Title: Accumulation of Globotriaosylceramide in Podocytes in Fabry Nephropathy is Associated with Progressive Podocyte Loss

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ABSTRACT:

Background: Males with classic Fabry disease have a high incidence of end stage renal disease (ESRD). The processes leading to ESRD are poorly understood. Defects in the α-galactosidase A gene lead to globotriaosylceramide (GL3) accumulation in various cell types, but in the glomerulus, this is progressive with age only in podocytes. Of concern, podocytes are relatively resistant to enzyme replacement therapy (ERT) and are poorly replicating when lost. **Methods:** In this study in 55 males aged 27±13 years with classic Fabry disease genotype and/or phenotype unbiased quantitative morphometric electron microscopic renal biopsy studies were performed. **Results:** There was increasing podocyte GL3 volume fraction with increasing age, which plateaued at about age 27. GL3 accumulation was associated with podocyte injury and loss as evidenced by increased foot process width and decreased podocyte number density per glomerular volume. Worsening podocyte structural parameters were also associated with increasing urinary protein excretion, a strong prognosticator of adverse renal outcomes in Fabry disease, and with glomerular filtration rate loss. **Conclusions:** Given the known association between podocyte loss and irreversible focal segmental and global glomerulosclerosis, this study supports an important role for podocyte injury and loss in the progression of Fabry nephropathy and argues for therapeutic intervention before critical podocyte loss has occurred.

Table of Abbreviations:

α-Gal-A	α-galactosidase A
ERT	Enzyme replacement therapy
FPW	Average foot process width
GL3	Globotriaosylceramide
GFR	Glomerular filtration rate
Nv(PC/glom)	Number density of podocytes per glomerular volume
UPER	Urinary protein excretion rate
VPC	Average podocyte volume
VPCN	Average podocyte nuclear volume
V(Inc/PC)	Podocyte GL3 volume; average total volume of GL3 inclusions per podocyte
Vv(Inc/Endo)	Endothelial GL3 volume fraction; the fraction of endothelial cytoplasmic volume occupied by GL3 inclusions
Vv(Inc/Mes)	Mesangial GL3 volume fraction; the fraction of mesangial cytoplasmic volume occupied by GL3 inclusions
Vv(Inc/PC)	Podocyte GL3 volume fraction; the fraction of podocyte cytoplasmic volume occupied by GL3 inclusions
Vv(PC/glom)	The fraction of the volume of the glomerulus occupied by podocytes
Vv(PCN/PC)	The fraction of the volume of podocytes occupied by podocyte nuclei

INTRODUCTION:

Fabry disease is caused by mutations in *GLA* gene leading to deficiency in the lysosomal enzyme α-galactosidase A (α-Gal-A) and accumulation of its substrates, primarily globotriaosylceramide (GL3), in various cell types and organs (1). Especially in males with more severe α-Gal-A gene mutations and little or no residual α-Gal-A enzyme activity, Fabry disease often results in severe vital organ injury manifesting as strokes, cardiomyopathy, arrhythmias, renal failure, neuropathy, and premature death (2).

Enzyme replacement therapy (ERT) may relatively quickly (within 5 months) eliminate microscopically detectable GL3 accumulation in endothelial cells in the skin, kidney, and heart as well as in additional cell types such as glomerular mesangial cells, and fibroblasts (3). However, some cell types such as glomerular podocytes, vascular smooth muscle cells and cardiac myocytes are considerably more resistant to ERT (3, 4). These are all complex, terminally differentiated, poorly replicating cells with great functional significance whose injury or loss can lead to severe organ dysfunction. Thus, although a long-term randomized placebo controlled ERT trial reported reductions in serious clinical events (5), there are substantial residual risks despite ERT (6). While playing a crucial role in preserving nephron structure and function, podocytes have little ability to replenish when lost (7). Podocyte dysfunction/loss is closely associated with proteinuria, the strongest available biomarker for predicting glomerular filtration rate (GFR) loss in patients with Fabry disease (8). In addition, there is a large and ever-increasing body of human and animal research which is consistent with the concept that substantial podocyte loss is associated with focal segmental and global glomerulosclerosis, important and irreversible lesions in the path to end stage renal disease (ESRD) (9, 10). In young ERT-naïve patients with Fabry disease, podocyte GL3 accumulation increases with age, whereas

in glomerular endothelial and mesangial cells it does not (11). Given that the development of Fabry disease clinical events is highly age-dependent (6, 12, 13), cells with progressive Fabry changes with increasing age (e.g,, podocytes) are more likely to contribute to these events. This study focuses primarily on podocyte changes over a wide age range in untreated male patients with Fabry disease.

METHODS:

Patients: We studied treatment-naïve male patients with Fabry disease. Renal biopsies were performed as baselines for clinical trials, as clinical assessments prior to the initiation of ERT or for diagnosis of clinical renal abnormalities. All patients provided written informed consent (or parental consent in the case of children). Kidney biopsies from seven living transplant donors obtained prior to organ removal were studied as controls. The research was performed in accordance with principles of the Declaration of Helsinki and was approved by the Institutional Review Boards of the Universities of Minnesota and Washington.

