Pediatric RESEARCH

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CLINICAL RESEARCH ARTICLE

# Urinary Cystatin-C, a marker to assess and monitor neonatal kidney maturation and function: validation in twins

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**BACKGROUND:** Nephrogenesis is a complex process of nephron formation and maturation that can be compromised by preterm delivery and intrauterine growth restriction. This study aimed to evaluate and compare urinary Cys-C levels with renal volume in a cohort of preterm and term twins, adequate for gestational age or intrauterine growth restricted, to investigate their values in different conditions of nephrogenesis.

**METHODS:** The study was performed on twins at 30–40 days of postnatal corrected age: renal volumes were measured by 3D ultrasound technology and urine samples were analyzed for Cystatin-C. A follow-up was performed by Cystatin-C.

**RESULTS:** Renal volumes in preterm and intrauterine growth-restricted twins showed values significantly lower than those observed in term twins and were inversely correlated to urinary Cystatin-C levels. During the follow-up, intrauterine growth-restricted twins showed amplified levels of urinary Cystatin-C; in contrast, invariable or decreased levels were observed in adequate for gestational age twins.

**CONCLUSIONS:** Urinary Cystatin-C, evaluated when intrauterine/extrauterine nephrogenesis could be considered completed, concurrently with renal volume assessment can improve the identification of neonates with initial kidney impairment. Its potential value as a useful marker in monitoring physiological/pathological renal conditions could be considered, mainly for neonates at elevated risk of developing long-term renal diseases.

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**IMPACT:**

- Urinary Cys-C levels are inversely correlated to renal volumes and reflect nephrogenesis conditions.
- No data in literature are reported regarding: (a) the concurrent assessment of renal volumes and urinary levels of Cystatin-C in preterm and term twins with different conditions of gestational life, i.e., AGA and IUGR and (b) the follow-up of IUGR and preterm neonates using the urinary Cys-C determination.
- The variations of urinary Cys-C levels, observed in the follow-up of preterm and/or IUGR neonates, support the usefulness of monitoring those neonates with altered nephrogenesis, who are later at risk for renal impairment and for long-term renal diseases.

## INTRODUCTION

Nephrogenesis is a long and complex process that is completed approximately by the 36th week of gestation, with the majority of nephrons formed in late gestation. Once the nephrogenesis has stopped, each kidney has a number of nephrons that can vary widely and there is no possibility of forming new nephrons later in life.[1,2] However, the maturation of the kidney continues through the postnatal period with significant functional changes. In the near term period, fetal kidney shows sufficient glomerular and tubular development to allow the adaptation to extrauterine life.[3–5] The main risk factors for impaired nephrogenesis are preterm delivery and intrauterine growth restriction,[6–8] which are two conditions associated with low birth weight, more frequently observed in twin pregnancies.[9,10]

In extremely preterm neonates in which birth occurs when nephrogenesis is often ongoing, autopsy studies showed active or even accelerated extrauterine nephrogenesis until 40 postnatal days with a high percentage of morphologically abnormal glomeruli, including atubular and cystic glomeruli, which would not be able to function. Among those infants who survived >40 days, a full complement of nephrons was never achieved compared to term newborns.[11,12] As far as intrauterine growth-restricted (IUGR) neonates are concerned, it is well documented that they have a significantly reduced nephron endowment compared to infants who are adequate for gestational age (AGA; whose body size is within the normal range for their GA). It has been estimated that neonates below the 10th centile of birth weight had 30% fewer glomeruli than the neonates with birth weights above the 10th centile.[13,14]

Renal development in fetal life has taken on a very important significance, as kidney immaturity or developmental impairment may not only have a short-term impact (with many postnatal

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complications) but also lifelong consequences with an increased risk of chronic kidney disease and hypertension in adulthood.[15–18] Kidney growth is currently evaluated using two- or three-dimensional (3D) ultrasound (US) scans and reference ranges for kidney size (length, area, or volume) in healthy singletons during fetal and neonatal life have been published.[19,20] Generally, at birth, kidney size and nephron number are proportional to body size and renal size, respectively. As a consequence, renal volume in preterm and IUGR neonates has been recognized as an essential parameter to evaluate kidney function and predict the clinical course of these infants.[21,22]

Recently, in addition to the anthropometric parameters, including renal volumes, various biochemical indicators that could identify neonates with kidney injury have been studied; among these, urinary Cystatin-C (uCys-C) looks like a promising biomarker.[23–30] Cys-C is an inhibitor of cysteine proteinases produced at a constant rate in all nucleated cells; it is freely filtered by kidney glomeruli due to its low molecular weight and its positive charge. In proximal tubular cells, it is predominantly reabsorbed and catabolized, so uCys-C levels are normally low, ranging between 0.03 and 0.18 mg/L,[31] In tubular diseases, Cys-C degradation would be reduced and its measurement has become a way to assess structural and functional renal tubular impairment independent of glomerular filtration rate.[31–33]

The aim of this observational study was to evaluate and compare uCys-C levels with renal volumes in a cohort of preterm and term twins, AGA or IUGR, to investigate their values in different conditions of nephrogenesis.

