Genetic variants at the chromosomal region 2q21.3 underlying inhibitor development in patients with severe haemophilia A

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Abstract

Introduction: Inhibitor development affects about 30% of patients with severe haemophilia A (HA) and results from different environmental and genetic risk factors. Previously, we identified the missense variant rs3754689 in the LCT gene linked with this predisposition. Since rs3754689 variant is benign and is located in a conserved haplotype region, we hypothesized that the association signal captured by this variant is located in co-inherited, neighbouring genes.

Aim: To identify novel genetic risk factors associated with inhibitor development in coding regions of R3HDM1, UBXN4, CXCR4, MCM6, DARS and miR128-1 genes.

Methods: Targeted sequencing was performed in 246 severe HA patients (72 with and 174 without inhibitor): 181 previously and 65 newly enrolled.

Results: Forty-one common and 152 rare variants passed the quality control. Logistic regression analysis of common variants identified rs3754689 and four additional variants (.011 < P < .047; FDR ranging .2-.38). Logistic regression analysis performed only in the 220 Italian patients showed similar results (.004 < P < .05; FDR ranging .12-.22). Three of these variants (rs3213892 and rs3816155 in the LCT intron 13 and rs961360 in the R3HDM1 intron10-exon11 junction) may affect the expression of UBXN4 and R3HDM1, respectively. Rare variants did not show association with inhibitor development. Identified variants were not replicated in the multi-ethnic SIPPET cohort of 230 severe HA patients.

Conclusion: Due to the limited sample size that may be responsible of the high FDR values, we could not confirm with certainty the analysed association. Further evaluation of the expression levels of analysed genes will confirm or not their role in inhibitor development.

KEYWORDS
haemophilia A, high-throughput DNA sequencing, neutralizing antibodies, risk factors, single nucleotide polymorphism

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1 | INTRODUCTION

Haemophilia A (HA, OMIM #306700) is a bleeding disorder characterized by the deficiency of coagulation factor VIII (FVIII). Patients with FVIII coagulant activity in plasma less than 1% have severe disease and suffer from frequent and spontaneous bleeding episodes (up to 30/year) mainly at joints and muscles. Treatment of bleeding episodes consists of on-demand or prophylactic replacement therapy with plasma-derived or recombinant FVIII products. About 30% of patients with severe HA develop, mainly in the first 20 days of therapy, anti-FVIII neutralizing alloantibodies (i.e. inhibitors) that decrease the efficacy of the replacement therapy. In these cases, haemostasis can only be achieved using FVIII-bypassing agents (recombinant activated FVII and activated prothrombin complex concentrate) or a bispecific antibody mimicking the function of activated FVIII (emicizumab). Since both alternative treatments are very costly, understanding the aetiology of inhibitor development is an urgent need. Inhibitor development is a multifactorial complication resulting from environmental and genetic factors. Null mutations in the FVIII coding gene (F8), leading to the complete lack of FVIII activity, have been described as the strongest genetic risk factors for inhibitor development. In addition, polymorphisms in antigen presenting HLA class II molecules and in immune regulatory IL-1, IL-2, IL-10, CTLA4, TNFa, HMOX1, MAPK9 and CD32 genes have been reported to be associated with this complication although their role is still under debate due to the poor reproducibility of the genotyping results, the small replication cohorts, and the different genetic background of the analysed populations.

Results from our recent study, point towards the presence of novel risk factor(s) for inhibitor development in a highly conserved haplotype region surrounding the LCT gene on chromosome 2q21.3 were six genes (R3HD1M, UBXN4, CXCR4, MCM6, DARS and miR128-1), previously linked with autoimmune disorders and pathways of the immune response and therefore potential clinically relevant targets, are localized.

In this study, the targeted next generation sequencing (NGS) of the coding regions of these genes located in the 586-kb region surrounding the LCT gene has been performed in a cohort of 246 patients with severe HA to identify novel variants/genes associated with inhibitor formation.

