

# Rapid and reproducible characterization of sickling during automated deoxygenation in sickle cell disease patients.

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## Abstract

In sickle cell disease (SCD), sickle hemoglobin (HbS) polymerizes upon deoxygenation, resulting in sickling of red blood cells (RBCs). These sickled RBCs have strongly reduced deformability, leading to vaso-occlusive crises and chronic hemolytic anemia. To date, there are no reliable laboratory parameters or assays capable of predicting disease severity or monitoring treatment effects. We here report on the oxygenscan: a newly developed method to measure RBC deformability (expressed as Elongation Index – EI) as a function of  $pO_2$ . Upon a standardized, 22 minute, automated cycle of deoxygenation ( $pO_2$  median 16mmHg $\pm$  0.17) and reoxygenation, a number of clinically relevant parameters are produced in a highly reproducible manner (coefficients of variation <5%). In particular, physiological modulators of oxygen affinity, i.e. pH and 2,3-diphosphoglycerate showed a significant correlation (respectively  $R=-0.993$  and  $R=0.980$ ) with Point of Sickling ( $Pos_{5\%}$ ), which is defined as the  $pO_2$  where a 5% decrease in EI is observed during deoxygenation. Furthermore, *in vitro* treatment with anti-sickling agents, including GBT440, which alter the oxygen affinity of hemoglobin, caused a reproducible left-shift of the PoS, indicating improved deformability at lower oxygen tensions. When RBCs from 21 SCD patients were analyzed, we observed a significantly higher PoS in untreated homozygous SCD patients compared to treated patients and other genotypes. We conclude that the oxygenscan is a state-of-the-art technique that allows for rapid analysis of sickling behavior in SCD patients. The method is promising for personalized treatment, development of new treatment strategies and could have potential in prediction of complications.

## 1. INTRODUCTION

In sickle cell disease (SCD) a single point mutation in the gene encoding for  $\beta$ -globin chain underlies the production of the abnormal hemoglobin S (HbS). HbS polymerizes upon deoxygenation which results in the formation of hemoglobin polymers and eventually gives the red blood cell (RBC) its characteristic sickled shape. Exacerbating the hypoxia-induced polymerization is the inherent low-affinity of HbS, presumably due to high levels of 2,3-diphosphoglycerate (2,3-DPG) present in sickle RBCs.<sup>1</sup> These sickled RBCs have strongly reduced deformability and show increased adherence to vascular endothelium, leading to vaso-occlusive crises.<sup>2-4</sup> Hydroxyurea (HU), regular blood transfusion and L-glutamine are the main treatment options in SCD, with allogeneic hematopoietic stem cell transplantation being the only curative therapy currently available.<sup>5</sup> Remarkably, clinical severity varies considerably despite the fact that all SCD patients carry the same homozygous point mutation in the  $\beta$ -globin gene. To date there is no reliable laboratory or clinical parameter available to predict severity and/or complications.

An individual patient's tendency to "sickle" can be tested *in vitro* using a so-called sickling assay: RBCs are incubated under hypoxic conditions and manually or digitally counted using a light microscope. This assay is still widely applied in research, and many preclinical and early phase pharmacologic and gene therapy trials use this assay as outcome variable to predict clinical effect.<sup>6-11</sup> However, there are many disadvantages: it is time consuming, it has low sensitivity and high variability, and the process is not automated. More importantly, morphological characteristics of sickle cells may not correlate well with physiologic characteristics, such as deformability.<sup>12</sup>

RBC deformability at normoxic conditions is compromised in patients with SCD due to irreversibly sickled cells, as well as altered hydration causing a decrease in surface/volume ratio.<sup>12-14</sup> Osmotic gradient ektacytometry, in which deformability is measured as a function of a continuous change in osmolarity, is a well-established method, in particular in hereditary disorders of the red cell membrane.<sup>15-18</sup> In SCD, osmotic gradient ektacytometry showed that increasing HbF induces a better surface-to-volume ratio thereby improving deformability in untransfused patients with SCD.<sup>19</sup> RBC deformability during normoxic conditions as a marker for complications has also been investigated in the past, and a negative correlation was found between an increase in RBC deformability and osteonecrosis or painful sickle cell crises.<sup>20-22</sup> In contrast to these findings, RBC deformability was found to be decreased during an acute vaso-occlusive crises when compared to steady state values.<sup>23</sup> Thus, in itself, RBC deformability during normoxic conditions gives inconsistent results which makes it unsuitable to be used as predictive biomarker.

