Changes in erythrocyte column width and cell free layer in sublingual microcirculation of patients with type 2 diabetes

Paper in preparation

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Abstract

Introduction: Perturbation of the glycocalyx is associated with an impairment of its red blood cell (RBC)-excluding properties. Spontaneous temporal variations of the microvascular RBC column width (RBCW) might reflect the level of penetration of RBCs into the glycocalyx. Evaluating microvascular alterations in subjects with increased cardiometabolic risk could therefore be a useful tool to monitor the level of microvascular disease.

Methods: Measurements of median RBCW, Perfused Boundary Region (PBR) and RBC Perfused Diameter (Dperf) were performed by SDF imaging in 20 non-smoking, male patients with diabetes mellitus type 2 without signs of macrovascular disease, age 56 ± 6. Subjects were divided in two groups: DM2 (N=11; without microalbuminuria) and DM2MA (N=9; with microalbuminuria) and compared to a control group of 14 normoglycemic, non-smoking, age-matched healthy males. Reproducibility data was acquired by performing SDF imaging on two separate days in 14 healthy young subjects, age 28.6 ± 1.7.

Results: Average values of RBCW were: YOUNG (9.58 micron; SEM 0.15 micron), OLD (9.85 micron; SEM 0.22 micron), DM2 (10.52 micron; SEM 0.22 micron; P < 0.05 vs. YOUNG and OLD), and DM2MA (10.51 micron; SEM 0.26 micron; P < 0.05 vs. YOUNG and OLD). Average values of median DPerf were: YOUNG (16.40 micron; SEM 0.35 micron), OLD (15.83 micron; SEM 0.38 micron), DM2 (16.01 micron; SEM 0.43 micron), and DM2MA (17.69 micron; SEM 0.44 micron; P < 0.05 vs. YOUNG, OLD and DM2). In addition, median PBR also showed significant changes in DM2 vs. YOUNG; P < 0.05 and DM2MA vs. OLD and DM2; both P < 0.05.

Conclusion: Median RBC column width was significantly increased without a change in Dperf in DM2 patients without microvascular complications. DM2MA patients demonstrated in addition to an increase in median RBC column width also an increase in Dperf. These findings imply that assessment of temporal variations in RBC column width can be used to detect microvascular changes in DM2, however additional research is needed in patients with increased risk for cardiovascular disease to determine the predictive value for future vascular complications.
Introduction

The microcirculation is vital to circulatory physiology, contributing to exchange of gas and fluid, delivery of nutrients, removal of waste products and defense against pathogens. Microvascular perturbation is associated with perfusion defects and, eventually, ischemia in vital organs such as heart and kidney. As a consequence, monitoring of microvascular alterations in subjects at increased cardiometabolic risk is a potentially attractive diagnostic and therapeutic tool. To date, a wide variety of non-invasive techniques to assess local organ perfusion is available, including magnetic resonance imaging (MRI), computed tomography (CT) and contrast-enhanced ultrasonography (CEUS). These techniques, however, require infusion of contrast agents or radio-isotopes, resulting in exposure to radiation (CT) and are very costly. Alternative approaches to assess microvascular properties, such as venous-occlusion plethysmography and laser Doppler fluxmetry of the skin, do not directly assess the microcirculation. In contrast, intravital microscopy does allow direct visualization of the microvasculature. For human studies, however, until recently, this technique could only be used for the assessment of nailfold and retinal perfusion.

