Activation of Glycolytic Enzymes in Red Blood Cells for Therapeutic Purposes

by
Alice Meyer-Delvendahl

(7787731)

Research Report

Master´s program:

Science and Business Management,

Graduate School of Life Sciences,

Utrecht University

First examiner: dr. Richard van Wijk

Second examiner: dr. Judith Jans

Daily Supervisors: drs. Carolina Hernandez-Quiroga & drs. Jonathan de Wilde

Start date internship: 03.10.2023

End date internship: 24.06.2024

Abstract

Background: Glucose-6-phosphate dehydrogenase (G6PD) is a crucial enzyme in the pentose phosphate pathway and pivotal for maintaining redox balance in red blood cells (RBCs). Deficiency in G6PD can lead to hemolytic anemia, particularly under oxidative stress. While therapeutic strategies are primarily preventive, recent advancements propose the activation of G6PD as a therapeutic approach.

Aim: This study investigated the activation of the glycolytic enzyme G6PD in RBCs using a small molecule activator, AG1, to evaluate its potential in mitigating oxidative stress and increasing cell survival.

Methods: RBCs from healthy donors were isolated and incubated with AG1 and Bay-11. Bay-11 was utilized to mimic G6PD deficiency. Oxidative stress was induced using diamide. The impact on G6PD and PK enzyme activity, RBC deformability, and hemolysis was assessed using spectrophotometric assays, RoxyScans and Osmoscans.

Results: AG1 did not significantly alter G6PD enzyme activity in healthy RBCs. Incubation with Bay-11 significantly decreased G6PD activity, but AG1 failed to protect or rescue this depletion. RoxyScan analysis revealed no significant protective effects of AG1 against oxidative stress induced by diamide. However, Osmoscan and spectrophotometric assay results indicated that AG1-incubated samples show slightly improved deformability and hydration status after prolonged storage and a notable reduction in hemolysis.

Conclusion: While AG1 showed potential in reducing hemolysis during RBC storage, it did not significantly increase G6PD activity or protect against oxidative stress in healthy RBCs. These findings suggest that AG1 might stabilize RBCs under storage conditions but is not effective in activating G6PD in the context of oxidative stress. Further research is necessary to elucidate its mechanism and potential therapeutic applications.

Keywords: G6PD deficiency, hemolytic anemia, oxidative stress, AG1, Bay-11, enzyme activity, RoxyScan, Osmoscan, diamide.

Acknowledgments

First and foremost, I would like to express my deepest gratitude to my supervisors, Carolina Hernandez-Quiroga and Jonathan de Wilde, for their invaluable guidance and patience throughout this internship. Thank you for answering my countless questions and supporting me throughout this journey. A heartfelt thank you to, Richard van Wijk, for including me in the meeting of the European Red Cell Society in Ameland and for always taking the time to listen and offer valuable advice. I am also grateful to my second examinor Judith for taking me on as a student and everyone in the URED group for their assistance in the lab and the valuable knowledge shared during our weekly work discussions. Additionally, I would like to extend my appreciation to the CDL. I always felt welcomed and appreciated, and I will sincerely miss my time here. The past ten months have been an incredible learning experience, for which I am deeply thankful.

Table of Content

List of Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BMSS	β-Mercapto stabilizing solution
G6P(D)	Glucose-6-phosphate (dehydrogenase)
GSH	Glutathione
HB	Hemoglobin
HCT	Hematocrit
LDH	Lactate dehydrogenase
LORRCA	Laser Optical Red Cell Analyzer
NADPH	Nicotinamide adenine dinucleotide phosphate
PC	Packed cells
PEP	Phosphoenol pyruvate
PPP	Pentose-Phosphate-Pathway
PK	Pyruvate kinase
RBC	Red blood cell
ROS	Reactive oxygen species
SCD	Sickle cell disease
WB	Whole blood
WBC	White blood cells

1. Introduction

1.1 Erythrocytes anatomy and function

The red blood cell (RBC) is the most common cell in the human body, where it makes up 25% of the total cells. Its primary functions include the transport of inhaled oxygen from the lungs to the body's tissues as well as deporting a quarter of the carbon dioxide waste from the tissues to the lungs for exhalation (1).

Due to the specificity of the RBC, several characteristics are unique and related to its function. The shape is a biconcave disk that is think in the outer edges and thin in the center. Thanks to the unique shape there is a greater surface area for gas exchange in relation to its volume. Within the narrow capillaries, RBCs must fold themselves to pass through. Their membrane proteins allow them to deform to a remarkable degree before bouncing back to their original shape as soon as they enter a wider vessel again (2). Moreover, RBCs lack most organelles, for example mitochondria, thus making them rely on anaerobic respiration. Due to the lack of organelles, there is more space for hemoglobin molecules to transport gases (3).

1.2 Role of G6PD in RBC Metabolism

Metabolism of RBCs is complex. The primary metabolic functions are to generate energy to maintain glycolysis (4) and homeostasis of electrochemical gradients of ions (5), sustain the deformability of red cell membrane (6) and maintain the antioxidative systems to cope with oxidative stress (7,8). The glycolysis and its related pathways within the RBC are presented in Figure 1.1. Glucose is the only source of energy for the RBC. RBCs lack nuclei and mitochondria; thus they are incapable of generating energy via the oxidative Krebs cycle. They depend on the anaerobic conversion of glucose by the Emden-Meyerhof pathway.

Glucose-6-phosphate dehydrogenase (G6PD) is the enzyme that catalyzes the first reaction in the pentose phosphate pathway (PPP) therefore the focus lies on this shunt (Figure 1.2). The reduction to glutathione (GSH) is fundamental for the PPP to maintain its antioxidative function. Reduced glutathione is a key factor in converting reactive oxygen species (ROS) into harmless water. Reactive oxygen species can harm the cell membrane and cause hemolysis within the cell. Without a functional G6PD enzyme, the concentration of ROS in the cell increases, subsequently leading to cellular damage. These damaged cells die prematurely, thus leading to intravascular hemolysis.

1.3 G6PD Deficiency and Diagnostics

G6PD deficiency is the most common enzymopathy and is estimated to affect 400-500 million people worldwide. It is more common in populations in sub-Saharan Africa, or Mediterranean and Southeast-Asian regions. The deficiency is inherited as an X-linked recessive condition, thus making men or homozygote woman to be affected.

The severeness of symptoms of a G6PD deficient patient depend on the acuteness of the deficiency and the presence of a trigger. The activity of the enzyme can be classified in Class A (severe deficiency, with chronic, hemolytic anemia), Class B (moderate deficiency, hemolysis only acute when triggered by stressors) and Class C (no hemolysis) (9). Most people with the enzymopathy can live without having medical issues or a hemolytic crisis. Many carriers of the G6PD mutation, including heterozygous females, are unaware of its presence in their bodies. While carriers may remain asymptomatic, they can develop symptoms and become patients when exposed to specific triggers.

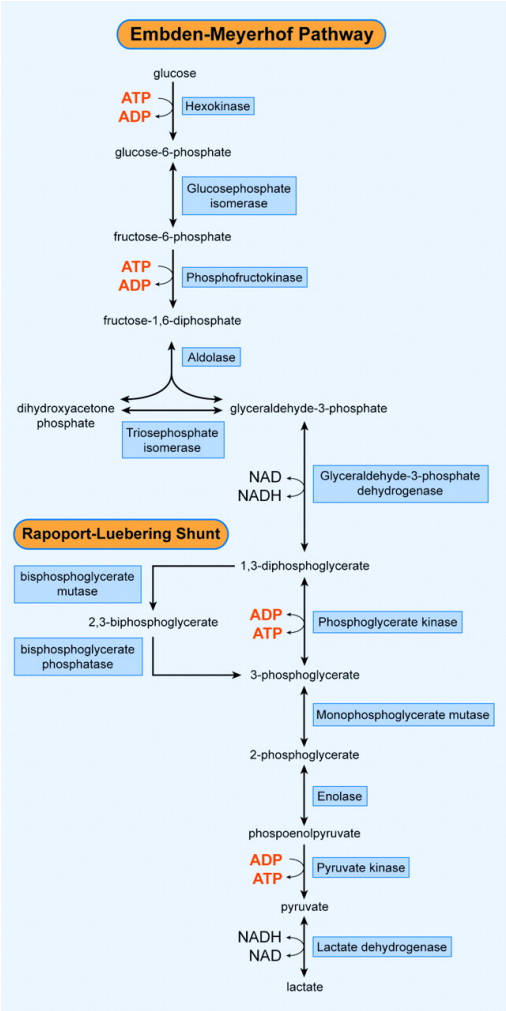


Figure 1.1 *Schematic overview of the Embden-Meyerhof pathway and the Rapoport-Luebering shunt. Illustration enhanced by A. V. Chen.*

Hemolysis is triggered by specific stressors such as infections, certain medications (including antimalarial drugs and some antibiotics), and the consumption of fava beans. One of the most severe complications arising from hemolysis is neonatal jaundice (icterus). The symptoms of hemolytic episodes, or crises, can be diverse and include hemolytic anemia, neonatal jaundice, dark-colored urine, fatigue, pallor, a rapid heart rate, shortness of breath, and splenomegaly (enlarged spleen).

Diagnostics are usually conducted in patients from certain ethnical backgrounds after developing anemia, jaundice or symptoms of hemolysis. This applies especially to patients with relatives that are already known to be positive. Diagnosis can be done via determining red cell enzyme activity with a quantitative assay or screening test (10).

1.4 Treatment

The development of therapeutics is hindered by the belief that common variants have a mild pathology that can be overcome by avoiding trigger foods and triggers drugs or the treatment with blood transfusions during a hemolytic crisis. Therefore, there aren't as many treatment options available as one would expect, considering G6PD-D is the most common enzymopathy worldwide (11).

For non-acute classes of G6PD prevention is the most common kind of 'treatment'. Avoiding certain triggers like fava beans, specific medicines like for instance malaria medication or environmental exposures, as well as daily stress. However, there is interventions with antioxidants like ascorbate (vitamin C) (12) in lower doses to treat methemoglobinemia in G6PD deficient patients (13) or α-Tocopherol (vitamin E), which helps to improve hematological parameters through for example a reduction in reticulocytosis in severe cases (14,15,16). Blood transfusions are also an effective treatment, which are often used as co-treatments with for example Vitamin C simultaneously (17,18).

An alternative treatment strategy is restoring the GSH pool by involving other metabolic pathways capable of generating GSH. G6PD-deficient erythrocytes have higher levels of glycolytic metabolites related to nicotinamide adenine dinucleotide phosphate (NADH) and adenosine triphosphate (ATP) production (19). When RBCs are exposed to the oxidant diamide, GSH is depleted, prompting a metabolic shift towards GSH biosynthesis. Despite these changes, G6PD-deficient cells fail to restore GSH levels to those seen in wild-type cells (20). A predictive model suggests that a 50% increase in plasma L-cysteine can improve GSH levels (21).

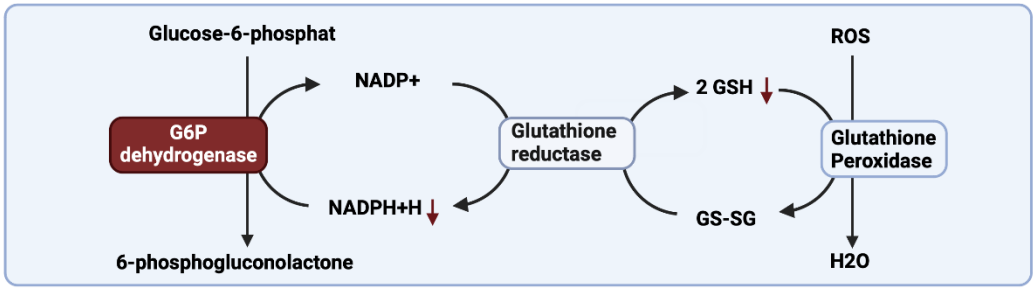


Figure 1.2 *Schematic overview of redox metabolism in RBCs. G6PD catalyzes reaction in PPP that leads to a reduction of GSH to maintain its antioxidative function. Created with BioRender.com*

Class A variants are rare but have a severe phenotype with a significant impact on the patient's life (22). For the severe Class A variants gene therapy is gaining more interest. Some gene therapies for other inherited genetic disorders have already been approved and some are in clinical trials. However, gene therapy for G6PD deficiency is facing challenges regarding the diversity of this disorder (23).

1.5 Novelties

In RBCs the stability of the enzyme is an important determinant of residual activity. In recent years a small molecule activator has been discovered and published by Hwang et al. (24) under the name AG1. It works by promoting oligomerization in G6PD Monomers, through stabilization of the dimeric G6PD state. This is important, because G6PD specifically is only catalytically active in the form of a dimer or tetramer.

Furthermore, a novel method has been developed to study the ability of RBCs to deal with oxidative stress under the name RoxyScan. The application of the technology makes it possible to compare the effect of oxidants in RBCs (25). Expected due to previous research we assume adding an oxidant can change the diffraction pattern.

Due to the successful trials of AG1 in patients, we presume to also detect an increased G6PD activity in healthy controls as well as a protective factor after applying an oxidant in the RoxyScan.

2. Methods

2.1 RBC Isolation

Red blood cells were isolated from whole blood (WB), implicating that white blood cells (WBCs) and platelets were discarded during isolation. For this, a PEGG column filled with a cellulose mixture was drained twice with saline and 3ml of blood were added to the column.

The separation is conducted via centrifugation. Within a solution the gravitational force from continuous revolutions pushes the denser heavier particles (here: RBCs) through the cellulose to the bottom of the tube, whilst WBCs remain trapped as they are less deformable and cannot pass through the cellulose. The RBCs are washed multiple times in saline to remove platelets and plasma proteins. The detailed protocol is attached in the appendix (Protocol A).

A complete blood count was performed on the CELL-DYN Sapphire Hematology Analyzer (Abbott, Illinois, US) to check if the RBC isolation was successfully isolated from WBC and platelets. Assuming an RBC count of 4.0, WBC had to be below 1.0 and platelets below 20.

2.2 Spectrophotometric Assays

The Spectramax iD3 (MolecularDevices) is a spectrophotometer with a microplate reader, which among other functions, includes a module to measure enzymatic reaction. The measurement is achieved by detecting and quantifying light signals within a certain wavelength and temperature. The workmechanisms of a spectrophotometer are schematicaly shown in figure 2.1.

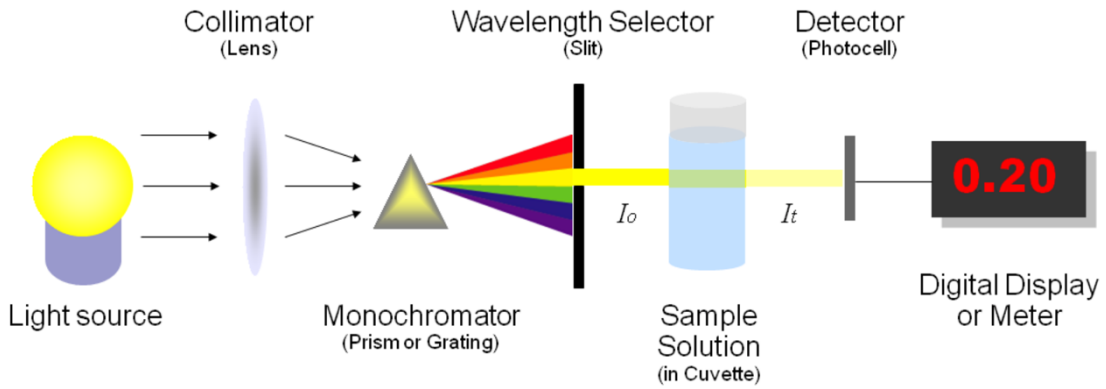


Figure 2.2 *Schematic basic structure of a spectrophotometer. The spectrometer produces the desired range of wavelength of light. The spectrometer generates, disperses, and measures light. It uses a collimator to direct a straight beam through a monochromator, to split it into several component wavelengths. A wavelength selector then isolates the desired wavelengths. The photometer measures the intensity of light. After light passes through a sample solution, the photometer detects the absorbed photons and sends a signal to a digital display for reading. (Heesung Shim via LibreTexts)*

2.2.1 Enzyme activity measurements

Enzyme activity measurements evaluate the activity of the present enzymes. To measure the enzyme activity RBCs need to be isolated to ensure that only the enzymes derived from the RBCs are included. The principle G6PD reaction is the catalyzation from Glucose-6-Phosphate to 6-phosphogluconolactone via the G6PD enzyme. The enzyme activity is measured through the function of time rate of the reduction of $NADP^+$ to NADPH.

$$\text{Glucose - 6 - P} + \text{NADP}^+ \xrightarrow{\text{G-6-PD}} \text{6 - PGA} + \text{NADPH} + \text{H}^+$$

In preparation for the measurement the hemolysate is incubated with glucose-6-P. A portion of the formed product is 6-phosphogluconate, which is mainly oxidized further in the 6-PGD reaction. More than 1 (nearly 2) mole of NADP is reduced for each oxidized mole of glucose-6-P (Beutler, 1975).

$$\text{6-PGA} + \text{NADP}^+ \Rightarrow \text{Ribulose-5-P} + \text{CO}_2 + \text{NADPH} + \text{H}^+$$

The activity of PK was measured to have a control enzyme that is not expected to react to the activator and inhibitor. PK is an allosteric enzyme; it changes its conformation upon binding of an allosteric modulator. This causes a change in the binding affinity at a different ligand binding site (26). Here adenosine diphosphate (ADP) is the allosteric modulator and PK catalyzes the phosphorylation of ADP to ATP through phosphoenol pyruvate (PEP).

$$\text{PEP} + \text{ADP} \Rightarrow \text{Pyruvate} + \text{ATP}$$

The amount of the formed pyruvate is linked to the rate of oxidized NADH in reaction with lactic dehydrogenase (LDH) to Lactate and NAD^+ (27).

$$\text{Pyruvate} + \text{NADH} + H^{+} \overset{\text{LDH}}{\rightleftharpoons} \text{Lactate} + NAD^{+}$$

In reference to Protocol D, E and G a lysate was made from packed cells diluted in beta-mercapto stabilizing solution (BMSS). BMSS consists amongst others of water and beta-mercapto; the water component lyses the RBCs and the beta-mercapto causes a small disruption in the disulfide bridges of the proteins (28). This ensures that the enzymes are more prone to be activated. The procedure is shown schematically in figure 2.2. The settings of the spectrophotometer are set on a wavelength of 340 nm at a temperature 37°C on each well in a 96 well microplate.

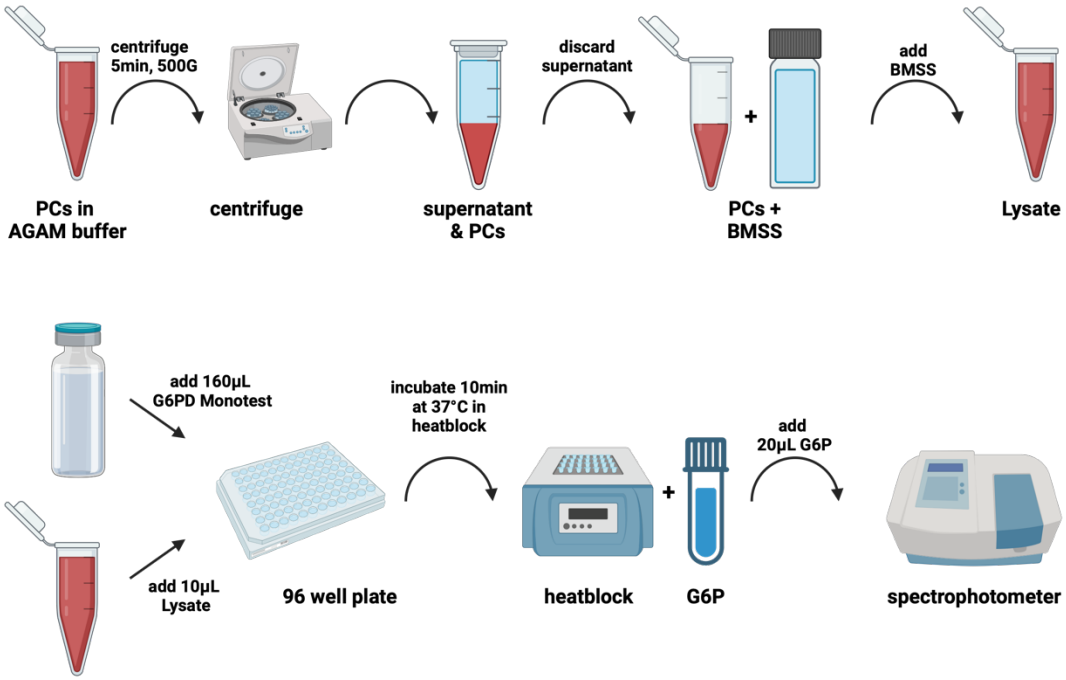


Figure 2.2 *Schematic representation of enzyme activity measurement protocol. Created with BioRender.com*

2.2.2 Measurement of Lysis

In addition to enzyme activity measurements, the plate reader can determine the amount of lysis based on the hemoglobin count. For that the same template was used as in the previously described enzyme activity measurement. However, for this analysis the settings of the second plate in the template are chosen. The spectrophotometer is set on a wavelength of 542 nm at a temperature 37°C on a 96 well microplate. The detailed parameters are portrayed in protocol H.