Therefore, an assay that investigates sickling during deoxygenation is required to monitor

treatment and disease state in patients with SCD. Importantly, this assay could identify individuals who are at increased risk for certain complications, and potentially predict response to therapy. Finally, it contributes to a better understanding of the clinical effect of new anti-sickling agents. In this study, we evaluate the oxygenscan, which investigates RBC deformability as a function of continuously changing  $pO_2$ , allowing us to characterize individual sickling behavior in SCD patients.

## 2. METHODS

### 2.1 Blood Specimens.

University Medical Center Utrecht (UMCU): Whole blood of patients with SCD who were included in 2 different clinical studies (Netherlands Trial Register [NTR] identifier, NTR 6779 and NTR 6462) was used. Additional samples were collected from discarded anonymized EDTA blood of patients with SCD who visited outpatient clinic. Whole blood from healthy controls was collected through the Mini Donor Service, which is approved by the ethical committee of the UMCU and in accordance to the declaration of Helsinki.

The Children's Hospital of Philadelphia (CHOP): Leftover EDTA blood samples from individuals with SCD after routine clinical visits were utilized for the traditional anti-sickling studies. The use of such samples, with informed consent, is in accordance to an approved IRB protocol.

### 2.2 Oxygen gradient ektacytometry: the oxygenscan

The Laser Optical Rotational Red Cell Analyzer (Lorrca, RR Mechatronics) is a next generation ektacytometer which measures different qualitative aspects of RBC deformability expressed as Elongation Index (EI). The  $pO_2$ scan is a new add-on of the Lorrca, allowing the measurement of red cell deformability as a function of a continuously changing gradient of  $O_2$  tension. In brief, a couette system is used to apply shear stress on the RBCs, which are diluted in a viscous solution (Iso Oxy), consisting of polyvinylpyrrolidone (PVP) with characteristics of whole blood (i.e. osmolarity and pH). Between a static cylinder (bob) and rotating cylinder (cup), there is a small gap in which the blood suspension is injected (Figure 1A). Light from the laser beam is scattered by the presence of RBCs and this diffraction pattern is projected and analyzed. In healthy controls, the diffraction pattern is circular at rest and elliptical shaped at higher shear stress (Figure 1B).<sup>15</sup>

To carry out the oxygenscan, 50 $\mu$ L of whole blood, standardized to a fixed RBC count of  $200 \times 10^6$  is suspended into 5ml Iso Oxy (osmolarity 282-286mOsm/kg, pH 7.35-7.45) at room

temperature ( $\pm 22^{\circ}\text{C}$ ). Approximately 2 ml of the sample solution is slowly injected into the gap between the cup and bob until a fixed level is attained (Figure 1C). The bob is maintained at a constant temperature of  $37^{\circ}\text{C}$ . Inlet of nitrogen gas from above facilitates deoxygenation (Figure 1C).  $\text{O}_2$  tension is measured every 20 seconds by means of a small oxygen ( $\text{O}_2$ )-spot which is covered with luminophore that is present in the wall of the cup. The  $\text{pO}_2$  is calculated from the amount of quenching of the signal that is sent from the fiber to the  $\text{O}_2$ -spot. One oxygenscan consists of approximately 80 measurements of EI, during one round of deoxygenation (1300 seconds), and followed by reoxygenation (280 seconds). Shear stress is fixed at 30 pascal (Pa). Ambient air  $\text{pO}_2$  in the cup was about 160mmHg and is gradually lowered to below 20mmHg (2.7kPa, 2,9%)  $\text{O}_2$ . Reoxygenation occurs due to passive diffusion of ambient air.

