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Altered red blood cell deformability—A novel hypothesis for retinal microangiopathy in diabetic retinopathy

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Abstract

Purpose: Impaired red blood cell (RBC) deformability impedes tissue perfusion. This study aims to investigate RBC biomechanics in type 2 diabetes mellitus (DM) patients with different grades of diabetic retinopathy (DR) and to correlate RBC deformability with hematological and serum biochemical markers.

Methods: This cross-sectional study included 86 type 2 DM patients (31 with no DR, 31 with non-proliferative DR [NPDR] and 24 with proliferative DR [PDR]) and 32 control subjects. RBC deformability was measured by a microfluidic cross-slot channel (elongation index, EI). Venous blood samples were taken for assessment of hematological and serum biochemical markers.

Results: RBC deformability showed significant reduction in diabetic patients, being lowest in the PDR group, followed by NPDR and DM with no DR groups, and highest in control group ($P = .018$). RBC deformability was not affected by age or gender but showed significant associations with certain hematological and serum biochemical markers. In the regression analysis controlling for DM status, urea concentration and reticulocyte count were shown to be negatively associated with EI.

Conclusion: Impaired RBC deformability measured by a microfluidic cross-slot channel in DM patients with different grades of DR underscores the contribution of RBC rheological properties to the pathogenesis and progression of DM related microangiopathy.

1 | INTRODUCTION

Type 2 diabetes mellitus (DM), one of the most prevalent metabolic disorders characterized by hyperglycemia, can result in macrovascular complications (eg, coronary artery disease, cerebrovascular disease and peripheral arterial disease) as well as microvascular angiopathies (eg, diabetic retinopathy, diabetic nephropathy and peripheral neuropathy[1,2]).

Diabetic retinopathy (DR) is the leading cause of vision loss in the working age population in the developed world, which results in significant socioeconomic burden.[3] Depending on the severity of retinal changes, it can be classified into non-proliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR).[4]

The pathogenesis of diabetic retinopathy is related to tissue ischemia and hypoxia, resulting in microvascular hyperpermeability

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and abnormal vessel growth.[5,6] The underlying pathophysiological events that lead to inadequate tissue perfusion are likely multifactorial and can be divided into disturbance to the vasculature,[7] abnormal hemorheology,[8] and impaired interaction between endothelial cells and blood cells.[9,10] While changes to the retinal vasculature can be directly visualized and have therefore been well studied,[11,12] the latter two are often overlooked in the past but are receiving more attention nowadays as new techniques for assessment are developed.

Hemorheology is the study of blood flow in vessels with particular attention to the behavior of red blood cells (RBC) or erythrocytes as they are the main carriers for oxygen and carbon dioxide and represents metabolically significant blood flow.[13,14] One essential rheological property of the RBC is its deformability, which is its ability to undergo cellular deformation under mechanical loading, allowing RBCs to pass through capillaries with a smaller diameter than itself and to minimize the resistance to flow at high shear rates in arteries.[15,16]

Impaired RBC deformability leads to disrupted perfusion at tissue level, and this is well documented in diseases that primarily affect RBCs (eg, malaria,[17] sickle cells anemia,[18] and hereditary RBC membrane disorders[19]). Studies on RBC deformability in DR patients are sparse in current literature despite the prevalence of DM and DR. A few reports have shown reduced RBC deformability in DR patient but their methodology varied and some of the techniques were labor intensive and difficult to replicate.[20-24] This necessitates the use of simpler and higher-throughput systems in order to collect larger sample sizes for more conclusive outcomes. Extensional rheometry using a microfluidic cross-slot channel is a relatively new technique for the assessment of single cell RBC deformability.[25] This technique allows for bulk sample processing and measurement, which is ideal for large clinical studies.

In this study, we aim to investigate the differences in RBC deformability between type 2 DM patients with different grades of DR and control subjects using high throughput extensional rheometry, and to correlate RBC deformability with hematological and serum biochemical markers.

2 | METHODOLOGY

This is a cross-sectional study conducted in an ophthalmology department from a tertiary hospital in Singapore. Informed consent was obtained from all participants. This study was approved by the institutional review board and adhered to the tenets of the declaration of Helsinki.

