

BMJ Open Fabry disease due to D313Y and novel GLA mutations

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To cite: Koulousios K, Stylianou K, Pateinakis P, *et al.* Fabry disease due to D313Y and novel GLA mutations. *BMJ Open* 2017;7:e017098. doi:10.1136/bmjopen-2017-017098

► Prepublication history and additional material for this paper are available online. To view these files please visit the journal online (<http://dx.doi.org/10.1136/bmjopen-2017-017098>).

Received 23 April 2017
Revised 14 June 2017
Accepted 19 July 2017



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ABSTRACT

Objectives Our aim is to report four novel α -gal A gene (*GLA*) mutations resulting in Fabry disease (FD) and provide evidence of pathogenicity of the D313Y mutation regarding which contradictory data have been presented in the literature.

Setting and participants Twenty-five family members of nine unrelated patients with definite FD diagnosis, 10 clinically suspected cases and 18 members of their families were included in this polycentric cohort study.

Primary and secondary outcome measures Genotyping and measurement of lyso-Gb₃ was performed in all individuals. The α -Gal A activity was measured in all men as well as plasma and urine Gb₃ concentration in selected cases. Optical and electron microscopy was performed in kidney biopsies of selected patients. All the above were evaluated in parallel with the clinical data of the patients.

Results Fourteen new cases of FD were recognised, four of which were carrying already described *GLA* mutations. Four novel *GLA* mutations, namely c.835C>T, c.280T>A, c.924A>C and c.511G>A, resulting in a classic FD phenotype were identified. Moreover, FD was definitely diagnosed in five patients carrying the D313Y mutation. Eight D313Y carriers were presenting signs of FD despite not fulfilling the criteria of the disease, two had no FD signs and two others were apparently healthy.

Conclusions Four novel *GLA* pathogenic mutations are reported and evidence of pathogenicity of the D313Y mutation is provided. It seems that the D313Y mutation is related to a later-onset milder phenotype than the typical phenotype with normal lysoGb₃ concentration. Our study underlines the significance of family member genotyping and newborn screening to avoid misdiagnoses and crucial delays in diagnosis and treatment of the disease.

INTRODUCTION

FD or Anderson-Fabry disease is an X-linked inherited metabolic disorder that results from mutations in the α -gal A gene (*GLA* gene), leading to reduction of the enzyme activity and subsequent accumulation of Gb₃ (or GL-3) in plasma, urine and cellular lysosomes throughout the body. These depositions cause a multisystemic pathology with life-threatening manifestations, including renal failure, cardiac and cerebrovascular disease.^{1,2}

Strengths and limitations of this study

- This is the largest series in the literature of clinically evaluated male and female carriers of the α -gal A gene (*GLA*) D313Y mutation supporting its possible pathogenicity that occasionally has been proved by renal biopsy.
- Novel *GLA* mutations resulting in a classic Fabry disease phenotype are presented.
- The main limitation is the lack of detailed clinical data in older participants.
- Biopsies of affected organs, the gold standard of definite diagnosis, are not available in all cases.

More than 900 currently known *GLA* mutations have been identified^{3,4} as causing a variety of clinical manifestations. Most of them are unique to a family (private) and therefore genotype-phenotype correlation is limited.⁵ Diagnosing FD is challenging due to the range of disorders that mimic the disease and the great variety of atypical clinical presentations. As a result, underdiagnosis and misdiagnosis of FD lead to late diagnosis, delays in appropriate treatment and a subsequent negative prognosis.⁶ Human genetic analysis must be performed to exclude or verify a *GLA* mutation.⁷ Once a diagnosis has been made, biochemical and molecular genetic analysis, as well as genetic counselling, should be made available to all family members.⁸ A detailed pedigree analysis for each patient presenting with FD is crucial,⁹ as it can inform the diagnosis of the proband and the identification of all at-risk relatives.¹⁰

Our aim is to report four novel *GLA* mutations resulting in FD and provide evidence of pathogenicity of the D313Y mutation regarding which contradictory data have been presented in the literature.

METHODS

Study design and setting

This is a polycentric population screening study of individuals from Greece either

demonstrating phenotypic traits suggestive of FD or belonging to families of patients with a definite FD diagnosis and fulfilling any one of the following inclusion criteria:

- ▶ Definite diagnosis of FD.
- ▶ Nephrological, cardiac or neurological symptoms suspicious of FD.
- ▶ Relatives of patients with definite FD diagnosis.