We believe that twins represent an excellent study population as they are at increased risk of prematurity and/or IUGR,[9,10] two conditions associated with reduced kidney development, impaired nephrogenesis, and long-term renal diseases.

## MATERIALS AND METHODS

All twins of this cohort were prospectively recruited from the newborn nursery at S. Maria della Misericordia's Hospital in Perugia, Italy. They were selected from healthy parents (non-obese, non-smokers, non-alcoholics, non-drug users, <40 years) and from mothers not affected by pre-eclampsia, hypertension, and/or gestational diabetes mellitus. Ninety-five percent of them delivered by cesarean section. Exclusion criteria for twins were: congenital anomalies, urinary tract infection, acute kidney injury (AKI), and twin–twin transfusion syndrome. Neonates eligible for the present study were twins with Apgar score value ≥7 at the first and fifth minute and normal physiological postnatal adaptation, negative for urine multiple test strip, serum creatinine, and azotemia. All neonates underwent routine biochemical analysis on day 3 of life. A total of 74 twins (all heterozygous: 30 males, 44 females) born at 32–39 weeks of gestation were included in the study: 58 were classified as AGA and 16 as IUGR twins. Since nephrogenesis is completed within the 36th week of gestation, the AGA and IUGR groups were sub-classified in twins born before the 36th week of gestation (premature group, $n = 24$) or after (term group, $n = 50$) to distinguish twins in whom nephrogenesis was still incomplete from those in whom nephrogenesis was considered concluded at the time of delivery. In this manner, four groups were designed: (1) AGA ≥36 weeks (term twins/control group); (2) $32 \le$ AGA $< 36$ weeks (preterm AGA twins); (3) IUGR ≥36 weeks (term IUGR twins); and (4) $32 \le$ IUGR $< 36$ weeks (preterm IUGR twins). The diagnosis of IUGR was assigned to neonates with early altered placental fetal hemodynamics (evaluated by Doppler US) and with a birth weight below the 10th centile for GA reference for twins.[34] All neonates were evaluated at 30–40 days of postnatal corrected age; at this time, nephrogenesis (that may continue ex utero after delivery for 20–40 days in preterm neonates) is considered concluded and an initial physiological adaptation of kidney to extrauterine life has occurred.[4,5,11] In this way, we evaluated all neonates in a set time to reduce the significant functional changes in the early postnatal period. This was done in an outpatient setting during the usual planned follow-up appointment.

### Ultrasonography examinations

Whole-renal and renal cortex volumes were evaluated by echo 3D combined with VOCAL (Virtual Organ Computer-Aided Analysis) II volume software, a general imaging 3D quantification software (GE Ultrasounds, USA), at 30–40 days of postnatal corrected age. The measurement was obtained as the average of four repeated measurements by the same sonographer, blinded to group assignment, with intra- and inter-operator variability equal to 4.0% and 5.1%, respectively.

### Urine Collection

Morning urine samples were collected using a U-bag collection device, on the same day as echo 3-D 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 5000 rpm, aliquoted, and stored in a −20 °C freezer for enzyme immunoassay (EIA) Cystatin-C quantification.

### Measurement of urinary Cys-C levels

Urinary Cys-C levels were measured with the DetectX® Human Cystatin C Kit (Arbor Assays, Catalog Number K012-H1; Ann Arbor, MI 48108, USA), an EIA designed to quantitatively measure human Cys-C present in biological samples and tissue culture media, according to the manufacturer's 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 a 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 was stopped after a third incubation. The intensity of the generated color was detected in a microtiter plate reader capable of measuring 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 inter-assay coefficient of variations of Cys-C using two urine specimens of 2.3 and 25.5 ng/ml were 5.3 and 7.9, respectively.

### Follow-up

In a preliminary follow-up study, 16 infants born at different GA, representative of different nephrogenesis conditions, were recruited with parents' consent, 1 month after the first Cys-C determination to monitor uCys-C levels. Urine collection and Cys-C measurement were achieved with the same procedures.

