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Increased Urinary Cystatin-C Levels Correlate with Reduced Renal Volumes in Neonates with Intrauterine Growth Restriction

Antonella Barbati[a] Benito Cappuccini[d, e] Maria Cristina Aisa[b] Chiara Grasselli[a] Mariarosalba Zamarra[a] Vittorio Bini[c] Gianni Bellomo[e] Aldo Orlacchio[b, e] Gian Carlo Di Renzo[a, b, e]

[a]Department of Surgical and Biomedical Sciences, Section of Obstetrics and Gynecology, [b]Centre of Perinatal Medicine, and [c]Department of Medicine, Section of Internal Medicine, Endocrine and Metabolic Sciences, University of Perugia, [d]Department of Neonatology, Hospital S.M. della Misericordia, and [e]GEBISA, Research Foundation, Perugia, Italy

Key Words

Neonate · Intrauterine growth retardation · Renal volume · Renal cortex volume · Urine · Cystatin-C

Abstract

Background: Exposure to intrauterine growth retardation (IUGR) can have a negative impact on nephrogenesis resulting in limited fetal kidney development and supporting the hypothesis that IUGR represents a risk for renal function and long-term renal disease. Cystatin-C (Cys-C), a strong inhibitor of cysteine proteinases, is freely filtered by the kidney glomerulus and is reabsorbed by the tubules, where it is almost totally catabolized; what remains is subsequently eliminated in urine. In tubular diseases and in hyperfiltration conditions, it seems reasonable to postulate that Cys-C degradation would decrease, and consequently an increase in its urinary elimination would be observed. ***Objectives:*** The aim of this study was to investigate the urinary excretion of Cys-C simultaneously with the assessment of renal volumes in adequate for gestational age (AGA) and IUGR neonates in order to identify its clinical value in IUGR. ***Methods:*** Urinary Cys-C levels were measured using the enzyme immunoassay DetectX® Human Cystatin C kit in IUGR and AGA neonates. Whole renal and renal cortex volumes were assessed with ultrasounds (Vocal II; Software, GE). ***Results:*** Urinary Cys-C levels in IUGR were significantly higher than those found in AGA and were negatively correlated to reduced whole renal and renal cortex volumes. ***Conclusions:*** The increased levels of Cys-C in the urine of neonates with IUGR were significantly associated with reduced renal/renal cortex volumes, suggesting that Cys-C could be taken as a surrogate of nephron mass. It also could be used as an early biochemical marker to identify IUGR neonates at high risk of developing long-term renal disease and to select patients for monitoring during childhood.

Introduction

Nephrogenesis starts early in pregnancy (at weeks 5–6 of gestation) and it is normally completed by the third trimester between 32 and 36 weeks of gestation; at the end of this period, each kidney has 900,000–1,000,000 nephrons, although the number can vary widely, in correlation

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Antonella Barbati
Dipartimento Scienze Chirurgiche e Biomediche
Polo Didattico, Ed. C, 3° P., Sant'Andrea delle Fratte
IT–06132 Perugia (Italy)
E-Mail antonella.barbati@unipg.it

with birth weight; once nephrogenesis has stopped, there is no possibility of forming new nephrons later in life. An increasing number of investigations are showing an association between intrauterine growth retardation (IUGR) and limited fetal kidney development, as first documented by autoptic studies [1–4].

In this regard, exposure to IUGR can negatively impact nephrogenesis, supporting the hypothesis that IUGR represents a risk factor for impaired renal function resulting from a reduced nephron number and decreased renal size, causing loss of filtration surface area, single nephron hyperfiltration, glomerular hypertension and long-term renal disease as clearly demonstrated by epidemiological studies [5–8]. The assessment of kidney growth is currently performed using 2D or estimating renal volume with 3D ultrasound scans [9–11]; however, to date, no biochemical marker has been evaluated in association with renal volume reduction and IUGR. Cystatin C (Cys-C) is a strongly basic secretory nonglycosylated protein produced at a constant rate in all nucleated cells [12]. Due to its low molecular weight (13.3 kDa), Cys-C is freely filtered in the kidney glomerulus with no retrieval back to the circulation; thus, serum Cys-C is a clinically established biomarker for glomerular filtration rate [13]. In proximal tubular cells, Cys-C is predominately reabsorbed and subsequently catabolized; therefore, in urine, the concentration of Cys-C is normally low and high levels reflect an abnormal reabsorption and degradation by tubular cells [14–17]. The aim of this study was to investigate the urinary excretion of Cys-C simultaneously with the assessment of renal volumes, in adequate for gestational age (AGA) and IUGR neonates (1) to evaluate its clinical value in IUGR newborns and (2) to identify a possible biochemical marker useful to select IUGR newborns at high risk for long-term renal disease.

