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Measuring endothelial glycocalyx dimensions in humans: a potential novel tool to monitor vascular vulnerability

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Abstract

Introduction: The endothelial glycocalyx is increasingly considered as an intravascular compartment that protects the vessel wall against pathogenic insults.

Objective: The purpose of this study was to translate an established experimental method of estimating capillary glycocalyx dimension into a clinically useful tool and to assess its reproducibility in humans.

Methods: We first evaluated by intravital microscopy the relation between the distance between the endothelium and erythrocytes, as measure of glycocalyx thickness, and the transient widening of the erythrocyte column upon glycocalyx compression by passing leukocytes in hamster cremaster muscle capillaries. We subsequently assessed sublingual microvascular glycocalyx thickness in 24 healthy males using orthogonal polarization spectral imaging. In parallel, systemic glycocalyx volume (using a previously published tracer dilution technique) as well as cardiovascular risk profiles were assessed.

Results: Estimates of microvascular glycocalyx dimension from the transient erythrocyte widening correlated well with the size of the erythrocyte-endothelium gap \( (r=0.63) \). Measurements in humans were reproducible \( (0.58 \pm 0.16 \) and \( 0.53 \pm 0.15 \mu m, \text{ coefficient of variance} 15 \pm 5\%) \). In univariate analysis, microvascular glycocalyx thickness significantly correlated with systemic glycocalyx volume \( (r=0.45) \), fasting plasma glucose \( (r=0.43) \) and HDL-cholesterol \( (r=0.40) \) and correlated negatively with LDL-cholesterol \( (r=-0.41) \) as well as body mass index \( (r=-0.45) \) (all \( p<0.05 \)).

Conclusion: The dimension of the endothelial glycocalyx can be measured reproducibly in humans and is related to cardiovascular risk factors. It remains to be tested whether glycocalyx dimension can be used as an early marker of vascular damage and whether therapies aimed at glycocalyx repair can protect the vasculature against pathogenic challenges.
Introduction

Until now, cardiovascular (CV) preventive strategies have mainly focused at lowering systemic risk factors, such as dyslipidemia and increased blood pressure. Despite the success of these strategies, more than 60% of CV events cannot be prevented 1. Hence, attention has shifted towards approaches that are primarily focused at increasing the resistance of the vessel wall itself against atherogenic insults 2. In search for novel targets at the level of the vessel wall, reports on the potential role of the endothelial glycocalyx in mediating vascular protection have offered novel and exciting opportunities 3, 4.

The endothelial glycocalyx is an intraluminal layer, mainly consisting of heparan sulphate, syndecan and hyaluronan. It has emerged as a central orchestrator of vascular permeability, leukocyte and thrombocyte adhesion as well as endothelial function 5, 6. Indeed, acute damage to endothelial glycocalyx in experimental models was closely correlated to the induction of a pro-atherogenic state, such as increased influx of lipoproteins 7, increased leukocyte and thrombocyte adhesion 8, 9, as well as the induction of endothelial dysfunction 8, 10-12. Conversely, reconstitution of glycocalyx restored protective abilities of the vessel wall 8, 13. Based on these findings, it has been put forward that an intact glycocalyx may contribute to the anti-atherogenic capacity of the vessel wall 3, 4,14.

To demonstrate a potential role for the glycocalyx in human pathophysiology, it is imperative to develop techniques that can reliably quantify or visualize the glycocalyx in vivo in humans. Early studies of more than 40 years ago have already revealed that most of the glycocalyx is severely impaired following even gentle handling of vascular material 15. However, endothelial glycocalyx visualization has been successfully achieved using experimental in vivo and flow-cultured in vitro settings, using e.g. the cremaster muscle preparation in animal models or human umbilical vein endothelial cells (HUVEC) under flow 13,15,17. Recently, we have focused on the assessment of systemic glycocalyx volume in humans, using a tracer dilution technique comprising labeled erythrocytes and dextran 40 2,17,18.