A cohort of 62 subjects from 19 unrelated families were involved in the study. Twenty-five were relatives of 9 patients with definite FD diagnosis and 18 were relatives of 10 individuals recruited as carriers of FD traits in whom a *GLA* mutation was detected. The presence of the D313Y mutation in a Greek population was examined by genotyping of 145 apparently healthy subjects (70 men, 75 women). Written informed consent was obtained from each subject or an accompanying relative if legally appropriate. The study was approved by the institutional review board of the University of Thessaly, Larissa.

Clinical assessment

Patients' medical records were evaluated and a detailed medical history of the family members was obtained, especially regarding heart or kidney disease, cerebrovascular events, death at young age and respective causes of death. All study participants underwent a physical examination particularly focused on cardiac, renal and neurological signs and symptoms. A detailed pedigree was constructed for every family and newborn screening was performed.

Laboratory evaluation

In DBS, we measured α -Gal A activity in all male subjects using tandem mass spectrometry,¹¹ lyso-Gb₃ in all subjects using high-performance liquid chromatography and tandem spectrometry,¹² as well as plasma and urine Gb₃ concentration in selected cases using tandem mass spectrometry.¹³

Optical and electron microscopy was occasionally performed for the study of kidney biopsies.

Genotyping

Genomic DNA was extracted from peripheral blood using the iPrep Pure Link DNA blood kit (Invitrogen, Thermofisher, USA) according to the manufacturer's instructions. All coding regions and exon-intron splice junctions of the *GLA* gene were analysed in a targeted custom next-generation sequencing (NGS) platform (Ampliseq custom panel, Thermo Scientific). Analysis of primary data was conducted with Ion Reporter software v.5.2 (Thermo Scientific). Common polymorphisms (UCSC Common SNPs) were excluded and pathogenicity of variations was predicted by bioinformatic analysis using PhyloP, SIFT, Grantham and PolyPhen tools, in comparison to their global (1000 Genomes Global Minor Allele Frequency, ExAC) and European frequency (5000 Exomes European Minor Allele Frequency). The characterisation of variants was based on the recommendations of the American College of Medical Genetics and

Genomics (ACMG) and the Association for Molecular Pathology.¹⁴

Novel variants were verified by PCR in combination with Sanger sequencing. Amplification of *GLA* exons (including exon-intron boundaries) was performed in five reactions corresponding to exons 1, 2, 3, 4, and 5–7, using the primers included in the supplementary material. A total of 100–200 ng of genomic DNA was amplified by PCR in a 30 μ L reaction mixture using 200 μ M of each deoxynucleoside triphosphate, 30 pmol of each primer, 1.5 mM MgCl₂ and 1.0 U Taq polymerase (Invitrogen, Thermofisher, USA) in a 10x buffer supplied by the manufacturer. Reaction conditions were as follows: for exons 1–3: 94°C for 2 min, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, and a final extension at 72°C for 5 min. For exons 4 and 5–7: 94°C for 2 min, followed by 32 cycles of 30 s at 94°C, 30 s at 54°C, 30 s for exon 4 or 75 s for exons 5–7 at 72°C, and a final extension at 72°C for 5 min. All PCR reactions were carried out in the Veriti 96-Well Thermal Cycler (Applied Biosystems, Thermofisher, USA). PCR engine apparatus and the emerging PCR products were purified using the Pure-Link PCR Purification Kit system (ThermoFisher Scientific, USA). Sequencing was performed using the primers described in the supplementary table, using a 3730 DNA Analyzer (Applied Biosystems, Thermofisher, USA) and BigDye Terminator DNA sequencing kit (Applied Biosystems, Thermofisher, USA), according to manufacturer's instructions.

RESULTS

Nine (all with definite FD) out of the 62 genotyped subjects were carrying five previously described *GLA* mutations: c.334C>T (p.Arg112Cys, R112C), c.644A>G (p.Asn215Ser, N215S), c.1153A>C (p.Thr385Pro, T385P), c.453C>G (p.Tyr151Ter, Y151X) and c.782G>T (p.Gly261Val, G261V).

