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Microvascular Glycocalyx Dimension Estimated by Automated SDF Imaging is not Related to Cardiovascular Disease

Fouad Amraoui, Rik H.G. Olde Engberink, Jaqueline van Gorp, Amal Ramdani, Liffert Vogt and Bert-Jan H. van den Born

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ABSTRACT

Objective: The endothelial glycocalyx (EG) regulates vascular homeostasis and has anti-atherogenic properties. Sidestream darkfield (SDF) imaging allows for non-invasive visualization of microvessels and automated estimation of EG dimensions. We aimed to assess whether microcirculatory EG dimension is related to cardiovascular disease.

Methods: Sublingual EG dimension was estimated by SDF imaging in healthy volunteers and in patients visiting an outpatient clinic for vascular medicine of a university hospital in Amsterdam, the Netherlands. EG dimension was compared among healthy volunteers, patients with cardiovascular disease (CVD) and patients at low (<10%) or high risk (≥10%) of CVD according to the Framingham algorithm.

Results: In total 120 patients and 30 healthy volunteers were included. Patients had a mean age of 59±14 years, 71 (59%) were male and 24 (20%) were black. Healthy volunteers were on average 28±4 years and 19 (63%) were male. EG dimension was similar in healthy volunteers (2.04±0.23µm), low risk patients (2.05±0.24µm, n=39), high risk patients (2.05±0.23µm, n=30) and in patients with CVD (2.09±0.21µm, n=51, p=0.79). EG dimension was not correlated with cardiovascular risk factors.

Conclusions: Microcirculatory EG dimension, as estimated by automated SDF imaging, is not associated with CVD, suggesting that this technique may not contribute to cardiovascular risk stratification.
INTRODUCTION

The endothelial glycocalyx (EG) is a dynamic layer, composed of membrane-bound proteoglycans and attached negatively-charged glycosaminoglycans, lining the vascular wall of the micro-and macrovasculature [1]. Thickness of this endothelial surface layer increases with the vessel diameter and ranges from 0.5-3μm [2]. Numerous pre-clinical studies have shown a fundamental role of the EG in vascular homeostasis, with potential anti-atherogenic properties. As a first-line barrier between blood flow and endothelium, the EG contributes to regulation of endothelial permeability [3, 4]. Shielding of the endothelium by the EG limits atherogenic transendothelial lipid migration and interaction of leucocytes with adhesion molecules on the endothelium [5-7]. By harbouring plasma proteins such as superoxide dismutase and antithrombin, the EG has anti-oxidative and anticoagulant properties that may protect against atherothrombotic sequelae [2]. In addition, the EG serves as a mechano-transductor, which is pivotal for shear-mediated nitric oxide production [8-10]. Interestingly, perturbation of the EG can be provoked by several atherogenic stimuli, such as infusion of oxidized low density lipoprotein (LDL), inflammatory cytokines, and hyperglycaemia [11-15]. Together, these data suggest that patients with diminished EG might be at increased risk for developing cardiovascular disease (CVD) [2, 16].

Until recently estimation of EG volume comprised of invasive, time-consuming methods [17]. Assessment of EG dimension in large population studies was therefore not feasible. Sidestream darkfield imaging (SDF) is a technique, which allows visualization of the sublingual microcirculation by using absorption of light by haemoglobin in erythrocytes. Acquired images are automatically analysed by integrated software, which estimates EG dimensions by assessing the erythrocyte-endothelium gap [18, 19]. This rapid and non-invasive measurement poses minimal burden to patients and is therefore suitable for investigating EG dimension in large cohorts.

In the present study, we aimed to assess whether EG dimension, as estimated by SDF imaging of the sublingual microcirculation, is associated with cardiovascular risk.

