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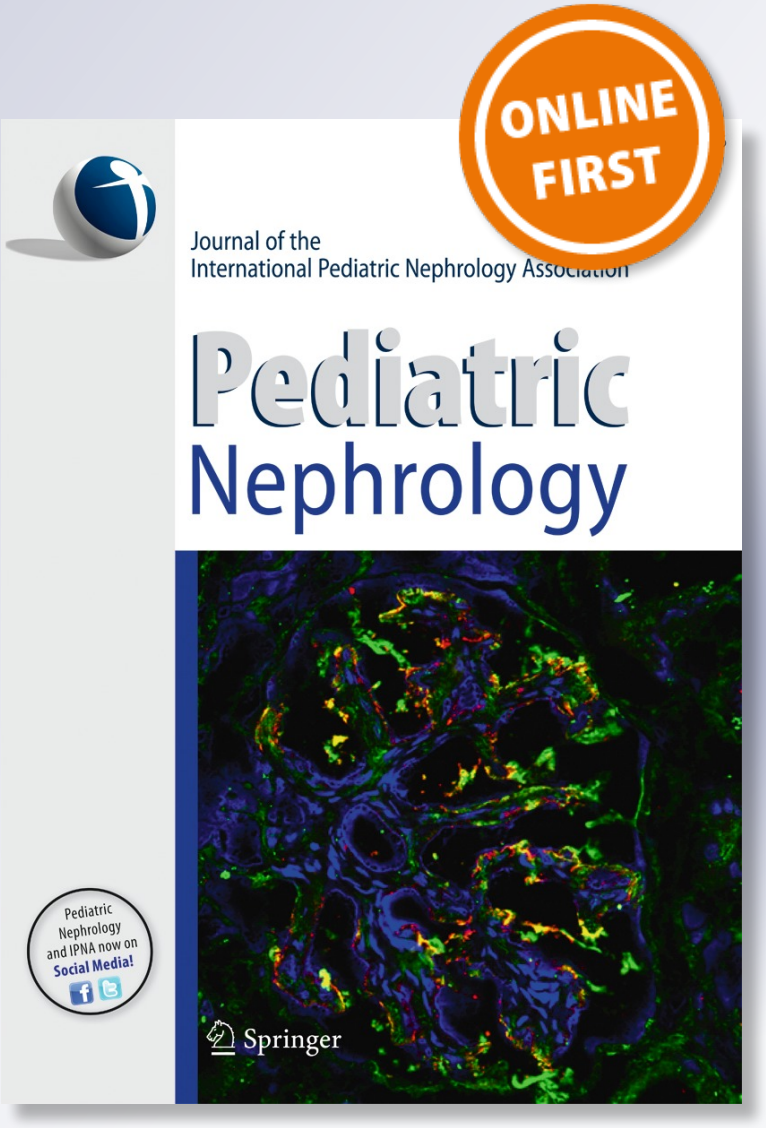

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ORIGINAL ARTICLE

Biochemical parameters of renal impairment/injury and surrogate markers of nephron number in intrauterine growth-restricted and preterm neonates at 30–40 days of postnatal corrected age

Maria Cristina Aisa[1,2] · **Benito Cappuccini**[3,4,5] · **Antonella Barbati**[1] · **Aldo Orlacchio**[4,5] · **Mauro Baglioni**[6] · **Gian Carlo Di Renzo**[1,4,5]

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Abstract
Background Premature and/or intrauterine growth-restricted neonates have an increased risk of developing postnatal renal injuries in later life. Studies on renal physiology in these neonates at a corrected age of 30–40 days are scarce and mostly relate to preterm infants. The data from these studies often lack the results of correlation analyses between biochemical parameters and nephron number—data which could provide additional insight and/or improve recognition of individuals at higher risk of renal failure.
Methods Urinary total protein and albumin levels and *N*-acetyl-β-D-glucosaminidase and cathepsin B activity were evaluated in preterm and intrauterine growth-restricted infants at a corrected age of 30–40 days and compared to data from a healthy control neonate population. The data were then associated with predominant susceptibility factors of renal damage related to low nephron number, such as gestational age, birth weight, total renal volume and renal cortex volume.
Results Compared to the control neonate population, we found significantly increased levels of all biochemical parameters tested in the intrauterine growth-restricted neonates, whereas in the preterm infants we observed a significant increase in cathepsin B activity, total protein level and, to a lesser extent, albumin level. Cathepsin B activity showed a significant, strong and inverse correlation with all surrogate markers of nephron number and was also strongly and positively correlated with urinary albumin level.
Conclusions At this postnatal age, we found that lower nephron number in low birth weight neonates was associated to tubular impairment/injury that could be concurrent with a dysfunction of glomerular permeability. Urinary cathepsin B activity may be a candidate marker for the early prediction of renal susceptibility to damage in low birth weight neonates.

Keywords Low birth weight · Nephron number · *N*-acetyl-β-D-glucosaminidase · Cathepsin B · Proteinuria · 3D-ultrasound imaging

✉ Maria Cristina Aisa
maria.aisa@unipg.it

[1] Present address: Section of Obstetrics and Gynecology, Department of Surgical and Biomedical Sciences, University of Perugia, Perugia, Italy

[2] Section of Biochemistry, Department of Agricultural Sciences, Food and Environment, University of Perugia, Perugia, Italy

[3] Department of Neonatology, Hospital S. M. della Misericordia, Perugia, Italy

[4] Centre of Perinatal Medicine, University of Perugia, Perugia, Italy

[5] GeBiSa, Research Foundation, Perugia, Italy

[6] Minerva Laboratory of Clinical Analysis, Perugia, Italy

Introduction

The incidence of low birth weight (LBW) neonates due to preterm (PR) birth and/or intrauterine growth restriction (IUGR) is high worldwide. The survival of these neonates has improved substantially over recent decades, but prematurity and/or IUGR are associated with an increased risk of developing postnatal renal injuries in later life [1–7]. In addition, newborns diagnosed with renal dysfunction and/or renal failure have a higher rate of mortality compared to their healthy counterparts [4, 8]. It has become evident that once renal functional impairment is established it is often difficult to retard further disease evolution [9, 10]. Given this

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knowledge, it is crucial to identify as early as possible those PR and intrauterine growth-restricted neonates who may be subject to future progression of renal disease in order to initiate preventative measures before renal dysfunction becomes established [11].

To date, the indirect assessment of total nephron number (using surrogate markers) in the postnatal period represents the main approach for evaluating the risk of future evolution of kidney disorders in LBW neonates [12–14]. Reduction of nephron number, via diminished nephrogenesis, is indeed believed to be a major cause of adverse renal outcomes in this group [5, 15]. Nephron number is a critical variable in defective renal maturation and progression to renal disease. As a consequence, it is an important indicator of kidney functional capacity, and low nephron number is considered to be a significant risk factor for renal disease [15, 16]. Reduced nephrogenesis may be associated to hyperfiltration, hypertension, glomerular damage and proteinuria, which in turn initiates a vicious cycle that leads to a loss of functioning units and renal failure over the long term [15–17].

Studies on renal physiology in PR and intrauterine growth-restricted neonates during the early postnatal period of 30–40 days corrected age are scarce and mostly relate to PR infants. The data from these studies often lack the results of correlation analyses between biochemical parameters and nephron number. Such data could provide additional insight into the renal physiology of this patient group and thereby enable a better understanding of the high variability of the data as well as improve recognition of individuals at higher risk of renal failure.

