Evaluation of X chromosome inactivation in endemic Tunisian pemphigus foliaceus

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Abstract

Background: Almost 5% of the world’s population develops an autoimmune disease (AID), it is considered the fourth leading cause of disability for women, who represent 78% of cases. The sex ratio when it comes to the most prevalent AID varies from 9:1 in systemic lupus erythematosus (SLE) to 13:1 in endemic Tunisian pemphigus foliaceus (PF).

Methods: To test the potential involvement of skewed x-inactivation in the pathogenesis of Tunisian PF, we analyzed the methylation status of a highly polymorphic CAG repeat in the androgen receptor gene and evaluated the x chromosome inactivation (XCI) patterns in peripheral blood-leukocyte-derived DNA samples of female patients with PF (n = 98) compared to healthy control (HC) subjects (n = 150), as well as female patients with SLE (n = 98) were enrolled as a reference group.

Results: XCI status was informative for 50 of the 98 PF patients (51%) and 70 of the 150 HC women (47%). Extremely skewed XCI patterns were more frequent in PF and SLE women than HC, but the difference was statistically significant only in women with SLE. No statistical difference was observed in XCI patterns between PF and SLE patients. PF phenotype-XCI correlation analysis revealed that (i) skewed XCI patterns may be involved in the disease’s subtype and (ii) it was more pronounced in the endemic group than the sporadic one. Furthermore, preferential XCI showed an increase in heterozygote genotypes of PF’s susceptibility polymorphisms in immunity-related X genes (FOXP3, AR, and TLR7) in PF patients compared to HC.

Conclusion: Our results suggest that skewed XCI could lead to hemizygosity of X-linked alleles that might unmask X-linked deleterious alleles.

KEYWORDS
autoimmune disease, pemphigus foliaceus, X chromosome inactivation
1 | INTRODUCTION

Pemphigus foliaceus (PF) is a common life-threatening autoimmune blistering skin disease mediated by anti-desmoglein1 (Dsg1) autoantibodies that are responsible for the intraepidermal formation of blisters through the loss of keratinocyte adhesion, the so-called acantholysis process (Waschke et al., 2005).

Previous data suggest that common genetic factors contribute to PF pathogenesis. Furthermore, the geographic distribution of pemphigus is very uneven, suggesting the existence of certain environmental factors favoring the disease. There are two forms of PF; a non-endemic form, that occurs throughout the world, and an endemic form reported in Brazil (Diaz et al., 1989), Peru, Colombia (Robledo et al., 1988), and Tunisia (Bastuji-Garin et al., 1995; Morini, 1993). Both endemic and non-endemic forms are clinically, histologically, and immunologically similar, but the endemic form has several unique epidemiological features. The common epidemiological feature is that patients with the endemic forms of the disease live in poor rural areas with modest socio-economic conditions. In Brazil, the incidence is 34 cases/million/year. Young adults and children are typically affected by the disease with no sex or racial predisposition. Interestingly, Brazilian women with Fogo Selvagem have a significant increase in PF susceptibility polymorphisms and are also more prone to develop generalized lesions and show resistance to disease remission (Salviano-Silva et al., 2017). In contrast, men account for 95% of patients in the El Bagre region of northern Colombia. Accumulated evidence suggests that PF occurs in two forms in Tunisia: sporadic and endemic, which occurs primarily in the southern regions and has a distinct genetic background and epidemiological features distinct from the sporadic form (Abida, Kallel-Sellami, et al., 2009; Abida, Masmoudi, et al., 2009; Abida, Zitouni, et al., 2009). Thus, in the southern Tunisian regions, PF spares children and affects predominately young adult women, with an average age of onset of 25–34 years and an annual incidence of 20 cases per million (Bastuji-Garin et al., 1995).

While genetic and hormonal factors may explain part of the female predominance of Tunisian endemic PF, there is still a need to define the gender-related factors that determine susceptibility (Ben Jmaa et al., 2017; Masmoudi et al., 2019; Toumi et al., 2016), this leads to the common question of defining the gender-related factors that influence susceptibility to PF.

Numerous factors have been reported contributing to the striking female predominance in autoimmune diseases (AID), including sex hormone influence, fetal microchimerism, X chromosome inactivation (XCI), and X chromosome abnormalities (Nussinovitch & Shoenfeld, 2012). Then, abnormalities in epigenetic processes, such as preferential XCI, have been reported in many female pre-dominant AIDS. X-inactivation is an epigenetic dosage mechanism in female cells via transcriptional silencing of one copy of a gene, where the paternally derived or the maternally derived X chromosome is randomly silenced during the early stages of embryogenesis (Lee, 2011). Preferential and skewed XCI are deviations from the 50:50 ratio of the cells inactivating the same X chromosome (Lambert, 2009). This deviation is thought to be the result of (i) genetic factors directly involved in the process of XCI, (ii) genetic defects (mutations, rearrangements, etc.) on the X chromosome leading to a selective process, (iii) aging-related tendency toward the monoclonal expansion of cells, or (iv) pure chance, due to the stochastic nature of the choice of which X chromosome to inactivate (Brown & Robinson, 2000; Puck & Willard, 1998). The most widely accepted explanation for biased XCI in autoimmunity is a mechanism through which loss of mosaicism has the potential to make X-linked self-antigens escape presentation in the thymus, leading to the breakdown of tolerance and causing the development of AID (Stewart, 1998).