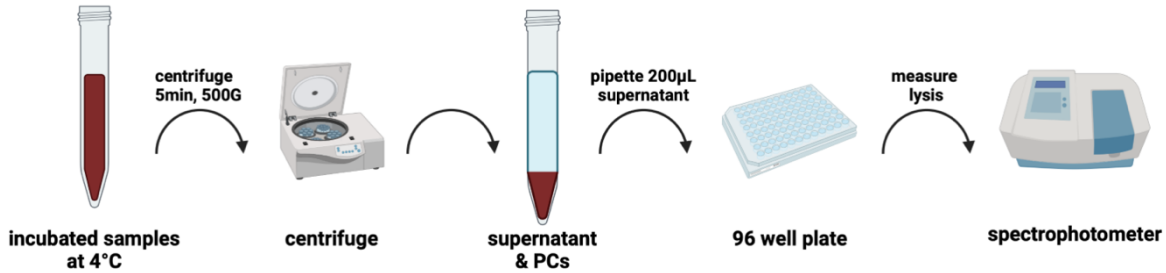


Figure 2.3 *Schematic presentation of lysis measurement. Created with BioRender.com*

2.3 Deformability and Oxidative- and Shear Stress Ektacytometry

Figure 2.4 *Set up of a Laser Optical Red Cell Analyzer (Lorrca MaxSis, RR Mechatronics, Zwaag, The Netherlands)*

The Laser Optical Red Cell Analyzer (Lorrca, RR Mechatronics, The Netherlands) is a machine that holds several modules to evaluate a variety of RBC parameters. The RoxyScan technology is a module that can evaluate oxidant effects on RBCs. RBCs exposed to shear or oxidative stress can only withhold it for a certain time until they deform, change their cell morphology and orientation, and intracellular and membrane characteristics.

The assay works by having a light pass through the RBCs. As the RBC are in a flow, they are exposed to shear stress. The laser beam produces a diffraction pattern, which changes from circular to elliptical as the shear increases.

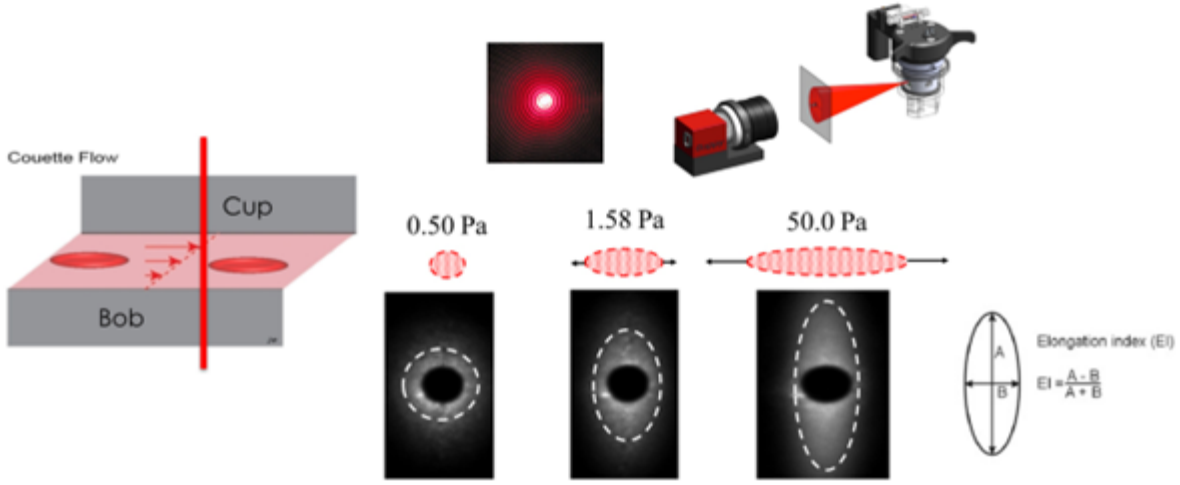


Figure 2.5 *Schematic principle of the Lorrca by creating a diffraction image that relates to the shape of the cell (Lorrca.com).*

The RoxyScan uses the following parameters, as listed in Figure 2.6. The T-Pod stands for the time point, where there has been a 10% decrease in the elongation Index (EI). A later time point means that the RBCs can withstand the stress longer before they are forced to deform. The maximum elongation index (EI_{max}) describes the maximum deformability of the RBC. A higher EI_{max}, is desirable as it indicates that the cell is more flexible. The minimum elongation index (EI_{min}) stands for the osmolality of the RBC. It shows which changes happened to the osmotic fragility and the surface-to-volume-ratio. It represents to what extent they can be shrunk or respectively the amount of damage that can be done to the RBCs, therefore a higher EI_{min} stands for a healthier RBC. Lastly the delta EI (ΔEI) is defined by the difference between the EI_{min} and EI_{max}, whereby a smaller ΔEI preferable is. However, it must be put in context, since the starting point can make big differences to the ΔEI (25).

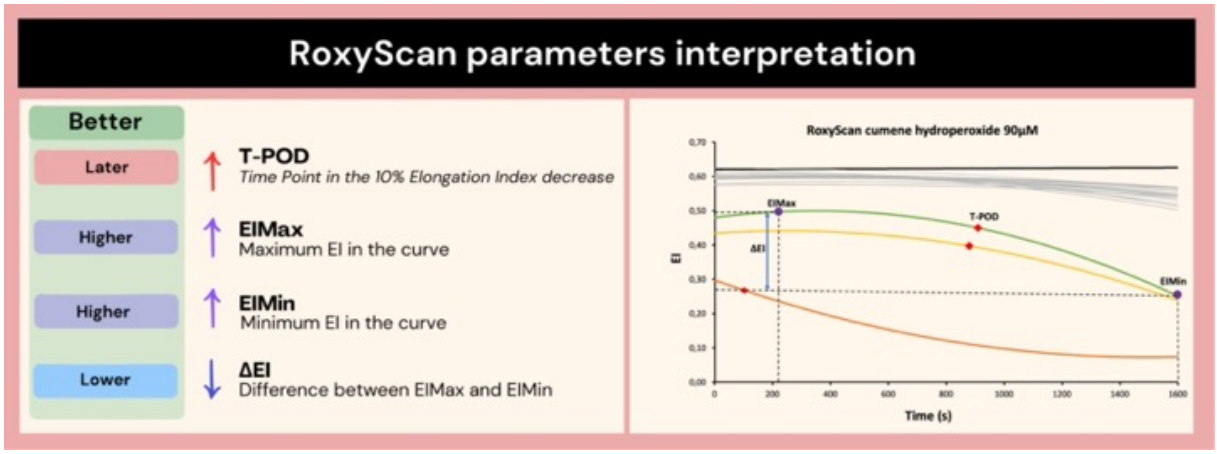


Figure 2.6. *Overview of RoxyScan parameters. (Friendly permission of Carolina Hernandez-Quiroga)*

The preparations and procedures for the RoxyScan are schematically shown in figure 2.7 and in protocol E,F and G in the appendix.

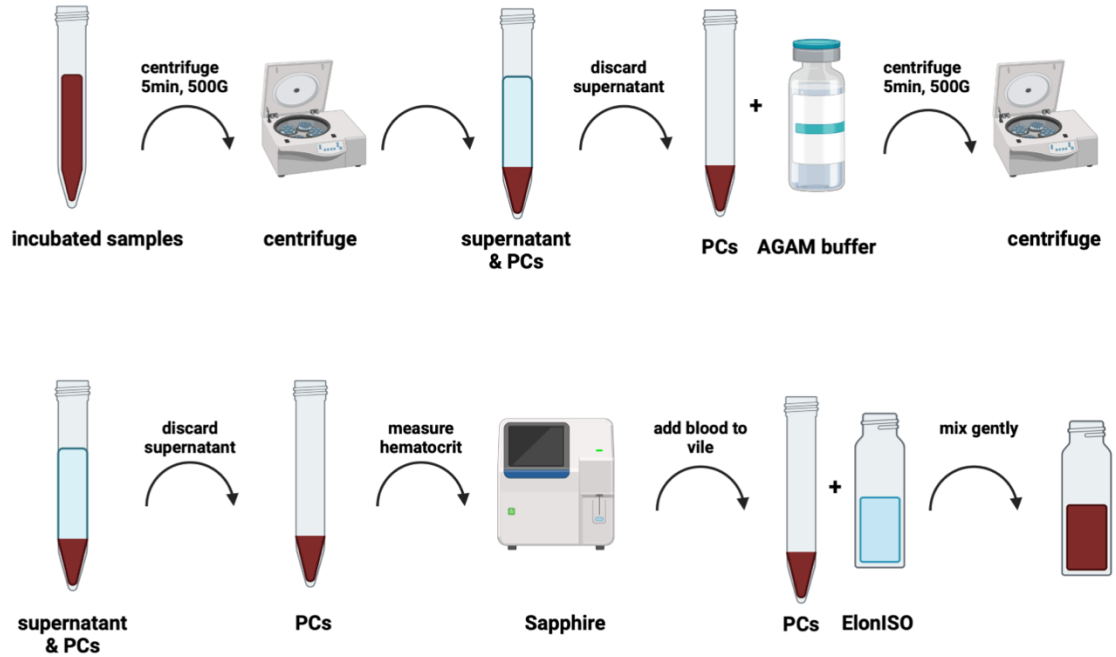


Figure 2.7 *Schematic overview of preparations for a RoxyScan assay. Created with BioRender.com*

2.4 Osmotic Gradient Ektacytometry

Osmotic gradient ektacytometry or Osmoscan is another module of the Lorrca. It allows the assessment of RBC deformability and hydration status.

The assay creates a curve entailing important parameters, allowing a deeper analysis of the sample This includes EImax, which represents maximum deformability, as previously described for the RoxyScan in paragraph 2.3. The osmolality (EI_{min}) has also been previously mentioned for the RoxyScan. It shows changes in the osmotic fragility and surface-to-volume ratio. The osmolality (O_{hyper}) stands for the mean cellular hydration status. In the hypertonic region is in correspondence to the 50% point of EI. If the cell is dehydrated the O_{hyper} is decreased, whereas overhydration increases the O_{hyper} (29).

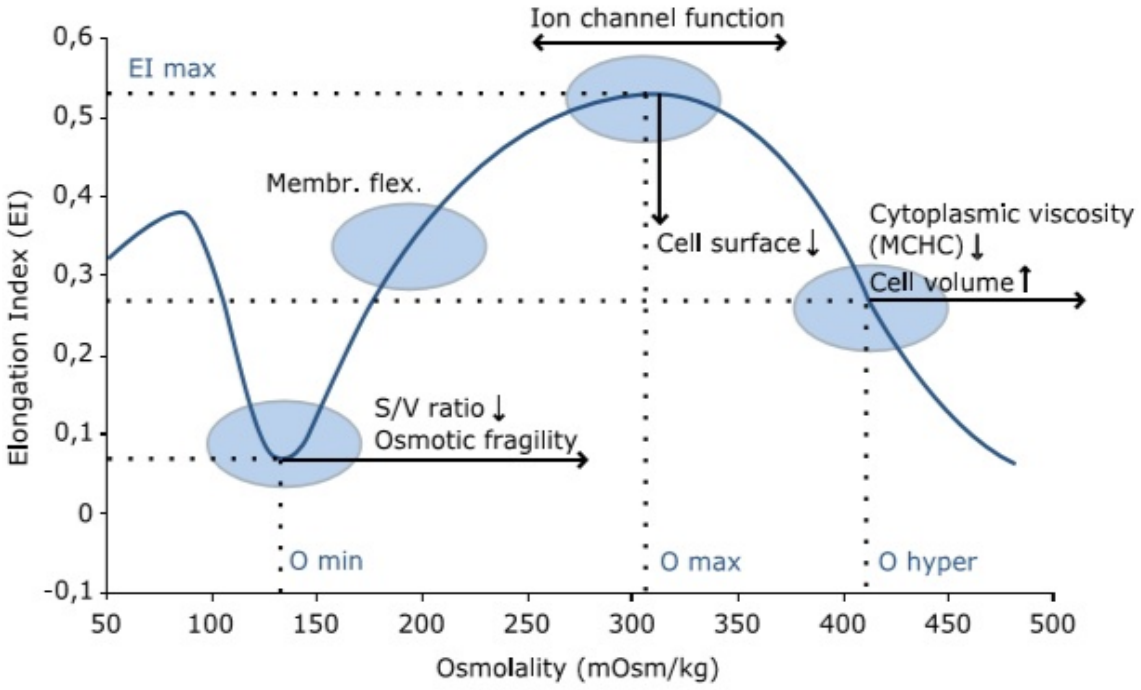


Figure 2.8 *Example curve of an Osmoscan (Lorrca.com).*

The preparation and incubation are shown in Figure 2.8 and can be found in the appendix in Protocol I.

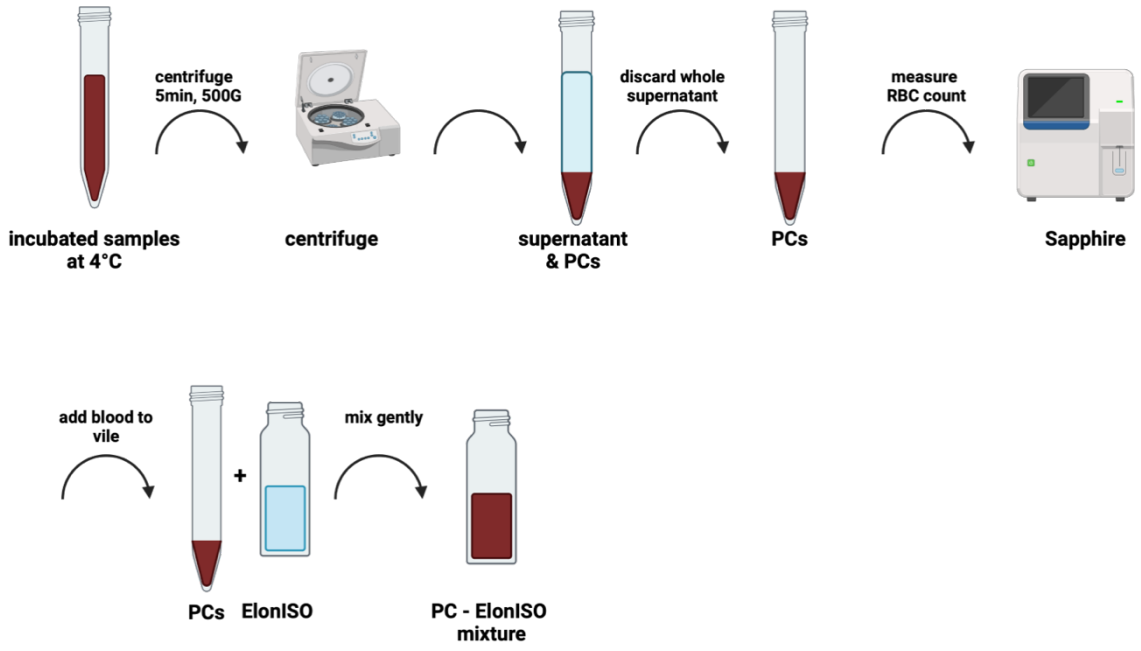


Figure 2.9 *Schematic overview of the Osmoscan protocol. Created with BioRender.com*

2.5 Incubations

To evaluate the effect of ex vivo treatment with the activator molecule on RBCs, PCs in a 5% RBC solution were incubated with AG1 (MedChemExpress, USA). A spectrophotometric assay was conducted to measure G6PD enzyme activity as described in section 2.2.1 Subsequently, the samples were incubated with diamide, and another enzyme activity measurement was performed.

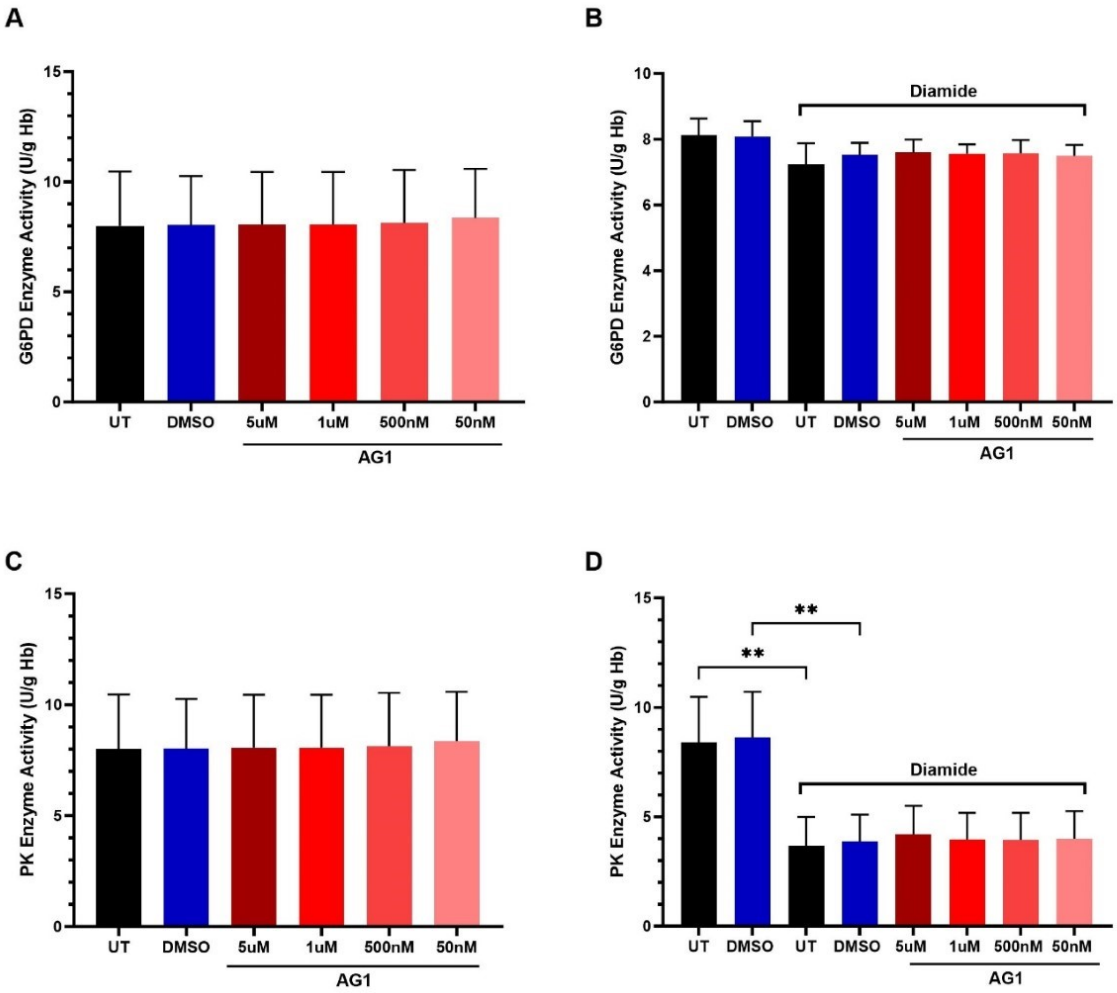
To assess the protective effects of AG1 on stressed RBCs, samples were incubated with AG1, followed by an enzyme activity measurement. The samples were then incubated with Bay 11-7082 (Sigma-Aldrich, USA) and another enzyme activity measurement was conducted following the procedure in section 2.2.1 RBCs were stressed using diamide (Sigma-Aldrich, USA) as an oxidant in a RoxyScan, as described in section 2.3. The same incubations and procedures were performed in reversed order for Bay-11 and AG1.