### Statistical analysis

Data analysis was carried out using the GraphPad Prism version 7.01 statistical software (GraphPad Software Inc., San Diego, CA). Shapiro–Wilk test was applied to assess the normality of variables. Owing to the non-normal distribution of variables, comparisons were performed using non-parametric Kruskal–Wallis one-way analysis of variance with Dunn's multiple comparisons test and/or Mann–Whitney's $U$ test, as deemed appropriate. Correlations among the variables under investigation were checked by Spearman's rho coefficient analysis. Predictive accuracy was quantified as the area under the receiver operating characteristic

**Table 1.** Comparison among variables studied in the different groups; values are expressed as mean (SD).

| Twins | | *n* | GA (weeks) | BW (g) | RCV (ml) | WRV (ml) | uCys-C (ng/ml) |
|---|---|---|---|---|---|---|---|
| AGA (58) | ≥36 weeks | 39 | 36.7 (0.74) | 2566 (305) | 10.91 (2.66) | 27.65 (4.74) | 7.27 (9.1) |
| | 32 ≤ weeks < 36 | 19 | 33.9 (1.19) | 2048 (240) | 9.03 (2.43) | 23.25 (5.55) | 13.88 (14.7) |
| IUGR (16) | ≥36 weeks | 11 | 36.8 (0.75) | 1962 (286) | 8.01 (1.24) | 18.83 (3.55) | 22.55 (11.0) |
| | 32 ≤ weeks < 36 | 5 | 33.6 (1.34) | 1565 (213) | 6.04 (0.70) | 17.18 (4.14) | 27.40 (6.9) |

*AGA* adequate for gestational age, *IUGR* intrauterine growth restriction, *GA* gestational age, *BW* birth weight, *RCV* renal cortex volume, *WRV* whole renal volume, *uCys-C* urinary Cystatin-C.

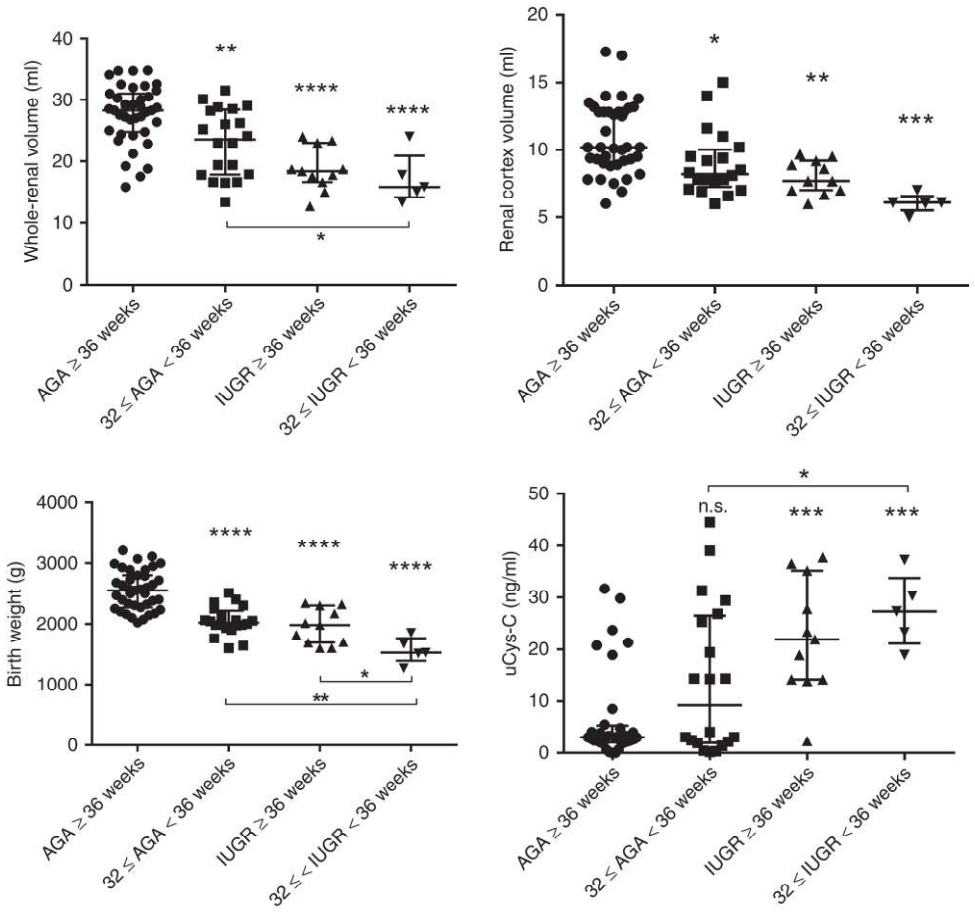


**Fig. 1 Variability of birth weight, whole renal volume, renal cortex volume, and uCys-C in all the studied groups.** Plots are presented as median with interquartile range. Analysis was performed using non-parametric Kruskal–Wallis one-way analysis of variance (ANOVA) with Dunn's multiple comparisons test (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

(ROC) curves (AUCs). A $p$ value of <0.05 was considered statistically significant.

