

Uromodulin: a new biomarker of fetal renal function?

Uromodulina: um novo biomarcador de função renal fetal?

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Submitted on: 05/12/2016.

Approved on: 08/08/2016.

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DOI: 10.5935/0101-2800.20160068

ABSTRACT

Introduction: Obstructive uropathies are main diseases affecting the fetus. Early diagnosis allows to establish the appropriate therapy to minimize the risk of damage to kidney function at birth. Biochemical markers have been used to predict the prognosis of renal function in fetuses. Uromodulin, also known by Tamm-Horsfall protein (THP) is exclusively produced in the kidneys and in normal conditions is the protein excreted in larger amounts in human urine. It plays important roles in kidneys and urinary tract. Also it participates in ion transport processes, interact with various components of the immune system and has a role in defense against urinary tract infections. Moreover, this protein was proved to be a good marker of renal function in adult patients with several renal diseases. **Objective:** To evaluate if uromodulin is produced and eliminated by the kidneys during fetal life by analyzing fetal urine and amniotic fluid and to establish correlation with biochemical parameter of renal function already used in Fetal Medicine Center at the Clinic Hospital of UFMG (CEMEFE/HC). **Methods:** Between 2013 and 2015, were selected 29 fetuses with indication of invasive tests for fetal diagnosis in monitoring at the CEMEFE/HC. **Results:** The determination of uromodulin was possible and measurable in all samples and showed statistically significant correlation with the osmolarity. **Conclusion:** There was a tendency of lower levels of Uromodulin values in fetuses with severe renal impairment prenatally. Thus, high levels of this protein in fetal amniotic fluid or fetal urine dosages possibly mean kidney function preserved.

Keywords: amniotic fluid; ultrasonography, prenatal; urine; urogenital abnormalities.

RESUMO

Introdução: Uropatias obstrutivas estão entre as principais doenças que acometem o feto. O diagnóstico precoce destas doenças permite estabelecer a terapêutica adequada, visando minimizar os riscos de danos à função renal no nascimento. Os marcadores bioquímicos têm sido utilizados na predição do prognóstico da função renal em fetos. A uromodulina, também chamada de proteína de Tamm-Horsfall (THP), é produzida exclusivamente nos rins, e em condições normais, é a proteína excretada em maior volume na urina humana. Ela desempenha importantes funções nos rins e trato urinário. Participa dos processos de transporte de íons, interage com vários componentes do sistema imunológico e possui papel na defesa contra infecções do trato urinário. Além disso, se mostrou um bom biomarcador de função renal em adultos portadores de diversas doenças renais. **Objetivos:** Avaliar se a uromodulina é produzida e eliminada pelos rins durante a vida fetal através da análise de urina fetal e líquido amniótico, além de estabelecer correlação com o parâmetro bioquímico de função renal já utilizado no Centro de Medicina Fetal do Hospital das Clínicas da UFMG (CEMEFE/HC). **Métodos:** Entre 2013 e 2015, foram selecionados 29 fetos com indicação de exames invasivos para diagnóstico fetal em acompanhamento no CEMEFE/HC. **Resultados:** A dosagem da uromodulina foi possível e quantificável em todas as amostras e mostrou correlação significativa com a osmolaridade. **Conclusão:** A uromodulina mostrou uma tendência em apresentar valores reduzidos em fetos com grave comprometimento renal no pré-natal. Assim, valores elevados desta proteína em dosagens de urina fetal ou líquido amniótico podem significar uma função renal preservada.

Palavras-chave: anormalidades urogenitais; líquido amniótico; ultrassonografia pré-natal; urina.

INTRODUCTION

Kidney and urinary tract malformations account for 20% of fetal anomalies detected through prenatal ultrasound examination.¹ Obstructive uropathy ranks first in the list of malformations and is the leading cause of chronic kidney disease (CKD) in children and teens.²

Obstructive uropathy is a congenital anomaly resulting from partial or complete urine flow obstruction in the urinary tract. It is initially characterized by hydronephrosis, a swelling of the kidney due to a build-up of urine, associated to other ultrasound findings depending on the type of obstruction.³ Prenatal diagnosis and early treatment may prevent or delay the onset of chronic kidney disease.^{4,5}

According to the Census of the Brazilian Society of Nephrology,⁶ approximately 100,397 individuals had end-stage renal disease in Brazil in 2013; 90.8% of these patients were on hemodialysis and 9.2% on peritoneal dialysis. Six percent of these individuals were under the age of 18 years. A collaborative study carried out in 13 centers analyzed the records for pediatric renal transplantation procedures performed in Brazil from 2004 to 2013. The data from 1,751 transplant procedures performed in individuals under 18 years of age showed that the most common cause of transplantation was obstructive uropathy, with 31% of the cases.⁷

