F8 intron 22 inversions and SNP rs73563631 in unrelated families with severe haemophilia A: clinical features and gene testing implications

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Dear Sirs,

One in 5,000 human males worldwide suffers from haemophilia A (HA), the commonest X-linked coagulopathy caused by deleterious mutations in the factor VIII gene (*F8*). Among them, intron 22 inversions (INV22) cause severe-HA (FVIII:C <1%) and result from non-allelic recombination between homologs within *F8* intron 22 (*int22h*-1, or *h1*) and an extra-*F8* Xq-telomeric copy (*h2* or *h3*) (1, 2). Involving *h3*, INV22 type I (INV22–1) causes 35% of severe-HAs and involving *h2*, INV22 type II (INV22–2), 7% (3, 4).

INV22 genotyping was initially achieved by *Bcl*I-Southern blot (2) and later on by rapid type-undefined approaches using long range-PCR (LR-PCR) and inverse shifting-PCR (IS-PCR) (5, 6).

Crossovers between equally-oriented *int22h*-copies originate deletions (DEL22) or duplications (DUP22) (7).

Reformulated versions of LR-PCR (8) and IS-PCR (IS-PCR/2008) (9) permitted type-specific genotyping of all *int22h*-rearrangements. IS-PCR/2008 detects all relevant *Bcl*I-fragments/circles: 21.5/17.5/15.5kb in the diagnostic test (IS-PCR/2008-DT) and 20/16/14kb in the complementary test (IS-PCR/2008-CT).

Herein, we performed an INV22 rescreening of our historical series of 308 families with severe-HA using IS-PCR/2008-DT/-CT. This allowed characterisation of 143 families with conventional INV22 (46%): 120 with INV22–1 (84% of INV22s) and 23 INV22–2 (16%). Two families (0.65%) showed unusual IS-PCR/2008 patterns characterised by no signals in the IS-PCR/2008-DT and conventional INV22–1/-2 patterns in the IS-PCR/2008-CT.

- *Family 1*: Familial severe-HA. It included three patients and an obligate carrier (►Figure 1A). Patient #502 showed low response inhibitor (4.2 BU) and HCV infection; #507, transient

