal-protein diet (NPD; 20%) or low-protein diet (LPD; 8%), to induce low birth weight, from 3 wk before mating until *postnatal day 21* (PN21), when kidneys from some male offspring were taken for quantitation of podocyte number and density in whole glomeruli using immunolabeling, tissue clearing, and confocal microscopy. The remaining offspring were fed a normal- or high-fat diet until 6 mo to induce catch-up growth and excessive weight gain, respectively. At PN21, podocyte number per glomerulus was 15% lower in low birth weight (LPD) than normal birth weight (NPD) offspring, with this deficit greater in outer glomeruli. Surprisingly, podocyte number in LPD offspring increased in outer glomeruli between PN21 and 6 mo, although an overall 9% podocyte deficit persisted. Postnatal fat feeding to LPD offspring did not alter podometric indexes or result in glomerular pathology at 6 mo, whereas fat feeding in NPD offspring was associated with far greater body and fat mass as well as podocyte loss, reduced podocyte density, albuminuria, and glomerulosclerosis. This is the first report that maternal diet can influence podocyte endowment. Our findings provide new insights into the impact of low birth weight, podocyte endowment, and postnatal weight on podometrics and kidney health in adulthood.

NEW & NOTEWORTHY The present study shows, for the first time, that low birth weight as a result of maternal nutrition is associated with low podocyte endowment. However, a mild podocyte deficit at birth did not result in glomerular pathology in adulthood. In contrast, postnatal podocyte loss in combination with excessive body weight led to albuminuria and glomerulosclerosis. Taken together, these findings provide new insights into the associations between birth weight, podocyte indexes, postnatal weight, and glomerular pathology.

developmental programming; kidney; podocyte; podocyte endowment

INTRODUCTION

The podocyte depletion hypothesis has emerged as a unifying concept for progressive glomerulosclerosis, proposing that reductions in podocyte number and/or density can result in persistent proteinuria, glomerulosclerosis, and nephron loss, leading to chronic kidney disease (CKD) and end-stage kidney disease (1). Human studies have shown associations between podocyte depletion and aging (2), hypertension (3), IgA nephropathy (4), and diabetes (5–7). Interestingly, the threshold of podocyte loss for subsequent glomerulosclerosis appears to be tightly regulated in the postnatal period. In a landmark paper, Wharram et al. (8) showed that loss of at least 20% of podocytes in rats led to sustained proteinuria, glomerulosclerosis, and decreased renal function. Subsequent studies confirmed these findings and characterized the compensatory capacity of the remaining podocytes in the context of podocyte loss (9–13).

Given the importance of podocyte number and density for glomerular health and maintenance as well as the limited

Correspondence: L. A. Cullen-McEwen (luise.cullen-mcewen@monash.edu); J. F. Bertram (john.bertram@monash.edu).
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evidence of podocyte gain in postnatal life (14), this raises the question as to whether low podocyte endowment can be developmentally programmed, thereby increasing susceptibility to CKD in later life. In other words, is the adult risk of CKD in some individuals due in part to their low podocyte endowment? It is well established that perturbations to the fetomaternal environment can developmentally program low nephron endowment in animal models (15–17) and humans (15, 18) and increase the subsequent risk of CKD and hypertension (15, 18). In most cases, but not all, this low nephron endowment is associated with low birth weight (LBW), which itself is associated with increased risk of CKD (15, 18–24), hypertension (15,16, 18), and focal and segmental glomerulosclerosis (FSGS) (25, 26).

In the present study, we examined whether LBW and low nephron endowment in rats as a result of a maternal low-protein diet (LPD) gives rise to offspring with low podocyte endowment. In addition, we examined whether postnatal catch-up growth and excess weight gain due to high fat feeding exacerbates the risk of proteinuria and FSGS in LBW offspring, given that excessive weight gain and obesity are risk factors for CKD (27–30) and lead to glomerular hypertrophy and podocyte depletion (31, 32). We found LBW offspring had low podocyte endowment but did not demonstrate albuminuria or FSGS following 6 mo of high fat feeding. In contrast, high fat feeding in normal birth weight offspring decreased podocyte number and led to albuminuria and FSGS.

MATERIALS AND METHODS

Animal and Diets

Animal experiments were conducted in accordance with guidelines provided by the Monash University Animal Research Platform (Ethics Approval No. MARP/2014/015). Briefly, female Sprague–Dawley rats were fed a normal-protein diet (NPD; 20% protein from casein, AIN93G, Specialty Feeds) or an isocaloric LPD (8% protein from casein, Low Protein Modification of AIN93G Rodent Diet, SF01-026, Specialty Feeds) from 3 wk before mating and throughout pregnancy and lactation. To control for litter size across the two groups, litters with less than four pups or greater than eight pups were excluded from analysis. At *postnatal day 21* (PN21), one male offspring/litter was euthanized and the kidneys removed for nephron and podocyte morphometrics (n = 8 litters/maternal diet). Two male offspring/litter were weaned at PN21, with one fed a normal-fat diet [NFD; 6% fat (wt/wt), SF04-057, Specialty Feeds) and the other fed a high-fat diet [HFD; 21% fat (wt/wt), SF00-219, Specialty Feeds) until 6 mo of age (Fig. 1), when they were euthanized and the kidneys were taken for analysis. The HFD had more digestible energy than the NFD. At all times, rats had ad libitum access to food and water. Diet compositions are shown in Table 1.

Perfusion Fixation and Tissue Collection

At PN21 or 6 mo of age, rats were anesthetized with 100% isoflurane and euthanized by exsanguination by perfusion of 1× PBS via the abdominal aorta, followed by perfusion fixation with 10% formalin. Both kidneys were then removed and immersion fixed in 10% formalin. Right kidneys were sliced with a series of razor blades evenly spaced at 800 μm, and one full-face midhilar slice from each kidney was randomly selected for thick slice immunofluorescence for podocyte counting. Whole left kidneys were embedded in paraffin for glomerular counting. Glomeruli were not counted at 6 mo.

Body Composition

At 6 mo of age, body composition (bone mineral content, bone mineral density, lean + bone mineral content, fat mass, total mass, and percent fat) were measured using dual energy X-ray absorptiometry.

