Glomerular Number and Function Are Influenced by Spontaneous and Induced Low Birth Weight in Rats

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A link exists between low birth weight and diseases in adulthood, such as hypertension, cardiovascular disease, and insulin resistance. Intrauterine growth restriction (IUGR) has been used to explain this association and has been shown to lead to a nephron endowment in humans. A reduction in glomerular number has been described in animal models with induced low birth weight as well but not in animals with spontaneous low birth weight. It therefore is debatable whether the models are suitable. The effect on glomerular number and size was studied in rats with naturally occurring IUGR and experimental IUGR, induced by bilateral uterine artery ligation. Design-based stereologic methods were used. Urinary protein excretion was determined as a measure of renal damage. Results showed a decrease of approximately 20% in glomerular number in both groups of IUGR (control 35,400, naturally occurring IUGR 30,900, and experimental IUGR 28,000 glomeruli per kidney). Mean glomerular volume was increased in both IUGR groups, which was associated with an increased proteinuria. It is concluded that IUGR leads to a nephron endowment with a compensatory glomerular enlargement. This compensation is associated with more proteinuria in the long run. Uterine artery ligation in the pregnant rat is a suitable model to study the effects of IUGR on the kidney.

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Low birth weight (LBW) is associated with an increased risk for chronic diseases such as hypertension, cardiovascular disease, and insulin resistance and diabetes in later life (1–5). Even though the causal pathways are not yet understood, the "fetal origins hypothesis" (1) suggests that an insult during fetal life that leads to intrauterine growth restriction (IUGR) not only results in LBW but also reprograms the development of organs. This reprogramming may have beneficial effects in the suboptimal uterine environment but predisposes to long-term health problems.

The number of nephrons in humans is determined *in utero*, with no new nephrons being formed after the 36th week of gestation (6–8). IUGR leads to a lower number of glomeruli in humans (9–11), which may predispose to development of glomerular and eventually systemic hypertension in later life according to the hyperfiltration theory (12–14). A relationship between low nephron number and raised BP has been confirmed in patients with "essential" hypertension: Compared with individuals with normal BP, patients with hypertension had fewer glomeruli at autopsy (15).

Various animal models support the association between LBW and a low glomerular number (16–20), but a correlation between naturally occurring LBW and nephron endowment has not been shown consistently (21). This suggests that the results may be more due to the method of obtaining LBW than the LBW *per se* and therefore do not provide a suitable model.

It is important to have an animal model that closely resembles the cause of IUGR in humans to study the association between LBW and kidney development. We used a model of uterine artery ligation in the pregnant rat resembling uteroplacental dysfunction, which is the main cause of IUGR in humans in the Western world (22). To examine the possible confounding effect of the method, we also examined a group of rats with naturally occurring IUGR. This study was designed to examine the relationship between birth weight and glomerular number in rats using appropriate design-based stereologic techniques (23,24) and the association between glomerular number and proteinuria in later life.

Materials and Methods

Timed pregnant Wistar rats were obtained from Harlan CPB (Horst, The Netherlands) and housed in an animal room in the Clinical Animal Laboratory of the VU University Medical Center individually per plastic cage with wood chips as bedding. A 12:12-h light-dark cycle was maintained (light on at 06:00 a.m.) in the room, at constant temperature (22 ± 1°C) and relative humidity. Rats had free access to tap water and were fed a standard rodent chow (ssniff R/M-H; Bio Services, Schaijk, The Netherlands). For all experiments, approval was obtained from the Animal Welfare Committee of the VU University Medical Center.

According to a modified method of Wigglesworth (25), IUGR was induced by bilateral ligation of the uterine arteries on day 17 of gesta-

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tion under general anesthesia with a mixture of ketamine HCl (75 mg/kg intraperitoneally) and xylazine (5 mg/kg intraperitoneally). Sham-operated dams underwent the same procedure except for the actual ligation. At days 21 to 22 of gestation, pups were born. IUGR was defined as a birth weight <-2 SD of the mean of control pups, born from sham-operated dams. From previous experiments, we know that this corresponds to a weight on day 2 (day of birth was defined as day 1) of 5.3 g or lower. Fetal count during the operation compared with pup count on day 2 revealed a survival rate of 92.1 and 43.9% in the sham-operated and ligated litters, respectively. After uterine artery ligation in 53 dams, 44 litters were produced with a total of 275 pups, from which 64 met the criteria for IUGR (Figure 1).

