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Evaluation of the eosin-5-maleimide flow cytometric test and other screening tests in the diagnosis of hereditary spherocytosis

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Date of submission:

30 June 2020

This research is submitted in partial fulfilment of the requirements in respect of the Degree Master of Medicine in Haematology, Department of Haematology and Cell Biology, Faculty of Health Sciences, University of the Free State.

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Plagiarism declaration:

I, Hemasha Mothi, declare that the coursework Master's Degree mini-dissertation that I herewith submit in a publishable manuscript format for the Master's Degree qualification MMed (Haematology) at the University of the Free State is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.

Signed:

Date: 30 June 2020

Place: Bloemfontein

Authors' Contributions (Publishable manuscript):

Hemasha Mothi wrote the protocol, recruited the subjects, collected blood samples, went through all the relevant hospital files, reviewed peripheral smears, analysed the data and wrote the first draft. Anneke van Marle and Jan Roodt conceived the study, were involved in the design and coordination of the study, analysed the data, and participated in the revision of the manuscript. All authors read and approved the final manuscript.

Acknowledgements:

We would like to acknowledge the patients for their willingness to participate in the study. Prof MJ Coetzee and Prof DK Stones provided patient records and contact details. All tests were conducted by Jan Roodt and Marianne van der Westhuizen of the flow cytometry bench and Emily Venter and Maria Vermeulen of the immunology bench at the National Health Laboratory Service in Bloemfontein. Mr Phillip Bester and Dr Dawid Venter assisted with statistical analysis.

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Abstract

Evaluation of the eosin-5-maleimide flow cytometric test and other screening tests in the diagnosis of hereditary spherocytosis

Background: Hereditary spherocytosis (HS) is a genetically determined haemolytic anaemia characterised by the spherical shape of affected red blood cells. With limited confirmatory tests currently available in South Africa, the diagnosis of HS is reliant on the clinical presentation and screening tests.

Objectives: The aim of this study was to compare the sensitivity and specificity of three screening tests: flow osmotic fragility test (FOFT), cryohaemolysis test (CHT) and the eosin-5-maleimide binding test (EMA-binding test) in diagnosing hereditary spherocytosis (HS).

Method: All three tests were performed on 18 subjects with confirmed HS. The negative control group was comprised of 10 subjects with haemolysis and spherocytosis, and either a positive direct antiglobulin test (DAT) or normal red cell membrane studies. The tests were also performed on samples submitted for cases of suspected HS during the study period.

Results: The EMA-binding test demonstrated superior sensitivity (88.9%) compared to the CHT (61.1%) and the FOFT (38.8%). The EMA-binding test specificity (90.0%) was equal to that of the FOFT and superior to the CHT (50%). Combined sensitivities and specificities for EMA-binding test and CHT, EMA-binding test and FOFT, and CHT and FOFT, were 100% and 33.3%, 94.4% and 80.0% and 88.9% and 50.0%, respectively.

Conclusion: EMA-binding test is the best screening test for cases of suspected HS. If there is a high clinical index of suspicion with a negative EMA-binding test, the CHT is recommended as a second screening test.

Key words: Hereditary spherocytosis, Eosin-5-maleimide, Flow osmotic fragility, Cryohaemolysis, Screening test, Red cell membrane, South Africa

Table of Abbreviations

EXPLANATION	ABBREVIATION
Acidified glycerol lysis test	AGLT
Autoimmune haemolytic anaemia	AIHA
Hypertonic cryohaemolysis test	CHT
Direct antiglobulin test	DAT
Eosin-5-maleimide	EMA
Eosin-5-maleimide flow cytometric test	EMA-binding test
Ethylene-diamine-tetra acetic acid	EDTA
Flow cytometric Osmotic fragility test	FOFT
Full blood count	FBC
Glycerol lysis test	GLT
Glucose-6-phosphate dehydrogenase	G6PD
Hereditary spherocytosis	HS
Mean corpuscular volume	MCV
Mean corpuscular haemoglobin	MCH
Mean corpuscular haemoglobin concentration	MCHC
National Health Laboratory Service	NHLS
Next-generation osmotic gradient ektacytometry	NG-OGE
Osmotic fragility test	OFT
Phosphatidylserine	PS
Red blood cell	RBC
Red cell distribution width	RDW
Rhesus	Rh
Sodium dodecyl sulphate-polyacrylamide gel electrophoresis	SDS-PAGE
Standard operating procedures	SOP
Surface-area-to-volume	SA/V
Universitas Academic Hospital	UAH

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Chapter 1: Literature Review

Introduction

Hereditary spherocytosis (HS) is a genetic disorder, characterised by spherical erythrocytes, that results in haemolytic anaemia which varies in severity from being asymptomatic to requiring recurrent blood transfusions and splenectomy. (1) The molecular defects are heterogeneous, involving genes that code for erythrocyte membrane proteins responsible for vertical associations between the membrane cytoskeleton and the lipid bilayer. (2)

The red cell membrane

Red blood cell (RBC) membrane is composed of a lipid bilayer with approximately 20 major and 850 minor proteins embedded within it. (3) The membrane attaches to an intracellular cytoskeleton by protein-protein and lipid-protein interactions. These interactions give the erythrocyte its characteristic biconcave shape and properties of stability, elasticity and deformability. (4)

Membrane lipids

The lipid bilayer, with inner and outer leaflets, is composed of cholesterol, phospholipids, free fatty acids and glycolipids. (5) Cholesterol is distributed evenly between the inner and outer leaflets, but the four major membrane phospholipids are asymmetrically distributed. (6) Neutral phospholipids (phosphatidylcholine and sphingomyelin) are found mainly in the outer monolayer, while negatively charged phosphatidylethanolamine and phosphatidylserine (PS), are confined to the inner monolayer. (7)

Membrane proteins

Protein-membrane associations are of two main types. Integral membrane proteins traverse the membrane and act as channels between the plasma and cytoplasm. Within the cytoplasm, integral membrane proteins interact with each other and with the second main group, the skeletal membrane proteins. The links provided by the integral proteins between the lipid bilayer and the membrane skeleton are called ‘vertical connections’, while the interactions of the membrane skeleton proteins are called ‘horizontal connections’. Spherocytosis is mainly caused by mutations affecting the vertical connections. (5)

Integral membrane proteins

There are up to 50 integral membrane proteins. They vary in abundance and are functionally diverse, functioning as transport proteins, adhesion molecules, and signalling receptors. (6) These proteins are embedded in the lipid membrane through hydrophobic interactions. They traverse the membrane and have distinct regions important for structure and function, both within the membrane and on either side of it. (6)

Band 3, glycophorins and Rh-associated glycoprotein (RhAG) are among the integral membrane proteins involved in vertical interactions that link the lipid bilayer to the membrane skeleton (see Figure 1).

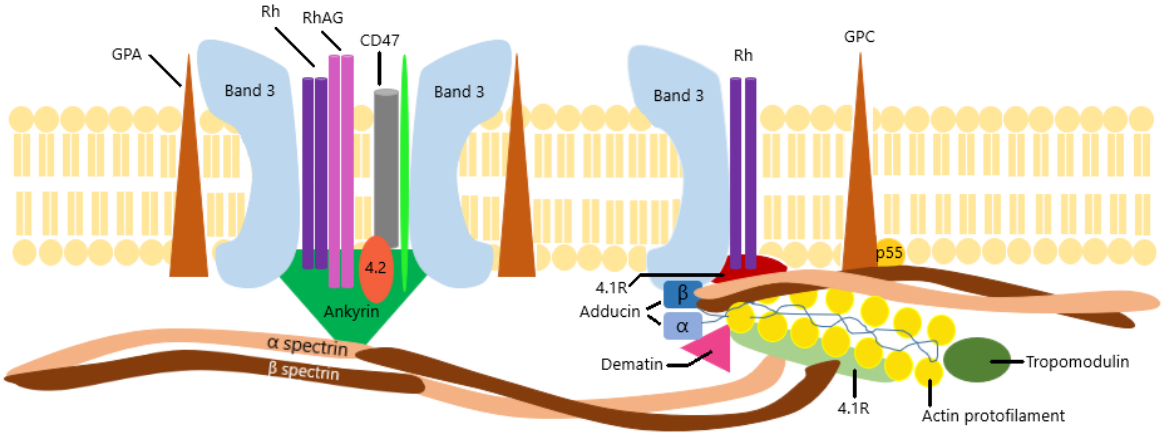

Figure 1: Structure of red cell membrane

Figure 1. Structure of the red cell membrane. Adapted from Postgraduate Haematology, Hoffbrand, V et al.(5)

GPA: Glycophorin A, Rh: Rhesus protein; RhAG: Rhesus associated glycoprotein; GPC: Glycophorin C

Band 3 is coded for by the *SLC4A1* gene on chromosome 17.(8) Each RBC has approximately 1.2 million copies, which is about one-fourth of total membrane protein. Its hydrophilic cytoplasmic domain interacts with proteins like ankyrin, band 4.2, protein 4.1 and adducin. (9) The band 3–protein-4.2-ankyrin-spectrin complex is important, as loss of any part of this complex results in membrane loss, reducing the RBC surface-area-to-volume (SA/V) ratio, leading to the characteristic spherocytes of HS. (5)

The glycophorins (A, B, C, and D) are glycoproteins that constitute approximately 2% of RBC membrane proteins. (10) They cause a strong negative charge on the RBC surface which reduces interactions of RBCs with each other and with the vascular endothelium. Glycophorin A is attached to band 3.(5) Glycophorin C binds to submembrane proteins p55 and protein 4.1 and regulates their abundance. (10)

The Rh polypeptides, the Rh-associated glycoprotein (RhAG), CD47 (the Landsteiner-Wiener glycoprotein) and glycophorin B dimers form the Rhesus (Rh) complex. Interaction of CD47 with protein 4.2 and the Rh polypeptides creates contact with ankyrin. Ankyrin binds to β-spectrin. Protein 4.2 has a role in regulating the interaction between band 3 and ankyrin and binds to both these proteins. (11)

Skeletal proteins

Skeletal proteins are located on the cytoplasmic surface of the lipid bilayer. They attach to the integral membrane proteins, not to the membrane itself, and maintain the structure, elasticity, and mechanical stability of the membrane. (11)

The major components of the membrane cytoskeleton are spectrin, ankyrin, protein 4.2, actin, tropomyosin, tropomodulin, protein 4.1, adducin, and dematin.

Spectrin, the major protein of the membrane skeleton, has two subunits, α and β, encoded by the *SPTA1* gene (chromosome 1) and the *SPTB* gene (chromosome 14), respectively. The amount of beta-spectrin synthesised is the rate-limiting step for the assembly of the spectrin tetramer as alpha-spectrin is synthesised in excess. (4) Therefore, variants affecting a single beta-spectrin allele are sufficient to cause spherocytosis, while variants affecting alpha-spectrin must be present at both alleles to cause HS. (4)

Spectrin is a flexible, rod-like protein with approximately 200,000 copies per RBC. The alpha and beta subunits are intertwined side-to-side to form heterodimers that join to other spectrin molecules by

flexible joining regions. The heterodimers associate head-to-head, forming tetramers approximately 200 nm in length. (12) (Figure 1).

The tail end of the spectrin dimer associates with actin. A junctional complex is formed between beta-spectrin, actin, and protein 4.1. This complex is stabilised by adducin and dematin. The association of spectrin and actin results in a hexagonal network of spectrin tetramers on the cytosolic surface of the membrane. (Figure 2).

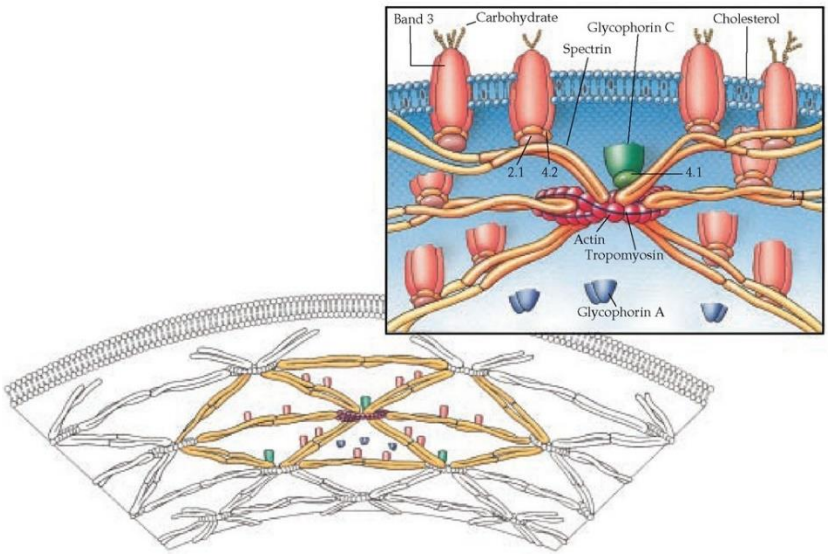

Figure 2. Spectrin-based cytoskeleton on the cytoplasmic side of the human red blood cell membrane. Copied from "What-When-How online tutorials; Hemoglobinopathies and Hemolytic Anemias Part 1."(13)

Pathophysiology of membranopathies

Conditions that interfere with normal membrane deformability, or stability, can reduce the normal SA/V ratio, alter RBC shape, and reduce the lifespan of the RBC.(14)

Lateral connections between spectrin dimers and the spectrin-actin-protein 4.1 complex regulate mechanical stability of the membrane. Weakening of either of these connections reduces membrane mechanical stability and results in cell fragmentation. (15)

Vertical connections between the lipid bilayer and membrane skeleton are essential for membrane cohesion, and weakening of these connections leads to membrane vesiculation and loss of surface area. (16)

To maintain its increased SA/V ratio, the red cell needs to conserve membrane cohesion and mechanical stability throughout its lifespan. (17) Inherited red cell membrane disorders are caused by mutations that result in membrane proteins that are less cohesive or less stable, which in turn results in membrane surface area loss, decreased red cell survival and anaemia. (17)

Hereditary Spherocytosis

Epidemiology

HS is seen in all populations but is more common in those of northern European ancestry. The prevalence in Caucasians is estimated to range from 1:2000 to 1:5000, making it the most common congenital haemolytic anaemia in this population. (4) It is thought to be less common in other

geographical regions such as Africa and Southeast Asia, although comprehensive population survey data are lacking. Most cases (approximately 75%) demonstrate autosomal dominant inheritance; the remaining are recessive. (18)

Aetiology

Membrane loss in hereditary spherocytosis is associated with defects in several membrane proteins. These include deficiencies of spectrin, ankyrin, band 3, protein 4.2 and proteins of the Rh complex. (15) Spectrin deficiency may result from defects in either α-spectrin or β-spectrin genes. (15) The prevalence of β-spectrin defects in northern European populations is about 15-30%. (15) α-Spectrin defects are found in about 5% of HS patients and only have clinical manifestations in the homozygous or compound heterozygous state. (19)

Ankyrin gene (*ANK1*) mutations causing clinical disease can be either dominantly or recessively inherited, and disease can range from mild to severe. Ankyrin has a crucial role in membrane stabilisation as it links β-spectrin to band 3. Ankyrin deficiency results in decreased spectrin assembly even though spectrin synthesis is normal. (15)

Band 3 deficiency occurs in about 33% of patients with HS. Inheritance is autosomal dominant, and disease is usually mild to moderate in severity. The reported range of band 3 mutations are spread throughout the *SLC4A1* gene. (19)

Homozygous mutations in the protein 4.2 gene (*EPB42)* result in a recessive form of HS. It occurs commonly in Japan but is rare in other parts of the world. (15) People with heterozygous mutations are asymptomatic.

Rh deficiency is rare. Rh antigen expression is either reduced (Rh mod) or absent (Rh null). Moderate haemolytic anaemia associated with spherocytes and stomatocytes is seen. (20)

Pathophysiology

Hereditary spherocytosis results from an intrinsic defect of the RBC membrane, and an intact spleen which retains and removes the defective RBCs. (15)

Although HS is caused by a heterogeneous group of molecular defects, all affected erythrocytes have weakening of the vertical linkages between proteins of the membrane skeleton and the integral proteins associated with the lipid bilayer. When vertical interactions are compromised, there is loss of cohesion between the lipid bilayer and membrane skeleton which destabilises the lipid bilayer, resulting in the release of lipid vesicles. These vesicles may contain integral membrane proteins such as band 3 but lack skeletal proteins. (21) Consequently, the membrane surface area is decreased, the SA/V ratio is reduced, and spherocytes are formed. (15)

Spherocytes have reduced cellular deformability and are unable to traverse easily from the splenic cords to the splenic sinuses and become trapped in the red pulp. While trapped in the spleen, abnormal erythrocytes undergo further damage and splenic conditioning resulting in additional loss of surface area and increase in cell density. (21) Retained spherocytes are ultimately removed from circulation. The severity of anaemia corresponds directly with the extent of membrane surface area loss and consequent increase in cell sphericity. (15)

Clinical Presentation

The symptoms and signs of HS include mild pallor, intermittent jaundice, and splenomegaly that is rarely marked. Clinical presentation is highly variable with the commonest forms presenting as mild anaemia and jaundice, with modest splenomegaly. The degree of haemolysis varies widely, from fully compensated to transfusion-dependent anaemia. (2)

Anaemia or hyperbilirubinaemia may be so severe that it requires exchange transfusion in the neonatal period or the disorder may be so mild that it escapes clinical recognition altogether. (22)(15)

Erythropoiesis is highly active before delivery, and splenic function becomes mature only in the postnatal period, so severe anaemia *in utero* is rare. In contrast, during the neonatal period, erythropoiesis enters a phase of decreased activity. Diminished production coupled with increased destruction of erythrocytes by the now mature spleen may result in severe anaemia, developing 5–30 days post-delivery. As compensatory erythropoiesis develops during the first year of life, the anaemia may greatly improve. (5)

Jaundice, rarely intense beyond the neonatal period, is usually intermittent, triggered by stressors such as fatigue, cold exposure, infections, or pregnancy. Undiagnosed adults usually have a very mild form of HS, which remains undetected until exposed to a stressor. (15)(23)

Pigment gallstones due to excess unconjugated bilirubin in bile occasionally occur in young children, but the incidence increases significantly with age. Presence of gallstones may be the first indicator of underlying HS. A family history of HS may be present. Splenectomy or cholecystectomy in a family member before the fourth decade of life is also suggestive. (24)

Management

Severe hyperbilirubinemia in neonates is a risk factor for kernicterus. Treatment is with phototherapy and/or exchange transfusion as clinically indicated. Aplastic crises secondary to an imbalance between red cell production and ongoing destruction of spherocytes, can cause a significant drop in haemoglobin levels and usually requires RBC transfusions. Folic acid is required to sustain erythropoiesis. It is recommended that patients with moderate or severe HS receive lifelong folic acid supplementation to prevent a megaloblastic crisis. (22)(15)

Splenectomy is the definitive treatment for HS. (22) Splenectomy usually eliminates haemolysis and the associated clinical features. (25) While it is clear that splenectomy is indicated in patients with severe anaemia and complications of HS, it can be safely deferred in patients with mild, uncomplicated HS. (25) Affected children in whom splenectomy is indicated, include those with severe disease requiring red cell transfusions and those with moderate disease who have growth failure or symptomatic anaemia. To reduce the risk of infection with encapsulated bacteria, splenectomy should be delayed, if possible, until the age of six years. (25) Partial splenectomy is an alternative approach in which splenic immunologic function is retained while the rate of haemolysis is reduced. (26)

Laboratory diagnosis of HS

The initial laboratory evaluation of patients with a suspected membranopathy includes a full blood count (FBC), reticulocyte count, red cell indices, review of the peripheral blood smear, and screening tests for haemolysis. In terms of red cell indices, a mean corpuscular haemoglobin concentration (MCHC) of ≥ 35 g/dL is consistent with the presence of spherocytes. (27) A high MCHC is more useful than a low mean cell volume (MCV), as MCV is affected by the degree of reticulocytosis. (27) Review of the peripheral blood smear may provide important information, for example, the presence of typical elements such as spherocytes, stomatocytes, ovalocytes, or elliptocytes, and the presence of polychromasia, the degree of which correlates with the degree of reticulocytosis. Spherocytes are dense, round, hyperchromic red cells that lack central pallor. In severe forms of HS, there are large numbers of spherocytes, but in the mild forms, they are fewer, accounting for less than 10-20% of red blood cells. (28) Specific morphological findings are seen in certain membrane protein defects, e.g. mushroom-shaped or pincered cells are suggestive of a band 3 mutation. (15)

The haemolytic screen should include lactate dehydrogenase (LDH) levels, indirect bilirubin and haptoglobin (in patients >3 months of age). A raised LDH and indirect bilirubin with low or absent haptoglobin together with reticulocytosis are consistent with haemolysis. If haemolysis is confirmed, direct antiglobulin testing (DAT) is indicated to exclude immune-mediated haemolysis. The DAT is negative when haemolysis is due to a membranopathy. (24)

Evidence of haemolysis and a negative DAT that cannot be explained by another condition warrants further testing, which may either be screening or confirmatory tests (Table 1).

Table 1: Screening and confirmatory tests for the diagnosis of HS (23)(18)

Screening tests	Confirmatory tests
Autohaemolysis test	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
Osmotic fragility tests	Osmotic gradient ektacytometry
Cryohaemolysis	Molecular analysis of membrane protein genes
Acid glycerol lysis test	
Eosin-5'-maleimide (EMA) binding test	

Screening tests

Autohaemolysis Test

The autohaemolysis test helps distinguish between membrane and enzyme defects. Blood samples are incubated both with and without sterile glucose at 37^0C for 48 hours. After this period, the ability of cells to withstand metabolic deprivation is measured by assessing the amount of haemolysis colourimetrically. If haemolysis is limited, an intrinsic red cell abnormality is unlikely. If the addition of glucose fully corrects abnormal haemolysis, a membrane abnormality is likely, and if abnormal haemolysis does not correct with glucose, a metabolic (enzyme) abnormality is more likely. (29)

Osmotic Fragility Tests (OFTs)

Osmotically fragile cells easily haemolyse in hypotonic solutions. Spherocytes, which have decreased ability to increase in volume due to their reduced surface area to volume ratio, cannot absorb hypotonic solutions. As a result, they haemolyse in higher concentrations of sodium chloride (NaCl) than normal biconcave RBCs. (2)

In the first OFT used in routine diagnostics, RBCs were exposed to serial solutions of saline at concentrations ranging from 0.1% to 0.9% NaCl. The degree of haemolysis was determined by using a spectrophotometer to measure the haemoglobin concentration in the solution. (29) The disadvantage of this test is a lack of specificity as false positives are observed with immune-mediated haemolytic anaemia, recent blood transfusions, RBC enzyme deficiencies, and unstable haemoglobin variants. (23) It was found that incubating the blood at 37^0C improved the sensitivity of the test. (30)(31)

Won et al. reported osmotic fragility testing based on flow cytometric assessment in 2009. (32) In their method, the vulnerability of RBCs to haemolysis is quantified, and a precise numerical value representing osmotic fragility is generated. The assay principle involves measurement of the RBC count in a 0.9% NaCl solution before and after the addition of water. The result of the test is determined from the ratio of the red cell count during the acquisition period (30 seconds) before the addition of water and the mean red cell count of two time periods (gates) after the addition of water (corrected for the dilution factor). (32)

Acidified glycerol lysis test (AGLT)

Acidified glycerol lysis test is a modification of the original glycerol lysis test (GLT). It determines the degree of lysis of RBCs suspended in a buffered glycerol solution. The original GLT measures the time taken for 50% haemolysis of a blood sample in a buffered hypotonic saline/glycerol solution. Glycerol hampers the entry of water into red cells, causing a prolonged lysis time. In the AGLT, the addition of sodium phosphate lowers the pH of the buffered solution to 6.85 which improves the sensitivity and the specificity of this test for HS. (29) Despite satisfactory sensitivity of ~95%, the AGLT test is time-consuming and has limited specificity as it can also give positive results in acquired spherocytosis, chronic renal failure, pregnancy, and myelodysplastic syndrome. (2)(23) The pink test is a modification of the AGLT, in which red cells obtained by heel/finger prick are added to a solution of acidic hypotonic glycerol with a slightly lower pH and assessed for haemolysis, which if present, imparts a pink colour. (29)

Cryohaemolysis test (CHT)

The observation by Streichman et al. (33) that spherocytes are "specifically susceptible to temperature changes while suspended in hypertonic solutions" is the basis of the CHT. When red cells are cooled from 37^0C to 7^0C, while suspended in a hypertonic medium, they undergo massive haemolysis. Under these conditions, the lipid bilayer undergoes a transition from a fluid to a gel phase, but under hypertonic conditions, the membrane cannot withstand the mechanical changes associated with the lipid phase transition and cell shrinkage and membrane rupture ensues. The degree of lysis is determined by spectrophotometrically measuring the amount of free haemoglobin released during cell lysis (>20% lysis is considered positive*).* (29) Because the principle of the test is haemolysis due to loss of integrity of membrane proteins instead of an unfavourable volume-to-surface area, it is reportedly more specific for HS.(34) The benefit of this test is, however, controversial as several studies report significantly different specificities and sensitivities. (34)(35)(36)

Eosin-5-maleimide (EMA-binding) test

EMA dye is a fluorescent dye that binds to Lys-430 (the 430^{th} lysine residue) in the extracellular loop of the integral membrane protein, band 3. It also binds to CD47 and RhAG. (37) The fluorescence of the dye can be detected on a flow cytometer equipped with an argon laser, and the fluorescence intensity is reduced in band 3 deficiency. Fluorescence intensity is also decreased with defects of cytoskeletal proteins such as spectrin and protein 4.2, most likely a result of the long-range modulation effect created by these defective proteins on the dye-binding site in the band 3 protein. (37) When the patient's RBCs demonstrate fluorescence approximately 85% or less than that of control samples, HS is highly probable. (38)

The major advantages of this test are the small volume of blood required (5 µL) and acceptable sample storage time (up to 7 days under refrigeration). (39)(40) Other advantages include that this test has a high sensitivity, a relatively short turn-around-time, is cost-effective and, can be performed in laboratories that are equipped with even a basic flow cytometer. (38) Additionally, the results of the EMA-binding test are not affected by blood transfusion. (41)

The disadvantage of this test is that the reporting of results varies among different laboratories. In some centres, the results are expressed as a percentage of the decrease in EMA fluorescence compared to control RBCs, (42) while others express results as the mean fluorescence intensity compared to a reference range (43) or an EMA "footprint" (44) established for their laboratory. (43)

Confirmatory tests

Plasma membrane protein electrophoresis

Although the screening tests described above have high sensitivity and specificity for the detection of HS, they do not provide information about which plasma membrane protein is deficient or where the genetic mutation has occurred. For detection of the plasma membrane protein abnormality,

electrophoresis of RBCs is performed. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) provides qualitative and quantitative information regarding abnormal plasma membrane protein content. (2)