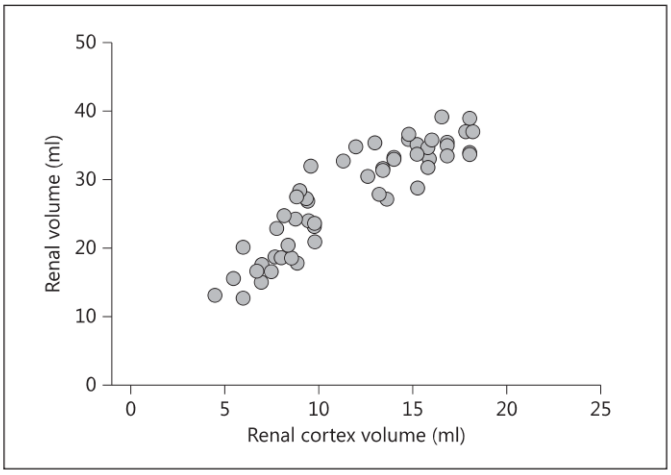

Fig. 1. Correlation between whole renal and renal cortex volume in AGA and IUGR neonates. Spearman's rho = 0.986; $p < 0.0001$.

Materials and Methods

Subjects

For this study, 20 neonates with IUGR (7 males and 13 females; gestational age ≥36 weeks to exclude the effects of prematurity on renal development) and 34 healthy controls (20 males and 14 females) defined as AGA (a newborn infant whose size is within the normal range for his or her gestational age), born of healthy parents (nonobese, nonsmokers, younger than 40 years), were prospectively recruited from the newborn nursery at S. Maria della Misericordia Hospital from June 2013 to March 2015. The diagnosis of IUGR was assigned to neonates with a birth weight below the 10th centile for gestational age and with early altered placental fetal hemodynamics (evaluated by Doppler US). Newborns with congenital anomalies or with a urinary tract infection were excluded. Informed consent was obtained from all parents, the protocol was approved by our institutional review board.

Ultrasonography Examinations

Whole renal and renal cortex volumes were evaluated by echo 3D combined with Vocal II volume software, a general imaging 3D quantification software (GE Ultrasounds, USA), at 30–40 days. The measurement was obtained as the average of four repeated measures by the same sonographer, blinded to group assignment, with intra- and interoperator variability equal to 4.0 and 5.1%, respectively (coefficient of variation).

Urine Collection

Morning urine samples were collected using a U-bag collection device, the same day of echo 3D assessment. Transferred to our laboratory, measurement of urinary proteins, leukocytes and nitrite were tested with a multiple test strip (Combi-Screen PLUS, Analyticon Biotechnologies AG) to exclude urinary infections and/or proteinuria. Then, the urine was centrifuged at 4°C for 30 min at 5,000 rpm, aliquoted and stored in a −20°C freezer for enzyme immunoassay Cys-C quantification.

Measurement of Urinary Cys-C Levels

Urinary Cys-C levels were measured with The DetectX® Human Cystatin C kit (Catalog No. K012-H1; Arbor Assays, Ann Arbor, Mich., USA), an enzyme immunoassay designed to quantitatively measure human Cys-C present in biological samples and tissue culture media, according to the manufacturers' instructions. The laboratory technician was blinded to group assignment as well as to the results of renal volume measurement.

Briefly, standards or diluted samples (1:10) were pipetted, in duplicate, into a clear microtiter plate coated with mouse anti-human Cys-C to capture the presence of Cys-C. After a 60-min incubation, the plate was washed and a monoclonal antibody to Cys-C labeled with peroxidase was added; the plate was again incubated for 30 min and washed. Then, substrate was added to the plate, to react with the bound Cys-C antibody conjugate, and the reaction