Study participants were recruited from June 2017 to October 2018. Patients with type 2 DM were previously diagnosed by endocrinologists or family physicians based on established criteria: fasting venous glucose ≥7.0 mmol/L and/or 2-hour glucose in oral glucose tolerance test ≥11.1 mmol/L.[26] Diagnosis of diabetic retinopathy was in accordance with the Early Treatment of Diabetic Retinopathy Study (ETDRS) and categorized into NPDR and PDR.[4] All study participants were at least 21 years of age. Exclusion criteria included subjects on anti-inflammatory or immunosuppressive treatment or on systemic medications affecting blood rheology, such as anticoagulants, anti-platelet agents, drugs affecting rheology of the blood (eg, pentoxifylline). Subjects with history of hypertension, hyperlipidemia, or chronic smoking were excluded from the control group. Baseline demographic information was collected. Past medical and ocular history of study participants was recorded.

2.1 | Collection of blood samples

Blood samples were collected using a vacutainer (Becton Dickinson). A tourniquet was briefly applied to locate the antecubital vein, and 30ml of blood was collected in different tubes based on the specific blood tests. A full blood count including hemoglobin, hematocrit, RBC count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cells distribution width (RDW), platelet count, mean platelet volume (MPV), white blood cell (WBC) count, neutrophil count, lymphocytes count, monocytes count, basophils, eosinophils, reticulocyte (% and absolute), erythrocyte sedimentation rate (ESR) was performed. Renal and liver function was assessed based on serum sodium, potassium, chloride, bicarbonate, urea, creatinine, estimated glomerular filtration rate, bilirubin, alkaline phosphatase, alanine transaminase, aspartate transaminase, total protein, albumin, globulin, C-reactive protein (CRP), random blood glucose, and hemoglobin A1c (HbA1c). Additional measurements included a lipid panel comprising total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides, and fibrinogen.

2.2 | Sample preparation

Blood samples were transported to the secondary study site via a cold chain at 4°C. Blood samples were fractionated by centrifugation at 2500 *g* for 10 minutes. The plasma and buffy coat layers were aspirated and discarded. The RBC pellet was then washed three times with phosphate-buffered saline (PBS, Biowest) by centrifugation at 2500 *g* for 10 minutes prior to use in the cross-slot experiments.

2.3 | Cross-slot microfluidic measurement of single RBC deformability

Deformation of RBCs under extensional flows is well documented in the literature.[25] Of these, stretching of RBCs in a hyperbolic cross-slot microfluidic channel allows for contactless measurement of single cell deformability at high throughput.[27-29] In brief, hydrodynamic focusing using a viscoelastic suspending medium is used to initialize all cells to the centerline of the channel. At the cross-slot region, a

counter-opposing flow generates a large extensional flow field at the stagnation point. This causes RBCs entering this stagnation point to be stretched uniaxially, from which the individual cell deformability may be calculated based on the resultant cell geometry:

$$\text{Elongation Index (EI)} = \frac{l}{l_0} \quad (1)$$

where l and l_0 refer to the stretched (stagnation point) and initial lateral widths of the RBCs respectively.

We adopted the protocol described by Cha et. al.[27] Washed RBCs were resuspended in a viscoelastic solution containing 6.8 wt % of polyvinylpyrrolidone (Sigma Aldrich, molecular weight = 360 000 g/mol) dissolved in 1X PBS (Biowest) at a hematocrit of 0.5%. The microfluidic channels were flushed with 1% bovine serum albumin (Sigma Aldrich) prior to use to minimize cell adhesion. The cell suspension was flowed through the channel at a flow rate of 1 μL/hour using a volumetric syringe pump (Harvard Apparatus) and imaged at 40× magnification using an inverted microscope (Olympus IX71, Olympus) fitted with a high-speed video camera (Photron). Image processing and EI measurements were performed using a custom MATLAB algorithm. The extensional stress was estimated to be 16 Pa based on previous simulations.[30] For each blood sample, a minimum sample size of 100 cells was collected. EI histograms were plotted for each sample and fitted with a Gaussian curve by non-linear regression, from which the goodness-of-fit was quantified by the R^2 parameter.