Among the disadvantages of SDS-PAGE are the large volume of sample required for analysis, that the technique is only available in specialised diagnostic centres and, interpretation requires expertise. It is also labour intensive and time-consuming as preparation of RBC ghosts and removal of haemoglobin from plasma membranes is necessary. (2)

Osmotic gradient ektacytometry test

Ektacytometry is a technique that measures the deformability of cells at constant shear stress but with gradually changing osmolality. The results depend on the SA/V ratio and viscosity of the cell membrane. (45) A next-generation osmotic gradient ektacytometry (NG-OGE) assay has allowed the test to be adapted from a primary research method into a clinical diagnostic tool. (46) Lazarova et al. (47) found that the NG-OGE can be used to distinguish between HS and other inherited anaemias. It cannot, however, differentiate between HS and autoimmune haemolytic anaemia. (47) While it has been suggested that NG-OGE can be utilised as an intermediate diagnostic step between screening tests and advanced confirmatory tests, its use is not yet popular. (23)(47)

Molecular diagnostics

Genetic mutations within genes that encode membrane proteins, the deficiency of which leads to HS, can be identified with gene sequencing techniques. (23) This diagnostic method is the most advanced and most expensive and, is reserved as a last option for subjects who present with symptoms, but in whom a direct diagnosis cannot be made using the previously mentioned methods. (23)

Comparison of available screening tests

Several studies have compared the different screening methods, both individually and in various combinations. Concerning the NaCl OFTs, Arora et al. (31) showed that OFT performed on samples incubated at 37^0C for 24 hours had a higher sensitivity (79.3%) than fresh samples at room temperature OFT (62.1%). (31) However, the two tests had similar specificities (83.6% versus 87.7%). The relatively low sensitivity of NaCl OFTs was also reported by Bianchi et al. (2) who found that the sensitivity of these tests was further reduced in cases of compensated HS. Therefore, despite the incubated OFT traditionally being the test of choice for diagnosing HS in patients with DAT negative haemolytic anaemias, (15) it has limited utility in mild and atypical cases. (2)

The AGLT test has been shown to have high sensitivity (~95%). (2) Mariani et al. (48) demonstrated that the sensitivity of this method was greater than that of the EMA-binding test. This study also showed that the combination of the AGLT and incubated OFT yielded a sensitivity of 99%. (48) Bianchi et al. (2) found similar sensitivity (97%) with this combination. In their study, the association of AGLT and EMA-binding identified all cases of HS. (2) The sensitivity also remained high in compensated cases. (2) However, as mentioned previously, this method is time-consuming and has limited specificity. (2)(23)

The flow cytometric osmotic fragility test is reported to be sensitive and specific for detecting HS. Sensitivities in different case series range from 85.7% to 100%.(49)(50)(51)(52). A specificity of 97.24 % was reported in a 2016 study involving 237 subjects. (52) Arora et al. (31) found that the combination of FOFT and EMA-binding test identified all cases of HS with 100% sensitivity and 94.5% specificity. (31)

Due to the discrepancy in sensitivity and specificity reported in different studies, (34)(35)(50) the utility of the CHT in the diagnosis of HS is controversial. Streichman and Gescheidt (34) reported that the CHT had a 90% specificity and 100% sensitivity. These sensitivities and specificities were correlated in a prospective study by Crisp et al. (35) On the contrary, Mariani et al. (48) found a much lower sensitivity of 53%, but in their study, CHT was performed on just 33 of 300 HS patients. Park et al. (50) found the CHT to be inferior to EMA-binding and FOFT, and reported that it was unable to discriminate between

HS and iron deficiency anaemia. (50) Crisp et al. (35) reported a sensitivity of 93.5% when a combination of CHT and the EMA-binding test were performed. (35) This is similar to the sensitivity of EMA-binding or AGLT when used alone and lower than EMA-binding in combination with AGLT (2) or EMA-binding in combination with FOFT. (31)

The sensitivity of the EMA-binding assay for detecting hereditary spherocytosis in reports ranges from 86.2% to 97% with specificities of 93-100%. (2)(41)(31)(53)(42) Sensitivity was found to be independent of the type of molecular defect and the clinical phenotype. (2) Furthermore, the sensitivity of the EMA-binding tests does not vary with clinical phenotype as even cases with compensated anaemia were identified. (2) When EMA-binding was performed in combination with either FOFT or AGLT, all cases of HS were identified. (2)(31) Lower sensitivity of 93.5% was obtained when it was performed in combination with the CHT. (35)

The rationale for this study

In South Africa, there are no centres that currently offer confirmatory tests. We, therefore, rely on the clinical presentation and screening tests for diagnosis**.** The Universitas Academic Hospital National Health Laboratory Service (NHLS) service laboratory currently offers two screening tests for suspected hereditary spherocytosis: the FOFT and the CHT. FOFT is reported to have a higher sensitivity when compared to CHT. (36) However, recently we have had several cases where there was a high clinical suspicion of HS with a positive CHT, but a negative FOFT screen. The sensitivity of the tests varies greatly, and each method fails to identify all cases of HS.

Since the EMA-binding test has been shown to have a relatively high sensitivity and specificity in diagnosing HS (which is further increased when combined with FOFT) and, as our laboratory is equipped with a flow cytometer, this study aimed to determine if this test would add value to our current diagnostic platform. Additionally, we aimed to determine which test or combination of tests yielded the most reliable results. This will allow us, in the absence of confirmatory tests, to provide the best screening for patients with suspected HS.

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Chapter 2: Publishable Manuscript

Evaluation of the eosin-5-maleimide flow cytometric test and other screening tests in the diagnosis of hereditary spherocytosis

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Abstract

Background: Hereditary spherocytosis (HS) is a genetically determined haemolytic anaemia characterised by the spherical shape of affected red blood cells. With no limited confirmatory tests currently available in South Africa, the diagnosis of HS is reliant on the clinical presentation and screening tests.

Objectives: The aim of this study was to compare the sensitivity and specificity of three screening tests: flow osmotic fragility test (FOFT), cryohaemolysis test (CHT) and the eosin-5-maleimide binding test (EMA-binding test) in diagnosing hereditary spherocytosis (HS).

Method: All three tests were performed on 18 subjects with confirmed HS. The negative control group was comprised of 10 subjects with haemolysis and spherocytosis, and either a positive direct antiglobulin test (DAT) or normal red cell membrane studies. The tests were also performed on samples submitted for cases of suspected HS during the study period.

Results: The EMA-binding test demonstrated superior sensitivity (88.9%) compared to the CHT (61.1%) and the FOFT (38.8%). The EMA-binding test specificity (90.0%) was equal to that of the FOFT and superior to the CHT (50%). Combined sensitivities and specificities for EMA-binding test and CHT, EMA-binding test and FOFT, and CHT and FOFT, were 100% and 33.3%, 94.4% and 80.0% and 88.9% and 50.0%, respectively.

Conclusion: EMA-binding test is the best screening test for cases of suspected HS. If there is a high clinical index of suspicion with a negative EMA-binding test, the CHT is recommended as a second screening test.

Key words: Hereditary spherocytosis, Eosin-5-maleimide, Flow osmotic fragility, Cryohaemolysis, Screening test, Red cell membrane, South Africa

Introduction

Hereditary spherocytosis (HS) is a genetically determined haemolytic anaemia characterised by the spherical shape of the affected red blood cells (RBCs). (1) HS occurs in all racial and ethnic groups but is particularly common in individuals of Northern European descent. (2) With an estimated prevalence ranging from 1:2000 to 1:5000, it is the most common congenital haemolytic anaemia in Caucasians. (3)

HS is caused by a myriad of molecular defects which affect genes encoding proteins involved in vertical connections of the RBC membrane cytoskeleton to the lipid bilayer. (4) Disruption of these vertical linkages result in destabilisation of the lipid bilayer and release of skeleton-free lipid vesicles (5) This loss in membrane surface area relative to the intracellular volume, is responsible for the spheroidal shape of RBCs. (2) Spherocytes are less deformable and thus, are selectively retained in the spleen where they undergo splenic conditioning and premature removal from circulation. (2)

Clinical presentation of HS depends on the severity of haemolysis, which varies from fully compensated anaemia to transfusion dependence. (4) Signs and symptoms include pallor, jaundice, pigment gallstones and splenomegaly, which is rarely marked. In neonates, jaundice may be the most important sign as splenomegaly is often absent. (6) Neonates with severe hyperbilirubinemia are at risk for kernicterus and should be treated with phototherapy and exchange transfusion as clinically indicated. Management of HS depends on disease severity. Mild cases may be managed only with folate supplementation, while severe forms may require recurrent transfusions and splenectomy. (7)

Currently, in South Africa, there are no confirmatory tests for the diagnosis of HS, so we rely on the clinical presentation and screening tests for diagnosis. The Universitas Academic Hospital (UAH) laboratory offers two screening tests for the investigation of suspected HS: the flow osmotic fragility test (FOFT) and the cryohaemolysis test (CHT). With FOFT, RBCs are subjected to a hypotonic solution which causes exaggerated haemolysis in osmotically fragile cells. Following incubation with distilled water, the proportion of residual RBCs is measured by a flow cytometer. (8) The CHT is based on the observation that red cells in HS are particularly susceptible to lysis when rapidly cooled in hypertonic conditions. (4) While the literature suggests that FOFT has a higher sensitivity when compared to CHT (9), we have had several cases where there was a high clinical suspicion of HS with a positive CHT but a negative FOFT screen. Given the discrepancy between the literature and our experience, there was a need to evaluate the sensitivity and specificity of these tests in our setting.

We also explored the possibility of introducing a third screening test; the eosin-5-maleimide flow cytometric test (EMA-binding test) in an effort to improve our screening platform. EMA is a fluorescent dye that binds to Lys-430 in the extracellular loop of band 3 protein. (10) Its fluorescence, detected by a flow cytometer, corresponds to the band 3 content on the surface of RBCs. A decrease in fluorescence is observed when there is a deficiency of band 3.

This study aimed to evaluate the EMA-binding test as a screening test, to compare the EMA-binding test to the other two screening tests currently performed in our laboratory, and to determine which test/combination of tests give the greatest sensitivity and specificity, which in the absence of a confirmatory test, can be reliably used to diagnose HS.

Methods

Study Design

This was an observational descriptive study conducted at the National Health Laboratory Service (NHLS) Universitas Academic Hospital (UAH) service laboratory from 07/11/2019 to 31/03/2020.

Sample / Study Participants

Sample size

Blood samples from eighteen known HS patients served as our positive controls. For each confirmed positive sample, we tested six normal controls concurrently. Our test subjects included the samples of five patients with suspected HS as well as two asymptomatic family members of patients with confirmed HS. Nine samples of patients with direct antiglobulin test (DAT) positive autoimmune haemolytic anaemia (AIHA) and one patient with spherocytosis, a negative DAT test but normal red cell membrane studies, served as negative controls. The negative DAT despite normal membrane studies in the one participant may be due to RBC-bound immunoglobulin below the limit of routine DAT, RBC-bound IgA, or low-affinity autoantibodies.(11)

Sample acquisition / Data collection

Positive control samples were obtained from patients with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) confirmed HS, known to the UAH Haematology and Paediatric Oncology clinics.

Normal control samples were randomly selected from ethylenediamine tetra-acetic acid (EDTA) specimens submitted for routine full blood count analysis at the NHLS UAH service laboratory. FBCs were checked and samples were included as normal controls if Hb, MCV and MCHC values were within normal range. Test subjects included all samples submitted to our laboratory for the investigation of suspected HS during the study period. DAT positive samples and a single DAT negative sample with negative membrane served as negative controls.

Inclusion criteria

All samples that were submitted to the NHLS UAH service laboratory for HS testing during the study period.

Exclusion criteria

Samples that were haemolysed, incorrectly labelled, collected in the incorrect specimen bottle or exceeded the acceptable time from collection to testing were excluded. (12)

Measurement

All laboratory tests were performed by the technical heads of the Immunology and Flow cytometry benches of the NHLS UAH service laboratory, with assistance from the first author.

A full blood count (FBC) and microscopy, DAT, and the three screening tests for HS (EMA-binding, CHT and FOFT) were performed on all test subjects. For controls with confirmed HS, the DAT was omitted.

Full blood count and microscopy

The full blood count was performed on the Advia® 20120i (Siemens Heathineers, Erlangen, Germany), and microscopic evaluation of the peripheral blood smear was performed by the first author.

Direct antiglobulin test (DAT)/ direct Coombs test

A drop of red cell suspension, prepared with the patient's washed red cells and saline, was mixed with two drops of Bioscot ^{R} (EMD Millipore, Burlington Massachusetts, United States) polyspecific anti-human globulin (AHG), centrifuged and then examined both macroscopically and microscopically for

agglutination. If the broad spectrum AHG was positive, further testing was performed to determine the type of antibody present.

Cryohaemolysis Test (CHT)

CHT testing was performed according to the method described by Streicman et al. (13) in which washed packed red cells, added to a hypertonic solution (2 mL of 0.7 m sucrose/phosphate buffer), were kept in the water bath at 37^0C for 10 minutes and then transferred to an ice bucket (0^0C) for another 10 minutes. The degree of lysis following rapid cooling was determined by measuring the free haemoglobin spectrophotometrically at 540 nm. A normal control sample (blood from a healthy donor), negative parallel control sample (kept at 37^0C for 20 minutes with no cooling stage) and a 100% lysis tube (prepared by adding 2 mL distilled water to 50 µL packed red cells) were included to improve interpretation of results.

- The mean value of the two duplicate optical density (OD) readings of the patient sample, normal control and parallel control were determined.
- The % lysis of each sample or control was determined as follows:

$$\% \text{ Lysis} = \frac{\text{OD of test (x)}}{(\text{OD of 100\% Lysis}) \times 6} \times 100$$

- ≥20% lysis was considered to be a positive result

Eosin-5-maleimide flow cytometric test (EMA-binding test)

EDTA blood samples from positive controls and test subjects were kept at 2-8^0C and tested within 24 hours of collection.

The normal control group consisted of randomly selected EDTA blood samples of the day. In each assay, patient samples were set up with six normal controls. RBC labelling with EMA (from Thermo Fisher™, Waltham Massachusetts, US) and flow cytometric testing on the Facs Canto™ II (Becton, Dickinson, and Company, Franklin Lakes, New Jersey), using FacsDiva™ acquisition and gating software, version 8 (Becton, Dickinson, and Company, Franklin Lakes, New Jersey), were carried out according to the procedure previously described by King et al. (14) Results were expressed as the percentage reduction of fluorescence of the patient to the mean fluorescence of the six controls (14)

$$\text{Decrease in fluorescence} = \frac{\{(\text{mean fluorescence of the six controls}) - (\text{fluorescence of the patient})\}}{\text{Mean fluorescence of the six controls}} \times 100$$

Decrease in fluorescence < 15.84% = Normal
Decrease in fluorescence ≥ 15.84% = Increased (positive)

.

Flow cytometric osmotic fragility test (FOFT)

The presence of spherocytes due to HS are detected by comparing the percentage red cells in the red cell/saline suspension acquired on the BD FACS Canto™ II flow cytometer (using FacsDiva™ acquisition and gating software, version 8) before and after the addition of distilled water. A normal control was run concurrently with each test sample. The method used was that described by Won and Suh**.** (15) and the formula for calculating percentage residual red cells was that used by Park et al. (16). A residual red blood cell percentage of <23% was considered positive for HS.

Data Analysis

Basic descriptive statistics including the sensitivity, specificity and predictive values for each assay were used to summarise the data. Receiver operator characteristic (ROC) and logistic regression curves were used to determine the optimal cut-off values for the EMA-binding test. For the combination of tests, parallel testing was performed. If one of the tests in a combination was positive, the result was considered to be positive, while both tests had to be negative for the result to be regarded as negative.

Other parameters evaluated included participant age and gender, the type of membrane defect, whether the participants had received any recent blood transfusions or had undergone splenectomy, the haemoglobin levels of participants, the mean corpuscular volumes (MCV) and the mean corpuscular haemoglobin concentrations (MCHC) of the positive and negative controls.

Results

Our findings are presented in the tables and figures that follow. Table 1 summarises the laboratory data and test results for the positive control, the test- and the negative control groups. Table 2 compares the sensitivity, specificity, and predictive values of each test and combinations of tests. Figures 1-3 illustrates the typical pattern of confirmed HS samples for each of the screening tests performed. Figures 4 and 5 are graphs used to determine the optimal cut-off value for % mean florescence intensity (MFI) decrease for the EMA-binding test in our laboratory.

Table 1: Laboratory Data and Test Results of the three Participant Groups

Participant Group	Positive Control(n=18)	Negative Control(n=10)*	Test (n=7)
Haemoglobin (g/dL)			
Range	6.8-18.9	4-15.7	6.2-15.7
Median	15.5	11.4	11.7
Mean	15.2	10.79	11.45
Mean corpuscular volume (fL)			
Range	65.6-98.9	80.7- 122.6	72.0-91.7
Median	89.9	100.1	84.45
Mean	87.3	98.62	82.4
Mean corpuscular haemoglobin concentration (g/dL)			
Range	29.7-36.6	27-33.8	33.6-37.2
Median	35.2	30.9	34.1
Mean	34.9	30.81	34.55
FOFT			
Number of positive cases	7	1	2
Number of negative cases	11	9	4
Range (% of residual red cells)	0.92-75.8	4.3-94.8	7.43-77.7
Median (% of residual red cells)	49.9	50.63	28.3
EMA-binding test			
Number of positive cases	16	1	4
Number of negative cases	2	9	2
Range (% decrease in MFI)	13.1-35.9	-1.35-31.79	4.31 – 48.31
Median (% decrease in MFI)	30.1	7.89	23.53
CHT			
Number of positive cases	11	3 (3 of 6*)	4

Number of negative cases	9	3 (3 of 6*)	2
Range (% red cell lysis)	4.1-33.9	2.9-35.7	13.7-31.8
Median (% red cell lysis)	22.4	19.3	24.05

*Sample size for the negative control group for CHT was 6 instead of 10

Table 2: Sensitivity, specificity, predictive values of each test

Test	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
EMA-binding test	88.9%	90%	94.1%	81.9%
FOFT	38.8%	90%	87.5%	45%
CHT	61.1%	50%	78.6%	30%
EMA + FOFT	94.4%	80%	89.4%	88.9%
EMA + CHT	100%	33.3%	81.9%	100%
CHT + FOFT	88.9%	50.0%	84.2%	60%

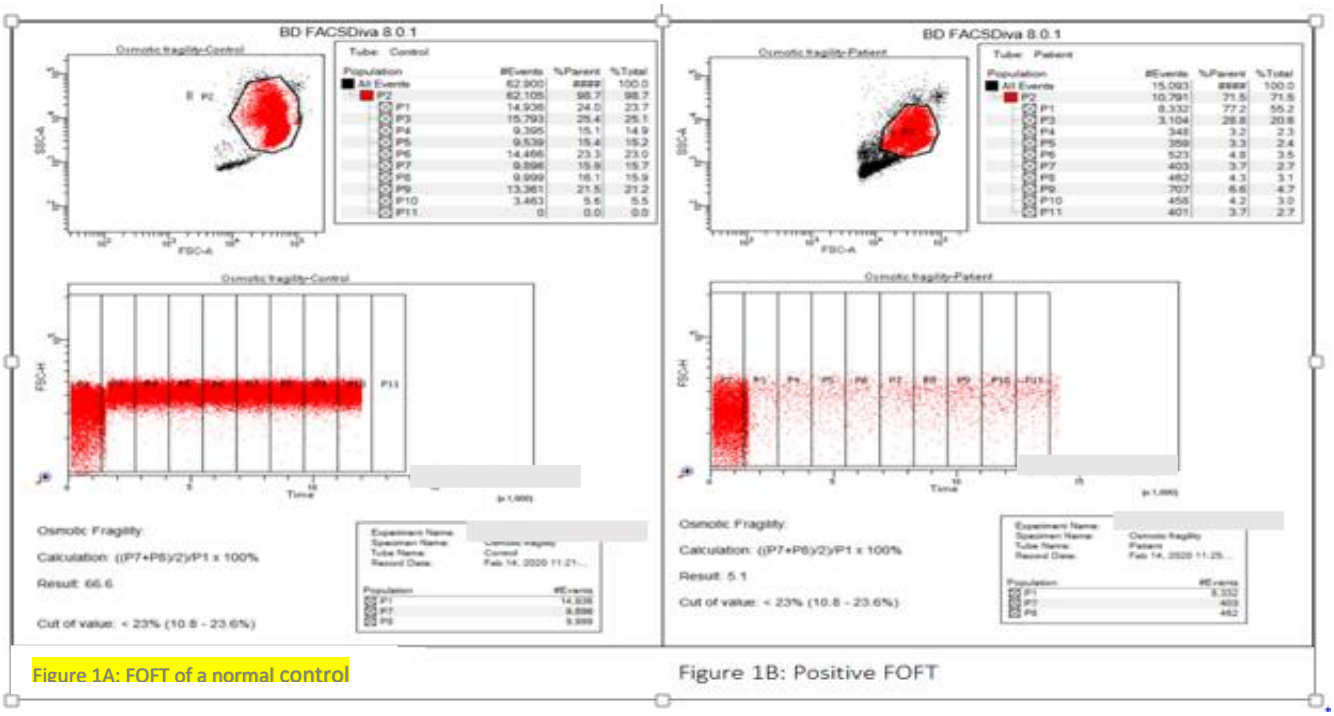

Figure 1A: FOFT of a normal control

Figure 1B: Positive FOFT

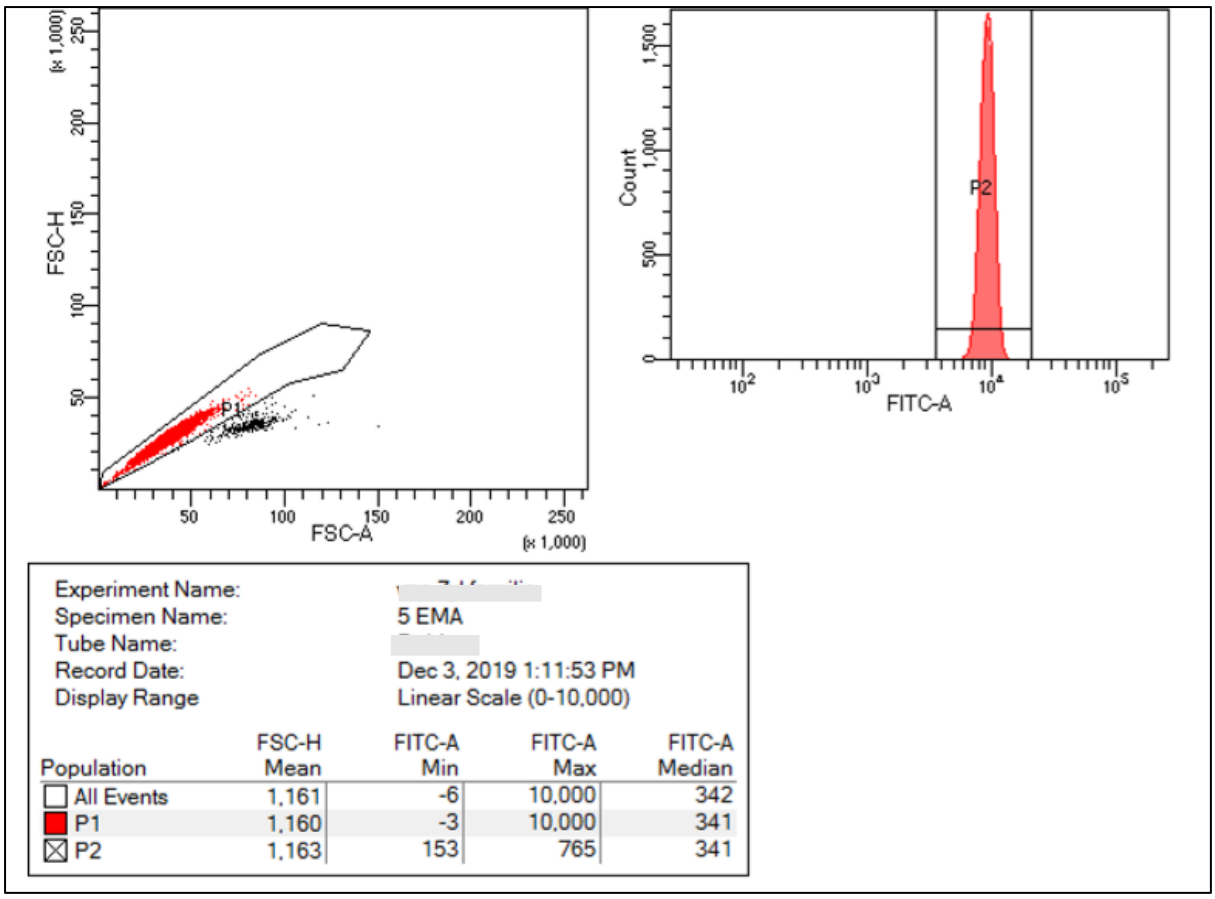

Figure 2a: Example of positive EMA-binding test graph (acquisition and gating performed on FacsDiva™ software version 8.0)

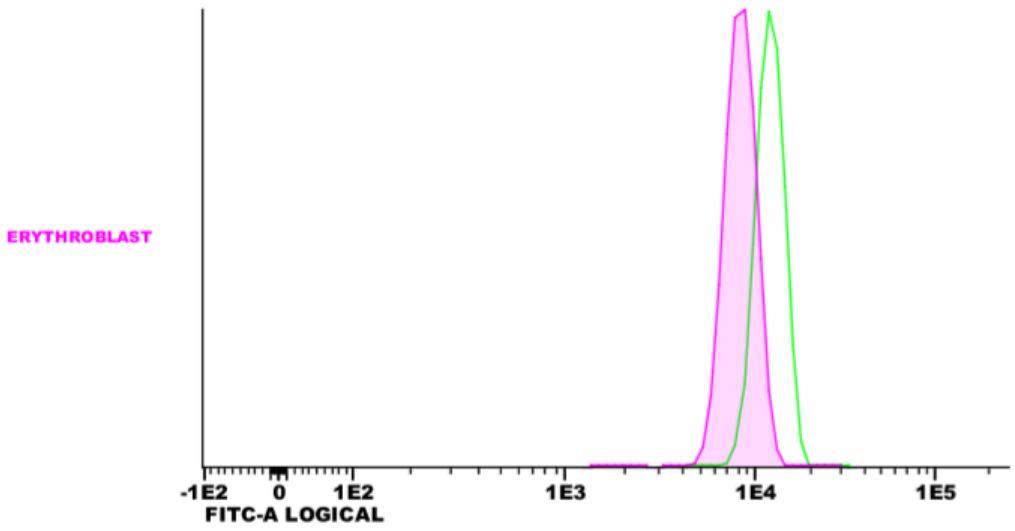

Figure 2b: Difference in MFI of a positive patient (purple) and normal control (green) [Graph generated using Infinicyt™ v1.7 software (Cytognos, Santa Marta de Tormes, Salamanca, Spain)]