Therefore, we evaluate the relation between the gap between the endothelium and erythrocytes and the change in erythrocyte width as a measure of glycocalyx thickness in hamster cremaster muscle capillaries using intravital microscopy. We subsequently assessed sublingual microvascular glycocalyx thickness as well as systemic glycocalyx volume in healthy male volunteers. Furthermore, we determined the relation between microvascular glycocalyx thickness and cardiovascular risk factors.
Materials and methods

Estimation of local microvascular glycocalyx thickness in hamsters

The width of flowing erythrocytes was measured in individual capillaries before and immediately after the passage of a leukocyte through the capillary. This method is based on the linear theory model 19, 20. In short, endothelial glycocalyx limits the proximity of erythrocytes to capillary endothelial cells 17, 21. In contrast, leukocytes, which are much more rigid, compress the capillary endothelial glycocalyx during their passage through the capillary lumen, thus allowing a transient ‘widening’ of the erythrocytes following the leukocyte passage (Figure 1a). Hence, the change in erythrocyte column diameter divided by two (referred to as delta erythrocyte width/2) is related to the dimension of the microvascular glycocalyx, that is transiently compressed by the passing leukocytes 19.

In six Syrian golden hamsters, the capillaries of the cremaster muscle were investigated under physiological conditions. Moreover, the effect of degradation of the endothelial glycocalyx by a single bolus of oxidized low-density lipoprotein (LDL)-cholesterol (dose 0.4 mg/100 g of body weight) on leukocyte passage and transient erythrocyte widening was investigated. The protocol was approved by the Institutional Review Board. Hamsters were anesthetized with intraperitoneal pentobarbital sodium (70 mg/kg body weight), and the trachea was cannulated to ensure a patent airway. The cremaster muscle was prepared as described by Vink et al. and observed with an intravital microscope (Olympus BHM) and a cooled ICCD video camera (GenIV ICCD, Princeton Instruments) 17. The tissue was transilluminated with a mercury lamp (100 W) equipped with a 435nm band pass interference filter (blue light) using an aplanat (lens free from spherical aberration), achromatic condenser set at numerical aperture (NA) 1.2 (U-AAC, Olympus). All preparations were examined with a ×60 water immersion objective lens (Olympus, UPlanApo NA 1.2W or LUMPlanFL NA 0.9 W) and a telescopic tube to give a final object-to-camera magnification of ×200. Images were displayed on a Philips CM 8833-II video monitor and recorded using a SVHS video tape recorder [JVC BR-S611E] and a time coding interface unit [JVC SA-F911E] for further off-line image analysis. Video images were digitized using a frame grabber [DT3152, PCI Local Bus] and Image-Pro Plus software [Image-Pro Plus version 3.0, Media Cybernetics, Silver Spring, PA, USA]. An onscreen caliper using a 1 mm/0.01mm stage micrometer was used for all calibrated dimensional measurements. The anatomical capillary diameter and the width of the flowing erythrocyte column were measured using digital calipers at the inside of the capillary wall to determine the dimension of the erythrocyte – endothelial cell (EEC) gap prior to, during and after spontaneous capillary leukocyte passage. Of note, the EEC gap is the gold standard to measure glycocalyx dimension in vivo 17. Finally, capillary erythrocyte velocity (µm/s) was calculated from the time required for a cell to travel a measured segment length of vessel between two fixed points as described previously 22.
Figure 1a. Determining the change in erythrocyte width in a capillary, as measure of the endothelial glycocalyx thickness. On screen image of microvascular glycocalyx thickness measurement with Image-Pro Plus.

A capillary between 3 and 7 µm is selected for analysis. Erythrocyte width is determined as line profile diameter in an image just before and after leukocyte passage at the same location. Leukocytes appear as moving blank areas in the capillary. The change in erythrocyte width divided by two provides the microvascular glycocalyx thickness. Green lines represent the calipers. On the Y-axis light intensity is plotted, on the X-axis the pixel number.