Some pups ($n = 10$) from the sham-operated dams (a total of eight litters with 93 pups) also had a weight <-2 SD and were kept as a naturally occurring IUGR group (natIUGR). Figure 2 shows the distribution of body weights from all pups from sham-operated dams. Pups were cross-fostered, and litters were reduced to eight to 10 pups. At day 28, pups were weaned and housed two (male rats) or three (female rats) per cage.

At the age of 18 mo, rats were placed individually in a metabolic cage for collection of 24-h urine. Afterward, they were anesthetized with a mixture of ketamine HCl (75 mg/kg intraperitoneally) and xylazine (5 mg/kg intraperitoneally) and transcardially perfused with 50 ml of physiologic saline followed by 200 to 250 ml of 4% phosphate-buffered formaldehyde. Both kidneys were removed and weighed after the capsule and perihilar fat was taken off.

The left kidney was used for estimating glomerular number and stored in 4% phosphate-buffered formaldehyde until tissue preparation within 2 wk of perfusion. The kidney was cut in half, dehydrated in graded ethanol, and embedded in glycolmethacrylate (Technovite 7100; Hereus Kulzer, Wehrheim, Germany). With the use of a Microm HM 355 microtome, each kidney was cut in 20-μm-thick sections. The first section sampled was determined by a random-number table. With the use of the fractionator technique (26), every 25th section and its adjacent section were selected for estimation of glomerular number, providing a section sampling fraction (SSF) of 1/25. The sampled sections were mounted on a slide and stained with periodic acid-Schiff and Mayer's hematoxylin before examination. There were 10 section pairs on average (range 8 to 14) per kidney.

Counting was performed using an Olympus BX-50 microscope (Tokyo, Japan) at a magnification of ×113 with an automated Märzhäuser Multi Control 2000 specimen stage (Märzhäuser Wetzlar GmbH, Steindorf, Germany) and a 3-CCD color video camera (JVC KY-F55B; JVC, Wayne, NY) connected to a computer (Dell Optiplex GX110; Dell, Round Rock, TX) with CAST software (version 2.1.5.8, Visiopharm, Horsholm, Denmark) to superimpose the counting frame and point-counting grid. A sampled section and its adjacent section were positioned together on the specimen stage, and the region of interest was drawn around both sections. Using small vessels as landmarks in both sections and marking them as "fixpoints," we identified corresponding areas by the CAST software. After x- and y-step lengths (3500 μm) were defined, the counting grid was randomly oriented and placed on the sections by the CAST software. Glomeruli were counted only in approximately five consecutive section pairs starting with the third because of the problem of artificial edges in the first two sections (27) and the last sections. Therefore, a sampling fraction Ps/Pf was introduced: Ps is the number of points that hit all kidney tissue, and Pf is the number of points that hit only kidney tissue used for glomerular counting.

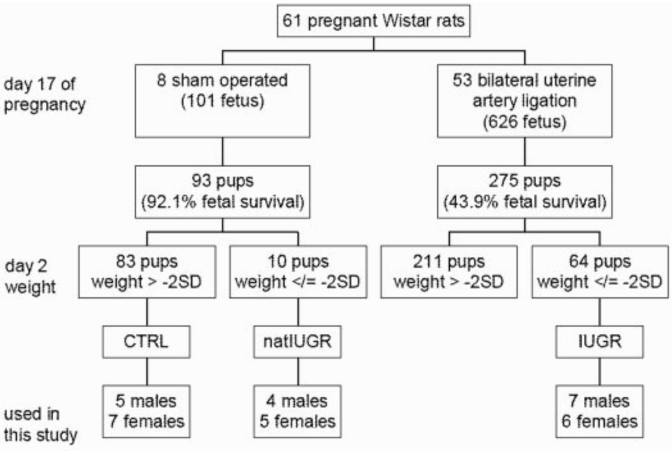

Figure 1. Study profile.