Albumin-to-Creatinine Ratio

At 6 mo of age, during tissue collection, urine was sampled directly from the bladder for analysis of the albumin-to-creatinine ratio. Albumin was measured using a direct competitive ELISA (Nephrat, Exocell, Philadelphia, PA). Creatinine

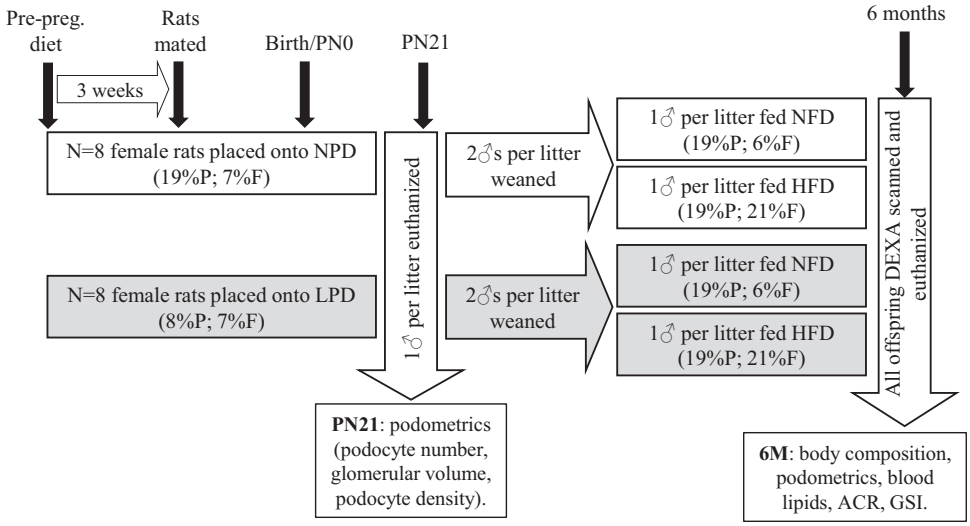

Figure 1. Schematic diagram of the study design. Female rats were fed a normal-protein diet (NPD; n = 8) or a low-protein diet (LPD; n = 8) from 3 wk before mating and up to weaning at *postnatal day 21* (PN21). One male offspring/litter was euthanized at PN21, and one male offspring/litter was weaned onto the normal-fat diet (NFD) and another male offspring was weaned onto the high-fat diet (HFD). These offspring were maintained on these diets until 6 mo of age. ACR, albumin-to-creatinine ratio; DEXA, dual-energy X-ray absorptiometry; F, fat; GSI, glomerulosclerotic index; P, protein.

Table 1. *Maternal and postnatal diet composition*

	AIN93G	SF01-026	SF04-057	SF00-219
	Normal-Protein Diet	Low-Protein Diet (8% Low Protein Modification of AIN93G)	Normal-Fat Diet (6% Fat, Semi-Pure Rodent Diet, Control for SF00-219)	High-Fat Diet (21% Fat, 0.15% Cholesterol, Semi-Pure Rodent Diet)
Calculated nutritional parameters				
Protein, %	19.4	8.4	19	19
Fat, %	7	7	6	21
Crude fiber, %	4.7	4.7	4.7	4.7
Adequate dietary fiber, %	4.7	4.7	4.7	4.7
Digestible energy, MJ/kg	16.1	16.2	16.1	19.4
Total calculated digestible energy from lipids, %	16	16	14	40
Total calculated digestible energy from protein, %	21	9	21	17
Ingredients				
Casein (acid), g/kg	200	87	195	195
Sucrose, g/kg	100	200	341	341
Canola oil, g/kg	70	70	60	
Clarified butter, g/kg (Ghee)				210
Cellulose, g/kg	50	50	50	50
Wheat starch, g/kg	404	417	306	145
DL-Methionine, g/kg	3.0	3.0	3.0	3.0
Calcium carbonate, g/kg	13.1	13.1	17.1	17.1
Sodium chloride, g/kg	2.6	2.6	2.6	2.6
AIN93 trace, g/kg Minerals	1.4	1.4	1.4	1.4
Potassium citrate, g/kg	2.5	2.5	2.5	2.6
Potassium, g/kg Dihydrogen Phosphate	6.9	6.9	6.9	6.9
Potassium sulphate, g/kg	1.6	1.6	1.6	1.6
Choline chloride, g/kg (75%)	2.5	2.5	2.5	2.5
AIN93 vitamins, g/kg	10	10		
SF00-219 vitamins, g/kg			10	10
Cholesterol, g/kg			0	1.5
Oxicap E2, g/kg			0.04	0.04

was measured using the Jaffe reaction of alkaline picrate with creatinine (Creatinine Companion, Exocell).

Estimation of Total Glomerular Number

Total glomerular number was estimated at PN21 using the physical disector/fractionator combination, a design-based stereological approach (33, 34). Briefly, whole left kidneys embedded in paraffin were exhaustively sectioned at 5 μm. Ten evenly spaced section pairs were systematically sampled and stained with lectin peanut agglutinin (L3165, Sigma-Aldrich, Castle Hill, NSW, Australia) to identify the plasma membrane of podocytes and counterstained with hematoxylin. Section pairs were projected using a light microscope, and all peanut agglutinin-positive glomeruli were counted using the disector counting principle (34).

Estimation of Total Podocyte Number in Whole Glomeruli

At PN21 and 6 mo of age, podocytes were counted in 20 whole glomeruli in a single 800-μm slice from each rat: 10 glomeruli from the outer cortex and 10 glomeruli from the inner cortex. Glomeruli were randomly selected by capturing fields close to the capsule (outer cortical zone) and the corticomedullary junction (inner cortical zone). Total podocyte number per glomerulus was estimated using a combination of immunofluorescence, optical clearing, and confocal microscopy (11). Podocytes were identified by their nuclear expression of Wilms' tumor 1 (WT-1; monoclonal mouse anti-human WT-1, M356101, clone 6 F-H2, Agilent, 1:50 dilution) and cytoplasmic expression of synaptopodin (SNP; polyclonal rabbit, Cat. No. 163002, Synaptic Systems, 1:1,000). Following immunofluorescence labeling, kidney slices were cleared with benzyl alcohol/benzyl benzoate and then imaged using a Leica SP8 confocal microscope fitted with a ×20 benzyl alcohol/benzyl benzoate objective lens. Serial optical sections were obtained at 1-μm intervals and stored in 1,024 × 1,024 pixel frames. Fiji imaging software (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany) was used to move through the complete *z*-series of 1-μm optical sections from each glomerulus to manually count absolute podocyte number in each of 20 glomeruli/sample. Podocytes were defined as $WT\text{-}1^+$ SNP^+ cells. Figure 2*A* shows immunofluorescence labeling for WT-1 in podocyte nuclei and SNP in the podocyte cytoplasm in a whole glomerulus reconstructed from confocal optical sections. Figure 2*B* shows a single optical section from the whole glomerulus shown in Fig. 2*A*.

Estimation of Glomerular Volume and Podocyte Density

The volumes of each of the 20 whole sampled glomeruli/rat used for podocyte counting were estimated using the Cavalieri principle (35). In brief, with a random start the area of every seventh optical section per glomerulus was measured using Fiji imaging software. Glomerular volume was calculated by multiplying the sum of the section areas per glomerulus by the thickness of the optical sections by the section sampling fraction. Podocyte density was calculated by dividing the total podocyte number for each glomerulus by the volume of that individual glomerulus.