## RESULTS

### Renal volumes and uCys-C for kidney assessment

The characteristics of each group and the values of all variables examined, expressed as media (SD), are reported in Table 1. Birth weight and renal volumes (both whole and cortical) in preterm AGA and/or IUGR groups, evaluated at 30–40 days of postnatal corrected age, were significantly lower than those observed in the control group (Fig. 1). The impact of reduced renal volume in IUGR is regardless of the degree of prematurity. In fact, both preterm and IUGR groups have a statistically significant reduction of renal volumes compared to those of the control group (AGA ≥36 weeks). In IUGR, the reduction of renal volumes compared to preterm is also evident, with statistically significant difference between the preterm and preterm IUGR groups. In concurrence with reduced renal volumes, increased levels of uCys-C were found; statistically significant differences were observed only in IUGR but not in preterm twins, compared to the control group.

Furthermore, all twins were distributed, according to GA at birth, to 8 groups (range: 32–39 weeks; median = 36; Fig. 2, upper box), and the mean values of uCys-C levels and renal

| Twins | Weeks | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 |
| AGA ($n = 58$) | 3 | 2 | 4 | 10 | 16 | 19 | 2 | 2 |
| IUGR ($n = 16$) | 1 | 2 | — | 2 | 4 | 5 | 2 | — |

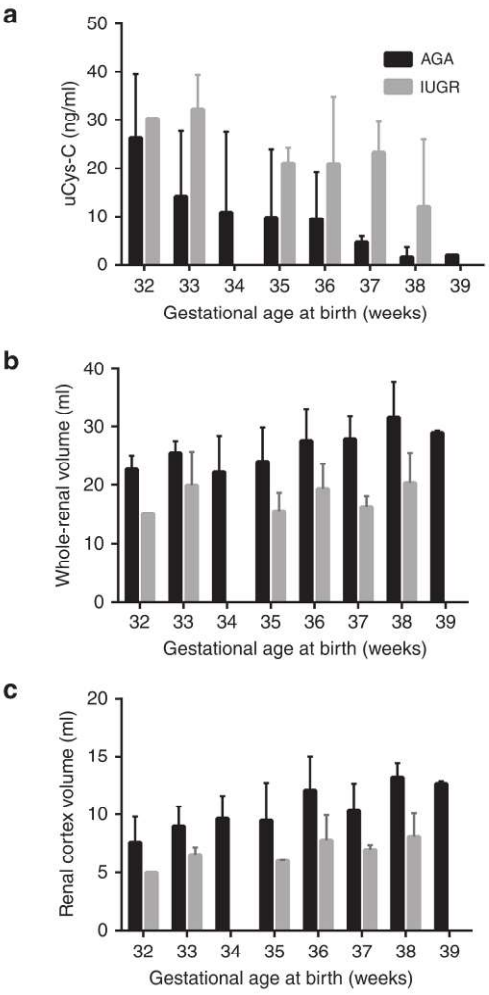


**Fig. 2 Twins distribution by gestational age at birth.** Mean values of urinary Cys-C (**a**), whole renal volume (**b**), and renal cortex volume (**c**) for each week of gestational age at birth (range 32–39; upper box) in AGA (adequate for gestational age) and IUGR (intrauterine growth restricted) twins, evaluated at 30–40 days of postnatal corrected age.

volumes for each week of GA at birth were calculated in AGA and IUGR twins, separately. The mean values of uCys-C in the AGA twins decreased with increasing GA at birth and were always manifestly lower than those observed in IUGR twins (Fig. 2a). Whole-renal and renal cortex volumes measured at 30–40 days of postnatal corrected age increased progressively with GA in both AGA and IUGR twins, showing a similar pattern, whereas values for IUGR were always lower than those for AGA twins (Fig. 2b, c).

### Analysis of correlation among the variables investigated

The analysis of correlation by Spearman rho values indicate renal volumes (whole and cortical) closely related to each other (0.856; $p < 0.0001$) and to birth weight (0.621 and 0.528, respectively; $p < 0.0001$); their association with GA was moderate ($p < 0.05$). uCys-C levels showed an inverse correlation with birth weight (−0.395; $p < 0.001$), renal volumes (−0.292 and −0.280 for whole and cortical renal volume, respectively; $p < 0.05$) and GA (−0.215; $p$ value = not significant).