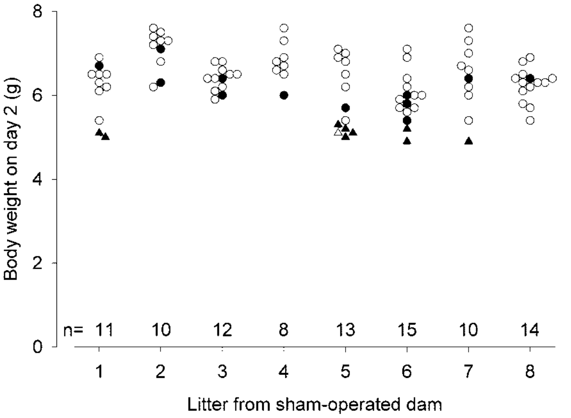

Figure 2. Scatter plot showing weights of all pups from sham-operated dams on day 2. ○, control pup not used for this study; ●, control pup used for this study; △, naturally occurring IUGR (natIUGR) pup not used for this study; ▲, natIUGR pup used for this study.

The number of glomeruli was estimated by the physical fractionator/disector (26,28). This technique consists of a three-dimensional counting rule using pairs of parallel sections. The glomeruli were counted when they were present inside the two-dimensional unbiased counting frame in one section (the sampling frame) but not in the adjacent section plane (the look-up section) and *vice versa*. On average, 136 glomeruli (ΣQ^-) were counted per kidney. The total number of glomeruli per kidney (N(glom)) was calculated using the following formula:

$$\mathrm{N(glom)} = \frac{1}{\mathrm{SSF}} \cdot \frac{1}{\mathrm{ASF}} \cdot \frac{P_S}{P_F} \cdot \frac{\Sigma Q^-}{2}$$

The factor ½ was introduced because glomeruli were counted both ways in the disector.

Area sampling fraction (ASF) was calculated as the counting frame area divided by the step lengths in the x- and y-direction ($dx \times dy$) of the counting frame. The coefficient of error of this technique used for counting glomeruli was estimated to be 8.8% (29).

Mean glomerular volume ($\bar{v}_n$(glom)) was calculated using the volume density of glomeruli in the kidney [V_V(glom/kid)] estimated with a random-oriented point-counting grid, divided by the numerical density of glomeruli in the kidney [N_V(glom/kid)]:

$$\mathrm{Vv(glom/kid)} = \frac{\Sigma \mathrm{P(glom)} \cdot \mathrm{p(kidney)}}{\Sigma \mathrm{P(kidney)} \cdot \mathrm{p(glom)}}$$

$$Nv(glom/kid) = \frac{\Sigma Q^{-}}{2 \cdot h \cdot (a/p) \cdot \Sigma Pf}$$

$$\bar{v}_n(glom) = \frac{Vv(glom/kid)}{Nv(glom/kid)}$$

where ΣP(glom) is the total number of points of the counting grid that hit glomeruli, p(kidney) is the number of points in the counting grid used for counting kidney tissue, ΣP(kidney) is the total number of points of the counting grid that hit kidney tissue, p(glom) is the number of points in the counting grid used for counting glomeruli, h is disector height, and (a/p) is the area per test point.

For evaluating tissue deformation, all kidneys were weighted after fixation. A weight-based volume before processing was calculated by dividing the kidney weight by 1.04 g/cm^3. The volume of the kidney after processing was estimated using Cavalieri's principle (23). There was no difference between the groups in tissue deformation. The volume of all of the kidneys before processing was on average 9.4% larger than the estimated volume of the kidneys after processing, which corresponds to a linear shrinkage of 2.1%. Glomerular volume data were not corrected for tissue deformation. During the counting procedure, the observer was blinded to the group and gender of the animal by using identification numbers.

To evaluate the kidney for focal glomerulosclerosis (FGS), we scored glomerulosclerosis semiquantitatively on a scale of 1 to 4 as described previously (30). Glomerulosclerosis was scored when mesangial cellularity, adhesion formation, and capillary obliteration were present in one segment. When 25% of the glomerulus was affected, a score of 1 was given, 50% was scored as 2, 75% was scored as 3, and 100% was scored as 4. A total of 50 glomeruli per kidney were scored by two blinded observers (M.F.S. and J.A.E.v.W.). For each glomerulus, a score was noted on the basis of consensus between the two observers. The ultimate score per kidney was obtained by multiplying the degree of change by the percentage of glomeruli with the same degree of injury and adding these scores. Urinary protein concentration was determined according to the protein assay described by Iwata *et al.* (31) on a Modular analytics (Roche Diagnostics, Mannheim, Germany).

