International Journal of
Translational Medicine

MDPI

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Role of Circulating X-Chromosome Inactivation and *Xist* as Biomarkers in Female Carriers of Fabry Disease

Salvatore Rossi [1], Arcangelo Fargnoli [2], Daniele Di Natale [1,†], Gianmarco Dalla Zanna [1], Antonio Funcis [1], Federica Re [3], Vincenza Gragnaniello [4,5], Elena Verrecchia [6], Alberto Burlina [4,5], Elisabetta Tabolacci [7,8] and Gabriella Silvestri [1,9,*]

1 Department of Neurosciences, Università Cattolica del Sacro Cuore, 00168 Rome, Italy
2 UOC Genetica Medica, Fondazione Policlinico Universitario "A. Gemelli" IRCCS, 00168 Rome, Italy
3 Cardiology Division, Cardiomyopathies Unit, St. Camillo Hospital, 00152 Rome, Italy
4 Division of Inherited Metabolic Diseases, Department of Diagnostic Services, University Hospital of Padua, 35128 Padua, Italy
5 Division of Inherited Metabolic Diseases, Department of Women's and Children's Health, University of Padua, 35128 Padua, Italy
6 Department of Aging, Orthopaedic and Rheumatological Sciences, Fondazione Policlinico Universitario "A. Gemelli" IRCCS, 00168 Rome, Italy
7 Dipartimento Scienze della Vita e Sanità Pubblica, Sezione di Medicina Genomica, Università Cattolica del Sacro Cuore, Fondazione Policlinico Universitario "A. Gemelli" IRCCS, 00168 Rome, Italy
8 Fondazione Policlinico Universitario "A. Gemelli" IRCCS, 00168 Rome, Italy
9 UOC Neurologia Dipartimento Neuroscienze, Organi Di Senso E Torace, Fondazione Policlinico Universitario "A. Gemelli" IRCCS, 00168 Rome, Italy
* Correspondence: gabriella.silvestri@unicatt.it
† Current address: Ospedali Riuniti Padova Sud-ULSS 6 Euganea, 35043 Padova, Italy.

Abstract: Background: Fabry Disease (FD) is an X-linked lysosomal disease, in which, unlike other X-linked disorders, most female carriers manifest signs or symptoms for unknown reasons. Objectives: Herein, we aimed to test the potential role of X-chromosome inactivation (XCI) in leukocytes as a prognostic biomarker of disease in FD female carriers. Moreover, we explored if levels of X-inactive-specific transcript (*Xist*), a long non-coding RNA driving XCI, were detectable in the leukocytes of FD female carriers. Methods: We tested the XCI pattern in leukocytes on 33 consecutive females carrying pathogenic *GLA* variants. Disease severity was defined using the Mainz Severity Score Index (MSSI). *Xist* levels in leukocytes were assessed by real-time PCR and compared to the levels of 22 controls. Results: XCI was obtained for 31 female patients, finding 16 skewed (51.6%) individuals. Global MSSI did not differ in skewed vs. non-skewed FD carriers. In skewed FD females, the renal function and mean cardiologic MSSI subscore were significantly worse, and systemic arterial hypertension was more frequent. *Xist* levels detected in leukocytes were similar between female patients and controls, and did not differ by phenotype or XCI status. Conclusions: A skewed XCI pattern in leukocytes may represent a prognostic biomarker of worse renal and cardiac outcomes in female FD carriers.

Keywords: Fabry disease; X-chromosome inactivation; *Xist*

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Citation: Rossi, S.; Fargnoli, A.; Di Natale, D.; Dalla Zanna, G.; Funcis, A.; Re, F.; Gragnaniello, V.; Verrecchia, E.; Burlina, A.; Tabolacci, E.; et al. Role of Circulating X-Chromosome Inactivation and *Xist* as Biomarkers in Female Carriers of Fabry Disease. *Int. J. Transl. Med.* **2024**, *4*, 618–630. https://doi.org/10.3390/ijtm4040043

Academic Editor: Kurt Showmaker

Received: 7 October 2024
Revised: 12 November 2024
Accepted: 20 November 2024
Published: 21 November 2024

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1. Introduction

Fabry Disease (FD; OMIM # 301500), also known as Anderson–Fabry Disease, is an X-linked inborn error of the glycosphingolipid metabolic pathway caused by mutations in the *GLA* gene, that encodes for the lysosomal enzyme alpha-galactosidase A (α-Gal A) [1]. The resulting α-Gal A deficiency causes lysosomal accumulation of globotriaosylceramide (Gb3) in a wide variety of cells in various tissues, organs, or systems, such as the kidney, the heart, the skin, the endothelial and smooth muscle cells, the central and peripheral nervous system, the eye, and the inner ear. Subsequently, the clinical manifestations of FD variably include kidney dysfunction, eventually leading to renal failure, hypertrophic

cardiomyopathy, angiokeratomas, acroparesthesias, acute cerebrovascular and cognitive manifestations, sweating abnormalities, corneal opacities, hearing loss, and gastrointestinal symptoms [2].

