In vitro hemorheological studies focusing on erythrocyte deformability and aggregation

Ph.D. dissertation

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I. Prologue

The Framingham Study and other epidemiological investigations have revealed numerous cardiovascular risk factors; while further studies have reported the basic principles of risk reduction. Those examinations have described the importance of the healthy food consumption including Mediterranean diet with moderate red wine intake. In spite of these fundamental observations, several factors have been remained in the pathomechanism of cardiovascular diseases which requires deeper investigations, such as the properties of circulating blood.

Blood is a non-Newtonian suspension containing cells, lipid components, proteins, carbohydrates and electrolytes. Blood flow is characterized by several hemorheological parameters, such as hematocrit, plasma and whole blood viscosity, plasma proteins, erythrocyte deformability and aggregation. Numerous investigations have presented that hemorheological parameters (e.g., hematocrit, plasma fibrinogen and blood viscosity) are cardiovascular risk factors, and alteration of these parameters can impair microcirculation. Other hemorheological parameters, such as red blood cell deformability and aggregation have also been under both basic science and clinical interest; reduced deformability and increased aggregation and can impair *in vivo* tissue perfusion.

There is a general agreement regarding the factors affecting erythrocyte deformability: cell shape and membrane surface area to volume ratio as "extrinsic" factors; membrane viscoelastic properties and cytosolic viscosity as "intrinsic" factors. Deviations from the normal resting biconcave shape, decreased area to volume ratio, higher membrane viscosity or elevated cytoplasmic viscosity tend to reduce deformability.

Erythrocyte deformability can be modified by several structural and functional alterations of erythrocytes. Changes in strictly regulated properties of blood, mechanical and oxidative damages, parasite infection and genetic disorders are associated with decreased red blood cell deformability. Abnormal red blood cell deformability is especially notable in sickle cell disease in which the erythrocytes become rigid at reduced oxygen levels due to intracellular polymerization of sickle hemoglobin leading to occlusions in microvessels and impaired tissue perfusion resulting in painful crisis and infarctions of various organs.

Erythrocyte aggregation is a major determinant of *in vitro* hemorheology occurring in either plasma or solutions with large polymers. During the process, red blood cells reversibly form linear or branched aggregates. Under *in vivo* circumstances erythrocyte aggregation occurs at low shear forces. Erythrocyte aggregation is characterized by red blood cell aggregability and the concentration of macromolecules.

At these days, two parallel models explain the process of aggregation. The bridging theory claims that erythrocyte aggregation occurs when disaggregating forces are not capable to interfere the adsorption of macromolecules to the nearby cell surfaces, while the depletion model suggests that the decreasing protein or polymer concentration creates an osmotic gradient between two adjacent erythrocytes leading to depletion interaction. Although increased erythrocyte aggregation has been observed in various clinical diseases (e.g., hypertension, diabetes mellitus), all mechanisms of the process have not been completely understood.

II. Aim of the studies

These studies were designed to investigate the possible alterations of erythrocyte deformability and aggregation in two *in vitro* experiments: 1) effects of red wine, alcohol-free red wine extract and ethanol was examined; 2) light scattering results of red blood cells in ektacytometry were also analyzed.

III. Methodology

In these experiments, erythrocyte deformability was determined by a LORCA ektacytometer (Laser-assisted Optical Rotational Cell Analyzer; R&R Mechatronics, Hoorn, Netherlands). In this instrument a dilute suspension of erythrocytes in a viscous medium is placed in the gap of a Couette shearing system having a laser-diode projected through the gap.

The presence of red blood cells in the gap diffracts the laser light that creates a diffraction pattern on a diaphragm changing from circular to elliptical as cells deform and elongate. The pattern is analyzed by a computer system that calculates an elongation index (EI) as the (length - width) / (length + width) of the pattern for each shear stress (SS).

Red blood cell aggregation can be determined with a LORCA aggregometer detecting the laser backscattering from the aggregating blood. Erythrocytes are placed in the gap of the instrument and disaggregated at a high shear rate (500 s^{-1}) which reduces rapidly to zero. During the process the intensity of the backscattered laser light changes, that is characterized by the aggregation index. Another sensitive parameter of red blood cell aggregation is called threshold shear rate describing the smallest shear rate which is required for the complete disaggregation of erythrocytes.

Erythrocyte aggregation can also be measured with a Myrenne aggregometer (model MA-1, Myrenne GmbH, Roetgen, Germany) that employs and measures the infrared light transmission through an erythrocyte suspension between a transparent plate and a cone. Cells are initially disaggregated by the cone at high shear (600 s^{-1}) following which shear is abruptly stopped or reduced to 3 s^{-1} and light transmission integrated for 10 seconds. The instrument provides two dimensionless indices of red blood cell aggregation (M, aggregation at stasis; M1, at very low shear); both M and M1 increase with enhanced aggregation.

IV. *In vitro* hemorheological effect of red wine, alcohol-free red wine and ethanol

1. The “French Paradox”

Cardiovascular diseases (CVD) are among the most frequent causes of morbidity and mortality in the developed countries. Several epidemiological studies have revealed that total mortality is not but CVD-related death is substantially lower in France than in other industrialized Western-European countries, although consumption of saturated fats and level of blood cholesterol are higher, while other major risk factors, such as smoking and hypertension are similarly prevalent in France as in other developed regions.

Further epidemiological studies have demonstrated a J-shape relationship between CVD mortality and consumed alcohol amount and shown that regular but moderate (i.e., not more than 10-20 g alcohol per day) red wine (RW) consumption results in a decreased risk of coronary heart disease, heart failure and stroke. According to other studies, wine consumption is associated with higher beneficial cardiovascular effects compared to other forms of alcohol. Furthermore, this beneficial protective effect depends on the type of wine; mortality rates in the RW drinking Mediterranean regions is lower than in Alsace, a white wine drinking area of France.

This phenomenon (i.e., beneficial cardiovascular effects of regular and moderate RW consumption) has been termed the "French Paradox".

2. Components of red wine

It is assumed that the favorable cardiovascular effects of RW originate in its non-alcoholic and alcoholic components. Non-alcoholic component of RW contains anthocyanins and polyphenols. Anthocyanins, such as delphidin and malvidin are responsible for the color of wines, while polyphenols are believed the main source of the cardiovascular protection. The most potent polyphenols of RW, such as resveratrol, katechin and quercetin have been extensively studied. In addition to the decreased oxidation of low-density lipoproteins (LDL) and expression of LDL receptors, polyphenols induce the nitric-oxide (NO) production and reduce the platelet aggregation plus the production of proinflammatory eicosanoids.