### 2.3 Oxygenscan parameters

When considering a typical oxygenscan curve (Figure 1F) six parameters are considered to reflect various features of the process of HbS polymerization or sickling: **Elmax**: maximum EI before deoxygenation that represents baseline position and shows overall deformability of the total RBC population. **Elmin**: minimum EI, representing minimal deformability resulting from changes in shape and orientation of (sickle) RBCs upon deoxygenation (Figure 1D). **DeltaEI**: difference between Elmax and Elmin, indicating sickling capacity during one round of deoxygenation. **Point of Sickling<sub>5%</sub> (PoS<sub>5%</sub>)**:  $\text{pO}_2$  (mmHg) at which 5% decrease of Elmax during deoxygenation is observed. This indicates the  $\text{pO}_2$  where sickling starts. **Area**: area under the curve which is defined by an integral calculation of the EI and  $\text{pO}_2$  measurements between minimum  $\text{pO}_2$  and 100mmHg. This is the result of Elmax, Elmin and PoS. **Recovery**: percentage of EI after reoxygenation compared to EI just before deoxygenation measured at the  $\text{pO}_2$  between 100-120mmHg. This represents the capacity of sickled cells to unsickle during reoxygenation.

### 2.4 Digital Microscopy.

Smears of peripheral blood of patients were analyzed using the CellaVision digital microscope DM96 (software 5.0.1build11), the traditional neural network of which classifies sickle cells based on manually designed features, such as shape, color and texture features. Sickle cell counts were expressed as percentage of absolute cell count analyzed by digital microscopy.<sup>24</sup>

### 2.5 In vitro addition of HbAA blood to HbSS blood, mimicking red cell transfusions.

To investigate the effect of adding RBCs of healthy donors, ABO and rhesus matched RBCs of a healthy donor was mixed with whole blood of a patient with SCD to reach the following

percentages of HbS blood: 100%, 75%, 50%, 25% and 0%.

## 2.6 Influence of 2,3-DPG levels, pH and temperature.

To assess the influence of 2,3-DPG levels on oxygenscan parameters, complete blood count and oxygenscans were performed within 10 minutes after blood collection, and after 24 hours. Whole blood was kept either at 4°C, room temperature ( $\pm 22^{\circ}\text{C}$ ) and 37°C for 24 hours. 2,3-DPG was measured by spectrophotometry (SpectraMax M2e, Molecular Devices) using the 2,3-DPG assay kit (Roche). 2,3-DPG levels were normalized to hemoglobin levels.

To assess the influence of temperature, oxygenscans were carried out with bob temperatures of 35, 37, 39, 41 and or 43°C.

To investigate the influence of pH, the pH of the Iso Oxy was set to 6.9, 7.1, 7.3, 7.7 and 7.9 before being used in the oxygenscan.

## 2.7 *In vitro* incubation with anti-sickling agents GBT440, 5-PMFC, and INN312.

GBT440, 5-PMFC and INN312 are a class of anti-sickling aromatic aldehyde agents that modulate hemoglobin oxygen affinity by stabilizing the higher oxygen affinity relaxed state (R-state) of hemoglobin and/or destabilizing the low oxygen affinity tense state (T-state) of hemoglobin. GBT440 (2-hydroxy-6 ((2-(1-isopropyl-1H-pyrazol-5-yl) pyridin-3-yl)-methoxy) benzaldehyde, MedChemExpress) is a novel, orally bioavailable agent currently in clinical trials for SCD.<sup>9,25</sup> 5-PMFC (5-(Phenoxymethyl)-2-furan carbaldehyde) is an aryl ether derivative of 5-(hydroxymethyl)furfural (5-HMF), with a potent anti-sickling effect.<sup>10</sup> Designated International Nonproprietary Name-312 (INN312) is a designed and synthesized derivate of vanillin.<sup>8,26</sup> Whole blood (20% hematocrit) was added to N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES)-buffer (pH 7.4), which contained 30mM TES-sodium salt and 30mM TES-free acid (both Merck KGaA), supplemented with 130mM NaCl, 5mM KCl, 10mM glucose and 0.2% bovine serum albumin. These suspensions were incubated with the agents for 1 hour at 37°C at normoxia. Concentrations used for incubation were 1mM and 2mM of GBT440, and 1 and 5mM of 5-PMFC and INN-312.<sup>7,10</sup> Anti-sickling agents were dissolved in dimethyl sulfoxide (DMSO), while incubation with DMSO alone was used as a negative control.

## 2.8 Morphological analysis after *in vitro* incubation with anti-sickling agents.

To compare the outcome of the effect of the agents mentioned above, sickling was also measured using previously reported methodology with slight modifications.<sup>7,10,27</sup> Briefly, whole blood was suspended (20% hematocrit) in isotonic Hemox buffer (TCS Scientific Corp), pH 7.4, supplemented with 10mM glucose and 0.2% bovine serum albumin and incubated at normoxia in varying concentrations of GBT440 (1, 2 and 5mM), 5-PMFC (1 and 5mM) and INN312 (1 and

5mM) at 37°C for 1 hour. Next, suspensions were incubated under hypoxic condition (2.5% Oxygen gas/97.5% Nitrogen gas) at 37°C for 1 hour. Cells were fixed and analyzed by light microscopy.

