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Quantitative measurement of red cell surface protein expression reveals new biomarkers for hereditary spherocytosis

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Running title: quantitative measurements of RBC membrane proteins
Sir, the red blood cell (RBC) contains numerous membrane and cytoskeletal proteins, some of which are important for its functional properties of transporting gases in the tissues. Many of the membrane proteins are located in multiprotein complexes connected intracellularly to cytoskeletal proteins\(^1\), forming a flexible network that enables the RBC to deform and pass even through the smallest capillaries of the human body. Hereditary spherocytosis (HS) is a heterogeneous disorder, mainly caused by mutations in the genes encoding band 3 (SLC4A1), ankyrin (ANK1), protein 4.2 (EPB42), α-spectrin (SPTA1), and β-spectrin (SPTB)\(^2\). These mutations can impact on membrane protein abundance in two ways, 1) by affecting the assembly of membrane complexes during erythropoiesis and nucleation\(^3\)–\(^5\) and 2) by also affecting anchoring of the cytoskeleton to the phospholipids bilayer, which causes membrane loss by vesiculation, a key feature of HS. Vesiculation results in the formation of spherocytes and poorly deformable RBCs that are prematurely cleared from the circulation causing a variable degree of hemolytic anemia\(^2\). A general marker for the loss of membrane in HS is the decrease in binding of eosin-5-maleimide (EMA)\(^6\). However, thorough investigations of the effect of membrane loss on the expression of individual extracellular or transmembrane proteins are currently lacking, in particular by correlation to the underlying causative mutation.

In this study we have explored expression levels of red cell surface proteins glycophorin A (GPA), glycophorin C (GPC), Rhesus-associated glycoprotein (RhAG), Rhesus factor (Rh), band 3 and CD47 by flow cytometry in 23 HS patients who have been characterized in detail at the molecular level. The results improve our understanding of the pathophysiological mechanism in HS and reveals new biomarkers. In addition, we show that a combination of expression levels of
RhAG, Rh and Band 3 may prove to be helpful in the diagnosis of HS of different molecular basis.

The patients with hereditary spherocytosis (HS) were enrolled for the CoMMiTMenT study by the CoMMiTMenT consortium (http://rare-anaemia.eu/). This study was approved by the Medical Ethical Research Board (MERB) of the University Medical Center Utrecht (UMCU), Utrecht, The Netherlands, under reference code 15-426M ‘disturbed ion homeostasis in hereditary hemolytic anaemia’. Patients eligible for inclusion in this study were all previously diagnosed with HS by morphological analysis, osmotic gradient ektacytometry by Laser-assisted Optical Rotational Cell Analyzer (Lorrca), eosin-5’-maleimide (EMA)-binding and osmotic fragility testing (OFT). The differential diagnosis of HS was performed by genetic analysis by Next-Generation Sequencing (NGS) of the seven genes most commonly mutated in HS (band 3 (SLC4A1), ankyrin (ANK1), protein 4.1 (EPB41), protein 4.2 (EPB42), α-spectrin (SPTA1), β-spectrin (SPTB) and Rhesus-associated glycoprotein (RhAG)). Exclusion criteria for enrollment in the CoMMiTMenT study were the use of transfusion in past 90 days, body weight less than 18 kg and age lower than 3 years. RBCs were purified from whole blood by using a cellulose column after collection of whole blood in EDTA vacutainers. RBCs were supplemented in PBS with 0.1% bovine serum albumin (BSA) and 0.2% glucose. RBCs were fixed using 1% paraformaldehyde + 0.0075% glutaraldehyde for 15 minutes. RBCs were incubated with mouse anti-human antibodies (IBGRL Reagents) (Filton, Bristol, UK) directed against glycophorin C (BRIC4), CD47 (BRIC32), Rhesus factor (BRIC69), band 3 (BRIC71), Rhesus-associated glycoprotein (LA1818) and glycophorin A (BRIC256). Subsequently, cells were stained with rat anti-mouse IgG1-APC and measured by flow
cytometry using BD FACSCanto™ II. After each measurement, the mean fluorescent intensity (MFI) of RBC membrane surface protein expression from patients was expressed relative to the average MFI from 6 healthy controls. Eosin-5′-maleimide (EMA)-binding was measured as previously described by King et al. Statistical analysis was carried out using IBM SPSS Statistics 21. One-way ANOVA (multiple comparison, post-hoc Tukey) was performed to test for significant differences between patient groups. To investigate whether the expression of RBC membrane proteins were significantly different in patients groups compared to healthy controls, the mean expression of RBC membrane proteins in patient groups was compared with the value of 100% using One-Sample T Test.

