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REVIEW

A Systematic review on diagnostic methods of red cell membrane disorders in Asia

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Abstract

Membranopathies are a group of inherited blood disorders where the diagnosis could form a challenge due to phenotype-genotype heterogeneity. In this review, the usage and limitations of diagnostic methods for membranopathies in Asian countries were evaluated. A systematic review was done using articles from PubMed, Google Scholar, and EBSCO from 2000 to 2020. Thirty-six studies conducted in seven Asian countries had used different diagnostic methods to confirm membranopathies. In 58.3% of studies, full blood count (FBC), reticulocyte count, and peripheral blood smear (PBS) were used in preliminary diagnosis. The combination of the above three with osmotic fragility (OF) test was used in 38.8%. The flowcytometric osmotic fragility (FC-OF) test was used in 27.7% where it showed high sensitivity (92%–100%) and specificity (96%–98%). The eosin-5-maleimide (EMA) assay was used in 68.1% with high sensitivity (95%–100%) and specificity (93%–99.6%). About 36.1% of studies had used sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a further diagnostic method to detect defective proteins. Genetic analysis to identify mutations was done using Sanger sequencing, next-generation sequencing (NGS), and whole-exome sequencing (WES) in 33.3%, 22.2%, and 13.8% of studies, respectively. The diagnostic yield of NGS ranged from 63% to 100%. Proteomics was used in 5.5% of studies to support the diagnosis of membranopathies. A single method could not diagnose all membranopathies. Next-generation sequencing, Sanger sequencing, and proteomics will supplement the well-established screening and confirmatory methods, but not replace them in hereditary hemolytic anemia assessment.

KEYWORDS
Asia, diagnostic methods, hereditary hemolytic anemia, membranopathies, next-generation sequencing

1 | INTRODUCTION

Hereditary hemolytic anemias (HHA) are a heterogeneous group of genetic disorders characterized by increased destruction of circulating red blood cells (RBC), causing mild-to-severe chronic anemia. The intrinsic red cell defects in HHA are due to red cell membrane disorders (membranopathies), red cell enzyme deficiencies (enzymopathies), and hemoglobin disorders (hemoglobinopathies).[1] HHA is clinically characterized by jaundice, recurrent anemia, cholelithiasis, splenomegaly, and hepatomegaly, with variable age at onset and severity.[2,3] In developing countries, HHA is an important cause of morbidity and mortality secondary only to malnutrition and infections.[4]

The RBC membrane cytoskeleton is a multiprotein complex that interacts with phospholipid bilayer via vertical and horizontal interactions. The mutation in genes coding for membrane proteins results in a decrease in RBC permeability and impaired RBC deformability.

These defects lead to membranopathies such as hereditary spherocytosis (HS), hereditary elliptocytosis (HE), hereditary stomatocytosis (HSt), hereditary pyropoikilocytosis (HPP), and Southeast Asian ovalocytosis (SAO).[1] Membranopathies account for more than half of HHA.[5,6]

Hereditary spherocytosis is the most common membranopathy with greater prevalence in North Europe and North America (1 in 2000 individuals).[3,7,8] Although HS is found in all racial and ethnic populations, it is comparatively less in Southeast Asians and African Americans.[7,8] A prevalence of 1:100 000 is reported in the Chinese population.[9] The inheritance pattern of HS is not uniform: In 75%, it is autosomal dominant, while in the rest, it is autosomal recessive.[10,11] HS is characterized by genotype-phenotype heterogeneity. The disease is caused by mutations in genes coding for membrane proteins, α spectrin, β spectrin, ankyrin, band 3, protein 4.1, and protein 4.2.[2,12–14] The deficiency of these proteins results in the loss of RBC membrane producing spherocytes with a short life span. They undergo splenic sequestration causing a varying degree of anemia, increased bilirubin, and cholelithiasis.[2] In HS, phenotypes vary from compensated anemia to severe transfusion-dependent anemia.[2,11,12]

Hereditary elliptocytosis is the next commonest and is transmitted in an autosomal dominant fashion. The prevalence varies across the malaria-endemic region with high prevalence in West Africa (0.6%–3%).[15,16] Its prevalence in United States is reported to range from 1 in 2000 to 1 in 4000.[16] However, HE is also found in Europe, Mediterranean, Middle East, Japan, and India.[17] It is caused by mutations in α-spectrin, β-spectrin, and protein 4.1 which cause a defect in horizontal interactions of RBC cytoskeleton. The presence of elliptical shaped RBC is a morphological trait of HE.[16,18]

Hereditary pyropoikilocytosis is a rare HHA that usually correlates with HE. It has characteristic poikilocytes, RBC fragments, and micro-spherocytes in the peripheral blood smear.[3,16,19] Both HPP and HE have increased or normal osmotic fragility and could be differentiated using genetic analysis.[20] Southeast Asian ovalocytosis (SAO) is an abnormal HE variant due to mutations in gene coding for band 3 protein.[18,19,21] The SAO which is common in malaria-endemic regions is usually found in Southeast Asian countries and Melanesia.[22] SAO is characterized by ovalocytes in the peripheral blood picture.[18,19]

Hereditary stomatocytosis, which is a rare membranopathy, is divided into two types, xerocytosis or dehydrated hereditary stomatocytosis (DHSt) with a prevalence of 1 in 100 000 births and overhydrated hereditary stomatocytosis (OHS) with a prevalence of 1 in million births. HSt is caused due to band 3 deficiency that leads to altered cation permeability causing change in cell volume. Both entities have autosomal dominant inheritance.[3,19]

In routine laboratories, hemolytic anemia (HA) is diagnosed based on clinical features, family history, PBS, screening, and confirmatory laboratory methods.[3,23,24] However, in rare hereditary hemolytic anemia, similar clinical traits with different etiologies make it difficult to characterize them using routine laboratory techniques.[25,26] As the classical approach in diagnosis has limitations, most of the symptomatic cases are treated based on their symptoms, while asymptomatic cases remain undiagnosed.[1]

The routine laboratory investigations for membranopathies, including HS, are based on family history, clinical findings, peripheral spherocytes, hematological parameters, retic count, OF, FC-OF test, auto hemolysis test, acid-glycerol lysis test (AGLT), and the EMA test.[2,12] Further, SDS-PAGE is used as a confirmatory test to determine protein deficiencies.[12,13,27,28] If OF test is used alone, it could miss mild protein deficiencies.[12,26]