Clinical Information: Patients' age at the biopsy, serum creatinine, urinary protein excretion rate (UPER) (based on urine protein creatinine ratio or 24 hours urine protein), Fabry-related symptoms, *GLA* mutation and/or α-Gal-A activity were extracted from medical records or from the clinical trial databases. Leukocyte and plasma α-Gal-A activity levels are strongly correlated(14). In order to make leukocyte and plasma α-Gal-A activity values measured in different labs and at different times comparable, we expressed α-Gal-A activity as percent of the lower limit of a given laboratory's normal range (Table 1). All protein changes were checked with the «Mutation Taster» software [http://www.mutationtaster.org/cgi-

bin/MutationTaster/MutationTaster69.cgi]. For mutation descriptions at the cDNA and protein levels, we followed the Human Genome Variation Society recommendations (15). GFR, where available, was measured by the plasma disappearance of iohexol, or was estimated based on serum creatinine values using the CKD-EPI equation if age≥18 (16) or the modified Schwartz' equation if age<18 years(17). Urinary protein excretion rate (UPER) was derived from timed urine collections or spot urine samples obtained close to the time of the biopsy.

Renal Biopsy Studies: 1 µm sections of 2.5% glutaraldehyde fixed plastic embedded tissues were stained with toluidine blue for identification of glomeruli (18). Random glomerular sections were prepared for stereological studies as described elsewhere (11). Overlapping digital low magnification (~8,000 x) images of entire glomerular profiles were obtained using a JEOL 1010 electron microscope for masked review by two observers (BN and MM) to select 2-5 non-sclerosed glomeruli per biopsy with minimal or no artifacts for stereological studies as described below. High magnification (~30,000 x) images were obtained according to a systematic uniform random sampling protocol for estimation using point counting of the fraction of the volume (Vv) of podocyte cytoplasm occupied by GL3 inclusions [Vv(Inc/PC)], hereafter called "podocyte GL3 volume fraction" for simplicity, (11) (Figure 1). This was also done for glomerular endothelial [Vv(Inc/Endo)] and mesangial cells [Vv(Inc/Mes)] (11). Average volume of podocyte nuclei (VPCN) was estimated using the point-sampled intercept method (19) with a modified sampling strategy to reduce the volume-weighted property of the method (Figure 1) (20). This provides shape and size independent volume estimates. The fraction of the volume of podocytes occupied by podocyte nuclei [Vv(PCN/PC)] and fraction of the volume of the glomerulus occupied by podocytes [Vv(PC/glom)] were estimated using point counting. The average volume of podocytes was calculated as: $\mathrm{VPC} = \frac{\mathrm{VPCN}}{\mathrm{Vv(PCN/PC)}}$. The total volume of GL3

inclusions per podocyte [V(Inc/PC)], hereafter called "podocyte GL3 volume" for simplicity, was then calculated as: VPC·Vv(Inc/PC). Number density of podocytes per glomerular volume [Nv(PC/glom)] was calculated as the fraction of the volume of the glomerulus occupied by podocytes divided by the average podocyte volume or Vv(PC/glom)/VPC(21). Podocyte average foot process width (FPW) was estimated as the reciprocal of slit length density as previously described (Figure 1) (11, 22). All stereological estimates were done by masked observers.

Statistical Analyses

Statistica 13.0 (Statsoft, Inc.) software was used. Parametric or non-parametric tests were used based on the variable characteristics and distribution. Comparison of variables in Fabry patients and normal controls was done using Student's t-test or Kolmogorov-Smirnov test. Relationships between variables were evaluated using Pearson correlation. Multiple regression analysis was performed to identify factors associated with podocyte loss and GFR. Piecewise linear regression analysis was performed to study nonlinear relationships. $p \leq 0.05$ was considered statistically significant.

RESULTS:

Patients' Characteristics

We aimed to study podocyte injury in kidney biopsies from ERT-naïve male patients with Fabry disease with classic genotype/phenotype. 67 ERT-naïve males with Fabry disease age 26 (4-60), median (range) years were initially considered for enrollment. The phenotype of the disease (classic *vs.* late-onset/cardiac variant) was definable in 60 patients based on the available

literature for known mutations and/or clinical findings; 48 (80%) had classic and 12 (20%) had late-onset/cardiac phenotypes of Fabry disease. The available information was insufficient for reliable classification in 7 patients. Clinical parameters (age, GFR, UPER) and Fabry specific structural data including GL3 inclusion volume fraction in podocytes and mesangial cells were not statistically different between these 7 patients and the patients with classic phenotype, and inclusion volume fraction in endothelial cells was greater in these 7 patients with unknown phenotype compared with the classic phenotype patients (Supplementary Table 1). We therefore combined these two groups for all subsequent analyses (n=55) and excluded the late-onset/cardiac variant subjects from the study. Subjects' characteristics are listed in Table 1.

Relationships between GL3 accumulation, podocyte injury and loss with age

The scatter plot of age vs. podocyte GL3 volume fraction suggested that the relationship between these two parameters may be best explained through two regression lines with a slope change somewhere between ages 20-30 years (Figure 2A). Piecewise linear regression analysis showed that 63% of podocyte GL3 volume fraction can be explained by age with the breakpoint at age 27±13 years [i.e., podocyte GL3 volume fraction correlated directly with age in patients younger than 27 years ($R=0.62$, $p=0.0001$), but did not increase with age thereafter (Figure 2A)]. This suggests that, beyond a threshold, increasing podocyte GL3 volume fraction may compromise the survival of these cells. In order to further examine this possibility we determined the relationship between GL3 inclusion volume fraction in podocyte profiles with a visible nucleus and the size of that podocyte profile as an indicator of podocyte size in 193 podocytes from 7 randomly selected biopsies. There was a statistically significant direct correlation between podocyte profile area and GL3 inclusion volume fraction in each biopsy consistent with larger

podocytes having greater fraction of cytoplasm filled with GL3, this suggesting that the rate of GL3 accumulation in these cells exceeds their rate of cellular enlargement (supplementary Table 2). Furthermore, the plot of pooled data from these biopsies showed an initial linear relationship between GL3 inclusion volume fraction and podocyte profile area followed by a plateau (Figure 2B). This supports a threshold for GL3 inclusion volume fraction and/or podocyte size beyond which podocyte survival is compromised (Figure 2B).

