INTRODUCTION

Fabry disease (FD) is an X-chromosomal disorder caused by mutations in the gene encoding alpha-galactosidase A (GLA). Impaired enzyme function leads to FD as a lysosomal storage disorder with cellular deposition of sphingolipids, among others also of globotriaosylceramide (Gb3), and subsequent multiorgan involvement (Germain, 2010). Although FD follows X-linked inheritance, women may reach any level of disease severity...
While the reason for this phenomenon is unclear, the impact of individual X-chromosomal inactivation patterns is assumed in analogy to other X-linked diseases such as Duchenne muscular dystrophy (Mercier et al., 2013) or hemophilia A and B (Garagiolì et al., 2021). In some studies, a potential link between X-inactivation patterns and clinical FD phenotype was described (Dobrovolny et al., 2005; Echevarria et al., 2016; Hossain et al., 2019); however, these findings were not confirmed by others (Elstein et al., 2012; Juchniewicz et al., 2019).

We investigated a large and clinically well-characterized cohort of women carrying GLA variants and analyzed the link of X-inactivation patterns with the clinical phenotype and blood parameters as a potential prognostic biomarker in clinical application.

2 | PATIENTS AND METHODS

2.1 | Recruitment

We recruited 154 study participants at our Fabry Center for Interdisciplinary Therapy (FAZIT), University Hospital of Würzburg, Germany, who were seen between August 2018 and March 2020. Our cohort consisted of 104 women with sequence variants in the GLA gene and 50 related men who were either healthy or FD mutation carriers. This setting allowed unequivocal determination of the mutated X-chromosome in the respective women. The Würzburg Medical Faculty Ethics Committee (#259/17) approved our study and written informed consent was obtained from all patients before inclusion.

2.2 | Classification of genetic sequence variants

Genotype was determined before the first interview at FAZIT and respective reports were provided by the patients or by Centogene (Rostock, Germany). The sequence variants were categorized by three classification systems: (1) Based on the observed clinical phenotype caused by the sequence variant, i.e., classic, late-onset, benign, and variant of unknown significance (VUS) (Arends et al., 2017). For individual classification, the Fabry database was used (http://www.dbfgp.org). (2) Based on the five pathogenicity classes of sequence variants according to the American College of Medical Genetics and Genomics (ACMG) (Richards et al., 2015), i.e., benign (equivalent to “class 1”), likely benign (“class 2”), VUS (“class 3”), VUS with probable pathogenicity (“class 3+”) (Kolokotronis et al., 2020), likely pathogenic (“class 4”), and pathogenic (“class 5”). The allocation to these classes was achieved by evaluating the information from Alamut Visual version 2.11 (Interactive Biosoftware, Rouen, France) providing the data of various population and mutation databases, different prediction tools, as well as information from the literature. (3) Based on the localization of missense variants (i.e., amino acid substitutions) in the final enzyme defining the categories of mutations found in the “active site”, “buried” mutations, and “other” mutations (Garman & Garboczi, 2004; Rickert et al., 2020); for this, the 3D-structure of GAL obtained from the Protein Data Bank (PDB) in Europe (https://www.rcsb.org/structure/1R46 2003) was analyzed in PyMOL 1.8 graphics system (Delano, 2015). For each missense variant, the individual localization of the amino acid exchange in the final enzyme was determined. We used these three classification systems for comprehensive stratification of our patients to draw attention to the diversity of classification systems and the heterogeneity of patient grouping depending on the systems applied.

2.3 | Clinical and laboratory phenotyping

Patients underwent a detailed medical interview, complete neurological and general medical examination, and filled in the Würzburg Fabry Pain Questionnaire (FPQ) (Magg et al., 2015). Additionally, medical reports were screened for past transient ischemic attacks (TIA) or cerebral stroke as hints of central nervous system involvement. The diagnosis of small fiber neuropathy was made in accordance with published criteria (Devigili et al., 2008). Cardiomyopathy was detected by signs of left ventricular hypertrophy in echocardiography and late gadolinium enhancement (LGE) in magnetic resonance imaging (MRI). Nephropathy was diagnosed by evaluation of the glomerular filtration rate and the albumin:creatinine ratio in morning spot urine. For better comparability of our subgroups, we calculated a numeric score that describes the clinical phenotype by symptom severity. We assigned one point for each involved organ system including kidneys, heart, central nervous system, and pain, resulting in: 0 points = no, 1 point = mild, 2 points = moderate, ≥3 points = severe clinical symptoms.

GAL enzyme activity was measured in leucocytes (Podskarbi Laboratory, Munich, Germany; normal range: 0.4–1.0 nmol/min/mg). Plasma lyso-Gb3 was assessed by Centogene (Rostock, Germany) using liquid chromatography and mass spectrometry (normal range: <0.9 ng/ml).