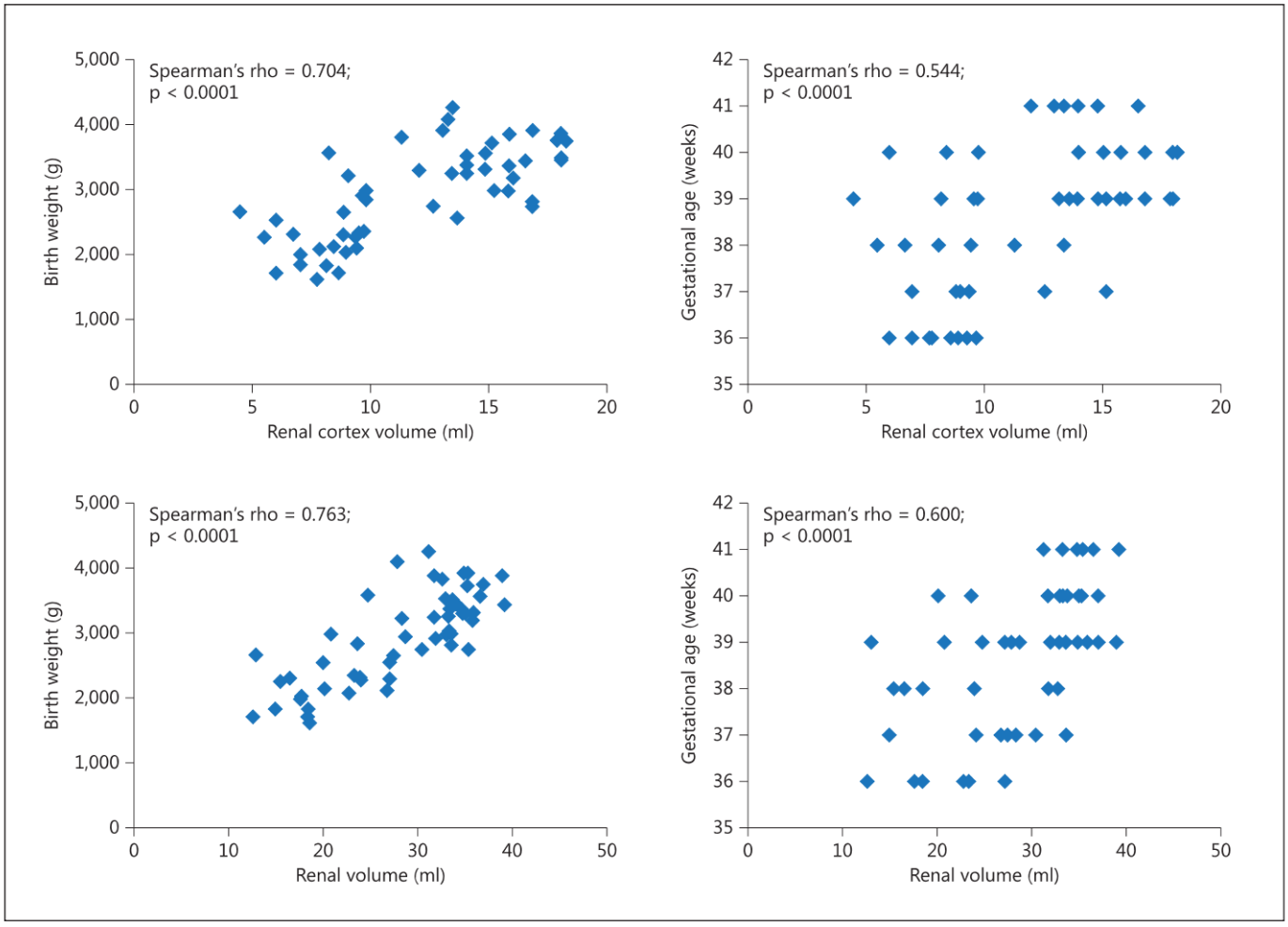

Fig. 2. Renal volume correlation with gestational age and birth weight.

was stopped after a third incubation. The intensity of the generated color was detected in a microtiter plate reader capable of measuring a 450-nm wavelength. The sensitivity of the assay was determined as 0.058 ng/ml. A human Cys-C standard was provided to generate a standard curve ranging from 0.156 to 10 ng/ml. Intra- and interassay imprecision (CV) of Cys-C using urine specimen of 5.07 ng/ml was 6.3 and 8.9, respectively.

Statistical Analysis

A preliminary power analysis indicated that a sample size of 20 cases and 30 controls (1/1.5 ratio) had a 84% chance of detecting a large effect size and a 50% chance of detecting a medium effect size (defined by Cohen, as 0.8 and 0.5 of a population standard deviation between the means, respectively) [18] between the two groups as significant at the 5% level (two tailed). Power analysis was performed by G*Power software version 3.1.7 (2013). The Shapiro-Wilk test was applied to assess the normality of variables. Due to nonnormal distribution of variables, the Mann-Whitney U test was used for comparisons of continuous data and correlations were checked by Spearman's rho coefficient analysis.

