Next generation sequencing panel for hereditary erythrocytosis in adults with otherwise unexplained erythrocytosis unveils additional genomic variants

Current diagnostic evaluation for erythrocytosis follows a stepwise algorithm that includes: i) *JAK2* exon 12-15 mutation testing; ii) exclusion of secondary causes (including medications); iii) assessment for high oxygen affinity hemoglobin (Hgb) variants; and iv) evaluation for oxygen-sensing pathway mutations.[1,2] Despite this systematic approach, a substantial proportion of patients remain without an identifiable etiology and are classified as having otherwise unexplained erythrocytosis (OUE). These patients are negative for *JAK2* exon 12-15 mutations, lack an identifiable acquired cause, and do not harbor high–oxygen-affinity Hgb variants or pathogenic alterations in the oxygen-sensing pathway (*VHL*, *EGLN1/PHD2*, *EPAS1/HIF2A*), *BPGM*, or *EPOR*. In the current study, we evaluated the diagnostic yield and clinical utility of a 24-gene hereditary erythrocytosis next-generation sequencing (NGS) panel (NGS-HEP) in adults with OUE, characterizing their clinical phenotypes, thrombotic outcomes, genetic findings, and management strategies.

Following Institutional Review Board (IRB) approval, we retrospectively evaluated adults (≥18 years) with OUE, defined as Hgb >16.5 g/dL or hematocrit (Hct) >49% in males, and Hgb >16 g/dL or Hct > 48% in females.[3] Study subjects were negative for *JAK2* exon 12-15 mutations, lacked an acquired cause, and did not harbor high-oxygen-affinity Hgb or pathogenic variants in the oxygen-sensing pathway, BPGM, or EPOR.[4-6] All included patients underwent NGS-HEP testing that detects single-nucleotide and copy-number variants in 24 genes implicated in hereditary erythrocytosis: *ACO1*, *ANKRD26*, *BHLHE41*, *BPGM*, *CYB5A*, *CYB5R3*, *EGLN1*, *EGLN2*, *EGLN3*, *EPAS1*, *EPO*, *EPOR*, *GFI1B*, *HIF1A*, *HIF1AN*, *HIF3A*, *JAK2*, *KDM6A*, *PFKM*, *PIEZO1*, *PKLR*, *SH2B3*, *SOCS3* and *VHL*. NGS testing was performed on DNA extracted from peripheral blood leukocytes. Non-myeloid tissue was not used as a germline comparator. Variant curation was performed using American College of Medical Geneticists (ACMG) guidelines, incorporating population frequency, conservation, and *in silico* prediction to classify variants as pathogenic or variants of uncertain significance (VUS). Baseline Hgb/Hct, serum erythropoietin (sEpo), family history, symptom burden, therapies (phlebotomy, antiplatelet, anticoagulation), and thrombotic events were recorded. Thromboses were adjudicated as arterial *versus* venous and provoked *versus* unprovoked.

A total of 40 adult patients with *JAK2* wild-type OUE (median age: 48 years, range: 21-79; 31 males [78%]) underwent NGS-HEP at the Mayo Clinic between March 2023 and June 2025 (Table 1, *Online Supplementary Table S1*). Bone marrow biopsy was performed in 18 of 40 patients (45%). Findings were predominantly normal or demonstrated mild, non-specific changes, including mild granulocytic hyperplasia or mild hypocellularity with mildly decreased trilineage hematopoiesis. None of the evaluated cases demonstrated morphologic features consistent with polycythemia vera (PV) or other myeloproliferative neoplasms (MPN). At baseline (at diagnosis), the median white blood cell count (WBC) was 7.35x10^9/L (range: 5-17.4), platelet count (PLT) was 236x10^9/L (range: 157-397), serum ferritin was 66 mcg/L (range: 8-981), and mean corpuscular volume (MCV) was 86.8 fL (range: 77.9-104.5). Variants were identified in 23 patients (58%), with a single variant detected in 17 (42.5%) and two variants in 6 (15%). All 23 variants were classified as heterozygous VUS and involved *PIEZO1* (N=8, 20%; median variant allele frequency [VAF] 47.1%), *HIF1A* (N=8, 20%; 46.2%), *ANKRD26* (N=4, 10%; 43.7%), *SH2B3* (N=3, 7.5%; 49.9%), *HIF3A* (N=3, 7.5%; 51.4%), *EPO* (N=1, 2.5%; 45.4%) and *BHLHE41* (N=1, 2.5%; 54.2%) (Figure 1). Compared with minor allele frequencies in the general population (gnomAD Browser: gnomad.broadinstitute.org), *PIEZO1* (20% *vs*. 5.2%, *P*=0.0015), *ANKRD26* (10% *vs*. 0.59%, *P*=0.0002) and *SH2B3* variants (7.5% *vs*. 0.21%, *P*=0.0003), were enriched in our cohort, while *HIF1A* variant frequency was comparable to population estimates (20% *vs*. 16%, *P*=0.51) consistent with a common polymorphism (Figure 2).

