The Effect of Intra-Uterine Environment and Low Glomerular Number on the Histological Changes of Diabetic Glomerulosclerosis

Running title: Intrauterine environment, glomerular number and diabetic nephropathy

Subject

Susan E Jones, Kathryn E White, Allan Flyvbjerg*, Sally M Marshall

Corresponding author: Professor Sally M Marshall
Diabetes Research Group
School of Clinical Medical Sciences
Floor 4, Leech Building
The Medical School
Framlington Place
Newcastle upon Tyne
NE2 4HH
UK

Telephone: +44 191 222 7019
Fax: +44 191 222 0723
Email: s.m.marshall@ncl.ac.uk

Affiliations: Diabetes Research Group, School of Clinical Medical Sciences, University Of Newcastle upon Tyne, UK; * Department of Experimental Medicine, University of Aarhus, Denmark.

Word count: 3754
ABSTRACT
We tested the hypothesis that diabetic individuals with fewer glomeruli are at increased risk of diabetic nephropathy than individuals with normal glomerular number in an experimental model. Female offspring of Wistar rats fed 6% low protein diet (LPD) or 18% normal protein diet (NPD) during pregnancy were studied. Streptozotocin diabetes was induced at 12 weeks and animals sacrificed at 40 weeks. Non-diabetic LPD animals were of lower birthweight than NPD animals (5.19±0.64 vs 6.45±0.67 g, p<0.001) and had fewer glomeruli (27402±3137 vs 34203±6471, p<0.05). Glomerular volume correlated inversely with glomerular number (r= -0.64, p=0.035) but total glomerular filtration surface area was reduced in the LPD animals (4770±541 vs 5779±1302 mm², p=0.05). Other renal structural and functional parameters were similar. Glomerular volume and basement membrane width were significantly increased in the LPD and NPD diabetic animals compared to their respective controls. However, there was no relationship between glomerular number and volume (r= -0.38, p=0.03). Podocyte density was lowest in the LPD diabetic animals and the area covered by each podocyte was greater in the LPD diabetic group (2.40±0.693 x10⁻³ mm²) compared to the LPD control (1.68±0.374 x10⁻³ mm², p<0.001) and the NPD diabetic animals (1.71±0.291 x10⁻³ mm², p<0.05). Thus a decrease in glomerular number with compensatory glomerular hypertrophy is not deleterious to renal structure and function. However, when diabetes is superimposed, the expected relationship between glomerular number and volume is lost and podocyte abnormalities are apparent. In the long term, these may accelerate the development of diabetic glomerulosclerosis.

Word count 248
KEYWORDS

Intrauterine environment; glomerular number; diabetic glomerulosclerosis; nephrin; podocyte; streptozotocin diabetes
INTRODUCTION
The Barker hypothesis suggests that an intra-uterine insult, leading to low birth weight, predisposes to the development of chronic diseases such as hypertension, cardiovascular disease and diabetes (1,2). Animals of low birth weight (LBW) induced by protein deprivation in utero have smaller kidneys with reduced numbers of glomeruli compared to animals of normal birth weight (NBW:3,4). Although some compensatory hypertrophy of the remaining nephrons occurs, with an increase in intraglomerular pressure, it is likely that total glomerular filtration surface area remains low. Hypertension is more common in individuals of low birth weight (1,2), probably arising because of a reduction in the glomerular filtration surface area (5). This combination of reduced glomerular number, reduced filtration surface area and intraglomerular and systemic hypertension results in an increased incidence and accelerated progression of non-diabetic renal diseases (6,7,8).

Diabetic nephropathy is characterised by progressive rises in urinary albumin excretion rate (UAER) and blood pressure, with declining glomerular filtration (GFR) and eventually glomerulosclerosis (9). Increased intraglomerular pressure is pivotal (10) and probably interacts with the metabolic abnormalities to drive the development of glomerulosclerosis. All components of the glomerular filtration barrier are susceptible to damage. There is accumulation of basement membrane and mesangial matrix, widening and effacement of the foot processes of podocytes, and loss of podocytes. The decline in glomerular filtration rate correlates with the reduction in glomerular filtration surface area (11).

