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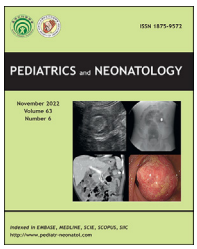

Original Article

Detection of urinary cystatin-c in IUGR neonates by immunoblot SDS-PAGE

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Abstract *Background:* it is known that intrauterine growth retardation (IUGR) represents a risk factor for the deterioration of renal function as it can adversely impact on the number of nephrons developed in the kidney during nephrogenesis. An interesting molecule is the Cystatin-C (cyst-C): it is considered to have the potency to detect both glomerular and proximal renal injury. Recently, using a quantitative EIA cyst-C detection kit, we found increased levels of cyst-C in the urine of neonates with IUGR. Since cyst-C molecules can be present in both monomer and/or polymer forms, the purpose of this study is to investigate in which forms this molecule is present in the urine of IUGR neonates by Immunoblot SDS-PAGE in order to verify if the presence or absence of a particular type of cyst-C conformation can give more information about the renal functioning.
Methods: urine samples were collected from 64 neonates with IUGR, and 86 healthy controls defined as appropriate for gestational age (AGA). Urinary cyst-C was investigated by the Immunoblot SDS-PAGE.
Results: in all urine samples, SDS-PAGE analysis showed a reactivity of the IgG anti cyst-C with a complex of about 70 kDa. The monomer form at 13 KDa appeared in 78% of IUGR neonates and in 12% of AGA neonates.
Conclusions: this study revealed the presence of monomer cyst-C in the urine of IUGR neonates, and suggests an insufficient and/or non-compensatory reabsorption by tubular cells. Monomeric cyst-C can be considered an early biochemical marker to identify and to select IUGR neonates who need to be monitored for risk of renal injury.

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1. Introduction

FLAThe total number of nephrons is a biological variable that is defined prior to birth. Approximately 60% of the nephron population develops during the third trimester of pregnancy, up to 36 weeks.[1] No new nephrons are formed after birth and the total number of nephrons in humans ranges between 300000 and 1,1 million, with a mean of approximately 600,000.[2] An increasing number of studies show that intrauterine growth retardation (IUGR) represents a risk factor for renal function deterioration as it can adversely impact on the number of nephrons developed in the kidney during nephrogenesis. The nephrons are the functional units of the kidney and their reduction results in a global renal reduction, leading to reduced surface filtration area, a glomerular hypertension, an impaired tubular function and long term renal disease as clearly demonstrated by epidemiological study.[3] A reduced number of nephrons at birth may be associated with a diminished resistance to any mechanism of renal damage in adult life. Intrauterine growth restriction is recognized as the second leading cause of perinatal mortality, and it is one of the most complex yet common problems of modern obstetrics.[4] It occurs in about 5—10% of all pregnancies and affects a high percentage of artificial inseminations.[5]

Evidence of the reduced volumes of the kidney in neonates with IUGR is well documented by ultrasonography,[6] while biochemical studies to identify an early renal impairment are in progress.[7—10] An interesting molecule is the Cystatin-C: a strong basic endogenous cysteine proteinases inhibitor belonging to the type 2 cystatin superfamily.[11] The mature, active form of human cyst-C is a single non-glycosylated peptide chain consisting of 120 amino acid residues, with a molecular mass of 13 KDa[12,13]; the cyst-C monomer is present in all human body fluids and it is produced at a constant rate by all karyocytes.[14—17] It is directly and freely filtrated from the blood into the glomerulus and is almost completely reabsorbed and catabolized in the proximal renal tubule.[18—20] The remaining cyst-C is eliminated in the urine and the urinary cyst-C concentration is normally very low.[21—23] Tubular damage induces impairment of cyst-C reabsorption by the proximal renal tubule and, in addition, glomerular alteration causes high molecular weight protein leakage which leads to competition with tubular uptake of cyst-C. Therefore, urinary cyst-C was considered to have the potency to detect both glomerular and proximal renal injury.[14,24—26] It is considered as a newly endogenous biomarker of kidney damage,[14,24,27—30] which can be used to detect early, as well as acute and chronic renal failure.[31] The measurement of cyst-C in the urine of newborns can be an early method for identifying those predisposed to renal function impairment. Pediatricians need to predict the glomerular and tubular function and renal mass, and select patients for prompt treatment in order to avoid long-term effects of kidney dysfunction on growth, tissue viability and quality of life.[32] Recently, using a quantitative EIA cyst-C detection kit, we found increased levels of cyst-C in the urine of neonates with IUGR.[6] Since cyst-C molecules can be present in monomer and/or polymer form,[32] the purpose of this study is to investigate in which forms this molecule is present in the urine of IUGR neonates by Immunoblot SDS-PAGE in order to verify if the presence or absence of a particular type of cyst-C conformation can give more information about the renal functioning.