The c.937G>T (p.Asp313Tyr, D313Y, NM_000169.2) mutation was revealed in 17 individuals (54±14, range 27–78 years), 7 men (61±11, range 45–78 years) and 10 women (49±15, range 27–70 years) but in none of the healthy subjects. Patients' clinical and laboratory findings are presented in table 1. All male patients (61±11, range 45–78 years) presented with an α -gal A activity decreased in a range of 56.2–87.5% compared with normal, with the exception of one patient in whom α -gal A activity was within the normal range. LysoGb₃ concentration was found normal (range 0.8–1.7 ng/mL) in all patients, while plasma and urine Gb₃ concentration varied, as shown in table 1.

The diagnosis of FD was definitely posed according to the recently published criteria of the disease¹⁵ in five carriers of this mutation (54±9, range 45–65 years). The first (patient no. 1, table 1), a 52-year-old man, was initially diagnosed at the age of 48 with chronic kidney disease (CKD) stage III and was not presenting cardiac or other FD signs. Kidney biopsy performed because of

Table 1 Characteristics of patients carrying the D313Y mutation. Plasma and urine Gb₃ concentration was measured only in patients 1, 4 and 5

Patient/gender/age (years)	FD-related clinical findings	Previous diagnosis
1/M/52	End-stage renal disease – dialysis, acroparesthesias, renal cysts, elevated plasma and urine Gb ₃ concentration	FSGS nephropathy
2/F/30	Healthy	No
3/F/70	Healthy	No
4/F/46	Three strokes of unknown origin, WML on brain MRI, micro-albuminuria, oedema, acroparesthesias, elevated urine Gb ₃ concentration	No
5/F/65	Multiple WML on brain MRI, depression, elevated plasma Gb ₃ concentration	MS, RA, spastic quadriplegia
6/F/62	Acroparesthesias, GI symptoms since adolescence	No
7/M/45	End-stage renal disease on dialysis, WML and vertebrobasilar vessel changes on brain MRI, increased cardiac interventricular septum echogenicity, hearing loss of higher frequencies	Nephropathy of unknown origin
8/M/62	End-stage renal disease on dialysis	No
9/F/36	WML on brain MRI, acroparesthesias	MS
10/M/78	End-stage renal disease on dialysis	RA
11/F/47	LVH, CKD	No
12/M/68	No FD signs	Myopathy
13/F/46	No FD signs	NMO
14/F/27	WML on brain MRI	MS
15/M/61	End-stage renal disease on dialysis	Diabetic nephropathy
16/M/63	CKD, hearing loss, multiple ischaemic infarcts on brain MRI	no
17/F/59	Cornea verticillata, hearing loss, LVH, acroparesthesias, GI symptoms (pain – diarrhoea) since adolescence, hypohidrosis, T2-WML or ischaemic infarcts on brain MRI	no

non-nephrotic proteinuria, microscopic haematuria and raised serum creatinine, revealed focal and segmental glomerulosclerosis (FSGS) – collapsing variant. At that time, enzyme activity and plasma lyso-Gb₃ concentration were normal. Three years after the initial presentation the patient had end-stage renal disease and extreme acroparesthesias. Evaluation of the kidney biopsy using a higher magnification uncovered focal cytoplasmic microvacuolisation of enlarged podocytes (figure 1), with α -gal A activity decreased by 50% and increased plasma (7.52 nmol/mL, reference: 0.8–4.52) and urine Gb₃ concentration (147.49 nmol/g, reference: <29.00). The patient commenced dialysis and enzyme replacement therapy (ERT) with rapid clinical improvement.

A second carrier of the D313Y mutation was a 46-year-old woman (patient no. 4, table 1) who had had a transient ischaemic attack and two ischaemic strokes that had been considered of unknown origin. On evaluation, 1 year after the last stroke, the patient appeared with microalbuminuria, oedema and acroparesthesias on both hands. The microalbuminuria was duplicated after 3 months and acroparesthesias worsened. Gb₃ concentration was pathological in urine (54.08 nmol/g) but normal in plasma. LysoGb₃ concentration was normal at that time and remained stable for one more year.