The introduction of new intravital microscopes, such as orthogonal polarization spectral (OPS) and sidestream dark-field (SDF) imaging, has rendered additional tissues accessible for evaluation of the microcirculation, such as the sublingual or buccal mucosa. Previously, we already applied these techniques to estimate changes in microvascular dimensions of the endothelial glycocalyx in humans. Patients with type 1 diabetes mellitus (DM1), who are characterized by an approximately 50% decrease in systemic glycocalyx volume, showed a 0.4 micron reduction in the transient widening of erythrocyte column upon glycocalyx compression by passing leucocytes in the sublingual microcirculation. These observations, combined with previous experimental studies have led to the concept that perturbation of the glycocalyx is associated with an impairment of its red blood cell (RBC)-excluding properties, thereby resulting in a different distribution of RBCs close to the vessel wall. The method by which glycocalyx dimensions were estimated from the transient widening of RBC column after leucocyte passage is, however, laborious, requires blinding of data and comes with a substantial inter-observer variability.

As an alternative, we previously reported that spontaneous temporal variations in the width of the RBC column in microvessels in patients with diabetes may reflect the level of penetration of RBCs into the luminal part of the glycocalyx. In the current paper, we applied this approach to investigate whether in vivo assessment of temporal RBC column width (RBCW) variations in the human sublingual microcirculation is reproducible and able to identify microvascular alterations in patients with diabetes. To test whether the previously reported loss of glycocalyx dimension in type II diabetes is indeed associated with increases in median RBCW, we analyzed diabetic patients with and without microalbuminuria and compared them to age- and sex-matched controls.
Methods

Study population
We enrolled 8 healthy males and 6 healthy females, mean age 28.6 ± 1.7, as reference group. Reproducibility data was acquired by performing SDF imaging on two separate days in these 8 male volunteers as well as 6 healthy female volunteers. Participants did not smoke, did not use any medication, and were free from any illness, including overt cardiovascular disease. All experiments were performed after an overnight fast. In parallel, 20 non-smoking, male patients with diabetes mellitus type 2 without signs of macrovascular disease [defined as a history of myocardial infarction, stroke, peripheral vascular disease or signs of macrovascular disease at physical examination] were studied, divided in two groups: DM2 (N=11; without microalbuminuria) and DM2MA (N=9; with microalbuminuria). As a control group, 14 normoglycemic, non-smoking, age-matched healthy males were studied. The study was approved by the institutional review board of the Academic Medical Center, Amsterdam, The Netherlands, and written informed consent was obtained from all volunteers. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Microcirculatory imaging
The sidestream dark field (SDF) Microscan (MicroVision Medical Inc., Wallingford, PA, USA.) was performed as described earlier. Briefly, subjects were sitting in front of the computer screen, holding the Microscan themselves. Images were collected with a 5x objective with a 0.2 NA providing a 325-fold magnification on screen and were sized 720 x 576 pixels. The frame rate was 23/s. Video sequences of 2 seconds each were recorded using Streampix software (Norpix Inc. Montreal, Canada) in at least 10 areas close to the frenulum.

Microvascular dimensions
Figure 1A shows a typical example of a single frame of a recorded movie. Movies consisted of 40 consecutive frames of 950 µm by 700 µm sublingual tissue surface area. The first frame of each movie was used to manually identify all available microvessels and measurement lines perpendicular to the vessel direction were placed automatically every 10 µm along each visible microvessel (Fig. 1A right panel). All vessels with a diameter of 50 um and larger were excluded. Each line represented a measurement site; at each measurement site a total of 21 parallel (every ± 0.5 µm) intensity profiles was plotted (using ImageJ, National Institutes of Health, Bethesda, MD) and RBC column width (full width half maximum) was determined at each line for all 40 consecutive frames in a movie, revealing a total of 840 RBC column width measurements at a measurement site [21 profiles X 40 frames]. As can be appreciated from figure 1B, RBC column width showed considerable variation in these 40 frames. The associated (cumulative) distribution of the RBC column widths for these 840 measurements was used to determine median RBC column width (P50), as well as lower and upper percentiles of the RBC column width distribution (Fig. 1A-C)). To assess the position of the outer edge of the RBC perfused lumen at each measurement site, the RBC Perfused Diameter (Dperf) was derived from the RBC...
Changes in erythrocyte column width and cell free layer in sublingual microcirculation of patients with type 2 diabetes

column width distribution by linear extrapolation of all RBC column width percentiles between P25 and P75 (Fig. 1C). The Perfused Boundary Region (PBR) was defined as the distance of median (P50) RBC column width to the outer edge of the extrapolated Dperf. Approximately 100 – 300 measurement sites were indentified per video recording, giving approximately 1000 – 3000 measurements of median RBC column width, PBR and Perfused Diameters per patient.

