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ORIGINAL ARTICLE

Podocyturia is significantly elevated in untreated vs treated Fabry adult patients

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Abstract

Background Proteinuria suggests kidney involvement in Fabry disease. We assessed podocyturia, an early biomarker, in controls and patients with and without enzyme therapy, correlating podocyturia with proteinuria and renal function.

Methods Cross-sectional study (n = 67): controls (Group 1, n = 30) vs. Fabry disease (Group 2, n = 37) subdivided into untreated (2A, n = 19) and treated (2B, n = 18). Variables evaluated: age, gender, creatinine, CKD-EPI, proteinuria, podocyte count/10 20× microscopy power fields, podocytes/100 ml urine, podocytes/g creatininuria (results expressed as median and range).

Results Group 1 vs. 2 did not differ concerning age, gender and CKD-EPI, but differed regarding proteinuria and podocyturia. Group 2A vs. 2B: age: 29 (18–74) vs. 43 (18–65) years (p = ns); gender: males n = 3 (16 %) vs. n = 9 (50 %). Proteinuria was significantly higher in Fabry treated patients, while CKD-EPI and podocyturia were significantly elevated in untreated individuals. Significant correlations: group 2A: age-proteinuria, $\rho = 0.62$ ($p = 0.0044$); age-CKD-EPI, $\rho = -0.84$ ($p < 0.0001$); podocyturia-podocytes/100 ml urine, $\rho = 0.99$ ($p = 0.0001$); podocyturia-podocytes/g creatininuria $\rho = 0.86$ ($p = 0.0003$), podocytes/100 ml urine-podocytes/g urinary creatinine, $\rho = 0.84$ ($p = 0.0004$); proteinuria-CKD-EPI, $\rho = -0.68$ ($p = 0.0013$). Group 2B: podocyturia-podocytes/100 ml urine, $\rho = 0.88$ ($p < 0.0001$); podocyturia-podocytes/g creatininuria, $\rho = 0.84$ ($p < 0.0001$); podocytes/100 ml urine-podocytes/g creatininuria, $\rho = 0.94$ ($p < 0.0001$); CKD-EPI-proteinuria, $\rho = -0.66$ ($p = 0.0028$).

Conclusions Patients with Fabry disease display heavy podocyturia; those untreated present significantly higher podocyturia, lower proteinuria and better renal function than those who are treated, suggesting that therapy may be started at advanced stages. Podocyturia may antedate proteinuria, and enzyme therapy may protect against podocyte loss.

Keywords Fabry disease · proteinuria · Podocyte · Podocyturia · A-galactosidase

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Introduction

Fabry disease is an X-linked lysosomal storage disease caused by the deficiency in α-galactosidase A activity, resulting in the accumulation of its substrates including the glycosphingolipids globotriaosylceramide (Gb3), galabiosylceramide, and globotriaosylsphingosine (lyso-Gb3). Kidney involvement is one of the main feared complications of the disease, affecting podocytes, endothelial cells, smooth muscle and tubular cells [1, 2]. Whereas enzyme replacement therapy with recombinant α-galactosidase has emerged as a therapeutic option for Fabry disease, its

effectiveness along with angiotensin converting enzyme inhibitors in preventing renal failure and admission to renal replacement therapy has been questioned, particularly when the diagnosis is made at advanced stages of the disease [3]. Besides, proteinuria may persist particularly as renal disease worsens, and its appearance may denote an advanced glomerular damage [2, 4–6].