The effects of ex vivo treatment on stored RBCs were evaluated by incubating samples with AG1 and measuring hemolysis and performing Osmoscans, as described in sections 2.2.2 and 2.4.

3. Results

3.1 Enzyme Activity Measurements

Enzyme Activity measurements were done to examine variations in G6PD and PK enzyme activity between AG1 and control samples. The blank was incubated with DMSO as a vehicle control. Diamide was next evaluated at a concentration of 1mM.



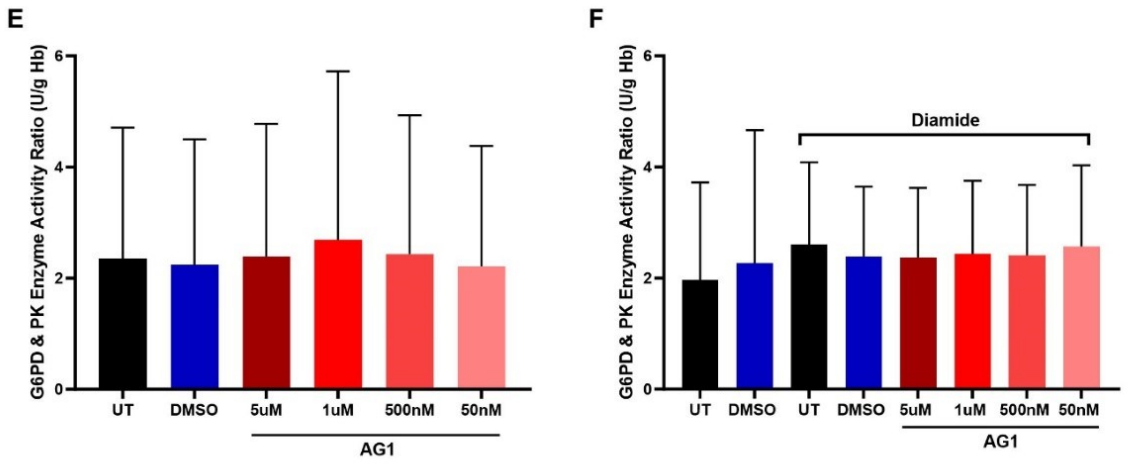
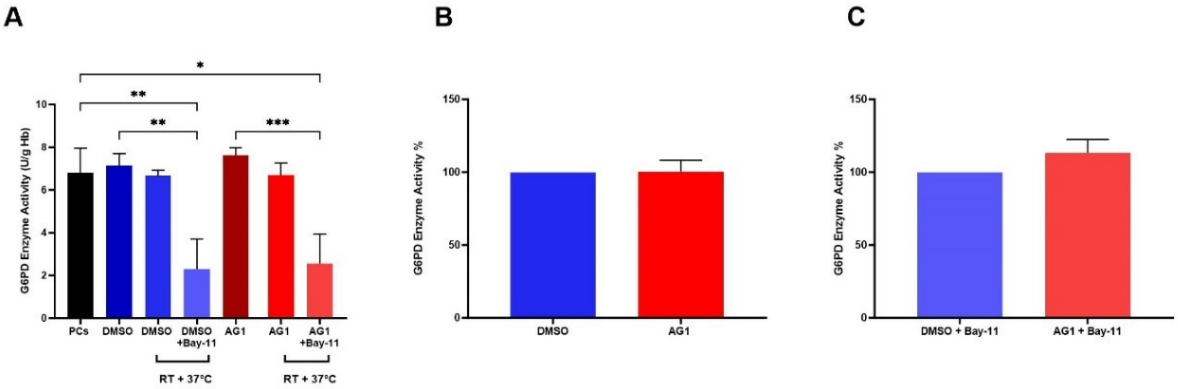


Fig. 3.1 *Enzyme activity measurements of five healthy donors that were incubated with AG1 and afterwards with Diamide 1mM.* ***A*** *G6PD enzyme activity in the untreated condition (UT), DMSO and at four different concentrations of AG1.* ***B*** *G6PD enzyme activity measurement in AG1 and Diamide treated samples* ***C*** *PK enzyme activity measurement in the untreated condition (UT), DMSO and at four different concentrations of AG1.* ***D*** *PK enzyme activity measurement in AG1 and Diamide treated samples, **p<0.001, Kruskal-Wallis test****.*** ***E*** *Ratio of G6PD and PK enzyme activity in AG1 incubations.* ***F*** *Ratio of G6PD and PK enzyme activity in AG1 and Diamide incubations.*

In regard to the G6PD and PK enzyme activity measurements there were no significant differences between the blank and the AG1 conditions, also when comparing the various AG1 concentrations to each other. Diamide treatment after AG1 incubation did not change the enzyme activity of G6PD or PK. Diamide incubations were shown to vary from pre-incubated samples without Diamide by reducing the enzyme activity of G6PD and PK. A Kruskal-Wallis test determined a significant decrease in PK activity, when comparing the UT and DMSO samples before and after the Diamide incubations (UT, mean decrease 65% (**p<0.001); DMSO, 66% (**p<0.001)) (Figure 3.1 D).



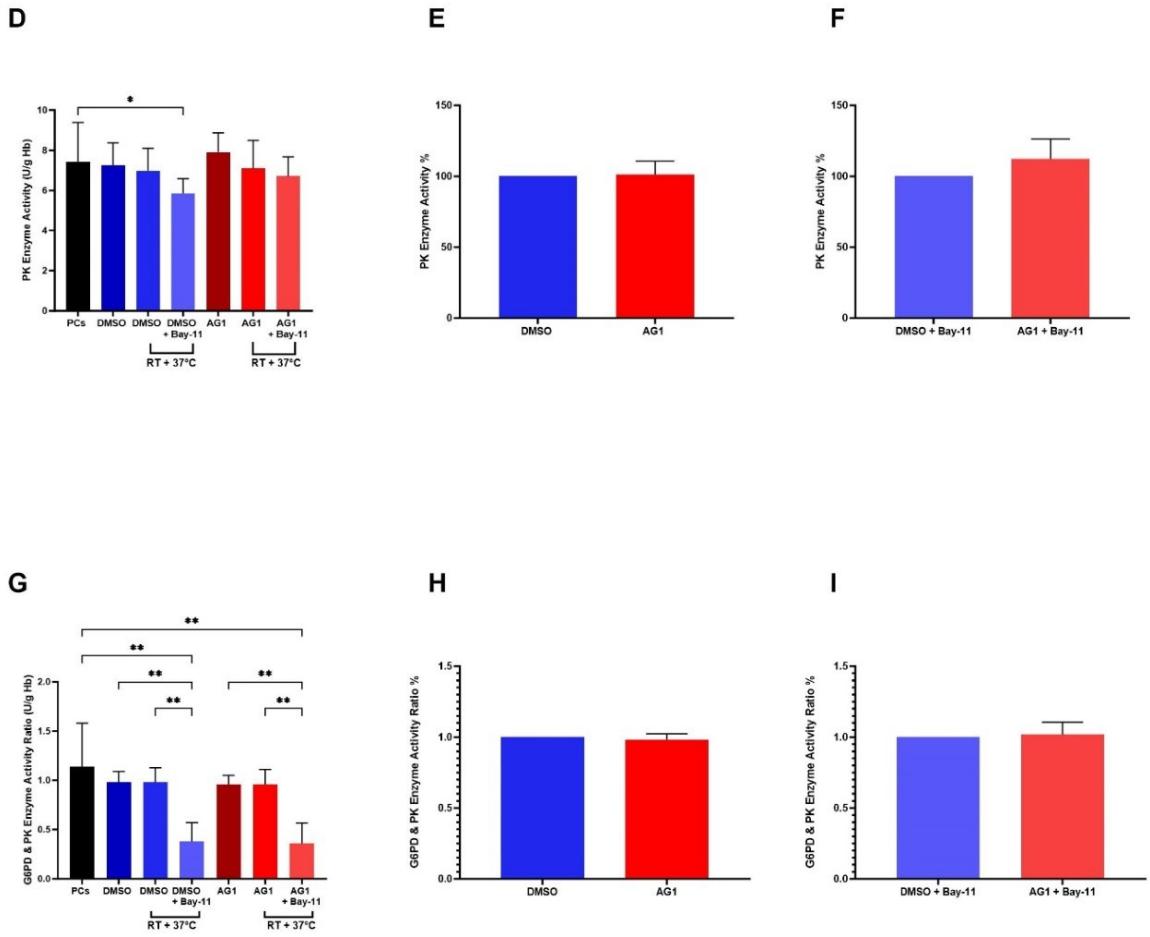


Fig. 3.2 *Enzyme activity measurements of five healthy donors. Samples were incubated with AG1 and Bay-11.* ***A*** *G6PD enzyme activity measurement of all samples, *p < 0.01, **p < 0.001, ***p < 0.0001, Kruskal-Wallis test.* ***B,C*** *G6PD enzyme activity in percentage with DMSO as a standard.* ***D*** *PK enzyme activity measurement of all samples *p < 0.01, Kruskal-Wallis test.* ***E,F*** *PK enzyme activity in percentage in comparison to DMSO as a standard.* ***G*** *Ratio of G6PD and PK enzyme activity in all samples, **p < 0.001, Kruskal-Wallis test* ***H,I*** *Ratio of G6PD & PK enzyme activity in comparison of AG1 and Bay-11 to the blank.*

A Wilcoxon test determined that G6PD and PK enzyme activity was not significantly different in the AG1 condition in comparison to the control (Figure 3.2 B,C,E,F) as well as the G6PD and PK enzyme activity ratio (Figure 3.2 H,I). Incubations with Bay-11 statistically significantly deplete the G6PD enzyme activity. Kruskal-Wallis tests found a significant decrease in G6PD enzyme activity, when comparing the DMSO and AG1 samples before and after Bay-11 incubations (DMSO, mean decrease 67% ($p<0.001$); AG1 69% ($p<0.0001$))(Figure 3.2 A). The G6PD and PK enzyme activity ratio was calculated with a Kruskal-Wallis tests. The results were similar to the G6PD enzyme activity and showed significant decreases in the G6PD and PK enzyme activity ratio in Bay-11 incubations (DMSO, mean decrease 61% ($p<0.001$); AG1 63% ($p<0.001$))(Figure 3.2 G). The DMSO sample that was incubated with Bay-11 showed a significant decrease in PK enzyme activity in comparison to the baseline measurement (DMSO, mean decrease 21% ($p<0.01$)(Figure 3.2 D).

The previous protocol was modified by reversing the order of AG1 and Bay-11. Based on the original protocol, it was assumed that AG1 protects against a stressor. By reversing the order, it could be tested if AG1 has a rescuing factor after depleting the enzyme activity with Bay-11. The results are shown in figure 3.3.

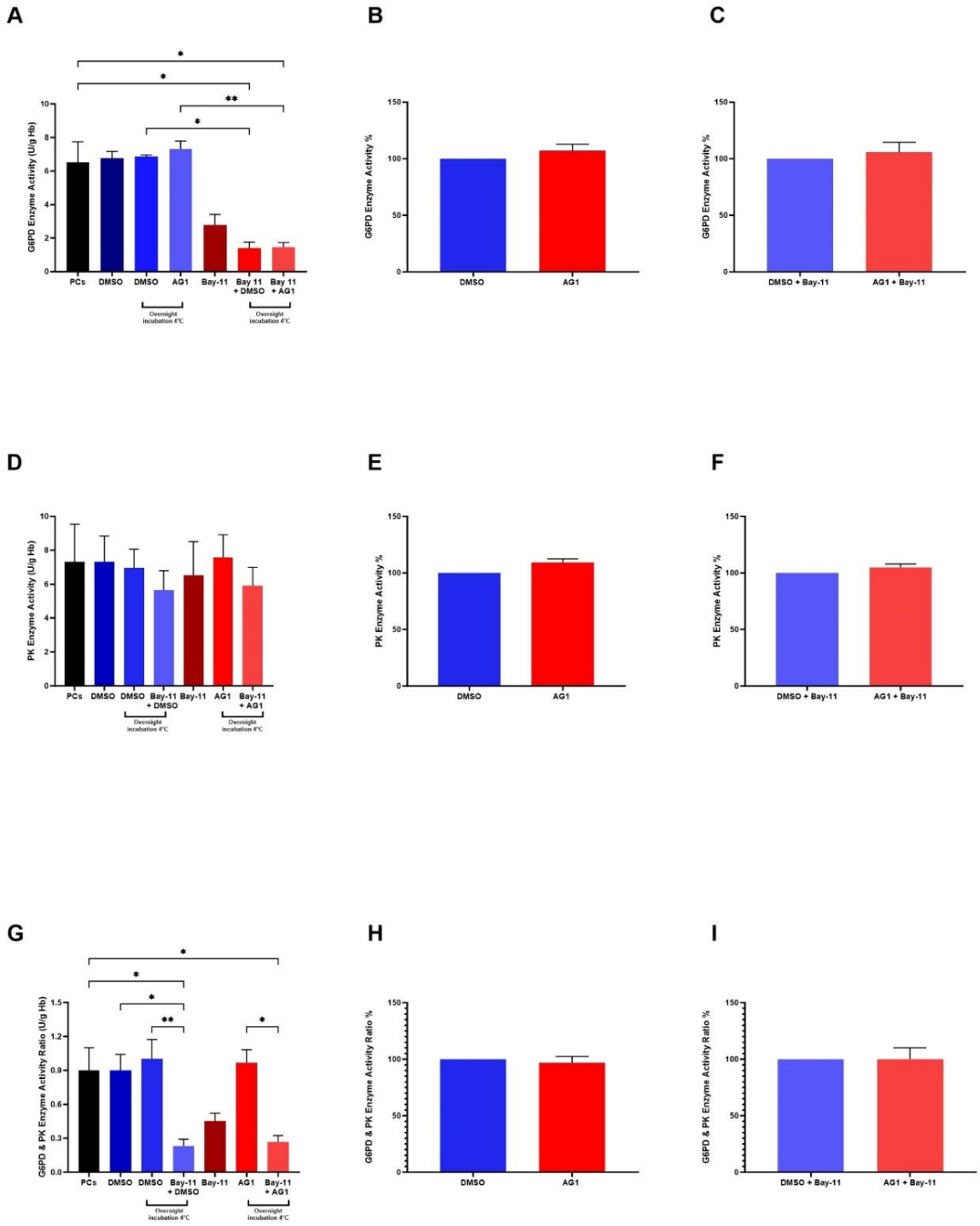


Fig. 3.3 *Enzyme activity measurements of three healthy donors. Samples were incubated in reversed order with Bay-11 and then AG1 Enzyme activity measurements of five healthy donors.* ***A*** *G6PD enzyme activity measurement of all samples*p < 0.01,** p < 0.001, Kruskal-Wallis test.* ***B,C*** *G6PD enzyme activity in percentage with DMSO as a standard.* ***D*** *PK enzyme activity measurement of all samples.* ***E,F*** *PK enzyme activity in percentage in comparison to DMSO as a standard.* ***G*** *Ratio of G6PD and PK enzyme activity in all samples *p < 0.01, **p < 0.001, Kruskal-Wallis test.* ***H,I*** *Ratio of G6PD & PK enzyme activity in comparison of Bay-11 and AG1 to the blank.*

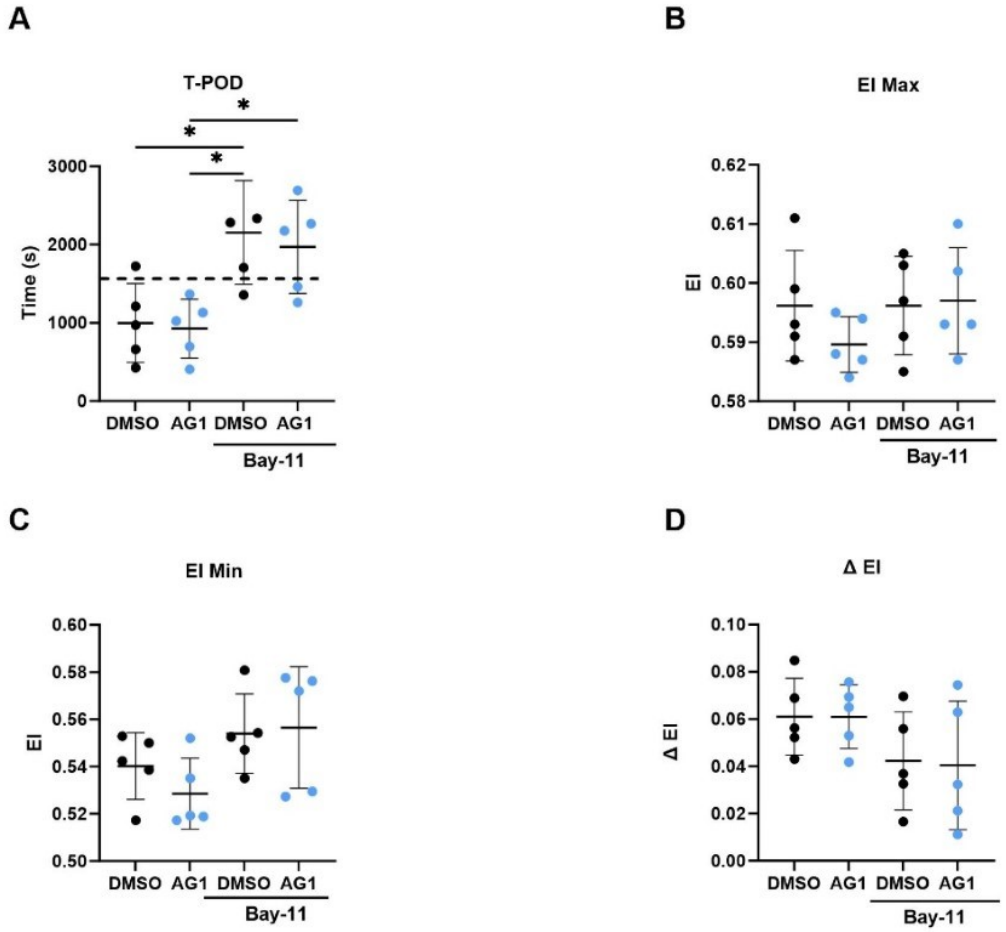
The results are comparable to the previous incubations. The AG1 results suggested no differences in relation to the control regarding the G6PD and PK enzyme activity. Kruskal-Wallis tests suggest a significant difference in comparing the AG1 conditions to AG1 that was incubated with Bay-11 (AG1

Bay-11, mean decrease 80% (p<0.001))(Figure 3.3 A). The G6PD and PK enzyme activity ratios show also significant decreases in the enzyme activity, when comparing samples before and after Bay-11 incubation (DMSO, mean decrease 77% (p<0.001), AG1 mean decrease 72% (p<0.01). PK enzyme activity remained similar over all conditions (Figure 3.3 D). Wilcoxon tests determined the differences between all DMSO and AG1 conditions for G6PD and PK enzyme activity and the ratio of G6PD and PK enzyme activity. The results suggest no significant differences (Figure 3.3 B,C,E,F,H,I).

3.2 RoxyScan

RoxyScans were conducted with PCs after incubations with AG1 and Bay-11 to see how the RBCs react under oxidative stress. Bay-11 was introduced as an agent to mimic G6PD deficiency. The RoxyScan test was used to measure the susceptibility to oxidative stress of the samples. As described in the methods diamide was added as an oxidant directly before the run.

The T-POD is the calculated point from a fitted curve in which a 10% loss of deformability is reached. The graphs show that the Bay-11 incubations have a significantly later T-POD, which was determined by an ordinary one-way ANOVA (DMSO, mean delay 54% (p<0.01), AG1, delay 53% (p<0.01) (Figure 3.4 A). The reversed incubated samples show the same pattern but are not significant (Figure 3.4 E). The measurements for the EImax have a lower variability and no significant difference between the control, AG1 or Bay-11. The EImin seems to be slightly elevated in both Bay-11 experimental groups, but not significantly. ΔEI suggest being slightly smaller in the Bay-11 incubations for the first five mini donors (Figure 3.4 D) and in the reversed incubated samples you can see a clear trend that the Bay-11 samples have a smaller ΔEI (DMSO, mean difference 40% (p<0.0997); AG1, mean difference 56%, (p<0.0997))(Figure 3.4 H). All measurements were analyzed with an ordinary one-way ANOVA.