To test the potential involvement of skewed X-inactivation in the pathogenesis of Tunisian PF, we analyzed the methylation status of a highly polymorphic CAG repeat in the androgen receptor gene (AR#OMIM 313700) and we evaluated the X-inactivation patterns in peripheral blood leukocyte-derived DNA samples of female patients with PF.

2 | MATERIALS AND METHODS

The research protocol was approved by the human research board of the ethics committee of the Habib Bourguiba University Hospital (protocol number of the ethics committee, 4/12).

2.1 | Subjects

A total of 98 patients were recruited at the Department of Dermatology of the Hedi Chaker University Hospital (Sfax, Tunisia). They were diagnosed as PF patients based on clinical presentation, histopathology, immunofluorescence, and enzyme-linked immunosorbent assay (Figure 1). The clinical presentation of PF was complemented with histopathology showing intraepidermal acantholysis and direct immunofluorescence of perilesional skin showing IgG and/or C3 deposits at the surface of keratinocytes.
To evaluate XCI patterns in AID other than PF, 98 women with Systemic Lupus Erythematosus (SLE) with a sex ratio of 9:1 similar to PF were also tested (Figure 1).

SLE patients were recruited at the Internal Medicine Department of the Hedi Chaker University Hospital, and all of them met at least four of the American College of Rheumatology (ACR) revised criteria for SLE.

A total of 150 healthy controls (HC) were matched by age (±5 years), sex, and geographical origin to patients who did not suffer from any autoimmune or inflammatory disease and were also enrolled.

2.2 | DNA extraction

After obtaining informed consent from the subjects, peripheral blood samples were taken in tubes containing EDTA-2Na. Genomic DNA was extracted from the blood samples using a standard proteinase K digestion and phenol/chloroform extraction procedure. After ethanol precipitation, DNA was dissolved and stored at −80°C.

2.3 | X chromosome inactivation assay

Assays currently used for determining X-inactivation patterns are indirect and distinguish maternal X chromosomes from the paternal by leveraging informative polymorphisms such as short tandem repeats (STR). The most extensively used method for evaluating XCI patterns is based on the assessment of differential DNA methylation of CpG dinucleotides between active (Xa) and inactive(Xi) X-chromosomes (Kutsche & Brown, 2000). We performed the human androgen-receptor X-inactivation assay as previously described (Allen et al., 1992; Uehara et al., 2001). The assay takes advantage of a highly polymorphic (CAG)_n repeat in the first exon of the androgen receptor gene X-linked gene located near several methylation-sensitive restriction enzyme sites that are differentially methylated on the Xa and Xi chromosomes. Briefly, to assess the differential methylation, samples are first treated with sodium bisulfite, and then PCR-amplified. After analysis of XCI patterns in women with PF and SLE, the incidence of preferential XCI and XCI patterns were compared with
those in control women and were also correlated with PF demographic stratification and clinical features.

2.3.1 | Heterozygosity selection

DNA samples (1 ng) were then amplified by PCR with primers flanking the polymorphic androgen receptor (AR) CAG repeat (Table 1). The forward primer was labeled with 6-FAM fluorescent labels. The fast-cycling master mix (Fast Cycling PCR Kit, QIAGEN) was used to amplify DNA, and the PCR products were separated by capillary electrophoresis on an ABI Prism 3100 Genetic Analyzer (Perkin-Elmer, CT, USA) and analyzed using Gene Scan software. Individuals for whom paternally derived and maternally derived AR gene alleles could not be distinguished (CAG repeat ≤ 2) were not included in the analysis and considered non-informative.

2.3.2 | Bisulfite modification of DNA

Since differential methylation can be assessed more precisely by the bisulfite method as compared to methylation-specific endonuclease methods (enzyme treatment results in incomplete digestion of DNA). Briefly, 100 ng of each female genomic DNA sample was treated with sodium bisulfite according to the manufacturer’s instructions (Cells-to-CpG™ Bisulfite Conversion Kit, Life technology). The area sizes of the heterozygous two-band peaks were measured using GeneScan Analysis software, and then the size ratios of the two-band peaks were calculated. Evaluation of XCI based on the differences in the size ratio of the heterozygous two-peak patterns indicates unequal methylation of the biparental X chromosomes. The percent XCI was calculated with the following formula: “percent inactivation = allele A peak height/(allele A peak height + allele B peak height) × 100”.

XCI skewing was typically defined as a ratio ≥ of 75–25 of cells inactivating the same X chromosome. Extreme skewed is a bias of ≥90. Testing for skewing is typically done using peripheral blood cells.

2.3.3 | Evaluation of XCI

The methods of PCR amplification and band pattern analysis were the same as described previously (Uehara et al., 2001). Patterns of XCI can be evaluated by assessing the differential methylation between active and inactive X-chromosomes. Because cytosines are altered to uracils by sodium bisulfite, whereas methyl cytosines are not, PCR products can be amplified using primers specific for methylated vs. unmethylated AR alleles after bisulfite treatment and the methylation status can be determined (Table 1).