### ROC curves

Based on these results, we evaluated the efficacy of uCys-C by ROC curve analysis. A comparison with renal volumes was performed (Fig. 3). For renal volumes, the AUC values in preterm and IUGR groups showed a statistically significant difference compared to term control. uCys-C appears to have a potential diagnostic value to discriminate IUGR (AUC = 0.858; $p < 0.0001$) from term neonates but not preterm from term neonates (AUC = 0.629; $p$ = NS).

### Urinary Cystatin-C follow-up

In eight couples of twins, representative of different nephrogenesis conditions (Table 2), uCys-C levels were further investigated 1 month after the first determination, as follow-up. Seven AGA twins with low levels of uCys-C at 30–40 days of postnatal of corrected age preserved low levels of the marker (range: 0.63–4.0 ng/ml); five AGA twins with initial elevated levels of uCys-C showed a marked reduction of their values in four cases. In contrast, 4 IUGR twins with high levels of uCys-C at 30–40 days of postnatal of corrected age showed increased (3 cases) or unchanged values (1 case) at the follow-up (Fig. 4).

### Chorionicity impact

A comparison of the parameters investigated in twins, classified for chorionicity and gender, is reported in Table 3. Statistically significant differences between the monochorionic (MC) and dichorionic (DC) groups were observed for GA and renal volumes, but not for uCys-C and birth weight. No differences were observed for gender ($p > 0.05$).

## DISCUSSION

In this prospective cohort study, 74 selected AGA and IUGR twins born at 32–39 weeks of gestation were investigated for renal conditions by measurements of uCys-C levels and renal volumes (both whole and cortical) at 30–40 days of postnatal corrected age when nephrogenesis was stopped and an initial physiological adaptation of kidney to extrauterine life occurred. Renal volume is considered a surrogate marker of nephron number, of which the association with chronic kidney disease in later life has been recently documented.[15,18,35,36] The strong correlation found between uCys-C levels and renal volumes in our twin population with no renal dysfunction allowed the testing of uCys-C as an early and prompt biochemical marker of impaired nephrogenesis.

In agreement with literature data and our previous studies regarding singleton neonates,[14,24] we observed a strong positive correlation between renal volumes and birth weight in twins. The close relationship between whole and cortex renal volumes recognizes the value and the reliability of the cortical volume measurement by echo 3D combined with Vocal II volume software. Thus, like the whole-renal volume, in absence of an inducible glomerular hypertrophy at 30–40 days of postnatal corrected age, the renal cortex volume can be considered indicative of nephron number, including 95% of glomeruli.

Even if nephrogenesis may continue ex utero after delivery for 20–40 days,[4,5,11] our results evidenced that renal volumes in preterm neonates ($32 \le$ AGA $< 36$ weeks), at 30–40 days of postnatal corrected age, were significantly lower than those found in the control group (AGA $\ge 36$ weeks). In twins grouped according to the single weeks of GA at birth, renal volumes increased progressively with advancing of GA, however, not reaching the volumes observed in term neonates. These results are in agreement with the concept that in premature neonates, the nephron number remains lower than normal even