Measurements in humans

Study population

24 healthy Caucasian male volunteers, aged 18-40 years, 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. Participants did not smoke, did not use any medication and were free from any illness, including specifically cardiovascular disease. Medical history, physical examination, routine laboratory examination and electrocardiogram were normal. All experiments were performed after an overnight fast. Measurements comprised microvascular glycocalyx thickness, systemic glycocalyx volume, endothelial function as well as biochemistry. In a subgroup of 12 volunteers, reproducibility of microvascular glycocalyx thickness (selecting new set of five capillaries) and systemic glycocalyx volume was assessed by performing two measurements at separate occasions.
Estimation of local microvascular glycocalyx thickness in humans

Thickness of the endothelial glycocalyx in individual capillary blood vessels was estimated using OPS imaging of the sublingual microcirculation based on the same principles as the previously described method in hamster capillaries. The OPS procedure is non-invasive, painless and takes approximately 15 minutes. Measurements were performed with a handheld OPS camera (Cytometrics, Philadelphia, PA, USA) with participants in the supine position. Pressure on the tissue was avoided to ensure normal flow. The region chosen for measurement was the central sublingual area. Images were collected with a 5× objective with a 0.2 numerical aperture providing a 325× magnification and were sized 720×576 pixels. The frame rate was 25 per second. All frames were recorded on Sony DSR-20P digital video recorder and transferred to a computer using Windows Movie Maker. Analysis of the images was performed with Image-Pro Plus by a single image analyst, who was blinded for the clinical details of the participants. Capillaries with a diameter between 3 and 7 µm were selected for analysis. The width of the flowing erythrocyte column was measured before and after spontaneous capillary leukocyte passage. An on-screen caliper using a 1 mm/0.01 mm stage micrometer was used for all calibrated dimensional measurements. The anatomical capillary diameter and the width of the flowing erythrocyte column were measured using digital calipers prior to and after spontaneous capillary leukocyte passage. Per participant, glycocalyx dimension was determined in at least 5 individual capillaries. The mean of these results was calculated and used in further analyses. In addition as indication of capillary density, the number of capillaries per field was counted as previously described.

![Figure 1b. Imaging the sublingual microcirculation using orthogonal polarization spectroscopy.](image)

Measurements were performed with a handheld OPS camera with participants in the supine position. The region chosen for measurement was the central sublingual area. The OPS procedure is non-invasive, painless and takes approximately 15 minutes. Images of the microvasculature are directly projected on screen.
Estimation of systemic glycocalyx volume

The endothelial glycocalyx limits access to plasma macromolecules and erythrocytes\textsuperscript{2, 18, 23}. Hence, the systemic glycocalyx volume can be estimated by subtracting the circulating plasma volume from the total intravascular distribution volume, which comprises both plasma volume and the (intravascular) glycocalyx.

In detail, we added 40mg sodium fluorescein [fluorescein-di-Na 25%, 250 mg/ml, AMC Pharmacy, Amsterdam, the Netherlands] to 20 ml of autologous erythrocytes for 5 minutes. After careful washing, labelled erythrocytes were resuspended in saline to the initial volume [60 ml] and re-infused. Subsequently, blood was drawn at 4, 5, 6, and 7 minutes after infusion. The fraction of labelled erythrocytes in the total erythrocyte pool was used to estimate the circulating erythrocyte volume \(V_{\text{ERY}}\). \textsuperscript{27} Pre-injection unlabeled erythrocytes served as negative control. The fraction of labelled erythrocytes in the blood was measured using a FACSscan analyzer (FACS Calibur\textsuperscript{\textregistered}, Becton Dickinson, Mountain View, USA). At least 100,000 cells were counted. Data were analyzed by Cellquest (Becton Dickinson, San Jose, CA, USA). Haematocrit \(\text{Ht}\) was measured after centrifugation of heparinized blood in a Hettich-Haematokrit centrifuge at 10,000 rpm during 5 minutes (Hettich, Tuttingen, Germany). Circulating plasma volume was calculated from \(V_{\text{ERY}}\) and large vessel \(\text{Ht}\) by the following formula: circulating plasma volume = \([1 – \text{Ht}] \times V_{\text{ERY}})/\text{Ht}\).

