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RESEARCH ARTICLE

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Multi-gene panel testing improves diagnosis and management of patients with hereditary anemias

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Abstract

Mutations in more than 70 genes cause hereditary anemias (HA), a highly heterogeneous group of rare/low frequency disorders in which we included: hyporegenerative anemias, as congenital dyserythropoietic anemia (CDA) and Diamond-Blackfan anemia; hemolytic anemias due to erythrocyte membrane defects, as hereditary spherocytosis and stomatocytosis; hemolytic anemias due to enzymatic defects. The study describes the diagnostic workflow for HA, based on the development of two consecutive versions of a targeted-NGS panel, including 34 and 71 genes, respectively. Seventy-four probands from 62 unrelated families were investigated. Our study includes the most comprehensive gene set for these anemias and the largest cohort of patients described so far. We obtained an overall diagnostic yield of 64.9%. Despite 54.2% of cases showed conclusive diagnosis fitting well to the clinical suspicion, the multi-gene analysis modified the original clinical diagnosis in 45.8% of patients (nonmatched phenotype-genotype). Of note, 81.8% of nonmatched patients were clinically suspected to suffer from CDA. Particularly, 45.5% of the probands originally classified as CDA exhibited a conclusive diagnosis of chronic anemia due to enzymatic defects, mainly due to mutations in *PKLR* gene. Interestingly, we also identified a syndromic CDA patient with mild anemia and epilepsy, showing a homozygous mutation in *CAD* gene, recently associated to early infantile epileptic encephalopathy-50 and CDA-like anemia. Finally, we described a patient showing marked iron overload due to the coinheritance of *PIEZO1* and *SEC23B* mutations, demonstrating that the multi-gene approach is valuable not only for achieving a correct and definitive diagnosis, but also for guiding treatment.

1 | INTRODUCTION

Hereditary anemias (HA) embrace a highly heterogeneous group of rare/low frequency disorders characterized by anemia of variable degree and by complex and often unexplained genotype-phenotype correlations. HA are genetic disorders caused by mutations in more than 70 genes controlling red blood cell (RBC) production and structure. Mutations in these genes can lead to alterations in hemoglobin (Hb) levels, RBC differentiation and proliferation, cell membrane structure, defective activity of erythrocyte enzymes. Within this large group of diseases, we included: (1) hyporegenerative anemias, as congenital dyserythropoietic anemias (CDA) and Diamond-Blackfan anemia (DBA); (2) erythrocyte membrane defects, as hereditary spherocytosis (HS) and hereditary stomatocytosis (HST); hemolytic anemias due to enzymatic defects, as glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase (PK) deficiency.[1–6]

Differential diagnosis, classification, and patient stratification among HA are often very difficult. Indeed, the variety of unspecific and overlapping phenotypes often hampers a correct clinical management of the patients. Particularly, the diagnosis of these conditions may

Roberta Russo and Immacolata Andolfo equally contributed to the study.

require several lines of investigation.[1–4] The first is based on clinical features (evaluation of complete blood count), positive familial history, and the observation of a peripheral blood smear. The second line comprises biochemical tests and highly specialized analyzes, as the ektacytometry for the diagnosis of erythrocytes membrane defects, which are available only in few laboratories in the world. The last line includes the molecular analysis of the causative gene by direct sequencing. Unfortunately, in several cases these investigations fail to achieve the correct diagnosis, especially when the family history is uninformative or when routine laboratory tests produce unclear data.

Due to the failure of the current diagnostic workflow to find a definitive and correct diagnosis of HA, next-generation sequencing (NGS) is making its way on this field. The major current application of NGS in diagnostics is through disease-targeted tests for which multiple causal genes are known. Some studies have already demonstrated the usefulness of targeted-NGS (t-NGS) approach in the investigation of specific subtypes of HA patients. The multi-gene panels described so far include a gene set ranging from 28 to 40 loci causative of CDA, DBA, hemolytic anemias due to enzymatic defects, HS, and sideroblastic anemia.[7–9]

We herein described the diagnostic workflow that we established for molecular diagnosis of hereditary hemolytic anemias, based on the development of two consecutive versions of a t-NGS panel, comprising 34 and 71 causative/candidate genes, respectively. We showed the results obtained by the analysis of 74 probands from 62 unrelated families. This is the largest cohort of patients and the most comprehensive gene set for this subset of anemias described so far.

2 | METHODS

2.1 | Patients and genomic DNA preparation

Seventy-four patients with clinical suspicion of different types of HA were included in the study. Particularly, 15 patients were originally suspected of erythrocyte membrane defects, comprising HS, hereditary elliptocytosis (HE), and hereditary pyropoikilocytosis (HPP); 20 probands were clinically suspected of HST, comprising dehydrated hereditary stomatocytosis (DHS), overhydrated hereditary stomatocytosis (OHS), sitosterolemia (STL), and familial pseudohyperkalemia (FP); 4 patients had clinical suspicion of DBA; finally, 35 cases were clinically classified as CDA, comprising CDA type I (CDAI), type II (CDAII), and unspecific CDA.

Diagnosis was based on history, clinical findings, laboratory data, morphological analysis of peripheral blood and/or aspirated bone marrow, whenever available.[1–4] For HS/HST patients, diagnosis was also based on indirect tests (osmotic fragility of erythrocyte, AGLT50, EMA binding test), as well as on ektacytometry. Local university ethical committees approved collection of patient's data from Medical Genetics Ambulatory in Naples (University Federico II, DAIMedLab).

DNA samples from the patients were obtained after signed informed consent and according to the Declaration of Helsinki. Whenever possible, affected and unaffected relatives were also enrolled to correctly assess the pathogenicity of each variant by the analysis of family segregation.

Genomic DNA (gDNA) preparation was performed as previously described.[10] To evaluate the quality of the extracted gDNA before the fragmentation, samples were quantified by NanoDrop 2000 (Thermo Scientific, Italy). Then, gDNA was loaded on 0.8% DNA agarose gel electrophoresis.