Since we observed a different pattern of relationship between fractional volume of inclusions per podocyte and age in patients younger than age 27 *vs*. those age 27 years or older, we also separately analyzed the data in each of these age groups. In contrast to podocyte GL3 volume fraction which, as noted above, reached a plateau at about age 27 years, both mean podocyte volume (VPC) and the podocyte GL3 volume [V(Inc/PC)] correlated directly with age in patients below age 27 (R=0.50; p=0.017 and R=0.59; p=0.004, respectively) and above age 27 (R=0.55; p=0.026 and R=0.51; p=0.046, respectively). Thus, although the podocyte GL3 volume fraction did not appear to increase beyond a certain level, podocyte cell volume and total GL3 content per podocytes continued to increase with increasing age. Comparison of a subset of Fabry patients (n=20) with similar ages to the living kidney donors showed that mean podocyte cell volume in Fabry patients was ~4 fold greater than these normal controls (Supplementary Figure 1).

While podocytes were enlarged in Fabry disease, there was progressive decline in the fraction of glomerular volume occupied by podocytes [Vv(PC/glom)] with age (R=-0.57; p=0.00006; Figure 2C). Likewise, by simple linear regression analysis, number density of podocytes per glomerular volume [Nv(PC/glom)] declined with age (R=-0.47, p=0.033 (Figure 2D), confirming podocyte

loss with increasing age. Inverse correlations between age and the fraction of glomerular volume occupied by podocytes and between age and podocyte number density were present both in patients who were younger and who were older than age 27 years. Moreover, in 33 subjects where values for both plasma or leukocyte α-Gal-A activity and podocyte number density were available, there was a direct relationship between α-Gal-A activity and podocyte number density ($r=0.46$, $p=0.007$), consistent with a relationship between α-Gal-A deficiency and podocyte loss. In order to identify factors associated with podocyte loss, multiple regression analysis was performed with podocytes number density [Nv(PC/glom)] as the dependent variable and age, podocyte GL3 volume and GL3 inclusion volume fraction per podocyte, endothelial cell and mesangial cell as predictor variables. With a tolerance of >0.01, since podocyte GL3 volume and mean podocyte volume were highly correlated and showed redundancy , mean podocyte volume was not included in the model. The model explained 40% (adjusted $R^2=0.40$, $p=0.003$) of podocyte number density variance and podocyte GL3 volume was the only independent predictor of podocyte number density ($p=0.0004$), consistent with a strong negative effect of podocyte GL3 accumulation on podocyte survival in Fabry disease. Importantly, the addition of α-Gal-A activity substantially improved the model, where now 61% (adjusted $R^2=0.61$, $p=0.0005$) of podocyte number density was explained with both podocyte GL3 volume ($p=0.00005$) and α-Gal-A activity ($p=0.001$) being independent predictors. Mean podocyte foot process width (FPW), a generally accepted structural marker of podocyte stress and injury, was ~1.5 fold greater in Fabry patients compared to normal controls ($p=0.004$) (Supplementary Figure 2). Also, FPW correlated with both podocyte GL3 volume fraction ($R=0.40$, $p=0.004$), and podocyte GL3 volume ($R=0.36$, $p=0.03$).

Relationships between Podocyte Structural Parameters and Renal Function

By simple linear regression analysis, urinary protein excretion rate (UPER) correlated with increasing podocyte GL3 volume fraction (R=0.44, p=0.003) and FPW (R=0.41, p=0.007) (Figure 3A-B). Using simple linear regression analysis, the fraction of glomerular volume occupied by podocytes (R=0.24; p=0.09), mean podocyte volume (R=0.30; p=0.07) and podocyte GL3 volume (R=0.30; p0.08) showed trends of relationship with GFR. . Using multiple regression analysis with a tolerance > 0.01, 13% of GFR variance (adjusted R^2=0.13, p=0.03) was explained by the fraction of glomerular volume occupied by podocytes, FPW and podocyte GL3 volume fraction, while the fraction of glomerular volume occupied by podocytes and FPW were independent predictors. Addition of age, podocyte GL3 volume, and average podocyte volume or podocyte number density made the model statistically insignificant.

The scatterplot of podocyte number density *vs.* podocyte GL3 volume showed that the relationship between these two parameters follows an initial steep downward slope followed by a milder slope, consistent with a two-phase exponential decay function (Figure 3C). Piecewise linear regression analysis identified podocyte GL3 volume of 2009 μm^3 as the breakpoint with maximum slope shift. Patients and biopsy characteristics in relation to this breakpoint are listed in Table 2. Aside from podocyte number density, podocyte GL3 volume, and average podocyte volume, the other clinical and structural parameters studied were not statistically different in patients with biopsies with podocyte GL3 volume above or below the breakpoint. Patients with podocyte GL3 volume greater than the breakpoint showed an inverse correlation between age and podocyte number density (r=-0.70, p=0.008) and direct correlations between age and podocyte GL3 volume (r=0.57, p=0.04) and mean podocyte volume (r=0.67, p=0.01). Also, in

patients with podocyte GL3 volume greater than the breakpoint, UPER correlated inversely with podocyte number density (r=-0.64, p=0.03) and directly with podocyte volume (r=0.79, p=0.002) while FPW correlated inversely only with podocyte number density (r=-0.74, p=0.04). Subjects with podocyte GL3 volume ≤ the breakpoint showed no statistically significant relationship between age or UPER and podocyte number density, podocyte GL3 volume, or podocyte volume. However, they showed a direct relationship between UPER and the podocyte GL3 fraction (r=0.60, p=0.009), this not found in subjects with podocyte GL3 volume > breakpoint.

