Genomic ancestry, *F8* variants, and immune tolerance in hemophilia A patients with inhibitors: exome sequencing insights

Hemophilia A is an X-linked recessive bleeding disorder caused by factor VIII gene (*F8*) mutations, affecting 1 in 5,000-10,000 male births globally. The primary treatment is FVIII protein replacement, but ~30% of patients develop FVIII-neutralizing inhibitors that reduce treatment efficacy.[1] Immune tolerance induction (ITI), effective in about two-thirds,[2] is the only eradication treatment. While *F8* genotype and inhibitor titers are established ITI outcome predictors,[3] the role of genomic ancestry remains underexplored, particularly in admixed populations such as Latin-Americans and North American-Hispanics.

We investigated the influence of autosomal and X-chromosome genetic ancestry on ITI outcomes in 193 Brazilian patients with hemophilia A and high-responding inhibitors, enrolled in the multicenter Brazilian Immune Tolerance Study (BrazIT). Participants were treated with low-dose ITI (50 IU/kg FVIII, 3x/week) using either recombinant (ADVATE, Takeda, Japan) or plasma-derived (mostly Octavi, Octapharma, United States) FVIII concentrates. ITI outcomes were classified as complete success, partial success, or failure, following the Hay and DiMichele criteria.[4] Clinical and genetic methodologies are detailed in Camelo *et al*.[2,5] and Zuccherato *et al*.[6] Statistical approaches for population structure and genetic ancestry inference are provided in *Online Supplementary Table S1*. The study was approved by institutional ethical committees (CAAE 52812415.8.0000.5149).

Demographic, clinical, and genetic characteristics of the BrazIT cohort are summarized in Table 1 and Figure 1. Median age at diagnosis was 0.9 years; 95% had severe hemophilia. ITI outcomes were: 64 (33%) complete success, 62 (32%) partial success, 67 (35%) failure. Exome sequencing showed high-quality data across ~34 Mb of targeted regions, with a median coverage of 123.66X and a transition/transversion ratio (Ts/Tv ratio, i.e., a measure of sequencing data quality) of 3.11, indicative of reliable variant calls. We identified 222,314 variants, including 91,599 singletons and 7,088 novel variants, reflecting genetic diversity (Table 2). *F8* haplotypes[7] frequencies, defined by R503H (rs35383156), D1260E (rs1800291) and M2257V (rs1800297), were: H1 (N=133, 68.5%), H2 (N=37, 19.2%), H3 (N=10, 5.2%), H4 (N=1), H5 (N=0), and missing haplotypes (N=13, 6.7%).

We confirmed minimal inbreeding (mean individual coefficient: 0.004, 95% confidence interval [CI]: -0.028 to 0.044) and relatedness (mean kinship coefficient: 0.00333, 95% CI: 0.000132-0.0092). Furthermore, we confirmed five cases of known relatedness between pairs of individuals sharing the same types of *F8* mutation and an unreported second-degree relatedness.

Population genetics analyses confirmed the BrazIT cohort is admixed, reflecting Brazil's post-Columbian ancestry, predominantly European (64%), African (17%), and Native American (13%; Table 1; Figure 1). Ancestry varied between individuals, Brazilian states, and mainly between Brazilian geographic regions (nested-ANOVA for autosomes and X-chromosome, F values always >4.065, *P* always <0.003; *Online Supplementary Table S2*). European ancestry was highest in the South (73% autosomal), Native American in the Northeast (20% autosomal), and African in the Southeast (30% autosomal).

In Latin-American populations, sex-ancestry bias is the rule: during the last five centuries, European admixture has been preferentially mediated by males, while African and Native-American admixture by females, producing higher European ancestries in autosomes than in X-chromosomes. As expected, BrazIT shows Native-American sex-ancestry bias (Autosome - X-chromosome bias: 0.06, *P*<0.001), but exceptionally, no African sex-ancestry bias (-0.005, not significant). BrazIT shows the lowest value of African sex-ancestry bias among 21 studied Latin American populations.[8,9,10] This result suggests that X-chromosomes of predominant African origin are underrepresented among hemophilia A patients with high-responding inhibitors. However, the composition of the BrazIT cohort does not allow us to extend this result to hemophilia A patients in general. Different scenarios, possibly concurrent, may explain this result. First, there may be genetic factors mapped in African X-chromosomes negatively associated with being a hemophilia A patient with high-responding inhibitor (or a hemophilia A patient in general), partially preventing the inclusion of X-chromosomes of African origin in the BrazIT cohort and eliminating the pervasive population-based sex-African ancestry bias. Although *F8*, located on the X-chromosome, is an obvious candidate, exome data do not have enough single nucleotide variants (SNV, i.e., unique changes in the DNA sequence) density to perform an admixture mapping to identify the causing locus. Second, the lack of African sex-ancestry bias in the BrazIT cohort may result from the X-linked inheritance of hemophilia A (with 30-60% of *de novo* mutations[11]), interacting with the demographic history of Brazilians during the last 500 years, characterized by intensive immigration of Europeans and Africans. Indeed, all the DNA fragments of European and African origins of the Brazilian genomes were introduced by

Table 1. Demographic, clinical, and genetic characteristics of the BrazIT cohort. (A) General characteristics of the BrazIT Cohort (N=193). (B) Inhibitor parameters and immune tolerance induction.