The aim of the study reported here was to determine possible early onset of renal damage in intrauterine growth-restricted and PR neonates at 30–40 days of postnatal corrected age by measuring a number of urinary indicators of glomerular and tubular impairment/injury and to assess the association of these indicators with predominant susceptibility factors of renal damage associated with low nephron number (i.e. gestational age, birth weight, total renal volume and cortical volume) [13, 14, 18]. As glomeruli constitute 95 % of the renal cortical volume, in the absence of an apparent inducible glomerular hypertrophy at this postnatal phase, renal cortical volume is considered to be indicative of nephron number [18].

For our purpose, we assessed the urinary levels of total protein and albumin and the activity of *N*-acetyl-β-D-glucosaminidase (NAG) and cathepsin B. Urinary total protein and albumin are well-established markers of renal damage, and their measurement can provide information on different aspects of renal activity. Specifically, the excretion level of total protein can be related both to glomerular and tubular function, while urinary albumin is a marker of glomerular permeability [19, 20] and represents a powerful predictor of kidney disease [21, 22]. NAG is an early, sensitive, persistent and robust marker of proximal tubule injury or dysfunction [23]. High levels of urinary NAG have been reported in PR infants, with an observed trend towards higher levels with increasing prematurity, and in fullterm and preterm neonates with renal tubular injury [24, 25]. Cathepsin B is a cysteine lysosomal proteinase which is very abundant in proximal tubule cells where it is involved in the digestion of proteins that are reabsorbed from the tubular fluid following glomerular filtration. Under normal physiological conditions, a small amount of cathepsin B can be found in urine, whereas levels increase following tubular damage [26] or dysfunction due to overloading of urinary proteins [27]. All biochemical parameters studied were expressed as the ratio to urinary creatinine in order to avoid differences in urinary flow rate.

Methods

Patients, samples and biochemical assays

A group of 110 neonates at a corrected age of 30–40 days were recruited into the study from the newborn nursery at "S. Maria della Misericordia" Hospital in Perugia, Italy. Of these neonates, 36 showed IUGR at 32–40 weeks of gestation (diagnosed early based on Doppler velocimetry technology and a birth weight of <10th centile); 36 were PR (32–36 weeks of gestation); 38 were healthy controls [37–41 weeks of gestation, defined as appropriate for gestational age (AGA: a newborn infant whose size is within the normal range for his/gestational age)]. Neonates were excluded from entry if they were affected by relevant morbidity (i.e. sepsis, urinary tract infections and urinary congenital abnormality), had received antenatal steroids during the gestation period and/or were born to mothers with a body mass index not in the normal range; additional exclusion factors were parents who were affected by diseases and/or were smokers or the presence of familial renal disease.

For each child, a first morning urine sample was obtained (using a U-bag collection device) which was immediately stored in ice to avoid denaturation. Once the sample had been transferred to our hospital laboratory, leukocytes and nitrite were measured using a multiple test strip (Combi-Screen PLUS; Analyticon Biotechnologies AG, Lichtenfels, Germany) to exclude possible urinary infections. Samples were then centrifuged at 5000 rpm for 20 min at 4 °C prior to storage at −80 °C until analysis. Urinary creatinine was measured using an enzymatic method (Advia ECREA_2, 04992596) performed on an Advia 1800 analyzer (Siemens AG, Munich, Germany).

Albumin and total protein levels (expressed in mg/ml) were determined by an immunonephelometric method using human albumin as the standard (BN II, Siemens AG) and the Lowry method using bovine serum albumin as the standard

(DC™ Protein assay; Bio-Rad, Hercules, CA). Values were expressed as the creatinine ratio (mg of albumin or total protein/mmol creatinine). With reference to urinary albumin level, values of <2.2 mg/mmol were defined as normal, whereas levels in the range of 2.2–22.6 or >22.6 mg/mmol were defined as microalbuminuria or macro-albuminuria, respectively [28].

Both NAG and cathepsin B activity were detected as previously described [29, 30] using the specific fluorescent substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Sigma-Aldrich, St. Louis, MO; 1 mM in 0.1 M citrate/0.2 M phosphate buffer pH 4.5) or Z-Arg-Arg-NH-MEC (Bachem AG, Bubendorf, Switzerland) (12 μg/ml in 0.1 M Na-phosphate buffer pH 6.3, containing 1 mM EDTA, 0.1 mM DTT), respectively. For the assays, the collected urine was appropriately diluted and incubated at 37 °C with the substrate solutions. The reaction was stopped by the addition of either 0.2 M glycine–NaOH buffer, pH 10.4 (NAG) or 0.1 M monoiodoacetic acid in 1 M Tris–HCl buffer (pH 8.0) (cathepsin B). Fluorescence of the liberated 4-methylumbelliferone (NAG) or 7-amino-4-methylcoumarin (cathepsin B) was measured on a LS3 fluorimeter (Perkin-Elmer, Waltham, MA) with excitation at 360 nm and emission at 446 nm for NAG activity and at an excitation of 370 nm and emission of 460 nm for cathepsin B activity; the fluorimeter was calibrated using 4-methylumbelliferone or 7-amino-4-methylcoumarin solution in 0.2 M glycine buffer (pH 10.4) or 0.1 M monoiodoacetic acid in 1 M Tris–HCl buffer (pH 8.0), respectively. The activity of each enzyme was corrected for urine creatinine concentration and then expressed as International Units (IU)/min mmol creatinine in the case of NAG and as IU/h mmol creatinine in the case of cathepsin B. One IU of activity is the amount of enzyme that hydrolyzes 1 μmol of substrate at 37 °C.

Evaluation of anthropometric parameters

The gestational age was expressed in gestational weeks and birth weight was expressed in grams. The diagnosis of IUGR was established during fetal life and confirmed by LBW.

Total renal volume and cortical volume were reconstructed and estimated using 3-D echo technology combined with Virtual Organ Computer-Aided Analysis (VOCAL) (Vocal II; GE ULTRASOUNDS, GE Healthcare, Little Chalfont, UK), a technology that has been shown to be highly reproducible and accurate for the assessment of organ volumes [31].

Measurements were obtained as an average of four repeated estimations by a blinded sonographer with an intra- and inter-operator variability of <5 %.

Statistical analysis

Data analysis was carried out and graphs were drawn using GraphPad Prism version 6.01 statistical software (GraphPad Software Inc., San Diego, CA). The normality of data distribution was tested using the D'Agostino–Pearson normality test. Results were presented as median and interquartile range. Multiple comparisons between more than two groups were performed using non-parametric Kruskal–Wallis one-way analysis of variance with Dunn's ad hoc posttest. Comparison within the LBW group, IUGR versus PR, was performed using the non-parametric Mann–Whitney test. Correlations between the variables under investigation were checked by Spearman's rho rank correlation coefficient analysis. Predictive accuracy was quantified as the area under the receiver operating characteristics (ROC) curve (AUC).

Results

Descriptive analysis of all parameters under investigation

The results of our analysis of all variables in AGA, intrauterine growth-restricted and PR neonates are shown in Table 1. Based on the urinary levels of albumin, microalbuminuria (albumin: 2.2–22.6 mg/mmol) was identified in all three neonate groups. Macroalbuminuria or proteinuria (albumin >22.6 mg/mmol) was observed in 22.7 % of neonates, the majority of whom were intrauterine growth-restricted neonates (38 %), followed by PR (27.7 %) and AGA neonates (2.6 %).