Presenting median Hgb/Hct values for males and females, respectively, were 16.7 g/dL/49.8% and 15.4 g/dl/46.8% in variant-positive cases, and 16.9 g/dL/50.1% and 16 g/dL/47.6% in variant-negative cases (*P*=0.73 and *P*=0.66, respectively). Median sEpo levels were 8.05 mIU/mL (range: <1-52.9) in variant-positive *versus* 8.8 mIU/mL (range: 1.6-54.7) in variant-negative cases, respectively (*P*=0.66). Notably, sEpo was <1 mIU/mL in a patient with *ANKRD26/HIF3A* VUS. Family history of erythrocytosis was documented in 4 patients (10%). Hyperviscosity-related symptoms were reported in 23 patients (58%), with no significant difference in prevalence between variant-positive and variant-negative cases (65% *vs*. 47%, *P*=0.25). Also, there was no significant association between the presence of symptoms and Hgb or Hct levels (*P*=0.11 and *P*=0.14, respectively). There was no significant difference in Hgb/Hct levels (*P*=0.99/0.92), symptom burden (76% *vs*. 33%, *P*=0.06), or thrombosis rates (18% *vs*. 33%, *P*=0.43) between patients harboring a single variant and those with two variants.

Eight thrombotic events were documented in 7 patients (18%; median age 44 years [71% males]; 4 arterial and 4 venous; 3 provoked deep venous thromboses) (*Online Supplemen-*

tary Table S2). Five (71%) of these 7 patients harbored VUS in *PIEZO1*, *PIEZO1/HIF1A*, *ANKRD26*, *SH2B3*, and *BHLHE41* (1 case each). Notably, 2 younger male patients experienced arterial events in the absence of identifiable risk factors: a 32-year-old with *PIEZO1/HIF1A* VUS (VAF 46.2%/47.6%; sEPO 10.3 mIU/mL) suffered a myocardial infarction, and a 35-year-old with *SH2B3* VUS (VAF 50.2%; sEpo 3.7 mIU/mL) had recurrent cerebrovascular accident despite phlebotomy, aspirin and apixaban. Overall, there was no significant association between thrombosis and the presence of a variant (13% in variant-positive *vs*. 12% in variant-negative, *P*=0.40). Active therapies included phlebotomy in 21 patients (53%), antiplatelet agents in 20 (50%), systemic anticoagulation in 6 (15%), and hydroxyurea in one patient (2.5%). Among those who received phlebotomy, 9 patients (43%) reported improvement in symptoms; however, symptom relief did not correlate with Hgb or Hct levels (*P*=0.27 and *P*=0.58, respectively).

A separate analysis of 8 patients with heterozygous *PIEZO1* VUS, none of whom had undergone splenectomy, showed a numerically higher incidence of symptoms (75% *vs*. 53%, *P*=0.25) and thrombosis (25% *vs*. 16%, *P*=0.54) compared to those without *PIEZO1* variants; however, these differences were not statistically significant.