Characteristics	Values
(A) General characteristics of the BrazIT Cohort (N=193)	
Male, N (%)	192 (99)
Age at diagnosis of hemophilia A, years Median (IQR) Missing data, N (%)	 0.90 (0.55-1.44) 10 (5)
Ethnoracial classification, N (%) Black Mixed[1] White Yellow, i.e. Asian Indigenous, i.e. Native-American	 15 (8) 73 (38) 105 (54) 0 (0) 0 (0)
Continental genomic ancestry by autosomes, mean (IQR) European African Native-American	 0.65 (0.53-0.77) 0.21 (0.09-0.29) 0.14 (0.08-0.19)
Continental genomic ancestry of X-chromosomes, mean (IQR) European African Native-American	 0.59 (0.37-0.82) 0.21 (0.03-0.30) 0.20 (0.02-0.33)
Continental sex-ancestry bias, mean in autosomes - mean in X-chromosomes European African Native-American	 0.065 - 0.005 -0.060
Distribution of BrazIT patients by Brazilian geographic region, N (%) West-Central Northeast North Southeast South	 13 (7) 47 (24) 8 (4) 53 (27) 72 (37)
Hemophilia A severity, N (%)[2] Severe Moderately-severe[3] Moderate4 Missing data	 173 (90) 8 (4) 4 (2) 8 (4)
F8 variant type, N (%) Large Deletion Frameshift Inversions Inversion intron 22 Inversion intron 1 Nonsense Missense Splice donor Missing data	 18 (9) 26 (13) 85 (44) 4 (2) 34 (18) 7 (4) 6 (3) 13 (7)
ITI outcomes, N (%) Failure Partial success Complete success	 67 (35) 62 (32) 64 (33)
(B) Inhibitor parameters and immune tolerance induction	
Age at inhibitor diagnosis, years Median (IQR) Missing data, N (%)	 2.16 (1.28-6.68) 3 (1.55)
Age at ITI start, years Median (IQR)	 6.48 (2.23-18.81)
Interval between diagnosis of hemophilia A and inhibitor development, years Median (IQR) Missing Data, N (%)	 0.87 (0.40-3.71) 13 (7%)

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Characteristics	Values
Historical inhibitor peak, BU/mL Median (IQR)	42.00 (14.80-119.40)
Inhibitor titer immediately before ITI starts, BU/mL Median (IQR)	7.20 (3.00-14.40)
Inhibitor peak during ITI, BU/mL Median (IQR)	30.00 (6.00-140.00)
ITI duration, years Median (IQR)	2.55 (1.67-3.13)

[1]The "Mixed" category corresponds to "pardo" in official Portuguese terminology. [2]The lowest plasma FVIII activity before inhibitor development was considered to classify hemophilia severity. [3]Defined when FVIII levels are between 0.01-0.02 IU/mL; [4]Defined when FVIII levels are between >0.02 and 0.05 IU/mL. BrazIT: Brazilian Immune Tolerance Study; F8: factor VIII; IQR: interquartile range; ITI: immune tolerance induction; IU: international units; BU: Bethesda units.

these immigrants, with the Native-Americans being the only population present in the Americas before 1492. Historical demography suggests that the male/female ratio among immigrants to the Americas was higher for Africans than for Europeans, and a ratio of 2-3 males for each female from Africa is broadly accepted (https://www.slavevoyages.org/). Because affected males were likely not included in the slave trade (or maybe, patients with severe disease have died before reaching adulthood), few X-chromosomes of African origins carrying hemophilia A mutations were brought to the Americas, mostly limited to the small proportion of carrier female immigrants. Therefore, this may have contributed to the absence of the African sex-ancestry bias in a hemophilia A cohort such as BrazIT.

F8 large deletions are prevalent in about 3-5% of individuals with severe hemophilia A.[12] We observed that *F8* large deletions are less frequent among individuals with higher autosomal (not for X-chromosome) Native-American ancestry, even after adjusting for inhibitor titers, geographical regions, and kinship coefficients (β=-0.081, 95% CI: -0.144 to -0.018; *P*=0.011). We also observed an association between H2 and H3 haplotypes and African ancestry, even after adjusting for peaks of inhibitor titers (X-chromosome: β=2.95-2.96; *P* always <0.001; autosomes: β=3.02-3.66 *P* always <0.04), consistent with observations in US racial groups[3].