2 | MATERIALS AND METHODS

2.1 | Patient cohorts

Two hundred and forty-six unrelated patients with severe haemophilia A (FVIII:C < 1%) regularly followed at the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Ca’ Granda, Ospedale Maggiore Policlinico (Milan, Italy) were enrolled (181 patients are from the replication cohort reported in Gorski et al and 65 are new patients). All patients received at least 50 infusions of FVIII during on-demand or prophylactic treatment. Of 246 patients, 72 experienced inhibitor development (cases) and the remaining 174 not (controls). The FVIII inhibitor was measured in patients’ plasma every 3–5 exposure days in children who started treatment, once a year or after a lack of response to the treatment or before any surgery in previously treated patients by means of the Bethesda assay with the Nijmegen modification. Inhibitor was defined as positive with two measured values > 5 Bethesda units (BU)/mL.

The replication study was conducted in an independent population of 230 patients with severe HA enrolled as part of the international SIPPET (Survey of Inhibitors in Plasma-Product Exposed Toddlers) study, of which 70 were inhibitor-positive and 160 inhibitor-negative.

Written informed consent was obtained from parents of underage patients and adult patients. The study was approved by the Ethics Committee of the Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico (Milan, Italy) and was carried out in accordance with the Declaration of Helsinki.

2.2 | NGS and data analysis

Genomic DNA was isolated from peripheral blood samples by the standard salting-out method. Coding regions including 10 bp of intron-exon boundaries and 100 bp of 5′ and 3′ untranslated regions of R3HD1M, UBXN4, CXCR4, MCM6 and DARS genes were amplified using TruSeq Custom Amplicon strategy (Illumina) (Table S1). Intrinsic single nucleotide polymorphisms (SNP) already reported in linkage disequilibrium (LD) with the missense variant rs375468921 and the intronic region of the R3HD1M gene encoding the miR128-1 were also included in the panel design producing a total target size of 19,737 bp (Table S1).

Paired-end sequencing was conducted on a MiSeq sequencing platform (Illumina). Obtained reads were mapped to the reference human genome hg19 using Burrows–Wheeler aligner (BWA-MEM algorithm). Variant calling and filtering was performed according to the guidelines reported by the Broad institute (https://software.broadinstitute.org/gatk/best-practices/). All 246 gVCF files were merged and cumulative VCF file was annotated by ANNOVAR. A second quality control step was performed by KGGSEquation (http://grass.cgs.hku.hk/limx/kgseq/): variants with low quality (Phred score Q < 30), Hardy-Weinberg equilibrium P < 1.0E-04 and genotypes with low depth (< 10) were excluded. VCFtools was used to select common/low-frequency (minor allele frequency, MAF ≥ 1%) and rare (MAF < 1%) variants. Variants with a call rate of < 80% were excluded from the analysis.

2.3 | Statistical analysis

Case control association test for common/low-frequency variants was performed by logistic regression model using Plink 1.07. Results were reported as odds ratios (ORs) with 95% confidence intervals.
In silico analyses

In silico analysis of identified variants

Identification of variants associated to inhibitor development

The replication study was performed using TaqMan SNP genotyping assays C_12054686_20 (rs1050115), C_27505260_10 (rs3816155), C_2104738_10 (rs3754689), C_15793430_10 (rs3087343) (Thermo Fisher Scientific) on a StepOnePlus RealTime Polymerase Chain Reaction system (Applied Biosystems) and direct sequencing of rs3213892 with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

2.4 | Replication

The replication study was performed using TaqMan SNP genotyping assays C_12054686_20 (rs1050115), C_27505260_10 (rs3816155), C_2104738_10 (rs3754689), C_15793430_10 (rs3087343) (Thermo Fisher Scientific) on a StepOnePlus RealTime Polymerase Chain Reaction system (Applied Biosystems) and direct sequencing of rs3213892 with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

2.5 | In silico analyses

Prediction of the pathogenicity of variants was estimated by CADD tool (https://cadd.gs.washington.edu/). The impact of variants on exonic splice enhancer (ESE) motifs and on splicing was evaluated with the ESE finder 3.0 software (rulai.cshl.edu/) and the Neural Network Splice Site prediction program (https://www.fruitfly.org/seqtools/splice.html), respectively. To assess localization of variants in functional elements in the human genome, Encyclopedia of DNA Elements (ENCODE) (https://www.encodeproject.org/) and Enhancer Atlas (http://enhanceratlas.org/) databases were examined.