Figure 1: New INV22–1/-2 BclI-RFLP patterns in families with severe-HA. A, B) Pedigree analysis of Family 1 (A) and Family 2 (B). Samples are denoted by the Lab ID number (#). Vertical lines indicate Xq28 STR-haplotypes: extra-F8 (3′), DXS7423 [10], DXS1073 [11]; intra-F8, Int25.3 [12], Int22 [13], Int21 [14], Int13 [12]; extra-F8 (5′) DSX1108 [10] STR genotyping (Suppl. Figure 1, available online at www.thrombosis-online.com). Upward black arrow indicates the F8. C) Schematic of int22h linked regions showing relevant BclI restriction fragments' size and IS-PCR DT primers associated with INV22–1 (17.5kb, primers ID and 3U, BclI sites B1 and B5) and INV22–2 in brackets (15.5kb, primers ID and 2U, BclI sites B1 and B4). The polymorphic BclI-site (B*) (SNP rs73563631) [A>G] associates with a BclI-RFLP, which destroys INV22-type specific and Normal fragments in the IS-PCR/2008-DT generating smaller fragments/circles, 15.5kb, 13.5kb and 19.5kb in INV22–1, INV22–2 and Normal patterns, respectively. Relevant PCR, IS-PCR products and primer-restriction site sizes are indicated in bp. D) Sanger sequencing of the 575bp-PCR product obtained using primers Bcl_N-up, 5′-GAGCGTTCATGGCAGCACTAT-3′ and Bcl_N-lo, 5′-ATGGCAGGGGAGGTAAATGTT-3′ from samples of Family 2 proband (#816) showing the BclI-RFLP (TGATCA); his carrier mother (#817); and non-carrier aunt (#331). E) Agarose gel electrophoresis (AGE) analysis of conventional IS-PCR/2008 DT (upper panel) and CT (lower panel) products as was described [9]. From lanes 1 to 11: INV22–2 [+/-] control; INV22–1 [+/-] control; #502; #507; #762; #763; #816; #817; #331; #1016 and negative control (no DNA). Unusual patterns from hemizygous patients of Family 1 and 2 characterised by lack of signals in DT and INV22–1 and INV22–2 patterns, respectively, in CT; while all females at-risk show the non-carrier pattern in the DT and properly discriminating patterns of INV22–1 (#763), INV22–2 (#1016, #817) and non-carrier (#331) in the CT. F) PCR-BclI-RFLP analysis by AGE. PCR products (Bcl_N-up+ Bcl_N-lo) with 575bp were either BclI digested (D) or not (U) and analysed in consecutive lane pairs U-D. PCR amplifications were performed using standard conditions (30 cycles, 53–55 °C annealing temperature) and RFLP analyses with 5 µl of PCR product using 10 units of BclI-restriction enzyme (Promega). Polymorphic BclI [+] allele shows signals of 418bp and 157bp (rarely observed). Lane 1: 100bp-ladder (Promega). Lanes 2–3, #502 B[+]; 4–5, #507 B[+]; 6–7, #762 B[+]; 8–9, #763 B[+/-];10–11, #331 B[-]; 12–13, #816 B[+]; 14–15, #817 B[+/-]. G. AGE analysis (2%) of a modified version of IS-PCR/2008 DT including primer Bcl_N-lo to the conventional IS-PCR/2008 DT primer set (ID, IU, 2U and 3U) [9]. From lanes 1 to 12: 100bp-ladder; INV22–1 control [+/-]; INV22–2 control [+/-]; #502; #507; #762; #763; #816; #817; #331; #1016 and negative control. This new version of the IS-PCR/2008 DT permits an accurate classification of rs73563631*G allele linked to INV22–1 (INV22–1x) and INV22–2 (INV22–2x) showing specific signals of 463bp and 515bp, respectively, in hemizygous patients and carriers. An eventual rs73563631*G allele linked to the normal pattern (not present in our study) would yield a signal of 617bp as result of IS-PCR amplification with Bcl_N-lo (157bp) and IU (460bp).