2.4 | Biomaterial collection

We investigated mouth epithelial cells, venous blood, and skin fibroblasts. Mouth epithelial cells were collected
using swabs (Copan Diagnostics Inc., Brescia, Italy). 2.7 ml of venous blood was drawn in a tube containing ethylene-diamine-tetra-acetate (EDTA). Skin fibroblasts were obtained from diagnostic 6-mm skin punch biopsies taken from the lower leg and cultured as described earlier (Karl et al., 2019; Üçeyler et al., 2010). In men, the mutated X-chromosome was determined only in mouth epithelial cells. Follow-up samples for the investigation of biological replicates (mouth epithelial cells, blood) were collected within a median of 1 year (range 0.2–3.0 years) from a subgroup of 49/95 (52%) women.

### 2.5 | DNA extraction

DNA was extracted with the QIAMP DNA mini kit (Quiagen, Venlo, Netherlands) following the manufacturer’s instructions with slight modifications. All DNA samples were stored at −20°C until X-inactivation analysis.

### 2.6 | X-inactivation analysis

X-chromosomal inactivation (XCI) analysis was performed by enzymatic methylations assessment of the human androgen receptor gene (AR, MIM 313700) located on Xq12 using methods and primers as described earlier (Allen et al., 1992) with minor modifications. After determining DNA concentrations with the Qubit fluorometer (Invitrogen AG, Carlsbad, California, USA), DNA dilution of 10 ng/μl in TE buffer (containing TRIS and EDTA) was prepared. Additionally, the methylation-sensitive restriction enzyme HhaI and the appropriate reaction buffer were added to the DNA dilution and incubated following the manufacturer’s instructions (Promega, Madison, Wisconsin, USA). Patient DNA was treated accordingly; however, no HhaI enzyme was added allowing comparison of digested (= non-methylated) versus non-digested (= methylated) DNA and exact calculation of X-chromosomal methylation degree. The X-chromosomal genes AR and in cases with uninformative AR also PCSK1N (located on Xp11.23) (Bertelsen et al., 2011) were amplified in both digested and non-digested DNA samples according to standard PCR conditions (Bertelsen et al., 2011). Fragment length analysis was performed on an ABI 3730 Genetic Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

To calculate the degree of XCI, fragment sizes and peak areas from each sample were analyzed with GeneMapper™ (Thermo Fisher Scientific, Waltham, Massachusetts, USA) including the digested and non-digested approaches. In an informative case, the software supplies two major peaks with different lengths for each approach: the short one labeled XC1 and the long one XC2. The area under these curves was used to determine the degree of XCI (X-inactivation ratio, XCR) as described earlier (Echevarria et al., 2016). XCR was defined “skewed,” if the distribution was ≥75:25%; all other cases were categorized as “random” (Dobrovolny et al., 2005; Echevarria et al., 2016). When more than one sample of tissue was collected (i.e., a biological replicate), we determined the XCR for each tissue and calculated the average of the XCI. This was the case for 21 mouth epithelial and 28 blood XCR. When the XCI pattern was skewed, we determined the active X-chromosome. For this, we compared the allele length of the PCR products with the ones in the samples of related men or related women where the mutation status was known and classified patients as “mutated X is active” and “mutated X is inactive.”

### 2.7 | Statistical analysis

Continuous data are given either as absolute numbers or median and range. Categorical data are presented as percentages. We used SPSS 27 for statistical analysis (IBM, Ehningen, Germany). Since data were not normally distributed, we used the Mann–Whitney-U test for group comparisons and the Chi²-test. Correlation analysis was performed by the Spearman test (ρ). p-values < .05 were assumed statistically significant.

### 3 | RESULTS

#### 3.1 | Patient cohort and biomaterial

We recruited 104 women and 50 related men. We excluded 9/104 (9%) women since six had intronic variants or 5’UTR substitutions and three were not informative for the genes AR and PCSK1N. Hence, further analysis was performed using biomaterial of 95 women and 50 men; 45/95 (47%) women were related. The median age of the female cohort was 53 years (range 18–77) and of the male cohort 42 years (range 18–74). From the female cohort, we collected 94/95 (99%) mouth epithelial cells, 94/95 (99%) blood, and 30/95 (32%) skin fibroblast samples. From the male cohort, we only collected 50/50 (100%) mouth epithelial cells (Figure 1).

#### 3.2 | Classification of sequence variants

We found 48 different sequence variants in the female cohort of 95 women (Table 1). 5/95 (5%) women had more than one variant, whereby all variants were on the same
allele. For categorization, we used the one with the putatively higher clinical impact (Supplementary Table S1). Table 2 summarizes details on the distribution of the mutations found. Most women had mutations categorized as classic (43/95, 45%) or class 5 (38/95, 40%). Of the 70/95 (74%) women carrying missense variants, the majority had “other” mutations (44/70, 62%). 18/95 (19%) women carried one of the sequence variants D313Y (9/95, 9%), A143T (8/95, 8%), and W399S (1/95, 1%), which are currently assumed apathogenic (Lenders et al., 2016; Lukas et al., 2016; Oder et al., 2016). Clinical data of the main patient cohort (“Group I”, $n = 77$) was assessed separately from the data of these women (“Group II”, $n = 18$).