The current study sought to evaluate the diagnostic utility of expanded NGS testing in OUE, as all patients had previously undergone systematic evaluation for *JAK2* exon 12-15 mutation, high oxygen affinity variants, mutations in the oxygen-sensing pathway, *BPGM* and *EPOR*. This is in contrast to findings from Europe's largest idiopathic erythrocytosis cohort, reported in abstract form,[7] in which pathogenic variants, predominantly involving oxygen-sensing pathway genes, were identified in 7.9% of 909 patients who had previously undergone a standardized workup.[1] Notably, many of the variants reported in that cohort would be expected to be captured through conventional erythrocytosis-focused diagnostic algorithms. Those reported were: *EPAS1* (N=20), *EGLN1* (N=13), *SH2B3* (N=10), *HBB/HBA* (N=8), *JAK2* (N=7), *EPOR* (N=5), *PIEZO1* (N=5), *VHL* (N=3), *BPGM* (N=1). Similarly, a separate study from the University of Padova which used a targeted 14-gene NGS panel, identified at least one variant in 66% of 118 patients with idiopathic erythrocytosis, most commonly involving *HFE*, followed by *EGLN1* and *EPAS1/EPOR/JAK2/TFR2*.[2] Additionally, pathogenic or likely pathogenic variants were identified in 15 of 55 patients (27.3%) with unexplained erythrocytosis evaluated at Careggi Hospital which included *EGLN1* (N=4), *EPAS1* (N=1), *HBB* (N=1), *MPL* (N=4), *SH2B3* (N=2), and *VHL* variants (N=2).[3]

We observed a relatively frequent detection of *PIEZO1*, *ANKRD26*, and *SH2B3* variants in our cohort exceeding those predicted by minor allele frequencies in the general population. Although all *PIEZO1* variants identified were classified as VUS, prior data support an association of gain of function *PIEZO1* mutations and erythrocytosis through disruption of cation flux and altered red cell hydration. Functionally, *PIEZO1*-associated hereditary xerocytosis impacts red cell energy metabolism and glycolysis, resulting in reduced 2,3-bisphopsoglycerate levels and increased oxygen affinity.[8,9] Consistent with this, *PIEZO1* mutations have been reported in up to 4% of patients with idiopathic erythrocytosis, frequently accompanied by clinical features of hereditary

Table 1. Baseline (at diagnosis) clinical and laboratory characteristics of 40 patients with otherwise unexplained erythrocytosis who underwent hereditary erythrocytosis 24-gene panel, next-generation sequencing.

Variables at time of erythrocytosis evaluation	N=40
Age, years, median (range)	47.5 (21-79)
Male gender, N (%)	31 (77.5)
VUS, N (%) *PIEZO* *HIF1A* *ANKRD26* *HIF3A* *SH2B3* *BHLHE41* *EPO*	 8 (20) 8 (20) 4 (10) 3 (7.5) 3 (7.5) 1 (2.5) 1 (2.5)
Family history of erythrocytosis, N (%)	4 (10)
Prior thrombosis, N (%) Major arterial thrombosis Major venous thrombosis	7 (17.5) 4 (10) 4 (10)
Baseline Hgb, g/dL, median (range) Male Female	16.45 (13-18.6) 16.9 (13-18.6) 15.4 (14.2-17.4)
Baseline Hct, %, median (range) Male Female	49.6 (37.8-56.6) 50 (37.8-56.6) 47.1 (42-52)
Peak Hgb, g/dL, median (range) Male Female	17.65 (15.5-21.2) 18.1 (16.3-21.2) 16.5 (15.5-17.5)
Peak Hct, %, median (range) Male Female	52.9 (45.4-61.3) 53.2 (46.7-61.3) 49.9 (45.4-57.4)
Serum ferritin, mcg/L, median (range) Ref range	66 (8-981) 24-336
PLT, x10^9/L, median (range)	236 (157-397)
WBC, x10^9/L, median (range)	7.35 (5-17.4)
MCV, fL, median (range)	86.8 (77.9-104.5)
sEpo, mIU/mL, median (range)	8.3 (<1-54.7)
Symptoms,* N (%)	21 (52.5)
Thrombosis during follow-up, N (%) Major arterial thrombosis Major venous thrombosis	1 (2.5) 1 (2.5) 0