Predictive accuracy was quantified as the area (AUC) under the receiver operating characteristics curve (ROC). Statistical analyses were performed using IBM-SPSS® version 22.0 (2013; IBM Corp., Armonk, N.Y., USA). A two-sided p value <0.05 was considered significant. MedCalc release 9.3.7.0 (2007; MedCalc Software, Mariakerke, Belgium) was used to plot ROC curves.

Results

Renal cortex volume was closely related to whole renal volume (fig. 1) and both of them correlated to gestational age and to birth weight ($p < 0.0001$; fig. 2). Their measurements in IUGR newborns were significantly lower than those found in AGA newborns (mean ± SD; whole renal volume: 19.83 ± 4.5 vs. 32.68 ± 4.16; renal cortex volume: 8.0 ± 1.95 vs. 14.1 ± 2.9); conversely, urinary Cys-C levels in IUGR were significantly higher than in AGA neonates

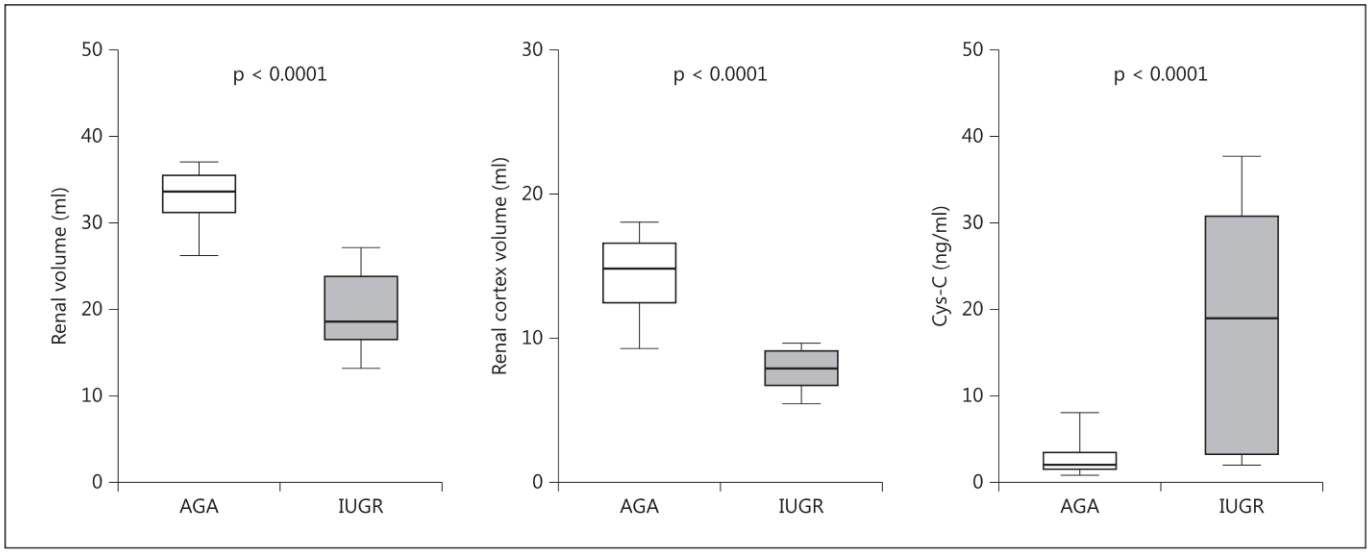

Fig. 3. Box and whisker plots of renal volume, renal cortex volume and urinary Cys-C concentrations in the AGA control group and neonates with IUGR. The box covers the middle 50% of data, between the lower and upper quartiles; the 'whiskers' extend out to the 10th and 90th percentiles, while the central line is at median. The differences between the two groups were statistically significant ($p < 0.0001$).