The mother of the above patient (patient no. 5, table 1), 65 years old, had been diagnosed at the age of 50 with multiple sclerosis and was receiving relative medication, without clinical benefit. She was also receiving treatment for pain in the extremities that was attributed to rheumatoid arthritis. During recent years the patient had mobility impairment (reported as spastic quadriplegia after a neurological examination), depression and dementia. Evaluation of the patient revealed pathological plasma Gb₃ concentration (4.7 nmol/mL), white matter lesion (WML) on brain MRI and normal kidney function. LysoGb₃ concentration was normal and remained so during a 6-month follow-up.

Another carrier of the D313Y mutation was a 45-year-old man (patient no. 7, table 1) on dialysis due to CKD by the age of 25. No kidney biopsy was performed at that time. He reported episodes of haematuria during childhood and adolescence, attributed at that time to vesico-ureteral reflux. Enzyme activity was slightly decreased (2.4 μ mol/L/hour, reference: $\geq$ 2.6) and plasma lyso-Gb₃ concentration was normal. Brain MRI revealed WML and vertebrobasilar vessel changes. Moreover, the patient had increased echogenicity of the cardiac interventricular septum on the echocardiogram and sensorineural hearing loss of higher frequencies. The patient commenced ERT.

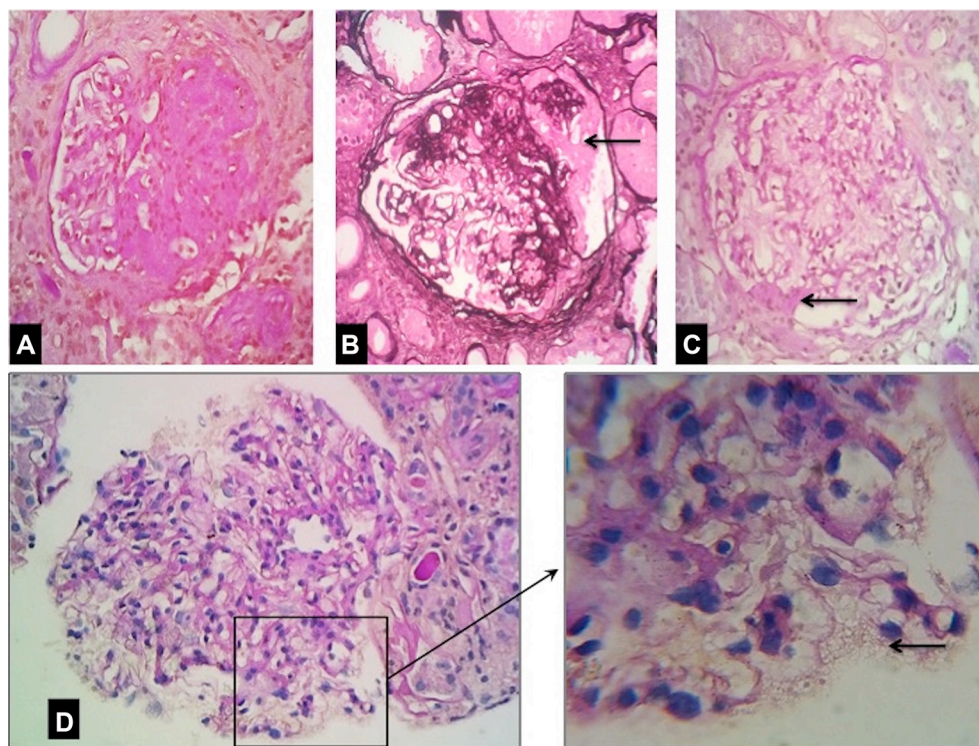


Figure 1 Optical microscopy findings of renal biopsy from a male patient with Fabry disease (FD) carrying the D313Y mutation of the *GLA*. (A) Glomerulus with segmental sclerosis – PAS ×400. (B) Segmental sclerosis with features of the 'collapsing' variant (arrow) – Jones' silver ×400. (C) Pale appearing glomerulus with a small area of sclerosis adhering to Bowman's capsule (arrow) – PAS ×400. (D) Cytoplasmic microvacuolisation of podocytes (arrow), suggestive of FD – PAS ×400.

The last patient with the D313Y mutation definitely diagnosed with FD was a 60-year-old woman (patient no. 17, [table 1](#)) who presented with cornea verticillata corneopathy, WML and ischaemic infarcts on brain MRI despite no stroke being reported, acroparesthesias and gastrointestinal (GI) symptoms (pain, diarrhoea) since adolescence, hypohidrosis, hearing loss and left ventricular hypertrophy (LVH).