## 2.9 Statistical Analysis.

Experiments were mainly carried out in duplicates, mean, standard deviation (SD) and coefficient of variation (CV) were calculated. Statistical analysis was performed using IBM Statistics SPSS (v21) and Graphpad Prism. One-way ANOVA (Post-hoc Tukey's test or Dunnett's test) or a Kruskal-Wallis test (Post-hoc Dunn's test) was used when appropriate. We normalized results in the experiments with 2,3-DPG because of known individual differences in 2,3-DPG levels. Results of incubation with anti-sickling agents was normalized to enable the comparison of DeltaEI at 18mmHg with the classical, microscopy based sickling assay. Pearson's correlation was used to determine correlations of oxygenscan parameters with laboratory and clinical parameters. A *p*-value of <0.05 was considered statistical significant.

## 3. RESULTS

### 3.1 The Oxygenscan reproducibly characterizes red blood cell sickling.

To validate the technique whole blood samples of 21 individuals with SCD, 5 individuals with sickle cell trait, and 8 healthy donors were used. A representative oxygenscan and its derived parameters is shown in Figure 1F. Importantly, parameters from duplicate measurements generally had a coefficient of variation (CV) <5% (median 1.83%). In case a CV >5% was obtained, a third measurement was performed. The parameters Elmax and Recovery appeared to be the most robust with median CVs <1%.

Measurements of pO<sub>2</sub> and EI are not performed at the same position in the cup. This ensures a better discrimination between the deoxygenation and reoxygenation curves and, hence, a better interpretation of them.

In order to confirm that the change in diffraction pattern during deoxygenation reflects sickling, cells were deoxygenated outside the Lorrca, under comparable conditions, fixed and analyzed by light microscopy as described. Compared to oxygenated conditions sickling is clearly induced upon deoxygenation (Figure 1E and Supplemental Methods).

Upon storage of whole blood at 4°C oxygenscan curves shifted slightly upwards. When stored for 24 hours at 4°C curves remained stable, with minor increases in Elmax (1.7%), PoS (2.7%),

DeltaEI (9.2%) and Area (6.7%). Minor decreases of 6.7% in Elmin and 0.6% in Recovery were observed under these conditions. Upon longer storage oxygenscans shifted upwards. (Supplemental Table 1).

### **3.2 The oxygenscan can adequately assess the effect of blood transfusion.**

In order to investigate the direct effects of blood transfusions on sickling behavior RBCs of a healthy control (HC) was mixed with whole blood of a SCD patient in different amounts (Supplemental Figure 2A). Supplemental Figure 2B and 2G shows that Elmax and Elmin shifted towards normal upon increasing percentages of HbAA blood. At the same time the PoS shifted to the left (Supplemental Figure 2D), whereas DeltaEI decreased and Area increased (Supplemental Figure 2C and E). Recovery also increased to 100% (Supplemental Figure 2F).

All oxygenscan parameters correlated significantly with the percentage of whole blood. Elmin showed a striking linear correlate of  $r=0.998$ ,  $p<0.0001$  (pooled data of 3 patients, individual correlations were above  $r=0.987$  and  $p<0.01$ , Supplemental Figure 2G- L).

### **3.3 Oxygenscan parameters reflect the influence of physiologic modulators of the Hb-dissociation curve.**

Temperature, pH and 2,3-DPG are important cellular modulators of the  $O_2$  affinity of hemoglobin and thereby of sickling. These parameters are therefore expected to exert effect on the oxygenscan. Oxygenscans and 2,3-DPG were essentially unchanged after 24 hours at 4 °C (Supplemental Figure 2A-G). When kept at room temperature or 37 °C, 2,3-DPG had decreased after 24 hours (normalized and pooled data is shown in Supplemental Figure 2B). 2,3-DPG showed a strong correlation with PoS ( $r=0.980$ ,  $p=0.020$ , Supplemental Figure 2F and K) and Elmin (Supplemental Figure 2D and I).