### 3.3 | XCI patterns and clinical phenotype

Table 3 summarizes the clinical phenotype of the patient cohort. 90/95 (95%) of women were informative (i.e., we obtained a result from the XCI analysis of the respective gene) for AR and 5/95 (5%) for PCSK1N. A total of 94 mouth epithelial cell and blood samples and 30 skin fibroblast samples were available for XCI pattern analysis. XCI patterns differed between the three biomaterials in individual cases (Table 4) and were skewed in 6/87 (7%) mouth epithelial cells (Group I: 5/75, 7%; Group II: 1/12, 8%), 31/88 (35%) blood samples (Group I: 29/76, 38%; Group II: 2/12, 17%), and 9/27 (33%) in skin fibroblasts (Group I: 8/22, 36%; Group II: 1/5, 20%) (Figure 1). The reciprocal number of women showed random XCI patterns each.

### 3.4 | Activation status of the mutated X-chromosome in the blood is not associated with disease burden

We continued our analysis by stratifying patient groups for the results of blood data since blood reflected the highest number of women with skewed XCI (31/88, 35%). In these 31 women, the mutated X-chromosome was active in 12/31 (39%) cases (Group I: 29/76, 38%; Group II: 1/2, 50%) (Figures 1–3).

In Group I, GAL showed a trend to lower activity and lyso-Gb3 to higher levels in women with the mutated X-chromosome active compared to those with the mutated X-chromosome inactive, however, differences did not reach statistical significance (Figures 2 and 4a,b). While cardiomyopathy and nephropathy showed a tendency to be more frequent in women with the mutated X-chromosome active (cardiomyopathy: 7/11, 64%; nephropathy: 6/11, 55%) versus those with the mutated X-chromosome inactive (cardiomyopathy: 10/18, 56%; nephropathy: 8/18, 44%), the difference did not reach statistical significance.
| Sequence variants | Clinical phenotype | Pathogenicity class | localization | Frequency |
|-------------------|--------------------|---------------------|--------------|-----------|
| c.137A>G//p.H46R  | classic            | 4                   | buried       | 1         |
| c.155G>C//p.C52S  | classic            | 3+                  | other        | 1         |
| c.188G>A//p.C63Y  | classic            | 4                   | other        | 2         |
| c.334C>T//p.R112C | classic            | 5                   | buried       | 1         |
| c.350T>G//p.I117S | classic            | 4                   | buried       | 1         |
| c.386T>C//p.L129P | classic            | 4                   | buried       | 1         |
| c.404C>T//p.A135V | classic            | 4                   | buried       | 5         |
| c.408T>A//p.D136E | late onset         | 2                   | other        | 1         |
| c.416A>G//p.N139S | benign             | 2                   | other        | 8         |
| c.471G>C//p.Q157H | VUS                | 3                   | buried       | 1         |
| c.484T>G//p.W162G | classic            | 3+                  | buried       | 1         |
| c.486G>T//p.W162C | classic            | 3+                  | buried       | 1         |
| c.515G>A//p.C172Y | classic            | 3+                  | active site  | 3         |
| c.559A>G//p.M187V | classic            | 3+                  | buried       | 1         |
| c.612G>T//p.W204C | classic            | 3+                  | buried       | 1         |
| c.644A>G//p.N215S | late onset         | 5                   | other        | 16        |
| c.806T>G//p.V269G | classic            | 4                   | buried       | 1         |
| c.860G>C//p.W287S | VUS                | 3+                  | buried       | 1         |
| c.902G>A//p.R301Q | late onset         | 5                   | other        | 3         |
| c.937G>T//p.D313Y | benign             | 2                   | other        | 9         |
| c.973G>A//p.G325S | late onset         | 3+                  | other        | 1         |
| c.1025G>T//p.R342L | classic            | 3                   | buried       | 1         |
| c.1184G>C//p.G395A | likely late onset  | 3                   | other        | 2         |
| c.1196G>C//p.W399S | benign             | 2                   | other        | 1         |
| c.1250T>C//p.L417P | VUS                | 3                   | buried       | 2         |
| c.648T>A//p.Y216* | VUS                | 5                   | NA           | 2         |
| c.934C>T//p.Q312* | classic            | 5                   | NA           | 2         |
| c.1196G>A//p.W399* | classic            | 5                   | NA           | 1         |
| c.290del//p.A977fs*24 | VUS          | 5                   | NA           | 1         |
| c.363del//p.N122fs*8 | classic        | 5                   | NA           | 1         |
| c.568del//p.A190Pfs*2 | classic       | 5                   | NA           | 1         |
| c.757del//p.L253fs*16 | VUS            | 5                   | NA           | 1         |
| c.718_719del//p.K240Efs*9 | classic     | 5                   | NA           | 1         |
| c.927del//p.L310fs*7 | VUS            | 5                   | NA           | 1         |
| c.994dup//p.R332Kfs*7 | classic        | 5                   | NA           | 1         |
| c.1223del//p.N408fs*10 | classic     | 5                   | NA           | 1         |
| c.35_58del//p.C12_A20delinsS | classic  | 3                   | NA           | 1         |
| c.354_368del//p.Q119_Y123del | classic | 3                   | NA           | 1         |
| c.963_964delinsCA//p. Q321_D322delinsHN | classic | 3                   | NA           | 1         |
| c.1072_1074del//p.E358del | classic | 4                   | NA           | 1         |
| c.369+1G>A    | classic            | 5                   | NA           | 1         |
| c.547+1G>A    | classic            | 3+                  | NA           | 1         |
| c.802-3_802-2del | classic            | 3+                  | NA           | 1         |

