Clinical impact of nonosmotic sodium storage
Olde Engberink, R.H.G.

Citation for published version (APA):

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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
Jacqueline van Gorp
Amal Ramdani
Liffert Vogt
Bert-Jan H. van den Born

*Microcirculation. 2014;21:499-505*
ABSTRACT

The endothelial glycocalyx regulates vascular homeostasis and has anti-atherogenic properties. Sidestream Dark Field (SDF) imaging allows for non-invasive visualization of microvessels and automated estimation of endothelial glycocalyx dimensions. We aimed to assess whether microcirculatory endothelial glycocalyx dimension is related to cardiovascular disease. Sublingual endothelial glycocalyx 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. Endothelial glycocalyx dimension was compared among healthy volunteers, patients with CVD, and patients at low (<10%) or high risk (≥10%) of CVD according to the Framingham algorithm. In total 120 patients and 30 healthy volunteers were included. Patients had a mean age of 59 ± 14 years, 71 (59%) were men and 24 (20%) were black. Healthy volunteers were on average 28 ± 4 years and 19 (63%) were men. Endothelial glycocalyx dimension was similar in healthy volunteers (2.04 ± 0.23 μm), low-risk patients (2.05 ± 0.23 μm, n=39), high-risk patients (2.05 ± 0.23 μm, n=30) and in patients with CVD (2.09 ± 0.21 μm, n=51, P=0.79). Endothelial glycocalyx dimension was not correlated with cardiovascular risk factors. We concluded that microcirculatory endothelial glycocalyx 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 to 3 \(\mu\)m\(^2\).

Numerous preclinical 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 leukocytes with adhesion molecules on the endothelium\(^5\)–\(^7\). By harbouring plasma proteins such as superoxide dismutase and antithrombin, the EG has antioxidative and anticoagulant properties that may protect against atherothrombotic sequelae\(^2\). In addition, the EG serves as a mechanotransductor, 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 LDL, inflammatory cytokines, and hyperglycemia\(^11\)–\(^15\). 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 Dark Field (SDF) imaging is a technique, which allows visualization of the sublingual microcirculation by using absorption of light by hemoglobin 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 this study, we aimed to assess whether EG dimension, as estimated by SDF imaging of the sublingual microcirculation, is associated with cardiovascular risk.

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: (i) coronary artery disease (including myocardial infarction, acute coronary syndrome requiring percutaneous coronary intervention or angina pectoris), (ii) cerebrovascular accidents including ischemic and/or hemorrhagic stroke, TIA or subarachnoid hemorrhage, (iii) heart failure or (iv) 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 nonfatal CVD within 10 years.

BP was measured three times while seated at the right arm after at least five minutes of rest using a validated semi-automatic device (Microlife®, Widnau, Switzerland). The last two measurements were averaged to represent office BP. Laboratory analyses included hemoglobin, hematocrit, platelet count, and plasma creatinine. Total cholesterol, LDL, 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. Renal function was estimated according to the MDRD formula. All laboratory results were performed in the hospital's central laboratory according to local protocols.

**Assessment of EG dimensions**
Dimensions of the EG were estimated non-invasively by imaging of the sublingual microcirculation using a hand-held SDF video microscope (Microvision Medical Inc., Wallingford PA, USA) with integrated software (GlycoCheck®, Maastricht, The Netherlands) for automatic analysis of the video recordings as previously published. During video recording, all visible microvessels with a diameter between 5 and 25 μm were automatically identified and measurement sites perpendicular to the vessel direction were selected every 10 micrometers 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 two to
three minutes. Three sequential measurement cycles were carried out in each participant. Measurements were performed in non-fasting state.

**Figure 1.** SDF image of the sublingual microcirculation.

![Typical SDF image of the sublingual microcirculation (A) with automated selection of microvessels (5–25 μm) by GlycoCheck software (B). Lines perpendicular to the vessel direction indicate the measurement sites. At least 3,000 measurement sites are obtained during each measurement.](image)

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 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\(^\text{19}\). For analysis, selected microvessels are automatically divided into subgroups of 5–9, 10–19, and 20–25 μm by the GlycoCheck software.