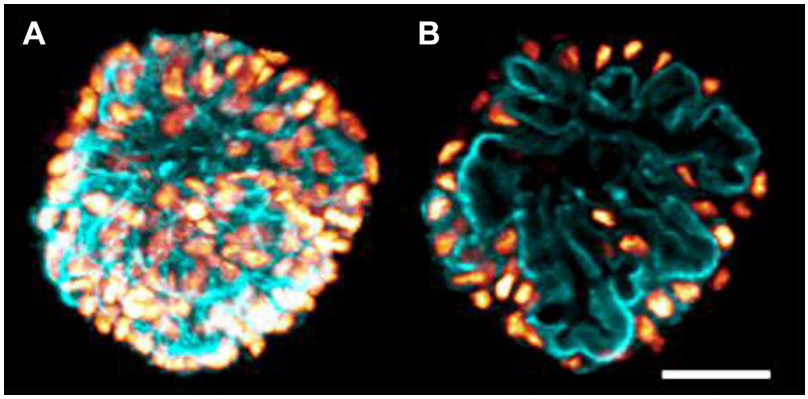

Figure 2. Double immunofluorescence labeling to identify podocytes at *postnatal day 21*. *A*: three-dimensional reconstruction of a whole glomerulus made from confocal optical sections showing podocyte nuclei (Wilms' tumor-1 positive) and cytoplasm (synaptopodin positive). *B*: confocal optical section from the glomerulus is shown in *A*. Scale bar = 25 μm.

Assessment of Podocyte Apoptosis and Proliferation

To determine whether differences in podocyte endowment between groups and subsequent postnatal changes in podocyte numbers involved podocyte apoptosis and/or proliferation, we conducted immunofluorescence analyses. Paraffin-embedded 2-μm sections were treated in Dako Target Retrieval Solution (S236784-2, Agilent) for 15 min with steamer heating followed by a cooling down period of 30 min at room temperature. Primary antibodies were incubated overnight at 4°C, and secondary antibodies were incubated for 1 h at room temperature. The following antibodies were used: guinea pig anti-SNP antibody (No. 163004, Synaptic Systems, 1:400), rabbit anti-K_i-67 antibody (ab15580, Abcam, 1:200), rabbit anti-cleaved caspase-9 antibody (No. 9506, Cell Signaling Technology, Danvers, MA, 1:100), goat anti-guinea pig IgG (H + L) Alexa Fluor 488-conjugated antibody (A-11073, Thermo Fisher Scientific, Waltham, MA, 1:200), and donkey anti-rabbit IgG (H + L) Alexa Fluor 555-conjugated antibody (A-31572, Thermo Fisher Scientific, 1:200).

For the analyses of both podocyte apoptosis and proliferation, between 20 and 50 glomeruli from each of 3 rats/group at both time points (PN21 and 6 mo) were examined. Every glomerulus was carefully examined for evidence of any cell that was K_i-67 or cleaved caspase-9 positive. If a positive cell was found, then the podocyte marker was also assessed. For every immunofluorescence round at both time points, we analyzed a positive control: archival rat tissue of experimental acute kidney injury where there was clear proliferation and apoptosis of proximal tubular cells.

Histopathology

One midhilar 2-μm section from each kidney was stained with periodic acid-Schiff and then imaged with Leica Aperio AT Turbo using a ×40 objective lens. Renal histopathology was assessed by two authors (K.H. and J.P.D.). Every glomerulus in these sections was viewed and scored for sclerosis to give a glomerulosclerotic index (GSI). A score of 0 was assigned to normal glomeruli, a score of 1 if sclerosis was present in up to 25% of the glomerulus, a score of 2 if sclerosis was present in 26–50% of the glomerulus, a score of 3 if sclerosis was present in 51–75% of the glomerulus, and a score of 4 if sclerosis was present in 76–100% of the glomerulus. The GSI was calculated using the following formula (36, 37):

$$\mathrm{GSI} = \frac{[(1 \times N1) + (2 \times N2) + (3 \times N3) + (4 \times N4)]}{(N0 + N1 + N2 + N3 + N4)},$$

where N is the number of glomeruli with each given score for a given section.

Statistical Analysis

One male offspring for each of eight NPD and eight LPD litters was studied at PN21. These datasets were tested for normality and analyzed by a two-tailed unpaired t test when two groups were compared and two-way ANOVA when four groups were compared. At 6 mo of age, four groups of male rats were analyzed: NPD/NFD, NPD/HFD, LPD/NFD, and LPD/HFD (Fig. 1). These data were analyzed by two- or three-way ANOVA, as appropriate. Statistical analyses were performed using GraphPad Prism 8. Data are presented as means ± SE; n refers to number of litters. P values presented following two- or three-way ANOVA have been adjusted for multiple comparisons using Sidak correction. Statistical significance was defined as $P < 0.05$.

RESULTS

LPD Offspring Have LBW and Low Nephron Number

Litter size between NPD and LPD was controlled, and data are shown in Table 2. At *postnatal day 2*, LPD offspring weighed on average 39% less than NPD offspring ($P < 0.0001$), and this weight differential persisted at PN21 ($P < 0.0001$; Table 2). At PN21, glomerular number was, on average, 30% lower in LPD than NPD offspring ($P < 0.0001$) and glomerular number/gram body weight was 20% higher in LPD than NPD offspring ($P < 0.05$).

LPD Offspring Have Low Podocyte Endowment at PN21

On average, LPD glomeruli were approximately half the size of NPD glomeruli ($P < 0.0001$; Fig. 3*A*) and contained 15% fewer podocytes ($P < 0.0001$; Fig. 3*B*). The greater reduction in glomerular size than in podocyte number increased podocyte density by 64% in LPD glomeruli compared with NPD glomeruli ($P < 0.001$; Fig. 3*C*).

The Deficit in Podocyte Endowment in LPD Offspring at PN21 Was Most Marked in Outer Glomeruli

During kidney development, inner glomeruli develop before superficial (outer) glomeruli. We therefore analyzed

Table 2. *Summary of morphometric data at PN2 and PN21*

	Normal-Protein Diet ($n = 8$)	Low-Protein Diet ($n = 8$)	*P* Value
Litter size, *n*	6.6 ± 0.6	6.0 ± 0.5	0.324
Body weight at PN2, g	6.053 ± 0.296	3.693 ± 0.142	<0.0001*
Body weight at PN21, g	53.3 ± 1.4	31.2 ± 1.5	<0.0001*
Kidney weight at PN21, mg	426 ± 20	229 ± 24	<0.0001*
Glomerular number at PN21	33,335 ± 1,446	23,223 ± 938	<0.0001*
Glomerular number/mg kidney weight at PN21	83 ± 4	119 ± 10	<0.01*
Glomerular number/g body weight at PN21	627 ± 32	751 ± 34	<0.05*

Values are presented as means ± SE; *n*, number of rats. PN2 and PN21, *postnatal days 2* and *21*, respectively. Data were analyzed by a two-tailed unpaired *t* test. *Statistically significant difference.

glomerular and podocyte depletion indexes by the cortical zone to determine if zonal differences in podocyte endowment were present. At PN21, glomeruli in the inner cortex were larger than those in the outer cortex in both NPD (25% larger, $P < 0.05$) and LPD (45% larger, $P < 0.05$; Fig. 3*D*) offspring. NPD glomeruli in both zones contained ~150 podocytes each. In contrast, LPD offspring had only 123 ± 4 podocytes/glomerulus in the outer cortex, significantly fewer than the 141 ± 4 podocytes/glomerulus in the inner cortex ($P < 0.01$). Although podocyte endowment per glomerulus in outer glomeruli was 18% lower in LPD than NPD offspring ($P < 0.0001$; Fig. 3*E*), the 8% lower podocyte number in LPD inner glomeruli compared with NPD inner glomeruli did not reach statistical significance ($P = 0.063$; Fig. 3*E*). Podocyte density was similar in both cortical zones in NPD offspring, but in LPD offspring it was greater in the outer cortex than the inner cortex ($P < 0.05$; Fig. 3*F*).

