Genotype-to-phenotype correlations in hereditary spherocytosis

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1. Abstract

The heterogenous nature of hereditary spherocytosis (HS), both genetically and regarding clinical expression, complicates the prediction of disease severity on an individual level. In this retrospective cohort study collected data used for diagnostics of 197 HS patients referred to University Medical Centre Utrecht (UMCU, the Netherlands) was used to study genotype-to-phenotype correlations in HS. From the included haematological parameters, Laser assisted Optical Rotational Cell Analyzer (LoRRca MaxSis) Osmoscan profiles and other diagnostic tests, the parameters red blood cell distribution width (RDW; r= 0.692, *P*= 0.000), maximal elongation index (EImax; r=-0.559, *P*= 0.000) and eosin-5'-maleimide assay (EMA; r=-0.433, *P*= 0.000) appeared to be the best predictors of clinical severity, respectively. Genetic analysis has identified a causative genetic defect for 185/197 (93.3%) of the patients included. With disease severity classified according to Eber's classification system (1990), no direct relationship between genotype and clinical severity could be detected (χ^2, *P*= 0.054). Nevertheless, both haematological parameters and Osmoscan parameters -correlated to disease severity though not included in this classification system- imply that SPTB-HS patients have a predominantly more severe clinical picture and SLC4A1-HS patients are generally less severely affected. Apart from the exploration of genotype-to-phenotype correlations in this cohort, a side study was performed on the carriers of Low Expression alleles Lyon (α^{LELY}) and PRAgue (α^{LEPRA}) to gain insight on the pathogenicity and inheritance patterns of these alleles. Finally, a first step has been taken in characterizing the subpopulation of HS patients showing distinctively different, non-classical, Osmoscan profiles identifiable by an increased Ohyper, and here defined as overhydrated HS.

2. Samenvatting

Deze scriptie gaat over hereditaire (erfelijke) sferocytose (HS). Dit is een vorm van bloedarmoede, wat betekent dat er te weinig hemoglobine in het bloed zit. Hemoglobine is een eiwit in rode bloedcellen dat ervoor zorgt dat zuurstof naar alle lichaamsdelen wordt gebracht. De lichaamscellen hebben zuurstof nodig om goed hun werk te kunnen doen.

Een rode bloedcel ziet eruit als een rond kussentje met aan beide kanten een holte. Deze vorm wordt behouden omdat elke rode bloedcel van binnen een soort skelet heeft gemaakt van eiwitten, dit heet het cytoskelet. Dit skelet zit verbonden aan het membraan, een dun laagje dat de cel omhult en bijeenhoudt. De samenwerking van dit skelet en het membraan zorgt ervoor dat de cel heel flexibel is, hij kan van vorm veranderen zonder dat hij kapotgaat. Dit is belangrijk om door alle kleine bloedvaatjes in het lichaam te passen zodat alle lichaamscellen van genoeg zuurstof worden voorzien.

Patiënten met HS hebben foutjes in hun DNA, mutaties. In sommige gevallen kan dit plotseling zijn ontstaan, maar meestal hebben ze dit geërfd van (een van) hun ouders. Een mutatie zorgt ervoor dat er niet genoeg eiwit wordt gemaakt of dat er een niet goed werkend eiwit wordt gemaakt. Elk eiwit heeft een eigen stukje DNA. Bij mensen met HS zitten de mutaties in DNA dat hoort bij de eiwitten die het cytoskelet vormen en de verbinding daarvan aan het membraan. Als deze eiwitten niet goed werken, raken de rode bloedcellen stukjes membraan kwijt door de afsnoering van kleine blaasjes. Het totale membraan wordt dus kleiner en de rode bloedcel krijgt de vorm van een balletje, een sferocyt.

Sferocyten zijn veel minder flexibel dan gezonde rode bloedcellen en ze kunnen blijven steken in de kleine bloedvaatjes. De kleinste bloedvaatjes zitten in een orgaan in de buik, de milt. De milt werkt als een zeef. De mismaakte rode bloedcellen blijven erin steken en worden opgegeten door cellen van het immuunsysteem. Er blijven dan minder rode bloedcellen en hemoglobine over voor zuurstoftransport.
Patiënten met HS zijn vaak vermoeid en hebben soms een gele huid. Dit komt door de ophoping van de afgebroken hemoglobine. Er zijn geen medicijnen voor deze ziekte. Wel kan een patiënt een bloedtransfusie krijgen, zodat hij meer gezonde rode bloedcellen krijgt van een andere patiënt. Ook kan de milt worden verwijderd, dit heet splenectomie. Na splenectomie kunnen sferocyten langer in het lichaam blijven, omdat ze niet meer worden weggevangen.

Er zijn veel verschillende eiwitten betrokken bij de flexibiliteit van de rode bloedcellen. Daarom zijn er veel verschillende DNA-foutjes die HS kunnen veroorzaken. Ook is er veel verschil in hoe ziek de patiënten met HS zijn. Sommige patiënten hebben elke maand een bloedtransfusie nodig of moeten al splenectomie ondergaan op kinderleeftijd. Anderen hebben bijna geen klachten.

In dit onderzoek is informatie verzameld van bijna tweehonderd HS-patiënten die zijn verwezen naar het Universitair Medisch Centrum Utrecht (UMCU). In het UMCU is hun bloed onderzocht en zijn de fouten in het DNA bepaald om te kijken of ze inderdaad HS hebben. Met deze informatie wilden we kijken of er verbanden zijn tussen de precieze mutaties in het DNA en wat voor klachten de patiënten hebben.

Een voorbeeld van bloedonderzoek dat is uitgevoerd is de LoRRca Osmoscan. Dit is een meting van de vervormbaarheid van rode bloedcellen. Door het uitvoeren van deze meting ontstaat een grafiek. Aan de vorm van deze grafiek kan je herkennen of iemand HS heeft of niet. Wij hebben gekeken of er een verband is tussen de mutaties en de vorm van de LoRRca Osmoscan-grafiek. Zo konden we aantonen dat de maximale vervormbaarheid bij sommige mutaties hoger is dan bij anderen.

Er is ook hematologisch bloedonderzoek gedaan. Hierbij wordt er gekeken naar het aantal en de vorm van verschillende typen bloedcellen en de hoeveelheid hemoglobine in het bloed. Omdat de patiënten met HS zulke diverse klachten hebben, hebben wij gekeken of er bloedwaarden zijn die kunnen voorspellen hoe ernstig ziek iemand is. Normaal zijn rode bloedcellen allemaal ongeveer even groot. Het bleek dat patiënten die rode bloedcellen hadden met veel verschillende groottes ernstiger ziek waren.

Tot slot waren er patiënten die wel HS hadden, maar een andere LoRRca Osmoscan-grafiek dan normaal. We hebben gekeken of deze patiënten andere bloedwaarden hadden dan de andere HS-patiënten. Zo zijn we erachter gekomen dat de HS-patiënten met een afwijkende grafiek minder hemoglobine hadden in hun rode bloedcellen. Toch lijkt het er niet op dat zij ook ernstiger ziek zijn.

De informatie over bloedwaarden en mutaties die wij hier hebben beschreven, kan worden gebruikt door doctoren om hun patiënten beter te begrijpen. Hopelijk draagt dit bij aan een beter leven voor alle patiënten met HS.

3. Table of contents

4. List of abbreviations

AD	Autosomal dominant
AQP1	Aquaporin 1
AQP3	Aquaporin 3
AR	Autosomal recessive
ATP	Adenosine triphosphate
B	Benign
EI	Elongation index
EI O290	Elongation index at physiological isotonic osmolality
EIhyper	0.5x the maximal elongation index in the hypertonic region
EImax	Maximal elongation index
EImin	Minimal elongation index in hypotonic region
EMA	Eosin-5'-maleimide assay
Hb	Haemoglobin
HK	Hexokinase
HPO	Hypochromic red blood cells
HPR	Hyperchromic red blood cells
HS	Hereditary spherocytosis
Ht	Haematocrit
IRF	Immature reticulocyte fraction
LB	Likely benign
LDH	Lactate dehydrogenase
LELY	Low expression allele Lyon
LEPRA	Low expression allele Prague
LoRRca	Laser assisted Optical Rotational cell analyser
LP	Likely pathogenic
LPC	Lysolecithin
MAC	Macrocytic red blood cells
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCHr	Mean corpuscular haemoglobin of reticulocytes
MCV	Mean corpuscular volume
MCVr	Mean corpuscular volume of reticulocytes
MIC	Microcytic red blood cells
NGS	Next generation sequencing
O	Osmolality
OFT	Osmotic fragility test
OHSp	Overhydrated hereditary spherocytosis
OHSt	Overhydrated stomatocytosis
Ohyper	Osmolality at 0.5x the maximal elongation index in the hypertonic region
Omax	Osmolality at maximal elongation index
Omin	Osmolality at minimal elongation index in hypotonic region
P	Pathogenic
PK	Pyruvate kinase
PKD	Pyruvate kinase deficiency
RBC	Red blood cells
RDW	Red blood cell distribution width
RET	Reticulocyte
RhAG	Rh-associated glycoprotein
RPI	Reticulocyte production index
TSAT	Transferrin saturation fraction
UMCU	University Medical Centre Utrecht
VUS	Variant of unknown significance
α^{LELY}	α-spectrin LELY
α^{LEPRA}	α-spectrin LEPRA
ΔEI	Difference between EImax and EImin
ΔO	Difference between Ohyper and Omin

5. Introduction

Hereditary spherocytosis (HS), also known as Minkowski Chauffard syndrome, is the most common type of congenital haemolytic anaemia in Western Europe and North America. Depending on the degree of severity in which patients are considered cases, the estimated prevalence ranges from 1:2000 to 1:5000 in the Caucasian population (1).
Biochemically, HS is hallmarked by a disruption of the complex physiology of the red blood cell (RBC) membrane, due to mutations in genes encoding the involved proteins.

5.1. Anatomy of the healthy red blood cell

To provide RBCs with elasticity to circulate through the narrowest blood vessels of the human body, yet being also resistant to the shear stress they concurrently experience, their cell membranes are uniquely constructed. The required sturdy, yet flexible membrane consists of an intracellular skeleton and an overlying lipid bilayer. The intracellular cytoskeleton contains α- and β-spectrin heterodimers, protein 4.1 and actin filaments. Proteins embedded in the outer lipid bilayer are anchored to this construct via ankyrin and 4.1R protein complexes (2). The ankyrin complex mainly consists of blood group antigens (Rh, RhAG), a thrombospondin receptor (CD47) and a band 3 tetradimer that also functions as an anion exchanger (3–5)(Figure 1). The RhAG and band 3 proteins interact with intracellular protein 4.2 connected to the cytoskeleton through ankyrin. The 4.1R complex consists of other blood group antigens linked to intracellular protein 4.1 and a band 3 dimer which is connected to adducin (6). This interplay of the cytoskeleton and the phospholipid membrane grounds the typical eight micrometre-wide biconcave disk shape of healthy RBCs and supports the deformability needed during the repeated passages through two-to-three micrometre capillaries of the microvasculature (7).

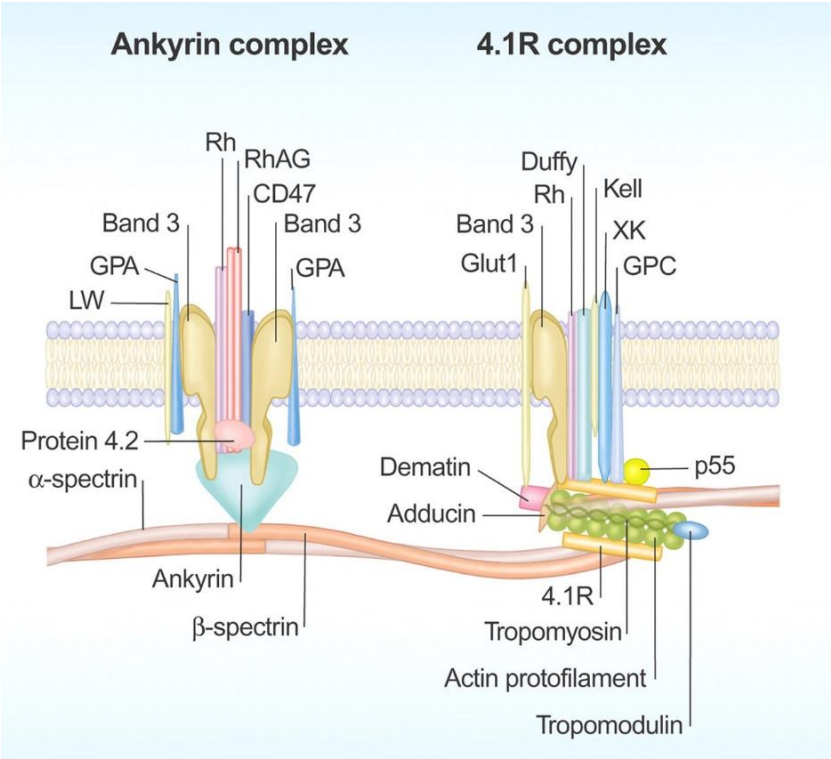


Figure 1 Schematic representation of the red cell membrane. Original illustration by Paulette Dennis. Retrieved from (8).

5.2. Molecular and physiological pathophysiology of hereditary spherocytosis

HS is a disorder of reduced RBC deformability predominantly caused by pathogenic mutations in genes coding for proteins involved in the cytoskeleton and accessory anchoring complexes. The five main genes - and corresponding proteins- associated with HS are ANK1 (ankyrin), SPTB (β-spectrin), SPTA1 (α-spectrin), SLC4A1 (band 3) and EPB4.2 (protein 4.2). Because the synthesis of β-spectrin proteins is the rate-limiting step in cytoskeleton assembly, mutations resulting in dysfunctional or deficient synthesis of this protein are clinically apparent in the heterozygous state (9). Contrarily, α-spectrin molecules are synthesized abundantly relatively to the required 1:1 ratio with β-spectrin (10). Therefore, heterozygotes for a mutation in SPTA1 usually do not express characteristics of HS. Although formation of the spectrin-network may proceed normally, mutations associated with ankyrin deficiency often lead to decreased incorporation of spectrin in the membrane, therefore resulting in a deficiency of both spectrin and ankyrin (11). Likewise, since ankyrin and protein 4.2 are connected in the intricate membrane structure, deficiencies in these proteins are often mutually correlated (9). Altogether, mutations in these HS-associated genes and the consequent synthesis of deficient or dysfunctional proteins cause destabilization of the vertical linkages between the cytoskeleton and the overlying membrane (1,12). This intrinsically unstable membrane is prone to micro vesiculation, which causes a reduction in the surface to volume ratio of the affected RBCs. Consequently, the characteristic biconcave disk shape converts into a poorly deformable sphere shape, hence the name spherocytosis (Figure 2). The ability of these abnormal RBCs to pass the narrow splenic vasculature is limited resulting in extravascular haemolysis due to entrapment in the spleen and phagocytosis by resident macrophages (13,14).

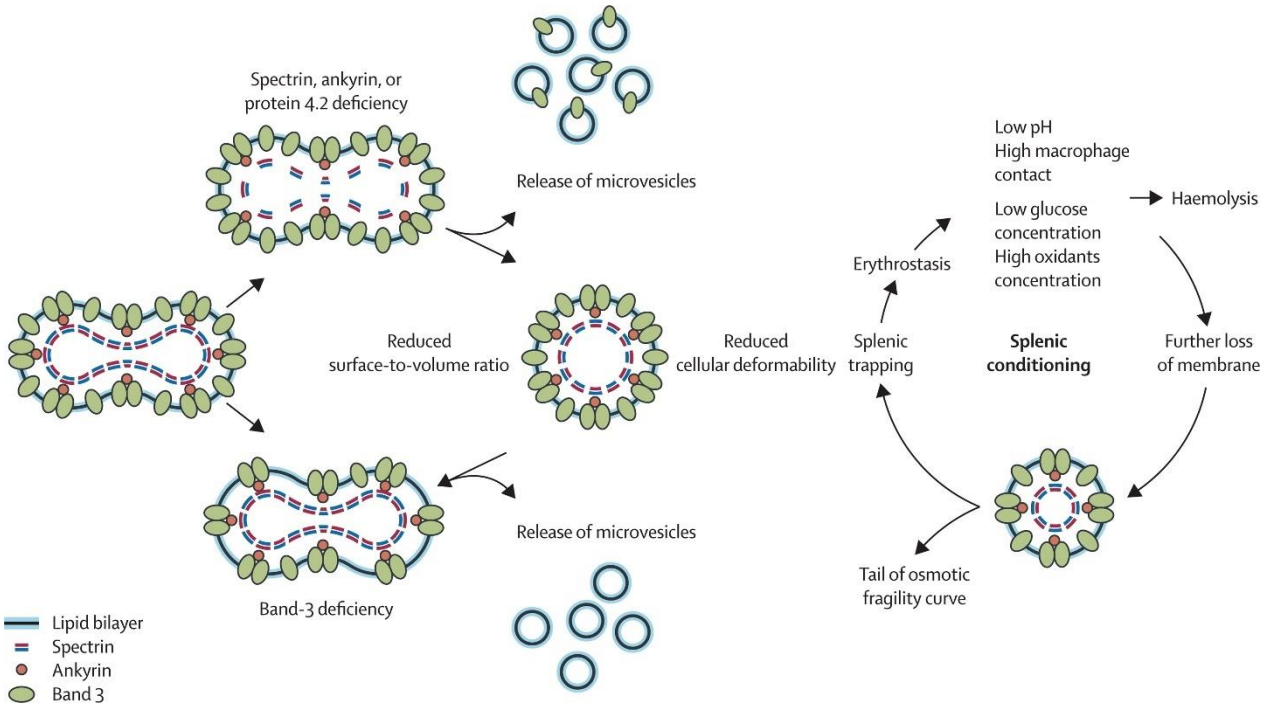


Figure 2 Schematic overview of pathophysiology of hereditary spherocytosis. Retrieved from (1).

5.3. Diagnosis of hereditary spherocytosis

The diagnosis of HS is currently often based on a combination of various diagnostic tools. Essentially examined aspects are family and clinical history, blood smear morphology, laboratory investigations of haematologic and haemolysis-related parameters and *ex vivo* tests of RBC functionality.

5.3.1. Genetic testing

To substantiate the diagnosis of HS, genetic analysis is increasingly performed in daily practice (15). With the rapidly growing knowledge in this field, nowadays next generation sequencing (NGS) can be applied with gene panels specifically targeted on all known genes associated with hereditary haemolytic anaemias (12). Molecular characterization of disease may be especially helpful in atypical HS cases (16,17). Moreover, it is essential in exploring genotype-to-phenotype correlations.

