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**Author:** Djaberi, Roxana  
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CHAPTER 12

Non-Invasive Assessment of Microcirculation by Sidestream Dark Field Imaging as a Marker of Coronary Artery Disease in Diabetes


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ABSTRACT

Purpose
In diabetes, generalized microvascular disease and coronary artery disease (CAD) are likely to occur in parallel. We used sidestream-dark-field (SDF) handheld imaging device to determine the relation between the labial microcirculation parameters and CAD in asymptomatic patients with diabetes.

Methods
SDF imaging was validated for assessment of labial capillary density and tortuosity. Thereafter, mean labial capillary density and tortuosity were evaluated and compared in non-diabetic controls, and in asymptomatic patients with type 1- and type 2 diabetes. In diabetic patients, mean capillary density and tortuosity were compared according to the presence of CAD.

Results
Both type 1- and type 2 diabetes were associated with increased capillary density and tortuosity. In diabetes, mean capillary density was an independent predictor of elevated CAC ($P = 0.03$) and obstructive CAD on CT-angiography ($P = 0.01$). Using a cut-off mean capillary density of 24.9 (per 0.63 mm$^2$) the negative predictive value was 84% and 89% for elevated CAC and obstructive CAD. Likewise, capillary tortuosity was an independent predictor of increased CAC ($P = 0.01$) and obstructive CAD ($P = 0.04$).

Conclusion
Assessment of labial microcirculation parameters using SDF imaging is feasible and conveys the potential to estimate vascular morbidity in patients with diabetes, at bedside.
INTRODUCTION
Cardiovascular disease, especially coronary artery disease (CAD), is a predominant cause of morbidity and mortality in diabetes.\(^1\) As a result, recent research has aimed to determine additional risk factors and markers, to distinguish high risk diabetic patients.\(^2,3\) Likewise, the presence of microvascular co-morbidities, in form of nephropathy, retinopathy and neuropathy, has been previously associated with an increased risk of CAD as well as its worse prognosis in diabetes.\(^4,7\) Generalized microvascular disease and CAD may occur in parallel due to common pathogenic mechanisms initiated by hyperglycaemia.\(^8\) However, microvascular disease has also been suggested to contribute to CAD directly through angiogenesis of microvessels in the atherosclerotic plaque.\(^9\) As a consequence, a measure to quantify and qualify microvascular disease in diabetes may convey the potential to predict vascular morbidity and CAD more accurately than the traditional risk factors.

The orthogonal polarization spectral (OPS) and the more novel sidestream dark field (SDF) handheld imaging device allow direct visualization of blood in the microcirculation.\(^10,11\) Thereby, the microcirculatory network of arterioles and capillaries can be investigated non-invasively. In particular, the technique is suitable for the study of easily accessible tissues with a superficial microcirculatory network of the skin and mucous membranes. Accordingly, OPS and SDF imaging have been applied to assess the characteristics of the microcirculation and monitor its alterations in the nail fold as well as in sublingual and labial tissue of patients with heart failure, rheumatic diseases and sepsis.\(^12,13\) However, to our knowledge no previous studies have been performed in patients with diabetes.

In the current study we first sought to validate the assessment of labial microcirculation parameters, comprising of capillary density and tortuosity, using the SDF imaging device. Secondly, the labial capillary density and tortuosity were compared in non-diabetic controls and patients with diabetes. Finally, the relation between labial capillary density and tortuosity with CAD was evaluated in the sub-population of patients with diabetes.

