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Original Article 1

Genetic insights and therapeutic implications in Egyptian patients with Fabry Disease

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Background
Fabry disease (FD) is an X-linked lysosomal storage disorder resulting from pathogenic variants in the α-galactosidase (*GLA*) gene, leading to a deficiency in "α-galactosidase A" *(GLA)* activity. This enzyme deficiency leads to progressive accumulation of glycosphingo lipids in various tissues and organs.

Objectives
This study aims to conduct clinical, biochemical, and molecular diagnosis for three unrelated Egyptian families with Fabry disease.

Patients and methods
The *GLA* enzyme activity was measured in 3 males from 3 unrelated families. The *GLA* activity was measured by using the fluorogenic substrate "4-methylumbelliferyl-α-D-galactopyranoside" and the diagnosis of FD was established by *GLA* deficiency. The patients presented with variable renal, cardiac, ocular and dermatological manifestations. History and pedigree were done for 3 families. Molecular analysis of *GLA* gene was performed for 3 patients, the mother, two daughters and the sister of P1, the mother, wife and daughter of P2, and the mother, brother, and cousin of P3. Genetic tests by PCR of the gene-coding region followed by Sanger sequencing.

Results
We revealed 3 previously reported *GLA* variants in 3 Fabry families. Family 1, a nonsense variant c.627G>A, p.W209* was identified in patient 1 and in one of the patient's daughters. In Family 2, a splice-site variant c.370-2A>G was detected in patient 2 and in his mother and daughter. In Family 3, a missense variant c.334C>T, p.R112C was detected in patient 3.

Conclusion
This study expands the molecular genetic spectrum of the *GLA* gene associated with Egyptian FD patients. Our findings highlighted the importance of early diagnosis, proper genetic counseling and therapy for FD patients. To the best of our knowledge, the present study is the first to delineate the *GLA* variant profile in Egyptian FD patients. The patients are currently receiving Enzyme Replacement Therapy (ERT) at the National Research Centre (NRC).

Keywords : Fabry disease, *GLA* gene, α-Galactosidase A, X-linked lysosomal storage disorder, Enzyme Replacement Therapy.

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Introduction

Fabry disease (FD) is a rare X-linked lysosomal storage disorder due to deficient lysosomal enzyme "α-galactosidase A (α-Gal A)" that results from pathogenic variants in the α-galactosidase A (*GLA*) gene. The α-Gal A enzyme deficiency leads to progressive lysosomal accumulation of "glycosphingolipids" such as "globotriaosylceramide (Gb3)" and its derivative, "globotriaosyl sphingosine (lyso-Gb3)" within the lysosomes of all body tissues and organs, including cardiac muscle, ganglion cells of the nervous system, renal glomeruli and tubules, endothelial cells, smooth muscle cells, and corneal epithelial cells [1–3].

Fabry disease has a reported incidence of 1:40,000-1:170,000 worldwide [4,5]. In some of the Gulf countries, the prevalence of classical FD showed notable variability; in the United Arab Emirates (UAE) prevalence was estimated to be around 0.25 per 100,000 live births [5,6], while in the Eastern province of Saudi Arabia, a prevalence of 42.2 per

100,000 live births was reported [5,7]. A recent study reported the prevalence of FD among Saudi patients on haemodialysis in three large hospitals as 4.8 per 1000 [5,8].

The *GLA* gene encodes for the *GLA* enzyme, it is located on the long arm of the X chromosome, Xq22, spanning around 13 kb, and has seven exons. The *GLA* coding region consists of 1290 bp and is translated to a polypeptide protein of 429 amino acids with the signal sequence represented by the first 31 residues [9]. More than 1000 *GLA* pathogenic variants were reported, involving point variants "nonsense, missense, and splice site", and disease-causing short length rearrangements, mainly duplications and deletions affecting less than 65 nucleotides" [3,9].

In classical Fabry disease, or early onset FD, the patients are severely affected and have reduced or absent α-Gal A activity. The clinical manifestations appear in childhood in association with exercise or heat intolerance, involving acroparesthesias, gastrointestinal symptoms, anhidrosis, angiokeratoma, cornea verticillata, and microalbuminuria. Long-term FD clinical manifestations are characterized by progressive renal failure, proteinuria, hypertrophic cardiomyopathy, and stroke. Conversely, in non-classical Fabry disease, or late-onset FD, the patients have residual α-Gal A activity and are less severely affected, with the clinical manifestations mainly limited to a single affected organ, such as the heart [2]. However, even if the symptoms start in childhood, there is a significant delay in the diagnosis up to 20 years after the onset of symptoms [1,10–12].

The diagnosis of patients with Fabry disease is challenging, resulting in misdiagnosis and consequent diagnostic delay [3]. Challenges for diagnosing FD patients include; a lack of specific clinical manifestations of this multisystemic disorder, heterogeneity, little awareness of the disease, and the fact that the diagnostic laboratory tests are done only in specialized centers. Using "high-risk screening" approaches based on involving patients who express at least one phenotypic symptom of the disease is suggested to improve the diagnosis of FD [9,13].

The present study aims to genetically diagnose Egyptian families who presented with manifestations of FD, and to start their Enzyme Replacement Therapy (ERT), in an attempt to avoid irreversible complications. Enzyme replacement therapy (ERT) helps in slowing down the disease progression and decreasing the mortality rate.

Patients and methods

This study included three adult Fabry patients and their available family members. The study was approved by the Medical Research Ethics Committee at the National Research Centre (Approval no. 044101223). Written informed consent was obtained from the patients and their included family members.

Clinical assessment

Patients' medical records were evaluated and a detailed medical history of the family members was obtained, especially for heart or kidney disease, cerebrovascular events, death at a young age and the respective causes of death. A detailed pedigree was constructed for the families. General examination was performed at the time of presentation.