End-stage kidney disease due to Fabry disease accounts for 0.01 % of patients enrolled in European and US dialysis registries [1, 3, 7–9]. Despite the fact that enzymatic screening indicates that the real prevalence for male dialysis subjects may be 10- to 100-fold higher, mutations causing disease need to be differentiated from those with no clinical implications [10, 11]. The capability to evaluate and offer effective therapy is limited by a poor understanding of the pathogenesis of Fabry disease and by the absence of biomarkers that correspond directly to the presence and degree of kidney dysfunction. The glomerulus is particularly affected, podocytes being a major target [1–3, 12]. Podocytes do not proliferate and therefore continue accumulating α-galactosidase substrates throughout their whole lifespan. This progressive cytoplasmic accumulation of glycosphingolipids may lead to morphologic and functional alterations. In this respect, it has been speculated that the mechanical stress caused by large amounts of Gb3 could alter the distribution of synaptopodin [13]. Synaptopodin is an actin-associated protein highly expressed in podocytes' foot processes that is involved in cytoskeletal reorganization [14, 15]. A reduction in cellular synaptopodin concentration has been associated with foot process effacement and proteinuria [16]. Synaptopodin regulates podocyte contraction by interacting with actin filaments [16], and prevents the reorganization of the podocyte cytoskeleton into a migratory phenotype [17]. In turn, actin and actinin filaments interact mainly with the β_1 or β_3 subunits of integrin increasing podocyte contraction and migration [16, 18], eventually leading to detachment and urinary loss of podocytes, a phenomenon known as podocyturia [19–22]. In this respect, it is believed that when each glomerulus loses more than 40 % of its 500 podocytes, it undergoes obliteration [19–22].

In this study, we aimed to confirm the existence of a physiological podocyturia, and to compare it to untreated Fabry disease patients who presented without proteinuria and normal kidney function. Podocyturia between Fabry treated and non-treated cases was then compared.

Subjects and methods

This was a cross-sectional, observational study which included 67 individuals. Group 1 consisted of 30 healthy subjects without known clinical morbidities and not undergoing any pharmacologic treatment. Potential kidney donors and subjects with normal values at clinical examination formed this group. Group 2 included 37 patients with Fabry disease, subdivided into untreated patients (Group 2A, n = 19), and patients undergoing enzyme replacement therapy (Group 2B, n = 18) with agalsidase beta 1 mg/kg every fortnight (Fabrazyme, Genzyme Corp, Cambridge, MA, USA). Fabry disease was diagnosed in all cases by low enzymatic α-galactosidase A activity, α-galactosidase A concentration in peripheral leukocytes, and confirmed by the molecular enzyme mutation. Diabetic patients were excluded from the study. Patient characteristics are reported in Table 1.

Considered variables

Age, gender, arterial hypertension, glomerular filtration rate estimated by the Chronic Kidney Disease—Epidemiology Collaboration formula (CKD-EPI), 24-h proteinuria, podocyturia/10 20x fields observed by fluorescent microscopy, podocyturia/100 ml of urine, and podocyturia/g of creatininuria.

Podocyturia

Podocyturia was performed employing synaptopodin as the specific post-mitotic podocyte marker to identify podocytes in the urine, as described previously in cases of pre-eclampsia, lupus nephritis and healthy individuals [23–25]. Briefly, a mid-stream freshly voided urine sample was collected on-site after a minimum of 3 h without voiding; 20 ml of urine were centrifuged at 700*g* for 5 min using a cytospin; the supernatant was discarded and the obtained sediment was stored in 100 µl aliquots at room temperature mixed with a 1.5 ml solution made of 40 % paraformaldehyde diluted in phosphate-buffered saline (PBS) (pH 7.2–7.4) to reach a final 10 % concentration. Nuclei of podocytes were stained with 4′,6-diamidino-2-phenylindole. Podocytes were identified by immunofluorescence using synaptopodin as the primary antibody, (1:100, Abcam, Cambridge, MA, USA) and IgG anti-rabbit Alexa Fluor® 488 (1:100, Abcam, USA) as the secondary

Table 1 Patient characteristics

Variables	Group 1 (n = 30)	Group 2 (n = 37)	Group 2A (n = 19)	Group 2B (n = 18)
Age (years)	41 (24–76)	38 (16–74)	29 (16–74)	43 (18–65)
Gender (males)	14 (47 %)	12 (32 %)	3 (16 %)	9 (50 %)
Hypertensives	0	3	0	3 (17 %)

antibody. Samples were analyzed employing an epifluorescent microscope. Following our standardized technique, podocytes were counted in 10 randomly chosen 20× fields of the slides and the average of the counted podocytes in the microscopy fields was considered as the final count for each subject. The results were corrected based on the levels of urinary creatinine found in each sample [24].

