Received: 3 November 2022 | Revised: 16 February 2023 | Accepted: 20 February 2023

DOI: 10.1111/ejh.13951

ORIGINAL ARTICLE

European Journal of Haematology

WILEY

Clinical utility of targeted next-generation sequencing panel in routine diagnosis of hereditary hemolytic anemia: A national reference laboratory experience

Archana M. Agarwal[1,2] | Valarie McMurty[1,2] | Adam L. Clayton[2] | Ashini Bolia[2] | N. Scott Reading[1,2,3] | Coumarane Mani[2] | Jay L. Patel[1,2] | Anton Rets[1,2]

[1]Department of Pathology, University of Utah Health and ARUP Laboratories, Salt Lake City, Utah, USA

[2]ARUP Laboratories, Salt Lake City, Utah, USA

[3]Department of Internal Medicine, Hematology Division, University of Utah Health, Salt Lake City, Utah, USA

Correspondence
Archana M. Agarwal, Department of Pathology, University of Utah Health, Salt Lake City, UT, USA.
Email: archana.agarwal@hsc.utah.edu

Abstract

Introduction: Hereditary hemolytic anemias (HHA) comprise a heterogeneous group of disorders resulting from defective red blood cell (RBC) cytoskeleton, RBC enzyme deficiencies, and hemoglobin (Hb) synthesis disorders such as thalassemia or sideroblastic anemia.

Materials and Methods: Our hemolytic anemia diagnostic next-generation sequencing (NGS) panel includes 28 genes encoding RBC cytoskeletal proteins, membrane transporter, RBC enzymes, and certain bilirubin metabolism genes. The panel covers the complete coding region of these genes, splice junctions, and, wherever appropriate, deep intronic or regulatory regions are also included. Four hundred fifty-six patients with unexplained hemolytic anemia were evaluated using our NGS panel between 2015 and 2019.

Results: We identified pathogenic/likely pathogenic variants in 111/456 (24%) patients that were responsible for the disease phenotype (e.g., moderate to severe hemolytic anemia and hyperbilirubinemia). Approximately 40% of the mutations were novel. As expected, 45/456 (10%) patients were homozygous for the promoter polymorphism in the *UGT1A1* gene, $A(TA)_7TAA$ (*UGT1A1**28). 8/45 homozygous *UGT1A1**28 cases were associated with additional pathogenic mutations causing hemolytic anemia, likely exacerbating hyperbilirubinemia. The most common mutated genes were membrane cytoskeleton genes *SPTA1*, and *SPTB*, followed by *PKLR*. Complex interactions between SPTA1 low expression alleles, alpha-LELY and alpha-LEPRA alleles, and intragenic SPTA1 variants were associated with hereditary pyropoikilocytosis and autosomal recessive hereditary spherocytosis in 23/111 patients.

Conclusions: Our results demonstrate that hemolytic anemia is underscored by complex molecular interactions of previously known and novel mutations in RBC cytoskeleton/enzyme genes, and therefore, NGS should be considered in all patients with clinically unexplained hemolytic anemia and in neonates with hyperbilirubinemia. Moreover, low expression alleles alpha-LELY and alpha-LEPRA should be included in all targeted HHA panels.

KEYWORDS
hereditary hemolytic anemia, molecular diagnosis, next-generation sequencing, red blood cell

Novelty statements

What is the new aspect of your work?
Our study includes the largest cohort of patients with suspected hereditary hemolytic anemia (HHA) with targeted NGS.

What is the central finding of your work?
NGS should be part of the routine work up of HHA and neonatal hyperbilirubinemia due to significant overlap of clinical findings.

What is (or could be) the specific clinical relevance of your work?
Our large cohort of genetically defined HHA cases will help understand the genotype–phenotype correlation.

1 | INTRODUCTION

Hereditary hemolytic anemia (HHA) is a group of heterogeneous disorders with diverse genetic etiology, ultimately leading to an unstable and weakened cytoskeleton, resulting in premature breakdown of the RBC. RBC membrane disorders include defects in the RBC membrane structure and abnormal RBC volume regulation. Hereditary spherocytosis (HS), hereditary elliptocytosis (HE), and hereditary pyropoikilocytosis (HPP) are the most prevalent of the RBC membrane disorders.[1] These conditions are commonly associated with mutated *ANK1* (ankyrin-1) (40%–65%), *SPTB* (beta spectrin) (15%–30%), *SLC4A1* (band 3) (20%–35%), *EPB42* (protein 4.2) (<5%), and *SPTA1* (alpha spectrin) (<5%).[2–4] RBC volume disorders are broadly divided into overhydrated and dehydrated stomatocytosis. These disorders have been extensively reviewed in the past and will not be discussed in detail here.[1,5,6]