DISCUSSION:

This is the first study that addresses detailed structural changes in podocytes in a relatively large number of patients with Fabry disease. The podocyte (23) is among a group of terminally differentiated relatively poorly replicating important cell types that also includes vascular smooth muscle cells and cardiac myocytes (24) all of which have relatively poor responses to enzyme replacement therapy (ERT) (3, 25). Severe damage to these cell types can have serious clinical consequences including, for vascular smooth muscle cells, down-stream ischemia and tissue infarction, e.g., strokes, cardiac arrhythmias, myocardial fibrosis, renal interstitial fibrosis, and global glomerulosclerosis, and for cardiac myocytes, cardiomyopathy related cardiac failure (26). The podocyte is critical to the maintenance of glomerular permselectivity (27) and podocyte damage is associated with proteinuria which, in untreated males with Fabry disease, is a very powerful predictor of progressive GFR loss in untreated patients (8) as well as a predictor of the failure of long term ERT to prevent further GFR loss (6). As noted above, podocyte loss cannot be easily compensated for by podocyte regeneration and, if sufficiently severe, leads to irreversible global glomerulosclerosis and the spiral of chronic kidney disease progression

related to reductions in functioning nephron number (28).

We have previously shown that podocyte GL3 volume fraction (i.e. the fraction of podocyte cytoplasm filled with GL3 inclusions) increases in Fabry patients with classic GLA mutations below age 19 years (11). Our hypothesis regarding the relationships between aging, GL3 accumulation and podocyte loss is based on the findings in the present study which involved a large patient cohort across a wide age span is summarized in Figure 4. We found that the increase in podocyte GL3 volume fraction with increasing age continues until about age 25-30 years. Thereafter there is a plateau in this relationship suggesting that podocyte viability is compromised with greater proportions of podocyte cytoplasm filled with GL3 inclusions. The direct relationship between podocyte GL3 volume fraction and podocyte profile area clearly shows that podocyte GL3 accumulation cannot be adequately compensated for by podocyte enlargement, otherwise podocyte GL3 volume fraction would have remained constant while podocytes enlarged. The direct relationships between FPW and podocyte GL3 volume fraction is indicative of increasing podocyte stress and injury with increasing GL3 accumulation, this supported by the direct relationships between podocyte GL3 volume fraction and UPER. These results also support the validity of our sampling and measuring methodologies in that our structural results from 3 glomeruli are reflective of the permselectivity properties of all approximately 2 million glomeruli.

Importantly, this podocyte injury was associated with podocyte loss. Although mean podocyte volume was increased in these male ‘classic’ Fabry disease patients, in some instances to 300-400% above the upper limit of normal, the fraction of glomerular volume occupied by podocytes actually decreased with increasing age. These findings are most consistent with decreasing numbers of podocytes per glomerulus. Although increased number of podocytes in the urine (i.e.

podocyturia) has been shown in Fabry disease (29-31), the present study is, in fact, the first to directly document podocyte loss in biopsies from patients with Fabry disease. Also important was the finding of an inverse relationship between both podocyte size and GL3 content and number density of podocytes per glomerulus. Podocytes undergo compensatory hypertrophy as a result of glomerular enlargement and/or reduced number of podocytes. Surpassing the capacity of podocytes for hypertrophy leads to podocyte loss and segmental glomerular sclerosis (32, 33). Several mechanisms have been proposed for explaining podocyte injury in Fabry disease (34-36). Regardless of the injury process, the strong inverse relationship found between podocyte GL3 volume and podocyte number density supports clinical relevance of using quantitative measures of podocyte GL3 volume as an indicator of response to Fabry-specific treatments (20, 37, 38). Importantly, the relationship between podocyte GL3 content and podocyte number density followed an initial phase with steep slope and a later phase with milder slope with a breakpoint at the transition between these two phases. While the proposed value for this breakpoint in this study needs to be confirmed in studies with larger number of biopsies, a few important points can be derived from the pattern of relationship between these two parameters. Biopsies with greater GL3 content per podocyte showed more prominent podocyte loss and were also associated with more progressive podocyte GL3 accumulation as well as with increasing podocyte loss with aging. While these cross-sectional observations suggest that quantitative assessment of podocyte GL3 volume may be of prognostic value and may help identify patients with more renal severe phenotypes, it will be important to confirm such conclusion in longitudinal studies.

As this study strongly suggests, the process of podocyte loss begins relatively early in Fabry disease, arguing for earlier institution of treatment, certainly before significant proteinuria

develops. It will also be important to conduct longitudinal studies to document if a given treatment can lead to amelioration of podocyte loss. Nevertheless, since beyond a certain point in the process of podocyte loss, glomerular scarring is the regular outcome (28) and since with higher levels of proteinuria GFR loss continues despite the institution of ERT (6), a beneficial effect of earlier institution of ERT on podocyte survival would not be surprising. Our findings also lend support to implementing kidney biopsies in the ascertainment of the severity of renal injury and timing of therapeutic strategies in males with Fabry disease. While a baseline biopsy prior to initiation of treatment will provide valuable information, performing follow up biopsies may be even more informative in assessment of the efficacy of treatment on the injury to podocytes and other kidney structures(20, 39, 40). The situation for females is more complex, since their podocyte involvement and injury is also affected by mosaicism resulted from random X-inactivation (41).