Table 1. Comparison among variables studied in AGA and IUGR neonates

	AGA	IUGR	p
n	34	20	
Renal volume, ml	33.5 (21–39)	18.6 (13–27)	<0.0001
Renal cortex volume, ml	14.8 (8.2–18.2)	7.95 (4.5–13.6)	<0.0001
Cys-C, ng/ml	1.9 (0.4–25.9)	18.9 (1.7–37.8)	<0.0001
Birth weight, g	3,350 (2,640–4,250)	2,120 (1,600–2,650)	<0.0001
Gestational weeks	39 (37–41)	37 (36–40)	<0.0001
Placental weight, g	600 (486–800)	510 (350–720)	0.660
Mother's BMI at the start of pregnancy	22.5 (18.4–38.7)	21.4 (17.1–38.6)	0.966
Mother's BMI at the end of pregnancy	28.5 (22.8–41.6)	27.4 (22.9–37.1)	0.857

Data are expressed as medians and ranges.

(mean ± SD: 18.0 ± 13.7 vs. 3.46 ± 4.61; fig. 3). All values of variable examined are reported in table 1.

The overall data of Cys-C showed a significant inverse correlation with whole renal and renal cortex volumes, birth weight, and gestational age; Spearman's rho correlation coefficients were: –0.379 ($p = 0.005$), –0.434 ($p = 0.001$), –0.487 ($p < 0.001$) and –0.416 ($p = 0.002$), respectively (fig. 4). ROC analysis (fig. 5) was performed; AUC was 0.978, 0.954 and 0.853 for renal volume, renal cortex volume and urinary Cys-C, respectively. At the value of 3.46 ng/ml (the mean for AGA neonates), Cys-C showed a sensibility and specificity of 75 and 76.4%, respectively.

The measurements of urinary proteins, leukocytes and nitrite, tested by multiple test strips, were negative for all samples. There were no gender differences in the variables studied.

Discussion

The results of the present study support a strong correlation between whole renal volume, renal cortex volume, birth weight and gestational age; more interesting were the results obtained by the urinary Cys-C measure-