Alcohol-free red wine (AFRW) has been mostly used in animal models. An ischemia-reperfusion rat model measurement has proven that AFRW treatment improves the ventricular functions and reduces the area of postinfarction remodeling. Some other rat experiments have reported that AFRW feeding decreases the thrombotic tendency and the degree of oxidative stress, while a human study has shown that AFRW ingestion inhibits the oxidation of LDL.

Several studies of RW consumption have confirmed that ethanol also plays a role in the beneficial cardiovascular effects of moderate red wine intake. Ethanol favorably modifies hemostasis leading to reduced levels of certain coagulation factors and of platelet function; enhanced fibrinolysis due to elevated levels of tissue-type plasminogen activator has also been demonstrated. Alterations of plasma lipid profiles with an increase in high-density lipoprotein and a decrease in LDL cholesterol concentrations have been reported. Furthermore, ethanol also enhances the production of the vasodilator endothelial NO.

3. Effect of red wine and its components in hemorheology

Only a few experiments have been performed to evaluate the effects of RW, polyphenols and ethanol on hemorheological factors. *In vitro* studies exploring the effects of ethanol addition to blood indicate no changes of hematocrit or whole blood viscosity, alcohol consumption leads to dehydration with an elevation of whole blood and plasma viscosity. On the other hand, several studies have demonstrated that regular but modest alcohol ingestion is associated with a decreased level of plasma fibrinogen and reduction in plasma viscosity. In contrast, a recent study has reported no changes of fibrinogen, hematocrit or blood viscosity after moderate RW and vodka consumption for two weeks.

Given the current uncertainty and the lack of data regarding the hemorheological consequences, the present *in vitro* study was designed to further explore possible effects of red wine and its major components on red blood cell (RBC) deformability and aggregation.

4. Methods

Throughout the measurements, a 2002 Merlot (Polgar Winery, Villany, Hungary) red wine was applied. For alcohol-free red wine extract measurements wine sample was vacuum distilled until the disappearance of alcohol and rediluted. Alcohol experiments were performed with reagent grade ethanol (Sigma-Aldrich Co., St. Louis, MO, USA).

Venous blood was obtained from 13 healthy volunteers into Vacutainer tubes coated with lithium heparin for the red wine and polyphenol measurements. For the ethanol experiment blood was taken from 7 volunteers and anticoagulated with ethylenediamine-tetraacetic acid.

Red wine and blood samples were initially mixed to simulate final blood alcohol concentration of 0.10, 0.30 and 1%, while other samples were treated with AFRW or physiological saline in a similar manner. After 1 hour incubation erythrocyte deformability and aggregation were tested.

In a 2nd study, two general approaches were utilized to evaluate the ethanol effects on erythrocyte deformability: 1) direct addition of ethanol to whole blood (the final ethanol concentration was 0, 0.25, 0.50, 1 and 2%) followed by incubation and testing; 2) addition of ethanol *only* to the viscous suspending medium used for deformability measurements (the final ethanol concentration was 0, 0.25, 0.50, 1, 2, 3, 4, 5 and 6%), following which untreated red blood cells were suspended in these media then measured.

Aggregation measurements used 40% hematocrit suspensions of erythrocytes in autologous plasma or in a 70 kDa dextran solution (3% in phosphate buffered saline). Red blood cells were initially suspended in plasma at a 40% hematocrit, ethanol added at concentrations of 0, 0.25, 0.50, 1 and 2%. Erythrocyte-plasma samples were then tested, while cells to be suspended in 3% 70 kDa dextran were washed twice with phosphate buffered saline (PBS) then re-suspended in the dextran at 40% hematocrit.

In a 3rd experiment, *in vitro* effect of red wine, alcohol-free red wine extract and ethanol was examined in the presence of oxidative stress. Blood samples were pretreated with RW and AFRW at a concentration of 0.30% or ethanol at concentrations of 0, 0.25, 0.50, 1 and 2% and then the free radical generator phenazine methosulfate (PMS, Sigma) was added at a final concentration of 500 μM. Samples were incubated at 37^{o}C for 2 hours then erythrocyte deformability was tested. In one series, whole blood was treated only with PMS and alcohol added only to the viscous medium used for deformability measurements.

After ethanol treatment, RBC shape was evaluated by DIC light microscopy (model BX50F; Olympus, Tokyo, Japan).

For some deformability results, EI-SS data was fitted to a Lineweaver-Burke type non-linear equation that yields the maximum EI at infinite shear stress (EI_{max}) and the stress required to achieve one-half of this maximum value ($SS_{1/2}$). Data fitting and analysis were carried out using non-linear regression (GraphPad Prism, GraphPad Software, La Jolla, CA).

5. Results of the deformability measurements

Our results show that no erythrocyte deformability changes were observed in any concentrations of the red wine and alcohol-free red wine extract treated samples followed by incubation then testing with LORCA. Analysis obtained using the Lineweaver-Burke regression indicated that in case of the two agents neither EI_{max}, $SS_{1/2}$ nor their ratio differed from the saline treated control.

The effects of ethanol on red blood cell deformability depended on the manner in which cells were exposed to the alcohol: 1) addition to whole blood followed by incubation caused no change in deformability, no changes were observed in $EI_{max,}$ $SS_{1/2}$ and $SS_{1/2}$ / EI_{max}; 2) addition to the LORCA media and testing of non-incubated cells resulted in significant, dose-dependent deformability increase ($p<0.05$). EI_{max}, $SS_{1/2}$ and their ratio for non-incubated cells significantly decreased ($p<0.05$) with alcohol concentration of the LORCA media.

6. Results of the oxidative stress experiment

The effects of RW and its major components on erythrocyte deformability when cells were oxidatively stressed by the free radical generator phenazine methosulfate were also studied using the LORCA ektacytometer. As expected, incubation with 500 μM PMS alone caused a significant decrease ($p<0.05$) of erythrocyte deformability.

Although AFRW pretreatment at 0.30% concentration significantly prevented ($p<0.05$) erythrocytes from the PMS generated deformability impairment, 0.30% RW had no such effect. The Lineweaver-Burke analysis demonstrated that EI_{max} for AFRW plus PMS treated cells did not change, while $SS_{1/2}$ and the $SS_{1/2}$ / EI_{max} ratio significantly decreased ($p<0.05$). On the other hand, EI_{max}, $SS_{1/2}$ and their ratio showed no alterations for erythrocytes treated with 0.30% RW and PMS compared to the only PMS damaged cells.