![Figure 1](image)

**Figure 1.** Representative image [A – Top panel] acquired by SDF Imaging. Vessels were manually selected and measurement sites were automatically positioned at 10 micron distance along each selected vessel. At each measurement site, RBC column widths were measured at full width half maximum of multiple parallel positioned radial intensity profiles for 40 consecutive video frames [B – Middle panel]. All measurements of RBC column width at a given measurement site are presented as a cumulative frequency distribution of RBC column width values [C – Bottom panel] from which median RBC column width (RBCW), Perfused Boundary Region (PBR) and RBC Perfused Diameter (Dperf) are obtained.
Statistical analysis
All data are presented as mean ± SEM. Coefficients of variability (%CV), defined as the SD in differences between measurements as a percentage of the mean of the measurements, were used as indices of inter- and intra-observer reliabilities. Differences in median RBC column width and Perfused Diameter between groups are tested by an unpaired student’s t test (two-tailed). Since the Perfused Boundary Region has been shown to depend on vessel size, PBR data were also classified according to the RBCW P50 and tested by an unpaired T-test. A value of P<0.05 was considered statistically significant. Baseline differences between controls and patients with type 2 diabetes were tested using an unpaired T-test (two-tailed). An unpaired Mann-Whitney test (two-tailed) was used for not normally distributed values, presenting medians and [interquartile range].

Results

Baseline characteristics
Mean age of young controls was 28.6 ± 1.7. No clinical blood measurements were performed in this group. Baseline characteristics of the participants are listed in Table 1. Blood glucose levels, High Density Lipoprotein (HDL) and Triglycerides (Tg) were significantly altered in participants with DM2 compared to older controls. Participants with DM2MA also showed a significant increase in Body Mass Index (BMI) and Systolic Blood Pressure (SBP). BMI in older controls was already elevated above the normal range.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Old Controls</th>
<th>DM2</th>
<th>*</th>
<th>DM2MA</th>
<th>**</th>
<th>***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>56 ± 9</td>
<td>54 ± 6</td>
<td>p=ns</td>
<td>58 ± 5</td>
<td>p=ns</td>
<td>p=ns</td>
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<tr>
<td>BMI</td>
<td>26.3 ± 1.9</td>
<td>28.3 ± 4.9</td>
<td>p=ns</td>
<td>31.7 ± 5.2</td>
<td>p&lt;0.05</td>
<td>p=ns</td>
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<tr>
<td>SBP</td>
<td>133 ± 18</td>
<td>142 ± 14</td>
<td>p=ns</td>
<td>160 ± 21</td>
<td>p&lt;0.01</td>
<td>p=0.05</td>
</tr>
<tr>
<td>DBP</td>
<td>81 ± 8</td>
<td>88 ± 5</td>
<td>p&lt;0.05</td>
<td>90 ± 12</td>
<td>p=ns</td>
<td>p=ns</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.9 ± 0.7</td>
<td>6.9 ± 2.2</td>
<td>p&lt;0.05</td>
<td>7.8 ± 2.9</td>
<td>p&lt;0.05</td>
<td>p=ns</td>
</tr>
<tr>
<td>TC</td>
<td>5.7 ± 1.1</td>
<td>5.5 ± 0.9</td>
<td>p=ns</td>
<td>5.0 ± 1.3</td>
<td>p=ns</td>
<td>p=ns</td>
</tr>
<tr>
<td>LDL</td>
<td>3.9 ± 1.0</td>
<td>3.4 ± 0.74</td>
<td>p=ns</td>
<td>3.3 ± 1.0</td>
<td>p=ns</td>
<td>p=ns</td>
</tr>
<tr>
<td>HDL</td>
<td>1.3 ± 0.3</td>
<td>1.0 ± 0.4</td>
<td>p&lt;0.05</td>
<td>1.1 ± 0.1</td>
<td>p&lt;0.01</td>
<td>p=ns</td>
</tr>
<tr>
<td>Tg</td>
<td>1.0 [0.8-1.2]</td>
<td>1.7 [1.1-4.0]</td>
<td>p=0.01</td>
<td>1.2 [0.9-2.4]</td>
<td>p=ns</td>
<td>p=ns</td>
</tr>
</tbody>
</table>