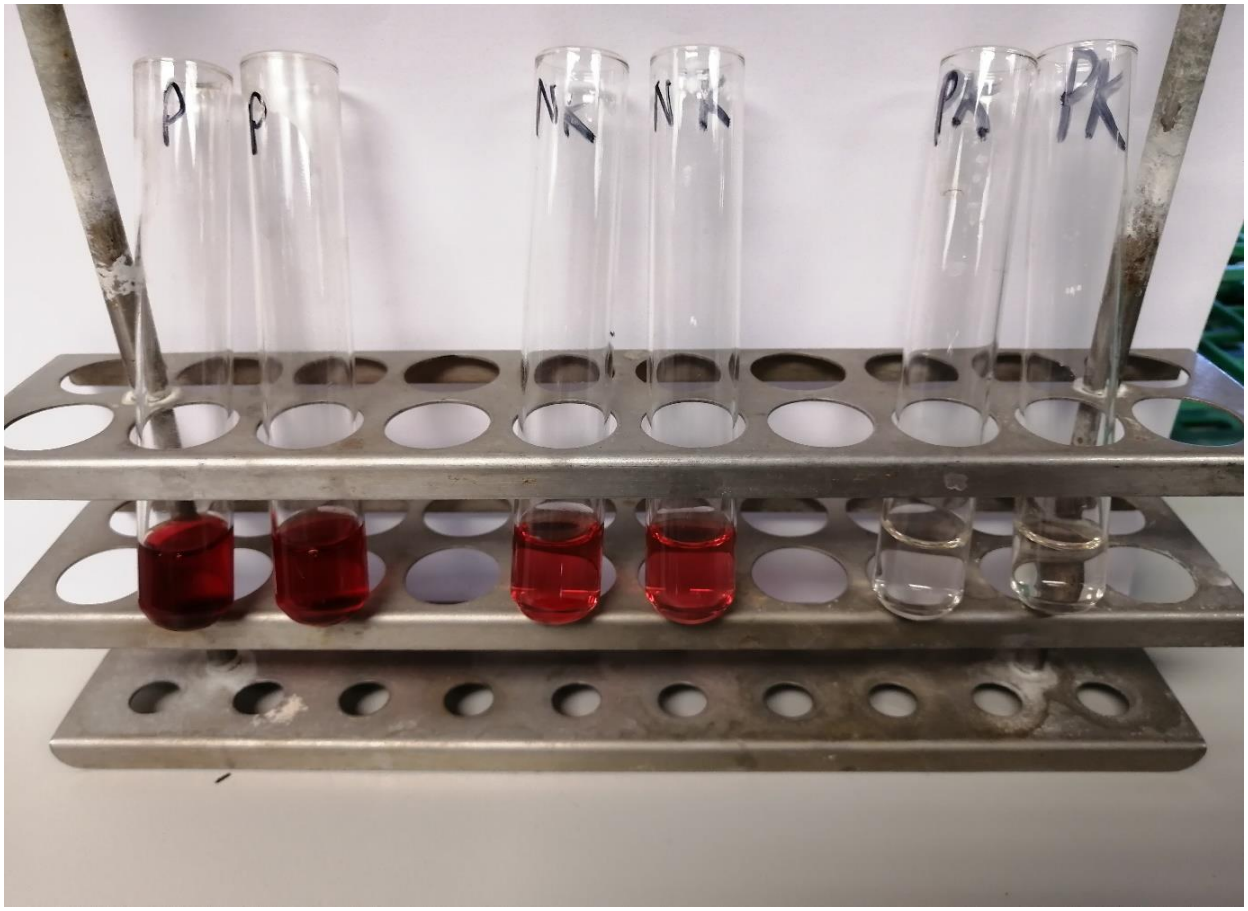

Figure 3: Image of a positive CHT (P= patient, NK= negative control, PK= parallel control)

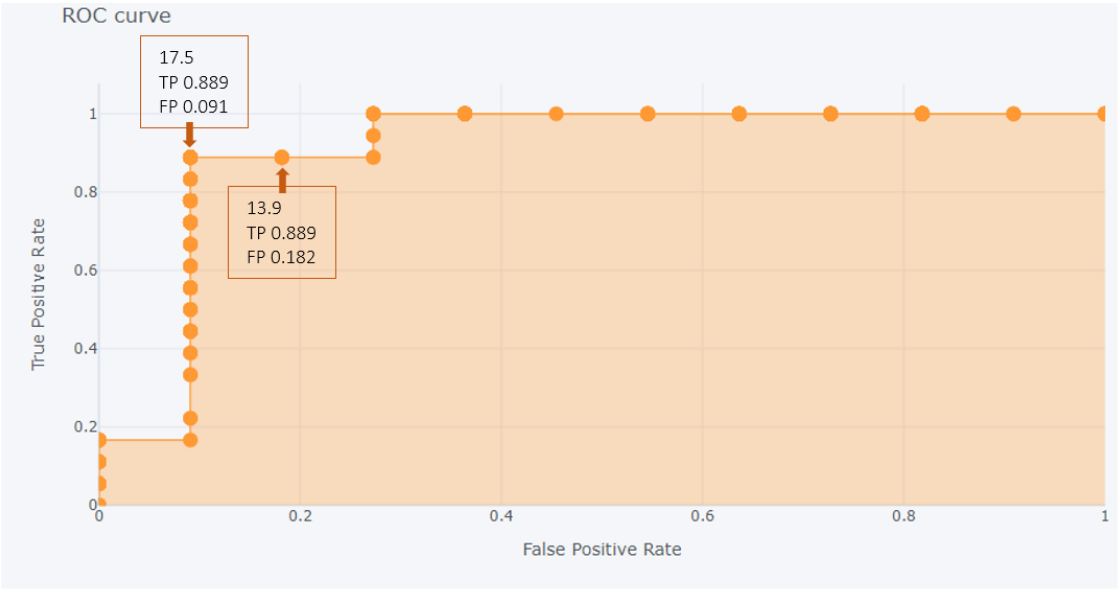

Figure 4: ROC curve analysis showing that a %MFI decrease threshold set to 17.5 has a true positive (TP) rate of 0.889 and a false positive (FP) rate of 0.091 while a threshold set to 13.9 has a TP rate of 0.889 and an FP rate of 0.182.

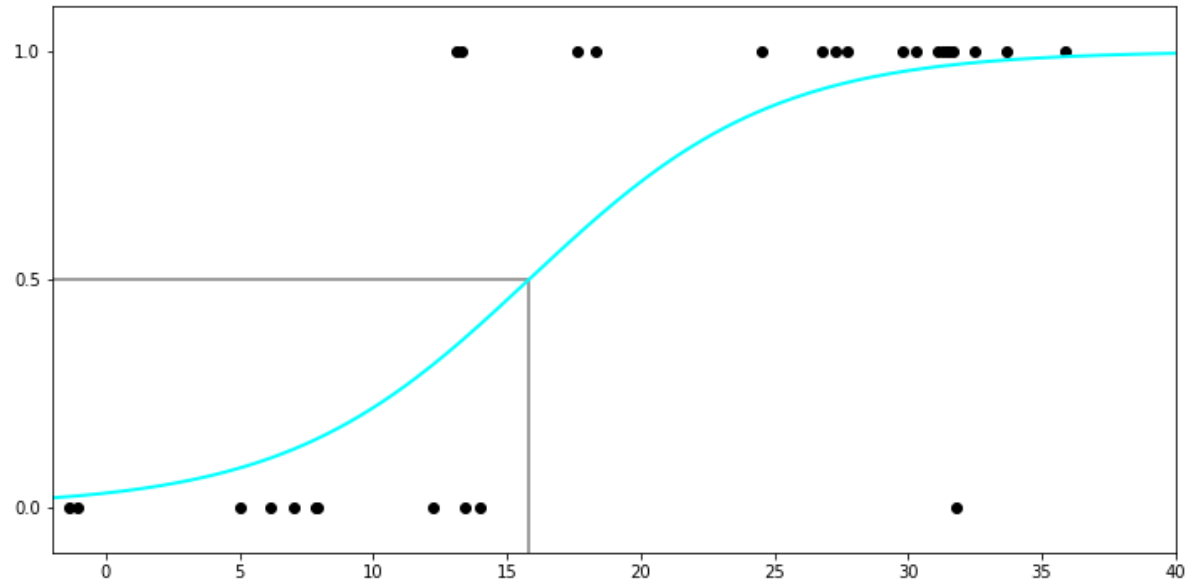

Figure 5: Logistic regression analysis indicating that %MFI decrease threshold of 15.84 best separates positive and negative samples

Positive control group:

There were 18 participants in the positive control group ranging in age from 12 months to 74 years with a male: female ratio of 5:4. Fifteen participants were splenectomised. One had received a blood transfusion within eight weeks of testing. A variety of confirmed membrane defects were present among the participants, the most common being beta spectrin deficiency (in 9 participants), followed by band 3 deficiency (in 5 participants). Alpha spectrin deficiency was identified in one participant, protein 4.2 deficiency in one participant, and combined spectrin and ankyrin deficiency in 2 participants.

The EMA-binding test identified 16 of the 18 samples as positive and two as negative. Of the two false negative samples, one had a beta spectrin deficiency while the other had a combined spectrin/ankyrin deficiency. The overall sensitivity of the test was 88.9%. The FOFT was positive in only seven samples with a sensitivity of 38.8%. The CHT generated a positive result for 11 of the samples, with 7 negative results (Table 1 and Table 2). The overall sensitivity of this test was 61.1%. The combination of the EMA-binding test and FOFT correctly identified 17 samples as positive, the combination of EMA-binding test and CHT identified all positive cases, and the combination of FOFT and CHT identified 16 as positive with sensitivities of 94.4%, 100% and 89.9% respectively (Table 2). Important to note, the two cases that were negative with the EMA-binding test were correctly identified by the CHT.

Negative control group

The negative control group was comprised of ten subjects, all of whom had features of haemolysis and spherocytes on peripheral blood. Nine of the samples had a positive DAT while one had a negative DAT but normal red cell membrane studies. The EMA-binding test and FOFT were performed on all ten participants in the negative control group; however, the CHT was only performed on six of the ten participants as four of the samples were insufficient for further testing. The EMA-binding test and FOFT were negative in 9 of the samples with a specificity of 90% while CHT was negative in 3 out of 6 samples resulting in a specificity of 50% (Tables 1 and 2). The combination of EMA-binding test and FOFT correctly identified eight out of the 10 participants as negative, while the combination of the EMA-binding test and CHT only identified two of six negative samples correctly resulting in

specificities of 80% and 33.3% respectively. The combination of FOFT and CHT correctly identified three of six negative samples with 50% specificity (Table 2).

Test group

The test group was comprised of four subjects with clinical and peripheral blood features suggestive of HS and two asymptomatic family members of known HS patients (Table 1). Of the four patients with clinical suspicion of HS, all had positive EMA-binding test and CHT results while two also had positive FOFT. Asymptomatic family members had normal full blood counts, without features of haemolysis on the peripheral smear. They were both negative for all three screening tests.

Cut-off values for the EMA-binding test

To determine the optimal cut-off values between HS and non-HS individuals, ROC curve analysis and logistic regression analysis were performed using % decrease in MFI obtained from the true positive group and the true negative groups (Figures 4 and 5). The ROC curve demonstrated that a cut off value between 13.9% and 17.5% was the best compromise between sensitivity and specificity. Logistic regression analysis showed that a % decrease in MFI threshold of 15.84% best separates positive and negative samples. We decided to implement this cut-off value when interpreting results of the EMA-binding test in our laboratory as this intermediate value gives equal weight to sensitivity and specificity. This value is within the range used in other studies where the cut-off value for % decrease in MFI ranges from 11-21%. (4)(17)(18)(18)(9) Some studies mention a "grey area" where there is an overlap of results with HS and non-HS cases. (19)(14) If we were to define that area in this study, it would be a % MFI decrease of between 13.1 and 15.84% which would include 2 participants from the true positive group and two from the true negative group. For the purpose of this study, values in this area were considered to be negative, however in practice, it is advisable that such borderline results be further investigated with family studies, another screening test or a confirmatory test if available. (19)

Discussion

In this study, we compared the performance of the three screening tests for HS, individually and in combination. Two of the three tests, the FOFT and the CHT were already being used at our centre while a third, the EMA-binding test was being considered for implementation.

Since first described in 2000 by King et al. (14), the EMA-binding test has become the screening test of choice in many specialised laboratories due to its high sensitivity and specificity. (4) In this method, the fluorescent probe, eosin-5-maleimide binds to transmembrane proteins band 3, CD47 and proteins of the Rh complex, which are reduced in erythrocytes of patients with HS. (10) Fluorescence intensity is also decreased with defects of cytoskeletal proteins such as spectrin and protein 4.2, most likely a result of the long-range modulation effect created by these defective proteins on the dye-binding site in the band 3 protein. (10)

The results of this study showed the EMA-binding test to have the highest sensitivity of the three tests evaluated and specificity that is equal to that of the FOFT and superior to the CHT. The sensitivity of the test in this study is similar to that reported in the literature where it ranges from 86.2% to 97.0%. (4)(14)(9)(18)

Reported specificity of the EMA-binding test ranges between 93 and 100%. (18)(9)(4)(14) We recorded a slightly lower specificity which may be attributed to our small sample size.

Ciepiela et al. (20) found that MCV affects the MFI of samples such that higher MCV results in increased MFI, while the converse is true for lower MCV values. Some authors even recommend using control samples with MCV within ±2 fL of patient RBCs to avoid false-negative results in cases of macrocytosis (e.g. newborns) and false-positive results in cases of microcytosis (e.g. coexistence of

iron deficiency anaemia). (21) We did not use MCV-matched control samples which may be one of the reasons for the lower the sensitivity and specificity observed in this study.

In agreement with other studies, the sensitivity of the EMA-binding test was not influenced by the type of molecular defect. (4) Although King et al. (14) reported the EMA-binding test to have reduced ability to detect ankyrin deficiency, a subsequent study by Girodon et al. (17) found all seven subjects in their study with ankyrin deficiency to have markedly reduced fluorescence, even more so than subjects with band 3 defects. We had no participants with isolated ankyrin defects. There were two participants from the same family with combined spectrin and ankyrin deficiency. One of these was correctly identified by the EMA-binding test while the other had a false negative result. The only other false negative result was in a participant with a beta spectrin deficiency. The other eight participants with beta spectrin deficiencies were correctly identified.

The EMA-binding test correctly identified HS participants with low haemoglobins who were transfusion dependant, as well as asymptomatic participants with normal haemoglobin values. This observation, similar to other reports, demonstrated that disease severity did not influence test performance. (9) (16)

While some studies have indicated that the EMA-binding test is more sensitive post-splenectomy (4), our results failed to confirm this. No difference in fluorescence intensity could be demonstrated between the splenectomised patients in our positive control group and the other confirmed HS (positive controls) and suspected HS (test subjects) cases with intact spleens.

In a case study of a neonate with clinical features of HS, Cheli et al. (22) showed that post-transfusion, the EMA-binding test could still identify a population of probable HS cells. The diagnosis of HS, in that case, was later confirmed by ektacytometry. In an *in vitro* experiment that involved mixing different volumes of normal red cells with red cells of HS patients, King et al. (14) concluded that even in a heterogeneous population of red cells, the EMA-binding test can still diagnose HS provided that the transfused blood is not more than one half of the total blood volume. In our study, four participants had received blood transfusions within three months of testing, all of whom had MFIs in the HS range.

One of the major disadvantages of the EMA-binding test is the lack of universal reference ranges and cut-off values to distinguish between HS and normal controls. (23) Currently, individual laboratories are encouraged to establish their own reference ranges according to the MFI scale portrayed by their particular flow cytometer. (23) There is also a lack of standardisation in the reporting of results. Some laboratories express results as a percentage decrease in EMA fluorescence compared to normal controls (24)(4)(18)(17) while others use MFI compared to a reference range (25) or an EMA "footprint"(26) established for their laboratory.

We attempted to establish a normal reference range for MFI by accumulating the MFI values of all the normal controls that were run with each test sample. Establishment of a normal reference range for our laboratory would negate the need for six normal controls for every sample tested in future. However, we noticed variability in fluorescence intensity when different operators performed the test. A possible explanation could be the decreased stability of the reconstituted EMA dye when exposed to light (14) as the time taken to perform each step in the procedure may vary between operators and hence the duration of the dye's exposure to light. Storage conditions are critical in maintaining the stability of the dye and studies have shown that storage at -20^{0}C results in a reduction in MFI over time but storage at -80^{0}C is reported to maintain stability over a four-month period. (27)(14). We observed a decrease in MFI as the dye aged even though the aliquots of reconstituted dye were stored in the dark at -80^{0}C. We, therefore decided that test samples need to be compared with normal controls tested with reconstituted dye of the same age and by the same operator to eliminate inter-assay variability.

Aside from the higher sensitivity and specificity observed in our study, another advantage of the EMA-binding test is the small volume of blood required (5 microlitres), which can even be obtained from capillary blood sampling. (28) Red cells can also be stored under refrigeration for up to 7 days before testing thus allowing time for samples to be transported to specialised laboratories. (4)(21)

Although more expensive than the FOFT and CHT, the EMA-binding test is still relatively cost-effective. The turn-around-time for all three tests is less than 3 hours, with the FOFT being the quickest. The advantage of the CHT over the other two is that it can be performed in laboratories without a flow cytometer. All three tests, however, need to be performed by experienced laboratory personnel.

The FOFT, introduced in 2009 by Won and Suh,(15) measures the percentage of residual red cells after the cells are exposed to distilled water. Since the red cells in HS are osmotically fragile, less intact red cells remain after a specified time. Different studies have shown sensitivities that range from 85.7% to 100% (29)(30)(31)(8), which is in stark contrast to our recorded sensitivity of only 38.8%. There is no apparent reason for our low sensitivity. We did have a high percentage of splenectomised participants who, with the exception of the presence of spherocytes and an increased MCHC, had near-normal laboratory values. However, we did not observe a difference in the percentage of residual red cells between splenectomised and non-splenectomised participants which is consistent with observations from other studies. (32)(15)

The cut-off value of 23% used in our laboratory was obtained by averaging the results of normal individuals and subtracting two standard deviations, a method used in many other studies. (29)(16)(31) Shim and Won (30), however, found that when receiver operating characteristic (ROC) analysis included both HS and non-HS groups, and when HS patients with a milder phenotype were included, the cut-off values were much different (61.9%) than when only normal controls were used (22-24%). (30) Perhaps revision of the cut-off values, taking into account values obtained from positive controls, will improve the sensitivity.

Furthermore, certain modifications of the conventional method have been shown to improve the sensitivity of the test. Ciepiela et al. (33) demonstrated that by increasing the events acquisition time after spiking with distilled water improved both the sensitivity and specificity of the test while Nobre et al. (32) showed that sensitivity of the test could be improved by incubating whole blood in saline for 24 hours prior to analysis and by adjusting the concentration of sodium chloride used.

The specificity in our study (90%) is also lower than the 97-98% reported in the literature (31)(29)(9) which may again be attributed to the small sample size of this study.

There is a discrepancy in the literature with regard to the sensitivity and specificity of the CHT. In our study, the sensitivity of the CHT was 61.1% with a specificity of 50.0%. This sensitivity is higher than the 48.5% and 53.0% reported by Mariani et al. (34) and Park et al. (8) respectively, but much lower than the 100% reported by Streichman and Gescheidt. (13) Park et al. (8) reported a specificity of 76.7% and found that the test was unable to discriminate between HS and iron deficiency anaemia. Higher specificities of between 90 and 95% were reported by Streichman and Gescheidt (13) and Crisp et al. (24). In the latter studies, specificity was largely determined by comparing HS patients and their asymptomatic relatives. In our study, we compared known HS patients to patients with confirmed autoimmune haemolysis and one symptomatic patient with negative membrane studies. The difference in patient profiles may explain the discrepancy in specificity.

The combination of EMA-binding test and CHT correctly identified all positive but only two out of six negative cases. Despite the low specificity, the sensitivity of this combination is higher than that of the combination of EMA-binding test and FOFT, and the FOFT and CHT, which had sensitivities of 94.4% and 88.9% respectively. The combination of EMA-binding test and FOFT had a specificity of 80% while the CHT and FOFT had a specificity of just 50%. Different to our results, Crisp et al., showed the

combination of the EMA-binding test and FOFT to have 100% sensitivity while the combination of the EMA-binding test and CHT had a sensitivity of 93.5%.

Conclusion

The EMA-binding test performed considerably better than the CHT and the FOFT. We recommend that for future cases of suspected HS, serial testing be performed with the EMA-binding test as the first-line test. In the absence of a confirmatory test, a positive result denotes a diagnosis of HS. If this test is negative, a second test will be performed, and since the combination of the EMA-binding test and CHT had the highest sensitivity, the CHT is the recommended second test. It should be noted that the acidified glycerol lysis test was not evaluated in this study and may be a more appropriate second screening test.

Ethical Aspects

Approval to conduct the research was obtained from the Health Sciences and Research Ethics Committee of the Faculty of Health Sciences, University of the Free State (Ethics clearance number: UFS-HSD2019/1322/2611). This study was executed in accordance with the declaration of Helsinki (1964) as amended in Tokyo (2008).

Informed consent was obtained from the patients in the positive control group.

Limitations

Due to the relative rareness of HS in the patient population at the UAH, the sample size of confirmed HS cases was small. Additionally, only a few cases of confirmed autoimmune haemolytic anaemia were identified during the study period, which resulted in a small sample size of true negative controls. This limitation reduces the likelihood of statistically significant results and necessitates cautious interpretation.

Ideally, the normal controls used for the EMA-binding test should have been healthy subjects. Unfortunately, since six such controls were required for each sample tested, it would have been challenging to ensure healthy volunteers for each test. Therefore, normal controls were randomly selected from samples submitted for full blood counts from inpatients or patients attending clinics. Whether the medical conditions these patients are afflicted with, affect the fluorescence intensity of the control group is not known.

The literature shows that the acid glycerol lysis test has good sensitivity and specificity when performed on its own and, when combined with the EMA-binding test, specificity and sensitivity are superior to most other test combinations. (4) Since this test is not performed at our laboratory, it was not included in the study but may be considered in future studies.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interests

The authors have no conflict of interest to declare.

Acknowledgements

All laboratory preparation and analyses were performed by the staff of the Immunology and Flow cytometry benches in the Department of Haematology and Cell Biology, NHLS in Bloemfontein, South Africa. Patient records and contact details were provided by Prof DK Stones and Prof MJ Coetzee.

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Appendices

RESEARCH PROTOCOL

Evaluation of the eosin-5-maleimide flow cytometric test in the diagnosis of hereditary spherocytosis as well as the combination of screening tests which yields the greatest sensitivity and specificity for diagnosis.

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1. SUMMARY IN LAY TERMS

Hereditary spherocytosis (HS) is a medical condition that runs in families. This condition is associated with a defect in the red blood cell membrane, which causes these cells to be destroyed by the spleen more readily than usual. As a result, patients can become anaemic and may require blood transfusions.

Testing for this condition is essential to confirm the diagnosis. Once the diagnosis is made, the correct treatment can be given, relatives can be tested for this condition and further laboratory tests, to look for the cause of anaemia, can be stopped as the cause is then known.

Several screening tests are available, of which two (Hypertonic cryohaemolysis test (CHT) and Flow cytometric Osmotic Fragility Test (FOFT)) are currently performed at the Universitas Academic Hospital (UAH) National Health Laboratory Services (NHLS) laboratory. As these are only screening tests and not definite tests (to confirm the diagnosis), they are not one hundred percent accurate and may give false positive and false negative results. We have also recently experienced different results between these two tests which further complicates matters. Unfortunately there are currently no definite test available in South Africa.

As a result, we are considering introducing a third HS screening test, the eosin-5-malemaide flow cytometric test (EMA test), to the UAH NHLS laboratory. In our study, we will be evaluating the performance of the EMA test, by using blood samples of patients known to have confirmed HS, we will then also compare the EMA binding test to the two screening tests currently offered and try to determine which combination of screening tests can most reliably diagnose HS.

Based on our results, we hope to be able to provide the best screening test or combination of tests that can effectively replace the need for a confirmatory test.

2. TABLE OF ABBREVIATIONS

EXPLANATION	ABBREVIATION
Acidified glycerol lysis test	AGLT
Auto-immune haemolytic anaemia	AIHA
Hypertonic cryohaemolysis test	CHT
Direct antiglobulin test	DAT
Eosin-5-maleimide	EMA
Eosin-5-maleimide flow cytometric test	EMA test
Ethylene-diamine-tetra acetic acid	EDTA
Flow cytometric Osmotic Fragility Test	FOFT
Full blood count	FBC
Glycerol lysis test	GLT
Glucose-6-phosphate dehydrogenase	G6PD
Hereditary spherocytosis	HS
Mean corpuscular volume	MCV
Mean corpuscular haemoglobin	MCH
Mean corpuscular haemoglobin concentration (MCHC)	MCHC
National Health Laboratory Services	NHLS
Next-generation osmotic gradient ektacytometry	NG-OGE
Osmotic fragility test	OFT
Phosphatidylserine	PS
Red blood cell	RBC
Red cell distribution width	RDW
Rhesus	Rh
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis	SDS-PAGE
Standard operating procedures	SOP
Surface area to volume	SA/V
Universitas Academic Hospital	UAH

3. INTRODUCTION

Hereditary spherocytosis (HS) is a genetically determined haemolytic anaemia characterised by the spherical shape of the affected red cells.(1) It is caused by highly heterogeneous molecular defects involving the genes that encode red blood cell (RBC) membrane proteins that vertically connect the membrane cytoskeleton to the lipid bilayer.(2)

Loss of cohesion between the bilayer and membrane skeleton occurs when these vertical linkages are compromised. This leads to destabilisation of the lipid bilayer and release of skeleton-free lipid vesicles.(3) Splenic destruction of abnormal erythrocytes is the main cause of haemolysis in these patients.