After bisulfite modification, DNA samples (50 ng) were subjected to both M-PCR and U-PCR, each in a total volume of 10 µl including 1x the fast-cycling master mix containing a HotStar Taq Plus DNA Polymerase (Fast Cycling PCR Kit, QIAGEN) added with 5 µmol of each primer (Invitrogen, CA, USA). Forward primers were fluorescently labeled with 6-FAM. The primer sequences are shown in Table 1. PCR reactions were performed in a thermal cycler (Simpli Amp Thermal Cycler, Thermo Fisher). Reactions were cycled 40 times with denaturation at 95°C for 5 s, hybridization for 5 s, and extension at 68°C for 5 s, according to manufacturer instructions.

The area sizes of the heterozygous two-band peaks were measured using GeneScan Analysis software, and then the size ratios of the two-band peaks were calculated. Evaluation of XCI based on the differences in the size ratio of the heterozygous two-peak patterns indicates unequal methylation of the biparental X chromosomes. The percent XCI was calculated with the following formula: “percent inactivation = allele A peak height/(allele A peak height + allele B peak height) × 100”.

XCI skewing was typically defined as a ratio ≥ of 75–25 of cells inactivating the same X chromosome. Extreme skewed is a bias of ≥90. Testing for skewing is typically done using peripheral blood cells.

2.4 | Statistical analyses

All study data were stored in a standard EXCEL database. All statistical analyses were performed using SPSS version 20.0 for Windows (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean (x) ± SD. Categorical variables were expressed as frequencies and percentages (n, %). Baseline characteristics were analyzed by chi-square test or Fisher exact tests, if appropriate. The Mann–Whitney U-test and Kruskal–Wallis, non-parametrical multiple comparison tests were applied for

| Table 1 | Primers flanking the polymorphic androgen receptor CAG repeat |
|---------|---------------------------------------------------------------|
| **Primer pair sequence** | **Annealing temperature** |
| For heterozygote selection | Forward GCGAGCGAGCACCTCCCGCCG² Reverse CAGGTAGCCTGTGGGGGCTCTACGT |
| | 59 |
| For XCI evaluation | M-PCR AR-M forward GCGAGCGTAGATTTCGC³ AR-M reverse AACCAAAATACCTATAAAAACCTCTACG |
| | U-PCR AR-U forward GTTGTAGTGATAGTTTTGGT³ AR-U reverse CAAATAACCTATAAAAACCTCTACA |
| | 58.9 54.6 |
| ²6-FAM- labeled primers. The primer pairs were designed to amplify the same region of the androgen receptor gene exon 1 (Uehara et al., 2001).
statistical analyses of the continuous XCI patterns for the experimental groups. \( p \leq .05 \) was considered statistically significant.

3 | RESULTS

3.1 | Sampling

The PF patient group enrolled 98 women, with a mean age of 35 years, ranging from 18 years to 84 years. All the patients were from the center and south of Tunisia. The HC group enrolled 150 women, with a mean age of 39 years, ranging from 14 years to 73 years. Ninety-eight women with SLE were also enrolled (Figure 1). Patients were matched to HC in age (\( p > .05 \)).

3.2 | Heterozygotes incidence

Among the 346 female subjects included in the study, a total of 50 women with PF (51%), 59 women with SLE (60%), and 70 healthy women (47%) were informative for the XCI assay and were considered in subsequent analyses (Table 2).

3.3 | Incidence of preferential XCI

Overall, XCI pattern distributions differed among women with PF and SLE when compared to healthy women (\( p \) values were, respectively, .2 and .042 (X2 test)) (Table 2). Extremely skewed XCI patterns were more frequent in PF and SLE women versus HC, but the difference was statistically significant only in women with SLE (respectively, 12% [6/50], 20% [12/59] versus 6% [4/70] with \( p \)-values of .2 and .012; respectively (X2 test)). XCI pattern did not differ between the SLE and PF women (\( p = .83 \), X2 test), as random, mildly skewed, and skewed patterns were present in 29 (50%), 18 (30%), and 12 (20%) of the 59 SLE patients assayed for XCI.

When modeling XCI patterns through a continuous approach, no statistical difference was observed in XCI patterns between women with PF (0.69 ± 0.13) and SLE (0.7 ± 0.15) patients compared to healthy women (0.68 ± 0.13) (\( p = .9 \) and .6; respectively, K–S). No statistical difference was observed in XCI patterns between PF and SLE patients (\( p = .8 \); X2 test and \( p = .86 \), K–S).

3.4 | PF epidemiological features-XCI correlation

Phenotypic features of PF patients are shown in Table 3. The PF patients' stratification based on the epidemi-demographic features of the disease in our country revealed many interesting results. Overall, XCI patterns differed between patient groups and their respective HC without reaching the significance level. Differences were even more pronounced when comparing frequencies of extremely skewed XCI patterns (90:10) and mildly skewed (80:20–89:11). Indeed, in women living in endemic versus sporadic localities of the disease, the incidence of Skewed XCI was 16.7% (3/18) and 9.4% (3/32); respectively, values that were much higher than those of their respective control groups (6.3% and 5.3%, respectively).