Concomitantly, total intravascular volume was calculated using the glycocalyx permeable tracer dextran 40 [Rheomacrodex, NPBI, Emmercompascum, the Netherlands] \textsuperscript{17, 28, 29}. 100 ml of dextran 40 was injected intravenously, after which repeated blood sampling at 3, 5, 7, 10, 15, 20 and 30 minutes was performed. The dextran 40 plasma concentration was calculated by measuring the increase in glucose concentration in the post infusion samples after hydrolyzation of the dextran glucose polymers, correcting for the individual plasma glucose levels. The glucose concentration was assessed in duplicate using the hexokinase method [Gluco-quant on Roche/Hitachi modular analyzer, Roche Diagnostics, Mannheim, Germany] and corrected for endogenous glucose concentration. The procedure was calibrated with known amounts of dextran 40 added to plasma in vitro. In order to estimate the initial intravascular distribution volume of dextran 40, the concentration at the time of injection was calculated by exponential fitting of the measured dextran 40 concentrations at \(t=0\) min.

Blood sampling and biochemistry

Blood samples were drawn after an overnight fast from all subjects at the baseline measurement. All measurements were performed at the Laboratory of Experimental Vascular Medicine and the Clinical Chemistry Laboratory of the Academic Medical Center. Baseline fasting plasma glucose was assessed in duplicate using the hexokinase method. Total cholesterol, HDL-cholesterol and triglycerides were measured by standard enzymatic methods [Roche Diagnostics, Basel, Switzerland]. LDL-cholesterol was calculated using the Friedewald formula. Leukocyte counts and subfractions were measured by flow cytometric analysis under standardized conditions and absolute counts were calculated.
**Statistical analysis**

All values are provided as means ± standard deviation (SD). To compare the two sets of microvascular and systemic glycocalyx measurements the paired two-tailed Student’s t-test was used. To determine the coefficient of variation (CV) the SD was divided by the mean. The agreement between successive measurements was evaluated by comparison of the measurements with the line of identity and by a Bland-Altman plot. Univariate correlations between microvascular glycocalyx and other parameters were calculated using linear regression analysis. Backward multivariate linear regression analysis was used to explore the relation between microvascular glycocalyx thickness and the parameters, which correlated significantly in univariate analysis. Sample size calculation was performed using the nomogram described by Altman. P<0.05 was considered to represent a statistically significant difference.

**Results**

**Endothelial glycocalyx thickness in hamster cremaster muscle capillaries**

In six separate experiments, we performed paired measurements of the anatomic capillary diameter, erythrocyte-endothelial cell gap and the maximal erythrocyte widening after leukocyte passage in cremaster capillaries with intravital microscopy. The gap between the membranes of flowing erythrocytes and endothelial cells, the gold standard of glycocalyx measurement, could be clearly identified. The anatomic capillary diameter did not change during the transient erythrocyte widening following leukocyte passage (data not shown). As reported previously, the EEC gap as well as the change in erythrocyte width were proportional to the anatomical capillary diameter in the range from 4 to circa 8 µm (Figure 2b). Furthermore, the measurement of erythrocyte column widening always provides an underestimation of the glycocalyx thickness, defined as EEC gap (Figure 2a). This underestimation is proportional to the EEC gap (Δ erythrocyte width/2 = 62% EEC gap), which in turn is related to the anatomic diameter of the capillary. Erythrocyte flow velocity before leukocyte passage was 92±0 µm/s, in line with previously published data. After leukocyte passage, it varied between 83 and 104 µm/s (Figure 2c). After degradation of the endothelial glycocalyx by oxLDL injection, no transient erythrocyte widening was observed upon leukocyte passage (0.72±0.01 to 0.75±0.014 µm, ns, Figure 2d).