3 | RESULTS

3.1 | Identification of variants associated to inhibitor development

Target sequencing has been performed in the discovery cohort of 246 patients with severe HA (72 inhibitor-positive and 174 inhibitor-negative). Data analysis revealed a total of 365 variants across all samples. After filtering, 193 variants (41 common; MAF ≥ 1% and 152 rare; MAF < 1%) passed the quality controls and included one in-frame deletion, two splicing, four nonsense, 42 synonymous, 54 missense and 90 intronic (Tables S2 and S3). One hundred and four variants are listed in dbSNP(150) and 89 are not reported. Case-control association analysis for common variants was carried out by logistic regression.

Association with inhibitor development was found for five common variants (Figure 1, Table 1): the missense variant rs3754689 (p.Val219Ile) in the LCT gene (P = .047; OR = .65; 95% CI = .43-.99), previously identified in our laboratory, and four additional common variants: rs1050115 (P = .011; OR = .56; 95% CI = .35-.87) in the UBXN4 gene, rs3213892 (P = .015; OR = .58; 95% CI = .37-.90) and rs3816155 (P = .023; OR = .60; 95% CI = .39-.93) in the LCT gene, and rs3087343 (P = .014; OR = .56; 95% CI = .36-.89) in the MCM6 gene (Table 1). To overcome the issue of genetic factors that may lead to confounding, logistic regression analysis was further performed in the sub-cohort of 220 patients with Italian ethnicity (i.e. the discovery cohort without the 26 non-Italian patients) (Supplementary Table 1). Similar results were obtained for the five common variants identified in UBXN4, LCT and MCM6 (Table 2) and additional three common variants with a nominal P < .05: rs961360 (P = .030; OR = .59; 95% CI = .36-.95) and rs12466487 (P = .030; OR = .57; 95% CI = .34-.95) in R3HDM1 and rs371309040 (P = .028; OR = .59; 95% CI = .37-.94) in DARS1 (Table 2).

Additional variants with no statistical significance were found in R3HDM1 (41 variants), DARS (23), CXCR4 (13), UBXN4 (18), LCT (60) and MCM6 (33) and no variants in the miR128-1 were identified (see Tables S2 and S3 for a comprehensive list of common and rare variants, respectively). The top variants are potentially protective against inhibitor development (.48 < OR < .64) despite the adjusted p-values were high (FDR ranging .12-.22) (Table 2). LD analysis evidenced that these variants were strongly associated with each other.

To test for independency of signal, a conditional logistic regression analysis was carried out for the most significant variant (rs1050115). All other variants lose the statistical significance (P > .05) (Tables 1 and 2).

Concerning rare variants, cumulative tests were carried out to evaluate the association between our target genes and the inhibitor development; none of them resulted significantly associated (Table S4).

3.2 | Replication of variants associated to inhibitor development

The replication study of five identified variants performed in the multiethic SIPPET cohort of 230 patients with severe HA (70 inhibitor-positive and 160 inhibitor-negative) showed no protection against inhibitor development (1.02 < OR < 1.19) (Table 1).

3.3 | In silico analysis of identified variants

To evaluate the potential pathogenic role of the identified variants, in silico analyses were performed. Due to the exonic localization of rs961360 (R3HDM1), rs3754689 (LCT) and rs1050115 (UBXN4) variants, the potential impact of nucleotide substitutions on ESE motifs was assessed. No ESE motifs were predicted in the region encompassing rs961360 (R3HDM1) and rs3754689 (LCT). Despite prediction of a binding site for the ESE-binding SRSF1 protein in the region encompassing rs.
DISCUSSION