On the other hand, 10.7% (3/28) of PF women with an age of onset under 35 years and 13.7% (3/22) of PF women with an age of onset above 35 years, compared to only 3.7% and 4.9% among their respective HC women, have skewed XCI (\( p = .2 \) and .4; respectively; Fisher exact test). On the other hand, the incidence of mildly skewed tends to increase in patients, and inversely, the random XCI was more frequent among HC compared to the patients' groups.

When modeling XCI patterns through a continuous approach, similar XCI patterns were observed in different PF patient stratification groups compared to healthy women (Table 3).

3.5 | PF clinical features-XCI correlation

Clinical classification data for 42 PF patients were available: (8/42) pemphigus erythematosus, (28/42) the more disseminated type referred to as generalized Pemphigus, and (6/42) pemphigus herpetiformis with anti-Dsg1 auto-Abs.

| Subjects groups | No. of heterozygotes (%) | Extreme skewing (90:10–100:0) (%) | Mildly skewed (75:25–89:11) (%) | Random (50:50–74:26) (%) | \( p \)-value |
|-----------------|-------------------------|-------------------------------|--------------------------------|------------------------|----------|
| PF (\( n = 98 \)) | 50 (51%)                | 6 (12)                        | 22 (44)                        | 22 (44)                | .2       |
| SLE (\( n = 98 \)) | 59 (60%)                | 12 (20)\( ^a \)               | 18 (30)                        | 29 (50)                | .042     |
| HC (\( n = 150 \)) | 70 (47%)                | 4 (6)                         | 25 (36)                        | 41 (58)                | –        |

\( ^a \)SLE patients exhibited significant extreme skewing XCI than HC (\( p = .01 \)).
XCI pattern differed between the different clinical forms of PF without reaching significance ($p = .1$, $\chi^2$ test), (i) as extremely skewed patterns were present in 4 (14.3%) of generalized form, 1 (16.7%) of herpetiformis form, and absent in the erythematosus clinical type, and (ii) as mildly skewed patterns were more frequent in erythematosus form (75%) compared to 42.9% (12/28) and 16.7% (1/6) in patients with generalized and herpetiformis types; respectively (Table 3).

3.6 X linked genes-XCI correlation

AR gene has not been studied in PF disease. We, therefore, compared the AR repeat length in control individuals and patients with PF. Patients and healthy subjects had AR CAG lengths that spanned the range from 9 to 29 (supplementary Table S1). The CAG22 and the CAG27 alleles were more expressed in PF patients (2.8% and 0.9%) compared to their matched HC (0.6% and 0%; respectively), in contrast to the CAG11 and the CAG20 alleles, which were significantly overexpressed in controls (4.4% with $p = .0017$, OR = 0.05, 95% CI 0.01–0.39 and 7.3% with $p = .0024$, OR = 0.36, 95% CI 0.15–0.91) compared to patients (0% and 2.8%; respectively) (supplementary Table S1). No difference was observed in the average length of the AR exon 1 CAG repeat between the HC group (16.25) and the PF subjects (16.38). To complement the allelic analysis, we classified the subject’s populations according to a dichotomous variable using the median (CAG repeat length-16) as a cut point. The SS, S/L, and L/L classes were similar in HC (27.5%, 50%, and 22.5%; respectively) than in PF patients (34.5%, 44.5%, and 20.5%; respectively).

Secondly, we tested whether the $AR$ CAG repeat length could be related to differences in clinical and/or in the characteristic and specific epidemiological features of Tunisian PF (supplementary Table S2). Women patients with the disseminated form exhibited significantly the heterozygote CAG repeat genotype (SL genotype) (57.4% vs 30.4%, $p = .03$). Furthermore, the SL genotype was more frequent in patients with disease onset under 35 years (45% vs 38.9%) and in women who originated from endemic vs. sporadic localities of the disease (50% vs 42.2%). Inversely, the LL genotype (long) was more frequent in patients in the chronic stage (25%) than the others in the remission stage (6.7%). Despite the absence of significant differences, shorter CAG repeats (SS) are more frequent in PF subjects with the seborrheic form vs the generalized clinical form (43.4% vs 23.4%; $p = .08$) and patients in the chronic disease phase vs those in remission (33.5% vs 25%).

Since skewed XCI leads to hemizygosity of X-linked alleles that can mask or unmask X-linked polymorphisms, it may have a role in disease susceptibility or development. We analyzed each subject’s X chromosomal
methylation to assess whether preferential expression of immunity-related genes (AR, TLR7 (Toll-Like Receptor 7), and FOXP3 (forkhead box P3)) might be more prevalent in pemphigus subjects. We examined in detail the 120 informative women (70 HC and 50 PF heterozygotes for AR gene). TLR7 and FOXP3 genotypes were described in our previous data (Abida et al., 2020; Ben Jmaa et al., 2017). Overall, our results showed that the preferential XCI skewed profile correlated with the heterozygote frequency distribution of those immunity-related genes in PF patients in comparison to their matched HC (Table 4). Thus, preferential XCI showed an increase in subjects with AR > CAGn SL genotype in PF patients (58%) compared to HC (45%). For FOXP3, which is located at Xp11.23, skewed XCI patterns showed an increase in heterozygote patients than HC in all studied polymorphisms (rs3761547, rs3761548, rs3761549, and rs2294021) (Table 4). Interestingly, a significant positive correlation between the skewed XCI profile and the FOXP3 > rs3761547G susceptibility allele ($r^2 = 0.22; p = .02$) was noted. Concerning TLR7 (Xp22.3), evidence of increasingly skewed and extremely skewed in TLR7 > rs3853839 GC heterozygotes of TLR7 genes was observed in patients (58%) compared to HC (45%).