(Continues)
Also, individual analysis of cardiac septum thickness and heart weight, as well as GFR and albuminuria did not reveal intergroup differences (Figure 4c–f). Similarly, the number of patients with the central nervous system (stroke, TIA) and peripheral nervous system symptoms (FD-associated pain, SFN, hypo-/anhidrosis, elevated thermal thresholds) was not different between groups (Figures 2 and 4g,h). This was also true for the overall clinical scores showing a tendency toward more severe phenotypes in the group of women with the mutated X-chromosome active, where, e.g., no case was found without organ involvement, but 4/11 (36%) women with severe disease burden compared to 7/18 (39%) each in the group of women with the mutated X-chromosome inactive (Figure 2). Patient subgroups did not differ in the number of women who received FD-specific treatment (Figure 2). In Group II, the mutated X-chromosome was active only in the blood sample of one patient, hence, no statistical analysis was possible (Figure 3).

3.5 | Skewed XCI with the mutated X-chromosome active is rare in mouth epithelial cells and skin fibroblasts

In mouth epithelial cells (Group I: 5/75, 7%; Group II: 1/12, 8%) and skin fibroblasts (Group I: 8/22,
### Table 4  Individual XCI patterns

| ID | Genotype | Mouth epithelial cells | Blood | Skin fibroblasts | Number of individually skewed tissues | Clinical score (0 = no symptoms; 1 = mild; 2 = moderate; 3 ≥ severe) |
|----|----------|------------------------|-------|-----------------|---------------------------------------|-------------------------------------------------------------------|
|    |          |                        |       |                 |                                       |                                                                   |
| **Group I** |          |                        |       |                 |                                       |                                                                   |
| FD 1005 | p.N139S  | 52:48                  | 68:32 | –               | 0/2                                   | 2                                                                 |
| FD 1019 | p.R332Kfs*7 | 54:46                  | **82:18 Xi** | –               | 1/2                                   | 3                                                                 |
| FD 1025 | p.G325S  | 72:28                  | 59:41 | –               | 0/2                                   | 2                                                                 |
| FD 1026 | p.A135V  | 29:71                  | 29:71 | –               | 0/2                                   | 1                                                                 |
| FD 1027 | p.A135V  | 21:79 Xa               | 33:67 | **0:100 Xa**    | 2/3                                   | 0                                                                 |
| FD 1032 | p.R342L  | 33:67                  | 23:77 Xi | 34:66          | 1/3                                   | 3                                                                 |
| FD 1035 | p.R342L  | 31:69                  | **24:76 Xi** | –               | 1/2                                   | 3                                                                 |
| FD 1036 | p.W204C  | 48:52                  | 70:30 | –               | 0/2                                   | 2                                                                 |
| FD 1040 | p.N122Ifs*8 | 60:40                  | 54:46 | 48:52           | 0/3                                   | 3                                                                 |
| FD 1044 | p.L129P  | 56:44                  | 52:48 | –               | 0/2                                   | 4                                                                 |
| FD 1045 | p.C52S   | 32:68                  | 57:43 | –               | 0/2                                   | 1                                                                 |
| FD 1048 | p. V269G | –                      | 95:5 Xi | 56:44          | 1/2                                   | 3                                                                 |
| FD 1049 | p.W399*  | 30:70                  | **20:80 Xi** | –               | 1/2                                   | 1                                                                 |
| FD 1056 | p.Q157H  | 59:41                  | 60:40 | –               | 0/2                                   | 3                                                                 |
| FD 1064 | p.N215S  | 45:55                  | **80:20 Xi** | –               | 1/2                                   | 0                                                                 |
| FD 1074 | p.1253Ifs*16 | 39:61                  | 54:46 | 30:70           | 0/3                                   | 2                                                                 |
| FD 1077 | p.W287S  | 36:64                  | 59:41 | **91:9 Xi**     | 1/3                                   | 1                                                                 |
| FD 1086 | p.W162G  | 47:53                  | 58:42 | –               | 0/2                                   | 4                                                                 |
| FD 1087 | p.A135V  | 50:50                  | 32:68 | –               | 0/2                                   | 1                                                                 |
| FD 1088 | p.A135V  | 31:69                  | 30:70 | –               | 0/2                                   | 1                                                                 |
| FD 1090 | p.R332Kfs*7 | 48:52                  | 57:43 | –               | 0/2                                   | 1                                                                 |
| FD 1092 | p.Q321_ D322delinsHN | 43:57                  | 28:72 | –               | 0/2                                   | 3                                                                 |
| FD 1099 | p.K240Ifs*9 | 68:32                  | **81:19 Xi** | 91:9 Xi        | 2/3                                   | 3                                                                 |
| FD 1104 | p.N215S  | 74:26                  | **75:25 Xi** | 94:6 Xi        | 2/3                                   | 2                                                                 |
| FD 1110 | p.Y216*  | 41:59                  | 52:48 | –               | 0/2                                   | 1                                                                 |
| FD 1111 | p.Y216*  | 43:57                  | 52:48 | –               | 0/2                                   | 1                                                                 |
| FD 1113 | p.N215S  | 47:53                  | 35:65 | –               | 0/2                                   | 0                                                                 |
| FD 1125 | p.R301Q  | 56:44                  | **21:79 Xi** | –               | 1/2                                   | 0                                                                 |
| FD 1127 | p.A135V  | 34:66                  | **298 Xa**     | –               | 1/2                                   | 3                                                                 |
| FD 1129 | p.D136E  | 24:76 Xi               | 46:54 | –               | 1/2                                   | 1                                                                 |
| FD 1131 | p.L417P  | 52:48                  | **24:76 Xi**     | –               | 1/2                                   | 3                                                                 |
| FD 1132 | p.L417P  | 49:51                  | 41:59 | –               | 0/2                                   | 3                                                                 |
| FD 1136 | c.369 +1G > A | 37:63                  | 45:55 | –               | 0/2                                   | 0                                                                 |
| FD 1139 | p.E358del | 39:61                  | 70:30 | 30:70           | 0/3                                   | 3                                                                 |
| FD 1141 | p.N215S  | 29:71                  | 27:73 | –               | 0/2                                   | 2                                                                 |
| FD 1146 | p.N215S  | 44:56                  | 60:40 | –               | 0/2                                   | 0                                                                 |
| FD 1152 | p.A190Pfs*2 | 41:59                  | 36:64 | 26:74           | 0/3                                   | 2                                                                 |
| FD 1156 | p.R112C  | 53:47                  | 43:57 | –               | 0/2                                   | 3                                                                 |
| FD 1159 | p.M187V  | 42:58                  | 29:71 | –               | 0/2                                   | 1                                                                 |
| FD 1160 | p.N215S  | 59:41                  | **92:8 Xi**     | 65:35          | 1/3                                   | 1                                                                 |