As mentioned earlier, there are five major genes associated to HS which are all included in the NGS panels. The prevalence of mutations in these genes varies for populations of different ancestry. In general, 20% of HS is caused by de novo mutations while 80% of HS is inherited (18). To current knowledge 75% of these mutations are inherited in an autosomal dominant matter. These are almost exclusively SLC4A1, ANK1 and SPTB mutations. Mutations in EPB4.2 and SPTA1 are the 25% assumed to be inherited recessively (11,19). However, for SPTA1 particularly, there is controversy about two common polymorphisms detected in this gene, namely the Low Expression Alleles PRAgue (α^{LEPRA}) and LYon (α^{LELY}).

5.3.1.1. Spectrin-α low expression alleles

Firstly, α^{LEPRA} is characterized by a c.4339-99C>T mutation in intron 30 resulting in the activation of an alternative acceptor splice site and consequent frameshift and premature termination of translation. For a single allele this reduces the residual α-spectrin production to only 16% of normal (20). This polymorphism can be found in approximately 5% of the white population (20). Secondly, the even more prevalent allele α^{LELY} is characterized by the c.6531-12C>T mutation in intron 45 (21,22). This mutation, which is in linkage disequilibrium with c.5572C>G in intron 40, is associated with partial skipping of exon 46 which results in a 50% decrease in α-spectrin production for one allele. This allele can be found in 25.5% of the world population (23).

Regarding the referred patients of recent years, it is unclear if these alleles cause HS in a dominant or recessively inherited manner. Besides, in some patients these alleles seem to be disease causing, whereas other patients are symptom-free carriers of these SPTA1-variants. As a side study we evaluated the presence of these two common polymorphisms in our cohort, and how they were associated with occurrence of HS.

5.3.2. Haematologic parameters

In HS patients, the general blood count is highly dependent on the body's capacity to compensate for the anaemia and the corresponding severity of disease. Typically, anaemia causes decreased haemoglobin (Hb) concentrations. As a compensatory mechanism, the production of erythrocytes can be accelerated, and reticulocytes can be released in the circulation prematurely. Typically, reticulocytosis is reflected by an increased red cell distribution width (RDW) (24). While the membrane surface area of the RBCs is reduced, the mean corpuscular volume (MCV) is usually sustained, resulting in an increased number of hyperdense or hyperchromic cells and an increase in mean corpuscular haemoglobin concentration (MCHC) (25). Furthermore, typical laboratory features of haemolytic anaemia in general are increased activity of lactate dehydrogenase (LDH), increased bilirubin levels in plasma, and decreased (often undetectably low) haptoglobin concentrations.

Lastly, enzyme activity for pyruvate kinase (PK) and hexokinase (HK) had been determined in some patients. These are important enzymes in the glycolytic pathway of RBCs for adenosine triphosphate (ATP) generation and interest in these enzymes has grown in recent years due to the emergence of pyruvate kinase activators for the indication pyruvate kinase deficiency. In addition, it has been shown that patients with HS may have reduced PK-activity and therefore may also benefit from treatment with these drugs. So, when available, data on PK and HK activity has also been collected.

5.3.3. Osmotic fragility test and eosin-5'-maleimide assay

Next to elementary haemocytometric analysis of RBCs, other laboratory tests can be performed to confirm the diagnosis HS. A classic laboratory test for diagnosing HS is the osmotic fragility test (OFT) (Figure 3). Due to the highly flexible membrane structure, healthy RBCs are usually reasonably resistant to changes in osmolality of the environment. On the contrary, RBCs of HS patients are characterized by increased osmotic fragility because of their low surface-to-volume ratio and consequent rigid spheroidal shape. This difference in RBC functionality can be demonstrated with the OFT.

Another test that is typically performed in HS diagnostics is the eosin-5'-maleimide (EMA)-test (Figure 4). In this test, EMA-mediated staining of band 3 proteins in the RBC membrane is used as an indirect measurement of surface area loss in RBCs.

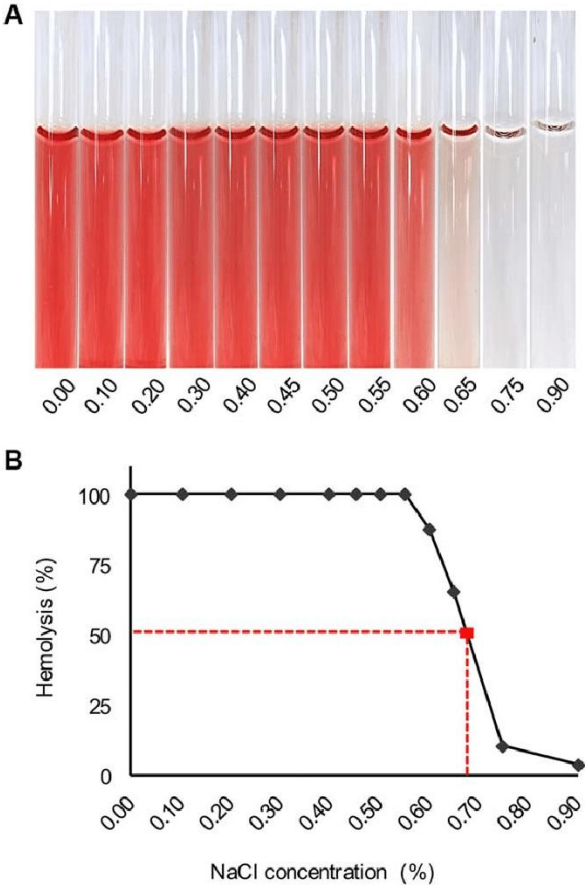


Figure 3 Osmotic fragility test. **A.** Exposure of red blood cells to hypotonic solutions leads to lysis and release of red-coloured haemoglobin. **B.** The point of 50% haemolysis is noted as the final test result. Healthy red blood cells are more resistant to a hypotonic environment and therefore have a lower 50% haemolysis point and a lower OFT test result. Retrieved from (26).

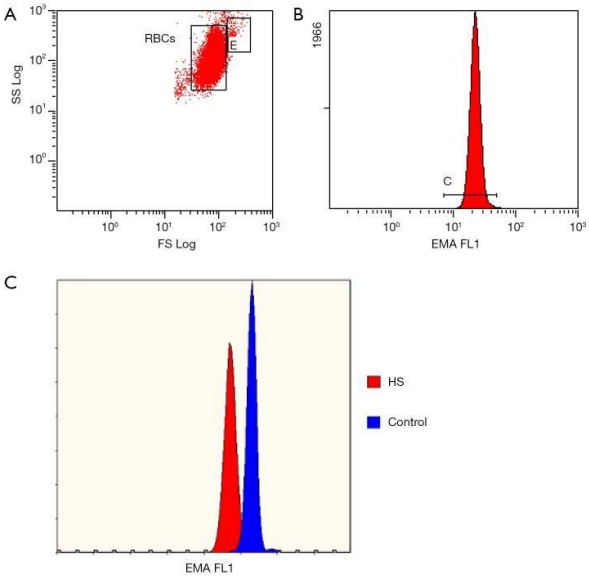


Figure 4 Eosin-5'-maleimide assay. **A.** Red blood cells are sorted with flow cytometry based on forward scatter and side scatter. **B.** The fluorescence of EMA bound to red blood cells is measured. **C.** Comparison of mean fluorescence intensity of an HS-patient compared to a healthy control. Retrieved from (27).

5.3.4. Osmotic gradient ektacytometry

Additionally, osmotic gradient ektacytometry is an increasingly used method to confirm the diagnosis of HS. This highly sensitive test for RBC membrane deformability is currently the golden standard in diagnosing RBC membranopathies. During this analysis, RBCs are exposed to shear stress in a viscous solution at a gradient of different osmotic conditions. The exposure to this shear stress forces light-reflecting RBCs to change into an elongated shape. The varying extent to which the RBCs are able to deform while exposed to the increasing osmotic gradient can be calculated by a fluctuating diffraction pattern of a laser. This procedure can be automatically performed with the laboratory instrument LoRRca MaxSis (Laser-assisted Optical Rotational Red Cell Analyzer, RR Mechatronics, Zwaag, The Netherlands). In the LoRRca, the vertical axis (A) and horizontal axis (B) of the diffraction pattern are used to calculate the elongation index by the formula (A-B)/(A+B), reflecting the RBC-deformability of the total population (28). The graphical presentation of the elongation index (EI) over the increasing osmolality (O) is called the Osmoscan.

Along with this, the software included in the LoRRca's Osmoscan module automatically calculates several diagnostically important parameters: Omin, corresponding to the osmolality at the minimal deformability (EImin) in the hypotonic region; EImax, the maximal EI of the total curve; O EImax, the osmolality at EImax; and Ohyper, the osmolality corresponding to 50% of the EImax in the hypertonic region; and the area under the curve (AUC) (29).

A typical Osmoscan profile of a healthy subject is provided in Figure 5. The Omin reflects the osmolality at which 50% of the RBCs are lysed in the classical osmotic fragility tests. An increased, right-shifted value of Omin represents a decline in the surface-to-volume ratio as typically seen in HS (Figure 6). With an increasing osmolality the deformability of RBCs then improves and reaches the EImax at normal physiological value. It then decreases again due to further increasing salt concentrations in the hypertonic region that negatively impact deformability. The Ohyper reflects the hydration status of the RBCs. On the one hand, a right-shifted Ohyper means that the RBCs are overhydrated. On the other hand, a decreased Ohyper reflects a relatively dehydrated RBC population. Typically, the rigid spheroidal RBCs of HS patients show an Osmoscan profile with an increased Omin, a decreased EImax and a decreased Ohyper (30).

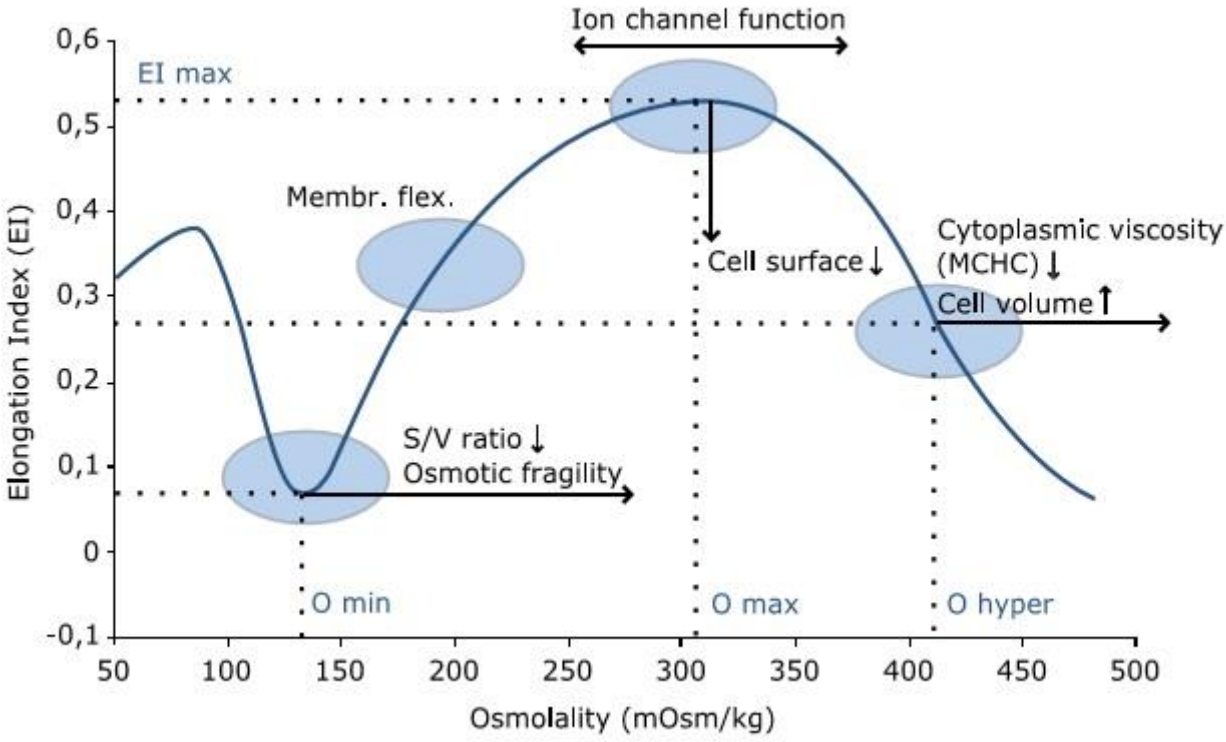


Figure 5 Classic parameters of an Osmoscan curve (28).

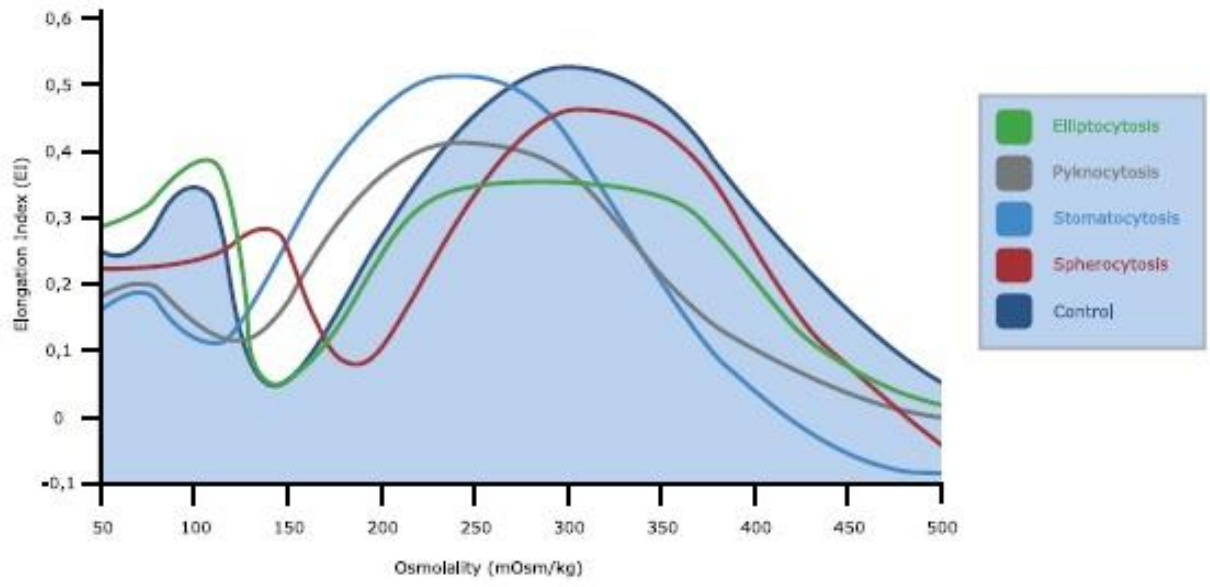


Figure 6 The characteristic Osmoscan profiles of different haemolytic anaemias make the Osmoscan a useful tool in diagnosing HS (28). A typical HS Osmoscan {red line} can be recognized by the increased Omin, decreased EImax and decreased Ohyper compared to healthy controls.

5.4. Clinical presentation of hereditary spherocytosis

Clinically, HS manifests in various ways, ranging from dependence on frequent blood transfusions to sustain a sufficient haemoglobin (Hb) level to completely asymptomatic, well-compensated anaemia. Besides, some patients might only be diagnosed with haemolytic anaemia because of a sudden additional condition such as the occurrence of an infection, substantial blood loss or pregnancy (1,31). Symptomatic patients might experience the classical triad of jaundice due to hyperbilirubinemia, splenomegaly, and pallor (1,13). The high turnover of red blood cells and associated accumulation of bilirubin leads to gallstones and consequent cholelithiasis in some patients. Consequently, common treatments in more severely affected HS patients are splenectomy and/or cholecystectomy (32). Regular blood transfusions may be necessary to maintain sufficient haemoglobin levels in severely affected patients.

5.4.1. Classification of disease severity

To predict the benefits of splenectomy in HS patients and to provide guidelines on the performance of this procedure, Eber and colleagues previously created a classification system of disease severity (Table 1). Patients are classified as mild, moderate, or severely diseased based on Hb concentration, percentage of reticulocytes (as a fraction of absolute RBC count), bilirubin concentration and the reticulocyte production index (RPI) (33). Moreover, spectrin content of the RBCs, osmotic fragility, and autohaemolysis of RBCs are included in this stratification method. Nowadays, this method of objectively scoring disease severity is still used in clinical practice (32). However, a number of methods has been disregarded in diagnostic practice (e.g., spectrin content) and other have been introduced (Osmoscan, EMA). Moreover, DNA analysis is now also available in most expertise centres.

Table 1 Classification of spherocytosis and indication for splenectomy. Retrieved from (33). The category Trait includes symptom-free relatives of patients with recessively inherited disease and are not included in the cohort studied here. † Normal with relation to age category. ‡ Values before blood transfusion. * Normal (mean ± SD): 226 ± 54 · 10^3 molecules per cell.

	Trait	Mild spherocytosis	Moderate spherocytosis	Severe spherocytosis‡
Haemoglobin (g/L)	Normal†	110 – 150	80 – 120	60 - 80
Reticulocyte count (%)	≤ 3	3.1 – 6	≥ 6	≥ 10
Bilirubin (µmol/L)	≤ 17	17 – 34	≥ 34	≥ 51
Reticulocyte production index (-)	< 1.8	1.8 – 3	> 3	
Spectrin per erythrocyte (% of normal)*	100	80 – 100	50 – 80	40 – 60
Osmotic fragility				
• Fresh blood	Normal	Normal to slightly increased	Distinctly increased	Distinctly increased
• Incubated blood	Slightly increased	Distinctly increased	Distinctly increased	Distinctly increased
Autohaemolysis				
• Without glucose (%)	<10	≥ 10	≥ 10	≥ 10
• Correctability (%)	> 60	> 60	0 – 80	50
Splenectomy	Not necessary	Usually not necessary during childhood and adolescence	Necessary during school age before puberty	Necessary, possibly not before year 3 of life

5.5. Research question

This retrospective cohort study of 197 HS patients is performed to improve the understanding of the complex genotype-to-phenotype correlations of this highly heterogenous disease. The study includes [1] the exploration of correlations between haematologic parameters and Osmoscan parameters to disease severity; [2] the comparison of haematologic parameters and Osmoscan parameters of patients according to their underlying genetic mutation; [3] an attempt to shed light on the α-spectrin alleles α^{LELY} and α^{LEPRA} and the corresponding presentation of new cases; and [4] a detailed analysis of a subgroup of HS patients with a distinctive Osmoscan profile from the classical HS profile. [5] Moreover, next to the analysis of classic Osmoscan parameters, some novel Osmoscan parameters are proposed. Altogether, these analyses contribute to unravelling the underlying mechanisms behind this clinically diverse disease.