### 4 DISCUSSION

Evidence is accumulating to consider that epigenetics, and especially, XCI could explain the remarkable female predominance in AID. Endemic PF constitutes a distinct variety, as the disease occurs mainly in young rural women in southern Tunisia and the female gender seems to influence the severity of the disease in Brazil (Abida, Kallel-Sellami, et al., 2009; Salviano-Silva et al., 2017).

Here, we tested the hypothesis that women with PF display preferential skewed XCI. XCI status was informative for 50 of the 98 PF patients (51%) and 70 of the 150 HC women (47%). To our knowledge, no studies have explored the role of the XCI patterns in PF. Thus, 98 female patients with SLE who had a sex ratio similar to PF (9:1) were enrolled as a reference group. In the present study, 59 of the 98 SLE patients (60%) were heterozygotes. Consistent with our results, several studies report similar heterozygosity incidence (Chabchoub et al., 2009; Uz et al., 2008).

On the contrary, extremely skewed XCI patterns were more frequent in PF women versus HC, and they were significant in women with SLE. Furthermore, the XCI pattern was not different between the SLE and PF women. Despite the absence of significance, probably due to the loss of data given the small number of cases after

### Table 4 XCI profile in PF patients and their matched HC heterozygote for the immune-related genes (AR, TLR7, and FOXP3)

| Gene / polymorphism | Extremely skewed XCI (90:10–100:0) n (%) | Skewed XCI (80:20–89:11) n (%) | Random XCI (50:50–79:21) n (%) | p-value |
|---------------------|----------------------------------------|--------------------------------|--------------------------------|---------|
| **AR gene**         |                                        |                                |                                |         |
| (CAG)$_n$ SL        |                                        |                                |                                |         |
| PF ($n = 38$)       | 4 (10.5)                               | 18 (47.5)                      | 16 (42)                        | .3      |
| HC ($n = 51$)       | 2 (4)                                  | 22 (41)                        | 28 (55)                        |         |
| **FOXP3 gene**      |                                        |                                |                                |         |
| rs3761547 > AG      |                                        |                                |                                |         |
| PF ($n = 11$)       | 1 (9)                                  | 2 (18)                         | 8 (73)                         | .8      |
| HC ($n = 16$)       | 1 (7)                                  | 2 (13)                         | 13 (81)                        |         |
| rs3761548 > CA      |                                        |                                |                                |         |
| PF ($n = 17$)       | 2 (12)                                 | 7 (41)                         | 8 (47)                         | .4      |
| HC ($n = 31$)       | 1 (3)                                  | 13 (42)                        | 17 (55)                        |         |
| rs3761549 > CT      |                                        |                                |                                |         |
| PF ($n = 12$)       | 2 (17)                                 | 5 (42)                         | 5 (42)                         | .2      |
| HC ($n = 31$)       | 1 (3)                                  | 13 (42)                        | 17 (55)                        |         |
| rs2294021 > TC      |                                        |                                |                                |         |
| PF ($n = 15$)       | 2 (13)                                 | 4 (27)                         | 9 (60)                         | .2      |
| HC ($n = 22$)       | 0 (0)                                  | 8 (36)                         | 14 (64)                        |         |
| **TLR7**            |                                        |                                |                                |         |
| rs3853839 > GC      |                                        |                                |                                |         |
| PF ($n = 19$)       | 2 (10.5)                               | 9 (47.5)                       | 8 (42)                         | .6      |
| HC ($n = 20$)       | 1 (5)                                  | 8 (40)                         | 11 (55)                        |         |
sub-group stratification, the PF phenotype-XCI correlation analysis revealed that (i) skewed XCI patterns may be involved in disease subtype and (ii) it was more pronounced in the endemic group than in the sporadic one.

It is perplexing that the XCI pattern does not clearly explain the female dominance in Tunisian endemic PF. The alternative hypothesis is that enhanced immunity-related X genes expression owing to hemizygosity contributes to the higher risk of developing PF. Peripheral blood cells could lose their biallelic expression of X-linked immunity-related genes under the maintenance of the skewed XCI to cause the hemizygosity with the mutant allele (Bianchi et al., 2012; Selmi, 2008). Specific X-linked genes have demonstrated an association with AID. In some cases, the gene in question has genetic mutations that alter the gene product function and/or the levels of gene expression.