The glucose-6-phosphate dehydrogenase (*G6PD*) variants are due to single amino acid missense mutations, and many are highly polymorphic. Loss-of-function mutations in *G6PD* are uncommon and are potentially lethal.[7] Pyruvate kinase (PK) deficiency is the most common enzymopathy of the glycolytic pathway. Mammals have two PK genes: PK muscle (*PKM*) and PK liver/RBC (*PKLR*). *PKLR* gene controls the expression of both PK-L (liver), PR-R (RBC) by two tissue specific promoters. Incomplete removal of leukocyte and platelets during pyruvate kinase enzyme testing can lead to false negative test as the reaction does not discriminate against non-erythrocytic PK.[8] Recently, an allosteric activator of both wild-type and mutant PK enzyme, AG-348 has been studied and resulted in FDA approval.[9] Its effect results in an increased PK enzymatic activity in patient's erythrocytes treated ex vivo.[10]

The third group of HHA include hemoglobinopathies (e.g., sickle cell disease), thalassemias, and unstable hemoglobins. Thalassemias are characterized by deficient synthesis of one or more Hb chains (e.g., α, β) leading to a relative excess of non-affected Hb chains and premature destruction of RBCs. Many unstable hemoglobins are dominantly inherited and are due to globin gene mutations leading to instability of globin chains due to defective folding, and subunit interactions.[11]

Inherited defects of bilirubin metabolism do not cause hemolytic anemia, but they result in hyperbilirubinemia, which potentially can lead to very high levels in conjunction with HHA and cause grievous complications especially in neonates. Uridine diphosphoglucuronate-glucuronsosyltransferase 1A1 (*UGT1A1*) gene deficiency can lead to Gilbert syndrome (GS) and Crigler–Najjar (CN) syndrome with variable increase in bilirubin.[12] GS syndrome causing mild chronic unconjugated hyperbilirubinemia is very common in the Caucasian population (7%–10%) and is typically caused by one or two additional TA repeats in the promoter region (Caucasians and Africans)[13] or by mutations in exon 1 (Asians) in the *UGT1A* gene. Other genes responsible are *SLCO1B1*/3.[12]

We herein report our findings based on a large cohort of 456 patients who were evaluated for HA using a 28-gene targeted NGS panel from 2015 to 2019 at ARUP Laboratories.

2 | MATERIALS AND METHODS

Retrospective data review of 456 patients with unexplained HA whose peripheral blood specimens were submitted to ARUP Laboratories for targeted NGS analysis from 2015 to 2019. The patients' ages varied from neonatal to >70 years, including 35 (7.7%) infants (1-year-old and younger). Their clinical presentation ranged from mild hemolysis to severe transfusion-dependent anemia, with many neonates presenting with mild to severe hyperbilirubinemia. Osmotic fragility (by spectrophotometry), band 3 reduction (by flow cytometry), G6PD activity (enzymatic assay in whole blood), and PK enzyme (enzymatic assay in whole blood) were correlated with molecular findings wherever possible. Our

TABLE 1 Genes included in the panel.