Several studies have investigated hemophilia-related traits using self-reported ethnoracial categories[13,14] (*Online Supplementary Table S3*); however, genomic ancestry has not yet been explored in this context. While ethnoracial classification (a categorical social construct) and genomic ancestry (a continuous biological variable) are conceptually distinct, they are statistically correlated in the BrazIT cohort. Specifically, we observed an adjusted R^2 of 0.32 between European genomic ancestry and the White ethnoracial group, and an adjusted R^2 of 0.39 between African genomic ancestry and the Black ethnoracial group (both *P*<0.001). Given the lack of comparable genomic ancestry-based studies in Brazilian studies on hemophilia-related traits, we contextualize our findings with US studies that use ethnoracial classification and benefit from larger sample sizes, enhancing statistical power. It is also important to note that African Americans tend to have a higher proportion of African ancestry compared to admixed Brazilian individuals.[8]

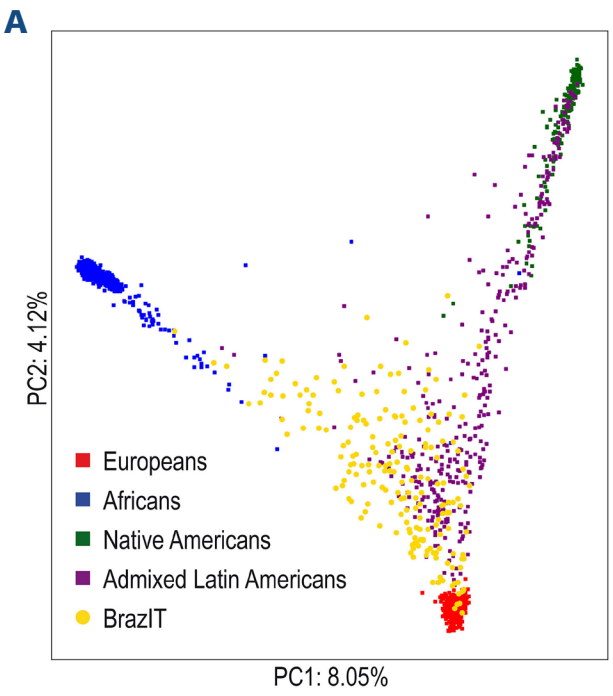
Unlike prior studies using self-reported race/ethnicity, we inferred genomic ancestry from exome data. We found no association between autosomal or X-chromosome ancestry (including African ancestry) and ITI outcomes, aligning with prior studies using self-identified race/ethnicity[13,14] (*Online Supplementary Table S3*). Similarly, no association was observed between inhibitor peak titers and genomic ancestry or *F8* haplotypes (*P* always >0.262). Genomic ancestry explained part of the variability in ethnoracial classification (adjusted-R^2: 0.32 for White, 0.39 for Black; *P*<0.001), but replacing genomic ancestry with reported race (White, Brown, Black) confirmed the lack of association with F8 large deletions, inhibitor titers, or ITI outcomes.

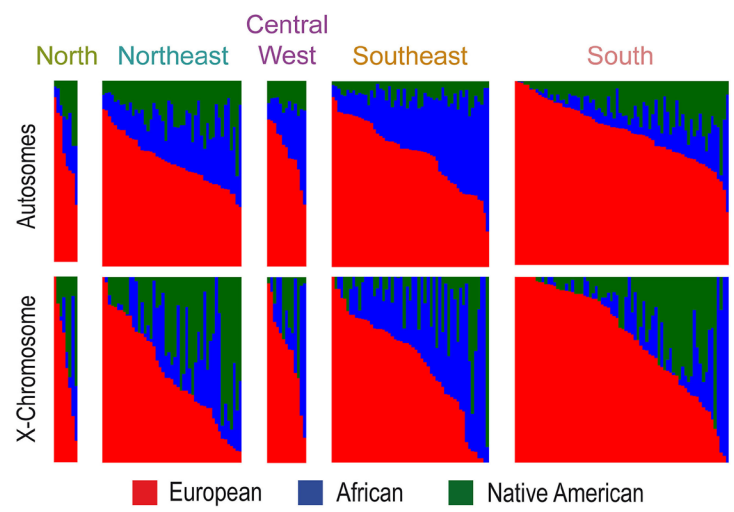
The relationship between reported race/ethnicity and genomic ancestry is complex, sensitive, and controversial. It varies in different geographic and social contexts, particularly in countries with a history of social inequalities and admixture, such as Brazil. A caveat in testing the association between health-related outcomes and reported racial categories or genomic ancestry is that in the US and Latin-America, Native-American and African ancestries (and the associated ethnoracial classes) are associated with poorer socio-economic conditions, a potential confounder. Thus, socioeconomic conditions should be included as covariates when testing the association between phenotypes and racial categories/ancestry. A limitation of BrazIT is that we do not have a variable for socioeconomic conditions.