Inhibitor formation is a multifactorial complication with a heterogeneous genetic component. Although the type of mutation in F8 gene is ascertained as the strongest risk factor in patients with severe HA, the mutation alone does not completely explain all the genetic components and increasing evidence supports a combined role of several other genetic factors. In this scenario, the present work continues our recent whole exome sequencing study that identified the missense variant p.Val219Ile (rs3754689) in the LCT (lactase-phlorizin hydrolase) gene associated with inhibitor development.\textsuperscript{21} Since this variant, a polymorphism with a predicted benign effect,\textsuperscript{27} is located in a conserved haplotype block surrounding the LCT gene at 2q21.3, we hypothesized that the association signal, captured by the rs3754689 variant, was located in co-inherited genes. Five neighbouring genes (R3HDM1, UBXN4, MCM6, DARS, CXCR4) that we selected for target sequencing are directly or indirectly involved in autoimmune diseases and/or in pathways of immune response. A reduced expression of R3HDM1 gene, coding a poorly characterized RNA binding protein, was recently found in patients with celiac disease.\textsuperscript{28} Moreover, the expression of MCM6, located in the R3HDM1 intron 18 (Figure 1), has been shown to be upregulated in naive CD4+ T cells from patients with multiple sclerosis\textsuperscript{29} and dysregulated in CD4+ lymphocytes of patients with systemic sclerosis,\textsuperscript{30} both autoimmune diseases. The UBXN4 protein is a member of the UBXN family; while UBXN1 is involved in innate immunity,\textsuperscript{31} UBXN4 has been demonstrated to function in endoplasmic reticulum–associated protein degradation (ERAD) and in degradation of the ERAD substrate CD3δ.\textsuperscript{32} The MCM6 protein is essential for the initiation of eukaryotic genome replication and is highly expressed in patients with systemic lupus erythematosus.\textsuperscript{33} The DARS gene, encodes the cytosolic aspartyl tRNA synthetase, with multiple non-canonical functions including mediation of inflammatory and immune responses.\textsuperscript{33,34} Finally, overexpression of chemokine receptor CXCR4 in patients with systemic lupus erythematosus has been reported to promote inflammatory cell infiltration into renal tissue.\textsuperscript{35} Given the alloimmune nature of inhibitor formation, the aforementioned genes and microRNA are interesting targets with putative role in FVIII inhibitor development.

To identify variants associated with inhibitor development, we performed target sequencing in the 586-kb chromosomal region 2q21.3-q22.1 encompassing the LCT gene in a large cohort of patients with a putative common genetic background (90% are of Italian ethnicity); 72.6% of patients are from the replication cohort reported in Gorski et al\textsuperscript{21} and 26.4% are new patients accounting for 1/3 of the here analyzed cases (patients with inhibitor). The protective effect of the previously identified LCT p.Val219Ile missense variant was confirmed by the odd ratio almost unchanged than previous one (OR = .65 vs .58 and .64 in total and Italian discovery cohorts, respectively). Moreover, we identified seven additional common variants, four in both cohorts and three only in the Italian cohort, with a protective effect
FIGURE 2  In silico analysis of rs961360 in R3HDM1 and rs3213892 and rs3816155 in LCT. A schematic representation of R3HDM1 (A) and LCT (B) genes is drawn to scale; exons are represented by gray boxes and introns by straight lines. (A) Physiologic and predicted acceptor (3'') and donor (5'') splice sites of R3HDM1 exon 11 are showed with the corresponding score (score range 0–1; threshold .6). (B) The LCT region with predicted regulatory elements is represented. The localization of rs3213892 and rs3816155 variants in intron 13 is shown.

(OR < 1). Three of these variants (rs1050115 in UBXN4, rs3087343 in MCM6 and rs3213892 in LCT) were previously found in LD with the missense variant p.Val219Ile, confirming the highly conserved haplotype region encompassing the LCT, the lactase enzyme responsible for the digestion of lactose. Persistence of LCT in adulthood is partly promoted by a single nucleotide polymorphism (rs4988235) at 14 kb upstream from LCT in the cis-acting enhancer element localized in the intron 13 of the MCM6 gene. Interestingly, two of the identified variants (rs3213892 and rs3816155) are located close together (10 nt distance) in the LCT intron 13, a region predicted as a potential regulatory element of the UBXN4 gene and localized 53 kb downstream from the start of transcription of the adjacent UBXN4 gene. Hence, the molecular mechanisms underlying the association between the identified variants and inhibitor development could rely on the different expression of the UBXN4 gene that, as ERAD protein, could be involved in FVIII antigen presentation of antigen-presenting cells. Since one of the variants identified in the Italian cohort (rs961360) is located in the R3HDYM1 exon 11 acceptor splice site and activation of a cryptic donor splice site around the mutated site was predicted in silico, an additional/alternative mechanism could rely on an alternative processing of the R3HDYM1 transcript and different synthesis/function of the corresponding protein. Since limitations of this study relies on (i) the low statistical power (51.5%) due to the disease rarity and the unavailability of the right sample size that may be responsible of the high FDR values (.12-.22) and (ii) the sequencing restricted to the coding regions of target genes that did not allow the identification of possible variants in regulatory regions not covered by sequencing, assessment of the expression levels of the analysed immunological target genes (R3HDM1, UBXN4, MCM6, DARS, CXCR4 and miR128-1) in patients with and without the identified variants should be undertaken to confirm or not our findings.

