Translational readthrough at *F8* nonsense variants in the factor VIII B domain contributes to residual expression and lowers inhibitor association

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Abstract

In hemophilia A, *F8* nonsense variants, and particularly those affecting the large factor VIII (FVIII) B domain that is dispensable for coagulant activity, display lower association with replacement therapy-related anti-FVIII inhibitory antibodies as retrieved from multiple international databases. Since null genetic conditions favor inhibitor development, we hypothesized that translational readthrough over premature termination codons (PTC) may contribute to immune tolerance by producing full-length proteins through the insertion of amino acid subset(s). To quantitatively evaluate the readthrough output *in vitro*, we developed a very sensitive luciferase-based system to detect very low full-length FVIII synthesis from a wide panel (n=45; ~60% patients with PTC) of *F8* nonsense variants. PTC not associated with inhibitors displayed higher readthrough-driven expression levels than inhibitor-associated PTC, a novel observation. Particularly, higher levels were detected for B-domain variants (n=20) than for variants in other domains (n=25). Studies on plasma from six hemophilia A patients with PTC, integrated by expression of the corresponding nonsense and readthrough-deriving missense variants, consistently revealed higher FVIII levels for B-domain variants. Only one B-domain PTC (Arg814*) was found among the highly represented PTC not sporadically associated with inhibitors, but with the lowest proportion of inhibitor cases (4 out of 57). These original insights into the molecular genetics of hemophilia A, and particularly into genotype-phenotype relationships related with disease treatment, demonstrate that B-domain features favor PTC readthrough output. This provides a potential molecular mechanism contributing to differential PTC-associated inhibitor occurrence, with translational implications for a novel, experimentally based classification of *F8* nonsense variants.

Introduction

Hemophilia A (HA [MIM: 306700]), caused by nucleotide variations in the *F8* gene (MIM: 300841) that encodes the multi-domain protein coagulation factor VIII (FVIII),[1] is one of the most frequent coagulation disorders.[2] Standard treatment of severe HA, associated with bleeding in soft tissues, joints, and muscles,[3] consists of frequent administrations of exogenous FVIII (replacement therapy),[4] whose most frequent drawback is the development of inhibitory (anti-FVIII) antibodies against the infused factor.[5,6] Among genetic components playing pathophysiological roles, which include the causative *F8* variant and the HLA alleles,[7] null genetic conditions such as *F8* large deletions have been found to confer a notable burden of risk.[8] Although it is well recognized that the more disruptive the *F8* variant, the more likely the development of inhibitor is, nonsense variants confer a lower risk for inhibitors than envisaged for a potentially null genetic condition.[8-10]

Nonsense variants are characterized by the introduction of premature termination codons (PTC) and are responsible for ~11% of human inherited disorders.[11,12] Their pathogenic effect is related to the synthesis of truncated molecules with decreased stability and/or loss-of-function, coupled with nonsense-mediated decay of PTC-bearing transcripts.[13,14] In this view, since residual FVIII levels have been predicted to likely protect against in-

hibitor development,[15] traces of full-length proteins arising from translational readthrough might confer lower risk for inhibitors after HA treatment. As a matter of fact, translational readthrough, a low-frequency event[16] driven by PTC misrecognition and incorporation of amino acid subsets,[17,18] might recode a nonsense to a sense codon producing traces of full-length proteins,[19] as demonstrated in a few *in vivo* studies.[20,21] Translational readthrough is dictated by: (i) the "leakiness" of termination signals and the PTC sequence context, with the downstream nucleotide as a major determinant,[22] and (ii) the type of amino acid incorporated at the PTC position,[17,18] which shapes the final protein output in terms of structure/function properties. However, the secreted and functional protein output of readthrough is driven by specific molecular determinants[22–27] even beyond the local nucleotide and protein contexts, as exemplified by the favorable localization of PTC in regions removed from the mature protein (i.e. in pre-peptides).[28]

Among the several FVIII domains (A1-A2-B-A3-C1-C2), the heavily glycosylated large B domain (908 residues) is removed upon processing/activation and is dispensable for coagulant activity,[1,29,30] which has led to therapeutic products with a modified B domain[31] or devoid of this domain.[32,33] The potentially lower impact of B-domain genetic variations on FVIII biosynthesis/trafficking/secretion is reflected by the low number of HA-causing missense variants in this region,[34,35] thus providing a paradigmatic model to test the hypothesis of a favored protein output arising from readthrough. However, despite the hints provided by all these elements, a systematic investigation of *F8* PTC, with a particular reference to B-domain PTC, and their association with inhibitor development, has not been performed so far.

Here, to interpret the lower inhibitor incidence reported for patients affected by B-domain nonsense variants, we give experimental evidence through an optimized luciferase-based expression system for a higher readthrough output of *F8* PTC affecting this domain. This opens the way for a new classification of *F8* nonsense variants in relation with inhibitors, thus providing new insights into the molecular genetics of HA, and particularly the genetic components of the main complication of replacement therapy.

Methods

Nomenclature

Nucleotides and residues are numbered in accordance with the Human Genome Variation Society (HGVS) nomenclature,[36] with numbering starting at the A (+1) nucleotide of the AUG (codon 1) translation initiation codon.

Reference *F8* sequence, databases and bioinformatic tools

Reference sequences for *F8* are NG_011403.2 and NM_000132.4 (GenBank), and NP_000123.1 (GenPept).

Genetic and epidemiological information related to the total number of patients with PTC (Pts_{tot}), patients with inhibitor (Pts_{inh}) and inhibitor-associated PTC (PTC_{inh}) were retrieved from EAHAD,[37] CHAMP,[38] and HMGD.[11] Inspection of gnomAD revealed the total number of annotated *F8* missense changes, either pathogenic or not (asymptomatic). Full information is reported in the *Online Supplementary Methods*.

The impact of amino acid substitutions at the PTC positions was predicted by taking advantage of the Protein Variation Effect Analyzer (PROVEAN),[39] Sorting Intolerant From Tolerant (SIFT),[40] Polymorphism Phenotyping v2 (Polyphen-2),[41] and Rare Exome Variant Ensemble Learner (REVEL)[42] tools.