6. Methods

6.1. Study population

The data examined in this retrospective cohort study originates from diagnostic tests performed between January 2012 and April 2023 on patients with a clinical background suggestive of HS. These patients were referred to University Medical Centre Utrecht (UMCU, The Netherlands) for genetic analysis and confirmation of the diagnosis HS. Patients were included in this cohort if they had a clinical diagnosis of HS based on a composite of available tests and if genetic testing was performed. The methods used in recent years for diagnosing HS are clinical/family history, visual examination of peripheral blood smears, eosin-5'-maleimide binding test (EMA), osmotic fragility test (OFT), osmotic gradient ektacytometry (Osmoscan) and (haematologic) parameters indicative of haemolytic anaemia.

For all included patients, all available data on pre-specified clinical characteristics, genetic analysis, haematologic parameters, EMA and OFT test results and Osmoscan parameters have been collected. Haematologic and Osmoscan parameters obtained in patients before reaching the age of 1 were excluded based on the distinct reference values for this age-group.

This study was approved by the institutional Research Ethics Board and a non-WMO declaration was granted to the research team by the quality assurance officer of the UMCU.

6.2. Clinical characteristics

For referral of patients for HS-diagnostics at the UMCU, physicians must fill in a standardized form. These forms have been used to collect data on clinical history of blood transfusions, splenectomy, and cholecystectomy. Furthermore, the forms require physicians to provide patients' recent laboratory results on classic haematologic parameters Hb, haematocrit (Ht), RBC count, MCV, mean corpuscular haemoglobin (MCH), RDW and haemolysis-associated parameters bilirubin, haptoglobin, LDH and ferritin. Depending on the completeness of these submitted forms, data on these subjects is included for all patients. During data collection, a distinction was made between parameters obtained before and after splenectomy.

For internal UMCU patients more information was accessed via electronic patient portal HiX 6.1 (Chipsoft, Amsterdam, The Netherlands) and laboratory information system GLIMS 9.9.6 (CliniSys, Gent, Belgium).

6.3. Next-generation sequencing

All patients included in this database were referred to the UMCU for genetic testing and therefore verification of the suspicion of HS. Next Generation Sequencing (NGS) has been performed in the genome diagnostics laboratory of the UMCU. Since this retrospective study includes data from over more than a decade, and genetics are a rapidly developing field of science, the extensiveness of this panel has changed over the years. Gene panel NGS7 that was used in the earlier years consisted of the genes: SPTA1, SPTB, ANK1, SLC4A1, EPB41, EPB42 and RHAG. Genes included in the currently used broader genetic panel NGS 46 are: ABCB6, ABCG5, ABCG8, ADA, AK1, ALAS2, ALDOA, ANK1, ATP11C, C15orf41, CD59, CDAN1, COL4A1, CYB5R3, EPB41, EPB42, G6PD, GATA1, GCLC, GPI, GPX1, GSR, GSS, HBA1, HBA2, HBB, HK1, KCNN4, KIF23, KLF1, NT5C3A, PFKM, PGD, PGK1, PGLS, PIEZO1, PKLR, RHAG, SEC23B, SLC2A1, SLC4A1, SPTA1, SPTB, TALDO1, TPI1 and XK. For all mutations found the clinical significance of these mutations was assessed as [1] Benign (B), [2] Likely Benign (LB), [3] Variant of Unknown Significance (VUS), [4] Likely Pathogenic (LP) or [5] Pathogenic (P) according to the guidelines of The American College of Medical Genetics and Genomics (ACMG) (34). For analysis of the genetic subgroups, patients with mutations in more than one HS-associated gene were categorized according to the mutation most likely to be disease causing.

In principle, genetic testing was only performed on the patients referred by their haematologists. Upon consultation, family members of the patients were sometimes also genotyped to confirm familial occurrence and to discover the inheritance pattern of the mutation.

In a few cases carrier status determination has been performed on one specific gene only, to confirm that the patient carried the same mutation previously detected in HS-affected relatives.

6.4. Osmotic gradient ektacytometry

6.4.1. Classic parameters

Deformability of RBCs was measured with the LoRRca MaxSis (Laser-assisted Optical Rotational Red Cell Analyzer, RR Mechatronics, Zwaag, The Netherlands). This method of osmotic gradient ektacytometry was performed at the Central Diagnostics Laboratory of the UMCU. Whole blood samples (EDTA tubes) were measured within three days of receival at the UMCU according to internal standard operating procedures (SOP). The volume of whole blood sample used for each measurement is standardized to $300 \cdot 10^6$ RBCs per vial based on the results of general hemocytometry. The shear stress is set constant at 30 Pa. During the total scanning time of approximately 315 seconds, the RBCs are exposed to an osmotic gradient from approximately 60 mOsmol/L to 600 mOsmol/L. The parameters automatically included in the Osmoscan's output and the corresponding reference values were recorded for all HS patients for whom an Osmoscan had been carried out. The Osmoscan-procedure of the UMCU specifically was performed as earlier described (35,36)

6.4.2. Novel parameters

In this study novel parameters for the Osmoscan are proposed. These have been calculated in a Microsoft Excel worksheet with the use of the Osmoscan raw data files. Firstly, the ΔO was calculated by subtracting the Omin from the Ohyper. Likewise, ΔEI was calculated by subtracting the EImin from the EImax. These parameters were included to gain insight into the total ranges of deformability on both the x- and y-axis, next to the absolute parameters of maximal deformability. Secondly, the elongation index at physiological isotonicity (290 mOsmol/L; EI O290) was calculated by interpolation of the nearest raw datapoints, to assess whether this is of added value compared to the EImax. Lastly, the Osharpness and Oboldness were calculated. The Osharpness is calculated as the difference in osmolality between the intersection points of y=EImin + 0.05 and the Osmoscan curve. For Oboldness the same method is used for the intersections of y=EImax – 0.05 and the Osmoscan curve. The rationale behind these parameters is to see how much the osmolality has to change to change the deformability of the RBCs away from the extremes.

The novel Osmoscan parameters were calculated and included in the database for all HS patients for whom raw Osmoscan data was available. Visual presentations of the newly proposed Osmoscan parameters are provided in Figure 7 and Figure 8.

The reference ranges of Oboldness and Osharpness have been calculated as mean ± SD using data of 12 healthy controls.

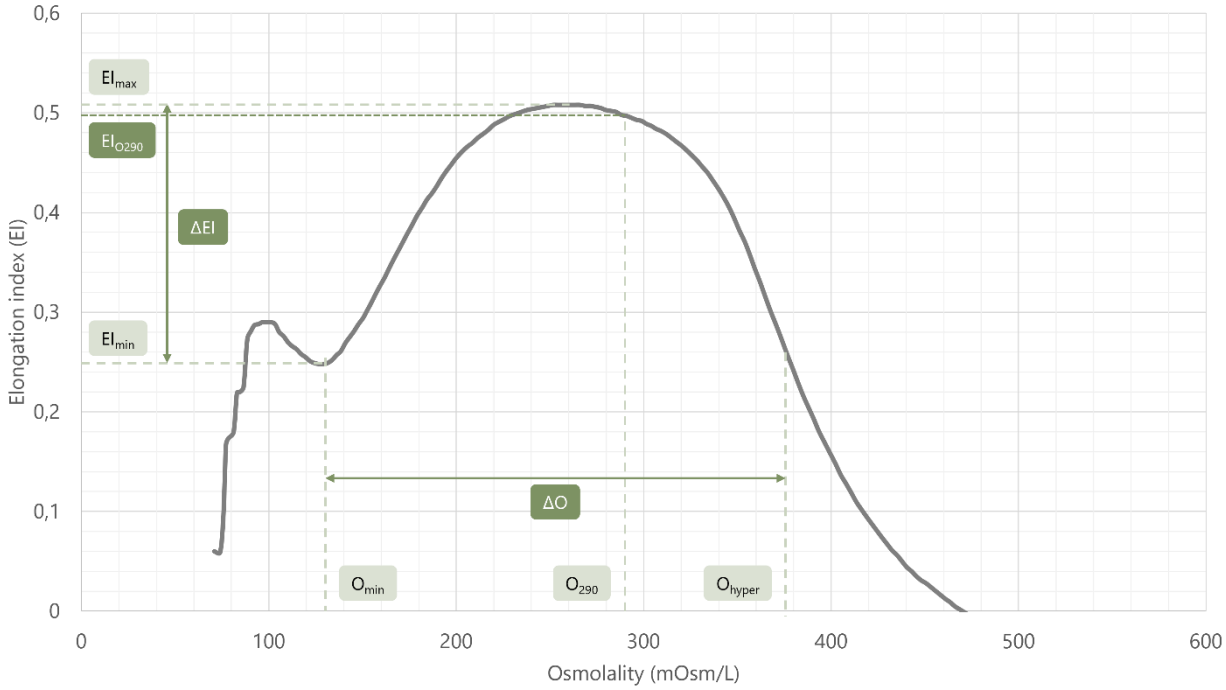


Figure 7 Schematic presentation of newly proposed Osmoscan parameters ΔEI, ΔO and EI O290.

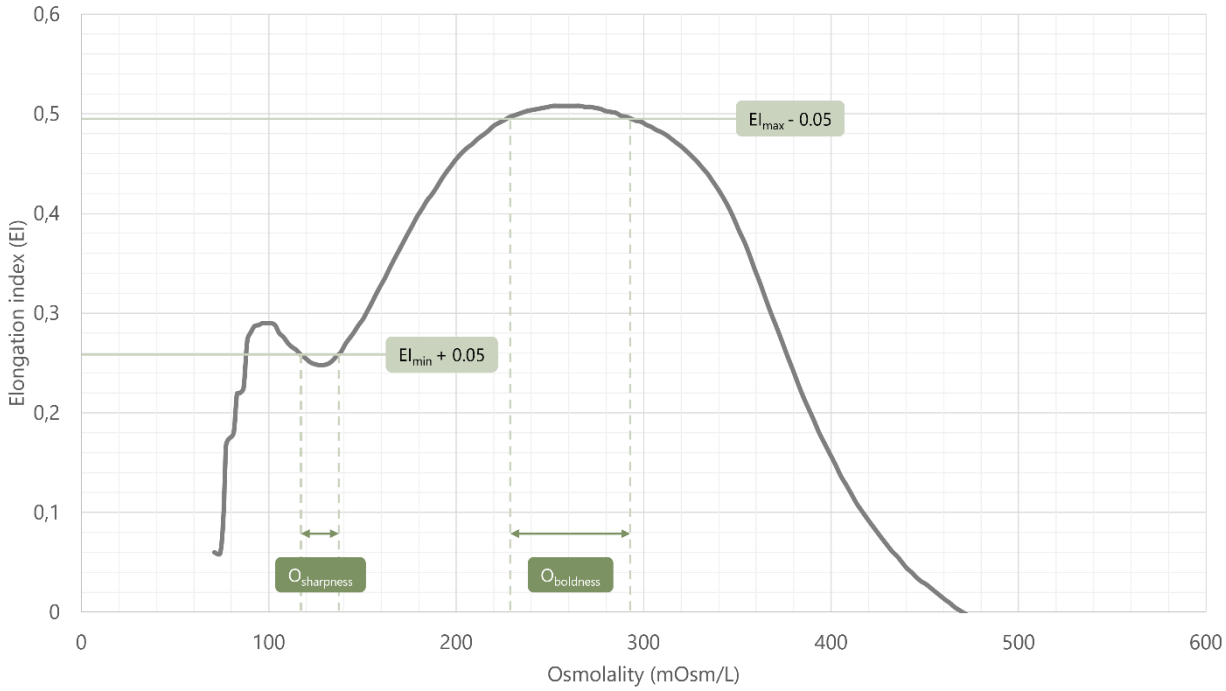


Figure 8 Schematic presentation of newly proposed Osmoscan parameters Osharpness and Oboldness.

6.5. Haematologic parameters

If available, haematologic laboratory parameters provided by the referring institutes were included for all patients. Since Osmoscan parameters need to be standardized on RBC count, complete blood count analysis was performed for all incoming samples using the Cell-DYN Sapphire Haematology Analyzer (Abbott Diagnostics Division, Santa Clara, CA, United States of America). Additional information on microcytic RBCs (MIC), macrocytic RBCs (MAC), hyperchromic RBCs (HPO), hyperchromic RBCs (HPR) and reticulocyte parameters was included from these analyses.

6.6. OFT and EMA

If requested by the referring haematologist, additional diagnostic tests OFT and EMA were performed at the Central Diagnostics Laboratory (CDL) of the UMCU following institutional protocols. For (pre-incubated) OFT the NaCl concentration (%) is documented at which half of RBCs are lysed compared to physiological conditions (37,38). This point is determined by spectrophotometric measurement of the Hb released from lysed RBCs in decreasing salt concentrations. For this test, pre-incubated blood (24 hours at 37 °C) whole blood from lithium-heparin tubes is used, whereas for the EMA-test, whole blood from EDTA-tubes is required. The latter test is based on flow cytometric analysis of the mean channel fluorescence of EMA mainly bound to the 430th residue (lysine) on the first extracellular loop of band 3 (39,40). This gives insight in the reduction of this protein in the affected RBC membrane.

6.7. Statistical analyses

Statistical analysis was performed using IBM SPSS Statistics 27 software and GraphPad Prism 9. Comparisons between two groups were made with independent samples *t*-tests or Mann-Whitney U tests for continuous variables when appropriate. Likewise, multiple comparisons were performed by one-way ANOVA or Kruskal-Wallis. For post-hoc analyses, Bonferroni adjusted *P*-values for multiple comparisons are provided. Categorical parameters have been examined with Chi-square testing. Spearman's correlation coefficients and corresponding *P*-values are given for haematologic parameters or Osmoscan parameters and disease severity. When appropriate, Pearson's or Spearman's correlation was used to determine correlations of haematologic parameters with Osmoscan parameters. Statistical significance was set at a two-sided $P<0.05$.

7. Results

7.1. General characteristics of study population

Our cohort included 197 patients referred to UMCU for diagnostic conformation of HS between January 2012 and April 2023. Clinical characteristics (Table 2) and laboratory parameters (Table 2) of these patients are provided. Full splenectomy was performed in 43 patients at the median age of 13 years old (IQR 9-19). In some patients splenectomy was indicated but not yet performed, because they either refused to have the surgery at all (n= 5), they wanted to wait if the surgery would still be necessary in the future (n= 2), or because they were too young or insufficiently vaccinated to have the operation (n= 5). 15 patients underwent both removal of the spleen and the gallbladder, of which 10 (66.7%) underwent these procedures simultaneously. In total cholecystectomy was performed in 29 patients at the median age of 13 years old (IQR 12-19).

With genetic analysis, a presumed causative mutation could be identified in 185 patients (93.9%), for 12 patients (6.1%) no mutation could be detected in the HS-associated genes. 76 (41.1%) of the total were missense mutations and 109 (58.9%) were non-missense. These proportions were similar for all genetic subgroups individually. The most mutations have been found in the ANK1 gene (n= 57; 28.9%), followed by SPTA1 (n= 47; 23.9%), SPTB (n= 46; 23.4%) and SLC4A1 (n= 34, 17.3%) (Figure 9). For the SPTA1 group, this also includes HS-diagnosed patients with either single α^{LEPRA} or α^{LELY} alleles and no detected mutation in the other major HS-associated genes. For only one patient (0.5%) a mutation was detected in EPB42. Non-missense mutations were relatively more prevalent in SPTB than in the other genes (χ^2, P= 0.000).

29 patients had mutations in a second HS-associated gene, mostly reported as VUS or LB. These included 11/46 (23.9%) SPTB-HS patients, 7/47 (14.9%) SPTA1-HS patients, 7/57 (12.3%) ANK1-HS patients, and 4/34 (11.8%) SLC4A1-HS patients.

Three specific disease-causing mutations were each identified in at least two patients who are not known to be related. Notably, all these mutations were detected in SPTA1: c.[5791C>T;5572C>G;6531-12C>T] p.[(Gln1931*);(Leu1858Val);(?)], c.[1462G>A] p.[(Val488Met)] and c.[83G>A] p.[(Arg28His)].

Most of the detected mutations were inherited in an autosomal dominant (AD) manner (131/185; 70.8%), and in 22 patients (11.3%) it was evident that the mutations were autosomal recessively (AR) inherited. These apparent AR mutations occurred significantly more in SPTA1 than in ANK1 (χ^2, Bonferroni adjusted P= 0.000). Furthermore ANK1, SLC4A1 and SPTB all showed significantly more AD inherited mutations compared to SPTA1 (χ^2, Bonferroni adjusted P= 0.000 for each comparison). Genetic analyses in some of the patients' relatives verified the occurrence of 3 de novo mutations (1.6%), of which two in ANK1 and one in SPTB. For 29 patients (15.7%) the inheritance pattern could not be confirmed because family members were not available for genetic analysis.

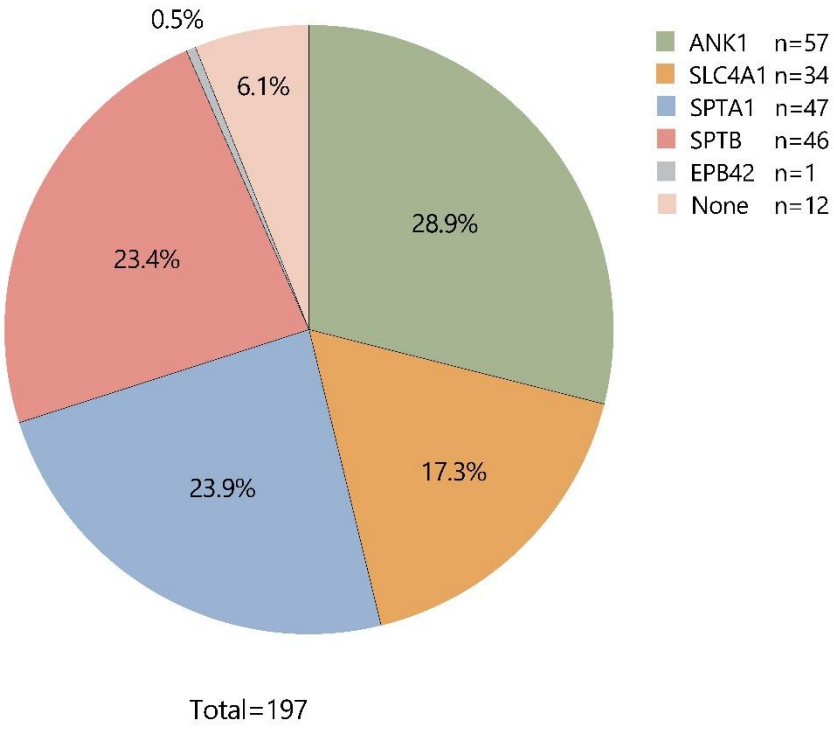


Figure 9 Overview of mutated genes. Categorization of patients diagnosed with HS according to their underlying genetic mutation.

Table 2 Clinical characteristics of baseline cohort. † Age at time genetic analysis was performed in UMCU. ‡ ≥1 blood transfusion received.