Multiple comparisons between variables in the AGA, IUGR and PR neonate groups

Multiple comparison analysis of the three neonate groups showed that gestational age, birth weight, total renal volume and cortical volume were significantly lower in the intrauterine growth-restricted and PR neonates than in the control neonates (Fig. 1a–d). With regard to biochemical parameters, in comparison with AGA infants, intrauterine growth-restricted infants exhibited a significant increase in urinary total protein and albumin levels and in urinary NAG and cathepsin B activity (Fig. 2a–d), whereas PR neonates displayed significantly higher levels of urinary total protein, cathepsin B activity and, to a lesser extent, albumin (Fig. 2a, b d). Urinary NAG activity was higher in the PR group than in the control group, but the differences were not statistically significant (Fig. 2c).

Table 1 Variability in the parameters under investigation in appropriate for gestational age, intrauterine growth-restricted and preterm neonates

Parameters tested	AGA ($n = 38$)		Intrauterine growth-restricted neonates ($n = 36$)		PR neonates ($n = 36$)	
	Median	IQR	Median	IQR	Median	IQR
GA (weeks)	40	38.50–41	36	33–37.25	35	34–36
BW (grams)	3310	3078–3790	1980	1760–2435	2260	2030–2425
RV (ml)	34.80	31.80–36	19.04	16.93–23.55	27.00	20.20–29.15
CV (ml)	15.20	13.40–17	7.41	6.075–9.325	10.00	9.15–12.20
TP (mg/mmol)	52.66	29.16–73.93	121.6	92.12–158.6	75.70	50.34–105.6
ALB (mg/mmol)	6.483	4.715–10.68	20.22	13.31–28.97	16.14	9.074–23.31
NAG activity (IU/min mmol)	2.456	1.475–4.351	5.119	3.525–12.24	4.217	2.454–4.780
CB activity (IU/min mmol)	1.044	0.8335–1.372	3.633	2.146–4.848	2.303	1.7–2.582

Values are presented as the median and interquartile range (IQR)

AGA, Appropriate for gestational age; PR, preterm; BW, birth weight; RV, total renal volume; CV, cortical volume; TP, total protein; ALB, albumin; NAG, *N*-acetyl-β-D-glucosaminidase; CB, cathepsin B

Comparison between variables within the LBW group: intrauterine growth-restricted versus PR neonates

In order to evaluate all possible significant differences between intrauterine growth-restricted and PR infants, including those which could not be clearly shown by multiple comparison analysis, we compared these two neonate groups using the non-parametric Mann–Whitney test. Infants with IUGR showed significantly increased levels of urinary total protein ($p < 0.001$), albumin ($p = 0.016$) and non-albumin total protein ($p = 0.002$), as well as increased cathepsin B ($p < 0.001$) and NAG activity ($p = 0.026$). Infants with IUGR also had a significantly increased gestational age ($p = 0.01$) and a significantly reduced birth weight ($p = 0.006$), total renal volume ($p < 0.001$) and cortical volume ($p < 0.001$).

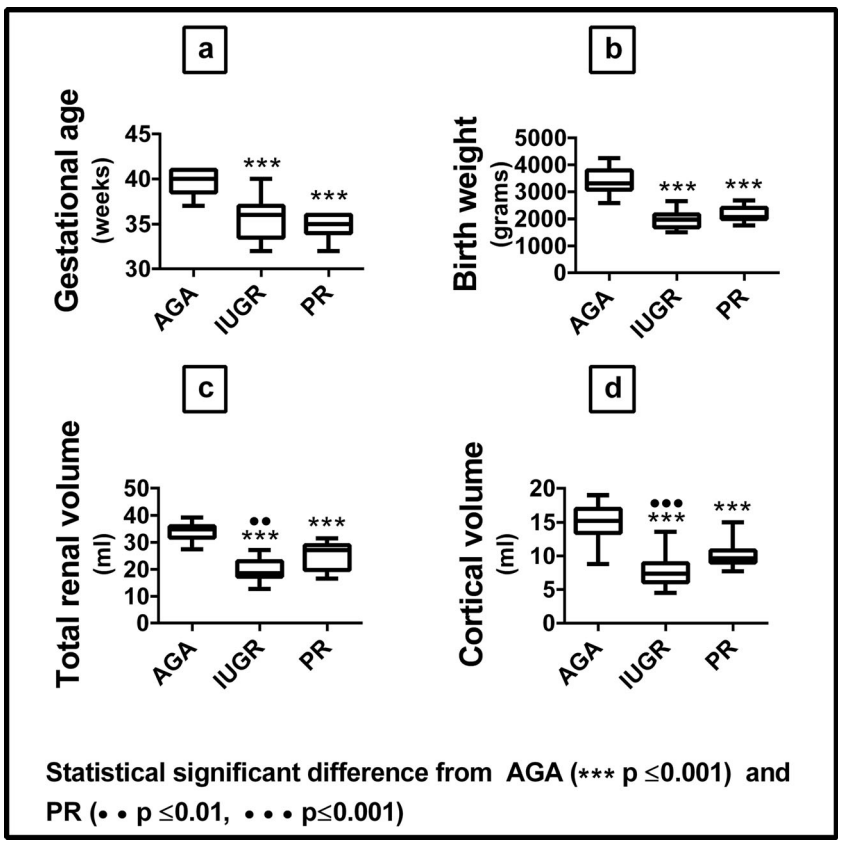

Fig. 1 Box plot of variability in gestational age (**a**), birth weight (**b**), total renal volume (**c**) and cortical volume (**d**) in neonates born appropriate for gestational age (*AGA*), preterm (*PR*) or with intrauterine growth restriction (*IUGR*). *Horizontal lines in boxes* Median, *boxes* interquartile range (IQR), *whiskers* range of values. Multiple comparison analysis was performed using non-parametric Kruskal–Wallis one-way analysis of variance (ANOVA) with Dunn's ad hoc posttest