Changes of erythrocyte deformability again depended strongly upon the manner in which cells were exposed to ethanol. Red blood cells incubated for 2 hours with alcohol plus PMS then tested in alcohol-free LORCA media exhibited significant decreases ($p<0.05$) of deformability from PMS alone. Under these conditions, EI_{max} for PMS treated cells was unaffected by the presence of ethanol during incubation, while both $SS_{1/2}$ and the $SS_{1/2}$ / EI_{max} ratio significantly increased ($p<0.05$).

However, PMS treated cells challenged with alcohol in the LORCA media exhibited significant improvements ($p<0.05$) of erythrocyte deformability compared to PMS alone, EI_{max} was not meaningfully altered, while $SS_{1/2}$ and the $SS_{1/2}$ / EI_{max} ratio were lower already at 0.25% ethanol.

7. Results of the aggregation measurements

Treatments with RW and AFRW inhibited erythrocyte aggregation in a dose dependent manner. In M and M1 mode of the Myrenne aggregometer the differences were significant ($p<0.05$) already at a concentration of 0.10%. Red wine had a tendency for stronger inhibition compared to AFRW which became significant ($p<0.05$) at a concentration of 1%.

LORCA aggregation index confirmed these results only at the highest concentration where the difference between RW and AFRW became significant ($p<0.05$). Changes in LORCA threshold shear rate were concordant with Myrenne parameters.

Further results show that erythrocyte aggregation in autologous plasma or in 3% 70 kDa dextran solution showed significant decreases ($p<0.05$) in a dose-dependent manner after ethanol treatment. The changes of the M and M1 indices were significant at 0.25% and above. At 2% ethanol the aggregometer was unable to detect RBC aggregate formation in either medium.

8. Microscopic analysis

Morphological analysis using DIC light microscopy demonstrated that normal discocytes (i.e., biconcave shaped cells) in PBS (Fig. 1A) became echinocytes (i.e., erythrocytes with spiky projections on the cell surface) with 2% ethanol (Fig. 1B). The viscous dextran medium used in the LORCA induces a slight stomatocytic (i.e., cup shaped red blood cells) transformation (Fig. 1C), while erythrocytes retain their normal, discocytic shape in dextran with 2% alcohol (Fig. 1D).

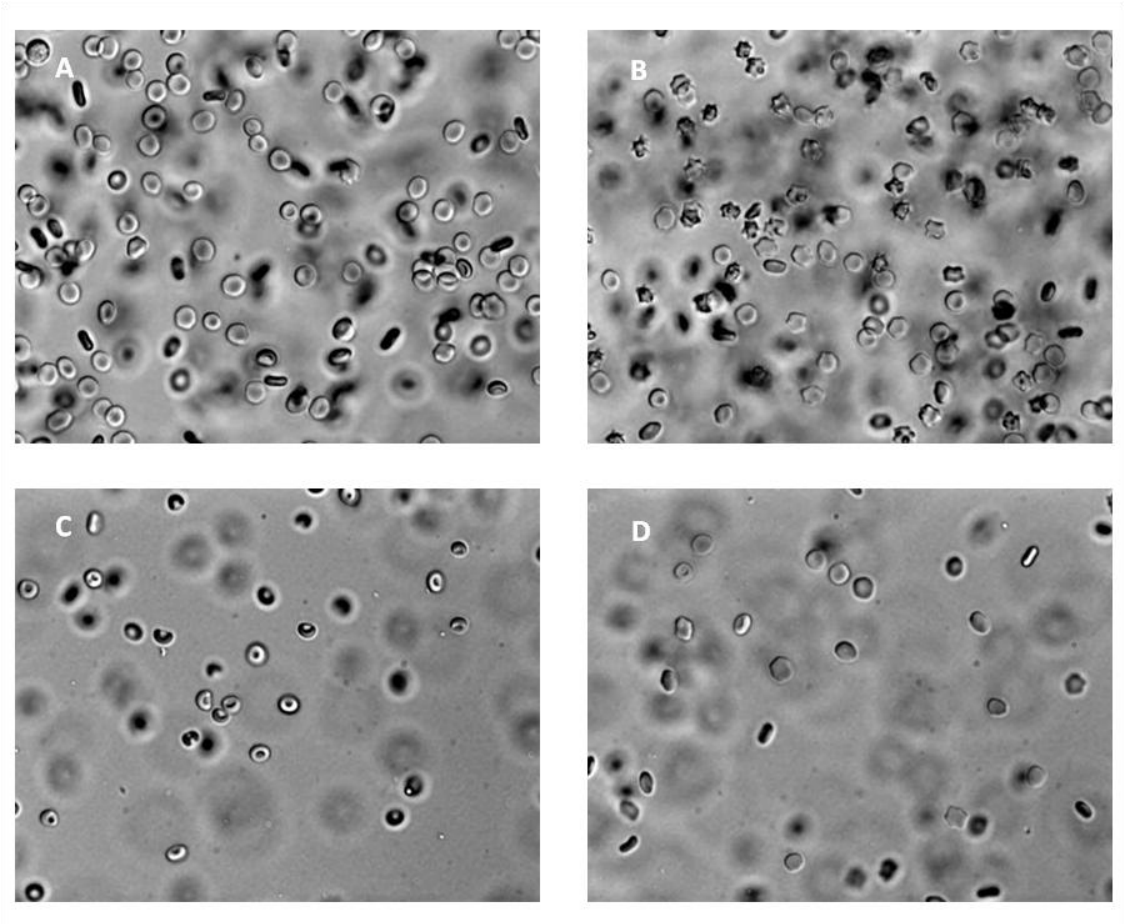

Fig. 1. *Morphological appearance of erythrocytes visualized by DIC light microscopy.* ***A)*** *Untreated erythrocytes in PBS demonstrating the normal, discoid shape of the cells.* ***B)*** *Echinocytes in PBS with 2% ethanol concentration.* ***C)*** *Stomatocytes in the viscous medium (dextran) of LORCA.* ***D)*** *Erythrocytes maintaining the normal, discocytic shape in dextran containing 2% ethanol.*

9. Discussion

In this *in vitro* experiment, hemorheological consequences of red wine and its major components were examined focusing on erythrocyte deformability measured by LORCA ektacytometer and aggregation determined by Myrenne and LORCA aggregometers.

