



Methodological paper

Targeted next-generation sequencing for the molecular diagnosis of hereditary angioedema due to C1-inhibitor deficiency



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ABSTRACT

SERPING1 genotyping of subjects suspicious for hereditary angioedema due to C1-INH deficiency (C1-INH-HAE) is important for clinical practice as well as for research reasons. Conventional approaches towards the detection of C1-INH-HAE-associated *SERPING1* variants are cumbersome and time-demanding with many pitfalls. To take advantage of the benefits of next-generation sequencing (NGS) technology, we developed and validated a custom NGS platform that, by targeting the entire *SERPING1* gene, facilitates genetic testing of C1-INH-HAE patients in clinical practice. In total, 135 different C1-INH-HAE-associated *SERPING1* variants, out of the approximately 450 reported, along with 115 negative controls and 95 randomly selected DNA samples from affected family members of C1-INH-HAE index patients, were included in the forward and reverse validation processes of this platform. Our platform's performance, i.e. analytical sensitivity of 98.96%, a false negative rate of 1.05%, analytical specificity 100%, a false positive rate equal to zero, accuracy of 99.35%, and repeatability of 100% recommends its implementation as a first line approach for the genetic testing of C1-INH-HAE patients or as a confirmatory method. A noteworthy advantage of our platform is the concomitant detection of single nucleotide variants and copy number variations throughout the whole length of the *SERPING1* gene, moreover providing information about the size and the localization of the latter. During our study, 15 novel C1-INH-HAE-related *SERPING1* variants were detected.

1. Introduction

Hereditary angioedema is a relatively rare, disabling and potentially

life-threatening disorder characterized by recurrent attacks of subcutaneous or submucosal edemas, which develop as a result of a transient and localized increase of bradykinin and increased permeability of

Abbreviations: C1-INH, C1-inhibitor; C1-INH-HAE, hereditary angioedema due to C1-INH deficiency; CI, confidence interval; MLPA, multiplex ligation-dependent probe amplification; NGS, next-generation sequencing; CNV, copy number variation; SNV, single-nucleotide variant; *SERPING1*-NGS platform, the custom NGS platform developed for *SERPING1* genotyping; U-HAE, unknown hereditary angioedema

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blood vessels. C1-INH-HAE is the most common and best-characterized form of hereditary angioedema (Cicardi et al., 2014). It is attributed to genetic alterations in the *SERPING1* gene encoding C1-INH, a regulatory protein of the contact system. *SERPING1* gene extends over a 17,159 bp genomic region located on chromosome 11q12-q13.1. Its pronounced allelic heterogeneity as well as the fact that approximately 20–25% of all unrelated patients with C1-INH-HAE represent sporadic cases carrying de novo *SERPING1* mutations with the same mutational spectrum as the familial ones, makes *SERPING1* a prime example of mutagenic lability. *SERPING1*'s proximity to the centromere and the high incidence of Alu repeat sequences and CpG sites explain its higher than average mutation rates (Pappalardo et al., 2002; Cicardi et al., 2005). Over 450 different *SERPING1* mutations related with C1-INH-HAE have been detected up to now, scattered over the eight exons and their flanking intronic sequences (Germeis & Speletas, 2016). The majority of C1-INH-HAE-related *SERPING1* mutations are SNVs (34% missense mutations, 10% splice-site defects, 7% nonsense mutations, 1% regulatory mutations) followed by CNVs (31% frameshift alterations and small indels, 17% large gene rearrangements) (Speletas et al., 2015a). In a small proportion of patients with C1-INH-HAE (approximately 5–10%) no *SERPING1* alterations can be detected even after meticulous analysis of the coding region (Speletas et al., 2015a; Pappalardo et al., 2008; Csuka et al., 2015) implying that the defect that modifies C1-INH expression may be located in an intronic or an untranslated region of the gene, like those recently reported (Maas et al., 2008; Kang et al., 2006; Colobran et al., 2014).

The diagnosis of C1-INH-HAE is based on its clinical appearance and is confirmed by the detection of low plasma levels of antigenic and/or functional C1-INH. However, with every passing day, *SERPING1* genotyping of patients who suffer from hereditary angioedema becomes more indispensable in the clinical practice (Farkas et al., 2017) as well as for research reasons. The conventional molecular analysis of *SERPING1* relies on with the prioritized amplification of all exons and the exon/intron boundaries by PCR and the detection of mutations by direct sequencing. If no mutation were found, further analyses for the identification of large gene rearrangements are performed, usually by two different techniques, namely the long-range PCR and the MLPA. This cumbersome and time-consuming approach is fraught with pitfalls as it is highly dependent on users' experience and knowledge. Sometimes, for example, a novel missense mutation is incorrectly held to be the causal genetic defect because its detection during the early steps of the analysis has prevented the discovery of an indel leading to premature truncation of protein synthesis. Moreover, deep intronic alterations that may affect pre-mRNA splicing and possibly contributing to C1-INH-HAE phenotype, as it is true for many other diseases (Vaz-Drago et al., 2017), escape the conventional approach.