Biochemical analysis

Analysis of Fabry disease can be performed in males by measuring the deficiency of "α-galactosidase A" activity in leukocytes. However, the enzymatic assay is unreliable for the detection of females, which can be detected accurately by molecular analysis. The *GLA* activity in leukocytes was determined in the three male patients and the sister's sons of patient 1, using the fluorogenic substrate, 4-methylumbelliferyl-α-D-galactopyranoside, as described by Desnick et al. [14]. For measurement of α-galactosidase A (*GLA*) activity in leukocytes, leukocytes were isolated from peripheral blood according to Skoog and Beck [15]. A mixture of 10 µl homogenized leukocyte with 40 µl of "4-methylumbelliferyl α-D-galactopyranoside" as the substrate solution was incubated at 37° C for half an hour. 950 µl of glycine buffer, pH 10.7, was added to stop the reaction. The end product "4-methylumbelliferone (MU)" was measured by a spectrofluorometer at emission and excitation wavelengths of 450 nm and 365 nm, respectively [16]. Before initiating enzymatic analysis, protein content was measured according to Lowry et al., against "bovine serum albumin (BSA)" standard [11].

Molecular analysis

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) extraction

Peripheral blood samples of the three Fabry patients and eleven participating relatives were obtained. Total RNA was extracted from the leukocytes of the withdrawn blood leukocytes by the "QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany)" according to the manufacturer's protocol. DNA extraction, from patients` blood lymphocytes, was done using the salting-out protocol [17].

α-galactosidase A (*GLA)* variantal analysis by complementary deoxyribonucleic acid (cDNA) and genomic sequencing approach

Reverse transcription was performed using 0.5 ug of extracted RNA and random hexamers in

accordance with the instructions of the manufacturer protocol, "High-Capacity cDNA Reverse Transcription Kit" (Thermo Fisher Scientific Inc.). Amplification of the *GLA* gene entire coding region "GenBank Ref Seq no. NG_007119.1" was done in three overlapped fragments. Designed primer pairs were aligned with the *GLA* gene reference sequence "GenBank Ref Seq no. NG_007119.1, NM_000169.3; Ensembl transcript ID ENST00000218516.4".

Genomic DNA was amplified in seven fragments of the *GLA* gene coding regions and exon-intron splice junctions using a designed primer pair. "Primer pair sequences are available upon request". Primers quality was checked using NetPrimer software, and the predicted PCR product specificity was examined against the reference database by NCBI nucleotide blast software. A typical PCR reaction of 2 µl cDNA or 100 ng genomic DNA, 25 pmol of each primer, 0.2 mmol/l dNTPs, 1.0 mmol/l $MgCl_2$**,** 1x ammonium sulphate reaction buffer, and 0.5µl (2.5 units) DNA polymerase "Fermentas, EU, Thermo Scientific" was set**.** The PCR cycling conditions were initial denaturation at 95 °C for 8 min, and 35 cycles of denaturation at 95 °C for 1 min, annealing for 1 min at 58 – 64 °C, extension for 1 min at 72 °C, and an additional extension for 10 min at 72 °C**.** The PCR products from both cDNA and genomic DNA were purified using the "QIA quick PCR purification kit" (Qiagen, Hilden, Germany), then sequenced in both directions using the "Big Dye Termination Kit" (Applied Biosystems, Foster City**,** California, USA), and analyzed on the "ABI Prism 310 Genetic Analyzer" (Applied Biosystems), in accordance with the instructions of the manufacturer**.** Detected variants were assigned following ACMG guidelines against the "NM_000169.3" reference sequence.

Database and *In-Silico* analyses

Querying the identified variants by browsing through several databases, including the "LOVD (Leiden Open Variation Database)" [18], "Human Gene mutation Database (HGMD)" ("HGMD® home page," 2023) [19] and "NCBI dbSNP (database of single nucleotide polymorphisms, ClinVar)" [20]. Identified variants frequency was compared against the reported general population frequencies on "1000 genomes" ("IGSR | samples," 2023) [1], the gnomAD ("GLA | gnomAD," 2023) databases [10], and Varsome database [21].

Results

Subjects from three unrelated consanguineous Egyptian families with Fabry disease were included in this study. The first family has an affected 33 years old male (F1/III-4), his mother (F1/II-10), and his two daughters (F1/IV-3 and F1/IV-4), who were apparently normal (Fig. 1). The second family has an affected 35 years old male (F2/III-2), his 70 years old mother (F2/II-5) with renal impairment, and his one and half years old daughter (F2/IV-1) (Fig. 2). The third family included an affected 27 years old male (F3/IV-9), his mother (F3/III-15), and his brother (F3/IV-10), who were apparently normal (Fig. 3).

Clinical report

Family #1

The proband is a 33- year- old male (F1/III-4), the second child of consanguineous parents. The condition started at the age of 12 years with neuropathic pain in the extremities (acroparathesia) as well as a skin rash in the lower extremities. The patient presented with myocardial hypertrophy, proteinuria, and renal failure that was diagnosed at the age of 25 years. He was on renal dialysis for three years, followed by kidney transplantation. He experienced frequent attacks of gastrointestinal disturbance in the form of diarrhea, flatulence, urgency, and abdominal pain for the last 10 years. His mother (F1/II-10) was the donor of the transplanted kidney at the age of 28 years. The wife of the patient's older brother (his cousin) (F1/III-2) died from heart problems at the age of 35 as did her father (his uncle) (F1/II-7), who died of the same reason at the age of 55 years (Fig. 1). The patient's mother (50 years old) and his three and five years old daughters (F1/IV-3 and F1/IV-4) did not display any relevant symptoms.

Clinical assessment of the proband (F1/III-4) at presentation revealed an average mentality, no dysmorphic features, hypertension, and a large number of angiokeratomous lesions all over the trunk and lower extremities. Anthropometric measurements were normal; height was 170 cm (Z score was -0.92 SD), weight was 75 kg (Z score was +0.37 SD), and head circumference was 57 cm (Z score was +1.32 SD).