Other variables

The values of serum creatinine and proteinuria were the ones obtained the same week that the urine was collected for podocyte count. Creatininuria was measured from the same specimen used for the podocytes search, as follows: the value of urinary creatinine obtained from the urine was then calculated for the initial urinary volume of 20 ml employed for the counting of podocytes. The same procedure was employed for the expression of podocytes per 100 ml of urine.

Ethics

The present protocol was approved by the Institutional Review Board of the Hospital Británico de Buenos Aires, Buenos Aires, Argentina. Informed consent was obtained from each study participant and the study was conducted in conformity with the International Conference of Harmonization (ICH) and Good Clinical Practice (GCP) recommendations.

Statistical analysis

Results are expressed as median and range. Variables were analyzed using the Wilcoxon–Mann–Whitney test. Correlations between variables were obtained with the Spearman correlation coefficient. Results were considered significant when $p \leq 0.05$.

Results

The most relevant results between controls and the two Fabry disease groups are reported in Table 2. Briefly, Groups 1 and 2 did not differ with regard to age, gender, or MDRD4, but they differed with respect to proteinuria, podocyte count, podocytes/100 ml urine and podocytes/g of urinary creatinine.

Group 2A vs. 2B results are presented in Table 3. The two subgroups did not differ with respect to age, but there were more males in the treated group. Proteinuria was

Table 2 Comparison of different variables between Controls and Fabry patients

Variables	Group 1	Group 2	P
Age (years)	41 (24–76)	38 (16–74)	0.1416
Serum Creatinine (mg/dl)	0.77 (0.56–1.12)	0.77 (0.47–12.50)	0.8105
CKD-EPI (ml/min)	95.70 (65.8–124)	101.90 (4.87–130)	0.8897
Proteinuria (gr/24 h)	0.05 (0.05–0.10)	0.37 (0.04–4.20)	<0.0001
Podocyte count (cells)	0.24 (0.00–0.92)	0.80 (0.20–6.84)	<0.0001
Podocytes/100 ml urine	1.45 (0.00–9.20)	5.0 (1.00–34.20)	<0.0001
Podocytes/g urinary creatinine	24.15 (0.00–108.00)	69.00 (8.00–910.30)	<0.0001

Table 3 Comparison of different variables between untreated and treated Fabry patients

Variables	Group 2A	Group 2B	P
Age (years)	29 (16–74)	43 (18–65)	0.0614
Duration of enzyme replacement therapy (months)	36.5 (26–52)	40.1 (29–55)	ns
Serum creatinine (mg/dl)	0.62 (0.47–1.10)	1.24 (0.48–12.50)	0.0049
CKD-EPI (ml/min)	95.54 (55.30–101)	59.12 (4.87–135)	0.0057
Proteinuria (gr/24 h)	0.10 (0.04–1)	0.66 (0.04–4.20)	0.0008
Podocyte count (cells)	1.48 (0.20–6.84)	0.54 (0.20–1.86)	0.0006
Podocytes/100 ml urine	7.65 (1–34.20)	3.35 (1–18.60),	0.0074
Podocytes/gr urinary creatinine	129.10 (15.70–910.30)	50.30 (8–235.60)	0.0164
Mutations	D33G; C281; C801; C1244T; T194I; N34D	D33G; C281; C801; C1244T; T194I; L180F; L415P; D155H	

Table 4 Significant correlations between treated and untreated Fabry patients

	Group 2A	Group 2B
Variable	Spearman; p	Spearman; p
Age-proteinuria	0.62; $p = 0.0044$	0.13; ns
Age-CKD-EPI	–0.84; $p < 0.0001$	−0.31; ns
Prot-CKD-EPI	−0.68; $p = 0.0013$	−0.66; $p = 0.0028$
Podocyte–podocyte/100 ml urine	0.99; $p < 0.0001$	0.88; $p < 0.0001$
Podocyte–podocyte/g creatinine	0.86; $p = 0.0003$	0.84; $p < 0.0001$
Podocyte/100 ml urine-Podocyte/g creatininuria	0.84; $p = 0.0004$	0.94; $p < 0.0001$

significantly higher in the treated group, while renal function was better in untreated individuals according to the CKD-EPI equation. Podocyturia, expressed in three different ways, was significantly elevated in untreated subjects. Hypertensives in Group 2A were nil compared to 3 (17 %) in Group 2B (Table 3). All Fabry patients were on enalapril and/or valsartan treatment as nephroprotection.