2.4 | Microchannel fabrication

The microchannels were fabricated using polydimethylsiloxane (PDMS) (Dow Corning) by the standard photolithography and replica molding procedures. SU-8 2075 negative photoresist (MicroChem) was spin-coated onto a polished silicon wafer to the desired thickness, after which the mold was subjected to subsequent soft baking, UV exposure, and postbaking. SU-8 developer (MicroChem) was then used to remove the un-crosslinked photoresist. To fabricate the microchannels, PDMS prepolymer and curing agent were mixed at a 10:1 ratio (w/w) and poured onto the silicon mold before degassing and baking for 2 hours at 70°C. The PDMS microchannels were then peeled off from the mold, and inlet and outlet ports were punched using a 1.5 mm biopsy punch. The microchannels were irreversibly bonded to microscope glass slides by oxygen plasma treatment. The device had a width and height of 50 μm throughout.

2.5 | Statistical analysis

Qualitative variables were expressed as numbers and percentages, while quantitative variables were expressed as mean and standard deviation (SD) if they followed a normal distribution or as median values (range) if not. The comparison of demographic, hematological and biochemical variables between the control group and study group was performed using Student's *t*-test for continuous parameters and chi-square test for categorical parameters. Univariate regression analysis was performed with the RBC deformability index as the dependent variable and each clinical variable as independent. The comparison of RBC deformability index across control and DR categories was performed using one-way analysis of variance (ANOVA).

All the analyses were performed using SPSS ver 20.0 (IBM Corp.), and the statistical significance was tested at 5% level.

3 | RESULTS

Eighty-six (86) patients with type 2 DM and 32 control subjects were included in this study (Figure 1 and Table 1). Of all the diabetic patients, 31 patients did not show signs of DR, 31 patients had NPDR, and 24 patients had PDR. Diabetic patients were older compared to control subjects (58.95 ± 8.92 years vs

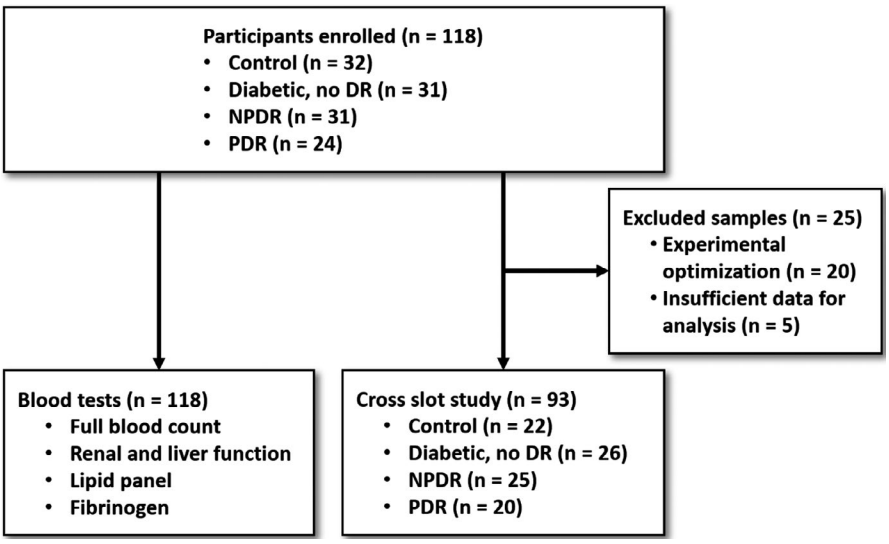


FIGURE 1 Summary of the participant enrolment and study components

TABLE 1 Descriptive statistics for demographic parameters in control and diabetes mellitus (DM) groups (n = 118)

Parameters	Group		*P*-value
	Control (n = 32)	DM (n = 86)	
Age (in years)			
Mean ± SD	50.28 ± 12.21	58.95 ± 8.92	.0007 (S)[a]
Minimum	22.00	34.00	
Maximum	69.00	78.00	
Gender			
Male	12 (17.91)	55 (82.09)	.009 (S)[b]
Female	20 (39.22)	31 (60.78)	
Total	32 (27.12)	86 (72.88)	

Note: Abbreviations: S, significant.

[a]Obtained using Student's t-test.