Although there is a genetic propensity in the development of diabetic nephropathy, environmental and intra-uterine factors may also play a role. We hypothesize that
individuals of low birth weight, and thus reduced glomerular number with increased intraglomerular and systemic hypertension, who develop diabetes will be at increased risk of nephropathy which will progress more rapidly than individuals of normal birth weight. There is some support for this, in that several studies have shown that Type 1 diabetic men with nephropathy (12) and men and women with microalbuminuria (13) are shorter than their peers without nephropathy, adult height being used as a surrogate for low birth weight. To test this hypothesis we have used a rat model of low birth weight and reduced glomerular number, achieved by protein deprivation in utero. In a short-term study, we have demonstrated previously that the acute renal adaptation to diabetes is altered in animals of low birth weight after one week of diabetes (14). We now explore the long-term consequences of diabetes in the same experimental model of low birth weight/ reduced glomerular number.
METHODS

Animal husbandry
Animal experiments were carried out in accordance with the 1986 UK Home Office Regulations. Female Wistar rats (Charles River, Kent, UK) were fed either 6% (low protein diet; LPD) or 18% casein diet (normal protein diet; NPD; Usine d’Alimentation Laboratories, France) for one week before timed-mating and during pregnancy. Diets were isocaloric with a similar source of protein, carbohydrate and lipid. The LPD diet contained supplemental methionine (0.3 vs 0.23 %).

Female offspring were weighed at birth and cross-fostered to NPD periparturient lactating dams so that the rats were only exposed to LPD in utero. Following weaning, at 21 days, rats were housed in cages at 21 ± 2°C with a cycle of 12 h light (0600-1800 h), and free access to water and standard rat chow.

Pre-diabetes procedures
Body weight was measured weekly from weaning until 12 weeks of age. UAER and fodder consumption were estimated at weeks 4, 8 and 12. Systolic blood pressure (SBP) was estimated at the same time points using an indirect tail cuff method.

Diabetes study
At 12 weeks of age, LPD and NPD rats were randomly assigned to Control (C) or Diabetic (D) groups. Diabetes was induced by intravenous injection of streptozotocin, 50 mg/kg body weight. During the first week of diabetes, weight and tail vein blood glucose [Accutrend meter (Roche Diagnostics, Welwyn Garden City, UK)] were measured between 0900 and 1000 h. Thereafter, weight, tail vein blood glucose and urinalysis for glucose and ketones were performed weekly with UAER, fodder
consumption and SBP estimated every four weeks. At 40 weeks of age (28 weeks post-induction of diabetes), tail vein blood was taken for fructosamine and creatinine estimation for the calculation of creatinine clearance.

**Perfusion fixation**

At 28 weeks post-diabetes, anaesthesia was induced using a cocktail of 1 ml Hypnorm (Janssen Pharmeutica, Beerse, Belgium) and 1 ml midazolam (5mg/ml; Roche products Ltd, Welwyn Garden City, UK) diluted in 2 ml water for injection, at a dose of 0.27 ml/100 mg body weight. The right kidney was removed and frozen in liquid nitrogen. Total body perfusion was performed using 4% phosphate buffered formalin and 1% glutaraldehyde. The left kidney was removed, stripped of its capsule and weighed before storage in 4% phosphate buffered formalin at 4°C. Eight kidneys from each group were processed for light and electron microscopy.

**Light microscopy**

Each left kidney was cut into 2 mm slices using a razor blade fractionator (15). Alternate slices were dehydrated in graded alcohol and embedded in methacrylate resin. Blocks was serially sectioned at 20μm intervals using a Universal Reichert Jung microtome. Every 10th section and its adjacent section was selected for the estimation of glomerular number using the disector fractionator technique (16). Sections were stained with PAS and viewed using an Olympus light microscope with video camera attachment.
Estimation of glomerular number

One section from each pair was designated the reference section and the other the look-up section. The reference section was moved systematically in 5 mm steps on the microscope stage and each image captured with the camera and Image-Pro software. The corresponding areas in the look-up slide were found and the two images viewed side by side on the computer screen at a magnification of x85. An unbiased 2-dimensional counting frame was superimposed on the images and the number of glomeruli appearing in the reference but not the look-up section counted. To improve efficiency, the roles of the reference and look-up sections were then reversed. The total glomerular number (GN) per kidney was then calculated from:

\[ GN = f_1 / f_1 * f_2 * f_a * (\Sigma Q - \Sigma P_f) / 2 \]

Where \( f_1 \) = sampling fraction 1; \( f_2 \) = sampling fraction 2; \( f_a \) = sampled area; \( Q \) = number of glomeruli counted. The numerical density of glomeruli (\( N_V \)) was calculated from:

\[ N_V = (\Sigma Q / 2) / a * t * P_f \]

Where \( P_f \) = points on reference area; \( a \) = area represented by \( P_f \); \( t \) = section thickness.