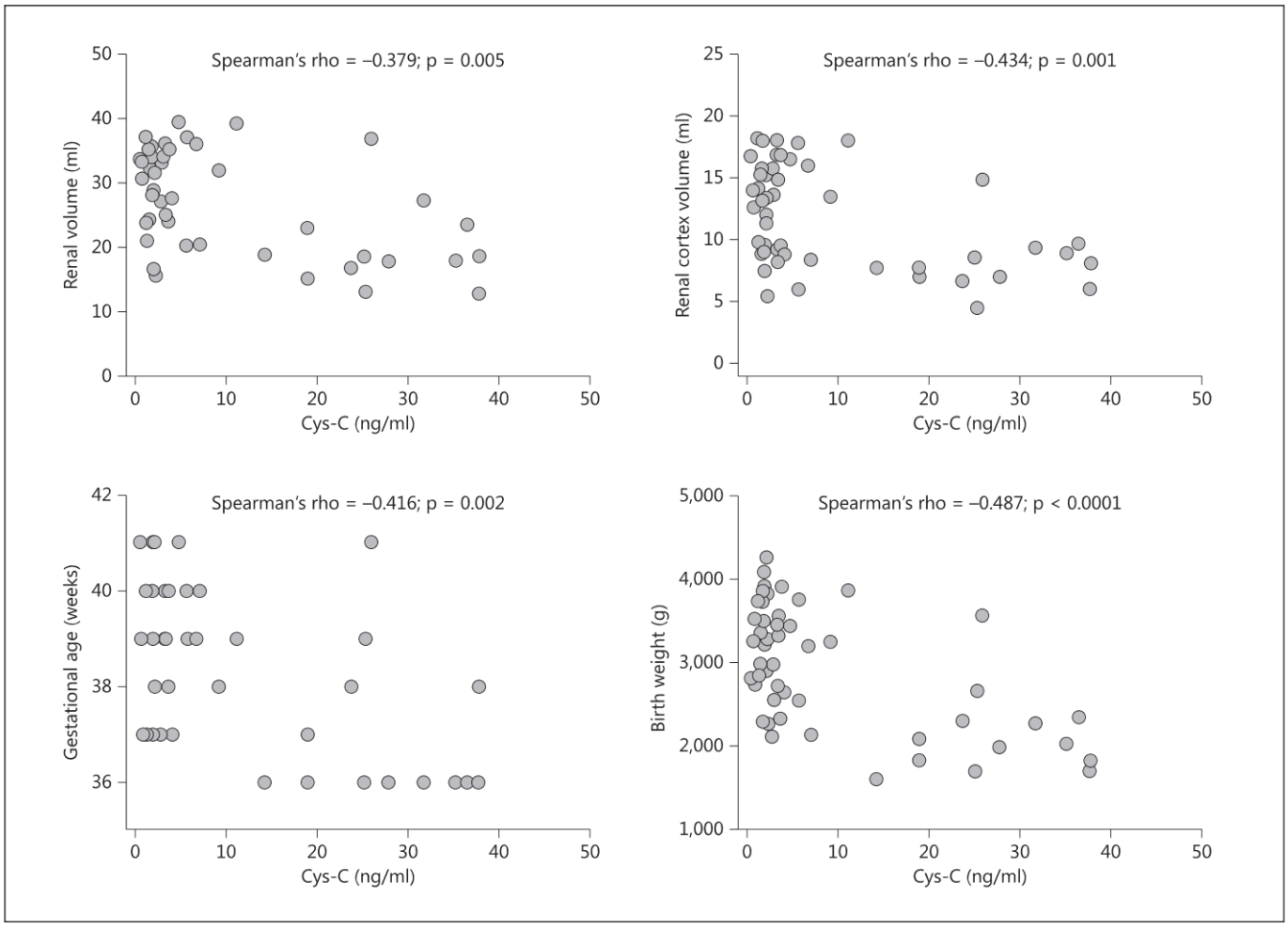

Fig. 4. Urinary levels of Cys-C were inversely correlated with renal volumes, gestational age and birth weight.

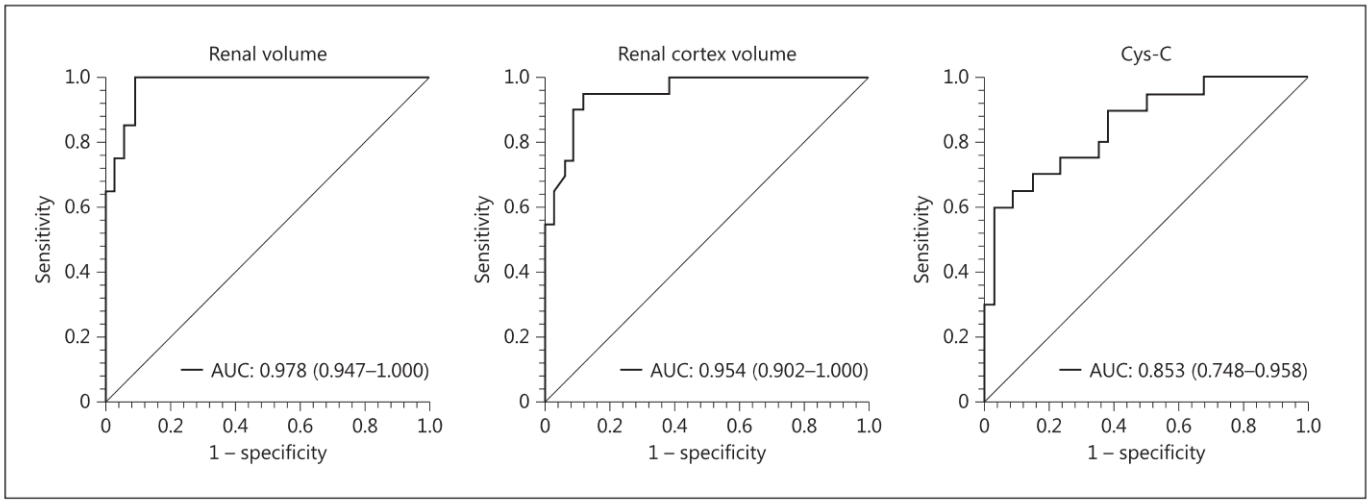

Fig. 5. ROC demonstrating the performance of renal volume, renal cortex volume and urinary Cys-C to detect IUGR condition. For each ROC plot, the AUC and 95% CI are reported in the boxes.

ment in association with renal volumes assessment in IUGR neonates.