Among the remaining 12 D313Y mutation carriers there were two patients with no FD signs and eight patients (55±17, range 27–78 years) presenting with other FD signs, mainly neurological; however, they cannot document a definite diagnosis of FD. Especially patient no. 6, [table 1](#) (62-year-old woman) had acroparesthesias and GI symptoms since adolescence and presented with lysoGb₃ at 1.7 ng/mL (reference: ≥1.8). Accordingly, patient no. 16, [table 1](#) (64-year-old man) presented with CKD, diabetes mellitus and hearing loss, while brain MRI showed multiple ischaemic infarcts despite no stroke being reported. The remaining two D313Y mutation carriers were apparently healthy.

Novel *GLA* mutations (4) ([figure 2](#)) were detected in 13 members (39±18, range 1–69 years) of four unrelated families ([table 2](#)), all fulfilling the diagnostic criteria of a definite diagnosis of FD [15]. The c.835C>T mutation (p.Gln279Ter, Q279X) in exon 6 of the *GLA* gene was identified in four members (30±24, range 1–60 years) of a Greek family. The proband, a 31-year-old man, presented at the age of 23 with proteinuria (3.5 g/24 hours),

microscopic haematuria, slightly deteriorated kidney function, right bundle branch block, mild hypertension and angiokeratomas in the arms and the loin area. He reported pain in the extremities, especially during infections, and inability to sweat. The α-gal A activity was close to zero. Cardiac MRI showed moderate LVH. The kidney biopsy showed cytoplasmic vacuolization and extended lysosomal accumulations in all types of kidney cells, especially in the podocytes ([figure 3](#)). The patient is currently under ERT with beneficial results in regard with kidney function, proteinuria and pain. Three other members of the family were revealed having with the same mutation and all presented clinical signs of FD. The proband's mother (60 years old) reported a possible TIA at the age of 32. Kidney examination showed albuminuria (>500 mg/24 hours), microscopic haematuria and normal kidney function. Cardiac MRI revealed severe LVH (cardiac interventricular septum over 19 mm) and on skin examination she showed angiokeratomas in the arms. Her mother (the proband's grandmother) had died at the age of 62, due to cardiac arrest. She had severe LVH and acroparesthesias, which at that time were attributed to Raynaud's phenomenon. The proband's sister (27 years old) had elevated lyso-Gb₃ concentration (2.7 ng/mL) and screening of her newborn daughter revealed the mutation too.

Four patients (46±11, range 30–56 years) belonging to another Greek family were carrying the c.280T>A (p.Cys94Ser, C94S) mutation. The proband, a 48-year-old