NGS overcomes many of shortcomings of traditional approaches. Here, we report the development and the validation of a custom NGS platform that targets the entire 11q12-q13.1 locus, including the promoter, coding, intron-exon boundary as well as intronic regions of the *SERPING1* gene, following the recently published professional standards and guidelines (Rehm et al., 2013; Weiss et al., 2013).

2. Material and methods

2.1. DNA samples

102 DNA samples from already diagnosed and genotyped C1-INH-HAE index patients (99 type I, 3 type II; 31 Hungarian, 33 Greek, 17 German, 11 Romanian, 10 Spanish) carrying different *SERPING1* variants were used for the forward validation of the newly developed NGS platform for *SERPING1* genotyping. The previously reported C1-INH-HAE associated SNVs and indels (76), and CNVs (22) are shown in Tables 1 and 2, respectively. Four additional samples in which the detection of a causal *SERPING1* defect had not been achieved were also included in the analysis. As negative controls, 115 DNA samples from

patients diagnosed with U-HAE (normal antigenic and functional C1-INH plasma levels) previously genotyped and free of *SERPING1* mutations were used.

Another cohort of 91 not genotyped patients (4 Greek, 30 Bulgarian, 36 Polish, 11 German, and 10 Romanian) already diagnosed with C1-INH-HAE (88 type I, 3 type II) belonging to 55 different families was used for the blind reverse validation of the developed *SERPING1*-NGS platform.

In order to test the repeatability of the *SERPING1*-NGS platform, 95 randomly selected DNA samples from C1-INH-HAE-affected family members of the above index patients were analyzed.

2.2. Next-generation sequencing

A NGS custom panel was designed using the Ion AmpliSeq Thermo Fisher Scientific Designer, in order to analyze *SERPING1* in its full length (all exons, introns, promoter, 5'- and 3'-untranslated regions –UTRs). 77 amplicons in two primer pools provide 100% coverage of all translated regions and UTRs with missing areas located only in intronic regions (overall coverage ≈83%). Additional amplicons (300 in total) for different genes in all chromosomes, selected among those encoding for proteins involved in bradykinin metabolism, were included in the panel, in order to achieve a more robust CNVs analysis.

DNA libraries for each sample were constructed using the Ion AmpliSeq Library Kit 2.0 (Thermo Scientific). Briefly, 10 ng of gDNA per primer pool was amplified and indexed with a unique adapter using Ion Xpress barcode adapters kit (Thermo Scientific). Equal volumes of the two pools were combined and barcoded libraries were purified using the Agencourt AMPure XP Beads (Beckman Coulter), quantified with Qubit 2.0 fluorometer (Thermo Scientific), diluted to 100 pM and pooled in equimolar proportion. Template preparation was carried out on OneTouch2 system using the Ion OT2 HI-Q Template kit (Thermo Scientific) and the enrichment of Ion Sphere Particles (ISPs) on Ion One Touch ES (Thermo Scientific). Sequencing was performed on PGM using Ion PGM Hi-Q sequencing kit (Thermo Scientific), on 318 chip. All procedures were performed according to the manufacturer's instructions.

Sequencing raw data were analyzed for base calling, demultiplexing, alignment to hg19 reference genome (GRCh37), coverage analysis and variant calling on Torrent Suite 5.2 software using default parameters. More specifically, variant calling was performed by the VariantCaller v.5.2 plug-in and coverage analysis by the coverageAnalysis v.5.2.1.2 plug-in. Annotation of variants and CNVs analysis was performed on Ion Reporter software v.5.2 (Thermo Scientific). Detected variants were annotated with the gene name and for their possible presence in the dbSNP database according the recommendations of the Human Genome Variation Society (HGVS) (<http://www.hgvs.org/mutnomen/>). In silico pathogenicity prediction of the variants was performed by the usage of SIFT (<http://sift.jcvi.org/>) and PolyPhen version 2 (<http://genetics.bwh.harvard.edu/pph2/>) bioinformatics tools. CNVs were detected by Hidden Markov Model algorithm on Ion Reporter software v.5.2 using paired CNVs analysis and default parameters. CNV analysis workflow uses depth of coverage and is limited by the number and extend of amplicons that cover the affected regions. Alignments and all obtained *SERPING1* sequences were visually inspected using the Integrative Genomics Viewer (IGV) v2.2 (Broad Institute).