METHODS
Study design and population
One hundred and thirty-one consecutive asymptomatic patients with diabetes were referred to the cardiology outpatient clinic for cardiovascular screening. The American Diabetes Association criteria were used to define diabetes and for further stratification in type 1 or 2.\(^14\) Patients were considered as having type 1 diabetes if laboratory analysis demonstrated auto-antibodies to islet cells, insulin and glutamic acid decarboxylase. Otherwise, patients were considered to have type 2 diabetes. Further cardiovascular risk
factors were assessed according to the following criteria: positive family history of CAD (defined as presence of CAD in first degree family members younger than 55 (men) or 65 (women) years of age), smoking (defined as current smoking or smoking in the last 2 years), hypertension (defined as a blood pressure >140/90 mmHg or treatment with antihypertensive medication), hypercholesterolemia (defined as a total cholesterol level >5.0 mmol/L or use of cholesterol lowering medication), degree of obesity (estimated by body mass index \[\text{BMI} = \text{Kg/m}^2\]), level of glycemic control defined by plasma glycated-haemoglobin (mmol/L) and presence of micro-albuminuria (defined by a urine albumin/creatinine ratio ≥3.5 mg/mmol). Second, non-invasive multi-slice CT (MSCT), including coronary artery calcium (CAC) scoring and coronary angiography, were performed as part of clinical work up. Also, all patients underwent non-invasive assessment of the labial microcirculation using SDF imaging, to determine capillary density and tortuosity. The latter was performed in a study setting, performed according to the Declaration of Helsinki and approved by the institutional review committee of the Leiden University Medical Centre, Leiden. All patients gave written informed consent. In addition, as part of the study setting, 50 asymptomatic healthy individuals comprising the non-diabetic control group, underwent a similar non-invasive assessment of the labial microcirculation using SDF. The healthy individuals comprising this control group had no history of diabetes or cardiovascular disease and were not known with related risk factors (hypertension, hypercholesterolemia, smoking or micro-albuminuria).

Validation study of the microcirculation parameters as assessed by SDF
The intra- and interobserver variability of the labial capillary density and structure was determined in the non-diabetic control group \(n = 50\). For this sub-population, SDF imaging of the four inner lip quadrants was performed by two experienced observers. SDF imaging was performed twice by each observer, on two different occasions. Each observer independently performed processing of own recordings followed by assessment of the capillary density and tortuosity.

Assessment of labial microcirculation

Data acquisition by Side-stream Dark Field imaging
Imaging of the capillaries was performed with SDF imaging with a handheld MicroScan Video Microscope (MicroVision Medical, Amsterdam, Netherlands). The SDF device was fitted with a sterile disposable 5x magnification lens. Video output was visualized on a monitor and connected to a computer via a signal converter (Canopus, ADVC110). Measurements were performed by two trained physicians blinded to clinical data. All subjects (patients with diabetes and non-diabetic controls) were instructed to refrain
from caffeine containing substances 2 h prior to the evaluation. Subjects were in supine position, in a temperature controlled room with a temperature of approximately 22°C. The tip of the SDF probe was placed on the inner lip. To prevent microcirculatory perfusion disturbance due to application of pressure on the imaging area, the probe was first placed on the labial tissue and then retracted to an extent which minimized contact but enabled visualization of the capillary bed. Illumination intensity and depth of focus were modulated to fine-tune image quality.

Continuous digital image recordings (duration 1 minute) were captured in four quadrants of the inner lip: upper right quadrant, upper left quadrant, lower right quadrant and lower left quadrant. Per quadrant, digital image recordings were saved on a hard drive as DV-AVI files to enable off-line analysis.

Assessment of microcirculation

For further assessment of capillary density and structure, 3 frozen microcirculatory imaging areas were selected from the digital image recordings for each quadrant. Microcirculatory imaging areas were selected to meet the following criteria: 1) representative capillary density and structure for the studied quadrant, 2) longitudinal axis view with full-length capillaries enabling structural as well as quantitative assessment of the capillaries, 3) clear, well-focused view of the capillaries. Each microcirculatory imaging area visualized by SDF corresponded with a tissue area of 0.63 mm² (0.9 mm x 0.7 mm) (Figure 1).

Capillary density

To determine capillary density, the number of capillaries was counted manually on each selected microcirculatory imaging area, on the monitor. All vessels identified as capillaries were included. Partially visible capillaries were included if the observer was certain that the vessel was a capillary due to its morphology. Capillary density was defined as the number of counted capillaries per microcirculatory image area (capillaries per 0.63 mm²) (Figure 1). Finally, capillary density of the 12 microcirculatory imaging areas (3 microcirculatory imaging areas per quadrant) were averaged to obtain the mean capillary density per subject.

Capillary tortuosity

To assess the capillary tortuosity score, the number of twists per capillary in the majority of capillaries was evaluated, on each selected microcirculatory imaging area. The number of twists was stratified as 0: no twists (or pinhead capillaries) to 4: 4 or more twists (Figure 1). Subsequently, the overall tortuosity score per subject was determined.
by selecting the most frequent tortuosity score in the 12 studied microcirculatory imaging areas.