(Continues)
| ID       | Genotype       | Mouth epithelial cells | Blood  | Skin fibroblasts | Number of individually skewed tissues | Clinical score (0 = no symptoms; 1 = mild; 2 = moderate; 3 ≥ severe) |
|----------|----------------|------------------------|--------|------------------|----------------------------------------|-------------------------------------------------|
| FD 1162  | p.Q312*        | 50:50                  | 46:54  | –                | 0/2                                    | 1                                               |
| FD 1163  | p.Q312*        | 47:53                  | 46:54  | –                | 0/2                                    | 2                                               |
| FD 1164  | p.C172Y        | 52:48                  | 49:51  | –                | 0/2                                    | 0                                               |
| FD 1166  | p.A977fs*24    | 38:62                  | 53:47  | –                | 0/2                                    | 1                                               |
| FD 1168  | p.N215S        | 46:54                  | 44:56  | –                | 0/2                                    | 0                                               |
| FD 1174  | p.G395A        | 60:40                  | 34:66  | 2:98 Xi          | 1/3                                    | 1                                               |
| FD 1175  | p.N215S        | 30:70                  | 22:78 Xi | –             | 1/2                                    | 1                                               |
| FD 1176  | p.Q119_Y123del | 39:61                  | 59:41  | 26:74 Xi         | 0/3                                    | 2                                               |
| FD 1182  | p.D136E        | 83:17 Xi               | 98:2 Xi | –                | 2/2                                    | 2                                               |
| FD 1185  | p.N215S        | 75:25 Xi               | 87:13 Xi | 96:4 Xi       | 3/3                                    | 0                                               |
| FD 1189  | p.R301Q        | 68:32                  | 87:13 Xa | –            | 1/2                                    | 1                                               |
| FD 1190  | p.R342L        | 35:65                  | 17:83 Xa | 57:43         | 1/3                                    | 2                                               |
| FD 1193  | c.547 + 1G > A | 55:45                  | 76:24 Xa | –             | 1/2                                    | 4                                               |
| FD 1196  | p.N408Ifs*10   | 68:32                  | 83:17 Xa | –            | 1/2                                    | 4                                               |
| FD 1198  | p.C63Y         | 50:50                  | 35:65  | –                | 0/2                                    | 2                                               |
| FD 1200  | p.N408Ifs*10   | 39:61                  | 15:85 Xa | –            | 1/2                                    | 2                                               |
| FD 1202  | p.N215S        | 58:42                  | 10:90 Xa | –            | 1/2                                    | 1                                               |
| FD 1203  | p.R301Q        | 39:61                  | 50:50  | –                | 0/2                                    | 0                                               |
| FD 1204  | p.G395A        | 31:69                  | 4:96 Xi | 13:87 Xi        | 2/3                                    | 0                                               |
| FD 1207  | p.N215S        | 54:46                  | 85:15 Xa | –            | 1/2                                    | 3                                               |
| FD 1209  | p.C172Y        | 44:56                  | 39:61  | –                | 0/2                                    | 2                                               |
| FD 1211  | p.C172Y        | 37:63                  | 28:72  | –                | 0/2                                    | 1                                               |
| FD 1212  | p.N215S        | 37:63                  | 6:94 Xi | –                | 1/2                                    | 3                                               |
| FD 1213  | p.I117S        | 23:77 Xa               | 16:84 Xa | 53:47         | 2/2                                    | 2                                               |
| FD 1214  | p.L310Ifs*7    | 52:48                  | 45:55  | 40:60           | 0/3                                    | 0                                               |
| FD 1216  | p.N215S        | 36:64                  | 59:55 Xa | 79:21 Xi     | 2/3                                    | 1                                               |
| FD 1217  | p.H46R         | 42:58                  | 34:66  | 32:68           | 0/3                                    | 2                                               |
| FD 1218  | p.W162C        | 51:49                  | 50:50  | –                | 0/2                                    | 0                                               |
| FD 1219  | p.R332Kfs*7    | 66:34                  | 63:37  | –                | 0/2                                    | 2                                               |
| FD 1220  | p.N215S        | 34:66                  | 30:70  | –                | 0/2                                    | 1                                               |
| FD 1222  | p.L253Lfs*16   | 40:60                  | 40:60  | 74:26           | 0/3                                    | 2                                               |
| FD 1223  | p.C63Y         | 36:64                  | 20:80 Xi | –            | 1/2                                    | 0                                               |
| FD 1225  | p.N215S        | 47:53                  | 76:24 Xa | –            | 1/2                                    | 1                                               |
| FD 1228  | p.N215S        | 44:56                  | 63:37  | –                | 0/2                                    | 0                                               |
| FD 1230  | p.I253Lfs*16   | 53:47                  | 77:23 Xi | –            | 1/2                                    | 2                                               |
| FD 1234  | p.C12_A20delinsS | 37:63                  | 46:54  | 57:43           | 0/3                                    | 2                                               |