Significant correlations were found in Group 2A for: age-proteinuria, $\rho = 0.62$ ($p = 0.0044$); age-CKD-EPI, $\rho = -0.84$ ($p < 0.0001$); podocyturia-podocytes/100 ml urine, $\rho = 0.99$ ($p = 0.0001$); podocyturia-podocytes/g of urinary creatinine $\rho = 0.86$ ($p = 0.0003$); podocytes/100 ml urine-podocytes/g of urinary creatinine, $\rho = 0.84$ ($p = 0.0004$); proteinuria-CKD-EPI, $\rho = -0.68$ ($p = 0.0013$). In Group 2B, significant correlations were: podocyturia-podocytes/100 ml urine, $\rho = 0.88$ ($p < 0.0001$); podocyturia-podocytes/g of urinary creatinine, $\rho = 0.84$ ($p < 0.0001$); podocytes/100 ml urine-podocytes/g of urinary creatinine, $\rho = 0.94$ ($p < 0.0001$); CKD-EPI-proteinuria, $\rho = -0.66$ ($p = 0.0028$) (Table 4).

Discussion

We confirm the existence of a physiological podocyturia (Table 2). Fabry patients display higher levels of podocyturia compared to the normal population (Table 2). In addition, untreated Fabry patients display significantly higher levels of urinary podocyte loss and lower proteinuria when compared to treated subjects, suggesting that clinically covert podocyturia may antedate proteinuria (Table 3; Fig. 1). Individuals on enzyme therapy present lower podocyturia, higher proteinuria and worse renal function, which may indicate that enzyme therapy is currently started at advanced stages of Fabry disease (Table 3).

One of the main implications of our study is that relatives of the index case of Fabry disease, who are considered free of kidney involvement due to the lack of proteinuria or renal insufficiency, present a silent but significant loss of urinary podocytes (Group 2A). Once podocytes are lost, the inability of contiguous podocytes to cover the glomerular basement membrane results in protein leakage in the urine and glomerulosclerosis [21, 22]. This silent process of progressive podocyte loss usually occurs in youth, when extrarenal symptoms may be absent, and also in the female population, due to the chromosomal mosaicism they present [26]. In the untreated group, the strong and significant negative correlation between age and renal function underscores the early renal subclinical involvement (Table 4).

The other provocative unanswered question of our study is whether podocyturia itself could be a reliable marker of kidney involvement, on which to base the decision whether to start enzyme replacement therapy. It has been reported that despite enzyme administration, kidney function deteriorates at a slower rate but patients nevertheless require renal replacement therapy [5, 27]. This clinical situation may be due to a delayed commencement of enzyme replacement therapy (Table 3), which in the case of kidney involvement is generally dependent on proteinuria and renal function decline, late markers of kidney damage [2]. Moreover, there is no gold-standard urinary biomarker in Fabry kidney disease. In this regard, it has recently been reported that an isoform of Gb-3 could be reliably employed to screen Fabry patients in chronic kidney disease cohorts [28]. Due to the small size of our population, we were unable to perform multivariate analysis to assess the predictive value of podocyturia.

An apparently conflicting observation that emanates from our study is that in treated Fabry individuals proteinuria is significantly higher than in untreated subjects, while they display significant lower podocyturia and glomerular filtration rates (Table 3). This finding could be in agreement with the literature, which states that in Fabry disease the initiation of enzyme replacement therapy is late, in part due to a delayed awareness of the disease, to the unavailability of electron microscopy, or to a minimization of clinical kidney involvement [3, 5, 27]. This situation explains the lower renal function displayed by our treated Fabry patients, and may also suggest that the delivery of the enzyme plus inhibition of the renin-angiotensin system could reduce podocyturia (Table 3). While α-galactosidase β could reduce the cytoplasmatic accumulation of glycosphingolipids, angiotensin