As in other X-linked diseases, hemizygous males are usually more severely affected than heterozygous females. Nevertheless, in FD, a condition of manifesting female carrier seems to be more frequent than in other X-linked recessive conditions, with significant phenotypic variation, also including the full classic FD expression characteristic of affected males [3]. Indeed, FD female carriers show a high prevalence of manifesting individuals (up to 70%), and the mechanisms of this finding are still largely unknown [4].

In humans, as females carry two X chromosomes compared to males holding only one X chromosome, X dosage compensation is needed in order to ensure equivalent expression levels of sex-linked genes. The phenomenon by which X dosage compensation is obtained is defined as X-chromosome inactivation (XCI), referring to the random selection and transcriptional silence of one of two X chromosomes in females at the early stages of embryonic development [5,6]. Because of the random XCI, some cells will have only the X chromosome with the defective gene activated and others the X chromosome with the functioning gene activated [7]. Opposed to random XCI, skewed (or non-random) XCI, a phenomenon by which more than 75% of cells in a tissue display the preferential inactivation of one of the two X chromosomes, has been postulated to explain the high rate of manifesting female carriers in FD. In other words, females with a higher percentage of cells with the inactivation of the non-mutated *GLA* allele will show FD symptoms because the X chromosome open to transcription is the one carrying the pathogenic *GLA* variant [6]. Thus, the degree of severity of the symptoms and organ involvement may depend on which organs or tissues have the mutated allele active in a substantial majority of cells, and the presence of clinical involvement in heterozygous females may be, at least in part, related to the occurrence of skewed (non-random) XCI [7].

XCI is mainly regulated by X-inactive-specific transcript (*Xist*), a long non-coding RNA (lncRNA) that is transcribed only in the inactive X chromosome and is responsible for the process of *cis* inactivation, promoting large-scale chromatin remodeling and the formation of a unique nuclear compartment, the inactive X chromosome, through the recruitment of chromatin-modifying proteins, transcriptional silencers, and other RNA-binding proteins [8]. However, *Xist* not only seems to have a role in physiological processes such as XCI, but it acts also as an effector of phenotypic variability or as a biomarker in several diseases, ranging from cancer to autoimmune and degenerative disorders, and it was never tested in FD [9].

Considering that effective treatments are available for FD, and that the early starting of therapy is associated with better outcomes, it is urgent to find easily accessible biomarkers able to detect organ damage before the clinical presentation of symptoms [10]. This is especially true for heterozygous females carrying pathogenic *GLA* variants, where symptoms may be more subtle and hard to recognize, and plasmatic α-Gal A activity may range from low to normal levels, as those found in healthy individuals [4,11]. Thus far, globotriaosylsphingosine (lyso-Gb3) is the only available, specific circulating disease biomarker in FD, but elevated blood lyso-Gb3 levels represent a reliable biomarker correlating with severity only in affected males, whereas data concerning female carriers suggest that it would not be as reliable as a marker of disease [4].

Therefore, the aim of this study is to assess the potential role of circulating XCI as a prognostic biomarker of disease in molecularly diagnosed FD female carriers, as the literature gives conflicting results regarding this latter issue in FD [12,13]. Moreover, given that a role has been shown for *Xist* upregulation in animal models of cardiac hypertrophy [14] and neuropathic pain [15], and in patients with diabetic nephropathy [16], being the heart, the sensory neurons, and the kidney the main targets of FD, we explored in a preliminary assessment if the levels of *Xist* in leukocytes are detectable in FD females. Further, we tested if *Xist* in leukocytes may be upregulated in symptomatic FD female

carriers vs. still asymptomatic pathogenic *GLA* variant carriers vs. controls, representing a possible prognostic biomarker in FD.

2. Materials and Methods

2.1. Study Design and Clinical Examinations

We designed a cross-sectional study on 33 consecutive females carrying pathogenic *GLA* variants, whose mean age ± standard deviation (SD) was 42.09 ± 16.04 years. Out of these 33 patients, 12 (36.4%) were asymptomatic mothers of children with pathogenic *GLA* variants found by a newborn screening program adopted in Veneto region. Twenty-three males (mean age 29.86 ± 26.15 years, median 29.08 years, min. 1.17 years, max 73.42 years) were used only as negative controls for determination of *Xist* levels, as, in them, *Xist* levels are expected to be absent or extremely low.

The patients and controls were recruited from the following centers: at Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome (n = 17 FD patients and n = 31 matched controls), at Unità Operativa Complessa Cardiologia, San Camillo Hospital, Rome (n = 4), and at Unità Operativa Complessa Malattie Metaboliche ed Ereditarie, Azienda Ospedale Università, Padova (n = 12). For diagnosis of FD, according to expert recommendations, a combination of phenotypic features, enzymatic assay (in men), and molecular analysis to disclose variants in *GLA* (in men and women) was used [17].