2.2 | In silico design of the custom gene panels, sample preparation

We created two custom gene panels: (1) the first, named RedPlex_rev.0, including 34 causative/candidate genes of CDA and erythrocyte membrane defects (Supporting Information Table S1a)[11]; (2) the second, named RedPlex_rev.1, composed by 71 genes causative or candidates of CDA, RBC membrane defects, DBD, hemolytic anemia due to enzymatic defects (Supporting Information Table S1b).

For the probe design, coding regions, 5′UTR, 3′UTR, 50 bp flanking splice junctions were selected as regions of interest. The probe design was performed by web-based tool SureDesign (https://earray.chem.agilent.com/suredesign.htm, Agilent Technologies, USA). Sequence length was set at 150 × 2 nucleotides. For RedPlex_rev.0, the target size was 239.764 kbp, with average target coverage of 99.9%. For RedPlex_rev.1, the target size was 324.879 kbp, with target coverage of 99.8%.

Sample preparation was performed following the instruction's manufacturer for HaloPlex Target Enrichment kit for Illumina Sequencing - Custom Design from 1 to 500 kb (Agilent Technologies).

2.3 | Sequencing and data analysis

High-throughput sequencing was performed by Illumina NextSeq 500. Agilent SureCall software (v 3.0.3.1, Agilent Technologies) was used for bioinformatic and computational analyzes.

According to the guidelines of American College of Medical Genetics and Genomics (ACMG), we evaluated the pathogenicity of each variant by gathering evidence from various sources: population data, computational and predictive data, functional data, and segregation data.[12] In agreement with the criteria of moderate evidence of pathogenicity, annotated variants were firstly filtered on the basis of their minor allele frequency (MAF). Due to the large range of prevalence in the population of these heterogeneous disorders, we selected both rare and low frequency variants (MAF < 0.01 and 0.05, respectively), as reported by 1000 Genomes (http://browser.1000genomes.org/index.html), ExAC (http://exac.broadinstitute.org/), and Exome Variant Server (EVS, http://evs.gs.washington.edu/EVS/). Computational and predictive data were obtained by using several in silico prediction programs. Particularly, coding variants were analyzed by the prediction tools PolyPhen2, SIFT, and the recently introduced classifier for rare missense variants, M-CAP. This latter is a clinical pathogenicity classifier that outperforms existing methods at all thresholds and correctly dismisses 60% of rare, missense variants of uncertain significance in a typical genome at 95% sensitivity.[13] Prediction analysis for splice sites mutations was also performed by web server tool Human Splicing Finder (http://www.umd.be/HSF/), as previously described.[14] Regarding the functional data for the new variants, we did not perform in vitro functional studies supportive of a damaging effect on the gene or gene

product, as requested to obtain strong evidence of pathogenicity. However, we selected and reported those variants with moderate pathogenic evidence, i.e., variants located in a mutational hot spot and/or critical and well-established functional domain.[12] All the prioritized variants were confirmed by Sanger sequencing and by the analysis of inheritance pattern. The validations were performed using 50 ng of genomic DNA. Custom primers were designed by Primer3 program (Primer3 v. 0.4.0, freeware online). Sequence primers are available on request (roberta.russo@unina.it). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of ATG translation initiation codon in the reference sequence, according to the nomenclature for the description of sequence variants of Human Genome Variation Society (www.hgvs.org/mutnomen). The initiation codon is codon 1.

3 | RESULTS

3.1 | Diagnostic workflow of RedPlex study

The diagnostic workflow used in the present study was depicted in Figure 1A. We analyzed 80 NGS libraries from 74 HA patients with different clinical suspicion. Both panels, RedPlex_rev.0 and its updated version RedPlex _rev.1, showed high sensitivity and specificity. Indeed, approximately 97% and 93% of analyzable target bases were covered by at least 20 reads in RedPlex_rev.0 and RedPlex_rev.1 respectively (Supporting Information Table S2).

Overall clinical features and diagnostic suspicion of the 74 HA patients included in the study were summarized in Supporting Information Table S3. Particularly, 27.0% of the enrolled patients (20/74) were clinically suspected to suffer from HST, while 20.3% of probands (15/74) exhibited a clinical diagnosis of red cell membrane defects. However, the majority of patients was originally classified as hyporegenerative anemias, mainly CDAs (35/74, 47.3%) (Supporting Information Table S3).

Twenty-nine patients from 25 unrelated families were analyzed by RedPlex_rev.0: 55.2% (16/29) of them were conclusively diagnosed, but 44.8% (13/29) received no diagnosis. During the second-round of t-NGS analysis, we analyzed 51 patients from 42 different families: 62.7% (32/51) of probands obtained a diagnosis, while 37.3% (19/51) of them were undiagnosed (Figure 1A). Overall, 64.9% (48/74) of analyzed patients were successfully diagnosed.

Among the 13 undiagnosed patients included in the first-round analysis, six of them underwent the second-round by RedPlex_rev.1: two patients obtained a conclusive diagnosis, while the remaining four probands resulted negative again. Of note, among the 19 undiagnosed patients analyzed by RedPlex_rev.1 we also counted the four undiagnosed patients previously analyzed. Overall, 35.1% (26/74) of investigated patients resulted undiagnosed. All the nondiagnosed patients were recommended for whole exome sequencing (WES) analysis (Figure 1A).