Ultrasound findings alone cannot be used to thoroughly assess fetal kidney function.⁸ Biochemical tests on fetal urine bridge that gap and allow for proper patient management and timely intervention.⁹

The placenta maintains fluid and electrolyte balance; therefore, tests based on fetal serum do not aid in the analysis of intrauterine renal function. However, electrolyte levels in fetal urine correlate directly with renal function¹⁰ and may be assessed through biochemical tests since week 15 of pregnancy, a time by which the fetus' kidneys are functioning.

Some authors believe electrolyte levels can be derived from amniotic fluid, since a significant portion of it contains fetal urine.¹¹ Biochemical tests on urine and amniotic fluid have been performed to assess fetal renal function, but none of the tested parameters is a proven accurate marker of fetal renal function.^{12,13}

UROMODULIN OR TAMM-HORSFALL GLYCOPROTEIN (THP)

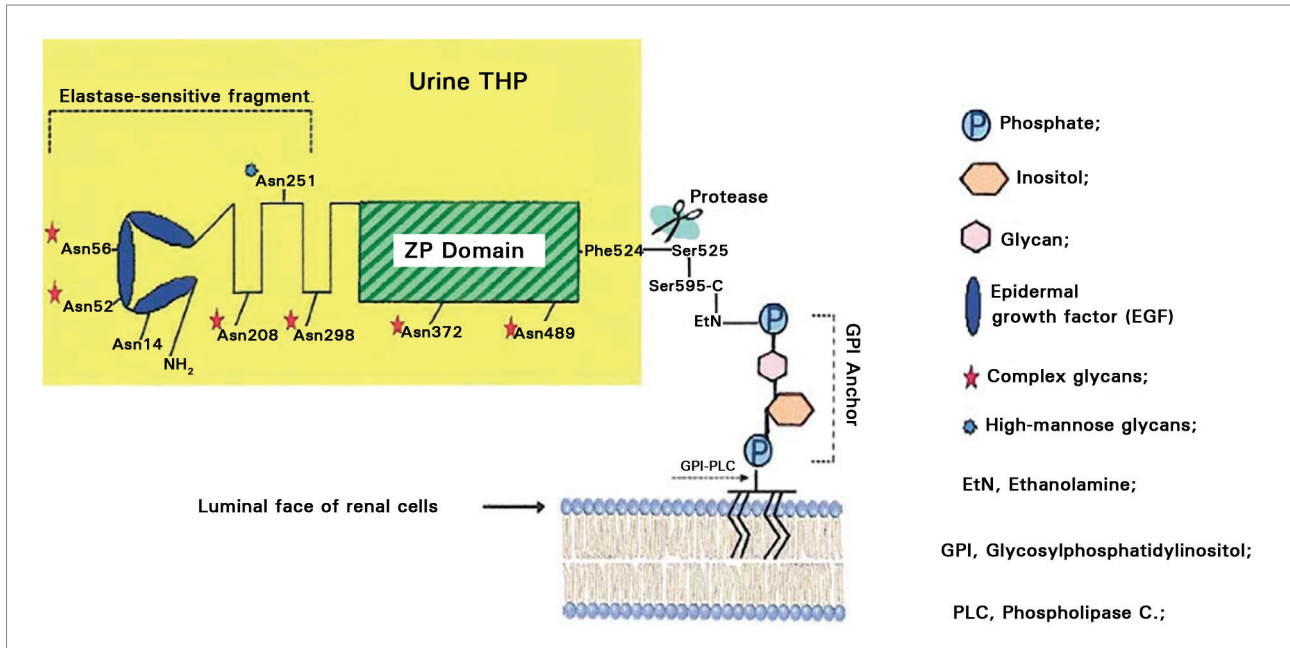
Tamm-Horsfall glycoprotein (THP) was discovered in 1950 by Igor Tamm and Frank L. Horsfall Jr. and characterized as a protein present in the urine of humans and animals that was able to inhibit viral hemagglutination.¹⁴ In 1985, Muchmore and Decker¹⁵ isolated a protein capable of inhibiting the proliferation of T-cells and monocytes. Because of its source of isolation and in-vitro immune response modulation property, they called it uromodulin. Two years later, researchers studying complementary DNA found that THP and uromodulin were one and the same protein.¹⁶

Rindler *et al.*¹⁷ showed that uromodulin is a glycosylphosphatidylinositol (GPI) anchor-linked protein. The mature protein contains 616 amino acids, including 48 cysteine residues involved in 24 disulfide bridges, which are important for its conformation (Figure 1).