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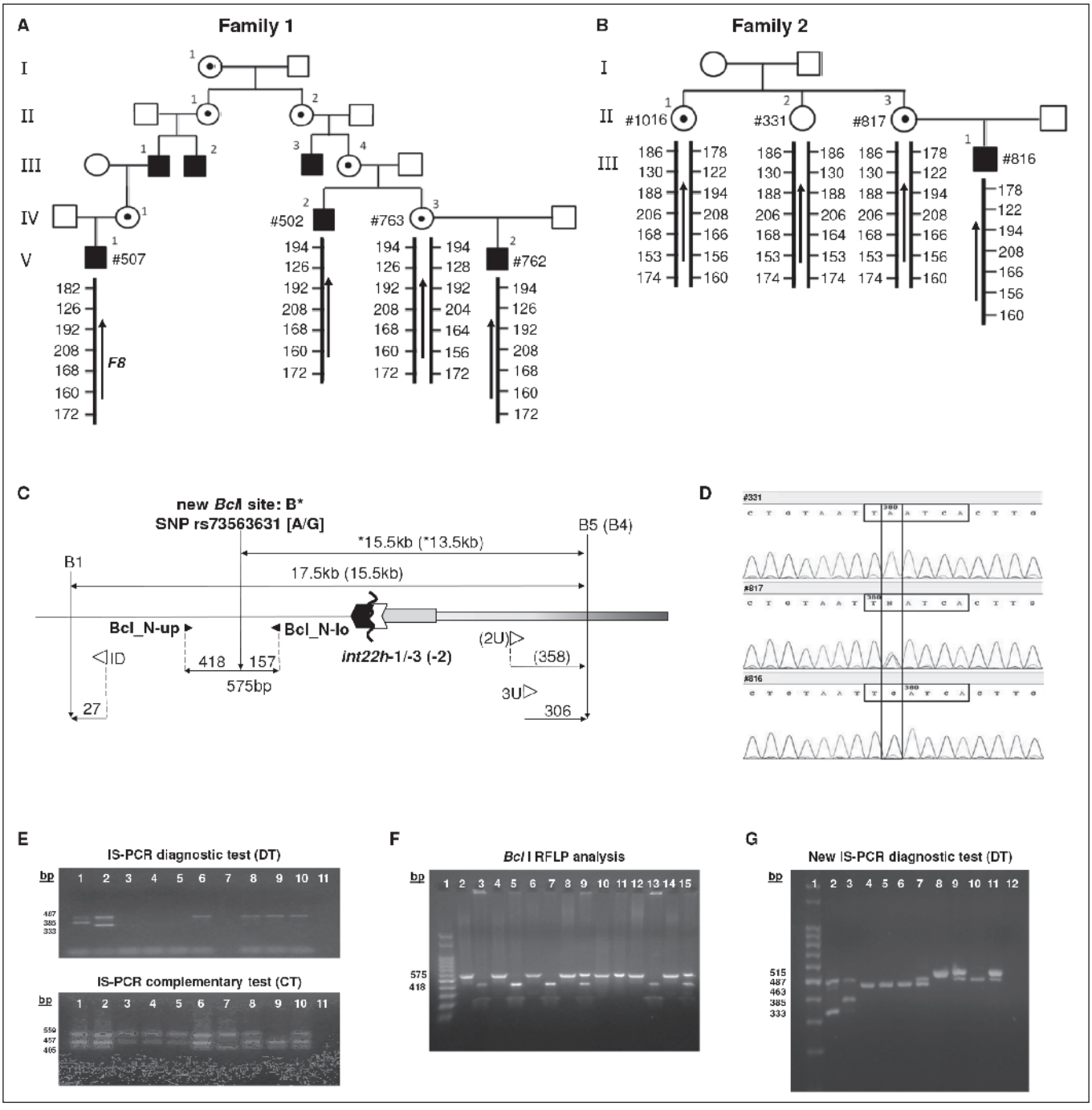
inhibitor (0.6 BU in 2008) and #762, no inhibitor. All patients showed no signals and a hemizygous INV22–1 pattern in the IS-PCR/2008-DT and -CT, respectively; whereas a patient's mother (#763) presented the normal pattern in IS-PCR/2008-DT and a heterozygous INV22–1 pattern in the IS-PCR/2008-CT (►Figure 1E).

- *Family 2*: Sporadic severe-HA. It included a patient and three female relatives (mother and two maternal aunts) (►Figure 1B). Patient #816 showed transient inhibitor, X-Fragile and Von Willebrand disease type 2N, and no signals in the IS-PCR/2008-DT and a hemizygous INV22–2 pattern in IS-PCR/2008-CT. His mother #817 and maternal aunt #1016 showed normal patterns in IS-PCR/2008-DT and heterozygous INV22–2 patterns in IS-PCR/2008-CT; and maternal aunt #331 showed normal non-carrier patterns in both tests (►Figure 1E).

Linkage analysis using seven *F8*-linked STRs confirmed the familial relationships and detected different haplotypes in-phase with severe-HA in both families (►Figure



1A, B and Suppl. Figure 1, available online at www.thrombosis-online.com). No additional *F8* mutations were identified in both families as was described earlier (15).

To investigate the cause of uncommon INV22 patterns, alternative hypotheses were tested: a mutation affecting (i) a terminal *Bcl*I site defining the 17.5kb-fragment (PCR-RFLP analysis); (ii) IS-PCR/2008-DT's primer target sites (PCR amplification using alternative primers); and (iii) eventual inversions involving the 17.5kb-fragment (IS-PCR analysis pairing combinations of equally-oriented primers). Consistent negative results of i, ii and iii tests allowed ruling out these hypotheses (Suppl. Figure 2, available online at www.thrombosis-online.com).