The EMA method requires an expensive dye, technical expertise, and a flow cytometer which is unavailable in most laboratories. In addition, the durability of the dye depends on storage temperature and duration.[1,26,29,30] Further, conventional diagnosis has failed in neonates and recently transfused patients due to morphological variability.[12,25]

Over the years, Sanger sequencing has been used to diagnose genetic variants. However, it is less feasible in large and complex genes with locus heterogeneity.[31] Next-generation sequencing has advantages over the traditional techniques and plays a vital role in identifying mutations, thereby facilitating differential diagnosis with high diagnostic yield.[7,24,31,32] Nevertheless, molecular diagnosis of membranopathies like HS is challenging due to co-inheritance with other HHA and due to the presence of a complex gene.[7,28] However, none of the diagnostic methods could diagnose all the patients as they all have their own limitations.[12,26]

In Asian countries, limited studies have been conducted to determine the use of different techniques to identify genetic defects and establish genotype-phenotype correlations in membranopathies.[7] Accordingly, in the present review, we compare and evaluate different diagnostic methods used in Asian countries for the definitive diagnosis of membranopathies.

2 | MATERIALS AND METHODS

2.1 | Search strategy

We searched databases of MEDLINE via PubMed, Google Scholar, and EBSCO for research studies published in English for the past 20 years (January 2000 to December 2020) using the following keywords in many combinations: Membranopathies, HS, HE, HPP, HSt, RBC membrane disorders, advanced diagnosis, Asia, India, Sri Lanka, China, Japan, and Korea.

2.2 | Inclusion criteria

Prospective studies, descriptive studies, and retrospective studies, including diagnostic methods of membranopathies in Asian countries (India, China, Japan, Korea, Turkey, Iran, Thailand, and Indonesia), were included in the present review.

2.3 | Exclusion criteria

Studies in membranopathies that did not describe their diagnosis were excluded. In addition, case reports, abstracts, unpublished studies, reviews, duplicates of previously included studies, and studies of non-Asian countries were excluded from the present review.

2.4 | Data extraction

Two researchers (Silva HJRL and Amarasinghe AADS) independently reviewed all abstracts of journal articles gathered to identify articles required for the review. All selected articles were discussed with a third independent reviewer (Perera PS). Data on study design, objectives, methodology, and results of the selected articles were methodically reviewed.

3 | RESULTS

We identified 245 citations through the search strategy, out of which 36 articles in agreement with inclusion criteria were selected for qualitative synthesis (Figure 1). Out of 36 articles, 15 (41.6%) originated from India, 11 (30.5%) from China, 5 (13.8%) from Korea, 2 (5.5%) from Thailand, 1 (2.7%) each from Turkey, Iran, and Indonesia. No eligible study was identified from Sri Lanka and other Asian countries. The majority of 61.1% (n = 22) articles were published during the last 5 years (2015–2020). The study designs excluding case studies and abstracts included prospective cohort studies (88.8%; n = 32) and retrospective studies (11.1%; n = 4). Summary descriptions of the selected studies are shown in Table 1.

Out of 15 prospective cohort studies done in India, 93.3% (n = 14) had used FBC, retic count, and PBS as first-line diagnostic methods. About 80% (n = 12) of studies had used clinical features, and only 40% (n = 6) had included family history. In addition, 11 (73.3%) studies had used direct agglutination test (DAT) to exclude autoimmune hemolytic anemia (AIHA), and 3 (20%) studies had used

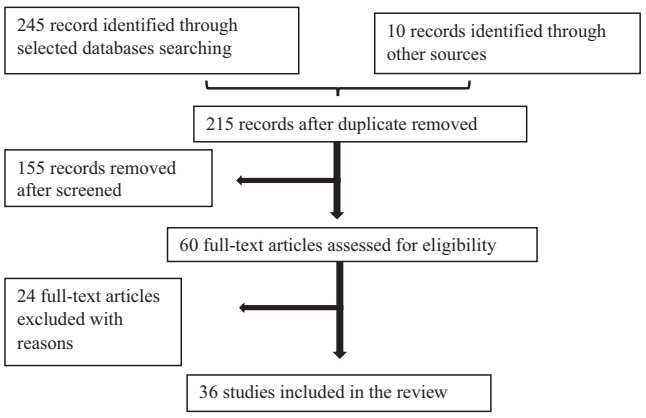

FIGURE 1 Flow diagram to show the article selection for the systematic review

hemoglobin (Hb) electrophoresis and enzyme assays to rule out hemoglobinopathies and enzymopathies. Osmotic fragility test was used in 10 (66.6%) studies, while FC-OF test was used in 5 studies. About 73.3% (n = 11) had used the EMA test as a screening method for HS, and from them, 33.3% revealed that it was a useful screening method. However, 2 studies showed that it was not appropriate for differentiation as there was no significant difference in mean channel fluorescence intensity between both diseased and the healthy control and between different membranopathies. Membrane protein analysis was done using SDS-PAGE in 4 studies, but two showed that it was not sensitive and had less diagnostic yield.[1,7]

Of 15 studies, 26.6 (n = 4) had used NGS in genetic analysis. All studies revealed the usefulness of NGS using customized gene panels in identifying defects in HS with high diagnostic yield. In 75% of them, Sanger sequencing was done to validate identified variants and silico analysis using computer software to analyze variant pathogenicity. In one study, matrix-assisted laser desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF) was used to identify different regulatory proteins in RBC membrane, and Hb depleted RBC cytosol.[33]

Of 11 Chinese prospective cohort studies, 81.1% (n = 9) had used FBC, and only 27.3% had used both retic and PBS. Tao et al.[34] (2015) used hematological parameters, comparing mean spherical corpuscular volume (MSCV) to mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC). The logarithm of these parameters along with FC-OF could be used as a screening tool for HS. Only eight cohort studies used clinical features, while five had used family history. In addition, seven studies had assessed bilirubin levels to determine the level of HA, while four used DAT, three used enzyme assays, 2 used Hb analysis, 2 used heat instability and unstable hemoglobin variant analysis, and 1 used sickle test to exclude other HA. Only five studies had used AGLT.[5,35,36] Further, four studies had used abdominal ultrasound scanning (USS) to diagnose hepatosplenomegaly in HA patients.[35–37] SDS-PAGE was used in three studies.[5,35,36] Ma et al.[37] (2018) stated that it should be used along with other routine tests to increase the diagnostic yield of HE.