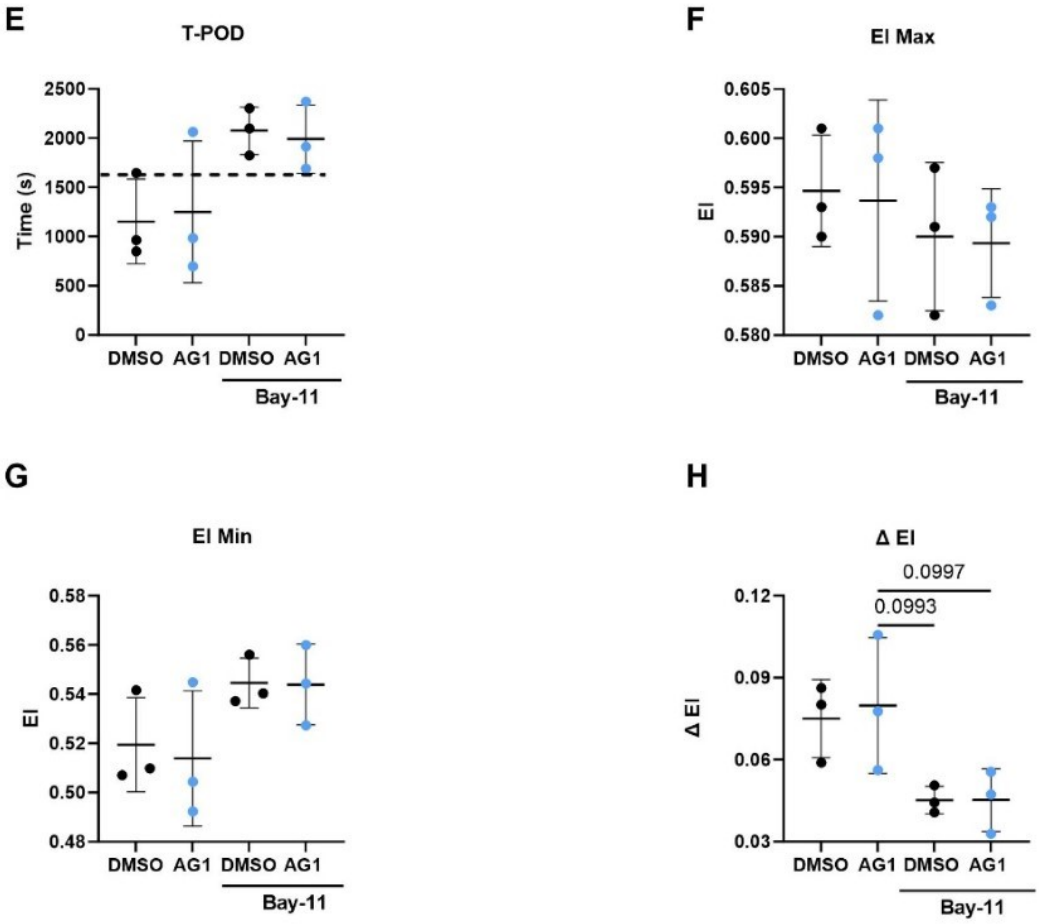
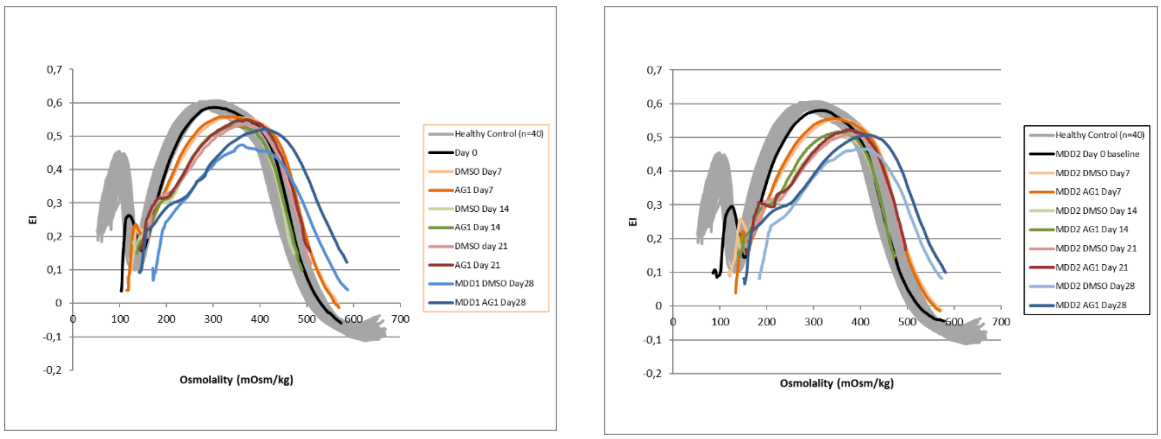


Fig. 3.4 *RoxyScan measurements of samples that were incubated with AG1 and/or Bay-11 in five healthy donors A-D and three healthy donors in reversed incubational order E-H.* ***A,E*** *Measurement of time point of oxidant induced decrease. Time points after 1600 seconds where calculated*p < 0.01, ordinary one-way ANOVA.* ***B,F*** *Measurement of EImax of all samples.* ***C,G*** *Measurement of EImin in all samples.* ***D,H*** *ΔEI results of all donors.*

3.3 Osmoscan and Hemolysis Assay

Osmoscans were performed to test the deformability of RBCs. PCs of three healthy donors were incubated with AG1 and DMSO as a vehicle control. The incubations took place over 28 days. The measuring points were set for day 0, 7, 14, 21 and 28.

A **B**



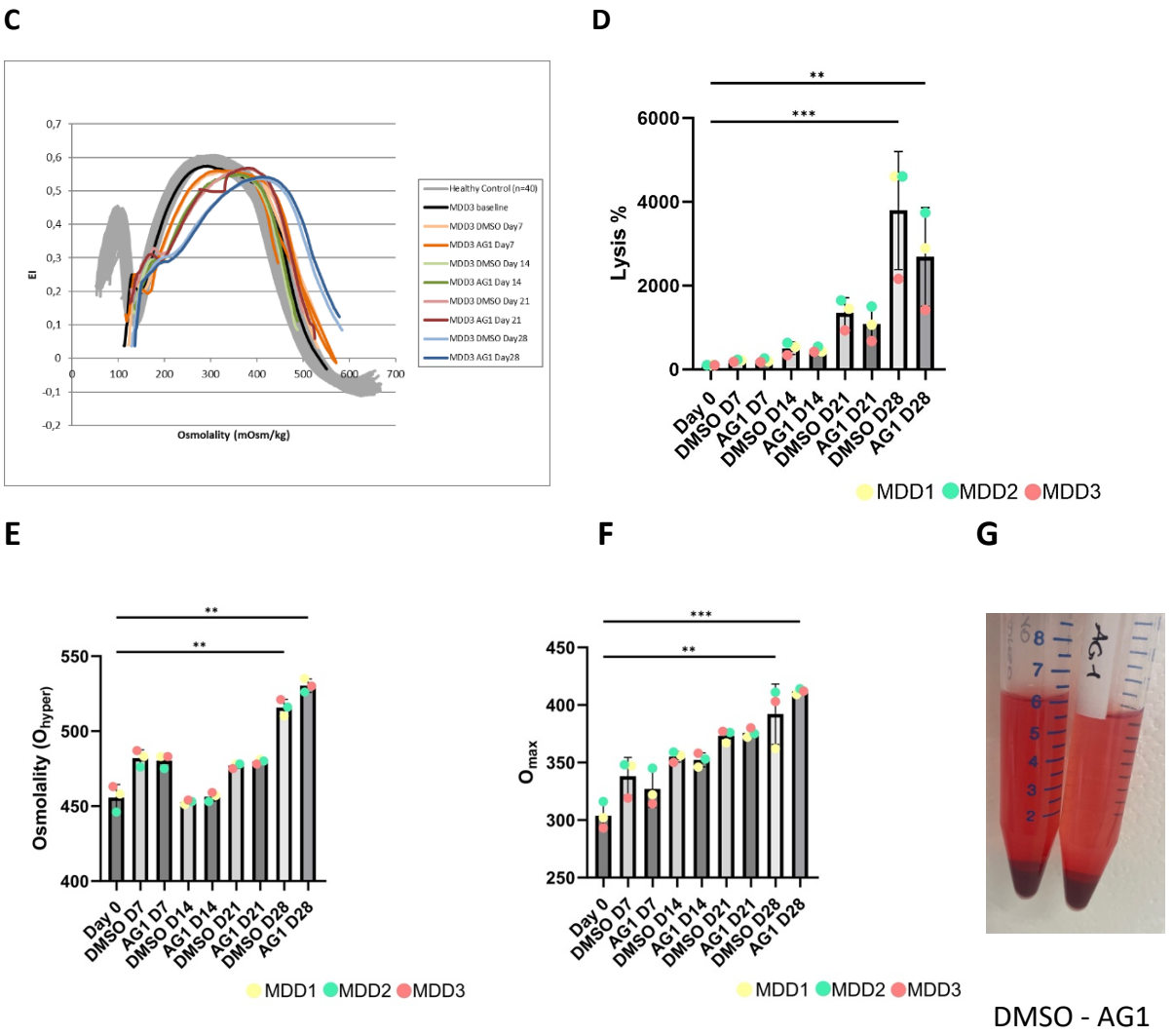


Fig. 3.5 *Measurement of healthy donor samples incubated with DMSO and AG1 at a concentration of 5µM over 28 with five time measuring points.* ***A*** *Osmoscans of donor 1* ***B*** *Osmoscans of donor 2* ***C*** *Osmoscans of donor 3* ***D*** *Measurement of amount of lysis based on the hemoglobin count, **p < 0.001, ***p < 0.0001, Kruskal-Wallis test.* ***E*** *Measurement of Ohyper in Osmoscans, **p < 0.001, **p < 0.001, Kruskal-Wallis test.* ***F*** *Measurement of O_{max} of Osmoscans, **p < 0.001, ***p < 0.0001, Kruskal-Wallis test. G Example of lysis in samples on day 28, DMSO left, AG1 right.*

There is a clear right shift in all samples over all five measuring points, which indicates a dehydration of the cells. However, the osmolality (O_{hyper}) shows that the AG1 incubated samples on day 28 are slightly more hydrated than the control (MDD1, increase 5%, MDD2 increase 2%, MDD3, increase 2%) (Figure 3.5 D). Compared to the baseline on day 0, AG1 samples and the control on day 28 show both a significant increase in osmolality (DMSO, mean increase 13% ($p < 0.001$), AG1 increase 15% ($p < 0.001$)(Figure3.5 E). The EImax shows in all three donors on day 28 a marginally higher deformability of the cells in the AG1 condition (MDD1, increase 9%, MDD2 increase 8%, MDD3, increase 1%) (Figure 3.5 D). The Omax is also slightly increased for all three mini donors in the AG1 condition on day 28, indicating a higher osmolality at EImax (MDD1, increase 11%, MDD2 increase 1%, MDD3, increase 2%) (Figure 3.5 D). However, mini donor 1 has the highest differences, whereas most of the results of mini donors 2 and 3 are minor. Compared to the baseline on day 0, AG1 samples and the control on day 28 show a significant increase in O_{max} (DMSO, mean increase 20% ($p < 0.001$), AG1 increase 26% ($p < 0.0001$)(Figure3.5 F).

Measurements of hemolysis were executed with the same samples as the Osmoscans. The measuring points on day 0, 7, 14, 21 and 28 are also in correspondence with the osmotic gradient ektacytometry assays.

The graph shows the amount of lysis over four weeks. The amount of lysis was calculated based on the hemoglobin count. There was a clear exponential increase in all samples over time. The DMSO and AG1 sample on day 28 had significantly more lysis compared to the baseline (DMSO, mean increase 3792 % ($p<0.001$), AG1, mean increase 2685%, ($p<0.0001$))(Figure 3.5 D). The AG1 samples have a lower amount of lysis compared to the control, especially on day 28 (AG1, mean decrease 71%), determined with a Kruskal-Wallis test. However, this difference was not significant. Although there was no significant difference in the measured amount of lysis between the samples on day 28, you could see differences in color of the supernatant. The higher amount of lysis in the DMSO sample darkened the color of the supernatant (Figure 3.5 G).

4. Discussion

G6PD deficiency can cause hemolytic anemia due to oxidative stress. Point mutations in the G6PD gene are the cause of this hereditary and in some cases chronic disease. Advances in research could improve millions of lives affected by this genetic enzymopathy. The goal of this research project was to increase G6PD enzyme activity with a novelly identified small molecule activator termed as AG1 in healthy RBCs, and to evaluate if this increase in G6PD activity could aid in protecting RBCs from oxidative stress.

Hwang et al. (24) published results of reducing oxidative stress in RBCs by correcting G6PD deficiency with AG1. They identified the small molecule activator via high-throughput screening and found that AG1 corrects G6PD deficiency by binding monomers. This increases the stability of the enzyme, and it elevates G6PD enzyme activity due to its catalytical activity only in the dimeric and tetrameric state. Via monitoring of NADPH production, they demonstrated a 1.7-fold increase of G6PD enzyme activity after incubating AG1 with RBC from the Canton variant. AG1 also reduced oxidative stress in human RBCs. Reduced hemolysis levels and increased G6PD enzyme activity in AG1 incubated samples were established by exposing RBCs to diamide induced oxidative stress. Hemolysis was monitored through hemoglobin release in the supernatant. G6PD activity was measured spectrophotometrically.

4.1 Conclusions

This research project could not produce similar findings in healthy donor RBCs as Hwang et al. (24) did in G6PD deficient patients. The enzyme activity measurements with AG1 (5µM, 1µM, 500nM and 50nM) based on the original protocol (24) did not demonstrate any significant differences in G6PD activity when treated with AG1. The same samples of AG1 were incubated with Diamide 1mM and failed to generate results with significant differences in G6PD enzyme activity in comparison to the control.

After the lack of conclusive results, changes to the protocol were proposed such as switching the buffer, changing the incubations, moving Diamide as an oxidant from enzyme activity measurements to RoxyScans and adding Bay-11 as a G6PD mimicking molecule. Lastly, AG1 and Bay 11 were reversed in the incubation order. The incubations were switched from a saline to an AGAM buffer to keep the RBCs in a stable environment. Buffered solutions are different to 0.9% saline regarding a lower sodium and chloride content, a stable pH of 7.4 and the presence of other ions such as magnesium (30). Higher temperatures cause a higher rate of enzyme activity due to more collision of the molecules and a therefore increased likelihood that the substrate will collide with the active site of the enzyme. AG1 incubations were originally incubated for 4 degrees overnight. After changing the protocol, the overnight incubation was followed by incubations for three hours at RT and 1,5 hours at 37 degrees.

These changes were chosen based on the afore-mentioned laws of kinetics in anticipation of seeing an increased activity of G6PD.

AG1 might not increase G6PD enzyme activity in healthy RBCs, because the enzyme is already at its maximum activity. There might be an insufficient number of monomers that can be oligomerized to reach significant levels of increased enzyme activity. Additionally, AG1 appears to function effectively in the Canton variant of G6PD deficiency (24,31). The mutation in the Canton variant leads to a weakened interaction between two helices, resulting in their displacement, the displacement of a proceeding loop and the attached residues. Notably, two residues (K171 and K172) are important in positioning G6P and NADP+ within the binding pockets. These residues are determinants of the enzyme's catalytic activity and the potential for AG1 as an activator molecule to bridge these. With estimate 186 known G6PD mutations, it is possible that AG1 may be effective for some but not all variants, as each have different structures. Findings of Pakparnich (31) support this hypothesis. Additionally, the exact structure of AG1 is currently unknown, which further complicates the prediction of its ability to stabilize the enzyme and promote oligomerization in the wild type and other G6PD mutations.

Bay-11 was introduced to mimic the deficiency and deplete the enzyme activity. AG1 and Bay-11 were incubated together, but significant results still failed to appear. The exact working mechanisms of Bay-11 are unknown. Therefore, it can only be assumed how it operates. It is only known that RBCs that were treated with Bay-11 showed depletion of GSH, due to inhibition of G6PDH activity (32). Presumably Bay-11 damages a part of the G6PD enzyme or sets a part out of place, which cannot be repaired or stabilized by AG1. The molecule activator works via oligomerization of monomers. Meaning it works by binding and stabilizing, but not repairing the enzyme.

RoxyScans in Diamide 1mM were not part of the original protocol but provided a novel possibility to test the response of RBCs under oxidative and shear stress. The samples used for the RoxsScans were incubated in AG1 and Bay-11 to evaluate if there was a protective effect of AG1. Afterwards, the incubational order of the two molecules was switched to prove a rescuing effect. Results were not statistically significant for any of the RoxyScan parameters (the T-Pod, nor for EI_{Min}, EI_{Max} and DEI).

The RoxyScan represents a novel technique, yet the reactiveness of RBCs to diamide in this assay is not much known about. Most of the RoxyScan research to date has been carried out with Cumene as an oxidant. It is established that exposing RBCs to Diamide leads to a decrease in cellular deformability, due to an increase in membrane rigidity (33). Additionally, Diamide exerts oxidative stress on RBCs by depleting NADPH levels.

Bay-11 depletes enzyme activity, which suggests a negative effect not just on the function but also the stability and morphology of the enzyme and its ability to deform under stress. Some RoxyScan results were counter-intuitive by showing that RBCs incubated in Bay-11 showed more resistance to Diamide as a stressor than samples not incubated with Bay-11. Leading to questions about the underlying mechanisms of Bay-11 and its effect in the cytoplasm and membrane of the RBC.

Considering that RBC populations are on a spectrum of younger and older cells, it can also be assumed that they vary in their sensitivity to stress. The range of older and younger populations and their reactiveness to stressors make interpreting the results increasingly difficult. Differences in the samples were only marginal and could also be traced back to the diversity of the sample's populations (25).

The original paper (24) conducted a 28-day experiment with stored blood of healthy donors and showed that the preincubation of the stored samples with AG1 decreased the amount of lysis in comparison to the control samples. A similar experiment was here performed in which samples of

healthy volunteers were incubated with AG1. Incubations produced similar results. Samples of three donors showed that the amount of lysis was significantly reduced in the AG1 samples after 28 days in comparison to the baseline on day 0.

Osmoscans were performed to test the deformability of RBCs. Hydration status of stored samples was also evaluated, in which Osmoscans all showed an expected right shift. This indicates a dehydration of the cells. The osmolality (O_{hyper}) shows that the AG1 incubated samples on day 28 are slightly more hydrated, have a marginally higher deformability according to the EImax and have an Omax that indicates a slightly higher osmolality at EImax. However, these differences were not significant. The storage assay showed some significant results in the lysis measurements. After 28 days the AG1 samples of three donors had an average of 71% lower amount of lysis compared to the control. Hwang et al. (24) showed just a decrease by an average of 12%, however they had 13 donors and more scatter of data points.

The observed differences in lower amounts of lysis and potentially higher deformability in the AG1 incubated samples could be due to the molecule activator's stabilizing ability. This is in line with the findings of Hwang et al. (24) and the hypothesis that AG1 is contributing to stabilizing the RBC membrane. During storage RBCs undergo structural and functional changes, which are referred to as storage lesions (34). Storage of blood in common practice is looked at as oxidative stress, because an increase in ROS over time and accumulation of oxidative biomarkers can be observed (35,36). Given that AG1 can improve preservation of RBCs, it could provide an opportunity to store blood longer, for instance for transfusions. This way a broader population could profit from it.

Testing the activator in a variety of assays like enzyme activity measurements, RoxyScans, Osmoscans and measurement of lysis, builds a strong foundation to conclude that the activator is not working as groundbreaking as it was promised in Hwang et al. (24). Critical voices over the functioning of the activator have been published (31). They tested the activator AG1 in a concentration of 10µM in nine different G6PD (cross-)mutation variants and the wild type. The findings showed it only stabilized the enzyme in half of the mutations, including the canton variant, which the Hwang et al. (24) research was based on. In comparison it even destabilized half of the other mutations of G6PD and notably also the wildtype. In addition, the mutations that could be activated only showed marginal increases of G6PD enzyme activity. This suggests a limitation to the functioning of AG1 only to a selection of G6PD mutations.

4.2 Limitations

The experiments in this research project have been conducted with PCs from healthy human donors. The research of the original paper (24) was based mainly on the G6PD Canton variant and did not publish results on healthy human donor RBCs with the G6PD wild type enzyme. Availability of Canton variant G6PD blood is limited, considering that it occurs predominantly in Asia.