Thirty-one controls (22 females, mean age 40.83 ± 11.75, and 9 males, mean age, 29.44 ± 10.08) were included to normalize *Xist* values.

All patients were evaluated by physical examination, and disease severity was defined using the Mainz Severity Score Index (MSSI), including total score and general, cardiac, renal, and neurological subscores [18].

For all patients, the following data obtained from medical charts were recorded for statistical analysis: age at evaluation (AE), sex at birth, type of *GLA* variant, phenotype (classic, late-onset, asymptomatic), age at onset (AAO) of FD symptoms and years of disease duration (DD) for symptomatic patients, type [enzymatic replacement therapy (ERT)/chaperone] and years of treatment, Lyso-Gb3 plasma levels before starting treatment, residual enzymatic α-GalA activity (measured on leukocytes and expressed as percentage of the normal mean), renal function parameters [Estimated Glomerular Filtration Rate (eGFR, expressed in ml/min), creatinine (mg/dL), Blood Urea Nitrogen (BUN, mg/dL), cystatin C (mg/dL), 24-h proteinuria (mg), and 24-h albuminuria (mg)], presence of renal dysfunction (eGFR < 90 mL/min or 24-h proteinuria > 300 mg/die or spot proteinuria > 30 mg/dL), severe renal dysfunction (eGFR < 30 mL/min/1.73 m^2, dialysis or renal transplantation), common vascular risk factors and comorbidities (hypertension and use of antihypertensive medications, dyslipidemia and use of statins, type 2 diabetes mellitus, and smoking habit), presence of cardiac hypertrophy and major cardiac events [atrial fibrillation or any major rhythm disturbance, congestive heart failure, implantation of an implantable cardioverter device (ICD) or pacemaker (PMK), myocardial infarction, coronary artery bypass graft (CABG) surgery, or a percutaneous transluminal angioplasty], major cerebral events [previous stroke or transient ischemic attack (TIA)], presence of subjective depressive symptoms and use of antidepressants, diagnosis of headache, severity of pain assessed by VAS (Visual Analogic Scale), use of medications for neuropathic pain, presence of acroparesthesias and/or dyshidrosis, presence of angiokeratomas and corneal abnormalities, tinnitus, vertigo, and/or hearing loss.

Plasma Lyso-Gb3 levels were measured, within 6 months before starting therapy (ERT or chaperone), by Tandem Mass Spectrometry 170 at an external laboratory (Centogene GmbH, Rostock, Germany). The eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation (CKD Work Group, 2013) [19].

A subset of symptomatic female patients (n = 21) had also undergone Nerve Conduction Studies (NCSs) in lower limbs to verify the presence of polyneuropathy. NCSs were performed using Natus Keypoint EMG equipment (Middleton, WI, USA). Antidromic

sural SNAP, Tibial CMAP, and F wave latency were measured and compared to our reference values.

For the same 21 symptomatic female patients, detailed echocardiographic parameters were recorded [interventricular septum (IVS) and left ventricle (LV) posterior wall thickness, end-systolic and end-diastolic LV diameter, 2D guided B-mode calculated Left Ventricle Mass index (LVMi), and Relative Wall Thickness (RWT)]. Left ventricular hypertrophy was defined as a left ventricular mass index (LVMi) higher than the upper normal limit (men $\geq$ 103 g/m^2; women $\geq$ 89 g/m^2).

2.2. X-Chromosome Inactivation (XCI) Assay

XCI assay was carried out on genomic DNA extracted from peripheral blood of the females included in our cohort of patients, using primers flanking the $(CAG)_n$ repeat in exon 1 of the human *Androgen Receptor* (*AR*) gene (Xq11-12), adapting the protocol of Allen et al. [20]. Approximately 200 ng of genomic DNA from each individual was divided in two separate tubes; one was single-digested with *Dde*I and the other was double-digested with *Dde*I and methylation-sensitive enzyme *Hpa*II. Twenty nanograms of digested DNA was amplified in a total volume of 20 µL using the forward unlabeled primer, AR-F (5′-GCT GTG AAG GTT GCT GTT CCT CAT), and the reverse FAM-conjugated primer, AR-R (5′-TCC AGA ATC TGT TCC AGA GCG TGC). Following PCR, 1 µL of the amplified product and GeneScanTM 500 LIZ size standard (Thermo Fisher Scientific, Waltham, MA, USA) were separated through gel capillary electrophoresis using an ABI3130 sequencer and fragments were analyzed with GeneMapper v4.0 (Thermo Fisher Scientific). XCI was calculated for informative heterozygous females as the ratio of the peak area of two alleles of the highly polymorphic CAG repeat of the AR gene both in single-*Dde*I-digested and in double-*Dde*I-*Hpa*II-digested sample. The active proportion of the X chromosome was determined by the ratio 1/(1 + 2), where 1 and 2 are the areas of the peaks corresponding to the allele 1 and allele 2, respectively. The range of XCI ratio can vary from 50:50 (random XCI) to 100:0 (completely skewed XCI) [21]. One female sample with known skewed XCI ratio (99:1) and one male sample (that of the unaffected control male) were used as controls to verify the complete digestion and replication. An a priori decision was taken to use the cut-off of a ratio of 75/25 or greater as skewed [22] and anything less (50/50–70/30) as random.