A total of seven studies had used OF test, while three had used both OF and FC-OF tests. In addition, 27.3% (n = 3) had shown the usefulness of scanning electron microscopy (SEM) in the morphological identification of spherocytes and acanthocytes that aid in diagnosis.[5,35,36] Xue et al.[36] (2020) showed that SEM was an ideal method compared to SDS-PAGE and Western blotting. Of 11 articles, 54.5% (n = 6) had used the EMA method to diagnose HS. Peng et al.[38] (2017) showed EMA had no correlation either with anemic levels or with disease severity.

In 36.3% (n = 4) of studies, NGS and Sanger sequencing were used to identify de novo mutations. The damaging effect of variants was analyzed by silico analysis. Two studies had utilized the WES technique.[9,39] Junying et al.[5] (2016) used single nucleotide polymorphism typing (SNPscan), high-throughput copy number variation (CNVPlex), and sequencing method, which revealed a diagnostic yield of 94%. Ma et al.[37] (2018) used MALDI-TOF mass spectrometry to confirm the variants identified by Sanger sequencing.

TABLE 1 Summary description of the selected studies excluding case reports

References	Study design	Sample Size and Test methods used	Related findings/Comments
Jamwal et al., 2020[23]	A cohort study in India to determine the genetic spectrum of unexplained HA using NGS.	Sample size = 43 patients Methods: FBC, retic count, Hb analysis, brewer's test, DAT, heat and isopropanol stability tests, Hb electrophoresis, enzyme assays, incubated osmotic fragility test (iOF), EMA method, commercial TruSight One Sequencing, and targeted NGS. Identified variants were verified by Sanger sequencing. SIFT (Scale-Invariant Feature Transform), PolyPhen, (Polymorphism Phenotyping) PROVEAN (Protein Variation Effect Analyzer), MutPred, Mendelian Clinically Applicable Pathogenicity, and Combined Annotation Dependent Depletion software were used to determine pathogenicity.	The combined genetic analysis provided a definitive diagnosis of 63%. However, 37% remain uncharacterized. About 33% of uncharacterized HA were membranopathies. Silico analysis identified variants as pathogenic. PBS was not useful in recently transfused patients due to heterogeneity. Sanger sequencing was a labor-intensive, time-consuming, and costly process as large number of complex genes were involved. NGS was useful in prenatal diagnosis, genetic counseling, and affect decision-making in splenectomy. Further, it was a less laborious and efficient method compared to Sanger sequencing.
More et al., 2020[53]	A retrospective study using NGS to determine the genotype-phenotype relationship in uncharacterized hereditary xerocytosis (HX).	Sample size = 7 unrelated patients. Methods: Family history, clinical findings, PBS, aspirate bone marrow, FBC, retic count, serum bilirubin, RBC electrolytes, DAT, EMA, NGS, and Sanger sequencing. PyMOL & Swiss PB computer software was used to determine structural traits of identified variants. Polyphen−2, PROVEAN, MutPred, and Mutation Taster were used to determine pathogenicity.	NGS identified eight mutations in the PIEZO1 (Piezo Type Mechanosensitive Ion Channel Component 1) gene with three novel variants. NGS was a cost-effective, rapid method with a high diagnostic yield to identify mutations in uncharacterized HHAs thereby enabling proper clinical management of patients. It facilitated the identification of conditions associated with multi-genes.
Qin et al., 2020[39]	A cohort study in China to identify novel mutations in HS patients using NGS.	Sample size = 35 patients Methods: FBC, retic count, serum bilirubin, family history, clinical traits, WES, and Sanger sequencing. The functional impact of variants was assessed using SIFT, Mutation Taster, PolyPhen−2, Condel, CADD (Combined Annotation Dependent Deletion), and dbscSNV (Single Nucleotide Variation) software.	Thirty-four mutations were identified using WES with 21 novel variants. HS patients with ANK1 (Ankyrin 1) mutations had high mean corpuscular hemoglobin (MCH) and MCV values with a lower number of spherocytes in PBS compared to the patients with SPTB (Spectrin Beta chain erythrocyte) mutation. NGS was a rapid, economic, and an accurate technique which could be use in clinical practice.

TABLE 1 (Continued)

References	Study design	Sample Size and Test methods used	Related findings/Comments
Xue et al., 2020[36]	A clinical and experimental study done in China on adult HS.	Sample size = 20 patients Methods: Family history, physical examination, retic count, Hb, PBS, USS, serum bilirubin, urine bilirubin, DAT, unstable hemoglobin, Hb electrophoresis, iron hemoglobin reduction test, glucose−6-phosphate dehydrogenase (G6PD) fluorescence spot test, OF, iOF, AGLT, SDS-PAGE, Western blotting, SEM, EMA dye method, NGS, and Sanger sequencing. The variant pathogenicity was analyzed using Polyphen, SIFT, Pmut, and Panther software.	The OF test was positive in only 15% while the AGLT50 test was positive in all cases. EMA helped in the differential diagnosis. SEM was a useful screening test. However, the result of a single test could not provide a definitive diagnosis. NGS identified a novel variant in 90% of the patients. The results of SDS-PAGE did not consist with the gene mutations and the method was complex, time-consuming and less sensitive in asymptomatic carriers. Splenectomy in HS patients had indirectly confirmed the diagnosis of HS.
Aggrawal et al., 2019[7]	A prospective study done North India.	Sample size = 113 patients Method: Clinical features, FBC, retic count, PBS, High-Performance Liquid Chromatography (HPLC), G6PD, EMA test, SDS-PAGE, PCR, and NGS.	About 60.3% had moderate phenotype with high reticulocyte count (12%). SDS-PAGE is not much sensitive as mild protein deficiencies will not be detected. Further it is labor-intensive. NGS had a diagnostic yield of 64.4% in HS diagnosis. It was a sensitive, rapid, and a cost-effective method.
Chois et al., 2019[28]	A prospective cohort study on HS patients in Korea.	Sample size = 59 patients Method: Family history, clinical features, PBS, FBC, retic count, DAT, serum bilirubin, TIBC (Total Iron-Binding Capacity), LDH, ferritin, OF, EMA, and multi-gene target sequencing.	Multi-gene target sequencing identified mutations in 84.7%. It was a feasible, and highly sensitive method for the accurate and definitive diagnosis of HS. OF was positive in 86.8%. OF and PBS together with genetic testing could diagnose HS with high sensitivity and specificity.
Ittiwut et al., 2019[15]	A prospective study done in Thailand to detect mutations using WES in HPP.	Sample size = 8 patients Methods: Clinical, hematological, and molecular characterization was done. The parent-offspring trio of all the families was subjected to WES to identify mutations.	Whole-exome sequencing was able to identify variants in all eight patients (100%). Seven patients had mutations in the SPTB gene, and one had mutations in SPTA1 (Spectrin Alpha chain erythrocyte 1).
Kedar et al., 2019[32]	A prospective study in India using targeted NGS to characterize HA with unexplained etiology.	Sample size = 21 patients Methods: FBC, retic count, PBS, DAT, G6PD assay, HAM test, Hb electrophoresis, enzyme assays, FC-OF, and EMA method, NGS, and Sanger sequencing. The damaging effects of the variant proteins were tested using SIFT, Mutation Assessor, Polyphen−2, and Mutation Taster software.	Only 9 out of 17 patients had membranopathies. About 81% patients were identified by NGS. It was a rapid, efficient, and a specific method. However, it failed diagnosis in 19%. Therefore, alternative like array base genome hybridization and copy number analysis could be used.