Gene symbol	Gene description	Transcript	OMIM gene	Disorder	Inh
ADA	Adenosine deaminase	NM_000022	608958	ADA deficiency	AR
AK1	Adenylate kinase 1	NM_000476	103000	AK1 deficiency	AR
ALDOA	Aldolase A	NM_000034	103850	ALDOA deficiency	AR
ANK1	Ankyrin 1	NM_000037	607008	Spherocytosis	AD/AR
CYB5R3	Cytochrome b5 reductase 3 (DIA1)	NM_000398	613213	Methemoglobinemia type 1, methemoglobinemia type 2	AR
EPB41	Erythrocyte membrane protein band 4.1	NM_004437	130500	Elliptocytosis	AR
EPB42	Erythrocyte membrane protein band 4.2	NM_000119	177070	Spherocytosis	AR
G6PD	Glucose-6-phosphate dehydrogenase	NM_001042351	305900	G6PD deficiency	XL
GCLC	Glutamate-cysteine ligase, catalytic subunit	NM_001498	606857	GCLC deficiency, hyperbilirubinemia, hemolytic anemia	AR
GPI	Glucose phosphate isomerase	NM_000175	172400	Acute/chronic hemolytic anemia	AR
GSR	Glutathione reductase	NM_000637	138300	GSR deficiency	AR
GSS	Glutathione synthetase	NM_000178	601002	GSS deficiency	AR
HK1	Hexokinase 1	NM_000188	142600	Hemolytic anemia	AR
NT5C3	Pyrimidine 5′ nucleotidase	NM_016489	606224	NT5C3 deficiency, hemolytic anemia	AR
PFKL	Phosphofructokinase, liver	NM_002626	171860		AR
PFKM	Phosphofructokinase, muscle	NM_000289	610681	PFKM deficiency, glycogen storage disease 7	AR
PGK1	Phosphoglycerate kinase 1	NM_000291	311800	PGK1 deficiency	XL
PIEZO1	Piezo-type mechanosensitive ion channel component 1	NM_001142864	611184	Xerocytosis (hereditary)	AR
PKLR	Pyruvate kinase (liver and RBC)	NM_000298	609712	PKLR deficiency, hemolytic anemia	AR
SLC4A1	Solute carrier family 4, anion exchanger, member 1, band 3	NM_000342	109270	Spherocytosis, blood group variation, anemia, stomatocytosis, acanthocytosis kernicterus (acute), ovalocytosis	AD/AR
SLCO1B1	Solute carrier organic anion transporter family, member 1B1	NM_006446	604843	Hyperbilirubinemia (rotor type), Rotor syndrome	AR
SLCO1B3	Solute carrier organic anion transporter family, member 1B3	NM_019844	605495	Hyperbilirubinemia (rotor type), Rotor syndrome	AR
SPTA1	Spectrin alpha	NM_003126	182860	Elliptocytosis, spherocytosis, pyropoikilocytosis, elliptopoikilocytosis	AD/AR
SPTB	Spectrin beta	NM_000347	182870	Elliptocytosis, spherocytosis	AD/AR
TPI1	Triosephosphate isomerase 1	NM_000365	190450	TPI1 deficiency	AR
UGT1A1	UDP glycosyltransferase 1 family, polypeptide A1	NM_000463	191740	Crigler–Najjar syndrome 1 and 2, hyperbilirubinemia (unconjugated), Gilbert syndrome	AR
UGT1A6	UDP glycosyltransferase 1 family, polypeptide A6	NM_001072	606431	UGT1A6 deficiency	AR
UGT1A7	UDP glycosyltransferase 1 family, polypeptide A7	NM_019077	606432	UGT1A7 deficiency	AR

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; inh, inheritance; XL, X-linked.

current diagnostic NGS panel of 28 genes (Table 1) includes cytoskeletal proteins and enzymes, and covers the complete coding region, splice junctions, and, wherever appropriate, deep intronic or regulatory regions. This study was approved under IRB #00077285, University of Utah.

2.1 | DNA extraction

Genomic DNA extraction was performed on EDTA-preserved whole blood samples using the Chemagic MSM 1, following the manufacturer's recommendations (Perkin Elmer, United States).

2.2 | NGS sequencing

Extracted genomic DNA (1.1 mg) from whole blood was fragmented by Covaris shearing. Truncated Illumina universal adapters containing an inline 3 bp unique molecular identifier (UMI) to mark PCR duplicates were added using KAPA Hyper Prep Kit reagents according to the manufacturer's instructions. Sample indices were added during library amplification with unique pairs of dual indexing primers. Adapter-ligated libraries then underwent 4–18-h hybridization with biotinylated DNA probes. Hybridized DNA targets of interest were captured using streptavidin-coated magnetic beads. Enrichment of the target occurred through a series of washes to remove the excess, untargeted genomic DNA from the hybridized, bead-bound DNA. DNA targets were amplified through PCR and then eluted. Samples were quantified on the TapeStation, pooled if molarity was >15000pM, and sequenced on the Illumina HiSeq 4000 instrument or the NovaSeq 6000 instrument.

2.3 | Bioinformatic analyses and variant annotation

The raw data from sequencing (binary base call or BCL) files were converted to paired end FASTQ files, which contains the sequencing reads. The quality reads from FASTQ were aligned to a human reference genome (GRCh37/hg19) using Burrows-Wheeler aligner with maximal extract match (BWA-MEM),[14] and the aligned bam was deduped for PCR duplicates using sambamba markedup.[15] Variant calling was performed on the deduped bam using GATK's HaplotypeCaller,[16] and the variants were annotated using Genome Oncology's custom annotation software. Sanger sequencing was performed when variant quality fell below established thresholds or where minimum coverage was not met. Genetic variants were classified according to the American College of Medical Genetics (ACMG) guidelines as benign, likely benign, uncertain significance, likely pathogenic, or pathogenic.