MATERIALS AND METHODS

Participants

We carried out an observational study by assessing EG dimensions in individuals with different cardiovascular risk profiles. Patients were recruited from the outpatient department of vascular medicine at a large teaching hospital in Amsterdam, The Netherlands, from May 2012 until August 2013. Healthy volunteers were recruited among the hospital staff.
Adult patients who were able to provide written informed consent were included. We excluded patients with any chronic inflammatory disease, familiar hypercholesterolemia or malignancy. Pregnant women and patients with end-stage renal disease requiring dialysis were also excluded. The study protocol was approved by the local ethics committee.

**Assessment of cardiovascular risk**
Cardiovascular risk estimation was routinely performed by physicians at the outpatient department of vascular medicine. Data on age, gender, length, body weight, ethnicity, a history of CVD and traditional risk factors such as hypertension, dyslipidemia, smoking habit and diabetes mellitus were obtained from the patient chart. Ethnicity was defined as self-reported white, black, or South-Asian.

CVD was defined as a documented episode of any of the following conditions: 1) coronary artery disease (including myocardial infarction, acute coronary syndrome requiring percutaneous coronary intervention or angina pectoris), 2) cerebrovascular accidents including ischemic and/or hemorrhagic stroke, transient ischemic attack or subarachnoid bleeding, 3) heart failure or 4) peripheral artery disease requiring surgical or endovascular treatment, aortic aneurysm and aortic dissection. In patients without CVD, the Framingham risk algorithm was used to stratify patients according to low (<10%) and high (>10%) risk of fatal and non-fatal CVD within 10 years.

Blood pressure (BP) was measured three times while seated at the right arm after at least 5 minutes of rest using a validated semi-automatic device (Microlife®). The last two measurements were averaged to represent office BP. Laboratory analyses included hemoglobin, hematocrit, platelet count and plasma creatinine. Total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and plasma glucose were assessed in fasting state. Microalbumin-creatinine ratio was assessed in random urine samples. Microalbuminuria was considered present if microalbumin-creatinine ratio was >2.5 mg/mmol in men or >3.5 mg/mmol in women [20]. Renal function was estimated according to the Modification of Diet in Renal Disease (MDRD) formula [21]. All laboratory results were performed in the hospital's central laboratory according to local protocols.

**Assessment of endothelial glycocalyx dimensions**
Dimensions of the EG were estimated non-invasively by imaging of the sublingual microcirculation using a hand-held SDF videomicroscope (Microvision Medical Inc., Wallingford PA, USA) with integrated software (GlycoCheck™, Maastricht, The Netherlands) for automatic analysis of the video recordings as previously published [19]. During video recording, all visible microvessels with a diameter between 5 and 25μm were automatically identified and measurement sites perpendicular to the vessel direction were selected every 10 microns along each microvessel (Figure 1). Data acquisition automatically started when image quality was within acceptable range and automatically stopped when data on a
minimum number of 3000 measurement sites had been obtained. Average duration of data acquisition was 2-3 minutes. Three sequential measurement cycles were carried out in each participant. Measurements were performed in non-fasting state.

Figure 1: Sidestream darkfield image of the sublingual microcirculation

Typical sidestream darkfield image of the sublingual microcirculation (A) with automated selection of microvessels (5-25µm) by GlycoCheck software (B). Lines perpendicular to the vessel direction indicate the measurement sites. At least 3000 measurement sites are obtained during each measurement.

The red blood cell column width is automatically determined at each measurement site. The distribution of the red blood cell column width of each vascular segment is used to calculate the perfused boundary region (PBR), which is defined as the distance between the median red blood cell column width and the estimated outer edge of the red blood cell column. The maximum red blood column width is extrapolated from the 25th and 75th percentile values of the red blood cell column width using the cumulative distribution curve. The PBR is considered to reflect EG thickness based on the assumption that loss of integrity of the
EG allows for deeper penetration of erythrocytes into the vessel wall, resulting in increased PBR values [18]. For analysis, selected microvessels are automatically divided in subgroups of 5-9μm, 10-19μm and 20-25μm by the GlycoCheck software.