LPD Offspring Fed the NFD From Weaning Underwent Significant Catch-Up Growth by 6 mo

Body weight for LPD and NPD offspring throughout the experimental period is shown in Fig. 4*A* LPD offspring weighed 60% less than NPD offspring at 1 wk of age. However, between 2 and 10 wk of age, LPD offspring underwent significant catch-up growth, after which their weight remained ~15% less than NPD offspring (Fig. 4*A* and Table 3). Overall, the percent weight gain between PN21 and 6 mo was 72% higher in LPD offspring than NPD offspring (Table 3). Weight gain, body weight, and body composition at 6 mo are shown in Table 3.

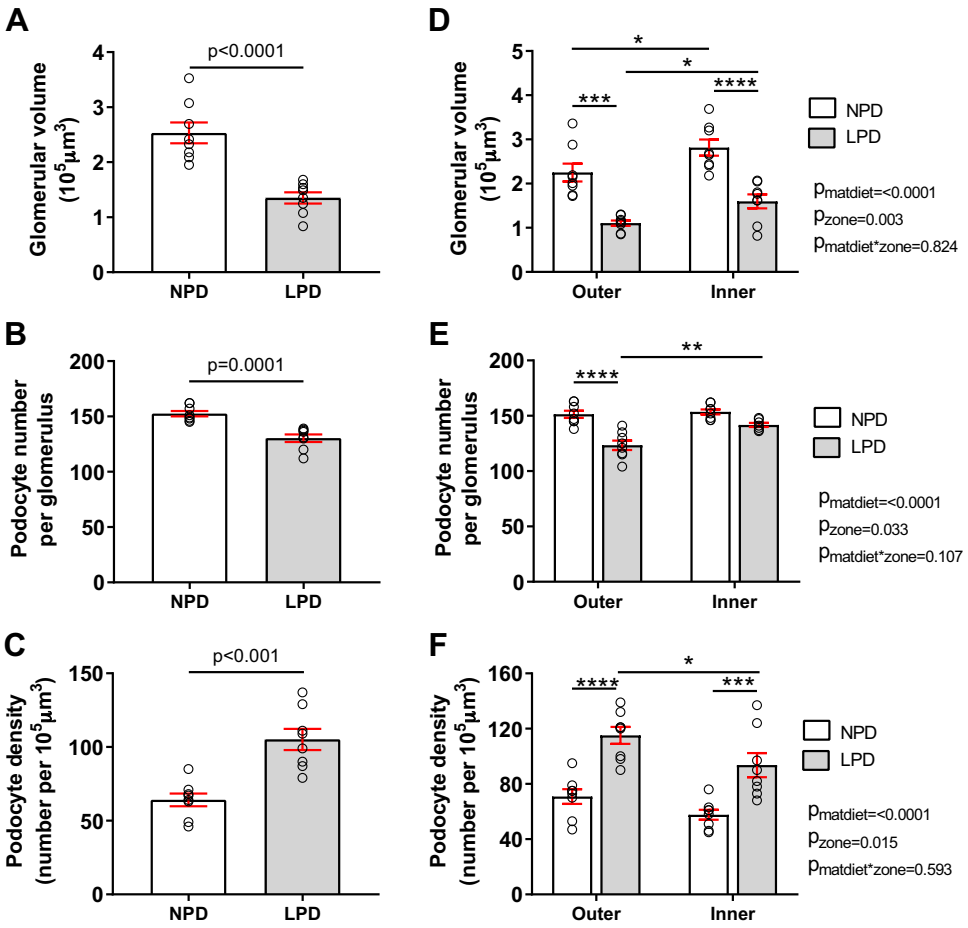

Figure 3. Morphometric data at *postnatal day 21*. Glomerular volume (*A*), podocyte number/ glomerulus (*B*), and podocyte density (*C*) in normal-protein diet (NPD) and low-protein diet (LPD) offspring. Glomerular volume (*D*), podocyte number per glomerulus (*E*), and podocyte density (*F*) in outer and inner cortical zones. Data were analyzed by a *t* test (*A*–*C*) or two-way ANOVA (*D*–*F*). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ following Sidak's correction for multiple comparisons. Values are presented as means ± SE. Each circle represents one offspring from one litter.