**Group II**

| ID       | Genotype       | Mouth epithelial cells | Blood  | Skin fibroblasts | Number of individually skewed tissues | Clinical score (0 = no symptoms; 1 = mild; 2 = moderate; 3 ≥ severe) |
|----------|----------------|------------------------|--------|------------------|----------------------------------------|-------------------------------------------------|
| FD 1003  | p.A143T        | 54:46                  | 45:55  | –                | 0/2                                    | 1                                               |
| FD 1053  | p.D313Y        | 42:58                  | 38:62  | –                | 0/2                                    | 0                                               |
| FD 1085  | p.A143T        | 44:56                  | 38:62  | 38:62           | 0/3                                    | 2                                               |
| FD 1098  | p.D313Y        | 49:51                  | 66:34  | 73:27           | 0/3                                    | 0                                               |
| FD 1114  | p.A143T        | 57:43                  | 49:51  | –                | 0/2                                    | 1                                               |
| FD 1135  | p.A143T        | 54:46                  | 73:27  | –                | 0/2                                    | 1                                               |
Table 4 (Continued)

| ID   | Genotype  | Mouth epithelial cells | Blood | Skin fibroblasts | Number of individually skewed tissues | Clinical score (0 = no symptoms; 1 = mild; 2 = moderate; 3 ≥ severe) |
|------|-----------|------------------------|-------|------------------|---------------------------------------|-------------------------------------------------------------------|
| FD 1147 | p.D313Y | 54:46                  | 51:49 | –                | 0/2                                   | 1                                                                  |
| FD 1188 | p.A143T | 48:52                  | 76:24 Xi | 45:55          | 1/3                                   | 0                                                                  |
| FD 1194 | p.D313Y | 47:53                  | 44:56 | –                | 0/2                                   | 1                                                                  |
| FD 1201 | p.A143T | 62:38                  | 47:53 | –                | 0/2                                   | 1                                                                  |
| FD 1232 | p.D313Y | 25:75 Xa               | 18:82 Xi | 95:5 Xi         | 3/3                                   | 2b                                                                 |

Note: Bold XCI shows skewing.

Abbreviations: FD, Fabry disease; ID, identification number; Xa, mutated X-chromosome active; Xi, mutated X-chromosome inactive; “–”, no sample available.

a Patient with chronic widespread pain and cerebral infarction.

b Patient with nephropathy due to arterial hypertension and chronic pain syndrome. Kidney biopsy without GB3 depositions.

36%; Group II: 1/5, 20%), the number of women with skewed XCI was lower compared to blood samples. Also, the mutated X-chromosome was active only in a few women (mouth epithelial cells: Group I: 2/5, 40%; Group II: 1/1, 100%; skin fibroblasts: Group I: 1/8, 13%; Group II: 0/1, 0%) (Figure 1; Supplementary Figures S1–S4). Hence, correlation analysis with the clinical phenotype was not possible. Table 4 gives a synopsis of the clinical phenotype related to patients' genotype and XCI status.