Reproducibility of the PBR in healthy volunteers

We assessed the reproducibility of the PBR values by calculating the coefficient of variation (CV) in 9 healthy volunteers after performing five consecutive measurements, while holding the SDF videomicroscope at the same sublingual quadrant. Measurements were carried out in four different sublingual quadrants adding up to a total of 20 measurements per individual. The coefficient of variation (CV) for overall PBR including microvessels with a diameter ranging from 5-25μm was 11.9%. CV increased with vessel size and was 9.1% for the smallest vessels with a diameter ranging from 5-9μm, 12.6% for vessels ranging from 10-19μm and 15.0% for vessels with a diameter ranging from 20-25μm.

Sample size calculation and statistical analysis

A decrease of 0.2μm in microcirculatory EG thickness was previously suggested to be relevant [22]. We performed a sample size calculation based on our measurements in healthy volunteers, showing that at least 21 participants should be included in each group to allow detection of a 0.2μm difference with a significance level of 0.05 and 80% power. We decided to include at least 30 subjects in each group. Continuous data are expressed as mean and standard deviation (SD) or median and interquartile range (IQR) for variables with a skewed distribution. Categorical data are expressed as number and percentages. Differences between groups for continuous variables were assessed by a one-way ANOVA with post-hoc LSD correction for parametric or Dunnets post-hoc correction for non-parametric distributions. Chi-square tests were used for categorical variables. Linear regression analyses were carried out to explore correlations between estimated EG dimension and separate cardiovascular risk factors. SPSS software was used for all analyses (Statistical Package for the Social Sciences, version 19.0, Inc. Chicago, Illinois, USA). A p-value <0.05 was considered significant.

RESULTS

Baseline characteristics

In total 120 consecutive patients and 30 healthy controls were enrolled in the study. The predicted 10-year risk of suffering from fatal and non-fatal CVD was <10% in 39 (33%) patients and ?10% in 30 (25%) patients, while 51 (43%) patients had a history of CVD. Baseline characteristics with comparison of patients in different cardiovascular risk categories are
summarized in Table 1. In the group of patients with history of CVD, 27 (23%) had coronary artery disease, 6 (5%) patients had heart failure and 21 (18%) patients had peripheral artery disease. Cerebrovascular accidents occurred in 25 (21%) patients, of whom 12 (10%) suffered from an ischemic stroke, 2 (2%) from hemorrhagic stroke and 11 (9%) patients had suffered a TIA. Healthy volunteers were 30 hospital employees, mean age was 28±4 years and 19 (63%) were male.

Table 1. Baseline Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Low risk</th>
<th>High risk</th>
<th>CVD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, No</td>
<td>39</td>
<td>30</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Male, No. (%)</td>
<td>12 (31)</td>
<td>25 (83)*</td>
<td>34 (67)*</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Age, years</td>
<td>50±12</td>
<td>64±11*</td>
<td>64±12*</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28±7</td>
<td>27±3</td>
<td>27±4</td>
<td>0.68</td>
</tr>
<tr>
<td>Ethnicity, No. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>22 (56)</td>
<td>22 (74)</td>
<td>41 (80)*</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Black</td>
<td>14 (36)</td>
<td>5 (17)</td>
<td>5 (10)</td>
<td></td>
</tr>
<tr>
<td>South-Asian</td>
<td>2 (5)</td>
<td>1 (3)</td>
<td>3 (6)</td>
<td></td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.41±0.03</td>
<td>0.41±0.05</td>
<td>0.41±0.05</td>
<td>0.92</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>147±18</td>
<td>147±19</td>
<td>143±20</td>
<td>0.41</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>90±14</td>
<td>82±13*</td>
<td>81±13*</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Antihypertensive drugs</td>
<td>2.6±1.4</td>
<td>2.7±1.2</td>
<td>3.0±1.8</td>
<td>0.55</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.4±1.2'</td>
<td>5.6±1.5'</td>
<td>4.6±1.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.2±1.1</td>
<td>3.1±1.3</td>
<td>2.6±1.1</td>
<td>0.07</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.7±0.6</td>
<td>1.4±0.5*</td>
<td>1.3±0.3*</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plasma glucose, mmol/L</td>
<td>5.7±1.4</td>
<td>6.6±1.7</td>
<td>6.4±2.5</td>
<td>0.14</td>
</tr>
<tr>
<td>Diabetes mellitus, No. (%)</td>
<td>6 (15)</td>
<td>10 (33)</td>
<td>10 (20)</td>
<td>0.18</td>
</tr>
<tr>
<td>Statin use, No. (%)</td>
<td>7 (18)</td>
<td>10 (33)*</td>
<td>34 (67)*</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Smokers, No. (%)</td>
<td>11 (28)</td>
<td>14 (47)</td>
<td>30 (59)*</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Plasma creatinine, µmol/l</td>
<td>92±27</td>
<td>111±45</td>
<td>101±29</td>
<td>0.10</td>
</tr>
<tr>
<td>eGFR mL/min/1.73m²</td>
<td>73±19</td>
<td>66±24</td>
<td>65±19</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Comparison of baseline characteristics of patients with cardiovascular disease (CVD), low risk patients (<10%) and high risk patients (<10%). Values are mean with standard deviation, median with interquartile range or numbers and percentage. eGFR indicates estimated glomular filtration rate. *p<0.05 versus low risk, §versus high risk, †versus CVD.