Several studies have found a link between PF and X-linked genes associated with autoimmune processes, such as CD40LG (a.k.a. CD154) at Xq24 (Caproni et al., 2007) and the interleukin-1 receptor-associated kinase1 gene (IRAK1) at Xq28 (Chatzikyriakidou et al., 2019). Our previous study reported significant associations of genetic variants of other X-linked genes like the FOXP3 gene (Xp11.23) with the risk for PF (Ben Jmaa et al., 2017). Concerning the TLR7 gene mapped in Xp22.3, we have noted an increase in the TLR7 rs3853839 > GG functional polymorphism in patients with PF compared to HC (Abida et al., 2020). Our results showed evidence of increasingly skewed and extremely skewed patterns among heterozygote patients in comparison to their matched HC in AR, TLR7, and FOXP3 gene polymorphisms were shown. Furthermore, the significant positive correlation between the skewed XCI profile and the FOXP3>rs3761547G susceptibility allele further consolidates our statement. Based on these results, we propose that the X chromosome in PF female lymphocytes is predisposed to become inactivated and to preferentially express susceptibility alleles of immunity-related genes to lose tolerance and enhance susceptibility to autoimmunity.

Although the present study was not initiated to examine allelic variation in the AR gene per se, the data provide an opportunity to address this question. AR gene has not been studied in PF disease. Our findings show that the AR (CAG) repeat length is significantly associated with the PF risk. Analysis using the weighted AR CAG length as a dichotomous variable revealed that PF patients with AR (CAG) repeat SL length showed more aggressive clinical forms and earlier disease onset. Thus, the AR (CAG)n polymorphism is barely related to the development of PF, but it could exacerbate the disease course (severe clinical damage and chronic phase of the disease). In this context, it has been shown that transcription of AR correlates inversely with repeat length. The relationships between AID clinical manifestation and CAG repeat length are contradictory. According to some studies, the longer CAG repeat length was related to lower androgen levels and to higher androgen concentrations according to others. Our previous data on Tunisian PF suggests that sex hormones are crucial for PF regulation, which, tends to worsen during pregnancy and to remit after menopause (Bastuji-Garin et al., 2002; Toumi et al., 2016). An imbalance between hormone relationships can result in lower immune-suppressive androgens and higher immune-enhancing estrogens. Women with PF tend to have lower androgen levels than healthy women. Further functional studies will be needed to address this point.

Skewed XCI is well-established in peripheral blood cells in women with AID (Invernizzi et al., 2005; Ozcelik, 2008). DNA methylation defects could be specific for a given cell subtype-skewed XCI can also vary among peripheral blood cells and tissue in AID (Özbalkan et al., 2005). Therefore, considering PF, XCI analysis in epidermal cells of the skin should be taken into consideration.

On the other hand, the XCI process achieves “dosage compensation” for many X chromosome genes by silencing one of the two copies in females, which is flexible to whether the region is inactivated or escaping XCI. In PF, CD40LG is overexpressed, which sets up an overly sensitive response. The mechanism of this CD40LG overexpression is not yet understood. However, epigenetic dysregulation is suspected since the demethylation of CD40LG on the inactive X chromosome of T cells in lupus patients has been reported (Lu et al., 2007). Future work is required to determine the specificity of the X-linked genes reactivation in PF patients.

In conclusion, the genetic dissection of PF and other AIDs is difficult because of the disease’s complexity which involves alterations of multiple biologic pathways. Our results underline the impact of the epigenetic dysregulation of the XCI process in PF. Additional studies on a larger scale in other endemic forms of PF are needed to replicate and confirm these preliminary findings.

**AUTHOR CONTRIBUTIONS**

All authors made substantial contributions to this study and approved the final version of the manuscript. Below is the contribution of each author to the manuscript: Olfa Abida conceived the project, performed enrolled methods and data gathering, and written original draft, Nesrine Elloumi provide DNA samples from SLE patients, their bisulfite treatment and the grammatical and spelling errors into the manuscript, Emna Bahloul, Khadija Sellami, and Hamida Turki provide the clinical characterization of PF and the clinical follow-up of patients, Hend Hachicha performed the biological assays for PF and SLE confirmation, Raouia Fakhfakh provide DNA samples from SLE
patients, Sameh Marzouk provide the clinical characterization of SLE and the clinical follow-up of patients, Ikhhlas Ben Ayed contribute to experimental design interpretation, Nadia Mahfoudh contributed to all genotypes analyses, Hatem Masmoudi critically revised the manuscript for important intellectual content.

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CONFLICT OF INTEREST
We declare having no conflicts of interest concerning published information in this study.

ETHICS STATEMENT
The research protocol was approved by the human research board of the ethics committee of the Habib Bourguiba University Hospital (protocol number of the ethics committee, 4/12).