With an estimated prevalence ranging from 1:2,000 to 1:5,000, HS is the most common congenital haemolytic anaemia in Caucasians. (4)

The signs and symptoms of HS depend on the severity of haemolysis, which can vary widely, from fully compensated anaemia to transfusion dependence.(2) The commonest forms of HS present as mild anaemia and jaundice, with splenomegaly that is rarely marked. Management of HS depends on clinical presentation. Neonates with severe hyperbilirubinemia are at risk for kernicterus and should be treated with phototherapy and/or exchange transfusion as clinically indicated. In aplastic crises, the ongoing destruction of spherocytes is not balanced by new RBC production. This can cause a drastic fall in haemoglobin levels and RBC transfusions often are necessary in these cases. (5) Folic acid is required to sustain erythropoiesis. In order to prevent a megaloblastic crisis, it is advised that patients with HS take supplementary folic acid for life. (5) Splenectomy, although rarely necessary, is indicated for severe haemolysis. Except in the unusual autosomal recessive variant of HS, splenectomy usually eliminates haemolysis and the associated signs and symptoms. (6)

4.1 The red cell membrane

The RBC membrane is composed of a bilayer of lipids in which approximately 20 major proteins and at least 850 minor ones are embedded.(7) The lipid bilayer is attached to an intracellular cytoskeleton by protein-protein and lipid-protein interactions that confer the erythrocyte shape, stability and deformability.(4)

Red blood cells are required to undergo extensive deformation as they travel through the circulation and squeeze through capillaries and splenic sinusoids that are often smaller than half their diameter. Deformability and stability of the RBC membrane are optimised by the biconcave disc shape and cytoplasmic viscosity of the RBC. The red cell membrane is highly elastic and rapidly responds to applied fluid stresses.

A normal red cell is capable of deforming with linear extensions of up to nearly 250%, however, a 3% to 4% change in surface area results in cell lysis. (8) Hence an important feature of induced red cell deformations is that they involve no significant change in membrane surface area. These unique membrane properties are the result of a composite structure in which a plasma membrane envelope, composed of cholesterol and phospholipids, is anchored to a 2-dimensional elastic

network of skeletal proteins through tethering sites on cytoplasmic domains of transmembrane proteins embedded in the lipid bilayer.(8)

4.1.1 Membrane lipids

The plasma membrane of the RBC is a lipid bilayer with inner and outer leaflets. The lipid bilayer is composed of equal proportions of cholesterol and phospholipids, and small amounts of free fatty acids and glycolipids. (9) Cholesterol is evenly distributed between the inner and outer leaflets, but the distribution of four major membrane phospholipids is highly asymmetric. (8) Phosphatidylcholine and sphingomyelin are predominantly located in the outer monolayer, while most phosphatidylethanolamine and all phosphatidylserine (PS), together with the minor phosphoinositide constituents, are confined to the inner monolayer.(10)

4.1.2 Membrane proteins

The normal biconcave shape and function of the red cell membrane are determined by the membrane proteins and their interactions with the lipid bilayer and with each other. There are two main types of protein–membrane associations. “Vertical connections” are formed by integral proteins that provide links between the lipid bilayer and the membrane skeleton. “Horizontal connections” are formed by interactions of the proteins of the membrane skeleton with each other. Genetic abnormalities that produce spherocytosis mainly have mutations affecting the vertical connections. These include genes encoding band 3, ankyrin-1, the rhesus complex, alpha and beta spectrin and protein 4.2. Mutations of the horizontal system usually produce elliptocytosis or more bizarre-shaped changes.(9)

The cytoplasmic domain of band 3 interacts with a variety of peripheral membrane and cytoplasmic proteins including ankyrin, band 4.2, protein 4.1, and adducin. These interactions provide cohesion between the plasma membrane and the underlying spectrin-based membrane skeleton and prevents loss of membrane surface. (11) Abnormalities affecting this domain are seen in hereditary spherocytosis and Southeast Asian ovalocytosis.

The Rhesus (Rh) complex contains the Rh polypeptides, the Rh-associated glycoprotein, CD47, the Landsteiner-Wiener glycoprotein, and glycophorin B dimer. CD47 interacts with protein 4.2 and the Rh polypeptides to establish a contact with ankyrin-1 which in turn binds to β-spectrin.

Protein 4.2 binds to both band 3 and ankyrin and can regulate the avidity of the interaction between band 3 and ankyrin. (12)

Ankyrin contributes to the mechanical coupling between the lipid bilayer and the submembrane cytoskeleton by binding to band 3 and β-spectrin. Protein 4.2 binds to both band 3 and ankyrin and strengthens this linkage.(13)

The main protein of the membrane skeleton is spectrin, consisting of two subunits, α and β. Functions of spectrin include maintaining the membrane composition of other proteins and lipids as well as the biconcave disc shape of RBCs.(9)

Spectrin–actin–protein 4.1 interactions provide much of the flexibility of the red cell membrane. Qualitative defects of spectrin that affect these horizontal interactions induce a loss of structural stability of the membrane and elliptocytosis.(9) The carboxyl-terminal region of β-spectrin molecule has a binding site for ankyrin. Ankyrin in turn binds to the cytoplasmic tail of band 3, and this provides the major linkage between the lipid bilayer and the membrane cytoskeleton. (9)

The amino-terminal region of the spectrin molecule binds protein 4.1. Protein 4.1 in turn binds to glycophorin C, and this provides a second linkage between the lipid bilayer and the membrane cytoskeleton. Therefore spectrin deficiency can cause hereditary spherocytosis due to weakened vertical associations.(9)

4.2 Pathophysiology of membranopathies

Conditions that interfere with normal membrane deformability and/or stability can reduce the normal surface area to volume (SA/V) ratio, alter RBC shape, and reduce the lifespan of the RBC. (14)

Dynamic unfolding and refolding of spectrin repeats confers to the red cell membrane its elasticity. Increased membrane rigidity is the result of structural changes in spectrin repeats that hinder the rapid unfolding. (14)

Membrane mechanical stability is regulated by the lateral linkages between spectrin dimers and by the spectrin-actin-protein 4.1R complex. Weakening of either of these linkages leads to decreased membrane mechanical stability and cell fragmentation. (14)

Maintenance of vertical linkages between lipid bilayer and membrane skeleton is critical for membrane cohesion, and any loss or weakening of these linkages leads to membrane vesiculation and surface area loss.(15)

In order for the red cell to maintain its excess surface area in relation to its volume, which enables it to undergo extensive cell deformations at constant surface area, the red cell needs to conserve its membrane cohesion and mechanical stability during its circulatory lifespan.(16)

Mutations in membrane proteins that lead to loss of either membrane cohesion or decreased membrane mechanical stability account for membrane surface area loss and resultant decreased red cell survival and anaemia. This occurs in hereditary spherocytosis and hereditary elliptocytosis.(16)

In HS, defective anchoring of the skeletal network to the membrane occurs as a result of loss of cohesion between the plasma membrane and the membrane skeleton. This causes loss of membrane and decreased surface area. Decreased membrane cohesion in HS is due to deficiencies of proteins that link the lipid bilayer to membrane skeleton (band 3 or RhAG), or of anchoring proteins (ankyrin or protein 4.2) or quantitative deficiency of spectrin. When red cells lose membrane surface area, they undergoes shape change to spherocytes. Spherocytes have reduced cellular deformability hence, are unable to traverse from the splenic cords to the splenic sinuses. They are sequestered in the spleen and prematurely removed from circulation. The severity of the anaemia is directly related to extent of membrane surface area loss.(17)

4.3 Laboratory diagnosis of HS

A preliminary laboratory diagnosis includes examination of red cell morphology and full blood count (FBC) results. Examination of the blood film is fundamental in individuals with suspected haemolytic anaemia as it gives important information, for example the presence of spherocytes, seen in both HS and immune mediated haemolysis. A direct Coombs test is necessary to exclude immune mediated haemolysis. (5)

Screening tests for red cell membrane defects include osmotic fragility test (OFT), flow cytometric osmotic fragility test (FOFT), acidified glycerol lysis test (AGLT), hypertonic cryohaemolysis test

(CHT), eosin-5-maleimide (EMA) flow cytometric test, and the autohaemolysis test. Confirmatory tests include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of red cell membrane proteins, osmotic gradient ektacytometry and molecular analysis of membrane protein genes.(12)

4.3.1 Red cell indices on FBC

Mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) can be in the normal range (due to the presence of macrocytic reticulocytes), but there is usually an increased red cell distribution width (RDW) and the mean corpuscular haemoglobin concentration (MCHC) is often increased above 35 g/dL. These changes result, not only from the reduction of the surface-area-to-volume ratio, but also from slight dehydration of HS cells. (9) Tao et al. reported sensitivity of only 41.07 %, and specificity 94.47 % when using MCHC >35.5 g/dL as diagnostic criteria. (34)

4.3.2 Red cell morphology

Examination of the peripheral smear is crucial in cases of suspected hereditary haemolytic anaemias. Careful examination of peripheral blood smears can give some clue to the correct diagnosis by demonstrating spherocytes which are dense, round, hyperchromic red cells, lacking central pallor. In severe forms of HS there is a large number of spherocytes, but in mild forms these usually account for less than 20% of red blood cells.(18) Molecular studies have shown that specific morphological findings are associated with certain membrane protein defects e.g. mushroom-shaped or pincered cells are suggestive of a band 3 mutation; acanthocytes or spiculated spherocytes are indicative of beta spectrin deficiency; microspherocytes, contracted erythrocytes and poikilocytes point toward an alpha spectrin deficiency; while ovalocytes and stomatocytes predominate in protein 4.2 deficiency. In HS due to defects of ankyrin 1, there is prominent spherocytosis without other morphological abnormalities. (17)

4.3.3 Screening tests

Osmotic Fragility Tests

Osmotic fragile cells easily haemolyse in hypo-osmotic solutions. Spherocytes, which are unable to increase in volume due to their surface-to-volume area, cannot absorb hypotonic solutions. As a result, they haemolyse in higher concentrations of sodium chloride than normal biconcave RBCs. (2)

The first osmotic fragility test (OFT) introduced into routine diagnostics was Dacie's method. In this method RBCs are placed in serial solutions of saline at concentrations ranging from 0.1% to 0.9% sodium chloride (NaCl). Haemolysis is evaluated by spectrophotometric measurement of the haemoglobin concentration in the solution(19). The drawback of the OFT is a lack in specificity, as other congenital red cell defects or conditions can also give a positive result. These include immune haemolytic anaemia, recent blood transfusion (i.e. lysis of recently transfused RBCs ex vivo due to depletion of ATP in these cells), RBC enzyme deficiencies (e.g. Glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase deficiencies), and unstable haemoglobin variants.(2)

Acidified glycerol lysis test (AGLT) is a modification of the original glycerol lysis test (GLT). It determines the extent of lysis of RBCs suspended in a buffered glycerol solution. The original GLT measures the time taken for 50% haemolysis of a blood sample in a buffered solution of hypotonic saline / glycerol solution. Glycerol retards the entry of water into red cells thus prolonging the lysis time. In the AGLT, addition of sodium phosphate lowers the pH of the buffered solution to 6.85 which improves the sensitivity and the specificity of this test for HS.(19) Despite satisfactory sensitivity of ~95%, the AGLT test is time consuming and has limited specificity as it could also give positive results in acquired spherocytosis, such as auto-immune haemolytic anaemia (AIHA), in

about one-third of the pregnant women, and in some patients with chronic renal failure and with myelodysplastic syndrome. (2)(12)

The pink test is a modification of the AGLT, in which red cells obtained by heel / finger prick are added to a solution of acidic hypotonic glycerol with a slightly lower pH and assessed for haemolysis, which if present, imparts a pink colour. (19)

In 2009, osmotic fragility testing based on flow cytometric assessment was introduced by Won et al. (20) In this method the vulnerability of red cells to haemolysis can be quantified and a precise numerical value representing osmotic fragility is generated. This assay is based on measurement of RBC count in a 0.9% NaCl solution before and after the addition of water. The ratio of the number of RBCs measured during a 30-second acquisition period before the addition of water to the mean RBC count during two gates (time periods) after the addition of water (fifth minute of the analysis), corrected for the dilution factor, represents the actual result of the test. (20) The FOFT has very high sensitivity and specificity for detecting HS. Sensitivities reported by studies were 100% (21), 93.9% (22), 91.3% (23), and 85.7% (24), respectively. A specificity 97.24 % was reported in a 2016 study involving 237 subjects.(24)

Cryohaemolysis test (CHT)

The CHT is based on an observation by Streichman et al.(25), who found that spherocytes are more susceptible to cold temperatures (0 °C) in hypertonic conditions. When red cells are cooled from 37°C to 0°C, while suspended in a hypertonic medium, they undergo massive haemolysis. Under these conditions, the cell's lipid bilayer undergoes a transition from a fluid to a gel phase but under hypertonic conditions, the membrane cannot withstand the mechanical changes associated with the lipid phase transition and cell shrinkage and membrane rupture ensues. The degree of lysis is determined by photospectometrically measuring the amount of free haemoglobin released during cell lysis (>20% lysis is considered positive).(19) The CHT is more specific for HS diagnosis, as the principle of the test is haemolysis due to affected integrity of membrane proteins rather than an unfavourable volume-to-surface area.(26) However, the utility of the CHT is disputable, as the sensitivities and specificities obtained in several studies differ significantly. Streichman and Gescheidt (26) reported 90% specificity and 100% sensitivity. These sensitivities and specificities were correlated by Crisp et al. in a prospective study .(27) On the contrary, Mariani et al.(28) found remarkably lower sensitivity of 53% however, in their study, CH test was performed only in 33 out of 300 HS patients. (28)

Eosin-5-maleimide flow cytometric test (EMA test)

EMA dye binds directly to Lys-430 in the extracellular loop of band 3 protein. Additionally, other erythrocytes integral membrane proteins (CD47, Rh and Rh- associated glycoproteins) were also found to bind EMA dye.(29) EMA dye is a fluorescent probe. Its fluorescence can be detected on a flow cytometer equipped with an argon laser. Its fluorescence corresponds to the plasma membrane protein content on the surface of RBCs. A decrease of mean fluorescence of the dye is indicative of cytoskeleton protein deficiency.(30). HS is highly probable when the patient's RBCs present approximately 85% or less fluorescence than EMA bound control samples. (31)

The volume of blood needed for the EMA test is a huge advantage of this test. A very small volume of blood (5 µL) is sufficient for straightforward diagnosis.(32) Additionally, it has been shown that RBCs stored under refrigeration can bind the same amount of EMA dye as freshly collected samples after up to 7 days of storage. Moreover, the stability of EMA-bound RBCs was demonstrated in stained samples kept refrigerated for 24 hours.(30)

The disadvantage of the test lies in the discrepancy in the presentation of EMA test results among different laboratories. Some prefer to express the result as a percentage of the decrease in EMA fluorescence compared to control RBCs,(33) while others use the mean fluorescence intensity obtained for EMA bound cells (34) or the EMA "footprint".(34) Despite these differences in presentation of EMA test results, it remains the most reliable screening test, with a relatively short turn-around-time, high sensitivity, cost effectiveness and availability in laboratories equipped with even the most basic flow cytometer. (31) Importantly, the results of the EMA test are not affected by splenectomy,(33) and even the smallest populations of RBCs with plasma membrane deficiency can be extracted, including after blood transfusion. (35)

The reported sensitivity of the EMA binding assay for detecting hereditary spherocytosis ranges from 89-93% with specificities of 96-98%.(2)(35) The sensitivity was independent of the type and amount of molecular defect and of the clinical phenotype.(2) Among the described screening tests for HS, the EMA test is the most specific for HS.(22)(29)(33)

Autohaemolysis Test

The autohaemolysis test helps distinguish between membrane and enzyme defects. Aliquots of blood are incubated both with and without sterile glucose at 37°C for 48 hours. After this period, the amount of haemolysis is measured colourimetrically and the ability of cells to withstand metabolic deprivation is measured. If the results are normal, an intrinsic red cell abnormality is unlikely. If abnormal haemolysis is fully corrected by the addition of glucose, a membrane abnormality is likely and if abnormal haemolysis does not correct with glucose, a metabolic (enzyme) abnormality is more likely. (19)

4.3.4 Confirmatory tests

Plasma membrane protein electrophoresis

Even though the screening tests for HS have high sensitivity and specificity, they do not indicate which plasma membrane protein is deficient or which genetic mutation is responsible for the observed symptoms. To detect plasma membrane deficiency, electrophoresis of disintegrated RBC ghosts is performed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) allows for detection of abnormal plasma membrane protein content, either quantitatively or qualitatively (2).

Disadvantages of SDS-PAGE include the large volume of sample needed for analysis, that the method is laborious and time consuming, and requires prior preparation of RBC ghosts and removal of haemoglobin from plasma membranes. In case of mutation within the ankyrin gene, interpretation of SDS-PAGE might be challenging because it presents as a thin band located directly under the spectrin band (69). Additionally, the technique is mostly available in highly specialized diagnostic centres. In South Africa, the only centre that performed this test was the University of Witwatersrand however, this test is now no longer being offered at that centre and is thus unavailable in the country.

Osmotic gradient ektacytometry test

Ektacytometry determines the deformability of cells at a constant shear stress but gradually changing osmolality, which depends on the inner viscosity, volume-to-surface area and viscosity of the cell membrane. (36) A next-generation osmotic gradient ektacytometry (NG-OGE) assay allows adaptation of the test from a primary research method into clinical diagnostics.(37) Lazarova et al. found that the NG-OGE is useful for distinguishing between HS and other inherited anaemias, however, it cannot differ between HS and AIHA.(38) Although, it has been suggested that NG-OGE could represent an intermediate diagnostic step between screening tests and advanced diagnostic

assays, the use of these analysers is not prevalent. NG-OGE is currently recommended as one of the screening tests for HS. (12) (38)

Molecular diagnostics

Molecular diagnostics allows for identification of the genetic mutation within genes encoding plasma membrane proteins, the deficiency of which leads to HS. The genetic defects confirmed to cause HS include the SPTA1 gene encoding alpha spectrin, SPTB gene encoding beta-spectrin, ANK1 gene encoding ankyrin, SLC4A1 gene encoding band 3 protein and/or EPB42 gene encoding protein 4.2.(12)

Molecular diagnostics is the most advanced and most expensive method, and is generally the last option, especially in subjects who present with symptoms, but the previously mentioned methods do not indicate a direct diagnosis. As the presence of a genetic mutation does not determine the severity of the disease or its course, it is not necessary to obtain precise information regarding the specific gene mutation.(12) Molecular diagnostics is used to distinguish between a polymorphism and a gene mutation. Moreover, it may help to predict the disease course if any detrimental polymorphisms are present. (39)

Previously, gene mutations were detected by polymerase chain reaction. (40) Advances in molecular diagnostics from Sanger sequencing to next generation sequencing allow screening for gene mutations over unlimited amounts of DNA. Whole genome sequencing allows the identification of new mutations that cause HS.(39)

Currently, in South Africa, there is very limited availability of confirmatory tests. We therefore rely on the clinical presentation and screening tests for diagnosis**.** The Universitas Academic hospital laboratory currently offers 2 screening tests for the investigation of suspected hereditary spherocytosis: the FOFT and the CHT. According to the literature, FOFT has a higher sensitivity when compared to CHT. (41) However, recently we have had several cases where there was a high clinical suspicion of HS with a positive CHT but a negative FOFT screen.

The EMA test has been shown to have sensitivity and specificity of 86.2% and 93.9% respectively, and Arora et al indicates that the combination of FOFT and EMA can increase sensitivity and specificity to 100% and 94.5% respectively. (41) The performance of the available screening and confirmatory tests has been mostly evaluated individually and on a limited number of cases. The sensitivity of the tests varies greatly and each method fails to identify all cases HS.

4. RESEARCH QUESTION/AIM AND OBJECTIVES

The aim of this study is to evaluate the eosin-5-maleimide flow cytometric test (EMA test) as a screening test in the diagnosis of HS and the usefulness of adding it to our current screening tests to improve our diagnostic platform.

Our objectives are:

- To evaluate the EMA test in the diagnosis of HS.
- To compare the EMA test to the other two screening tests currently performed in our laboratory (CH and FOFT) for suspected HS.
- To determine which test/combination of tests give the greatest sensitivity and specificity, which in the absence of a confirmatory test, can be reliably be used to diagnose HS.

5. METHODOLOGY

6.1 STUDY DESIGN

This will be an observational descriptive study.

6.2 SAMPLE / STUDY PARTICIPANTS

6.2.1 Sample size

We aim to include a minimum of ten patients with confirmed hereditary spherocytosis in our study. The blood samples collected from these patients will serve as our positive controls. All samples then submitted to the Universitas Academic Hospital NHLS Service Laboratory for the investigation of hereditary spherocytosis during the study period will be included. For every test sample, 6 negative controls will be tested concurrently.

6.2.2 Sample acquisition

The study group will consist of all samples submitted to the Universitas Academic hospital NHLS Service laboratory for HS testing during the study period.

Positive control samples will be obtained from patients known to the Universitas Academic hospital Haematology and Paediatric Oncology clinics, with SDS-PAGE confirmed HS.

Negative control samples will be randomly selected from ethylene-diamine-tetra-acetic acid (EDTA) specimens submitted for routine full blood count analysis at the Universitas Academic Hospital NHLS Service Laboratory.

6.2.3 Inclusion criteria

All samples submitted for HS testing during the study period.

6.2.4 Exclusion criteria

For both the study group and control groups, samples will be excluded according to the rejection criteria of the standard operating procedures (SOP) of the Haematology Laboratory.(42)(43)(44)

6.3 MEASUREMENT

All laboratory tests will be performed by the technical heads of the Immunology and Flow cytometry benches of the Universitas Academic hospital NHLS Service laboratory, with assistance from the researcher. The researcher will also be responsible for obtaining informed consent and blood samples from the patients in our positive control group.

The following will be performed on all study samples:

- Full blood count (FBC) and microscopy
- Direct antiglobulin test (DAT)
- The three screening tests for HS – EMA, CHT and FOFT

5.3.1 Full blood count and microscopy

Full blood counts and peripheral smear examinations will only be performed on new samples that are submitted for patients with suspected HS. This forms part of routine investigation for HS in these patients. Since the positive controls have already been diagnosed, full blood counts and examination of the peripheral smear is not required for these samples.

The full blood count will be performed on the Advia 20120I and microscopic evaluation of the peripheral blood smear will be performed by the researcher. The peripheral smear will be examined for spherocytes and polychromatic cells which are indicative of HS and for elliptocytes and stomatocytes which favour the diagnosis of other membranopathies. Features which may point to the specific molecular defect involved will also be searched for. These include mushroom-shaped or pincered cells; acanthocytes or spiculated spherocytes; microspherocytes, contracted erythrocytes and poikilocytes.

5.3.2 Direct antiglobulin test (DAT)/ direct Coombs test

The direct coombs test will be performed on new cases of suspected HS as part of the routine investigation of HS. Since the positive controls have already been confirmed to have HS, this test is not necessary in this group.

Spherocytes are not only found in HS, but also in immunologically-mediated haemolytic anaemias. The latter will, however have a positive DAT.

The direct Coombs test will be used to detect any antibodies on the surface of erythrocytes. A drop of the cell suspension, prepared with the patient's washed red cells and saline, will be mixed with two drops of polyspecific anti-human globulin (AHG), centrifuged and then examined both macroscopically and microscopically for agglutination. The presence of agglutination, indicates a positive DAT and therefore an immune-mediated haemolysis as the cause for the spherocytosis, and not HS. If the broad spectrum AHG is positive, further testing will be performed to determine the type of antibody present. (44)

6.3.3 Hypertonic Cryohaemolysis Test

Whereas osmotic fragility may be abnormal in any condition where spherocytes occur, it has been suggested that cryohaemolysis is more specific for HS (19). This is due to the latter being dependant on factors related to molecular defects of the red cell membrane rather than changes in the surface area-to-volume ratio. This test is currently considered the gold standard for HS testing at the Universitas Academic hospital NHLS Service laboratory.
Washed red cells, kept in the waterbath at 37°C for 10 minutes, are then placed in an ice bucket and rapidly cooled to 0'C while suspended in a hypertonic medium (sucrose/phosphate buffer). The mechanical changes exerted on the red cell membrane under these conditions, result in haemolysis, the extent of which is dependent on the tonicity and temperature of the medium. The free haemoglobin which is released when the cell undergoes lysis, is determined photospectrometrically and this is an indication of the degree of lysis (>20% lysis is considered positive). A normal control sample (blood from a healthy donor), negative parallel control sample (kept at 37'C and does not undergo a cooling stage) and a 100% lysis tube (prepared by adding distilled water to red cells) are included to improve interpretation of results. (43)

6.3.4 Flow cytometric osmotic fragility

Flow osmotic fragility testing (FOFT) has recently been added as a screening test for HS at the Universitas Academic hospital NHLS Service laboratory. It measures the osmotic fragility of red cells by using distilled water to create a hypotonic environment. Normal red cells will undergo a shape change in a hypotonic environment, but the membrane will remain intact. The red cells of patients with HS, however, will not be able to tolerate the shape change, leading to lysis of the cells. The prepared red cell/saline suspension is run on the FACS Canto II flow cytometer for 14 seconds. The sample is then removed from the instrument, distilled water is added and the sample is returned to the flow cytometer for further acquisition. These steps are repeated for the 6 control samples used. The percentage residual red cells are calculated using a formula. A residual red blood cell percentage of <23% will be considered positive for HS. (42)

6.3.5 Eosin-5-maleimide flow cytometric test

The test measures the fluorescence intensity of intact red cells labelled with dye eosin-5-maleimide, which reacts covalently with Lys-430 on the first extracellular loop of band 3 protein.

In each assay a patient sample will be set up together with six normal controls (the same steps for patient and all 6 control samples will be followed). Washed red cells are incubated with the EMA solution for an hour and then run on the FACS Canto flow cytometer and the fluorescence intensity measured. Reduction in binding, measured by reduced fluorescent intensity, corresponds to a quantitative reduction in band 3. Results are expressed as the percentage reduction of fluorescence of the patient to the mean fluorescence of the 6 controls (45)

$$\frac{\{(\text{mean fluorescence of the 6 controls}) - (\text{fluorescence of the patient})\} \times 100}{\text{Mean fluorescence of the 6 controls}}$$

Decrease in fluorescence < 16% = Normal
Decrease in fluorescence 16-21% = Grey area
Decrease in fluorescence > 21% = Increased

6. METHODOLOGICAL AND MEASUREMENT ERRORS

7.1 Intra- and inter observer variation

Inter-observer variation will be minimised in that all laboratory preparation and analysis will only be performed by the technical heads of the Immunology and flow cytometry benches respectively. All data will be collected by the principal investigator using a data collection tool, thereby minimising inter-observer data collection variation.

Intra-observer variation will be minimised by performing all tests according to the relevant SOPs.

7.2 Data collection errors

Daily routine quality control checks will be performed on the FACS Canto II flow cytometer, on which the FOFT and the EMA binding test will be performed. Sample testing will only be performed if all quality checks are in place.

All tests performed, will also include control samples to improve interpretation of the results.