The lack of association of the identified variants with inhibitor development in our available replication cohort can rely on the different genetic background (i.e. ethnicity) of the SIPPET cohort consisting of several populations (mainly Indian, Egyptian and Iranian). Recently, the association of the LCT p.Val219Ile missense variant (rs3754689) to inhibitor development in patients with severe HA has been found in Afghans but not confirmed in Brazilian and Iranian populations. Since the allele frequencies of all the identified variants reported in the 1000 Genome project are similar in the European and the South Asian populations but are markedly different in the Italian population (Table 1), a replication study performed in an independent cohort of Italian patients could be suitable to overcome the problem of poor reproducibility of genotyping results.
### TABLE 1  
Statistical analysis of variants identified by NGS in the discovery and replication cohorts

| dbSNP ID  | Positiona | Variant | Gene | Ex/Int | cDNA | aa variation | Minor allele | MAFAb | MAFc | MAFF | MAFc | Plogistic | OR | 95%CI | P adj-adjusted | P cond^a | P logistic | OR | 95%CI | p-adjusted | P cond^a |
|------------|------------|---------|------|--------|------|--------------|--------------|-------|-------|-------|-------|------------|----|-------|----------------|----------|------------|----|-------|-------------|----------|
| rs1050115  | 136511817C | A > G   | UBXN4| Ex 4   | c.303A > G | G   | .21           | .41           | .25   | .38   | .011  | .56   | .35- .87   | .20 |       | (.024)         | .58      | 1.13       | .73- 1.74 | .93     |
| rs3213892  | 136552517G | G > A   | LCT  | Int 13 | c.4977-172C > T | /   | A            | .21           | .41   | .26   | .38   | .015  | .58        | .37- .90 | .20   | .93   | (.071)       | .84      | 1.02       | .67- 1.55 | .93     |
| rs3816155  | 136552526G | A > C   | LCT  | Int 13 | c.4977-181T > G | /   | A            | .21           | .41   | .27   | .38   | .023  | .60       | .39- .93 | .24   | .59   | (.049)       | .75      | 1.19       | .79- 1.80 | .93     |
| rs3754689  | 136590746G | C > T   | LCT  | Ex 2   | c.655G > A  | p.Val219lle | T    | .22           | .43   | .29   | .38   | .047  | .65       | .43- .99 | .38   | .92   | (.051)       | .93      | 1.09       | .72- 1.65 | .93     |
| rs3087343  | 136622543G | T > G   | MCM6 | Int 7   | c.1078+40A > C | /   | G            | .22           | .43   | .25   | .38   | .014  | .56       | .36- .89 | .20   | .89   | (.071)       | .79      | 1.06       | .70- 1.62 | .93     |

Abbreviations: aa, aminoacid; CHI_SQ, chi square; CI, confidence interval; ctr, controls; EU, European; Ex, exon; Int, intron; MAF, minor allele frequency; N, number of patients; OR, odds ratio; p-adjusted, multiplicity adjusted p-value, calculated using the Benjamini and Hochberg’s (FDR_BH) method.

^aGenomic coordinates of chromosome 2 (hg19).

^bFrequency from 1000 Genomes Project Phase 3_European population.

^cFrequency from 1000 Genomes Project Phase 3_Italian Tuscany population.

^dP (logistic) conditioned rs1050115.