Expression studies

The *F8* cDNA, cloned in the pCMV6-XL4 expression plasmid with the natural termination codon removed, was joined to the naturally secreted Gaussia luciferase (GL), obtaining the wild-type (WT) FVIII-GL chimera.[43,44] The sequences of primers used to generate all variants are listed in *Online Supplementary Table S1*. Full details of the cloning strategy are provided in the *Online Supplementary Methods* and *Online Supplementary Figure S1*.

Site-directed mutagenesis of *F8* cDNA to produce FVIII nonsense/missense variants, as well as transient transfection of human embryonic kidney (HEK293) cells, were essentially as described elsewhere.[24,28] Briefly, HEK293 cells, seeded in 48-well culture plates, were transfected with 400 ng of FVIII-GL-encoding plasmids, either WT or bearing nonsense ($FVIII^{PTC}$)/missense ($FVIII^{missense}$) changes, in serum-free medium (Opti-MEM, Gibco, Life Technologies, USA) with Lipofectamine 2000 (Life Technologies) in a 1:1 DNA (µg):lipofectamine (µL) ratio. Transfection efficiency was evaluated by co-transfection with a GFP-coding construct (150 ng). The coefficient of variation for transfection efficiency calculated in our expression system was 12%.

Optimization of the luciferase-based expression system

Luciferase activity was revealed by the Gaussia Luciferase Flash Assay Kit (Thermo Fisher Scientific, USA). Luciferase activity, as relative luminescence units (RLU), was measured on a Glomax 20/20 luminometer (Promega, USA) by mixing 10 µL of medium or cell lysate with 50 µL of coelenterazine diluted in the provided assay buffer, as indicated by manufacturer's instructions. Media and cell lysates, prepared according to the manufacturer's instructions with the provided lysis buffer,

were evaluated after collection at the tested time points (24, 36 and 48 hours) after transfection. Full details on the luciferase-based system as well as its optimization are provided in the *Online Supplementary Methods* and *Online Supplementary Figures S2* and *S3*.

Patients

Plasma from HA patients was collected at Castelfranco and Careggi Hospital Hemophilia Centers during scheduled routine follow-ups and after a 4-day wash-out. All procedures, approved by the local ethics committees, were in accordance with the Helsinki Declaration, and patients gave written informed consent to participation in the study. FVIII antigen levels in plasma were evaluated by a polyclonal anti-human FVIII enzyme-linked immunosorbent assay (F8C-EIA, Affinity Biologicals).

Statistical analysis

Data were analyzed by correlation analyses and unpaired *t*-tests through the GraphPad Prism software (San Diego, USA). The value of $P<0.05$ was considered as statistically significant.

Results

The combined data obtained from the international EAHAD, CHAMP and HGMD reference databases revealed a similar distribution of HA-associated PTC throughout the FVIII sequence (Figure 1A left panel, blue bars). Within the FVIII domains, the mean number of PTC per 50 amino acids (Figure 1A left panel, horizontal black line) ranged from eight (A1 and B domains) to ten (C2 domain). However, the overall number of patients with PTC suffering from inhibitors (Pts_{inh}) as well as PTC associated with inhibitors (PTC_{inh}) was low for the B domain, particularly considering its large size. The number of PTC found in at least one patient with inhibitor (PTC_{inh}), and