Estimation of glomerular volume

One slide from each pair was selected randomly and areas sampled as above. A grid of coarse and fine points was superimposed and the number of coarse points hitting the reference area (\( P_{\text{kid}} \)) and fine points hitting glomeruli (\( P_{\text{glom}} \)) counted. The glomerular volume density \( V_V \) was calculated from:

\[ V_V = P_{\text{glom}} / P_{\text{kid}} * R \] where \( R \) = ratio of coarse to fine points

Mean glomerular volume (MGV) was calculated from the formula:

\[ MGV = V_V / N_V \]
Electron microscopy

Small pieces of cortical tissue (1 mm³) were post-fixed in osmium tetroxide, dehydrated in graded alcohol and embedded in epoxy resin. Ultrathin sections were taken from three glomeruli per animal, stained with uranyl acetate and lead citrate, and examined using a Philips CM100 electron microscope. The whole glomerular profile was photographed for the estimation of mesangial volume fraction (V$_{\text{mes/glom}}$) by point counting (17), podocyte number by the method of Weibel (18, 19) and surface densities by line intercept method (20). To obtain absolute surfaces, densities were multiplied by glomerular volume. The surfaces measured were the filtration surface (peripheral basement membrane), mesangio-capillary and mesangio-urinary surfaces. The sum of the filtration and mesangio-urinary surfaces were calculated in order to estimate the total surface area of glomerular basement membrane (GBM) underlying the podocytes. Higher magnification micrographs were systematically randomly sampled for the estimation of GBM width, and foot process width (FPW) (17, 21).

Laboratory Methods

Aliquots of 24 hour urine were stored at −40°C before estimating the urine albumin concentration by single-antibody radio-immunoassay with polyethylene glycol precipitation (22). The intra-assay and inter-assay coefficients of variation were less than 5% and 10%, respectively. Serum and urine creatinine were measured by an automated Jaffé reaction, with correction for the prevailing glucose concentration. Fructosamine was assayed using the Fructosamine Test Plus (Hoffman-La Roche, Basel, Switzerland), as described previously (23).
Real-Time Quantitative PCR (RT-QPCR)

After homogenization, total cellular RNA was extracted from renal cortical tissue using a 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA). Good RNA quality was ensured in all samples on a 0.5 % agarose gel. Reverses transcription from RNA to cDNA was performed with a MultiScribe Reverse Transcription kit. Each RT-QPCR reaction was performed using 1 µg total RNA and 25 µl TaqMan Universal PCR MasterMix (4304437 AB) prepared according to protocol. The forward, reverse and TaqMan middle primes for rat nephron (Rn00575235_ml AB) and 18S (4319413 AB; housekeeping gene) were purchased from Applied Biosystems. Forty cycles at 95 °C for 15 seconds and 60 °C for 1 minute were performed with an ABI Prism 7000 Sequence Detection System from Applied Biosystems. All experiments were carried out in triplicate; three readings for each individual sample were made and the average calculated. The relative RNA amount was calculated as \( \Delta Ct = Ct (\text{target sample}) - Ct (18S \text{ sample}) \) and the relative amount expressed as \( 2^{-(\Delta Ct)} \).

Statistical analyses

Albumin excretion rate was log transformed before analysis to correct for skewed distribution. Values are given as mean±SD or median (range). Comparisons between groups were carried out using one-way analysis of variance (ANOVA) with the Bonferroni correction for multiple corrections. A two-tailed \( p \) value <0.05 was considered statistically significant. All analyses were performed using SPSS version 11.
RESULTS

Effects of Low Protein Diet - Comparison of Control LPD and Control NPD

Animals

LPD animals were of significantly lower birth weight than NPD animals (Table 1) and the weight of the LPD control animals remained lower than that of the NPD control rats throughout the study (Table 2 and Figure 1a). Fodder consumption, SBP and UAER were similar in the LPD and NPD groups throughout (Figure 1c). Creatinine clearance at the end of the study was similar (Table 2).