(Continues)

TABLE 1 (Continued)

References	Study design	Sample Size and Test methods used	Related findings/Comments
Anil et al., 2019[49]	A prospective study to determine the use of imaging flow cytometry (IFC) in the diagnosis of membranopathies.	Sample size = 162 suspected cases and 50 healthy individuals Methods: Clinical features, FBC, PBS, retic count, DAT, OF test, EMA dye method, and image analysis including shape ratio, and circularity score using IDEAS 6.2 software.	MFI values of HS patients were low. HE was not able to diagnose using MFI value. It was diagnosed using shape ratio which was elevated with high sensitivity (100%) and specificity (93.7%). IFC was a simple, effective, and rapid method which allowed differentiation between membranopathies.
Xue et al., 2019[35]	A prospective study on HS patients in Nanjing First Hospital, China.	Sample size = 10 patients Methods: Blood tests, urine tests, serum bilirubin, USS, OF, acidified glycerol lysis test 50 (AGLT50), PBS, SEM, EMA, targeted gene enrichment, sequencing and bioinformatics and structural changes analyzed by SWISS-MODEL.	Targeted gene enrichment and sequencing methods were an efficient tool for determining genetic etiologies of RBC membrane disorders with 90% specificity. AGLT50 was positive in all cases. Positive detection rate of OF was low (40%).
Arora et al., 2018 [30]	A prospective study on suspected patients of HS and HHA from the New Delhi hospital, India.	Sample size = 112 patients Methods: Clinical features, family history, PBS, retic count, bilirubin, DAT, LDH, abdominal USS, iOF, FC-OF, EMA test, MCV, MCHC, MSCV, and MRV (Mean Reticulocyte Volume).	MCHC > 35g/dl had a sensitivity of 44.8% and a specificity of 94.3%. ΔMCV−MSCV > 10 fl had a sensitivity of 82.8% and specificity of 95.9%. In a resource poor setting a logarithm of ΔMRV−MSCV < 25 with sensitivity of 68.9% and specificity of 98.8% could be used to differentiate HS from HHA.
Lin et al., 2018[51]	The first Asian study done in Taiwan using WES to elucidate mutated gene membranopathies.	Sample size = 7 patients with their family members. Methods: FBC, PBS, EMA assay, WES, and Sanger sequencing. PolyPhen, SIFT & CADD_PHRE software were used to predict the damaging effect of variants.	WES identified mutations with novel variants in 57.1%. It enabled definitive diagnosis of membranopathies and proper genetic counseling.
Ma et al., 2018 [37]	A cohort study was done to determine new mutations in patients with HE.	Sample size = 95 patients, 95 healthy individuals and a proband with family members Methods: Clinical feature, FBC, retic count, DAT, total and direct bilirubin, USS, enzyme assays, urine analysis, Hb electrophoresis, SDS-PAGE, EMA method, Sanger sequencing, MALDI-TOF mass spectrometry. Identified genes were screened using high-resolution melt analysis (HRM).	EMA could be used as a screening test for HE. Mutations were identified and confirmed by MALDI-TOF mass spectrometry. SDS-PAGE showed normal protein levels in the proband and their family members. HRM was an efficient, inexpensive, high-throughput procedure with greater specificity and sensitivity for screening gene mutations. EMA and SDS-PAGE methods together with morphology and routine lab tests increased the diagnostic yield of HE.

TABLE 1 (Continued)