Reference (Ref) ranges - hemoglobin (Hgb): females 11.6-15 g/dL, males 13.2-16.6 g/dL; hematocrit (Hct): females 35.5-44.9%, males 38.3-48.6%; serum erythropoietin (sEpo): 2.6-18.5 mIU/mL. *Headache, fatigue, dizziness, pruritus, tingling, chest pain, epistaxis, brain fog. MCV: mean corpuscular volume; N: number; PLT: platelet count; VUS: variants of unclear significance; WBC: white blood cell count.

xerocytosis (iron overload, splenomegaly, hemolysis).[10] While overt xerocytosis features were not present in our cohort, causality cannot be inferred, and the observed arterial event warrants further investigation.

Variants in *SH2B3* (LNK) and *ANKRD26* also merit consideration in the context of unexplained erythrocytosis. *SH2B3* mutations have been well-described in patients with *JAK2* wild-type erythrocytosis and are generally associated with subnormal sEpo levels.[11-13] By contrast, none of the patients harboring *SH2B3* variants in our cohort demonstrated subnormal sEpo levels. Conversely, a patient with *ANKRD26* variant exhibited subnormal sEpo levels, consistent with

A

NHEP Comprehensive Panel		
ACO1	EGLN3	JAK2
ANKRD26	EPAS1	KDM6A
BHLHE41	EPO	PFKM
BPGM	EPOR	PIEZO1
CYB5A	GFI1B	PKLR
CYB5R3	HIF1A	SH2B3
EGLN1	HIF1AN	SOCS3
EGLN2	HIF3A	VHL