Kidney weight and kidney weight:body weight ratio were similar in LPD and NPD control animals (Table 3). Glomerular number was significantly reduced in the LPD animals (Table 3), with a significant correlation between birth weight and glomerular number in the combined cohort of LPD and NPD animals (Figure 2; r=0.5; p=0.002). There was also a significant negative correlation between mean glomerular number and mean glomerular volume (r=-0.64, p=0.035, Figure 3a). Mean glomerular volume, GBM width, volume fraction of mesangium and mesangio-urinary surface and the filtration surface area per glomerulus of peripheral GBM were all similar in LPD and NPD control animals (Table 3). However, the total glomerular filtration surface area per kidney (GBM surface area X number of glomeruli) was lower in the LPD animals (4770±541 vs 5779±1302 mm$^2$; p=0.05).

Podocyte number, density and the area covered by each podocyte were similar in NPD and LPD control animals, as were foot process width on mesangium and GBM and nephrin mRNA.
**Effects of Diabetes – Comparison of Control and Diabetic Animals within Each Diet Group**

Body weight in LPD and NPD diabetic animals was significantly lower, and fodder consumption significantly higher, than in their respective control groups (Table 2 and Figure 1a). Blood glucose throughout the study (Figure 1b) and serum fructosamine at the end of the study (Table 2) were significantly higher than in the control animals. UAER increased throughout the study, and creatinine clearance were significantly increased in the 2 groups of diabetic animals (Table 2 and Figure 1c). Blood pressure did not change. Kidney weight and kidney weight:body weight ratio were higher in both diabetic groups (Table 3). Mean glomerular volume was increased in the diabetic groups compared to their respective controls (p<0.05) as was GBM width, although this difference did not reach statistical significance (p=0.06 for both; Table 3). In contrast to the relationship found in non-diabetic animals, there was no correlation between glomerular number and mean glomerular volume in the diabetic animals (Figure 3b; r= -0.38, p= 0.2). GBM width correlated with UAER at week 28 of diabetes in both LPD (r=0.53, p= 0.008) and NPD animals (r= 0.42, p= 0.046). The greater mesangio-urinary surface area in the diabetic animals was significant in the LPD group only, suggesting that changes were occurring in the mesangium.

The absolute number of podocytes was not altered by diabetes (Table 3). However, because of the increase in glomerular volume, podocyte density was lower (ANOVA P=0.024) and the total surface covered by the podocytes greater (ANOVA P=0.046) in the diabetic animals, although the differences between individual groups did not reach conventional statistical significance. The area covered by each podocyte was increased in the LPD diabetic animals compared to their respective control group.
Foot process width on mesangium and GBM was similar in diabetic and control animals, as was nephrin mRNA.

**Interaction of Diabetes and LP: Comparison of LPD and NPD Diabetic Animals**

Body weight was significantly lower in the LPD diabetic animals compared to the NPD diabetic animals (Table 2 and Figure 1a). There were no differences in fodder consumption, serum fructosamine, blood pressure, UAER, and creatinine clearance between LPD and NPD diabetic animals. Kidney weight was significantly lower in the LPD diabetic animals, but kidney weight:body weight ratio was similar. By design, glomerular number was significantly lower in the LPD diabetic animals. Mean glomerular volume was similar (1.84±0.24 vs 1.69±0.22 x10⁶ µm³, p=0.271) and no differences were found in GBM width, volume fraction mesangium and mesangio-urinary surface or podocyte number (138±37 vs 174±35, p=0.095). Podocyte density was reduced (ANOVA P=0.024), although the difference in podocyte density between the LPD and NPD diabetic animals did not reach significance (95±29 vs 133±35 per 10⁶ µm³, p=0.305). However, the area covered by each podocyte was significantly greater in the LPD diabetic compared to NPD diabetic animals (2.40±0.693 vs 1.71±0.281 x10⁻³ mm², p<0.05). In the LPD diabetic animals, podocyte density (r=-0.597, p=0.002) and area covered by each podocyte (r=0.481, p=0.017) correlated with UAER at 28 weeks diabetes. No such relationships were seen in the NPD diabetic animals (density r=-0.255, p=0.24; area r=0.269, p=0.214). Foot process width on mesangium and GBM and nephrin mRNA were similar in diabetic LPD and NPD animals.
In the whole cohort of animals, UAER at 28 weeks diabetes correlated with mean glomerular volume ($r=0.67$, $p<0.001$) and GBM width ($r=0.50$, $p<0.001$). There was also a significant relationship between mean glomerular volume and creatinine clearance ($r=0.48$, $p<0.05$).
Discussion