3.2 | Phenotype-genotype matching

Tables 1 and 2 summarize pathogenic/likely pathogenic variants we identified in HA diagnosed patients by both multi-gene panels, showing moderate/strong evidence of pathogenicity as established by the ACMG guidelines. The molecular data obtained by our t-NGS analysis confirmed the clinical suspicion in 54.2% of patients (26/48) (matched phenotype-genotype). Nevertheless, the multi-gene approach modified the original diagnosis in 45.8% of patients (22/48) (nonmatched phenotype-genotype) (Figure 1B). Of note, the majority of patients with matched phenotype-genotype are included in the subgroup of erythrocyte membrane defects. Indeed, 84.6% (22/26) of them were originally suspected of HST or HS. Conversely, 81.8% (18/22) of nonmatched patients were clinically suspected to suffer from CDA. Accordingly, among 22 patients originally classified as CDA, only 18.2% (4/22) of them showed matched phenotype-genotype (Figure 1B). Particularly, two probands originally suspected of CDAII, RP0_36, and RP0_39, were conclusively diagnosed as CDAIa and CDAIb, respectively; while, RP0_59 and RP1_41, both initially suspected of CDAI, were diagnosed as CDAIV and CDA variant due to a heterozygous mutation in *ALAS2*, as recently described (Tables 1 and 2; Supporting Information Tables S3).[17]

Interestingly, four patients originally classified as CDA exhibited mutations in *PIEZO1*, the causative gene of DHS1. The first case was RP0_38, a 13-year-old boy showing mild anemia (Hb 11.7 g/dL) with macrocytosis (MCV 94 fl) and reticulocytosis (164 220/μL). The second, RP1_63, was a 0.8-year-old girl with normocytic severe anemia (MCV 80.0 fl, Hb 6.2 g/dL), increased MCHC, and transfusion-dependency (Supporting Information Table S3). Both patients exhibited heterozygous missense mutations in the C-terminal intracellular domain of PIEZO1 protein that forms the pore of the channel, mediating the ion conduction and the cation selectivity.[18,19] Of note, RP1_63 patient also coinherited a heterozygous *G6PD* mutation, namely the G6PD Sierra Leone, that was categorized with enzymatic activity of class III (Tables 1 and 2).[20] The remaining cases, RP1_16 and RP1_17, were two siblings carrying a missense variant in homozygous state that falls in one of the last transmembrane domains of the protein (Table 2). Both probands exhibited similar clinical features most resembling those of DHS1 patients: mild anemia (Hb 11.7–12.8 g/dL) with macrocytosis (MCV 91 fl) and reticulocytosis (145 920-266 880/μL) (Supporting Information Table S3).

3.3 | Congenital hemolytic anemias misdiagnosed as CDAs

Among 22 patients originally classified as CDA, 45.5% (10/22) of them exhibited a final diagnosis of chronic anemia due to enzymatic defects (Figure 1B). The first case was a patient originally suspected of CDAI, RP1_71, showing macrocytic anemia (Hb 8.9 g/dL, MCV 111.0 fl) with slight reticulocytosis (112 000/μL) (Supporting Information Table S3), carrying two recently described pathogenic missense mutations in *GPI*, the causative gene of hemolytic nonspherocytic anemia due to glucose phosphate isomerase deficiency (Table 2).[16] The second case, RP1_85, was a 29-year-old girl with severe normocytic anemia (Hb 6.6 g/dL, MCV 86.0 fl) and reticulocytosis (218 000/μL) (Supporting Information Table S3), exhibiting a nonsense mutation in *AK1* gene, the causative locus of hemolytic anemia due to adenylate kinase deficiency (Table 2).

Interestingly, 36.4% (8/22) of patients clinically referred as CDA showed mutations in *PKLR*, the causative gene of PK deficiency. Of