2. Materials and methods

2.1. Subjects

All newborns from the period June 2014—March 2015 were recruited; the parents agreeing to participate in the study.

64 neonates with IUGR (gestational age $\geq$36 weeks to exclude the effects of prematurity on renal development) and 86 healthy controls defined as appropriate for gestational age (AGA: a newborn infant whose size is within the normal range for his or her gestational age), born of healthy parents (no obese, no smokers, younger than 40 years) have been analyzed.

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.

2.2. Collection of the samples

Nurses at S. Maria della Misericordia Hospital took urine samples from all newborns in the period June 2014—March 2015. These samples were tested by the measurement of urinary proteins, leukocytes and nitrite with a multiple test strip (Combi-Screen Plus, Analytican Biotechnology AG) to exclude urinary infections and/or proteinuria. One hundred and fifty urine samples were selected for the present study and analysed by Immunoblot SDS-PAGE. The present study adheres to the World Medical Association Declaration of Helsinki regarding the ethical conduct of research; informed consent was obtained from all parents and the protocol was approved by our institutional review board.

2.3. Urinary cyst-C assay

Urinary cyst-C was determined by Immunoblot SDS-PAGE. The present study performed the qualitative analysis of cyst-C, with the aim of identifying its different molecular forms in the urine of newborns. The electrophoretic system used is the Mini-Protean Tetra Cell Biorad. The time of run was 50 min at 200 V and the time of blotting was 30 min at 100 V. The immunoreactivity was displayed by a subsequent incubation for 1 h at RT with the secondary antibody conjugated with horseradish peroxidase (anti-mouse IgG-HRP goat: sc-2005, Santa Cruz Biotechnology, Inc.). Protein bands were detected by exposing the sensitive plates for 2 min to a signal emitted by chemiluminescent products to nitrocellulose. The chemiluminescent products were obtained by the use of peroxidase enzyme substrate (Immunoblot Luminol Reagent: sc- 2048, Santa Cruz Biotechnology, Inc.).

2.4. Bradford assay

In acidic solution, the triarylmethane dye (Coomassie Brilliant Blue G-250) binds to proteins by developing a blue color. These bonds determine a shift of the maximum of the dye absorption from 465 nm (red) to 595 nm (blue) in acidic solutions (Bradford, 1976). The intensity of the coloration, therefore, determined at 595 nm, is directly proportional to the protein concentration, into a defined range. The calibration curve of the assay is constructed by reacting the reagent of Bradford with increasing amounts of a bovine serum albumin solution of known concentration. The absorbance reading of the solution was carried out with Bio Photometer – Eppendorf.

2.5. Statistical calculations

Statistical analysis and figures were performed by Excel 2010 (Microsoft Office). In the present study, the reference intervals used are in agreement with recommendations of The International Federation of Clinical Chemistry on the statistical treatment of reference values. Quantitative variables were compared using chi-square test, carried out by Excel 2010 (Microsoft Office); statistical significance was set at p less than 0.001.

3. Results

The molecular characterization of urinary cyst-C detected two different molecular forms: a reactivity of IgG anti cyst-C at 13 KDa and a reactivity at about 70 KDa (Fig. 1). The urine samples showed two different profiles: a reactivity at about 70 KDa in all samples and a reactivity at 13 KDa in 60 samples, of which 50 IUGR neonates and 10 AGA neonates (Fig. 2). The reactivity of IgG anti cyst-C at about 70 KDa was observed in 100% of samples, in both IUGR and AGA neonates, therefore it is not relevant to the aim of the study.

The blotting has been tested without a primary antibody to ensure the signal at 70 KDa is not due to any reactivity of the secondary anti-mouse IgG with human IgG heavy chain

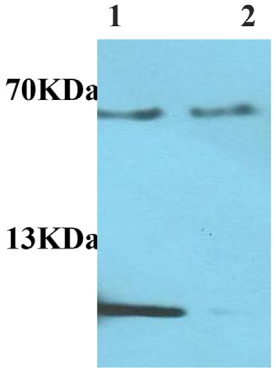

Figure 1 Picture of SDS-PAGE separation of cyst-C for urine samples of newborns that showed representative examples of the two different forms found: reactivity with a complex at 70 KDa and with the monomer at 13 KDa (1) and reactivity with a complex at 70 KDa (2).