As expected from previous studies (24), animals fed LPD in utero were of lower birth weight and had fewer glomeruli than animals fed NPD. Protein restriction in pregnancy is associated with increased apoptosis of mesenchymal cells at the start of rat metanephrogenesis, so that fewer precursors are available to differentiate into nephrons (25). Angiotensin may also play a role, as renal expression of the angiotensin II type 2 receptor is reduced in animals fed LPD in utero, with a greater and more prolonged pressor response to infusion of angiotensin II (26).

Glomerular number was reduced by approximately 20%. The interrelationship of glomerular number and volume is supported by the correlations between glomerular number and mean glomerular volume. Despite this attempt at compensatory glomerular hypertrophy, total kidney filtration surface area was reduced in the LPD control animals. However, at 40 weeks of age, no deleterious effects were observed on renal function or structure. Thus a reduced number of glomeruli is not deleterious per se to the kidney, at least for the duration of this study. It is likely that intraglomerular pressure is increased in the LPD animals. However, glomeruli appear to be able to resist this insult for some time. A parallel may be drawn with renal transplant donors, where longitudinal studies have shown no adverse effects in the early years after nephrectomy (27) but an increased prevalence of microalbuminuria and hypertension after 12-31 years (28).

Individuals of birth weight <2.5 kg are at increased risk of developing end-stage renal disease (7,29) or albuminuria (6) compared to those of birth weight >2.5 kg. Low birth weight, with fewer glomeruli, may provide a partial explanation for the excess end-stage renal disease in African Americans compared to Caucasians (7). However,
a second insult may be necessary before the detrimental effects of the reduced nephron mass become apparent. Congenital nephron deficits may interact with environmental and genetic factors to generate glomerulosclerosis (30). In children with nephrotic syndrome (31) or IgA nephropathy (32), those with intrauterine growth retardation had higher blood pressure and more rapidly progressive disease than those of normal birth weight.

Our LPD streptozotocin-diabetic animals were subjected to such a second insult. The LPD and NPD diabetic animals showed the classical functional and structural changes associated with early human and experimental diabetic glomerulosclerosis. UAER, creatinine clearance, mean glomerular volume and basement membrane thickness were all increased, and mesangio-urinary surface area and volume fraction of mesangium non-significantly. Changes in these parameters were generally similar in the LPD and NPD diabetic animals. However, podocyte density was lower and the average area covered by each podocyte higher in the LPD diabetic animals compared to the NPD diabetic group. This would suggest that the podocyte is particularly sensitive to the double insult of reduced nephron mass and diabetes.

The podocyte is sensitive to both pressure (stretch) and metabolic perturbations. When differentiated mouse podocytes are subjected to mechanical stress, foot processes become thin and elongated due to a reversible re-organization of the actin cytoskeleton (33). Mechanical stretch also induces changes in cell cycle regulatory proteins leading to decreased growth (34) and reduced expression of the β1 subunit of the α3β1 integrin in the foot process (35). A reduction in nephron number and the induction of diabetes both elevate intraglomerular pressure, so it is likely that
intraglomerular pressure in the LPD diabetic animals was significantly higher than in the NPD animals. This increased pressure may at least in part account for the changes in the podocyte in the LPD diabetic animals.

Hyperglycaemia also has structural and functional effects on podocytes. In cultured cells and in vivo, there is reduced expression of the $\beta_1$ subunit of the $\alpha_3\beta_1$ integrin (35,36), the combination of mechanical stretch and hyperglycaemia having an additive effect (36). A reduction in nephrin mRNA and protein has been reported in advanced human diabetic nephropathy with proteinuria and hypertension (37,38) and in experimental models with both diabetes and hypertension (39,40). Reductions in nephrin are much less in patients with microalbuminuria (37) and are not observed in animal models without hypertension (39). Thus the lack of change in nephrin mRNA in our current study is not surprising.

Thus hyperglycaemia, in addition to haemodynamic factors, has contributed to the podocyte changes seen in our LPD diabetic animals. Significant correlations between both podocyte density and the area covered by each podocyte with urine albumin excretion were observed only in the LPD animals, suggesting that podocyte damage does contribute to the proteinuria of diabetic glomerulosclerosis. Morphological changes, including foot process broadening and effacement and eventually a reduction in podocyte number are recognized (41-44).