Statistical Analyses

Results are presented as mean (coefficient of variation). Differences between groups were analyzed using ANOVA with a Student-Newman-Keuls *post hoc* correction. Correlations between variables were estimated by calculating the Pearson correlation coefficient. SPSS was used as statistical analysis system. $P < 0.05$ was considered to be statistically significant.

Results

For this study, 12 control (five male, seven female), nine natIUGR (four male, five female), and 13 IUGR (seven male, six female) rats were used. Body weights from day 2 until the age of 18 mo are presented in Figure 3. Birth weight was significantly reduced in natIUGR rats (5.1g [0.027]; $P < 0.001$) and IUGR rats (4.8g [0.088]; $P < 0.001$) as compared with control rats (6.2g [0.075]).

Kidney weight and relative kidney weight to body weight did not differ between groups (data not shown). Analysis showed no influence of gender on the glomerular number so data from male and female rats were combined. Figure 4A shows the significant difference in glomerular number between control (35,400 [0.080]) and natIUGR (30,900 [0.054]) and IUGR (28,000 [0.13]) rats. Figure 4B shows the distribution of glomerular number by birth weight.

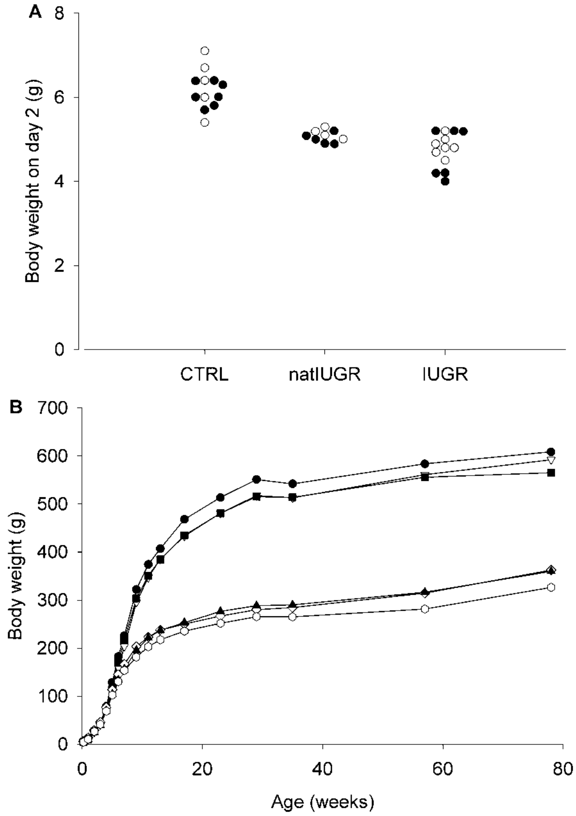

Figure 3. (A) Body weight on day 2 of the three experimental groups combining female (●) and male (○) rats. Control (CTRL) *versus* natIUGR, $P < 0.001$; CTRL *versus* IUGR, $P < 0.001$. (B) Body weight until the age of 18 mo for the three experimental groups per gender (male CTRL [●], male natIUGR [▽], male IUGR [■], female CTRL [◇], female natIUGR [▲], and female IUGR [○]).

The glomerular volume did differ between genders and was higher in male than in female rats. For both male and female rats, natIUGR and IUGR rats showed a significant higher mean glomerular volume than control rats ($P < 0.001$ for both groups; Figure 5A). Regression analysis showed a significant negative association between glomerular number and volume for both genders, as can be seen in Figure 5B.

FGS scores are presented in Figure 6. As compared with their respective female groups, IUGR and natIUGR male rats had a higher FGS score ($P < 0.05$). No statistical differences were found between the female groups. However, in the male groups, there was a trend toward a higher FGS score in the IUGR rats compared with control rats ($P = 0.07$).

Figure 7A shows the urinary protein excretion per group and gender. Because the data were not normally distributed, logarithmic data were used. Results showed a significant difference between genders and between control rats and both groups of LBW rats. The significant regression lines for male and female rats are shown in Figure 7B.