Family#2

The proband is a 35- year- old male (F2/III-2), the offspring of second-degree consanguineous parents. The condition started with severe visual impairment at the age of 6 years that was treated by the implantation of an intraocular lens at the age of 12 years. At the age of 17 years, the patient complained of acroparathesia; paresthesia and numbness in the hands and feet with no sweating. In addition, he experienced intermittent, unexplained headache attacks. The patient suffered from muscle cramps at the age of 25 years. He developed hypertension, renal function impairment, cherry skin granules, enlarged heart muscle and body asthenia at the age of 33 years. Additionally, he experienced bilateral hearing impairment, weak leg muscles, and intolerance to effort. The patient

has poor appetite, gait imbalance**,** and teeth problems. His mother (70 years old) (F2/II-7) has renal function impairment. His younger brother (25 years old) (F2/III-5) suffers from heart problems. His cousin (17 years old) (F2/III-8) has paresthesia and numbness in the extremities (hands and feet). His four uncles died; the youngest (F2/II-4) uncle died at the age of 29 years with heart failure. The oldest uncle (F2/II-1) died at the age of 50 years with a kidney problem and the other two uncles (F2/II-2 and F2/II-3) died at the ages of 37 and 42 years with renal failure (Fig. 2).

Clinical assessment at presentation revealed an average mentality, hypertension, no dysmorphic features, and scattered angio-keratomtous lesions mainly over the back and both arms. Anthropometric measurements were normal; height was 187 cm (Z score was +1.47 SD), weight was 76 kg (Z score was +0.43 SD), and head circumference was 56 cm (Z score was +0.52 SD).

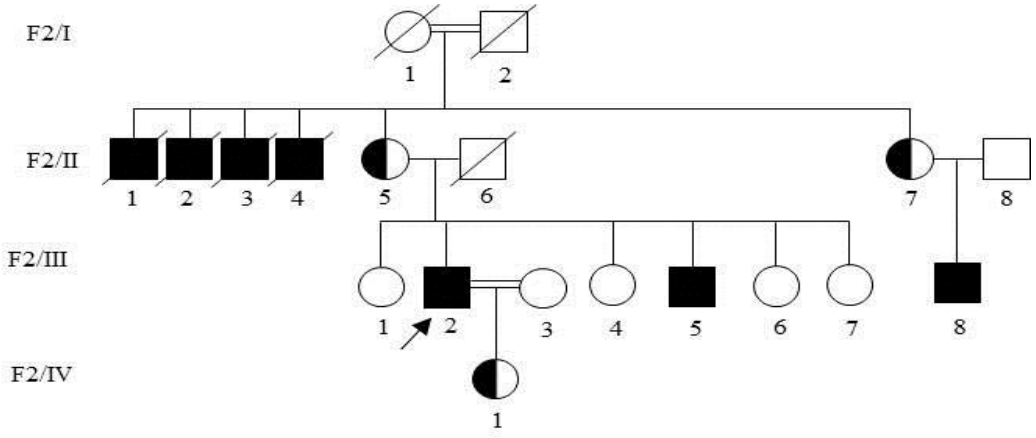

Fig. 1 Family#1 Pedigree. The pedigree represents affected male (F1/III-4), a heterozygous Female (F1/IV-4), and suspected heterozygous females (F1/II-10, F1/III-2, and F1/IV-3). White color rectangles and circles indicate apparently normal family members, while black color indicates affected cases.
(Suspected heterozygous females not confirmed by molecular analysis)

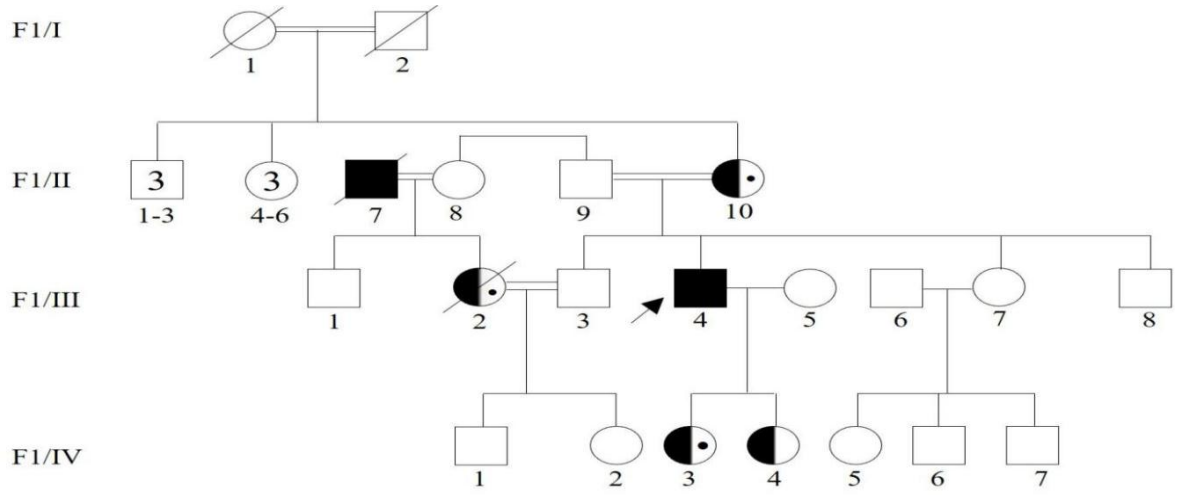

Fig. 2 Family#2 Pedigree. The pedigree represents affected males (F2/III-2, F2/III-5, and F2/III-8), heterozygous Females (F2/II-5, F2/II-7, and F2/IV-1). White color rectangles and circles indicate apparently normal family members, while black color indicates affected cases.

Family #3

The proband is a 27- year- old male (F3/IV-9), the offspring of a first-degree consanguineous parent. The patient presented with a history of neuropathic pain in the extremities (acroparesthesia) with no sweating since the age of 13 years, recurrent attacks of headache, hypertension and cherry red granules in the abdomen. Impairment of renal functions and enlarged heart muscle were noted at 26 years of age. The patient's mother (F3/III-15) (50 years old) did not display any relevant symptoms as well as his brother (F3/IV-10). The patient's cousin (F3/IV-13) (18 years old) suffers from heart problems, atrophy in one kidney, and enlargement in the other kidney, and his other cousin (F3/IV-14) (14 years old) has paresthesia and numbness in her extremities (hands). The patient's aunt (F3/III-1) (62 years old) has partial nephrectomy (had one

kidney removed). The patient has three uncles F3/II-8), (F3/II-9), and (F3/II-10), who died of renal impairment (Fig. 3)**.**

Clinical examination revealed an average mentality and minimal angio-keratomtous skin lesions over the trunk. Anthropometric measurements were normal; height was 182 cm (Z score was +0.76 SD), weight was 72 kg (Z score was +0.18 SD), and head circumference was 55 cm (Z score was -0.07 SD).