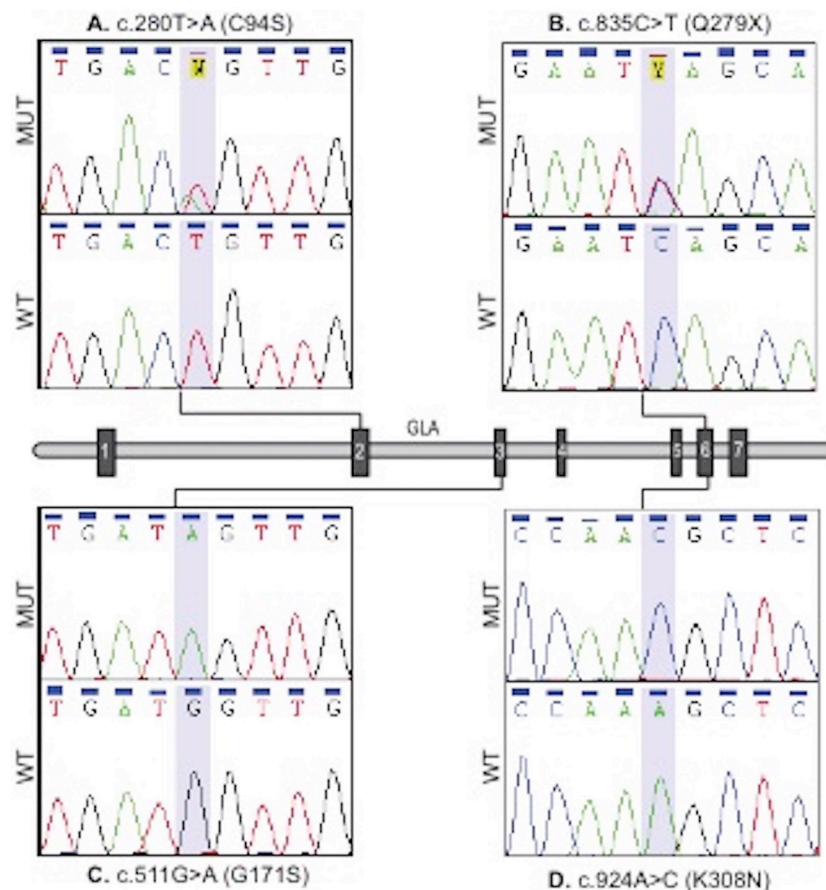


Figure 2 Sanger confirmation of novel mutations.

woman, was diagnosed with FD a year before after presenting with increased lyso-Gb₃ concentration (5.4 ng/mL), increased plasma Gb₃ concentration (6.14 nmol/mL), microalbuminuria of no other origin, cornea verticillata corneopathy, acroparesthesias in both hands and dyshidrosis. Clinical and laboratory data of the other affected members of the family are presented in table 2.

The c.924A>C mutation (K308N – p.Lys308Asn) in exon 6 of the *GLA* gene was identified in three members of a Greek family (44±26, range 17–69 years). The proband, a 46-year-old man, presented at the age of

34 with albuminuria (0.5–0.6 g/24 hours), had microscopic haematuria for 10 years and slight hypertension. The kidney function was normal. Kidney biopsy showed slight mesangial proliferative damage and cytoplasmic microvacuolisation of podocytes. The α-gal A activity was almost zero. After a 10-year follow-up he had LVH and proteinuria (1.8 g/24 hours). The patient is currently under ERT. His mother and daughter carry the same mutation. The probands grandmother had died at the age of 68, with severe LVH and end-stage heart failure.

Table 2 Clinical characteristics of patients with the four novel mutations

Mutation (NM_000169.2)	Clinical data
c.835C>T p.Gln279Ter Q279X	CKD, haematuria, proteinuria, pain in extremities, dyshidrosis, angiokeratomas, zero activity of a-gal A, LVH, kidney biopsy consistent with FD, TIA, pathological elevated lyso-Gb ₃
c.280T>A p.Cys94Ser C94S	Microalbuminuria, end-stage renal disease – dialysis, cornea verticillata corneopathy, acroparesthesias, dyshidrosis, LVH, WML on brain MRI, zero activity of a-gal A, pathological elevated lyso-Gb ₃ , pathological elevated Gb ₃ in plasma/urine
c.924A>C p.Lys308Asn K308N	CKD, haematuria, kidney biopsy findings related to FD, zero activity of a-gal A, LVH
c.511G>A p.Gly171Ser G171S	Proteinuria, end-stage renal disease – dialysis, LVH, valvulopathy, cornea verticillata corneopathy, extremely low activity of a-gal A, pathological elevated lyso-Gb ₃

