# DNA Methylation Analysis and Phenotype Severity in Fabry Disease

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**Abstract**

Fabry disease (FD) is an X-linked inborn error of glycosphingolipid metabolism characterized by progressive lysosomal deposition of partially metabolized substrates within various tissues. This condition results in significant morbidity and mortality for both men and women. However, the severity and progression of the disease differ by sex due to potential factors that modulate the phenotype in women, such as X chromosome inactivation. In this study, we conducted methylation assays on peripheral blood samples from seven women diagnosed with FD and examined the correlation between these assays and the clinical severity of the disease. The results showed no correlation, underscoring the importance of selecting appropriate tissues for analysis.

**Keywords:**

Fabry disease, X-chromosome inactivation, HUMARA, DNA methylation, heterozygotes.

## Introduction

Fabry disease (FD) is a multisystemic and progressive X-linked disease caused by deficient activity of the enzyme α-galactosidase A, leading to harmful lysosomal accumulation of macromolecules associated with glycosphingolipid metabolism[1]. When enzyme activity is undetectable, this systemic deposition triggers a cascade of pathological processes that manifest in early childhood with symptoms and signs such as neuropathic pain, gastrointestinal issues, autonomic nervous system impairment, and angiokeratomas, among others[2,3]. As the disease progresses, patients may develop renal, cardiac, and central nervous system complications, which are the leading causes of premature mortality in FD patients[2,4,5]. In addition to this classic presentation of the disease, atypical clinical variants exist, characterized by residual enzyme activity, isolated organ affection, and later onset[2,6]. The disease affects both men and women; however, while the progression and severity may be lessened in women, it still causes significant morbidity and reduced life expectancy[7,8].

Due to the inheritance pattern, inactivation of the X chromosome has been proposed as a critical factor in modulating the clinical presentation of FD in heterozygous women[5,7]. This process, which occurs early during embryonic development, results in the silencing of one X chromosome, which is maintained and clonally propagated for the lifetime of the cells[9,10]. This epigenetic mechanism leads to two distinct populations of cells, each expressing either the maternal or paternal X chromosome, which may contribute to the clinical heterogeneity observed in affected women[9,11].

X-chromosome inactivation (XCI) is a poorly understood process that occurs alongside unpredictable waves of cell division and migration, typical of the early embryonic stage[10,12]. As research advances, the mechanisms involved are being clarified, and a growing number of associated molecules are being identified[13–15]. Consequently, our understanding of XCI's role in X-linked diseases is expected to evolve and become more refined over time.

Allen *et al.* developed a methylation-based assay that analyzes a tandem trinucleotide repeat in exon 1 of the *AR* gene, a technique that remains widely used[16,17]. However, the relationship between XCI and the severity of FD, based on the results of this assay, has been questioned due to inconsistent findings reported in various studies[18–23]. Furthermore, certain X-linked genes have been identified as escaping silencing to varying degrees[24–26], which underscores the necessity for studies that specifically analyze the methylation of the *GLA* gene.

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In the present study, we describe the phenotypic heterogeneity observed in seven women with FD and present the results of methylation assays on peripheral blood samples.

## Methods

### *Subjects and Samples*

This cross-sectional study included seven heterozygous women diagnosed with FD, from two family cases, all of whom consented to participate. The Mainz Severity Score Index (MSSI) was applied for a specific clinical evaluation of FD. To assess health-related quality of life, the SF-36 v.2 questionnaire was administered to the adult participants. Additionally, peripheral blood samples were collected. This study was approved by the Ethics Committee Review Board of the Universidad Nacional de Colombia, in accordance with the ethical standard set by the Declaration of Helsinki and later amendments. Written consent forms were obtained prior to participation.

### *X Chromosome Inactivation Analysis*

Genomic DNA was extracted from blood samples using the ReliaPrep Blood gDNA Miniprep System kit (Promega Corporation, Madison, WI). DNA quality and quantity were assessed using a NanoDrop Spectrophotometer (ThermoFisher Scientific).