IUGR can adversely affect the number of nephrons in the newborn's kidney [1–3]; neonates below the 10th centile of birth weight had 30% fewer glomeruli than those with birth weights above the 10th centile [4]. However, if the growth restriction occurs at a late stage of gestation, when nephrogenesis is completed (or nearing completion), such a relationship disappears [7]. It has been described that a reduced nephron endowment at the beginning of life, in infants born IUGR, is associated with an adaptive single nephron glomerular hyperfiltration that may increase the risk of kidney damage and lead to chronic kidney disease later in life [19, 20]. When the number of nephrons is reduced, the compensatory hypertrophy pushes the glomeruli to function under increased intracapillary hydraulic pressure, which, over time, causes damage to the capillary walls [21, 22]. Nevertheless, reduced nephron number is not systematically associated with hypertension and impaired glomerular filtration rate, especially when it is moderate. This mechanism is still being discussed and recent experimental studies have failed to show such a link. Boubred et al. [23] reported that the reduced nephron number is not sufficient by itself to induce long-term renal diseases, but that it constitutes a factor of vulnerability when additional factors, in particular a rapid postnatal growth or overfeeding, promote the early onset of diseases through a complex combination of various pathophysiological pathways. The authors suggest that future research may aim to clarify early biomarkers of nephron endowment and early renal injury in order to determine optimal perinatal nutrition and eventual prophylactic measures to be applied to infants at increased risk of developmentally programmed adult diseases [23].

To our knowledge, this is the first study to evaluate urinary Cys-C levels in addition to whole renal/renal cortex volume in IUGR neonates compared to AGA. We found that IUGR newborns showed a statistically significant elevation of urinary Cys-C, inversely correlated with whole renal/renal cortex volume, compared to controls.

Urinary Cys-C has been validated as a good reflection of tubular function [15]. In proximal tubules, filtered Cys-C is reabsorbed by megalin-facilitated endocytosis, and its increased urinary elimination in IUGR could be indicative of reduced degradation and reabsorption [17].

How IUGR and/or the reduced renal volumes adversely impact tubular reabsorption of Cys-C is not clear. We can try to explain this association by suggesting different hypotheses: (1) in IUGR, tubular function is not adequate for a glomerular hyperfiltration adaptive system, and then the tubular cells are not able to reabsorb and degrade the increased Cys-C filtered, and (2) filtered albumin is also reabsorbed by megalin receptor-mediated endocytosis, and independently from tubular injury, competition for receptor-mediated transport between albumin and other low molecular weight proteins could be responsible for a significant increase of urinary Cys-C in the presence of proteinuria [24]. A recent experimental study demonstrated that rats with induced albuminuria and proteinuria produced parallel increases of urinary Cys-C excretion. Importantly, urinary Cys-C excretion decreased again when albuminuria and proteinuria returned to baseline. These observations are consistent with the hypothesis that albuminuria reduces the absorption of low molecular weight proteins by competing for common transport mechanisms [25, 26]. Nevertheless, in our study we found increased levels of urinary Cys-C in the absence of proteinuria. We might also speculate that such a finding could be explained by the difference in the analytical sensitivity of the tests. The enzyme immunoassay DetectX® Human Cystatin C kit is able to detect very low levels of Cys-C (detection limit: 0.058 ng/ml), showing a very high analytical sensitivity, whereas the lower detection limit for proteinuria using a multiple test strip is considered to be >150 mg/l. A third hypothesis is that the megalin-facilitated endocytosis system in IUGR neonates could be not completely developed, thus being responsible for the increased urinary Cys-C levels. Finally, and alternatively, in a condition of normal glomerular function, and in a condition without proteinuria, we can suppose an impairment of proximal tubular cells leading to an increase of urinary Cys-C levels.

The increased levels of Cys-C in the urine of neonates with IUGR, significantly associated with reduced renal volumes, suggest that Cys-C could be taken as a surrogate of nephron mass. Also, since the reduction of nephron number, per se, could be not enough to determine hypertension and/or renal disease, a new approach with the assessment of Cys-C combined with whole renal volume or renal cortex volume assessment would be useful to identify IUGR neonates at high risk to develop long-term renal disease and to select patients for monitoring during childhood. Additional studies for the follow-up of IUGR newborns with high levels of urinary Cys-C are in progress.

Furthermore, Cys-C determination could easily be used as an adjunct to the standard panel to screen kidney

functionality, even in emergency situations, which is supported by the observation that urinary Cys-C measurement in spot urine samples is possible, as no circadian rhythm was demonstrated for Cys-C excretion in urine [15]; hence, the 24-hour urine collection, which is difficult to handle, is not necessary.

In conclusion, detection of high levels of urinary Cys-C in IUGR neonates could be considered as an early precursor of renal injury and a promising biochemical marker to identify and to select IUGR neonates that could be monitored for risk of renal injury.

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Disclosure Statement

None of the authors report a conflict of interest. The process of obtaining informed consent was approved by the appropriate institutional review committee.

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