A limitation of the original paper is that AG1 itself has mostly been tested in the G6PD Canton variant, but rarely in other variants. Although, when they did, the authors claimed to see a significant increase in activity there as well (24). Other researchers however could replicate these results only in some of the other variants (31).

The research project was set for nine months. This limited period allowed enough time to change certain parameters, incubations, concentrations and solutions, but perhaps not enough time to find the optimal protocol for AG1 in healthy controls. There are some parameters that can still be switched, such as trying a different oxidant like chloroquine that was also used in the original article (24) or introducing other assays from the LORRCA to examine more membrane properties.

Bay-11 is utilized to mimic G6PD deficiency and is a reliable tool to deplete G6PD enzyme activity. However, mimicking a disease does not equal using actual G6PD deficiency samples. The work mechanism of Bay-11 is still not well understood, which complicates finding a starting point in improving the assay. It is unknown where the molecule influences the enzyme and its activity.

4.3 Future perspectives

The activation of glucose-6-phosphate dehydrogenase (G6PD) presents significant potential, particularly for individuals with G6PD deficiency and other hereditary conditions leading to hemolytic anemia. Despite the global prevalence of these conditions, the underlying mechanisms remain inadequately understood. Current therapeutic strategies for G6PD deficiency are predominantly preventative and focuse on the avoidance of known triggers such as specific foods, medications, and infections are far from ideal. In Class A G6PD deficient patients chronic hemolysis occurs and some are transfusion dependent. The development of a reliable activator could improve the quality of millions of lives. This stretches the importance of further research in this area.

Establishing a robust procedure that is applicable for AG1 in healthy controls, could possibly be a few-year long project. Following researchers could build on the foundation that was laid here by optimizing the protocol. Looking at research on PK activators may offer valuable insight for further development of a protocol and for avoiding potential mistakes. PK activators like AG-946 have been trialed successfully and are presumably beneficial across a broad range of anemias (37). Notably, PK is an allosteric enzyme, which differs strongly from the work mechanisms of G6PD. Consulting more experts in the field and extending the literature research is necessary for the continuation of the project.

The past nine months of this research project indicate that the potential positive effects of the AG1 activator cannot be adequately assessed through G6PD activity measurements in healthy human samples, therefore it is strongly suggested to switch the type of assay. Transitioning from enzyme activity measurements to for example more Osmoscans could provide new insights into AG1. This research could also be extended to examine the work mechanism of AG1 in different G6PD variants, such as the Canton variant and to identify any variant-specific differences.

Proceeding research on finding or creating a molecule that is an exact duplication of the mutated enzyme would simplify the work by not being limited to blood donations of the actual patients. Another possibility is investigating the functioning of Bay-11. Follow-up experiments on why Bay-11 appears to increase the RBCs capability to deform would be intriguing. This could involve incubating samples with Bay-11 in Osmoscans to gather more evidence on its potential protective effects on the RBC membrane and its deformability. Understanding the membrane properties in this context is crucial to determining further implications of Bay-11 treatment. Learning more about the workings of Bay-11 could provide information about how it influences G6PD activity and stability. This could improve future experiments by increasing their specificity. A deeper investigation into Bay-11 and its mechanisms might be even more insightful regarding G6PD deficiency than continuing working with AG1, when looking at this study's results.

Ultimately, G6PD disease is a diverse enzymopathy. It might be due to the diversity of the disease and the specificity of the molecule activator that AG1 only works for patients carrying the Canton variant. Hence, the optimal protocol for AG1 in healthy controls may not exist. At least not with AG1 as a molecule activator. Stepping away from AG1 and focusing resources on Bay-11 and alternative treatments could be considered.

References

1. Pretini, V., Koenen, M. H., Kaestner, L., Fens, M. H. A. M., Schiffelers, R. M., & Bartels, M. (2019). Red blood cells: Chasing interactions. Frontiers in Physiology, 10, 945. https://doi.org/10.3389/fphys.2019.00945
2. Huisjes, R., Bogdanova, A., van Solinge, W. W., Schiffelers, R. M., & Kaestner, L. (2018). Squeezing for life - properties of red blood cell deformability. Frontiers in Physiology, 9, 656. https://doi.org/10.3389/fphys.2018.00656
3. Lux, S. E. (2016). Anatomy and physiology of red cell membranes. In Hematology: Basic Principles and Practice (6th ed.).
4. Surgenor, D. M. (1974). The red blood cell. New York, NY: Academic Press.
5. Benesch, R., & Benesch, R. E. (1967). The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin. Biochemical and Biophysical Research Communications, 26(2), 162-167.
6. Valentine, W. N., & Paglia, D. E. (1980). The primary cause of hemolysis in enzymopathies of anaerobic glycolysis: A viewpoint. Blood Cells, 6(4), 819-829.
7. Miwa, S. (1983). Hereditary disorders of red cell enzymes in the Embden-Meyerhof pathway. American Journal of Hematology, 14(4), 381-391.
8. Möller, M. N., Orrico, F., Villar, S. F., López, A. C., Silva, N., Donzé, M., Thomson, L., & Denicola, A. (2022). Oxidants and antioxidants in the redox biochemistry of human red blood cells. ACS Omega, 8(1), 147-168. https://doi.org/10.1021/acsomega.2c06768
9. Global Malaria Programme, Malaria Policy Advisory Group. (2022). Meeting report of the technical consultation to review the classification of glucose-6-phosphate dehydrogenase (G6PD). World Health Organization. Retrieved from https://www.who.int/publications/m/item/WHO-UCN-GMP-MPAG-2022.01
10. Beutler, E. (2008). Glucose-6-phosphate dehydrogenase deficiency: A historical perspective. Blood, 111(1), 16-24. https://doi.org/10.1182/blood-2007-04-077412
11. Garcia, A. A., Koperniku, A., Ferreira, J. C. B., & Mochly-Rosen, D. (2021). Treatment strategies for glucose-6-phosphate dehydrogenase deficiency: Past and future perspectives. Trends in Pharmacological Sciences, 42(10), 829-844. https://doi.org/10.1016/j.tips.2021.07.002
12. Pearson, A. G., et al. (2021). Peroxiredoxin 2 oxidation reveals hydrogen peroxide generation within erythrocytes during high-dose vitamin C administration. Redox Biology, 43, 101980.
13. Kuipers, M. T., et al. (2020). G6PD deficiency-associated hemolysis and methemoglobinemia in a COVID-19 patient treated with chloroquine. American Journal of Hematology, 95(6), E194-E196.
14. Spielberg, S. P. (1979). Improved erythrocyte survival with high-dose vitamin E in chronic hemolyzing G6PD and glutathione synthetase deficiencies. Annals of Internal Medicine, 90(1), 53.
15. Corash, L., et al. (1980). Reduced chronic hemolysis during high-dose vitamin E administration in Mediterranean-type glucose-6-phosphate dehydrogenase deficiency. New England Journal of Medicine, 303(8), 416-420.
16. Hafez, M., et al. (1986). Improved erythrocyte survival with combined vitamin E and selenium therapy in children with glucose-6-phosphate dehydrogenase deficiency and mild chronic hemolysis. Journal of Pediatrics, 108(4), 558-561.
17. Reeves, D. J., et al. (2016). I.V. ascorbic acid for treatment of apparent rasburicase-induced methemoglobinemia in a patient with acute kidney injury and assumed glucose-6-phosphate dehydrogenase deficiency. American Journal of Health-System Pharmacy, 73(23), e238-e242.

18. Rehman, A., et al. (2018). Severe acute haemolytic anaemia associated with severe methaemoglobinaemia in a G6PD-deficient man. BMJ Case Reports, 2018. https://doi.org/10.1136/bcr-2017-223369
19. Francis, R. O., et al. (2020). Donor glucose-6-phosphate dehydrogenase deficiency decreases blood quality for transfusion. Journal of Clinical Investigation, 130(5), 2270-2285.
20. Tang, H., et al. (2015). Inability to maintain GSH pool in G6PD-deficient red cells causes futile AMPK activation and irreversible metabolic disturbance. Antioxidants & Redox Signaling, 22(9), 744-759.
21. Raftos, J. E., et al. (2010). Glutathione synthesis and turnover in the human erythrocyte: Alignment of a model based on detailed enzyme kinetics with experimental data. Journal of Biological Chemistry, 285(31), 23557-23567.
22. Watanabe, M., et al. (2019). Laparoscopic cholecystectomy and postoperative pain control in a patient with chronic non-spherocytic hemolytic anemia from glucose-6-phosphate dehydrogenase deficiency. Journal of Clinical Anesthesia, 54, 128-129.
23. Carr, D. R., & Bradshaw, S. E. (2016). Gene therapies: The challenge of super-high-cost treatments and how to pay for them. Regenerative Medicine, 11(4), 381-393.
24. Hwang, S., Mruk, K., Rahighi, S., Raub, A. G., Chen, C. H., Dorn, L. E., Horikoshi, N., Wakatsuki, S., Chen, J. K., & Mochly-Rosen, D. (2018). Correcting glucose-6-phosphate dehydrogenase deficiency with a small-molecule activator. Nature Communications, 9(1), 4045. https://doi.org/10.1038/s41467-018-06447-z
25. Larkin, S. K., Hernández, C., van Beers, E. J., et al. (2024). The RoxyScan is a novel measurement of red blood cell deformability under oxidative and shear stress. Scientific Reports, 14, 6344. https://doi.org/10.1038/s41598-024-56814-8
26. Monod, J., Wyman, J., & Changeux, J. P. (1965). On the nature of allosteric transitions: a plausible model. Journal of Molecular Biology, 12(1), 88–118. https://doi.org/10.1016/s0022-2836(65)80285-6. PMID 14343300.
27. Beutler, E. (1975). Reduced glutathione (GSH). In H. V. Bergmeyen (Ed.), Red Blood Cell Metabolism: A Manual of Biochemical Methods (2nd ed., pp. 112-114). Grune and Stratton.
28. Thermo Fisher Scientific. (n.d.). Protein denaturing and reducing agents. https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-labeling-crosslinking/protein-denaturing-reducing-agents.html.
29. Bianchi, P., Zaninoni, A., Fermo, E., Vercellati, C., Anna Paola, M., Zanella, A., Cortelezzi, A., & Barcellini, W. (2015). Diagnostic power of Laser Assisted Optical Rotational Cell Analyzer (LoRRca MaxSis) evaluated in 118 patients affected by hereditary hemolytic anemias. Blood, 126(23), 942. https://doi.org/10.1182/blood.V126.23.942.942.
30. Orbegozo, C. D., Rayo, B. A., & Vincent, J. L. (2014). Isotonic crystalloid solutions: a structured review of the literature. British Journal of Anaesthesia, 112(6), 968-981. https://doi.org/10.1093/bja/aet517
31. Pakparnich, P., Sudsumrit, S., Imwong, M., Suteewong, T., Chamchoy, K., Pakotiprapha, D., Leartsakulpanich, U., & Boonyuen, U. (2021). Combined effects of double mutations on catalytic activity and structural stability contribute to clinical manifestations of glucose-6-phosphate dehydrogenase deficiency. Scientific Reports, 11(1), 24307. https://doi.org/10.1038/s41598-021-03800-z
32. Ghashghaeinia, M., Giustarini, D., Koralkova, P., Köberle, M., Alzoubi, K., Bissinger, R., Hosseinzadeh, Z., Dreischer, P., Bernhardt, I., Lang, F., Toulany, M., Wieder, T., Mojzikova, R., Rossi, R., & Mrowietz, U. (2016). Pharmacological targeting of glucose-6-phosphate dehydrogenase in human erythrocytes by Bay 11-7082, parthenolide and dimethyl fumarate. Scientific Reports, 6, 28754. https://doi.org/10.1038/srep28754

33. Safeukui, I., Buffet, P. A., Deplaine, G., Perrot, S., Brousse, V., Sauvanet, A., Aussilhou, B., Dokmak, S., Couvelard, A., Cazals-Hatem, D., Mercereau-Puijalon, O., Milon, G., David, P. H., & Mohandas, N. (2018). Sensing of red blood cells with decreased membrane deformability by the human spleen. Blood Advances, 2(20), 2581–2587. https://doi.org/10.1182/bloodadvances.2018024562
34. Garcia-Roa, M., et al. (2017). Red blood cell storage time and transfusion: current practice, concerns and future perspectives. Blood Transfusion, 15, 222–231.
35. Kucukakin, B., et al. (2011). Storage-induced increase in biomarkers of oxidative stress and inflammation in red blood cell components. Scandinavian Journal of Clinical and Laboratory Investigation, 71, 299–303.
36. Orlov, D., & Karkouti, K. (2015). The pathophysiology and consequences of red blood cell storage. Anaesthesia, 70, 29–37.
37. De Wilde, J. R. A., Ruiter, T. J. J., van Oirschot, B. A., Jans, J. J. M., Dang, L., Wind-Rotolo, M., Thielen, N., van Solinge, W. W., van Rhenen, A., van Wijk, R., & Rab, M. A. E. The Pyruvate Kinase (PK) Activator AG-946 Improves PK Properties and Red Blood Cell (RBC) Characteristics upon Ex Vivo treatment of RBCs from Patients with Myelodysplastic Syndromes. https://doi.org/10.1097/01.HS9.0000969772.59102.e2

Appendix

Protocols

Protocol A – RBC isolation

Department of Clinical Chemistry and Haematology	Webautorisatie:	29-jun-2017
Code: S-RH128e, Versie: 004, 02-jun-2017	Herziening:	02-jun-2022
Isolation of Red Blood Cells		

unattended copy

Department of Clinical Chemistry and Haematology (DCCH)
University Medical Center Utrecht

Writing		
Name	***Profession***	***Date***
B.A. van Oirschot-Hermans	Research technician	24-04-2017

Verification		
Name	***Profession***	***Date***
Dr. R. van Wijk	Staff member	26-04-2017
M. Soeters	Quality and clerical staff	02-06-2017

Authorization	
Name	***Profession***
Dr. R. van Wijk	Staff member
Prof.dr. G. Pasterkamp	Head Research & Education

Distribution list					
Name			***Profession***	***Date of issue***	***Date of intake***
QA	Front page	Archive	QA-manager		
Section RH	Chest of drawers	G03.409	Employees		

Changes according to former revision	
Revision date	***Paragraph***
1: 04-12-2008	New document
2: 24-03-2011	5.2 (R1.1)
3: 23-04-2013	5.1, 6.1, 8.1
4: 02-06-2017	3, 4.1, 4.2, 5.2, 6.1, 6.2, 8.1, 8.2, 9.2

Department of Clinical Chemistry and Haematology	Webautorisatie:	29-jun-2017
Code: S-RH128e, Versie: 004, 02-jun-2017	Herziening:	02-jun-2022
Isolation of Red Blood Cells		

7.2 *Interferences (and errors)*
NA

7.3 *Storage conditions*

Pre analysis	*Post analysis*
Blood, maximal 1 week at 4°C	Long term storage -80°C

Table 2

8. Working method

8.1 *Procedure*
- Place PEGG Elution Column (6.2.1) on 50 mL tube
- Pour well mixed homogeneous Cellulose mixture (R2.1) in column, fill the column at once till the edge, drain NaCL 0,9%. The Cellulose mixture should remain wet
- Add 5 mL NaCL 0,9% (R1.1) and drain. The Cellulose mixture should remain wet
- Add 5 mL blood maximal on Cellulose mixture
- Place column in clean 50 mL tube
- Centrifuge at 50 g for 5 minutes at RT, acceleration fast (=9), brake medium (=5)
- Add 5 mL NaCL 0,9% (R1.1)
- Centrifuge at 50 g for 5 minutes at RT, acceleration fast (=9), brake medium (=5)
- Remove column and fill tube (with RBC) with NaCL 0,9%
- Centrifuge at 1000 g for 5 minutes at RT, acceleration fast (=9), brake slow (=2)
- Wash cells with NaCL 0,9%, 1000 g for 5 minutes at RT
- Check presence and distribution of cells by Cell-Dyn Sapphire analysis; dilute PCs 2x with NaCl 0,9%
- Depending on follow up: continue directly or aliquot sample and store at -80°C
- PEGG Columns need to be cleaned and dissembelled prior to cleaning with water

8.2 *Remarks*
Aliquoting and storage at -80 °C will lyse the cells. This does not interfere with further enzyme kinetics determination.
PEGG columns are re-usable until flow is blocked.
Because of increased osmotic fragility, patient RBCs may lyse during the procedure.

9. Data processing

9.1 *Calculation*
NA

9.2 *Interpretation*
RBCs: 4 – 6 E12/L, WBC < 1E9/L and Plt < 20E9/L

9.3 *Linearity/detection range*
NA

10. Report
Registration / archiving: see W-5201e Registration of research data DCCH.

Protocol B – Enzyme activity measurement G6PD

G6PD activity 96 well system for Spectramax iD3.
Jonathan de Wilde, adapted from protocol Jennifer and Carolina.

Reagentia and Solutions

Name	Firm	Cat nr	MW	code UMCU
Tris	Roche	10708976001	121,1	T017

$EDTA\text{-}Na_2.2H_2O$	RdH	34549	372,24	E003
$MgCl_2.6H_2O$	RdH	31413	203,3	M004
G6P	Roche	10127647001	304.2	G007
NADP	Roche	10128031001	787,4	N081
β-Mercapto Ethanol	Sigma	7522		

Tris-HCl + $EDTA\text{-}Na_2$, pH 8.0	1 M + 5 mM	(12,1 g Tris + 0,186 g $EDTA\text{-}Na_2$)/90 mL dest Adjust to pH 8.0 Add dest till 100 mL
$MgCl_2$	1 M	20,33 g/100 mL dest
Glucose	40 mM	0,720 g/100 mL
G6P	6 mM	18.2 mg/10 mL
NADP	2 mM	15.7 mg/10 mL
β-Mercapto Stabilising Solution		100 mg $EDTA\text{-}Na_2$/90 mL dest Adjust NaOH till pH 7.0 Add 5 uL β-Mercapto Ethanol Add dest till 100 mL

G6PD Monotest

	Cuvet or 24 well	96 well
Dest	67 mL	64 mL
Tris-HCL1M, EDTA 5 mM pH 8.0	10 mL	10 ml
MgCl2 1 M	1 mL	1 mL
NADP 2 mM	10 mL	10 mL
	--------+	--------+
	88 mL	85 mL

Method

Thaw the monotests, PEP 50 mM and 5 mM and the β-Mercapto Stabilising Solution (keep this last one on ice).

Isolate RBCs (see SOP 'Isolation of Red Blood Cells'S-RH128e Version 004). Isolate 20mL of WB.

Pre-heat heatblock at 37*C. Spectramax iD3 at 37*C. Open SoftMax Pro 7.1.0 via the protocol:T:\lkch\Rode-Cel\Jonathan\2022\Protocollen\Enzymes\Spectramax or Versamax and choose appropriate protocol. For settings, see end of protocol.

Prepare the lysate from the RBCs. After RBC isolation, take 70 uL PCs
and 70 uL NaCl 0.9% (=140uL PCNaCl for Sapphire). Measure Hb. Then, prepare lysate by combining β-Mercapto Stabilising Solution with PCs. You generally want an end volume of 2000uL with a Hb concentration of 0.6-0.8 mmol/L. So, to calculate how much volume you need of the PCs and the β-Mercapto Stabilising Solution:

Measured Hb ---> 2x = actual Hb of PCs
Measured Hb / 0.65 = times to dilute
2000 / times to dilute = volume of PCs
2000 – volume of PCs = volume of β-Mercapto Stabilising Solution

Measure lysate on Sapphire to determine Hb concentration.