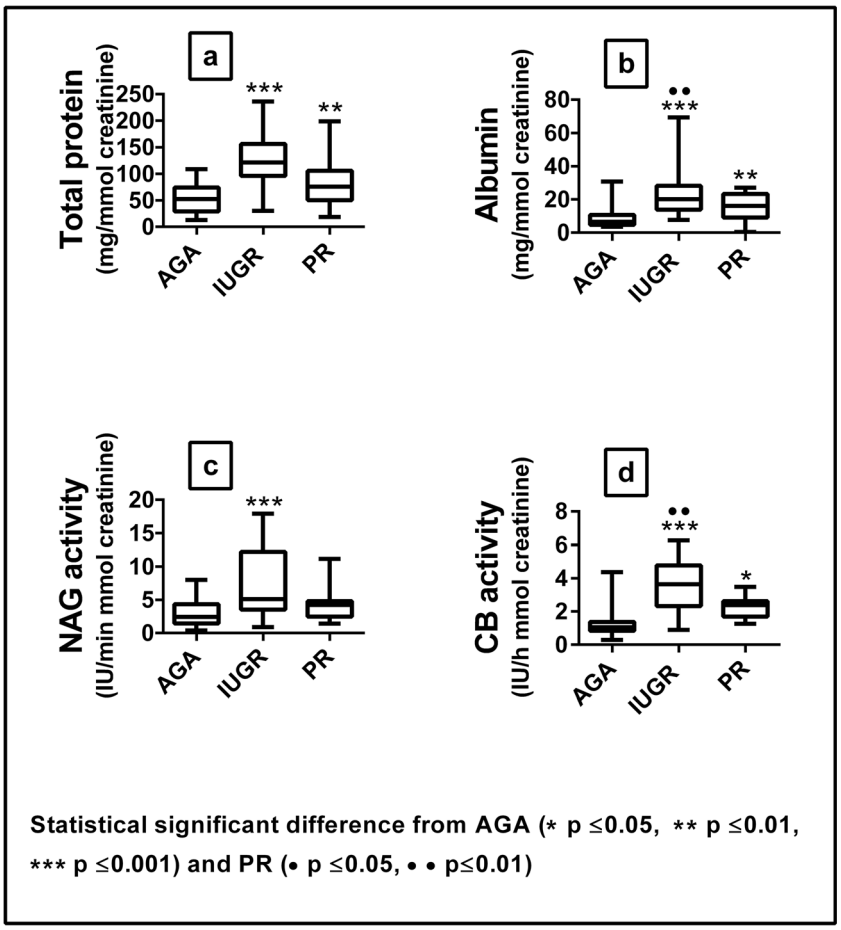

Fig. 2 Box plot of variability in urinary total protein (**a**) and albumin (**b**) levels and in *N*-acetyl-β-D-glucosaminidase (*NAG*) activity (**c**) and cathepsin B (*CB*) activity (**d**) in neonates born AGA, PR or with IUGR. *Horizontal lines in boxes Median* boxes IQR, *whiskers* range of values. Multiple comparisons analysis was performed using non-parametric Kruskal–Wallis one-way ANOVA with Dunn's ad hoc posttest *AGA*, appropriate for gestational age; *PR*, preterm; *IUGR*, intrauterine growth restriction

Multiple comparisons of variables between AGA and the subgroups of LBW neonates

In order to identify possible distinctive effects related to prematurity (in terms of renal development) and/or growth restriction in the heterogeneous group of LBW neonates, we made a more specific sub-classification in the intrauterine growth-restricted and PR infants, based on the assumption that nephrogenesis was completed by the 36th week of gestation. It is well known that nephrogenesis in human is completed between 35 to 36 weeks gestation. We classified neonates with IUGR into two subgroups, namely, those with a gestation age of ≤35 weeks (PR-IUGR; n = 12) and ≥36 weeks (IUGR; n = 24), respectively. With this study design we were able to examine the effects of prematurity and growth restriction together or growth restriction alone. The two subgroups were then compared to that of PR infants of ≤35 weeks of gestation (n = 22), mostly affected by prematurity (in terms of renal development), and to the AGA group, characterized by the completion of nephron development. PR infants of 36 weeks of gestation, being borderline cases, were excluded from this study to avoid possible bias.

Compared to AGA infants, the PR and PR-IUGR subgroups, as expected, showed a significant decrease in gestational age (Fig. 3a), whereas all subgroups (i.e. PR, PR-IUGR and IUGR) displayed reduced or significant lower levels of birth weight, total renal volume and cortical volume (Fig. 3b–d). Furthermore, total renal volume and cortical volume values were significantly diminished or lower in the PR-IUGR subgroup than in the PR and IUGR subgroups (Fig. 3c, d). In addition, compared to the AGA group, all the subgroups of LBW infants showed increased or statistically higher levels of all the biochemical parameters evaluated (Fig. 4), with those for the PR-IUGR subgroup being higher or significantly enhanced in comparison to those for the PR and IUGR subgroups (Fig. 4). No significant differences in any of the parameters tested were found between the IUGR and PR subgroups (Figs. 3, 4), with the exception of significantly reduced levels of total renal volume (Fig. 3c) and the expected higher values of gestational age in the IUGR subgroup (Fig. 3a).

Analysis of correlations between variables in overall data

Spearman rho values from the analysis of correlation between variables in the overall dataset are reported in Table 2. These values indicate that total protein and albumin levels and both NAG and cathepsin B activity were inversely associated to gestational age, birth weight, total renal volume and cortical volume, albeit to different extents.

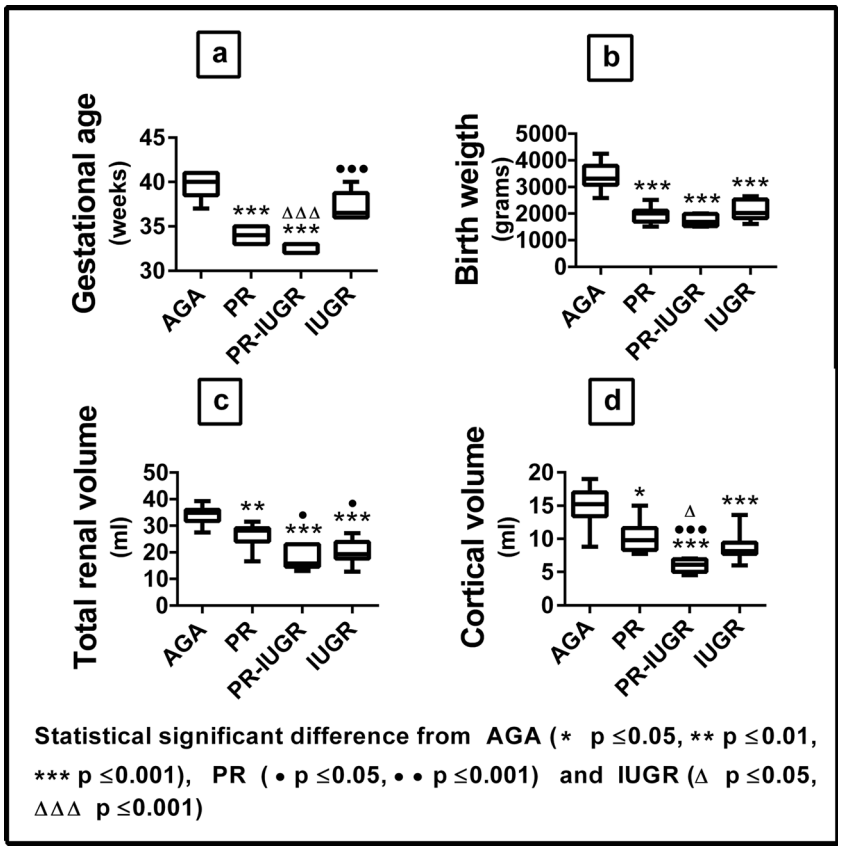

Fig. 3 Box plot of variability in gestational age (**a**), birth weight (**b**), total renal volume (**c**) and cortical volume (**d**) in AGA group and in the three subgroups of LBW neonates (*PR* 32–35 weeks of gestation, *PR-IUGR* 32–35 weeks of gestation, *IUGR* 36–40 weeks of gestation). *Horizontal lines in boxes* Median, *boxes* IQR, *whiskers* range of values. Multiple comparison analysis was performed using non-parametric Kruskal–Wallis one-way ANOVA with Dunn's ad hoc posttest *PR*, preterm; *AGA*, appropriate for gestational age; *IUGR*, intrauterine growth restriction

In detail, total protein level showed a moderate negative correlation with gestational age, birth weight, total renal volume and cortical volume as well as a moderate positive correlation with albumin level and NAG and cathepsin B activity. Albumin showed a moderate inverse association with all surrogate markers of nephron number, a positive association with total protein level (moderate) and NAG activity (weak) and a strong positive correlation with cathepsin B activity. Cathepsin B activity showed a strong inverse correlation with all surrogate markers of nephron number tested and a strong positive correlation with NAG activity. NAG activity was inversely and moderately correlated with gestational age, birth weight, cortical volume and total renal volume. Total renal volume showed a direct association to gestational age (moderate), birth weight (very strong) and cortical volume (very strong).