EG dimensions as estimated by PBR

EG dimension as estimated by calculation of the PBR was similar among patients from different cardiovascular risk categories and healthy volunteers (Table 2). Linear regression analyses showed no correlation between age and overall PBR in patients (r=0.05, p=0.57) and in healthy volunteers (r=0.10, p=0.59). PBR was similar in males (2.04±0.20µm) and females (2.11±0.25µm, p=0.10) and across different ethnic groups. Patients with coronary
artery disease (2.07±0.20µm) had similar PBR compared to those without coronary artery disease (2.06±0.23µm, p=0.87). PBR was also similar among patients with (2.05±0.20µm) and without cerebrovascular accidents (2.07±0.23µm, p=0.74).

There was no difference in PBR between smoking patients (n=55) and non-smokers (n=65, p=0.81). Patients with and without hypertension, diabetes mellitus or overweight (BMI > 30kg/m²) had comparable PBR values and there was no correlation between PBR and systolic BP, total cholesterol, LDL cholesterol, HDL cholesterol and eGFR. Diastolic BP (r=0.22, p=0.02) and hematocrit (r=0.33, p=0.02) were inversely correlated with PBR. PBR was similar in patients with microalbuminuria (2.10±0.25µm, n=27) compared to those without microalbuminuria (2.08±0.22µm, n=32, p=0.74).

PBR was similar in diabetic patients with a combination of oral medication and insulin (1.87±0.19µm, n=6) compared to diabetic patients with oral medication only (2.04±0.22µm n=16, p=0.11). Use of statins had no effect on PBR, as patients with CVD and statin treatment had similar PBR (2.09±0.21µm, n=34) compared to CVD patients without statin treatment (2.08±0.23µm, n=17, p=0.26).

### Table 2. Estimated EG Dimension in the sublingual microcirculation

<table>
<thead>
<tr>
<th>Vessel diameter</th>
<th>Healthy volunteers</th>
<th>Low risk</th>
<th>High risk</th>
<th>CVD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants, No</td>
<td>30</td>
<td>39</td>
<td>30</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>5-9µm</td>
<td>1.14±0.09</td>
<td>1.15±0.10</td>
<td>1.14±0.12</td>
<td>1.12±0.09</td>
<td>0.76</td>
</tr>
<tr>
<td>10-19µm</td>
<td>2.21±0.25</td>
<td>2.20±0.27</td>
<td>2.19±0.25</td>
<td>2.24±0.26</td>
<td>0.80</td>
</tr>
<tr>
<td>20-25µm</td>
<td>2.52±0.37</td>
<td>2.55±0.35</td>
<td>2.56±0.35</td>
<td>2.62±0.32</td>
<td>0.55</td>
</tr>
<tr>
<td>Overall</td>
<td>2.04±0.23</td>
<td>2.05±0.24</td>
<td>2.05±0.23</td>
<td>2.09±0.21</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Comparison of EG dimension in microvessels ranging from 5-25 µm among healthy volunteers, low risk patients (<10%), high risk patients (<10%) and patients with cardiovascular disease (CVD). Values are mean with standard deviation.