### TABLE 2  
Statistical analysis of variants identified by NGS in the discovery cohort of Italian patients

| dbSNP ID  | Positiona | Variant | Gene | Ex/Int | cDNA | aa variation | Minor allele | MAFAb | MAFc | MAFF | MAFc | Plogistic | OR | 95%CI | P adj-adjusted | P cond^a | P logistic | OR | 95%CI | p-adjusted | P cond^a |
|------------|------------|---------|------|--------|------|--------------|--------------|-------|-------|-------|-------|------------|----|-------|----------------|----------|------------|----|-------|-------------|----------|
| rs961360   | 136393658C | A > G   | R3HDM1| Ex 11  | c.808A > G | p.Met270Val | G   | .22           | .41   | .25   | .37   | .030  | .59       | .36- .95 | .18   | .98   | (.011)       | .67      | 1.26       | .72- 2.06 | .83     |
| rs12466487 | 136407078C | T > C   | R3HDM1| Int 16 | c.1624-222T > C | /   | C            | .19           | .38   | .22   | .33   | .030  | .57       | .34- .95 | .18   | .65   | (.035)       | .79      | 1.26       | .72- 2.06 | .83     |
| rs1050115  | 136511817G | A > G   | UBXN4| Ex 4   | c.303A > G | G   | .21           | .41   | .23   | .38   | .004  | .48       | .29- .79 | .12   |       | (.044)       | .52      | 1.33       | .70- 2.39 | .73     |
| rs3213892  | 136552517G | G > A   | LCT  | Int 13 | c.4977-172C > T | /   | A            | .21           | .41   | .25   | .38   | .012  | .54       | .33- .87 | .12   | .56   | (.046)       | .54      | 1.26       | .70- 2.20 | .77     |
| rs3816155  | 136552526G | A > C   | LCT  | Int 13 | c.4977-181T > G | /   | A            | .21           | .41   | .25   | .38   | .012  | .54       | .33- .87 | .12   | .56   | (.046)       | .54      | 1.26       | .70- 2.20 | .77     |
| rs3754689  | 136590746G | C > T   | LCT  | Ex 2   | c.655G > A  | p.Val219lle | T    | .22           | .43   | .28   | .38   | .050  | .64       | .40-1.0  | .22   | .44   | (.052)       | .70      | 1.18       | .71- 1.79 | .84     |
| rs3087343  | 136622543G | T > G   | MCM6 | Int 7   | c.1078+40A > C | /   | G            | .22           | .43   | .24   | .38   | .011  | .52       | .32- .86 | .12   |       | (.069)       | .78      | 1.26       | .70- 2.20 | .77     |
| rs371309040| 136690425G | T > C   | DARS1| Int 6   | c.505-13T > C | /   | C            | .21           | .44   | .28   | .39   | .028  | .59       | .37- .94 | .18   | .71   | (.069)       | .78      | 1.26       | .70- 2.20 | .77     |

Abbreviations: aa, aminoacid; CHI_SQ, chi square; CI, confidence interval; ctr, controls; EU, European; Ex, exon; Int, intron; MAF, minor allele frequency; N, number of patients; OR, odds ratio; p-adjusted, multiplicity adjusted p-value, calculated using the Benjamini and Hochberg’s (FDR_BH) method.

^aGenomic coordinates of chromosome 2 (hg19).

^bFrequency from 1000 Genomes Project Phase 3_European population.

^cFrequency from 1000 Genomes Project Phase 3_Italian Tuscany population.

^dP (logistic) conditioned rs1050115.
In conclusion, with the forementioned limitations, there is some evidence that LCT locus is a susceptibility locus for inhibitor development in Italian patients with severe HA. Further deeper investigations are needed to understand the effective contribution of R3HDH1 and UBXN4 on FVIII inhibitor development. Apart from their possible immunological role, the identified association between the genetic variants in the LCT locus and the risk of inhibitor development may be useful to better predict the risk of inhibitor development in Italian patients with severe HA.

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CONFLICTS OF INTEREST
RG reports participation in advisory boards for Biomarin, Pfizer, Bayer and Takeda and participation at educational seminars sponsored by Pfizer, Sobi and Roche. FP reports participation in advisory boards for Roche, Sanofi, Sobi, Takeda and Bologna and participation at educational meeting sponsored by Grifols and Roche. The other authors have no disclosures.

AUTHOR CONTRIBUTIONS
SS performed in-silico analyses, interpreted the results and drafted the manuscript. AC performed data analysis, interpreted the results and participated in manuscript preparation. EP performed NGS experiments. MMG designed the research and critically reviewed the manuscript. IG collected data, helped with the interpretation of the results and critically reviewed the manuscript. SH helped with the interpretation of the results and critically reviewed the manuscript. RG collected patients’ data. FP supervised the research, interpreted the results and critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Additional supporting information may be found in the online version of the article at the publisher’s website.

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