7. ANALYSIS

Specificity and sensitivity will be determined for each assay using Excel. The following formulas will be used:

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives + false negatives}}$$

$$\text{Specificity} = \frac{\text{True negatives}}{\text{True negatives + false positives}}$$

We will do descriptive data analyses, where the test/combination of tests, with the best sensitivity and specificity, will be considered the test of choice for all future HS requests. The data analyses doesn't require analyses from an expert biostatician and all analyses will be performed by the researcher and her supervisors.

8. IMPLEMENTATION OF FINDINGS

The results of this study will be used to determine which individual test or combination of tests provide the best specificity and sensitivity for the diagnosis of HS. Should the EMA test perform better than the current tests being used at our centre, it will be included in our scope of screening tests for HS. The combinations of tests that provided the greatest sensitivity and specificity will then be routinely performed on any future requests.

9. TIME SCHEDULE

Writing protocol: March 2019 - June 2019

Submission to the Ethics Committee of the Faculty of Health Sciences of the UFS: July 2019

Protocol approval/amendments: August 2019

Time period for specimen collection and laboratory testing: September 2019 – December 2019

Data analysis: January 2020

Write of research project: February 2020

Total time needed for the study: Twelve months

11. BUDGET

Item	Cost per test	Number of tests	Total
For FOFT: Distilled water Pipette tips	R8.00	± 200	R 1600
For CHT: Sucrose/phosphate buffer	R10.00	± 200	R 2000
For EMA: EMA dye	R80.00	± 200	R16 000
Transport money (remuneration): R300 per patient with confirmed HS (positive control group) (± 10) Once off payment for clinic visit to obtain blood sample for testing			R 3000
			R22 600

Full blood counts and the direct Coombs test will only be performed on new samples received for patients being investigated for HS. As these tests form part of the routine investigations for HS, they have not been included in the budget. Full blood counts and direct Coombs test will not be performed on the positive controls as these patients already have a confirmed diagnosis.

The project will be financed by the Department of Haematology and Cell Biology.

12. ETHICAL ASPECTS

This study is subject to the approval of the Ethics Committee of the University of the Free State. Approval will also be obtained from the Head of the Department of Haematology and Cell Biology (Prof MJ Coetzee), the head of the School of Pathology (Prof J Naicker) and the business manager of the NHLS Universitas (Mr P Letanta). This study will be executed in accordance with the declaration of Helsinki (1964) as amended in Tokyo (2008).

Informed consent will be obtained from the patients in our positive control group before blood is collected from them. A single blood sample will be collected from each patient for testing. There will be no remunerations offered other than assistance with transport money to the haematology clinic should it be requested. Negative control samples will be randomly selected from EDTA specimens submitted for routine full blood count analysis at the Universitas Academic Hospital NHLS Service Laboratory. Should any of these samples test positive for HS, the patient details will be traced and the treating physician of the patient concerned, will be informed.

Upon completion of tests, all blood samples will be disposed of as per the relevant NHLS SOP.

The primary investigator is a registrar haematopathology, employed by the NHLS and based primarily in the laboratory. As such she is not involved in clinical patient management, therefore the management of patients who chose not to participate in the study cannot be influenced by the primary investigator.

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14. APPENDICES

Appendix 1: Signature page

APPENDIX 1: SIGNATURE PAGE

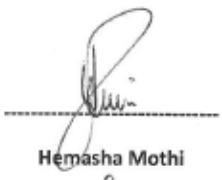
June 2019

To whom it may concern

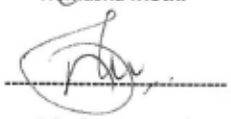
We, the undersigned authors, hereby declare that we will all actively contribute to the execution of the study:

Evaluation of the eosin-5-maleimide flow cytometric test in the diagnosis of hereditary spherocytosis as well as the combination of screening tests which yields the greatest sensitivity and specificity for diagnosis.

We also declare that none of the undersigned has any financial or other conflicts of interest that may affect the performance or reporting of the results of this study.



Hemasha Mothi — 08/07/2019 — **Date**

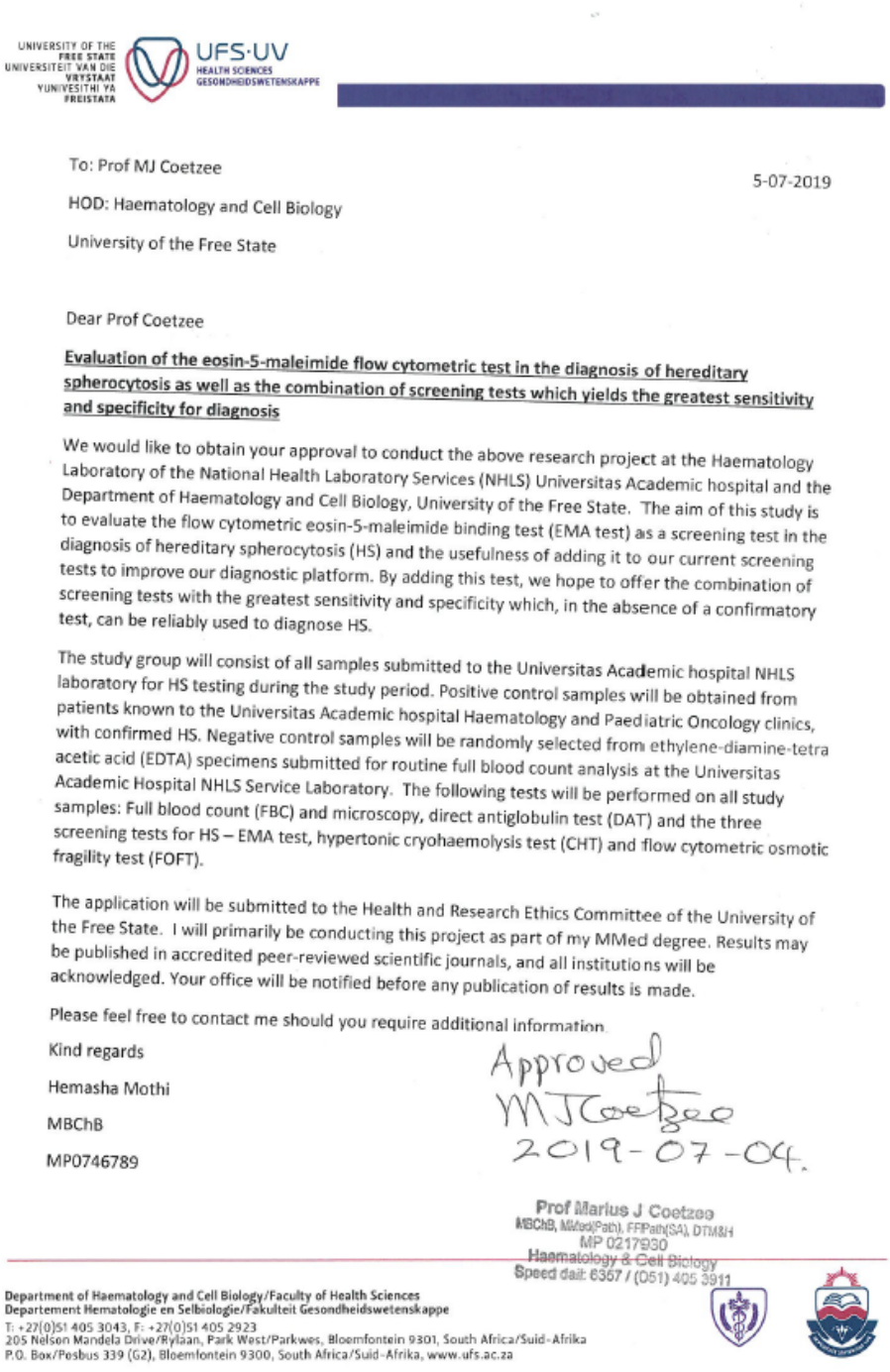


Anneke van Marle — 8/07/2019 — **Date**

Jan Roodt — 8/7/2019 — **Date**

Appendix 2: Permission letters

- Prof MJ Coetzee (signed)



UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

To: Prof MJ Coetzee

5-07-2019

HOD: Haematology and Cell Biology

University of the Free State

Dear Prof Coetzee

Evaluation of the eosin-5-maleimide flow cytometric test in the diagnosis of hereditary spherocytosis as well as the combination of screening tests which yields the greatest sensitivity and specificity for diagnosis

We would like to obtain your approval to conduct the above research project at the Haematology Laboratory of the National Health Laboratory Services (NHLS) Universitas Academic hospital and the Department of Haematology and Cell Biology, University of the Free State. The aim of this study is to evaluate the flow cytometric eosin-5-maleimide binding test (EMA test) as a screening test in the diagnosis of hereditary spherocytosis (HS) and the usefulness of adding it to our current screening tests to improve our diagnostic platform. By adding this test, we hope to offer the combination of screening tests with the greatest sensitivity and specificity which, in the absence of a confirmatory test, can be reliably used to diagnose HS.

The study group will consist of all samples submitted to the Universitas Academic hospital NHLS laboratory for HS testing during the study period. Positive control samples will be obtained from patients known to the Universitas Academic hospital Haematology and Paediatric Oncology clinics, with confirmed HS. Negative control samples will be randomly selected from ethylene-diamine-tetra acetic acid (EDTA) specimens submitted for routine full blood count analysis at the Universitas Academic Hospital NHLS Service Laboratory. The following tests will be performed on all study samples: Full blood count (FBC) and microscopy, direct antiglobulin test (DAT) and the three screening tests for HS – EMA test, hypertonic cryohaemolysis test (CHT) and flow cytometric osmotic fragility test (FOFT).

The application will be submitted to the Health and Research Ethics Committee of the University of the Free State. I will primarily be conducting this project as part of my MMed degree. Results may be published in accredited peer-reviewed scientific journals, and all institutions will be acknowledged. Your office will be notified before any publication of results is made.

Please feel free to contact me should you require additional information.

Kind regards

Hemasha Mothi

MBChB

MP0746789

Approved
MJ Coetzee
2019-07-04.

Prof Marius J Coetzee
MBChB, MMed(Path), FFPath(SA), DTM&H
MP 0217930
Haematology & Cell Biology
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Department of Haematology and Cell Biology/Faculty of Health Sciences
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- Prof J Naicker (signed)

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UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

06 July 2019

The Chair
Heath Sciences Research Ethics Committee
Faculty of Heath Sciences
University of the Free State.

Dear Dr Le Grange

Research Proposal: **Evaluation of the eosin-5-maleimide flow cytometric test in the diagnosis of hereditary spherocytosis as well as the combination of screening tests which yields the greatest sensitivity and specificity for diagnosis.**

Researcher: Dr H Mothi
Supervisor: Dr AC Van Marle
Co-Supervisor: Mr J Roodt

I grant approval for the above laboratory-based MMed research study to be conducted within the Department of Haematology and Cell Biology, School of Pathology.

Permission will have to also be obtained from the NHLS Business Manager in order to use the NHLS patient archival information and samples for additional testing.

I wish Dr Mothi much success with her study.

Yours Sincerely

Jocelyn Naicker
Head: School of Pathology
Faculty of Health Sciences
Tel: 051 405 2914 | Cell: 0829071925
jocelyn.naicker@nhls.ac.za | www.nhls.ac.za

NATIONAL HEALTH LABORATORY SERVICE

- Mr Letanta (signed) (NHLS Business Manager)

NATIONAL HEALTH LABORATORY SERVICE

Practice No. 5200296

Office of the Business Manager
UNIVERSITAS ACADEMIC LABORATORIES
PO BOX 339(G3)
C/O: CHEMICAL PATHOLOGY
1st FLOOR
BLOCK C
FACULTY OF HEALTH SCIENCES
UNIVERSITY OF FREE STATE
BLOEMFONTEIN
9301

REQUEST FOR APPROVAL OF LABORATORY RESOURCES FOR ACADEMIC PURPOSES

Date: 05 July 2019

Requestor: Dr Hemasha Mothi

Project Name: "Evaluation of the eosin-5-maleimide flow cytometric test in the diagnosis of hereditary spherocytosis as well as the combination of screening tests which yields the greatest sensitivity and specificity for diagnosis."

Dear Dr. Mothi,

Your request for use of laboratory facilities / data is hereby granted under following conditions:

1) That University Ethical Committee approval and approval from the Universitas Hospital management is obtained
2) All laboratory data remain confidential to the patient and doctor (anonymity is maintained)
3) This Office must be notified before any publication of any results / findings are made.
4) NHLS is recognised in all publications
5) That a successful K-Project application be made and relevant NHLS project cost centre be created to utilise testing at NHLS as per your protocol.

May your project be successful

Regards

NATIONAL HEALTH LABORATORY SERVICE
08 JUL 2019
Acting.
BUSINESS MANAGER
UNIVERSITAS ACADEMIC LABORATORIES

Mr. Pakiso Letanta
Acting Business Manager

- Dr Malherbe (Clinical Haematology)

To: Dr JleR Malherbe

5-07-2019

Head of Clinical Haematology

Universitas Academic Hospital

University of the Free State

Dear Dr Malherbe

Evaluation of the eosin-5-maleimide flow cytometric test in the diagnosis of hereditary spherocytosis as well as the combination of screening tests which yields the greatest sensitivity and specificity for diagnosis

We would like to obtain your approval to conduct the above research project at the Haematology Laboratory of the National Health Laboratory Services (NHLS) Universitas Academic hospital and the Department of Haematology and Cell Biology, University of the Free State. The aim of this study is to evaluate the flow cytometric eosin-5-maleimide binding test (EMA test) as a screening test in the diagnosis of hereditary spherocytosis (HS) and the usefulness of adding it to our current screening tests to improve our diagnostic platform. By adding this test, we hope to offer the combination of screening tests with the greatest sensitivity and specificity which, in the absence of a confirmatory test, can be reliably used to diagnose HS.

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The application will be submitted to the Health and Research Ethics Committee of the University of the Free State. I will primarily be conducting this project as part of my MMed degree. Results may be published in accredited peer-reviewed scientific journals, and all institutions will be acknowledged. Your office will be notified before any publication of results is made.

Please feel free to contact me should you require additional information.

Kind regards

Hemasha Mothi

MBChB

MP0746789

Dr. JLeR Malherbe

MP0598739

E-mail: malherbejlr@ufs.ac.za

Short Dial: 6803 Cell: 072 124 3048

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- Dr Jan du Plessis (Paediatric Oncology)

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YUNIVESITHI YA FREISTATA

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HEALTH SCIENCES
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To: Dr J du Plessis

5-07-2019

Head of Pediatric Oncology

Universitas Academic Hospital

University of the Free State

Dear Dr du Plessis

Evaluation of the eosin-5-maleimide flow cytometric test in the diagnosis of hereditary spherocytosis as well as the combination of screening tests which yields the greatest sensitivity and specificity for diagnosis

We would like to obtain your approval to conduct the above research project at the Haematology Laboratory of the National Health Laboratory Services (NHLS) Universitas Academic hospital and the Department of Haematology and Cell Biology, University of the Free State. The aim of this study is to evaluate the flow cytometric eosin-5-maleimide binding test (EMA test) as a screening test in the diagnosis of hereditary spherocytosis (HS) and the usefulness of adding it to our current screening tests to improve our diagnostic platform. By adding this test, we hope to offer the combination of screening tests with the greatest sensitivity and specificity which, in the absence of a confirmatory test, can be reliably used to diagnose HS.

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The application will be submitted to the Health and Research Ethics Committee of the University of the Free State. I will primarily be conducting this project as part of my MMed degree. Results may be published in accredited peer-reviewed scientific journals, and all institutions will be acknowledged. Your office will be notified before any publication of results is made.

Please feel free to contact me should you require additional information.

Kind regards

Hemasha Mothi

MBChB

MP0746789

Department of Haematology and Cell Biology/Faculty of Health Sciences
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P.O. Box/Posbus 339 (G2), Bloemfontein 9300, South Africa/Suid-Afrika, www.ufs.ac.za

Appendix 3: Example of consent forms

- ### Informed consent (English)

APPENDIX 3: EXAMPLE OF PARTICIPANT CONSENT FORM (ENGLISH VERSION) CONSENT TO PARTICIPATE IN RESEARCH

Dear participant

You have been asked to participate in a research study named: **Evaluation of the eosin-5-maleimide flow cytometric test (EMA test) in the diagnosis of hereditary spherocytosis (HS) as well as the combination of screening tests which yields the greatest sensitivity and specificity for diagnosis.**

The purpose of this study is to determine the best way to diagnosis HS. There are currently only screening tests but no definite tests available in South Africa to diagnose HS. Screening tests may identify the possible presence of HS, however, some patients with HS can still be missed whilst others may wrongly be identified as having the disorder. We have two screening tests available at Universitas Academic hospital NHLS laboratory and want to evaluate the performance of a third screening test, the EMA test, to see how well it can pick up the presence of HS. We also want to see which combination of the three screening tests, can most reliably diagnose HS.

As a patient known with HS, your blood can be used to see if our screening tests can pick up your disorder.

Your contribution will allow us to offer patients with suspected HS the best test/combination of tests to make the diagnosis.

If you agree to take part in this study, we will draw a single tube of blood from you on a single occasion. Your personal information will be kept confidential and, after testing your blood, it will be safely discarded and not be used for any other purposes.

Your participation in this research is voluntary and you may withdraw from this study at any time, should you want to. If you choose not to participate in the study, you will not be penalised in any way and your treatment will not be affected.

Participation in this study is free, and participants receive no remuneration or compensation for taking part in this study. If you agree to participate, you will be given a signed copy of this document as well as the participant information sheet.

The results of this study may be published and/or presented at a meeting or congress. You have been informed about the study by Dr Hemasha Mothi, whom you can contact at 051-4053043 at any time should you have questions about the research.

The research study, including the above information has been verbally described to me. I understand what my involvement in the study means and I voluntarily agree to participate.

_____	_____
Signature of Participant	Date
_____	_____
Signature of Translator (where applicable)	Date
_____	_____
Signature of Witness	Date

- Informed consent (Afrikaans)

Geagte proefpersoon,

Jy is gevra om deel te neem aan 'n navorsingsprojek getitel: **Evaluation of the eosin-5-maleimide flow cytometric test (EMA test) in the diagnosis of hereditary spherocytosis (HS) as well as the combination of screening tests which yield the greatest sensitivity and specificity for the diagnosis.**

Vertaal lees dit as volg: ***Evaluasie van die eosien-5 maleimied vloeisitimetriese toets (EMA toets) in die diagnose van Oorerflike Sferositose (OS) sowel as die kombinasie van siftingstoetse wat die grootste sensitiwiteit en spesifisiteit vir die diagnose het.***

Die doel van die studie is om die beste manier te bepaal om OS te diagnoseer. Membraanstudies, die enigste erkende diagnostiese toets om die diagnose te maak, is nie meer in Suid Afrika beskikbaar nie en gevolglik moet ons staat maak op siftingstoetse om die diagnose te maak. Siftingstoetse mag die moontlike teenwoordigheid van OS identifiseer, maar die resultaat mag negatief wees in pasiënte met OS, terwyl ander pasiënte 'n positiewe resultaat mag hê en gevolglik verkeerdelik gediagnoseer kan word met OS. Daar is twee siftingstoetse vir OS by Univertsitas Akademiese Hospitaal NGLD Laboratorium beskikbaar en ons wil dit saam met 'n derde metode, die EMA toets evalueer, sodat ons kan bepaal of dit OS kan diagnoseer. Ons wil ook kyk watter kombinasie van die drie toetse die betroubaarste diagnose van OS gee.

As 'n bekende pasiënt met OS kan ons jou bloed gebruik om te sien of die siftingstoetse die diagnose kan maak. Jou bloed sal ons in staat stel om die beste moontlike kombinasie van siftingstoetse aan ander, tot dusver onbevestigde pasiënte, beskikbaar te stel.

As u instem om aan die studie deel te neem, sal ons 'n enkele 5 mL buis bloed van u trek op 'n eenmalige basis. U persoonlike inligting sal vertroulik hanteer word en daar sal veilig van die bloed ontslae geraak word en dit sal vir geen ander doel aangewend word nie.

U deelname aan die studie is vrywillig en u mag op enige stadium van die studie onttrek. Sou u verkies om nie aan die studie deel te neem nie, sal u geensins benadeel word nie en u behandeling sal nie beinvloed word nie. Deelname is gratis en u gaan geen vergoeding vir deelname ontvang nie. As u instem om aan die studie deel te neem sal u 'n getekende kopie van hierdie dokument en die deelnemer inligtingstuk ontvang.

Die resultate van die studie mag in vakjoernale gepubliseer word of op vergaderings en kongresse voorgedra word. U is ingelig oor die studie deur Dr. Hemasha Mothi, wie u kan kontak by 051 405 3043 op enige tyd sou u enige vrae oor die studie hê

Die navorsingsprojek, insluitende bostaande inligting is mondelings aan my verduidelik. Ek verstaan wat my betrokkenheid by die studie behels en ek stem toe om vrywillig hieraan deel te neem.

_____	_____
Handtekening van Proefpersoon	Datum
_____	_____
Handtekening van Vertaler (waar van toepassing)	Datum
_____	_____
Handtekening van Getuie	Datum

- Informed consent (Sesotho)

APPENDIX 3: TUMELLONO YA HO KENYA LETSOHO DIPATLISISONG

Morupeluoa ya ratehang

O memelwa ho kenya letsoho phuputsong ya dipatlisiso tsa: **Hlahlobo ya eosin-5-maleimide flow cytometric test (EMA test) bakeng sa lefu la Hereditary Spherocytosis (HS) ha mmoho le diteko tse fanang ka kutloisiso e kholo e hlakileng bakeng sa ho hlahlobo.**

Morero wa thuto ena ke ho fumana tsela e hlakileng ya ho hlahloba HS. Hona jwale ho na le diteko feela tsa ho hlahloba empa ha ho diteko tse hlakileng tse fumanehang Afrika Boroa ho hlahloba HS. Diteko tse teng di kgona ho bontsha ho ba teng ha HS, leha ho le jwalo, bakudi ba bang ba nang le HS ba ntse ba hlolohelwa ha ba bang ba kanna ba fumanwa ka phoso hore ba na le lefu lena. Re na le diteko tse pedi tsa ho hlahloba lipatlisiso tse fumanoang sepetleleng sa Universitas laboratoring ya NHLS mme re batla ho hlahloba teko ea boraro ea ho hlahloba, tlhahlobo ea EMA, ho bona kamoo e ka fumanang boteng ba HS. Re boetse re batla ho bona hore na hara ha diteko tse tharo, ke tsefe tseo ka ho fetisisa di ka fumanang HS.

Jwaloka mokudi ya tsejwang a na le lefu lena la HS, madi a hao a ka sebediswa ho bona haeba diteko tsa rona tsa ho hlahloba di ka nka boloetse ba hau.

Monehelo wa hao o tla re dumella hore re fane ka teko tse nepahetseng hlahlobong ya bakudi ba solwang ka hoba le lefu lena la HS.

Haeba o dumela ho kenya letsoho thutong ena, re tla hula tubu e lengwe ya mali ho wena ka nako e le ngwe. Tlhahisoleseding ya hao ya botho e tla bolokwa e le lekunutu mme, kamora ho hlahloba madi a hao, e tla lahlwa ka mokhwa o sireletsehileng, mme e se ke ya sebediswa bakeng sa morero ofe kapa ofe. Haeba o kgetha ho se nke karola thutong ena, o ke ke wa fuwa kotlo mme le kalafo ya hao e ke ke ya ameha.

Ho kopanela ha hao patlisisong ena ke ka boithatelo mme o ka tlohela thuto ena ka nako leha e le efe, ha o batla. Ho kenya letsoho thutong ena ke mahala, mme barupeluwa ha ba fumane moputso kapa matshediso bakeng sa ho nka karolo thutong ena. Haeba o dumellana ho nka karolo, o tla fuwa kopi e saennweng ya tokomane ena hammoho le leqephe la tlhahiso leseding ho ba barupeluoa.

Diphetho tsa thuto ena di ka phatlalatsoa/ kapa di hlahiswe dibokeng kapa dikopanong. O tsebisitswe ka thuto ena ke Dr Hemesha Mothi, eo o ka ikopanyang le eena dinomorong tsa 051-4053040 nako engwe le engwe ha ona le dipotso ka dipatlisiso tsena.

Ke hlaloseditswe ka mantsoe ka phuputso ya dipatlisiso, ho kenyelletsa le boitsebiso bo ka hodimo. Kea utlwisisa hore na ho ameha ha ka thupelong ho bolelang, mme ke ithaopela ho dumela ho nka karolo.

_____	_____
Mosaeno waka	Letsatsi
_____	_____
Mosaeno wa mofetoleli	Letsatsi
_____	_____
Mosaeno wa Paki	Letsatsi

Appendix 4: Example of patient information document

- Patient information document (English)

APPENDIX 4: Example of Participant Information Document (English Version)

Evaluation of the eosin-5-maleimide flow cytometric test in the diagnosis of hereditary spherocytosis as well as the combination of screening tests which yields the greatest sensitivity and specificity for diagnosis.

Dear participant

You are invited to take part in a research project conducted by Dr Hemasha Mothi, a registrar at the Department of Haematology and Cell Biology, University of the Free State.

Why are we doing this study?

Previously, hereditary spherocytosis (HS) could be diagnosed by a definite test that was offered by the University of Witwatersrand. However this test is no longer available in South Africa and patients with suspected HS have to rely on screening tests for diagnosis. Screening tests may identify the possible presence of HS, however, some patients with HS can still be missed whilst others may wrongly be identified as having the disorder. We currently have two screening tests available at our centre, but neither of them are able to pick up all cases of HS and they sometimes give different results. We want to evaluate the performance of a third screening test, the EMA test, to see how well it can pick up the presence of HS and also see which combination of the three screening tests, can then most reliably diagnose HS.

How do we do this study?

We will take one tube of blood (3 mL) from you on a single occasion and then perform the three screening tests on this sample in the laboratory.

What are the risks?

The risk of being involved in the study is minimal, except for mild discomfort or pain at the site where the blood is drawn. Sometimes a small bruise may develop at the site where the blood was taken from, but this will heal in a few days.

What are the benefits?

Your participation in this study will allow us to establish the best screening test/combination of screening tests to diagnose hereditary spherocytosis. While it will not be of direct benefit to you, your participation will help to offer patients with suspected HS the best chance of making the correct diagnosis. Participation in this study is free, and participants receive no remuneration or compensation for taking part in this study.

Will my blood results be treated confidentially?

All efforts will be made to keep your personal information confidential. As soon as you enrol in this study, a number gets assigned to your personal information and blood specimens so that no direct connection can be made between the individual and the information obtained.

Results of this study may be published and/or presented at a meeting or congress. Participation is voluntary and you will not be penalized in any way if you choose not to participate. You may withdraw from this study at any time.