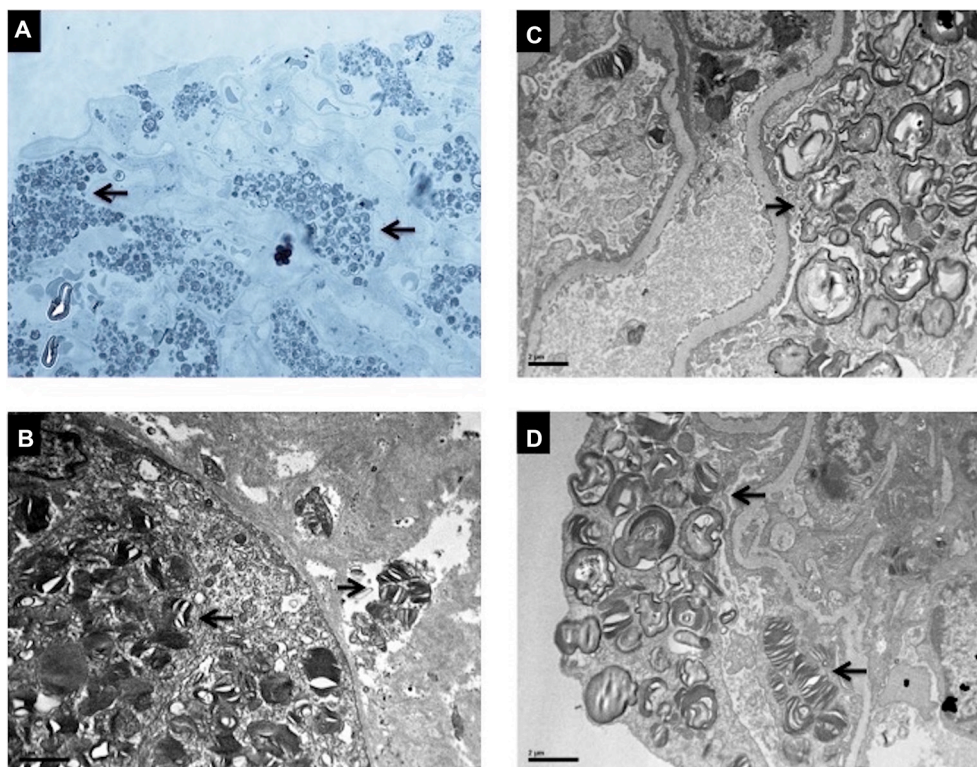


Figure 3 Electron microscopy findings of renal biopsy from a male patient with Fabry disease (FD) carrying the Q279X mutation. Multi-lamellated myelin figures ('zebra' bodies), a typical finding of FD, are marked with black arrows in (A) methylene blue semithin section, (B) tubular cells and a fibroblast and (C,D) podocytes.

Lastly, the c.511G>A mutation (G171S – p.Gly171Ser) in exon 3 of the *GLA* gene was identified in two members (37±4, range 34–39 years) of an Albanian family living in Greece. The proband, a 39-year-old man, was diagnosed at the age of 32 with severely deteriorated kidney function and proteinuria. No biopsy was performed at that time due to the small size of the kidneys. After nearly a year he presented with severe clinical and laboratory findings of acute renal failure and needed dialysis. Normal kidney function was never restored. FD was definitely diagnosed at the age of 37, as the α -gal A activity was extremely low and lyso-Gb₃ concentration was 11.9 ng/mL. The patient had LVH, increased pulmonary artery diameter, dilatation of the ascending aorta and aortic valve stenosis, due to which he underwent a valve replacement surgery. Ophthalmological evaluation indicated lipid deposition with blurriness of the cornea. The patient is currently under ERT. His 34-year-old brother was identified with the same mutation and has extremely low α -gal A activity, lyso-Gb₃ concentration 12.9 ng/mL and albuminuria of no other aetiology.

DISCUSSION

The D313Y mutation

Contradicting results about the pathogenicity of this mutation have been reported in the literature since its first description in 1993.¹⁶ The mutation has been detected in many series of patients presenting signs of FD.^{17–24} However, Niemann *et al*²⁵ describes this variant

as non-pathogenic, although his two patients were presenting with decreased α -gal A activity. Similarly, Oder *et al*²⁶ support that the D313Y genotype does not lead to severe organ manifestations as seen in genotypes known to be causal for classical FD and Froissart *et al*²⁷ characterise the mutation as a 'pseudodeficient allele', implying that it is a sequence variant which encodes an enzyme that is transported to the lysosomes, where it has about 75% of normal enzymatic activity. The D313Y mutation has also been referred to as polymorphism,¹⁹ despite the fact that, according to the ExAC and 1000 Genomes databases, its frequency in the World and European population is below 1%. Finally, it must be mentioned that bioinformatics analysis shows that this mutation is predicted as probably damaging (PolyPhen-2) or damaging (SIFT).