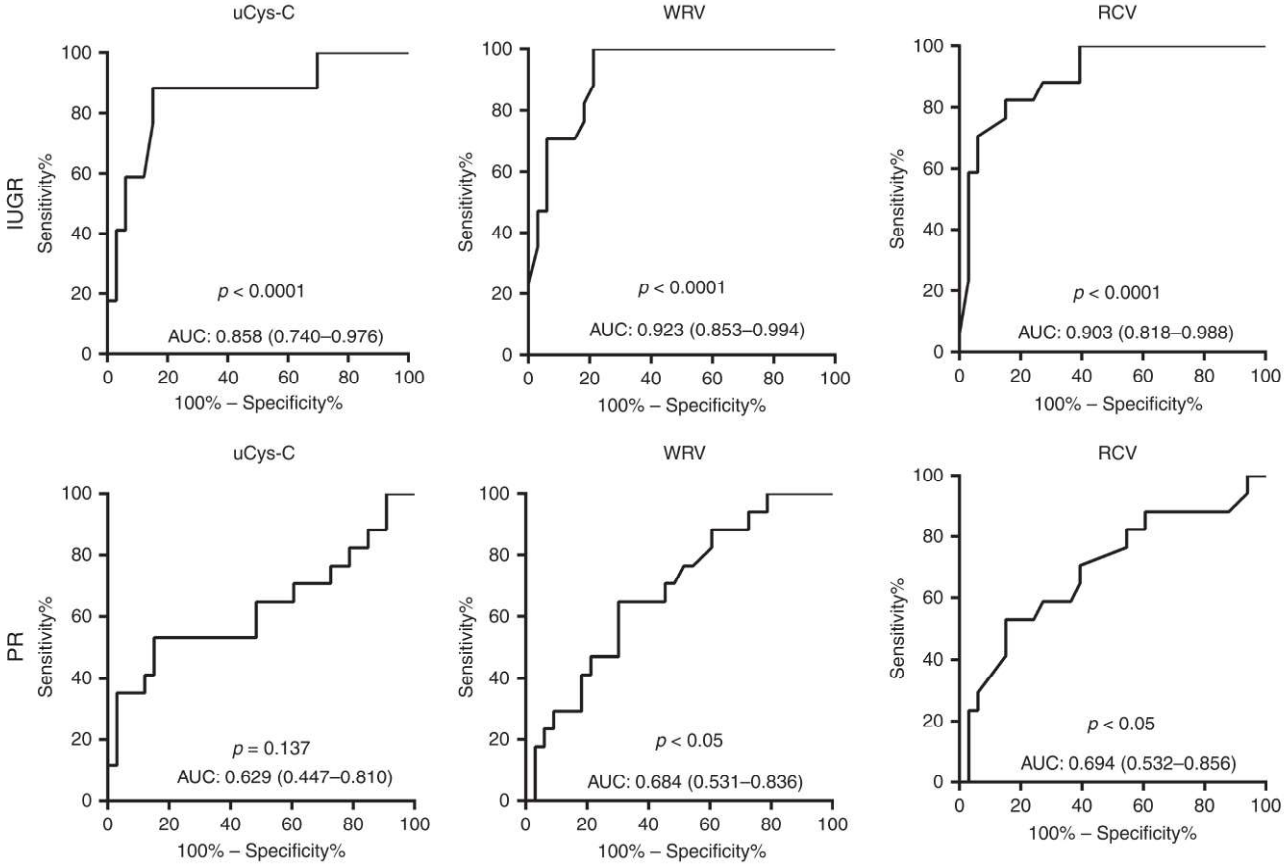


**Fig. 3 Receiver operating characteristic (ROC) curve demonstrating the performance of renal volume, renal cortex volume, and urinary Cystatin-C in IUGR and preterm (PR) condition.** For each ROC plot, area under the curve (AUC), 95% confidence interval, and *p* value are reported in the boxes. The ROC analysis was performed by comparing IUGR or preterm twins with those of the AGA term group.

though nephrogenesis may continue postnatally for approximately 40 days.[11,12]

In parallel, our data on uCys-C determinations at 30–40 days of postnatal corrected age showed levels inversely related to renal volumes. In AGA twins ≥36 weeks, in whom nephrogenesis can be considered concluded, high levels of uCys-C were found in a limited number of neonates, probably due to a physiological and inter-individual postnatal renal adaptation. In literature, a variability between 35 and 37 weeks of gestation in the timing of the cessation of nephrogenesis was reported.[4] In AGA twins <36 weeks, increased levels of uCys-C were found in a higher number of neonates compared to the control group. These data suggest, in AGA twins, it could be physiological to find temporarily high levels of uCys-C corresponding to a stabilization phase of renal function, especially in preterms, presumably as a consequence of immature kidney with ongoing nephrogenesis at delivery, which may continue ex utero. This condition may impose a longer time of adaptation, in agreement with Gubhaju et al.[23] who demonstrated that renal function in preterm neonates during the first month of life is significantly affected by GA at birth and postnatal age.

Recently, various authors have investigated urine biomarkers (such as albumin, neutrophil gelatinase-associated lipocalin, beta-2-microglobulin (B2M), Cystatin C, epidermal growth factor, Osteopontin, uromodulin) in preterm infants.[23–30] In these neonates, AKI is reported to occur in 8–24% of them.[37] Hanna et al.[27] found that urinary Cys-C increased significantly in preterm infants who developed AKI. Saedi et al.[25] showed that postnatal age affects urine biomarkers measured in the first 4 days of life also in preterm infants without AKI. Urinary levels of B2M have also been shown to be significantly greater in preterm infants compared with term-born infants throughout the first month of life and they decrease with increasing gestational and postnatal age.[38] A high variation of urinary albumin levels in preterm neonates, with the highest levels exhibited by those with a low GA at birth and those who are clinically unstable, was reported.[39–41] In most publications, urine markers of tubular damage are reported following correction for urine creatinine (uCreat). In this study, uCys-C was not corrected by uCreat, in agreement with recommendation by Conti et al.[42] who explained that correction with uCreat can be performed only in pure glomerulopathy, when specific markers of glomerular function are measured (i.e., urinary albumin). In all other cases, such correction is inappropriate and should be avoided.[43] Furthermore, uCys-C measurement in spot urine samples is possible, as no circadian rhythm was demonstrated for Cys-C excretion in urine;[30] hence, the 24-h urine collection, which is difficult to handle, is not necessary.