To investigate the potential involvement of a *Bcl*I-RFLP disrupting the 17.5kb-fragment/circle, a comprehensive bioinformatics screening of all encompassed SNPs was performed. The screening consisted in performing a theoretical *Bcl*I restriction map of the 17.5kb-fragment *in silico* on the updated X-chromosome sequence (NC_000023.11) including all 85 allele variants annotated in SNP databases (dbSNP: http://www.ncbi.nlm.nih.gov/SNP/, HapMap: http://hapmap.ncbi.nlm.nih.gov/ and ENSEMBL: http://www.ensembl.org/index.html). The analysis revealed a SNP (rs73563631) (A/G) as a potential *Bcl*I-RFLP placed 228bp downstream *int22h*-1. Consequently, the rs73563631*G allele was investigated in both families by PCR-*Bcl*I-RFLP analysis. Unexpectedly, it was found in all four affected patients and three females at-risk from both families indicating that the *Bcl*I-RFLP (TGATCA) is independently associated with INV22–1 and INV22–2 breaking the 17.5kb- and 15.5kb-fragment/circle, respectively, revealing the cause of IS-PCR/2008-DT failure (►Figure 1C, E, F).

The addition of Bcl_N-lo (5'-ATGGCAGGGGAGGTAAATGTT-3') in the IS-PCR/2008-DT primer set (ID+IU+2U+3U) allowed identification of the new patterns of INV22–1/-2 (INV22–1x/-2x), and all canonical types (►Figure 1G). Modified IS-PCR/2008-DT identifies INV22–1× and INV22–2× with specific signals of 463bp and 515bp, respectively. Consequently, the modified IS-PCR/2008 achieved an accurate molecular diagnosis of INV22–1x/-2× in Family 1 and 2 patients, carriers and non-carriers (►Figure 1G).

PCR-*Bcl*I-RFLP analysis in 404 X-chromosomes from the Argentinean general population (120 males and 142 females) indicated the absence of the rs73563631*G allele estimating a frequency q<0.24% (Suppl. Figure 3, available online at www.thrombosis-online.com).

The potential involvement of INV22–1x/-2× in different international populations of severe-HA patients was estimated by referencing the frequencies of the rs73563631*G allele in the 1000 Genomes Project, which estimates a global average of 1.27% (16). The rs73563631*G allele showed some discrepancy in the global frequencies between hemizygotes (qm=0.9%) and heterozygotes (qf=1.5%) although the Hardy-Weinberg equilibrium departure cannot be properly analysed due to the reduced number of individuals in each particular population and the low frequencies of the rs73563631*G allele (e.g. Sierra Leone, n=85, qf=0.19, qm=0.05). Frequencies of the rs73563631*G allele estimate moderate involvement of the *Bcl*I-RFLP in Africans (2–15%), as it was reported in African Americans linked to a Normal *F8* and to an INV22 allele (17), low in Mesoamericans (0.7–1.4%), and negative in Asians and Europeans.

In conclusion, we have presented two rare patterns of the INV22 in four out of 149 patients with the INV22 (2.7%) and out of 306 Argentinean patients with severe-HA (1.3%). Patients with INV22–1x/-2× presented no appreciable differences in clinical/biochemical severity or inhibitor development risks, as one out of four patients showed permanent inhibitors, similar to our comprehensive severe-HA series, 24%(18–29%) (15).

None of the so far reported non-canonical Southern blot patterns (18) or *int22h*-rearrangements associated with genomic gain and/or loss (19-22) match with INV22–1x/-2x. *In silico* predicted changes, in *Bcl*I-Southern blot patterns associated with rs73563631*G allele (►Figure 1C), are disturbingly similar to the Normal pattern and may lead to misdiagnosis or underestimation of the INV22 mutation. The inspection of LR-PCR-based approaches (5, 9) and the former IS-PCR approach (6) predicts no problems for INV22 detection.