The results of our *in vitro* study indicate that direct addition of RW and AFRW to blood followed by incubation do not alter erythrocyte deformability, while ethanol can improve it. These improvements were *only* observed when ethanol was in the viscous media used for ektacytometry testing and were *not* present when cells were incubated with the alcohol but tested in alcohol-free viscous media. Our *in vitro* results thus indicate that the ethanol-induced deformability improvement requires the presence of ethanol and the changes in the cell membrane are reversible. Our results indicate that the greatest enhancement of deformability, indexed by $SS_{1/2}$ and the $SS_{1/2}$ / EI_{max} ratio, was observed at 4% and 5% ethanol concentration, while deformability at 6% was significantly lower and similar to the 3% ethanol results. These findings thus indicate a bi-phasic effect of ethanol: improved deformability followed by decreased benefits with increasing concentration.

Based on the general agreement, four factors (e.g., morphology, geometry, membrane rheologic properties and cytoplasmic viscosity) can affect erythrocyte deformation behavior. Although ethanol can cause a discocyte-echinocyte shape change, cells suspended in dextran + ethanol had discoidal morphology. Ingested alcohol increases plasma osmolality, thereby reducing cell volume, increasing surface to volume ratio and elevating cytoplasmic viscosity; the increased ratio favors deformability, while the greater cytoplasmic viscosity has the opposite effect. Given that cells were always suspended in isotonic media, thus it is assumed that ethanol affects the mechanical behavior of the membrane with its attached cytoskeleton.

Ethanol has a polar hydroxyl group soluble in aqueous media and hence must distribute within the exterior glycocalyx and the interior of the cell, while the non-polar part of the molecule is preferentially found in the lipid bilayer. The fluidity of the lipid portion can be altered by ethanol in a dose dependent manner. Note, that the less viscous lipid bilayer has only minimal effect on cell deformability. Thus the cytoskeleton must be reversibly altered; the most likely changes occur in the spektrin-aktin linkages and in the interactions between transmembrane proteins and cytoskeletal components.

RBC deformability alterations induced by red wine, alcohol-free red wine extract and ethanol were also examined in the presence of oxidative stress generated by phenazine methosulfate. PMS is a well-known oxygen free radical generator that causes lipid peroxidation and structural modifications in the membrane skeletal protein network, leading to increased membrane rigidity and decreased deformability.

Determination of erythrocyte deformability after RW or AFRW pretreatment demonstrated that AFRW significantly decreased the PMS generated RBC deformability impairment thus prevented erythrocytes from oxidative stress. Our *in vitro* results confirm prior *in vivo* results about the antioxidant properties of polyphenols where plasma antioxidant capacity was increased by the consumption of AFRW in human volunteers. Although AFRW could partially protect erythrocytes, red wine pretreatment had no such preventive influence in this model.

This red wine observation is presumably supported by the *in vitro* ethanol experiment, where pure ethanol + PMS were added together to whole blood and tested in alcohol-free LORCA media; ethanol enhanced the effect of oxidative stress with increasing concentration. Based on these results it seems reasonable that the protective effect of polyphenols is attenuated by the presence of ethanol in the red wine portion.

The ethanol + PMS results also showed that the deformability of oxidatively damaged erythrocytes could be improved when ethanol was present in the LORCA media. Ethanol presumably acts in a manner similar to the effects on normal erythrocytes.

In this experiment, red wine and alcohol-free red wine extract were incubated with whole blood then tested with Myrenne and LORCA aggregometers demonstrating a dose-dependent reduction in erythrocyte aggregation and indicating that RW is a more potent inhibitor than AFRW. This decrease may be a consequence of the changes in RBC membrane and in plasma components especially modifications of plasma proteins; polyphenols can bind to plasma proteins due to their poor water solubility. Based on the bridging theory, the phenol-protein interactions presumably alter the properties of proteins leading to reduced capability to form cross links between cellular components leading to decreased aggregation.

The alcohol experiment indicated decreased erythrocyte aggregation when ethanol was added to whole blood and when alcohol was added to a suspension of erythrocytes in 3% 70 kDa dextran. This experiment presumably gave the explanation why RW and not AFRW showed the greater inhibitory effect on RBC aggregation. Decreased aggregation in plasma may be partially due to the ethanol-induced echinocytic shape transformation and to alteration of plasma proteins that promote aggregation. Reduced RBC deformability also tends to reduce aggregation; however, our results indicate an increased cellular deformability. It seems most likely that ethanol-induced changes of the RBC glycocalyx are involved. Based on the depletion layer model for aggregation, the scale of a protein or polymer depletion zone near the membrane depends strongly on the ability of the macromolecule to penetrate the glycocalyx; increased penetration would reduce aggregation. It is interesting to note, that this presumed change of glycocalyx properties is irreversible, since reduced RBC aggregation was observed for cells incubated with ethanol but suspended in ethanol-free dextran.

10. Conclusion

Our investigations proved that AFRW can protect erythrocytes and preserve their deformability from oxidative stress mediated impairment, while RW had no such effect. Both RW and AFRW reduce RBC aggregation although RW is the more potent inhibitor. Furthermore, ethanol reversibly improves erythrocyte deformability and irreversibly decreases RBC aggregation. The presence of ethanol enhances the oxidative stress induced RBC deformability impairment and improves the deformability of the previously damaged cells.

It is important to note that the cardiovascular risk reduction associated with moderate red wine drinking is most likely related to the combined beneficial effects of red wine components. In our opinion, the found beneficial hemorheological changes enhance the tissue perfusion and may play a role in the cardiovascular protective effects of moderate red wine consumption.

V. Analysis of light scattering by red blood cells in ektacytometry

1. Introduction

RBC deformability is the ability of erythrocytes to deform in response to mechanical forces which is essentially required for traversing capillaries. Pathological red blood cell deformability can be seen in several disease states especially in sickle cell disease (SCD) characterized as a genetic disorder due to an amino acid substitution (valine for glutamic acid) at the 6^{th} position in the β-globin chain forming hemoglobin S (HbSS).

At low oxygen tension HbSS starts polymerizing leading to increased intracellular viscosity and diminished erythrocyte deformability with the typical distorted and elongated cell shape. Blood of patient with SCD contains different sub-populations of erythrocytes including well-deforming discocytes, fairly rigid sickled cells (reversibly sickled cells (RSC)) and not deformable erythrocytes (irreversibly sickled cells (ISC)).

Based on the general agreement, irreversibly sickled erythrocytes can be recognized with microscopic analysis because their length is twice as much as their width (Fig. 2). These rigid cells are fragile causing continuous hemolysis and anemia. Furthermore, sickled cells are also responsible for other main symptoms of SCD including capillary occlusions, painful crisis, infarctions of different organs and increased blood flow resistance in the lungs.