2.3. Xist Determination

Lymphocytes were isolated from fresh peripheral venous blood by density-gradient centrifugation using human lymphocyte separation medium. Total RNA of the lymphocytes was extracted using TRIzol, chloroform, isopropyl, and ethanol method. Total RNA was quantified by NanoDrop ND-2000 (Thermo Fisher Scientific). Afterwards, 500 ng of total RNA was retro-transcribed into cDNA by SensiFAST cDNA Synthesis kit (Bioline, UK), according to the manufacturer's instructions. For a relative quantification of *Xist* transcript using ABI7900HT (Applied Biosystems by Life Technologies™, Thermo Fisher Scientific), the following pre-developed TaqMan® assays were employed: *Xist* (Hs.PT.58.38958533, IDT) and *GAPDH* (glyceraldehyde-3-phosphate-dehydrogenase) (Hs.PT.39a.22214836, IDT), the latter being constitutively expressed in every cell and thus used as endogenous control. The cycle parameters were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles with 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing/extension). The relative quantification of target transcript vs. endogenous transcript was calculated as follows: $2^{-(\Delta Ct(Xist))-\Delta Ct(GAPDH))} = 2^{-\Delta Ct}$, where ΔCt is the difference (Ct(*Xist*)–Ct(*GAPDH*)) and Ct is the cycle at which the detected fluorescence overcomes the threshold. The ΔCt calculated from the control samples were arbitrarily set at 1 (or 100%) to calculate relative expression of the target transcript in the patients expressing the values as $2^{-\Delta\Delta Ct}$. Each sample was evaluated in triplicate and three independent technical replicates were performed.

2.4. Statistical Analysis

The sample was characterized in its clinical and demographic features using descriptive techniques. Quantitative variables were described using mean and standard deviation (SD), median, minimum (min.), and maximum (max.). Qualitative variables were summarized with absolute and percentage frequency tables. Normality of continuous variables was checked using the Kolmogorov–Smirnov test. N-value was specified for each variable. Patients with some missing values have been included in the study and maintained as missing. Mann–Whitney U test was used to evaluate the presence of differences for continuous variables between FD patients' subgroups (classic vs. late-onset FD females, non-skewed vs. skewed FD females). Kruskal–Wallis test was used to evaluate the presence of differences for continuous variables between >2 FD patients' subgroups (classical vs. late-onset vs. asymptomatic individuals) with subsequent post hoc analysis with pairwise comparisons by Bonferroni test. The χ^2 test, or Fisher two-tailed exact test when required, was used to compare categorical variables between the groups. *Xist* levels were correlated by Pearson's correlation test (or Spearman correlation test if required) with demographic variables (AE), FD-related features (MSSI and its subscores, AAO, DD, diagnostic delay, years of treatment, enzymatic activity percentage, and LysoGb3 levels), cardiac (IV septum thickness, posterior wall thickness, LV end-diastolic diameter, LV end-systolic diameter, and RWT), and renal indices (serum creatinine, cystatin C and BUN levels, eGFR, and 24-h proteinuria and albuminuria levels).

3. Results

3.1. Overall Findings

The demographic and disease-related features of our cohort of 33 females carrying pathogenic *GLA* variants are shown in Table 1.

Table 1. Demographic and disease-related features of the cohort. All the frequencies were calculated on the whole sample of females (n = 33) unless otherwise specified. Abbreviations: AAD, age at diagnosis; AAO, age at onset; AE, age at evaluation; BUN, blood urea nitrogen; DD, disease duration; eGFR, estimated glomerular filtration rate; ERT, enzymatic replacement therapy; IVS, interventricular septum; LV, left ventricular; LVMI, left ventricular mass index; MSSI, Mainz Severity Score Index; PNP, polyneuropathy; RWT, relative wall thickness; sAH, systemic arterial hypertension; VAS, Visual Analog Scale; XCI, X-chromosome inactivation; WMH, white matter hyperintensities; y, years.