The modified IS-PCR/2008 presented here permits an accurate classification of all *int22h*-rearrangements, INV22 types and patterns reported thus far.

Our findings and the severe clinical consequences of large deletions (e.g. DEL22 [23–25]) suggest that a missing signal, or differential pattern, in a genotyping test for an X-linked disease-causative rearrangement should be further investigated to determine its actual structure and extent to attain reliable conclusions about its potential association with differential clinical features in hemizygous patients.

Consequently, it is advisable that all haemophilia gene testing laboratories using IS-PCR-based approaches apply the modified IS-PCR/2008 version presented here at first line in severe-HA.

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Author contributions

M. M. Abelleyro and L. C. Rossetti designed and performed research, analysed data and final approval of the manuscript. M. A. Curto, C. P. Radic and V. D. Marchione performed research, analysed data and final approval of the manuscript. C. D. De Brasi designed research, analysed data and wrote the paper.

Conflicts of interest

None declared.

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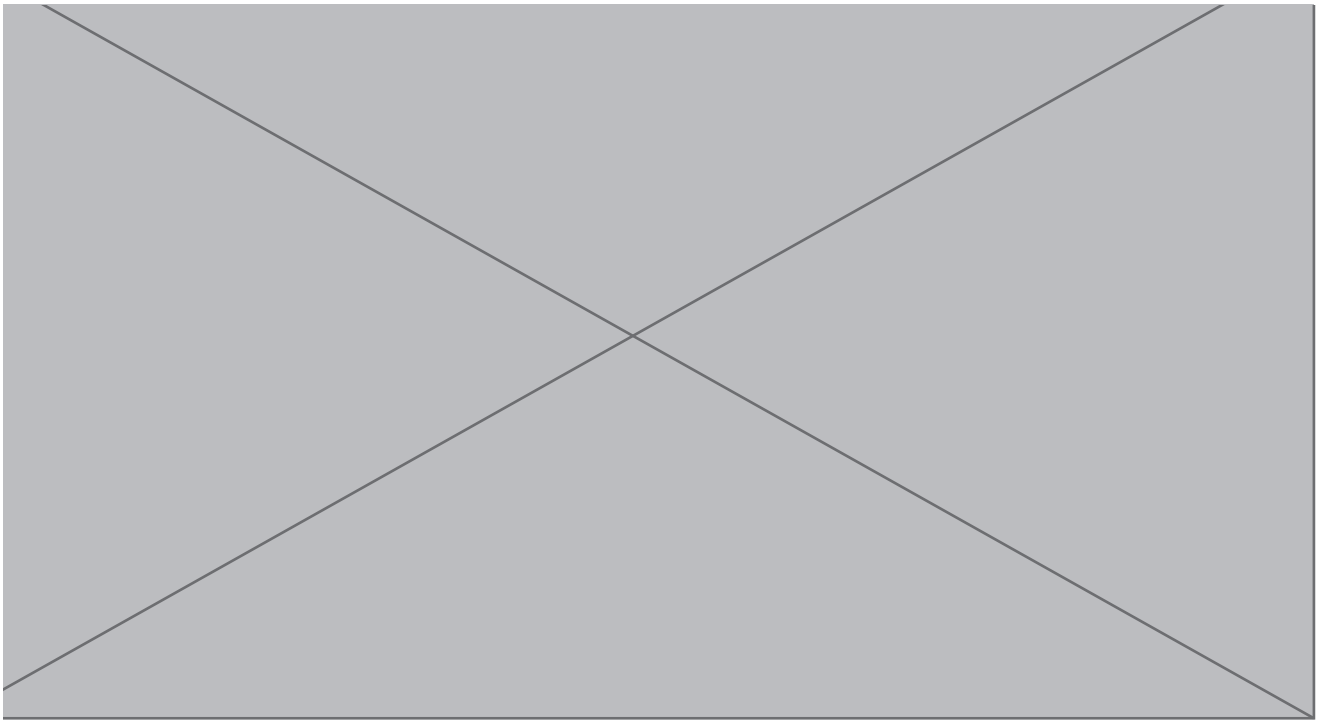

