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New biomarkers defining a novel early stage of Fabry nephropathy: A diagnostic test study

Patrício Aguiar [a,*], Olga Azevedo [b], Rui Pinto [c], Jacira Marino [d], Robert Baker [d], Carlos Cardoso [c], José Luís Ducla Soares [a,1], Derralynn Hughes [d,1]

[a] Medicine 1 Department, Centro Hospitalar Lisboa Norte, Lisbon, Portugal
[b] Department of Cardiology, Reference Center on Lysosomal Storage Disorders, Hospital Senhora da Oliveira, Guimarães, Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, ICVS/3Bs PT Government Associate Laboratory, Braga/Guimarães, Portugal
[c] JCS. Dr Joaquim Chaves, Lab Análises Clínicas, Miraflores, Portugal
[d] Lysosomal Storage Disorders Unit, Royal Free London NHS Foundation Trust and University College London, London, United Kingdom

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ABSTRACT

Background: Renal involvement in Fabry disease is a major determinant of overall disease prognosis and early enzyme replacement therapy seems effective in preventing progression of kidney injury. Gb3 storage, glomerular sclerosis and tubulo-interstitial fibrosis may occur with minimal or no changes on standard renal tests, hence alternative markers of renal dysfunction are crucial. In this study we compared several biomarkers with albuminuria in the identification of incipient Fabry nephropathy and their diagnostic accuracy to identify chronic kidney disease (CKD) stage ≥ 2.

Methods: In this multicentre, prospective, cross-sectional and diagnostic test study, a cohort of 78 Fabry patients and 25 healthy controls was consecutively recruited. Patients were grouped by severity of nephropathy: 1) albuminuria < 30 mg/g; 2) albuminuria 30–299 mg/g; 3) albuminuria > 300 mg/g; 4) glomerular filtration rate (GFR) < 60 mL/min/1.73 m^2. Several index tests, namely biomarkers of glomerular (transferrin and type IV collagen) and tubular (α1-microglobulin, *N*-acetyl-β-glucosaminidase and alanine aminopeptidase) dysfunction were compared with the reference standard (albuminuria).

Results: Significant increase of all tested biomarkers in Fabry patients, even in the subgroup of patients without evidence of nephropathy. We also found inverse significant correlations between estimated GFR and collagen type IV ($\rho = -0.289$; $p = 0.003$) or *N*-acetyl-β-glucosaminidase ($\rho = -0.448$; $p < 0.001$), which were stronger than with albumin ($\rho = -0.274$; $p = 0.019$). There was also better diagnostic accuracy of *N*-acetyl-β-glucosaminidase to predict CKD stage ≥ 2.

Conclusions: These results suggest that studied biomarkers may overcome the limitations of albuminuria as sensitive marker of early renal dysfunction and as marker for CKD progression risk. These biomarkers may also define novel early stages of nephropathy characterized by mesangial expansion and/or tubular damage.

1. Introduction

Anderson-Fabry disease (FD) is an X-linked lysosomal storage disorder caused by mutations in the GLA gene that encodes the enzyme α-galactosidase A. The deficiency of this enzyme leads to the lysosomal accumulation of neutral glycosphingolipids (mainly globotriaosylceramide [Gb3]) and organ failure. Involvement of the heart, kidney and brain causes significant morbidity and premature death [1,2].

In the past, before renal dialysis and transplant became widely available, kidney failure was the main cause of death in male patients with FD [3,4]. More recent studies reported cardiovascular disease as the main cause of death. However, the majority of patients dying from cardiovascular events had previously received renal replacement therapy [4,5].

In FD nephropathy Gb3 storage occurs in all renal cells and the disease progression results in the development of glomerulosclerosis, tubular atrophy and interstitial fibrosis [6]. There is limited knowledge about the renal histology of young patients with incipient nephropathy. However, heavy storage in podocytes and distal tubules, as well as segmental foot process effacement have been shown in children and patients with minimal or no alterations in standard renal tests [7–12]. Moreover, mesangial and endothelial cells inclusions,

* Corresponding author at: Medicine 1 Department, Centro Hospitalar Lisboa Norte, Avenida Professor Egas Moniz, 1649-035 Lisbon, Portugal.
E-mail address: patricio.aguiar@campus.ul.pt (P. Aguiar).
[1] Drs Hughes and Ducla Soares contributed equally.

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glomerulosclerosis, tubule-interstitial fibrosis and arteriopathy have also been described in this group of patients [7,10]. There is massive storage in the podocytes and early podocytopathy, with increased podocyturia and reduced expression of nephrin in the slit diaphragm in patients without increased albuminuria [13–15].

Albuminuria is usually considered to be the gold standard and a sensitive marker of early renal dysfunction in FD [16–18]. In fact, proteinuria is one of the most important indicators of renal disease progression in adult FD patients [19,20] and there is a significant correlation between urinary protein excretion rates and foot process width and fractional volume of Gb3 inclusions in the podocytes [8]. Nevertheless, significant histological changes may occur without pathological albuminuria and/or proteinuria [7–12], thus the sensitivity of albuminuria and/or proteinuria to identify incipient FD nephropathy is questionable.

Nowadays, enzyme replacement therapy (ERT) is the gold standard for the treatment of FD. ERT has shown to slow or halt the deterioration of renal function in patients with mild to moderate renal impairment [21–26]. These benefits may be limited patients with advanced renal disease [23,24,26–28]. Moreover, early ERT has a good safety profile (even in Paediatric population) [29] and is effective preventing kidney injury progression and reversing early pathological changes previously described [9,10].