An inverse correlation was observed between glomerular number and mean glomerular volume in control animals, a reflection of compensatory enlargement in animals with fewer glomeruli in an attempt to increase the total available glomerular
filtration surface area. Such a relationship was not seen in the diabetic animals. This suggests that in the LPD animals, glomerular changes had already occurred so that the glomerular hypertrophy expected in response to hyperglycaemia was in some way attenuated. Over a prolonged period of time, this inability of the glomerulus to expand further in response to diabetes may be deleterious, preventing the glomerular tuft to accommodate increasing mesangial matrix accumulation.

The finding that animals of low birth weight and thus a reduced number of glomeruli develop podocyte abnormalities when diabetes is induced may have implications for human diabetes. Indeed, one explanation for the very high prevalence and rapid progression of diabetic nephropathy in Pima Indians may be the co-existence of glomerulomegaly and glomerulopenia (45).

In conclusion, animals of low birth weight have reduced number of glomeruli and total glomerular filtration surface area. However, over the duration of this study, renal structural or functional abnormalities did not develop. When a second renal insult, diabetes, is added, the expected relationship between glomerular volume and number is lost, suggesting that glomerular response to hyperglycaemia is abnormal. Perhaps because of this and the greater increase in intraglomerular pressure, podocyte changes are more marked in low birth weight diabetic than normal birth weight animals. Podocyte density is decreased and the area covered by each podocyte increased. Long-term, this stress on the podocyte may lead to podocyte loss and accelerate the development of diabetic glomerulosclerosis.
Acknowledgements

The study was funded by project grants from Diabetes UK, the Danish Diabetes Association and the Danish Medical Research Council. We are grateful to Denise Reid and Rob Stewart for their excellent animal husbandry, and to Heather Gilbert, Karen Mathiassen, Kirsten Nyborg, Vivian Thompson and Tracey Davey for technical help.
References


42. White KE, Bilous RW on behalf of the Diabiopsies Study Group. Structural alterations to the podocyte are related to proteinuria in type 2 diabetic patients. *Nephrol Dial Transplant* 19;1437-1440, 2004


Figures

1. Changes in body weight (A), blood glucose (B) and urinary albumin excretion rate (C) throughout the study in female offspring of animals fed normal protein diet (NPD) or low protein diet (LPD) during pregnancy. Diabetes was induced at 12 weeks.
2. Relationship of birth weight and glomerular number in female offspring of animals fed normal protein diet (NPD) or low protein diet (LPD) during pregnancy
3. Relationship of glomerular number and mean glomerular volume in control and diabetic groups of female offspring of animals fed normal protein diet or low protein diet during pregnancy.
4. Relationship of podocyte density and urine albumin excretion rate in female offspring of animals fed low protein diet (LPD; A) or normal protein diet (NPD; B) during pregnancy.
Table 1 Pre-Diabetes Data

<table>
<thead>
<tr>
<th></th>
<th>Low Protein Diet</th>
<th>Normal Protein Diet</th>
<th>ANOVO P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
<td></td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>5.19±0.64**</td>
<td>4.68±0.67***</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bodyweight (g) at 12 weeks</td>
<td>233±18.1*</td>
<td>235±13.7*</td>
<td>0.004</td>
</tr>
<tr>
<td>SBP (mmHg) at 12 weeks</td>
<td>122±9</td>
<td>120±10</td>
<td>0.180</td>
</tr>
<tr>
<td>UAER (mg/24 h)</td>
<td>0.28 (0.12-1.45)</td>
<td>0.46 (0.12-1.68)</td>
<td>0.164</td>
</tr>
</tbody>
</table>

Mean±SD or median (range)
* P<0.05 vs respective NPD group
*** P<0.001 vs respective NPD group
SBP systolic blood pressure
UAER urine albumin excretion rate
Table 2 Metabolic parameters after 28 weeks diabetes (study week 40)