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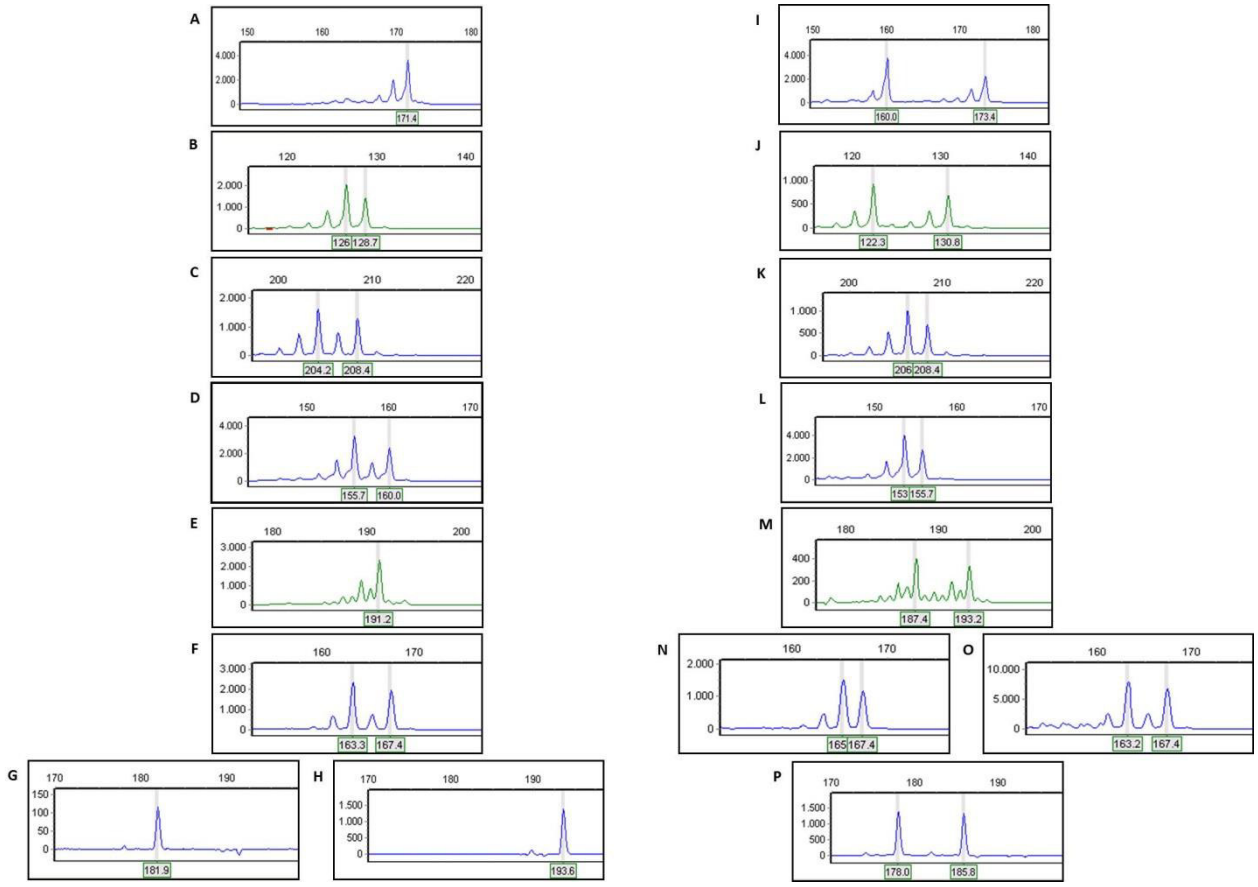