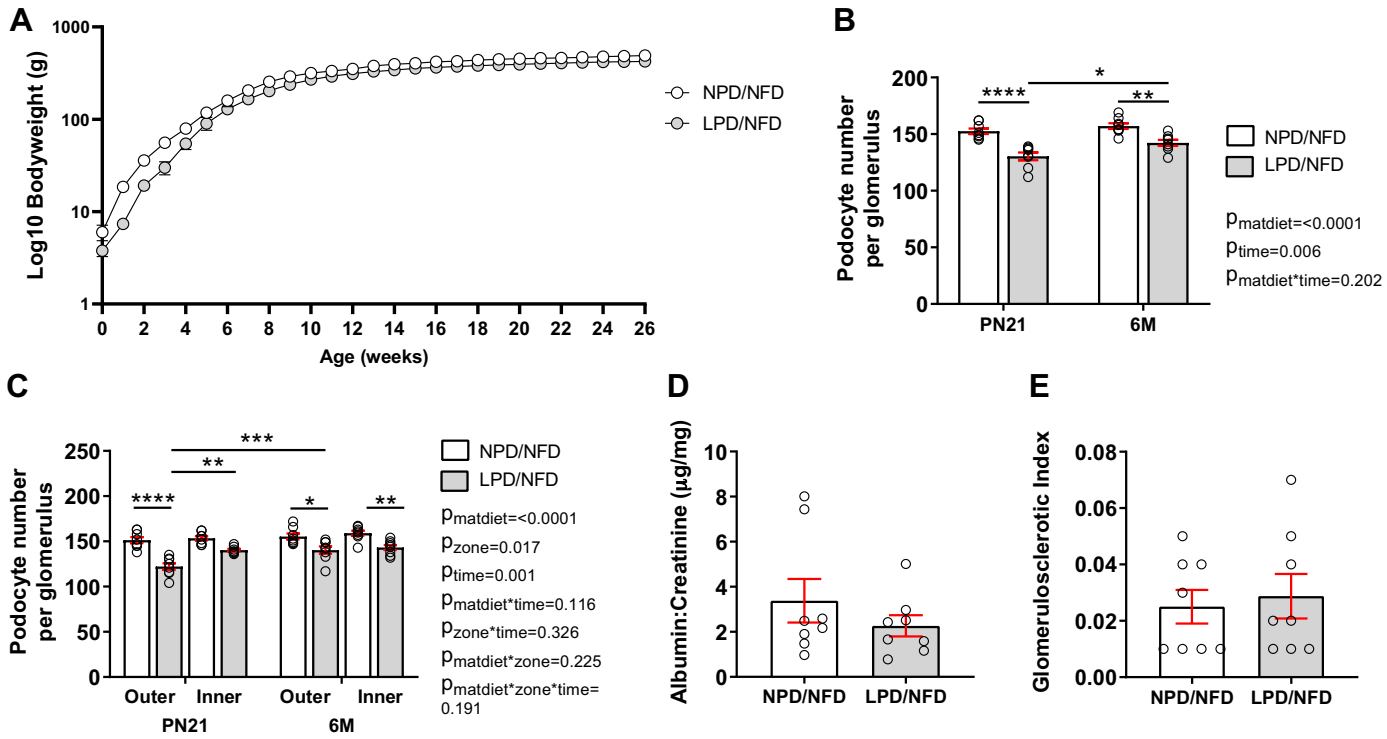

Figure 4. Catch-up growth, podocyte indexes, and renal pathophysiology at 6 mo following normal fat feeding. *A*: $\log_{10}$ body weight in low-protein diet (LPD) offspring compared with normal-protein diet (NPD) offspring throughout the experimental period. *B*–*E*: podocyte number per glomerulus (*B*), podocyte number per glomerulus by the cortical zone (*C*), albumin excretion (*D*), and glomerulosclerotic index (*E*) for maternal NPD and LPD offspring at 6 mo following normal fat feeding. Data were analyzed by two-way ANOVA (*B*), three-way ANOVA (*C*), or a *t* test (*D* and *E*). *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$ following Sidak's correction for multiple comparisons in ANOVA. Values are presented as means ± SE. Each circle represents one offspring from one litter. NFD, normal-fat diet; PN21, *postnatal day 21*.

Podocyte Number Increased in LPD Offspring Fed the NFD After PN21

Surprisingly, podocyte number increased by 9% (~12 podocytes/glomerulus) in LPD offspring between PN21 and 6 mo of age, a finding not observed in NPD offspring (Fig. 4*B*). Despite this increase, LPD offspring at 6 mo still had 9% fewer podocytes per glomerulus than NPD offspring ($P < 0.01$; Fig. 4*B*). This overall increase in podocyte number in LPD offspring was the result of an increase in podocyte number in outer glomeruli, with podocyte number in inner glomeruli remaining unchanged (Fig. 4*C*).

Table 3. *Body composition and blood lipid profiles at 6 mo of age*

	Normal-Protein Diet ($n = 8$)	Low-Protein Diet ($n = 8$)	Normal-Protein Diet/High-Fat Diet ($n = 8$)	Low-Protein Diet/ High-Fat Diet ($n = 8$)	$P_{matdiet}$	$P_{postdiet}$	$P_{matdiet \times Postdiet}$
Body weight, g	487 ± 12	424 ± 11[a]	580 ± 19[b]	505 ± 19[e,h]	0.0001*	<0.0001*	0.811
Lean mass, g	370 ± 9	349 ± 9	365 ± 11	368 ± 10	0.362	0.501	0.238
Bone mineral content, g	13.6 ± 0.3	11.9 ± 0.3[b]	15.7 ± 0.4[c]	13.8 ± 0.3[f,h]	<0.0001*	<0.0001*	0.827
Fat mass, g	106 ± 5	68 ± 4[a]	198 ± 12[d]	123 ± 10[g,i]	<0.0001*	<0.0001*	0.045*
Fat, %	22 ± 1	16 ± 1[b]	34 ± 1[d]	24 ± 1[g,j]	<0.0001*	<0.0001*	0.060
Weight gain since PN21, g	434 ± 11	394 ± 10	525 ± 18[b]	472 ± 18[h]	0.004*	<0.0001*	0.678
Weight gain since PN21, %	783 ± 20	1,345 ± 77[d]	1,002 ± 56	1,471 ± 58[g]	<0.0001*	0.0051*	0.418
Kidney weight, g	2.284 ± 0.143	2.011 ± 0.095	2.368 ± 0.164	2.206 ± 0.127	0.117	0.3106	0.684
Triglycerides, mmol/L	1.40 ± 0.15	1.35 ± 0.08	3.36 ± 0.41[c]	3.06 ± 0.43[h]	0.576	<0.0001*	0.689
Total cholesterol, mmol/L	2.575 ± 0.124	2.575 ± 0.140	3.013 ± 0.151	2.813 ± 0.208	0.534	0.0425*	0.534
Low-density lipoprotein, mmol/L	0.583 ± 0.034	0.598 ± 0.025	0.811 ± 0.063[a]	0.636 ± 0.086	0.175	0.0275*	0.110
High-density lipoprotein, mmol/L	1.351 ± 0.128	1.359 ± 0.144	0.620 ± 0.158[a]	0.824 ± 0.290	0.584	0.0025*	0.611

Values are presented as means ± SE. Data were analyzed by two-way ANOVA for maternal diet and postnatal diet. Post hoc analysis using Sidak's correction for multiple comparisons was used. $P_{matdiet}$, main effect of maternal diet; $P_{postdiet}$, main effect of postnatal diet; $P_{matdiet \times postdiet}$, interaction between maternal diet and postnatal diet. [a,b,c,d]Adjusted *P* values following multiple comparisons, where [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$, and [d]$P < 0.0001$ compared with the normal-protein diet group. [e,f,g]*P* values following multiple comparisons, where [e]$P < 0.05$, [f]$P < 0.01$, and [g]$P < 0.0001$ compared with the normal-protein diet/high-fat diet group. [h,i,j]Adjusted *P* values following multiple comparisons, where [h]$P < 0.01$, [i]$P < 0.001$, and [j]$P < 0.0001$ compared with the low-protein diet group. *Statistically significant difference.

LPD Offspring Fed the NFD After PN21 Have Normal Albumin Excretion and Renal Morphology

Despite the significant catch-up growth in LPD offspring fed the NFD between PN21 and 6 mo, albumin excretion at 6 mo was within the normal range (P = 0.32; Fig. 4*D*). Similarly, the GSI in LPD offspring was similar to NPD offspring at 6 mo of age, indicating normal renal morphology (P = 0.71; Fig. 4*E*).

Effect of High Fat Feeding on Body Composition and Lipid Profiles at 6 mo

Body composition and lipid profiles for the four groups of rats at 6 mo of age are shown in Table 3. Six months of high fat feeding increased body weight, fat mass, and percent body fat and altered plasma lipid profiles compared with offspring fed the NFD. Overall, NPD offspring fed the HFD were 15% heavier than LPD offspring fed the HFD, with 61% more body fat and 42% greater percent body fat. High-density lipoprotein was significantly lower and low-density lipoprotein significantly higher in HFD-fed NPD offspring than in littermates fed the NFD (Table 3). However, lipid profiles in LPD offspring fed the HFD were similar to those in LPD offspring fed the NFD.

High Fat Feeding Induced a Decrease in Podocyte Number, Albuminuria, and Glomerulosclerosis in NPD Offspring by 6 mo

Although NPD offspring fed the NFD to 6 mo of age had the same number of podocytes/glomerulus as at PN21, NPD offspring fed the HFD appeared to lose 15% of their podocytes by 6 mo of age (Fig. 5*A*). This podocyte loss occurred in both cortical zones (Fig. 5*B*). Fat feeding did not alter glomerular volume (Fig. 5*C*). Podocyte density was 22% lower in NPD offspring following high fat feeding than in NPD offspring following normal fat feeding (P = 0.03; Fig. 5*D*). Fat feeding in NPD offspring significantly increased albumin excretion (Fig. 5*F*) and glomerulosclerosis (Fig. 5, *G* and *H*).