		Cohort N= 197
Sex (n, %)	Female	93 (47.2)
	Male	104 (52.8)
Age† (median, IQR)		23 (9-43)
Splenectomy in medical history (n, %)	Yes	43 (21.8)
	Partial	1 (0.5)
	No	130 (66.0)
	No, but indicated	12 (6.1)
	Unknown	11 (5.6)
Cholecystectomy in medical history (n, %)	Yes	29 (14.7)
	No	116 (58.9)
	Unknown	52 (26.4)
Dependency on blood transfusions in medical history‡ (n, %)	Yes	50 (25.4)
	No	79 (40.1)
	Unknown	68 (34.5)

Currently, no pharmacotherapeutic options are available for the indication of HS. Besides blood transfusions to sustain sufficient haemoglobin levels, splenectomy can be indicated to eliminate the extravascular site of haemolysis. The positive effects of splenectomy can be seen in the differences in haematologic parameters of patients before or without and after splenectomy (Table 3). The means of the parameters Hb, Ht, RBC count, immature reticulocyte fraction (IRF) are all restored to within reference range after splenectomy compared to before/without splenectomy. An improvement in the haemolysis-related parameters could be observed as well. These observations are in line with previous work from Berrevoets *et al.* (2021) who performed a longitudinal case-control study on HS patients undergoing splenectomy (35).

Table 3 Haematologic and haemolysis-related parameters of baseline cohort. A distinction was made for patients before or without splenectomy and patients on which splenectomy had been performed.

	Reference values	Before or without splenectomy n= 167		After splenectomy n= 30	
		Mean	SD	Mean	SD
Haematologic parameters					
Haemoglobin (g/dL)	13.7 – 17.2	11.9	2.4	14.5	2.0
Haematocrit (L/L)	.41 – .50	.33	.07	.42	.06
Red blood cell count ($\cdot 10^{12}$/L)	4.20 – 5.50	3.85	.68	4.93	.75
Mean corpuscular volume (fL)	80 – 97	87	9	86	10
Mean corpuscular haemoglobin (fmol)	1.75 – 2.25	1.92	.20	1.85	.21
Red blood cell distribution width (% CV)	10.5 – 13.5	18.2	5.1	15.6	4.6
Microcytic RBCs (%)	.33 – 1.28	7.37	8.71	7.04	15.11
Macrocytic RBCs (%)	0 – 2	3.86	4.22	2.95	4.99
Hypochromic RBCs (%)	0.2 – 3.16	5.78	9.69	3.66	7.19
Hyperchromic RBCs (%)	0 – .5	8.38	8.02	8.12	5.89
Absolute reticulocyte count ($\cdot 10^{9}$/L)	25 – 120	344.8	205.6	184.8	113.8
Immature reticulocyte fraction (-)	.09 – .31	.36	.11	.17	.10
Haemolysis-related parameters					
Ferritin (ug/L)	20 – 150	240	300	156	154
Transferrin saturation fraction (-)	.25 – .60	.34	.20	.29	.13
Total bilirubin (µmol/L)	3 – 21	50	35	20	12
Haptoglobin (g/L)	.3 – 2	.11	.15	.52	.59
Lactate dehydrogenase (IU/mL)	0 – 325	271	104	243	81

7.2. Correlations with clinical severity of hereditary spherocytosis

7.2.1. Classification of clinical severity

The disease severity of all patients in this cohort with available data on at least one of the parameters Hb, bilirubin, reticulocyte percentage or RPI has been determined according to Eber's classification (33). Among the patients before or without splenectomy, 57 (41.0%) were classified as mild cases, 66 (47.5%) as moderate and 16 (11.5%) as severe. Splenectomy and cholecystectomy were performed significantly more often in severely compared to mildly affected patients (Bonferroni adjusted P= 0.000 for both comparisons). Blood transfusion occurred more in both groups with moderate (Bonferroni adjusted P= 0.006) and severe (Bonferroni adjusted P= 0.000) disease compared to the milder affected patients.

7.2.2. Haematologic parameters

Correlations were investigated between phenotype and underlying genetic mutation, haematologic parameters and EMA and OFT test results. Because splenectomy affects most of these factors, only data from patients without or before splenectomy (n= 139) is included. All correlations coefficients are provided in Appendix 1 Table 6 to Table 9.

As to be expected, the laboratory markers for haemolytic anaemia included in Eber's classification system were found to be significantly different between the different phenotypes (Appendix 1, Table 5). Among these parameters is the percentage of reticulocytes which shows a strong positive correlation to disease severity (r= 0.746, P= 0.000). Accordingly, the absolute reticulocyte count is also positively correlated to the severity of HS (r= 0.679, P= 0.000) (Figure 10A). Another reticulocyte parameter that increases in more severely affected patients is the IRF (r= 0.556, P= 0.000); indicating a larger contribution of very young reticulocytes in the total blood count (Figure 10B). The presence of many and relatively young reticulocytes illustrates an appropriate compensatory reaction to the more severe anaemia. This severe anaemia is also reflected by a higher percentage of MIC (r= 0.585, P= 0.000), because of a higher extent of membrane shedding (Figure 10C). The combination of many relatively large reticulocytes and microcytic cells gives a more heterogenous population of RBCs, reflected by the positive correlation of disease severity to the RDW (r= 0.692, P= 0.000) (Figure 10D).

Next to determining these haematologic parameters, blood samples were used to perform EMA and OFT in 106 and 41 patients, respectively. The EMA-mediated analysis of membrane-bound proteins was found to be negatively correlated to disease severity (r= -0.433, P= 0.000), again indicating that membrane vesiculation is more prominent in severe non-splenectomised cases of HS (Figure 10E). Confirmingly, OFT was found to be positively correlated to disease severity (r= 0.476, P= 0.002) (Figure 10F). Which means that RBCs from severely affected HS patients are less resistant to increasing hypotonic salt concentrations.

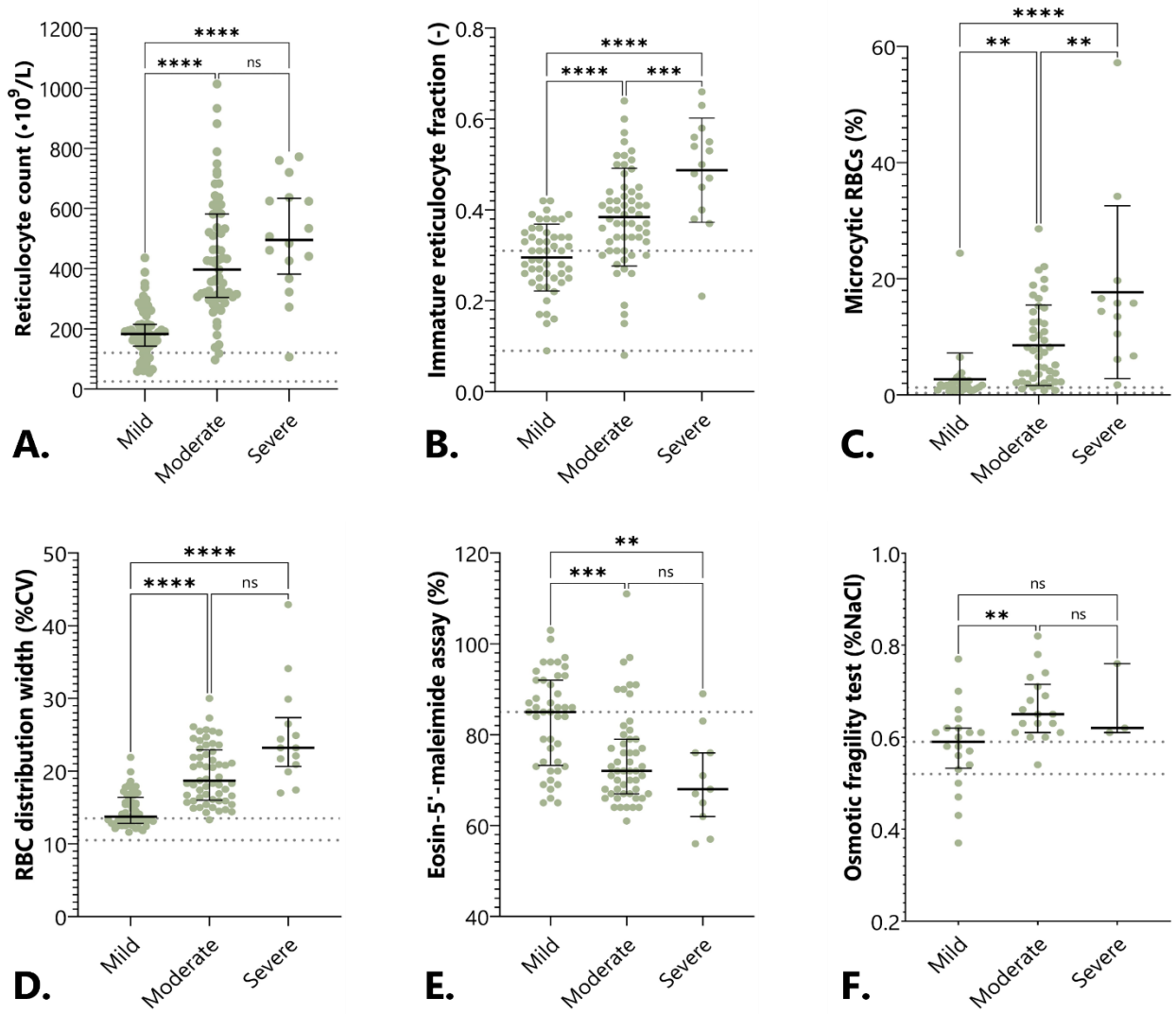


Figure 10 Haematologic parameters according to phenotype of unsplenectomised hereditary spherocytosis patients. Classification of disease severity according to Eber *et al.* (1990) (33). Green dots represent individual measurements. Black horizontal bars represent median with IQR (A, C-F)/mean with SD (B) within phenotype-subgroup. Dotted lines represent reference values used in the UMCU. **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; ns: no significant difference. **A.** Absolute reticulocyte per litre whole blood increases with increasing disease severity. Moderate and severe are not significantly different. **B.** Immature reticulocyte fraction increases with increasing disease severity. **C.** The percentage of microcytic RBCs increases with increasing disease severity. **D.** RDW increases with increasing disease severity. **E.** EMA-binding decreases with increasing disease severity. Reference value for healthy controls >85%. **F.** RBCs of patients with moderate disease severity are less resistant to hypotonic solutions compared to mild disease severity.

7.2.3. Osmoscan and disease severity

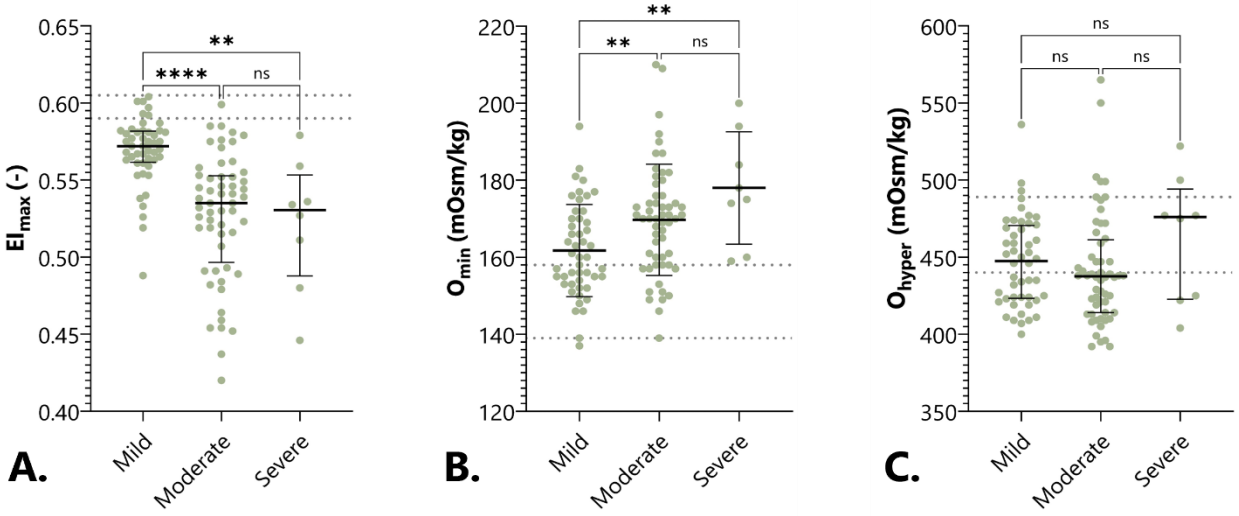
To further validate the use of the LoRRca Osmoscan in diagnosing patients with HS, and exploring its use in predicting disease severity, correlations between Osmoscan parameters and disease severity were evaluated. Again, only data from patients without or before splenectomy were included, i.e., 131 available Osmoscans with corresponding reference values.

Considering the entire cohort, independent of disease severity, the AUC was below the mean of the reference limit for all patients but one (99.2%). The same applies to the EImax, substantiating the distinctive value of these parameters in diagnosing HS. Furthermore, for most patients the Omin and O EI max were above the reference value (86.3% and 79.4%, respectively). The Ohyper was usually lower than the reference limit in the HS patients of this cohort (76.3%).

In comparison of the Osmoscan values and disease severity, the EImax shows the strongest correlation (r= -0.559, *P*= 0.000) to disease severity, meaning that the most severely affected patients have the least deformable RBCs (Figure 11A). With the EIhyper marking exactly half of the EImax, a similar correlation was found. For the Omin which is typically increased in HS-Osmoscans, the observed correlation to disease severity was less prominent (r= 0.346, *P*= 0.000) (Figure 11B). Contrarily, for the Ohyper, which is usually decreased in typical Osmoscan profiles for HS patients no correlation was detected (r= -0.023, *P*= 0.808) (Figure 11C). The observation of a decreased Ohyper in the majority of the Osmoscan profiles, but the absence of a correlation between Ohyper and disease severity shows that this is a suitable parameter for diagnosing HS, but not for subsequent prediction of disease severity.

Regarding the newly explored Osmoscan parameters Osharpness and Oboldness, the found correlations to disease severity are less prominent compared to the classic Osmoscan parameters. But generally, the Osharpness is higher in HS patients compared to control, whereas the Oboldness is usually below the reference range (Figure 11D-E).

All correlation coefficients are provided in Appendix 1, Table 9.



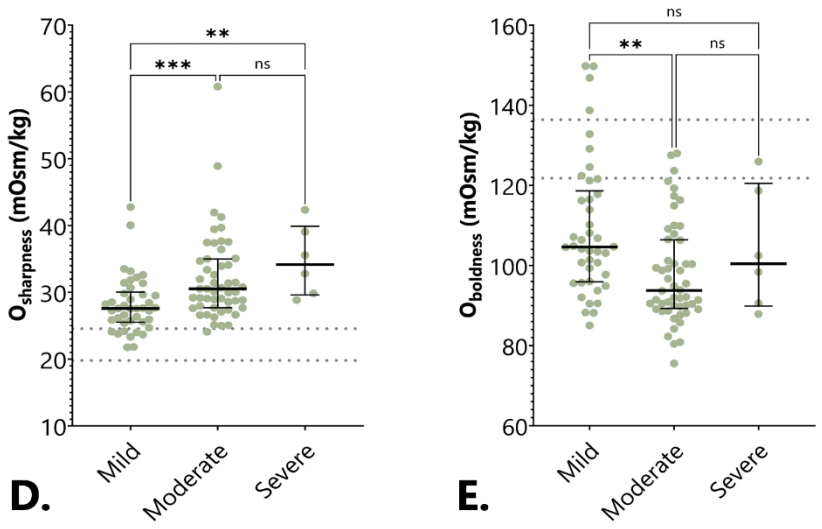


Figure 11 Osmoscan parameters according to phenotype of unsplenectomised hereditary spherocytosis patients. Classification of disease severity according to Eber *et al.* (1990) (33). Green dots represent individual measurements. Black horizontal bars represent median with IQR (A, C, E)/mean with SD (B, D) within phenotype-subgroup. Dotted lines represent reference values used in the UMCU. **** *P*< 0.0001; *** *P*< 0.001; ** *P*< 0.01; ns: no significant difference. **A.** EImax decreases with increasing disease severity. In the severe subgroup, all EImax-values are below reference range. **B.** Omin increases with disease severity. In the severe subgroup, all Omin-values are above reference range. **C.** No significant differences could be found for Ohyper between the phenotype subgroups. **D.** Osharpness gradually increases with increasing disease severity. No reference values are known yet. **E.** Oboldness is significantly lower in moderate disease compared to mild.

Combining the haematologic parameters and Osmoscan parameters correlated to disease severity shows that severe HS is characterized by a heterogenous RBC population with many reticulocytes and microcytic cells that altogether show decreased maximal deformability (EImax) upon ektacytometric analysis. With a similar Hb concentration but a decreased cell volume, these microcytic cells can be detected as hyperchromic cells and represent a population of RBCs with high density. Consequently, a negative correlation was found for these hyperchromic cells and the EImax (r= -0.594, *P*= 0.000) as well (Figure 12A). Moreover, it was found that these hyperchromic cells are negatively correlated to Oboldness (r=-0.773, *P*= 0.000), illustrating a narrower peak around the EImax (Figure 12B). This indicates that these cells relatively quickly lose their ability to deform with an increasing salt concentration in the hypertonic region, which can be explained by their already high intracellular viscosity and limited ability to lose water to the environment.

Diagnostic parameters EMA and OFT have also been compared to the Osmoscan parameters (Appendix 2, Table 11) Both these diagnostic tests correlate to disease severity. EMA is positively correlated to the EI-related Osmoscan parameters, whereas OFT is negatively correlated to these same indicators of decreased deformability. Moreover, OFT was found to be positively correlated to Osharpness (r= 0.652, *P*= 0.000), illustrating the width of the valley around the point of minimal deformability. This indicates that healthy RBCs that are more resistant to lysis in hypotonic solutions have a more quickly increasing ability to deform towards their maximal deformability upon increasing osmotic values. Interestingly, Osharpness stronger correlates to OFT than the Omin, which is generally assumed to reflect the 50% lysis point (Figure 12C-D).