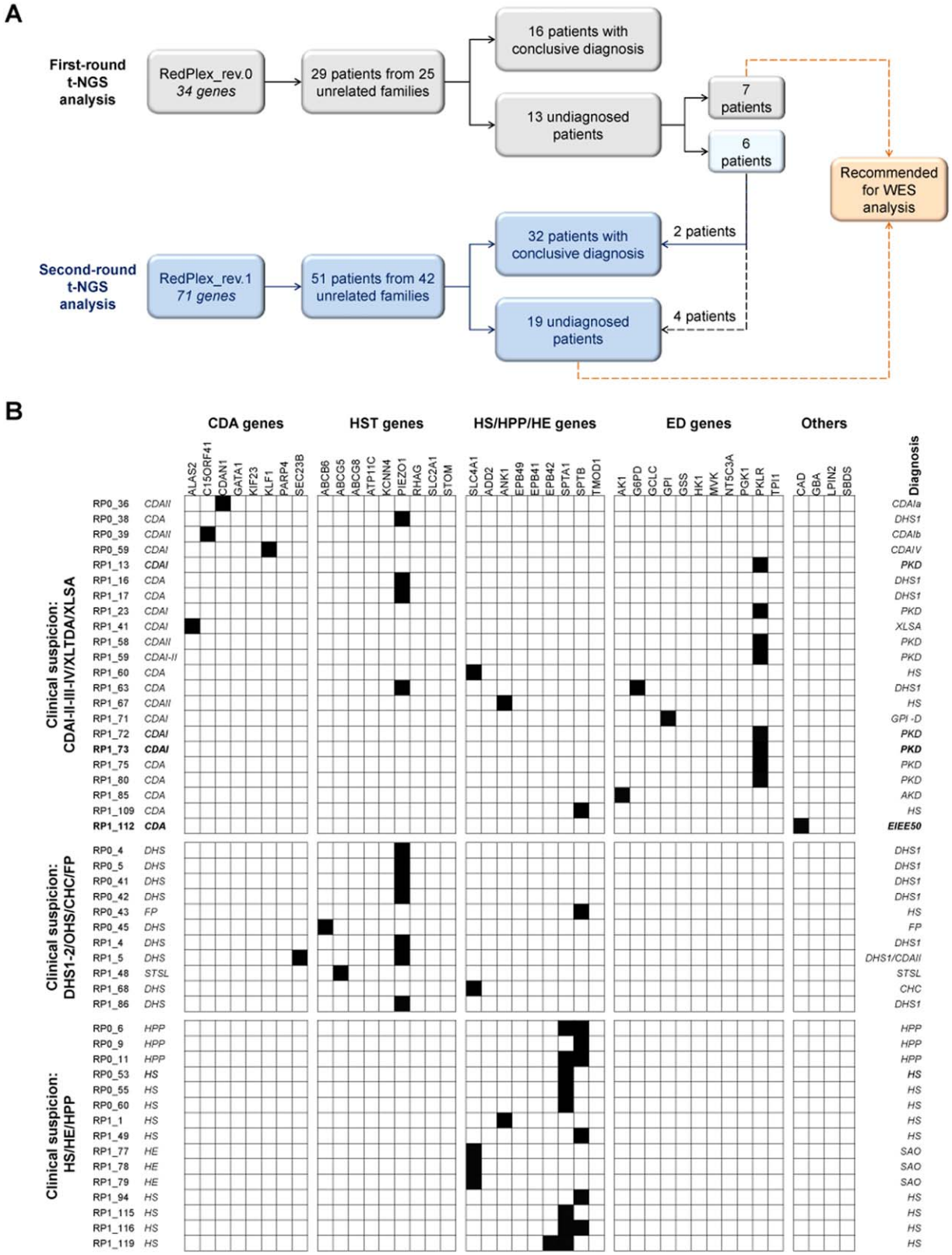

FIGURE 1 RedPlex study on HA patients. (A) Diagnostic workflow used in RedPlex study. WES, whole exome sequencing. (B) Schematic representation of the 48 HA patients with conclusive diagnosis. In the column the causative genes divided according to different HA phenotypes; in the row the investigated patients divided according to their clinical suspicion (indicated on the left). On the right, the conclusive diagnosis of each patient is shown. Dark gray squares highlight the mutated genes in each proband. AKD, adenylate kinase deficiency; CDAIa, congenital dyserythropoietic anemia Ia; CDAIb, congenital dyserythropoietic anemia Ib; CDAII, congenital dyserythropoietic anemia II; CDAIV, congenital dyserythropoietic anemia IV; CHC, cryohydrocytosis; DHS1, dehydrated hereditary stomatocytosis 1; EIEE50, Epileptic encephalopathy, early infantile, 50; FP, familial pseudohyperkalemia; GPI-D, glucose phosphate isomerase deficiency; HPP, hereditary pyropoikilocytosis; HS, hereditary spherocytosis; PKD, pyruvate kinase deficiency; SAO, Southeast Asian ovalocytosis; STSL, sitosterolemia; XLSA, X-linked sideroblastic anemia [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Genetic variants identified by RedPlex_rev.0

Family ID	Patient ID	Gene	HGVS (Coding)	HGVS (Protein)	Status	Inheritance	SIFT (score)	PolyPhen2 (score)	M-CAP (score)	RefSeq ID	MAF (1000G)	MAF (ExAC)	HGMD
F2	RP0_4[b]	*PIEZO1*	c.6796G>A	p.Val2266Ile	Het	M allele	T (0.11)	B (0.369)	P (0.291)	rs546338962	0.0004	0.0003	New variant
F2	RP0_5[b]	*PIEZO1*	c.6796G>A	p.Val2266Ile	Het	-							
F3[a]	RP0_6[c]	*SPTA1*	c.5029G>A	p.Gly1677Arg	Het	M allele	T (0.32)	P (0.935)	P (0.084)	rs771033064	-	0.00003	New variant
		SPTB	c.6175G>C	p.Ala2059Pro	Hom	Het parents	D (0.01)	P (0.673)	P (0.059)	-	-	-	New variant
	RP0_9[c]	*SPTB*	c.6175G>C	p.Ala2059Pro	Hom	Het parents							
F5	RP0_11	*SPTA1*	c.2542C>T	p.Arg848Cys	Het	P allele	D (0.00)	D (0.967)	P (0.046)	rs781647129	-	0.00007	New variant
		SPTB	c.2639T>A	p.Leu880Gln	Het	M allele	D (0.00)	D (0.996)	P (0.140)	rs770698912	-	0.00005	New variant
F12	RP0_36	*CDAN1*	c.1945C>T	p.Arg649Trp	Comp het	M allele	D (0.00)	D (0.999)	P (0.135)	rs751778010	-	0.00003	New variant
		CDAN1	c.1189C>T	p.Arg397Trp	Comp het	P allele	D (0.00)	D (0.999)	P (0.411)	rs756376237	-	0.00003	New variant
F13	RP0_38	*PIEZO1*	c.7180G>A	p.Gly2394Ser	Het	P allele	T (0.24)	B (0.218)	P (0.551)	rs201950081	0.0002	0.0007	New variant
F14	RP0_39	*C15orf41*	c.689A>C	p.His230Pro	Hom	Het parents	D (0.11)	B (0.058)	B (0.009)	-	-	-	New variant
F16	RP0_41	*PIEZO1*	c.7367G>A	p.Arg2456His	Het	-	D (0.00)	D (0.998)	P (0.945)	rs587776988	-	-	CM127746
F17	RP0_42	*PIEZO1*	c.5694G>C	p.Glu1898Asp	Het	-	T (0.63)	B (0.000)	P (0.028)	rs201829917	0.004	0.003	New variant
F18	RP0_43	*SPTB*	c.1606G>A	p.Asp536Asn	Het	-	D (0.00)	D (0.969)	P (0.025)	rs145675502	0.0005	0.0006	New variant
F19	RP0_45	*ABCB6*	c.826C>T	p.Arg276Trp	Het	-	D (0.00)	D (1.000)	-	rs57467915	0.004	0.02	CM128904
F22	RP0_53	*SPTA1*	c.1703G>C	p.Arg568Pro	Het	-	T (0.13)	P (0.926)	P (0.035)	rs200829664	0.0004	0.001	New variant
F23	RP0_55	*SPTA1*	c.33G>C	p.Glu11Asp	Het	-	T (0.72)	B (0.000)	B (0.013)	rs41273533	0.001	0.004	New variant
F24	RP0_59	*KLF1*	c.973G>A	p.Glu325Lys	Het	de novo	D (0.00)	D (1.000)	-	rs267607201	-	-	CM099513
F25	RP0_60	*SPTA1*	c.5029G>A	p.Gly1677Arg	Het	P allele	T (0.32)	P (0.996)	-	-	-	-	New variant
		SPTA1	c.2319C>A	p.Cys773Ter	Het	M allele	-	-	-	-	-	-	New variant
		SPTA1	c.6531-12C>T	-	Het	M allele	-	-	-	rs28525570	0.23	0.26	Alpha LELY
		SEC23B	c.1254T>G	p.Ile418Met	Het	M allele	D (0.00)	B (0.262)	P (0.078)	rs772896622	-	-	CM108329