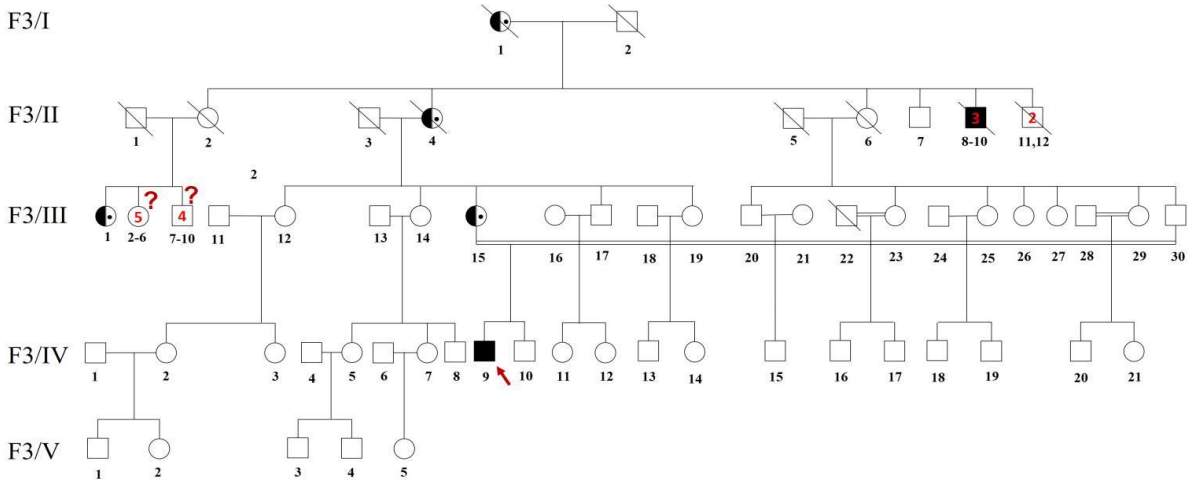

Fig. 3 Family#3 Pedigree. The pedigree represents affected male (F3/IV-9), suspected affected males (F3/II-8, F3/II-9, and F3/II-10) and suspected heterozygous Females (F3/I-1, F3/II-4, F3/III-1, F3/III-15). White color rectanglesr and circles indicate apparently normal family members, while black color indicates affected cases.

- (Suspected heterozygous females not confirmed by molecular analysis)

? Have no clinical data

Biochemical findings

The three affected unrelated males from the three families showed deficient (α-Gal A) activity that confirmed the diagnosis of Fabry disease, while the other two males of the first family (patient's 1 sister's sons) showed normal (α-Gal A) activity as well as six relative males and one female of the third family (Table 1).

Table 1 Represents age, gender, and biochemical findings in the three affected Fabry families.

Patient no.	Gender	Status	Age at study (years)	Age at diagnosis (years)	GLA level (µmol/l/h) "the reference range of GLA activity (10-45 µmol/l/h)"
F1/III-4	Male	Affected	33	12	1.2
FI/IV-6	Male	Normal	7	-	23
FI/IV-7	Male	Normal	2	-	16.4
F2/III-2	Male	Affected	35	7	1.9
F3/IV-8	Male	Normal	19	-	16.4
F3/IV-9	Male	Affected	27	13	0.52
F3/IV-10	Male	Normal	19	-	15.5
F3/IV-13	Male	Normal	18	-	28.5
F3/V-1	Male	Normal	2	-	20.6
F3/V-3	Male	Normal	2	-	11.35
F3/V-4	Male	Normal	1/2	-	13

Molecular results

The Sanger sequencing revealed a nonsense hemizygous variant NM_000169.3: c.627G>A, p.W209* in exons 4 of the *GLA* gene in the affected male patient of the first family. One of his two daughters showed the same variant in a heterozygous status, however both his mother and the other daughter showed homozygous wild allele inheritance, and the defected allele could not be detected in their blood DNA samples (Table 2, Fig. 4).

The nonsense c.627G>A, p.W209* variant was reported in publicly available mutational analysis databases (rs869312350) (14,18,22,23) as a likely pathogenic variant on Chrx at position 101400678 with a pathogenicity score of 2 and a conservation score of 0.941. This variant was not detected in exomes, gnomAD, or 1000 genome databases (Varsome.org) [1,21].

Sanger sequencing in family 2, revealed an intronic NM_000169.3, c.370-2A>G variant, detected in the splice-site region at intron 2 of the *GLA* cDNA of the *GLA* gene, in the affected male patient in a hemizygous form and heterozygous in his daughter (Fig. 5). In the current study, the splice site NM_000169.3, c.370-2A>G variant, leads to the skipping of exons 3 and 4 from the *GLA* gene transcript by removing the region from T391 to C662 (271 pb) (Table 2, Fig. 6).

The splice site NM_000169.3, c.370-2A>G variant was reported in publicly available mutational analysis databases (rs730880444) (14,18,22,23) as a pathogenic variant on Chrx at position 100656799 with a pathogenicity score of 5 and a conservation score of 7.91. This variant was not detected in exomes, gnomAD, or 1000 genome databases (Varsome.org) [1,21].