Our study has several strengths. First, this is the largest study of Latin-American individuals with hemophilia A and high-responding inhibitors treated with ITI to date, and the first study to perform exome sequencing of this population. The study is a well-characterized cohort that assessed relevant clinical, immunological, and genetic factors with little missing data. All BrazIT participants were followed up until the end of ITI, which was performed according to a national standardized ITI protocol.

In conclusion, exome sequencing of BrazIT contributes to

the need for genomic data on patients with rare diseases and more diverse ancestries. Our data, harmonized and integrated with other cohorts from different ancestries to be studied, will allow us to gain statistical power and identify genetic variants and genes associated with hemophilia-related outcomes such as inhibitor titers and ITI success. We did not observe an association between genomic ancestries and inhibitor titers or ITI response.





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Sex-ancestry bias

	N	European	African	Native American
Central-West	13	0.011	0.001	-0.013
North	8	0.047	0.027	-0.073
Northeast	47	0.085	0.030	-0.115
Southeast	53	0.072	-0.021	-0.050
South	72	0.059	-0.022	-0.037
BrazIT	193	0.065	-0.005	-0.060

Figure 1. Geographic distribution, genetic structure, and admixture patterns across Brazilian regions of the BrazIT cohort. (A) The first and second principal components (PC1 and PC2, respectively) of autosomal genotypes distinguish individuals from European, African, Native-American, and Asian parental populations. Individuals from the BrazIT cohort are scattered across the principal component analysis (PCA) plot, indicating admixture. The numbers in parentheses next to PC1 and PC2 indicate the percentage of variance explained by each Principal Component. (B) Geographic distribution of BrazIT participants across the 5 Brazilian regions. Different colors represent the regions. States that include BrazIT individuals are shaded darker in each region. Below are vertical barplots depicting the individual proportions of continental ancestry in autosomes and X-chromosomes, estimated by the ADMIXTURE method. Each of the 10 blocks of vertical bars (e.g., autosomal ancestry for the North region in the upper left) consists of thin, adjacent vertical bars. Each vertical bar represents the percentage of Native-American (green, at the top), African (blue, in the middle), and European (red, below) ancestries for each individual. The width of each block of vertical bars is proportional to the number of individuals. (C) Sex-ancestry bias (mean autosomal ancestry - mean X-chromosome ancestry) is divided by region (Central-West, North, Northeast, Southeast, South) and by BrazIT as a total. We used 42,542 and 654 unlinked (linkage disequilibrium estimator r2<0.4) single nucleotide variants (SNV) in the autosomes and X-chromosomes for PCA and ADMIXTURE analyses.

Table 2. Exome diversity of the target region (34,156,490 bp) of the BrazIT cohort.

SNV	INDELS	Variants not present in dbSNP	Singletons	Doubletons	Transition/ Transversion ratio (Ts/Tv)	Total (SNV and INDELS)
216,531	5,783	7,088	93,657	25,761	3.110	222,314

The transition/transversion ratio (Ts/Tv) is used to assess mutation patterns and genomic integrity in sequencing data. It reflects the number of base substitutions that are transitions (A↔G, C↔T) divided by those that are transversions (e.g., A↔C, A↔T, G↔C, G↔T). A Ts/Tv ratio of 3.110 in this cohort indicates a predominance of transitions, consistent with a stable genomic profile and high-quality variant calls. SNV: single nucleotide variants; INDELS: insertions or deletions of nucleotides of <50 bp in DNA; Singletons: variants present at an absolute allele frequency of 1; Doubletons: variants present at an absolute allele frequency of 2.

Still, Native-American ancestry was negatively associated with *F8* large deletion, and H2-H3 haplotypes were associated with African ancestry. An intriguing result is that X-chromosomes of predominant African origins are underrepresented among Brazilian hemophilia A patients with high-responding inhibitors, exemplifying the unexplored issue of how sex-ancestry bias in post-Columbian migrations from Europe and Africa to the Americas may have differently shaped the patterns of genetic diversity of X-chromosome and autosomal Mendelian diseases in different populations of the Americas.

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Disclosures

No conflicts of interest to disclose.

Contributions

SMR conceived the study. SMR and RMC supervised the study and provided funding. HS, RT, ET-S and SMR designed the experiments/analyses and wrote the manuscript. HS RT, LF, BM and LWZ conducted analyses. RPS provided statistical input. All other authors coordinated the study in each of the centers, contributed to the acquisition of data, and contributed to the critical reading of the manuscript. All authors approved the final version of the manuscript.

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Data-sharing statement

The data that support the findings of this study are available from the corresponding author upon request.

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