2.3. Variants verification

All identified variants were verified by Sanger sequencing as previously described (Speletas et al., 2015a). Emerged CNVs were verified by a commercial MLPA kit (MRC-Holland for *SERPING1*) and by long-range PCR. In MLPA, 60 ng of gDNA from each sample were analyzed. Three reference samples (without CNVs), one positive and one negative control were included. All procedures were performed according to

Table 1The SNVs carried by the previously genotyped patients used in the forward validation of the *SERPING1*-NGS platform.

cDNA	Protein	Location	Type of mutation	Reference
c.1A > G	M1V	Exon 2	Regulatory	(Gösswein et al., 2008)
c.1A > C ^a	M1L	Exon 2	Regulatory	(Speletas et al., 2015b)
c.2T > G	M1R	Exon 2	Regulatory	(Speletas et al., 2015b)
c.51 + 1G > A		Intron 2	Splice defect	(Gösswein et al., 2008)
c.51 + 5G > A		Intron 2	Splice defect	(Verpy et al., 1996)
c.65C > G	S22X	Exon 3	Nonsense	(Speletas et al., 2015a)
c.94C > T	Q32X	Exon 3	Nonsense	(Kalmár et al., 2003)
c.121delG	G41 fs	Exon 3	Frameshift	(Speletas et al., 2015b)
c.124G > T	E42X	Exon 3	Nonsense	(Speletas et al., 2015b)
c.235 delA	T79 fs	Exon 3	Frameshift	(Speletas et al., 2015b)
c.239C > G	A80G	Exon 3	Missense	(Speletas et al., 2015b)
c.325_337delCTCCCAACAGATT	T111 fs	Exon 3	Frameshift	(Speletas et al., 2015b)
c.347delA	Q116fs	Exon 3	Frameshift	(Speletas et al., 2015b)
c.357_358insGGGTCCTCTGCCCAGGACC	V127 fs	Exon 3	Frameshift	(Speletas et al., 2015b)
c.389G > A	C130Y	Exon 3	Missense	(Kalmár et al., 2003)
c.392_393delCT	S131X	Exon 3	Nonsense	(Speletas et al., 2015a)
c.435_476delTTTGGTAGATTTCTCCCTGAAGCTCTACCACGCCTTCTCAGC	L145_S158del	Exon 3	Deletion	(Kalmár et al., 2003)
c.448 T > C	S150P	Exon 3	Missense	(Speletas et al., 2015b)
c.467C > A	A156D	Exon 3	Missense	(Gösswein et al., 2008)
c.498C > A	N166 K	Exon 3	Missense	(Cagini et al., 2016)
c.500T > A	M167 K	Exon 3	Missense	(Speletas et al., 2015b)
c.508delT	S170 fs	Exon 3	Frameshift	(Speletas et al., 2015b)
c.550G > A	G184R	Exon 3	Missense	(Verpy et al., 1996)
c.550 + 2,550 + 3insT		Exon 3	Frameshift	(Kalmár et al., 2003)
c.550 + 1G > A		Intron 3	Splice defect	(Kalmár et al., 2003)
c.550 + 2T > C		Intron 3	Splice defect	(Speletas et al., 2015b)
c.550 + 5G > A		Intron 3	Splice defect	(Roche et al., 2005)
c.551-1G > A		Intron 3	Splice defect	(Speletas et al., 2015b)
c.553G > C	A185P	Exon 4	Missense	(Speletas et al., 2015a)
c.586-589DelATCC	I195fs	Exon 4	Frameshift	(Speletas et al., 2015b)
c.600insC	K201 fs	Exon 4	Frameshift	(Speletas et al., 2015b)
c.650_651delGT	V218 fs	Exon 4	Frameshift	(Speletas et al., 2015b)