AUC under the ROC curves

Based on the results of our correlation analysis showing a strong correlation between cathepsin B activity and all surrogate markers of nephron number (inverse) or albumin (direct) and with the knowledge that low nephron number and urinary albumin level are significant risk factors for renal disease [15, 16, 21, 22], we evaluated the diagnostic efficacy of cathepsin B activity by assessing the corresponding areas under the ROC curves in intrauterine growth-restricted and PR infants. Figure 5 shows the corresponding areas under the ROC curves of cathepsin B, total renal volume and cortical volume, including their respective values in IUGR and PR groups.

Discussion

Due to reduced nephrogenesis, neonates with a LBW due to IUGR and/or PR birth have an increased risk of developing renal injuries in later life [1–7].

Our results show that compared to the kidneys of infants born AGA (37–41 weeks of gestation), those of intrauterine growth-restricted neonates (32–40 weeks of gestation) and PR infants (32–36 weeks of gestation), both at 30–40 days of corrected age, are characterized by decreased nephron number. This reduced nephrogenesis, moreover, associates to a tubular impairment/injury and could be concurrent with a dysfunction of glomerular permeability, an event that could progress to renal disease over the long term [1, 15, 16].

To date, the main approach used to identify at an early age those LBW neonates at risk of future kidney disorders is the assessment of low nephron number during the postnatal period [12–14]. Low nephron number is considered to be a significant risk factor for kidney disease [15, 16]. At the present time there are no specific markers of injury, and in previous studies measures of biochemical parameters of renal function

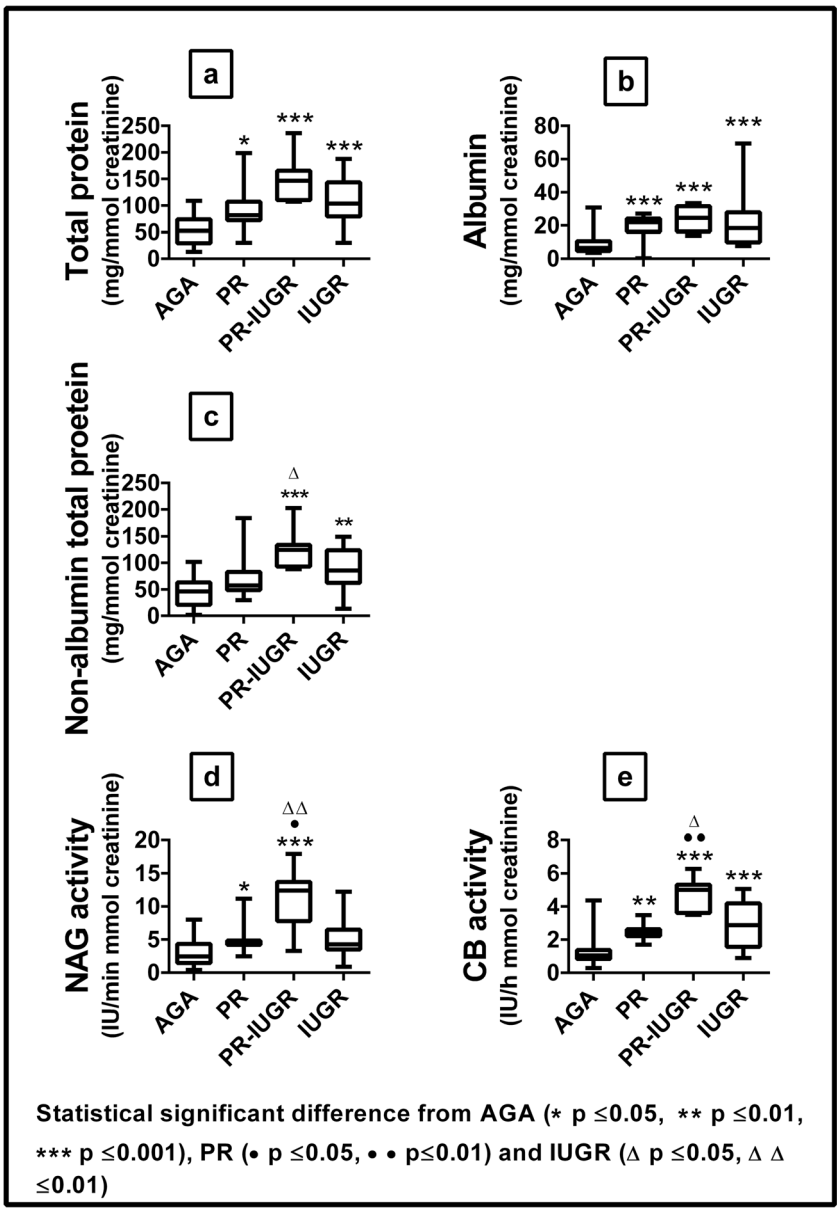

Fig. 4 Box plot of variability in total protein (**a**), albumin (**b**) and non-albumin total protein (**c**) levels and in NAG (**d**) and CB activity (**e**) in the AGA group and in the three subgroups of LBW neonates (see caption to Fig. 3 for gestation ages). *Horizontal lines in boxes* Median, *boxes* IQR, *whiskers* range of values. Multiple comparison analysis was performed using non-parametric Kruskal–Wallis one-way ANOVA with Dunn's ad hoc posttest *NGA*, *N*-acet-β-D-glucosaminiclase, *CB*, cathepsin B; *AGA*, appropriate for gestational age; *LBW*, low birth weight

Table 2 Correlation between variables (Spearman rho values)

Variables	GA	BW	CV	RV	TP	ALB	NAG activity	CB activity
GA		0.796***	0.555***	0.594***	−0.475***	−0.550***	−0.534***	−0.650***
BW	0.796***		0.753***	0.809***	−0.507***	−0.560***	−0.568***	−0.665***
CV	0.555***	0.753***		0.880***	−0.409***	−0.562***	−0.431***	−0.661***
RV	0.594***	0.809***	0.880***		−0.427***	−0.488***	−0.436***	−0.645***
TP	−0.475***	−0.507***	−0.409**	−0.427***		0.406***	0.439***	0.471***
μALB	−0.550***	−0.560***	−0.562***	−0.488***	0.406***		0.362***	0.633***
NAG activity	−0.534***	−0.568***	−0.431***	−0.436***	0.439***	0.362***		0665***
CB activity	−0.650***	−0.665***	−0.661***	−0.645***	0.471***	0.633***	0.665***	

GA, gastational age; BW, birth weight; CV, cortical volume; RV,total renal volume; TP, total protein; NAG, *N*-acetyl-β-D-glucosaminidase; CB, cathepasin; ALB, albumin