BMI = body mass index, SBP = Systolic blood pressure, DBP = Diastolic blood pressure, TC = Total cholesterol, LDL = Low density lipoprotein, HDL = High density lipoprotein, Tg = Triglycerides. * = Old controls versus DM2, ** = Old controls versus DM2MA, *** = DM2 versus DM2MA
**Median RBC column width (RBCW)**

Figure 2A displays the pooled median RBC column width distributions for YOUNG (total number of measurements: 24,087), OLD (43,332), DM2 (17,303), DM2MA (53,706). Data are shown for the range of 4 – 18 microns to more clearly demonstrate the right shift of values compared to YOUNG for DM2 and DM2MA.

Average values of individual median RBC column widths are depicted in Figure 2B, being YOUNG (9.58 micron; SEM 0.15 micron), OLD (9.85 micron; SEM 0.22 micron), DM2 (10.52 micron; SEM 0.22 micron; P < 0.05 vs. YOUNG and OLD), and DM2MA (10.51 micron; SEM 0.26 micron; P < 0.05 vs. YOUNG and OLD).

**Figure 2.** (A – Top panel) Pooled distribution of median RBC column width measurements, depicted as a percentage of all measurements categorized in classes of 1 um. (B – Bottom panel) Median RBC column widths for individual subjects in all groups.
Perfused Boundary Region (PBR)

Figure 3A depicts the values of PBR for YOUNG, OLD, DM2 and DM2MA as function of RBC column width (RBCW) to illustrate the increases in PBR from 2 to 4 microns in vessels with RBCW $\geq 6 - 16$ microns. To compare PBR values between groups independent of the above mentioned increases in RBCW, PBR values were classified to RBCW bins for each individual subject and average values of PBR (+ SEM) per RBCW are displayed in figure 3A. PBR is significantly reduced in OLD compared to YOUNG for RBCW bins 7 – 12 microns; PBR is significantly reduced in DM2 vs. YOUNG for RBCW bins 7 – 14 microns and DM2 vs. OLD for RBCW bins 9 – 15 microns; PBR is significantly increased in DM2MA vs. DM2 in RBCW bins 8 – 16 microns.

Average values of individual median PBR values are depicted in Figure 3B, being YOUNG (3.13 micron; SEM 0.14 micron), OLD (2.83 micron; SEM 0.12 micron), DM2 (2.52 micron; SEM 0.19 micron; P < 0.05 vs. YOUNG), and DM2MA (3.34 micron; SEM 0.10 micron; P < 0.05 vs. OLD and DM2).

Figure 3. (A – Top panel) Perfused Boundary Region of all groups categorized by median RBC column width (RBCW) values. (B – Bottom panel) Median values of Perfused Boundary Regions of individual subjects for all groups.
Changes in erythrocyte column width and cell free layer in sublingual microcirculation of patients with type 2 diabetes

RBC Perfused Diameter ($D_{\text{perf}}$)

Figure 4A displays the pooled distributions of Perfused Diameters for YOUNG (total number of measurements: 24,087), OLD (43,332), DM2 (17,303), DM2MA (53,706). Data are shown for the range of 5 – 35 microns and clearly demonstrates the right shift of DM2MA values compared to YOUNG, OLD and DM2.