c.675-76TC > AA	F225X	Exon 4	Nonsense	(Speletas et al., 2015b)
c.667C > T	Q223X	Exon 4	Nonsense	(Kalmár et al., 2003)
c.671 T > A	I224N	Exon 4	Missense	(Gösswein et al., 2008)
c.728 T > C	L243P	Exon 5	Missense	(Speletas et al., 2015a)
c.871A > C	N291H	Exon 5	Missense	(Gösswein et al., 2008)
c.878 T > C	I293T	Exon 5	Missense	(Pappalardo et al., 2000)
c.889 + 4delAGGGT		Intron 5	Splice defect	(Speletas et al., 2015b)
c.890-14C > G		Intron 5	Splice defect	(Speletas et al., 2015b)
c.896G > A ^b	W299X	Exon 6	Nonsense	(Speletas et al., 2015b)
c.908 T > C	F303S	Exon 6	Missense	(Gösswein et al., 2008)
c.1029 + 1G > A		Intron 6	Splice defect	(Gösswein et al., 2008)
c.1030-1G > A		Intron 6	Splice defect	(Speletas et al., 2015b)
c.988 T > G	Y330D	Exon 7	Missense	(Speletas et al., 2015a)
c.1036C > T	Q346X	Exon 7	Nonsense	(Verpy et al., 1996)
c.1038_1052delGCTGCAGCTCTCCCA	Q346_S350del	Exon 7	Deletion	(Speletas et al., 2015b)
c.1106delA	D369fs	Exon 7	Frameshift	(Speletas et al., 2015b)
c.1127_1128insC	S377 fs	Exon 7	Frameshift	(Speletas et al., 2015a)
c.1180delA	T394 fs	Exon 7	Frameshift	(Speletas et al., 2015b)
c.1187 T > C	L396P	Exon 7	Missense	(Gösswein et al., 2008)
c.1193 T > C	L398P	Exon 7	Missense	(Gösswein et al., 2008)
c.1223A > T	D408V	Exon 7	Missense	(Kalmár et al., 2003)
c.1243delA	K416 fs	Exon 7	Frameshift	(Gösswein et al., 2008)
c.1250-2A > G		Intron 7	Splice defect	(Gösswein et al., 2008)
c.1341_1342insGAACTGAC_dupl	E451fs	Exon 8	Frameshift	(Speletas et al., 2015b)
c.1346 T > G	L449R	Exon 8	Missense	(Speletas et al., 2015b)
c.1353_1354delGA	E451fs	Exon 8	Frameshift	(Gösswein et al., 2008)
c.1356_1357delTG	T454 fs	Exon 8	Frameshift	(Speletas et al., 2015a)
c.1367C > A	A456Q	Exon 8	Missense	(Skriver et al., 1991)
c.1373C > T	A458V	Exon8	Missense	(Siddique et al., 1992)
c.1382_1383insGGGGTGGAGGCGGCTGCAGCCTCCGC	I462fs	Exon 8	Frameshift	(Speletas et al., 2015a)
c.1391_1392delTG	V464 fs	Exon 8	Frameshift	(Speletas et al., 2015a)
c.1396C > T	R466C	Exon 8	Missense	(Skriver et al., 1991)
c.1396C > G	R466G	Exon 8	Missense	(Gösswein et al., 2008)
c.1397G > A	R466H	Exon 8	Missense	(Faiyaz-Ul-Haque et al., 2010; Rijavec et al., 2013)
c.1409insG	V470 fs	Exon 8	Frameshift	(Speletas et al., 2015b)
c.1417G > A	V473 M	Exon 8	Missense	(Verpy et al., 1996)
c.1418 T > G	V473G	Exon 8	Missense	(Blanch et al., 2002)
c.1418 T > A	V473E	Exon 8	Missense	(Kalmár et al., 2003)
c.1420C > T	Q474X	Exon 8	Nonsense	(Speletas et al., 2015b)
c.1446G > A	W482X	Exon 8	Nonsense	(Speletas et al., 2009)
c.1466delC	P489fs	Exon 8	Frameshift	(Speletas et al., 2015a)
c.1478G > A	G493E	Exon 8	Missense	(Blanch et al., 2002)