Upon lowering the pH of Iso Oxy, an increase of Elmax, DeltaEI and PoS was observed, and Elmin, Area and Recovery decreased (Supplemental Figure 3A-G). Upon increasing the pH the opposite effect was observed (Supplemental Figure 3A-G). PoS appeared to correlate best with pH change ( $r=-0.993$ ,  $p<0.001$  (pooled data, Supplemental Figure 3K), indicating that lowering pH results in sickling that starts at higher  $pO_2$ .

Next, we performed series of oxygenscans of a single sample at different cup and bob temperatures. Results were similar as those found with pH adjustments (Supplemental Figure 4). Elmax decreased upon increased temperature and correlated best (linear correlate  $r=0.996$ ,  $p<0.001$ ), indicating RBC deformability at normoxic conditions is temperature dependent (Supplemental Figure 4G).

### **3.4 The oxygenscan provides a means to evaluate the efficacy of anti-sickling agents.**

Currently several new drugs for SCD are being tested in clinical trials. With blood of 6 SCD patients we investigated 3 of those drugs (GBT440, 5-PMFC and INN312) for their efficacy on directly preventing hypoxia-induced sickling using the oxygenscan as readout. GBT440, showed a large improvement in oxygenscan parameters at a concentration of 1mM and an even larger effect at 2mM (Figure 2A). After incubation with GBT440, Elmax slightly decreased. In contrast, Elmin increased with increasing doses of GBT440, which was significant for 2 and 5mM ( $p=0.005$  and  $0.001$ , Figure 2B). Similar to these findings, DeltaEI and PoS significantly improved with increasing GBT440 concentration ( $p<0.001$ , Figure 2C and D). Area significantly improved as well ( $p<0.001$ ). The same pattern was observed with 5-PMFC and INN312, with the latter showing the higher efficacy of the two (Figure 2B-E). However, at dosages of 1mM the effect of these 2 compounds was lower compared to GBT440 (Figure 2B-E): Elmin increased with 1mM and significantly increased with 5mM ( $p<0.001$ ), DeltaEI decreased significantly with 5-PMFC 5mM ( $p<0.001$ ) and INN312 1 and 5mM ( $p<0.01$ ). PoS decreased in both concentrations of 5-PMFC and INN312, with 5mM being significant in both ( $p<0.001$ ). Importantly, when oxygenscan parameters were compared with the conventional sickling assay (microscopy) the results showed similar patterns and were correlated ( $r=0.934$   $p=0.002$ , Figure 2F and G). However at a lower concentration of GBT440, and to lesser extent also 5-PMFC and INN312, a slightly larger anti-sickling effect was observed with the conventional sickling assay (Figure 2F).

### **3.5 The oxygenscan is able to discriminate between different genotypes and treatment regimens.**

In order to investigate if the oxygenscan can detect differences between genotypes and treatment regimens, oxygenscans were performed with whole blood of 21 individuals with SCD, 5 individuals with HbS trait, and 5 healthy controls (HC). Homozygous patients without treatment (HbSS untreated,  $n=7$ ) were compared to HbSS patients treated with HU ( $n=5$ ), HbSS patients treated with exchange transfusion ( $n=3$ ), HbS/ $\beta^0$ thalassemia ( $n=1$ ), HbSS patients with  $\alpha$ -thalassemia ( $n=2$ ), hemoglobin SC disease patients (HbSC,  $n=3$ ), HbS traits ( $n=5$ ) and HC ( $n=5$ ). Representative curves of groups with 3 or more patients are shown in Figure 3A-D, with the mean of the different groups of Elmax, Elmin, PoS and Recovery shown in Figure 3E-H. Absolute values of all oxygenscan parameters are reported in Supplemental Table 2. A significant decrease of PoS was seen in all groups compared to untreated HbSS patients, except HbSS individuals who had been recently transfused. Recovery was significantly lower in HbSC patients compared to all the other groups. These results show that the oxygenscan is able to discriminate between different genotypes and treatment regimens.

When levels of HbF and HbS of patients with HbSS, HbS $\beta^0$ , HbSS/ $\alpha$ -thalassemia with/without HU, were correlated with oxygenscan parameters, Elmin showed the strongest correlations ( $r=0.833$  and  $r=-0.852$ , both  $p<0.001$ , Figure 4A-B and Supplemental Figure 5). Digital microscopy findings of these patients were also correlated with oxygenscan parameters. Elmax showed the strongest correlation with sickled cells at normoxia ( $r=0.596$ ,  $p=0.019$ , Figure 4C).