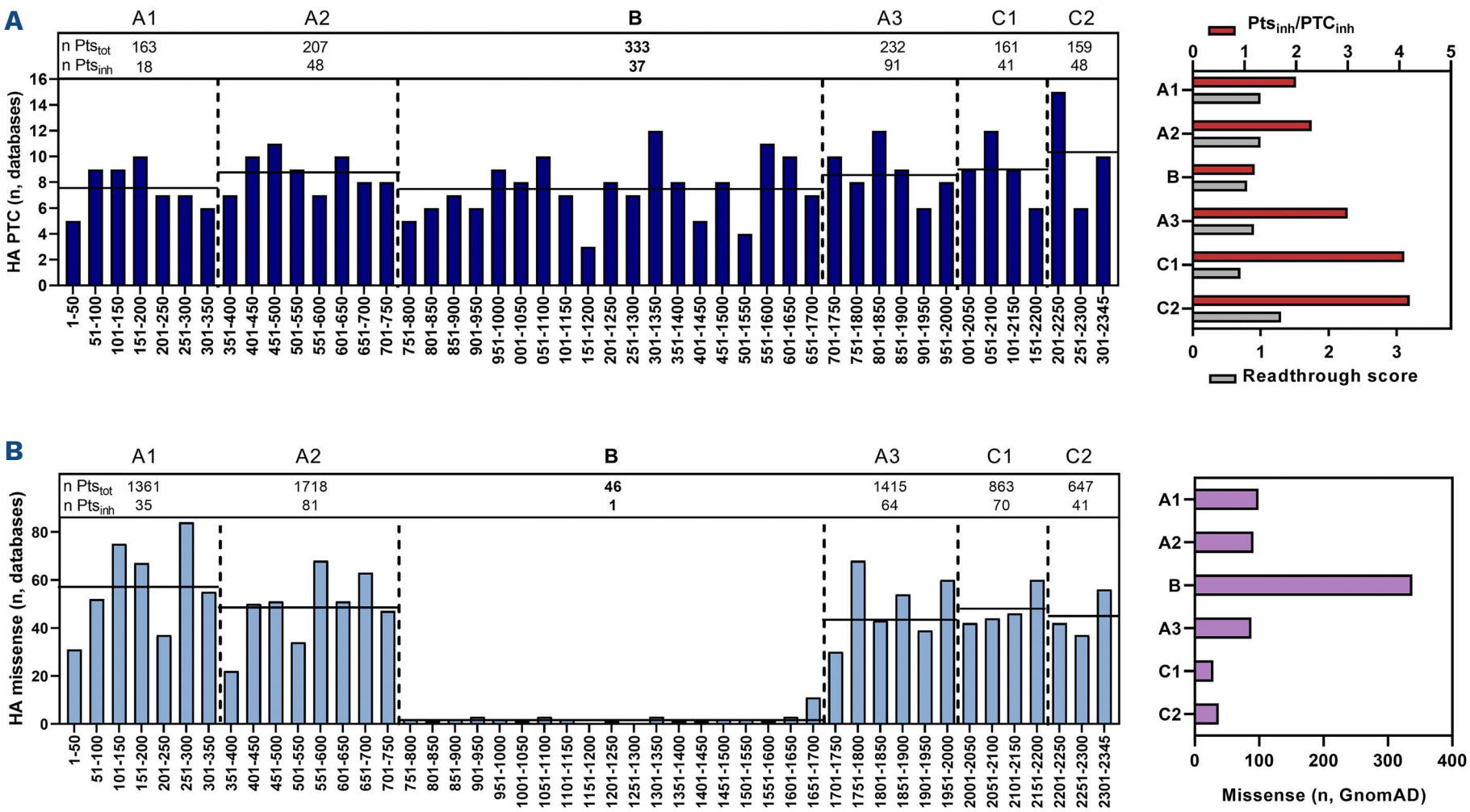

Figure 1. Distribution of *F8* nonsense and missense variants in the factor VIII sequence. (A) Left panel. Domains are compared for the total number of patients (n Pts_{tot}) with *F8* premature termination codons (PTC). The number of patients with an inhibitor is defined as n Pts_{inh}. Hemophilia A (HA) PTC (bars) reports the number of PTC depicted per 50 amino acids, namely the sum of each single PTC reported in the variant databases (see text for details) for each factor VIII (FVIII) sequence interval indicated. Black lines: mean number of PTC indicated per 50 amino acids for each domain. Right panel. Ratio (Pts_{inh}/PTC_{inh}, red bars) between the number of patients with PTC who have developed inhibitors (Pts_{inh}) and the number of PTC found in at least one patient with inhibitor (PTC_{inh}). Readthrough score (gray bars; according to Manuvakhova *et al.*[22]) predicted for all *F8* PTC, indicated as the mean score for each FVIII domain. (B) Left panel. Domains are compared for the total number of patients (n Pts_{tot}) with missense variants and the relative number of those with inhibitors (n Pts_{inh}). Bars represent the number of HA-associated missense variants, grouped as intervals of 50 amino acids in the FVIII sequence, reported in the international databases. Black lines: mean number of missense variants indicated per 50 amino acids for each domain. Right panel. Number of missense nucleotide changes reported in gnomAD.

the number of inhibitor patients for each PTC (Pts_{inh}), were used to calculate the Pts_{inh}/PTC_{inh} ratio to preliminarily estimate susceptibility to inhibitor development as a function of FVIII domain localization. Interestingly, this ratio was lower for the FVIII B domain (Figure 1A right panel, red bars). Since the PTC nucleotide sequence context is a main determinant of readthrough, for each *F8* PTC we further analyzed the predicted scores,[22] which estimate the relative susceptibility of PTC to undergo readthrough, and thus potentially predict the level of production of full-length proteins. Readthrough scores were similar in the different domains, thus not supporting a preferential intrinsic susceptibility of B-domain PTC to readthrough (Figure 1A, right panel, gray bars; *Online Supplementary Figure S4*).

Further inspection of these databases showed a very low number of B-domain missense variants associated with HA (Figure 1B, left panel), whereas numerous missense changes in this region are annotated in gnomAD (Figure 1B, right panel). This observation might be explained by the low degree of causality of most B-domain missense changes, and thus their low association with clinically relevant hemophilia.[34,35]

Integration of these database-derived data prompted us to hypothesize that the synthesis of full-length FVIII traces by translational readthrough, with potential missense changes introduced having a minor impact, might contribute to the lower association of B-domain nonsense variants with inhibitors.

B-domain premature termination codons display a high readthrough output

Intrigued by these observations, we experimentally investigated readthrough over a wide panel (n=45) of *F8* PTC, including the most frequent at CpG sites,[45,46] overall representing ~60% of HA patients with PTC (Figure 2A). In particular, we selected PTC as: (i) frequent (n. of patients >5) in the whole *F8* sequence (PTC n=20), includ-