Het, heterozygous; Hom, homozygous; Hem, hemizygous; Comp het, compound heterozygous; M allele, maternal allele; P allele, paternal allele.
PolyPhen2 (B, benign; P, possibly damaging; D, probably damaging).
SIFT (D, damaging; T, tolerated).
M-CAP (P, possibly pathogenic; B, likely benign).
[a]Family described by Al-Riyami AZ et al. Am J Hematol. 2017 Oct;92(10):E607-E609 [Ref. 11].
[b]RP0_5 is the affected mother of RP0_4.
[c]RP0_6 and 9 are two affected siblings.

TABLE 2 Genetic variants identified by RedPlex_rev.1

Family ID	Patient ID	Gene	HGVS (Coding)	HGVS (Protein)	Status	Inheritance	SIFT (score)	PolyPhen2 (score)	M-CAP (score)	RefSeq ID	MAF (1000G)	MAF (ExAC)	HGMD
F26	RP1_1	*ANK1*	c.856C>T	p.Arg286Ter	Het	*de novo*	-	-	-	-	-	-	New variant
F27	RP1_4	*PIEZO1*	c.1369C>T	p.Arg457Cys	Het	-	D (0.01)	D (0.997)	P (0.489)	-	-	-	New variant
F28	RP1_5	*SEC23B*	c.325G>A	p.Glu109Lys	Hom	-	D (0.00)	D (1.000)	P (0.149)	rs121918221	0.0002	0.0003	CM094612
		ABCB6	c.575G>A	p.Arg192Gln	Hom	-	D (0.00)	D (0.775)	P (0.205)	rs150221689	0.001	0.003	New variant
		PIEZO1	c.7505A>G	p.Lys2502Arg	Het	-	D (0.05)	D (0.999)	-	rs34830861	0.002	0.005	Pathogenic[a]
F30	RP1_13	*PKLR*	c.1349A>G	p.Asp450Gly	Hom	het parents	D (0.05)	B (0.072)	P (0.269)	-	-	-	New variant
F31	RP1_16/17[b]	*PIEZO1*	c.5389C>T	p.Arg1797Cys	Hom	Het parents	D (0.00)	D (0.995)	P (0.929)	rs561936787	0.0001	0.0008	New variant
		PKLR	c.257G>T	p.Arg86Leu	Het	M allele	D (0.01)	P (0.877)	P (0.440)	rs375471342	-	0.00004	CM981550
		GBA	c.1223C>T	p.Thr408Met	Het	M allele	T (0.11)	B (0.089)	-	rs75548401	0.007	0.002	CM960697
F33	RP1_23	*PKLR*	c.1117-1G>C	-	Hom	het parents	-	-	-	-	-	-	New variant
F36	RP1_41	*ALAS2*	c.1306C>T	p.Arg436Trp	Het	-	D (0.00)	B (0.260)	P (0.729)	-	-	-	CM062420
F38	RP1_48	*ABCG5*	c.161G>A	p.Trp54Ter	Hom	het parents	-	-	-	-	-	-	New variant
F39	RP1_49	*SPTB*	c.5651C>T	p.Ala1884Val	Het	P allele	D (0.00)	P (0.674)	B (0.015)	rs148337824	0.001	0.002	CM984610
F40	RP1_58	*PKLR*	c.1116 + 2T>G	-	Hom	het parents	-	-	-	rs768823171	-	0.000008	CS991485
F41	RP1_59	*PKLR*	c.67_68delTA	p.Leu23Cysfs*55	Comp het	P allele	-	-	-	-	-	-	New variant
		PKLR	c.287C>A	p.Pro96Gln	Comp het	M allele	D (0.00)	D (0.999)	P (0.441)	-	-	-	New variant
F42	RP1_60	*SLC4A1*	c.1334C>T	p.Ala445Val	Het	-	D (0.00)	D (0.731)	P (0.162)	-	-	-	New variant
F45	RP1_63	*G6PD*	c.311G>A	p.Arg104His	Het	-	T (0.62)	B (0.016)	-	rs181277621	0.002	0.0012	CM082725
		PIEZO1	c.7558A>G	p.Lys2520Glu	Het	-	D (0.00)	B (0.158)	P (0.160)	rs570744198	0.0002	0.0007	New variant
F47	RP1_67	*ANK1*	c.4385C>T	p.Ala1462Val	Het	-	T (0.08)	B (0.017)	P (0.356)	rs34664882	0.0098	0.02	New variant
F48	RP1_68	*SLC4A1*	c.2386G>C	p.Gly796Arg	Het	*de novo*	D (0.00)	D (1.000)	P (0.271)	-	-	-	CM094498
		GBA	c.1448T>C	p.Leu483Pro	Het	P allele	D (0.00)	D (1.000)	-	rs421016	0.0034	0.0031	CM870010
F49	RP1_71	*GPI*	c.478T>C	p.Ser160Pro	Het	-	D (0.00)	D (0.997)	P (0.453)	-	-	-	Pathogenic[c]
		GPI	c.1414C>T	p.Arg472Cys	Het	-	D (0.01)	P (0.583)	P (0.424)	-	-	-	Pathogenic[c]
F50	RP1_72	*PKLR*	c.1492C>T	p.Arg498Cys	Het	-	D (0.03)	P (0.560)	P (0.128)	rs551883218	0.0002	0.000008	CM981579
		PKLR	c.994G>A	p.Gly332Ser	Het	-	D (0.00)	D (0.998)	P (0.408)	rs773626254	-	0.00006	CM941163
F15	RP1_73	*PKLR*	c.353A>G	p.Asn118Ser	Hom	Het parents	D (0.00)	D (1.000)	P (0.549)	-	-	-	New variant
F52	RP1_75	*PKLR*	c.1594C>T	p.Arg532Trp	Comp het	M allele	D (0.02)	D (0.911)	P (0.211)	rs201255024	-	0.00002	CM941171