High Fat Feeding in LPD Offspring Did Not Alter Podometric Indexes by 6 mo

Podocyte depletion indexes in LPD offspring fed the HFD to 6 mo of age were similar to littermates fed the NFD (Fig. 5, *A*–*D*). Although mean albumin excretion was greater in LPD offspring fed the HFD than in littermates fed the NFD, this difference was not statistically significant (P = 0.35; Fig. 5*F*). Similarly, fat feeding in LPD offspring did not alter the GSI compared with LPD offspring fed the NFD (P = 0.82; Fig. 5*G*).

Assessment of Podocyte Apoptosis and Proliferation

Immunofluorescence labeling was used to identify apoptotic and proliferating podocytes at PN21 and 6 mo. At both time points, no K_i-67$^+$ or cleaved caspase-9$^+$ podocytes were observed in any group (Supplemental Fig. S1; all Supplemental material is available at https://doi.org/10.6084/m9.figshare.14891355.v1). In the positive control, proliferation and apoptosis of proximal tubule cells was observed in rats with acute kidney injury at both time points.

Impact of Body Weight on Podocyte Density

To further assess the impact of postnatal weight gain on podometric indexes, we examined podocyte density adjusted for body weight at 6 mo. Podocyte density adjusted for body weight was 40% lower in NPD offspring fed the HFD than NPD offspring fed the NFD (Fig. 6*A*). In contrast, podocyte density adjusted for body weight in LPD offspring fed the HFD was similar to that in LPD offspring fed the NFD (Fig. 6*A*). When data from all four groups at 6 mo were examined, a strong inverse correlation between body weight and podocyte density was observed ($P < 0.01$, $r = -0.45$; Fig. 6*B*).

DISCUSSION

The main findings of this study are *1*) compared with NPD offspring, LPD offspring with LBW and low nephron number also have low podocyte endowment at PN21; *2*) podocyte number per glomerulus increased unexpectedly in LPD/LBW offspring between PN21 and 6 mo, with this increase only observed in glomeruli in the outer cortex; and *3*) although postnatal fat feeding did not alter podometric indexes or result in albuminuria or glomerulosclerosis in LPD/LBW offspring, postnatal fat feeding in NPD offspring was associated with podocyte loss, decreased podocyte density, higher body fat mass, albuminuria, and glomerulosclerosis. Thus, although we expected that the LPD/LBW offspring with low nephron and podocyte endowment would be the most susceptible to the effects of a postnatal HFD, NPD offspring fed the HFD were the only group to demonstrate albuminuria or glomerulosclerosis.

Altered maternal diet and caloric intake has been used in numerous studies to assess developmental programming in the offspring. It is well established that maternal diets low in protein induce LBW and low nephron number. Several studies have also reported ultrastructural changes to podocytes in LPD offspring (38–40). This is the first report that maternal diet (LPD) can influence the number of podocytes that form during glomerular development and maturation. Offspring of mothers fed the LPD before mating, throughout gestation, lactation, and until weaning at PN21 contained 15% fewer podocytes per glomerulus than did offspring of mothers fed the NPD. This developmental programming of lower podocyte number per glomerulus raises the following question: are other adverse maternal environments such as placental insufficiency, maternal exposure to glucocorticoids, or hyperglycemia also associated with low podocyte number? (41). To our knowledge, the only previous report of low podocyte endowment was our recent report of podocytopenia in male mouse offspring at PN21 exposed to mild maternal hypoxia from *embryonic day 14.5* to birth (42) Male hypoxic offspring had significantly lower birth weight, nephron number, and podocyte endowment than normoxic male offspring. In contrast, hypoxic female offspring had LBW, but their nephron and podocyte endowment were the same as normoxic female offspring. Interestingly, Menendez-Castro et al. (38) observed increased glomerular immunoreactivity and expression of WT-1 in intrauterine growth restriction rats and an increase in the proportion of glomerular cells with WT-1$^+$ nuclei. They concluded that this increased glomerular expression of WT-1 was more likely due to overexpression of WT-1 by podocytes than an increase