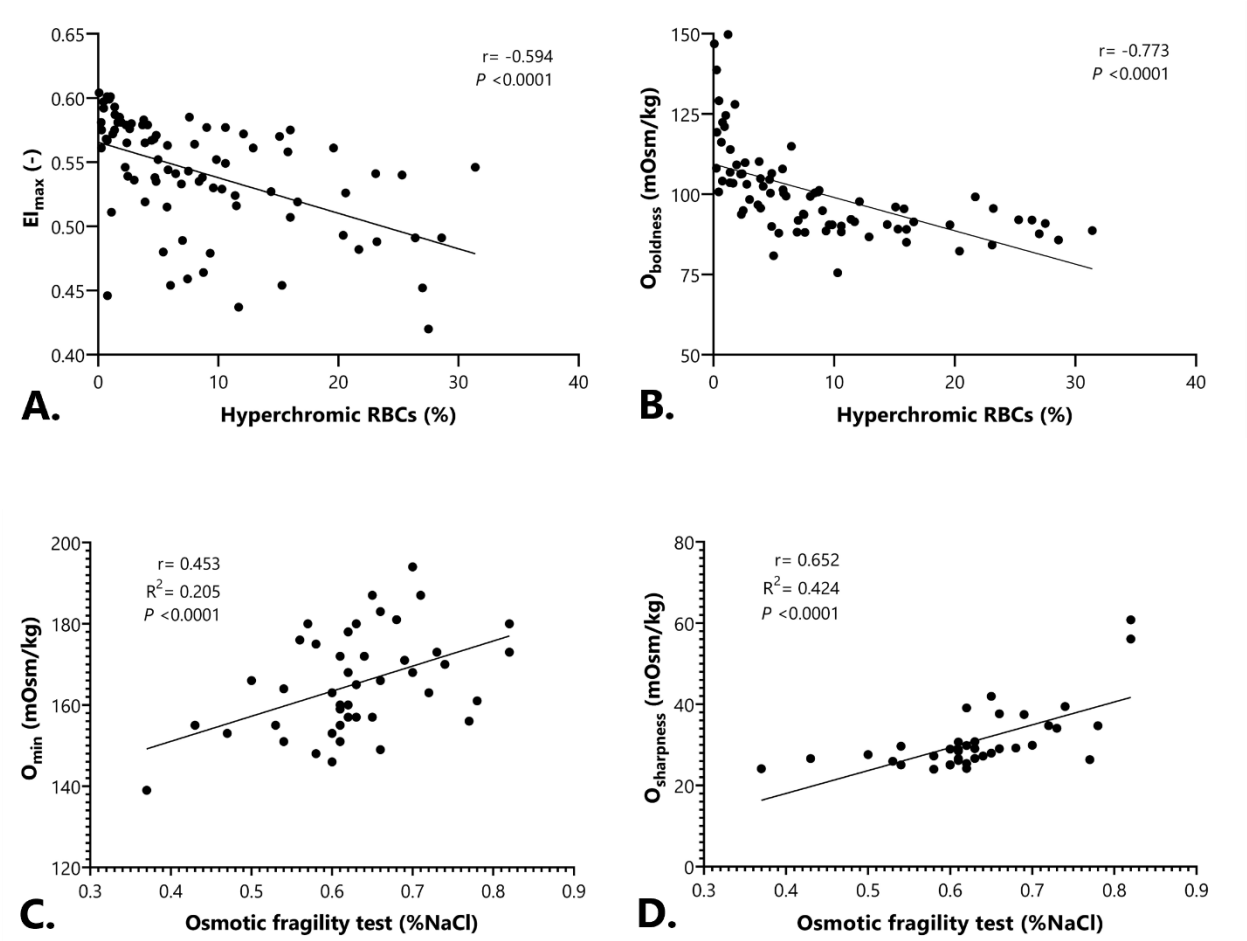


Figure 12 Osmoscan parameters and their relationship to haematological and diagnostic parameters in unsplenectomised hereditary spherocytosis patients. **A, B.** Spearman correlation coefficients for EImax and Oboldness against percentage of hyperchromic red blood cells, respectively. The higher the percentage of hyperchromic red blood cells, the lower the maximal deformability of the RBCs and the narrower the Osmoscan peak around the EImax. **C, D.** Pearson correlation coefficients for Omin and Osharpness. Osharpness shows stronger correlation to the 50% lysis point than the classic Osmoscan parameter Omin.

7.3. Genotype-to-phenotype correlations in hereditary spherocytosis

In exploring genotype-to-phenotype correlations in HS, the genetic subgroups were compared. Because the group of EPB42 consisted of only one patient, this group could not be included in post-hoc analyses. Furthermore, it should be noted that all patients with at least one mutation in SPTA1 were included in this subgroup, including patients with α^{LEPRA} or α^{LELY} alleles.

No differences could be detected in sex, occurrence of splenectomies or cholecystectomies, or receival of blood transfusions. The age at which patients were referred to the UMCU for genetic analysis was significantly lower for ANK1-HS patients compared to SLC4A1 (Bonferroni adjusted P= 0.003) and SPTA1 (Bonferroni adjusted P= 0.014), however it must be noted that this is not per se the actual age at official diagnosis. SPTB patients were genotyped earlier than SLCA4A1 (Bonferroni adjusted P= 0.001) and SPTA1 (Bonferroni adjusted P= 0.006) patients as well. No relationship could be found for the genetic subgroups and disease severity (χ^2, P= 0.054; Figure 13), neither for the occurrence of missense/non missense mutations and disease severity (χ^2, P= 0.554).

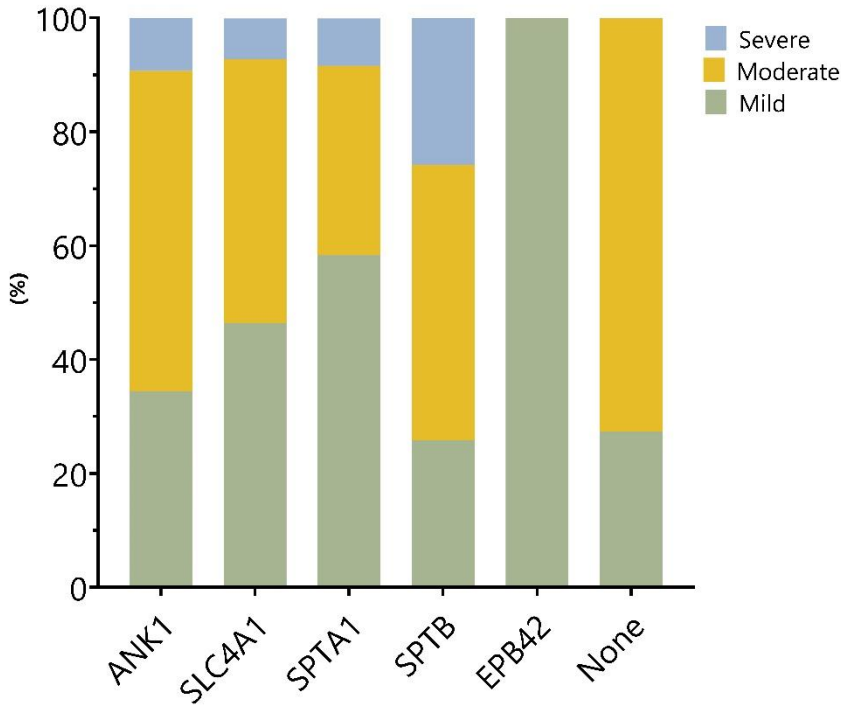


Figure 13 Overview of mutated genes and phenotypes. Distribution of HS phenotypes according to the classification system of Eber *et al.* (1990) (33) categorized by underlying genetic mutation. All data is from patients before or without splenectomy.

7.3.1. Haematologic parameters

Part of the exploration of genotype-to-phenotype correlations within this cohort is the comparison of haematologic parameters for the subgroups of HS patients with different underlying mutations (Appendix 3). Although no statistically significant differences were found, a pattern was observed with SPTB having the lowest mean values for Hb, Ht and RBC count and SLC4A1 the highest (Appendix 3, Table 12). The same trend applied to MCV and MCH, despite all being within the reference values for healthy controls.

Looking into the individual genetic subgroups, SPTB-HS patients had a significantly higher RDW compared to SPTA1-HS patients (Bonferroni adjusted *P*= 0.011) (Figure 14A), indicating a more heterogenous RBC population. This might be result of the higher percentage of small, microcytic RBCs compared to SLC4A1 (Bonferroni adjusted *P*= 0.001), and the significantly higher absolute count of reticulocytes compared to SLC4A1 and SPTA1 (Bonferroni adjusted *P*= 0.048 and 0.005, respectively), since reticulocytes are relatively large cells compared to mature RBCs.

Contrarily, SLC4A1 was found to have the most homogenous RBC population with a high percentage of hyperchromic cells compared to SPTA1 (Bonferroni adjusted *P*= 0.007), and the least hypochromic cells out of ANK1, SPTA1 and SPTB (Bonferroni adjusted *P*= 0.005, 0.000 and 0.001, respectively). Since Hb is measured for detecting hypo-/hyperchromic cells, this is in line with the observation of the highest mean Hb concentration in this patient group. Accordingly, the mean corpuscular Hb content of SLC4A1-HS reticulocytes (MCHr) was significantly higher than in the ANK1 group (Bonferroni adjusted *P*= 0.018). Furthermore, although not statistically significant, this group of patients seemed to perform better on the OFT, illustrated by the lowest mean salt concentration at which 50% of the RBCs lysed (Figure 14C). This would indicate that this RBC population of relatively dense cells is better resistant to a hypotonic environment.

However still exceeding the reference range for healthy controls, the group of SPTA1-HS patients was mainly characterized by the low number of reticulocytes, especially compared to SPTB (Bonferroni adjusted *P*= 0.005). Additionally, this reticulocyte population was observed to consist of distinctly large cells, illustrated by a high MCVr compared to ANK1 (Bonferroni adjusted *P*= 0.024). Regarding the EMA-test, it was observed that the SPTA1 group had significantly higher staining of band 3 molecules in the RBC membrane, with the median even exceeding the cut-off value for healthy controls (Figure 14B). The mean EMA staining in SPTA1 was significantly higher than SPTB (Bonferroni adjusted *P*= 0.016). This challenges the suitability of the EMA to diagnose HS patients with SPTA1 defects in this cohort.

In ANK1-HS patients, besides having the smallest reticulocytes, a higher percentage of microcytic cells was found compared to SLC4A1 (Bonferroni adjusted *P*= 0.013), similar to SPTB.

For patients in which no genetic defect could be detected within the targeted gene panel, no significant differences were found in any of the tested parameters compared to the subgroups with known genetic defects. This is most likely due to the diversity within this group, resulting in high coincidence with all other, better specified groups.

In spite of these clear differences in haematologic parameters, suggesting varying compositions of the RBC populations in these genetic subgroups, no differences were found in haemolysis-related parameters bilirubin, haptoglobin, LDH, ferritin or transferrin saturation factor (TSAT). Furthermore, the IRF and RPI are comparable for these groups. Altogether this indicates an equal extent of haemolytic anaemia and corresponding compensatory reactions in all genetic subgroups. This is in line with the absence of a clear correlation between disease severity and underlying genetic defect.

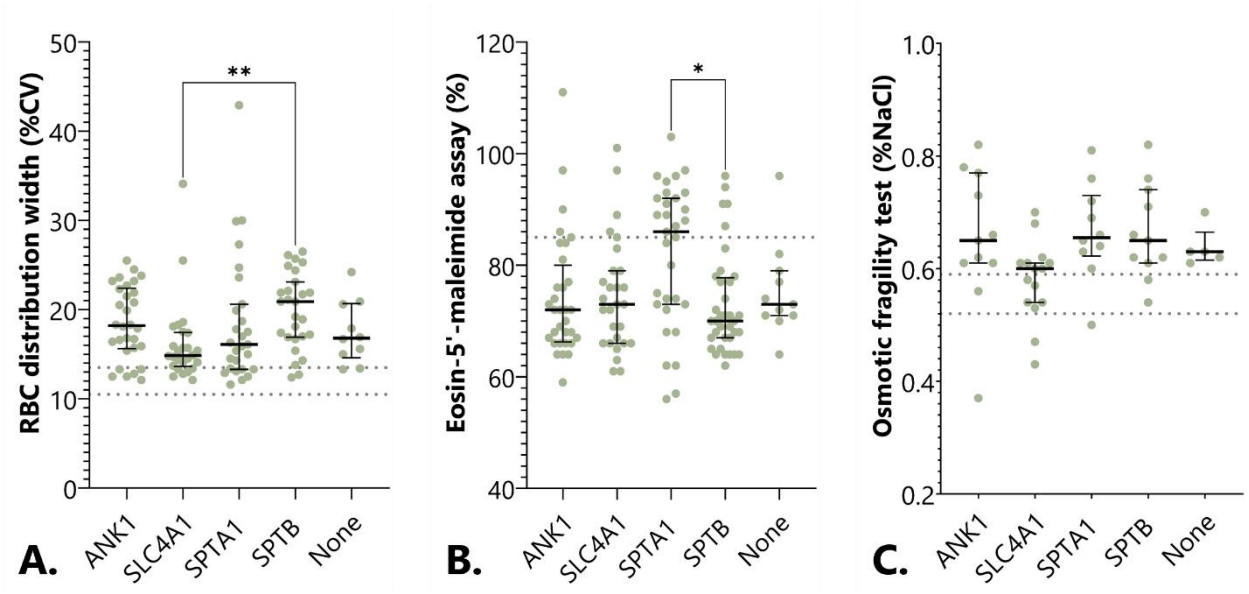


Figure 14 Haematologic and diagnostic parameters according to underlying genetic mutation of unsplenectomised hereditary spherocytosis patients. Green dots represent individual measurements. Black horizontal bars represent median with IQR genotype-subgroup. Dotted lines represent reference values used in the UMCU. ** $P < 0.01$, * $P < 0.05$. **A.** Patients with a β-spectrin defect have a more homogenous population of RBCs regarding cell-size compared to SLC4A1-HS patients. **B.** SPTA1 patients have significantly higher EMA-mediated staining of membrane proteins compared to patients with β-spectrin defects. Reference value for healthy controls >85%. **C.** No significant differences could be detected for the osmotic fragility test.

7.3.2. Osmoscan parameters

Differences between genetic subgroups were assessed for the Osmoscan parameters as well. No significantly differences could be found for the parameters related to osmolality: Omin, Omax and Ohyper were comparable for all underlying genetic defects. Only the Osharpness was found to be significantly higher in the SPTB group compared to SPTA1 (Bonferroni adjusted *P*= 0.045), indicating a steep increase of the elongation index towards the EImax with increasing osmolality (Figure 15A). This observation is somewhat unexpected since this novel Osmoscan parameter is positively correlated to OFT results for the entire cohort, though the OFT results for SPTA1 and SPTB are similar. Looking at the correlation between these two parameters for the genetic subgroups individually, the correlation indeed holds up for the SPTB group (n=9, r= 0.910, *P*= 0.001), but not for the other genetic subgroups, explaining this discrepancy.

More differences were found in the EI-related parameters. The SPTB group shows both the least maximum deformability and the highest minimal deformability of all subgroups. The EImax and consequently the EIhyper is lower compared to both SPTA1 and SLC4A1 (Bonferroni adjusted *P*= 0.025 and 0.003, respectively) (Figure 15B), whereas the EImin is significantly higher than SPTA1 only (Bonferroni adjusted *P*= 0.006) (Figure 15C).

Similar to the haematologic and haemolysis-related parameters, no significant differences could be detected in any of the Osmoscan parameters for the group of patients without detected genetic defects in the targeted gene panel.

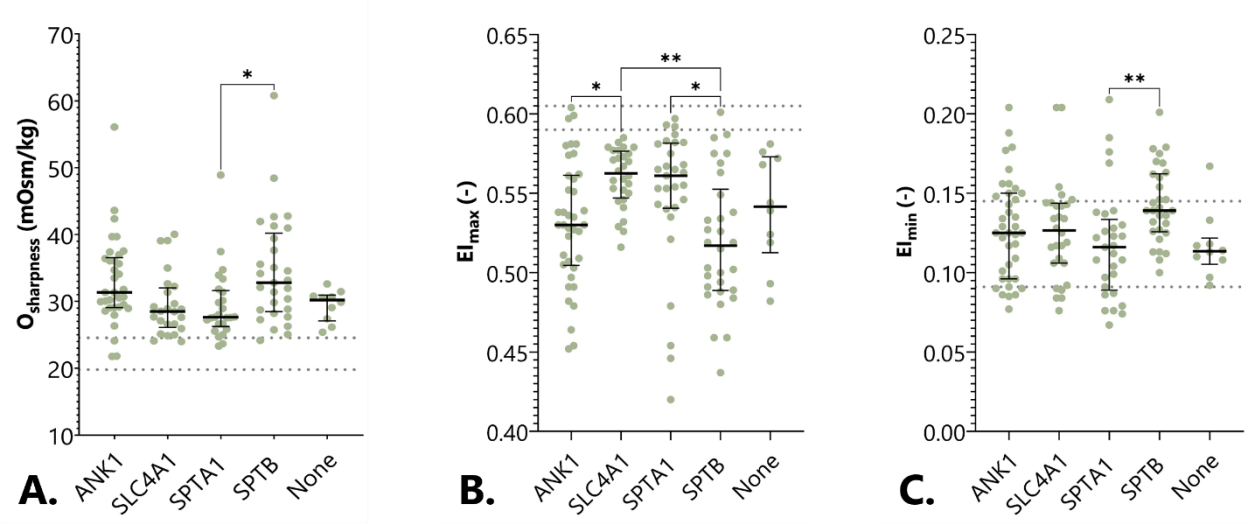


Figure 15 Osmoscan parameters according to underlying genetic mutation of unsplenectomised hereditary spherocytosis patients. Green dots represent individual measurements. Black horizontal bars represent median with IQR (A, C)/mean with SD (B) within genotype-subgroup. Dotted lines represent reference values used in the UMCU. ** *P*< 0.01, * *P*< 0.05. **A.** Patients with a β-spectrin defect have a higher Osharpness, indicating a wider valley around EImin. **B.** SLC4A1-HS patients have a higher maximal deformability compared to ANK1- and SPTB-HS patients. Patients with an α-spectrin defect have a higher EImax compared to β-spectrin defects. **C.** Many EImin values lay within the reference range, however SPTB-HS patients show a higher minimal deformability compared to SPTA1.

7.4. Low expression alleles of the SPTA1 gene

Because there is some controversy on the pathogenicity and mainly the inheritance patterns of alleles α^{LEPRA} and α^{LELY}, an overview was created on the carriers of these specific alleles in the HS cohort studied here. The presence of these alleles was examined for the majority of patients in this cohort.

Firstly, the α^{LEPRA} allele could be detected in 23 patients (23/197, 11.7%) genotyped for the SPTA1 gene. Of these patients, 19 (82.6%) are (compound) heterozygotes and 4 (17.4%) are homozygotes. Secondly, the α^{LELY} allele was covered in genetic analysis of 175 patients. The majority tested negative for this polymorphism (96/175, 54.9%), 21 (12%) patients were homozygotes and 58 (33.1%) were (compound) heterozygous. No relation could be found between genotype and α^{LELY} status (χ^2, P= 0.184), nor for disease severity and α^{LELY} status (χ^2, P= 0.780). Meaning that the prevalence of α^{LELY} alleles is similar for all genetic subgroups and for the three groups with mild, moderate and severe expression of disease.