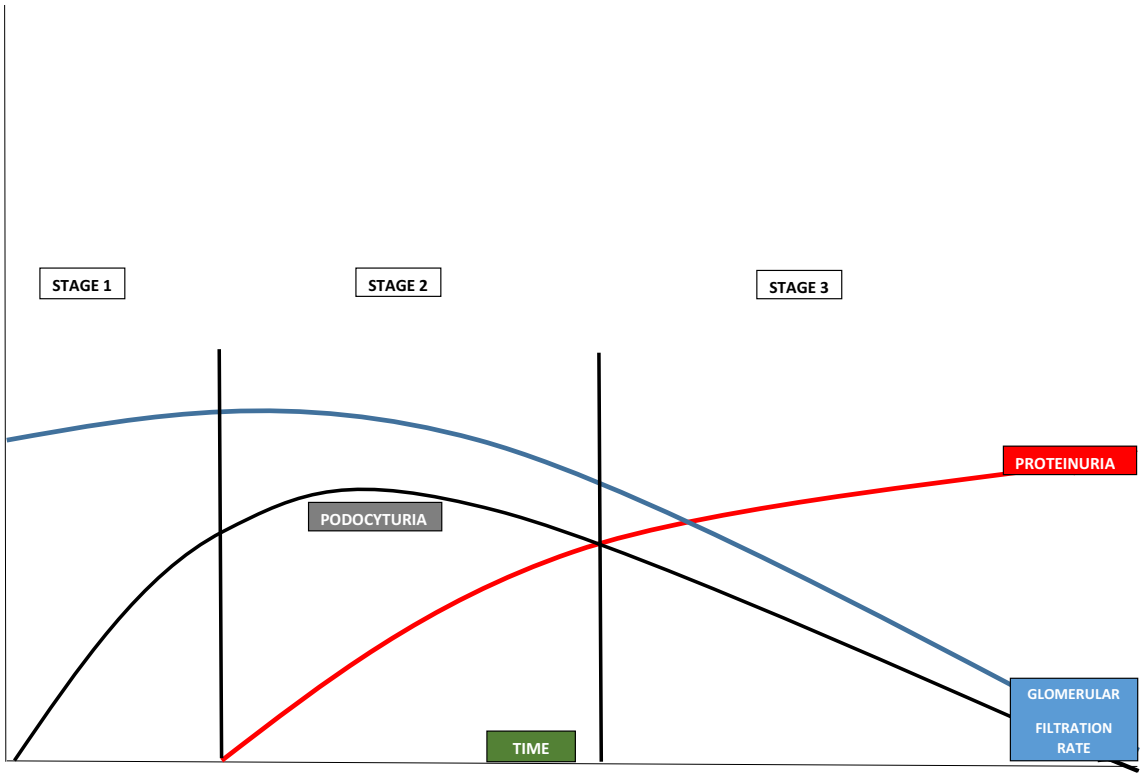

Fig. 1 Proposed speculative model of podocyturia, proteinuria and glomerular filtration rate dynamics in Fabry disease. In Stage 1, there is podocyturia without proteinuria and normal renal function and probably hyperfiltration. No podocyturia-proteinuria correlation exists. In Stage 2, podocyturia continues but is accompanied by proteinuria. There is a positive correlation between podocyturia and proteinuria, and a progressive decline in renal function. In Stage 3, podocyturia declines while proteinuria increases, and renal function continues to fall. No podocyturia-proteinuria correlation exists

converting enzyme inhibitors and angiotensin receptor blockers could stabilize the anchoring of podocytes to the glomerular basement membrane [2]. Finally, a lower filtration rate could correspond to proteinuria due to glomerulosclerosis. Due to the lack of kidney biopsies, in our study we could not confirm this hypothesis.

We believe podocyturia is an ideal biomarker to assess early glomerular involvement especially in hereditary diseases such as Fabry disease, because it can be employed in relatives in whom an enzymatic and genetic diagnosis has been made and no proteinuria exists [12]. Moreover, it can also be used during the follow-up of patients undergoing enzyme replacement therapy in order to assess more precisely the response to treatment. We have shown that patients on enzyme therapy display lower podocyturia, suggesting that this treatment may stabilize podocytes attachment and reduce their irreversible loss.