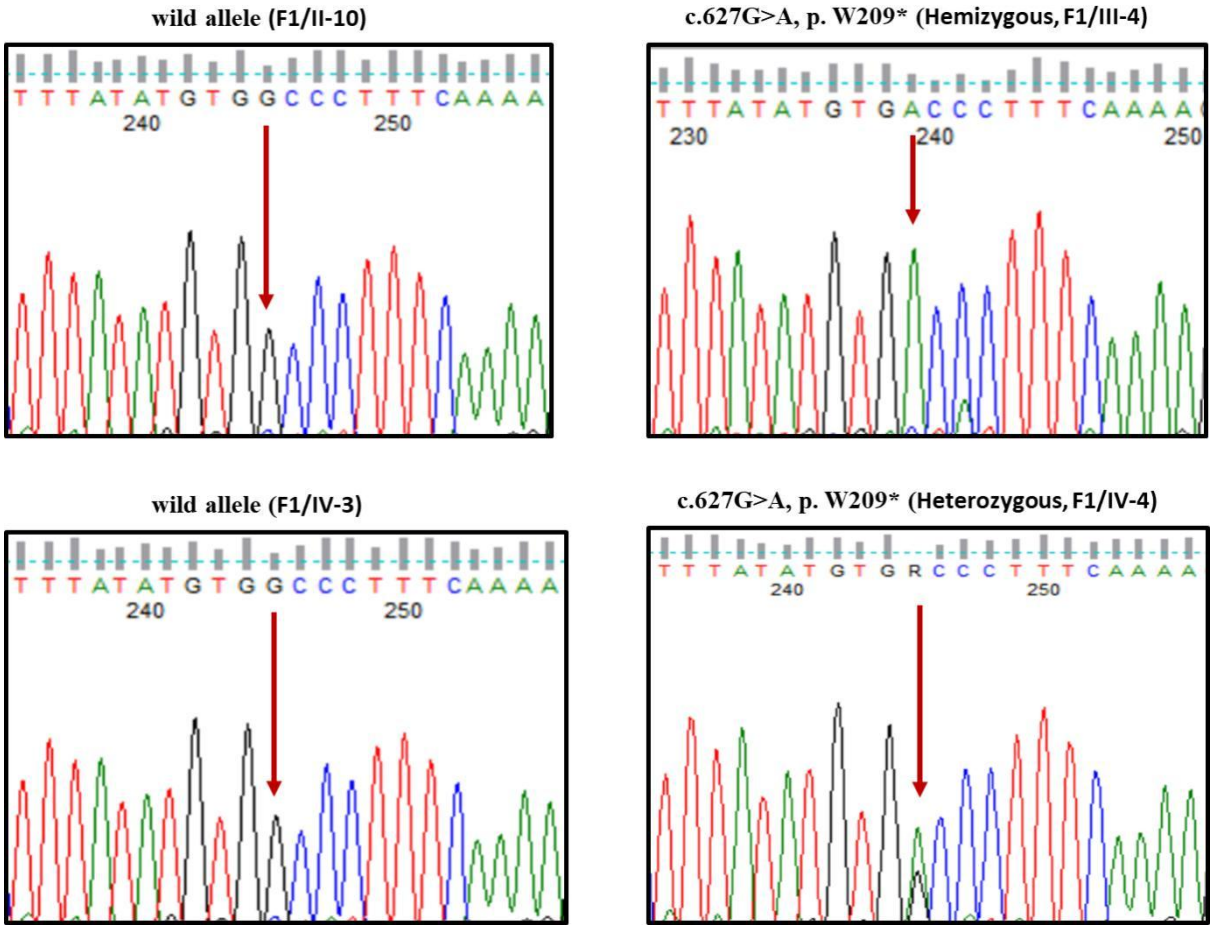

Fig. 4 Sequencing electropherograms showing NM_000169.3 (GLA): c.627G>A, p. W209* variant in the GLA gene in family 1. (A): Homozygous wild allele in the mother (F1/II-10); (B): Hemizygous male patient (F1/III-4); (C): Homozygous wild allele in the patient's daughter (F1/IV-3), and (D): Heterozygous daughter (F1/IV-4). The arrow indicates the site of base substitution.

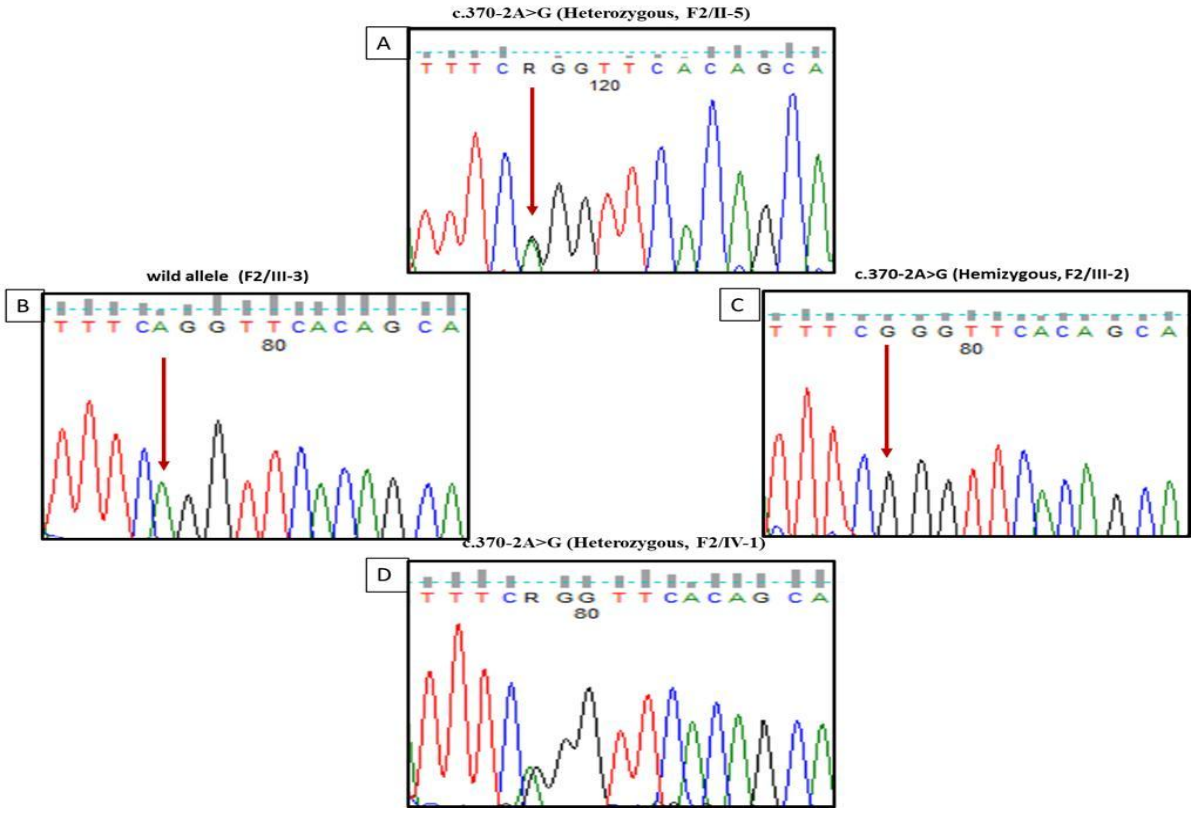

Fig. 5 Sequencing electropherograms showing NM_000169.3 (*GLA*): c.370-2A>G variant in the *GLA* gene in the family 2: (A): Heterozygous mother (F2/II-5); (B): Homozygous wild allele in the proband's wife (F2/III-3); (C): Hemizygous male patient (F2/III-2); and (D): Heterozygous daughter (F2/IV-1). The arrow indicates the site of base substitution.