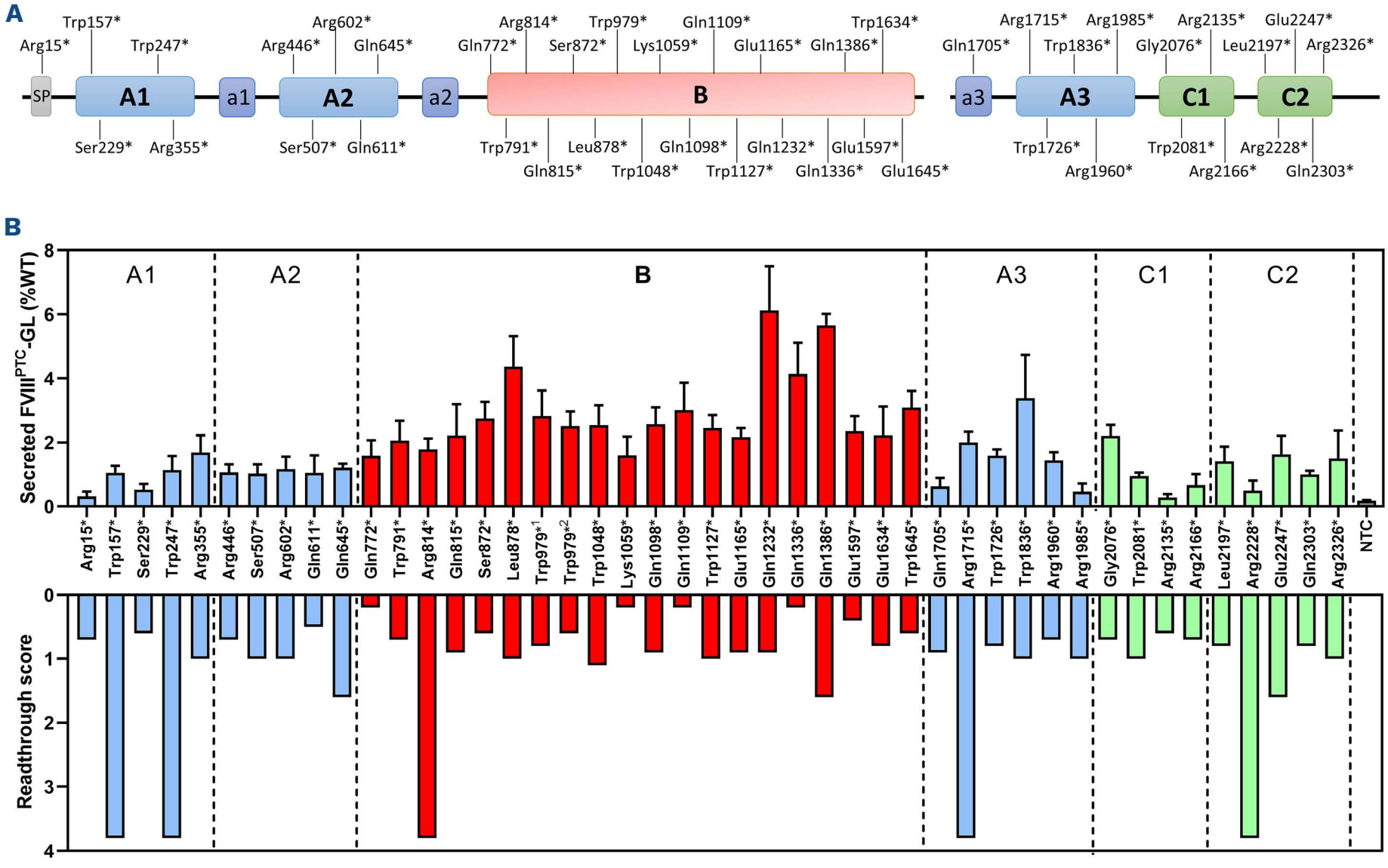

Figure 2. Investigation of *F8* premature termination codons through expression studies with the FVIII-GL fusion protein. (A) Schematic representation of the factor VIII (FVIII) domain organization and relative position of the *F8* premature termination codons introduced in the FVIII-GL fusion protein. The Trp979* variant was analyzed as TGA and TAG PTC, both frequent in the B domain. (B) Upper panel. Luciferase activity levels arising from readthrough of *F8* PTC measured in medium from HEK293 cells expressing the PTC-bearing FVIII-GL chimeras. The last column on the right (gray) indicates the luciferase activity measured for the FVIII-GL chimera bearing the natural *F8* termination codon (NTC) interposed between FVIII and Gaussia expressed as negative control. [1]Trp979* TGA PTC; [2]Trp979* TAG PTC. Results (mean ± standard deviation; n=4 replicates) are expressed as percentage of WT FVIII-GL. Lower panel. Readthrough score of investigated *F8* PTC (according to Manuvakhova *et al.*[22]).

ing those at CpG sites; (ii) frequent (n. of patients ≥3) in the B domain (PTC n=16); and (iii) PTC at which re-insertion of the WT residue is not predicted (PTC n=8), including the Trp979* PTC that was analyzed as frequent TGA and TAG PTC.

To evaluate the occurrence of readthrough *in vitro*, we optimized several components of a very sensitive luciferase-based system. In particular, we assessed: (i) time-dependent expression; (ii) signal detection by including or not the Gaussia signal peptide as well as (iii) by separating fusion partners through a flexible linker; and (iv) background expression by interposing the *F8* natural, two or three termination codons between FVIII and Gaussia (*Online Supplementary Methods and Supplementary Figure S3*). This allowed us to select the FVIII-GL direct fusion, joining FVIII and the mature Gaussia luciferase, and the time point at 48 hours after transfection to discriminate low/very low levels of readthrough-deriving full-length FVIII. Facilitated by the high sensitivity of the optimized expression platform, we detected large differences in expression levels among FVIII^{PTC}-GL variants, ranging from 6.1±1.4% of WT FVIII-GL for the Gln1232* (B domain) to 0.3±0.1% of the Arg2135* (C1) PTC variants (Figure 2B, upper panel). The relationship between secreted levels and readthrough scores for the selected PTC variants (Figure 2B, lower panel) was negligible (r=0.04, *P*=0.86).

Notably, secreted FVIII^{PTC}-GL levels for B-domain nonsense variants (range, 1.6-6.1%, mean 2.9%, confidence interval (CI): 2.3-3.4% of WT FVIII-GL) were significantly higher (*P*<0.0001) (Figure 3A, left panel) than those in the other domains (range, 0.3-3.4%, mean 1.2%, CI: 0.9-1.4%), even in domain-domain comparisons (Figure 3A, right panel). Differently, intracellular levels of FVIII^{PTC}-GL variants, ranging from 0.1% to 20% of WT, were slightly higher for PTC in the B domain (mean 7.4%, CI: 5.8-9.1%) than those in the A1-C2 group (mean 5.2%, CI: 4.1-6.3%) (Figure 3B).

Analysis of secreted levels as a function of the total readthrough products (secreted/secreted+intracellular)