Figures were constructed using R: A language and environment for statistical computing, version 4.1.0 "Camp Pontanezen" (Vienna, Austria) and ggplot2 (Springer-Verlag New York, 2016).[17]

3 | RESULTS

Clinically relevant mutations (pathogenic and likely pathogenic) were detected in 111 of 456 patients (24.3%). The top four genes with the highest incidence of clinically relevant mutations were *SPTA1* (27/111 patients, 23%), *SPTB* (21/111 patients, 18.9%), *PKLR* (20/111 patients, 18%) and *ANK1* (13/111 patients, 11%) (Figure 1). *G6PD* (12/111 patients, 10%), *SLC4A1* (11/111, 10%), *PIEZO1* (6/111), *EPB41* (2/111) and *TP1* (2/111) represented the rest (Figure 1). The mutations in genes encoding membrane proteins and enzymes affected 80 patients (72.1%) and 34 patients (30.6%), respectively. Three patients had mutations in genes from both categories (RBC membrane and enzyme). All three of them had *SPTA1* and *G6PD* variants. Two of these three patients were female, so the presence of G6PD variant was likely clinically asymptomatic. Of 111 patients, 44 (39.6%, 9.6% of the studied cohort) had novel pathogenic/likely pathogenic mutations. Figure 2 shows all the mutations, both novel and known.

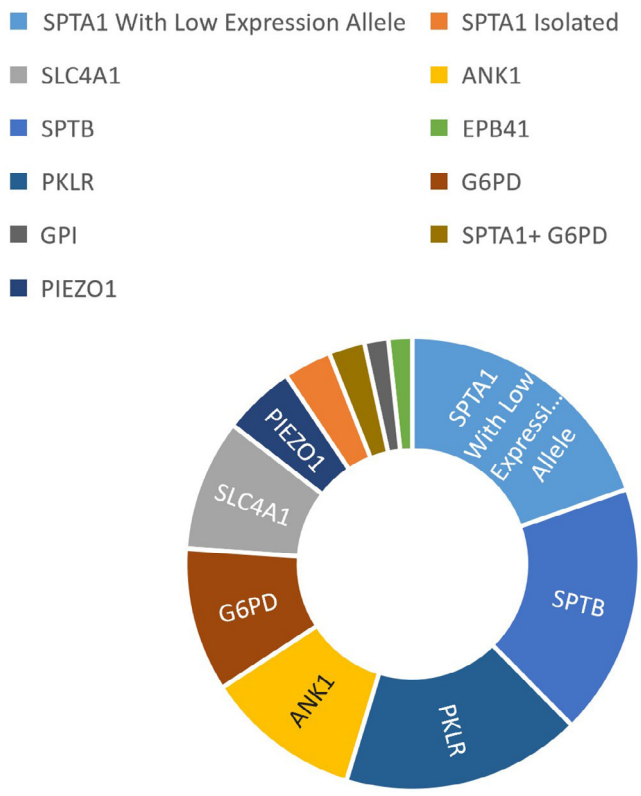

FIGURE 1 Incidence of clinically relevant mutations in our cohort.

Mutations affecting RBC membrane integrity dominated the cohort and included *SPTA1* (27 patients), *SPTB* (21 patients), *ANK1* (13 patients), and *SLC4A1* (11 patients). As previously observed in other studies, a significant number of *SPTA1*-mutated individuals (23/27) also had the common low expression *SPTA1* allele, either alpha-*LELY* and/or alpha-*LEPRA*. Interestingly, several of the pathogenic/likely pathogenic mutations identified in this group of genes were also novel: *SPTB* (15/21), *SPTA1* (9/27), *ANK1* (8/13), *SLC4A1* (5/11), *EPB41* (2/2), and *PIEZO1* (1/6) (Figure 3). Most of the novel mutations were point mutations followed by small insertion and deletions (Figure 4).

Mutations causing RBC enzymopathies were identified in 34 patients comprising three genes: *PKLR* (32 mutations in 20 patients), *G6PD* (13 mutations in 12 patients), and *GPI* (3 mutations in 2 patients) (Figure 2). Missense mutations were the most common variants in the *PKLR* gene. While all 20 *PKLR*-mutated individuals carried at least one missense variant, 4 (20%) of them were novel. *PKLR* c.1529G>A variant occurred more frequently, followed by *PKLR* c.1456C>T (Figure 4). None of the *G6PD* mutations detected in this study were novel. However, 6 individuals had the common pathogenic complex variant (c.202G>A; c.376A>G), also known as the *G6PD A*-allele. Due to lower