Eight potential sites for N-glycosylation are also present. The structure of uromodulina also contains three epidermal growth factors (EGF) and a zona pellucida-like domain. The C-terminus of the protein includes a stretch of hydrophobic amino acids that acts as a signal for transpeptidase within the endoplasmic reticulum of cells in the thick ascending limb (TAL) of the loop of Henle - where uromodulin is produced - to attach a preformed GPI anchor to the protein.

Following this addition, the anchor-linked protein is transported to the Golgi complex, where glycans are processed, then delivered to the luminal cell surface, and finally released into the urine by proteolytic cleavage.¹⁸ Ontogenetic studies indicated that the presence of uromodulin is closely related to the development and functional maturation of the loop of Henle.¹⁹ In normal conditions, uromodulin is the protein excreted by the kidneys in larger quantities, at a rate of approximately 50 mg/day, with levels varying as a function of a number of factors such as urine volume, nutrition, and physical exercise.²⁰

Studies have shown a number of biological roles for uromodulin in the urinary system. Since it is produced in the cells of the TAL of the loop of Henle, uromodulin is believed to play a role in ion transport.^{21,22} Uromodulin has also been recognized for its role in the regulation of the immune system

Figure 1. Structural model for urine uromodulin (yellow area) and GPI anchor (Source: Serafini-Cessi *et al.*, 2003).

due to the interactions it maintains with various component of the immune system;²³⁻²⁵ it is also known for helping in the defense against urinary tract infections, particularly the ones caused by *Escherichia coli*.²⁶⁻²⁸ Uromodulin also acts in the prevention against the formation of kidney stones by reducing the clustering of calcium crystals.²⁹

Mutations on the gene encoding uromodulin (UMOD) have been associated with various autosomal dominant kidney diseases.^{30,31} Fifty-eight mutations to the UMOD gene have been described in the literature.³² These conditions are assumed to produce defective proteins that are not released in the cell membrane. In-vitro studies of kidney cells with UMOD mutations revealed that uromodulin is retained inside the cell,^{33,34} causing further damage to the cells of the TAL of the loop of Henle.

According to Prajczar *et al.*³⁵, urinary levels of uromodulin were lower in individuals with CKD when compared to healthy controls and correlated positively with glomerular filtration rates (GFR).

Zhou *et al.*³⁶ reported a correlation between urine uromodulin levels and GFR in a group of patients with IgA nephropathy, with individuals showing lower levels of uromodulin as the disease progressed. These findings agree with previous studies in which uromodulin levels decreased in individuals with diseases affecting kidney function or integrity.³⁷⁻⁴⁰

With these evidences and the lack of studies on the production and release of uromodulin in fetal life in mind, this study aimed to verify whether uromodulin is produced during fetal life and if the protein may be used as a biomarker of renal function in fetuses with kidney malformations.

OBJECTIVES

This study aimed to check whether uromodulin is produced and excreted by the kidneys during fetal life by analyzing samples of fetal urine and amniotic fluid collected from patients followed at the Center for Fetal Medicine of the Hospital and Clinics of the Federal University of Minas Gerais (CEMEFE/HC) and describe the correlations between uromodulin levels and osmolarity, the biochemical marker of renal function used at CEMEFE/HC.

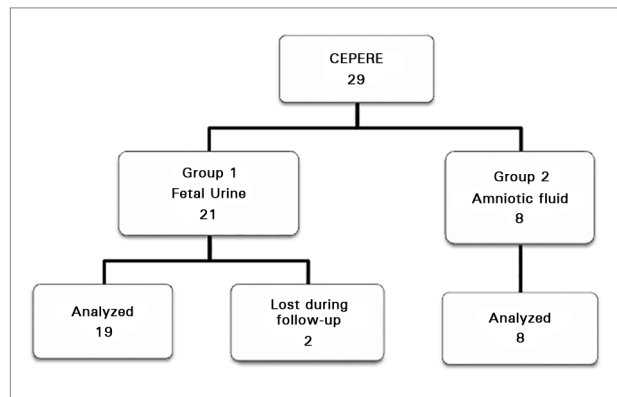
PATIENTS AND METHODS

PATIENTS

Twenty-nine fetuses with gestational ages ranging from 17 to 34 weeks diagnosed with disease and selected as candidates for ultrasound-guided invasive procedures were selected from a group of patients seen at CEMEFE/HC from March 2013 to March 2015. Twenty-one fetuses with obstructive uropathy (Group 1) were prescribed kidney function biochemical testing requiring urine collection (Figure 2).