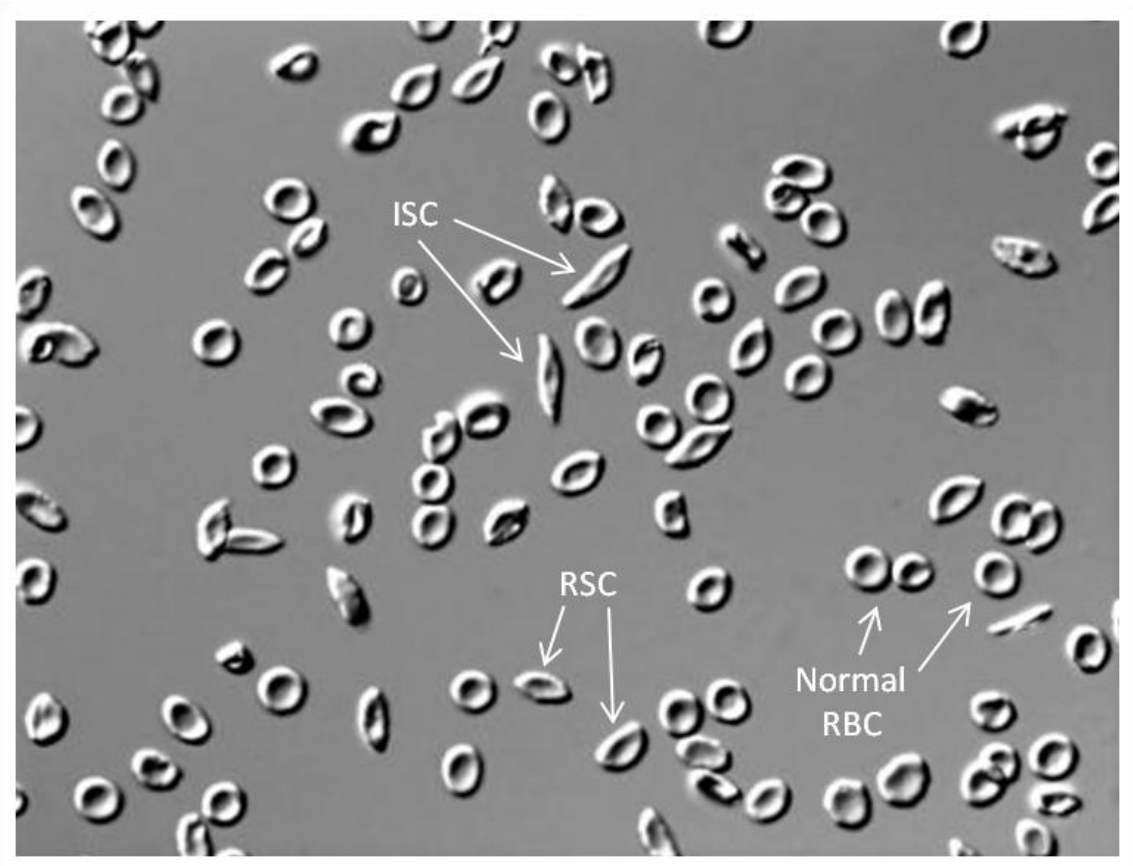

Fig. 2. *Different shapes of erythrocytes (i.e., normal RBC, reversibly (RSC) and irreversibly (ISC) distorted sickled cells) in blood obtained from a patient with sickle cell disease.*

2. Previous analysis of diffraction patterns

The technique of ektacytometry has already been described in detail. In brief, it analyzes the laser diffraction patterns of red blood cells subjected to shear stress while suspended in a fluid. At low shear stress the essentially circular cells generate a circular diffraction pattern, while the increasing stress forces the cells to progressively deform into ellipsoidal shapes and thereby generate elliptical diffraction patterns (Fig. 3).