Researcher: Dr Hemasha Mothi, Department Haematology and Cell Biology

Telephone number: 051-4053043; E-mail: hemasha.mothi@gmail.com

- Patient information document (Afrikaans)

APPENDIX 4: Example of Participant Information Document (Afrikaans Version)

Evaluasie van die eosien-5 maleimied vloeisitometriese toets (EMA toets) in die diagnose van Oorerflike Sferositose (OS) sowel as die kombinasie van siftingstoetse wat die grootste sensitiwiteit en spesifisiteit vir die diagnose het.

Geagte deelnemer,

Jy word uitgenooi om deel te neem aan 'n navorsingsprojek wat uitgevoer word deur Dr. Hemasha Mothi, 'n kliniese assistant by die Departement van Hematologie en Selbiologie aan die Universiteit van die Vrystaat.

Hoekom doen ons hierdie studie?

Vroeër is oorerflike sferositose (OS) gediagnoseer deur spesifieke membraanstudies wat gedoen was by die Universiteit van die Witwatersrand. Die toets is egter nie meer in Suid Afrika beskikbaar nie, en pasiënte met vermoedelike OS moet staat maak op siftingstoetse vir 'n diagnose. Siftingstoetse mag die teenwoordigheid van OS identifiseer, maar in sommige pasiënte mag dit gemis word of verkeerdelik positief gediagnoseer word. Daar is tans twee siftingstoetse by die Universitas laboratorium beskikbaar, maar nie een van die twee is in staat om al die gevalle van OS te diagnoseer nie en resultate tussen die toetse verskil soms. Ons wil 'n derde siftingstoets evalueer om te sien hoe goed dit die teenwoordigheid van OS kan optel en ook kyk watter kombinasie van die drie toetse die betroubaarste is om die diagnose maak.

Hoe doen ons die studie?

Ons gaan eenmalig een buisie bloed van jou trek en dan al drie siftingstoetse daarop doen in die laboratorium.

Wat is die risiko?

Die risiko is minimaal en is beperk tot matige ongemak of pyn op die plek waar die bloed getrek word, maar dit sal volkome binne 'n paar dae herstel.

Wat is die voordele?

U deelname in die studie sal ons in staat stel om die beste diagnostiese platform daar te stel vir die diagnose van KS. Alhoewel daar geen direkte voordeel vir u in die deelname is nie, sal dit die beste moontlike kans vir 'n akkurate diagnose bied aan ander pasiënte met vermoedelike OS. Deelname aan die studie is gratis en u sal geen betaling of vergoeding daarvoor ontvang nie.

Sal my resultate vertroulik hanteer word?

Ons sal alles in ons vermoë doen om jou persoonlik inligting vertroulik te hou. Sodra jy aanmeld vir die studie word 'n nommer aan jou persoonlike inligting en bloedmoster toegeken sodat geen verbinding tussen die individu en en inligting gemaak kan word nie.

Resultate van die studie mag gepubliseer word in vakjoernale of mag voorgedra word by kongresse en vergaderings. Jy mag op enige stadium uit die studie onttrek sonder enige verduideliking en sonder om 'n rede te verskaf.

Navorser: Dr Hemasha Mothi, Department Hematologie en Selbiologie

Telefoon nommer: 051-4053043; E-pos: hemasha.mothi@gmail.com

- Patient information document (Sesotho)

APPENDIX 4. Mohlala wa tumellano ya boitsebiso ba barupeluoa

Ho hlahloba tlhahlobo ya cytometer ya eosin-5-malemide ho hlalojoa ha spherocytosis e nang le lefa hammoho le ho kopanya diteko tsa ho hlahloba tsa fanang ka kutliwisiso e kholo le tsa Khethehileng.

Morupeluoa ya ratehang

U memeloa ho kenya letsoho phuputsong ya dipatlisiso e hatisitsoeng ke Dr Hemasha Mothi lengolo la ngodiso Lefapheng la Haematology le Cell Biology, Univesithing ya Free State.

Ke hobaneng ha re ntse re esta thuto ee?

Nakong e fetileng, Hereditary Spherocytosis ene e fumanoa ka teko e netefatsang ene e fanoa ke Univesithi ya Witwatersrand. Leha ho le joalo, teko ena ha e sa fumaneha Afrika Boroa le bakudi ba belaellestoeng hore ba na le lefutso la Hereditary Spherocytosis ba tlameha ho itsetleha ka diteko tsa ho hlahlojoq bakeng sa ho hlahlojoa. Har'a diteko tsa ho hlahloba hona joale tsa fumanehang setsing sa rona, ha ho motho ya khonang ho hlahloba mefuta ohle ya Hereditary Spherocytosis. Re batla ho bapisa di kutloisiso le botumo ba tlhahlobo ya cytometer ya eosin-5-malemide ho diteko tsa rona tsa hlahlobo, le hape re bone haeba e sebetsa hantle, re tsepile ho e kenya ts'ebetsong ho laboratori ya rona ho ntlafatsa sethala sa rona sa ho hlahloba.

Re etsa thuto ee joang?

Re tla nka tube ele'ngoe ya madi me re etse diteko tsa laboratori.

Dikotsi ke tsefe?

Kotsi ya ho kenella thuthong ena e nyane, ntle le bohloko bo bobe kapa bohloko bo teng setseng moo madi a hoheloang teng. Ka dinako tse ding ho langoa ha nyenyane ho ka hlaha setseng seo madi a nkiloeng ho sona, empa sena se tla fola ka matsatsi a 'maloa.

Melemo ke efe?

Ho kenya letsoho thutong ena ho tla re dumella hore re thehe diteko tsa ho hlahloba ka ho fetisisa bakeng sa Hereditary Spherocytosis. Le hoja e ke ke ya ba le molemo o tobileng ho uena, ho ba le Karolo ho tla thusa ho hlahloba ba bang ka boema bona. Ho kenya letsoho thutong ena ke mahala ebile barupeluoa ha ba fumane moputso kapa matsediso bakeng sa ho nka Karolo thutong ena.

Na diphetho tsa madi tsa ke di tla tsoaroa ka sephiri?

Boiteko bohle bo tla etsoa ho boloka boitsebiso ba hau bo lekunutu. Itang ha u ngodisa thutong ena, palo e fuoa boitsebiso ba hau ya botho le di specimens tsa madi e le hore ho se ke ha e-ba le puisano e tabileng pakeng tsa motho ka mong le boitsebiso bo fumanoeng.

Diphetho tsa thuto ena di ka phatlalatsoa dibokeng kapa dikopanong. Ho nka karolo ke ka boithatelo me u ke ke ua fuoa phoso ka tsela leha e le efe haeba u khetha ho se nke karolo. U ka tlohela thuto ena ka nako leha e le efe.

Mofuputsi: Dr Hemasha Mothi, Lefapheng la Haematology and Cell Biology

Nomoro ya mohala: 051 405 3043; E-mail: hemasha.mothi@gmail.com

Nomora ya mohala ya Ethics komiti: 051 405 2821

Appendix 5: Example of assent form

- Assent form (English)

CHILD ASSENT

You are being asked to take part in a research study being done by Dr H Mothi at the Universitas Academic Hospital. We have asked your parent or caregiver whether it is okay for you to participate, but now we want to ask if it is okay with you.

The study is on hereditary spherocytosis, a disease that people are born with, and which occurs in families. It causes blood cells to be broken down quicker than is normal. We want to find the best test to decide if someone has hereditary spherocytosis so that they can get the help they need as soon as possible. If you agree, a doctor will take a single sample of blood from you and do different tests on your blood to find out which is the best test.

By signing this you are showing that you understand what is going to be happening and have asked any questions you may have about the research. You can also ask questions later if you cannot think of them now. Signing this form does not mean that you have to finish the study as you can pull out from the study at any time without providing a reason.

Please note that if you decide not to participate, your treatment will not be affected in any way.

....

Signature of child Date

....

Witness Date

- Assent form (Afrikaans)

Minderjarige instemming

Jy word gevra om deel te neem aan 'n navorsingstudie wat gedoen word deur Dr H Mothi by die Universitas Akademiese Hospitaal. Ons het jou ouer of voog gevra of dit aanvaarbaar is vir jou om deel te neem, maar nou wil ons by jou weet of jy bereid is om deel te neem.

Die studie word gedoen op persone met ooreflike sferositose, 'n siekte waarmee mens gebore word en wat in sekere families voorkom. Dit veroorsaak dat rooibloedselle vinniger as normal afgebreek word. Die doel van hierdie studie is om die beste toets te vind om te bepaal of iemand oorerflike sferositose het, sodat hul so gou moontlik die nodige hulp kan ontvang. As jy instem om aan die studie deel te neem sal 'n dokter net een bloedmonster van jou versamel.

Deur hierdie vorm te teken wys jy dat jy verstaan wat met jou gaan gebeur en dat enige vrae wat jy gehad het oor die studie deur die dokter tot jou tevredenheid geantwoord is. Jy kan op enige stadium van die studie verdere vrae vra wat jy nie nou aan kon dink nie. Deur hierdie vorm te teken, beteken nie dat jy enduit aan die studie moet deelneem nie. Jy kan op enige stadium onttrek sonder om 'n rede daarvoor te gee.

As jy besluit om nie deel te neem nie, sal jou behandeling glad nie beïnvloed word nie.

…… ……

Handtekening van minderjarige Datum

…… ……

Getuie Datum

- Assent form (Sesotho)

TUMELLO YA NGWANA

O kopuwa ho nka karolo dipatlisisong tsa thuto e etswang ke Dr H Mothi sepetleleng sa Universitas. Re kopile batswadi kappa mohlokomedi wa hao hore na o ka nka karolo, mme jwale re kopa tumellona ho tswa ho wena.

Thuto ena e mabapi le Hereditary Spherocytosis, e leng lefu leo batho ba tswalwang le lona, mme le hlaha malapeng. Lefu lena le etsa hore disele tsa madi di robehe kapele ho feta ka tsela e tlwalehileng. Re batla ho fumana teko e betere ya ho etsa qeto ya hore na motho o na le lefutso la spherocytosis, hore ba fumane thuso e hlokahalang kapele. Haeba o dumela, ngaka e tla nka mali ho wena, mme ho etswe diteko tsa mali ho fumana hore na ke tlhahlobo efe e ntle ka ho fetisisa.

Kaho saena o bontsha hore o utlwisisa se tlo etsahala, ebile o botsitse le dipotso mabapi le patlisiso ena. O ka botsa dipotso tse ding ka morao haeba di le siyo ha jwale. Ho saena foromo ena ha ho bolele hore o tlameha ho qetela thuto, o ka tlohela neng kappa neng ntle le ho itlhalosa.

Haeba o kgetha ho se nke karola thutong ena, o ke ke wa fuwa kotlo mme le kalafo ya hao e ke ke ya ameha.

_____ _____

Mosaeno wa ngwana Letsatsi

_____ _____

Mosaeno wa mofetoleli Letsatsi

_____ _____

Mosaeno wa paki Letsatsi

Appendix 6: Example of request for permission from the Free State Department of Health (Dr Motau)

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

16 August 2019

To: Dr David Motau

HOD: Department of Health

Dear Dr Motau

Evaluation of the eosin-5-maleimide flow cytometric test in the diagnosis of hereditary spherocytosis as well as the combination of screening tests which yields the greatest sensitivity and specificity for diagnosis

I, Hemasha Mothi, am a haematology registrar at the Department of Haematology and Cell Biology at the University of the Free State. I kindly request your permission to conduct the above research project that involves patients from the paediatric and adult haematology clinics at the Universitas Academic Hospital.

The aim of this study is to evaluate the flow cytometric eosin-5-maleimide binding test (EMA test) as a screening test in the diagnosis of hereditary spherocytosis (HS) and the usefulness of adding it to our current screening tests to improve our diagnostic platform. By adding this test, we hope to offer the combination of screening tests with the greatest sensitivity and specificity which, in the absence of a confirmatory test can be reliably used to diagnose HS.

The study group will consist of all samples submitted to the Universitas Academic hospital NHLS laboratory for HS testing during the study period. Positive control samples will be obtained from patients known to the Universitas Academic hospital Haematology and Paediatric Oncology clinics, with confirmed HS. Negative control samples will be randomly selected from ethylene-diamine-tetra acetic acid (EDTA) specimens submitted for routine full blood count analysis at the Universitas Academic Hospital NHLS Service Laboratory. The following tests will be performed on all study samples: Full blood count (FBC) and microscopy, direct antiglobulin test (DAT) and the three screening tests for HS – EMA test, hypertonic cryohaemolysis test (CHT) and flow cytometric osmotic fragility test (FOFT).

The application has been submitted to the Ethics Committee of the University of the Free State. I will primarily be conducting this project as part of my MMed degree. Results may be published in accredited peer-reviewed scientific journals, and all institutions will be acknowledged. Your office will be notified before any publication of results is made.

Please feel free to contact me should you require additional information.

Kind regards

Hemasha Mothi
MBChB
MP0746789
Contact number: 0798904617
Email: hemasha.mothi@gmail.com

Department of Haematology and Cell Biology/Faculty of Health Sciences
Departement Hematologie en Selbiologie/Fakulteit Gesondheidswetenskappe
T: +27(0)51 405 3043, F: +27(0)51 405 2923
205 Nelson Mandela Drive/Rylaan, Park West/Parkwes, Bloemfontein 9301, South Africa/Suid-Afrika
P.O. Box/Posbus 339 (G2), Bloemfontein 9300, South Africa/Suid-Afrika, www.ufs.ac.za

Appendix 7: Form A (nontherapeutic study on minors)

health
Department:
Health
REPUBLIC OF SOUTH AFRICA

NATIONAL HEALTH
RESEARCH ETHICS
COUNCIL

Private Bag X828, PRETORIA, 0001, Cnr Struben and Thabo Sehume Street
Tel: +27 (0) 12 395 8124/8113 Fax: +27 (0) 12 395 9249

MINISTERIAL CONSENT
FOR NON-THERAPEUTIC HEALTH RESEARCH WITH MINORS:
OPERATIONAL GUIDELINES
2015

1 PURPOSE

To provide guidance to health Research Ethics Committees (RECs) and health researchers regarding Ministerial Consent for 'non-therapeutic' health research with minors.

2 BACKGROUND

1. Section 71(3)(a)(ii) of the National Health Act (NHA) requires the Minister of Health to consent to 'non-therapeutic' health research with minors, after considering whether four criteria are met (see Appendix 1).
2. The Minister may delegate authority, in terms of s 92(a), to any person in the employ of the state, a council, board or committee established in terms of the Act to give this consent.
3. The Minister has delegated authority to provide Ministerial Consent for 'nontherapeutic' health research with minors to RECs who have been found to be compliant with the audit and have achieved full registration with the NHREC. Correspondence in this regard was sent to relevant RECs on 14 October 2014. As further RECs become fully registered, their authority to exercise the delegated power will be communicated by the NHREC through the Secretariat.
4. Regulations for research with human participants, published on 19 September 2014 (R 719)(see Appendix 2),contain Form A that sets out the four criteria to be met for the additional review of 'non-therapeutic' health research with minors. Proper use of Form A should provide adequate evidence that these reviews are performed appropriately by RECs(see Appendix 3).

3 RECOMMENDATIONS FOR RESEARCHERS

1. Researchers should consider carefully whether their planned research involving minors holds out the prospect of direct benefit to participants ('therapeutic' research); or whether it holds out no prospect of direct benefit to participants but holds out the prospect of generalizable knowledge ('non-therapeutic' research).
2. Researchers conducting 'non-therapeutic' research with minors must attach Form A to the application for ethics approval.
3. The content supplied in Form A should draw on relevant sections of the protocol or ethics application, for example, the sections that deal with the scientific justification for enrolling minors; how knowledge will be advanced by enrolling minors; the benefits to society in terms of knowledge gained by enrolling minors; and the potential risks to enrolled minors and risk minimization.
4. Whether Ministerial Consent for 'non-therapeutic' health research with minors has been granted will be communicated, as part of the overall feedback about the application from the REC.

4 RECOMMENDATIONS FOR REGISTERED RECS

1. RECs with delegated authority to grant Ministerial Consent must draw to the attention of researchers the following requirements:
    a. That researchers must consider carefully whether their planned research involving minors holds out the prospect of direct benefit to participants ('therapeutic research'); or whether it holds out no prospect of direct benefit to participants but holds out the prospect of generalizable knowledge ('nontherapeutic research').
    b. That 'non-therapeutic' research must meet four criteria to be eligible for Ministerial Consent.
    c. That the ethics application for 'non-therapeutic' health research with minors must include Form A completed appropriately.
    d. That where the REC judges that the research involves 'non-therapeutic' health research with minors, this view will be communicated to the researcher with a request to complete Form A accordingly.
    e. That the content supplied in Form A should draw on relevant sections of the protocol or ethics application, for example, the sections that deal with the scientific justification for enrolling minors; how knowledge will be advanced by enrolling minors; the benefits to society in terms of knowledge gained by enrolling minors; and the potential risks to enrolled minors and risk minimization.

f. That the outcome (whether consent for non-therapeutic health research with minors is granted) will be communicated by the REC, as part of the overall feedback about the application.

g. That 'therapeutic' health research with minors does not require this additional review but is reviewed in the usual way to ensure norms and standards are met.

2. RECs should only grant Ministerial Consent after review of the application leads to the decision to grant ethics approval, and the careful review of Form A satisfies the REC that the four criteria are met.
3. RECs should maintain specific records of such applications, and outcomes, for reporting purposes. A traceable link to each application must be maintained.
4. RECs may devise Standard Operating Procedures (SOPs) to integrate the additional review into the overall ethics review process to facilitate efficient use of time.

5 RECOMMENDATIONS FOR NON-REGISTERED RECs

1. Two types of REC might not be registered with the NHREC.
2. The first type is an REC that reviews health research but has not been granted delegated authority because it is not (yet) fully registered with the NHREC. RECs that fall into this category are urged to complete the registration process as soon as possible.
3. The second type is an REC that does not review 'health research' with human subjects and is – because of the remit of its review and oversight authority – not registered and is unlikely to seek registration. While such RECs are not required to address the issue of Ministerial Consent, they should carefully review non-health research including the justification for the involvement of minors.

6 CONCLUSIONS

1. It is hoped that the delegation of authority from the Minister to fully registered RECs to grant Ministerial Consent for 'non-therapeutic' research with children will resolve an important issue in the short-term, while appropriate law reform of section 71 is pursued in the medium term.
2. RECs and researchers are requested to send feedback to the NHREC about the process of granting Ministerial Consent and the adequacy of these operational guidelines, so that improvements can be made.

Appendix 1: CURRENT WORDING: S 71 OF THE NATIONAL HEALTH ACT

Research on or experimentation with human subjects
(1) Notwithstanding anything to the contrary in any other law, research or experimentation on a living person may only be conducted- *(a)* in the prescribed manner; and
(b) with the written consent of the person after he or she has been informed of the objects of the research or experimentation and any possible positive or negative consequences on his or her health.

(2) Where research or experimentation is to be conducted on a minor for a therapeutic purpose, the research or experimentation may only be conducted- *(a)* if it is in the best interests of the minor;
(b) in such manner and on such conditions as may be prescribed;
(c) with the consent of the parent or guardian of the child; and
(d) if the minor is capable of understanding, with the consent of the minor.

(3) *(a)* Where research or experimentation is to be conducted on a minor for a non-therapeutic purpose, the research or experimentation may only be conducted- (i) in such manner and on such conditions as may be prescribed;
(ii) with the consent of the Minister;
(iii) with the consent of the parent or guardian of the minor; and (iv) if the minor is capable of understanding, the consent of the minor.
(b) **The Minister may not give consent in circumstances where- (i) the objects of the research or experimentation can also be achieved if it is conducted on an adult; (ii) the research or experimentation is not likely to significantly improve scientific understanding of the minor's condition, disease or disorder to such an extent that it will result in significant benefit to the minor or other minors; (iii) the reasons for the consent to the research or experimentation by the parent or guardian and, if applicable, the minor are contrary to public policy;**
(iv) the research or experimentation poses a significant risk to the health of the minor; or (v) there is some risk to the health or wellbeing of the minor and the potential benefit of the research or experimentation does not significantly outweigh that risk.

Appendix 2: CURRENT WORDING: S 7 OF THE REGULATIONS FOR RESEARCH WITH HUMANN PARTICIPANTS

Ministerial consent for non-therapeutic research with minors

7. Protocols for human participants' research that propose non-therapeutic research with minors must have ministerial consent in terms of section 71(3)(a)(ii) of the Act **or, where appropriate, consent from a delegated authority in terms of section 92(a) of the Act.**

(a) Applications for ministerial consent must be made on Form A;

(b) the application should be considered by the Minister or the delegated authority after the protocol is reviewed by a registered health research ethics committee to assess whether it meets the required norms and standards of the health research ethics committee;

(c) in granting ministerial consent, relevant bodies or experts may be consulted;

(d) the researcher must be notified of the outcome in writing within 60 days; and

(e) the researcher may appeal the outcome including by approaching the National Health Research Ethics Council in terms of section 72 (6) (d) of the Act.

Appendix 3: FORM A AS PUBLISHED IN THE REGULATIONS FOR RESEARCH WITH HUMAN PARTICIPANTS

DEPARTMENT OF HEALTH

APPLICATION FOR MINISTERIAL CONSENT
FOR NON-THERAPEUTIC RESEARCH WITH MINORS

1 INSTRUCTIONS

1.1 This application form must be completed for all protocols that are classified as "non-therapeutic" and involve the participation of minors. *Non therapeutic research is defined in the regulations relating to research on human participants as "research that does not hold out the prospect of direct benefit but holds out the prospect of generalizable knowledge". Minors are defined as persons under the age of 18 by section17 of the Children's Act (No. 38 of 2005).*

1.2 This application form should be submitted with a copy of the protocol and supporting documents.

1.3 This application should be submitted to the Minister of Health or the delegated authority in terms of section 92(a) of the Act.

1.4 This application form should describe how 'non-therapeutic' research protocols with minors meet the conditions set out in section 71 (3)(b) of the Act (described below).

1.5 All sections of the form must be completed in full.

1.6 Ministerial Consent may be granted for non-therapeutic health research with minors when certain conditions set out in section 71 (3)(b) of the Act are met and these conditions are:

(a) The research objectives cannot be achieved except by the enrolment of minors;

(b) The research is likely to lead to an improved scientific understanding of conditions, or disorders affecting children;

(c) Any consent given to the research must be in line with public policy; and

(d) The research does not pose a significant risk to minors, and if there is some risk, the benefit of the research outweighs the risk.

PLEASE COMPLETE AND PRINT THESE LAST TWO PAGES AND SUBMIT WITH YOUR PROTOCOL

2. INVESTIGATORS' DETAILS

Name of principal investigator	Dr Hemasha Mothi
Title of research protocol	Evaluation of the eosin-5-maleimide flow cytometric test in the diagnosis of hereditary spherocytosis as well as the combination of screening tests which yields the greatest sensitivity and specificity for diagnosis.
Institutional affiliation	University of the Free State National Health Laboratory Services (NHLS)
Postal Address	PO BOX 339 Bloemfontein 9300
Physical Address	205 Nelson Mandela Drive Park West Bloemfontein 9301
Email Address	hemasha.mothi@gmail.com
Phone	0798904617 / 051-4053043
Fax	051-4052923
Date of Application	08/07/2019
Signature of Applicant	

3. **APPLICATION**

3.1 **Condition 1: The research objectives cannot be achieved except by the participation of minors**
Describe the scientific justification for the enrolment of minors. Explain why this research must be done with minors as participants:

Hereditary spherocytosis is a relatively rare disorder therefore the exclusion of minors will greatly reduce the number of positive cases available for the study. This will have a negative impact on the sample size.

3.2 Condition 2: The research is likely lead to an improved scientific understanding of certain conditions, diseases or disorders affecting minors

Describe how the research might, or aims to, advance knowledge affecting the health and welfare of minors as a class. Note that 'condition' is defined in the Regulations as 'physical and psychosocial characteristics understood to affect health' allowing that this research does not only involve children with an illness.

Since hereditary spherocytosis is inherited and congenital, it is often diagnosed in childhood. This project aims to improve the diagnosis of this condition which means that affected children can be timeously diagnosed and treated.

3.3 Condition 3: Any consent given to the research is in line with public policy

Consent given by authorised persons must be in line with public policy considerations. Describe how consent to the research will be in line with public policy or would be acceptable, for example, show how the research poses acceptable risks and promotes the rights of minors:

Informed consent will be obtained from parents or caregivers and assent will be obtained from minors. Information will be provided in simple language that is easy to understand by both parents and minors. There is minimal risk involved as only a sample of blood is required from participants on a single occasion and blood will be drawn by a qualified medical practitioner.

3.4 Condition 4: The research does not pose a significant risk to minors; and if there is some risk, the benefit of the research outweighs the risk.

Describe how the potential risks from the research procedures and/or intervention to minor participants will be minimized and describe any possible benefits from the research to society in the form of knowledge:

The risks involved are those associated with phlebotomy. This include pain or bruising at the site of puncture, haematomas, nerve damage and infection. However these are rare complications and every precaution will be taken to minimise the chances of these occurring.

The benefit of this research is that in the absence of a diagnostic test, the best screening tests will be offered to patients with suspected hereditary spherocytosis.

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

Health Sciences Research Ethics Committee

07-Nov-2019

Dear **Miss Hemasha Mothi**

Ethics Clearance: **Evaluation of the eosin-5-maleimide flow cytometric test in the diagnosis of hereditary spherocytosis as well as the combination of screening tests which yields the greatest sensitivity and specificity for diagnosis.**

Principal Investigator: **Miss Hemasha Mothi**

Department: **Haematology and Cell Biology Department (Bloemfontein Campus)**

APPLICATION APPROVED

Please ensure that you read the whole document

With reference to your application for ethical clearance with the Faculty of Health Sciences, I am pleased to inform you on behalf of the Health Sciences Research Ethics Committee that you have been granted ethical clearance for your project.

Your ethical clearance number, to be used in all correspondence is:**UFS-HSD2019/1322/2611**

The ethical clearance number is valid for research conducted for one year from issuance. Should you require more time to complete this research, please apply for an extension.

We request that any changes that may take place during the course of your research project be submitted to the HSREC for approval to ensure we are kept up to date with your progress and any ethical implications that may arise. This includes any serious adverse events and/or termination of the study.

A progress report should be submitted within one year of approval, and annually for long term studies. A final report should be submitted at the completion of the study.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this proposal for ethical clearance and we wish you every success with your research.

Yours Sincerely

Dr. SM Le Grange
Chair : Health Sciences Research Ethics Committee

health
Department of
Health
FREE STATE PROVINCE

28 October 2019

Miss H Mothi
Dept. of Haematology and Cell Biology
UFS

Dear Miss H Mothi

Subject: Evaluation of the eosin-5-maleimide flow cytometric test in the diagnosis of hereditary spherocytosis as well as the combination of screening tests which yields the greatest sensitivity and specificity for diagnosis.