[b]Obtained using Pearson's Chi-square test.

50.28 ± 12.21 years, $P = .0007$). There were more males in the DM group and more females in the control group, and this difference was statistically significant ($P = .009$; Table 1). However, both age and gender were not significantly associated with RBC deformability indices (Table S1).

The DM and control groups differed in the following hematological and biochemical markers in the blood (Table 2). As expected, random blood glucose and HbA1c were both significantly higher in the DM group, each with *P*-values < .0001. Of note, the WBC count was significantly higher in DM patients (8.08 ± 2.48 vs 6.92 ± 0.97, $P = .015$). RBC count was lower in DM patients (4.56 ± 0.65 vs 5.02 ± 0.56, $P = .019$) and so were hemoglobin level (12.88 ± 1.67 vs 14.47 ± 1.58, $P = .005$) and hematocrit (39.1 ± 4.97 vs 43.24 ± 4.11, $P = .006$). Interestingly, DM patients showed significantly lower total, HDL, and LDL cholesterol levels in the blood compared to the control group (all $P < .0001$). The DM group also had lower albumin ($P = .03$), higher urea ($P = .033$), higher CRP level ($P = .044$), and higher ESR ($P = .016$). There were no statistically significant differences in the rest of the hematological and serum biochemical markers between DM and control groups.

RBC deformability was available in 93 subjects (Figure 1). Twenty-five (25) were excluded due to experimental optimization (n = 20) and insufficient data collected (n = 5). RBC *EI* from > 90% of the samples were normally distributed, with no consistent apparent subpopulations observed (Figure 2). Gaussian-fitted curves demonstrated high goodness-of-fit ($R^2 = 0.87 \pm 0.09$, 0.93 ± 0.08, 0.91 ± 0.08 and 0.93 ± 0.07 for the control, DM with no DR, NPDR and PDR groups respectively) with no significant differences between the groups ($P = .075$).

RBC deformability showed significant associations with certain hematological and serum biochemical markers (Table 3). In the univariate regression analysis controlling for DM status, serum urea concentration was negatively associated with EI ($P = .003$). In addition, reticulocyte count, both in % and absolute count, was also negatively associated with EI ($P = .041$ and .026, respectively; Table 3).

After adjusting for hematological and serum biochemical factors, RBC deformability as measured by EI showed a significant reduction in diabetic patients, being lowest in PDR group (2.43 ± 0.41), followed by NPDR (2.62 ± 0.29) and DM with no DR (2.64 ± 0.41), and highest in the control group (2.77 ± 0.27; $P = .018$; Table 4 and Figure 2C).

4 | DISCUSSION

RBC deformability, as measured by a microfluidic cross-slot platform, was compared between control subjects and DM patients with different grades of DR in the current study. RBC deformability was significantly reduced in diabetic patients. This reduction was also associated with DR severity, being significantly lower in PDR patients compared to no DR and NPDR patients.

Impaired RBC deformability was hypothesized to be a cause of microangiopathic complications of DM since the 1980s.[31] It was thought that intrinsic stiffening of RBC impeded blood flow in the microcirculation. Depending on whether autoregulatory mechanism was intact, this could either lead to vascular occlusion if there was inadequate vasodilation, or result in transudation if there was vasodilation accompanied by increased perfusion pressure. This hypothesis was backed up by early studies that showed impaired RBC deformability in DM patients using filtration[32-34] and micropipette[35] techniques. However, conflicting results did exist in literature. There was no difference found between DM and control subjects in terms of RBC deformation under shear stress in one study using a rheoscope.[36]

More recently, using a microfluidic ektacytometer, it was reported that RBC deformability was reduced in DM patients and was further decreased in DM patients with microangiopathic complications such as retinopathy and nephropathy.[23,24] Using optical tweezers stretching technique, Agrawal et al showed that RBC deformation was significantly impaired in DM and DR patients.[20] Similarly, in DM patients with other vascular complications, for example, coronary artery disease,[37] nephropathy,[38] and diabetic foot ulcer,[39] RBC deformability was reported to be impaired.