Significant at: $^{**}p < 0.05$; $^{***}p < 0.001$

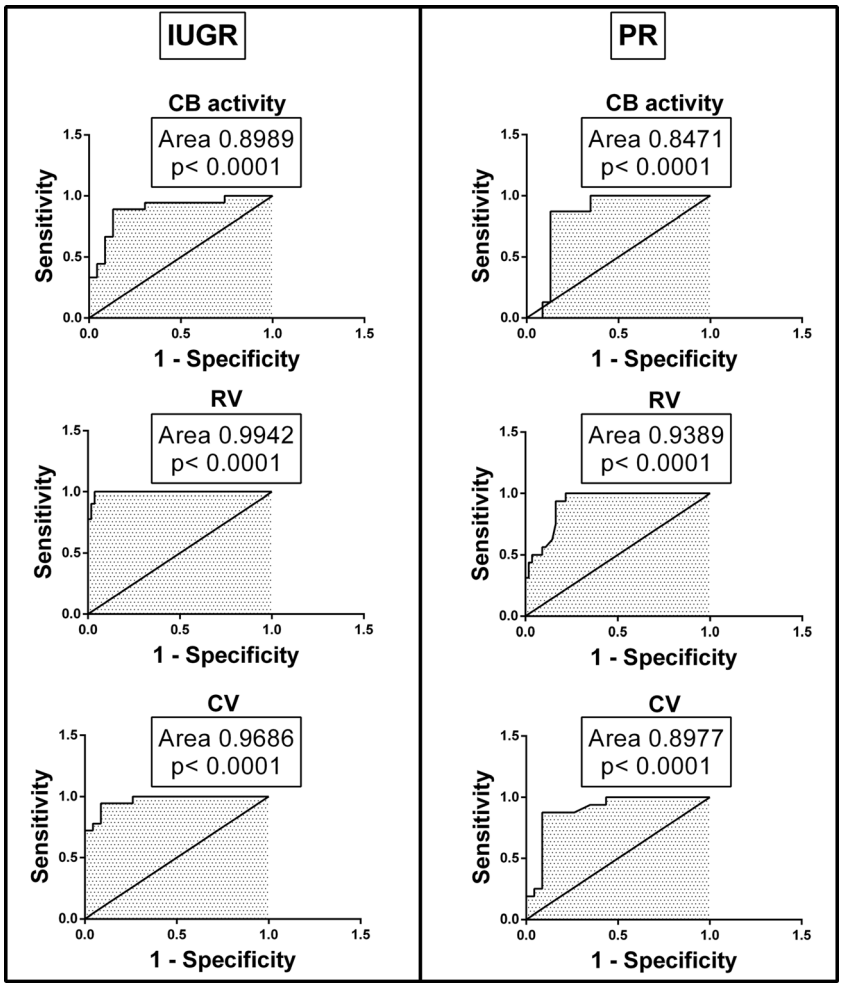

Fig. 5 Areas under the receiver operating characteristics (ROC) curve for CB activity, total renal volume (*RV*) and cortical volume (*CV*) in PR infants and those with IUGR. The ROC curve analysis was performed by comparing IUGR or PR infants to those in the AGA group

failed to distinguish between immaturity (which similarly affects the kidneys of healthy full-term infants) and injury. In addition, the biochemical values reported in these studies were often highly variable and mostly related to PR neonates at birth or in the first days of life. Data relating to renal function in intrauterine growth-restricted and PR newborns at 30–40 days of corrected age are limited. The aim of our study was to associate the assessment of biochemical parameters of glomerular and tubular impairment/injury to the main surrogate markers of nephron number in order to better evaluate the physiological significance of data at this postnatal phase.

Compared to the IUGR and PR groups, we found that AGA infants displayed higher levels of all surrogate markers of nephron number (i.e. gestational age, birth weight, cortical volume and total renal volume) showing, as expected, a greater nephron mass. In terms of kidney function/dysfunction, in agreement with previous data, we found that reduced nephrogenesis in the intrauterine growth-restricted and PR infants, compared to the AGA infants, was clearly associated with increased impairment/injury of tubules [32–35]. Indeed, compared to the AGA infants, the intrauterine growth-restricted and PR infants had significantly higher levels of the two lysosomal markers of tubular impairment/injury. In addition, cathepsin B activity, which was significantly increased in both IUGR and PR groups, showed a strong negative correlation with all surrogate markers of nephron number (gestational age, birth weight, total renal volume and cortical volume). Data of NAG activity found in the urine of PR neonates were in accordance with previous reports [25, 34, 36–38]. Intrauterine growth-restricted neonates presented the highest levels of urinary total protein and albumin, with the highest percentage of proteinuria. PR neonates also exhibited a significant increase in albumin levels compared to AGA neonates. Previous data showed a high variation in urine albumin levels between individual PR neonates [39, 40], with the greatest levels exhibited by those with a low gestation age at birth and those who were clinically unstable [34, 39–42].

There were also differences in nephrogenesis and renal function among LBW neonates. Compared to the PR group of LBW neonates, the IUGR group exhibited significantly lower levels of total renal volume and cortical volume, a significant increase in albumin level and more severe tubular impairment/injury, as evidenced by the significantly higher levels of cathepsin B and NAG activity as well as of non-

albumin total protein level (which presumably refers to tubular function). Such differences, however, mostly concerned the subgroup of PR-IUGR neonates characterized by coexistence of prematurity and growth restriction. Indeed, we found that, with the exception of total renal volume and the expected gestational age, moderate prematurity only (in PR infants of 32–35 weeks of gestation) and growth restriction only (in intrauterine growth-restricted infants of 36–40 weeks of gestation) did not induce significant differences in the parameters evaluated. However, when present concomitantly, as in infants in the PR-IUGR subgroup (32–35 weeks of gestation), their effects tended to be cumulative.

Increased levels of urinary albumin were found in both intrauterine growth-restricted and PR neonates, but it is not clear whether such increases are exclusively due to a limited reabsorptive capability of tubules or to a concomitant glomerular dysfunction, or both. However, in the intrauterine growth-restricted and PR neonates examined in our study, an increased glomerular permeability, concomitant with the tubular impairment/injury, cannot be excluded. Experimental studies have shown that a reduction in renal volume causes hyperfiltration, a compensatory mechanism that prevents glomerular filtration rate (GFR) decline, and that the consequences of this state are proteinuria, hypertension and a decrease in the GFR. Albuminuria represents a predominantly hemodynamic effect of glomerular hypertension and hyperfiltration [43]. It is now well established that the reduced nephron mass at birth of LBW neonates due to PR birth and/or IUGR can induce hyperfiltration, albuminuria, glomerulosclerosis and a decline in renal function in later life [15–17]. The time at which these events occur is unknown. Our data suggest that they could start, at subtle levels, during the early postnatal phase of 30–40 days of corrected age.

Interestingly, a strong correlation between urinary albumin level and cathepsin B activity was shown. In intrauterine growth-restricted and PR infants at this postnatal age, these increases could represent the initial phase at which proteinuria initiates the progressive renal disease via tubulointerstitial damage [44]. In support of this notion, it has been recently demonstrated that the excessive reabsorption of ultrafiltered proteins by proximal tubular cells induces tubular damage and apoptosis/necrosis by exhaustion of the lysosomal degradation pathway and spillage of lysosomal enzymes, including cathepsin B, into the cytoplasm and urine [27]. Alternatively, with reference to the "tubular hypothesis" of hyperfiltration, this increases could represent the early phase at which the damaged tubule may initiate glomerular dysfunction, resulting in an increased amount of urinary albumin [45, 46]. Very recent data indicate that, after utero-placental insufficiency, kidneys of intrauterine growth-restricted rats are characterized by an altered expression of the genes involved in tubular development and function, both at birth and in the early postnatal period [47]. Further studies are needed to further clarify the mechanisms implicated in these events. In particular, it is essential to elucidate whether the higher activities of urinary cathepsin B and NAG directly reflect cell damage or an increased cell turnover, as well as whether they are related to kidney damage or to a greater extent of renal immaturity. In addition, a possible competition between the two hydrolases and urinary albumin/protein for megalin/cubilin receptors should be evaluated. Recent data have suggested that the increased urinary excretion of lysosomal enzymes and their concomitant reduction in the proximal tubule may be due to competition with urinary albumin/protein for megalin/cubilin-mediated uptake in overload proteinuria conditions [48]. A normal physiological tubular reabsorption of circulating cathepsin B, similar to that of other lysosomal enzymes, by means of these receptors has indeed been demonstrated and is thought to be the main source for tubular cells to renew the lysosomal enzyme pool [48].