HUMARA assay was performed as previously described[27]. Briefly, both undigested and digested DNA with the HhaI methylation-sensitive restriction enzyme (ThermoFisher Scientific), were amplified to analyze the polymorphic STR in the first exon of the *AR* gene, using fluorochrome-coupled primers: F 5'-FAM TCCAGAATCTGTTCCAGAGCGTGC-3', R 5'-GCTGTGAAGGTTGCTGTTCCTCAT-3'[16]. The amplification products were separated on an ABI 3500 Automatic Genetic Analyzer (Applied Biosystems). The areas under the curve were used to calculate the XCI ratio[27]. For this study, we classified skewed inactivation as occurring when the proportion of cells exhibiting inactivation of any allele was between 0.75 and 0.89, and extremely skewed when the proportion was ≥0.9. In all other cases, inactivation was classified as random, as has been done by other groups[5,21,28].

Additionally, to study locus-specific methylation, bisulfite-treated DNA, using the EZ DNA Methylation-Direct Kit (Zymo Research), was subjected to nested amplification to achieve greater specificity and to be able to genotype informative SNPs. The first amplification was meant to be insensitive to methylation using the following primers: F 5'- GACGATTAGAATTATTTTTGTTTA-3' and R 5'-TATCCAATACTCTAACCCCAAAA-3'. In one case (F1) due to the degradation of bisulfite-converted DNA, a shorter amplicon was obtained using primers previously reported in the literature: F 5′-GGTTTATTTTTGGGGATAATTGTT-3′ and R 5′-CAACTATTCCCATTAAAACTCTCC-3′[29]. For the subsequent methylation-sensitive amplification, the following primers were used: F methylated 5'-ATT**CGCG**GAAATTTATGTTGTT**C**-3'; R methylated 5'-AAAAAA**CG**AAA**CG**CAAA**CGCG**-3'; F unmethylated 5'-TTATT**TGTG**GAAATTTATGTTGTT**T**-3'; R unmethylated 5'- ACCAAAAAA**CA**AAA**CA**CAAA**CACA**-3', which include at least three CpG cytosines (in bold) and four non-CpG cytosines. The latter amplification was done exclusively in informative cases for any of the SNPs located in the 5' untranslated region of the *GLA* gene, which allowed differentiation of the alleles. Hot start AmpliTaq Gold DNA Polymerase with Buffer II and MgCl2 (Applied Biosystems) were used with the thermal profile according to its recommendation. The amplified DNA was purified and sequenced using 3.1 BigDye Terminator cycle sequencing kit (Applied Biosystems) in an ABI 3500 analyzer using POP7 and 50 cms capillary.

### *Statistical Analysis*

Normality of continuous data was tested using the Shapiro-Wilk test. A non-parametric Spearman correlation test was applied to evaluate a correlation between the MSSI scores and the inactivation ratios. Statistical significance was set at p-value ≤0.05. Statistical analyses were performed using Jamovi software 1.6.15.

## Results

The first case F1, is a 50-year-old woman diagnosed with FD through cascade screening. Genetic testing identified a heterozygous variant c.1A>G (p.Met1Val), rs869312265 in the *GLA* gene, classified as likely pathogenic and previously described in the literature[30] (HGMD database CM078384). She reported abdominal pain and concentric left ventricular hypertrophy with preserved ejection fraction as the only FD manifestations. Her MSSI score indicated mild severity (score: 13). The SF-36 questionnaire showed her mental health dimension was the most affected. Family history reveals a sister with end-stage renal disease as the only reported FD complication, in whom the molecular study established the diagnosis. Point variant screening in their mother was negative. The father had a personal history of neuropathic pain, renal disease, and stroke, but no sample was available to determine the paternal origin of the variant (Figure 1A).

HUMARA assay was possible in F1, given the patient's zygosity, and reported an extremely skewed XCI, with a 0.9 proportion of peripheral blood cells presenting the superior allele of the STR on the active X chromosome (Figure 1B). Although it was possible to estimate the proportion of inactivation using the HUMARA assay, its direction remained unknown due to the unavailability of family samples necessary for assessing allele segregation. This extremely skewed inactivation was consistent with the findings at the *GLA* gene. This assay was enabled by the presence of the FD-associated variant (c.1A>G), and the identification of a SNP (c.-12G>A; rs3027585; in heterozygosis)

in the amplified region. This locus-specific assay revealed preferential detection of the wild-type alleles in the unmethylated state (c.1A; c.-12G) (Figure 1C).