	n (%)	Mean	SD	Median	Min.	Max.
AE, y	33	42.09	16.04	38.83	16.25	85.50
AAO, y	21	27.81	17.65	24.00	8.00	79.00
AAD, y	33	34.82	14.87	32.00	8.00	79.00
DD, y	21	17.21	17.10	11.00	0	60.00
Diagnostic delay, y	33	6.33	11.40	0	0	39
Phenotype	• classic n = 13 (39.4%) • late-onset n = 8 (24.2%) • asymptomatic n = 12 (36.4%)					
***GLA* variant**	• missense n = 28 (84.8%) • other n = 5 (15.2%)					
Treatment	treated n = 15 (45.5%), n = 11 ERT n = 4 chaperone					

Table 1. *Cont.*

	n (%)	Mean	SD	Median	Min.	Max.
Treatment duration, y	15	7.47	4.05	8.00	1	18
MSSI	33	7.33	8.12	5.00	0	30
general	33	2.03	2.34	1.00	0	7
renal	33	1.06	2.18	0	0	8.00
cardiological	33	1.58	3.12	0	0	15.00
neurological	33	2.30	2.94	1.00	0	11.00
lyso-Gb3 (before treatment, ng/mL	28	2.55	2.39	1.65	0.30	8.40
α-Gal A activity, %	20	42.93	17.75	45.20	12.90	73.8
XCI status (n = 31)	• non-skewed n = 15 (48.4%) • skewed n = 16 (51.6%)					
	Renal features					
Renal dysfunction	7/33 (21.2%)					
Creatinine, mg/dL	33	0.79	0.39	0.69	0.55	2.69
Cystatine C (n = 22), mg/dL	22	0.89	0.57	0.81	0.30	3.26
BUN, mg/dL	31	16.00	10.09	14.00	7.00	58.00
eGFR, ml/min	33	102.30	27.48	112.70	20.00	156.00
24 h proteinuria, mg	21	47.64	118.57	5.0	0	520.00
Proteinuria (spot urine, mg/dL	16	9.25	16.26	3.0	0	62.00
24 h albuminuria, mg	25	90.74	317.12	9.70	0	1587.00
Microalbuminuria	5/25 (20.0%)					
Severe renal dysfunction	1/33 (3.0%)					
	Cardiovascular features					
Loop-recorder implantation	3/33 (9.1%)					
Major cardiovascular events	1/33 (3.0%)					
sAH	9/33 (27.3%)					
Dyslipidemia	7/33 (21.2%)					
Statin treatment	4/33 (12.1%)					
Antithrombotic drug treatment	3/33 (9.1%)					
Smoking habit	9/33 (27.3%)					
Diabetes mellitus	0/33 (0%)					
Lower limb oedema	2/33 (6.1%)					
IVS thickness, mm	21	10.72	2.43	9.00	8.20	15.00
LV end-diastolic diameter, mm	21	44.19	5.66	45.00	31.00	55.00
LV end-systolic diameter, mm	21	25.48	3.80	24.00	20.80	36.0

Table 1. *Cont.*

	n (%)	Mean	SD	Median	Min.	Max.
LV posterior wall thickness, mm	21	10.01	2.40	9.00	7.00	15.00
LVMI, g/m^2	21	92.67	25.52	86.00	68.00	154.00
LV hypertrophy	6/21 (28.6%)					
RWT	21	0.38	0.13	0.31	0.23	0.62
Neurological features						
Previous stroke	2/33 (6.1%)					
Brain MRI WMH (n = 16)	8/16 (50.0%)					
Brain MRI pulvinar hyperintensity	0/16 (0%)					
Brain MRI basilar dolichoectasia	0/16 (0%)					
Fazekas score						
0	10/16 (62.5%)					
1	5/16 (31.3%)					
2	0/16 (0%)					
3	1/16 (6.3%)					
PNP	0/21 (0%)					
Depression	5/33 (15.2%)					
Anti-depressive treatment	2/33 (6.1%)					
Headache	7/33 (21.2%)					
VAS score	33	1.06	1.50	0	0	5.00
Tinnitus	5/33 (15.2%)					
Vertigo	4/33 (12.1%)					
Hearing loss	5/33 (15.2%)					
Acroparesthesias	16/33 (48.5%)					
Dysidrosis	12/33 (36.4%)					
Other features						
Recurrent fever	2/33 (6.1%)					
Angiokeratomas	5/33 (15.2%)					
Corneal abnormalities	7/33 (21.2%)					
Gastrointestinal manifestations	13/33 (39.4%)					

The mean age was 42.09 ± 16.04 years, and the mean age at diagnosis was 34.82 ± 14.87 years with a mean diagnostic delay of 6.33 ± 11.40 years. Of the 33 females studied, 13 had a classic phenotype (39.4%), 8 were late-onset cases (24.2%), and 12 (36.4%) were asymptomatic carriers of pathogenic *GLA* variants. The mean AAO for symptomatic females (n = 21) was 27.81 ± 17.65 years, with a mean DD of 17.21 ± 17.10 years.