Our results, however, provide evidence that urinary cathepsin B activity may be an early candidate marker of susceptibility to renal disease in intrauterine growth-restricted and PR neonates. The activity of this lysosomal cysteine proteinase showed a strong correlation with all of the surrogate markers of nephron number (negative) and with urinary albumin level (positive) and also exhibited an excellent diagnostic profile. We have recently drawn similar conclusions with urinary cystatin C in intrauterine growth-restricted neonates [49]. These two notable molecules are the enzyme and its inhibitor, respectively. In the case of reduced nephrogenesis in intrauterine growth-restricted neonates, their variability could be coordinated and possibly dependent on the same event.

The biological importance of cathepsin B in the kidney

Under normal conditions, cathepsin B is highly expressed in the S1 segment of the proximal tubule where it is the main enzyme involved in the lysosomal digestion of protein reabsorbed from the glomerular ultrafiltrate via endocytosis. Urinary cathepsin B has long been considered a marker of tubular injury, as levels of cathepsin B are found to be reduced in tubules and increased in urine in response to injuries [26]. Such trends have also been found in diabetes and renal hypertensive hypertrophy. As mentioned above, in overload proteinuria conditions, the augmented urinary excretion of this enzyme has also been related to a possible competition between circulating cathepsin B and urinary albumin/protein for megalin/cubilin-mediated uptake in tubular epithelial cells [48] or to a lysosomal dysfunction in these cells [27].

An emerging possible role for this proteinase in the pathogenesis of chronic renal diseases has recently been highlighted based on its involvement in kidney cancer and, in particular, in the onset and progression of tubulointerstitial inflammation and

fibrosis induced by proteinuria. In vivo and in vitro experiments have indeed shown that the prominent cytoplasmic release of cathepsin B in tubular epithelial cells subsequent to lysosomal membrane permeabilization induced by urinary protein/albumin overload [27, 50] is essential for the concurrent tubular cells injury [50] and for the activation of the inflammasome NLRP3, a cytoplasmic macromolecular complex that has been involved in the progression of kidney diseases [50].

These newly emerging data further support the plausibility that cathepsin B can be a good marker of the risk for renal disease development in intrauterine growth-restricted and PR neonates. In addition, cathepsin B in the kidney (as well as lysosomal membrane stabilization) deserves additional evaluation as a potential therapeutic target to prevent or reduce renal tubular damage induced by overload proteinuria. This could be of great importance for LBW neonates if such damage were demonstrated to initiate very early, at 30–40 days of postnatal corrected age.

Conclusions

The findings of this study indicate that at 30–40 days of corrected age, the kidneys of PR and intrauterine growth-restricted neonates are characterized by low nephron number that is associated to both tubular impairment/injury and higher levels of urinary albumin, possibly in combination with concurrent increased glomerular permeability. This state augments the risk of glomerular proteinuria, which could contribute to hyperfiltration, hypertension, glomerulosclerosis and renal disease in later life [15–17]. These data also demonstrate that growth restriction alone and prematurity alone are capable of modulating both nephrogenesis and renal function and that when they are concurrent their effects tend to be cumulative.

Together with the main surrogate markers of nephron number (i.e. gestational age, birth weight, renal volume and cortical volume) urinary cathepsin B activity may provide an early indication of the risk for renal disease development and may represent an inexpensive and sensitive indicator for early preliminary screening. Further evaluations to confirm the prognostic role of urinary cathepsin B activity for renal disease in LBW neonates are therefore needed.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Hoy WE, Rees M, Kile E, Mathews JD, Wang Z (1999) A new dimension to the Barker hypothesis: low birthweight and susceptibility to renal disease. Kidney Int 56:1072–1077
2. Lackland DT, Bendall HE, Osmond C, Egan BM, Barker DJ (2000) Low birth weights contribute to high rates of early-onset chronic renal failure in the Southeastern United States. Arch Intern Med 160:1472–1476
3. Fan Z, Lipsitz S, Egan B, Lackland D (2000) The impact of birth weight on the racial disparity of end-stage renal disease. Ann Epidemiol 10:459–466
4. Drukker A, Guignard JP (2002) Renal aspects of the term and preterm infant: a selective update. Curr Opin Pediatr 14:175–182
5. Abitbol CL, Bauer CR, Montane B, Chandar J, Duara S, Zilleruelo G (2003) Long-term follow- up of extremely low birth weight infants with neonatal renal failure. Pediatr Nephrol 18:887–893
6. Choker G, Gouyon JB (2004) Diagnosis of acute renal failure in very preterm infants. Biol Neonate 86:212–216
7. Rodríguez-Soriano J, Aguirre M, Oliveros R, Vallo A (2005) Long-term renal follow-up of extremely low birth weight infants. Pediatr Nephrol 20:579–584
8. Andreoli SP (2004) Acute renal failure in the newborn. Semin Perinatol 28:112–123
9. Klahr S, Schreiner G, Ichikawa I (1988) The progression of renal disease. N Engl J Med 318:1657–1666
10. Remuzzi G, Benigni A, Remuzzi A (2006) Mechanisms of progression and regression of renal lesions of chronic nephropathies and diabetes. J Clin Invest 116:288–296
11. Taal MW, Brenner BM (2006) Predicting initiation and progression of chronic kidney disease developing renal risk scores. Kidney Int 70:1694–1705
12. Kandasamy Y, Smith R, Wright IM, Lumbers ER (2013) Extrauterine renal growth in preterm infants: oligonephropathy and prematurity. Pediatr Nephrol 28:1791–1796
13. Tsuboi N, Kanzaki G, Koike K, Kawamura T, Ogura M, Yokoo T (2014) Clinicopathological assessment of the nephron number. Clin Kidney J 7:107–114
14. Charlton JR, Springsteen CH, Carmody JB (2014) Nephron number and its determinants in early life: a primer. Pediatr Nephrol 29: 2299–2308
15. Schreuder MF (2012) Safety in glomerular numbers. Pediatr Nephrol 27:1881–1987
16. Brenner BM, Mackenzie HS (1997) Nephron mass as a risk factor for progression of renal disease. Kidney Int Suppl 63:124–127
17. Hoy WE, Bertram JF, Douglas-Denton R, Zimanyi M, Samuel T, Hughson MD (2008) Nephron number, glomerular volume, renal disease and hypertension. Curr Opin Nephrol Hypertens 17:258–265
18. Cappuccini B, Torlone E, Ferri C, Arnone S, Troiani S, Bini V, Di Renzo GC (2013) Renal echo-3D and microalbuminuriauria in children of diabetic mothers: a preliminary study. J Dev Orig Health Dis 4:285–289
19. Rowe DJF, Dawnay A, Watts GF (1990) Microalbuminuria in diabetes mellitus: review and recommendations for the measurement of albumin in urine. Ann Clin Biochem 27:297–312
20. Shihabi ZK, Konen JC, O'Connor ML (1991) Albuminuria vs urinary total protein for detecting chronic renal disorders. Clin Chem 37:621–624
21. Iseki K, Ikemiya Y, Iseki C, Takishita S (2003) Proteinuria and the risk of developing end-stage renal disease. Kidney Int 63:1468–1474
22. Viazzi F, Leoncini G, Conti N, Tomolillo C, Giachero G, Vercelli M, Deferrari G, Pontremo R (2010) Microalbuminuria is a predictor

of chronic renal insufficiency in patients without diabetes and with hypertension: the MAGIC study. Clin J Am Soc Nephrol 5:1099–1106