To measure G6PD Activity, pipette the following (multichannel the G6P in each well just before measurement):

1 well:

G6PD Monotest	170 uL
Lysate	10 uL
	--------+
	180 uL

Incubate at 37°C, 10 min

Start the reaction

G6P 6 mM	20 µL

Protocol C – Enzyme activity measurement PK (HK)

Pyruvate Kinase and Hexokinase Activity Assay, Pyruvate Kinase Thermobstability Assay 96 Well systes PUMA Study **(ONLY FOR HX!)**

Reagentia and Solutions

Name	Firm	Cat nr	MW	code UMCU
NaCl	Merck	1.06404.1000	58,44	
Tris	Roche	10708976001	121,1	T017
EDTA-$Na_2.2H_2O$	RdH	34549	372,24	E003
KCl	RdH	31248	74,55	K037
$MgCl_2.6H_2O$	RdH	31413	203,3	M004
ADP	Sigma	A2754	427,2	A172
PEP	Sigma	P7252	465,6	P079
LDH 5500 U/mL	Roche	10127230001		L017
NADH	Roche	10128023001	709,4	N080
Glucose	VWR	101174Y	180,16	G001a
ATP	Roche	10127523001	605,2	A024a
NADP	Roche	10128031001	787,4	N081
G6PD 700 U/mL	Roche	10127671001		G046
β-Mercapto Ethanol	Sigma	7522		
DMSO	Sigma	D8418	78.13	D045
AG348	Agios		548.63	
AG946 (formerly known as 416)	Agios		392.44	

Solutions

Tris-HCl + EDTA-Na_2, pH 8.0	1 M + 5 mM	(12,1 g Tris + 0,186 g EDTA-NA_2)/90 mL dest (NB EDTA dissolves slowly)
		Adjust to pH 8.0
		Add dest till 100 mL
NaCl	0.9%	9 g/1000 mL dest
KCl	1 M	7,46 g/100 mL dest
$MgCl_2$	1 M	20,33 g/100 mL dest
ADP	30 mM	0,128 g/8 mL dest
		Adjust to pH 7.0
		Add dest till 10 mL
PEP	50 mM	0,233 g/10 mL dest
PEP	5 mM	PEP 50 mM, 10x diluted in dest
LDH	110 U/mL	50x diluted in dest
NADH	4.2 mM	3 mg/mL dest
Glucose	40 mM	0,720 g/100 mL
ATP pH 7,0	100 mM	0,605 g/8 mL Add NaOH till pH 7,0 Add dest till 10 mL
NADP	2 mM	0,0157 g/10 mL
G6PD	14 U/mL	50x diluted in dest
β-Mercapto Stabilising Solution		100 mg EDTA-Na_2/90 mL dest
		Add 5 uL β-Mercapto Ethanol (NB in fume hood)
		Adjust NaOH till pH 7.0 Add dest till 100 mL
AG348	10uM	In stock (-20, Freezer 409V2)
AG946	10uM **AND** 5uM	In stock (-80, Freezer 409V2)

Preparation of Monotests

PK Monotest

Tris-HCL1M, EDTA 5 mM pH 8.0	10 mL
MgCl2 200 mM	5 mL

KCL	1 M	10 mL
ADP	30 mM pH 7,0	5 mL
Dest		40 mL ---> **Ensure that before adding all the Dest the pH is 8.0**
		--------+
		70 mL

HK Monotest

Tris-HCL1M, EDTA 5 mM	pH 8.0	10 ml
MgCl2	1 M	1 mL
Glucose	40 mM	5 mL
ATP	100 mM pH 7,0	10 mL
NADP	2 mM	10 mL
Dest		49 mL ---> **Ensure that before adding all the Dest the pH is 8.0**
		--------+
		85 mL

Both the monotests, the β-Mercapto Stabilising Solution, the PEP high and the PEP low solutions can be kept at -20*C.
The NADH, LDH and G6PD should always be prepared freshly.

Equipment and appliances

Microplate 96 well, PS, F-bottom	Greiner	655101
Microplate 96 well, Conical well	Thermo Scientific	442587
Digital Heatblock	VWR	460-3273
Spectramax iD3	Molecular Devices	
Thermomixer comfort	Eppendorf	No.5355
Centrifuge	Hettich	Rotina 420
CELL-DYN Sapphire Hematology Analyzer	Abbot	
Timer		
Tumbler		
Multichannel	(10-100uL and 20-300uL)	
Eppendorf tubes (1.5 and 2.0mL)		

Methods

Thaw the monotests, PEP 50 mM and 5 mM and the β-Mercapto Stabilising Solution (keep this last one on ice).

Isolate RBCs (see SOP 'Isolation of Red Blood Cells'S-RH128e Version 004). Isolate 20mL of WB.

Pre-heat heatblock at 37*C. Pre-heat thermomixer comfort at 53*C (in case of PK-thermostability). Start-up and pre-heat Spectramax iD3 at 37*C. Open SoftMax Pro 7.1.0 via the protocol:T:\lkch\Rode-Cel\Jonathan\2022\Protocollen\Enzymes\Spectramax or Versamax and choose appropriate protocol. For settings, see end of protocol.

Prepare the lysate from the RBCs (common method for both activity and thermostability). After RBC isolation, take 70 uL PCs and 70 uL NaCl 0.9% (=140uL PCNaCl for Sapphire). Measure Hb. Then, prepare lysate by combining β-Mercapto Stabilising Solution with PCs. You want an end volume of 6000uL with a Hb concentration of 0.6-0.8 mmol/L. So, to calculate how much volume you need of the PCs and the β-Mercapto Stabilising Solution:

Measured Hb ---> 2x = actual Hb of PCs
Measured Hb / 0.65 = times to dilute
6000 / times to dilute = volume of PCs
6000 – volume of PCs = volume of β-Mercapto Stabilising Solution

Also, prepare two Eps of 'Controle Enzymes' (found in the -80 freezer KV01A on Lab 2). For one (**Contr1**), add 1893uL of BMSS (in order to have a Hb of ~0.65). For the other (**Contr2**), add 2000uL of BMSS so you have a final volume of ~2100uL of lysate. Measure **Contr1** on Sapphire and keep on ice (with the 6000uL lysate of the patient).

Incubations for PK thermostability – PLEASE NOTE: for total PCs, D-fraction and H-fraction all of the following steps!

Before starting the enzyme activity assay, incubate the lysate with the PK activators (or with DMSO) for the PK thermostability assay. Prepare the different concentrations of activator freshly: all stocks can be found in the freezer of Jonathan (Lab 1, 409V1, close to the Versamax).

- AG946 1mM by taking 1uL AG946 10mM and 9uL DMSO

Take 1000uL of lysate of the patient in two different 1.5mL Eps: add 1uL DMSO or 1uL AG946 1mM. Incubate (whilst tumbling) for 2 hours at 37 degrees.
NB: do this for the total PCs simultaneously with the D-fraction; start the H-fraction later (at least 30 minutes later)

After this, see **PK Thermostability assay.**

Enzyme activity assay (PLEASE NOTE only for total PCs)

Add the following to the 96-well flatbottom plate (plate should be in the Digitial Heatblock). Add the lysate (for HK) and the PEP (for PK) last. As for the order; start with adding lysate to HK, then adding PEP 5mM and lastly PEP 50mM.

PK-activity	Well:	Plate
PK Monotest	140 µL	14 mL
LDH 110 U/mL	10 µL	1,0 mL (= 980 uL dest + 20 uL LDH 5500 U/mL)
NADH 4.2 mM	10 µL	1.0 mL (= 3mg in 1000uL dest)
	--------+	--------+
	160 uL/well	16 mL
Lysate	20 µL	
Incubate at 37°C, 10 min		
Start the reaction		
PEP 50 mM **OR** 5 mM	20 µL	
	--------+	
	200 µL	

HK-activity	Well:	Plate:
HK Monotest	170 µL	17 mL
G6PD 14 U/mL	10 µL	1,0 mL (= 980 uL dest + 20 uL G6PD 700 U/mL)
	--------+	--------+
	180 µL	18 mL
Start the reaction		
Lysate	20 µL	
	--------+	
	200 µL	

After pipetting in the lysate (for HK) and the PEP (for PK), the measurement should start as soon as possible. Put the plate in the Spectramax iD3 and let the assay run.

After the run, save the file as a data-file. Then, adjust the 'Reduction'. In this way, you can adjust the time-points that are being taken along

when calculating the Vmax. You want the R^2 to be >0.990, so adjust the reduction until you achieve this (but keep having enough measurement points). For the PK-PEP high (5mM end concentration) and the PK-PEP low (0.5mM end concentration) usually don't use the first 300 seconds and not the last 200-500 seconds (especially for PK-PEP high). For HK, you can usually use the measurement after 1000 seconds.

PK thermostability assay → total PCs and D-fraction

Open correct protocol-file on Spectramax iD3.
Place a 96 well plate, (Conical well Plate) at ice water
Pipet 150 uL of lysate from each condition (which has 1000uL per Ep) in well of 96 well plate at ice water
 T = 0 min
Place the remaining 850 uL lysate in thermomixer of 53°C – 600 rpm, start timer
Take samples of 140 uL (pipet in well of 96 well plate at ice water) after 5 – 10 – 20 – 40 and 60 min.

Centrifuge plate 3 min – 600 g – 9/\3 - 4°C (Rotina 420: Program 24)

Pipet supernatant in clean well and keep on ice!

	PEP high						PEP low					
	0	5	10	20	40	60 min	0	5	10	20	40	60 min
Total-DMSO	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
Total 9461u	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
D-DMSO	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
D-9461u	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
	1	2	3	4	5	6	7	8	9	10	11	12

Add the following to the 96-well flatbottom plate (plate should be in the Digitial Heatblock). Order the lysates based on the time-moment:
so 0-5-10-20-40-60 and measure in duplo (so for each lysate 2x PEP high and 2x PEP low). As for the order; start with adding PEP 5mM to 60 min, then 40 min, etc. Lastly add PEP 50mM to 60 min, then 40 min, etc.

PK-activity	Well:	Plate
PK Monotest	140 µL	14 mL
LDH 110 U/mL	10 µL	1,0 mL (= 980 uL dest + 20 uL LDH 5500 U/mL)
NADH 4.2 mM	10 µL	1.0 mL (= 3mg in 1000uL dest)
	--------+	--------+
	160 uL/well	16 mL
Lysate	20 µL	
Incubate at 37°C, 10 min		
Start the reaction		
PEP 50 mM **OR** 5 mM	20 µL	
	--------+	
	200 µL	

After the run, save the file as a data-file. Then, adjust the 'Reduction'. In this way, you can adjust the time-points that are being taken along when calculating the Vmax. You want the R^2 to be >0.990, so adjust the reduction until you achieve this (but keep having enough measurement points). For the PK-PEP high (5mM end concentration) and the PK-PEP low (0.5mM end concentration) usually don't use the first 300 seconds and not the last 200-500 seconds (especially for PK-PEP high).

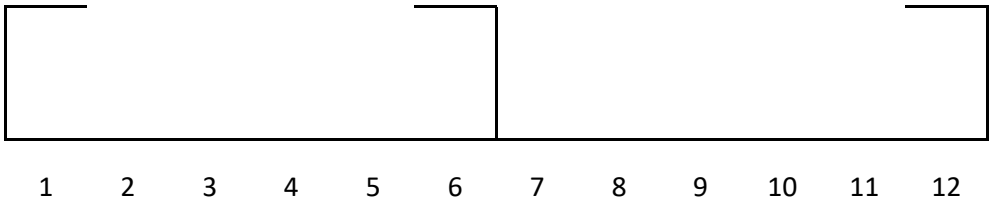
PK thermostability assay → H-fraction

Open correct protocol-file on Spectramax iD3.
Place a 96 well plate, (Conical well Plate) at ice water
Pipet 150 uL of lysate from each condition (which has 1000uL per Ep) in well of 96 well plate at ice water
 T = 0 min
Place the remaining 850 uL lysate in thermomixer of 53°C – 600 rpm, start timer
Take samples of 140 uL (pipet in well of 96 well plate at ice water) after 5 – 10 – 20 – 40 and 60 min.

Centrifuge plate 3 min – 600 g – 9/\3 - 4°C (Rotina 420: Program 24)

Pipet supernatant in clean well and keep on ice!

	PEP high						PEP low					
	0	5	10	20	40	60 min	0	5	10	20	40	60 min
H-DMSO	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
H-9461u	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx



Add the following to the 96-well flatbottom plate (plate should be in the Digitial Heatblock). Order the lysates based on the time-moment:
so 0-5-10-20-40-60 and measure in duplo (so for each lysate 2x PEP high and 2x PEP low). As for the order; start with adding PEP 5mM to 60 min, then 40 min, etc. Lastly add PEP 50mM to 60 min, then 40 min, etc.

PK-activity	Well:	Plate
PK Monotest	140 µL	14 mL
LDH 110 U/mL	10 µL	1,0 mL (= 980 uL dest + 20 uL LDH 5500 U/mL)
NADH 4.2 mM	10 µL	1.0 mL (= 3mg in 1000uL dest)
	--------+	--------+
	160 uL/well	16 mL
Lysate	20 µL	
Incubate at 37°C, 10 min		
Start the reaction		
PEP 50 mM **OR** 5 mM	20 µL	
	--------+	
	200 µL	

After the run, save the file as a data-file. Then, adjust the 'Reduction'. In this way, you can adjust the time-points that are being taken along when calculating the Vmax. You want the R^2 to be >0.990, so adjust the reduction until you achieve this (but keep having enough measurement points). For the PK-PEP high (5mM end concentration) and the PK-PEP low (0.5mM end concentration) usually don't use the first 300 seconds and not the last 200-500 seconds (especially for PK-PEP high).

PK thermostability assay → H-fraction

Open correct protocol-file on Spectramax iD3.
Place a 96 well plate, (Conical well Plate) at ice water
Pipet 150 uL of lysate from each condition (which has 1000uL per Ep) in well of 96 well plate at ice water
 T = 0 min
Place the remaining 850 uL lysate in thermomixer of 53°C – 600 rpm, start timer
Take samples of 140 uL (pipet in well of 96 well plate at ice water) after 5 – 10 – 20 – 40 and 60 min.

Centrifuge plate 3 min – 600 g – 9/\3 - 4°C (Rotina 420: Program 24)

Pipet supernatant in clean well and keep on ice!

Calculation of enzyme activity and thermostability.

Use the Template which is integrated in the PUMA results file '20220908_PUMA results template' found at T:\lkch\Rode-Cel-Patienten\Studies\2022_PUMA Study NL79316.041.21\B. EXPERIMENTS AND RESULTS **OR USE the template** '20211217_Template berekenen enzymactiviteit en thermostabiliteit 96 wells' found at T:\lkch\Rode-Cel\Jonathan\2022\Templates to calculate both activity and thermostability. Always note the reduction and the measured Hb.

Settings Spectramax iD3

Kinetics		
Temperature		37°C
Wavelength		340 nm
Timing	Time	00:35:00
	Interval	00:00:28
Plate type		96 Well Standard clrbtm Height 14.6 mm
Automix and blanking		Before first read, 30 sec
	Pre Read plate	Off
Autocalibrate		Off
Column Wavelength Priority		Column Priority
Carriage Speed		Normal
AutoRead		Off

Hb concentration
Measure at the Spectramax iD3 the Absorbance at 542 nm of every well.

Endpoint (Absorbance)	
Temperature	37°C
Wavelength	542 nm
Plate type	96 Well Standard clrbtm Height 14.6 mm
Automix and blanking	No mix

Protocol D – First protocol about AG1 with original four conditions

G6PD Activity Protocol in Purified RBCs with AG1 and Diamide

Reagentia

Name	Firm	Art nr	UMC Code	MW
NaCl			N020	58.44
DMSO	Sigma	D8418	D045	78.13
AG1 (*Fridge G626 KK1)*	MedChemExpress	#HY-123692		438.65
β-Mercapto Ethanol	Sigma	7522		
Diamide				

Microplate Conical Well	Label for G6PD, HK, Patient, Control, Conditions, Hb
2mL eps	Label
15ml tubes	
Incubator	
Rotator	
Microfuge 16	500G, 5min, soft brake
CELL-DYN Sapphire Hematology Analyzer	Abbot
Heatblock	Pre-heat at 37 degrees
Spectramax	Turn on, heat up to 37 degrees

Solutions:

NaCl 0.9%	Dissolve 9 grams of NaCl in 1L of aqua dest.

AG1 10mM (Stock)	Dissolve 0.43865 (0.439) mg in 100uL DMSO. Split in twenty different Eps with 5uL containing in each. (Freezer Jonathan Lab 1)
AG1 5mM (Fresh)	Dilute AG1 10mM in DMSO: 4uL DMSO and 4uL AG1 10mM.
AG1 1mM (Fresh)	Dilute AG1 5mM in DMSO: 8uL DMSO and 2uL AG1 5mM.
AG1 500uM (Fresh)	Dilute AG1 1mM in DMSO: 4uL DMSO and 4uL AG1 1mM.
AG1 50uM (Fresh)	Dilute AG1 500uM in DMSO: 9uL DMSO and 1uL AG1 500uM.

Diamide 10mM (Fresh) Dissolve 1.72 mg in 1mL aqua dest. Final concentration during incubation will be 1mM.

Preparations Day 0:

- Collect reagentia, blood and prepare solutions for AG1
- collect and pre-heat Equipment

Preparations Day 1:

- collect reagentia, incubated samples & prepare Diamide
- collect and pre-heat Equipment

Method:

RBC isolation according to protocol ("Isolation of RBCs'S-RH128e Version 004").

Preparation for 3 plates 96 well system:

1st plate G6PD and Hexokinase activity **only** on patient blood and healthy controls

- Make lysate from purified RBCs and measure G6PD activity and HK according to protocol (20230815_G6PD Activity Assay_96 wells system_V1_JdW).

2nd plate G6PD and Hexokinase activity with **AG1** in different concentrations on patient blood

- Dilution of purified RBCs in NaCl 0.9% to 5% RBC solution 1:20 (1mL RBC with 19mL NaCl 0.9%, based on original article by Hwang et al., 2018. When assuming an RBC count of 4.0*10^12/L, this 5% should correspond with ±0.35 RBCs*10^12L and a Hb of ±0.6mmol/L.-> corresponding with an RBC count of ±0.3535*10^{12}/L and a Hb of ±0.65 mmol/L in RBCs of a healthy control)
- Measure on sapphire for definite RBC and Hb concentration
- Take 6 individual 2mL eps and add 2mL of the RBC solution
- Add to each ep 2uL of:

Untreated	DMSO (vehicle control)	AG1 5mM	AG1 1mM	AG1 500uM	AG1 50uM

Incubate these overnight (approx. 4PM until 8AM) at 4 degrees in a rotator in a cooling chamber (make sure fluid moves, but in a gentle angle to not damage Cells)

- Day 1: Centrifuge eps (500G, 5 min, soft brake on Beckman Coultier Microfuge 16)
  Following all eps besides control:
- To check lysis: take off 100uL (min. 50 - max. 200uL) of supernatant and pipet it into a well on the plate (f.e. right side of plate, label)
- The remaining rest in the eps are gently being mixed via resuspension with a cut pipet tip
- Take of 300uL of this and pipet each into new Ep (label these Eps according to the condition)
- Save the rest in the original eps for the third plate
- centrifuge the 300uL in the new eps (500G, 5 min, soft brake)
- take of the whole supernatant (gently),
- add 300uL β-Mercapto stabilizing solution (BMSS)
- this lysate is now being used for the enzyme activity measurements after incubation
- measure the Hb concentration of the lysates and measure G6PD activity and HK according to protocol (20230815_G6PD Activity Assay_96 wells system_V1_JdW).

3rd plate G6PD and Hexokinase activity with **AG1** in different concentrations and also oxidative stress through **diamide** on patient blood

- take 450uL of diluted RBCs from each condition (that is the remaining rest that we saved from the original Eps for the 2nd plate) and add it to a new ep each, label eps
- add 50uL of diamide 10mM to each ep, ultimately leading to a final concentration of 1mM diamide per condition
- incubate for 3 hours at 37 degrees in rotator (make sure fluid moves, but in a gentle angle to not damage cells)
- Centrifuge eps (500G, 5 min, soft brake)
- To check lysis: take off 100uL (min. 50 - max. 200uL) of supernatant and pipet it into a well on the plate (f.e. right side of plate, label)
- Also add 100uL of the control condition & 100uL NaCl each into a well (min. 50 - max. 200uL)
- take the rest of the supernatant off (gentle)
- add 450uL β-Mercapto stabilizing solution (BMSS)
- this lysate is now being used for the enzyme activity measurements
- measure Hb of lysate and G6PD activity and HK according to protocol (20230815_G6PD Activity Assay_96 wells system_V1_JdW).

<u>Aims</u>

- To test whether we see an increase in G6PD activity.
- To calculate the dilution factor based on RBC count, instead of making a 5% RBC solution. (*Assuming that the general RBC count of purified RBCs is ±7*10^12/L, the final concentration should be around 0.35*10^{12}/L, so aim for that in the calculation with a range of 0.3 – 0.4 *10^{12}/L.)*
- To test whether this incubation can also be done in AGAM buffer instead of NaCl 0.9%, and if it has similar results.
- If the results in the AGAM buffer are similar to NaCl 0.9%, the next step will be to test the results of AG1 when the concentration of RBCs is 0.1*10^{12}/L instead of 0.35*10^{12}/L.
- Lastly, to test the effect when combining both AG946 (10uM and 2uM) and AG1 (concentration still unknown) on PK, HK and G6PD activity (and possibly GPI?).