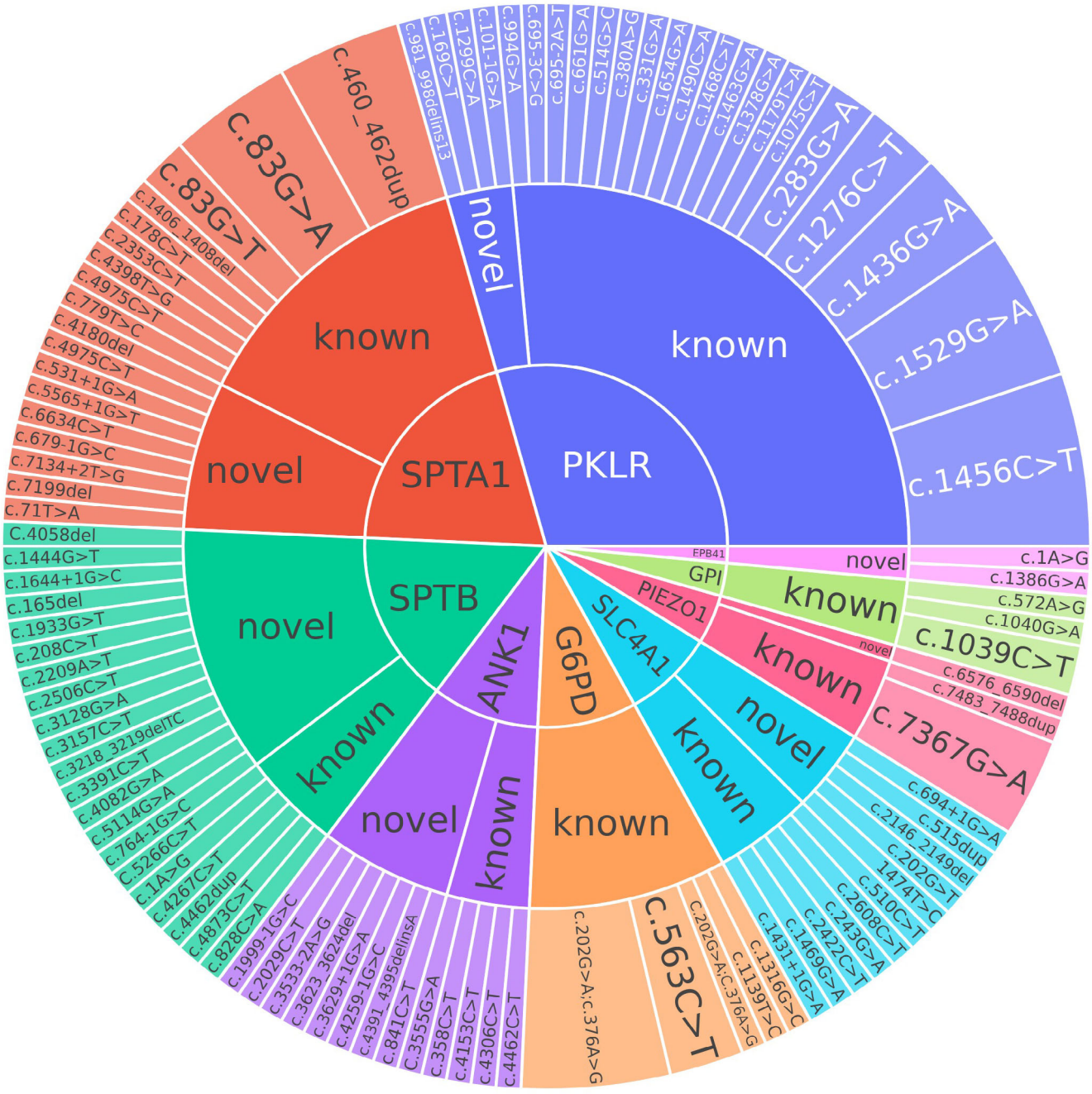

FIGURE 2 Pie diagram showing all the mutations (novel as well as known).

incidence and smaller cohort size, other well-established pathogenic *G6PD* alleles were not detected among our patients. Within our cohort there were 45/456 patients that carried the homozygous *UGT1A1*28* allele, causing Gilbert's syndrome. Eight of these patients also carried comorbid mutations in RBC membrane/enzyme genes.

4 | DISCUSSION

Targeted NGS, as well as whole-genome sequencing (WGS) and whole-exome sequencing (WES), are well-established technologies for the diagnosis of genetic diseases.[12,18,19] Although targeted NGS simultaneously interrogates multiple established disease-causing genes, WGS and WES offer a superior potential to uncover additional cellular pathways not known to contribute to disease etiology and progression. The utilization of WGS and WES, however, is associated with longer turnaround time, requiring sophisticated bioinformatics support and the evaluation of many more variants, potentially of uncertain significance.