Linear regression coefficients with several models showed

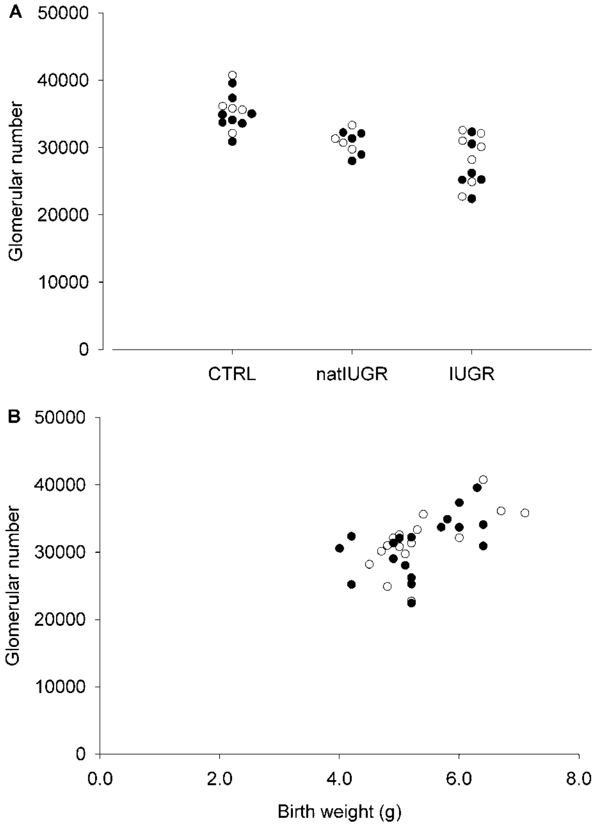

Figure 4. (A) Number of glomeruli per kidney in the three experimental groups combining female (●) and male (○) rats. CTRL *versus* natIUGR, $P < 0.01$; CTRL *versus* IUGR, $P < 0.001$. (B) Scatter plot showing the distribution of glomerular number by birth weight.

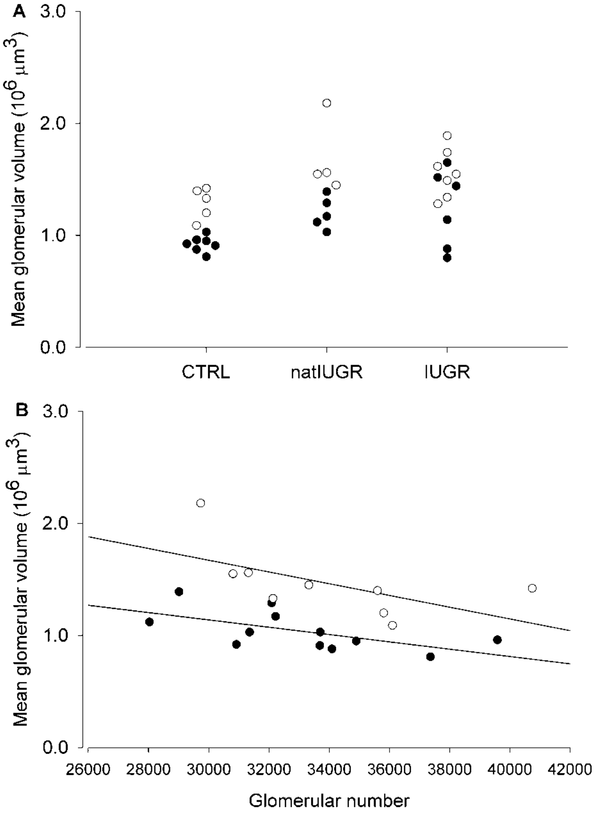

Figure 5. (A) Mean glomerular volume in the female (●) and male (○) experimental groups. CTRL *versus* natIUGR, $P < 0.01$; CTRL *versus* IUGR, $P < 0.005$. (B) Scatter plot showing the distribution of mean glomerular volume by glomerular number and the regression line for female (●; $r = -0.78$, $P < 0.001$) and male (○; $r = -0.48$, $P < 0.05$) rats.

that the amount of proteinuria was associated mainly with the gender, experimental group, and mean glomerular volume but not with glomerular number and birth weight (Table 1). Correlation analysis showed that birth weight contributed only very little to the correlation between glomerular volume and gender, experimental group, and glomerular number.

