

Podocyturia in Fabry disease

Podocitúria na doença de Fabry

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ABSTRACT

Introduction: Fabry disease is a lysosomal storage disorder due to abnormalities in the GLA gene (Xq22). Such changes result in the reduction/absence of activity of the lysosome enzyme α -GAL, whose function is to metabolize globotriaosylceramide (Gb3). Renal disease is a major clinical outcome of the accumulation of Gb3. Podocyte injury is thought to be a major contributor to the progressive loss of the renal function and may be found altered even before the onset of microalbuminuria. **Objective:** The aim of this study was to quantify the urinary excretion of podocytes in Fabry disease patients (V269M, n = 14) and healthy controls (n = 40), and to correlate podocyturia with the variables gender, age, time of therapy and albumin: creatinine ratio (ACR). **Methods:** Urinary podocytes were stained using immunofluorescence to podocalyxin and DAPI. The number of podocalyxin-positive cells was quantified and the average number was taken (normal range 0-0.6 podocytes/mL). **Results:** The average number of podocytes in the urine of Fabry disease patients was significantly higher than in healthy controls ($p < 0.0001$). We observed a positive correlation between podocyturia and ACR ($p = 0.004$; $r^2 = 0.6417$). We found no correlation between podocyturia and gender, age or duration of therapy. **Conclusion:** Podocyturia is an important parameter in the assessment of renal disease in general, and it may serve as an additional early tool for monitoring Fabry disease nephropathy even before changes in ACR are seen. This may prove to be a useful tool to assess disease progression in patients expected to have a more aggressive phenotype.

Keywords: fabry disease; fluorescent antibody technique, indirect; podocytes.

RESUMO

Introdução: A doença de Fabry (DF) é uma desordem lisossômica ligada ao cromossomo X ocasionada por mutações no gene que codifica a enzima lisossômica α -galactosidase A (α -GAL). A redução ou ausência da atividade dessa enzima leva ao acúmulo progressivo de gb3. A doença renal é uma importante consequência clínica da acumulação de Gb3. Podócito é o tipo celular mais afetado na doença renal, que mostra apenas uma resposta parcial à Terapia de Reposição Enzimática. Além disso, a disfunção podocitária é a principal contribuinte para a perda progressiva da função renal e pode ser encontrada alterada mesmo antes do início da microalbuminúria. Assim, a podocitúria na DF pode ser uma ferramenta importante para prever a doença renal. **Objetivo:** O objetivo deste estudo foi quantificar a excreção urinária de podócitos em pacientes com DF (V269M, n = 14) e controles saudáveis (n = 40), e relacioná-las com as variáveis sexo, idade, tempo de terapia e a razão albumina: creatinina (AUC). **Métodos:** Podócitos urinários foram identificados utilizando imunofluorescência para podocalixina e DAPI. O número de células podocalixina positivo foi contado e o número médio foi utilizado (faixa normal 0-0.6 podócitos/mL). **Resultados:** O número médio de podócitos na urina de pacientes com DF foi significativamente maior do que os controles saudáveis ($p < 0.0001$). Observou-se uma correlação positiva entre podocitúria e AUC ($p = 0.004$; $r^2 = 0.6417$). **Conclusão:** A podocitúria pode ser uma ferramenta adicional para avaliar a progressão da doença renal em pacientes que se espera que tenha um fenótipo mais agressivo.

Palavras-chave: doença de fabry, podócitos, técnica indireta de fluorescência para anticorpo.

INTRODUCTION

Fabry disease is a lysosomal storage disorder caused by abnormalities in the GLA gene,^{1,2} which is mapped to the X chromosome in humans (Xq22).^{3,4} Such changes result in either a reduction or an absence of the activity of the hydrolase α -galactosidase A (α -gal A) or the inability of α -gal A to enter the lysosome.⁵ This enzyme metabolizes neutral glycosphingolipids with D-galactosyl residues, mainly globotriaosylceramide (Gb3), which is the major lipid that accumulates in Fabry disease.⁶

Progressive glycosphingolipid accumulation within cells results in multi-organ dysfunction. Fabry nephropathy is one of the most severe clinical consequences of this accumulation.^{7,8} Podocytes are the most affected kidney cell type and show a suboptimal response to enzyme replacement therapy (ERT).⁹ Whereas the natural history and histopathological findings of Fabry nephropathy have been largely described over the past decades, strikingly, the precise mechanism linking the initial biochemical insult and kidney failure remains poorly understood.