**Reproducibility of measurement of microvascular sublingual glycocalyx thickness**

In contrast to intravital microscopy, OPS imaging is easily applicable in humans. Unfortunately, its use is limited to measuring the change in erythrocyte column width. Endothelial cells, lacking hemoglobin, cannot be visualized. However, comparable to the EEC gap measurements, measuring the delta erythrocyte width/2 using OPS in vivo has been proposed to provide an estimate for glycocalyx thickness.
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Figure 2a. Erythrocyte-endothelial cell gap versus delta erythrocyte width/2 in hamster capillaries

The EEC gap is the gold standard of glycocalyx measurement. Delta erythrocyte width/2 correlates with this standard, but always underestimates glycocalyx thickness.

Figure 2b. Erythrocyte-endothelial cell gap and delta erythrocyte width/2 as function of anatomic capillary diameter

The size of the EEC gap and the delta erythrocyte width/2 were determined in capillaries of six hamsters. Both are related to the anatomic capillary diameter, i.e. larger glycocalyx dimensions are related to capillaries with larger diameters.
Figure 2c. Erythrocyte flow velocity before and after leukocyte passage

Figure 2d. Effect of leukocyte passage on the erythrocyte-endothelial cell gap before and after degradation of the endothelial glycocalyx layer by oxLDL

Before oxLDL

After glycocalyx degradation by oxLDL
Baseline characteristics of volunteers are listed in Table 1. All study procedures were well-tolerated and no serious adverse events occurred. The mean width of the capillary erythrocyte columns prior to leukocyte passage was almost identical on both study days (day 1: 4.22 ± 0.27 mm, day 2: 4.44 ± 0.46 mm), as was the maximal erythrocyte diameter (day 1: 5.37 ± 0.45 mm, day 2: 5.50 ± 0.35 mm). Thus, the mean microvascular glycocalyx thickness was 0.58 ± 0.16 mm and 0.53 ± 0.15 mm respectively, which was not significantly different from each other (mean difference 0.05 mm, n=12). The intersession CV was 15 ± 5%. Agreement between the two measurements was acceptable, as shown by the approximation of the values to the line of identity and the limits of the Bland-Altman plot (Figure 3). Finally, sublingual capillary density was fairly identical in all volunteers (59.2 ± 12 capillaries per field) and did not correlate with determined microvascular glycocalyx thickness in univariate analysis (r = 0.05, p=0.4).

Table 1. Baseline characteristics (mean ± SD)

| Characteristic                      | mean ± SD     |
|------------------------------------|---------------|
| Age, years                         | 25.6 ± 6.4    |
| BMI, kg/m²                         | 22.9 ± 1.6    |
| Systolic blood pressure, mmHg      | 125.0 ± 10.2  |
| Diastolic blood pressure, mmHg     | 70.4 ± 8.4    |
| Heart rate, bpm                    | 57.7 ± 9.1    |
| Total cholesterol, mmol/L          | 4.1 ± 0.6     |
| LDL-cholesterol, mmol/L            | 2.2 ± 0.7     |
| HDL-cholesterol, mmol/L            | 1.5 ± 0.3     |
| Triglycerides, mmol/L              | 0.5 ± 0.2     |
| Fasting plasma glucose, mmol/L     | 4.9 ± 0.4     |
| C-reactive protein, mg/L           | 0.5 ± 0.3     |
| Leukocytes, x10⁹/L                 | 5.1 ± 1.3     |
| Neutrophils, x10⁹/L                | 2.9 ± 0.9     |
| Eosinophils, x10⁹/L                | 0.12 ± 0.1    |
| Basophils, x10⁹/L                  | 0.03 ± 0.02   |
| Lymphocytes, x10⁹/L                | 1.7 ± 0.4     |
| Monocytes, x10⁹/L                  | 0.4 ± 0.1     |
| Thrombocytes, x10⁹/L               | 244 ± 30      |
| Microvascular glycocalyx, µm       | 0.7 ± 0.2     |
| Systemic glycocalyx volume, L      | 1.6 ± 0.5     |