### DISCUSSION

In the present study we demonstrate that microcirculatory EG dimension, as estimated by SDF imaging and automated PBR analysis, is not associated with cardiovascular risk. Similar PBR values were observed among patients with and without CVD, in patients at high and low cardiovascular risk and in healthy controls. This suggests that estimation of microcirculatory EG dimension by SDF imaging may not be useful for cardiovascular risk prediction.

The first study that used imaging of the sublingual microcirculation for estimation of EG dimension in humans showed promising results with regard to implementation of this novel technique in cardiovascular risk stratification [17]. Fairly reproducible estimates of EG dimension were significantly correlated with traditional cardiovascular risk factors such
as LDL cholesterol and BMI, providing a potential novel diagnostic tool for early detection of CVD. Several fundamental differences in approach may explain the discrepancies in our study and the previous one. Firstly, measurement of the erythrocyte-endothelium gap, on which estimation of EG dimension is based [23], was performed using a different method. In the earlier study, microvessels ranging from 3-7µm were selected manually and width of the erythrocyte column was assessed before and after passage of a leukocyte, based on the assumption that these larger and more rigid blood cells compress the EG, allowing following erythrocytes to reach the endothelium more closely [17]. In the present study, microvessels ranging from 5-25µm are automatically selected for analysis of the erythrocyte column width distribution, enabling inclusion of at least 3000 measurements sites. Although this automated method seems more reproducible with lower intersession CV values compared to the manual method, the principle for estimation of EG dimension is completely different. Another major difference between the studies relates to the study population. The previous study was carried out in fasting healthy volunteers with BMI and lipid levels all within the normal range. We compared estimates of EG dimension among non-fasting healthy controls and patients with an increased cardiovascular risk. The observed associations between EG dimension and BMI and lipid levels in healthy volunteers could not be reproduced in patients with higher BMI and unfavourable lipid profiles.

In the smallest microvessels (5-9µm) of healthy controls, we observed an EG dimension of 1.1µm, whereas a previously reported estimate using the manual technique of sublingual EG dimension was 0.8µm [24]. The estimated sublingual EG dimension reported in that study, was closely correlated with systemic glycocalyx volume and associated with circulating glycocalyx degradation products, suggesting accurate estimation of EG dimension.

A few recent studies have used the same approach of sublingual SDF imaging combined with the novel GlycoCheck software for automatic estimation of EG dimension. In contrast to our findings in patients with CVD, patients with premature atherosclerosis were shown to have a decreased EG dimension compared to age-, and sex matched healthy controls [22]. Patients with premature atherosclerosis were on average 18 years younger, more often female and had lower blood pressure, LDL cholesterol and BMI compared to patients with CVD in the present study, suggesting that the observed decrease in EG dimension was not related to traditional cardiovascular risk factors.