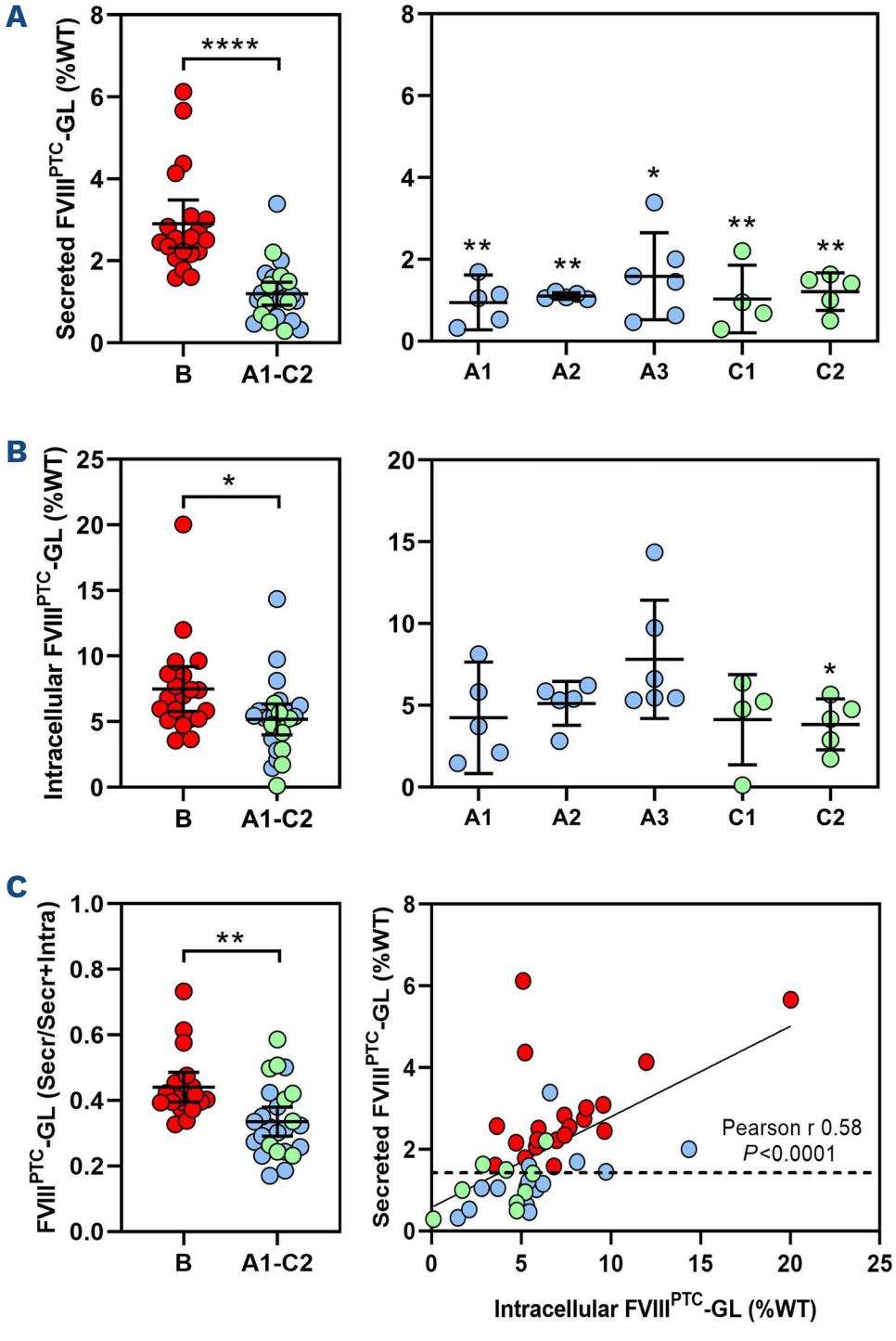

Figure 3. Expression studies and analysis of *F8* premature termination codon readthrough. (A) Comparison of luciferase activity levels of secreted full-length FVIII-GL between factor VIII (FVIII) B and A1-C2 domains (left panel) and in domain-domain comparisons (right panel). (B) Comparison of luciferase activity levels of intracellular FVIII-GL, measured in cell lysates, between FVIII B and A1-C2 domains (left panel) and in domain-domain comparisons (right panel). (C) Left panel. Comparison between secreted levels as a function of the total readthrough products (secreted/secreted+intracellular). Right panel. Correlation between luciferase activity levels measured in cell lysates (x axis) and in medium (y axis). Results in (A-C) left panel (mean ± 95% confidence interval) are expressed as percentage of WT FVIII-GL. *P<0.05; **P<0.005; ****P<0.0001.

(Figure 3C, left panel), and particularly the good correlation between intracellular and secreted FVIII levels (r=0.58, *P*<0.0001) (Figure 3C, right panel) corroborates the qualitative/quantitative features of readthrough over *F8* PTC, and further supports the higher readthrough output for those in the B domain.

Consistent *in vivo* and *in vitro* data support higher readthrough protein outputs for B-domain premature termination codons

To corroborate *in vitro* findings, we evaluated FVIII levels in plasma from six HA patients, four of them bearing highly frequent PTC at CpG sites (Table 1). The B-domain p.Arg814* (1.5±0.2% of reference plasma) and p.Lys1289* (2.1±0.1%) nonsense variants were associated with appreciable antigen levels that were significantly higher (*Online Supplementary Table S2*, upper part) than those of the p.Arg446* (0.2±0.04%), p.Trp1726* (0.7±0.2%), p.Arg1985* (0.4±0.1%) and p.Arg2135* (0.3±0.1%) variants (Figure 4A, yellow bars; Figure 4B, left panel). In FVIII^{PTC}-GL expression studies with the six nonsense variants (Table 1), the B-domain Arg814* (1.9±0.3% of WT FVIII-GL) and Lys1289* (2.1±0.2%) showed the highest levels (Figure 4A, light blue bars and *Online Supplementary Table S2*, middle part; Figure 4B, right panel).

To estimate the impact of amino acid substitutions at PTC positions, we took advantage of the PROTEAN, SIFT, PolyPhen-2 and REVEL bioinformatic tools. This analysis showed variable impacts for amino acid substitutions at B-domain PTC and consistently detrimental effects for changes at the other PTC positions (Table 1).

To obtain experimental findings, we expressed the most provide readthrough-deriving missense variants (FVIII^{missense}-GL) (Table 1). We detected intermediate/high (30-100%) secreted levels for the Arg814Trp, Lys1289Tyr/Gln and Arg446Trp, and low (5-15%) levels for the Trp1726Tyr, Arg1985Trp and Arg2135Trp missense variants (Figure 4A, blue bars). B-domain missense variants were those with the highest levels in single (*Online Supplementary Table S2*, lower part) as well as in group (Figure 4C) comparisons. In addition, even the non-conservative arginine-to-tryptophan substitution, predicted for the frequent TGA PTC at CpG sites, permitted significantly higher secreted levels for the B-domain Arg814Trp substitution as compared with Arg446Trp, Arg1985Trp and Arg2135Trp in the A2-A3-C1 domains (*Online Supplementary Figure S5*).

To compare the overall profile of plasma and recombinant nonsense/missense variants, we performed correlation analyses. Notably, a good correlation was observed between plasma (yellow) and FVIII^{PTC}-GL (light blue) levels (r=0.90, *P*=0.0056) (Figure 4D), as well as between secreted FVIII^{PTC}-GL and secreted FVIII^{missense}-GL (blue) (r=0.93, *P*=0.0022) (Figure 4D). These correlations further highlighted the higher

Table 1. Nonsense variants in patients with hemophilia A under study and prediction of amino acid substitutions at premature termination codon positions.