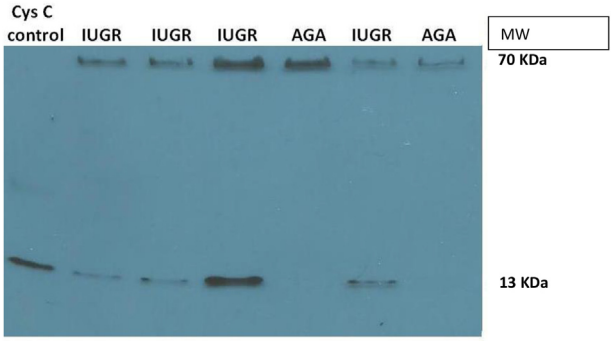

Figure 2 Different molecular profiles of cyst-C in IUGR and AGA neonates detected by Immunoblot SDS-PAGE: the monomer form at 13 KDa appeared in IUGR neonates. The reactivity at 13 KDa clearly indicates the presence of cyst-C in monomeric form, as demonstrated by the correspondence with the positive control cyst-C Human E. Coli (Biovendor).

in the urine samples. The reactivity of the complex at about 70 KDa has been analyzed in our laboratory and the study has been published.[33] The reactivity at 13 KDa clearly indicates the presence of cyst-C in monomeric form, as demonstrated by the correspondence with the positive control cyst-C Human E. Coli (Biovendor) (Fig. 2). The monomeric form was observed in 78% of IUGR neonates and in 12% of AGA neonates (Table 1). The results obtained were analyzed by chi-square test. The null hypothesis admits that the presence of monomeric cyst-C is independent of the condition of uterine growth retardation. Therefore, the expected data were calculated considering the samples with monomer cyst-C are 40% of the total. The null hypothesis was rejected with a p-value < 0.001. We can conclude that the correlation between monomer cyst-C and intrauterine growth retardation is statistically significant at the 99,9% probability level. There is no correlation between the amount of proteins present in the samples, determined by Bradford assay, and the presence of monomer cyst-C.

4. Discussion

Immunoblot SDS-PAGE revealed, on urine samples, the presence of different forms of cyst-C: the well-known native monomeric form of the protein and an aggregate form with a molecular weight of about 70 KDa. The presence of the70 KDa complex in all of the samples, in both IUGR and AGA neonates, does not allow us to differentiate neonates with intrauterine growth retardation from healthy

Table 1 Results obtained by Immunoblot SDS-PAGE.

Results	Number of samples with monomeric cyst-C	Number of samples without monomeric cyst-C	% neonates with monomer cyst-C
IUGR neonates	50	14	78%
AGA neonates	10	76	12%

$\chi^2 = 4$, $k = 1$, $p < 0.001$

ones and it is not relevant to the aim of the study. However, this complex is more interesting because it is not yet known in official literature,[32] but it has been analyzed in our laboratory and the study has been published.[33] Current literature and data supports the conclusion that cyst-C can form dimers, tetramers and oligomers in conditions of low pH, high temperature and in the presence of denaturing agents,[34—36] but our studies have confirmed that the 70 KDa complex is not related to the denaturing conditions used, but is naturally present in the urine of newborns.[33] This complex was investigated also by LC—MS/MS analysis, because no cyst-C aggregate with this molecular weight has been published.[32] The presence of cyst-C in the complex has been demonstrated through the identification of the cyst-C tryptic unique peptides[37] performed by Ultra High-Performance Liquid Cromatography (UHPLC) on Agilent Technologies 6540 UHD Accurate Mass Q-TOF LC/MS, 1290 Infinity Series.[33,38] In our SDS-PAGE, the molecular weight of about 70 KDa might coincide with the weight of pentameric cyst-C aggregate. In the study of Mussap M et al. about cyst-C conformations, no pentameric aggregates were detected, suggesting that the only forms known were the dimeric and the tetrameric.[32] For this reason, we posit that our signal at 70 KDa indicates a protein complex in which cyst-C is bound to another protein. Further studies will be able to identify which molecules are part of the complex. Initially the types of Cathepsin (H, L and S) were excluded from our study, and should be investigated.

The present study has focused on monomeric cyst-C. Due to its low molecular weight (13.3 KDa), monomeric cyst-C is freely filtered in the kidney glomerulus with no retrieval back to the circulation. In proximal tubular cells, cyst-C is predominately reabsorbed and subsequently catabolized, so, in urine, the concentration of cyst-C is normally low.[14,21,29,30] The absence of monomeric form of cyst-C in most of AGA neonates confirms that it is reabsorbed by renal tubule since the early months of life.[21—23] Its presence in a small percentage (12%) of AGA neonates can be explained considering that the maturation of the glomerular filtration and tubular function of absorption/degradation, which occurs in the first year of life, can be completed at different times in neonates; these newborns, therefore, may not absorb and degrade cyst-C because they have not as developed yet a fully mature renal function. In fact, we repeated the analysis of these samples after six months and we found that the monomeric form of cyst-C disappeared or was significantly reduced.