<table>
<thead>
<tr>
<th></th>
<th>Low Protein Diet</th>
<th>Normal Protein Diet</th>
<th>ANOVO P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
<td>Control</td>
</tr>
<tr>
<td>Bodyweight (g) week 40</td>
<td>333±19.9*</td>
<td>234±20.8**a</td>
<td>353±56.7</td>
</tr>
<tr>
<td>Fodder consumption (g)</td>
<td>21.3±3.6</td>
<td>45.5±5.1**a</td>
<td>20.5±3.1</td>
</tr>
<tr>
<td>Fructosamine (mmol/l)</td>
<td>296±54</td>
<td>362±69**a</td>
<td>301±26</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>111±16</td>
<td>110±10</td>
<td>111±11</td>
</tr>
<tr>
<td>UAER (mg/24 h)</td>
<td>2.94 (0.40-68.4)</td>
<td>27.5a (2.51-99.1)</td>
<td>2.62 (0.37-45.0)</td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>49±7</td>
<td>45±10</td>
<td>49±6</td>
</tr>
<tr>
<td>CrCl (ml/min)</td>
<td>1.5±0.4</td>
<td>2.6±0.6a</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>Nephrin mRNA</td>
<td>1.005±0.197</td>
<td>1.542±1.034</td>
<td>1.004±0.150</td>
</tr>
</tbody>
</table>

* P<0.05 vs respective NPD group
a P<0.001 vs respective control group
SBP systolic blood pressure
UAER urine albumin excretion rate
CrCl creatinine clearance
Table 3 Renal Parameters

<table>
<thead>
<tr>
<th></th>
<th>Low Protein Diet</th>
<th>Normal Protein Diet</th>
<th>ANOVO</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.93±0.08</td>
<td>1.53±0.15*a</td>
<td>1.02±0.17</td>
<td>1.83±0.24a</td>
</tr>
<tr>
<td>Kidney weight: body weight ratio</td>
<td>0.273±0.024</td>
<td>0.656±0.070*a</td>
<td>0.287±0.031</td>
<td>0.672±0.062a</td>
</tr>
<tr>
<td>Glomerular number</td>
<td>27402±3137*</td>
<td>26971±3540*</td>
<td>34203±6471</td>
<td>33615±2875</td>
</tr>
<tr>
<td>Mean glomerular volume (x10^6 µm³)</td>
<td>1.38±0.23</td>
<td>1.84±0.24*a</td>
<td>1.20±0.35</td>
<td>1.69±0.22a</td>
</tr>
<tr>
<td>GBM width (nm)</td>
<td>263±28</td>
<td>294±13*b</td>
<td>272±18</td>
<td>304±26*b</td>
</tr>
<tr>
<td>Vvmes/glom</td>
<td>0.168±0.029</td>
<td>0.181±0.022</td>
<td>0.160±0.014</td>
<td>0.175±0.030</td>
</tr>
<tr>
<td>Mes-urinary surface (mm²)</td>
<td>0.070±0.014</td>
<td>0.102±0.023a</td>
<td>0.075±0.019</td>
<td>0.094±0.020</td>
</tr>
<tr>
<td>Podocyte number</td>
<td>157±42</td>
<td>138±37</td>
<td>163±26</td>
<td>174±35</td>
</tr>
<tr>
<td>Podocyte density (/10^6 µm³)</td>
<td>136±38</td>
<td>95±29</td>
<td>158±48</td>
<td>133±35</td>
</tr>
<tr>
<td>Surface underlying podocytes (mm²)</td>
<td>0.252±0.030</td>
<td>0.314±0.056</td>
<td>0.254±0.046</td>
<td>0.294±0.057</td>
</tr>
<tr>
<td>Area covered by each podocyte</td>
<td>1.68±0.374</td>
<td>2.40±0.693*a</td>
<td>1.60±0.400</td>
<td>1.71±0.281</td>
</tr>
<tr>
<td>FPWmes (nm)</td>
<td>490±85</td>
<td>499±41</td>
<td>412±74</td>
<td>464±84</td>
</tr>
<tr>
<td>FPWgbm (nm)</td>
<td>408±58</td>
<td>472±74</td>
<td>409±46</td>
<td>416±25</td>
</tr>
</tbody>
</table>

* P<0.05 vs respective NPD group
a P<0.001 vs respective control group
b P=0.06 vs respective control group
GBM glomerular basement membrane
Vvmes/glom volume fraction of mesangium
Mes-urinary surface mesangio-urinary surface
FPWmes foot process width on the mesangium
FPWgbm foot process width on the glomerular basement membrane