- Please ensure that you read the whole document, Permission is hereby granted for the above – mentioned research on the following conditions:
- Serious Adverse events to be reported to the Free State department of health and/ or termination of the study
- Ascertain that your data collection exercise neither interferes with the day to day running of **Universitas Hospital** nor the performance of duties by the respondents or health care workers.
- Confidentiality of information will be ensured and please do not obtain information regarding the identity of the participants.
- **Research results and a complete report should be made available to the Free State Department of Health on completion of the study (a hard copy plus a soft copy).**
- Progress report must be presented not later than one year after approval of the project to the Ethics Committee of the University of the Free State and to Free State Department of Health.
- Any amendments, extension or other modifications to the protocol or investigators must be submitted to the Ethics Committee of the University of the Free State and to Free State Department of Health.
- **Conditions stated in your Ethical Approval letter should be adhered to and a final copy of the Ethics Clearance Certificate should be submitted to** sebeelats@fshealth.gov.za / makenamr@fshealth.gov.za **before you commence with the study**
- No financial liability will be placed on the Free State Department of Health
- **Please discuss your study with Institution Manager on commencement for logistical arrangements see 2nd page for contact details.**
- Department of Health to be fully indemnified from any harm that participants and staff experiences in the study
- Researchers will be required to enter in to a formal agreement with the Free State department of health regulating and formalizing the research relationship (document will follow)
- **As part of feedback you will be required to present your study findings/results at the Free State Provincial health research day**

Trust you find the above in order.

Kind Regards

pp **Dr D Motau**
HEAD: HEALTH
Date: 4/11/2019

FREE STATE PROVINCIAL TREASURY
RECEIVED
29 -10- 2019
10h40
OFFICE OF THE HOD

TO: MEMBERS OF THE EXECUTIVE COUNCIL
HEADS OF DEPARTMENTS
STAFF IN THE DEPARTMENT OF HEALTH

APPOINTMENT AS ACTING HOD: DEPARTMENT OF HEALTH

In terms of the provisions of section 32 of the Public Service Act, 1994 (Proclamation No. 103 of 1994), I wish to inform you that I have appointed Mr M.N.G. Mahlatsi as Acting Head of the Department of Health from 28th October to 13th November 2019, as the Head of Department, Dr David Motau will not be available during this period.

This official will be responsible for the performance of the duties of the abovementioned post and the concomitant delegated powers attached to the said post.

Yours sincerely

MS S.H. NTOMBELA, MPL
PREMIER: FREE STATE

DATE: 28th October 2019
PREMIER2019.650 ACTING HOD HEALTH

The Premier of the Free State
Private Bag X20538, Bloemfontein, 9300
OR Tambo House, 4th Floor, Cnr St Andrew and Markgraaff Streets, Bloemfontein
Tel: (051) 405 5799 **Fax:** (051) 405 4803
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Literature Review Introduction Hereditary spherocytosis (HS) is a genetic disorder, characterised by spherical erythrocytes, that results in haemolytic anaemia which varies in severity from being asymptomatic to requiring recurrent blood transfusions and splenectomy. (1) The molecular defects are heterogeneous, involving genes that code for erythrocyte membrane proteins responsible for vertical associations between the membrane cytoskeleton and the lipid bilayer. (2) The red cell membrane Red blood cell (RBC) membrane is composed of a lipid bilayer with approximately 20 major and 850 minor proteins embedded within it. (3) The membrane attaches to an intracellular cytoskeleton by protein-protein and lipid-protein interactions. These interactions give the erythrocyte its characteristic biconcave shape and properties of stability, elasticity and deformability. (4) Membrane lipids The lipid bilayer, with inner and outer leaflets, is composed of cholesterol, phospholipids, free fatty acids and glycolipids. (5) Cholesterol is distributed evenly between the inner and outer leaflets, but the four major membrane phospholipids are asymmetrically distributed. (6) Neutral phospholipids (phosphatidylcholine and sphingomyelin) are found mainly in the outer monolayer, while negatively charged phosphatidylethanolamine and phosphatidylserine (PS), are confined to the inner monolayer. (7) Membrane proteins Protein-membrane associations are of two main types. Integral membrane proteins traverse the membrane and act as channels between the plasma and cytoplasm. Within the cytoplasm, integral membrane proteins interact with each other and with the second main group, the skeletal membrane proteins. The links provided by the integral proteins between the lipid bilayer and the membrane skeleton are called 'vertical connections', while the interactions of the membrane skeleton proteins are called 'horizontal connections'. Spherocytosis is mainly caused by mutations affecting the vertical connections. (5) Integral membrane proteins There are up to 50 integral membrane proteins. They vary in abundance and are functionally diverse, functioning as transport proteins, adhesion molecules, and signalling receptors. (6) These proteins are embedded in the lipid membrane through hydrophobic interactions. They traverse the membrane and have distinct regions important for structure and function, both within the membrane and on either side of it. (6) Band 3, glycophorins and Rh-associated glycoprotein (RhAG) are among the integral membrane proteins involved in vertical interactions that link the lipid bilayer to the membrane skeleton (see Figure 1). Figure 1. Structure of the red cell membrane. Adapted from Postgraduate Haematology (5) GPA: Glycophorin A, Rh: Rhesus protein; RhAG: Rhesus associated glycoprotein; GPC: Glycophorin C Band 3 is coded for by the SLC4A1 gene on chromosome 17.(8) Each RBC has approximately 1.2 million copies, which is about one-fourth of total membrane protein. Its hydrophilic

cytoplasmic domain interacts with proteins like ankyrin, band 4.2, protein 4.1 and adducin. (9) The band 3– protein4 .2-ankyrin-spectrin complex is important, as loss of any part of this complex results in membrane loss, reducing the RBC surface-area-to-volume ratio, leading to the characteristic spherocytes of HS. (5) The glycophorins (A, B, C, and D) are glycoproteins that constitute approximately 2% of RBC membrane proteins. (10) They cause a strong negative charge on the RBC surface which reduces interactions of RBCs with each other and with the vascular endothelium. Glycophorin A is attached to band 3.(5) Glycophorin C binds to submembrane proteins p55 and protein 4.1 and regulates their abundance. (10) The Rh polypeptides, the Rh-associated glycoprotein (RhAG), CD47 (the Landsteiner-Wiener glycoprotein) and glycophorin B dimers form the Rhesus (Rh) complex. Interaction of CD47 with protein 4.2 and the Rh polypeptides creates contact with ankyrin. Anykrin binds to β-spectrin. Protein 4.2 has a role in regulating the interaction between band 3 and ankyrin and binds to both these proteins (11) Skeletal proteins Skeletal proteins are located on the cytoplasmic surface of the lipid bilayer. They attach to the integral membrane proteins, not to the membrane itself, and maintain the structure, elasticity, and mechanical stability of the membrane. (11) The major components of the membrane cytoskeleton are spectrin, ankyrin, protein 4.2, actin, tropomyosin, tropomodulin, protein 4.1, adducin, and dematin. Spectrin, the major protein of the membrane skeleton, has two subunits, α and β, encoded by the SPTA1 gene (chromosomes 1) and the SPTB gene (chromosome 14), respectively. The amount of beta-spectrin synthesised is the rate-limiting for the assembly of the spectrin tetramer as alpha- spectrin is synthesized in excess. (4) Therefore, variants affecting a single beta-spectrin allele are sufficient to cause spherocytosis, while variants affecting alpha-spectrin must be present at both alleles to cause HS. (4) Spectrin is a flexible, rod-like protein with approximately 200,000 copies per RBC. The alpha and beta subunits are intertwined side-to-side to form heterodimers that join to other spectrin molecules by flexible joining regions. The heterodimers associate head-to-head, forming tetramers approximately 200 nm in length. (12) (Figure 1). The tail end of the spectrin dimer associates with actin. A junctional complex is formed between beta- spectrin, actin, and protein 4.1. Thisl complex is stabilised by adducin and dematin. The assoaciation of spectrin and actin results in a hexagonal network of spectrin tetramers on the cytosolic surface of the membrane. (Figure 2). Figure 2. Spectrin-based cytoskeleton on the cytoplasmic side of the human red blood cell membrane. Adapted from “What-When-How online tutorials; Hemoglobinopathies and Hemolytic Anemias Part 1” Pathophysiology of membranopathies Conditions that interfere with normal membrane deformability, or stability, can reduce the normal surface area to volume (SA/V) ratio, alter RBC shape, and reduce the lifespan of the RBC.(13) Lateral connections between spectrin dimers and the spectrin-actin-protein 4. 1 complex regulate mechanical stability of the membrane. Weakening of either of these connections reduces membrane mechanical stability and results in cell fragmentation. (14) Vertical connections between the lipid bilayer and membrane skeleton is essential for membrane cohesion, and weakening of these connections leads to membrane vesiculation and loss surface area. (15) To maintain its increased surface area to volume ratio, the red cell needs to conserve membrane cohesion and mechanical stability throughout its lifespan. (16) Inherited red cell membrane disorders are caused by mutations that result in membrane proteins that are less cohesive or less stable, which in turn results in membrane surface area loss, decreased red cell survival and anaemia. (16) Hereditary Spherocytosis Epidemiology HS is seen in all populations but is more common in those of northern European ancestry. The prevelance in Caucasians is estimated to be between 1:2 000 to 1:5000, making it the the most common congenital haemolytic anaemia in this population (4) It is thought to be less common in other geographical

regions such as Africa and Southeast Asia, although comprehensive population survey data are lacking. Most cases (approximately 75%) demonstrate autosomal dominant inheritance; the remaining are recessive. (17) Aetiology Membrane loss in hereditary spherocytosis is associated with defects in several membrane proteins. These include deficiencies of spectrin, ankyrin, band 3, protein 4.2 and proteins of the Rh complex. (14) Spectrin deficiency may result from defects in either α-spectrin or β-spectrin genes. (14) The incidence of β-spectrin defects in northern European populations is about 15-30%. (18) α-Spectrin defects are found in about 5% of HS patients and are only have clinical manifestations in the homozygous or compound heterozygous state. (19) Ankyrin gene (ANK1) mutations causing clinical disease can be either dominantly or recessively inherited and disease can range from mild to severe. Ankyrin has a crucial role in membrane stabilisation as it links β-spectrin to band 3., Ankyrin deficiency results in decreased spectrin assembly even though spectrin synthesis is normal. (14) Band 3 deficiency occurs in about 33% of patients with HS. Inheritance is autosomal dominant and disease is usually mild to moderate in severity. The reported range of band 3 mutations are spread throughout SLC4A1 gene. (19) Homozygous mutations in the protein 4.2 gene (EPB42) result in a recessive form of HS. It occurs commonly in Japan but is rare in other parts of the world. (14) People with heterozygous mutations are asymptomatic. Rh deficiency is rare. Rh antigen expression is either reduced (Rh mod) or absent (Rh null). A moderate haemolytic anaemia associated with spherocytes and stomatocytes is seen. (20) Pathophysiology Hereditary spherocytosis results from an intrinsic defect of the RBC membrane, and an intact spleen which retains and removes the defective RBCs. (14) Although HS is caused by a heterogenous group of molecular defects , all affected erythrocytes have weakening of the vertical linkages between proteins of the membrane skeleton and the integral proteins associated with the lipid bilayer. When vertical interactions are compromised, there is loss of cohesion between the lipid bilayer and membrane skeleton which destabilises the lipid bilayer, resulting in release of lipid vesicles. These vesicles may contain integral membrane protiens such as band 3 but lack skeletal proteins (21) Consequently, membrane surface area is decreased, the SA/V ratio is reduced, and spherocytes are formed. (14) Spherocytes have reduced cellular deformability and are unable to traverse easily from the splenic cords to the splenic sinuses and become trapped in the red pulp. While trapped in the spleen, abnormal erythrocytes undergo further damage and splenic conditioning resulting in additional loss of surface area and increase in cell density. (21) Retained spherocytes are ultimately removed from circulation. The severity of anaemia corresponds directly with the extent of membrane surface area loss and consequent increase in cell sphericity. (14) Clinical Presentation The symptoms and signs of HS include mild pallor, intermittent jaundice, and splenomegaly that is rarely marked. Clinical presentation is highly variable with the commonest forms presenting as mild anaemia and jaundice, with modest splenomegaly. The degree of haemolysis varies widely, from fully compensated to transfusion-dependent anaemia. (2) Anaemia or hyperbilirubinaemia may be so severe that it requires exchange transfusion in the neonatal period or the disorder may be so mild that it escapes clinical recognition altogether. (22)(14) Erythropoiesis is highly active before delivery and splenic function becomes mature only in the postnatal period, so severe anaemia in utero is rare. In contrast, during the neonatal period, erythropoiesis enters a phase of decreased activity. Diminished production coupled with increased destruction of erythrocytes by the now mature spleen may result in severe anaemia, developing 5–30 days post-delivery. As compensatory erythropoiesis develops during the first year of life, the anaemia may greatly improve. (5) Jaundice, rarely intense beyond the neonatal period, is usually intermittent, triggered by stressors such as fatigue, cold

exposure, infections, or pregnancy. Undiagnosed adults usually have a very mild form of HS, which remains undetected until exposed to a stressor. (14)(23) Pigment gallstones due to excess unconjugated bilirubin in bile occasionally occur in young children, but the incidence increases significantly with age. Presence of gallstones may be the first indicator of underlying HS. A family history of HS may be present. Splenectomy or cholecystectomy in a family member before the fourth decade of life is also suggestive. (24) Management Severe hyperbilirubinemia in neonates is risk factor for kernicterus. Treatment is with phototherapy and/or exchange transfusion as clinically indicated. Aplastic crises secondary to an imbalance between red cell production and ongoing destruction of spherocytes, can cause a significant drop in haemoglobin levels and usually requires RBC transfusions. Folic acid is required to sustain erythropoiesis. It is recommended that patients with moderate or severe HS receive lifelong folic acid supplementation to prevent a megaloblastic crisis. (22)(14) Splenectomy is the definitive treatment for HS.(22) Splenectomy usually eliminates haemolysis and the associated clinical features. (25) While there is clear that splenectomy is indicated in patients with severe anaemia and complications of HS, it can be safely deferred in patients with mild, uncomplicated HS. (25) Affected children in whom splenectomy is indicated, include those with severe disease requiring red cell transfusions and those with moderate disease who have growth failure or symptomatic anaemia. To reduce the risk of infection with encapsulated bacteria, splenectomy should be delayed, if possible, until the age of six years. (25) Partial splenectomy is an alternative approach in which splenic immunologic function is retained while at the the rate of haemolysis is reduced. (26) Laboratory diagnosis of HS The initial laboratory evaluation of patients with a suspected membranopathy includes a full blood count (FBC), reticulocyte count, red cell indices, review of the peripheral blood smear, and screening tests for haemolysis. In terms of red cells indices, a mean corpuscular haemoglobin concentration (MCHC) of ≥ 35 g/dL is consistent with the presence of spherocytes. (27) A high MCHC is more useful than a low mean cell volume (MCV), as MCV is affected by the degree of reticulocytosis.(27) Review of the peripheral blood smear may provide important information, for example, the presence of typical elements such as spherocytes, stomatocytes, ovalocytes, or elliptocytes, and the presence polychromasia, the degree of which correlates with the degree of reticulocytosis. Spherocytes are dense, round, hyperchromic red cells that lack central pallor. In severe forms of HS, there are large numbers of spherocytes, but in the mild forms, they are fewer, accounting for for less than 10-20% of red blood cells. (28) Specific morphological findings are seen in certain membrane protein defects, e.g. mushroom-shaped or pincered cells are suggestive of a band 3 mutation. (14) The haemolytic screen should include lactate dehydrogenase (LDH) levels, indirect bilirubin and haptoglobin (in patients >3 months of age). A raised LDH and indirect bilirubin with low or absent haptoglobin together with reticulocytosis are consistent with haemolysis. If haemolysis is confirmed, direct antiglobulin testing (DAT) is indicated to exclude immune-mediated haemolysis. The DAT test is negative when haemolysis is due to a membranopathy. (29) Evidence of haemolysis and a negative DAT that cannot be explained by another condition warrants further testing, which may either be screening or confirmatory tests (Table 1). Table 1: Screening and confirmatory tests for the diagnosis of HS (23)(17) Screening tests Confirmatory tests Autohaemolysis test Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Osmotic fragility tests Osmotic gradient ektacytometry Cryohaemolysis Molecular analysis of membrane protein genes Acid glycerol lysis test Eosin-5’-maleimide (EMA) binding test Screening tests Autohaemolysis Test The autohaemolysis test helps distinguish between membrane and enzyme defects. Blood samples are incubated both with and without sterile glucose at 370C for 48 hours. After

this period, the ability of cells to withstand metabolic deprivation is measured by assessing the amount of haemolysis colourimetrically. If haemolysis is limited, an intrinsic red cell abnormality is unlikely. If the addition of glucose fully corrects abnormal haemolysis, a membrane abnormality is likely, and if abnormal haemolysis does not correct with glucose, a metabolic (enzyme) abnormality is more likely. (30) Osmotic Fragility Tests (OFTs) Osmotic fragile cells easily haemolyse in hypotonic solutions. Spherocytes, which have decreased abilty to increase in volume due to their reduced surface area to volume ratio, cannot absorb hypotonic solutions. As a result, they haemolyse in higher concentrations of sodium chloride (NaCl) than normal biconcave RBCs. (2) In the first OFT used in routine diagnostics, RBCs were exposed to serial solutions of saline at concentrations ranging from 0.1% to 0.9% NaCl. The degree of haemolysis was determined by using a spectrophotometer to measure the haemoglobin concentration in the solution(30). The disadvantage of this test is a lack in specificity as false positives are observed with immune-mediated haemolytic anaemia, recent blood transfusions, RBC enzyme deficiencies, and unstable haemoglobin variants. (23) It was found that incubating the blood at 37°C improved the sensitivity of the test. (31)(32) Won et al. reported osmotic fragility testing based on flow cytometric assessment in 2009. (33) In their method, the vulnerability of RBCs to haemolysis is quantified, and a precise numerical value representing osmotic fragility is generated. The assay principle involves measurement of the RBC count in a 0.9% NaCl solution before and after the addition of water. The result of the test is determined from the ratio of the red cell count during the acquisition period (30 seconds) before the addition of water and the mean red cell count of two time periods (gates) after the addition of water (corrected for the dilution factor). (33) Acidified glycerol lysis test (AGLT) Acidified glycerol lysis test is a modification of the original glycerol lysis test (GLT). It determines the degree of lysis of RBCs suspended in a buffered glycerol solution. The original GLT measures the time taken for 50% haemolysis of a blood sample in a buffered hypotonic saline/glycerol solution. Glycerol hampers the entry of water into red cells, causing a prolonged lysis time. In the AGLT, addition of sodium phosphate lowers the pH of the buffered solution to 6.85 which improves the sensitivity and the specificity of this test for HS.(30) Despite satisfactory sensitivity of ~95%, the AGLT test is time- consuming and has limited specificity as it can also give positive results in acquired spherocytosis, chronic renal failure, pregnancy, and myelodysplastic syndrome. (2)(23) The pink test is a modification of the AGLT, in which red cells obtained by heel/finger prick are added to a solution of acidic hypotonic glycerol with a slightly lower pH and assessed for haemolysis, which if present, imparts a pink colour. (30) Cryohaemolysis test (CHT) The obseravation by Streichman et al. (34) that spherocytes are "specifically susceptible to temperature changes while suspended in hypertonic solutions" is the basis of the CHT. When red cells are cooled from 37° C to 7 °C, while suspended in a hypertonic medium, they undergo massive haemolysis. Under these conditions, the lipid bilayer undergoes a transition from a fluid to a gel phase, but under hypertonic conditions, the membrane cannot withstand the mechanical changes associated with the lipid phase transition and cell shrinkage and membrane rupture ensues. The degree of lysis is determined by photospectometrically measuring the amount of free haemoglobin released during cell lysis (>20% lysis is considered positive). (30) Because the principle of the test is haemolysis due to loss of integrity of membrane proteins instead unfavourable volume-to-surface area, it is reportedly more specific for HS.(35) However the benefit of this test is controversial as several studies report significantly different specificities and sensitivities. (35)(36)(37) Eosin-5-maleimide (EMA-binding) test EMA dye is a fluorescent dye that binds to Lys-430 (the 430th lysine residue) in the extracellular loop of the integral membrane protein, band 3. It also binds to CD47 and RhAG.(38) The fluorescence of

the dye can be detected on a flow cytometer equipped with an argon laser, and the fluorescence intensity is reduced in band 3 deficiency. Flourescence intensity is also decreased with defects of cytoskeletal proteins such as spectrin and protein 4.2, most likely a result of the long-range modulation effect created by these defective proteins on the dye-binding site in the band 3 protein.(38)When the patient's RBCs of the patient demonstrates fluorescence approximately 85% or less than that control samples, HS is highly probable. (39) The major advantages of this test are the small volume of blood required (5 µL) and acceptable sample storage time (up to 7 days under refrigeration). (40).(41) Other advantages include that this test has a high sensitivity, a relatively short turn -around-time, is cost effective and, can be performed in laboratories that are equipped with even a basic flow cytometer. (39) Additionally, the results of the EMA- binding test are not affected by blood transfusion. (42) The disadvantage of this test is that the reporting of results varies among different laboratories. In some centres, the results are expressed as a percentage of the decrease in EMA fluorescence compared to control RBCs, (43) while others express results as the mean fluorescence intensity compared to a reference range(44) or an EMA "footprint"(45) established for their laboratory. (44) Confirmatory tests Plasma membrane protein electrophoresis Although the screening tests described above have high sensitivity and specificity for the detection of HS, they do not provide information about which plasma membrane protein is deficient or where the genetic mutation has occurred. For detection of the plasma membrane protein abnormality, electrophoresis of RBCs is performed. Sodium dodecyl sulphate -polyacrylamide gel electrophoresis (SDS-PAGE) provides qualitative and quantitative information regarding abnormal plasma membrane protein content. (2) Among the disadvantages of SDS-PAGE are the large volume of sample is required for analysis, that the technique is only available in specialised diagnostic centres and, interpretation requires expertise. It is also labour intensive and time-consuming as preparation of RBC ghosts and removal of haemoglobin from plasma membranes is necessary. (2) Osmotic gradient ektacytometry test Ektacytometry is a technique that measures the deformability of cells at constant shear stress but with gradually changing osmolality. The results depend on the SA/V ratio and viscosity of the cell membrane. (46) A next-generation osmotic gradient ektacytometry (NG-OGE) assay has allowed the test to be adapted from a primary research method into a clinical diagnostic tool. (47) Lazarova et al. (48) found that the NG-OGE can be used to distinguish between HS and other inherited anaemias. It cannot, however, differentiate between HS and autoimmune haemolytic anaemia. (48) While it has been suggested that NG-OGE can be utilised as an intermediate diagnostic step between screening tests and advanced confirmatory tests, its use is not yet popular. (23)(48) Molecular diagnostics Genetic mutations within genes that encode membrane proteins, the deficiency of which leads to HS, can be identified with gene sequencing techniques. (23) This diagnostic method is the most advanced and most expensive and, is reserved as a last option for subjects who present with symptoms, but in whom a direct diagnosis cannot be made using the previously mentioned methods. (23) Comparison of available screening tests Several studies have compared the different screening methods, both individually and in different combinations. Concerning the NaCl OFTs, Arora et al. (32) showed that OFT performed on samples incubated at 370C for 24 hours had a higher sensitivity (79.3%) than fresh samples at room temperature OFT (62.1%). (32) However, the two tests had similar specificities (83.6% versus 87.7%). The relatively low sensitivity of NaCl OFTs was also reported by Bianchi et al. (2) who found that the sensitivity of these tests was further reduced in cases of compensated HS. Therefore, despite the incubated OFT traditionally being the test of choice for diagnosing HS in patients with DAT negative haemolytic anaemias,(14) it has limited utility in mild and atypical cases.

(2) The AGLT test has been shown to have high sensitivity (~95%). (2) Mariani et al. (49) demonstrated that the sensitivity of this method was greater than that of the EMA-binding test. This study also showed that the combination of the AGLT and incubated OFT yielded a sensitivity of 99%. (49) Bianchi et al. (2) found similar sensitivity (97%) with this combination. In their study, the association of AGLT and EMA-binding identified all cases of HS.(2) The sensitivity also remained high in compensated cases. (2) However, as mentioned previously, this method is time-consuming and has limited specificity. (2)(23) The flow cytometric osmotic fragility test is reported to be sensitive and specific for detecting HS. Sensitivities in different case series range from 85.7% to 100%.(50) (51)(52)(53). A specificity of 97.24 % was reported in a 2016 study involving 237 subjects. (53) Arora et al. (32) found that the combination of FOFT and EMA-binding tested identified all cases of HS with 100% sensitivity and 94.5% specificity. (32) Due to the discrepancy in sensitivity and specificity reported in different studies,(35)(36)(51) the utility of the CHT in the diagnosis of HS is controversial. Streichman and Gescheidt(35) reported that the CHT had a 90% specificity and 100% sensitivity. These sensitivities and specificities were correlated in a prospective study by Crisp et al. (36) On the contrary, Mariani et al. (49) found a much lower sensitivity of 53%, but in their study, CHT was performed on just 33 of 300 HS patients. Park et al. (51) found the CHT to be inferior to EMA-binding and FOFT and reported that it was unable to discriminate between HS and iron deficiency anaemia. (51) Crisp et al. (36) reported a sensitivity of 93.5% when a combination of CHT and the EMA-binding test were performed. (36) This is similar to the sensitivity of EMA-binding or AGLT when used alone and lower than EMA-binding in combination with AGLT(2) or EMA-binding in combination with FOFT. (32) The sensitivity of the EMA-binding assay for detecting hereditary spherocytosis in reports ranges from 86.2% to 97% with specificities of 93-100%. (2)(42)(32) (54) (43) Sensitivity was found to be independent of the type of molecular defect and the clinical phenotype. (2) Furthermore, the sensitivity of the EMA-binding tests does not vary with clinical phenotype as even cases with compensated anaemia were identified. (2) When EMA-binding was performed in combination with either FOFT or AGLT, all cases of HS were identified. (2)(32) Lower sensitivity of 93.5% was obtained when it was performed in combination with the CHT.(36) The rationale for this study In South Africa, there are no centres that currently offer confirmatory tests. We, therefore, rely on the clinical presentation and screening tests for diagnosis. The Universitas Academic Business Unit laboratory currently offers two screening tests for suspected hereditary spherocytosis: the FOFT and the CHT. FOFT is reported to have a higher sensitivity when compared to CHT. (37) However, recently we have had several cases where there was a high clinical suspicion of HS with a positive CHT, but a negative FOFT screen. The sensitivity of the tests varies greatly, and each method fails to identify all cases of HS. Since the EMA-binding test has been shown to have a relatively high sensitivity and specificity in diagnosing HS (which is further increased when combined with FOFT) and, as our laboratory is equipped with a flow cytometer, this study aims to determine if this test will add value to our current diagnostic platform. Additionally, we aim to determine which test or combination of tests yields the most reliable results. This will allow us, in the absence of confirmatory tests, to provide the best screening for patients with suspected HS.