Targeted panel-based molecular assays, which may include from 28 to 217 genes, have been employed for HHA diagnosis since 2012.[18,20,21] This methodology offers a number of advantages as

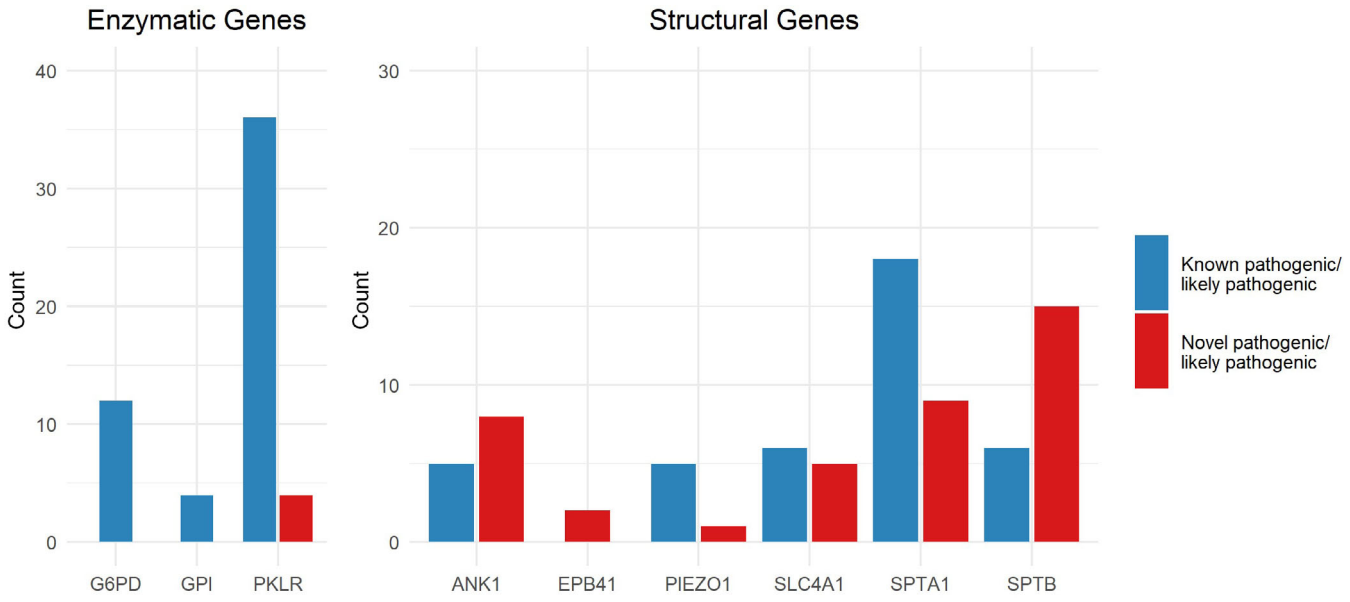

FIGURE 3 Novel and known pathogenic and likely-pathogenic mutations identified: Pathogenic and likely-pathogenic mutations are grouped for each category. Novel mutations identified in this study are colored red and those known are colored blue.

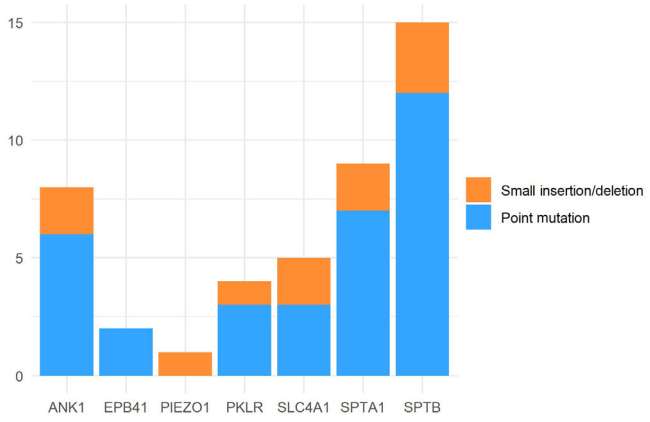

FIGURE 4 Breakdown of novel mutations (pathogenic and likely-pathogenic mutations): Most of them were point mutations.

compared to the "traditional" tests. Firstly, a significant overlap in the phenotype of various causes of HA makes it very difficult to make an accurate diagnosis relying solely on the hematological and biochemical tests (e.g., complete blood count (CBC), osmotic fragility, etc.). Moreover, these tests require a higher volume of blood sample, which could be problematic in a neonatal setting. False negativity in a post-transfusion setting is another consideration. Quantitative enzyme testing, although reliable, is also prone to false interpretations. For example, post-transfusion enzyme levels usually appear normal, and falsely elevated enzyme activity may be seen shortly after a hemolytic episode, etc.