A comparison of our results with those found in literature appears difficult for different reasons: Cys-C in neonatal urine has been studied by few authors, the study population age was variable and usually referred to AKI, different detection methods, and values often expressed as uCys-C/creatinine.[26,43,44] Nevertheless, the low uCys-C levels found in the control group of our twins cohort were concordant with reference values reported in literature.[27,31] DeFreitas et al.[26] reported that uCys-C decreased

**Table 2.** uCys-C levels in eight couples of twins (A–H) determined at 30–40 days of postnatal corrected age and 1-month follow-up.

| TWINS | (A) 32 weeks | | (B) 33 weeks | | (C) 34 weeks | | (D) 35 weeks | | (E) 36 weeks | | (F) 36 weeks | | (G) 36 weeks | | (H) 37 weeks | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AGA | AGA | AGA | IUGR | AGA | AGA | IUGR | AGA | AGA | IUGR | AGA | AGA | AGA | IUGR | AGA | AGA |
| Birth weight (g) | 2000 | 1976 | 1698 | 1510 | 1860 | 2050 | 1850 | 2220 | 2500 | 1600 | 2570 | 2680 | 2300 | 1980 | 2380 | 2600 |
| RCV/WRV (ml) | 6.6/23 | 6.9/23 | 7.8/26 | 7/24 | 8.3/17.8 | 11.0/24 | 6.1/17.8 | 12/25 | 12.8/26.8 | 7.7/18.7 | 9.3/28.8 | 10.2/29 | 10/21.2 | 7/17.6 | 7.8/24.8 | 11.4/32 |
| uCys-C at 30–40 days (ng/ml) | 44.5 | 19.4 | 25.30 | 22.9 | 3.09 | 0.72 | 19 | 2.9 | 0.63 | 14.21 | 3.0 | 3.8 | 4.0 | 27.8 | 17.7 | 21.8 |
| uCys-C (ng/ml) at 1-month follow-up | 24.5 | 18.0 | 2.4 | 37.2 | 2.1 | 1.2 | 23.5 | 2.1 | 0.7 | 15.1 | 1.6 | 1.0 | 3.1 | 28.0 | 2.4 | 2.8 |

*AGA* adequate for gestational age, *IUGR* intrauterine growth restriction, *BW* birth weight, *RCV* renal cortex volume, *WRV* whole renal volume, *uCys-C* urinary Cystatin-C.

significantly in preterm infants from birth to 3 months of age to levels similar to those of term infants. This was in agreement with the decreased values of uCys-C that we observed in AGA preterm infants during the follow-up. Considering the IUGR groups, renal volumes were lower than those of preterm AGA; simultaneously elevated levels of uCys-C were more frequently observed suggesting that this condition can negatively impact on nephrogenesis more than preterm birth; and when IUGR and prematurity are associated, the impact is somehow additive. We can suppose that the combined assessment of renal volumes with uCys-C levels could be of interest to understand which infants are most vulnerable to develop renal disease in later life as part of the fetal origins of health and disease model. The variations of uCys-C levels, observed in the follow-up of twins with different nephrogenesis conditions, advocate that its increased levels in IUGR neonates could be considered a risk factor to develop renal diseases. In fact, the amplified (three cases) or unchanged values (one case) of uCys-C levels in IUGR twins seem to reveal the presence of a permanent renal injury and/or a worsening of renal function. On the contrary, a reduction of the urinary levels of marker in AGA twins during the follow-up seems to reflect their renal adaptation at birth. As recent studies showed that kidney immaturity or developmental impairment may have not only a short-term impact but also lifelong consequences with an increased risk of chronic kidney disease in adulthood,[15–18] Cys-C determination could provide important monitoring in long-term follow-up of these children. So the identification by uCys-C of at-risk newborns could have both a diagnostic value as an early precursor of renal injury and an important role in their monitoring. Furthermore, we consider it reasonable to extend the potential value of uCys-C also to monitoring the physiological nephrogenesis since its determination could provide information on the renal maturity achieved in AGA twins. One of the strong points of this study is that our results are less influenced by the variables of GA and individual maternal conditions, given that in our study population twins of the same couple were often distributed in different groups according to AGA or IUGR condition.