Method according to paper: *Blood sample assay. De-identified blood samples were obtained from the Stanford Blood Center. Erythrocytes were collected by filtering the samples through a cellulose slurry to remove platelets and leukocytes and then washed with saline. G6PD activity was measured spectrophotometrically by the Beutler*

method. The activity of all the samples used in this study was in a normal range (5–9Ug−1 Hb), suggesting that the subjects have WT G6PD. 5% erythrocyte suspension was preincubated with 1–5 μM AG1 at 4 °C overnight, which was followed by treatment with (or without) either 1 mM chloroquine (CQ) or 1 mM diamide for 3–4 h at 37 °C (for hemolysis assay with chloroquine, the mixture was incubated under light). Then centrifugation at 100 × g for 5 min was followed. Hemoglobin release in the supernatant was monitored by measuring absorbance at 540 nm. Saline was used as a negative control (0% hemolysis) and a sample treated with 0.1% Triton X100 was used as a positive control (100% hemolysis). For ROS measurement, erythrocyte mixture was washed with saline by centrifugation after treatment and incubated with chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CMH2DCFDA) at a final concentration of 5 μM in saline at 37 °C for 15 min. After wash, the samples were lysed with 0.1% Triton X-100 (final concentration), and the florescence was analyzed with excitation/emission at 485/525 nm. GSH measurement was determined using a Cayman glutathione assay kit (Cayman Chemicals, 703002). Briefly, 50 μL of diluted erythrocyte lysate samples were mixed with 150 μL of assay reagents including glutathione reductase, 5',5-dithiobis-(2-nitrobenzoic acid) (DTNB) and NADPH, which was followed by incubation for 25 min at room temperature. The absorbance was read at 412 nm. For storage assay, 5% erythrocyte suspension was stored at 4 °C with and without 1 μM AG1, and hemolysis and G6PD activity were monitored every week for 28 days to examine whether AG1 improves preservation of erythrocytes over time. Protein leakage was also examined by measuring the absorbance of the supernatant of samples at 280 nm. The samples were re-treated with AG1 every week.

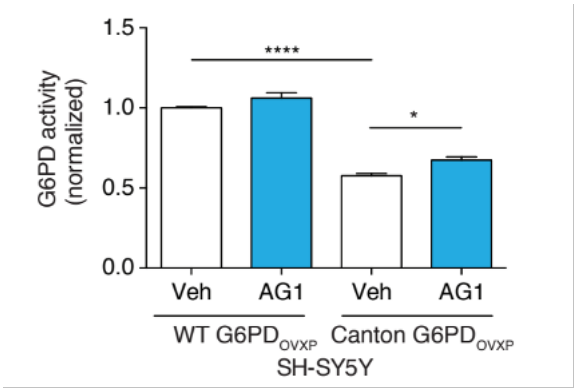


Figure 1 – Acquired from supplemental data of Hwang et al. No clear increase in activity in this wildtype G6PD model.

Protocol E – Changed AG1 protocol with RoxyScan and Bay-11

G6PD Activity Protocol in Purified RBCs with AG1 and Diamide

Reagentia:

Name	Firm	Art nr	UMC Code	MW
DMSO	Sigma	D8418	D045	78.13
AG1 (*Fridge G626 KK1)*	MedChemExpress	#HY-123692		438.65
AG348	Agios			548.63
AG946 (416)	Agios			392.44
Diamide				
Elon ISO	Boom B.V.	030901		
Oxy ISO				
PBS	Sigma	P3813	P134	
Glucose 45%	Sigma	G8679	D160	180.16
Adenine	Sigma	A2786	A171	135.15
Mannitol (-D)	Sigma	M1902	M026a	182.17

Equipment and appliances:

Microplate Conical Well	Label for G6PD, HK, Patient, Control, Conditions, Hb
2mL eps	Label
15ml tubes 50ml tubes	
Rotator	Cenco Testtube Rotator
Centrifuge 16	500G, 5min, soft brake
CELL-DYN Sapphire Hematology Analyzer	Abbot
Heatblock RBC Analyzer	VWR heatblock, pre-heat at 37 degrees LORRCA MaxSis 7
Microplate Reader	SpectraMax iD3, turn on, heat up to 37 degrees

Solutions:

PBS 10x — Dissolve a packet in 100 mL dest → 0.1 M PBS pH 7.4
Or 3 packets in 300mL
Store at -20°C

Adenine 10x — Dissolve 170 mg Adenine in 90 mL dest
(510 in 270mL)
Add HCl till Adenine goes fully into solution
Add dest till 100 mL (or 300), final concentration 12mM
Store at -20°C

Mannitol 10x — Dissolve 5.25 g Mannitol in 100 mL dest
(or 15.75 in 300mL)
Store at -20°C

AGAM buffer pH 7.4

Dest	67.8	2034	
PBS 10x	10	300	→PBS 1x (conc: 0.01M)
Glucose 45%	2.2	66	→Glucose 1% (conc: 55mM)
Adenine 10x	10	300	→Adenine 170mg/L(conc:1.2mM)
Mannitol 10x	10	300	→Mannitol 5.25g/L (conc: 30mM)
	------+	------+	
	100mL	3000mL	

Sterilize trough a 0.20 um filter
Store at -20°C

AG1 10mM (Stock) — Dissolve 0.43865 (0.439) mg in 100uL DMSO. Split in twenty different Eps with 5uL containing in each. (Freezer Jonathan Lab 1)

AG1 5mM (Fresh) — Dilute AG1 10mM in DMSO: 4uL DMSO and 4uL AG1 10mM.

Diamide 10mM (Fresh) — Dissolve 1.72 mg in 1mL aqua dest. Final concentration during incubation will be 1mM.

Preparations:

Day 0:

- Collect reagentia, blood and prepare solutions for AG1
- Collect equipment

Day 1:

- Collect reagentia, incubated samples, prepare Bay-11 & Diamide
- Collect and pre-heat Equipment

Methods:

Day 0:

- RBC isolation according to protocol ("Isolation of RBCs'S-RH128e Version 004").
- For the incubations with AG1, we dilute the PCs (isolated RBCs) in the AGAM buffer to a Ht of ±0.03 (0.027-0.037). We use the following calculation:

  Prepare the diluted RBCs in AGAM buffer:
  Hematocrit / 0.032 = *XXX* times dilution.
  30.000 uL (=30mL) / *XXX* = volume of PCs in uL.
  30.000 uL – volume of PCs = volume of AGAM in uL.

- Check RBC count at Sapphire
- Take 2 15mL tubes and
  prepare the DMSO/activators diluted 1:100 in AGAM buffer:
  992,5uL AGAM buffer + 7,5 uL DMSO / 7,5 uL AG1 5mM

Prepare each condition:
14.000 uL RBCs in AGAM + 1000uL AGAM w/ DMSO
14.000 uL RBCs in AGAM + 1000uL AGAM w/ AG1 5mM → final concentration 5uM

- Place the tubes in the rotator and let tumble slightly, 16 hours (overnight) at 4 degrees. (Ensure that you see that the sample flows through the tube**,** but in a gentle angle to not damage cells)

Day 1:

- Incubation at RT for 3 hours
- Incubate at 37 degrees for 1,5 hours:
  Split each condition in two tubes (7.5mL RBC suspension in each, so four tubes in total)
  After 30 minutes: add 9,75 uL of Bay-11 100mM to one of the DMSO and one of the AG1 conditions, for the blanks add 9,75 uL (the same amount DMSO as Bay-11)
  Incubate at 37 degrees for the remaining hour

  Now you have the following conditions:

DMSO	AG1	DMSO w/Bay-11	AG1 w/Bay-11

Step 1: Enzyme activity
- Take 100uL of each condition and place it in a conical well
- Centrifuge for 5 min, 600G, 9/\3
- Discard supernatant
- Add 100uL of beta-mercapto stabilizing solution to each well
- Alternatively prepare the lysates in Eps and proceed according to the protocol (20230815_G6PD Activity Assay_96 wells system_V1_JdW)
- Measurement of G6PD and PK activity (in triplo) for each condition
- Make enough mastermix for 15 wells + extra for each the G6PD and PK assay
- G6PD and PK enzyme activity measurement and measurement Hb of lysate according to protocol (20230815_G6PD Activity Assay_96 wells system_V1_JdW)

Step 2: Concentrate RBCs
- Switch on LORRCA & PC, log in on T-disk, start LORRCA software
- Prepare Diamide: 4 LORRCA RUNS -> 100ml Diamide 1mM
- RBCs need to be concentrated, final hematocrit should be around 0.45 with a final volume of 500uL.
- Centrifuge the tubes 350G, 5 min, 9/\2, soft brake on Centrifuge, program 22
- After the centrifugation 7.5ml diluted RBCs should correspond with ±500uL RBCs of volume remaining

- Take of the whole supernatant and fill up the tube with AGAM buffer (to wash away the rest of the Bay-11)
- Centrifuge the tubes again 350G, 5 min, 9/\2, soft brake on Centrifuge, program 22
- Take of the supernatant until you have 500uL of RBCs left
- Measure samples on Sapphire for definite RBC count and hematocrit
- If necessary dilute the sample to reach a final hematocrit around 0.45
- Procedure RoxyScan according to protocol (Procedure RoxyScan 1st Draft)

Protocol F – RoxyScan

Name	Ct nr	MW	Code UMCU	Precautions
Cumene Hydroperoxide (G03.626 Koelkast1)	247502	152.19	C186	
Elon ISO (G03.626 Koelkast1)			NA	NA

Procedure RoxyScan:

Start up

- First switch On the Lorrca and secondly switch On the PC, login on T-disk
- Start Lorrca software, the Lorrca MaxSis needs 5 minutes to warm up
- Make sure the D-Ionized water bottle is filled with MilliQ-water (H1)
- Change at the position of the aspirator; the tube containing X-Clean (R4) for a tube containing Demi water
- Lower the Bob in the Cup

- Make sure the connector on the Bob is closed
  Use a Luer Lock Plug
- Prior to use, Oxidant* was very well mixed (for example: Cumene minimum 20min)

- Connect the Oxidant bottle to the Osmo LOW tube/PVP LOW tube.
- Connect the High tube to the MiliQ bottle.

- Go to **Settings** in the Cell Stability Test and ensure the following settings:

General options
- Enable Cell Stability Test: Enable

Cell Stability Test options:
- Cell Stability Test shear stress (Pa): 30.00
- Measurement duration (s): 1600
- Determine deformability every (s): 5

- Valid determinations per measuring points: 10

- Save settings by closing the window; press OK, OK

- Go to **Main Menu** and then click **Hardware check**
- Go to **Lorrca IO** and ensure the following settings:

Cup:
M3: Cup Velocity: 1,00rps
Pumps:
M4: Gradient pump velocity: 0,2 rps

- Standardize measurements to 1.000E6 RBCs/vial:
125 µL of RBC 4.0E12/L in 2.5 mL Elon ISO (R1)
→ Calculation: 4.0/RBC x 125 = *xx* µL blood / 2.5ml vial Elon ISO
Note: Maximum of 175 µL / 2.5ml vial Elon ISO
- Pipet calculated sample volume (xx µL blood) into Elon ISO (R1) 5 mL
Pre-wet tip by gently re-suspending blood 3 times
Note: When using a 1-200 µL tip, cut the tip at the 10 µL mark to ensure a wide opening

- Gently mix until a homogeneous solution (± 25 - 40x)
- Place the blood/Elon-Isomolar under the aspirator

Place a stopwatch for the following Steps:

- In the same **Lorrca IO** settings:

Pumps:
M4: Gradient pump on/off: Turn it on (Click it)
Start the timer as soon as you turn on the gradient pump and wait till **2:35 minutes.** *(This time is related to when the sample from the 2.5ml Sample is almost complete aspirated)*

- Click on **Main Menu** to stop the pump.
- Go to **Cell Stability Test:**

1. Click **New…** and fill in ‘Sample ID, ‘Remarks’, ‘Donation time’ and ‘Viscosity’
Fill in the viscosity value of ElonISO used on the standardization of blood.
- Press **OK** and click **Start now.** *For this step the stopwatch should show no more than 3:10 minutes (Time standardized since the oxidant is already affecting the cells).

- After measurement: ‘Print report?’; click **No**

- After every run <’Rinse Gradient System’>
- Remove the blood/Elon-Isomolar sample tube and place a tube with Demi water under the aspirator

Before placing the Demi water tube, remove any remaining blood/Elon-Isomolar with Tork wiper paper (to prevent pollution of the Demi water tube)
- Press **Rinse**; Lift the aspiration needle and lift the Demi water tube up and down under the aspiration needle

The air bubbles will provide a proper flush of the sample

- <'Empty Cup'> Clean, will start automatically after the rinse cycle

→ Lorrca is ready for next

End of the day wash:

Preliminary shut down:
- Go to **Hardware check → Lorrca IO**
- Press **Prime all**, press **Start**, do this 2 times.
Ignore remark in between "dry Bob and Cup with soft cloth"

Lorrca general shut down:
When the program is shut down a pop-up screen appears with <'End of day wash'>
- Place a tube with X-Clean (R4) under the aspirator
- Connect the tubes of Osmo LOW and HIGH via the Y-piece to a bottle of X-Clean
- press **Start** to start
- Place a tube with DI water (R4) under the aspirator
- Connect the tubes of Osmo LOW and HIGH via the Y-piece to a bottle of DI water
- press **Start** to start
- Remove the DI water (R4) under the aspirator
- Disconnect the tubes of Osmo LOW and HIGH via the Y-piece to a bottle of DI water
- press **Start** to start
- Dry Bob and Cup and keep Bob above Cup
- Remove and clean the waste and close the Lorrca lid

  **Since the Peroxide is used during the tests please discharge the blood in the corresponding waste and not to the sink. (Cumene in Cat 3 , rinse with water at least x2 and also disposed every rinse in the corresponding waste)*

- Switch off the computer and Lorrca

Protocol G – Reversing the order AG1 Bay-11

G6PD Activity Protocol in Purified RBCs with AG1 and Diamide

Reagentia:

Name	Firm	Art nr	UMC Code	MW
DMSO	Sigma	D8418	D045	78.13
AG1 (*Fridge G626 KK1)*	MedChemExpress	#HY-123692		438.65
AG348	Agios			548.63
AG946 (416)	Agios			392.44
Diamide				
Elon ISO	Boom B.V.	030901		
Oxy ISO				
PBS	Sigma	P3813	P134	
Glucose 45%	Sigma	G8679	D160	180.16
Adenine	Sigma	A2786	A171	135.15

Mannitol (-D)	Sigma	M1902	M026a	182.17

Equipment and appliances:

Microplate Conical Well	Label for G6PD, HK, Patient, Control, Conditions, Hb
2mL eps	Label
15ml tubes 50ml tubes	
Rotator	Cenco Testtube Rotator
Centrifuge 16	500G, 5min, soft brake
CELL-DYN Sapphire Hematology Analyzer	Abbot
Heatblock RBC Analyzer	VWR heatblock, pre-heat at 37 degrees LORRCA MaxSis 7
Microplate Reader	SpectraMax iD3, turn on, heat up to 37 degrees

Solutions:

PBS 10x — Dissolve a packet in 100 mL dest → 0.1 M PBS pH 7.4
Or 3 packets in 300mL
Store at -20°C

Adenine 10x — Dissolve 170 mg Adenine in 90 mL dest
(510 in 270mL)
Add HCl till Adenine goes fully into solution
Add dest till 100 mL (or 300), final concentration 12mM
Store at -20°C

Mannitol 10x — Dissolve 5.25 g Mannitol in 100 mL dest
(or 15.75 in 300mL)
Store at -20°C

AGAM buffer pH 7.4

Dest	67.8	2034	
PBS 10x	10	300	→PBS 1x (conc: 0.01M)
Glucose 45%	2.2	66	→Glucose 1% (conc: 55mM)
Adenine 10x	10	300	→Adenine 170mg/L(conc:1.2mM)
Mannitol 10x	10	300	→Mannitol 5.25g/L (conc: 30mM)
	------+	------+	
	100mL	3000mL	

Sterilize trough a 0.20 um filter
Store at -20°C

AG1 10mM (Stock) — Dissolve 0.43865 (0.439) mg in 100uL DMSO. Split in twenty different Eps with 5uL containing in each. (Freezer Jonathan Lab 1)

AG1 5mM (Fresh) — Dilute AG1 10mM in DMSO: 4uL DMSO and 4uL AG1 10mM.

Diamide 10mM (Fresh) — Dissolve 1.72 mg in 1mL aqua dest. final concentration during incubation will be 1mM.

Preparations:

Day 0:

- Collect reagentia, blood and prepare solutions for AG1

- Collect reagentia prepare Bay-11
- Collect equipment

Day 1:

- Collect and pre-heat Equipment

- Collect reagentia, incubated samples, prepare Diamide

Methods:

Day 0:

- RBC isolation according to protocol ("Isolation of RBCs'S-RH128e Version 004").
- G6PD and PK enzyme activity measurement and measurement Hb of lysate according to protocol (20230815_G6PD Activity Assay_96 wells system_V1_JdW)
- For the incubations with AG1, we dilute the PCs (isolated RBCs) in the AGAM buffer to a Ht of ±0.032 (0.027-0.037). We use the following calculation:

  Prepare the diluted RBCs in AGAM buffer:
  Hematocrit / 0.032 = *XXX* times dilution.
  30.000 uL (=30mL) / *XXX* = volume of PCs in uL.
  30.000 uL – volume of PCs = volume of AGAM in uL.