Genetic data[a]						Inhibitor status[b]		Bioinformatic prediction of amino acid substitutions[c]							
Patients 'number	Protein variant	FVIII domain	Nucleotide change	PTC	CpG site	Study patients	International databases[a,b]	PROVEAN[d]	Impact	SIFT[e]	Impact	Poly-Phen-2[f]	Impact	REVEL[g]	Impact
42	p.Arg446*	A2	c.1336C>T	TGA	Yes	No	+++	-3.14	+	0.030	+/++	1.000	+++	0.605	++
57	p.Arg814*	B	c.2440C>T	TGA	Yes	No	–/+	-0.46	–	0.020	++	0.960	+++	0.398	–
2	p.Lys1289*	B	c.3865A>T	TAA	No	No	–	-1.16	–	0.030	+/++	0.320	–	0.320	–
4	p.Trp1726*	A3	c.5177G>A	TAG	No	Yes	+++	-11	+++	0.000	+++	1.000	+++	0.852	+++
49	p.Arg1985*	A3	c.5953C>T	TGA	Yes	No	+++	-4.98	++	0.000	+++	1.000	+++	0.700	++
52	p.Arg2135*	C1	c.6403C>T	TGA	Yes	Yes	+++	-5.94	++	0.000	+++	1.000	+++	0.704	++

[a]EAHAD, CHAMP and HGMD databases (see text). [b]Inhibitor status, indicated as frequency (inhibitor patients/total patients), was arbitrarily defined as + (frequency 0.01-0.1), ++ (0.1-0.2) and +++ (>0.2). [c]For PROVEAN and SIFT (*http://provean.jcvi.org/protein_batch_submit.php?species=human*) and Polyphen-2 (*http://genetics.bwh.harvard.edu/pph2/*) the scores are referred to readthrough-mediated amino acid insertions (TGA, Trp/Cys/Arg; TAG/TAA, Tyr/Gln/Lys), including those derived from non-single nucleotide changes; REVEL (*https://sites.google.com/site/revelgenomics/*) scores are referred to precomputed amino acid changes resulting from single nucleotide changes, not including readthrough-deriving insertions but indicating the impact of possible natural substitutions at these positions. [d]Cutoff scores were arbitrarily defined as tolerated (–, >-2.5), slightly damaging (+, from -2.5 to -4.5), moderately damaging (++, from -4.5 to -6.5), and severely damaging (+++, <-6.5). [e]Cutoff scores were arbitrarily defined as tolerated (–, >0.05), slightly damaging (+, 0.05-0.03), moderately damaging (++, 0.03-0.01), and severely damaging (+++, <0.01). [f]Cutoff scores were arbitrarily defined as tolerated (–, ≤0.45), slightly damaging (+, 0.45-0.7), moderately damaging (++, 0.7-0.9), and severely damaging (+++, >0.9). [g]Cutoff scores were arbitrarily defined as tolerated (–, ≤0.45), slightly damaging (+, 0.4-0.6), moderately damaging (++, 0.6-0.8), and severely damaging (+++, >0.8). FVIII: factor VIII; PTC: premature termination codon.