Our findings of impaired RBC deformability in DM patients with or without DR are consistent with majority of current literature and are supportive of the hypothesis that RBC rheological properties were a determinant of DM associated microangiopathy. In addition, RBC deformability appeared to be associated with severity of DR as evidenced by RBCs from PDR patients having lower deformability compared to NPDR and patients with no DR.

The mechanisms behind the impaired deformability of RBCs in diabetes is well established and has been reviewed elsewhere.[40] Briefly, hyperglycemia and oxidative stress rigidify RBCs through various parallel agencies. An increase in saturated and decrease in polyunsaturated fatty acids have been reported in the main phospholipid fractions (phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and phosphatidylethanolamine), along with increased cholesterol and sphingomyelin concentrations.[41,42] This

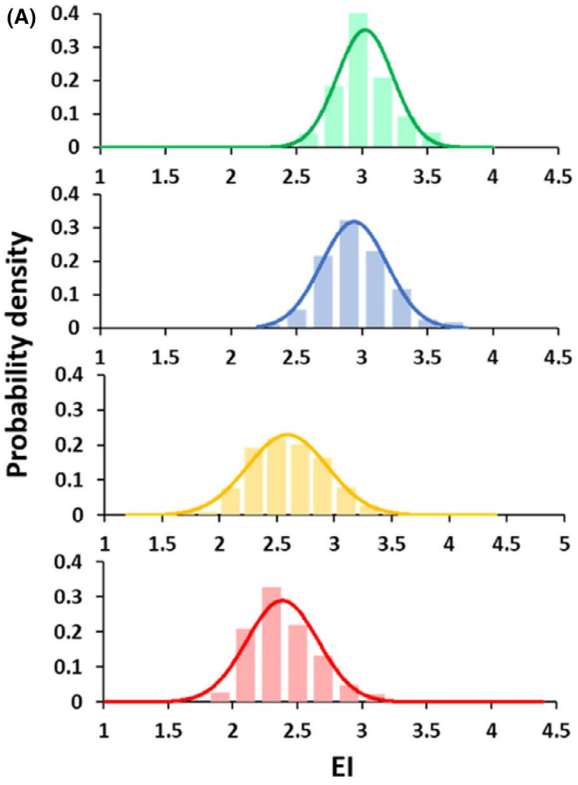
TABLE 2 Descriptive statistics for hematological and serum biochemical markers in control and diabetes mellitus (DM) groups (n = 118)

Clinical parameters	Group [Mean ± SD]		*P*-value[a]
	Control	DM	
White blood cell count (*10^9/L)	6.92 ± 0.97	8.08 ± 2.48	**.015**
Red blood cell count (*10^{12}/L)	5.02 ± 0.56	4.56 ± 0.65	**.019**
Hemoglobin (g/dL)	14.47 ± 1.58	12.88 ± 1.67	**.005**
Mean corpuscular volume (fL)	86.46 ± 6.28	86.3 ± 7.54	.943
Mean corpuscular hemoglobin (pg)	28.92 ± 2.66	28.48 ± 2.83	.616
Mean corpuscular hemoglobin concentration (g/dL)	33.38 ± 1.04	32.95 ± 0.83	.129
Hematocrit (%)	43.24 ± 4.11	39.1 ± 4.97	**.006**
Platelet count (*10^9/L)	273.38 ± 39.04	255.5 ± 82.46	.454
Mean Platelet Volume (fL)	7.98 ± 0.64	8.45 ± 0.94	.097
Red blood cell distribution width (%)	13.72 ± 1.65	14.55 ± 1.82	.151
Neutrophils (%)	55.32 ± 16.54	62.84 ± 11.04	.061
Lymphocytes (%)	28.45 ± 6.33	24.78 ± 9.23	.186
Monocytes (%)	7.68 ± 1.87	8.01 ± 2.92	.703
Eosinophils (%)	4.33 ± 3.87	3.87 ± 4	.715
Basophils (%)	0.63 ± 0.29	0.53 ± 0.35	.320
Erythrocyte sedimentation rate (mm/h)	10.01 ± 11.00	22.47 ± 20.00	**.016**
Sodium (mmol/L)	139.10 ± 1.52	138.06 ± 2.08	.081
Potassium (mmol/L)	4.14 ± 0.35	4.09 ± 0.41	.716
Urea (mmol/L)	4.48 ± 1.27	5.85 ± 3.24	**.033**
Creatinine (umol/L)	83.58 ± 12.62	96.98 ± 49.36	.089
Total protein (g/L)	72.20 ± 3.99	72.81 ± 5.64	.755
Albumin (g/L)	42.22 ± 2.82	37.94 ± 5.47	**.030**
Globulin (g/L)	30 ± 5.22	33.54 ± 5.19	.087
C-reactive protein (mg/L)	1.83 ± 2.02	11.85 ± 26.83	**.044**
Random blood glucose (mmol/L)	5.01 ± 1.17	11.03 ± 3.62	**<.0001**
HbA1C (%)	5.10 ± 1.52	8.34 ± 1.88	**<.0001**
Triglycerides (mmol/L)	1.91 ± 1.43	1.99 ± 1.28	.855
Total cholesterol (mmol/L)	5.95 ± 1.35	4.05 ± 0.87	**<.0001**
HDL cholesterol (mmol/L)	1.45 ± 0.26	1.02 ± 0.22	**<.0001**
LDL cholesterol (mmol/L)	3.82 ± 1.15	2.24 ± 0.8	**<.0001**
Fibrinogen (g/L)	3.33 ± 0.64	3.72 ± 1.09	.264
Reticulocytes (%)	1.23 ± 0.38	1.41 ± 0.59	.346
Absolute Reticulocytes (*10^9/L)	61.62 ± 19.11	65.05 ± 25.22	.673