References	Study design	Sample Size and Test methods used	Related findings/Comments
Nixon et al., 2018[44]	A retrospective study in Indonesia to determine the accuracy of the light microscope in SAO.	Sample size = 971 blood smears. Methods: Blood smears were analyzed by three microscopists. PCR was performed for the genetic identification. The diagnosis was based on knizocyte-centric diagnostic criteria. Mixed modeling was used to measure specificity, sensitivity, PPV, and NPV.	Knizocyte-centric diagnostic criteria showed a high sensitivity (89%) and a specificity (93%). Observational analysis of knizocytes was dependent on microscopists. Light microscopy was a cost-effective, simple procedure that could be used as an alternative to molecular methods in the diagnosis of SAO.
Wang et al., 2018[53]	A prospective study in China to confirm the molecular diagnosis in HS families using exome sequencing.	Sample size = 38 families Methods: HS was diagnosed according to King et al., 2015, WES, Sanger sequencing, annotation by ANNOVAR (Annotate Variation), damaging effect by REVEL software, and splicing effect by HSF (Human Splicing Finder).	WES determined definitive diagnosis in all 38 families (100%). The parental genetic testing revealed de novo mutations. Sequencing and parent-offspring trio increased the efficiency of diagnosis.
Charis and Prasad, 2017[8]	A prospective study to evaluate the EMA compared to OF test in HS diagnosis.	Sample size = 51 patients Methods = Clinical findings, PBS, DAT, FBC, retic count, serum bilirubin, OF, cryohemolysis, and EMA method were used to screen HS.	Positive OF and EMA was shown by 20% while 4% showed positive EMA with normal OF results. The EMA method was a simple, effective, reproducible, less labor-intensive, and a rapid technique that provide definitive diagnosis.
He et al., 2017 (54)	An evaluation to determine the genotype-phenotype relationship in membranopathies using NGS.	Sample size = 15 individuals from 3 unrelated families Methods: Clinical features, FBC, PBS, retic count, total and indirect bilirubin, OF test, USS, Hb electrophoresis, NGS, and Sanger sequencing. The damaging effect of the variants was assessed using SIFT, and PolyPhen−2.	NGS was a sensitive, high-throughput, rapid, and a cost-effective procedure. Genetic analysis together with the medical history, clinical findings, hematological, biochemical, and pedigree analysis enabled diagnosis of uncharacterized HA and facilitates patient management and genetic counseling.
Manivannam et al., 2017[47]	A prospective observational study in India to evaluate the use of FC- OF in the diagnosis of HS compared to iOF.	Sample size = 40 healthy individuals, 40 HS patients, and 20 beta thalassemia trait patients. Methods: Clinical features, Family history, PBS, FBC, Hb analysis, reticulocyte count, DAT, iOF test, FC-OF test. Percentage of residual red cells was measured, and %RRC ratio was calculated.	FC-OF had high test efficacy (96%) with high sensitivity (92.5%), specificity (98.3%), PPV (97.3%), and NPV (95.1%). iOF had a test efficacy of 89% with low sensitivity (82.5%), specificity (93.3%), PPV (89.1%), and NPV (88.8%). Therefore, FC-OF which is a rapid, sensitive, specific, and a cost-effective method could be used as a screening method for HS replacing iOF test.

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TABLE 1 (Continued)

References	Study design	Sample Size and Test methods used	Related findings/Comments
Guangxin et al., 2017[37]	A prospective study to determine the correlation between EMA method and HS severity.	Sample size = 258 patients Methods: FBC, retic count, absolute retic count, indirect bilirubin levels, LDH, AGLT50, OF, and EMA dye-binding method.	The intensity of EMA fluorescence was 29.7%, which showed a correlation between MCV and MCHC. There was no significant relationship between decreased fluorescence intensity and Hb levels, retic count, absolute retic count, and indirect bilirubin levels. EMA assay did not correlate either with anemic levels or with the disease severity.
Li et al., 2016[5]	A prospective study in China to determine the systemic analysis and differential diagnosis of HHA.	Sample size = 1506 patients Methods: Clinical features, family history, FBC, PBS, DAT, AGLT, sucrose lysis test, heat instability test, isopropanol test, Hb analysis, sickle test, enzyme assays, SEM, OF, SDS-PAGE, high-throughput SNPscan, high-throughput CNVPlex, and high-throughput sequencing method.	About 94% were diagnosed as HHA with 26% mixed deficiencies. Commonest heterozygous complex HA was hemoglobinopathy with membranopathy (50%) and commonest single complex HA was double membranopathy (15%). Differential diagnosis required a combination of hematological, morphological, biochemical, molecular, and genetic analysis.
Tao et al., 2016[48]	A prospective cohort study to evaluate the use of FC-OF for diagnosing HS in Chinese patients.	Sample size = 237 patients Methods: Clinical features, FBC, biochemical tests, PBS, OF test, and FC-OF.	The FC-OF had higher sensitivity (85.71%) and specificity (97.24%) in diagnosing HS. However, FC-OF test showed negative result in 14.2%. FC-OF test together with PBS could be used as a simple and accurate screening method for HS.
Tao et al., 2014[34]	A cohort study in China to evaluate three screening tests in diagnosing HS.	Sample size = 56 patients and 95 healthy individuals and 86 thalassemia patients Methods: Clinical features, FBC, PBS, OF test, Hb electrophoresis, and FC-OF test.	MCHC > 355 g/L had high specificity (94.47%) and low sensitivity (41.07%) while, MSCV < MCV had high sensitivity (89.28%) and specificity (96.14%). FC-OF had high sensitivity (85.71%) and specificity (97.24%). Comparing MSCV to MCV and FC-OF test could be used as accurate screening methods for HS.
Warang et al., 2015[50]	A cohort study in India to determine the spectrum of red cell abnormalities in undiagnosed HA and methemoglobinemia.	Sample size = 231 patients with 167 suspected for HS Methods: Clinical features, family history, FBC, PBS, retic count, FC-OF, enzyme assays, isopropanol, and heat instability test, nicotinamide adenine dinucleotide cytochrome b5 reductase (NADH-cytochrome b5 reductase) activity, spectroscopic analysis of HbM variants, SDS-PAGE, and gene sequencing.	Of study population 35.82% had membranopathies diagnosed by EMA and FC-OF test. However, 64.17% showed normal EMA and FC-OF results. Therefore, SDS-PAGE and molecular methods should be carried out for all undiagnosed cases. Advanced methods like proteomics and WES should be established for the definitive diagnosis.

TABLE 1 (Continued)