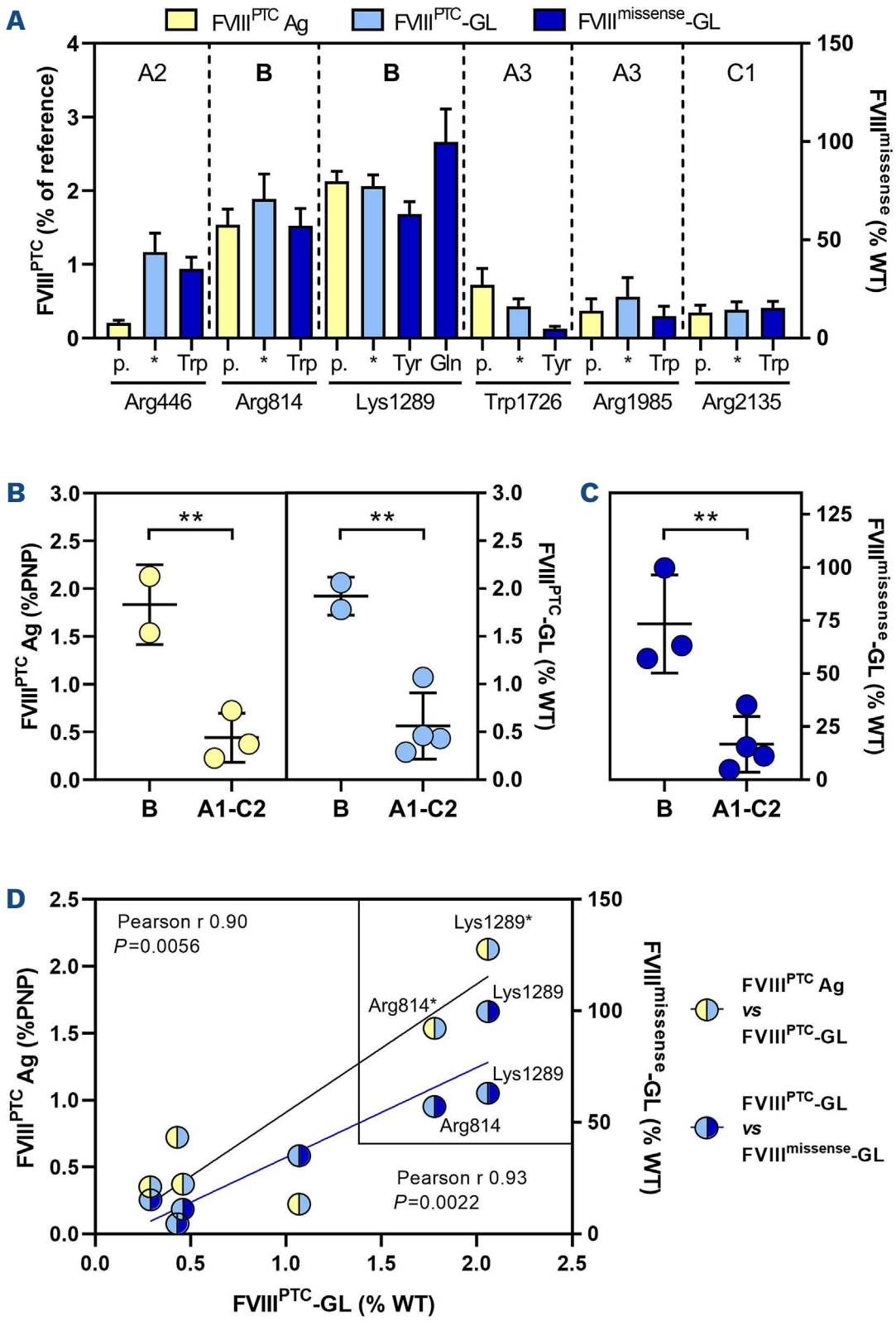

Figure 4. Studies in the plasma of patients with hemophilia A and expression studies on corresponding nonsense and missense variants. (A) Comparison among factor VIII (FVIII) residual levels found in the plasma of patients with hemophilia A (HA) (FVIII^{PTC} Ag, yellow bars), luciferase activity levels of secreted FVIII-GL chimeras bearing patients' premature termination codons (PTC) (FVIII^{PTC}-GL, light blue bars), and FVIII-GL chimeras bearing the most frequent amino acid(s) inserted by readthrough (FVIII^{missense}-GL, blue bars) over the six PTC found in patients (for the Lys1289* PTC both the Lys1289Tyr and Lys1289Gln missense variants, predicted to be comparably frequent,[17,18] were expressed). (B, C) Comparison of FVIII levels (as reported in A) between FVIII B and A1-C2 domain groups. (D) Correlation analysis of FVIII levels measured in HA plasma (yellow) *versus* luciferase activity levels of secreted FVIII^{PTC}-GL variants (light blue), and secreted levels of FVIII^{PTC}-GL variants *versus* secreted FVIII^{missense}-GL variants (blue). The box highlights the B-domain nonsense and missense variants showing the highest levels in all comparisons. Results (mean ± standard deviation; n=4 replicates) are expressed as percentage of reference (pooled normal plasma, PNP, for patients' plasma; WT FVIII-GL for recombinant variants). **P<0.01.

secretion profile of B-domain nonsense and missense variants (Figure 4D, boxed circles).

Secreted FVIII-GL levels are higher for premature termination codons associated with low inhibitor incidence

We compared the secreted levels of all expressed PTC variants in relation to the reported inhibitor status. To this purpose, we categorized *F8* PTC as not associated with inhibitors (NO inh) or, to exclude confounding effects of sporadic events, reported to be inhibitor-associated in more than one patient (inh>1). Mean secreted levels of the nonsense variants belonging to the NO inh group were significantly higher than those of the inh>1 group (Figure 5A, left panel). Among the PTC variants in the NO inh group (total n=21), those in the B domain (n=14) represented the major component. The inhibitor patients' group included only two B-domain PTC variants (Arg814* and Lys1059*) *versus* 17 A1-C2 PTC. The highly frequent (n. of Pts$_{tot}$>10) CpG PTC variants, collectively affecting more than 100 HA patients with inhibitors, were in the lowest tertile of the overall distribution of FVIII-GL levels (Figure 5A, right panel). Interestingly, the B-domain Arg814* is highly frequent (57 patients) (Table 1) and displayed the lowest proportion of patients with inhibitors (4 out of 57). The Arg814* variant produced relatively low expression levels among B-domain PTC, and relatively high levels among inhibitor-associated PTC. We further categorized highly frequent PTC to highlight those most associated with inhibitors (Pts$_{inh}$>10). Interestingly, secreted recom-

binant FVIII levels were significantly higher in the Pts_{inh}<10 than in the Pts_{inh}>10 group (Figure 5B).

The analysis of expression levels coherently suggests that a higher readthrough-mediated protein output, resulting in higher residual levels of full-length FVIII, is associated with a lower inhibitor occurrence for B-domain PTC.

Discussion

Nonsense variants alter gene expression and shape patients' phenotypes through multiple mechanisms, including nonsense-mediated mRNA decay and/or the formation of truncated proteins with reduced or no function.[13,14,47] However, PTC suppression mediated by translational readthrough might produce traces of full-length proteins through insertion of amino acid subsets. Albeit occurring with low efficiency,[16] this recoding process might have an impact on the outcome of human genetic disorders, and particularly on HA as a two-layer modulator of the resulting disease phenotypes, the bleeding tendency and the immune response to exogenous FVIII infused for therapy. *F8* nonsense variants are associated with a lower risk of developing inhibitors than that expected for a potentially null genetic condition.[9,10] Since residual FVIII expression is predicted to disfavor inhibitor development,[15] readthrough emerges as a plausible candidate to lower the association of nonsense variants with inhibitors.

Based on premises such as the: (i) lower occurrence of inhibitors associated with PTC in the B domain as compared with those in the other domains; (ii) very low number of HA-causative B-domain missense variants; and (iii) high number of annotated but not HA-associated missense nucleotide changes in the B domain, we hypothesized a higher degree of FVIII protein output upon translational readthrough at B-domain PTC. This hypothesis was tested through a sensitive system based on the FVIII-GL chimera, instrumental to discriminate low or very low full-length FVIII levels, hardly feasible through expression of the native FVIII. The optimized luciferase-based expression platform enabled the systematic investigation of a wide panel of *F8* PTC representing a large proportion (~60%) of HA patients affected by PTC. The distribution of most B-domain nonsense variants in the higher expression range indicated a higher degree of readthrough for B-domain PTC, resulting from improved biosynthesis and secretion. This finding is modestly explained by the PTC sequence context, a component contributing to PTC readthrough susceptibility, as evaluated through the predicted score[22] of each investigated PTC.

In vitro data were consistent with residual FVIII levels measured in plasma from the investigated HA patients, with higher FVIII expression levels for B-domain PTC variants. To interpret the readthrough output in terms of amino acid substitutions inserted at the PTC, based on previous experimental evidence on relative frequencies of amino acid insertions,[17,18] we expressed the most probable missense variants predicted to arise from readthrough. Biosynthesis/secretion of missense variants, driven by the features of the inserted residue(s) as well as by structural effects on the resulting protein variant, might provide information on the tolerance to amino acid substitutions in each affected domain. The correlation between secreted levels of (plasma/recombinant) nonsense and read-