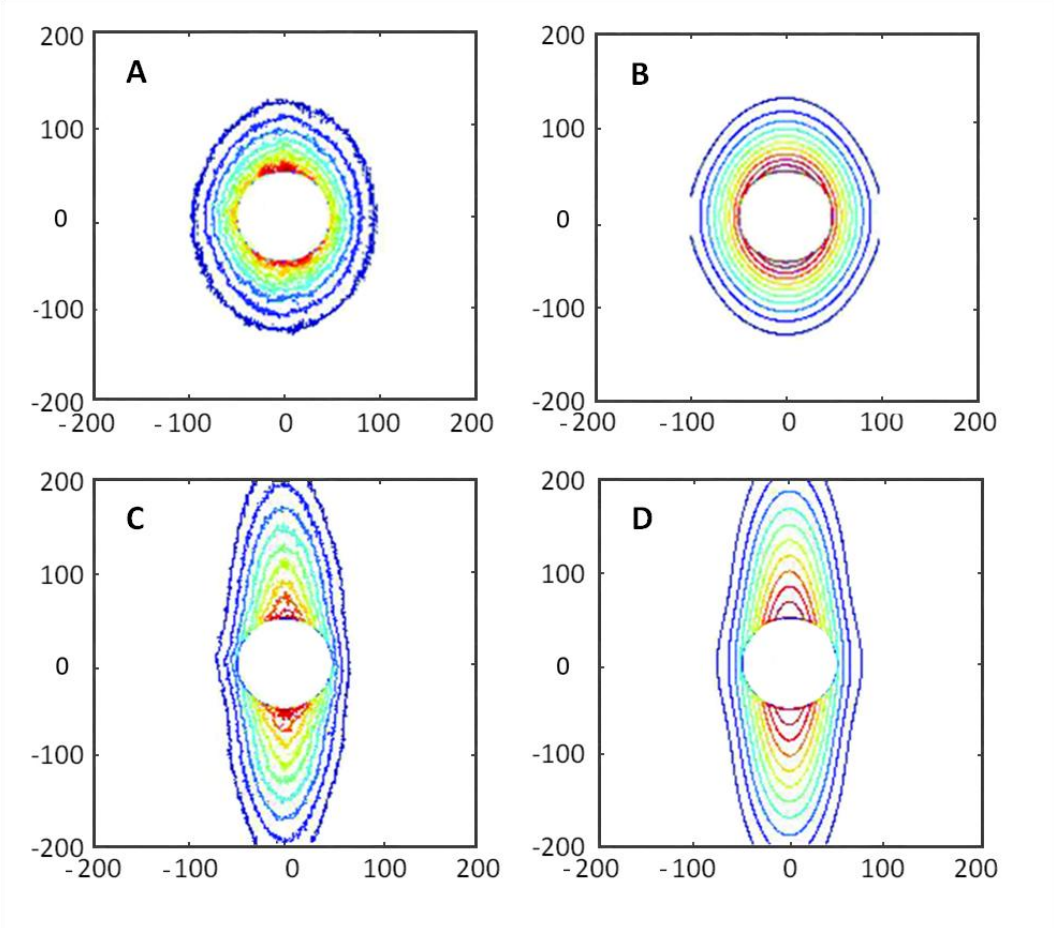

Fig. 3. *Contour plots of intensity diffraction patterns of a mixture of 100% normal red blood cells. Low shear stress (0.5 Pa):* ***A)*** *Observed diffraction pattern.* ***B)*** *Best fit of that diffraction pattern using a Bessel function. High shear stress (50 Pa):* ***C)*** *Observed diffraction pattern.* ***D)*** *Best fit of that diffraction pattern using an anomalous diffraction function.*

For analyzing the laser diffraction patterns, ektacytometry takes a single level slice through the measured laser intensity pattern and fits the resulting contour to an ellipse. If the major and minor axes of the fitted ellipse have lengths "*a"* and "*b"* respectively, then for each shear stress an elongation index; EI = (*a* - *b*) / (*a* + *b*) can be assigned to the cells.

However, in patients with sickle cell disease, the red blood cells are a mixture of normal cells together with a sub-population of poorly deformable sickle cells. With such blood, the resulting laser diffraction pattern is a weighted average of the diffraction pattern of rigid, non-deforming cells together with the normally-deforming cells.

Under increasing shear stress, normal cells progressively deform and yield elliptical diffraction curves, while the poorly deformable cells exhibit rigid body rotation and consequently produce an essentially circular diffraction pattern. The combined diffraction pattern of these two kinds of cells has a cross-like appearance; it is a distorted ellipse with a bump at its center (Fig. 4). Applying the commercial ellipse-fitting routines to such patterns yields incorrect values for the elongation index.

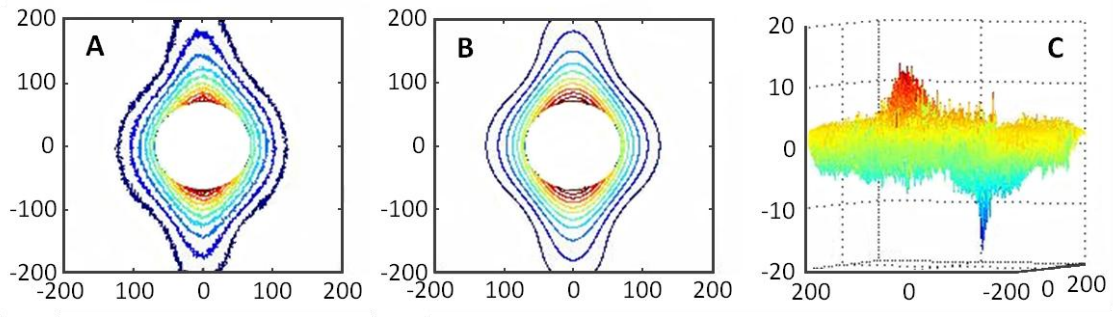

Fig. 4. *Contour plots of intensity diffraction patterns of a mixture of 70% normal + 30% rigid red blood cells.* ***A)*** *Measured diffraction pattern at high shear stress (50 Pa).* ***B)*** *Best fit to the measured diffraction pattern.* ***C)*** *Digital difference between the two patterns. Note the bumps in the center of the diffraction patterns due to the presence of the rigid cells.*

In this study, a new method is described to analyze the diffraction patterns produced by a sickle cell blood model; a mixture of normal and abnormal red blood cells. The method relies on global curve fitting, in which a series of diffraction patterns taken at different shear stresses are analyzed simultaneously.

3. Theory

An incident laser beam diffracts from the cell and travels to a distant screen whose *x-y* plane is perpendicular to the direction of the incident laser beam and is located at a distance *z* from the scattering cell. The intensity of the diffraction pattern observed on the screen is:

$$I(x,y,z) = \left(\frac{I_0}{k^2 r^2}\right)\left|S(x,y,z)\right|^2 \qquad \text{Eq. 1}$$

Here I_o is the laser intensity, $r = (x^2 + y^2 + z^2)^{1/2}$ is the distance from the red blood cell to any point (x, y, z) on the viewing screen, and k is the wavevector of the laser light in air.

The scattering function $S(x, y, z)$ and the diffraction pattern produced depends on the shape of the cell and varies with shear stress. At zero shear stress a RBC is a biconcave disc. It is assumed, that *at low shear stress* a normal RBC transforms from a biconcave disc into an elliptical disc having major and minor diameters a and b respectively, and having a uniform thickness, c (resembling a stretched hockey puck). In this case, the scattering function can be evaluated using Bessel function of the first kind.

It is assumed, that *at high shear stress* the red blood cell no longer resembles a disc of a uniform thickness and instead is modeled by an ellipsoid with axis diameters $a > b > c$. The resulting scattering function is a so-called "anomalous" diffraction pattern.

On the other hand, combinations of cells create sums of diffraction patterns. In general, when a laser beam passes through a sample containing both rigid and deformable cells, the observed laser diffraction pattern is the incoherent sum of two scattered light waves. In the present study, a least-squares fit of this composite calculated function was performed to the *entire* measured diffraction pattern.

4. Global computer fits of observed diffraction patterns

At low shear stress values, both the normal and the rigid cells are described as discs of uniform thickness, and the fitting Bessel-function is proportional to the projected shadow area of the normal and of the rigid cells. In this case, there are seven adjustable parameters as well:

q1 - concentration of normal red blood cells
q2 - mean diameter of normal red blood cells
q3 - ratio of minor to major axes of normal red blood cells
q4 - concentration of rigid red blood cells
q5 - mean diameter of rigid red blood cells
q6 - ratio of minor to major axes of rigid red blood cells
q7 - uniform background of the photodetector

However, since the volume of a red blood cell as well as its surface area do not change as the cell is stretched, the thickness of the red blood cell can be computed if the cell's eccentricity and mean diameter are known; ~100 μm^3 and ~140 μm^2 for the fixed values of the cell's volume and surface area were used, respectively.