Generally, risk factors such as hypertension, smoking and dyslipidemia seem of less great importance in the onset of CVD at young age [25], whereas the role of heritability is more pronounced in this population [26]. The diminished EG dimension in patients with premature atherosclerosis might thus reflect an innate predisposition to CVD without being related to cardiovascular risk factors. The estimated EG dimension and standard deviation among healthy volunteers were similar to our observation.
Another previous study examined sublingual EG dimensions in patients with and without a history of lacunar stroke [19]. No difference in estimated EG dimension was observed. Although we did not differentiate between lacunar stroke and cortical stroke, we also could not find any difference in EG dimension in patients with and without cerebrovascular disease. Interestingly, a subgroup analysis in the previous study showed that lacunar stroke patients with white matter lesions had smaller estimated EG dimensions compared to lacunar stroke patients without these lesions and compared to healthy controls. White matter lesions are associated with cerebral small vessel disease [27], suggesting that reduced sublingual EG dimensions may reflect microvascular abnormalities, but not macrovascular disease. Indeed, type 1 diabetic patients with microalbuminuria, as marker of microvascular damage, were shown to have smaller EG dimensions compared to diabetic patients without microalbuminuria, as measured by the tracer-dilution method [24]. This technique compares the intravascular distribution volume of an EG-permeable tracer (dextran 40) with that of an EG-impermeable tracer (labelled erythrocytes). With SDF imaging of the sublingual microcirculation, estimated EG dimension was also shown to be decreased in a small group of patients with type 2 diabetes compared to healthy controls [28]. However, we could not reproduce these findings in our study with SDF imaging comprising a larger number of subjects with diabetes both with and without CVD. In addition, we observed similar PBR values in patients with and without microcirculatory abnormalities as indicated by the presence of microalbuminuria.

Finally, a recent publication showed that sublingual EG dimensions are diminished in dialysis patients [18], who have a 10-30 fold increased risk of dying from CVD compared to the general population [29]. The decrease in EG dimension coincided with increased circulating levels of EG degradation products such as hyaluronan and syndecan-1, consistent with shedding of the EG. Although the estimated EG dimension was smaller in a subgroup of six dialysis patients with a history of CVD compared to those without CVD, no difference in plasma levels of EG breakdown products could be detected. The observed decrease in EG dimension among dialysis patients might have been influenced by rheology changes as postulated by the investigators [18]. Hemodialysis augments hematocrit and blood viscosity [30], which could affect erythrocyte dynamics and thus the erythrocyte-endothelium gap on which estimation of EG dimension is based. Our finding that PBR was significantly correlated with hematocrit corroborates this hypothesis. Interpretation of PBR values in future studies might therefore improve with adjustment for differences in hematocrit. We did not observe correlations with any of the cardiovascular risk factors, except for diastolic BP. The correlation between diastolic BP and PBR disappeared after excluding 4 (3%) outliers with a diastolic BP > 120 mmHg (r=0.14, p=0.13). Given the absence of any association with systolic BP, pulse pressure and mean arterial pressure, the observed correlation of PBR with diastolic BP might be the result of random chance due to multiple testing.
Our study has some limitations. Firstly, the novel approach of EG dimension estimation with SDF imaging-based PBR measurements has not yet been validated against an alternative technique for EG dimension measurement. Secondly, comparison of EG dimension between different studies that used a similar automated method of PBR measurements might be hampered by differences in image analysis. Vessels up to 50 µm were previously selected automatically, while currently only vessels between 5-25 µm are included and analysed in separate vessel size categories. Nevertheless, our main conclusions rely on differences between groups rather than on absolute PBR values. Thirdly, the lack of any differences in EG dimension might have been attributed to a type II error. However, our own and previously reported power analyses indicate that our sample size was sufficient to detect relevant differences in EG dimension between groups [17]. Finally, previous studies performed measurements after an overnight fast, while we included fasting as well as non-fasting subjects. Treatment modality for diabetes mellitus has been shown to affect glycocalyx dimension [31, 32], indicating that the non-fasting state of participants in this study might have influenced our results. However, we did not observe any difference in glycocalyx dimension among diabetes patients with and without insulin treatment.

**PERSPECTIVES**

Estimation of EG dimension in the sublingual microcirculation by SDF imaging and automated PBR analysis is not related to cardiovascular risk. Although diminished EG in the sublingual microcirculation may reflect microvascular damage in patients with diabetes and kidney disease, our data suggest absence of any relation with overt macro- or microvascular disease. Assessment of EG dimension in the sublingual microcirculation might therefore not be helpful in cardiovascular risk stratification.
REFERENCES