Podocytes are highly specialized epithelial cells that serve as key components of the glomerular filtration barrier, and podocyte injury leads to loss of the integrity of the barrier and progression to chronic kidney disease.¹⁰ Foot process effacement has been shown to precede microalbuminuria in Fabry disease, and this phenomenon seems to be reversible by early clearance of the Gb3 deposits in podocytes.¹¹ Podocytes can detach from the glomeruli, move into the urinary space, traverse the tubules and enter the urine, a phenomenon called podocyturia. In fact, podocyte loss to levels below a certain threshold is known to promote glomerulosclerosis. Ichikwa and colleagues have speculated that injuries can be fully reversed by correcting the initial insult only when damage occurs in no more than 25% of the podocyte population.¹² Thus, podocytopenia predicts the prognosis of kidney glomerular diseases in both experimental and human clinical scenarios.¹⁰

Podocytes can be retrieved from the urine in a minimal amount in a healthy state but can be found in greater numbers as an early finding of glomerular diseases.¹³ The assessment of podocyturia by cytological or molecular methods allows for a valuable, noninvasive and inexpensive approach to assess glomerular disease status and progression.

Determining the status and extent of podocyturia in Fabry disease can be a relevant issue for predicting renal disease during phases in which histological and functional reversibility might still be achieved. In addition, podocyte loss via the urine in FD can be useful in monitoring disease progression and renal failure to respond to standard therapies. The aim of this study was to quantify the urinary excretion of podocytes in FD patients (V269M, n = 14) and healthy controls (n = 40) and to correlate podocyturia with the variables gender, age, time of therapy and the albumin: creatinine ratio (ACR).

MATERIALS AND METHODS

This study was approved by the Universidade Federal do Piauí Institutional Board Review (0404.0.045.000-10), and all patients have provided written informed consent prior to enrollment. Demographics and clinical data were obtained from reviewing the patients' medical records.

SUBJECTS

The subjects of the study were 14 patients diagnosed with Fabry disease receiving ERT (0.2 mg/Kg biweekly), all of them from a single family in Piauí, Brazil. The patients were traced back from a pedigree composed of 610 members, seventy nine of which have a confirmed molecular diagnosis of FD (V269M). Each member of the pedigree was traced based using the index case identified in 2005 in Teresina - Piauí, Brazil.

PODOCYTURIA

Clean-catch midstream urine specimens were collected in sterile vials and processed immediately. For processing, approximately 50 mL of urine was transferred to a tube and centrifuged at 350 g for 10 min at room temperature. After centrifugation, the supernatant was discarded, and the pellet was resuspended, washed in HDF (137 mMNaCl, 5 mMKCl, 5.5 mM glucose, 4 mM NaHCO₃, and 0.2% EDTA in dH₂O), and cytocentrifuged on microscope slides. The prepared slides were fixed in 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 10 min. Samples were then blocked for 1 h in a solution containing 0.2% BSA, 50 mM NH₄Cl, and 1% goat serum in PBS.

Next, the samples were incubated with the primary antibody Mouse anti-Podocalyxin (Invitrogen) for 1 h and with the secondary antibody Alexa Fluor® 647 Goat Anti-Mouse IgG (Invitrogen) for 45 min. All steps were performed at room temperature. DAPI was used to stain nucleic acids. Slides were viewed with a fluorescent microscope. Two independent healthcare providers evaluated each sample, and the average value of the two measurements was taken. Podocyturia was defined as the number of podocalyxin positive cells per slide per mL of urine.

ALBUMIN/CREATININE RATIO (ACR)

The albumin and creatinine concentrations in the urine samples were determined using the LabTestAlbumin kit and the LabTestCreatinine Kit (Minas Gerais, Brazil), respectively, according to the manufacturer's protocol. The urine ACR was calculated from the concentrations of urine albumin and urine creatinine, and the values are expressed in mg/g.

STATISTICAL ANALYSIS

Statistical analysis was performed using IBM SPSS Statistics software v.21. A t-test was performed to assess differences in the median, considering a significance level of $p < 0.05$, and a Pearson correlation coefficient was calculated to explore the linear correlation between variables.

RESULTS

The patients' demographics can be found in Table 1. Fourteen patients (seven males and seven females) with the V269M mutation in exon 6 of the GLA gene were included in the study. Their ages ranged from 8-74 years, and the duration of therapy with agalsidase alfa ranged from 1-24 months. We also obtained urine from 40 healthy individuals with no known history of kidney disease, who served as controls.