Eight fetuses (Group 2) with non-nephrounary anomalies were prescribed genetic or infectious disease testing requiring amniotic fluid collection (Figure 2).

Figure 2. Study follow-up flowchart.



The low volumes of amniotic fluid seen in the mothers of the subjects in Group 1 warranted the collection of fetal urine. The protocol in effect for the individuals in Group 2 included amniotic fluid analysis. The patients and their fetuses were exposed to no risks other than the ones inherent to the prescribed collection procedures. At the time of the procedure, a portion of the sample was set aside to test for uromodulin levels.

ETHICS

The Research Ethics Committee of the Federal University of Minas Gerais (UFMG) approved this study and assigned it permit CAAE 35559214.1.0000.5149. The enrolled pregnant women were informed of the protocol and voluntarily joined the study after signing an informed consent term. They were followed through to labor and puerperium, according to the protocols in effect at CEMEFE, and were allowed to leave the study if they so wished.

The neonates with uropathy were followed at the Neonatal Unit and the Pediatric Nephrology Unit of the UFMG Hospital and Clinics. Only two of the 29 pregnant women initially enrolled in the study were lost during follow-up, and their data were excluded from the final analysis.

STUDY PROTOCOL

The samples of fetal urine (Group 1) and amniotic fluid (Group 2) were centrifuged at 4°C and 300 G for 20 minutes, frozen at -80°C, and sent for analysis

at the Interdisciplinary Medical Research Laboratory of the School of Medicine at UFMG. The biochemical parameters analyzed included osmolarity, and uromodulin and creatinine levels.

Creatinine levels were measured to adjust uromodulin urine levels for creatinine urine clearance levels according to the recommendations for the measurement of markers in urine samples.⁴¹ Uromodulin levels were expressed in nanograms of uromodulin per milliliter of urine or amniotic fluid (ng/ml) or in nanograms of uromodulin per microgram of urine creatinine (ng/μg). Creatinine levels were expressed in micrograms of creatinine per milliliter of urine (μg/ml).

An OSMOMAT 030 (AUTOMATIC CRYOSCOPIC OSMOMETER) osmometer calibrated with a standard specimen of 300 mOsm/Kg, made by Gonatec GmbH, was used in the analysis of fetal urine osmolarity. Samples with values greater than 210 mOsm/l⁴² were deemed anomalous, as described in the protocol in effect at the service.

Uromodulin levels were measured from samples of fetal urine and amniotic fluid using an ELISA kit designed to perform quantitative measurements of human uromodulin. Biovendor RD191163200R Human Uromodulin ELISA kits manufactured by BioVendor Research and Diagnostic Products were used in this study.

A standard curve was built plotting absorbance values *versus* standard uromodulin values; unknown sample levels were determined based on this standard curve. Absorbance values were captured from an ELISA reader (MOLECULAR DEVICES, USA). A straight line was used to calculate the uromodulin levels in each sample.

Creatinine levels from fetal urine samples were measured with the aid of a modified Jaffee method kit made by Bioclin. The tests were run in accordance with manufacturer instructions. The method consists of a colorimetric reaction between creatinine and picric acid, producing a reddish-yellow color. An ELISA reader was used to assess the absorbance of the formed compound (MOLECULAR DEVICES, USA) at a wavelength of 510 nm.

The values observed in the samples were related to the values obtained from the standard and the straight line was calculated. Then the absorbance values for each sample were interpolated onto the straight line to yield the creatinine levels for certain volumes of urine.

Fetal urine osmolarity is calculated routinely at CEMEFE and analyzed jointly with ultrasound findings, amniotic fluid volumes, and aspects of renal parenchyma to aid in the prognostic assessment of fetal renal function. The results of uromodulin and creatinine biochemical tests of the two groups - fetuses with normal osmolarity and fetuses with altered osmolarity - were compared.

STATISTICAL ANALYSIS

The Shapiro-Wilk test was used to assess the distribution of variables. Parametric variables were expressed in terms of their mean values and standard errors, whereas non-parametric variables were expressed in terms of their median values and interquartile ranges (25th percentile - 75th percentile).