Average values of individual Median Perfused Diameters are depicted in Figure 4B, being YOUNG (16.40 micron; SEM 0.35 micron), OLD (15.83 micron; SEM 0.38 micron), DM2 (16.01 micron; SEM 0.43 micron), and DM2MA (17.69 micron; SEM 0.44 micron; $P < 0.05$ vs. YOUNG, OLD and DM2).

Figure 4. (A – Top panel) Pooled distribution of RBC Perfused Diameters. (B – Bottom panel) Median values of RBC Perfused Diameters of individual subjects for all groups.
Discussion

In the current paper we find a significant increase in median RBC column width without a change in Dperf in DM2 patients without microvascular complications. These findings imply a decreased dimension of the cell free layer located between the RBC column and the vessel wall. In contrast, DM2 patients with microvascular complications demonstrated an increase in both median RBC column width as well as Dperf. This indicates increased penetration of RBCs also into the cell impermeable part of the cell free layer, causing a concomitant increase in the PBR in these patients [Figure 5].

Overall, our findings imply that assessment of temporal variations in RBC column width can be used as a monitoring tool to detect pathophysiological changes in the microvasculature of patients with DM2. The predictive value of these changes for micro- and macrovascular complications in DM2, however, remains to be evaluated.

**Figure 5.** Schematic illustration of changes in median RBC column width (RBCW), Perfused Boundary Region (PBR) and RBC Perfused Diameter (Dperf) in YOUNG, OLD, DM2 and DM2MA subjects.
Methodological considerations

SDF results in images where RBCs in the microcirculation are depicted as dark globules against a white/grayish background (Figure 1). The sublingual microvascular network was well visualized by the SDF imaging (Figure 1A). The distributions of the median RBC column width and Dperf show that in the majority of vessels the RBC column is flowing through vessels with functional diameters of approximately 15 microns, comprising predominantly the larger capillaries as well as pre- and postcapillary arterioles and venules. The observed low number of small capillary sized vessels with a median RBCW of 4-6 micron indicate the limited ability of the SDF camera to image vessels of this size. To obtain data on variations in the radial distribution of the RBC, we determined RBC column width from the full width at half maximum of plotted intensity profiles. From the cumulative frequency distribution of RBC column width values, the median RBC column width (RBCW) was derived as well as the Perfused Diameter (Dperf), following extrapolation of P25 – P75 percentiles of the RBC column width distribution. PBR was defined as half the difference between Dperf and RBCW (see also Figure 1C and Figure 5). The high frequency radial excursions of the RBC column from which PBR and Dperf are derived reflect radial excursions of the flowing RBCs within the vessel lumen rather than anatomical variations in vessel wall position, since changes in vessel wall position itself have been shown to occur at a much slower rate. Hence, the PBR reflects the erythrocyte accessible part of the ‘cell free layer’ (CFL; Figure 5). The latter results from the phase separation of RBCs and plasma in the blood stream of microvessels, and is explained by the tendency of RBCs to migrate in the axial direction of the microvessel, leaving a sleeve of plasma in the zone between the RBCs and the wall. The physiological relevance of this phenomenon is attested to by its contribution to a decrease in apparent blood viscosity and local hematocrit with decreasing vessel diameter (Fahraeus-Lindqvist and Fahraeus effect, respectively). In the YOUNG group, PBR measurements were reproducible, and PBR dimensions were on average ~3 micron. They ranged between 2.5 in the small sized vessels and ~4 micron in the vessels of 12 micron and larger (Figure 4), which is in line with reported dimensions of the cell free layer in rodent microcirculation. It was recently recognized that, in addition to the time-averaged estimation of the cell free layer, detailed assessment of the temporal variations in CFL may also provide useful information on microvascular function in vivo because of its potential influence on effective blood velocity and potential for NO-scavenging. Kim and co-workers observed very rapid variations in CFL width along rat cremaster arterioles, with occasionally the RBCs column appearing to extend to the vessel wall. These observations mirror our estimations of PBR with the SDF camera and direct measurements of CFL with brightfield microscopy.