(continued on next page)

Table 1 (continued)

cDNA	Protein	Location	Type of mutation	Reference
c.1480C > T	R494X	Exon 8	Nonsense	(Verpy et al., 1996)
c.1483-7delGTATA	V495X	Exon 8	Nonsense	(Speletas et al., 2015b)
c.1493C > G	P498R	Exon 8	Missense	(Speletas et al., 2015a)
c.1499_1505delCCTGAGA	A500fs	Exon 8	Frameshift	(Speletas et al., 2015b)

^a Previously reported by conventional genotyping as large insertion in exon 5.

^b Previously reported by conventional genotyping as large deletion in exon 6.

manufacturers' instructions. Fragment analysis was carried out on ABI 3730xl (Applied Biosystems) and MLPA data analysis in Coffalyser.Net software (MRC-Holland). Long-range PCR was performed as previously described (Pappalardo et al., 2000).

3. Results

3.1. Validation

Our NGS panel generates 300 amplicons of 204 bp on average, covering all translating regions, exon-intron boundaries, UTRs and most of the intronic regions of *SERPING1* gene, with overlapping amplicons across the gene. In the 102 DNA samples from conventionally genotyped C1-INH-HAE patients analyzed (48 SNVs, 28 indels, 22 large defects, and 4 without variants of *SERPING1*), a mean of 313,270 reads was produced per sample. On average, 91.5% of these reads mapped to the targeted regions, with a mean depth of coverage of 270 × and 90% uniformity. For variant calling, a minimum 20 × coverage was set as threshold.

Forward analysis of DNA samples with our *SERPING1*-NGS platform was concordant with conventional *SERPING1* typing in 97/102 cases, i.e. 47/48 SNVs, 26/28 indels, 20/22 large defects and 4/4 with no defect. Out of the five observed discrepancies, the conventional approach had miss-assigned two substitutions as large defects, i.e. a large insertion located in exon 5 and a large deletion located in exon 6. NGS analysis identified in the first of these samples a SNP altering the start codon of *SERPING1* (c.2T > G, M1R) and in the second a mutation resulting in an early termination codon (c.896G > A, W299X). The presence of both of these mutations was confirmed by Sanger sequencing, whereas large defects were not detected by MLPA and long-range

PCR. CNV analysis by our *SERPING1*-NGS platform did not uncover large defects in these samples.

The other three discrepancies were a 4 bp deletion (c.586-589delATCC, 1196fs), a missense mutation (c.553G > C, A185P) and a deletion of a single base (c.347delA, Q116fs). The first one was identified by VariantCaller as c.585C > G. However, by visually reviewing the IGV file, we found a complex variation with a 4 bp deletion and a single nucleotide substitution (c.585_589delCATCCinsG) assigned as S195 fs that thereafter was confirmed by Sanger sequencing. After adjustment of the VariantCaller by setting the allow_complex parameter to 1 instead of 0 and re-analysis, this complex variation was correctly recognized. Similarly, VariantCaller did not detect the other two variants (c.553G > C and c.347delA) that were again recognized after visually reviewing the IGV files. In fact, VariantCaller had highlighted these positions but as high background that was filtered out due to strand bias (< 1% in + strand). Adjustment of VariantCaller strand bias settings and re-analysis uncovered the presence of these mutations; however, this adjustment resulted in a high proportion of false positive variants which precluded its permanent use.

It has to be noted that all the 20 real CNVs of the initial cohort used for the forward validation of our *SERPING1*-NGS platform were correctly detected regardless of their type (deletion, duplication) and location. Moreover, information about their size and CytoBand localization was provided (Table 2). Similarly, in the 4 samples initially reported as not carrying any defect, the *SERPING1*-NGS platform also did not detect a defect.

Reverse validation of our *SERPING1*-NGS platform by blindly genotyping 91 C1-INH-HAE patients from 55 unrelated families detected *SERPING1* variants or their absence in all cases that were a posteriori confirmed by appropriate methods when required. Forty-four different

Table 2

The CNVs carried by the previously genotyped patients used in the forward validation of the *SERPING1*-NGS platform. (Two samples initially reported as CNVs by the conventional genotyping but not finally confirmed are not shown. See footnotes of Table 1).