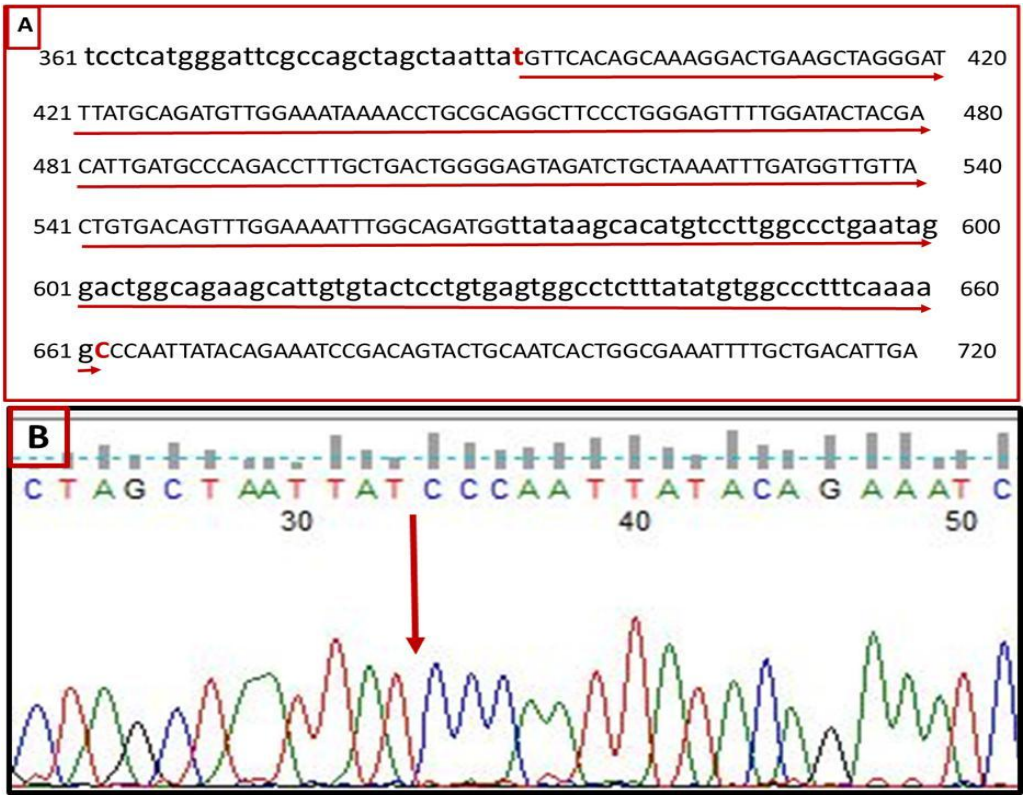

Fig. 6 (A) Base Pairs sequencing and skipping of exon 3 & 4 of the GLA gene transcript including the region from T391 to C662 (271 pb). (B) Sequencing electropherograms showing the skipping of exons 3 and 4 of the mRNA of the GLA gene by removing the region started at T391 and ended at C662 (271 pb) as a consequence of the splice site c.370-2A>G variant.

The missense c.334C>T, p.R112C variant was reported in publicly available mutational analysis databases (rs104894834) (14,18,22,23) as a pathogenic variant on Chrx at position 101403846 with a pathogenicity score of 11 and a conservation score of 4.275. This variant was not detected in exomes, gnomAD, or 1000 genome databases (Varsome.org) [1,21].

Sanger sequencing in family 3 revealed a missense hemizygous variant NM_000169.3: c.334C>T, p.R112C in exons 2 of the *GLA* gene in the affected male patient. However his mother showed homozygous wild allele inheritance, and the defected allele could not be detected in her blood DNA samples (Table 2, Fig. 7).

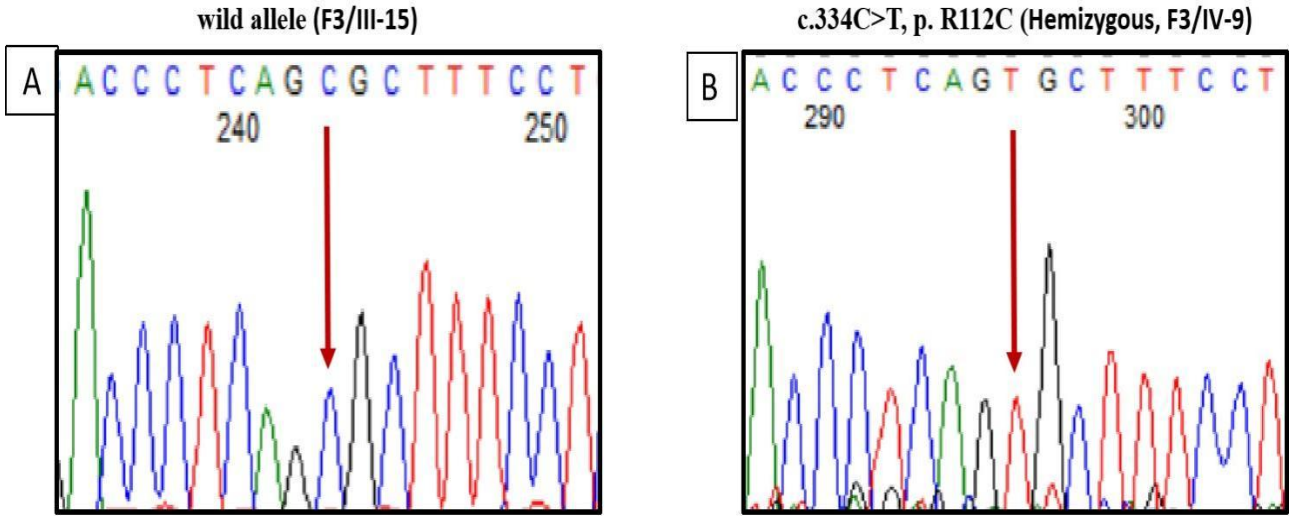

Fig. 7 Sequencing electropherograms showing NM_000169.3 (GLA): c.334C>T, p.R112C variant in the GLA gene in the family 3: (A): Homozygous wild allele in the mother (F3/III-15), brother (F3/IV-10), and cousin (F3/IV-13); and (B): Hemizygous male patient (F3/IV-9). The arrow indicates the site of base substitution.