Podocytes that detach into the urinary space and are retrieved in the urine can be visualized and quantified using cytological methods combined with immunofluorescence staining for podocyte surface proteins.¹⁴ As observed in other glomerulopathies,¹⁵⁻¹⁸ we observed a significant loss of podocytes via the urine ($p = 0.0001$) of the FD patients (Median \pm SEM: 0.8193 ± 0.1090), which was greater than what we observed in healthy controls (Median \pm SEM: 0.4450 ± 0.03754) (Figure 1).

Furthermore, the number of podocytes in the urine positively correlated ($r^2 = 0.6417$) with the urine ACR ($p = 0.0006$, 95% CI = 0.4703 to 0.9345) (Figure 2). However, no correlation was found with variables age and time of therapy (Figure 3).

DISCUSSION

Nephrologists treating Fabry disease are faced with two important questions: the first is concerned with the optimum time to start ERT. In fact, there is no medical consensus regarding this issue because some physicians start the treatment soon after the diagnosis and others wait for the appearance of the first symptoms.¹⁹⁻²² Because the current gold standard for investigating nephropathy is proteinuria and proteinuria is a consequence of podocytopenia, we realized that testing for podocyturia could yield an earlier diagnosis.

In this study, we investigated the degree of podocyturia in FD patients with the classic phenotype. Our results suggest that FD patients of both genders presented significant urinary podocyte excretion compared to healthy controls. Determining the status and extent of podocyturia in Fabry disease can be relevant in predicting renal disease during phases in which histological and functional reversibility might still be achieved.

This pilot study demonstrated the feasibility and clinical relevance of assessing podocyturia in patients with Fabry disease. However, this study was cross-sectional and involved a limited number of patients, all with the same disease genotype. Further longitudinal follow-up studies are needed, especially with the inclusion of a genotypically diverse population of patients with Fabry disease before the development of clinically overt proteinuria. These studies could be used to better assess the predictive value of podocyturia in relation to disease progression and histopathological changes, as well as these events' temporal relationship with known markers of glomerular disease, in the context of Fabry disease.

CONCLUSION

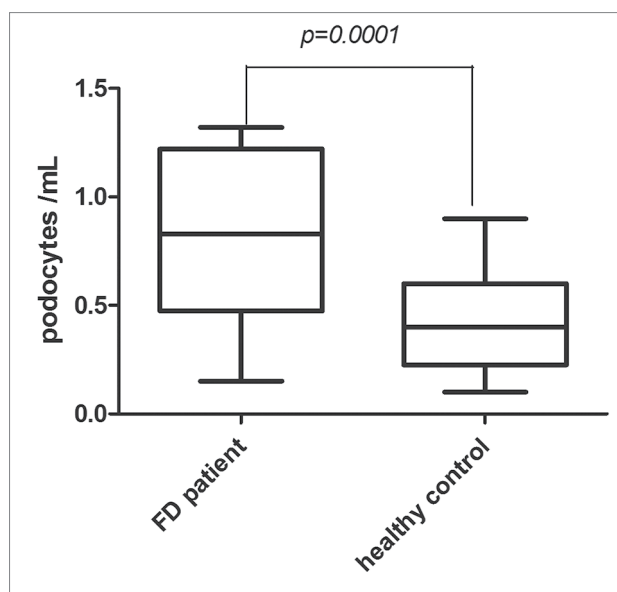
In summary, urinary detection of detached podocytes by cytology and immunofluorescence is a feasible noninvasive test for glomerular and podocyte dysfunction. In a cross-sectional cohort of patients with Fabry disease with a classic

TABLE 1 DEMOGRAPHIC AND CLINICAL OF FABRY PATIENTS

FD Patients	Age	Sex	Angiokeratoma	ECG abnormalities	eGFR < 80 mL/min/1.73m ²	Radiographic Lesions
1	27	M	No	Yes	Yes	Yes
2	33	F	No	Yes	Yes	Yes
3	8	F	No	No	No	No
4	20	F	No	No	No	No
5	8	M	No	No	No	No
6	31	M	No	Yes	No	No
7	23	F	No	No	No	No
8	53	F	No	No	Yes	No
9	22	M	No	No	No	No
10	35	M	No	No	No	No
11	46	F	Yes	Yes	Yes	No
12	74	M	Yes	Yes	Yes	Yes
13	37	F	No	No	No	Yes
14	35	F	No	Yes	No	No