References	Study design	Sample Size and Test methods used	Related findings/Comments
Golafshan et al., 2014[18]	A prospective study using EMA for screening membranopathies in South Iran.	Sample size = 84 patients and 15 healthy controls Methods: FBC, PBS, DAT, SDS-PAGE, and EMA were used to diagnose and confirm patients with HS.	There was no significant difference in SDS-PAGE results between HS and control and PBS showed only 10% to 15% of spherocytes. In EMA assay, there was a significant reduction in MFI with high sensitivity (95%), specificity (93%) and PPV and NPV (93%) which makes it a reliable screening method compared to routine tests.
Park et al., 2014[27]	A prospective study in Korea to compare the EMA method, FC-OF test, and cryohemolysis test in HS diagnosis.	Sample size = 153 patients and 140 healthy controls Methods: Family history, clinical features, hematological indices, retic count, serum bilirubin, PBS, LDH, DAT, iron studies, SDS-PAGE, EMA dye-binding test, FC-OF test, and cryohemolysis test.	Satisfactory performance was shown by EMA and FC-OF tests compared to cryohemolysis as it showed false-positive results with iron deficiency anemia. EMA and FC-OF tests could be used as screening methods as they showed high sensitivity (97%, 93.9%), specificity (97.5%, 97.5%), PPV (99.2%, 98.3%), and NPV (91.4%, 91.2%). The Hb/MCHC ratio reflected the clinical severity.
Park et al., 2013[42]	A survey done in Korea on the prevalence and traits of patients diagnosed with HHA.	Sample size = 95 patients Methods: Clinical features, family history, FBC, PBS, retic count, iron studies, DAT, serum bilirubin, enzyme assays, glycerol lysis test, isopropanol and heat instability test, serum haptoglobin, LDH, Hb electrophoresis, OF test, iOF test, sequencing and multiple ligation dependent probe amplification (for hemoglobinopathy), SDS-PAGE, and EMA assay.	About 64% were diagnosed as membranopathies, 1.5% as undiagnosed HHA and the rest as enzyme and Hb disorders. The diagnosis of enzymopathies and hemoglobinopathies had increased due to improved diagnostic methods. However, sensitive tests including flow cytometry are needed for diagnosis of membranopathies.
Ayhan et al., 2012[11]	A prospective cohort study on RBC membrane protein defects among HS patients in Turkey.	Sample size = 50 patients and 42 controls. Methods: Family history, clinical findings, PBS, FBC, retic count, iOF, DAT, serum haptoglobin, serum ferritin, TIBC, enzyme assays, Hb electrophoresis, serum bilirubin, and SDS-PAGE.	Protein efficiencies were determined only in 42% and not in 58% of HS patients. There was no significant relationship between protein deficiency and Hb levels. More advanced techniques are required as SDS-PAGE is unable to diagnose mild protein deficiencies.
Saha et al., 2011[33]	A prospective study done India to identify different proteome profiles in the membrane and hemoglobin-depleted cytoplasm of RBC in HS patients.	Sample size = 8 patients and 10 normal controls Methods: Clinical features, FBC, retic count, serum bilirubin, PBS, OF test, 2-D gel electrophoresis, and MALDI mass spectrometry. Transmission electron microscope (TEM) was done on the RBC membrane using the erythrocyte ghost.	TEM revealed destructed RBC membrane cytoskeleton. Proteomics techniques allowed detection of changes in cytoskeletal protein organization, protein levels, oxidative stress, and redox regulation thereby facilitating the identification of pathophysiology and disease severity of HS.

(Continues)

TABLE 1 (Continued)

References	Study design	Sample Size and Test methods used	Related findings/Comments
Warang et al., 2011[29]	A prospective study in India to compare the FC-OF test with EMA to diagnose membranopathies.	Sample size = 54 patients with IHA and 50 healthy individuals Methods: FBC, PBS, retic count, Hb analysis, FC-OF, and EMA dye test were performed on suspected patients with red cell disorders. SDS-PAGE was used in few cases.	FC-OF test had high sensitivity (100%) and specificity (98%) in diagnosing HS. EMA also had high sensitivity and a specificity (100%). However, FC-OF is simple and cost-effective compared to EMA assay and had high discriminating power between HS and HE. Therefore, FC-OF could be used as an effective first-line screening method for membranopathies.
Kar et al., 2010[45]	A prospective cohort study in India to evaluate the use of the EMA test in the diagnosis of HS.	Sample size = 114 patients Methods: Family history, clinical findings, FBC PBS, retic count, Coomb's test, Hb analysis, iOF test, and EMA method.	EMA assay diagnosed 40% of patients with suspected HS, while iOF showed negative or equal results in all the suspected population. EMA assay had high sensitivity (96.4%), specificity (94.2%), PPV (84.4%), and NPV (98.8%) compared to iOF test which had low sensitivity (71.4%), specificity (74.1%), PPV (74.1%), and NPV (71.4%). EMA assay is a cost-effective and rapid method that required small blood volume.
Preethi et al., 2010[4]	A hospital-based retrospective study in India on HHA.	Sample size = 40 patients Methods: Clinical features, FBC, PBS, retic count, blood grouping, DAT, urine analysis, erythrocyte sedimentation rate (ESR), bone marrow aspiration, sickle test, Hb F analysis, serum bilirubin, Hb electrophoresis, and OF test.	HS was diagnosed in only 10% with increased retic count and OF test with mild anisopoikilocytosis and spherocytes covering 40%–60% of RBC in PBS. Hematological investigations could be used as first-line diagnostic techniques in HA. Further, OF test established the diagnosis of HS.
Kar et al., 2009[46]	A retrospective study to determine the Clinico-hematological profile of HS in a tertiary care center in North India.	Sample size = 70 patients Methods: Clinical features, Family history, FBC, retic count, PBS, iOF, DAT, iron studies, serum bilirubin, liver enzymes, Hb analysis, G6PD screening test, bone marrow examination, and EMA dye-binding test.	OF test was positive in 88.2%. However, 8 cases that showed negative or equal OF results were confirmed by EMA assay with high sensitivity (96.4%) and specificity (94.2%). The EMA method should be used in combination with other tests to diagnose asymptomatic HS.
Tachavanich et al., 2009 [43]	A cohort study in Thailand to diagnose membranopathies using EMA assay.	Sample size = 202 patients and 142 healthy controls Methods: Family history, clinical features, FBC, PBS, DAT, Hb electrophoresis, enzyme assays, autohemolysis test, OF test, and EMA assay. SAO patients were diagnosed and confirmed with a molecular diagnosis of band 3 mutation.	Mean channel fluorescence (MCF) in HS, SAO, and HE patients were low compared to controls and other analyzed HA. EMA assay showed high sensitivity (100%) and specificity (99.6%). EMA method was a simple, reliable, sensitive, specific, and rapid method that required small blood volume. It could be used as a screening method for HS and SAO.