23. Skálová S (2005) The diagnostic role of urinary *N*-acetyl-B-D-glucosaminidase (NAG) activity in the detection of renal tubular impairment. Acta Med (Hradec Kralove) 48:75–80
24. Kojima T, Sasai-Takedatsu M, Hirata Y, Kobayashi Y (1994) Characterization of renal tubular damage in preterm infants with renal failure. Acta Paediatr Jpn 36:392–395
25. Tsukahara H, Hori C, Tsuchida S, Hiraoka M, Sudo M, Haruki S, Suehiro F (1994) Urinary *N*-acetyl-beta-D-glucosaminidase excretion in term and preterm neonates. J Paediatr Child Health 30:536–538
26. Schaefer L, Gilge U, Heidland A, Schaefer RM (1994) Urinary excretion of cathepsin B and cystatins as parameters of tubular damage. Kidney Int Suppl 46:64–67
27. Liu WJ, Xu BH, Ye L, Liang D, Wu HL, Zheng YY, Deng JK, Li B, Liu HF (2015) Urinary proteins induce lysosomal membrane permeabilization and lysosomal dysfunction in renal tubular epithelial cells. Am J Physiol Renal Physiol 308:639–649
28. Smulders YM, Slaats EH, Rakic M, Smulders FT, Stehouwer CD, Silberbusch J (1998) Short-term variability and sampling distribution of various parameters of urinary albumin excretion in patients with non-insulin-dependent diabetes mellitus. J Lab Clin Med 132:39–46
29. Beccari T, Mancuso F, Costanzi E, Tassi C, Barone R, Aisa MC, Orlacchio O (2000) beta-hexosaminidase, alpha-D-mannosidase, and beta-mannosidase expression in serum from patients with carbohydrate-deficient glycoprotein syndrome type I. Clin Chim Acta 302:125–132
30. Aisa MC, Rahman S, Senin U, Maggio D, Russell RG (1996) Cathepsin B activity in normal human osteoblast-like cells and human osteoblastic osteosarcoma cells (MG-63): regulation by interleukin-1 beta and parathyroid hormone. Biochim Biophys Acta 21:29–36
31. Rousian M, Verwoerd-Dikkeboom CM, Koning AH, Hop WC, Van der Spek PJ, Exalto N, Steegers EA (2009) Early pregnancy volume measurements: validation of ultrasound techniques and new perspectives. BJOG 116:278–285
32. Aperia A, Broberger O, Elinder G, Herin P, Zetterstrom R (1981) Postnatal development of renal function in pre-term and full-term infants. Acta Paediatr Scand 2:183–187
33. Tsukahara H, Yoshimoto M, Saito M, Sakaguchi T, Mitsuyoshi I, Hayashi S, Nakamura K, Kikuchi K, Sudo M (1990) Assessment of tubular function in neonates using urinary beta 2-microglobulin. Pediatr Nephrol 4:512–514
34. Awad H, El-Safty I, El-Barbary M, Imam S (2002) Evaluation of renal glomerular and tubular functional and structural integrity in neonates. Am J Med Sci 324:261–266
35. Gubhaju L, Sutherland MR, Horne RS, Medhurst A, Kent AL, Ramsden A, Moore L, Singh G, Hoy WE, Black MJ (2014) Assessment of renal functional maturation and injury in preterm neonates during the first month of life. Am J Physiol Renal Physiol 307:149–158
36. Chen JY, Lee YL, Liu CB (1991) Urinary beta 2-microglobulin and *N*-acetylbeta-D-glucosaminidase (NAG) as early markers of renal tubular dysfunction in sick neonates. J Formos Med Assoc 90:132–137
37. Hayashi M, Tomobe K, Hirabayashi H, Hoshimoto K, Ohkura T, Inaba N (2001) Increased excretion of *N*-acetyl-beta-D-glucosaminidase and beta-2-microglobulin in gestational week. Am J Med Sci 321:168–172
38. Perrone S, Mussap M, Longini M, Fanos V, Bellieni CV, Proietti F, Cataldi L, Buonocore G (2007) Oxidative kidney damage in preterm newborns during perinatal period. Clin Biochem 40:656–660
39. Clark PM, Bryant TN, Hall MA, Lowes JA, Rowe DJ (1989) Neonatal renal function assessment. Arch Dis Child 64:1264–1269
40. Fell JM, Thakkar H, Newman DJ, Price CP (1997) Measurement of albumin and low molecular weight proteins in the urine of newborn infants using a cotton wool ball collection method. Acta Paediatr 86:518–522
41. Galaske RG (1986) Renal functional maturation: renal handling of proteins by mature and immature newborns. Eur J Pediatr 145:368–371
42. Tsukahara H, Fujii Y, Tsuchida S, Hiraoka M, Morikawa K, Haruki M, Sudo M (1994) Renal handling of albumin and beta-2-microglobulin in neonates. Nephron 68:212–216
43. Pflueger AC, Larson TS, Hagl S, Knox FG (1999) Role of nitric oxide in intrarenal hemodynamics in experimental diabetes mellitus in rats. Am J Physiol 277:725–733
44. Abbate M, Zoja C, Remuzzi G (2006) How does proteinuria cause progressive renal damage? J Am Soc Nephrol 17:2974–2984
45. Thomson SC, Deng A, Bao D, Satriano J, Blantz RC, Vallon V (2001) Ornithine decarboxylase, kidney size, and the tubular hypothesis of glomerular hyperfiltration in experimental diabetes. J Clin Invest 107:217–224
46. Vallon V, Blantz RC, Thomson S (2003) Glomerular hyperfiltration and the salt paradox in early (corrected) type 1diabetes mellitus: a tubulo-centric view. J Am Soc Nephrol 14:53–59
47. Nusken E, Spencer L, Wohlfarth M, Lippach G, Lechner F, Dotsch J, Nusken KD (2015) Whole-transcript expression analysis identifies new candidate genes involved in renal tubular programming after utero-placental insufficiency in rats. J Dev Orig Health Dis 6[Suppl 2]:S140
48. Nielsen R, Courtoy PJ, Jacobsen C, Dom G, Lima WR, Jadot M, Willnow TE, Devuyst O, Christensen EI (2007) Endocytosis provides a major alternative pathway for lysosomal biogenesis in kidney proximal tubular cells. Proc Natl Acad Sci USA 104:5407–5412
49. Barbati A, Cappuccini B, Aisa MC, Grasselli C, Zamarra M, Bini V, Bellomo G, Di Renzo GC (2016) Increased urinary cystatin-C levels correlate with reduced renal volumes in neonates with intrauterine growth restriction. Neonatology 109:154–160
50. Liu D, Wen Y, Tang TT, Lv LL, Tang RN, Liu H, Ma KL, Crowley SD, Liu BC (2015) Megalin/cubulin-lysosome-mediated albumin reabsorption is involved in the tubular cell activation of NLRP3 inflammasome and tubulointerstitial inflammation. J Biol Chem 290:18018–18028