(Continues)

TABLE 2 (Continued)

Family ID	Patient ID	Gene	HGVS (Coding)	HGVS (Protein)	Status	Inheritance	SIFT (score)	PolyPhen2 (score)	M-CAP (score)	RefSeq ID	MAF (1000G)	MAF (ExAC)	HGMD
		PKLR	c.1529G>A	p.Arg510Gln	Comp het	P allele	D (0.01)	D (0.990)	P (0.177)	rs113403872	0.0002	0.0004	CM941170
F54	RP1_77[d]	*SLC4A1*	c.166A>G	p.Lys56Glu	Het	-	T (1.00)	B (0.000)	-	rs5036	0.06	0.04	CM921015
		ABCB6	c.574C>T	p.Arg192Trp	Het	-	D (0.00)	D (0.940)	P (0.304)	rs149202834	0.001	0.002	CM128903
F54	RP1_78[d]	*SLC4A1*	c.166A>G	p.Lys56Glu	Het	M allele							
		ABCB6	c.574C>T	p.Arg192Trp	Het	M allele							
F54	RP1_79[d]	*SLC4A1*	c.166A>G	p.Lys56Glu	Het	-							
		ABCB6	c.574C>T	p.Arg192Trp	Het	-							
F55	RP1_80	*PKLR*	c.1528C>T	p.Arg510Ter	Hom	Het parents	-	-	-	-	-	-	CM981581
F56	RP1_85	*AK1*	c.319C>T	p.Arg107Ter	Hom	Het parents	-	-	-	rs104894102	-	-	CM990133
F57	RP1_86	*PIEZO1*	c.6328C>T	p.Arg2110Trp	Het	-	D (0.00)	D (0.990)	P (0.847)	rs776531529	-	-	New variant
		G6PD	c.988C>T	p.Arg330Cys	Hem	-	D (0.01)	P (0.480)	P (0.476)	rs782699946	-	0.00002	New variant
F59	RP1_94[e]	*SPTB*	c.4208G>A	p.Arg1403Gln	Het	P allele	T (1.00)	B (0.001)	-	rs17180350	0.03	0.06	New variant
		SPTA1	c.6531-12C>T	-	Het	P allele	-	-	-	rs28525570	0.23	0.26	Alpha LELY
F59	RP1_115[e]	*SPTA1*	c.4490G>A	p.Gly1497Glu	Het	P allele	T (1.00)	B (0.440)	-	rs41273523	0.006	0.015	New variant
		SPTA1	c.6531-12C>T	-	Het	P allele							
F59	RP1_116[e]	*SPTA1*	c.4490G>A	p.Gly1497Glu	Het	-							
		SPTB	c.4208G>A	p.Arg1403Gln	Het	-							
		SPTA1	c.6531-12C>T	-	Hom	-							
F60	RP1_109	*SPTB*	c.26A>C	p.Asn9Thr	Comp het	P allele	D (0.04)	B (0.202)	-	rs138437526	0.002	0.001	New variant
		SPTB	c.1606G>A	p.Asp536Asn	Comp het	M allele	D (0.01)	P (0.765)	P (0.025)	rs145675502	0.0004	0.0006	New variant
F9	RP1_112	*CAD*	c.5366G>A	p.Arg1789Gln	Hom	Het parents	T (0.33)	D (0.988)	P (0.200)	-	-	-	New variant
F62	RP1_119	*EPB42*	c.1369C>T	p.Arg457Cys	Het	-	D (0.03)	B (0.081)	B (0.015)	rs45594632	0.002	0.003	New variant
		PKLR	c.1456C>T	p.Arg486Trp	Het	-	D (0.01)	D (0.995)	P (0.187)	rs116100695	0.002	0.003	CM950958
		SPTA1	c.1958A>G	p.Tyr653Cys	Het	-	T (0.05)	B (0.414)	-	rs148912436	0.005	0.008	New variant

Het, heterozygous; Hom, homozygous; Hem, hemizygous; Comp het, compound heterozygous; M allele, maternal allele; P allele, paternal allele.
PolyPhen2 (B, benign; P, possibly damaging; D, probably damaging).
SIFT (D, damaging; T, tolerated).
M-CAP (P, possibly pathogenic; B, likely benign).
[a]Del Orbe Barreto R et al. Ann Hematol. 2016;95:1545–1546 [Ref. 15].
[b]Mojzikova R et al. Blood Cells Mol Dis. 2017 [Ref. 16].
[c]RP1_16/17 are siblings.
[d]RP1_77 is the mother of RP1_78; RP1_79 is the affected brother of RP1_77.
[e]RP1_116 is the father of both siblings RP1_94 and RP1_115.

note, these patients presented clinical data and morphological features of the bone marrow mostly resembling those of both CDAI and CDAII (Table 3). It is important to underline that almost all the patients originally classified as CDA and subsequently diagnosed as chronic hemolytic anemia due to enzyme defects were transfusion-dependent, with subsequent reduced reliability of the enzyme assays.