At high shear stress values, the normal cells are ellipsoids, while the rigid cells remain discs. The form of the fitting function is then a mixture of "anomalous" and Bessel functions, so the intensity of the light is determined by not only the length of minor and major axes but by the thickness of the cell as well, which changes with applied shear stress. However, as in the case of the discs, the area and the volume of the cell remain constant under shear, and the thickness *c* can be calculated from the other two dimensions of the ellipsoid.

In principle, five of the above seven parameters should not vary with shear stress. In particular, the concentration of normal cells (q1), the concentration (q4), mean diameter (q5), and axes ratio (q6) of rigid cell as well as the background counts of the photodetector (q7) should all stay fixed as the shear stress is varied. This condition was employed by requiring that all of the fitting parameters except for the mean diameter (q2) and the axes ratio (q3) of normal cells maintain fixed values for *all* shear stress, and then minimize the *global* sum of the least-squares differences for *all nine shear stresses simultaneously*. Thus, the minimized following global sum over all nine shear stresses:

$$Global\ Sum = \sum_{\substack{shear\ stresses \\ j=1}}^{9} \sum_{\substack{all\ pixels \\ (x,y)}} \left[I^{j}{}_{measured}(x,y) - I^{j}{}_{computed}(x,y) \right]^2 \quad \text{Eq. 2}$$

Only the two parameters, (q2) and (q3) are allowed to vary as the shear stress varies; the other five parameters are locked at their optimum values as determined by the computer. In effect, this method performed 23 parameter fit for all of the nine shear stress patterns simultaneously.

4. Methods

Venous blood samples were obtained from healthy adult subjects into Vacutainer tubes and anticoagulated with ethylenediamine-tetraacetic acid. Blood samples were centrifuged at 1,400 x g for 5 minutes. The plasma and the white cell layer were removed. Erythrocytes were washed twice with phosphate buffered saline (PBS, 290 mOsm/kg, pH = 7.4) then re-suspended in PBS.

For preparing rigid erythrocytes a dilute red blood cell/phosphate buffered saline suspension was carefully added to an equal volume of 1% glutaraldehyde (Sigma Chemical Co., St Louis, MO, USA) in PBS followed by gentle stirring for 60 minutes at room temperature. Rigid cells were washed to remove any unreacted glutaraldehyde, then re-suspended in phosphate buffered saline.

Mixtures of normal and rigid erythrocytes were prepared containing 0, 5, 10, 20, 30 and 50% rigid cells. The experimental protocol involved adjusting the normal red blood cell/phosphate buffered saline suspensions to a cell concentration equal to the rigid cell suspension using an automated hematology analyzer (Micros, Horiba-ABX, Irvine, CA, USA) to determine cell concentrations.

The above mentioned global-fitting approach was tested with a LORCA ektacytometer measuring erythrocyte deformability of rigid and normal RBC mixtures. These different cell populations were added directly to a viscous, isotonic 70 kDa dextran solution (Sigma, 297 mOsm/kg, η=31.4 mPa.s in PBS), and mixed well to obtain a uniform suspension before being measured.

Throughout the measurements, diffraction patterns of the deforming erythrocytes were captured by a video camera and digitally stored. The central region of the diffraction pattern also contains the undiffracted laser spot and so is very bright; it is physically blocked by an opaque dot leading to a “hole” in the middle of the diffraction pattern (Figs. 3 and 4). For each sample, 10 patterns were digitally averaged at each shear stress and used for further processing of global parameter fitting.

5. Results and Discussion

Throughout the erythrocyte deformability measurements, nine different shear stresses from 0.5 Pa to 50 Pa were used for the global fit for each RBC sample, but only the lowest and the highest shear stresses of the laser diffraction patterns generated by normal erythrocytes (0% rigid cells) are shown (Fig. 3). The seven fitting parameters (qi) are varied to minimize simultaneously the least-square difference between the nine measured diffraction patterns and the calculated patterns.

Data of red blood cell samples containing 30% rigid and 70% normal erythrocytes are demonstrated as well (Fig. 4).

Note, that at high shear stress the measured contours produced by this mixture are non-elliptical, however are faithfully reproduced by the calculated pattern and showed the computed elongation index for red blood cells at nine different values of applied shear stress using two different RBC samples (Fig. 5).

The EI values computed using global fits are shown, as well as the EI values obtained using the LORCA's elementary ellipse-fitting routine. For normal RBC samples the global fits and the LORCA analysis produce identical results. For the normal-plus-rigid cell population the global fits still yield the correct EI values for the normal sub-population of cells present in the sample. Note, however, that the EI curve obtained using the LORCA's ellipse fitting routine is displaced downward due to the presence of the rigid cells.

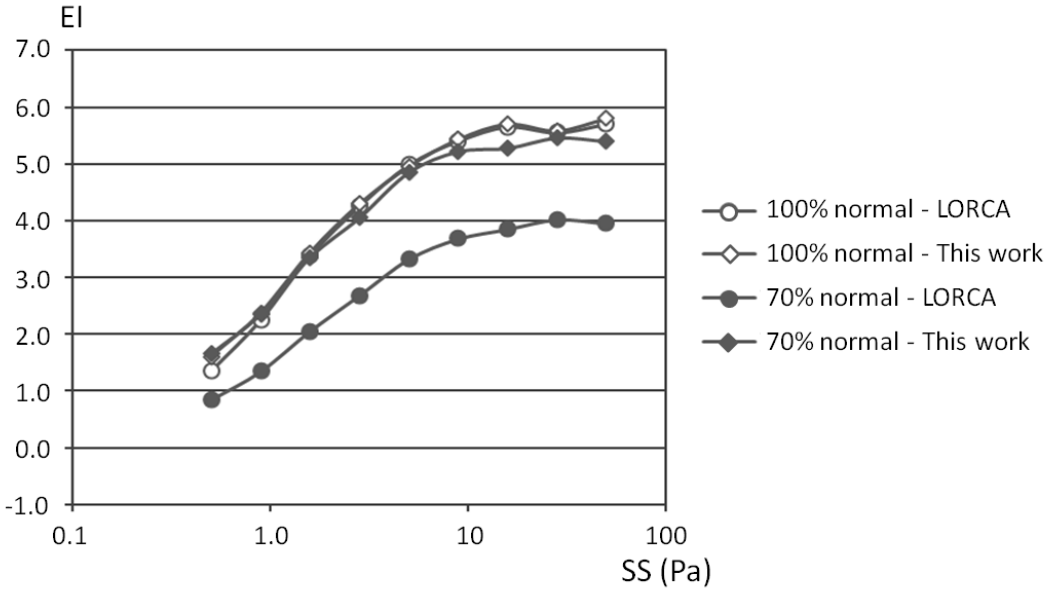

Fig. 5. *Computed Elongation Index (EI) using the LORCA's software and using the techniques presented herein. Values of two blood samples are presented here: (1) 100% normal cells shown with unfilled (open) markers. For these cells our global fits and the LORCA's ellipse-fitting routine give essentially identical EI values. (2) Mixture of 70% normal cells / 30% rigid cells. For the mixed cells, our global fits provide the correct EI of the normal cells in spite of the presence of the rigid cells. In contrast, the LORCA's ellipse-fitting routine gives EI values that are markedly reduced.*