Student's *t*-test for unpaired data was applied to parametric variables; the Mann-Whitney U test was used with non-parametric variables to compare between median values. Pearson's correlation coefficient was used to test the correlations between the following variables:

Uromodulin level vs. osmolarity;

Uromodulin level vs. creatinine level;

Uromodulin adjusted for creatinine vs. osmolarity.

The following values were used to define the strength of the correlations:

Positive or negative 0.70 - indicative of strong correlation;

Positive or negative 0.30 to 0.70 - indicative of moderate correlation;

Zero to 0.30 - indicative of poor correlation.

In statistical hypothesis testing, differences with a $p < 0.05$ were deemed statistically significant. The analyses were run on statistical package MINITAB version 14.13 (MINITAB, STATE COLLEGE, PA, USA).

RESULTS

The study enrolled 21 fetuses with gestational ages of 24.4 ± 1.2 weeks diagnosed with obstructive uropathy (Group 1) and eight fetuses with gestational ages of 26.4 ± 1.5 weeks free of obstructive uropathy (Group 2). No statistically significant differences were observed between the gestational ages of the individuals in groups 1 and 2.

The mothers of 16 of the 21 fetuses with obstructive uropathy (76%) had moderate to sharp decreases in amniotic fluid volume. Only five fetuses in Group 1 (24%) were exposed to adequate volumes of amniotic

fluid. In contrast, all fetuses in Group 2 were exposed to normal volumes of amniotic fluid.

Five neonatal deaths occurred in Group 1 as a result of pulmonary hypoplasia. None of the neonates in Group 2 died. Posterior urethral valve (PUV), the leading cause of obstructive uropathy in the subjects in Group 1, was present in 19 of the 21 fetuses (90%).

The median level of fetal urine uromodulin in the fetuses on Group 1 was 399.4 ng/ml (interquartile range - 319.4 to 1139); the median creatinine level seen in the same urine samples was 11.35 ng/ μ g (interquartile range - 7.1 to 35.7).

The mean fetal urine osmolarity measured for 19 of the 21 fetuses included in Group 1 was 206.7 ± 23.4 mOsm/l. When analyzed for normal urine osmolarity (< 210 mOsm/l), ten of the 19 urine samples were normal (52.6%) and nine were anomalous (47.4%).

Figure 3 shows a comparison between urine uromodulin levels (ng/ml) of fetuses with obstructive uropathy with urine osmolarity below 210 mOsm/l (normal) *versus* fetuses with altered urine osmolarity. The absolute uromodulin urine levels were significantly different between the subgroups (1152 ± 282.1 ng/ml in the fetuses with normal urine osmolarity *vs.* 428.1 ± 44.1 ng/ml in the group with altered urine osmolarity; $p = 0.01$; Figure 3).

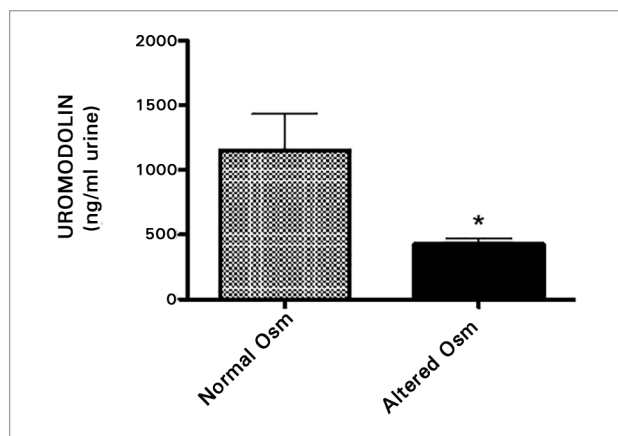
The same comparison using uromodulin levels related to urine creatinine levels in the two subgroups (normal *vs.* altered osmolarity) failed to yield statistically significant differences [11.3 (interquartile range 5.7 to 23.3) *versus* 13.3 (interquartile range 7.9 to 25.3) ng/ μ g; $p = 0.77$; Mann Whitney U test].

A moderate-to-significant inverse correlation was found between uromodulin levels and osmolarity (Pearson's correlation coefficient $r = -0.675$; $p = 0.02$). The correlations between uromodulin *vs.* creatinine and uromodulin adjusted for creatinine *vs.* osmolarity were not statistically significant (data not shown).

In contrast, the median level of uromodulin in amniotic fluid samples (Group 2) from fetuses free of renal involvement was 1164 (interquartile range: 528.5 to 1348) ng/ml, while the median level of uromodulin in the urine of fetuses with obstructive uropathy (Group 1) was 399.4 (interquartile range: 319.4 to 1139) ng/ml.