Discussion

This study shows that IUGR is associated with a decrease in glomerular number and an increase in mean glomerular volume and protein excretion. Our results suggest that birth weight determines glomerular number. This glomerular number influences glomerular volume, and the volume in itself is associated with the amount of proteinuria.

Another finding of this study is that spontaneous LBW is associated with the same renal sequelae as experimentally induced LBW. Birth weight was lower in the IUGR rats than in the natIUGR rats, as was the glomerular number. This shows that there is a difference in severity of the growth restriction between these two groups. However, the effects on glomerular volume and number were in the same range in the two groups of LBW rats. This indicates that the results in the IUGR rats may have been influenced partially by the method itself but do represent the effect of LBW on the kidney. We therefore conclude that our model of uterine artery ligation in the pregnant rat is a suitable model for the study of the consequences of IUGR on the kidney.

The number and the size of glomeruli were determined using design-based stereologic methods. These methods allow us to perform measurements without an assumption about the shape, size, or orientation of the glomeruli in the kidney. Esti-

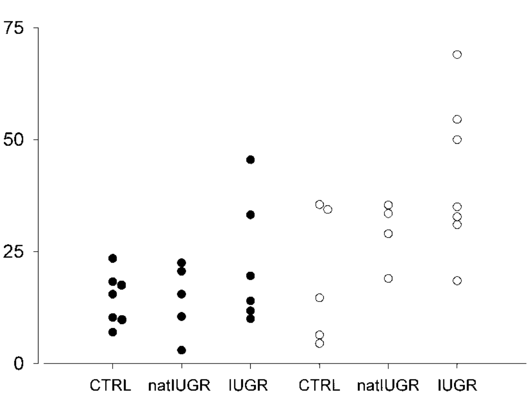

Figure 6. Focal segmental sclerosis (FGS) scores in the female (●) and male (○) experimental groups.

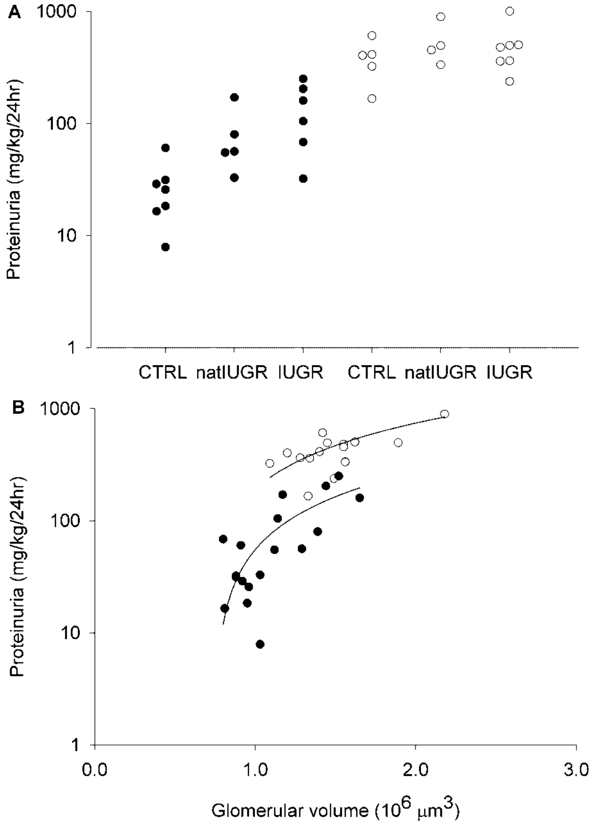

Figure 7. (A) Proteinuria in the female (●) and male (○) experimental groups per gender. CTRL *versus* natIUGR, $P < 0.01$; CTRL *versus* IUGR, $P < 0.001$. (B) Scatter plot showing the distribution of proteinuria by glomerular volume and the regression line for female (●; $r = 0.78$, $P < 0.001$) and male (○, $r = 0.68$, $P < 0.005$) rats.

mation of glomerular volume, however, can be influenced by tissue shrinkage (32). This effect is minimized by using perfusion fixation and embedding in methacrylate (33). The average linear shrinkage was 2.1%, implicating only minor tissue deformation. Glomerular number can be influenced by a loss of glomeruli, for instance as a result from glomerulosclerosis. This sclerosis has been associated with a low glomerular number (12–14). However, examination of the sections provided no evidence of sclerotic glomeruli. Although we cannot rule out the possibility that the loss of glomeruli was not detected, we believe that our results are valid.