- Check RBC count at Sapphire
- Take 2 tubes and
- Prepare 18,75uL Bay-11 for 14ml and 18,75uL DMSO and incubate for 1h at 37 degrees
- G6PD and PK enzyme activity measurement and measurement Hb of lysate according to protocol (20230815_G6PD Activity Assay_96 wells system_V1_JdW)
- Then prepare the DMSO/activators diluted 1:100 in AGAM buffer:
  992,5uL AGAM buffer + 7,5uL of AG1 10mM/7,5uL of DMSO

  Now you have the following 4 conditions:

DMSO	AG1	DMSO w/Bay-11	AG1 w/Bay-11

Prepare each condition:
14.000 uL RBCs in AGAM + 1000uL AGAM w/ DMSO
14.000 uL RBCs in AGAM + 1000uL AGAM w/ AG1 5mM → final concentration 5uM

- Place the tubes in the rotator and let tumble slightly, 16 hours (overnight) at 4 degrees. (Ensure that you see that the sample flows through the tube**,** but in a gentle angle to not damage cells)

Day 1:

- Incubation at RT for 3 hours
- Incubate at 37 degrees for 1 hour:

Step 1: Enzyme activity
- Take 100uL of each condition and place it in a conical well
- Centrifuge for 5 min, 600G, 9/\3
- Discard supernatant
- Add 100uL of beta-mercapto stabilizing solution to each well
- Alternatively prepare the lysates in Eps and proceed according to the protocol (20230815_G6PD Activity Assay_96 wells system_V1_JdW)
- Measurement of G6PD and PK activity (in triplo) for each condition
- Make enough mastermix for 15 wells + extra for each the G6PD and PK assay
- G6PD and PK enzyme activity measurement and measurement Hb of lysate according to protocol (20230815_G6PD Activity Assay_96 wells system_V1_JdW)

Step 2: Concentrate RBCs
- Switch on LORRCA & PC, log in on T-disk, start LORRCA software
- Prepare Diamide: 4 LORRCA RUNS -> 100ml Diamide 1mM

- RBCs need to be concentrated, final hematocrit should be around 0.45 with a final volume of 500uL.
- Centrifuge the tubes 350G, 5 min, 9/\2, soft brake on Centrifuge, program 22
- After the centrifugation 7.5ml diluted RBCs should correspond with ±500uL RBCs of volume remaining
- Take of the whole supernatant and fill up the tube with AGAM buffer (to wash away the rest of the Bay-11)
- Centrifuge the tubes again 350G, 5 min, 9/\2, soft brake on Centrifuge, program 22
- Take of the supernatant until you have 500uL of RBCs left
- Measure samples on Sapphire for definite RBC count and hematocrit
- If necessary dilute the sample to reach a final hematocrit around 0.45
- Procedure RoxyScan according to protocol (Procedure RoxyScan 1st Draft)

Protocol H – Storage Assay

RBC Hemolysis Storage Assay

Reagentia

Name	Firm	Art nr	UMC Code	MW
NaCl			N020	58.44
DMSO	Sigma	D8418	D045	78.13
AG1 (*Fridge G626 KK1)*	MedChemExpress	#HY-123692		438.65

15ml tubes	
Centrifuge	350G, 5min, 9/\2 (check the radius)
CELL-DYN Sapphire Hematology Analyzer	Abbot

LORRCA

Calculations:

4 million cells per run needed

3ml diluted RBCs -> 300uL PCs
75uL -> sapphire (1:1 dilution with NaCl 0.9%)
Ca.125uL -> Osmoscan

- 3 ml need for each time point
- 5 measuring points: Day 0, 7, 14, 21, 28
- 3*5 = 15 ml per condition

Conditions:

DMSO	AG1 5uM

Preperations:

- RBC isolation according to protocol (Isolation of RBCs'S-RH128e Version 004)

- Dilution of purified RBCs in NaCl 0.9% to a 5% RBC (→ this should correspond with an RBC count of ±0.4*10^{12}/L and a Hb of ±0.65 mmol/L in RBCs of a healthy control)
- Prepare 40ml of diluted RBCs, 20ml per condition

Procedure:

Lysis measurement

Day 0:

- Add 3ml to a 15ml tube and centrifuge 350G, 5min, 9/\2 (check the radius)
- Take 200uL of supernatant of and use for baseline measurement
- Discard 2,3ml of the supernatant off
- Add 2,5ml of dest.
- Lyse cells for 10 min at RT (on a rollerbench)
- Centrifuge 350G, 5min 9/\2
- Take 200uL of the supernatant and use for total lysis measurement

Day 0,7,14,21,28

- Take 3ml from the 15ml placed in the fridge and place in new tube
- Centrifuge 350G, 5min, 9/\2 (check the radius)
- Take of 200uL of supernatant off and use for total measurement
- Measure lysis on Spectramax and take along 200uL NaCl 0,9% according to plate 2 in protocol (20230815_G6PD Activity Assay_96 wells system_V1_JdW), settings are at the end of protocol

Percentage of lysis measurement

Before every measurement mix the tube well and take out 3ml of the blood mixture

- Centrifuge the tubes 350G, 5 min, 9/\2, soft brake on Centrifuge, program 22
- Take off whole supernatant
- Prepare 1:1 NaCl 0,9% dilution in 1,5ml Eps
- Measure blood on sapphire to determine RBC count
- Perform Osmoscan according to protocol 'S-RH191e - LoRRca Oxygenscan - v-001'
  *changes to protocol: using 2,5ml Elon ISO instead of 5ml and adjusting the formular accordingly

Hb concentration
Measure at the Spectramax iD3 the Absorbance at 542 nm of every well.

Endpoint (Absorbance)	
Temperature	37°C
Wavelength	542 nm
Plate type	96 Well Standard clrbtm
	Height 14.6 mm
Automix and blanking	No mix

Protocol I - Osmoscan

Central Diagnostic Laboratory	Webautorisatie: 01-apr-2020
Code: S-RH191e, Versie: 001 - 001, 26-mrt-2020	Herziening: 26-mrt-2021
LoRRca Oxygenscan	

Writing		
Name	***Profession***	***Date***
B.A. van Oirschot - Hermans	Associate Researcher	04-02-2020

Verification		
Name	***Profession***	***Date***
Dr. R. van Wijk	Staff member	02-03-2020
M. Soeters	Quality and clerical staff	26-03-2020

Authorization	
Name	***Profession***
Dr. R. van Wijk	Staff member
Dr. I.E. Höfer	Head ARCADIA

Distribution list					
Name			***Profession***	***Date of issue***	***Date of intake***
QA	Front page	Archive	QA-manager		
Section RH	Chest of drawers	G03.409	Employees		

Changes according to former revision	
Revision date	***Paragraph***
1: 26-03-2020	New document

11.1 Start up

- Switch on PC and LoRRca, open nitrogen cylinder (ensure minimum pressure of 50 bar), Login T-disk
- Fill the D-Ionized water bottle with Milli Q Dest (H2)
- Lower the bob in the cup
- Start program: LoRRca pO_2 spot - shortcut.
- LoRRca: Make sure the gas valve is open! **OK**
- LoRRca: pO_2 Self check, select **Start (enter)**,
  If it fails, rerun **Self check** in **Hardware check --> pO_2 --> Self check**
  The Self check will take ± 8 minutes and is automatically saved at C:\LoRRcaMaxSis\
- Choose **pO_2-scan**
- Check settings

Files	
Storage directory	T:\.....
Auto export method	Enumerate file number (*Measurement ID0.CSV, ...
Auto export file name	Results
Store diffraction patterns	Off - Off
General options	
Fill/empty speed (rps)	1,0
Shear stress for camera adhustment (Pa)	30,00
Bob temperature (°C)	37,0
Auto start	Off
pO_2 scan options	
Logarithmic pO_2 axis	Off
Minimum aspiration time (s)	60
pO_2 scan shear stress (Pa)	30,00
Determine pO_2 every (s)	20
Moving average size	2
Calc. Area between (mm Hg)	10 and 100
Scan steps; Edit	0 – OFF 120 – ON 1420 – OFF 1760 – OFF
pO_2 control	Off

- Save; press **OK**, **OK**

- Begin by performing a test measurement (with a SCD patient or Healthy Control sample) to warm up the bob and cup and ensure the N2 flow.

11.2 Sample Preparation

- Make sure blood has been stored at least 30 minutes at 4 °C
- Mix gently sometimes until cells are in solution
- Let sample warm up to room temperature on a roller bench
  Large tube (9 - 10 mL): Long period at 4°C: 20 min, < 1 hr at 4°C: 10 min
  Small tube (2 - 6 mL): Long period at 4°C: 10 min, < 1 hr at 4°C: 5 min

- Sapphire: S-CH419 Hemocytometer Cell-Dyn Sapphire
- Measure CBC+RETC [L+] (in case NRBCs present, CBC + RETC, Resistant RBC
  Print results (plots) in color

- Standardisation: 50 µL of RBC 4.0E12/L in 5 mL Oxy ISO (--> 200E6 RBCs/vial)
  Calculation: 4.0/RBC x 50 = xx µL blood / vial Oxy-Isomolar

11.3 Procedure Oxygenscan

- Pipet calculated sample volume (xx µL blood) into Oxy ISO 5 mL
  Use a pipette tip with a wide opening (1-200 µL tip cutted at 10 µL mark)
  Prewet tip by gently resuspending blood 3 times
  Open Oxy-Isomolar vial for as short a time as possible
- Gently mix until a homogeneous solution (± 25 - 40x)

- Slowly draw 2.0 mL of the blood/Oxy-Isomolar into a 2 mL syringe
- Remove any visible air bubbles and excessive sample volume until 1.8 ml is left in syringe

- Inject the total sample volume (1,8 mL) slowly and evenly in the Bob through the connector. Make sure the level of the sample is above the suction point. Do not leave any sample solution in the syringe

- Click New and fill in Sample ID, Remarks and Donation time (= date of donation) and Viscosity of Oxy-Isomolar.
- Click **Aspirate**, (this will take 75 seconds). Click **OK**
- Close the LoRRca lid. Check that the Gain is set to 300
- Click **Start now**.
  The run of pO_2 scan will take about 30 min.

- Print report?; **No**

- Rinse; Remove the sample syringe and replace it with a syringe filled with aqua dest
- Press **Clean**, slowly flushing the connector during rinsing, make sure to flush in both directions.
- Remove the syringe and lift bob.
- Dry Bob, Cup and Connector with soft cloth. Use a large syringe on the inlet to flush (2 times), and block the lower inlet/outlet of the bob to get back pressure in the tubes

- Lower the Bob in the Cup --> LoRRca ready for next measurement

11.4 Procedure shut down

- Rinse after the last run as described above
- Place Cleaning Solution under the aspirator and connect the tubes (Y-piece) to the Cleaning Solution
- Close the software; press **X** (Close), and press **Start** to start <'End of day wash'**>**
- Remove syringe and lift the bob.
- Dry the bob, connector and cup with a soft cloth. Flush the connector with a large syringe on the inlet (2 times), block the lower inlet/outlet of the bob to get back pressure in the tubes
- Empty the waste bottle
- Close the lid of machine. Close the nitrogen cylinder. Turn off the computer and the machine

11.5 Remarks

- Check the LoRRca Reagents Certificates of Analysis of Oxy ISO to ensure that the measured values falls within the strict limits; Osmolality 282 – 286 mOsm/kg, pH (at 25°C) 7.35 – 7.45 and Viscosity (at 37°C ± 0.5°C) 28 – 30 cP.
  https://support.rrmechatronics.com/index.php/home/lorrca/lorrca-reagents-coa/

Central Diagnostic Laboratory	Webautorisatie:	01-apr-2020
Code: S-RH191e, Versie: 001 - 001, 26-mrt-2020	Herziening:	26-mrt-2021
LoRRca Oxygenscan		

- The PO2S Luminophores are delivered in black plastic bags, protect them against light
- Nitrogen 5.0 T10 Cylinders are ordered via Facilitair Service Centrum, tel. 66000
  An extra spare cylinder is in space G.03.4.12
- Check for scratches on surface projection screen and cup
- Clean the cup with Tork wiper paper by turning the cup at the upper ring, do not touch the outside of the cup at the middle part where the laser emits and don't touch the O_2-spot at the inside of the cup.
- If the self-check fails, consider replacing the O_2-spot. The O_2-spot is replaced by gently pushing the spot out from the inside of the cup with a fingertip. A new spot is placed by gently pushing the spot from the outside into the cup. Check the batch number of the O_2-spot and download the new pO_2 sensor batch software.
  https://support.rrmechatronics.com/index.php/home/lorrca/po2s-luminophore-batch-information/
  Or : Go to lorrca.com, Choose 'Our products' – 'Products' in the menu bar, Click on 'Batch information Oxygen sensitive spot', Click 'Automatic installation file' for the regarding batch, You will be asked: 'Do you want to run .exe file? Click 'Save'. The information is downloaded to your computer (Drive location C:\LoRRca MaxSis). *Note: if the computer blocks the run/download, ignore those messages and run the program/download anyway.*
  Open the LoRRca program, Choose 'pO2scan 'in the menu, Click on Settings, click on 'Instrument settings' - 'Hardware settings'. Select the wanted pO_2 sensor batch number

11.5.1 General remarks

Oxygen gradient ektacytometry is able to determine the oxygen tension at which RBCs from a patient with sickle cell disease start to sickle. This oxygen tension is patient specific and can serve as a biomarker for disease severity.
Oxygen gradient ektacytometry is a functional assay that assesses the individual sickling tendency. To date, no other functional assays are available or used.
In sickle cell disease, this technique can be used to monitor treatment efficacy and it can be used for the development of new treatments.

The technique is very sensitive to sample preparation and sample handling. There are numerous factors that influence the measurement. It is important to work as standardized as possible and to develop a routine.

12. Data processing

12.1 Calculation

NA

Sapphire Measurements

Sapphire Measurements for EXP-24-AP6502 - Enzyme Activity and RoxyScan with AG1 & Bay-11

Date	Name	RBC	HGB	HCT
02.04.2024	**MDD1** WB	4.89	9.43	.464
	PC	4.18	8.43	.407
	Lysate	.228	.580	.010
	AGAM dil.	.320	.751	.032
03.04.2024	Lys. DMSO	.330	.658	.020
	Lys. AG1	.280	.549	.014
	DMSO RoxyScan	2.67	5.72	.260

	AG1 RoxyScan	2.08	3.98	.204
	DMSO B-11 Rox.	2.26	4.12	.226
	AG1 B-11 Rox.	2.41	4.27	.239
	Lys. DMSO	.265	.584	.017
	Lys. AG1	.284	.633	.017
	Lys. DMSO B-11	.261	.557	.012
	Lys. AG1 B-11	.273	.575	.014
10.04.2024	**MDD2** WB	5.82	9.60	.504
	PC	4.35	7.85	.387
	Lysate	.301	.608	.015
	AGAM dil.	.384	.768	.035
11.04.2024	Lys. DMSO	.334	.644	.018
	Lys. AG1	.339	.603	.019
	DMSO RoxyScan	3.06	5.73	.271
	AG1 RoxyScan	2.05	3.98	.180
	DMSO B-11 Rox.	2.32	4.42	.208
	AG1 B-11 Rox.	3.03	5.48	.266
	Lys. DMSO	.366	.716	.021
	Lys. AG1	.331	.686	.017
	Lys. DMSO B-11	.355	.726	.020
	Lys. AG1 B-11	.343	.685	.016
15.04.2024	**MDD3** WB	5.01	9.58	.484
	PC	4.12	8.01	.408
	Lysate	.280	.618	.014
	AGAM dil.	.312	.730	.031
16.04.2024	Lys. DMSO	.290	.593	.017
	Lys. AG1	.265	.537	.016
	DMSO RoxyScan	2.54	4.79	.250
	AG1 RoxyScan	2.67	3.39	.265
	DMSO B-11 Rox.	1.95	3.75	.197
	AG1 B-11 Rox.	2.47	4.74	.246
	Lys. DMSO	.259	.612	.016
	Lys. AG1	.268	.574	.016
	Lys. DMSO B-11	.272	.597	.015
	Lys. AG1 B-11	.269	.552	.015
23.04.2024	**MDD4** WB	4.62	8.20	.428
	PC	4.85	7.38	.460
	Lysate	.308	.635	.018
	AGAM dil.	.382	.797	.037
24.04.2024	Lys. DMSO	.262	.535	.009
	Lys. AG1	.282	.531	.011
	DMSO RoxyScan	2.03	3.88	.199
	AG1 RoxyScan	2.32	4.25	.229

	DMSO B-11 Rox.	2.57	4.63	.238
	AG1 B-11 Rox.	2.30	3.72	.226
	Lys. DMSO	.301	.628	.017
	Lys. AG1	.314	.596	.018
	Lys. DMSO B-11	.311	.616	.018
	Lys. AG1 B-11	.322	.622	.018
29.04.2024	**MDD5** WB	5.50	9.48	.569
	PC	4.73	7.81	.499
	Lysate	.221	.684	.012
	AGAM dil.	.354	.850	.038
30.04.2024	Lys. DMSO	.295	.682	,017
	Lys. AG1	.297	.644	.016
	DMSO RoxyScan	2.22	5.05	.232
	AG1 RoxyScan	2.46	3.78	.263
	DMSO B-11 Rox.	.195	3.95	.208
	AG1 B-11 Rox.	2.43	4.32	.259
	Lys. DMSO	.323	.718	.020
	Lys. AG1	.302	.699	.019
	Lys. DMSO B-11	.297	.712	.019
	Lys. AG1 B-11	.308	.703	.018

Sapphire Measurements for EXP-24-AP6503 - Enzyme Activity and RoxyScan with Bay-11 & AG1

Date	Name	RBC	HGB	HCT
07.05.2024	**MDD1** WB	4.28	8.23	.394
	PC	5.16	7.19	.484
	Lysate	.214	.659	.012
	AGAM dil.	.337	.933	.032
08.04.2024	Lys. DMSO			
	Lys. Bay-11			
	DMSO RoxyScan	1.90	3.52	.177
	AG1 RoxyScan	1.99	4.06	.185
	DMSO B-11 Rox.	1.95	3.61	.182
	AG1 B-11 Rox.	1.99	2.91	.188
	Lys. DMSO	.309	.668	.018
	Lys. AG1	.298	.561	.017
	Lys. DMSO B-11	.318	.652	.018
	Lys. AG1 B-11	.284	.612	.014
13.05.2024	**MDD2** WB	6.20	7.48	.540
	PC	5.38	7.10	.481
	Lysate	.328	.693	.013
	AGAM dil.	.369	.784	.034
14.05.2024	Lys. DMSO	.361	.703	.020

	Lys. Bay-11	.340	.702	.014
	DMSO RoxyScan	2.52	4.84	.224
	AG1 RoxyScan	2.36	4.53	.211
	DMSO B-11 Rox.	2.95	4.07	.270
	AG1 B-11 Rox.	2.36	4.50	.206
	Lys. DMSO	.279	.636	.015
	Lys. AG1	.292	.606	.016
	Lys. DMSO B-11	.253	.580	.011
	Lys. AG1 B-11	.259	.557	.014
15.05.2024	**MDD3** WB	5.21	8.40	.446
	PC	5.80	7.71	.694
	Lysate	.331	.666	.019
	AGAM dil.	.478	1.05	.042
16.05.2024	Lys. DMSO	.364	.681	.020
	Lys. Bay-11	.301	.646	.014
	DMSO RoxyScan	2.26	3.89	.196
	AG1 RoxyScan	2.95	4.13	.257
	DMSO B-11 Rox.	2.67	4.47	.233
	AG1 B-11 Rox.	2.35	3.89	.207
	Lys. DMSO	.325	.590	.018
	Lys. AG1	.290	.583	.014
	Lys. DMSO B-11	.272	.617	.014
	Lys. AG1 B-11	.264	.588	.011

Sapphire Measurements for Storage Assay

Date	Name	RBC	HGB	HCT
10.05.2024	MDD1 WB	6.31	9.91	.546
	MDD2 WB	5.72	10.5	.527
	MDD3 WB	6.40	10.6	.578
	MDD1 PC	5.77	6.95	.507
	MDD2 PC	6.16	7.77	.578
	MDD3 PC	5.48	6.65	.507
	MDD1 dil. RBC	.433	.815	.038
	MDD2 dil. RBC	.414	.863	.039
	MDD3 dil. RBC	.406	.806	.038
	MDD1 Osmoscan	5.45	7.64	.480
	MDD2 Osmoscan	4.89	6.71	.459
	MDD3 Osmoscan	4.32	7.15	.401
17.05.2024	MDD1 DMSO D7	4.66	5.01	.429
	MDD1 AG1 D7	5.31	5.44	.490
	MDD2 DMSO D7	2.94	5.47	.287
	MDD2 AG1 D7	5.03	6.81	.490

	MDD3 DMSO D7	4.36	5.57	.421
	MDD3 AG1 D7	3.72	5.92	.357
24.05.2024	MDD1 DMSO D14	4.43	6.03	.418
	MDD1 AG1 D14	4.64	5.39	.440
	MDD2 DMSO D14	4.61	6.09	.460
	MDD2 AG1 D14	5.02	5.65	.501
	MDD3 DMSO D14	4.92	4.82	.484
	MDD3 AG1 D14	5.01	5.76	.494
31.05.2024	MDD1 DMSO D21	4.21	4.78	.409
	MDD1 AG1 D21	4.29	6.24	.420
	MDD2 DMSO D21	5.10	5.78	.518
	MDD2 AG1 D21	4.75	5.11	.488
	MDD3 DMSO D21	3.03	4.18	.352
	MDD3 AG1 D21	3.47	4.85	.352
07.06.2024	MDD1 DMSO D28	3.73	4.77	.355
	MDD1 AG1 D28	4.35	4.63	.437
	MDD2 DMSO D28	3.99	4.55	.392
	MDD2 AG1 D28	4.17	4.91	.429
	MDD3 DMSO D28	4.28	5.11	.439
	MDD3 AG1 D28	4.55	5.08	.471

Mini Donor Overview

Experiment	Date Begin	Name	Donor number	Sex	Age
Enzyme activity measurement AG1	19.02.2024	MDD1	2019741	M	35
	19.02.2024	MDD2	2017212	F	59
	21.02.2024	MDD3	2023989	F	27
	21.02.2024	MDD4	20231004	F	41
Enzyme activity measurement AG1 + RoxyScan	02.04.2024	MDD1	2023957	M	30
	10.04.2024	MDD2	2017552	F	61
	15.04. 2024	MDD3	2017510	F	49
	23.04.2024	MDD4	20231006	F	29
	29.04.2024	MDD5	2017481	F	66
Enzyme activity measurement AG1 + RoxyScan, reversed order	07.05.2024	MDD1	201757	F	51
	13.05.2024	MDD2	2018675	F	56
	15.05.2024	MDD3	2017339	F	36
Storage Assay	10.05.2024	MDD1	2017467	M	28
	10.05.2024	MDD2	2022919	M	29

	10.05.2024	MDD3	2023997	F	27