In our study, 5 of 17 carriers of the D313Y mutation (54±9, range 45–65 years) proved to have definite FD according to the recently published criteria of the disease.¹⁵ The presentation of the disease in our patients indicates that the mutation results in a milder phenotype, with later onset of symptoms. This phenotype, also including milder mono- or oligosymptomatic cases,¹⁹ is characterised as atypical or type 2.²⁸ The late onset of clinical symptoms and the milder than the typical FD phenotype in these patients can be partly explained by the high α -gal A residual activity, since there is evidence that the mutated α -gal A reaches the lysosomes intracellularly.²⁹ Actually, in all but one male D313Y carrier of our study, α -gal A activity was decreased. Another reason for the

decreased activity of the D313Y enzyme in plasma could be a functional intolerance to blood plasma neutral pH conditions. This effect is irreversible and, once in contact with a neutral or basic pH environment, the D313Y enzyme remains inactive, even if transferred to optimal pH.^{21 29}

Interestingly, the vast majority of our patients with D313Y presented with neurological symptoms and signs. Moreover, one of them was misdiagnosed with MS while two carriers of the mutation, in whom a definite FD diagnosis had not been established yet, were diagnosed with MS and treated accordingly. Similarly the detailed study of the renal biopsy in one of our cases underlies the significance of early detection of Fabry-specific findings in cases with FSGS.

Novel mutations

The pathogenicity of the above-mentioned novel mutations has been undoubtedly proved as all carriers had definite FD. Of these carriers, the c.835C>T (p.Gln279Ter, Q279X) is a nonsense mutation causing an interruption of the reading frame by a premature stop codon, which results in a truncated protein. A truncated protein and the subsequent loss of its functionality is strong evidence that the mutation is probably pathogenic for FD [14]. No amino acid change at this position has ever been described in the NCBI and Fabry Database (<http://fabry-database.org/>).³⁰

As far as the c.280T>A (C94S), c.924A>C (K308N), c.511G>A (G171S) mutations are concerned, bioinformatics analysis supports their disease-causing effect as they are predicted as probably damaging (PolyPhen-2) and/or damaging (SIFT). Further evidence of their pathogenicity could be the fact that the same aminoacid changes at the corresponding positions but in different nucleotides have already been described as disease causing.^{31–33}

CONCLUSIONS

We report four novel *GLA* mutations causing FD. Moreover, we offer strong evidence that the D313Y mutation could be pathogenic. It seems that this mutation is related to a later-onset milder phenotype than the typical phenotype with normal lysoGb₃.

Additionally our study underlines the significance of family member genotyping, newborn screening and genetic counselling in avoiding misdiagnoses and crucial delays in diagnosis and treatment of the disease. Finally, we confirm the fact that heterozygous women may develop mild to severe FD and that the genotype–phenotype correlation does not exist even among members of the same families.

Acknowledgements We appreciate the help and advice from A Andrikos, S Kouzouka, A Triantafyllou, D Kaltsidou, A Kallantzoglou, A Lysitska, K Papadopoulos, K Zoganas, G Tsigoulis, Z Tegou, A Fountoglou, T Karapanagiotidis, S Patsialis.

Contributors KK: collection, analysis and interpretation of the data, pedigree analysis, literature review, drafting and revision of the manuscript. KS: renal biopsy and analysis on electron microscope. PP: renal biopsy and analysis on optical

microscope. MS, MZ and GL: genotyping and bioinformatics analysis. KS, PP, EM, PK, CK, AO, JK: treating physicians of patients. AEG: design and coordination of the study, revision of the manuscript. All authors approved the final version of the manuscript.

Funding The study has been partially supported by a grant from the Research Committee of the University of Thessaly.

Competing interests KK received travel assistance from Shire and Genzyme and speaker's honoraria from Shire. KS received travel assistance and speaker's honoraria from Shire. PP and AO received travel assistance from Genzyme and Shire. CK received travel assistance from Shire. AEG received research grants from Shire. The authors MZ, GL, MS, EM, PK, JK, report no competing interests.

Ethics approval This report was approved by the institutional review board of the University of Thessaly, Larissa. Written informed consents for participation were obtained from patients.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement All data supporting our findings are included in the manuscript.

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Correction: *Fabry disease due to D313Y and novel GLA mutations*

Koulousios K, Stylianou K, Pateinakis P, *et al.* Fabry disease due to D313Y and novel GLA mutations. *BMJ Open* 2017;7:e017098. doi:10.1136/bmjopen-2017-017098.

The footnotes should include the following statement:

Patient consent Written consent to publish these case reports has been obtained from the patient or next of kin.

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BMJ Open 2018;8:e017098corr1. doi:10.1136/bmjopen-2017-017098corr1