Finally, we also identified a case of syndromic CDA due the homozygous mutation p.Arg1789Gln in *CAD* gene, encoding a trifunctional protein involved in the pathway of pyrimidine biosynthesis (Table 2). The proband, RP1_112, was a 6.5-year-old boy with mild macrocytic anemia (Hb 11.3 g/dL, MCV 90.0 fl) and increased RDW (19.0%), suffering also from epilepsy (Supporting Information Table S3).

3.4 | Additive effect of multiple disease-causing genotypes

Among the patients originally diagnosed as DHS we identified a 35-year-old male, RP1_5, showing a causative *PIEZO1* mutation in heterozygous state, thus confirming the clinical suspicion of DHS1.[4] Interestingly, the patient exhibited also homozygosity for the most common causative mutation of CDAII, the Glu109Lys-*SEC23B* (Table 2).[1] His clinical picture was suggestive of DHS1; indeed, he presented mild anemia (Hb 13.5 g/dL) with macrocytosis (MCV 97.9 fL) and reticulocytosis (117 000/μL) (Supporting Information Table S3). Nevertheless, ektacytometry analysis highlighted a peculiar bell shape curve with reduced DImax and right shift of the Omin point. Interestingly, the coinheritance of *PIEZO1* and *SEC23B* causative mutations resulted in marked iron overload, with very high ferritin levels (1938 ng/mL) and increased transferrin saturation (TSAT 88%).

4 | DISCUSSION

Clinical and molecular definition of patients with HA may be very hard to obtain. For some conditions, the great phenotypic variability is partially explained either with high genetic heterogeneity or with reduced penetrance and variable expressivity.[1–4] In the last decade, remarkable progresses have been made in discovering new disease genes involved in red blood disorders. This increasing genetic heterogeneity underlines the problem of a very complex differential diagnosis.[3] It is proper in this context that the multi-gene panel testing takes place. Some t-NGS panels for the diagnosis of HA have been already described. These studies analyzed a cohort of patients ranging from 19 to 57 cases by means of custom panels comprising a smaller gene set of 28–40 loci causative of some RBC membrane defects, CDA, DBA, hemolytic anemias due to enzymatic defects, sideroblastic anemia.[7–9] We herein described the largest cohort of patients and the most comprehensive gene set for this subset of anemias described so far.

Clinical diagnosis of hereditary hemolytic anemias is often inaccurate due to overlapping phenotypes. Of note, our multi-gene panel analysis modified the original clinical diagnosis in 45.8% of patients. Our data demonstrated that CDAs are the most difficult to diagnose at clinical level. This is not surprising if we consider that dyserythropoiesis appears to be a common morphologic feature in several conditions.[1] Indeed, 45.5% of patients classified as CDA showed a conclusive diagnosis of chronic anemia due to enzymatic defects. This observation is in agreement with a recently reported case of CDA conclusively diagnosed as PK deficiency.[7] Nevertheless, our data pointed out even more clearly the problem of overlapping phenotypes among these disorders, since 36.4% of CDA patients within our cohort exhibited mutations in *PKLR* gene. Of note, one of these patients, the case RP1_23, was recently transplanted for a myelodysplastic syndrome due to transfusion dependency. Despite no target therapies are available for PK deficiency, it has been recently demonstrated that an allosteric activator of both wild type and mutant PK enzymes, AG-348, is able to increase the enzymatic activity in patient erythrocytes treated *ex vivo*, thus opened a new way for clinical management of PK deficient-patients.[21]

Interestingly, in our cohort of patients we identified a case classified as CDA carrying a homozygous mutation in *CAD* gene, which encodes a multifunctional enzyme complex that catalyzes the first steps of de novo pyrimidine biosynthesis. We originally included *CAD* in the list of candidate loci for CDA in RedPlex_rev.1 panel, since it was reported to be a candidate gene for congenital disorders of glycosylation.[22] Of note, biallelic mutations in this gene have been recently associated to early infantile epileptic encephalopathy-50 in five patients, a severe neurodegenerative disease which can be lethal in childhood. Interestingly, all patients showed also mild CDA-like anemia with marked anisopoikolocytosis.[22,23] Similarly, our patient showed mild macrocytic anemia with increased RDW. A careful revaluation of the case showed that the proband suffered also from epilepsy. Importantly, a supplementation with oral uridine has been recently suggested as treatment for CAD-deficient patients.[23] Thus, we suggest including molecular analysis of *CAD* gene in the diagnostic workflow of CDA patients with syndromic phenotype. These paradigmatic cases pointed out even more that molecular diagnosis is pivotal in the diagnostic workflow of these disorders. Thus, beyond achieving a definitive diagnosis, knowing the genetic basis of these patients is valuable also for guiding treatment.

Among patients clinically classified as CDA, we identified also four cases with new variants in *PIEZO1* gene. Despite *PIEZO1* is a large and highly polymorphic gene and has a very large tolerance for missense variants,[24] all the variants we identified were likely pathogenic, since they were located in the C-terminal region of the protein that represents a mutational hot spot, as well as a critical and well-established functional domain.[12,18,19] Additionally, a careful revaluation of the cases showed clinical features most resembling those of DHS1 patients. Interestingly, we identified also two siblings with a homozygous variant in *PIEZO1*. Although DHS1 is an autosomal dominant disorder, biallelic mutations in dominantly inherited disorders have been already reported, for example in familial pseudohyperkalemia.[25] Nevertheless, the two siblings were from Turkey, a geographical region with high percentage of inbreeding. Unfortunately, we were not able to establish if the homozygous variant in *PIEZO1* accounted for a codominant phenotype or a recessive one, since clinical data of the parents of the two siblings were not available. Our data were in agreement with previous ones demonstrating the frequent misdiagnosis among CDA and other hereditary hemolytic anemias, particularly between CDAII