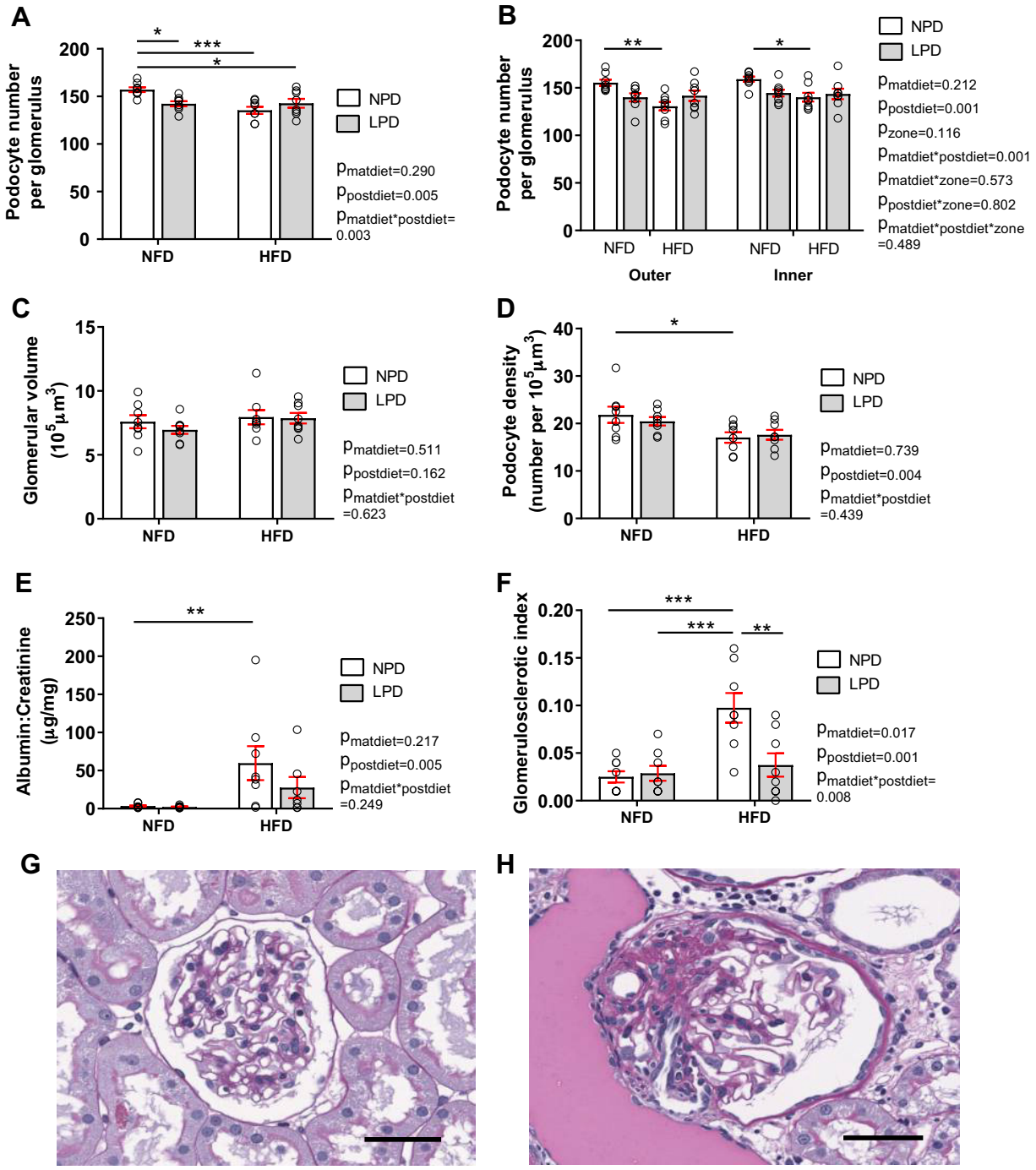

Figure 5. Podocyte indexes, albumin excretion, and glomerulosclerosis at 6 mo following high fat feeding. *P*odocyte number per glomerulus (*A*), podocyte number per glomerulus by the cortical zone (*B*), glomerular volume (*C*), podocyte density (*D*), albumin excretion (*E*), and glomerulosclerotic index (*F*) at 6 mo of age for offspring of maternal normal-protein diet (NPD) and low-protein diet (LPD) following postnatal normal-fat diet (NFD) or high-fat diet (HFD) feeding. *G* and *H*: representative images of a normal glomerulus (*G*) and a glomerulus with a segmental sclerotic lesion (*H*). Staining was by periodic acid-Schiff. Scale bar = 50 µm. Data were analyzed by two-way ANOVA (*A* and *C*–*F*) or three-way ANOVA (*B*). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ following Sidak's correction for multiple comparisons in ANOVA. Values are presented as means ± SE. Each circle represents one offspring from one litter.

in podocyte number. It is difficult to reconcile the present finding of lower podocyte endowment in LBW rats with the findings of Menendez-Castro et al. (38), but it is worth bearing in mind that the two studies used very different podocyte counting techniques. Nevertheless, further studies on these intriguing findings of podocyte endowment are warranted.

Interestingly, our data suggest that podocyte number per glomerulus increased significantly in LPD offspring between PN21 and 6 mo of age, such that the deficit in podocyte

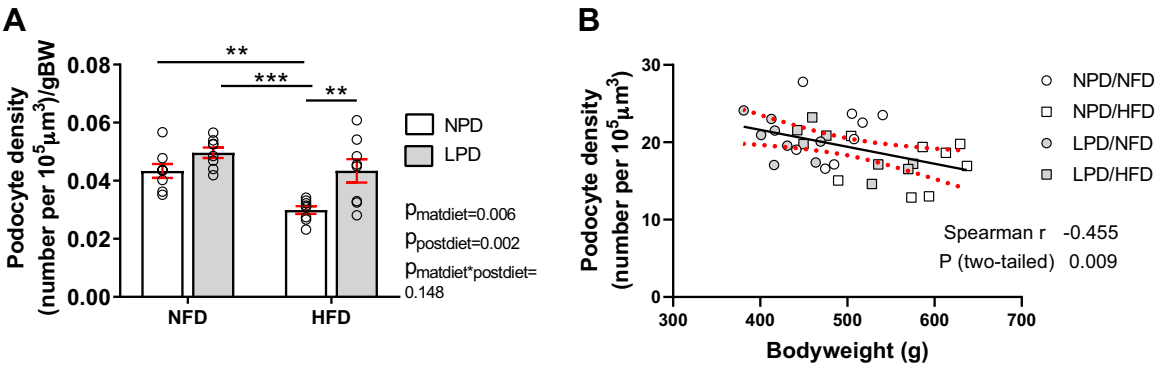

Figure 6. Impact of body mass on podocyte density. *A*: podocyte density adjusted for body weight. Data were analyzed by two-way ANOVA. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ following Sidak's correction for multiple comparisons in ANOVA. Values are presented as means ± SE. Each circle represents one offspring from one litter. *B*: correlation between podocyte density and bodyweight. Each circle or square represents one animal from one litter. HFD, high-fat diet; HPD, high-protein diet; NFD, normal-fat diet; NPD, normal-protein diet.

number per glomerulus compared with NPD offspring was now only 9%. No such increase in podocyte number was found in NPD offspring between PN21 and 6 mo, suggesting that podocyte endowment is complete in NPD offspring before PN21 as expected, but not in LPD offspring. The source of these apparent "new" or "additional" podocytes in LPD offspring remains unknown. However, given that outer glomeruli at PN21 were smaller than inner glomeruli and contained fewer podocytes, and that the increase in podocyte number at 6 mo occurred in outer glomeruli, our findings suggest that some podocytes in outer glomeruli were either not present or were not fully differentiated at PN21. It is worth keeping in mind here that during kidney development, outer glomeruli are the last to form, so possibly at PN21 in LPD offspring the outer glomeruli were demonstrating some developmental delay in terms of the completion of podocytogenesis. Our immunofluorescence analyses found no evidence of podocyte proliferation in any group at any time point. However, these analyses were only performed at two defined time points so it is possible that any podocyte proliferation in LPD offspring that occurred between 21 days and 6 mo was missed. An alternative explanation for this apparent increase in podocyte number between PN21 and 6 mo of age in LPD offspring is that new podocytes migrated to the glomerular tuft. Evidence suggests that additional podocytes can arise in postnatal life from several sources (14) including parietal epithelial cells on Bowman's capsule (43–47) and from cells of renin lineage (48, 49). Interestingly, Appel et al. (43) showed in mice that a subpopulation of parietal epithelial cells termed parietal podocytes can migrate from Bowman's capsule to the glomerular tuft in the first 12 wk of postnatal life. Perhaps this phenomenon also occurs to some degree in rats, and especially in rats born with small glomeruli that undergo marked glomerular hypertrophy in postnatal life. In the present study, LPD glomeruli at PN21 were half the size of NPD glomeruli, but by 6 mo glomeruli were the same size in all groups: the volume of NPD glomeruli increased approximately threefold between PN21 and 6 mo, whereas LPD glomeruli increased approximately sixfold in size. This finding of apparent podocyte recruitment between PN21 and 6 mo of age in LPD offspring is intriguing and can be expected to motivate new studies into the regulation and cessation of podocyte generation.

It is also worth noting at this point that at least two studies have reported increases in podocyte number in postnatal life. Olivetti et al. (6) found that young rats (defined as 35–45 g) contained on average 67 podocytes/glomerulus, whereas 35 days later this number had increased to 102 podocytes/glomerulus. In humans, Puelles et al. (50) found in four children that the median podocyte count per glomerulus was 452, whereas in 12 adults the median count was 558 podocytes/glomerulus, with the highest podocyte counts found in the largest adult glomeruli. Again, the postnatal origin of these additional podocytes remains unknown.