The evident presence of monomeric cyst-C in 78% of IUGR neonates confirmed an insufficient and/or non-compensatory reabsorption by tubular cells. IUGR can adversely affect the number of nephrons in the newborn's kidney[7—9]; neonates below the 10 th centile of birth weight had 30% fewer glomeruli than those with birth weights above the 10th centile.[10] 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 [48—49]. IUGR represents a risk factor for impaired renal function resulting from a reduced nephron number and decreased renal size, causing the loss of filtration surface area, single nephron hyperfiltration, glomerular hypertension and long-term renal disease as clearly demonstrated by epidemiological study.[3] Urinary cyst-C has been validated as a good reflection of tubular function.[14] In proximal tubules, filtered cyst-C is reabsorbed and its increased urinary elimination in IUGR reflect an abnormal reabsorption and degradation by tubular cells.[14,15,21,29,30] The absence of monomeric cyst- C in 22% of IUGR neonates can be explained by the following considerations, based on published data:

(1) if the growth restriction occurs at a late stage of gestation, when nephrogenesis is completed or nearing completion, there is no significant reduction in the number of nephrons;[6]
(2) reduced number of nephron, especially when it is moderate, is not systematically associated with hypertension and impaired glomerular filtration rate, but 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.[39]

5. Conclusion

Chronic kidney disease is a major health problem worldwide with dramatically rising incidence and prevalence.[40] Early detection of impaired kidney function is imperative to optimize the efficacy of disease-modifying therapy and the prevention. Determination of cyst-C can diagnose early-stage renal dysfunction and monitor renal function over time.[31] Since cyst-C molecules can be present in monomer and/or polymer form, we developed a qualitative analysis to investigate in which forms this molecule is present in the urine of IUGR in order to verify if cyst-C conformation can give more information.

Differently from quantitative immunometric assay, SDS-PAGE immunoblotting allows a qualitative analysis of the urinary conformation of cyst-C. This study revealed the absence of the monomeric form of cyst-C in most of the urine of healthy neonates (88% of AGA neonates) and we observed that the monomeric cyst-C, present in a small percentage (12%) of AGA neonates, disappeared or was significantly reduced in about six months, as explained in the discussion of the data.

In fact, the maturation of the glomerular filtration and tubular function of absorption/degradation occurs in the first year of life, and AGA neonates can reach renal maturity at different times. These results confirm that in normal condition of renal functionality, cyst-C is reabsorbed by renal tubule since the early months of life.

Conversely, its presence in the urine of IUGR neonates suggests an insufficient and/or not compensatory reabsorption by tubular cells and an increased risk of chronic kidney disease later in life. Moreover, urinary cyst-C can be measured in spot urine samples and the 24-h urine collection is not necessary because cyst-C excretion in urine is not regulated by circadian rhythm.[14] These results are supported by our other published study, conducted simultaneously whit the present study and on the same samples: Increased Urinary Cystatin-C Levels Correlate with Reduced Renal Volumes in

Neonates with Intrauterine Growth Restriction.[6] This paper is the foundation of the present study: it supports a strong correlation between whole renal volume, renal cortex volume, birth weight and gestational age and it demonstrates that the measurements of whole renal volume and renal cortex volume in IUGR newborns were significantly lower than those found in AGA newborns. Furthermore, this study demonstrates urinary levels of cyst-C, measured by EIA, were inversely correlated with renal volumes, gestational age and birth weight. The same samples have been analyzed, for a second time, by Immunoblot SDS-PAGE in the present study and the obtained results are in accordance with those of the published study. Through the analysis of the urinary conformation of cyst-C, we found that the detection of monomeric cyst-C, repeated twice during the first years of life, allows to differentiate between AGA neonates and IUGR neonates with renal injury or risk of renal injury.

The evident presence of monomeric cyst-C in 78% of IUGR neonates confirmed cyst-C can be taken as a surrogate of nephron mass. Seen that the reduction in the number of nephrons could be insufficient to determine renal disease, a new approach based on the detection of monomer urinary cyst-C by Immunoblot SDS-PAGE in IUGR newborns, repeated twice during the first years of life, combined with the measurement of whole renal volume and renal cortex volume would be useful for early identification and monitoring of IUGR neonates at high risk of developing long term renal disease.

Therefore, monomeric urinary cyst-C detection can easily be used as an adjunct to the standard panel to screen kidney functionality in IUGR neonates and can be considered an early biochemical marker to select IUGR neonates who need to be monitored for the risk of renal injury.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The present study is dedicated to Michele Bellucci, with love.

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