TABLE 3 Clinical features of CDA patients conclusively diagnosed as PK deficiency

	RP1_13	RP1_23	RP1_58	RP1_59	RP1_72	RP1_73	RP1_75	RP1_80
Age (years)	1.4	5.2	2.0	1.7	7	0.8	1.6	14
Onset symptoms (years)	At birth	Neonatal	Neonatal	At birth	4	At birth	At birth	At birth
Gender	Male	Female	Male	Male	Female	Female	Male	Male
Ethnicity	Turkish	Turkish	Colombian	Turkish	Italian	Turkish	Hungarian	Venezuelan
Complete blood count								
RBC ($10^6/\mu L$)	2.1	2.9	2.6	2.9	3.2	1.7	1.7	2.7
Hb (g/dL)	6.8	7.7	7.6	7.9	9.6	5.5	6.1	9.5
Ht (%)	18.0	23.4	21.6	23.3	29	15.8	17.5	32
MCV (fL)	104.9	80.6	82.0	81.2	89.6	90.1	103.6	117.8
MCH (pg)	32.5	26.1	29.0	28.1	33	31.4	35.3	35.2
MCHC (g/dL)	-	32.4	35.0	34.4	36.8	34.9	34.3	29.9
RDW (%)	-	13.7	14.0	13.2	-	14.9	16.7	18.2
PLT ($10^3/\mu L$)	387.0	287.0	361.0	276.0	295	284	362	1010
Retics %	0.6	0.1	3.2	1.8	7.2	2.0	8.56	18.2
Retics abs count ($x10^3/\mu L$)	12.8	3.8	83.5	51.5	23.3	35.2	144.7	215.0
Transfusion rate	8/year	7–8/year	25/year	12/year	-	6/year	10/year	12/year
Bone marrow examination	Erythroid hyperactivity, 10% double nucleated normoblasts (asymmetric nuclei)	Hypercellular with megaloblastic changes in erythroid cells	-	Erythroid hyperactivity, megaloblastic elements (bi- and multi-nucleated with internuclear bridges)	Erythroid hyperactivity with dyserythropoiesis, mostly bi- and multi-nucleated with internuclear bridges	Normoblasts with double nuclei and internuclear bridges	Hypercellular with megaloblastic changes and bi-nucleated normoblasts	Erythroid hyperactivity with dyserythropoiesis
Laboratory data								
Total bilirubin (mg/dL)	1.7	1.9	3.7	6.1	5.6	3.5	2.2	7
Unconjugated bilirubin (mg/dL)	0.5	1.5	3.1	5.4	5	3.1	2.1	6.3
Ferritin (ng/mL)	554	2554	1042	389	132	-	198	238
PKLR molecular analysis								
HGVS (coding[a]; protein; status)	c.1349A>G; p.Asp450Gly; Hom	c.1117-1G>C; Hom	c.1116 + 2T>G; Hom	c.67_68delTA; p.Leu23Cysfs*55c.287C>A; p.Pro96Gln; Comp het	c.1492C>T; p.Arg498Cysc.994G>A;p.Gly332Ser Comp het	c.353A>G; p.Asn118Ser; Hom	c.1594C>T; p.Arg532Trpc.1529G>A; p.Arg510Gln; Comp het	c.1528C>T; p.Arg510Ter; Hom

Hom, homozygous; Comp het, compound heterozygous.
[a]Reference Transcript ID: NM_000298.

and HS, and between CDAI and DHS.[1,3] Of note, a subtype of HST with overlapping features of CDA was already associated to a specific de novo mutation, p.Gly796Arg, in *SLC4A1* gene, encoding band 3.[3] In our cohort of patients we identified a second case, RP1_68, carrying the same mutation, although with a different nucleotide replacement (Table 2).

Contrary to CDAs, most of the patients (84.6%) originally classified as erythrocyte membrane defects showed conclusive diagnosis fitting well to the clinical suspicion. This could be explained by the availability of well-defined lines of investigations, as well as of reliable laboratory assays.[3,4]

Finally, the multigenic approach also allowed us identifying patients showing multiple disease-associated variants, suggesting complex inheritance. This was the case of a DHS1 patient that resulted also affected by CDAII. Interestingly, the coinheritance of *PIEZO1* and *SEC23B* causative genotypes accounted for marked iron overload in this patient. Indeed, both DHS1 and CDAII presented iron loading anemia even in absence of transfusion regimen, although with different pathomechanisms.[4,26]

NGS studies have identified a greater-than-expected number of genetic variations in the human genome, suggesting that existing monogenic testing systematically miss relevant information. Our analyzes demonstrated that multigenic approach represent a reliable diagnostic tool for HA patients, overcoming for sensitivity and specificity the gene-by-gene strategy. One of the most important aspects of the use of custom gene panels in clinical practice is their ability to be easily upgradable in view of novel discoveries. This aspect is important not only for the chance to extend the diagnostic spectrum, as described in the present study, but also for the possibility to incorporate noncoding regulatory regions that may harbor pathogenic mutations, as described for GATA1 *cis* elements disrupted in human erythroid disorders.[27,28]

Our paradigmatic cases highlighted how the multi-target sequencing diagnosis may be valuable not only for achieving a correct and definitive diagnosis, but also for guiding treatment. Moreover, it allowed the identification of polygenic conditions, in which the phenotypic variability could be explained by the coinheritance of multiple disease genotypes.

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CONFLICT OF INTERESTS

The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS

RR, IA, and AI designed and conducted the study, and prepared the manuscript; RR and IA performed in silico design of the NGS panels; FM and PP performed libraries enrichment; AG, PC, and VP performed clinical evaluation of the patients; RM and BER performed Sanger sequencing analysis; GT performed ektacytometry analysis; KR, SU and GLF provided samples and cared for the patients.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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