Reproducibility of measurement of systemic glycocalyx volume

Throughout all infusion protocols, blood pressure and heart rate remained unaffected (data not shown). Infusion of the dextran 40 solution had no significant effect on hematocrit values. Circulating plasma volumes were (3.1 ± 0.4 liters versus 3.0 ± 0.4 liters, ns) and systemic dextran 40 distribution volumes (4.7 ± 0.8 liters versus 4.6 ± 0.5 liters, ns) during the baseline study visits. Accordingly, systemic glycocalyx volumes were reproducible between visits (day 1: 1.6 ± 0.8 liters versus day 2: 1.6 ± 0.6 liters, ns) resulting in an inter-session coefficient of variance of 15.8 ± 11.9%. Again, agreement between the two measurements was good as shown by the Bland-Altman plot (Figure 4).
Figure 3a. Comparison of two successive microvascular glycocalyx measurements per individual

Figure 3b. Bland-Altman plot of the means of the 2 measurements of microvascular glycocalyx thickness against their difference
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Figure 4a. Comparison of two successive systemic glycocalyx measurements per individual

Figure 4b. Bland-Altman plot of the means of the 2 measurements of systemic glycocalyx volume against their difference
Correlation between microvascular sublingual glycocalyx and cardiovascular risk factors

In univariate analysis, OPS measured microvascular glycocalyx was positively correlated with systemic glycocalyx volume \( (r = 0.45, p<0.05) \). Furthermore, glycocalyx thickness correlated with fasting plasma glucose \( (r = 0.43, p<0.05) \) and HDL-cholesterol \( (r = 0.40, p<0.05) \). In contrast, it was negatively correlated with LDL-cholesterol \( (r = -0.41, p<0.05) \) and body mass index [BMI] \( (r = -0.45, p<0.05) \). After multivariate analysis, only systemic glycocalyx volume \( (r = 0.44, p<0.02) \) and BMI \( (r = -0.44, p<0.02) \) retained significance (Table 2). It should be noted only 21% \( (r^2) \) of the variation in systemic glycocalyx volume is reflected by the variance of the microvascular glycocalyx.

### Table 2. Univariate and multivariate analyses of microvascular glycocalyx thickness

| Parameters                              | Univariate \( \beta \) coefficient | \( P \)-value | Multivariate \( \beta \) coefficient | \( P \)-value |
|-----------------------------------------|-------------------------------------|---------------|--------------------------------------|---------------|
| Age, years                              | 0.17                                | Ns            |                                      |               |
| BMI, kg/m²                               | -0.45                               | 0.03          | -0.44                                | 0.018         |
| Systolic blood pressure, mmHg           | 0.37                                | Ns            |                                      |               |
| Diastolic blood pressure, mmHg          | 0.18                                | Ns            |                                      |               |
| Heart rate, bpm                         | -0.16                               | Ns            |                                      |               |
| Total cholesterol, mmol/L               | -0.10                               | Ns            |                                      |               |
| LDL-cholesterol, mmol/L                 | -0.41                               | 0.05          |                                      |               |
| HDL-cholesterol, mmol/L                 | 0.40                                | 0.05          |                                      |               |
| Triglycerides, mmol/L                   | -0.17                               | Ns            |                                      |               |
| Fasting plasma glucose, mmol/L          | 0.43                                | 0.04          |                                      |               |
| CRP, mg/L                               | -0.16                               | Ns            |                                      |               |
| Leukocytes, \( x10^9/L \)               | -0.34                               | Ns            |                                      |               |
| Neutrophils, \( x10^9/L \)              | -0.10                               | Ns            |                                      |               |
| Eosinophils, \( x10^9/L \)              | -0.15                               | Ns            |                                      |               |
| Basophils, \( x10^9/L \)                | 0.21                                | Ns            |                                      |               |
| Lymphocytes, \( x10^9/L \)              | 0.17                                | Ns            |                                      |               |
| Monocytes, \( x10^9/L \)                | -0.19                               | Ns            |                                      |               |
| Thrombocytes, \( x10^9/L \)             | -0.21                               | Ns            |                                      |               |
| Systemic glycocalyx volume, L           | 0.45                                | 0.026         | 0.44                                 | 0.018         |