Based on the two assumptions that early kidney involvement is clinically silent and early treatment is more likely to prevent progressive kidney injury, alternative markers of kidney dysfunction are mandated. So, the identification of biomarkers correlated to the earliest pathological findings is paramount, as these biomarkers may become a non-invasive, diagnostic method of pre-clinical renal involvement by FD.

In this study, we investigated several markers of glomerular and tubular damage in a large cohort of FD patients (within the entire spectrum of FD nephropathy severity), with an emphasis on the usefulness of these markers in incipient FD nephropathy.

2. Material and methods (Appendix 1 for extended methods)

2.1. Study design and population

In this multicentre, cross-sectional, prospective and diagnostic test study, a cohort of 78 FD patients and 25 healthy controls was consecutively recruited, between February 2013 and June 2014.

For FD patients the sole inclusion criteria were diagnosis of FD and age ≥ 18 years old. The control group included individuals with none of the exclusion criteria hereinafter listed and a normal kidney function (estimated GFR ≥ 60 mL/min/1.73 m^2 and albuminuria <30 mg/g).

The exclusion criteria defined for the control group included diseases with possible kidney involvement: systemic hypertension, diabetes, nephritic or nephrotic syndrome, immunoglobulin A nephropathy, systemic vasculitis, systemic lupus erythematosus, hepatitis C, amyloidosis and multiple myeloma. The exclusion criteria for FD patients were the same, except systemic hypertension, because it may be manifestation of FD nephropathy.

To ensure the entire spectrum of severity of FD nephropathy, the recruitment was made in accordance to subgroups of increasing severity: 1) albuminuria <30 mg/g and GFR ≥ 60 mL/min/1.73 m^2 (objective: 25 patients); 2) albuminuria 30–299 mg/g and GFR ≥ 60 mL/min/1.73 m^2 (objective: 20 patients); 3) albuminuria ≥300 mg/g and GFR ≥ 60 mL/min/1.73 m^2 (objective: 10 patients); 4) GFR < 60 mL/min/1.73 m^2 (objective: 20 patients). The control group was age- and sex-matched with the less severe FD subgroup.

The study protocol was approved by the local or national Ethical Committees of each participating centre and study was conducted in accordance with this protocol and the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from all participants before enrolment.

2.2. Clinical work-up, renal function assessment and outcomes

For each recruited patient, clinical data were collected and renal function was evaluated by the determination of serum creatinine, albuminuria and measurement or estimation of GFR.

Albuminuria (albumin-to-creatinine ratio) was used as the reference test to compare the index tests, as it is considered to be the gold standard and a sensitive marker of early renal dysfunction in FD [16–18]. It was quantified from spot urine, in random urine samples. Increased albuminuria was defined as ≥30 mg/g [30].

In FD patients, serum creatinine was assessed at routine follow-up (collected in the same day of the urine used to determine the albuminuria and the index tests). GFR was estimated by the 2009 CKD-$EPI_{creatinine}$ formula [30]. In a single centre GFR is often measured using Cr-51 EDTA clearance in FD patients. CKD staging by GFR categories [30] was based in estimated GFR, except in patients in which radioisotopic measurement of GFR was available.

Laboratories assessing the renal function were unaware of the measurements of the investigated glomerular and tubular damage biomarkers and clinical data.

The prespecified outcomes were to compare the index tests with albuminuria in the identification of incipient Fabry nephropathy (comparing subgroup 1 patients with controls) and their diagnostic accuracy to identify chronic kidney disease (CKD) stage ≥ 2.

2.3. Novel biomarkers of kidney injury analysis/index tests

Two markers of glomerular damage (transferrin and type IV collagen [ColIV]) and three markers of tubular injury (α1-microglobulin [A1MG], *N*-acetyl-β-D-glicosaminidase [NAG] and alanine aminopeptidase [AAP]) were quantified from spot urine. Each spot urine has been splitted from the sample used to determine albuminuria and immediately stored at −20 °C, transported to the laboratory at the same temperature conditions in dry ice and thawed just prior to the assay.

Transferrin, col IV, A1MG and AAP were evaluated by an ELISA method, according to manufacturer instructions and measured at 450 nm (SunRed®, Shanghai, PRC); NAG was evaluated by a colorimetric assay at 580 nm (Roche® Diagnostics GmbH, Mannheim; Germany) (detailed description in Supplementary methods). Duplicate determinations were made for each individual and mean results are presented. All biomarkers levels were normalized for the urinary creatinine concentration. Laboratory researchers were blinded to clinical data and renal function assessment (reference test).

Given that there are no validated reference values for the tested biomarkers, the upper limit of 95% confidence interval for mean of the control group was assumed to be the upper limit of the reference value.

2.4. Statistical analysis

Statistical analysis was performed with SPSS® (Statistical Package for the Social Sciences, version 21) software. Continuous variables were expressed as medians and interquartile range (IQR) and as number and percentage for categorical variables. Normal distribution of continuous variables was tested using Shapiro-Wilk test.

For continuous variables, comparison of means/medians was performed using Student *t*-test for variables that followed a normal distribution and Mann–Whitney test/related samples Wilcoxon signed rank test for variables who did not.

If the qualitative variable had >2 categories, an ANOVA test (post-hoc analysis with Bonferroni correction) was used for variables with normal distribution, and a Kruskal–Wallis test was used for those without. For categorical variables, the comparison of the variables distribution between groups was done using the Qui-square or Fisher exact tests.

To evaluate the correlation between the several biomarkers and the quantitative variables, Pearson's correlation coefficient was determined