Note: Bold *P*-value indicate statistical significance.

[a]Obtained using Student's t-test.

altered membrane lipid profile has been correlated to reduced RBC deformability.[43,44] Elevated oxidative stress and augmented generation of reactive oxygen species due to glucose and fatty acid accumulation within muscle, adipose tissue and pancreatic cells is typically associated with DM.[45] In RBCs, this manifests in increased lipid peroxidation,[46] and decreased antioxidizing glutathione[47] and membrane thiol groups.[48] Oxidative damage also extends to the skeletal proteins, with direct implications on cell deformability. Beta-spectrin, ankyrin, and protein 4.2 have been shown to be profoundly glycosylated while amino acid analysis revealed significant oxidative damage of spectrin.[49] These have also been linked to increased echinocytosis due to altered protein folding and spectrin-hemoglobin cross-links.[50] RBC ionic imbalances have also been observed in diabetic patients. Reduced functionality of Na/K-ATPase and Ca-pumping ATPase has been reported to be more pronounced in retinopathy and neuropathy groups.[51] Apart from impairing ionic homeostasis and regulation of cell volume,[52] this has also been linked to an accumulation of cytoplasmic Ca^{2+}, which was associated with increased cross-linking of membrane proteins.[53]



(B)

Parameter	Group				P-value
	Control	DM no DR	NPDR	PDR	
R^2	0.87 ± 0.09	0.93 ± 0.08	0.91 ± 0.08	0.93 ± 0.07	0.075