Nephrogenesis in the human is complete at approximately the 36th week of gestation (8). In the rat, however, new nephrons are formed until postnatal day 8 (34). IUGR in humans suggests that there is no opportunity for compensatory development of nephrons, as the growth restriction is present until the end of nephrogenesis. In our model of IUGR, the adverse environment does not last until the end and could possibly allow for postnatal catch-up formation of nephrons. However, surgical reduction of nephron mass during nephrogenesis (*i.e.*, neonatal uninephrectomy in the rat) has been shown not to lead to extra nephron formation (35). It therefore is unlikely that IUGR does lead to formation of extra nephrons in the neonatal rat. LBW in the rat leads to a reduction of 20% in the number of glomeruli. If there had been an increase in postnatal nephrogenesis after IUGR, then the reduction in nephrons as a result of IUGR can be expected to be even more than this 20%. The effect of postnatal growth restriction on nephrogenesis in the rat could be a focus of future research.

Nephrogenesis is a highly complex process that requires an adequate supply of nutrients and various growth factors, including IGF-I (36) and an intact renin-angiotensin system (RAS) (37). IGF-I and the RAS interact, and blocking the RAS leads to an inhibition of IGF-I action (38), which is associated with a nephron deficit (39). LBW is associated with low fetal IGF-I levels (40) but an increased plasma renin activity (41). This suggests that the low IGF-I levels are the cause of the nephron endowment, with the RAS unable to compensate. This leads to an increase in apoptosis in the developing kidney, which has been shown to be associated with IUGR (16).

In total, 10 control pups of 93 had a birth weight <−2 SD below the mean. This number (10.8%) is higher than the expected 2.3% corresponding with the −2 SD cutoff point in normal distributed data. However, when all groups of rats that have been used in our study group in the last years are combined, only 14 (3.2%) of 432 control pups had a weight on day 2 of 5.3 g or less. This suggests that the relatively high number

Table 1. Correlation coefficients between various models and proteinuria, glomerular volume, and glomerular number

	Proteinuria	Glomerular Volume	Glomerular Number
Group and gender	$r=0.885$[a]	$r=0.768$[a]	$r=0.762$[a]
Group, gender, and birth weight	$r=0.899$[a]	$r=0.778$[a]	$r=0.772$[a]
Group, gender, and glomerular number	$r=0.887$[a]	$r=0.824$[a]	
Group, gender, and glomerular volume	$r=0.913$[a]		
Group, gender, glomerular number, and birth weight	$r=0.900$[a]	$r=0.827$[a]	
Group, gender, glomerular volume, and birth weight	$r=0.921$[a]		
Group, gender, glomerular volume, glomerular number, and birth weight	$r=0.922$[a]		

[a] $P < 0.001$.

of natural occurring IUGR in pups in the described study population was due to chance.

Most pups are delivered in the early morning. We measured body weight of the pups on day 2 in the morning, around the time that they are 24 to 30 h of age. This method was chosen to optimize survival of the pups, especially the LBW rats, during the handling and cross-fostering.

Analysis of the urinary protein excretion revealed heavy proteinuria in male rats at the age of 18 mo, as has been described previously (42), but even in this group, a significant relationship between glomerular volume and proteinuria was found, similar to the association in female rats. Although gender seems to be the main determinant of the heavy proteinuria in the adult rat, glomerular volume influences the amount of protein loss. Because the glomerular volume is related to glomerular number, which is associated with birth weight, there is an indirect link between birth weight and proteinuria. Nephron endowment and raised BP in adult life have been linked (14), and we have shown a rise in systolic BP in the described model of IUGR (43).

In summary, our study shows that both experimentally induced IUGR and naturally occurring IUGR result in a lower glomerular number. This glomerular number is associated with an increase in glomerular volume, which, in turn, is associated with an increased proteinuria. Uterine artery ligation in the pregnant rat is a suitable model to study the effects of IUGR on the kidney.

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