In Fabry disease, the mechanisms of podocyte detachment are caused by the accumulation of Gb3, which interacts with actin causing cell contraction, slit diaphragm widening and the coupling with integrins. In this regard, $\alpha_v\beta_3$ integrin and $\alpha_3\beta_1$ are some of the main molecules that anchor podocytes to the basement membrane. In Fabry disease, when the $\alpha_v\beta_3$ becomes activated, it triggers podocytic contraction and migration, finally contributing to podocyte detachment from the glomerulus and podocyturia [20, 29]. Of note, Utsumi et al. found elevated levels of $\alpha_v\beta_3$ integrin in the urine of Fabry individuals, localized mainly in podocytes and in Bowman's capsule epithelial cells [29]. Finally, the amount of vitronectin was moderately increased in the kidney of Fabry patients [29]. This finding is very interesting, as vitronectin couples with the urokinase-plasminogen activator receptor (uPAR). uPAR actively participates in podocyte signal transduction via the $\alpha_3\beta_1$ integrin, which in turn interacts with actin to cause podocyte contraction [4, 30–34]. Therefore, the activation of certain integrins appears to be actively involved in podocyte detachment from the glomerular basement membrane in Fabry disease [12, 29–34]. With respect to the progression of kidney disease and proteinuria, in vitro studies of Fabry cultured human podocytes have shown that the accumulation of Gb3 or lyso-Gb3 stimulated the secretion of transforming growth factor-β1 and fibrosis [35]. Moreover, autophagy is dysregulated in Fabry podocytes due to the inhibition of mammalian target of rapamycin (mTOR), a key enzyme that inhibits autophagy

and an additional cause of podocyte depletion and proteinuria [36]. Recently, Sanchez-Niño et al. reported the involvement of lyso-Gb3 deposition in the activation of Notch1 as another cause of podocyte injury in Fabry disease [37]. Finally, Group 2A was free of any treatment, including converting enzyme inhibitors or angiotensin receptor blockers. The addition of such therapy to this patient population in order to reduce podocyturia could be a hypothesis to test, as these drugs stabilize podocytes, decrease their contraction and reduce the size of pores of the glomerular basement membrane [38].

Regarding the methodology employed for the search of podocytes, we first calculated them in absolute numbers as per 10 randomly chosen 20× fields, and then corrected the cell count of each patient for the urinary creatinine and per 100 ml of urine as explained above. We found that there was a strong and significant correlation amongst the three different ways podocyturia was expressed (Tables 2, 3, 4). Nonetheless, we believe—as previously reported—that the expression of podocytes is better expressed by a qualitative common nominator for correction purposes, as is urinary creatinine [24]. In this respect, a quantitative expression of podocyturia could be defined, based on our findings. Finally, we chose 10 20× as the standard method for the counting of cells, based on the results obtained when the urine samples from the control population were studied. There are sufficient data to consider synaptopodin as a reliable and specific marker of podocytes [23–25, 39].

Our paper presents several limitations. We suggest that podocyturia could be employed to assess the early degree of kidney damage despite the absence of proteinuria and for follow-up purposes. However, while the technique is non-invasive and simple, it is also time-consuming (B Najafian, personal communication) [12, 40]. Moreover, it needs to be validated with studies adjusted for kidney function, age and probably also to the type of glomerulopathy, among other variables. In our study, only one marker was employed to identify podocytes, but certainly the addition of other podocyte markers could have identified podocytes at other cell-cycle stages [25]. All these considerations underscore the need to standardize the study of podocyturia. Ours was a cross-sectional study, but the effectiveness of therapy and the evolution of podocyturia, proteinuria and renal function in all patients requires a longitudinal follow-up. This underscores the fact that Fig. 1 is a mere hypothesis that needs to be proven. We had to exclude kidney biopsy as a variable to analyze because some patients did not have one performed. Fabry disease being a genetic disease, the impact of the different mutations on kidney function is a relevant factor that was not analyzed ad-hoc in this study with respect to podocyturia (Table 3). Nevertheless, all these mutations are related to the classical phenotype and not to late-onset variants of the disease. Considering the fact that Fabry disease is a rare entity, we nevertheless managed to include a relatively large number of subjects employing the same drug and dose, and the promising results of our study compel us to continue our investigations.

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Compliance with ethical standards

Conflict of interests Hernán Trimarchi is a consultant to Genzyme for the product alpha galactosidase-β.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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