CytoBand ^a	Length ^a	Location ^a	MLPA or long range PCR	Reference
11q12.1(57364831-57382476)x1	17.645 kb	exon 1–8	deletion exons 1–8	(Roche et al., 2005)
11q12.1(57364831-57382476)x1	17.645 kb	exon 1–8	deletion exons 1–8	(Roche et al., 2005)
11q12.1(57364831-57382476)x1	17.645 kb	exon 1–8	deletion exons 1–8	(Speletas et al., 2015a)
11q12.1(57364831-57382476)x1	17.645 kb	exon 1–8	deletion exons 1–8	(Speletas et al., 2015a)
11q12.1(57364831-57367229)x1	2.398 kb	upstream-intron 2	deletion exons 1–2	(Roche et al., 2005)
11q12.1(57366421-57369726)x1	3.305 kb	intron 2-intron 4	deletion exons 3–4	(Speletas et al., 2015a)
11q12.1(57367936-57369930)x1	1.994 kb	intron 3-intron 4	deletion exon 4	(Speletas et al., 2015a)
11q12.1(57368466-57369930)x1	1.464 kb	intron 3-intron 4	deletion exon4	(Speletas et al., 2015a)
11q12.1(57368466-57370856)x1	2.39 kb	intron 3-intron5	deletion exon 4	(Roche et al., 2005)
11q12.1(57368466-57370856)x1	2.39 kb	intron 3-intron 5	deletion exon 4	(Roche et al., 2005)
11q12.1(57368904-57369930)x1	1.026 kb	intron 3-exon 4	deletion exon 4	(Speletas et al., 2015a)
11q12.1(57369000-57370856)x3	1.856 kb	intron 3-intron 4	duplication exon 4	(Speletas et al., 2015a)
11q12.1(57369000-57370856)x3	1.856 kb	intron 3-intron 4	duplication exon 4	(Roche et al., 2005)
11q12.1(57369219-57370856)x1	1.637 kb	intron 3-intron 4	deletion exon 4	(Speletas et al., 2015a)
11q12.1(57371760-57376644)x1	4.884 kb	intron 4-intron 6	deletion exons 5–6	(Roche et al., 2005)
11q12.1(57373059-57378387)x3	5.328 kb	intron 4-intron 6	duplication exon 5–6	(Roche et al., 2005)
11q12.1(57375659-57382476)x1	6.817 kb	intron 6-exon 8	deletion exon 7–8	(Speletas et al., 2015a)
11q12.1(57377605-57380942)x1	3.337 kb	intron 6-intron 7	deletion exon 7	(Roche et al., 2005)
11q12.1(57379394-57380190)x1	796b	exon 7-intron 7	deletion exon 7	(Speletas et al., 2015a)
11q12.1(57379785-57382169)x1	2.384 kb	intron 7-exon 8	deletion exon 8	(Roche et al., 2005)

^a Final information obtained by CNV analysis using the *SERPING1*-NGS platform.

Table 3The different *SERPING1* variants detected during the reverse validation of *SERPING1*-NGS platform.

Origin	cDNA	Protein	Location	Type	Reference
PL	c.550G > A	G184R	Exon 3	Missense	(Verpy et al., 1996) ^a
BL	c.550 + 1G > A		Intron 3	Splice defect	(Roche et al., 2005) ^a
BL	c.600_601insC	K201 fs	Exon 4	Frameshift	(Speletas et al., 2015b) ^a
ROM	c.988 T > G	Y330D	Exon 6	Missense	(Speletas et al., 2015a) ^a
PL	c.1396C > T	R466C	Exon 8	Missense	(Skriver et al., 1991) ^a
GER	c.1396C > G	R466G	Exon 8	Missense	(Gösswein et al., 2008) ^a
GR	c.1480C > T	R494X	Exon 8	Nonsense	(Verpy et al., 1996) ^a
BL	c.-22-1G > A		Intron 1	Splice defect	(Verpy et al., 1996)
GR	c.55A > T	R19X	Exon 3	Nonsense	Novel
PL	c.94delC	Q32fs	Exon 3	Frameshift	Novel
PL	c.152C > T	S51F	Exon 3	Missense	Novel
BL	c.218delA	N73 fs	Exon 3	Frameshift	Novel
PL	c.301C > T	Q101X	Exon 3	Nonsense	Novel
ROM	c.424_425insGT	L142 fs	Exon 3	Frameshift	Novel
PL	c.425 T > C	L142S	Exon 3	Missense	Novel
BL	c.473C > G	S158X	Exon 3	Nonsense	(López-Lera et al., 2011)
PL	c.485_498delAGGTGGAGACCAACinsTGCTGAGA	K162 fs	Exon 3	Frameshift	Novel
ROM	c.503C > A	A168D	Exon 3	Missense	(Suffritti et al., 2014)
GER	c.508 T > A	S170 T	Exon 3	Missense	Novel
PL	c.551-2insG		Intron 3	Splice defect	Novel
PL	c.622C > T	Q208X	Exon 4	Nonsense	Novel
PL	c.793 T > C	T265R	Exon 5	Missense	(Bafunno et al., 2014)
BL	c.813_818delCAACAA	N271_N272del	Exon 5	Nonsense	Novel
GER	c.890-2A > G		Intron 5	Splice defect	(Bowen et al., 2001)
ROM	c.860 T > C	L287P	Exon 6	Missense	Novel
BL	c.897G > A	W299X	Exon 6	Nonsense	(Gösswein et al., 2008)
GR	c.1012C > T	Q338X	Exon 6	Nonsense	(Iwamoto et al., 2012)
GER	c.1027insC	K343 fs	Exon 6	Frameshift	Novel
ROM	c.1042C > T	Q348X	Exon 7	Nonsense	(Gösswein et al., 2008)
ROM/PL	c.1223A > G	D408G	Exon 7	Missense	(Martinho et al., 2013)
GR	c.1247 T > A	L416X	Exon 7	Nonsense	(Pappalardo et al., 2008)
GER	c.1250-1G > A		Intron 7	Splice defect	(Gösswein et al., 2008)
BL	c.1283G > A	C428Y	Exon 8	Missense	(López-Lera et al., 2011)
BL	c.1330C > T	Q444X	Exon 8	Nonsense	(Uyguner & Kesim, 2008)
PL	c.1333A > C	T445P	Exon 8	Missense	Novel
GER	c.1346 T > C	L449P	Exon 8	Missense	(Pappalardo et al., 2008)
PL	c.1372G > A	A458T	Exon 8	Missense	(Levy et al., 1990)
GER	11q12.1(57368466-57369930)x1	1.464 kb	Exon 4	Large defect	^b
ROM	11q12.1(57368466-57369930)x1	1.464 kb	Exon 4	Large defect	^b
ROM	11q12.1(57368466-57371760)x4	3.294 kb	Exon 4	Large defect	^b
BL	11q12.1(57368904-57370856)x1	1.952 kb	Exon 4	Large defect	^b
PL	11q12.1(57368904-57369726)x1	822b	Exon 4	Large defect	^b
PL	11q12.1(57368466-57369930)x1	1.464 kb	Exon 4	Large defect	^b
PL	11q12.1(57379590-57382476)x1	2.886 kb	Exon 8	Large defect	^b