DATA AVAILABILITY STATEMENT
The datasets generated and analyzed for the current study are available at the affiliated Hospital (Habib Bourguiba University Hospital of Sfax). The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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REFERENCES
Abida, O., Bahloul, E., Elloumi, N., Toumi, A., Fakhfakh, R., Mokni, S., Turki, H., & Masmoudi, H. (2020). Toll-like-receptor gene polymorphisms in Tunisian endemic pemphigus foliaceus. BioMed Research International, 2020, 1–11. https://doi.org/10.1155/2020/6541761
Abida, O., Kallel-Sellami, M., Joly, P., Ben Ayed, M., Zitouni, M., Masmoudi, A., Makni, M., Fezzaa, B., Ben Osman, A., Kammoun, M., Gilbert, D., Makni, H., Tron, F., Masmoudi, H., & Turki, H. (2009). The familial feature of Tunisian endemic pemphigus foliaceus. British Journal of Dermatology, 161(4), 951–953. https://doi.org/10.1111/j.1365-2133.2009.09386.x
Abida, O., Zitouni, M., Kallel-Sellami, M., Mahfoudh, N., Kammoun, A., Ben Ayed, M., Masmoudi, A., Mokni, M., Fezzaa, B., Ben Osman, A., Kammoun, M. R., Turki, H., Makni, H., Gilbert, D., Joly, P., Tron, F., Makni, S., Masmoudi, H., & the Franco-Tunisian Group for Survey and Research on Pemphigus. (2009). Tunisian endemic pemphigus foliaceus is associated with the HLA-DR3 gene: Anti-desmoglein 1 antibody-positive healthy subjects bear protective alleles. British Journal of Dermatology, 161(3), 522–527. https://doi.org/10.1111/j.1365-2133.2009.09207.x
Allen, R. C., Zoghbi, H. Y., Moseley, A. B., Rosenblatt, H. M., & Bellmont, J. W. (1992). Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. American Journal of Human Genetics, 51(6), 1229–1239.
Bastuji-Garin, S., Souissi, R., Blum, L., Turki, H., Nouira, R., Jomaa, B., Zahaf, A., Osman, A. B., Mokhtar, I., Fazaa, B., Revuz, J., Roujeau, J.-C., & Kamoun, M. R. (1995). Comparative epidemiology of pemphigus in Tunisia and France: Unusual incidence of pemphigus Foliacius in young Tunisian women. Journal of Investigative Dermatology, 104(2), 302–305. https://doi.org/10.1111/1523-1747.ep12612836
Bastuji-Garin, S., Turki, H., Mokhtar, I., Nouira, R., Fazaa, B., Jomaa, B., Zahaf, A., Ben Osman, A., Souissi, R., Hémon, D., Roujeau, J.-C., & Kamoun, M. R. (2002). Possible relation of Tunisian pemphigus with traditional cosmetics: A multicenter case-control study. American Journal of Epidemiology, 155(3), 249–256. https://doi.org/10.1093/aje/155.3.249
Ben Jmaa, M., Abida, O., Bahloul, E., Toumi, A., Khilf, S., Fakhfakh, R., Elloumi, N., Sallami, K., Masmoudi, A., Turki, H., & Masmoudi, H. (2017). Role of FOXP3 gene polymorphism in the susceptibility to Tunisian endemic pemphigus Foliacius. Immunology Letters, 184, 105–111. https://doi.org/10.1016/j.imlet.2017.02.005
Bianchi, I., Lleo, A., Gershwin, M. E., & Invernizzi, P. (2012). The X chromosome and immune associated genes. Journal of Autoimmunity, 38(2–3), J187–J192. https://doi.org/10.1016/j.jaut.2011.11.012
Brown, C. J., & Robinson, W. P. (2000). The causes and consequences of random and non-random X chromosome inactivation in humans. Clinical Genetics, 58(5), 353–363. https://doi.org/10.1034/j.1399-0004.2000.580504.x
Caproni, M., Antiga, E., Torchia, D., Volpi, W., del Bianco, E., Cappetti, A., Feliciani, C., & Fabbri, P. (2007). The CD40/CD40 ligand system is involved in the pathogenesis of pemphigus. Clinical Immunology (Orlando, Fla.), 124(1), 22–25. https://doi.org/10.1016/j.clim.2007.04.007
Chachhoub, G., Uz, E., Maalej, A., Mustafa, C. A., Rebai, A., Mnif, M., Bahloul, Z., Farid, N. R., Ozcelik, T., & Ayadi, H. (2009). Analysis of skewed X-chromosome inactivation in females with rheumatoid arthritis and autoimmune thyroid diseases. Arthritis Research & Therapy, 11(4), R106. https://doi.org/10.1186/ar2759
Chatziki-riakidou, A., Kyriakou, A., Melzanidou, P., Lambropoulos, A., & Patsatsi, A. (2019). Association of NFKB1 -94ATTG ins/del polymorphism (rs28362491) with pemphigus vulgaris. Experimental Dermatology, 28(8), 972–975. https://doi.org/10.1111/exd.13957
Diaz, L. A., Sampaio, S. A. P., Rivitti, E. A., Martins, C. R., Cunha, P. R., Lombardi, C., Almeida, F. A., Castro, R. M., Macca, M.
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L., Lavrado, C., Filho, G. H., Borges, P., Chaul, A., Minelli, L., Empinotti, J. C., Friedman, H., Campbell, I., Labih, R. S., & Anhalt, G. J. (1989). Endemic pemphigus Foliaceus (Fogo Selvagem): II. Current and historic epidemiologic studies. *Journal of Investigative Dermatology, 92*(1), 4–12. https://doi.org/10.1111/1523-1747.ep13070394

Invernezzi, P., Miozzo, M., Selmi, C., Persani, L., Battezzati, P. M., Zuin, M., Lucchi, S., Meroni, P. L., Marasini, B., Zeni, S., Watnik, M., Grati, F. R., Simoni, G., Gershwin, M. E., & Podda, M. (2005). X chromosome monosomy: A common mechanism for autoimmune diseases. *The Journal of Immunology, 175*(1), 575–578. https://doi.org/10.4049/jimmunol.175.1.575