The present study has some limitations. The cross-sectional nature of this study limits our ability to draw firm conclusions regarding prognostic significance of the podocyte injury findings. The available α-Gal-A activity values included plasma or leukocyte values measured in different laboratories over a wide time span, requiring a normalization process in an attempt to derive comparative values. Also, these observations in untreated males with 'classic' Fabry disease should not be generalized to patients with atypical forms of Fabry disease or to females or ERT or otherwise treated patients

In summary, podocyte GL3 accumulation in Fabry disease is associated with podocyte injury which progresses with age and this is associated with proteinuria and podocyte loss.

AUTHOR CONTRIBUTIONS:

B.N and M.M designed the study and contributed equally to the writing of the manuscript. B.N. performed all of the statistical analyses. C.T, E.S, M-C. G, J-P.O contributed renal biopsies and clinical data for this study and critically reviewed and edited the manuscript.

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FINANCIAL DISCLOSURES:

B.N. is a recipient of investigator-initiated grants from Sanofi/Genzyme and Amicus Therapeutics, has research contracts with Sanofi/Genzyme and Avrobio, and is a consultant to Sanofi/Genzyme, Amicus Therapeutics and Freeline Therapeutics. M. M. is a recipient investigator-initiated Sanofi/Genzyme research grants. His lab performs kidney/skin biopsy

lab studies for Sanofi-Genzyme. He is also a consultant to Sanofi/Genzyme for clinical trial design. He is a speaker at Sanofi/Genzyme educational meetings. These interests have been reviewed and managed by the University of Minnesota in according to its conflict of interest policies. M.M. is also a consultant to and his lab performs kidney biopsy studies for Amicus Therapeutics and he is a consultant to Freeline Therapeutics. C. T. has received speakers' fees and travel support from Sanofi/Genzyme, Amicus Therapeutics and Shire, and has participated in studies supported by Sanofi/Genzyme, Shire, Protalix and Freeline Therapeutics. E. S. has received speakers' fees and travel support from Sanofi/Genzyme, Amicus Therapeutics and Shire, and has participated in Sanofi/Genzyme and Amicus Therapeutics Advisory Boards. J-P. O. has received financial support for research from Sanofi/Genzyme; honoraria for lecturing from Sanofi/Genzyme and Shire; honoraria for consultancies and advisory board membership from Sanofi/Genzyme.

The Supplemental Table of Contents:

1. Supplemental Table 1. Comparison of clinical and Fabry specific renal structural data in Fabry patients with known 'classic' phenotype and those with unknown phenotype.

2. Supplemental Table 2. Relationships between Vv(Inc/PC) and podocyte profile area in individual biopsies.

3. Supplemental Figure 1. Comparison of podocyte volume in male Fabry patients and healthy controls

4. Supplemental Figure 2. Comparison of foot process width in Fabry and healthy control subjects.

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Table 1. Clinical and renal functional characteristics in male 'classic' patients with Fabry disease.

Case	Age (years)	Protein Change	cDNA Mutation	Mutation Category	α-Gal-A activity (%LNL)	Plasma GL3	GFR	UPER	RAAS Blockade	Non-renal Fabry Features
1	4	p.(Tyr216 Asp)	c.646T>G	missense	NK	NK	92	NK	N	NK
2	5	p.(Arg112 Cys)	c.334C>T	missense	0%	NK	122	0.460	N	None
3	7	p.(Met267 Arg)	c.800T>G	missense	14%	7.5	106	0.250	N	CO, PN, GI,
4	11	p.(His302 Ala fs*13)	c.903_904ins G	frameshift	0%	NK	121	0.150	N	NK
5	11	NK	NK	NK	20%	NK	NK	0	N	CO, AK
6	12	NK	NK	NK	38%	NK	NK	0.039	N	CO, AK
7	13	NK	NK	NK	0%	NK	122	0.169	N	NK
8	15	NK	NK	NK	3%	NK	93	0.082	N	NK
9	15	NK	NK	NK	0%	NK	103	0.088	N	NK
10	15	NK	NK	NK	3%	NK	134	0.079	N	NK
11*	15	NK	NK	NK	NK	NK	NK	0.350	N	CO, AK, PN
12	16	p.(Arg404 del)	c.1212_1214 delAAG	deletion	49%	13.4	112	0.090	N	CO, AK, PN, GI
13	17	p.(Arg227 Ter)	c.679C>T	nonsense	2%	17.1	190	0.229	N	CO, AK, PN,
14	17	p.(Asn272 Lys)	c.816C>A	missense	2%	NK	107	0.110	N	PN, GI
15	17	NK	NK	NK	0%	NK	97	0.082	N	NK
16	17	NK	NK	NK	3%	NK	127	0.069	N	NK
17	18	p.(Met267 Arg)	c.800T>G	missense	19%	4.8	96	0.220	N	CO, AK, PN, GI
18	18	p.(Arg227 Ter)	c.679C>T	nonsense	2%	19.3	156	0.167	N	CO, AK, PN
19	20	NK	IVS5-2,3delCA (c.802-3_802-2delCA)	intronic deletion / splicing	3%	NK	153	0.167	N	CO, AK,
20	20	p.(Arg220 Lys)	c.658C>T	nonsense	2%	7.7	70	0.211	Y	CO, AK, PN
21	20	p.(Asn272 Lys)	c.816C>A	missense	<24%	27.3	131	0.024	N	CO, AK, PN, GI
22	21	p.(Arg227 Ter)	c.679C>T	nonsense	2%	15.8	130	0.102	N	AK, PN, GI