More recently, CFL formation has been suggested to be influenced by the presence of the endothelial glycocalyx on the luminal surface of the vessel wall because of the blood-excluding properties of the glycocalyx. In the original experimental studies in rodent cremaster muscle capillaries, glycocalyx dimensions of 0.5 – 1 micron have actually been derived from the distance between the RBC column width and the endothelial lining. Subsequent studies using EM and two photon and confocal laser
scanning microscopy using tracers directed at glycocalyx elements, have suggested glycocalyx dimensions of a few micrometer in large vessels \(^{15}\). In our previous approach to delineate microvascular glycocalyx dimensions in humans using sublingual imaging, we evaluated the transient widening of the erythrocyte column after leukocyte passage as a manner to compress the glycocalyx. This approach, based on changes in the median RBC width, revealed cell compressible glycocalyx dimensions of approximately 0.6 micron that were \(\approx 65\%\) of the RBC-endothelial gap in hamster cremaster capillaries \(^{16}\).

In the present study, assessment of the temporal and spatial variations in RBC column width in OLD subjects revealed that RBCs more frequently move towards the outer edge of the perfused lumen compared to the YOUNG subjects, resulting in a modest widening of the RBC column width by \(\approx 0.3 \mu m\) (Figure 2B). In contrast, median RBC column width increased by \(\approx 0.9 \mu m\) in DM patients, which is consistent with our earlier report of widening of the (median) RBC column width with a concomitant loss of dimension of the leukocyte compressible part of glycocalyx in type 1 diabetes. Widening of the median RBC column width is consistent with a reduced dimension of the most luminal part of the CFL, which includes the cell permeable part of the glycocalyx. Loss of the luminal part of the CFL and associated widening of RBCW towards the luminal surface of the vascular wall has been suggested to indicate an increased vulnerability of the vasculature to atherogenic damage, including increased thrombogenicity and inflammatory activation \(^{17}\). At the same time, RBCW widening will increase the effective microvascular blood filling \(^{6}\) and the corresponding increases in hematocrit and viscosity may impair microvascular perfusion by increases in hemodynamic resistance.

On top of the widening of the median RBC column width, DM2 patients with microalbuminuria also demonstrated an increase in RBC Perfused Diameter, consistent with increased penetration of RBCs into the cell-impermeable part of the cell free layer, which has been reported to be more tightly associated with the endothelial cell membrane. Loss of the protective barrier properties of the cell impermeable part of CFL causes an increase in the dimension of the CFL that can be penetrated by RBCs. Counter intuitively, the latter is associated with an increase in the Perfused Boundary Region in these patients compared to DM2 without microalbuminuria. In patients with microvascular complications, RBCs can penetrate deeper towards the vessel wall (Figure 4C and Figure 5). A potential explanation for this phenomenon could be that in DM2MA patients, the membrane bound tight region of the glycocalyx becomes more permeable. In support, we recently showed that administration of hyaluronidase in rats \(^{18}\) as well as inhibition of hyaluronan synthesis with 4ME in mice \(^{19}\), both leading to profound glycocalyx damage, resulted in a significant widening of the RBC Perfused Diameter, quite similar to our observation in DM2MA patients. Alternatively, widening of erythrocyte columns in DM2 may also relate to remodeling of microvascular network architecture, changes in red cell mechanical properties or rheological changes. These issues need closer attention in follow up studies.
In conclusion, automated assessment of RBC column width distribution demonstrates distinct changes in diabetic patients. Further studies are needed to determine the usefulness of this technique for the identification and monitoring of early loss of microvascular function in diabetic patients as well as other patient groups at increased cardiovascular risk.
Reference List