Kutsche, R., & Brown, C. J. (2000). Determination of X-chromosome inactivation status using X-linked expressed polymorphisms identified by database searching. *Genomics, 65*(1), 9–15. https://doi.org/10.1006/geno.2000.6153

Lambert, N. C. (2009). The price of silence. *Arthritis and Rheumatism, 60*(11), 3164–3167. https://doi.org/10.1002/art.24962

Lee, J. T. (2011). Gracefully ageing at 50, X-chromosome inactivation becomes a paradigm for RNA and chromatin control. *Nature Reviews Molecular Biology Cell, 12*(12), 815–826. https://doi.org/10.1038/nrm3231

Lu, Q., Wu, A., Tesmer, L., Ray, D., Yousif, N., & Richardson, B. (2007). Demethylation of CD40LG on the inactive X in T cells from women with lupus. *The Journal of Immunology, 179*(9), 6352–6358. https://doi.org/10.4049/jimmunol.179.9.6352

Masmoudi, H., Abida, O., Masmoudi, A., & Turki, H. (2019). Update on immunogenetics of Tunisian endemic pemphigus foliaceus. *Journal of Leukocyte Biology, 105*(2), 257–265. https://doi.org/10.1002/JLB.MR0318-132R

Morini, J. P. (1993). Pemphigus Foliaceus in young women: An endemic focus in the Sousse area of Tunisia. *Archives of Dermatology, 129*(1), 69. https://doi.org/10.1001/archderm.1993.0168020081019

Nussinovitch, U., & Shoenfeld, Y. (2012). The role of gender and... (Fogo Selvagem): II. Current and historic epidemiologic studies. *Journal of Investigative Dermatology, 92*(1), 4–12. https://doi.org/10.1111/1523-1747.ep13070394

Özbalkan, Z., Bağışlar, S., Kiraz, S., Akyerli, C. B., Özer, H. T. E., Yavuz, Ş., Birlik, A. M., Çalışgneri, M., & Özçelik, T. (2005). Skewed X chromosome inactivation in blood cells of women with scleroderma. *Arthritis and Rheumatism*, 56(3), 348–351. https://doi.org/10.1007/s12016-007-8051-0

Ozcelik, T. (2008). X chromosome inactivation and female predisposition to autoimmunity. *Clinical Reviews in Allergy & Immunology, 34*(3), 348–351. https://doi.org/10.1007/s12016-007-8051-0

Puck, J. M., & Willard, H. F. (1998). X inactivation in females with X-linked disease. *The New England Journal of Medicine, 338*(5), 325–328. https://doi.org/10.1056/NEJM199801297431380611

Robledo, M. A., Stema Prada, C., Jaramillo, D., & Leon, W. (1988). South American pemphigus foliaceus: Study of an epidemic in El Bagre and Nechi, Colombia 1982 to 1986. *British Journal of Dermatology, 118*(6), 737–744. https://doi.org/10.1111/j.1365-2133.1988.tb02590.x

Salviano-Silva, A., Petzl-Erler, M. L., & Boldt, A. B. W. (2017). CD59 polymorphisms are associated with gene expression and different sexual susceptibility to pemphigus foliaceus. *Autoimmunity, 50*(6), 377–385. https://doi.org/10.1080/08916934.2017.1329830

Selmi, C. (2008). The X in sex: How autoimmune diseases evolve around sex chromosomes. *Best Practice & Research Clinical Rheumatology, 22*(5), 913–922. https://doi.org/10.1016/j.berrh.2008.09.002

Stewart, J. J. (1998). The female X-inactivation mosaic in systemic lupus erythematosus. *Immunology Today, 19*(8), 352–357. https://doi.org/10.1016/s0167-5699(98)01298-5

Toumi, A., Chaabouni, K., Abida, O., Masmoudi, A., Turki, H., Ayedi, F., & Masmoudi, H. (2016). Elevated prolactin levels in patients with pemphigus Foliaceus. *Clinical Dermatology & Therapy, 3*(1), 1–6. https://doi.org/10.24966/CDT-8771/100017

Uehara, S., Sato, K., Hashiyada, M., Obara, Y., Matsuizaki, S., Nata, M., & Okamura, K. (2001). X chromosome inactivation patterns in 45, X/46, XX mosaics. *Journal of Human Genetics, 46*(3), 126–131. https://doi.org/10.1038/s100380170099

Uz, E., Louibiere, L. S., Gadi, V. K., Ozbalkan, Z., Stewart, J., Nelson, J. L., & Ozcelik, T. (2008). Skewed X-chromosome inactivation in scleroderma. *Clinical Reviews in Allergy & Immunology, 34*(3), 352–355. https://doi.org/10.1007/s12016-007-8044-2

Waschke, J., Bruggeman, P., Baumgartner, W., Zillikens, D., & Drenckhahn, D. (2005). Pemphigus foliaceus IgG causes dissociation of desmoglein 1-containing junctions without blocking desmoglein 1 transinteraction. *Journal of Clinical Investigation, 115*(11), 3157–3165. https://doi.org/10.1172/JCI23475

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