In the second family case F2-F7 (Figure 2A), cascade screening identified the pathogenic null variant c.298A>T (p.Arg100X) rs869312272[31], in all six women. Their clinical presentation varied in severity (Figure 2B). Patient F3 experienced multiple FD complications, including chronic kidney disease, left ventricular hypertrophy, incomplete bundle branch block, tricuspid regurgitation, and stroke (MSSI score: 54). Three women

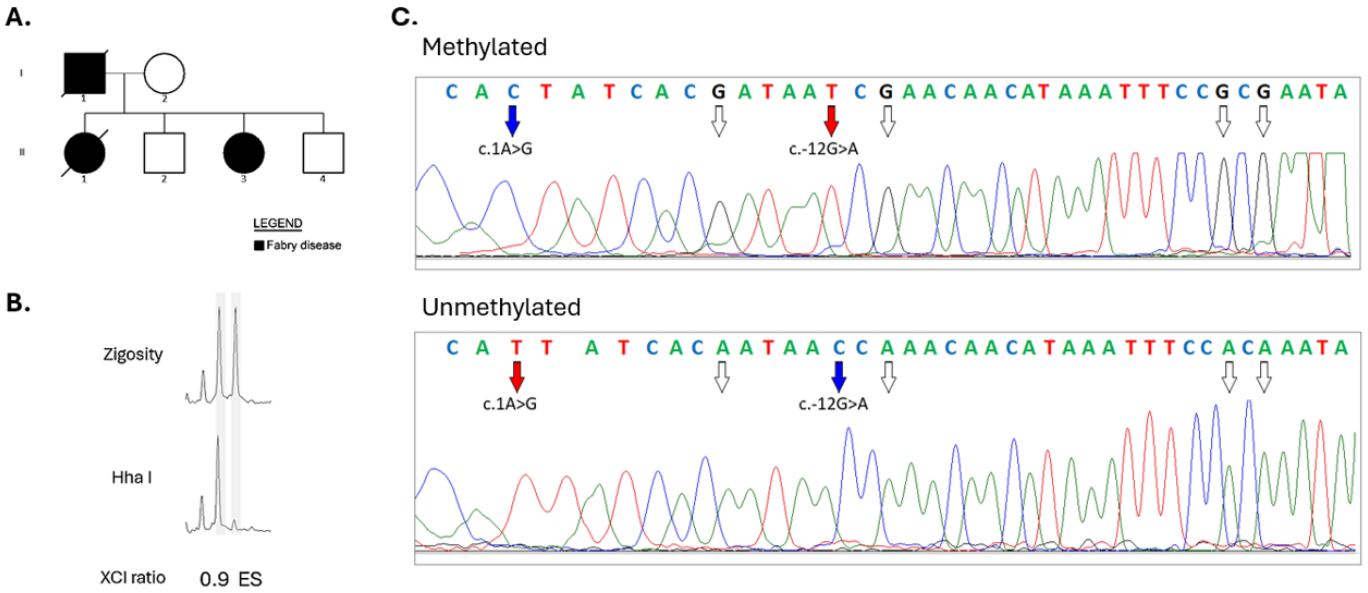
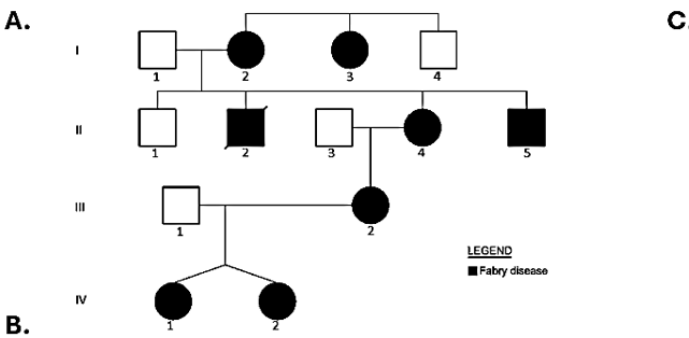



**Figure 1.** Case F1. A. Family pedigree. B. Electropherograms of the HUMARA assay before and after digestion with a methylation-sensitive restriction enzyme. XCI ratio is specified. ES: extremely skewed. C. Methylation-specific amplification of the *GLA* gene. Electropherograms of the reverse strand sequencing are shown; CpG cytosines are marked with white arrows; the SNP and the FD-associated variant are marked with blue and red arrows, respectively. Sample: peripheral blood.