## 4. DISCUSSION

The oxygenscan is a rapid and reproducible technique, that is developed to assess RBC sickling under automated continuous deoxygenation and re-oxygenation. We report for the first time on the potential of the oxygenscan to monitor clinical conditions and parameters that influence HbS polymerization. We found strikingly strong correlations of oxygenscan parameters with physiological and pharmacological modulators of HbS polymerization that are much stronger than in previously reported assays.<sup>11</sup> Importantly, diverse physiologic and pharmacologic conditions correlated best with particular oxygenscan parameters. This underlines the specificity of the various oxygenscan parameters: conditions mimicking exchange blood transfusion were found to specifically affect Elmin, similarly to HbF and HbS levels (Figure 4A-B). Physiological modulators of oxygen affinity predominantly affected PoS (Supplemental Figures 2 and 3). Regarding *in vivo* treatment of SCD, our findings show that RBCs of untreated HbSS patients start to sickle at higher oxygen tensions. Treatment with either HU or transfusion lowers the PoS indicating a lower  $pO_2$  at which cells start to sickle. In case of HbSC patients, Recovery is significantly lower compared to the other groups indicating distinct processes of unsickling.<sup>28</sup> We speculate that this reduced capacity to recover could be involved in the distinct vaso-occlusive phenotype of HbSC patients.

### 4.1 Elmin is a reliable biomarker of the efficacy of blood transfusion.

Our results show a strong correlation of Elmin with the distribution of HbS, HbF and HbA, and their effect on polymerization (Figure 2B, 3F and 4A-B). Elmin shows the deformability at the point of minimal  $pO_2$  during an oxygenscan and reflects how many cells are sickled. The physiological ranges of intravascular  $pO_2$  tension differ between tissues and blood vessel diameters, with the lowest values found in the bone marrow of live animals (i.e. 17.7mmHg).<sup>29,30</sup> Oxygenscans curves reach at least 20mmHg in >95% of measurements, with a mean of 15.9mmHg (SEM 0.17). The minimal  $pO_2$  during the oxygenscan is yet another relevant

functional characteristic. In contrast to other sickling assays, in which a non-physiologically low  $pO_2$  is used, often during several hours. Other assays use sodium metabisulfite, which depletes RBCs fully from oxygen. Both techniques result in full sickling of RBCs, even in sickle cell trait. The fact that oxygenscans of individuals with sickle cell trait do not show any significant sickling behavior compared to patients with SCD underlines the physiological applicability of the oxygenscan (Figure 3A-D and F).

Elmin therefore is a potent parameter for assessing the direct effects of blood transfusion and distribution of HbS, HbF and HbA. We suggest Elmin is mostly predicted by the percentage of cells that are not able to sickle at all and therefore is most influenced by blood transfusion and F-cells.

#### **4.2 The potency of anti-sickling agents is captured by DeltaEI.**

DeltaEI can be used as outcome parameter in the assessment of the efficacy of new treatments that inhibit sickling. As shown by the experiments mimicking blood transfusion (Supplemental Figure 1), DeltaEI reflects the percentage of RBCs that sickle upon deoxygenation and as such is comparable with the conventional sickling assays (microscopy). In our experiments with the anti-sickling agents GBT440, 5-PMFC and INN312, data of the conventional assay was compared to DeltaEI and a similar pattern was observed (Figure 2E-F). Overall, the effects were more pronounced in the conventional assay, which could be due to a longer exposure to hypoxic conditions in the latter. This results in a higher amount of sickled cells at the start, and thus a more pronounced effect of anti-sickling agents. Importantly, cells that sickle instantly do not have the 'sickle' shape but a more irregularly shaped or 'star' shaped appearance, also called 'mosaic' cells.<sup>31</sup> These cells have a different orientation in the flow in the Lorrca than fully sickled RBCs and, hence, behave differently in deformability measurements. Moreover, such cells are regarded as sickled cells but they likely are still able to deform to a certain extent. This could account for the less pronounced effect of anti-sickling agents as determined by the oxygenscan.