3.6 | XCI patterns remain stable for at least 2 years

To answer the question, if female XCI patterns change over time, we assessed ≥2 biosamples from one tissue of 49 patients collected at two time points with a median latency of 1 year (range 0.2–3.0). Overall, we collected 42 mouth epithelial cell samples obtained from 21/95 (22%) women and 60 blood samples obtained from 28/95 (29%) women. After a median latency of 0.6 years (range

![XCI patterns in blood](image1)

**Figure 2** XCI patterns and clinical phenotype based on stratification via blood samples in group I. Group I: women carrying potentially pathogenic variants in the alpha-galactosidase A gene. ERT, enzyme replacement therapy; GAL, alpha-galactosidase A; SFN, small fiber neuropathy; TIA, transient ischemic attack; XCI, X-chromosomal inactivation.
between the first and second mouth epithelial sample collection, the median difference of the XCR was 6% (range 0–19; \( \rho = 0.86, p < .001 \)) (Figure 5). After a median of 1.6 years (range 0.2–3.0) upon first blood sample collection, XCR also showed a median shift of 6% (range 0–25%; \( \rho = 0.91, p < .001 \)) (Figure 5). Analysis of 65 technical replicates from 29/95 (31%) patients (i.e., a second or third analysis of the XCI pattern from the same sample) revealed robust results with a median shift in XCR of 5% (range 0–48; \( \rho = 0.87, p < .001 \)) (Figure 6). Hence, the used method shows high reliability considering the technical replicates. This enables the individual and inter-individual comparison of the XCR. Also, taking this fact into account, the XCR in biological replicates remains stable over time without relevant changes in the XCI.

4 | DISCUSSION

We have investigated XCI patterns related to the activation status of the mutated X-chromosome in a clinically well-characterized, single-center cohort of women carrying genetic variants in the \( \text{GLA} \) gene and using DNA from three different tissues and additionally determining the activation status of the mutated allele. While venous blood was most informative, no correlation was found between the XCI patterns and patients’ clinical phenotype. Also, GAL activity and lyso-Gb3 levels did not correlate with the female XCI pattern.

Although of X-chromosomal inheritance, women with FD may reach every level of disease severity, and the reason for this is unknown. XCI is a mechanism that may influence disease penetrance, which was already shown for Duchenne muscular dystrophy and hemophilia A and B (Garagiola et al., 2021; Mercier et al., 2013). In previous studies, XCI patterns were also investigated in women with FD giving contradictory results. While some studies found data suggesting a role of XCI in symptom severity of women with FD (Dobrovolny et al., 2005; Echevarria et al., 2016; Hossain et al., 2019), others did not (Elstein et al., 2012; Juchniewicz et al., 2018; Rossanti et al., 2021; Viggiano & Politano, 2021).

In a previous study, XCR determined in blood and skin fibroblasts correlated with symptom severity scores in FD.

![Figure 3](image1.png)

**FIGURE 3** XCI patterns and clinical phenotype based on stratification via blood in group II. Group II: women carrying variants in the alpha-galactosidase A gene currently classified apathogenic. ERT, enzyme replacement therapy; GAL, alpha-galactosidase A; SFN, small fiber neuropathy; TIA, transient ischemic attack; XCI, X-chromosomal inactivation.

![Figure 4](image2.png)

**FIGURE 4** Inter-group comparison stratified for XCI patterns in group I and based on blood. Scatter plots illustrate the distribution of alpha-galactosidase A activity (a), lyso-Gb3 levels (b), cardiac septum thickness (c), heart weight (d), glomerular filtration rate (e), albumin/creatinine ratio (f), cold detection thresholds (g), and warm detection thresholds (h) between women with skewed XCI and the mutated X-chromosome active or inactive and a random XCI pattern. CDT, cold detection threshold; GAL, alpha-galactosidase A; Gb3, globotriaosylceramide; GFR, glomerular filtration rate; WDT, warm detection threshold.
(Dobrovolny et al., 2005). Similar results were reported in a study investigating XCI patterns in mouth epithelial cells, blood, urine, and skin fibroblasts (Echevarria et al., 2016). However, data cannot be compared directly, since Echevarria et al. (2016) defined a “skewed XCI pattern” when more than one tissue showed skewed XCR. We did not find a correlation between the individual XCI patterns of FD women and symptom severity in line with several other studies (Elstein et al., 2012; Juchniewicz et al., 2018; Rossanti et al., 2021), albeit the number of investigated FD women was small in these studies (n = 12 and n = 9) and a skewed XCI pattern was found only in one of these patients (Juchniewicz et al., 2018; Rossanti et al., 2021). Further, without determining whether the mutated or the healthy allele is active (Elstein et al., 2012), data interpretation is not possible. In our study, blood was the biomaterial most frequently revealing skewed XCI. Skin fibroblasts gave similar results, but biological relevance remains obscure due to the low number of skin punch biopsies available. Mouth epithelial cells were obtained from almost the entire study cohort, however, XCI patterns were mostly not skewed. This diversity of XCI patterns found in different cell types is a known phenomenon (Zito et al., 2019), the reason for which is elusive.

Our data show the relevance of genetic subclassification in clinical practice: women carrying classic, class 3+ and higher, and the active site or buried mutations (Arends et al., 2017; Rickert et al., 2020) also had a more severe clinical phenotype (Figure 2, Tables 1 and 4). However, XCI patterns did not show additive effects in our cohort.