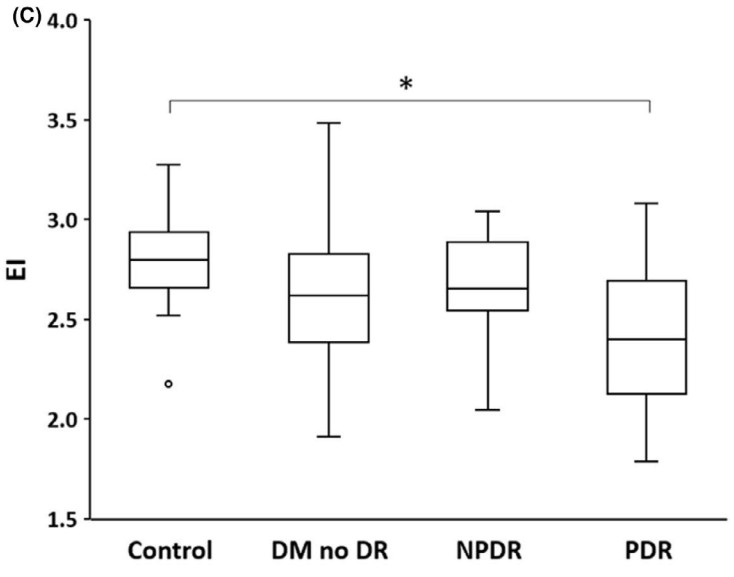


FIGURE 2 Distribution of red blood cell (RBC) elongation index (EI) in study groups. A, Representative histograms of the measured RBC EI with a Gaussian envelope (green: control; blue: DM, no DR; orange: NPDR; red: PDR). >90% of the samples produced unimodal Gaussian distributions. B, Mean and SD of the R2 of the unimodal Gaussian-fitted curves from each patient cohort. One-way ANOVA revealed no significant differences between the groups. C, Box plot of the mean EI calculated for each sample

The observation that PDR patients have more impaired RBC deformability compared to DM patient with no DR or NPDR might reflect a more pronounced disturbance to retinal hemodynamics in PDR. Reduced RBC deformability, along with abnormal microvasculature and endothelial dysfuntion, likely exert a synergistic effect that severely hamper retinal perfusion and trigger a cascade of events that lead to neovascularization.

How impaired RBC deformability affects blood flow can be explained from basic principles governing fluid mechanics. The assumption of Poiseuille flow is often adopted by researchers studying hemodynamics.[54] The resistance to flow R, a ratio of pressure gradient Δp to flow rate Q, is given by.

$$R = \frac{\Delta p}{Q} = \frac{128\mu L}{\pi D^4} \quad (2)$$

where μ is the viscosity of the fluid, L and D are length and diameter of the vessel.[54] As blood is comprised of both cells and plasma, its viscosity varies depending on local RBC concentration and shear rate, which are in turn influenced by RBC deformability and aggregation.[55] Therefore, similar to the effect of reduced vessel caliber, higher blood viscosity contributed to by impaired RBC deformability would increase local resistance to blood flow, leading to the downstream cascade of events that result in diabetic microangiopathy. The direct relation between RBC deformability and the microcirculation impairment has been alluded to in several studies,[20,56-58] although there has only been a handful of empirical evidence of this. Sosa et. al. demonstrated ~ 15% reduction in perfusion with reduction in RBC deformability by utilizing an artificial microvascular network.[58] More recently, Barshstein et. al. measured skin blood flow in patients who received transfusions of packed RBCs and observed a correlation between the skin blood flow and the deformability of the packed RBCs.[59]

Impaired RBC deformability in patients with DR as shown in our study might also add to existing knowledge on the differences in retinal blood flow measured by conventional fluorescein angiography (FA) and the newer optical coherence tomography angiography (OCTA). It was observed that OCTA often demonstrated areas of capillary non-perfusion that were not appreciable in FA in retinal vascular disorders.[60,61] It was assumed that FA was the gold standard and that OCTA overestimated the area of retinal non-perfusion because slow flow was commonly misinterpreted as non-perfusion. Our results, however, suggest an alternative explanation. As RBC deformability was decreased in DR, the transit of RBC through certain capillary plexus was slowed or absent but the flow of plasma was still intact. As a result, these areas would show up as non-perfusion in OCTA but normal in FA. Because RBCs are the main carrier of oxygen and carbon dioxide, the non-perfused area on OCTA would actually be the true metabolically hypoxic region. In this regard, the

TABLE 3 Correlation analysis between hematological, serum biochemical markers and erythrocyte deformability index measured by cross-slot microfluidics (n = 93)