TABLE 1 (Continued)

References	Study design	Sample Size and Test methods used	Related findings/Comments
Won and Sue, 2009[41]	A cohort study in Korea to establish a flowcytometric method to detect RBC osmotic fragility.	Sample size = 24 patients and 25 healthy controls Methods: Clinical features, FBC, PBS, OF, and EMA binding test. FC-OF was performed, and optimal parameters {normal saline (NS): distill water, NS contact time, flow rate consistency, added RBC number, temperature, and reproducibility} were established for the new procedure.	The protocol showed discrimination between patients and normal control and between immediate analysis and analysis after 24 h stored sample. The FC-OF was increased in all the tested HS patients. It had high sensitivity (100%) and specificity (96%). FC-OF was a simple, rapid, effective, inexpensive, and quantitative method.
Kedar et al., 2003[1]	A cohort study in India to determine the usefulness of the EMA dye-binding method in the diagnosis of membranopathies.	Sample size = 45 patients and 120 healthy controls Methods: Clinical features, FBC, PBS, retic count, DAT, bilirubin, OF, AGLT, autohemolysis test, Hb analysis, Hb electrophoresis, enzyme assays, SDS-PAGE, and EMA test. The stability of the dye was measured by storing it in dark at three temperatures, 4°C, −20°C, and −80°C for a period of 4 months.	SDS-PAGE was done in 4 HS patients which confirmed the diagnosis. MCF was low in HS and HE compared to controls and other RBC disorders. A rapid decrease in MCF was observed in dye stored at 4°C, while at −20°C and −80°C, there was a gradual reduction over a period of 4 months. EMA was a simple, practical, sensitive, specific, cost-effective, and rapid method.
Lee et al., 2000[40]	A cohort study done in Korea to determine abnormalities associate with RBC membrane proteins in HS.	Sample size = 27 patients Methods: Family history, clinical findings, PBS, OF, and SDS-PAGE.	Different single and combined protein deficiencies were identified in 66.6%, and no protein deficiency was detected in 33.3% by SDS-PAGE. It does not require complex instruments. Protein loading that affects the results could be overcome by considering protein band ratios.

All the five prospective cohort studies in Korea have used clinical features, while family history was considered in only four articles.[27,28,40–42] Of 5 studies, 60% ($n = 3$) used hematological indices, retic count, and PBS in the diagnosis of HS. Lee et al. (2000) used only PBS, and Won and Sue (2009) used only FBC and PBS.[40,41] Coomb's test, serum bilirubin, lactate dehydrogenase (LDH), and iron studies were done in three studies.[27,28,42] OF test was used in four studies, and the FC-OF test was used in two studies.[28,42] In addition, 80% ($n = 4$) of studies used the EMA dye method.[27,28,41] The EMA and the FC-OF tests were efficient in differentiating HS from other anemias compared to hypertonic cryohemolysis HCH test. Of 5 cohort studies, three used SDS-PAGE.[27,40,42] Multi-gene target sequencing was used in one study where it was a feasible and highly sensitive molecular diagnostic method for HS.[28]

Of 2 prospective cohort studies done in Thailand, 1 used WES to identify mutations in HPP while the other used EMA assay, which showed that it could be used for screening HS and SAO.[15,43] A retrospective study done in Indonesia used a light microscope in the diagnosis of SAO, where it showed its both advantages and disadvantages.[44] Two prospective cohort studies done in Iran and Turkey used a combination of clinical findings, hematological, biochemical, and molecular analysis to diagnose membranopathies.[11,18] The study in Iran showed the drawbacks of SDS-PAGE and the usefulness of EMA dye assay.[18]

4 | DISCUSSION

Hemolytic anemias are a group of genetic disorders with phenotype and genotype heterogeneity. Based on the literature review, different studies have used different diagnostic work flows for the definitive diagnosis of membranopathies. Screening and confirmatory methods integrated in these workflows have both advantages and limitations.

Studies done in different countries have used clinical features in the preliminary diagnosis of membranopathies.[11,28,36,43,45,46] Few

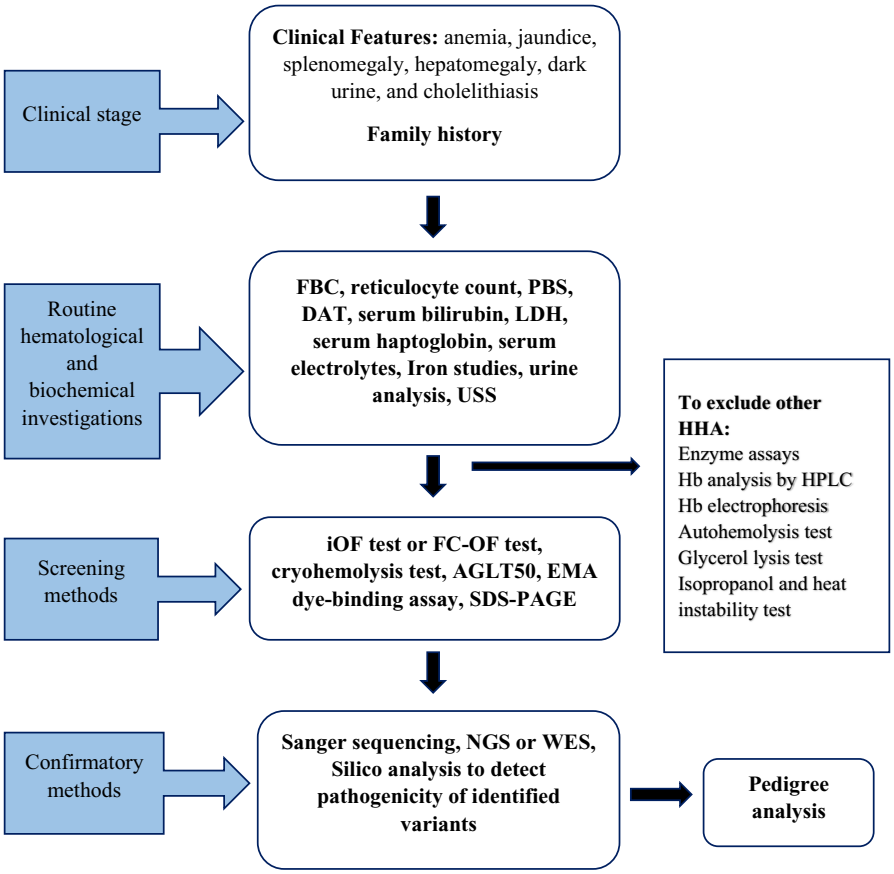

FIGURE 2 A common diagnostic workflow for membranopathies

studies have shown the usefulness of hematological parameters to diagnose membranopathies in a resource poor setting. At different cutoff values, MSCV < MCV showed different sensitivities (82.8% and 89.3%) with similar specificity (~ 96%). Similarly, the parameter MCHC > 35% showed greater specificity (~ 94%) with low sensitivity (41% and 44%). Despite the diagnosis, RBC parameters enabled distinguishing membranopathies from other HHA with a significantly high sensitivity (68.9%) and a high specificity (98.8%).[4,30] Further, Preethi et al.[4] (2010) showed that reticulocytosis was associated with all the diagnosed cases of HS.