23	21	p.(Arg227 Ter)	c.679C>T	nonsense	NK	2	289	0.140	N	CO, PN, GI
24	23	p.(Asn298 Lys fs*2)	c.893_894ins G	insertion	6%	7	112	0.102	N	CO, AK, GI
25	23	NK	NK	NK	5%	8.5	178	NK	N	CO, AK, LVH, PN
26	23	p.(Gly132 Arg)	c.394G>A	missense	<24%	9.9	88	NK	N	CO, AK,
27	23	p.(Arg404 del)	c.1212_1214 delAAG	deletion	14%	8.5	113	0.240	N	CO, PN, GI
28	23	NK	NK	NK	6%	10.5	148	NK	N	CO, AK, PN
29	24	NK	NK	NK	4%	20	126	0.140	N	CO, AK, PN
30	25	NK	NK	NK	<24%	13.9	146	0.402	N	CO, AK, AR, PN, GI
31	25	NK	NK	NK	6%	NK	110	NA	N	CO, AK,
32	25	p.[(Asp55 Val; Gln57Leu)]	c.[164A>T; 170A>T]	double missense	0%	NK	114	0.018	NK	NK
33	26	p.(Trp204 Ter)	c.612G>A	nonsense	6%	9.9	96	NK	N	CO, AK, PN, GI
34	30	p.(Arg404 del)	c.1212_1214 delAAG	deletion	28%	10.2	86	0.220	N	CO, AK, PN, GI
35	31	NK	c.639+4A>T	splicing	3%	5.3	167	1.150	N	CO
36	33	p.(Asp244 Asn)	c.730G>A	missense	NK	NK	115	0.023	NK	NK
37	33	p.(Val339 Alafs*32)	c.1016_1026d elTGTGGGA ACGA	deletion	0%	7.7	103	0.383	N	CO, AK, PN
38	34	p.(Tyr216 Cys)	c.647A>G	missense	0%	NK	119	0.029	NK	NK
39	34	NK	c.639+4A>T	splicing	3%	29.5	137	0.297	N	CO, AK, LVH, AR, MI, PN
40	35	p.(Ser102 Glnfs*19)	c.304delC	deletion	3%	16.9	102	NK	Y	CO, AK, PN
41	35	p.(Gly144 Val)	c.431G>T	missense	0%	NK	105	0.009	NK	NK
42	36	p.(Val281_ Thr282deli nsAla)	c.842_844del TAA	deletion	<24%	10.8	102	1.267	N	CO, AK, PN
43	37	p.(Ser148 Arg)	c.444T>G	missense	6%	13.7	101	0.359	N	CO, AK, PN
44	38	NK	NK	NK	<24%	35.5	135	1.615	N	CO, AK, PN
45	38	p.(Ser148 Arg)	c.444T>G	missense	6%	12	101	NA	N	CO, AK, MI,
46	40	p.(Asp153 del)	c.457_459del GAC	deletion	0%	NK	76	3.380	Y	LVH
47	40	p.(Arg112 Cys)	c.334C>T	missense	NK	4.8	79	0.230	N	CO, AK, LVH

48	40	p.(Arg301 Ter)	c.901C>T	nonsense	0%	19.2	133	0.462	N	CO, AK, GI
49	45	p.(Pro259 Arg)	c.776C>G	missense	3%	NK	104	0.027	NK	NK
50	45	p.(Ala156 Thr)	c.466G>A	missense	NK	NK	74	0.016	NK	NK
51	45	p.(Leu243 Phe)	c.729G>C	missense	0%	NK	104	0.008	NK	NK
52	46	NK	NK	NK	<24%	25.3	154	0.209	N	CO, AK, LVH, PN, GI
53	52	p.(Asp33 Gly)	c.98A>G	missense	0%	NK	83	0.016	NK	NK
54	53	p.(Trp44Cys)	c.132G>T	missense	NK	NK	75	0.090	Y	CO, AK, LVH, GI
55	60	p.(Asp322 Glu)	c.966C>G	missense	1%	NK	NK	NK	NK	NK

Abbreviations: AK=angiokeratoma; AR=arrhythmia; CO=corneal opacity; GFR= glomerular filtration rate in ml/min/1.73m^2; GI=gastrointestinal; LNL=lower normal limit; LVH: left ventricular hypertrophy; MI=myocardial infarction; NK=not known; PN=peripheral neuropathy; UPER=urine protein excretion rate, mg/g if urine protein/creatinine ratio or g/day if 24 hour collection. * Fabry disease diagnosis confirmed by clinical history of periodic lower extremity pain crises with and without fevers, angiokeratomas, cornea verticillata, mild proteinuria at age 14 years, kidney biopsy findings consistent with Fabry nephropathy in the absence of lysomotrophic medications, and a maternal uncle with acroparasthesias and cardiac disease who died prematurely at age 40 years.

Table 2. Clinical and renal structural characteristics of male 'classic' patients with Fabry disease whose total volume of GL3 inclusions per podocyte [V(Inc/PC)] is below or above the breakpoint (2009 μm^3) determined by piecewise linear regression analysis of relationship between the volume fraction of GL3 inclusions per podocyte [V(Inc/PC)] and number density of podocytes per glomerular volume [Nv(PC/glom)].