In this study of 456 patients with unexplained HA, pathogenic and likely pathogenic variants were identified in a significant number of patients (≈25%). Among those, we found previously unreported novel variants in almost 40% of patients. Like previous reports, the majority of variant (80/111 patients) were in the RBC membrane genes. Complex interactions among *SPTA1* variants, including low expression alpha-*LELY* and alpha-LEPRA alleles, were associated with HPP and autosomal recessive HS in 23/111 patients. Disease-causing pathogenic/likely pathogenic mutations accounting for mild anemia with elliptocytosis were detected in 6/456 patients (4 with *SPTA1* and 2 with *EPB41*).

In our cohort, approximately one-third of the patients had mutations potentially causing enzyme deficiencies: 20 patients with *PKLR*, 12 patients with *G6PD*, and 2 patients with *GPI*. As described previously, missense mutations were common in the *PKLR* gene. Genetic variants, particularly missense in nature, in enzyme coding regions should be evaluated in conjunctions with any available pre-transfusion enzyme testing. It is important to note that 3 patients with *PKLR* variants identified in our study were initially classified as variants of unknown significance (VUS). However, all three were subsequently reclassified as likely pathogenic when the recommended PKLR enzyme testing resulted in decreased activity. It is frequently observed that many such individuals remain asymptomatic for a prolonged period and are usually diagnosed in adulthood due to other precipitating factors. It becomes more pertinent to accurately diagnose PK deficiency nowadays considering the recent FDA approval of Mitapivat, based on phase 2 and phase 3 trials where a statistically significant increase in Hb, reduction in transfusion burden, and improved quality of life was noted in approximately half of the patients.[9,22] Interestingly, some level of PK protein with at least one missense mutation is necessary for this compound to activate the enzyme, further necessitating the genetic testing. 2/12 G6PD positive patients were female and had concurrent SPTA1 mutations likely

explaining the hemolytic anemia. One male patient out of 12 G6PD positive also had concurrent SPTA1 mutation, likely exacerbating the hemolysis. The degree and nature of hemolysis, that is, acute versus chronic was not apparent in patients with G6PD mutations.

As expected, 10% (45/456) of the patients in our cohort were homozygous for a promoter polymorphism in the *UGT1A1* gene A(TA)$_7$TAA (UGT1A1*28), causing Gilbert syndrome. Of the 45 individuals with homozygous *UGT1A1**28 allele, 8 individuals, including 2 neonates, were shown to carry additional pathogenic mutations in another gene causing hemolytic anemia and, while not confirmed, were expected to present with exaggerated hyperbilirubinemia[23] Two of these eight patients were neonates and likely presented with significant hyperbilirubinemia. In adult patients, Gilbert syndrome is asymptomatic or may cause mild jaundice. However, in neonates, this can exacerbate an existing mild to moderate hyperbilirubinemia, potentially leading to neurotoxicity.

Compared to other published data, the diagnostic yield using our targeted NGS panel appears to be lower,[6,21,24] owing to several contributing factors such as the population tested due to reference laboratory set up, and number of target genes covered (fewer than currently recommended). As a tertiary national reference laboratory, we experience limited access to all pertinent clinical information and the results of the external biochemical testing, which hinders accurate classification of detected variants. At our center, most of the tested samples are submitted for "unexplained hemolytic anemia" workup. In the current report, we only comment on the variants which were confidently classified as either likely pathogenic or pathogenic based on the ACMG guidelines. Our current panel is also limited by a lack of information on copy number changes including large deletions. However, we believe our study encompasses the largest cohort of patients with suspected HA. It also includes neonates presenting with hyperbilirubinemia. Our large cohort of genetically defined HHA cases will help to better understand future genotype–phenotype correlation.

AUTHOR CONTRIBUTIONS

Archana M. Agarwal, Valarie McMurty and Anton Rets have analyzed the data. Archana M. Agarwal has written the first draft of the paper. All other authors performed data collection, data analysis and, edited the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Valarie McMurty https://orcid.org/0000-0003-1304-2843
N. Scott Reading https://orcid.org/0000-0003-0806-5661
Jay L. Patel https://orcid.org/0000-0001-6531-3225