One crucial question is if XCR individually remains stable over time. The biological replicates we investigated showed XCR stability over 2 years, which was the longest period possible in our study (Figure 5). Assessment of XCI patterns in healthy women revealed that the rate of skewing rises from 16% to 38% between the age of ≤32 to ≥60 years (Busque et al., 1996). In another study, a skewing ratio of approximately 40% was described in healthy aging women when assessed in blood samples (Busque et al., 2009). Also, high skewing rates were more frequent...
in blood samples obtained from women ≥60 years (Sharp et al., 2000). Hence, prospective and long-term studies are needed to answer the question of at which age and after which interval XCI pattern analysis would be most informative.

Our data further show that the method used to determine XCI patterns results in similar XCR within a median difference of 5% when repeatedly analyzing the same bio-sample (Figure 6). This enables the comparison of samples collected from one patient between the same and other tissues and the comparison with the XCR among all patients. Our results are in line with those of a previous study which determined repeatability of 3% for the assay used (Busque et al., 2009).

Another question is if XCI patterns differ between biomaterials. We found that the XCI pattern was not different between mouth epithelial cells, blood, and skin fibroblast samples considering the classification in skewed and random XCI in approximately 60–70% cases (data not shown), which is in line with data from previous studies (Dobrovolny et al., 2005; Echevarria et al., 2016; Rossanti et al., 2021). Also, the incidence of random XCI patterns found in blood and skin fibroblast samples reported in other studies is similar to ours (70% versus 65–67%) (Dobrovolny et al., 2005; Echevarria et al., 2016; Elstein et al., 2012). When comparing XCI patterns in all three tissues, we found a deviation to skewing mainly in blood samples. The median age of the subpopulation of women with skewed XCI between the tissues (36/95, 38%) was 57 years (range 26–74), which fits data of a previous study investigating XCI patterns of healthy women: here, a higher frequency of skewed XCI was found at >60 years and also larger inter-tissue deviations of XCI patterns were described with aging (Sharp et al., 2000).

Further, it is crucial to determine the X-chromosome that carries the mutated allele to conclude the usefulness of XCI patterns in clinical practice. This was also achieved in some previous studies through comparison with relatives (Dobrovolny et al., 2005; Echevarria et al., 2016; Juchniewicz et al., 2018). We and others used biomaterial that is easily available but not primarily affected by FD such as heart, kidneys, and nervous tissue. Hence, it remains elusive if and to which extent results obtained in mouth epithelial cells, blood, and fibroblasts can reflect disease severity. Another crucial issue to be considered is that tissues from different embryonic layers can have different X-inactivation patterns (Viggiano et al., 2013). Further, the phenomenon that some genes may “escape” X-chromosomal inactivation needs attention, since this might also contribute to the diversity in clinical phenotypes of women with FD (Carrel & Willard, 2005).

One limitation of our study is that we could not apply validated clinical scoring systems such as the Mainz Severity Score Index (MSSI) due to missing data, since the majority of patients were recruited during follow-up visits that included a focused investigation program. Another limitation of our study is that we used a scoring system that does not reflect mono- versus multiorgan involvement. Also, our patient cohort includes variants of FD with organ-specific clinical phenotypes such as N215S, which may have influenced the clinical composite score used. However, we have also investigated organ-specific parameters and believe that the impact on the overall results is minor if any. Approximately half of the study population was on FD-specific treatment, which we could not stop for our study for ethical reasons and which may have contributed to modify the clinical phenotype.

We show that blood as an easily available biomaterial most frequently reflects skewed XCI patterns in women carrying genetic variants in GLA, while these patterns do not correlate with disease severity. We conclude that further studies investigating larger patient cohorts including longitudinal investigation, and using biomaterial obtained from organs primarily affected by FD are needed to understand the pathophysiological role XCI patterns may play in the disease development of women with FD.

AUTHOR CONTRIBUTIONS
Nurcan Üçeyler and Simone Rost created the study concept and research design. Laura Wagenhäuser collected data and performed analysis. Vanessa Rickert collected data. Laura Wagenhäuser, Simone Rost, Nurcan Üçeyler performed analysis and data interpretation. Nurcan Üçeyler, Claudia Sommer, Christoph Wanner, Peter Nordbeck performed patient examination. Nurcan Üçeyler, Laura Wagenhäuser, Simone Rost wrote the manuscript. All authors reviewed the manuscript and approved the final version of the paper.

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CONFLICT OF INTEREST
The authors declare the following conflicts of interest: L.W., V.R., and S.R. report no conflicts of interest. C.S. has received honoraria for lectures from Amicus and Takeda. P.N. has received travel grants and honoraria for lectures from Amicus Therapeutics, Chiesi, Idorsia, Greenovation, Sanofi Genzyme, and Takeda Shire, and research funds from Amicus Therapeutics, Idorsia Sanofi Genzyme, and Takeda Shire. CW has received honoraria from Amicus, Chiesi, Idorsia, Sanofi-Genzyme, and Shire-Takeda for advisory board activities and lecturing. N.Ü. has received travel grants and honoraria for lectures from Sanofi Genzyme and Takeda Shire, and research funds from Sanofi Genzyme, Takeda Shire, and Idorsia.

DATA AVAILABILITY STATEMENT
Data are available upon request from the corresponding author. Data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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