^a Detected in forward validation.^b Large defects in the same region (exons 4 and 8) have been reported. However, data on their exact extend and localization are not available; therefore, it is unknown whether these are exactly the same defects.

variants were detected of which 15 (34.1%) were missense, 11 (25%) nonsense, 7 (15.9%) large defects, 6 (13.6%) frameshift mutations and 5 (11.4%) splice-site defects (Table 3). No *SERPING1* alteration was detected in 8 out of the 55 families (14.5%), neither by the *SERPING1*-NGS platform nor the confirmatory conventional genotyping. Twenty two out of the 37 SNVs detected during reverse validation had been already reported in the literature, while 15/37 were not yet reported alterations (Table 3). Interestingly, one large defect reported by the *SERPING1*-NGS platform as a large duplication of 3.3 kb [11q12.1(57368466–57371760) × 4, including exon 4] was confirmed by long-range PCR, but not by MLPA. Possibly, the observed duplication consists only of intronic regions and, as a result, the CNVs analysis of *SERPING1*-NGS platform led to reporting a more extended defect.

In total, 135 different C1-INH-HAE-associated *SERPING1* variants were included in the overall validation process of our *SERPING1*-NGS platform and the platform failed to detect only two variants. Further, analysis of negative controls did not reveal any C1-INH-HAE-associated variants, although there were some polymorphisms of the *SERPING1* gene (e.g. V480 M). As such, the *SERPING1*-NGS platform's analytical sensitivity was 98.96% (95% CI 96.31–99.87%) with a false negative rate 1.05%, its analytical specificity was 100% (95% CI

96.84–100.00%) with a false positive rate equal to zero, and its accuracy was 99.35% (95% CI 97.67–99.92%). The repeatability of the *SERPING1*-NGS platform was found to be 100%.

3.2. Novel *SERPING1* mutations

Out of the 15 novel mutations detected during the reverse validation, 5 (33.3%) were missense, 4 (26.6%) nonsense, 5 (33.3%) frameshift mutations and 1 (6.6%) splice-site defect. All novel missense mutations were predicted to be deleterious by SIFT and PolyPhen prediction tools; however, none had been detected in > 65,000 Europeans of the ExAC Database (except for S51F, with an allele frequency of < 0.001%). Pathogenicity of eight of the novel mutations was supported by family segregation studies. Family segregation also proved that three of nonsense variants represented de novo mutations. The fact that one novel missense mutation (c.508 T > A) occurred at amino acid residue where a different missense change has been previously observed in association with C1-INH-HAE [c.508 T > C (Pappalardo et al., 2000)] is further evidence of that mutation's pathogenicity (Richards et al., 2015). Similarly, the novel mutation c.551-2insG occurs at the –2 position of the acceptor site of exon 4, where a

substitution (c.551-2A > G) was previously reported in four patients from three C1-INH-HAE families (Gösswein et al., 2008). Finally, the observed c.301C > T (Q101X) has been previously detected in three affected members of a Serbian family (Andrejević et al., 2015), but it was reported as c.300C > T (Q101X).