**Reproducibility of the PBR in healthy volunteers**

We assessed the reproducibility of the PBR values by calculating the CV in nine healthy volunteers after performing five consecutive measurements, while holding the SDF video microscope 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 CV for overall PBR including microvessels with a diameter ranging from 5 to 25 μm was 11.9%. CV increased with vessel size and was 9.1% for the smallest vessels with a diameter ranging from 5 to 9 μm, 12.6% for vessels ranging from 10 to 19 μm, and 15.0% for vessels with a diameter ranging from 20 to 25 μm.
Sample size calculation and statistical analysis

A decrease of 0.2 μm in microcirculatory EG thickness was previously suggested to be relevant. 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 0.2 μm 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 SD or median and 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 nonparametric distributions. Chi-squared 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, IL, 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 nonfatal 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 men.

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 men (2.04 ± 0.20 μm) and in women (2.11 ± 0.25 μm, P=0.10) and across different ethnic groups. Patients with coronary artery
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 CVD, low-risk patients (<10%) and high-risk patients (>10%). Values are mean with SD, median with IQR or numbers and percentage. *p < 0.05 versus low risk, †versus CVD.

disease (2.07 ± 0.20 µm) had similar PBR compared to those without coronary artery disease (2.06 ± 0.23 µm, P=0.87). PBR was also similar among patients with (2.05 ± 0.20 µm) and without cerebrovascular accidents (2.07 ± 0.23 µm, 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 ≥ 30 kg/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 ± 0.25 µm, n=27) compared to those without microalbuminuria (2.08 ± 0.22 µm, n=32, P=0.74). PBR was similar in diabetic patients with a combination of oral medication and insulin.
(1.87 ± 0.19 μm, n=6) compared to diabetic patients with oral medication only (2.04 ± 0.22 μm 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 ± 0.21 μm, n=34) compared to CVD patients without statin treatment (2.08 ± 0.23 μm, 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>5-9 μm</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>10-19 μm</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>20-25 μm</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 to 25 μm among healthy volunteers, low-risk patients (<10%), high-risk patients (>10%) and patients with CVD. Values are mean with SD.

**DISCUSSION**

In this 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 stratification17. 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 this study and the previous one. First, measurement of the erythrocyte–endothelium gap, on which estimation of EG dimension is based13, was performed using a different method. In the earlier study, microvessels ranging from 3 to 7 μm 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 closely17. In this study, microvessels ranging from 5 to 25 μm 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 unfavorable lipid profiles.

In the smallest microvessels (5–9 μm) of healthy controls, we observed an EG dimension of 1.1 μm, whereas a previously reported estimate using the manual technique of sublingual EG dimension was 0.8 μm²⁴. 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 controls²². Patients with premature atherosclerosis were on average 18 years younger, more often women and had lower BP, LDL cholesterol and BMI compared to patients with CVD in this 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 age²⁵, whereas the role of heritability is more pronounced in this population²⁶. The diminished EG dimension in patients with premature atherosclerosis might thus reflect an innate predisposition to CVD without being related to cardiovascular risk factors.

Another previous study examined sublingual EG dimensions in patients with and without a history of lacunar stroke¹⁸. 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 disease²⁷, 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 method²⁴. 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. However, we could not reproduce these findings in this 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, who have a 10–30 fold increased risk of dying from CVD compared to the general population. 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. Hemodialysis augments hematocrit and blood viscosity, 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 haematocrit.

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.

This study has some limitations. First, 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. Second, 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 $\mu$m were previously selected automatically, while currently only vessels between 5 and 25 $\mu$m are included and analysed in separate vessel size categories. Nevertheless, our main conclusions rely on differences between groups rather than on absolute PBR values. Third, 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.

Previous studies performed measurements after an overnight fast, while we included fasting as
well as non-fasting subjects. Finally, treatment modality for diabetes mellitus has been shown to affect glycocalyx dimension\textsuperscript{31, 32}. However, the presence of diabetes or diabetes treatment did not influence our results.

**PERSPECTIVE**

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**