B.

| | Age (yo) | Main signs and symptoms of FD | MSSI |
|---|---|---|---|
| F2 I-3 | 70 | Chronic kidney disease, ventricular hypertrophy, dyspnea, atrial fibrillation | 39 |
| F3 I-2 | 74 | Chronic kidney disease, ventricular hypertrophy, stroke, bundle branch block | 54 |
| F4 II-4 | 50 | Neuropathic pain, proteinuria, vertigo, depression | 29 |
| F5 III-2 | 29 | Neuropathic pain, bradycardia, depression | 23 |
| F6 IV-1 | 10 | Neuropathic pain, impaired sweating | 16 |
| F7 IV-2 | 10 | Grastrointestinal symptoms, impaired sweating | 10 |

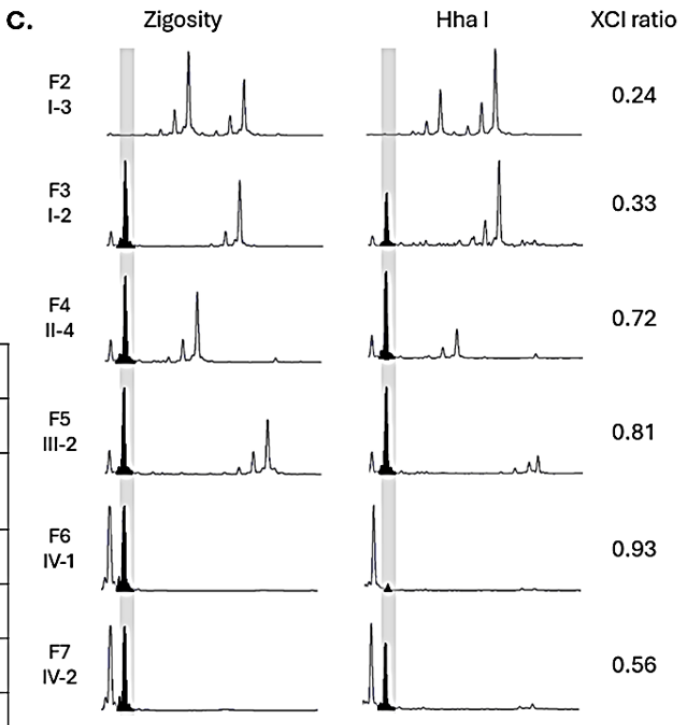



**Figure 2.** Second family case. A Pedigree including the six women that participated in the study. B. Clinical scores and main signs and symptoms of FD reported. C. Electropherograms of the HUMARA assay before and after digestion with a methylation-sensitive restriction enzyme. XCI ratios are specified; family segregation is highlighted. Sample: peripheral blood.

(F2, F4, F5) had moderate severity (MSSI scores: 39, 29, 23, respectively), reporting cardiac issues such as arrhythmias (atrial fibrillation, bradycardia), valvular heart disease (atrioventricular valve insufficiency), proteinuria, and a history of neuropathic pain in childhood. Additionally, all patients reported a negative impact on their mental dimension, as assessed by the SF-36 scale. The two pediatric cases (F6, F7) reported changes in bowel habits and pain in the palms and soles, which worsened with exercise or fever (MSSI scores: 16, 10, respectively). Among the comorbidities reported by the patients were obesity, chronic gastritis, type 2 diabetes mellitus, and sleep apnea-hypopnea syndrome.

All six women were informative for the HUMARA assay, and allele segregation was observed in 5 out of 6 family members. In two cases (F2, F5) skewed XCI was documented, while F6 exhibited extremely skewed inactivation. In the remaining cases, random inactivation was detected. The XCI ratios are detailed in Figure 2C. None of the subjects in this family case were heterozygous for any of the SNPs located in the amplified region at the *GLA* locus. Consequently, due to the inability to differentiate the alleles, a *GLA*-specific methylation assay was not performed.

No significant correlation was found between the inactivation ratio of the phenotype-associated allele from the HUMARA assay results and the MSSI score (Spearman's rho -0.2, p-value: 0.6).