Discussion

Fabry disease (FD) is an X-linked lysosomal storage disease caused by variants in the "α-galactosidase A (*GLA*)" gene that result in the absence or deficiency of "α-galactosidase A (α-Gal A)" activity. Affected males are classified as having classical FD when they meet the following criteria: the specific *GLA* variant, enzyme activity ≤ 5% (up to 20% in plasma in markedly multisystemic affected patients) of the mean reference range, and one or more characteristic FD manifestations such as neuropathic pain, angiokeratoma, and cornea verticillata. In the absence of these criteria, affected males are categorized as having non-classical FD (late-onset FD) [3]. The present study involved three unrelated consanguineous families, with three affected males having a classical FD phenotype. They presented with variable manifestations of multisystem affection. α-Gal A enzyme activity in the affected subjects was deficient.

The non-classical Fabry disease males who have higher residual enzyme activity are likely to have later disease-onset with mainly involvement of a single affected organ, whereas females are likely to have slowly progressive and milder disease phenotypes as well as a wide spectrum of disease severity (ranging from asymptomatic to severely affected phenotype) [33]. Despite the early onset of symptoms in classic FD males enhancinng early diagnosis and management, the diagnosis of our probands was much later (in their thirties and late twenties) although the symptoms started in childhood (at 7 years, 12 years and 13 years subsequently). This emphasizes the importance of awareness and screening studies that can lead to early diagnosis of patients of both classical and non-classical FD and subsequent carrier detection [3]. Measurement of the enzyme activity is not diagnostic in females with FD. Fabry disease can be analyzed in affected males by demonstrating a deficiency of α-galactosidase A in leukocytes. However, the enzymatic assay is unreliable for the detection of carrier females, which can be detected accurately by variantal analysis. The 70- year- old molecularly confirmed heterozygous mother of the second family had renal problems, as evidenced by the disturbed kidney function tests. This variability in the phenotype and disease course in female patients has been attributed to the variants' pathogenicity and X-chromosome inactivation pattern [33]. More severely affected females are mostly expected to have the X-chromosome with the wild-*GLA* gene inactivated while the X-

chromosome carrying the pathogenic variant is actively expressed in the affected organs [10,12]. Heterozygous detection in the non-manifesting females in this study, the daughters in families 1 and 2, is very important to arrange for periodic follow-up and early management of subsequent complications. Moreover, it can prevent early, unexplained death, as seen in case F1/III-2.

It is noteworthy that pedigree analysis of the three families revealed many affected males and even symptomatic females from both maternal and paternal sides, which clarifies the masking effect of the high rate of consanguinity in our community on the pedigrees showing the X-linked mode of inheritance of Fabry disease.

The molecular analysis of the *GLA* gene in the first family showed a nonsense variant c.627G>A, p. W209*, detected in two genotypes: a hemizygous pattern in the affected male (F1/III-4) and a heterozygous pattern in one of his two daughters (F1/IV-4). The heterozygous variant could not be detected in both the mother (F1/II-10) and daughter (F1/IV-3) (mother and daughter are potential heterozygous of the inherited variant, p. W209*) (Fig. 1). This could be explained by somatic mosaicism, where DNA derived from another tissue needs to be tested. Fabry disease is inherited in an X-linked manner, and therefore an affected male would be obligated to transmit the pathogenic disease-causing variant to all of his daughters [37]. Consistent with our results, Barriales-Villa et al. revealed a variant in a Fabry male patient however, he couldn't detect the variant in his daughter [9]. The nonsense c.627G>A variant leads to "a premature stop codon" at Tryptophan 209, resulting in a non-functional truncated protein. This nonsense variant is located in the N-terminal domain that contains the enzyme active site and leads to a shorter polypeptide chain or produces no protein due to the mRNA degradation. The nonsense variant motivates "the nonsense-mediated mRNA decay (NMD) " pathway, a quality control system that regulates the removal and breakdown of mutant mRNA transcripts, resulting in mRNA instability and subsequently producing truncated proteins with reduced enzyme activity [34]. The nonsense c.627G>A, p. W209* variant was previously reported, for the first time, in a Japanese patient [38] and then detected in European countries [24].

The molecular analysis of the *GLA* gene in the second family showed a splice site variant (c.370-2A>G) that was hemizygous in the affected male and heterozygous in his mother and daughter. The splice variant affecting the splicing of mRNA results in a significant alteration in the protein because of either exon skipping, shortening, or the insertion of intronic sequences. There were no previous reports on the impact of this splice site variant on the mRNA transcript. However, in our study, the identified splice site variant resulted in the skipping of exons 3 and 4 from the *GLA* gene transcript by removing the region from T391 to C662 (271 pb) (Fig. 5), producing a shorter transcript (1016 bp). The mutant transcript might be translated into a truncated protein that would lack at least 90 amino acids.

In Fabry disease only 4.6% of the total reported disease-causing variants were found in the splice site areas of the *GLA* gene. In the present study, the splice site variant NM_000169.3 (*GLA*): c.370-2A>G was previously reported, for the first time, in England [27], then identified in other European countries [39, 40].