	V(Inc/PC) ≤ Breakpoint N=21[†]	V(Inc/PC) > Breakpoint N=13[†]	p-value
Age, years	30 ± 11	28 ± 11	0.64
GFR, ml/min/1.73m^2	110 ± 25	124 ± 30	0.15
UPER*	0.14 ± 0.14	0.39± 0.54	0.16
α-Gal-A activity**	0.07 ± 0.13 %	0.07 ± 0.06 %	0.98
Vv(PC/glom)	0.31 ± 0.05	0.35 ± 0.03	0.05
Nv(PC/glom)	0.000157 ± 0.000082	0.0000477 ± 0.000022	**0.0003**
Vv(Inc/PC)	0.41 ± 0.06	0.43 ± 0.06	0.47
V(Inc/PC), μm^3	990 ± 486	3514 ± 1415	**10^{-8}**[‡]
VPC, μm^3	2460 ± 1327	8326 ± 3238	**3×10^{-8}**
FPW, nm	703 ± 200	795 ± 271	0.28
Vv(Inc/Endo)	0.13 ±0.04	0.11 ± 0.03	0.27
Vv(Inc/Mes)	0.06 ± 0.03	0.05 ± 0.04	0.40

[†]Data for this analysis was available for 34 patients. UPER=Urine protein excretion rate, * mg/g if urine protein/creatinine ratio or g/day if 24 hour collection; ** % lower limit of normal range; Vv(PC/glom)=fractional volume of podocytes per glomerulus; Vv(Inc/PC)=fractional volume of inclusions per podocyte; [‡]By design; FPW=foot process width. Vv(Inc/Endo)=fractional volume

of inclusions per endothelial cell; Vv(Inc/Mes)=fractional volume of inclusions per mesangial cell.

Figure 1

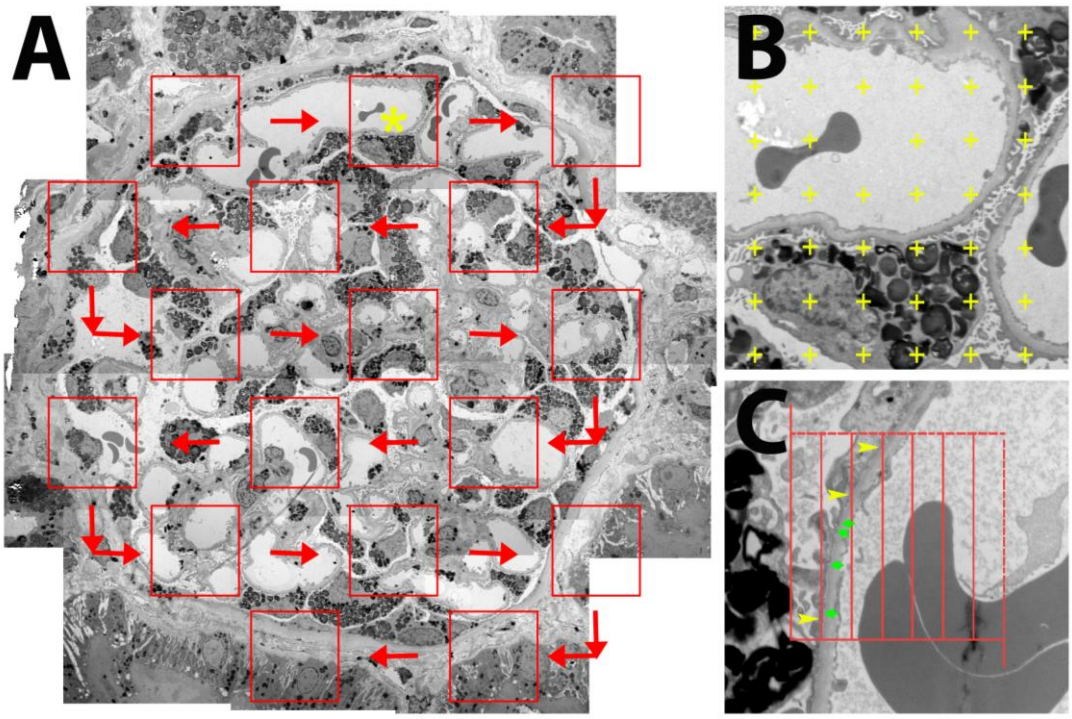

Figure 1. (A) Systematic uniform random sampling of a glomerular profile for stereological measurements from a patient with Fabry disease. Red boxes represent locations where higher magnification images are obtained by transmission electron microscopy. Red arrows show the path of sampling. Asterisk marks the box that is magnified in B (montage low magnification about 8,000x). **(B)** Magnified view of a portion of glomerular tuft with a superimposed point grid used for fractional volume estimation. **(C)** Higher magnification (~30,000x) image with an unbiased counting frame superimposed for estimation of foot process width based on the number of line intercepts with the glomerular basement membrane (yellow arrowheads) and number of slits (green arrows).