Clinical parameters	Coefficient[a]; *P*-value
White blood cell count (*10^9/L)	0.0241; .865
Red blood cell count (*10^{12}/L)	−0.0269; .850
Hemoglobin (g/dL)	−0.0209; .883
Mean corpuscular volume (fL)	−0.0054; .969
Mean corpuscular hemoglobin (pg)	−0.0268; .850
Mean corpuscular hemoglobin concentration (g/dL)	0.1217; .390
Hematocrit (%)	−0.0399; .778
Platelet count (*10^9/L)	0.2191; .119
Mean Platelet Volume (fL)	−0.1092; .441
Red blood cell distribution width (%)	−0.0965; .496
Neutrophils (%)	−0.0037; .979
Lymphocytes (%)	0.0645; .649
Monocytes (%)	−0.1281; .366
Eosinophils (%)	−0.1092; .441
Basophils (%)	−0.2078; .139
Erythrocyte sedimentation rate (mm/h)	−0.0987; .534
Sodium (mmol/L)	0.0476; .730
Potassium (mmol/L)	0.0332; .808
Urea (mmol/L)	−0.4099; **.003**
Creatinine (umol/L)	−0.2081; .124
Total protein (g/L)	−0.3033; .068
Albumin (g/L)	0.0037; .981
Globulin (g/L)	−0.2824; .100
C-reactive protein (mg/L)	0.1711; .298
Random blood glucose (mmol/L)	−0.1964; .155
HbA1C (%)	−0.0839; .554
Triglycerides (mmol/L)	−0.0676; .648
Total cholesterol (mmol/L)	0.1046; .479
HDL cholesterol (mmol/L)	0.2045; .163
LDL cholesterol (mmol/L)	0.2151; .161
Fibrinogen (g/L)	−0.1381; .402
Reticulocytes (%)	−0.3164; **.041**
Absolute Reticulocytes (*10^9/L)	−0.3431; **.026**

Note: *P*-values in bold indicate statistical significance.

[a]Pearson's correlation coefficient.

current acceptance of FA being the gold standard might require review and reconsideration.

Association between hematological and serum biochemical markers and RBC deformability was not systemically studied previously. Several reports had noted that RBC deformability was correlated with serum creatinine,[24] plasma glucose,[37] glycated hemoglobin,[62] and reticulocyte count.[63] Reticulocytes are immature erythrocytes which might be stiffer than their mature counterparts. Our observation of reticulocyte count being negatively associated with elongation index was in agreement with previous literature. In our study, serum urea concentration was also found to be negatively associated with RBC deformability. However, there was no previous literature reporting similar findings. The true effect of these hematological and biochemical markers on RBC deformability remain elusive and might require large population studies to fully elucidate.

Our study has a few limitations. Firstly, there were differences in the age and gender between control and DM group. However, as mentioned earlier, both were not shown to have an effect on RBC deformability. Similarly, there was significant difference in the lipid profile between control and DM patients, with DM patients having lower total, HDL, and LDL cholesterol. Although cholesterol level was not associated with RBC deformability in the current study, it is one of the components of cell membrane and has been correlated with RBC rheology changes in previous studies.[64] Secondly, the microfluidic approach used in our study evaluated RBC deformability in an artificial microenvironment. The stress profile imposed on the cells may not accurately mimic actual in vivo conditions. However, this technique has the advantage of measuring RBC extensional deformability in a contactless manner under flow, and the flow rate used subjected the RBCs to a shear stress level within the physiological range. Lastly, the sample size might have been inadequate for subgroup analysis and association between hematological and serum biochemical markers and RBC deformability due to the effect of confounding factors; follow-up studies should consider larger cohorts in order to glean deeper correlations.

In conclusion, we have shown impaired RBC deformability in DM patients with different grades of DR. Our results underscore the contribution of RBC rheological properties to the pathogenesis and progression of DM related microangiopathy. This might shine light on novel diagnostic and therapeutic interventions in the management of DR based on RBC hemorheology. Future studies with larger sample sizes and a combination of markers of vasculature,

TABLE 4 Comparison of erythrocyte deformability index among control and diabetes mellitus (DM) subgroups

	Group				
Parameter	Control	DM no DR	NPDR	PDR	*P*-value[a]
Elongation index	2.77 ± 0.27[a]	2.64 ± 0.38[a,b]	2.62 ± 0.29[a,b]	2.43 ± 0.41[b]	.018

Note: Abbreviations: DR, diabetic retinopathy; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

[a]Using one-way analysis of variance; Superscripts across groups with same alphabet imply statistical insignificance as per Tukey's post hoc test.

hemorheology, and endothelium-blood interaction would be required to understand the complex pathophysiology of DM related vascular complications.

PERSPECTIVES

In this study, we have shown impaired RBC deformability in DM patients with different grades of DR. Our results underscore the contribution of RBC rheological properties to the pathogenesis and progression of DM related microangiopathy. This might shine light on novel diagnostic and therapeutic interventions in the management of DR based on RBC hemorheology.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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