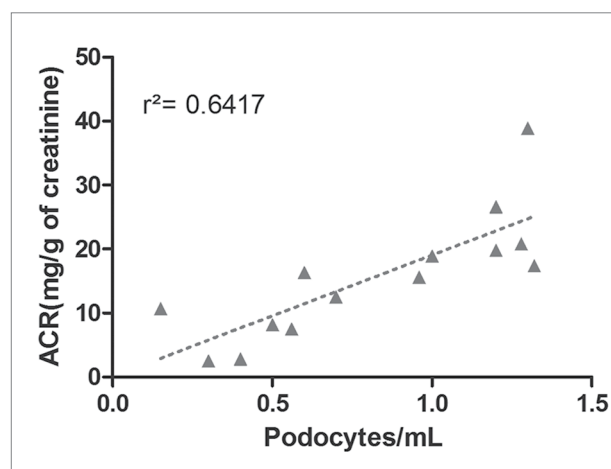
FD: Fabry disease; M: Male; F: Female; ECG: Echocardiogram; eGFR: Estimated glomerular filtration ratio (calculated using the CDK-EPI formula).

Figure 1. Fabry disease patients lose more podocytes in the urine than health controls. A fresh sample of the first urine (50 - 100 mL) void was used. Urinary podocytes were stained using immunofluorescence to podocalyxin and DAPI. The number of podocalyxin-positive cells was counted by two healthcare professionals, and the average number was taken.



phenotype, podocyte loss via the urine was significantly greater than for healthy controls and was strongly correlated with the patients' urine ACR. Determining podocyuria in patients with Fabry disease with a classic phenotype may serve as a relevant tool to assess and predict renal disease in these patients, even before changes in the urine ACR are observed. However, future prospective

Figure 2. Podocyuria correlates with the albumin-creatinine ratio in Fabry disease patients. Podocytes were identified through immunofluorescence staining against podocalyxin in fresh urine samples of FD patients, and the number of positive cells was quantified (podocytes/mL). The dotted line represents the linear regression.

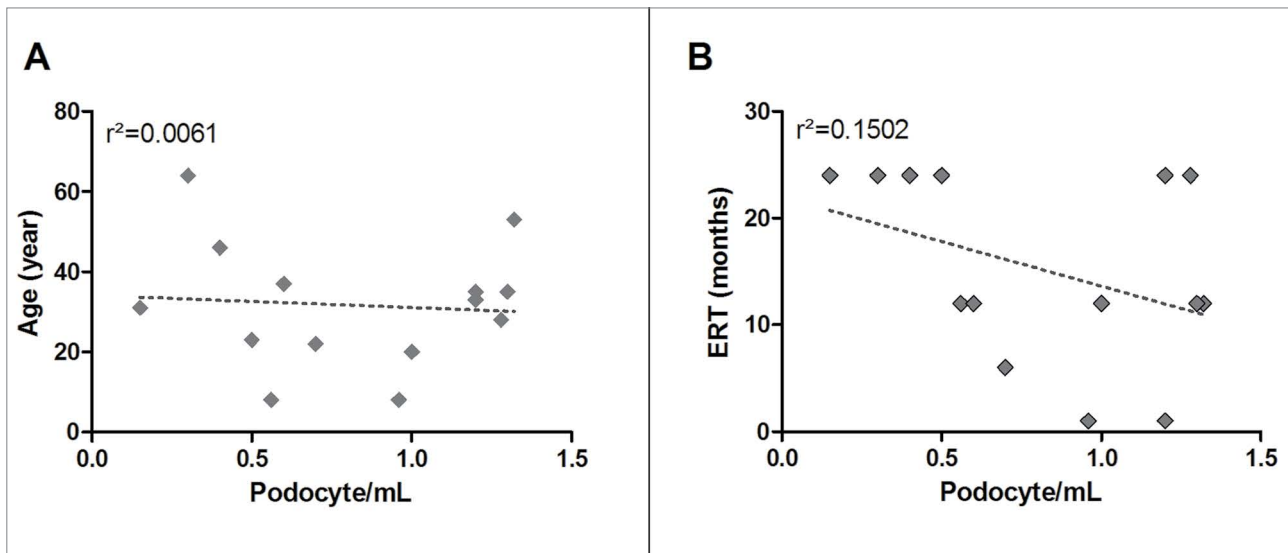


studies should be conducted to better examine the temporal relationship with known markers of glomerular disease and to validate our findings in a larger population of patients with early-stage Fabry nephropathy.

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Figure 3. There is no correlation between podocyuria and the variables age and time of treatment in Fabry disease patients. Podocytes were identified through immunofluorescence staining against podocalyxin in fresh urine samples of FD patients, and the number of positive cells was quantified (podocytes/mL) and correlated with A) age and B) enzyme replacement therapy (ERT). The dotted line represents the linear regression.



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