**Figure 1.** Visualization of the labial micro-vasculature by SDF. Capillaries are identified as loops emerging from the wider arterioles in the background (1A, 1C-E). To assess capillary density the number of capillaries was determined in a visual field of 0.63 mm$^2$ (1A). To assess capillary tortuosity, the number of twists per capillary in the majority of capillaries, was evaluated for each patient. Number of twists was stratified as 0: no twists (or pinhead capillaries) to 4: 4 or more twists (1B). A relatively low capillary density and tortuosity score was observed in non-diabetic controls (1C). In contrast, a higher capillary density and tortuosity score was observed in patients with diabetes (1D), often accompanied by dilation, branching and malformation of the capillaries (1E).

**Assessment of coronary artery disease by MSCT in patients with diabetes**

**MSCT data acquisition**

Imaging was performed with a 64-slice MSCT scanner (Aquilion64, Toshiba Medical Systems, Japan). If necessary and tolerated, oral beta-blockers (metoprolol 50 mg or
100 mg) were provided 1 hour preceding the scan to achieve a heart rate <65 beats per minute. Initially, a non-enhanced prospective electrocardiographically gated scan, triggered at 75% of the R-R interval with 4 x 3.0 mm collimation was obtained to measure CAC score and determine the start and end position of the helical scan. Thereafter, MSCT angiography was performed using the following parameters: collimation 64 x 0.5 mm, tube rotation time 400, 450 or 500 ms depending on the heart rate, tube current 300 or 350 mA, tube voltage 120 kV. Non-ionic contrast material was administered in the antecubital vein at a flow rate of 5 ml/L and the amount of 90–105 ml (depending on the total scan time), followed by 50 ml of saline solution flush. Automated bolus-tracking in the aortic root was used for the timing of the scan. Images were acquired with simultaneous ECG registration during a single breath hold of approximately 10 seconds. Segmental reconstruction algorithm was applied to generate a single image from the data of one, two or three consecutive heartbeats. Images were reconstructed in the cardiac phase showing least motion artifacts. In general, the end-diastolic phase was used. However, additional reconstructions were made throughout the entire cardiac cycle if necessary to improve image quality. Subsequently, the images were transferred to a remote workstation (Vitrea 2, Vital Images, Minnetonka, USA) for post-processing.

Assessment of CAD

Coronary artery calcium score
All data were evaluated with a remote workstation using dedicated software (Vitrea2, Vital Images, Minnetonka, USA). In each patient, coronary calcium was identified as a dense area in the coronary artery exceeding the threshold of 130 Hounsfield units. The total Agatston score was determined for each patient. Patients with a CAC score >100 were classified as having increased CAC.

Coronary atherosclerosis
MSCT coronary angiography images were interpreted by two experienced observers blinded to the patient characteristics. Discrepancies in interpretation were directly resolved in consensus. The presence of coronary atherosclerosis was visually evaluated on axial images and curved multiplanar reconstructions in at least two orthogonal planes. Obstructive coronary atherosclerosis was defined as the presence of luminal narrowing ≥50%.
Statistical analysis
Continuous variables were expressed as means ± standard deviation. Categorical variables were expressed as numbers (percentages).
First, for the validation of the mean labial capillary density assessment in non-diabetic controls, the interobserver for the first and second session, and the intraobserver for observer-1 as well as observer-2, were determined by calculating the Pearson coefficient of correlation ($r$). For the validation of the capillary tortuosity assessment in the control group, the interobserver for the first and second session, and the intraobserver for observer-1 as well as observer-2, were determined by calculating the agreement percentage and the kappa value.
Second, the mean capillary density was compared in non-diabetic controls and patients with type 1- and type 2 diabetes. For this purpose, the average capillary density and standard deviation were calculated in each group. The independent T-test was used to assess the difference in mean capillary density between the three groups. In addition, the relation between type 1 diabetes (versus non-diabetic controls as reference) and type 2 diabetes (versus non-diabetic controls as reference) with the capillary density was tested in a backward multivariate linear regression analysis, to correct for the influence of other cardiovascular risk factors.
Third, the distribution of capillary tortuosity was compared among healthy individuals, patients with type 1- and type 2 diabetes, by calculating the percentage of patients per tortuosity score for each group. Subsequently, the relation between type 1 diabetes (versus non-diabetic controls as reference) and type 2 diabetes (versus non-diabetic controls as reference) was also tested with the capillary tortuosity in a backward multivariate linear regression analysis.
Finally, in the sub-population of patients with diabetes, the relation of capillary density and tortuosity score with the presence of CAD was evaluated. Initially, patients with diabetes were stratified as having a CAC score 0-100 or as those with an elevated CAC score of >100. Average capillary density and standard deviation were calculated in each group. The independent T-test was used to assess the difference in mean capillary density between the two groups. A similar procedure was performed to compare the mean capillary density between diabetic patients with no obstructive CAD and those with obstructive CAD (luminal narrowing ≥50%).
To identify the potential predictors of an elevated CAC score in diabetes, a univariate logistic regression analysis of baseline cardiovascular risk factors, capillary density and capillary tortuosity was initially performed. Thereafter, all risk factors as well as capillary density and tortuosity were entered in a backward stepwise multiple logistic regression analysis model to identify the independent predictors of an elevated CAC.
Statistical analyses were performed using SPSS software (version 12.0.1, Inc., Chicago, Illinois). $P$ values <0.05 were considered statistically significant.