We initially hypothesized that low podocyte endowment may be an important mechanism that leads to the onset of podocyte-associated glomerular pathology later in life. However, our findings suggest that LPD offspring with low nephron endowment and a 9% lower podocyte endowment at 6 mo do not develop significant albuminuria or glomerulosclerosis despite accelerated early postnatal growth (catch-up growth), a known risk factor for renal disease and hypertension (51). This may be due in part to their still smaller body size (despite significant catch-up growth) and/or with the timing of our analysis, given that 6 mo is a relatively young age in rats (52). In addition, the podocyte deficit in LPD offspring was 9%, and several studies have shown that loss of <20% of podocytes results in a transient response, whereas loss of >20% of podocytes is associated with proteinuria and development of glomerular pathology (8, 11, 47). However, these previous studies differ from the present study in that here podocytes do not appear to have been lost but rather failed to develop in sufficient numbers during glomerulogenesis. Nonetheless, our results suggest that LPD offspring are able to adapt to 9% fewer podocytes to maintain renal filtration and/or the lower body size reduces the impact of the podocyte deficit. Studies of LPD offspring in older age will be important in confirming the role of podocyte endowment in the development of renal disease in the longer term.

As expected, high fat feeding from PN21 to 6 mo of age resulted in greater body weight and larger fat mass in both NPD and LPD offspring compared with offspring fed the NFD. However, although LPD offspring fed the HFD did not demonstrate significantly greater albumin excretion or glomerulosclerosis at 6 mo of age, NPD offspring fed the HFD

had the greatest body weight, fat mass, and percent body fat at 6 mo (well above that of LPD rats fed the HFD) and showed excess albumin excretion and glomerulosclerosis. Reduced podocyte density is considered a key factor in the development of albuminuria and glomerulosclerosis (1, 8, 11, 53, 54); however, in this study, both high fat-fed groups showed similar podocyte densities at 6 mo of age. Interestingly, podocyte density decreased between PN21 and 6 mo in NPD offspring fed the HFD but not in LPD offspring fed the HFD. Importantly, however, of the four groups of offspring at 6 mo, podocyte density adjusted for body weight was lowest in the NPD/HFD offspring, being almost half the value in LPD/HFD offspring. This finding may demonstrate the impact of body mass on glomerular health and function.

Podocyte number per glomerulus decreased by 15% in NPD offspring fed the HFD between PN21 and 6 mo. In contrast, podocyte number did not decrease in this period in NPD offspring fed the NFD or in LPD offspring fed the HFD, suggesting that the HFD in itself was not toxic to podocytes. It is possible that the sclerotic segments present in some glomeruli in the NPD offspring fed the HFD contributed in part to this reduction in podocyte number, although the level of sclerosis was mild. Podocyte phenotype, function, and survival are known to be susceptible to a range of perturbations including immunological factors, various cytokines and growth factors, high glomerular blood pressure, high glomerular blood flow, and hyperglycemia (1, 53, 55). Podocyte injury has also been linked to adiponectin levels (56–58) as well as lipids, inflammation, and oxidative stress (59–61), all characteristics of obesity and metabolic syndrome. However, we found no evidence of podocyte apoptosis in any group at any time point.

The protection of podocytes in LPD offspring against HFD-induced damage and loss is plausible given previous studies have shown offspring suckled by a dam fed a protein-restricted diet have elevated levels of sirtuin 1 (62), which has been shown to protect podocytes and prevent glomerular injury (63, 64). In addition, Martin-Gronert et al. (62) reported an increase in antioxidant enzymes in offspring exposed to LPD in the early postnatal period, with offspring being less albuminuric (62), a finding consistent with those of Petry et al. (65). The increase in antioxidant enzymes in this LPD model was also associated with a lack of telomere shortening in the kidney with age (66), perhaps indicating that increased expression of antioxidant enzymes in kidneys of LPD offspring provides protection from oxidative stress, age-related telomere shortening, and age-associated renal damage, a finding that may also apply to HFD-induced renal injury. It is important to note that 34% of the bodyweight in NPD offspring was composed of fat compared with only 24% in LPD offspring following high fat feeding. NPD offspring fed the HFD also showed increased low-density lipoprotein and reduced high-density lipoprotein levels, indicating NPD offspring were more vulnerable to the HFD in this study. Future studies to investigate the basis of this podocyte loss in normal weight offspring following high fat feeding are warranted given the current epidemic of obesity and metabolic disease.

The present findings, albeit in rats, suggest that babies born with LBW and low nephron endowment may also have low podocyte endowment. If this permanent nephron deficit occurs in combination with a permanent podocyte deficit, this would indeed be a double hit for those born small.

Whether LBW babies, like the LPD offspring in the present study, increase their podocyte endowment in the first months or years of postnatal life is unknown, although the findings of Puelles et al. (50) presented above suggest this might be possible.

Most recently, Haruhara et al. reported nephron number and podocyte number in the same human kidneys for the first time. The number of nonsclerotic glomeruli per kidney tended to be directly correlated with podocyte number per glomerulus, although this did not reach statistical significance ($P = 0.055$), most likely due to the relatively small sample size. Nevertheless, the data suggested that those subjects with low nephron endowment also had low podocyte number. Additional studies on the relationship between nephron number and podocyte number are certainly warranted, including whether any such associations are developmentally linked.

This study has several limitations. First, podocyte identification was primarily based on immunostaining for specific markers (WT-1 and SNP). If for any reason the expression of these markers was influenced by the diets, this could have influenced our data and findings, and, importantly, as mentioned above, if these markers are not present in immature podocytes these would not have been counted. In future studies, genetic cell tracing could be used to avoid any such issue. Second, if analyses had been performed at later time points than 6 mo, the effects of the HFD on albuminuria and glomerulosclerosis may have been more marked or differentiated between the LPD and NPD groups. Additional physiological stressors such as uninephrectomy or diabetes may also have revealed phenotypic differences. Finally, the analyses of podocyte apoptosis and proliferation were only conducted at two time points (PN21 and 6 mo). Any podocyte apoptosis or proliferation occurring at other time points would not have been detected.

In conclusion, this study has revealed novel insights into the complexity of the interplay between maternal and postnatal diets, podocyte number and density, birth weight and postnatal weight, and the risk of glomerular pathology in adulthood. We expect our findings will motivate future studies into podocytogenesis and links with adult disease.

SUPPLEMENTAL DATA

All Supplemental Material: https://doi.org/10.6084/m9.figshare.14891355.v1.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.A.C., F.H., J.C. Y.O., J.A.A., M.J.B., W.E.H., V.G.P., and J.F.B. conceived and designed research; L.A.C., J.v.W., K.H., L.T., J.P.D., F.H., J.C., Y.O., W.E.H., and J.F.B. performed experiments; L.A.C., M.G.B., and R.D. analyzed data; L.A.C., F.H., J.A.A., M.J.B., V.G.P., and J.F.B. interpreted results of experiments; L.A.C. prepared figures; L.A.C. drafted manuscript; L.A.C., J.v.W, F.H., J.C., J.A.A., M.J.B., V.G.P., and J.F.B. edited and revised manuscript; L.A.C., J.v.W., K.H., L.T., J.P.D., M.G.B., R.D., F.H., J.C., Y.O., J.A.A., M.J.B., W.E.H., V.G.P., and J.F.B. approved final version of manuscript.

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