Fifteen out of 33 females (45.5%) were under specific FD treatment, 11/15 (73.3%) with ERT and 4/15 (26.7%) with chaperone, with a mean duration of treatment of 7.47 ± 4.05 years. The *GLA* variants found in our cohort were mainly missense (84.8%, see Supplementary Table S1).

The mean MSSI global score (n = 33) was 7.33 ± 8.12, indicating an overall low disease burden. The MSSI subscores are detailed in Table 1.

The mean plasma lyso-Gb3 level before specific FD treatment initiation, available for 28 patients, was 2.55 ± 2.39 ng/mL, and the mean residual α-Gal A activity, available for 20 female patients, was 42.93 ± 17.75%. As expected, plasma lysoGb3 was significantly different in the three subgroups of patients, with asymptomatic cases having lower levels compared to late-onset ($p = 0.005$) and classic cases ($p < 0.001$, Supplementary Table S2).

3.2. Renal Features

Seven out of 33 female FD patients (21.2%) had renal dysfunction, and only 1 patient (3.0%) had a severe renal insufficiency requiring hemodialysis; 5 out of 25 FD patients had microalbuminuria (20.0%). The renal function parameters are summarized in Table 1.

Comparing the renal function parameters in classic vs. late-onset cases, only 24-h albuminuria was significantly higher in late-onset cases ($p = 0.033$, Supplementary Table S2).

3.3. Cardiac Features

Echocardiography data, as detailed in Table 1, were collected from 21 symptomatic FD female patients. Of these, 6 (28.6%) had cardiac hypertrophy. A comparison of the echocardiographic parameters between classic and late-onset cases showed that only the LV tele-systolic diameter was significantly higher in late-onset cases ($p = 0.030$, Supplementary Table S2).

Only 1 out of 33 patients (3.0%) had had a major cardiac event, namely, had undergone ICD implantation for sustained ventricular tachycardia. Three out of 33 FD females (9.1%) had implanted a loop recorder for syncope or palpitations, and an equal number (3/33, 9.1%) were taking an antithrombotic drug (1 was taking low-dose acetylsalicylic acid for a previous stroke, 1 was taking low-dose acetylsalicylic acid as primary prevention, and 1 was taking an anticoagulant for atrial fibrillation). Nine out of 33 FD females (27.3%) had systemic arterial hypertension and were under specific treatment. Comparing classic and late-onset cases, none of these cardiovascular features significantly differed (Supplementary Table S2).

Among other cardiovascular risk factors non-related to FD, 7/33 FD females had dyslipidemia (21.2%) and only 4 were taking statins, none had diabetes mellitus, and 9/33 (27.3%) were smokers. Comparing classic, late-onset, and asymptomatic cases for these risk factors, only smoking resulted more frequent in late-onset cases (Supplementary Table S2).

3.4. Neurological Features

Only 2 patients out of 33 (6.1%) had suffered from ischemic stroke: one had had a large-vessel ischemic stroke and was taking low-dose acetyl-salicylic acid as secondary prevention, while the other one had had an atherothrombotic stroke and was not taking any antithrombotic drugs as numerous, diffuse cerebral microbleeds were found in brain MRI.

Sixteen patients have undergone brain MRI. Eight of these (50%) had white matter hyperintensities, mostly punctate and scattered, with a low burden measured by Fazekas score (Table 1). None showed pulvinar hyperintensity or basilar dolichoectasia.

Out of 21 patients, who had undergone electromyography, none had polyneuropathy; 12 out of 33 FD female patients (36.4%) had dyshidrosis, and 16 (48.5%) had acroparesthesias.

Seven patients (21.2%) suffered from chronic headache. Only 5 patients out of 33 (15.2%) had tinnitus, 4 (12.1%) vertigo, and 5 (15.2%) hearing loss. Five out of 33 FD female patients (15.2%) manifested depressive symptoms, but only 2 patients were taking antidepressants.

None of the neurological features was significantly more frequent in classic vs. late-onset cases (Supplementary Table S2).

3.5. Non-Skewed vs. Skewed Pathogenic GLA Variants Carriers

XCI was obtained for 31 female patients, finding 15 non-skewed and 16 skewed individuals. This method does not allow the identification of which X chromosome carries the *GLA* mutation, whether the active one or the inactivated one.

There was a similar number of symptomatic patients among non-skewed and skewed individuals (60.0% and 68.8%, respectively, Supplementary Table S3), and global MSSI and lyso-Gb3 levels did not differ in the two subgroups (Supplementary Table S3). DD was longer in skewed individuals (23.91 ± 18.81 years) vs. non-skewed ones (6.61 ± 6.46 years, $p = 0.004$, Supplementary Table S3).