Sample size calculation

Based on these results, we calculated that for future evaluation of glycocalyx dimensions in a disease state vs. healthy controls, 17 subjects per group would enable detection of a difference of 0.2 \( \mu \)m in microvascular glycocalyx thickness or 0.5 L in systemic glycocalyx volume with a significance level of 5% and 80% power. 10 participants would be necessary in a paired design.
Discussion

In the present study we demonstrate that change in erythrocyte width correlates with the size of the erythrocyte-endothelium gap, which is the gold standard of glycocalyx measurement. Using OPS imaging, we are able to visualize erythrocyte width in the sublingual microvasculature in humans, reflecting microvascular glycocalyx thickness. Both this microvascular glycocalyx thickness as well as systemic glycocalyx volume can be quantified reproducibly. Furthermore, we confirm the correlation between these two techniques.18 Interestingly, also in the present cohort, we observed an inverse relation between the presence of cardiovascular risk factors and microvascular glycocalyx thickness.

Microvascular glycocalyx thickness measurement

It has now been widely acknowledged that erythrocytes are separated from the endothelial surface by a gap that is about an order of magnitude larger than the dimension of the fluid film that is minimally required for lubrication. The width of the erythrocyte column was shown to increase following local disruption of the glycocalyx in the absence of changes in the anatomical capillary diameter. Markedly, the gap between erythrocytes and the endothelium has usually been found to be larger in vivo than predicted from theoretical considerations and in vitro observations, yet tends to be similar in size compared to previous reports by Pries et al.31

Under physiological conditions in vivo, fluid shear stress and leukocytes have a profound effect on endothelial glycocalyx morphology, structure and function.34,35 The findings that erythrocytes are not able to compress endothelial glycocalyx, whereas leukocytes can compress endothelial glycocalyx, provided the basis for the linear theory model, the theoretical background of the microvascular glycocalyx thickness measurements.17,19 According to this model, there are two phases in endothelial glycocalyx recoil upon leukocyte passage. At the initial phase of leukocyte compression, the endothelial surface layer thickness is <0.36 of its undisturbed thickness (glycocalyx fibers parallel to the capillary wall). During the second phase the glycocalyx recoils after passage of leukocyte.19,20 It is expected that the force restoring glycocalyx thickness after leukocyte passage is generated by plasma proteins adsorbed to the glycocalyx.31,36,37 Most likely via tension in membrane-bound glycoprotein chains.32,38 Recently, Han et al. were able to actually measure the time dependent restoration of the endothelial glycocalyx after compression following the passage of a leukocyte, thus further underscoring the validity of this theory.39

Concerns may arise that transient widening of erythrocytes following capillary leukocyte passage may not be related to the presence of a compressible glycocalyx per se, since erythrocyte widening is also observed when erythrocyte velocity is reduced in glass tubes without glycocalyx.39,40 However, our experiments and those reported by Vink7 on the leukocyte-induced erythrocyte widening in capillaries with intact vs. disrupted glycocalyx clearly demonstrate that the effect of capillary leukocyte passage...
is dependent on the presence of an intact glycocalyx. Leukocyte passage did not result in erythrocyte widening when glycocalyx dimension was significantly reduced, despite unaltered anatomical capillary dimensions and leukocyte induced changes in erythrocyte hemodynamics.

Similarly, we previously demonstrated that, in fact, erythrocyte velocity cannot account for erythrocyte widening. Thus, the relation between erythrocyte velocity and the gap between erythrocytes and the endothelial surface was predominantly explained by that fact that the average erythrocyte velocity is higher in capillaries with a larger anatomical diameter. By grouping capillaries of similar anatomical diameter or collecting erythrocyte width data from an individual capillary, we could no longer find a relationship between erythrocyte velocity and erythrocyte width. Apparently, larger gaps are found in capillaries with greater diameters.