The molecular analysis of the *GLA* gene in the third family showed a missense c.334C>T, p. R112C variant in exon 2 of the *GLA* gene, that was detected in a hemizygous pattern in the affected male (F3/IV-9). The heterozygous variant could not be detected in his mother (F3/III-15) (the mother is a potential heterozygous of the inherited variant, p. R112C), which could be explained by somatic mosaicism. The X-inactivation mechanism produces a mosaic of healthy cells "a non-pathogenic variant of the activated *GLA* gene" and affected cells "a pathogenic variant of the activated *GLA* gene" in different proportions. Severe symptoms in the tissue concerned in Fabry females results from the expression of a high percentage of the X chromosome expressing the pathogenic variant.

In this variant, the sequence change replaces arginine, which is basic and polar, with cysteine, which is neutral and slightly polar, at codon 112 of the *GLA* protein. The change from arginine to cysteine lead to destabilization of the outer loops on the active site of *GLA* protein. Functional analysis for the missense c.334C>T, p. R112C variant in a previous study revealed that this variant did not impact directly on the active site of *GLA* protein, however it was located at the base of a loop that protruded from the N-terminal domain. Arginine is a basic and polar amino acid that supports and stabilizes the disulfide bond which stabilizes the outer loops on the active site. The changing from arginine to cysteine, which is a neutral and slightly polar amino acid, would destabilize this loop and the disulfide bond on the active site [41].

The missense c.334C>T, p.R112C variant, detected in the third family, has been observed in individuals with FD and was previously reported for the first time by Barman et al. in Turkish patients [28], Afterwards, more than 40 studies reported this variant in FD patients suggesting that it is a common variant [29, 30]. To the best of our knowledge, the three variants c.627G>A (p. W209*), c.370-2A>G, and c.334C>T (p. R112C)

reported here are described for the first time in Fabry disease patients from Egypt or other Arab countries.

In Fabry disease, the presence of human *α-GAL* point variants causes one of the two major classes of FD protein defects: active site and folding-related protein defects. The active site point variant reduces the enzymatic activity by altering the active site configuration without affecting the overall *α-GAL* structure. The folding-related variants may reduce the stability of *α-GA*L by disrupting its hydrophobic core. The active site is considered a hotspot for the variants causing FD [42].

The current approaches of therapy for Fabry disease rely on the reduction of the accumulated "glycosphingolipids" through "Enzyme Replacement Therapy (ERT)". This approach has demonstrated a modifying effect on the organs' complications and mortality rate [43]. ERT has been shown to be effective in neuropathic pain reduction, thermal sensation threshold, and sweat function improvement [43]. Early initiation of treatment is crucial in Fabry disease to avoid multi-organ complications that can significantly reduce life expectancy [23,44].

The patients in this study have currently started ERT in the form of agalsidase beta (Fabrazyme®, Sanofi Genzyme, Cambridge, MA, USA). ERT is administered at a dose of 1.0 mg/kg body weight once every two weeks as an IV infusion at the Enzyme Replacement Unit, NRC. The probands of the first (F1/III-4) and second (F2/III-2) families started the ERT at the same time two years ago, while the proband (F3/IV-9) of family three has just started it recently. ERT has demonstrated improvements in kidney function, cardiac function, neuropathic pain, and quality of life in the proband of the first family (F1/III-4) after two years of treatment, as he started the therapy after his kidney transplantation. However, the proband of the second family (F2/III-2) developed renal failure after one year of treatment with ERT, as he started the therapy at the End Stage Kidney Disease (ESKD), and the ERT delayed the patient's renal failure for one year. In agreement with our study, FD patients described in other Arab and Gulf countries [31], particularly in Tunisia [32] and Saudi Arabia [5,31], revealed that the clinical phenotype of the patients was of the classical FD symptoms [5,31,32]. Nine Tunisian patients with FD and renal complications were diagnosed as having α-Gal A enzyme deficiency [32]. These patients underwent ERT; after a 10-year follow-up period, five patients developed renal failure, while four died of cardiovascular complications [32]. In Saudi Arabia, ten Fabry patients from a single family were post-kidney transplants [5]. Some of them showed cardiac disease and End-Stage Kidney Disease (ESKD). They were all referred to ERT. Both studies [5, 32] concluded that screening and early diagnosis for Fabry disease are crucial, particularly in the groups of neonates, patients on dialysis, and/or the kidney transplant population [5] for starting the ERT and can potentially halt the progression of renal failure or other organ damage [32].

Conclusion

Our study is the first study of FD with molecular analysis of the *GLA* gene in Egyptian patients. This study has identified three pathogenic variants; a nonsense c.627G>A, p.W209*, a splice-site c.370-2A>G, a missense c.334C>T, p.R112C variants in the *GLA* gene in three independent Egyptian families with Fabry disease. The nonsense p.W209* and the splice site c.370-2A>G variants are considered very rare. Early genetic diagnosis is strongly recommended for Egyptian families with Fabry disease for better management of the affected cases and periodic follow-up of molecularly confirmed asymptomatic late-onset males and females.

Authors' contributions

This work was carried out in collaboration between all authors. E.F. and E.E.A.M. designed, wrote the protocol and coordinated the study. E.F. and Z.Y.A. performed the biochemical studies, and provided patients' samples for molecular analysis. M.A. and R.M.E. performed the clinical study for the patients and their families. E.E.A.M. and N.N.A. performed the molecular experiments, provided data analysis, results interpretation, literature searches, wrote the first draft, and revised the various versions of the manuscript. E.F. and M.A. revised the manuscript. All authors read, revised, and approved the submitted manuscript. E.E.A.M. (guarantor) has the responsibility for the integrity of the work as a whole from inception to published article.

The manuscript has been read and approved by all the authors, and the requirements for authorship as stated earlier in this document have been met, and that each author believes that the manuscript represents honest work.

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Availability of data and materials

Sequencing generated in this study is available upon a reasonable request made to the corresponding authors.

Human Ethics and Consent to Participate declarations

- The Medical Research Ethics Committee at the National Research Centre, Egypt has approved the present study and the informed consent.
- The name of the Approval Committee that approved the study: **"The Medical Research Ethics Committee"**.
- Written informed consent was obtained from the parents of the patients according to the guidelines of **"The Medical Research Ethics Committee"** at the National Research Centre (NRC) (approval no. 044101223).

Consent for publication

Participants or legal guardians have consented for the publication.

Competing interests

The authors declared that no conflict of interest exists.

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