Membranopathies have unique RBC morphological characteristics that, together with other methods, help in differentiation. A study in Indonesia has used only microscopic analysis along with polymerase chain reaction (PCR) to diagnose SAO. The light microscopic diagnosis was a cost-effective and a rapid method with high sensitivity (89%) and specificity (93%). However, the observational analysis depended on the microscopist.[44]

In few studies, FC-OF test was used as a screening method along with other routine hematological and biochemical investigations. The FC-OF method was reported to have a high sensitivity (92%–100%) and a specificity (96%–98%).[29,41,47,48] Further, it showed high positive predictive value (PPV; 97.3%) and negative predictive value (NPV; 95.1%). The FC-OF test showed high test efficacy of 96% compared to the conventional OF test (89%). In developing countries, FC-OF could be a better first-line screening method in the presence of a flow cytometer.[47] Further, it is a simple, rapid, accurate, and a quantitative screening method with good discriminating power between HS and HE.[29,41,47,48] However, percentage residual red cells (%RRC) or %RRC ratio had no significant relationship with the disease severity.[47]

Several studies have used EMA dye method along with routine investigations and other screening methods to diagnose membranopathies.[1,8,18,38,42,43,45,46] A significant reduction in mean fluorescence intensity (MFI) was seen in HS, HE, and SAO compared to healthy controls. It was reported that EMA assay had confirmed HS patients with normal or equal OF results.[8,46] The EMA assay is a reliable screening method for HS with high sensitivity (95%–100%), specificity (93%–99.6%), PPV (84%–93%), and NPV (93%–99%).[18,42,43,45,46] Different sensitivities and specificities emphasized the need to establish own MFI cutoff value for each laboratory. Similar to FC-OF test, MFI value did not correlate with anemic level or disease severity.[38] Except for initial installations, EMA is a cost-effective, rapid, less labor-intensive, simple, and a reproducible method that is useful in neonates due to the use of small sample volume.[1,8,43,45,46] Advanced EMA assay where it combined with imaging flow cytometer in a single platform provided an effective and a rapid diagnosis of HE with high sensitivity (100%) and specificity (93.7%).[49] However, the stability of EMA dye was critical, where it had greater stability at −80°C in the dark over 4 months than at −20°C and 4°C.[11] In an Indian study, the diagnostic workflow which used only screening methods showed that EMA assay and FC-OF test had satisfactory performance with high sensitivity (97%, 93.9%), specificity (97.5%),

PPV (99.2%, 98.3%), and NPV (~ 91%) compared to cryohemolysis test (48.5%, 76.7%, 84.4%, and 36.4%, respectively).[27] However, a study by Warang et al.[50] (2015) showed that EMA and FC-OF methods had diagnosed only 35.82% of membranopathies.

Few studies have used SDS-PAGE as the ultimate diagnostic method in combination with routine investigations. The diagnostic efficacy of SDS-PAGE was reported as only 40% by Ayhan et al. (2012) and 66.6% by Lee et al. (2000).[11,40] There was no significant relationship between protein deficiency and Hb levels.[11] SDS-PAGE was a complex, labor-intensive, and a time-consuming method.[7,11,36] Further, the study by Golfshan et al.[18] (2014) showed that there was no difference in SDS-PAGE results between the diseased and healthy individuals.

Definitive diagnosis of membranopathies could not be achieved using single screening method. Therefore, based on the literature review, most of the studies had used considerably a common stepwise diagnostic method. Accordingly, the diagnostic work flow of membranopathies is based on the combine qualitative and quantitative results of clinical features, family history, routine hematological and biochemical investigations, first-line screening methods, and genetic analysis for the definitive diagnosis (Figure 2). These studies had highlighted the significance of integrating genetic analysis in the differential diagnosis of red cell membrane disorders. Sanger sequencing was useful in identifying variants when the affected gene is known.[37] It was also useful in validating already identified mutations.[24,32,37,39,51,52] Further, in new era, NGS was incorporated which is an improved technique to detect mutations and novel variants and identify their genotype-phenotype correlation. Different countries have shown varying diagnostic yields with the NGS method (63%–100%).[7,23,24,28,32,35,36,53] Further, it was a sensitive (90%), specific (90%), rapid, and accurate method. It could be used as an alternative when conventional lab methods failed to diagnose HA with unknown etiology.[24,28,39,53] It also enabled prenatal diagnosis, genetic counseling, patient management, and decision-making in splenectomy.[23,53] In parallel with NGS, in silico analysis was performed using different software programs to determine the pathogenicity of identified variants.[23,24,32,35,39,51,53] However, considerable amount of cases (19%–37%) were still uncharacterized using NGS which could be due to large base deletions or due to mutation occurred in pseudogenes.[7,23,32] This emphasized the requirement of advanced alternative techniques like array base genome hybridization and copy number analysis.[32] A study by Iittiwut et al.[15] (2018) had used whole-exome sequencing directly along with clinical features and hematological investigations for the diagnosis of HPP where it identified mutations in all cases. In addition, few studies had used proteomics to support their diagnosis. Ma et al. (2018) used MALDI-TOF mass spectrometry along with screening methods and sequencing to confirm identified mutations, while Saha et al. (2011) used MALDI-TOF mass spectrometry along with only screening methods to detect changes in cytoskeletal protein organization, protein levels, oxidation stress, and redox regulation to determine disease pathophysiology.[33,37]

In summary, studies done in different Asian countries had used different diagnostic criteria. Conventional lab methods do not provide a definitive diagnosis in all cases due to their limitations. The introduction of NGS has increased the diagnostic yield in identifying mutations with novel variants. Therefore, a common work flow using a combination of conventional and advanced techniques could be used for the definitive diagnosis of membranopathies. Supplementing, not replacing the "old" with the "new" techniques will increase the diagnostic yield in most diagnostic services.

CONFLICT OF INTERESTS

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

HJRL Silva and AADS Amarasinghe independently reviewed all abstracts of journal articles gathered to identify articles required for the review. All selected articles were discussed with a third independent reviewer, PS Perera. HJRL Silva prepared primary draft of the manuscript with editing by PS Perera and AP Premawardhana. All authors reviewed and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no data sets were generated or analyzed during the current study.

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