From the 23 HS patients that were included in this study, seven patients were found to display mutations in SLC4A1 (Band 3), eight patients with mutations in ANK1 (ankyrin), six patients with mutations in SPTA1 (α-spectrin) and two patients with mutations in SPTB (β-spectrin). An overview of the individual patient mutations is given in Table and an overview of the quantitative membrane proteins measurements by flow cytometry of glycophorin C, Rh factor, band 3, RhAG, glycophorin A and CD47 is depicted in Figure 1a to Figure 1f.

All patients exhibited decreased binding of eosin-5′-maleimide (EMA). EMA-binding, which involves the ε-NH₂ group of the lysine at position 430 from band 3, correlated with band 3 expression measured by BRIC71 (Figure 2a). However, patients with defects in SLC4A1 were found to have a significantly more pronounced reduction in expression of band 3 compared to
patients with defects in ankyrin, α-spectrin or β-spectrin (Figure 1c and Figure 2a). Within the erythrocyte membrane band 3 exists in different oligomeric states: as ankyrin mediated tetramers at the core of the ankyrin associated multiprotein complex and in a dimeric state, both freely mobile and cytoskeletally associated as part of the junctional complex. The increased loss of band 3 in patients with defects in \textit{SLC4A1} compared to those with other molecular defects likely results from the direct impact of the \textit{SLC4A1} mutation on band 3 expression e.g encoding of an unstable or misfolded protein product, which impacts upon band 3 in multiple oligomeric conformations and complexes\textsuperscript{3,5}. The milder loss of band 3 in RBCs with \textit{ANK1}-defects can be attributed to the indirect impact upon band 3 stability resulting from impaired cytoskeletal attachment on a specific proportion of band 3 population, which in the case of \textit{ANK1} defects is confined to the ankyrin associated band 3 tetrameric population.

HS patients with defects in \textit{SLC4A1}, \textit{ANK1}, and \textit{SPTA1} all showed significantly decreased expression of Rh, Band 3, RhAG and glycophorin A compared to healthy controls (=100%). Hence, decreased expression of these membrane proteins represents novel general biomarkers for HS.

A previous study has shown reduced expression of both RhAG and Rh in a small cohort of ankyrin-deficient HS patients\textsuperscript{4}. We also observed significant reductions in the expression of these proteins in HS patients with \textit{ANK1} mutations. Both datasets confirm the Rh complex residency within the ankyrin-based multiprotein complex and the reported interaction of ankyrin, as well as the reported interaction with the C-terminus of RhAG\textsuperscript{8}. 
Neither glycophorin C nor CD47 were found to be significantly reduced in HS patient erythrocytes with mutations in $SLC4A1$, $ANK1$, $SPTA1$ or $SPTB$ indicating that non-specific gross membrane protein loss is not a feature in these patients and that the reductions in protein observed are not an effect solely of reduced cell surface area. CD47 expression has been previously shown to be reduced in rare HS patients with mutations in $EPB42$ resulting in complete absence of protein 4.2$^{9,10}$ and in Rh null syndrome$^{10}$. However, deficiency of protein 4.2 is a more rare cause of HS and we unfortunately did not have the opportunity to study CD47 expression in this type of HS. Mutations in the cytoplasmic domain of band 3 within the vicinity of the protein 4.2 binding site$^{11}$) that could influence protein 4.2 abundance within affected erythrocytes may be expected to correlate with an indirect reduction in CD47$^{1}$. We, however, did not identify an extensive reduction of CD47 in two patients with mutations in the cytosolic domain of band 3 (CD47 expression of 89% and 132% in respectively patient 1 and patient 2). We conclude therefore that reduction in CD47 expression is not a general feature of HS erythrocytes.