Given the strong similarity between the compositions of amniotic fluid and fetal urine, uromodulin levels in both bodily fluids were compared. Although uromodulin levels were higher

Figure 3. Comparison of uromodulin levels in fetuses with normal and altered osmolarity (osm). * $p = 0.01$, T-test for unpaired data.



in the fetuses in Group 2, the difference in relation to the levels seen in the individuals in Group 1 was not statistically significant ($p = 0.23$; Mann Whitney U test; Figure 4).

DISCUSSION

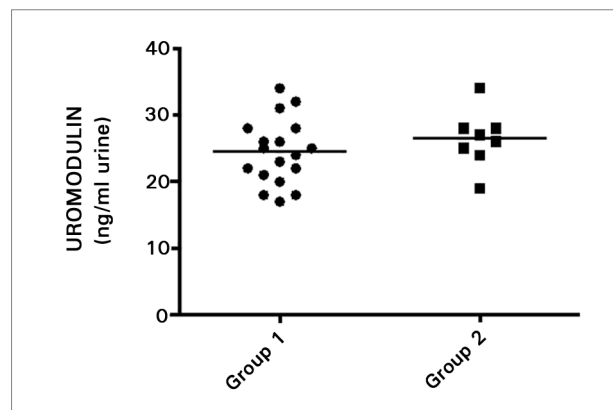
One of the main challenges in the prenatal care of obstructive uropathy revolves around finding markers able to accurately predict fetal renal function. This information allows the medical team to establish a more suitable treatment plan with the parents designed to preserve renal function through to birth. Obstructive uropathy is known for its prevalence and treatment options. Biomarkers able to effectively predict early prenatal renal function have not been reported to date.

In this study, uromodulin levels were measured in amniotic fluid and fetal urine, indicating it is produced and excreted by fetal kidneys. Batchelder *et al.*,⁴³ in a study carried out with Rhesus monkey fetuses, found uromodulin expression in cells of the TAL of the loop of Henle starting on the second trimester of pregnancy.

THP or uromodulin has been attributed numerous roles in renal physiology and protection against urinary tract infection, in addition to associations with genetic renal diseases. Adult individuals diagnosed with conditions leading to kidney involvement were shown to have minute uromodulin levels.

Uromodulin has also been used as a marker of acute kidney injury (AKI) in neonates. Individuals with AKI had lower uromodulin levels than injury-free neonates.⁴⁴

Figure 4. Comparison of urine uromodulin levels of fetuses with obstructive uropathy (group 1) and uromodulin levels in amniotic fluid of fetuses without obstructive uropathy (group 2). $p = 0.23$, Mann Whitney U test.



With such information in mind, the authors of this study compared uromodulin levels in fetuses with renal involvement (Group 1) and fetuses without renal involvement (Group 2). In Group 2, uromodulin levels in amniotic fluid were higher than the levels seen in the individuals in Group 1, thus supporting the findings in the literature on adult and neonate health in which individuals with probable loss of renal function have lower uromodulin levels.

Thus far low uromodulin levels have almost unanimously been considered the consequence of damage to cells of the TAL of the loop of Henle and correlated with decreased renal function.

Uromodulin levels measured from fetal urine were sorted into subgroups with normal or altered osmolarity. A statistically significant inverse correlation was found between these markers. Uromodulin was shown to be similar to osmolarity in diagnosing fetal renal function.

The results reported in this study showed that uromodulin levels were lower in the fetuses with severe prenatal renal involvement, indicating possible cell injury and compromised excretion. Uromodulin might possibly become a chemical biomarker of fetal renal function and contribute to the early diagnosis and better management of fetuses with severe obstructive uropathy. Uromodulin levels may be measured from fetal urine and amniotic fluid.

However, more studies with different methods and larger series - in which uromodulin is measured from the same fluid - comparing individuals with preserved renal function and subjects with obstructive uropathy are required to further observe the behavior of uromodulin as a marker.

CONCLUSIONS

Uromodulin levels may be derived and quantified from fetal urine or amniotic fluid samples with the aid of ELISA kits for human uromodulin. All tested samples were read and measured quantitatively based on calculated uromodulin absorbance.

A statistically significant inverse correlation was found between uromodulin levels and osmolarity.

A trend toward lower uromodulin levels in urine was observed in fetuses with prenatal renal involvement. Thus, high uromodulin levels in fetal urine or amniotic fluid may mean preserved renal function.

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