Figure 2

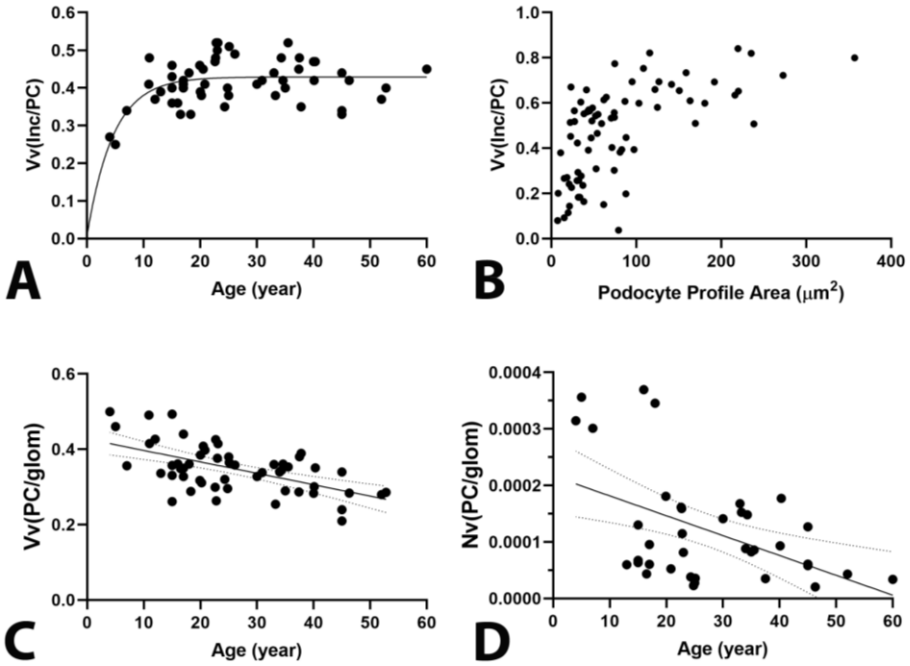

Figure 2. (A) Relationship between the podocyte inclusion volume fraction [Vv(Inc/PC)] and age. Regression line shows an exponential model with plateau. **(B**) Relationship between podocyte profile area and Vv(Inc/PC). **(C)** Inverse relationship between the fractional volume of podocytes per glomerulus [Vv(PC/glom)] and age (r=-0.54, p=0.0001). **(D)** Relationship between number density of podocytes per glomerulus [Nv(PC/glom)] and age. Solid lines = regression lines. Dashed lines = 95% confidence interval.

Figure 3

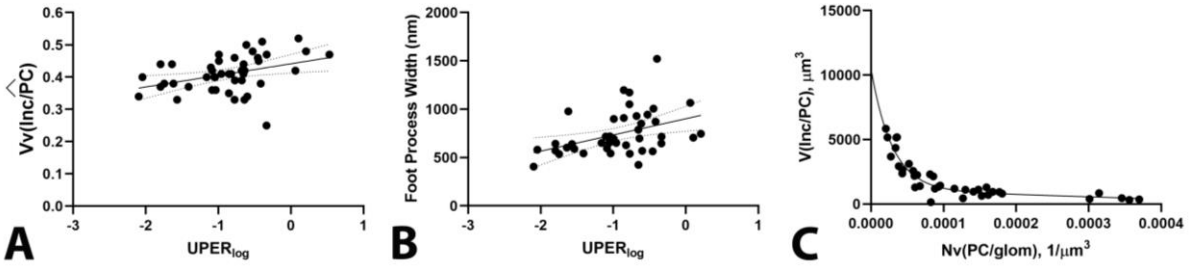

Figure 3. (A) Relationship between urine protein excretion rate (UPER; logarithmic scale, in mg/g if urine protein/creatinine ratio or g/day if 24 hour collection) and podocyte inclusion volume fraction [Vv(Inc/PC); R=0.44, p=0.003]. **(B)** Relationship between UPER (logarithmic scale) and podocyte foot process width (FPW), (R=0.41, p=0.007). **(C)** Relationship between number density of podocytes per glomerular volume [Nv(PC/glom)] and podocyte total inclusion volume. Regression line shows an exponential model with two-phase decay. Solid lines = regression lines. Dashed line = 95% confidence interval.

Figure 4

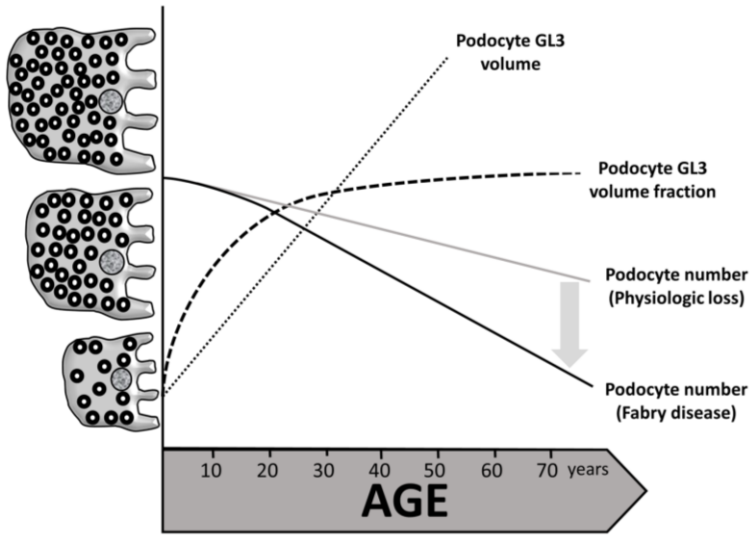

Figure 4. Relationships between aging and podocyte GL3 volume (dotted line), podocyte GL3 volume fraction (dashed line) and podocyte loss in Fabry disease (black bold line). The grey line represents physiologic podocyte loss with aging. Initially, the rate of GL3 accumulation is greater than the rate of podocyte enlargement, this leading to increasing podocyte GL3 volume fraction with increasing age up to age 25-30 years. Thereafter, podocyte GL3 volume fraction plateaus while GL3 accumulation continues in parallel with podocyte enlargement, and this is associated with podocyte loss from aging aggravated by additional podocyte loss from Fabry disease.