Glycophorin C associates with protein 4.1 and p55 within the junctional complex$^{12}$ and has been demonstrated to be absent in hereditary elliptocytosis patients with complete protein 4.1 deficiency$^{13}$. The normal glycophorin C expression in HS patients with a variety of molecular defects studied here indicate that glycophorin C expression is not affected by heterozygous mutations in $SLC4A1$, $ANK1$ or $SPTA1$. Glycophorin C, therefore, seems to be a specific biomarker for HS due to protein 4.1 deficiency.
In summary, we show that by measuring RBC membrane protein expression by flow cytometry, quantitative differences in RBC membrane proteins can be identified. In particular decreased expression of RhAG, Rh-factor and glycophorin A represent novel general biomarkers for HS, whereas the more pronounced decrease in expression of band 3 is a biomarker for HS due to mutations in SLC4A1. Additional studies on the use of combined measurements of RBC surface proteins are warranted to further explore its use for the differential diagnosis of HS. Thus the results presented within this study suggest that quantitative measurement of RBC surface protein expression by flow cytometry could be used to categorize HS patients according to the underlying genetic defects in either SLC4A1, ANK1 or SPTA1. Figure 2b shows a 3D-scatterplot for expression levels of Rh factor, band 3 and RhAG. Using this 3D-scatterplot, patients could be assigned to having a genetic defect in either SLC4A1, ANK1 or SPTA1. This approach could complement and enhance existing initial HS diagnostic tools by providing a preliminary categorization of HS molecular defect or initial lead for confirmatory DNA analysis. In particular, this methodology enables rapid confirmation or exclusion of phenotypic effects of novel polymorphisms or Variants of Unknown clinical Significance (VUS) identified through next-generation sequencing approaches.
**Author contribution:** RH, TS, AT and RvW designed the research. RH, TS and LV performed the research. RH and LV analyzed the data. RH, TS, RS, WvS, AT and RvW wrote the paper.

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References


Table and figure legends

Table 1: overview of mutations identified in patients with hereditary spherocytosis (HS).

Splenectomized patients are noted with # and mutations not previously reported are depicted in bold face. αLELY represents the c.[5572C>G;6531-12C>T] p.[(Leu1858Val);?] mutations in SPTA1.

Figure 1 a, b, c, d, e, and f) RBC surface protein expression in patients with hereditary spherocytosis (HS) categorized according to the underlying molecular defect (SLC4A1 (Band 3), ANK1 (ankyrin), SPTA1 (α-spectrin) and SPTB (β-spectrin). Significant differences in expression compared with healthy controls (=100%) are depicted with * (P≤0.05), † (P≤0.01) or ‡ (P≤0.001) (One-Sample T Test). Significant differences between patients groups are depicted with horizontal lines.

Figure 2 a) significant correlation (R²=0.51, P≤0.001) between eosin-5’-maleimide (EMA)-binding and band 3 expression on RBCs from HS patients. Despite the correlation between EMA-binding and band 3 expression, EMA binding lacks sensitivity to discriminate HS patients with different gene defects. b) 3D-scatterplot of RBC membrane protein expression of Rh factor, band 3 and Rh-associated glycoprotein (RhAG) in HS patients with molecular defects in SLC4A1 (Band 3) (depicted in blue), ANK1 (ankyrin) (depicted in red) and SPTA1 (α-spectrin)(depicted in green). RBC membrane expression levels of Rh factor, band 3 and Rh-associated glycoprotein (RhAG) provides an initial lead to the pathological mutation that caused HS.