## Discussion

Previously, it was believed that women with FD were asymptomatic carriers who did not require specific treatment[7]. However, recent research has shown that women experience a significant burden of the disease, and often exhibit greater clinical heterogeneity than what is described in hemizygous males[7,8,32].

Additionally, studies have documented considerable differences in disease progression among FD patients within the same family who share the same pathogenic variant in the *GLA* gene[33–36]. This variability is evident in the cases discussed in this study. For instance, one woman with the variant c.1A>G (p.Met1Val) developed end-stage renal disease, while her sister exhibited ventricular hypertrophy. A similar degree of heterogeneity was noted in the second family case, characterized by a notable delay in diagnosis despite the presence of typical FD complications in two family members (F2, F3). This underscores the need to maintain a high index of suspicion for FD manifestations, as this disease is a cause of renal and heart failure, and stroke, with available treatment[37,38]. Consequently, cascade screening remains an important diagnostic strategy for women with FD[39,40]. Initially, the first patient F1 claimed to be asymptomatic, but further evaluation revealed significant health issues. Similarly, emotional compromise was not initially apparent in most patients until a health-related quality-of-life measurement scale was applied.

The high burden of morbidity and mortality observed in male relatives, along with decades of undiagnosed manifestations[41,42], the heritability of the trait[43], heterogenous expression among women in the same family, and the pain[44,45], contributes to uncertainty, guilt, fatigue, and a great emotional impact[43,44,46]. To address this, it is critical to systematically include quality-of-life scales[44,47,48].

It has been proposed that XCI may influence the phenotypic expression of FD in women by favoring either the wild-type or the mutated allele expression[5]. However, studies on XCI-phenotype correlation in FD have yielded inconsistent results[49,50], likely due to factors such as differing definitions of skewed versus random inactivation[27], allelic heterogeneity among the patients studied[5], the inclusion of young women who may not have fully manifested the disease[49], the use of different clinical assessment scores, and the varying tissues examined in the assays[5,49]. In the present study, no correlation was found between XCI and the MSSI scores. While the first case described showed mild severity of clinical presentation and skewed methylation of the mutated allele, systemic involvement cannot be correlated with a methylation assay performed in a single tissue, or in a pair of tissues unrelated to the evaluated manifestations. Furthermore, it remains unclear how differentiation events, such as cellular proliferation and migration during early development, impact XCI patterns in different tissues[51]. Consequently, caution should be taken when extrapolating ratios[51].

The HUMARA assay represents a practical approach for analyzing XCI, although it is important to acknowledge its limitations[17,50]. Notably, there is the possibility of uninformative zygosity, even when analyzing a highly polymorphic STR[52]. Additionally, the assay requires familial samples to determine the direction of inactivation and may be affected by the occurrence of STR expansions or contractions[53], as presumably observed in one subject from the second family case. Moreover, the specific escape profiles of genes associated with X-linked diseases[14] present further challenges for interpreting results[23].

Our findings suggest that results from the HUMARA assay conducted on peripheral blood samples are concordant with those from the *GLA*-specific methylation assay. This result correlates with previous studies[23], suggesting that, similar to the *AR* locus, the *GLA* gene does not consistently escape inactivation, at least in the tissue analyzed. The *GLA* gene has also been classified as subject to inactivation by other authors[24,54–56].

Lastly, several SNPs located in the 5' untranslated region of the *GLA* gene have been associated with variations in plasma enzyme activities[57–59]. In particular, the c.-12A allele has been reported to exhibit a neutral effect[57,60,61]; however, further investigation is required to fully understand the implications of this variant on the overall clinical presentation of FD.

## Conclusion

This study provides insights into the application of methylation assays in women diagnosed with FD. The variable manifestations of the disease within family cases and the impact of this diagnosis on quality-of-life scores are highlighted in our study. In addition, our results showed that the HUMARA assay results are concordant with those from the *GLA* gene-specific methylation assay, suggesting that this gene does not consistently escape inactivation in peripheral blood cells. XCI-phenotype correlations must be refined by noting the importance of the tissue analyzed.

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### Declaration of Conflicting Interests

The authors declare that the is no conflict of interest.

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