The mean serum creatinine levels were significantly higher in skewed female individuals (n = 16, 0.93 ± 0.53 mg/dL) vs. non-skewed ones (n = 15, 0.66 ± 0.08, $p = 0.03$), and similar results were obtained for mean cystatin C (1.10 ± 0.74 mg/dL in 11 skewed individuals vs. 0.70 ± 0.17 mg/dL in 10 non-skewed individuals, $p = 0.036$, Supplementary Table S3). Accordingly, eGFR was significantly lower in 16 skewed female individuals (89.60 ± 29.67 mL/min) compared to 15 non-skewed ones (112.61 ± 19.83 mL/min, $p = 0.003$).

The mean cardiologic MSSI subscore was significantly worse in skewed female individuals (n = 16, 2.75 ± 4.11) vs. non-skewed ones (n = 15, 0.33 ± 1.05, $p = 0.041$, Supplementary Table S3). Moreover, skewed individuals were more frequently affected by SAH under treatment (7/16, 43.8%) compared to non-skewed ones (1/15, 6.7%, $p = 0.037$, Supplementary Table S3).

No differences between non-skewed and skewed individuals were found for neurological and other FD-related features (Supplementary Table S3).

3.6. Xist Evaluation

Xist was undetectable in males (n = 23), compared to females (n = 29), whose mean value was 3.71 ± 6.85 (median 1.74, min. 0.01, max. 35.40).

Xist was similar in FD female carriers (n = 29, 1.74 ± 3.25) vs. controls (n = 22, 1.12 ± 1.34). In the female group (n = 29), *Xist* levels did not differ by phenotype (Supplementary Table S2), nor by XCI status (Supplementary Table S3). *Xist* levels did not correlate with any studied variable (AE, AAO, DD, creatinine, BUN, cystatin C, eGFR, echocardiographic parameters, MSSI and its subscores, and LysoGb3; Supplementary Table S4).

4. Discussion

Historically, recessive X-linked disorders have been considered detrimental only for male patients, where full penetrance is expected, while females were asymptomatic or only mildly symptomatic due to random XCI [23]. More recently, this dogma has been challenged by the evidence for many X-linked diseases of a high degree of symptomatic females carrying pathogenic variants [24]. This is especially true for FD, as female patients are symptomatic in more than 70% of cases, also bearing a reduced life expectancy [12]. As there are different treatment options available in FD, it is of the utmost importance to promptly recognize FD-related signs and symptoms to start specific therapy and avoid late, irreversible organ dysfunction [2]. In female FD patients, this may be challenging, as they may display symptoms later and more variably than male FD patients [3]. Therefore, the main aim of this work was to possibly identify an easily accessible and reliable prognostic biomarker, able to recognize those still asymptomatic female FD patients being at a higher risk of developing FD-related symptoms and subsequently amenable to specific treatment.

We had some interesting results from the XCI status in the leukocytes of FD female patients. Thus far, data regarding the XCI status in FD females have been conflicting, as underscored in a recent meta-analysis regarding this issue [12]. In particular, we found that FD female patients with a skewed XCI pattern in leukocytes had a worse cardiologic MSSI subscore, were more frequently affected by systemic arterial hypertension, and had worse renal function. This is of the utmost importance, suggesting the utility of

comparing follow-up data from non-skewed and skewed heterozygous females carrying *GLA* pathogenic variants by means of non-invasive, extensively available tools (i.e., frequent home-based measurement of arterial systemic blood pressure and creatinine determination), in order to verify the putative role of X-inactivation as a predictor of clinical involvement in FD carriers.

We decided to study XCI in leukocytes as we assumed that performing biopsies of the target organs of FD is not feasible, especially in children carrying FD pathogenic variants, as they are invasive, expensive, and time-consuming procedures. Therefore, we chose to investigate XCI in blood, as it requires only a venipuncture, which is a risk-free, fast, and also well-tolerated procedure in some categories of patients (e.g., children or patients on anticoagulants). Even if we studied XCI only in leukocytes, this should not be seen as a limiting factor, as two other studies demonstrated that the XCI status in blood may actually reflect that of other less accessible tissues. As a matter of fact, the study by Echevarria at al. [25] on 56 females with FD found, with some exceptions, concordance between XCI in blood and buccal smears, urine, and skin samples. Similarly, the XCI status among different tissues from the same patient did not differ significantly in the study by Dobrovolny et al. [22].

More importantly, as in our work, Echevarria et al. [25] also found that, in 10 FD female patients with the preferential inactivation of the wild-type allele, the global MSSI and renal and cardiac function were worse. Concordantly, Dobrovolny et al. [22] found 11 FD female patients (out of 38) with a significantly skewed XCI status, and, among these, patients with the wild-type *GLA* allele inactivated scored worse in global MSSI and deteriorated faster than the non-skewed females. Conversely, in a study by Maier et al. [26] on 28 FD female patients, 15 of them had either moderate or high skewing, but no correlations were found between the extent of XCI and MSSI scores. This was also the case in another study by Elstein et al. [27] on 77 FD females, where no correlations were found between XCI ratios and MSSI, age, or other FD-related manifestations in the minority of skewed FD female patients found.