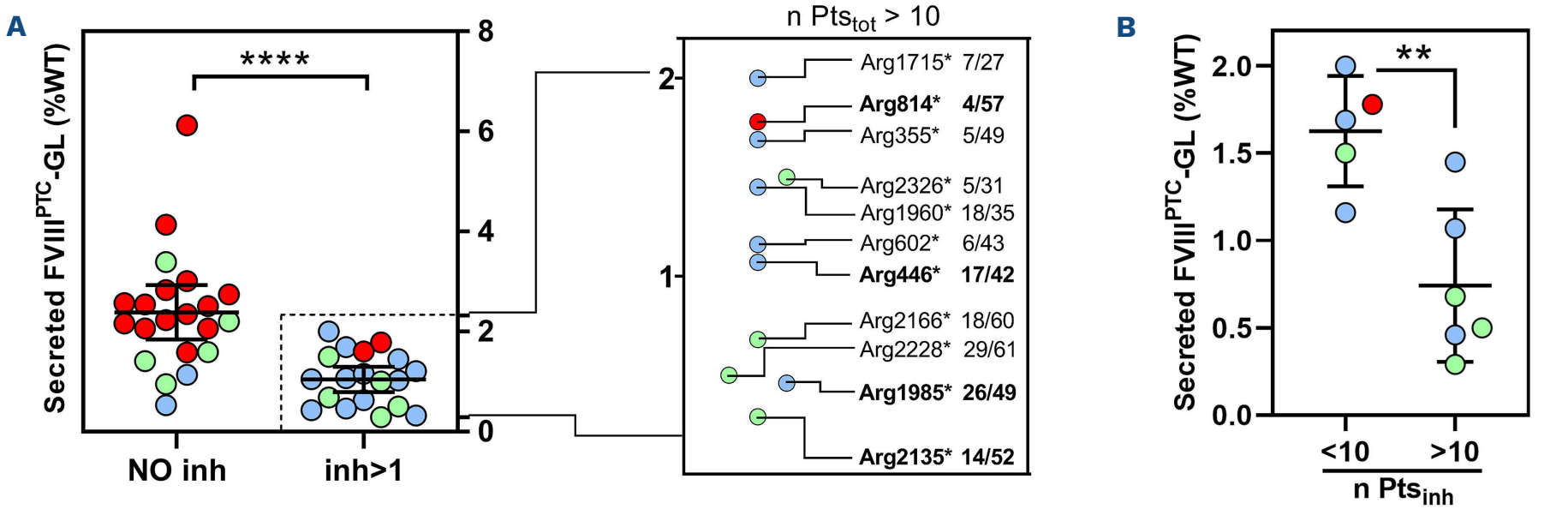

Figure 5. Association between residual factor VIII levels and inhibitor occurrence. (A) Left panel. Analysis of secreted levels obtained in expression studies on premature termination codons (PTC) grouped as not associated (NO inh) or associated in at least two patients with inhibitors (inh>1). Right panel. Magnification of the inh>1 group considering frequent PTC (CpG sites) with total patient number (n Pts_{tot}) >10. The relative occurrence of patients with inhibitors on the total number of patients is indicated by the ratio on the right. PTC variants found in the investigated hemophilia A (HA) patients are highlighted in bold. (B) Comparison of secreted levels, obtained in recombinant expression studies, of PTC variants shown in the magnified inh>1 group and further categorized as PTC associated with <10 (inh<10) or >10 (inh>10) of HA patients with inhibitors. Results in (A) (mean ± 95% confidence interval) and (B) (mean ± standard deviation) are expressed as percentage of WT FVIII-GL. ***P*<0.01; *****P*<0.0001.

through-deriving missense variants supported the hypothesis of a favorable protein output for B-domain variants.

Concerning the relationship of our experimental findings with the occurrence of inhibitors, *F8* PTC associated with inhibitors (inh>1) displayed lower readthrough-deriving expression levels than those not inhibitor-associated (NO inh), a novel observation in the field. It is worth noting that, in the inh>1 group, only two B-domain PTC are present, both showing the lowest secreted levels among all expressed B-domain PTC. Notably, among the highly represented PTC at CpG sites only one B-domain PTC (Arg814*) was present, albeit with the lowest proportion of cases with inhibitors (4 out of 57) and with relatively high expression levels. Importantly, expression studies suggest that PTC in the A1-C2 domains show lower readthrough levels, as also observed after readthrough induction by drugs,[27] and higher association with inhibitors, with particular reference to the frequent CpG PTC reported in hundreds of HA patients.

We are aware of the limits of our study, namely the limited number of HA patients validating the recombinant system to evaluate *F8* PTC readthrough, which however was indispensable to assess very low readthrough-related FVIII synthesis, hardly assessable in patients. *Vice versa*, the highly sensitive expression system might have slightly overestimated residual levels resulting from the most severe variants, particularly those (i.e., Arg1960* and Arg1985*, A3 domain; Arg2228*, C2 domain) strongly associated with inhibitors (~50% of patients).

Notwithstanding, our data provide experimental evidence for FVIII traces arising from readthrough that might have translational implications. It is worth noting that B-domain PTC readthrough, by favoring FVIII biosynthesis/secretion and reverting a null condition, would further reduce the immunogenicity of therapeutic FVIII by ensuring the synthesis of the native A3-C2 FVIII domains. As a matter of fact, genetic defects associated with the lack or alterations of the A3-C2 domains increase the development of inhibitory antibodies targeting these highly immunogenic regions on the infused FVIII.[48-50] This further contributes to explain the lower association of B-domain PTC, favoring readthrough, with anti-FVIII inhibitory antibodies.

In conclusion, through a systematic approach exploring a relevant number of *F8* PTC, we provide original: (i) findings on molecular mechanisms potentially contributing to the low inhibitor occurrence in patients with PTC localized in the FVIII B domain; (ii) insights into HA molecular genetics, with a novel classification of *F8* nonsense variants, which might assist genetic counseling in HA; and (iii) *F8* genotype-phenotype relationships related with individual HA patients' response to replacement therapy.

Disclosures

No conflicts of interest to disclosure.

Contributions

MFT created constructs and performed preliminary studies to optimize the expression system, performed expression studies with nonsense/missense fusion constructs, analyzed data and compiled figures; SL created plasmids encoding nonsense/missense FVIII-GL fusion proteins; MF performed enzyme-linked immunosorbent assays on patients' plasma samples; DB, PR and GC enrolled patients, collected and processed blood samples, and collected written consents; AB conceived the study and designed research; and AB, FB and MP analyzed data and wrote the manuscript. All authors revised and approved the final version of the manuscript.

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Data-sharing statement

For original data, please contact brnlss@unife.it (AB) or pnm@unife.it (MP).

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