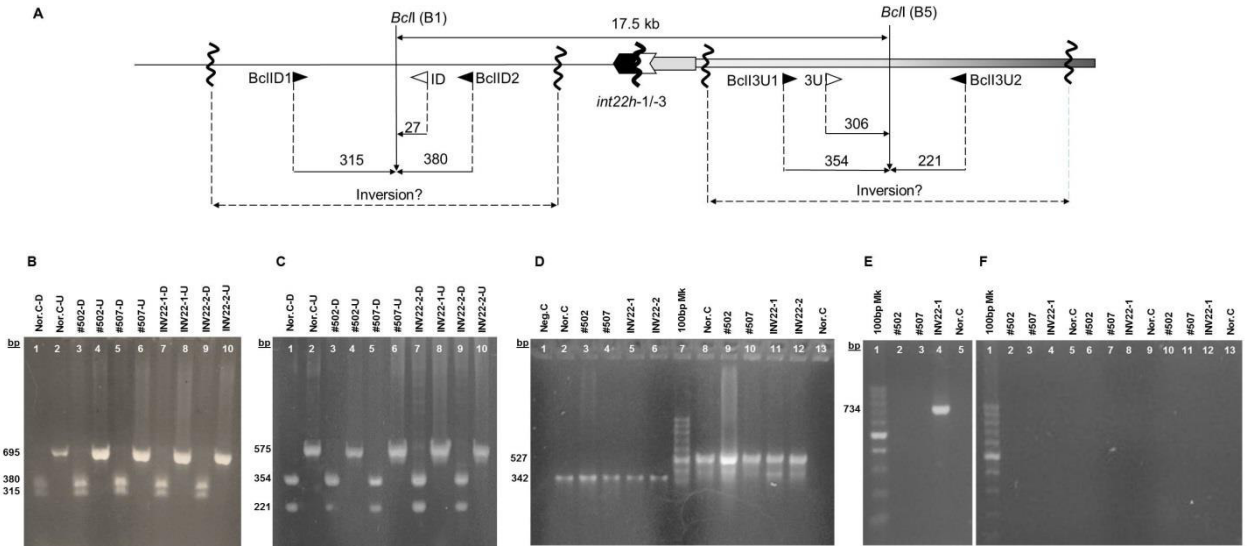
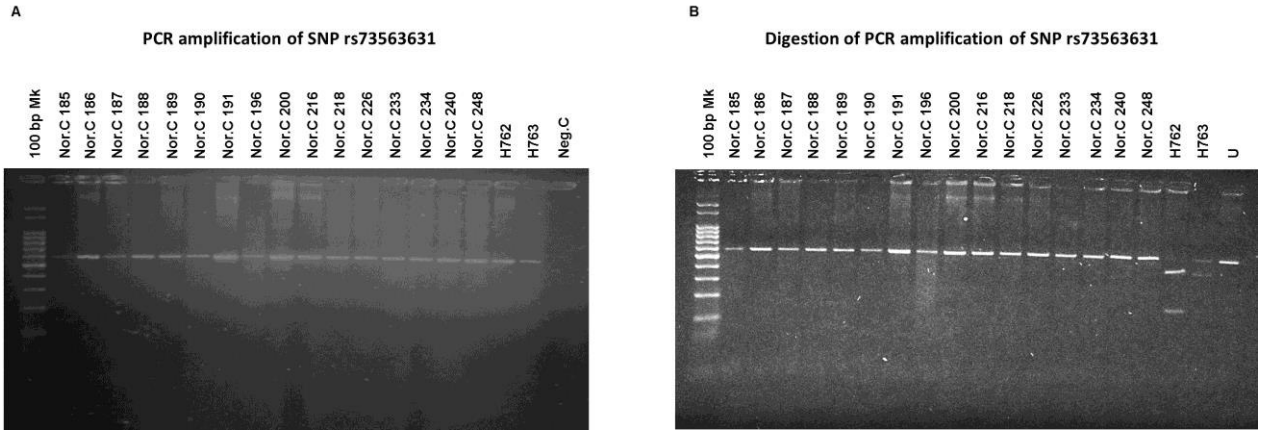