DeltaEI has a strong correlation with percentage HbSS blood (Supplemental Figure 1J) and can distinguish between a mixture of HbSS and HbAA RBCs and a population of HbAS RBCs (HbS trait, Supplemental Figure 1C, 3D and 4D-F). A feature that could be very helpful for upcoming gene-therapy trials in which an assay is needed that discriminates between mixed chimerism of fully corrected cells (50% HbAA and 50% HbSS) and fully engraftment of half corrected (100% HbAS) cells. Therefore DeltaEI is an useful parameter, in particular in the evaluation of individuals with HbS trait and the development of new treatment strategies.

#### **4.3 PoS discriminates between genotypes and different treatment regimens.**

We show that PoS correlates with 2,3-DPG levels and other modulators that have an effect on the hemoglobin dissociation curve, such as pH and temperature (Supplemental Figure 2A, B, F and J). PoS is unique in its ability to determine the  $pO_2$  at which RBCs start to sickle, and is therefore of high value in the development and clinical testing of new drugs, in particular anti-sickling agents. When considering individuals with HbS trait, PoS has a distinct value in this group compared to controls and SCD patients (Figure 3G). PoS also is the most informative parameter when considering different treatment in groups of SCD patients. Based on these observations we conclude that PoS likely reflects properties of an individual's hemoglobin dissociation curve.

#### **4.4 Elmax reflects percentage of sickled cells at normoxia and RBC hydration.**

In whole blood, the percentage of sickled cells at normoxia compared to relatively healthy cells seem to have the most pronounced effect on Elmax. The number of sickled cells at normoxia correlated to Elmax (Figure 4C), indicating that already sickled cells lower the overall deformability and thereby Elmax. We suggest that Elmax reflects the percentage of non-deformable cells at ambient air, which are the dense cells containing HbS polymer and sickled cells at normoxia.

#### **4.5 Comparison with other hypoxic deformability assays.**

In the past several approaches to measure RBC deformability under hypoxic conditions have been developed. All but one were based on a deoxygenation step that took place outside the ektacytometer.<sup>32–35</sup> In contrast to the oxygenscan, these approaches involved either a deoxygenation or reoxygenation step, or used hypoxia and normoxia as different conditions. A very recent study demonstrated a strong effect of GBT440 on RBC deformability, but no  $pO_2$  could be determined and related to the measurements.<sup>36</sup> The oxygenscan allows now for the first time the study of sickling behavior during both automated deoxygenation and reoxygenation in a reproducible and fast manner.

One of the strengths of the oxygenscan is that deformability is measured at a whole range of different oxygen tensions. Furthermore, the technique has an excellent reproducibility, the time to perform an oxygenscan measurement is relatively short and it takes only a small amount of whole blood. More importantly, the oxygenscan is a functional assay that determines various aspects of the sickling process, each represented by different parameters.

A limitation of the technique is that measurements are performed on the total RBC population. Therefore, individual differences in cellular behavior or subpopulations of cells are not considered and, consequently, outcome parameters always reflect the mean behavior of all cells.

Also, the minimal  $pO_2$  during deoxygenation does not reach 0mmHg. Although this  $pO_2$  is not a physiologically relevant value, it would be of interest to measure in order to see how much the EI would further decrease.

Our data show that apart from the influence of HbF, HbS and HbA, this assay also takes individual hemoglobin dissociation curves and percentage of sickled cells at normoxia into account. Therefore, the oxygenscan is an assay which simultaneously determines a number of clinically important factors involved in SCD pathophysiology. As such it may represent a better alternative for the conventional sickling assay and fill the clinical need for a reliable biomarker. Current studies are aimed at investigating the applicability of the oxygenscan in the determination of clinical severity and vaso-occlusive crisis, as well as a more comprehensive analysis of the effect of current and new treatment strategies.

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## Authorship

Contribution: M.A.E.R, B.A.O, E.J.B. and R.W. designed the experiments. M.A.E.R., B.A.O., J.B., T.H.M., A.C.W.W., O.A., A.B.V., M.E.H., and J.R. collected samples and/or performed experiments. M.S. and O.A. provided new drugs. M.A.E.R., E.J.B. and R.W. analyzed the data and wrote the manuscript with input and revisions of B.A.O., O.A., M.S., M.E.H., M.H.C., M.B., R.E.G.S., and G.P.

## Conflict-of-interest disclosure

The authors declare no conflicts of interest

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