To gain insight if presence of either allele contributes to the clinical expression of other mutated genes (i.e., SPTB, SLC4A1, SPTB), patients solely carrying either of these alleles α^{LEPRA} or α^{LELY} without other mutations in HS-associated genes were examined. Information on these patients is provided in Table 4. For the α^{LEPRA} allele, 8 cases could be identified of patients and their relatives who had no mutations in the HS-related genes apart from at least one α^{LEPRA} allele. Among these cases are two symptom-free heterozygous carriers of the allele, three heterozygous HS patients and three homozygous HS-patients. The Osmoscan profiles available for four of these subjects were found to have high specificity in distinguishing the clinically unaffected relatives from the HS patients. Notably, the severity of disease in these patients is predominantly mild, with the exception of one patient who was classified as moderately diseased. Additionally, one patient (subject 5) was classified as mild after undergoing concurrent splenectomy and cholecystectomy, implying that this patient was initially probably more severely affected. Accordingly, for both these patients a reduced EMA-mediated staining of membrane proteins was observed, whereas the more mildly affected patients have EMA-results within the reference range. The absence of reduced EMA-staining, despite the diagnosis of SPTA1-HS was observed earlier when comparing the genetic subgroups. The OFT results that were available for two HS subjects showed minimal exceedance of the reference range, independent of the number of α^{LEPRA} alleles inherited. This indicates that α^{LEPRA} barely diminishes the resistance of RBCs to hypotonic salt concentrations.

For the α^{LELY} allele, 14 cases were identified. Half of them are symptom-free relatives, among which five heterozygous and two homozygous α^{LELY} carriers. The other half consists of patients diagnosed with HS, mostly mildly affected, with an equal distribution of hetero- and homozygosity. Again, the available Osmoscan profiles proved their worth in distinguishing healthy relatives from diagnosed HS patients. Both of the homozygote HS patients and three out of five heterozygote HS patients tested positive for decreased EMA binding. Out of the eight subjects with available OFT results, three subjects, all diagnosed with HS, were observed to have more fragile RBCs than healthy controls.

Based on this overview with both diseased and healthy carriers of alleles α^{LEPRA} and α^{LELY} without the presence of other HS-related genes, the contribution of these alleles to disease severity remains inconclusive.

Table 4 Overview of patients in this HS cohort and their relatives who had no mutations in the HS-related genes apart from at least one α^{LEPRA} or α^{LELY} allele. - Data not available. & Cases are related. * Data from splenectomised and cholecystectomised patient; HS categorized as mild after these procedures. # No available data for classification of disease severity. † Expansion of research panels 7 and 46, including more genes associated with hereditary haemolytic anaemias.

Subject	Genotype	HS	EMA Ref > 0.85	OFT Ref: 0.52-0.59	Osmoscan	Genetic test panel
LEPRA						
1	α^{LEPRA} / α	No	95	0.56	Normal	Carrier status SPTA1
2[&]	α^{LEPRA} / α	No	-	-	Normal	Carrier status SPTA1
3	α^{LEPRA} / α	Yes, mild	97	0.61	Typical HS	Genetic panel analysis EMSV00v16.1
4	α^{LEPRA} / α	Yes, moderate	74	-	Typical HS	NGS 7
5	α^{LEPRA} / α	Yes, mild*	75	-	Typical HS	Genetic panel analysis EMSV00v17.1
6[&]	α^{LEPRA} / α^{LEPRA}	Yes, mild	96	0.60	Typical HS	Genetic panel analysis EMSV00v16.1
7	α^{LEPRA} / α^{LEPRA}	Yes, mild	-	-	-	NGS 7
8	α^{LEPRA} / α^{LEPRA}	Yes, mild	-	-	-	Genetic panel analysis EMSV00v16.1
LELY						
9	α^{LELY} / α	No	-	-	-	Carrier status SPTA1
10	α^{LELY} / α	No	100	0.53	Normal	NGS 7
11	α^{LELY} / α	No	98	0.53	Normal	NGS 7
12	α^{LELY} / α	No	-	0.55	Normal	Carrier status SPTA1
13	α^{LELY} / α	No	-	-	Normal	NGS 7
14	α^{LELY} / α	Yes, moderate	73	-	Typical HS	NGS 7
15	α^{LELY} / α	Yes, mild	96	-	Typical HS	NGS 7
16	α^{LELY} / α	Yes, [#]	71	0.70	Typical HS	NGS 46
17	α^{LELY} / α	Yes, mild	79	0.61	Typical HS	NGS 7, NGS 46
18	α^{LELY} / α	Yes, mild	96	-	Typical HS	NGS 7
19	α^{LELY} / α^{LELY}	No	93	Normal	Normal	Carrier status SPTA1
20	α^{LELY} / α^{LELY}	No	-	Normal	Normal	Carrier status SPTB
21	α^{LELY} / α^{LELY}	Yes, mild	Decreased	-	Typical HS	NGS 7
22	α^{LELY} / α^{LELY}	Yes, mild	82	Increased	Typical HS	NGS 7, NGS 46, NGS 87[†]

7.5. Classic versus overhydrated HS

While examining the available Osmoscans of patients without or before splenectomy, a subgroup could be distinguished because of showing a Ohyper shifted to the right on the osmolality axis (Figure 16). Where typical HS Osmoscans are not only characterized by a decreased maximal deformability (Elmax) and an increased Omin, but also by a decreased Ohyper compared to control patients (41), these scans showed a variety of Ohyper values ranging from within the reference range of healthy controls to even exceeding the upper limit of this reference range. Similar subgroups of so called HS2 patients with distinctive Osmoscan profiles have been described before (16,17), without mentioning a specific cut-off value or other form of definition. In this study several cut-off values have been explored, resulting in the definition of this subgroup of HS as having an Ohyper above the upper reference limit of healthy controls.

To further describe this subgroup of HS patients, the term HS2 was considered to be confusing because "HS type 2" is used by The Online Mendelian Inheritance in Man (OMIM)® compendium to depict HS caused by SPTB mutations (42). Therefore, for further reference the HS2 subgroup will be named overhydrated HS (OHSp), whereas the patients with classic Osmoscan profiles will be referred to as classic HS.

Out of the 131 Osmoscans in total, 17 (13.0%) Osmoscan profiles could be designated as overhydrated HS, whereas 114 (87.0%) were classic HS Osmoscan profiles. The two groups are comparable in terms of age, sex, received blood transfusions and the occurrence of splenectomy and cholecystectomy. Moreover, there was no significant correlation between phenotype nor genotype and type of HS (χ^2, P= 0.094 and 0.650, respectively).

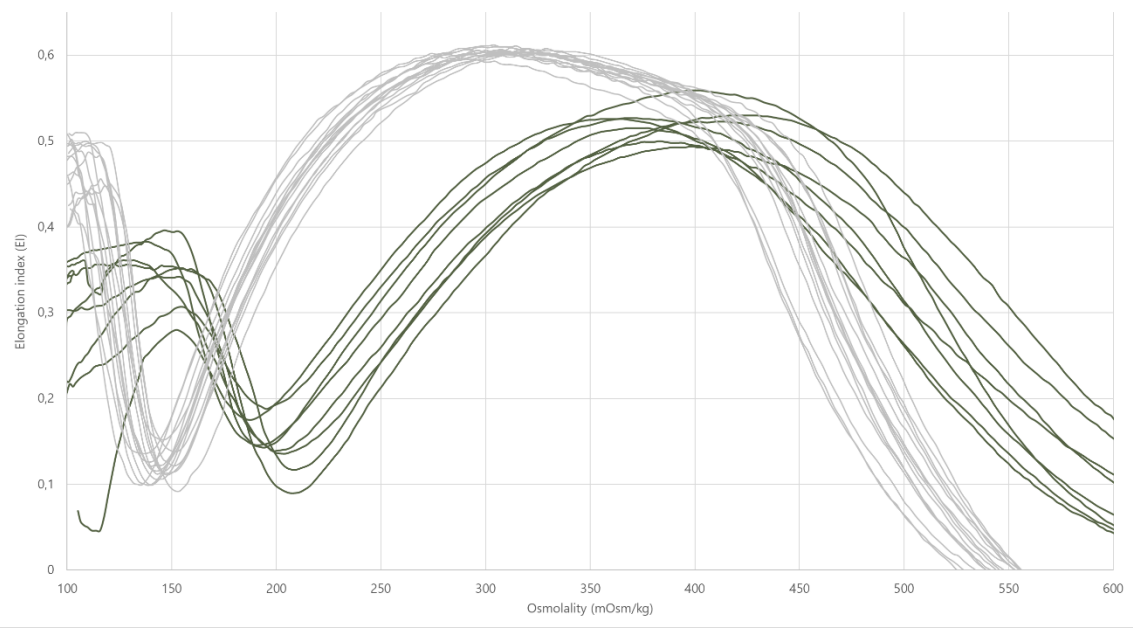
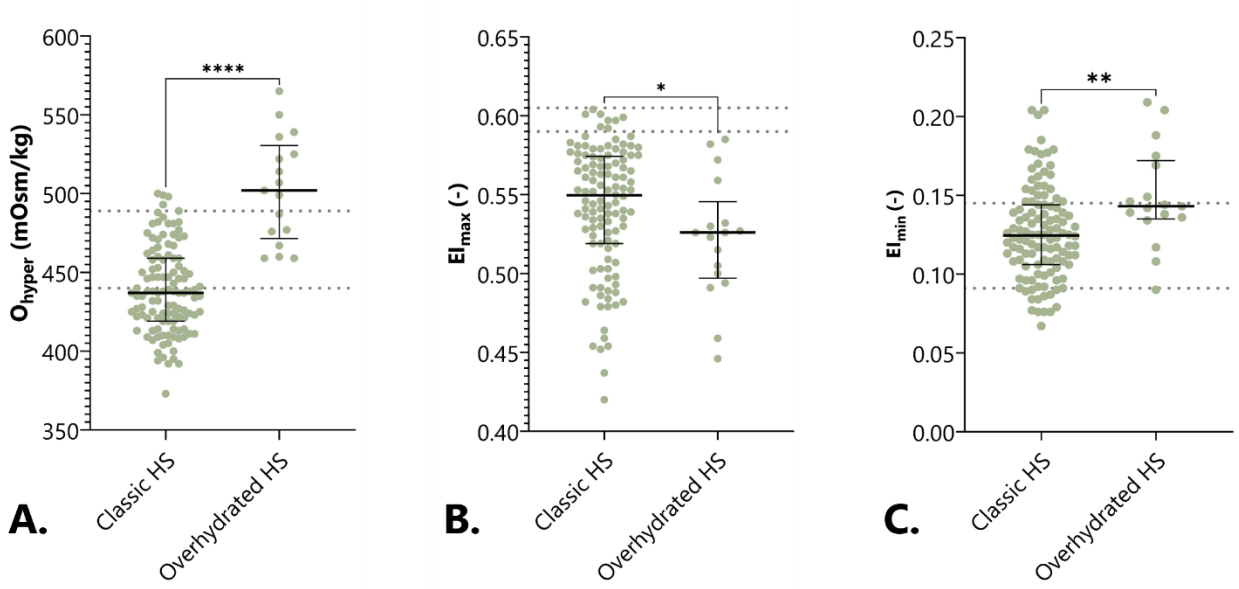


Figure 16 Presentation of several Osmoscan profiles of overhydrated HS patients {green}, compared to healthy controls {grey}. Both classic HS (not shown here) and overhydrated HS are characterized by an increased Omin and decreased Elmax compared to control. Overhydrated HS can be distinguished by the Ohyper exceeding the reference range of healthy controls.

7.5.1. Osmoscan parameters

Looking at other Osmoscan parameters than the distinctive Ohyper (Figure 17A), OHSp is associated with a lower EImax and consequently EIhyper compared to classic HS (*P*= 0.045) (Figure 17B-C). Together with an increased EI min in OHSp (*P*= 0.004), this results in a smaller total range of deformability (ΔEI). Furthermore, the Osmoscan profile of OHSp is shifted to the right as seen by the increased Omin and O EImax (*P*= 0.000 for both parameters) (Figure 17D-E). The higher ΔO for OHSp (*P*= 0.000) shows that, besides being shifted to the right, these curves are also more stretched out on the osmolality axis. This was also seen in the increased Osharpness and Oboldness (*P*= 0.000 for both parameters) (Figure 17F-G).



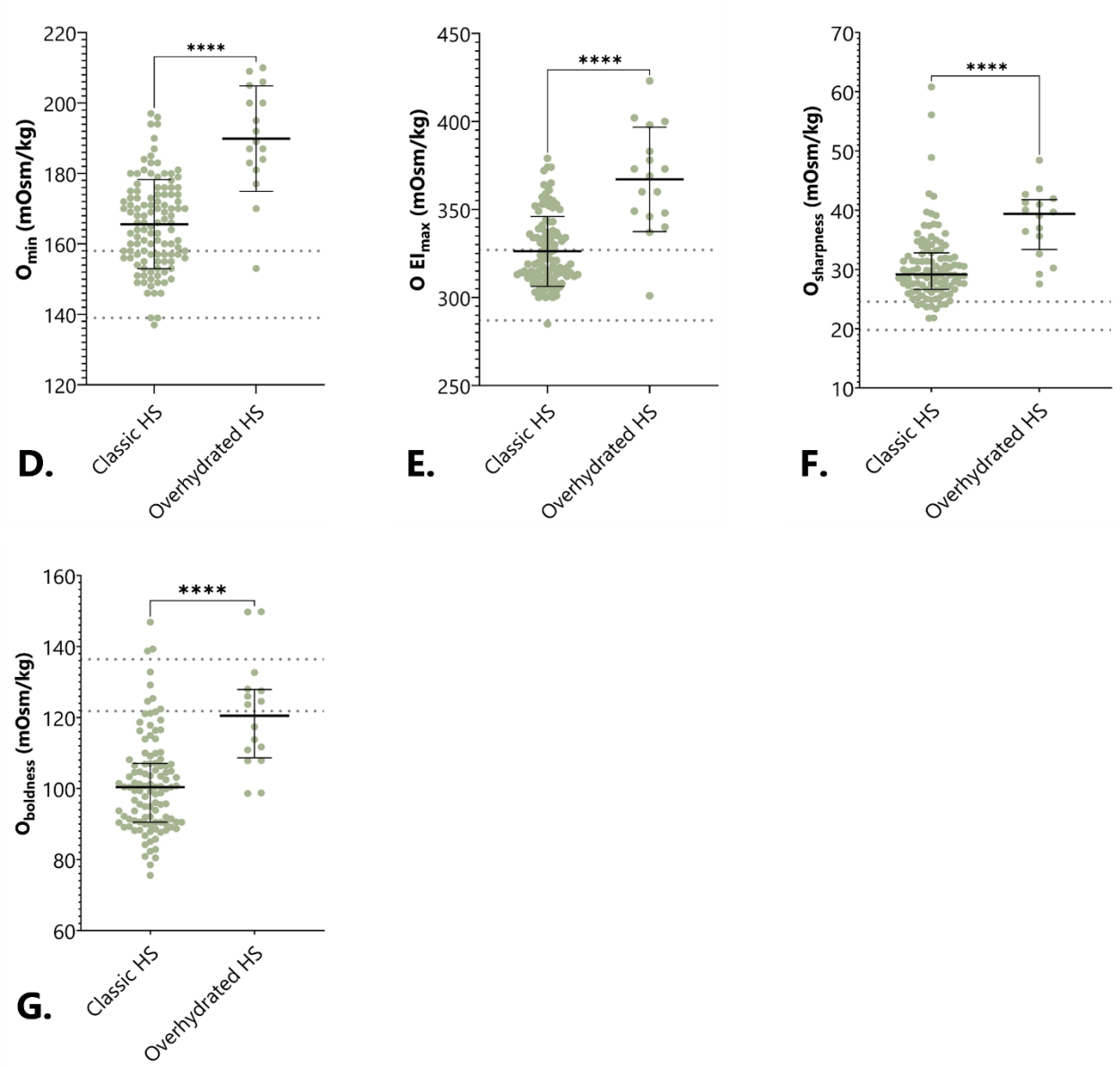


Figure 17 Comparison of Osmoscan parameters between classic and overhydrated hereditary spherocytosis patients. All data are from patients without or before splenectomy. Green dots represent individual measurements. Black horizontal bars represent median with IQR (B, C, F, G)/mean with SD (A, D, E) within subgroup. Dotted lines represent reference values used in the UMCU. **** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$. Overhydrated HS patients have distinctive higher values for Ohyper (**A**), minimal deformability and corresponding osmolality (**C, D**), osmotic value at maximal deformability (**E**), Osharpness (**F**) and Oboldness (**G**). The maximal deformability of OHSp patients is lower compared to classic HS (**B**).

7.5.2. Haematologic parameters

In addition to Osmoscan parameters, haematological parameters of classic and overhydrated HS were examined. Both subgroups had an equal absolute count of RBCs and reticulocytes and equal Ht. Although the mean MCV for both groups is similar, the RDW of OHSp patients is higher (*P*= 0.006), illustrated by a higher percentage of macrocytic RBCs and a higher MCVr (*P*= 0.006 and 0.004, respectively) (Figure 18A-C). Classic HS is associated with higher Hb levels (*P*= 0.029), also reflected by higher MCH (*P*= 0.011) compared to overhydrated HS (Figure 18D-E). Although it must be noted that not too many data are available (overhydrated HS; n= 5), a striking observation is the significantly lower PK/HK ratios of OHSp patients (*P*= 0.019) covering the lower area of the reference range of healthy controls (Figure 18F). There were no statistically significant differences in any of the haemolysis-related parameters.