Concerning the chorionicity analysis, we found a significant reduction of both whole and cortical renal volumes in MC compared to DC twins. Presumably, this reduction could be related to the placenta sharing in MC twins, given its importance and its impact on fetal organogenesis/nephrogenesis. Nevertheless, renal volume reduction is also compatible with the statistically significant reduction of GA in MC compared to DC twins, so further studies will need to elucidate these aspects. Finally, the analysis by gender did not show any statistically significant difference for the variables examined.

In conclusion, renal volumes in IUGR and preterm neonates are lower than those estimated for the control group; in IUGR, the reduction of renal volumes compared to preterm is also evident, with statistically significant difference between the preterm and preterm IUGR groups. Concurrently, the elevated levels of uCys-C more frequently observed in IUGR at 30–40 days of postnatal corrected age, when intrauterine/extrauterine nephrogenesis could be considered completed, suggest that this condition can negatively impact on nephrogenesis more than preterm birth. When these disorders are associated, the impact is somehow additive. This was supported by follow-up results: the increased or unchanged values of uCys-C levels in IUGR twins, compared to the reduced uCys-C levels in preterm and/or term AGA, can improve the identification of those neonates with an initial kidney impairment. These variations suggest that uCys-C could be valuable as a marker to verify the renal postnatal adaptation and monitor IUGR neonates with potential renal impairment. Further evaluations on the follow-up of these neonates are in progress to evaluate their renal conditions in the near future and verify the urinary Cys-C predictivity for long-term renal diseases.

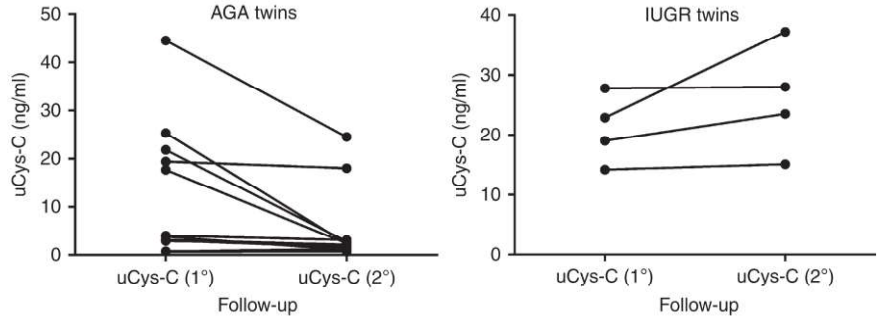


**Fig. 4 Variations of urinary Cys-C in AGA and IUGR twins during the follow-up.** Cys-C (1°): first determination at 30–40 days of postnatal corrected age; Cys-C (2°): at 1 month of follow-up.

**Table 3.** Comparison of variables in twins classified for chorionicity and gender.

| Twins | MC ($n = 12$) | DC ($n = 62$) | $p$ Value | Males ($n = 30$) | Females ($n = 44$) | $p$ Value |
|---|---|---|---|---|---|---|
| BW (g) | 2050 (1850–2350) | 2260 (1510–3210) | 0.242 | 2155 (1510–3210) | 2250 (1272–2890) | 0.382 |
| WRV (ml) | 20.40 (15.8–26.4) | 26.9 (15.0–34.8) | 0.016* | 24.0 (15.0–34.8) | 25.0 (12.7–34.7) | 0.534 |
| RCV (ml) | 6.95 (6.0–9.2) | 9.5 (6.1–17.0) | 0.001** | 9.35 (6.1–14.0) | 9.05 (6–17) | 0.997 |
| uCyst-C (ng/ml) | 2.33 (0.34–44.5) | 3.93 (0.37–39.0) | 0.499 | 8.97 (0.43–44.5) | 3.96 (0.34–39.0) | 0.936 |
| GA (weeks) | 35 (32–36) | 36 (32–39) | 0.026* | 36 (32–38) | 36 (32–39) | 0.280 |

The values are expressed as median (range).
*BW* birth weight, *WRV* whole renal volume, *RCV* renal cortex volume, *uCys-C* urinary Cystatin-C, *GA* gestational age, *MC* monochorionic, DC dichorionic.
*$p < 0.05$; **$p < 0.01$

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## AUTHOR CONTRIBUTIONS

A.B.: substantial contributions to conception and design, acquisition of data, analysis and interpretation of data; drafting the article and revising it critically for important intellectual content. M.C.A.: analysis and interpretation of data. B.C.: contributions to conception. M.Z.: acquisition of data. S.G.: revising the article. G.C.D.R.: final approval of the version to be published

## ADDITIONAL INFORMATION

**Competing interests:** The authors declare no competing interests.

**Consent statement:** Infants were recruited with parents' consent and a written informed consent was obtained. Participation in the study was voluntary and the parents were informed verbally and by a written information sheet about the aim of the study.

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