4. Discussion

In the vast majority of patients, the laboratory diagnosis of C1-INH-HAE is based on the measurement of antigenic and/or functional C1-INH plasma levels. However, genetic testing is often helpful or required. For example, patients younger than 1 year-of-age present with C1-INH levels lower than in adults (Craig et al., 2012; Nielsen et al., 1994), making diagnosis by traditional means difficult. Similarly, genetic testing is useful in the workflows for prenatal and preimplantation diagnostics (Farkas et al., 2017), as well as for confirmation of causal *SERPING1* variants carried by apparently healthy individuals whose relative(s) have been diagnosed with C1-INH-HAE (Kasami et al., 2018). Further, peculiarities of the C1-INH-HAE-associated *SERPING1* variants frequently make the conventional approach towards their detection quite laborious and time-demanding. Confirmation of the results obtained by some widely used methods, like MLPA for the detection of large defects, is usually recommended. The NGS platform we developed and validated takes advantages of the benefits of NGS technology, targets the entire *SERPING1* gene, and facilitates genetic testing of C1-INH-HAE patients in clinical practice.

The developed *SERPING1*-NGS platform was validated against 135 C1-INH-HAE-associated variants of all types (SNVs and CNVs) representing about 1/3 of the up to now reported variants (≈ 450). Its performance and estimated analytical parameters combined with its time and cost saving advantages justify its use in the clinical practice as a first line approach for the genetic testing of C1-INH-HAE patients. A significant advantage of our platform is that it screens the whole length of the *SERPING1* gene, simultaneously detecting SNVs and CNVs, while also providing information about the size and the localization of CNVs. According to our findings, confirmation of the results obtained by the *SERPING1*-NGS platform would be required mainly in cases of negative results in patients suspected for C1-INH-HAE. It is true that large defects remain a demanding issue, but no single method can cover the requirements for the detection of all types of large defects. Finally, the *SERPING1*-NGS platform provides automated assignment of variants in a user-friendly manner, thereby minimizing the possibility of nomenclature errors that commonly occur with traditional methods for cases of multiple nucleotide variants.

The fact that we did not use a standard conventional procedure for *SERPING1* analysis to validate the performance of our platform might represent a limitation of our study. However, such a “gold standard” has not yet been proposed by the community with different researchers following various workflows using classical techniques. Thus, we decided to compare the performance of a high-throughput approach against the overall bias presented by conventional methodologies.

Overall coverage of our *SERPING1*-NGS platform is $\approx 83\%$; however, all of the missing regions are in deep intronic regions. Given that deep intronic variants have not yet been associated with C1-INH-HAE, we did not make any further effort to improve the coverage. Besides, the existing coverage of intronic regions already gives our platform a significant advantage in accurate detection of the extent and localization of CNVs.

During the forward validation of the *SERPING1*-NGS platform, we discovered that up to 2.94% (3/102) of variants are miss-assigned by conventional genotyping. Clearly, even the results of the conventional approaches require confirmation, which can be provided by our platform. Alternatively, the *SERPING1*-NGS platform could be the primary genotyping approach for C1-INH-HAE. After all, our study detected a large number of novel *SERPING1* mutations and analysis by our *SERPING1*-NGS platform provided substantial information on

SERPING1 polymorphisms, including within intronic regions and synonymous variants (data not presented).

5. Conclusion

We present a novel and validated NGS platform that targets the entire *SERPING1* gene, facilitating genetic testing of C1-INH-HAE patients in clinical practice. The platform screens the entire *SERPING1* gene, simultaneously detecting SNVs and CNVs and providing information about the size and the localization of CNVs. Obviously, potential users must address the special demands and quality prerequisites of NGS technology (in general and with respect to the specific platform), especially if employing it as the primary approach for genetic testing. Nevertheless, the *SERPING1*-NGS platform offers a powerful approach for clinical analysis of patients with respect to C1-INH-HAE. Fifteen novel C1-INH-HAE-related *SERPING1* variants were detected during the validation process of the platform.

Author contributions

G.L., M.Z.: Designed the experiments, performed experiments, analyzed the data, writing of the manuscript; F.P., S.V.: Performed experiments; C.D., A.L.-L., M.L.-T., M.S. H.F.: Provided conventionally genotyped samples; F.P., G.P., K.O., A.V., M.S., D.M., M.M., M.M.: Provided non-genotyped samples; A.E.G. Conceived and coordinated the study, analyzed the data, writing of the manuscript. All authors reviewed the manuscript.

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Conflict of interest statement

Authors have no conflicts of interest to declare.

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