REFERENCES

1. Russo R, Marra R, Rosato BE, Iolascon A, Andolfo I. Genetics and genomics approaches for diagnosis and research into hereditary anemias. *Front Physiol*. 2020;11:613559.
2. da Costa L, Galimand J, Fenneteau O, Mohandas N. Hereditary spherocytosis, elliptocytosis, and other red cell membrane disorders. *Blood Rev*. 2013;27(4):167-178.
3. Narla J, Mohandas N. Red cell membrane disorders. *Int J Lab Hematol*. 2017;39(Suppl 1):47-52.
4. Kim Y, Park J, Kim M. Diagnostic approaches for inherited hemolytic anemia in the genetic era. *Blood Res*. 2017;52(2):84-94.
5. Iolascon A, Andolfo I, Russo R. Advances in understanding the pathogenesis of red cell membrane disorders. *Br J Haematol*. 2019;187(1):13-24.
6. Andolfo I, Martone S, Rosato BE, et al. Complex modes of inheritance in hereditary red blood cell disorders: a case series study of 155 patients. *Genes (Basel)*. 2021;12(7):958.
7. Minucci A, Moradkhani K, Hwang MJ, Zuppi C, Giardina B, Capoluongo E. Glucose-6-phosphate dehydrogenase (G6PD) mutations database: review of the "old" and update of the new mutations. *Blood Cells Mol Dis*. 2012;48(3):154-165.
8. Zanella A, Fermo E, Bianchi P, Chiarelli LR, Valentini G. Pyruvate kinase deficiency: the genotype–phenotype association. *Blood Rev*. 2007;21(4):217-231.
9. Al-Samkari H, Galacteros F, Glenthoj A, et al. Mitapivat versus placebo for pyruvate kinase deficiency. *N Engl J Med*. 2022;386(15):1432-1442.
10. Kung C, Hixon J, Kosinski PA, et al. AG-348 enhances pyruvate kinase activity in red blood cells from patients with pyruvate kinase deficiency. *Blood*. 2017;130(11):1347-1356.
11. Gallagher PG. Diagnosis and management of rare congenital nonimmune hemolytic disease. *Hematology Am Soc Hematol Educ Program*. 2015;2015:392-399.
12. Rets A, Clayton AL, Christensen RD, Agarwal AM. Molecular diagnostic update in hereditary hemolytic anemia and neonatal hyperbilirubinemia. *Int J Lab Hematol*. 2019;41(Suppl 1):95-101.
13. Bosma PJ, Chowdhury JR, Bakker C, et al. The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. *N Engl J Med*. 1995;333(18):1171-1175.
14. Li H. *Aligning Sequence Reads, Clone Sequences and Assembly Contigs With BWA-MEM*; 2013. arXiv:1303.3997.
15. Tarasov A, Vilella AJ, Cuppen E, Nijman IJ, Prins P. Sambamba: fast processing of NGS alignment formats. *Bioinformatics*. 2015;31(12):2032-2034.
16. McKenna A, Hanna M, Banks E, et al. The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res*. 2010;20(9):1297-1303.
17. R RCT. *A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing; 2016.
18. Agarwal AM, Nussenzveig RH, Reading NS, et al. Clinical utility of next-generation sequencing in the diagnosis of hereditary haemolytic anaemias. *Br J Haematol*. 2016;174(5):806-814.
19. Bianchi P, Vercellati C, Fermo E. How will next generation sequencing (NGS) improve the diagnosis of congenital hemolytic anemia? *Ann Transl Med*. 2020;8(6):268.
20. Russo R, Andolfo I, Manna F, et al. Multi-gene panel testing improves diagnosis and management of patients with hereditary anemias. *Am J Hematol*. 2018;93(5):672-682.
21. Vives-Corrons JL, Krishnevskaya E, Rodriguez IH, Ancochea A. Characterization of hereditary red blood cell membranopathies using combined targeted next-generation sequencing and osmotic gradient ektacytometry. *Int J Hematol*. 2021;113(2):163-174.

22. Grace RF, Rose C, Layton DM, et al. Safety and efficacy of Mitapivat in pyruvate kinase deficiency. *N Engl J Med*. 2019;381(10):933-944.
23. Agarwal A, Patel JL, Clayton A, Reading NS. Use of next generation sequencing panel for routine diagnosis of hereditary hemolytic anemias. *Blood*. 2018;132(Suppl 1):2325.
24. van Vuren A, van der Zwaag B, Huisjes R, et al. The complexity of genotype–phenotype correlations in hereditary spherocytosis: a cohort of 95 patients: genotype–phenotype correlation in hereditary spherocytosis. *Hemasphere*. 2019;3(4):e276.

How to cite this article: Agarwal AM, McMurty V, Clayton AL, et al. Clinical utility of targeted next-generation sequencing panel in routine diagnosis of hereditary hemolytic anemia: A national reference laboratory experience. *Eur J Haematol*. 2023;1-8. doi:10.1111/ejh.13951