B

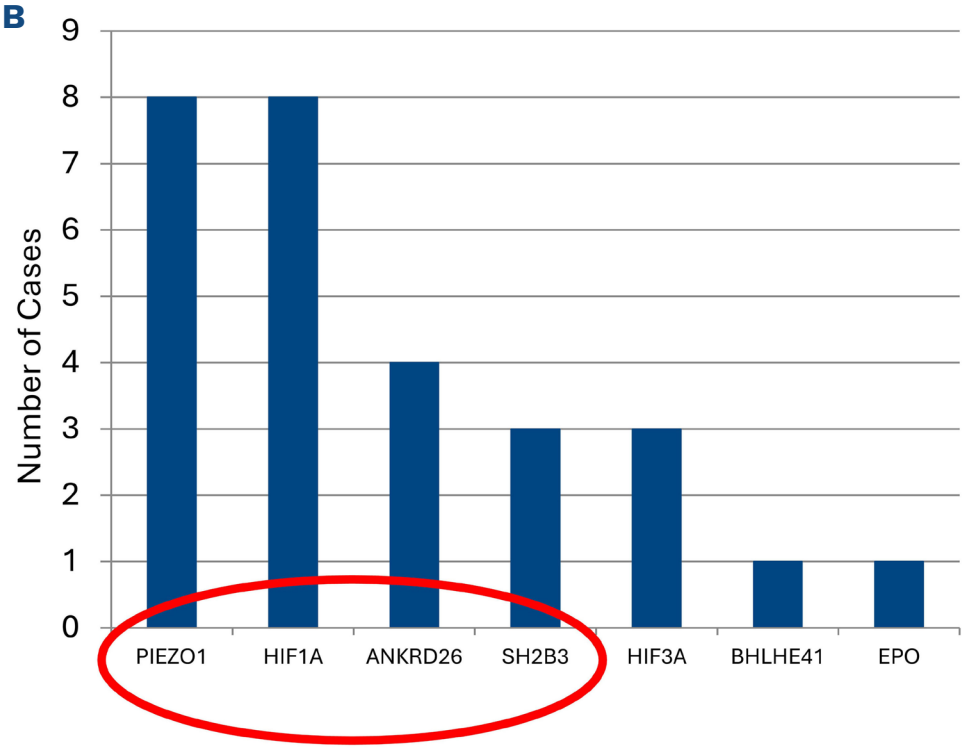


Figure 1. Next-generation sequencing panel for hereditary erythrocytosis in adults with otherwise unexplained erythrocytosis. Subjects were negative for *JAK2* exon 12-15 mutations, lacked an acquired cause, and did not harbor high–oxygen-affinity hemoglobin variants or pathogenic alterations in *VHL*, *EGLN1/PHD2*, *EPAS1/HIF2A*, *BPGM*, or *EPOR*. (A) Comprehensive hereditary erythrocytosis panel (NGS-HEP). (B) Genomic variants were identified in 23 of 40 (58%) study subjects. (See Figure 2 for a comparison of the *PIEZO1*, *HIF1A*, *ANKRD26* and *SH2B3* variant frequencies in the study cohort; circled in red.) MAF: minor allele frequency; VAF: variant allele frequency.

A

PIEZO1
Variants of uncertain significance
Study cohort 20%
General population ~ 5.2%
P=0.0015

	Variant	VAF %	%
#1	c.7180G>A (p.Gly2394Ser)	48.5	0.11
#2	c.3602C>T (p.Thr1201Met)	46.2	0.08
#3	c.6506_6508del (p.Lys2169del)	51	0.1
#4	c.7529C>T (p.Pro2510Leu)	42.7	0.68
#5	c.7505A>G (p.Lys2502Arg)	46.7	0.6
#6	c.226G>A (p.Ala76Thr) c.5988A>G (p.Ser1996)	47.5 51	0.0017 0.02
#7	c.7529C>T (p.Pro2510Leu)	49.1	0.68
#8	c.5195C>T (p.Thr1732Met)	46	1.0

B

HIF1A
Variants of uncertain significance
Study cohort *20%*
General population ~16%
P=0.51

Variant	%
c.1744C>T (p.Pro582Ser)	8.6

C

ANKRD26
Variants of uncertain significance
Study cohort 10%
General population ~ 0.59%
P=0.0002

	Variant	%	%
#1	c.1153A>G (p.Thr385Ala)	44	0.0028
#2	c.245C>T (p.Thr82Met)	55.7	0.0036
#3	c.440A>G (p.Asn147Ser)	43.5	Not reported
#4	c.679C>T (p.Pro227Ser)	32.9	0.29

D

SH2B3
Variants of uncertain significance
Study cohort 7.5%
General population ~ 0.21%
P=0.0003

	Variant	VAF %	%
#1	c.586C>T (p.Arg196Cys)	49.7	0.0065
#2	c.452A>G (p.Gln151Arg)	50.2	0.0011
#3	c.232G>A (p.Glu78Lys)	49.2	0.1

Figure 2. Comparison of (A) *PIEZO1*, (B) *HIF1A*, (C) *ANKRD26* and (D) *SH2B3* variant frequencies in the study cohort. The study cohort consisted of adults with otherwise unexplained erythrocytosis (N=40) and minor allele frequencies reported in the general population.

prior reports implicating germline *ANKRD26* mutations in erythrocytosis.[14]

Overall, NGS-HEP identified genomic variants in 58% of adults with stringently defined OUE, all of which were classified as VUS, most commonly heterozygous *PIEZO1* variants (20%), followed by *ANKRD26* (10%) and *SH2B3* (7.5%). No consistent association was observed between variant status and presence of hyperviscosity-related symptoms or thrombotic events, suggesting the complexity of genotype–phenotype relationships in non-clonal erythrocytosis and supporting an individualized management approach. In this context, routine phlebotomy may not mitigate thrombotic risk in OUE, underscoring the need for prospective, genotype-informed studies to define optimal antithrombotic strategies and clarify when, if at all, phlebotomy is beneficial.

Limitations of the current study include the small sample size and the predominance of VUS, which limit statistical power and preclude establishing causality. In addition, Hgb thresholds were derived from PV criteria and have not been validated for hereditary erythrocytosis. Therefore, expanded NGS findings should be interpreted with caution (*Online Supplementary Figure S1*).

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NG has served on the advisory board for DISC Medicine and Agios. All the other authors have no conflicts of interest to disclose.

Contributions

MR collected and analyzed data. JH, AR and XZ provided laboratory expertise. AP provided patient care. NG and AT wrote the paper and provided patient care.

Data-sharing statement

Please email the corresponding author.

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