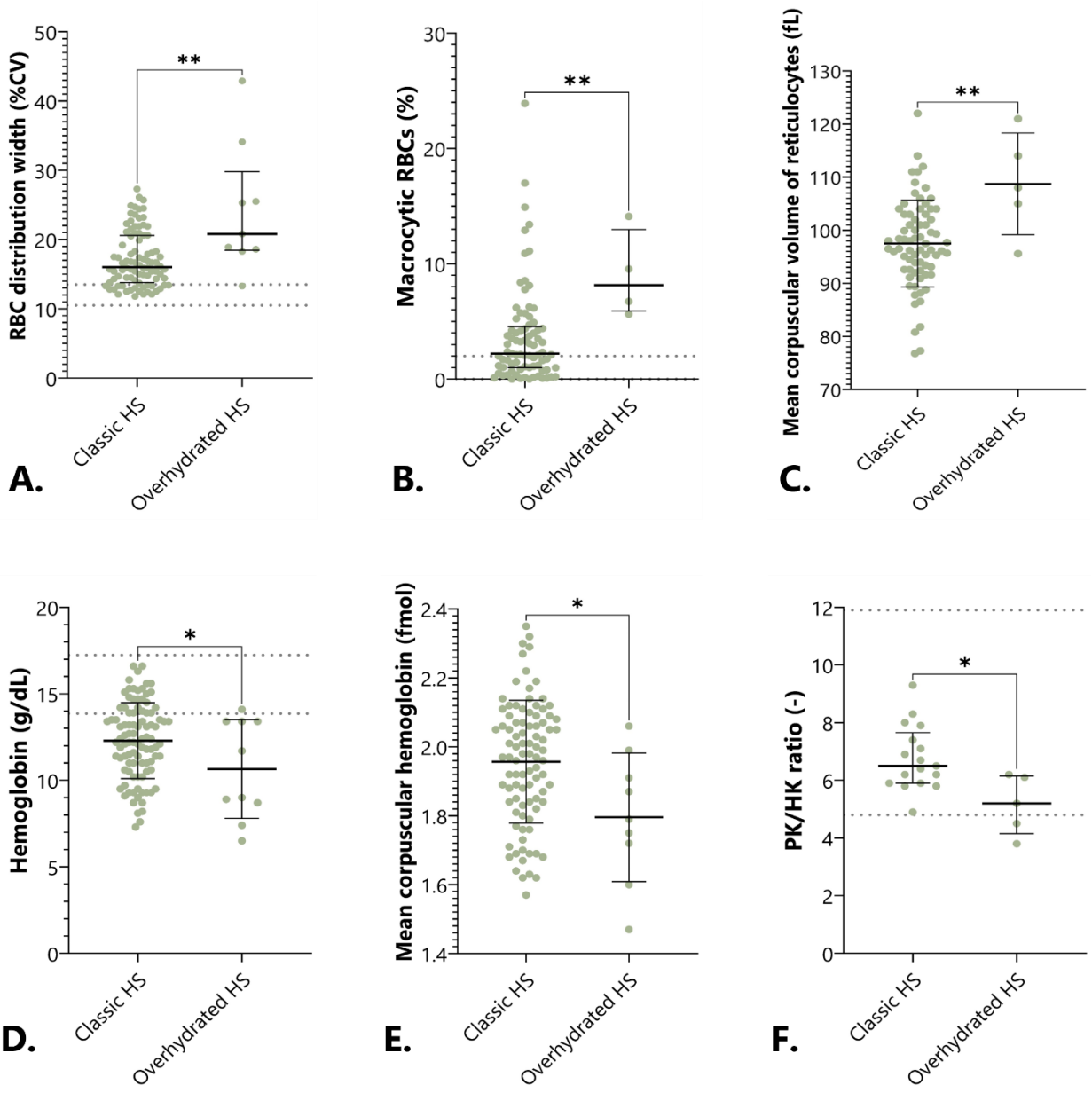


Figure 18 Comparison of haematological parameters between classic and overhydrated hereditary spherocytosis patients. All data are from patients without or before splenectomy. Green dots represent individual measurements. Black horizontal bars represent median with IQR (A, B, F)/mean with SD (C, D, E) within subgroup. Dotted lines represent reference values used in the UMCU. ** $P < 0.01$; * $P < 0.05$. Overhydrated HS patients have a more heterogenous RBC population (**A**), with more macrocytic RBCs (**B**) and a higher MCVr (**C**). Hb-levels (**D**) and MCH (**E**) are lower in OHSp patients compared to HS patients with typical Osmoscan profiles. So is the PK/HK-ratio (**F**).

8. Discussion

This retrospective study describes a cohort of 197 HS patients with high diversity in genotypes and clinical expression of disease. A presumed causative genetic defect was detected for 93.9%, similar to recent genotype-to-phenotype studies in HS patients (18,43). Although the absolute prevalence of mutations in the most common HS-associated genes depends on the descent of the patient population, molecular defects in ankyrin seem to be the most common, followed by spectrin-defects and dysfunctions in band 3 synthesis (13,18,44). The same was found in this cohort, as was the occurrence of multiple gene defects for individual patients independent of the main, causative mutation (18,45).
If available, laboratory parameters were obtained from the referring institutes. For interpretation of these data, it must be kept in mind that different institutes may use different reference values. For patients for whom Osmoscans were performed, basic haemocytometric analysis has been performed in the UMCU. Because this is the case for the majority of patients, little bias of the haematological parameters is expected.

8.1. Disease severity in hereditary spherocytosis

Disease severity of the patients in this cohort was classified using the laboratory parameters in the classification system of Eber (33). Due to overlapping reference values between the classes and patients that would be classified differently according to different parameters, attaining an unambiguous classification was challenging. Furthermore, the parameters spectrin content per erythrocyte and the extent of autohaemolysis included in the original system, were not used here because of insufficient data-availability. The fact that these tests are no longer frequently used in HS-diagnostics nowadays demonstrates that this classification system is outdated and in need of revision.

The parameter RDW might be a suitable addition to the revised classification system, since its strong positive correlation to disease severity, as previously shown by Rocha *et al.* (2011) and Huisjes *et al.* (2020)(24,46). Furthermore, a high RDW might reflect an increasing fraction of microcytic RBCs, directly related to membrane loss and thus spherocytosis, also strongly predicting severe HS also stated by Van Vuren *et al.* (2019) (43). The expected concurrent increase in hyperchromic cells with microcytic cells was only found in mild to moderate cases, followed by a decrease from moderate to severe, substantiating the theory that RBCs of severe HS patients do not reside in the circulation long enough to reach a dense state (46). The high density and low surface-to-volume ratio limits the deformability of these RBCs as reflected by a decreased EImax. Additionally, a correlation was found between the fraction of hyperchromic cells and the Oboldness, indicating that these cells relatively quickly lose their ability to deform upon an increasing salt concentration in the hypertonic region, which can be explained by their already high intracellular viscosity and limited ability to lose water to the environment. Similarly, Berrevoets *et al.* (2021) have demonstrated that fractions of RBCs with increasing density show less wide Osmoscan curves (35), hereby quantified by the parameter Oboldness. Regarding the Osmoscan parameters, it was confirmed that the classic parameters EImax, AUC and Omin are the best predictors of disease severity in HS in descending order (47).

Next to RDW and EImax, EMA might be a valuable addition to the revised classification system. Contrary to Huisjes *et al.* (2020) who could not distinguish 21 mild and moderate-severe cases of HS based on this diagnostic test (46), in this cohort with 106 EMA tests, lower EMA-staining predicted higher disease severity. This outperforms the OFT for which no differences could be detected between mild and severe cases. Though it is commonly accepted that the Osmoscan's Omin reflects the 50% lysis point in the OFT (30), these two parameters were not found to be strongly correlated in this cohort. Contrarily, high values of Osharpness were found to indicate the more fragile RBCs. This demonstrates that the slope of the decline from EImax to EImin better reflects the osmotic fragility than the osmolality at minimal deformability on its own. Thus, the novel parameters Oboldness and Osharpness are of added value in reflecting the proportion of hyperchromic RBCs and the osmotic fragility of the RBC population, respectively.

Altogether, the haematologic parameters RDW and MIC are the best predictors of disease severity and should be considered for inclusion in a revised classification system. Moreover, the classic Osmoscan parameter EImax and the diagnostic EMA seem promising replacements of the outdated determination of spectrin content. Ideally, a prospective study should be designed in which a new cohort of HS patients can be extensively phenotyped, not only using haemocytometry, Osmoscans and diagnostic tests, but also surveying the burden of disease experienced by the patients themselves, through questionnaires. This would establish a well-validated, standardized and contemporary classification system, to be used by both physicians as well as researchers.

8.2. Genotype-to-phenotype correlations in hereditary spherocytosis

Regarding the genetic subgroups in this cohort, no significant differences were found in clinical characteristics such as splenectomy and the receival of blood transfusions. This is not in line with findings by Mariani *et al.* (2008), who observed a higher prevalence of splenomegaly and gallstones in SLC4A1-HS patients (48). In a recent literature review by Yang *et al.* (2022) including thirteen genotype-to-phenotype studies regarding HS, a higher prevalence of splenectomies in patients with an SPTA1 defect is reported (49). Additionally, Tole *et al.* (2020) found that the SPTA1-HS patients needed blood transfusions more often (18). As in this cohort, no significant difference in the performance of cholecystectomies was found, although Tole *et al.* (2020) does demonstrate that SPTA1-HS patients undergo this procedure at a younger age (18). Due to the retrospective data-inclusion of patients from extern institutes, insufficient data was available to perform a similar analysis. Although no obvious differences are observed in clinical expression of HS depending on the causative genetic defect, as reported by Van Vuren *et al.* (2019), the highest proportions of moderate-severe HS were found in the groups with SPTB and ANK1 mutations (43).

Despite the absence of a significant correlation between genotype and disease severity classified by Eber *et al.* (1990), the relatively high Hb concentrations and low absolute reticulocyte counts seem to indicate a milder phenotype for SLC4A1-HS patients, as also reported by Yang *et al.* (2022) (49). Furthermore, the osmotic fragility of RBCs is least affected by SLC4A1 mutations and these cells show the greatest maximal deformability upon osmotic gradient ektacytometry. As also reported by Van Vuren *et al.* (2019), SPTB-HS patients seem to be more severely affected, reflected by lower Hb, higher RDW and higher reticulocyte counts (43). Consequently, this subgroup shows the smallest total range of deformability on the Osmoscan, illustrated by the highest EImin and the lowest EImax. Additionally, the higher Osharpness of SPTB-HS compared to SPTA1, indicates a more gradual increase in deformability at increasing osmolalities from the Omin.

In this cohort, SPTA1-HS patients have not been shown to be more severely affected than other patients, on the contrary it was shown that SPTA1 has higher EMA staining than the other subgroups, as previously reported (43,46), indicating less loss of membrane proteins and thus less spherocytosis. This contrasts the higher indication for splenectomy and blood transfusions, the lower Hb and higher RDW values found in other cohorts (18,44,50). This discrepancy might be explained by the differing views on the pathogenicity of the α-spectrin LELY allele and therefore selection of patients for the SPTA1 subgroup. Since the α^{LELY} variant is more prevalent than the incidence of HS, it is logical to assume that it is not pathogenic (18). Moreover, it has been demonstrated that the α^{LELY} allele *in trans* to an SPTA1-null mutation does not cause sufficient α-spectrin deficiency for HS to be clinically expressed (51,52). On the other hand, cases with this α^{LELY}/α^{0} genotype are presented that are diagnosed with HS and it is shown that they are more severely affected than their relatives who have only inherited the null-mutation (21,22). Likewise, two related HS-patients (mother and son) with a dominant pathogenic ANK1-mutation have been included in this cohort. Both are severely affected and have been splenectomised. However, even after splenectomy the son still has a more severe phenotype than his mother, perhaps contributed to by the α^{LELY} allele he has inherited from his symptom-free father. These case reports all support the role of the α^{LELY} allele as a disease modifier for genes other than SPTA1. While this seems to indicate that the α^{LELY} allele is not disease-causing on its own,

in this cohort, HS has been demonstrated to manifest in even heterozygous carriers of this allele. Similarly, the α^{LEPRA} allele is generally believed to be a recessive pathogenic mutation (13,51,52), whereas this cohort includes patients diagnosed with HS despite only heterozygous carriership of this allele. Based on this variety of clinical expression in carriers of alleles α^{LELY} and α^{LEPRA}, the pathogenicity and inheritance patterns of these alleles remain inconclusive. Besides, it suggests a theory of gradual gene expression, rather than the binary system of fully pathogenic mutations and benign polymorphisms. Whole genome sequencing could be used to definitely rule out the presence of yet undiscovered HS-associated genes. Furthermore, proteomics could give insight into the direct molecular relationship between genotype and phenotype in these patients. Together these techniques could assist in resolving the controversy around these intriguing alleles.

8.3. Overhydrated hereditary spherocytosis

To our knowledge, this is the first study in which a subpopulation of HS patients with distinctive Osmoscan profiles has been extensively investigated. Although this observation of right-shifted Osmoscan profiles was previously described by Llaudet-Planas *et al.* (2018) and Zaninoni *et al.* (2018) (16,17), no exact definition was mentioned to distinguish this subgroup from the classic Osmoscan profiles. The hereby proposed subgroup of overhydrated HS patients consists of 13% of this cohort, similar to the 10.3% declared by Zaninoni *et al.* (2018)(17). In line with these previous reports, no significant relation could be detected between disease severity and the distinctive Osmoscan profiles, however it must be noted that the percentage of moderate-severe cases in the OHSp group (80%) exceeds that of the classic HS patients (53.6%). Moreover, the lower Hb and higher RDW values seem to imply that overhydrated HS is associated with a somewhat more severe anaemia. Larger numbers of patients with overhydrated HS should be studied to gain insight into possible differences in clinical expression and characteristic laboratory parameters of this subgroup.

One of the interesting parameters for future research would be the PK/HK-ratio, which was found to be significantly lower in the OHSp group compared to classic HS, despite the little data available. This raised the question if HS-B patients may have a secondary subclinical pyruvate kinase deficiency (PKD) influencing RBC deformability. Notably, PKLR gene is included in the NGS gene panel, hence hereditary PK deficiency is excluded. Earlier cases of HS with heterozygous PKD have rarely been reported. A case report from Zarza *et al.* (2000) first described a young boy with SLC4A1 and EPB42 HS-causing mutations inherited from his more severely affected father in combination with a mutated PKLR allele inherited from his symptom-free mother (53). This led the authors to the conclusion that defective pyruvate kinase does not aggravate HS phenotype. Another young boy with concomitant HS and heterozygous PKLR mutation was described by Vercelatti *et al.* (2013) (54). The index patient inherited both HS and the PKLR mutation from his father who showed reduced PK activity but no haemolytic signs. Furthermore, he inherited an assumed benign polymorphism of PKLR caused by a nucleotide -148C>T substitution from his mother who had normal PK activity. Despite the difference in clinical presentation of HS in these family members, the authors conclude that PKD does not contribute to the haemolytic process. This is conform the observed similarity in haemolysis markers between classic and overhydrated HS. The drawback of these case reports is the absence of ektacytometric measurements, which were first performed by Van Zwieten *et al.* (2015) (55). His team studied an adult male HS patient who inherited a PLKR mutation from his heterozygous mother who was free of clinical symptoms. The index patient's daughter inherited the SLC4A1 mutation expressing as mild HS. Conflicting with the earlier findings, this suggests that low ATP levels negatively affect the phenotypic expression of HS. Comparing the Osmoscan profiles of OHSp with PKD patients from literature, many similarities can be found. Both patient groups show a decreased EImax and increased Omin and Ohyper (55,56). For this typical increase in Ohyper it has been hypothesized that the decrease in ATP levels causes diminished ion channel activation leading to accumulation of sodium ions within the RBCs and consequent increased hydration. However, the osmoscan performed on the earlier described HS-patient with only 70% of normal ATP levels does not show this increased Ohyper (55). In addition, it is commonly thought that the

decreased ATP levels due to PKD lead to overactivation of the RBC Gardos channels resulting in potassium efflux, water-loss and a dehydrated state (57–59). Moreover, the incidence of silent heterozygous PKD is ~1% (55), thus co-inheritance of HS and partial PKD does not seem a comprehensive explanation for the distinctive osmoscan profiles found in the 10% HS-B cases. Additionally, the PKLR gene is included in the NGS 46 panel, ruling out mutations for the patients in this cohort for which this panel was used.

Since the hydration status of RBCs is regulated by many different ion channels apart from the Gardos channel, a plausible theory seems that of Llaudet-Planas and colleagues who state that the differences in OHSp and classic HS are related to channel polymorphisms affecting ion homeostasis (16). An example of important channels for volume changes and therefore deformability of RBCs in changing osmotic environments are aquaporins (60). These aquaporins 1 (AQP1) and 3 (AQP3) present on human RBCs (61) regulate loss of free water in hyperosmotic conditions and swelling in hypo-osmotic conditions. Mathai *et al.* (1995) performed osmotic gradient ektacytometry on a patient with the so-called Colton-null phenotype; homozygous AQP1 deficiency (62). This showed a decreased EImax, reflecting a reduction in cell surface area, possibly due to loss of the area normally occupied by AQP1 (62,63). Furthermore, AQP1-deficiency results in a slightly right-shifted Omin compared to healthy controls, indicating a reduced surface-to-volume ratio of the affected RBCs, also observed in OHSp. Whereas, exclusive AQP1-deficiency causes a rare mild form of haemolytic anaemia (62), Crisp *et al.* (2016) studied AQP1 in HS patients and demonstrates an increasing reduction of AQP1-channels with severe HS independent of RBC size (64). Additionally, expression of AQP1 was found to be positively correlated to the Hb concentration, which is in line with the decreased Hb found in OHSp compared to classic HS. Moreover, AQP1-inhibitors Hg^{2+} and Cu^{2+} did not affect the osmotic fragility of these cells, comparable to the similar OFT results for OHSp and classic HS. Despite the similarities between OHSp and the reduced AQP1 expression in HS patients, the absence of a clearly increased Ohyper compared to healthy controls makes this speculation less plausible.

The observation of a right-shifted Osmoscan curve with an increased Ohyper can also be seen in another type of haemolytic anaemia known as overhydrated stomatocytosis (OHSt) (65,66). Caused by mutations in ammonium transporter Rh type A (RHAG), this disease is known for RBCs with increased intracellular cation content and consequent overhydration (67,68). This results in a decreased surface-to-volume ratio also seen in HS RBCs. Although HS and OHSt are both associated with an increased Omin upon osmotic gradient ektacytometry, in HS this is due to loss of membrane proteins (normal to decreased MCV (7,13)), whereas in OHSt it is caused by the superfluous water content reflected by an increased MCV. Besides the similar increased Omin and Ohyper in OHSt and OHSp, an important difference must be noted in the decreased EImax on the one hand and the increased EImax on the other (65). The inclusion of the RHAG gene in the HS-NGS panel, the extreme rarity of OHSt (with only 20 cases reported worldwide), and the differences in pathophysiology, clinical presentation and Osmoscan profiles debunk the plausibility of a relation between OHSt and OHSp.

An even more similar Osmoscan profile to those of OHSp patients can be obtained by treating RBCs with lysolecithin (LPC). Incubating RBCs with LPC induces membrane vesiculation and consequent loss of surface area and reduced surface-to-volume ratio, as seen in echinocytes (type III) (30). Renoux *et al.* (2019) demonstrated that incubation with increasing LPC-concentrations causes decreased maximal deformability and an increasing right-shift of the Osmoscan curve (69). The induced membrane shedding is conform the observed lower EMA-staining in OHSp patients, suggesting that OHSp may be associated with presence of high numbers of echinocytes, as also seen in PKD blood smears (70,71).

Altogether, these theories have in common that differences regarding hydration status underlie the distinct Osmoscan profiles of OHSp compared to classic HS curves. As previously suggested, investigation of larger cohorts with more OHSp patients would improve statistical power for detailed characterization of this subgroup regarding haematologic parameters and clinical features. Morphological examination of blood smears and genetic analysis of genes associated with water-homeostasis of RBCs could help further elucidate the mechanism underlying this distinct behaviour of RBCs in osmotic gradient ektacytometry.