Series of blood samples were prepared and analyzed containing different fractions of rigid cells varying between 0 and 50%. The fitting parameters (q1) and (q4) reveal the concentrations of normal and rigid cells in each sample, and the ratio q4/(q1 + q4) yields the percentage of rigid cells in each sample. A correlation between the computed fraction of rigid cells determined by the global fits and the prepared fraction of rigid cells in that sample is found (Fig. 6). At each prepared concentration, two sets of data were obtained using different apertures on the LORCA's video camera; the reproducibility of the computed results can be seen on the figure.

Although the slope of the straight-line fit is near unity, the intercept is not zero due to spillover of the un-diffracted portion of the laser beam as well as scattering from other objects. If necessary, the intercept can be brought closer to zero by excluding a larger central region of the intensity pattern from the least-square fit.

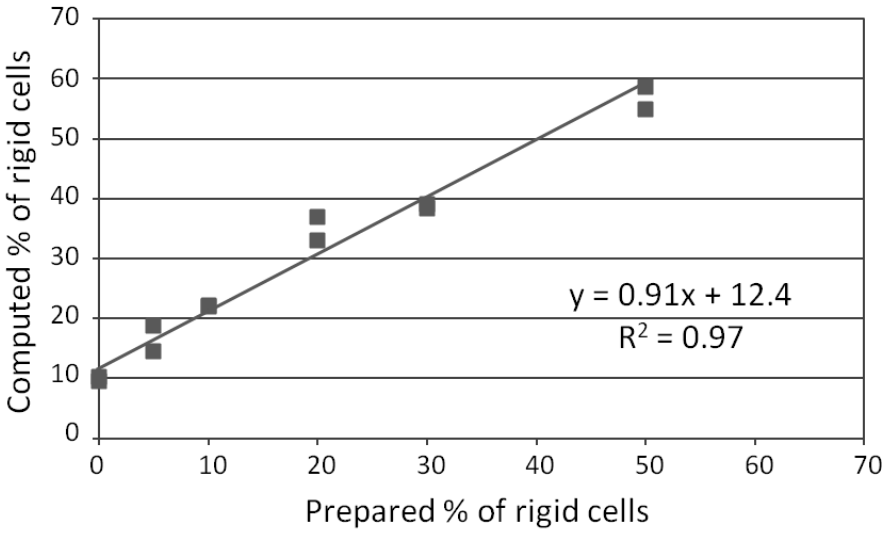

Fig. 6. *Computed fraction of rigid cells vs. the prepared fraction of rigid cells for each sample. A straight line fits to the data. The intercept of the line is not zero due to additional scattering of laser light from sources other than the rigid cells deliberately added to the sample.*

6. Future analysis of sickled blood

In sickled blood, it is expected that there is a continuous distribution in red blood cell deformability, while in this experiment only two cell populations were used. Several studies have presented that the knowledge of cell deformability has clinical value. The number of circulating irreversibly sickled cells has been confirmed to be strongly correlated with the extent of hemolysis. Furthermore, the number of rigid cells can provide information about the efficacy of therapy and may have predictive value for estimating the probability of a painful sickle crisis.

Nevertheless, our preliminary experiments with sickled blood show that our global curve-fitting technique can accurately extract the elongation index-shear stress behavior of the normally-deforming cells in the sample. It would be also desired if this technique could estimate the percentage of rigid or barely deformable sickled cells in the blood sample.

7. Conclusion

Using a combination of Bessel functions and anomalous scattering functions to simultaneously fit ektacytometry data for multiple shear stresses can reveal the elongation index of erythrocytes over the entire range of shear stresses, even in the presence of rigid, non-deformable cells (Fig. 5). In addition, this global fitting technique can yield the concentration of non-deformable cells in the sample (Fig. 6).

It is thus suggested that this technique will be useful in determining the curve of elongation index versus shear stress of the normal cells, as well as the concentration of rigid cells in mixed red blood cell populations, for example, in sickle cell disease.

VI. Summary of new scientific results

1. Effects of red wine, alcohol-free red wine extract and ethanol

[1] Our *in vitro* measurements have showed that both red wine and alcohol-free red wine extract reduce red blood cell aggregation in plasma. Red wine showed stronger inhibitory effect.

[2] Ethanol reversibly improves erythrocyte deformability and irreversibly decreases RBC aggregation.

[3] Our experiments have revealed that alcohol-free red wine extract protects erythrocytes and preserves their deformability from oxidative stress mediated impairment.

[4] The presence of ethanol enhances the oxidative stress induced erythrocyte deformability impairment and improves the deformability of the previously damaged cells.

2. Analysis of light scattering of red blood cells in ektacytometry

[1] A new theoretical analyzer model was designed for accurately examining the diffraction patterns of ektacytometry technique.

[2] It has been proven that combination of Bessel and anomalous scattering functions reveals the elongation index of the normally-deforming red blood cells over a wide range of shear stresses in the presence of non-deformable cells.

[3] Moreover, the global curve-fitting technique yields the concentration of non-deformable cells in the blood sample.

VII. List of abbreviations

AFRW	alcohol-free red wine
CVD	cardiovascular disease
EI	elongation index, parameter of LORCA ektacytometer
EI_{max}	maximum of the elongation index at infinite shear stress
HbSS	hemoglobin S
ISC	irreversibly sickled cell
LDL	low-density lipoprotein
LORCA	Laser-assisted Optical Rotational Cell Analyzer
M	erythrocyte aggregation at stasis, parameter of Myrenne
M1	erythrocyte aggregation at very low shear, parameter of Myrenne
NO	nitric-oxide
PBS	phosphate buffered saline
PMS	phenazine methosulfate
RBC	red blood cell
RSC	reversibly sickled cell
RW	red wine
SCD	sickle cell disease
SS	shear stress
$SS_{1/2}$	shear stress required for the one-half of the maximal elongation

VIII. Acknowledgement

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IX. Publications of the author

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Cumulative impact factor: 33.051

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