It must be noted that measuring the transient widening of the erythrocyte column after leukocyte passage underestimates the glycocalyx thickness, since compression of the glycocalyx by passing leukocytes is incomplete. Furthermore, it is smaller than the EEC gap, since this gap also includes the lubricating plasma layer between the erythrocyte membrane and the glycocalyx surface. Our data confirm that erythrocyte widening measured using intravital microscopy and/or OPS underestimates glycocalyx thickness by approximately 30%.

Both the OPS method and the tracer dilution method provide estimations of the dimension and volume of the endothelial glycocalyx in humans, each with its own limitations and technical challenges. The systemic glycocalyx volume does not inform about heterogeneity of glycocalyx properties between organs. The sublingual glycocalyx measurement only gives information on capillary blood vessels. Despite these limitations, these independent techniques are able to detect differences between the healthy and diseased state, e.g. diabetes mellitus and are correlated. Interestingly, when applying this method to measure glycocalyx dimension in various species (i.e. mouse, dog, goat and human), we find a fairly consistent glycocalyx volume between 15 and 20 ml/kg body weight (data not shown).

**Correlations between glycocalyx dimension and cardiovascular risk factors**

With respect to cholesterol levels, it is known that degradation of the glycocalyx, resulting in shedding of its main constituent hyaluronan, is increased after exposure to oxidized LDL-cholesterol. Oxidized LDL-cholesterol plasma levels are known to be inversely associated with HDL-cholesterol and positively correlated with LDL-cholesterol plasma levels. Thus, it is expected that cholesterol metabolism (and indirectly BMI) is closely associated with glycocalyx dimensions in humans. This conclusion is underscored by the fact that glycocalyx harbors LDL- as well as HDL-cholesterol, probably via binding to endothelial heparan sulphates.
Less understood, but even more intriguing is our finding of a positive correlation between fasting normoglycemic plasma glucose levels and glycocalyx dimension. In contrast, we have previously reported that hyperglycemia was associated with loss of endothelial glycocalyx in healthy volunteers. This loss is probably mediated by the generation of reactive oxygen species \textsuperscript{18}. However, glycolysis renders important compounds for synthesis of hyaluronan and other proteoglycans \textsuperscript{44}. Moreover, none of the healthy volunteers had fasting glucose plasma levels over 5.6 mmol/L. Therefore, it is tempting to speculate that in the physiological range glucose provides substrate for glycocalyx synthesis, while pathogenic hyperglycemia results in glycocalyx degradation due to generation of reactive oxygen species.

**Endothelial glycocalyx as a biomarker: future in patient related research**

The investigation of novel circulating plasma biomarkers in relation to endothelial glycocalyx in patients with cardiovascular disease has been accelerating at fast pace \textsuperscript{45}. Therefore, criteria for appraisal of novel biomarkers need to be structured around three fundamental questions involving reproducible measurement, consistent association between biomarkers and early detection of otherwise subclinical disease. If these three criteria are fulfilled, long term studies will have to be performed in order to study modification of these biomarkers upon specific therapy \textsuperscript{46}. With the current study, we have made the first steps in fulfilling two of the three criteria to establish the endothelial glycocalyx as a biomarker. Furthermore, it has shown us that relatively small sample sizes are sufficient to perform exploratory studies. This opens the way for further research in vivo in humans.

**Conclusions**

The dimension of the endothelial glycocalyx can be measured reproducibly in humans and is related to cardiovascular risk factors. Together with the advent of glycobiomics, these techniques will enable further research in human endothelial glycobiology. They will help us assess whether and to what extent interventions aimed at normalizing glycan enzymatic regulation systems will have the capacity to modulate the endothelial glycocalyx and thereby the atherogenic vulnerability of the vessel wall, which is to become an important area in the search for novel therapeutic targets to reverse atherogenesis \textsuperscript{47}. 
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