In conclusion, the size and diversity of this cohort are a good reflection of the heterogenous disease HS in real life, highlighting the complexity of genotype-to-phenotype correlations. To standardize classification of disease severity for both clinical practice and research, a revision of the current classification system is proposed, with use of the parameters RDW, EImax and EMA. Furthermore, the use of novel Osmoscan parameters Oboldness and Osharpness gains insight in the presence of hyperchromic RBCs and osmotic fragility of the RBC population, respectively. Without conclusive evidence, SPTB-caused HS appears to be associated with the most severe clinical expression, whereas SLC4A1 defects were associated with the mildest forms of HS within this cohort. Further research is needed on the pathogenicity and inheritance patterns of low expression alleles α^{LELY} and α^{LEPRA} of the SPTA1-gene to reach uniformity in categorization carriers of these alleles. Moreover, we have provided the first step for research into characterization of the subpopulation with overhydrated HS.

9. References

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10. Appendix 1

Clinical severity of hereditary spherocytosis

Table 5 Haematologic and haemolysis-related parameters for subgroups of HS patients with increasing disease severity. Classification of disease severity according to Eber *et al.* (1990) (33). † ANOVA, ‡ Kruskal-Wallis. All data are from patients before or without splenectomy.

	Classification of severity of HS according to Eber *et al.* (1990)						
	Mild		Moderate		Severe		*P*-value
	Mean	SD	Mean	SD	Mean	SD	
Haematologic parameters							
Haemoglobin (g/dL)	13.1	1.8	11.4	2.1	9.4	2.5	.000†
Haematocrit (L/L)	.37	.05	.32	.06	.27	.07	.000†
Red blood cell count (·10^{12}/L)	4.14	.58	3.71	.59	3.34	.90	.000‡
Mean corpuscular volume (fL)	91	7	86	9	82	10	.000†
Mean corpuscular haemoglobin (fmol)	1.98	.16	1.91	.21	1.79	.23	.004†
Red blood cell distribution width (% CV)	14.6	2.3	19.6	4.0	24.9	6.9	.000‡
Microcytic RBCs (%)	3.10	4.53	8.55	6.93	17.69	14.88	.000‡
Macrocytic RBCs (%)	3.46	4.14	4.13	4.33	4.20	4.28	.297‡
Hypochromic RBCs (%)	2.91	5.65	5.48	7.16	16.75	18.65	.000‡
Hyperchromic RBCs (%)	6.27	7.22	10.71	8.28	6.61	7.76	.004‡
Absolute reticulocyte count (·10^{9}/L)	188.8	83.6	437.6	198.3	503.6	184.0	.000‡
Immature reticulocyte fraction (-)	.29	.07	.38	.11	.49	.11	.000†
Reticulocyte production index (-)	3.5	1.8	6.1	3.0	5.9	2.8	.000‡
Mean corpuscular volume of reticulocytes (fL)	98.5	8.6	97.9	8.2	95.5	11.1	.594†
Mean corpuscular haemoglobin of reticulocytes (fmol)	2.05	.18	2.04	.22	1.96	.21	.548‡
Haemolysis-related parameters							
Ferritin (ug/L)	200	383	280	258	180	189	.250‡
Transferrin saturation fraction (-)	.28	.17	.40	.22	.30	.03	.198‡
Total bilirubin (µmol/L)	20	8	62	34	72	32	.000‡
Haptoglobin (g/L)	.14	.15	.09	.15	.02	.02	.163‡
Lactate dehydrogenase (IU/mL)	223	55	281	87	342	177	.006‡

Table 6 Spearman's correlation coefficients for haematologic parameters and disease severity according to Eber *et al.* (1990) (33). ** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). All data are from patients before or without splenectomy.

		Hb	Ht	RBC count	MCV	MCH	RDW	MIC	MAC	HPO	HPR	RET count	RET (%)	IRF	RPI	MCVr	MCHr
Disease severity	Correlation Coefficient	-.475**	-.544**	-.375**	-.361**	-.264**	.692**	.585**	.139	.442**	.183	.679**	.746**	.556**	.450**	-.119	-.041
	Sig. (2-tailed)	.000	.000	.000	.000	.002	.000	.000	.165	.000	.069	.000	.000	.000	.000	.261	.693
	N	138	135	132	138	130	121	101	101	99	99	136	131	125	130	92	96

Table 7 Spearman's correlation coefficients for haemolysis-related parameters and disease severity according to Eber *et al.* (1990) (33). ** Correlation is significant at the 0.01 level (2-tailed). All data are from patients before or without splenectomy.

		Ferritin	TSAT	Total bilirubin	Haptoglobin	LDH
Disease severity	Correlation Coefficient	.112	.237	.653**	-.304	.401**
	Sig. (2-tailed)	.406	.184	.000	.063	.002
	N	57	33	63	38	60

Table 8 Spearman's correlation coefficients for diagnostic parameters and disease severity according to Eber *et al.* (1990) (33). ** Correlation is significant at the 0.01 level (2-tailed). All data are from patients before or without splenectomy.

		OFT	EMA
Disease severity	Correlation Coefficient	.476**	-.433**
	Sig. (2-tailed)	.002	.000
	N	41	106

Table 9 Spearman's correlation coefficients for Osmoscan parameters and disease severity according to Eber *et al.* (1990) (33). ** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). Osharp: Osharpness, Obold: Oboldness. All data are from patients before or without splenectomy.

		EI min	O min	EI max	O EImax	EI hyper	O hyper	AUC	EI O290	O sharp	O bold	ΔEI	ΔO
Disease severity	Correlation Coefficient	.221*	.346**	-.559**	.028	-.559**	-.023	-.449**	-.535**	.425**	-.308**	-.503**	-.173
	Sig. (2-tailed)	.019	.000	.000	.766	.000	.808	.000	.000	.000	.002	.000	.068
	N	112	112	112	112	112	112	112	101	99	100	111	112

11. Appendix 2

Correlation coefficients haematologic and diagnostic parameters with Osmoscan parameters

Table 10 Correlation coefficients for haematologic parameters and Osmoscan parameters. ** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). Osharp: Osharpness, Obold: Oboldness. All data are from patients before or without splenectomy.

		EI min	O min	EI max	O EImax	EI hyper	O hyper	AUC	EI O290	O sharp	O bold	ΔEI	ΔO
Hb	Pearson r	-.229*	-.200*	.252**	-.060	.250**	-.213*	.037	.279**	-.260**	-.102	.298**	-.142
	Sig. (2-tailed)	.015	.035	.007	.530	.008	.024	.697	.005	.009	.312	.001	.136
	N	112	112	112	112	112	112	112	101	99	100	111	112
Ht	Pearson r	-.202*	-.169	.348**	.026	.347**	-.063	.188*	.309**	-.238*	.077	.350**	.009
	Sig. (2-tailed)	.035	.078	.000	.787	.000	.515	.049	.002	.019	.450	.000	.925
	N	110	110	110	110	110	110	110	99	97	98	109	110
RBC count	Spearman r	-.135	-.130	.203*	.002	.209*	-.189	.015	.205*	-.249*	-.069	.232*	-.152
	Sig. (2-tailed)	.165	.183	.036	.980	.031	.051	.878	.045	.016	.504	.017	.117
	N	107	107	107	107	107	107	107	96	94	95	106	107
MCV	Pearson r	-.186*	.028	.382**	.233*	.381**	.245**	.373**	.211*	-.023	.329**	.367**	.304**
	Sig. (2-tailed)	.049	.772	.000	.013	.000	.009	.000	.034	.823	.001	.000	.001
	N	112	112	112	112	112	112	112	101	99	100	111	112
MCH	Pearson r	-.234*	-.048	.181	.020	.178	-.113	.037	.184	-.089	-.102	.249*	-.053
	Sig. (2-tailed)	.016	.623	.064	.841	.067	.250	.703	.074	.397	.329	.010	.590
	N	106	106	106	106	106	106	106	95	93	94	105	106
RDW	Spearman r	.321**	.420**	-.635**	.038	-.638**	.100	-.448**	-.659**	.470**	-.239*	-.628**	-.092
	Sig. (2-tailed)	.001	.000	.000	.702	.000	.318	.000	.000	.000	.020	.000	.359
	N	102	102	102	102	102	102	102	95	93	94	101	102
MIC	Spearman r	.344**	.182	-.548**	-.238*	-.549**	-.144	-.455**	-.458**	.180	-.385**	-.572**	-.269*
	Sig. (2-tailed)	.001	.094	.000	.027	.000	.187	.000	.000	.111	.000	.000	.012
	N	86	86	86	86	86	86	86	81	80	81	86	86

~ Table continues on next page ~

		EI min	O min	EI max	O EImax	EI hyper	O hyper	AUC	EI O290	O sharp	O bold	ΔEI	ΔO
MAC	Spearman r	.110	.324**	-.167	.382**	-.170	.432**	.010	-.299**	.492**	.171	-.210	.335**
	Sig. (2-tailed)	.313	.002	.125	.000	.118	.000	.929	.007	.000	.126	.053	.002
	N	86	86	86	86	86	86	86	81	80	81	86	86
HPO	Spearman r	.287**	.263*	-.224*	.197	-.227*	.349**	-.017	-.333**	.376**	.158	-.318**	.235*
	Sig. (2-tailed)	.008	.015	.040	.073	.038	.001	.880	.003	.001	.165	.003	.031
	N	84	84	84	84	84	84	84	79	78	79	84	84
HPR	Spearman r	-.069	.225*	-.594**	-.162	-.593**	-.491**	-.741**	-.542**	.115	-.773**	-.381**	-.620**
	Sig. (2-tailed)	.536	.039	.000	.141	.000	.000	.000	.000	.315	.000	.000	.000
	N	84	84	84	84	84	84	84	79	78	79	84	84
RET count	Spearman r	.108	.381**	-.720**	.023	-.719**	-.151	-.669**	-.691**	.406**	-.545**	-.540**	-.348**
	Sig. (2-tailed)	.261	.000	.000	.812	.000	.115	.000	.000	.000	.000	.000	.000
	N	110	110	110	110	110	110	110	99	97	98	109	110
IRF	Pearson r	.088	.312**	-.251*	.283**	-.251*	.291**	-.081	-.346**	.277**	.002	-.223*	.180
	Sig. (2-tailed)	.375	.001	.010	.004	.011	.003	.415	.001	.007	.987	.024	.069
	N	103	103	103	103	103	103	103	95	93	94	102	103
RPI	Spearman r	.038	.305**	-.567**	.026	-.566**	-.199*	-.583**	-.571**	.322**	-.526**	-.392**	-.371**
	Sig. (2-tailed)	.698	.001	.000	.793	.000	.041	.000	.000	.002	.000	.000	.000
	N	106	106	106	106	106	106	106	95	93	94	105	106
MCVr	Pearson r	.024	.251*	-.041	.457**	-.044	.353**	.051	-.091	.196	.223	-.039	.294**
	Sig. (2-tailed)	.833	.027	.725	.000	.701	.001	.656	.443	.100	.058	.736	.009
	N	78	78	78	78	78	78	78	73	72	73	77	78
MCHr	Spearman r	.062	.179	-.198	.150	-.197	-.221*	-.334**	-.152	.110	-.270*	-.132	-.297**
	Sig. (2-tailed)	.587	.111	.078	.185	.079	.049	.002	.194	.354	.020	.245	.007
	N	80	80	80	80	80	80	80	75	73	74	79	80

Table 11 Correlation coefficients for diagnostic parameters and Osmoscan parameters. ** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). Osharp: Osharpness, Obold: Oboldness. All data are from patients before or without splenectomy.

		EI min	O min	EI max	O EImax	EI hyper	O hyper	AUC	EI O290	O sharp	O bold	ΔEI	ΔO
EMA	Spearman r	-.262**	-.410**	.663**	-.033	.661**	.179	.709**	.576**	-.321**	.368**	.582**	.347**
	Sig. (2-tailed)	.004	.000	.000	.720	.000	.050	.000	.000	.001	.000	.000	.000
	N	120	120	120	120	120	120	120	115	113	114	119	120
OFT	Pearson r	.317*	.453**	-.781**	.099	-.781**	.002	-.689**	-.785**	.652**	-.384*	-.707**	-.235
	Sig. (2-tailed)	.034	.002	.000	.518	.000	.987	.000	.000	.000	.014	.000	.121
	N	45	45	45	45	45	45	45	41	39	40	45	45

12. Appendix 3

Table 12 Haematologic and haemolysis-related parameters for HS patients categorized by their underlying genetic mutation. † ANOVA, ‡ Kruskal-Wallis. All data are from patients before or without splenectomy. For EPB42 some data was unavailable, because there was only one patient in this group.

	ANK1		SLC4A1		SPTA1		SPTB		EPB42		None		*P*-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Haematologic parameters													
Haemoglobin (g/dL)	11.7	2.3	12.9	2.0	11.7	2.1	11.2	2.8	14.8	.	11.7	2.2	.096†
Haematocrit (L/L)	.33	.07	.36	.05	.34	.07	.31	.08	.41	.	.32	.06	.064†
Red blood cell count ($\cdot 10^{12}$/L)	3.82	.64	4.06	.60	3.85	.68	3.68	.78	4.58	.	3.76	.67	.352†
Mean corpuscular volume (fL)	85	9	91	7	90	10	85	8	90	.	86	9	.015†
Mean corpuscular haemoglobin (fmol)	1.89	.22	2.02	.16	1.92	.21	1.87	.17	2.02	.	1.94	.19	.106†
Red blood cell distribution width (% CV)	18.5	4.1	16.2	4.6	18.5	7.2	19.9	4.2	11.8	.	17.5	3.5	.009‡
Microcytic RBCs (%)	9.42	7.33	2.80	2.44	7.56	13.17	10.59	7.25	1.06	.	5.99	6.74	.001‡
Macrocytic RBCs (%)	2.89	4.90	3.40	3.29	5.51	5.31	3.45	2.82	.37	.	4.31	3.69	.171‡
Hypochromic RBCs (%)	5.94	9.90	1.55	3.55	9.63	11.88	7.09	11.65	.19	.	3.41	4.48	.000‡
Hyperchromic RBCs (%)	7.98	8.59	11.10	7.79	4.54	7.10	9.96	7.79	.74	.	10.07	7.56	.005‡
Absolute reticulocyte count ($\cdot 10^{9}$/L)	369.5	203.0	263.4	89.1	274.0	193.8	443.6	228.1	215.0	.	424.5	257.2	.004‡
Immature reticulocyte fraction (-)	.38	.14	.30	.09	.38	.10	.38	.11	.34	.	.33	.09	.054†
Reticulocyte production index (-)	5.0	2.9	4.4	1.7	4.2	2.8	6.2	3.2	4.3	.	5.8	3.0	.070‡
Mean corpuscular volume of reticulocytes (fL)	94.8	8.5	98.5	6.8	101.8	9.6	95.4	8.9	.	.	97.5	6.8	.023‡
Mean corpuscular haemoglobin of reticulocytes (fmol)	1.98	.22	2.17	.16	2.00	.18	2.00	.23	.	.	2.09	.16	.010‡
Haemolysis-related parameters													
Ferritin (ug/L)	131	197	265	245	421	519	192	177	35	.	170	87	.152‡
Transferrin saturation fraction (-)	.28	.13	.42	.27	.31	.10	.25	.11	.38	.	.73	.	.489‡
Total bilirubin (µmol/L)	48	30	55	36	43	43	55	35	14	.	57	29	.469‡
Haptoglobin (g/L)	.16	.18	.05	.04	.22	.20	.08	.14	.	.	.03	.03	.054‡
Lactate dehydrogenase (IU/mL)	252	65	270	140	231	65	296	90	190	.	368	214	.275†

13. Appendix 4

Classic versus overhydrated hereditary spherocytosis

Table 13 Haematologic and haemolysis-related parameters for subgroups of HS patients with distinctive Osmoscan profiles. Overhydrated HS patients are selected by the Osmoscan parameter Ohyper exceeding the reference range of healthy controls. † Independent samples *t*-test, ‡ Mann-Whitney U tests. All data are from patients before or without splenectomy.

	Classic HS		Overhydrated HS		*P*-value
	Mean	SD	Mean	SD	
Haematologic parameters					
Haemoglobin (g/dL)	12.3	2.2	10.6	2.8	.035†
Haematocrit (L/L)	.34	.06	.33	.09	.599†
Red blood cell count ($\cdot 10^{12}$/L)	3.92	.65	3.76	.74	.528†
Mean corpuscular volume (fL)	88	8	90	11	.333†
Mean corpuscular haemoglobin (fmol)	1.96	.18	1.80	.19	.010†
Red blood cell distribution width (% CV)	17.2	4.0	24.2	9.2	.006‡
Microcytic RBCs (%)	6.51	6.72	11.00	15.56	.604‡
Macrocytic RBCs (%)	3.70	4.24	9.00	3.78	.009‡
Hypochromic RBCs (%)	3.51	3.92	13.81	15.01	.148‡
Hyperchromic RBCs (%)	9.01	8.07	2.37	2.28	.061‡
Absolute reticulocyte count ($\cdot 10^{9}$/L)	343.4	201.0	351.8	197.7	.872‡
Immature reticulocyte fraction (-)	.35	.09	.35	.16	.999†
Reticulocyte production index (-)	5.1	2.8	5.3	3.8	.811†
Mean corpuscular volume of reticulocytes (fL)	97.5	8.2	108.7	9.6	.004†
Mean corpuscular haemoglobin of reticulocytes (fmol)	2.08	.18	1.97	.17	.149‡
Haemolysis-related parameters					
Ferritin (ug/L)	228	309	499	381	.264‡
Transferrin saturation fraction (-)	.33	.18	.86	.	.125‡
Total bilirubin (µmol/L)	48	36	71	14	.254‡
Haptoglobin (g/L)	.13	.16	.03	.03	.390†
Lactate dehydrogenase (IU/mL)	247	64	435	245	.318†
Enzyme-related parameters					
Pyruvate kinase (U/g Hb)	10.6	1.8	13.2	1.5	.006†
Hexokinase (U/g Hb)	1.6	.3	2.7	.8	.035†
PK/HK ratio (-)	6.8	1.1	5.1	1.0	.038†

Table 14 Diagnostic parameters for subgroups of HS patients with distinctive Osmoscan profiles. Overhydrated HS patients are selected by the Osmoscan parameter Ohyper exceeding the reference range of healthy controls. All parameters are tested with Mann-Whitney U tests. All data are from patients before or without splenectomy.

	Classic HS		Overhydrated HS		*P*-value
	Median	IQR	Median	IQR	
Eosin-5'-maleimide assay (%)	74	69-86	67	64-73	.002
Osmotic fragility test (%NaCl)	.62	.58-.69	.67	.65-.68	.404