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Processed on: 22-Jun-2020 11:46 SAST
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Evaluation of the eosin-5-maleimide flow cytometric test and other screening tests in the diagnosis of hereditary spherocytosis Introduction Hereditary spherocytosis (HS) is a genetically determined haemolytic anaemia characterised by the spherical shape of the affected red blood cells (RBCs). (Ma et al., 2018) HS occurs in all racial and ethnic groups but is particularly common in individuals of Northern European descent. (Kaushansky et al., 2016) With an estimated prevalence ranging from 1:2000 to 1:5000, it is the most common congenital haemolytic anaemia in Caucasians. (Andolfo et al., 2016) HS is caused by a myriad of molecular defects which affect genes encoding proteins involved in vertical connections of the RBC membrane cytoskeleton to the lipid bilayer. (Bianchi et al., 2012) Disruption of these vertical linkages result in destabilisation of the lipid bilayer and release of skeleton-free lipid vesicles (Gallagher, 2005) This loss in membrane surface area relative to the intracellular volume, is responsible for the spheroidal shape of RBCs. (Kaushansky et al., 2016) Spherocytes are less deformable and thus, are selectively retained in the spleen where they undergo splenic conditioning and premature removal from circulation. (Kaushansky et al., 2016) Clinical presentation of HS depends on the severity of haemolysis, which varies from fully compensated anaemia to transfusion dependence. (Bianchi et al., 2012) Signs and symptoms include pallor, jaundice, pigment gallstones and splenomegaly, which is rarely marked. In neonates, jaundice may be the most important sign as splenomegaly is often absent. (Da et al., 2013) Neonates with severe hyperbilirubinemia are at risk for kernicterus and should be treated with phototherapy and exchange transfusion as clinically indicated. Management of HS depends on disease severity. Mild cases may be managed only with folate supplementation, while severe forms may require recurrent transfusions and splenectomy. (Hoffbrand et al., 2016) Currently, in South Africa, there are no confirmatory tests for the diagnosis of HS, so we rely on the clinical presentation and screening tests for diagnosis. The Universitas Academic Hospital (UAH) laboratory offers two screening tests for the investigation of suspected HS: the flow osmotic fragility test (FOFT) and the cryohaemolysis test (CHT). With FOFT, RBCs are subjected to a hypotonic solution which causes exaggerated haemolysis in osmotically fragile cells. Following incubation with distilled water, the proportion of residual RBCs is measured by the flow cytometer. (Park et al., 2014a) The CHT is based on the observation that red cells in HS are particularly susceptible to lysis when rapidly cooled in hypertonic conditions. (Bianchi et al., 2012) While the literature suggests that FOFT has a higher sensitivity when compared to CHT (Arora et al., 2018), we have had several cases where there was a high clinical suspicion of HS with a positive CHT but a negative FOFT screen. Given the discrepancy between the literature and our experience, there was a need to evaluate the specificity and sensitivity of these tests in our setting. We also explored the possibility of introducing a third screening test; the eosin-5-maleimide flow cytometric test (EMA-binding test) in an effort to improve our screening platform. EMA is a fluorescent dye that binds to Lys-430 in the extracellular loop of

band 3 protein. (King, Smythe and Mushens, 2004) Its fluorescence, detected by a flow cytometer, corresponds to the band 3 content on the surface of RBCs. A decrease in fluorescence is observed when there is a deficiency of band 3. This study aimed to evaluate the EMA-binding test as a screening test, to compare the EMA-binding test to the other two screening tests currently performed in our laboratory, and to determine which test/combination of tests give the greatest sensitivity and specificity, which in the absence of a confirmatory test, can be reliably be used to diagnose HS. Methods Study Design This was an observational descriptive study conducted at National Health Laboratory Services (NHLS) Universitas Academic Hospital (UAH) Service Laboratory from 07/11/2019 to 31/03/2020. Sample / Study Participants Sample size Blood samples from seventeen known HS patients served as our positive controls. For each confirmed positive sample, we tested six normal controls concurrently. Our test subjects included the samples of five patients with suspected HS as well as two asymptomatic family members of patients with confirmed HS. Ten samples of patients with direct antiglobulin test (DAT) positive autoimmune haemolytic anaemia (AIHA) served as negative controls. Sample acquisition / Data collection Positive control samples were obtained from patients with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) confirmed HS, known to the UAH Haematology and Paediatric Oncology clinics. Normal control samples were randomly selected from ethylenediamine tetra-acetic acid (EDTA) specimens submitted for routine full blood count analysis at the NHLS UAH Service Laboratory. Test subjects included all samples submitted to our laboratory for the investigation of suspected HS during the study period. DAT positive samples served as negative controls. . Inclusion criteria All samples that were submitted to the NHLS UAH service laboratory for HS testing during the study period. Exclusion criteria In the study group and control groups, samples were excluded according to the rejection criteria of the standard operating procedures (SOP) of our Haematology Laboratory. (van der Westhuizen, 2018; Venter, 2018a, 2018b)(Venter, 2018b) Measurement All laboratory tests were performed by the technical heads of the Immunology and Flow cytometry benches of the NHLS UAH service laboratory, with assistance from the researcher. A full blood count (FBC) and microscopy, DAT, and the three screening tests for HS (EMA-binding, CHT and FOFT) were performed on all test subjects. For patients with confirmed HS, the DAT was omitted. Full blood count and microscopy The full blood count was performed on the Advia 20120i, and microscopic evaluation of the peripheral blood smear was performed by the researcher. Direct antiglobulin test (DAT)/ direct Coombs test A drop of the cell suspension, prepared with the patient's washed red cells and saline, was mixed with two drops of Bioscot R polyspecific anti-human globulin (AHG), centrifuged and then examined both macroscopically and microscopically for agglutination. If the broad spectrum AHG was positive, further testing was performed to determine the type of antibody present. Cryohaemolysis Test (CHT) CHT testing was performed according to the method described by Streicman et al. (Bain, Bates and Laffan, 2017) in which washed packed red cells, added to a hypertonic solution (2 mL of 0.7 m sucrose/phosphate buffer), were kept in the water bath at 370C for 10 minutes and then transferred to an ice bucket (00C) for another 10 minutes. The degree of lysis following rapid cooling was determined by measuring the free haemoglobin photospectrometrically at 540 nm. A normal control sample (blood from a healthy donor), negative parallel control sample (kept at 370C for 20 minutes with no cooling stage) and a 100% lysis tube (prepared by adding 2 mL distilled water to 50 µL packed red cells) were included to improve interpretation of results. ? The mean value of the two duplicate optical density (OD) readings of the patient sample, normal control and parallel control were determined. ? The % lysis of each sample or control was determined as follows: % Lysis = _____OD of test (x) _____ x 100 (OD of 100% Lysis) x 6 ? ≥20% lysis was considered to be a positive result Eosin-5-maleimide binding test EDTA blood samples from positive controls and test subjects were kept at 2-8°C and tested within 24 hours of collection. The control group consisted of randomly selected EDTA blood samples of the day. In each assay, patient samples were set up with six normal controls. RBC labelling with EMA (from Thermo FisherTM) and flow cytometric testing (on the Facs Canto II) were carried out according to the procedure previously described by King et al. (King et al., 2000) Results were expressed as the percentage reduction of fluorescence of the patient to the mean fluorescence of the six controls (King et al., 2000) Decrease in fluorescence = {(mean fluorescence of the 6 controls) – (fluorescence of the patient)} x100 Mean fluorescence of the six controls Decrease in fluorescence < 15.84% = Normal Decrease in fluorescence ≥ 15.84% = Increased . Flow cytometric osmotic fragility test The presence of spherocytes due to

HS are detected by comparing the percentage red cells in the red cell/saline suspension acquired on the FACS Canto II flow cytometer before and after the addition of distilled water. A normal control was run concurrently with each test sample. The method used was that described by Won and Suh. (Won and Suh, 2009) and the formula for calculating percentage residual red cells was that used by Park et al. (Park et al., 2014b). A residual red blood cell percentage of <23% was considered positive for HS. Data Analysis Basic descriptive statistics including the sensitivity, specificity and predictive values for each assay were calculated. Receiver operator characteristic (ROC) and logistic regression curves were used to determine the optimum cut-off values for the EMA-binding test. For the combination of tests, parallel testing was performed. If one of the tests in a combination was positive, the result was considered to be positive, while both tests had to be negative for the result to be considered as negative. Other parameters evaluated included participant age and gender, the type of membrane defect, whether the participants had received any recent blood transfusions or had undergone splenectomy, the haemoglobin levels of participants, the mean corpuscular volumes and the mean corpuscular haemoglobin concentrations of the positive and negative controls. Results Our findings are presented in the tables and figures that follow. Table 1 summarise the laboratory data and test results for the positive control, the test- and the negative control groups. Table 2 compares the sensitivity, specificity, and predictive values of each test and combinations of tests. Figures 1-3 illustrates the typical pattern of confirmed HS samples for each of the screening tests performed. Figure 4 and 5 are graphs used to determine the optimum cuff-off value for % MFI decrease for the EMA-binding test in our laboratory. Table 1: Laboratory Data and Test Results of the three Participant Groups Participant Group Confirmed Positive Confirmed Negative Test Group Haemoglobin (g/dL) Range Median Mean 6.8-18.9 15.5 15.2 4-15.7 11.4 10.79 6.2-15.7 11.7 11.45 Mean corpuscular volume (MCV) Range Median Mean 65.6-98.9 89.9 87.3 80.7- 122.6 100.1 98.62 72.0-91.7 84.45 82.4 Mean corpuscular haemoglobin concentration (MCHC) Range Median Mean 29.7-36.6 35.2 34.9 27-33.8 30.9 30.81 33.6-37.2 34.1 34.55 FOFT (% of residual red cells) Positive Negative Range Median 7 11 0.92-75.8 49.9 1 10 4.3-94.8 50.63 2 4 7.43-77.7 28.3 EMA-binding test (% decrease in mean fluorescence intensity) Positive Negative Range Median 16 2 13.1-35.9 30.1 9 1 -1.35-31.79 7.89 4 2 4.31 – 48.31 23.53 CHT (% red cell lysis) Positive Negative Range Median 11 9 4.1-33.9 22.4 3 3 2.9-35.7 19.3 4 2 13.7-31.8 24.05 Table 2: Sensitivity, specificity, predictive values of each test Test Sensitivity Specificity Positive Predictive Value Negative Predictive Value EMA-binding test 88.9% 90% 94.1% 81.9% FOFT 38.8% 90% 87.5% 45% CHT 61.1% 50% 78.6% 30% EMA + FOFT 94.4% 80% 89.4% 88.9% EMA + CHT 100% 33.3% 81.9% 100% CHT + FOFT 88.9% 50.0% 84.2% 60% Figure 2a: Example of positive EMA-binding test graph Figure 2b: Difference in MFI of positive patient (purple) and normal control (green) Figure 3: Image of a positive CHT Figure 4: ROC curve analysis showing that a % decrease in MFI between 14.1 and 17.5 separates positive from negative samples Figure 5: Logistic regression analysis indicating that %MFI decrease threshold of 15.84 best separates positive and negative samples Positive control group: There were 18 participants in the positive control group ranging in age from 12 months to 74 years with a male: female ratio of 5:4. Fifteen participants were splenectomised. One had received a blood transfusion within eight weeks of testing. A variety of confirmed membrane defects were present among the participants, the most common being beta spectrin deficiency (in 9 participants), followed by band 3 deficiency (in 5 participants). Alpha spectrin deficiency was identified in one participant, protein 4.2 deficiency in one participant, and combined spectrin and ankyrin deficiency in 2 participants. The haemoglobin levels ranged from 6.8 g/dL to 18.9 g/dL with a median of 15.5 g/dL. The MCV ranged from 65.6 fL to 98.9 fL with a median of 89.9 fL. The MCHC had a range of 29.7 g/dL to 36.6 g/dL with a median of 35.4g/dL. (Table 1) The EMA-binding test identified 16 of the 18 samples as positive and two as negative. The overall sensitivity of the test was 88.9%. The FOFT was positive in only seven samples with a sensitivity of 38.8%. The CHT generated a positive result for 11 of the samples, with 7 negative results (Table 1 and Table 4). The overall sensitivity of this test was 61.1%. The combination of the EMA-binding test and FOFT correctly identified 17 samples as positive, the combination of EMA-binding test and CHT identified all positive cases and the combination of FOFT and CHT identified 16 as positive with sensitivities of 94.4%, 100% and 89.9% respectively (Table 2). The two cases that were negative with the EMA-binding test were correctly identified by the CHT. Negative control group The negative control group was comprised of ten subjects, all of whom had features of haemolysis and spherocytes on peripheral

blood. Nine of the samples had a positive DAT while one had a negative DAT but normal red cell membrane studies. The EMA-binding test and FOFT were performed on all ten participants in the negative control group, however the CHT was only performed on six of the ten participants. The EMA-binding test and FOFT were negative in 9 of the samples with a specificity of 90% while CHT was negative in 3 out of 6 negative samples resulting in a specificity of 50%. (Tables 1 and 2). The combination of EMA-binding test and FOFT identified eight out of the 10 participants as negative, while the combination of the EMA-binding test and CHT only identified two of six negative samples correctly. The combination of FOFT and CHT correctly identified three of six negative samples resulting in a specificities of 80%, 33.3% and 50% respectively (Table). Test group The test group was comprised of four subjects with clinical and peripheral blood features suggestive of HS and two asymptomatic family members of known HS patients (Table 2). Of the four patients with clinical suspicion of HS, all had positive EMA-binding test and CHT results while two also had positive FOFT. Asymptomatic family members had normal full blood counts, without features of haemolysis on the peripheral smear. They were both negative for all three screening tests. Cut-off values for the EMA-binding test To determine the optimum cuff -off values between HS and non-HS individuals, ROC curve analysis and logistic regression analysis were performed using % decrease in MFI obtained from the true positive group and the true negative groups (Figures 4 and 5). The ROC curve demonstrated that a cut off value between 14.18% and 17.5% was the best compromise between sensitivity and specificity. Logistic regression analysis showed that a Þcrease in MFI threshold of 15.84% best separates positive and negative samples. We decided to implement this cut off value when interpreting results of the EMA-binding test in our laboratory as this intermediate value gives equal weight to sensitivity and specificity. This value is within the range used in other studies where the cut off value for % decrease in MFI ranges from 11-21%(Bianchi et al., 2012)(Girodon et al., 2008)(Joshi et al., 2016) (Joshi et al., 2016)(Arora et al., 2018). Some studies mention a "gray area" where there is an overlap of results with HS and non-HS cases (Hunt et al., 2015)(King et al., 2000) If we were to define that area in this study, it would be a % MFI decrease of between 13.1 and 15.84% which would include 2 participants from the true positive group and two from the true negative group. For the purposes of this study, values in this area were considered to be negative, however in practice, it is advisable that such borderline results be further investigated with family studies, another screening test or a confirmatory test if available.(Hunt et al., 2015) Discussion In this study, we compared the performance of the three screening tests for HS, individually and in combination. Two of the three tests, the FOFT and the CHT were already being used at our centre while a third, the EMA-binding test was being considered for implementation. Since first described in 2000 by King et al. (King et al., 2000), the EMA-binding test has become the screening test of choice in many specialised laboratories due to its high sensitivity and specificity. (Bianchi et al., 2012) In this method, the fluorescent probe, eosin-5'-maleimide binds to transmembrane proteins band 3, CD47 and proteins of the Rh complex, which are reduced in erythrocytes of patients with HS. (King, Smythe and Mushens, 2004) Fluorescence intensity is also decreased with defects of cytoskeletal proteins such as spectrin and protein 4.2, most likely a result of the long-range modulation effect created by these defective proteins on the dye-binding site in the band 3 protein. (King, Smythe and Mushens, 2004) The results of this study show the EMA-binding test to have the highest sensitivity (88.9%) of the three tests evaluated and specificity that is equal to that of the FOFT (90%) and superior to the CHT. The sensitivity of the test in this study is similar to that reported in the literature where it ranges from 86.2% to 97.0%. (Bianchi et al., 2012)(King et al., 2000)(Arora et al., 2018)(Joshi et al., 2016) Reported specificity of the EMA-binding test ranges between 93 and 100%. (Joshi et al., 2016)(Arora et al., 2018)(Bianchi et al., 2012)(King et al., 2000) We recorded a slightly lower specificity which may be attributed to our small sample size. Ciepiela et al. (Ciepiela et al., 2015) found that MCV affects the MFI of samples such that higher MCV results in increased MFI while the converse is true for lower MCV values. Some authors even recommend using control samples with MCV within ±2 fL of patient RBCs to avoid false negative results in cases of macrocytosis (eg. newborns) and false positive results in cases of microcytosis (eg. coexistence of iron deficiency anaemia). (Ciepiela, 2018) We did not use MCV-matched control samples which may be one of the reasons for the lower the sensitivity and specificity observed in this study. In agreement with other studies, the sensitivity of the EMA-binding test was not influenced by the type of molecular defect. (Bianchi et al., 2012) Although King et al. (King et al., 2000)

reported the EMA-binding test to have reduced ability to detect ankyrin deficiency, a subsequent study by Girodon et al. (Girodon et al., 2008) found all seven subjects in their study with ankyrin deficiency to have markedly reduced fluorescence, even more so than subjects with band 3 defects. We had no participants with isolated ankyrin defects, however, the two participants with combined spectrin and ankyrin deficiency, were both correctly identified with the EMA-binding test. Similar to other reports, disease severity did not influence test performance. (Arora et al., 2018) (Park et al., 2014b) The EMA-binding test correctly identified HS participants with low haemoglobins who were transfusion dependant, as well as asymptomatic participants with normal haemoglobin values. . While some studies have indicated that the EMA-binding test is more sensitive post splenectomy(Bianchi et al., 2012), our results failed to confirm this. No difference in fluorescence intensity could be demonstrated between the splenectomised patients in our positive control group and the other confirmed HS (positive controls) and suspected HS (test subjects) cases with intact spleens. In a case study of a neonate with clinical features of HS, Cheli et al. (Cheli et al., 2017) showed that post-transfusion, the EMA-binding test could still identified a population of probable HS cells. The diagnosis of HS in that case was later confirmed by ektacytometry. In an in vitro experiment that involved mixing different volumes of normal red cells with red cells of HS patients, King et al. (King et al., 2000) concluded that even in a heterogeneous population of red cells, the EMA-binding test can still diagnose HS provided that the transfused blood is not more than one half of the total blood volume. In our study, there were four participants who had received a blood transfusion within three months of testing, all of whom had MFIs in the HS range One of the major disadvantages of the EMA-binding test is the lack of universal reference ranges and cut-off values to distinguish between HS and normal controls. (King et al., 2015) Currently, individual laboratories are encouraged to establish their own reference ranges according to the MFI scale portrayed by their particular flow cytometer. (King et al., 2015) There is also lack of standardisation in the reporting of results. Some laboratories express results as a percentage decrease in EMA fluorescence compared to normal controls (Crisp et al., 2011)(Bianchi et al., 2012)(Joshi et al., 2016)(Girodon et al., 2008) while others use mean fluorescence intensity compared to a reference range (Kar, Mishra and Pati, 2010) or an EMA "footprint"(Agarwal et al., 2016) established for their laboratory. We attempted to establish a normal reference range for MFI by accumulating the MFI values of all the normal controls that were run with each test sample. Establishment of a normal reference range for our laboratory would negate the need for 6 normal controls for every sample tested in future. However, we noticed variability in fluorescence intensity when different operators performed the test. This could be explained by the decreased stability of the reconstituted EMA dye when exposed to light (King et al., 2000) as time taken to perform each step in the procedure may vary between operators and hence the duration of the dye's exposure to light may also vary. Storage conditions are critical in maintaining stability of the dye and studies show that storage at -200C results in reduction in MFI over time but storage at -800C is reported to maintain stability over a 4 month period. (Kedar et al., 2003)(King et al., 2000). We observed a decrease in MFI as the dye aged even though the aliquots of reconstituted dye were stored in the dark at -800C. We, therefore decided that test samples need to be compared with normal controls tested with reconstituted dye of the same age and by the same operator to eliminate inter-assay variability. Aside from the higher sensitivity and specificity observed in our study, other advantages of the EMA- binding test over the other two screening tests include the small volume of blood required (5 micolitres), which can even be obtained from capillary blood sampling (Crisp et al., 2012), and that red cells can stored under refrigeration for up to 7 days before testing thus allowing time for samples to be transported to specialised laboratories for testing. (Bianchi et al., 2012)(Ciepiela, 2018) Although more expensive than the FOFT and CHT, the EMA-binding test is still relatively cost effective. The turn-around time for all three tests is less than 2 hours, with the FOFT being the quickest. The advantage of the CHT over the other two is that it can be performed in laboratories without a flow cytometer. All three tests, however, need to be performed by experienced laboratory personnel. The FOFT, introduced in 2009 by Won and Suh,(Won and Suh, 2009) measures the percentage of residual red cells after the cells are exposed to distilled water. Since the red cells in HS are osmotically fragile, less intact red cells remain after a specified time period. Different studies have shown sensitivities that range from 85.7% to 100% (Warang et al., 2011)(Shim and Won, 2014)(Tao et al., 2016)(Park et al., 2014a), which is in stark contrast to our recorded sensitivity of only 38.8%. There is no clear reason for our low sensitivity. We did have a high percentage of

splenectomised participants who, with the exception of the presence of spherocytes and an increased MCHC, had near normal laboratory values. However, we did not observe a difference in the percentage residual red cells between splenectomised and non-splenectomised participants. This is consistent with observations from other studies which showed that splenectomy did not affect test performance.(Nobre et al., 2018)(Won and Suh, 2009) The cut-off value of 23% used in our laboratory was obtained by averaging the results of normal individuals and subtracting two standard deviations, a method used in many other studies.(Warang et al., 2011)(Park et al., 2014b)(Tao et al., 2016) Shim and Won(Shim and Won, 2014), however, found that when receiver operating characteristic (ROC) analysis included both HS and non-HS groups, and when HS patients with a milder phenotype were included, the cut-off values were much different (61.9%) than when only normal controls were used (22-24%).(Shim and Won, 2014) Perhaps revision of the cut-off values, taking into account values obtained from positive controls, will improve the sensitivity. Furthermore, certain modifications of the conventional method have been shown to improve sensitivity of the test. Ciepiela et al (Ciepiela et al., 2018) demonstrated that by increasing the events acquisition time after spiking with distilled water improved both the sensitivity and specificity of the test while Nobre et al(Nobre et al., 2018) showed that sensitivity of the test could be improved by incubating whole blood in saline for 24 hours prior to analysis and by adjusting the concentration of sodium chloride used. The specificity in our study (90%) is also lower than the 97-98% reported in literature. (Tao et al., 2016)(Warang et al., 2011)(Arora et al., 2018) This may again be attributed to the small sample size of this study. There is discrepancy in the literature with regard to the sensitivity and specificity of the CHT. In our study, the sensitivity of the CHT was 61.1% with a specificity of 50.0%. This sensitivity higher than the 48.5% and 53.0% reported by Mariani et al (Mariani et al., 2008) and Park et al(Park et al., 2014a) respectively but much lower than the 100% reported by Steichman and Gescheidt. (Streichman and Gescheidt, 1998) Park et al(Park et al., 2014a) reported a specificity of 76.7% and found that the test was unable to discriminate between HS and iron deficiency anaemia. Higher specificities of between 90 and 95% were reported by Striechman and Grescheidt(Streichman and Gescheidt, 1998) and Crisp et al(Crisp et al., 2011). In the latter studies, specificity was largely determined by comparing HS patients and their asymptomatic relatives. In this study we compared known HS patients to patients with confirmed autoimmune haemolysis and one symptomatic patient with negative membrane studies. The difference in patient profiles may explain the discrepancy in specificity. Furthermore, as mentioned before, this study is limited by the small sample size. The combination of EMA-binding test and CHT correctly identified all positive but only two out of six negative cases. Despite the low specificity, the sensitivity of this combination is higher than that of the combination of EMA-binding test and FOFT and the FOFT and CHT which had sensitivities of 94.4% and 88.9% respectively. The combination of EMA-binding test and FOFT also had a specificity of 80% while the CHT and FOFT had a specificity of just 50%. Different to our results, in a study by Crisp et al, it was the combination of the EMA-binding test and FOFT that identified all positive cases while the combination of the EMA- binding test and CHT had a sensitivity of 93.5%. Conclusion The EMA-binding test performed considerably better than the CHT and the FOFT. We recommend that for future cases of suspected HS, serial testing be performed with the EMA-binding test as the first line test. If this test is positive, the patient has HS. If this test is negative, a second test will be performed. If both tests are negative, the diagnosis is unlikely. Since the combination of the EMA- binding test and CHT test had the highest sensitivity, the CHT is the recommended second test. Ethical Aspects Approval to conduct the research was obtained from the Ethics Committee of the Faculty of Health Sciences, University of the Free State (Ethics clearance number: UFS- HSD2019/1322/2611). This study was executed in accordance with the declaration of Helsinki (1964) as amended in Tokyo (2008). Informed consent was obtained from the patients in the positive control group. Limitations Due to the relative rareness of HS in the patient population at the UAH, the sample size of confirmed HS cases was small. Additionally, only a few cases of confirmed autoimmune haemolytic anaemia were identified during the study period, which resulted in a small sample size of true negative controls. This limitation reduces the likelihood of statistically significant results and necessitates cautious interpretation. Ideally, the normal controls used for the EMA-binding test should have been healthy subjects. Unfortunately, since six such controls were required for each sample tested, it would have been challenging to ensure healthy volunteers for each test. Therefore, normal controls were randomly selected from samples submitted for full

blood counts from inpatients or patients attending clinics. Whether the medical conditions these patients are afflicted with, affect the fluorescence intensity of the control group is not known. The literature shows that the acid glycerol lysis test has good sensitivity and specificity when performed on its own and, when combined with the EMA-binding test, specificity and sensitivity are superior to most other test combinations. (Bianchi et al., 2012) Since this test is not performed at our laboratory, it was not included in the study but may be considered in future studies.
Funding This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Competing interests The authors have no conflict of interest to declare. Acknowledgements All laboratory preparation and analyses were performed by the staff of the Immunology and Flow cytometry benches in the Department of Haematology and Cell Biology, National Health Laboratory Service (NHLS), in Bloemfontein, South Africa. Patient records and contact details were provided by Prof DK Stones and Prof MJ Coetzee.

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Acknowledgements: Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution. Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named.
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