All these works underscore two issues. First, the XCI status in leukocytes may be of clinical utility, as it may help in managing heterozygous FD females by scheduling closer follow-up visits. However, it is certain that the clinical variability of FD cannot be explained only by XCI, as only a fraction of FD female patients shows skewed XCI in different tissues, raising the need to seek other molecular mechanisms able to explain such clinical diversity. One possible reason for this may be sought in the altered cross-correction mechanisms in FD, as demonstrated in the work by Fuller at al [28]. They show that, compared to another lysosomal storage disorder such as mucopolysaccharidosis type II, in artificially generated FD fibroblasts, the cells expressing wild-type *GLA* alleles were less able to secrete the normal enzyme. Moreover, possibly because of the lack of the mannose 6-phosphorylated form of *GLA*, mutant cells were less able to efficiently endocytose normal enzyme.

We found fewer interesting results on the lncRNA *Xist*. Previous studies demonstrated that it mainly regulates XCI, resulting in the silencing of one of the X chromosomes during female cell development [29]. However, evidence showed a role for it also in human diseases, especially in numerous types of cancer [30,31], and as a biomarker for disorders like polycystic ovary syndrome [32], diabetic polyneuropathy [33] and nephropathy [16], coronary artery disease [34], osteoporosis [8], and pneumonia [35]. Given its pivotal role in XCI, we assumed that a dysregulation of XCI may be driven by either the overexpression or downregulation of *Xist*, and, therefore, we tested its levels in the same blood samples obtained for XCI determination. Thus, before testing the plasma or serum levels of *Xist*, we wanted to verify whether *Xist* was detectable in leukocytes, and if its levels could correlate with any of the investigated variables, yet obtaining negative results. In fact, *Xist* levels in leukocytes from FD female patients did not differ by phenotype or XCI status, and they did not correlate with any FD-related features. This may have resulted from the fact that we decided to measure *Xist* in leukocytes instead of its free levels in the serum of patients, as we thought that the intracellular levels of an lncRNA could have been stabler and easier

to analyze also in non-expert laboratories. Alternatively, we may assume that *Xist* is not dysregulated in FD, and, therefore, other molecular mechanisms for such a variability in female FD patients should be sought in other molecular mechanisms. We will test in a further future study if *Xist* in serum, not being affected by its levels in leukocytes, may possibly represent a prognostic biomarker in pre-manifesting FD females, reflecting its release in the bloodstream as a consequence of the ongoing tissue damage.

However, our study had some limitations. Firstly, it is a cross-sectional study: this study design is optimal for measuring the prevalence of a specific clinical outcome or to describe features of a population, while it cannot establish a cause-and-effect relationship or analyze behavior over a period of time. Secondly, we were not able to distinguish, in skewed FD female patients, if the inactivated X chromosome was the mutant or the wild-type one. More sophisticated methods, such as those utilized by Hossain et al., who analyzed the methylation-sensitive restriction enzyme sites throughout the *GLA* gene rather than the *HUMARA* gene, may be more accurate for performing genotype/phenotype correlations and possibly inferring outcomes about prognosis [36]. Thirdly, even if we chose to test XCI and *Xist* in leukocytes, as these cells are easily accessible by a venipuncture, it is possible that other tissues may have different patterns of XCI and *Xist* expression, especially those that can be studied only by invasive methods. Taken together, our data need further insights to better understand the role of XCI in FD.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijtm4040043/s1. Supplementary Table S1: *GLA* variants found in our cohort. Supplementary Table S2: Classic vs. late-onset vs. asymptomatic carrier females of pathogenic *GLA* variants. Supplementary Table S3: Non-skewed vs. skewed carrier females of pathogenic *GLA* variants. Supplementary Table S4: Spearman's correlations between *Xist* levels in leukocytes and the variables studied in out cohort of carrier females of pathogenic *GLA* variants.

Author Contributions: Conceptualization, G.S.; methodology, A.F. (Arcangelo Fargnoli) and E.T.; formal analysis, S.R.; investigation, E.T., E.V., D.D.N., G.D.Z., A.F. (Antonio Funcis), F.R., A.B. and V.G.; data curation, D.D.N. and S.R.; writing—original draft preparation, S.R.; writing—review and editing, S.R., E.T. and G.S.; supervision, E.T. and G.S.; project administration, G.S.; funding acquisition, S.R. and G.S. All authors have read and agreed to the published version of the manuscript.

Funding: This project was funded by Società Italiana di Neurologia (SIN), Grant "Bando SIN Neurologia di Genere 2019".

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee CET Lazio AREA 3 (protocol number 0034427/21, study ID 4001, date of approval 5 October 2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available upon reasonable request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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