Suppl. Figure 1: **Linkage analysis in Family 1 and 2 using seven STR markers.** The figure shows an example of each marker analysed to perform the haplotype of each families. Left panel: INV22-1x (family 1), samples #762 (A), #763 (B, C, D, H, F), #507 (E, G). Right panel: INV22-2x (family 2), samples #817 (I, J, K), #1016 (L, M,N,P), #331 (O). Each line of the figure is associated with a specific genetic marker: DXS7423 (G, H, P), DXS1073 (B, J), STR25.3 (E, M), STR22 (C, K), STR21 (F, N, O), STR13 (D, L), DXS1108 (A, I). Seven *F8*-linked short tandem repeats (STR) (i.e., DXS7423, DXS1073, *F8*Int25.3, *F8*Int22, *F8*Int21, *F8*Int13 and DXS1108) were analysed by fluorescent capillary electrophoresis as was described [12].



Suppl. Figure 2: **Experiments to investigate the absence of IS-PCR dt signals on the Family 1 affected patients #502 and #507**. **A**: Schematic of the *Bcl*I restriction fragment (17.5kb) INV22 showing primer target locations and relevant molecular sizes of PCR products and restriction fragments. **B**: Agarose (1.5%) gel electrophoresis analysis of the PCR-RFLP of the B1 site. Undigested PCR product with primers BclID1 (5'-CCTGTTTCGTCTAGCTACCTCCTG-3') and BclID2 (GATCCTTTTTCCTTTCTTTTCCAG) yields 695bp and *Bcl*I digestion, 380bp and 315bp. **C**: Agarose (1.5%) gel electrophoresis analysis of the PCR-RFLP of the B5 site. Undigested PCR product with primers Bcl3IU1 (5'- AACTTTACTTTGATCCCATACA-3') and BclI3U2 (5'-ACTCATGCCTACAATCCCAG-3') yields 575bp and *Bcl*I digestion, 354bp and 221bp. **D**: Analysis of IS-PCR dt primer target sites ID and 3U. Agarose (1.5%) gel electrophoresis, lane 1-6: amplification with alternative primer BclI3U1 and conventional ID, PCR product yields 342bp, Lane 8-13: PCR amplification with conventional primer 3U and alternative primer BclI3U2, PCR product yields 527bp. **E and F**: Analysis of the potential inversions on the terminals of the 17.5kb fragment by combining equally oriented primers. Agarose (1.5%) gel electrophoresis analysis, **E**: Lanes 2-5: IS-PCR analysis performed on *Bcl*I circles with primers BclID2 and BclI3U1. **F**: Lanes 2-5: IS-PCR analysis (*Bcl*I circles) with primers BclID1 and BclI3U1, lanes 6-9: IS-PCR analysis (*Bcl*I circles) with primers BclID2 and BclI3U2. lanes 9-13: IS-PCR analysis (*Bcl*I circles) with primers BclID1 and BclI3U2. Lane sample code: 100bp Marker (Mk) (PB-L, Argentina), Nor.C (normal control), #502 and #507 (Family 1 affected males), INV22-1 and INV22-2 (affected patients with conventional IS-PCR dt signals), Neg.C (no-input negative contamination control), D (*Bcl*I digested PCR product), U (undigested/mock digested).



Suppl. Figure 3: **PCR-RFLP experiments to examine the frequency of the SNP associated with the Inv22 variants in our normal population. A:** Shows the amplification with primers Bcl_N-up/lo containing the SNP number (rs:73563631) on seven females, nine males of the general population as well as Normal Controls (Nor.C) and two members (a male with severe HA and his mother) of the family 1. **B:** Shows the digestion of the PCR product shown in A. Females of the general population (Nor.C: 187, 188, 191, 196, 200, 233, 240), males of the general population (Nor.C: 185, 186, 189, 190, 216, 218, 226, 234, 248), male with the INV22-1v (#762) and females carrier of the INV22-1v (#763) as a positive digestion control, Neg.C (no-input negative contamination control), U (undigested/mock digested), 100bp Marker (Mk) (PB-L, Argentina).