**RESULTS**

**Characteristics of non-diabetic controls and patients with diabetes**

Characteristics of the sub-populations of non-diabetic controls, patients with type 1- and type 2 diabetes are provided in Table 1. No significant difference was observed among the gender distribution of patients with diabetes (type 1 and type 2) as compared to non-diabetic controls. Patients with type 1 diabetes were relatively younger than non-diabetic controls. In contrast, patients with type 2 diabetes were similar in age to non-diabetic controls. Patients with type 2 diabetes had a higher mean BMI than non-diabetic controls. As compared to non-diabetic controls, patients with diabetes (type 1 and type 2) were more often smokers, more often had a positive family history for CAD, and were frequently treated with statins and anti-hypertensive medication.

<table>
<thead>
<tr>
<th>Non–Diabetic Controls N = 50</th>
<th>Type 1 Diabetes N = 61</th>
<th>Type 2 Diabetes N = 70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 57 ± 14</td>
<td>46 ± 12</td>
<td>55 ± 12</td>
</tr>
<tr>
<td>Male sex 27 (53%)</td>
<td>37 (61%)</td>
<td>33 (47%)</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>24 ± 14</td>
<td>10 ± 7</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>11 (22%)</td>
<td>28 (46%)</td>
</tr>
<tr>
<td>Smoking</td>
<td>0 (0%)</td>
<td>20 (33%)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24 ± 3</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>HbA1c (mmol/L)</td>
<td>7.8 ± 1.5</td>
<td>8.3 ± 1.6</td>
</tr>
<tr>
<td>Micro-albuminuria</td>
<td>9 (13%)</td>
<td>18 (26%)</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>36 (59%)</td>
<td>50 (71%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>29 (48%)</td>
<td>46 (66%)</td>
</tr>
<tr>
<td>Statin use</td>
<td>0 (0%)</td>
<td>22 (36%)</td>
</tr>
<tr>
<td>Anti-hypertensive medication</td>
<td>0 (0%)</td>
<td>19 (31%)</td>
</tr>
</tbody>
</table>

* Difference in distribution between non-diabetic controls and patients with type 1 diabetes.
** Difference in distribution between non-diabetic controls and patients with type 2 diabetes. CAD = Coronary artery disease; HbA1c = glycated haemoglobin;
Validation study

Capillary density
The interobserver correlation for the assessment of capillary density during the first session and second session were reasonable with a regression coefficient of 0.75 ($P < 0.001$) and 0.72 ($P < 0.001$) respectively. The intraobserver regression correlation coefficients for the assessment of capillary density were 0.80 ($P < 0.001$) and 0.72 ($P < 0.001$) for observer-1 and observer-2.

Figure 2. Mean capillary density in non-diabetic controls, versus type 1 diabetic patients, and type 2 diabetic patients. Mean capillary density was significantly higher in patients with type 2 diabetes.
Capillary tortuosity

The interobserver values for the assessment of capillary tortuosity were excellent and similar for the first and second session with an agreement of 88% in tortuosity score ($\kappa = 0.83$). The intraobserver evaluation for tortuosity score revealed an agreement of 90% ($\kappa = 0.85$) for observer-1 and 88% ($\kappa = 0.83$) for observer-2.

Labial capillary density in patients with diabetes as compared to non-diabetic controls

Capillary density in type 1 diabetes

The mean capillary density in patients with type 1 diabetes (25 ± 4 per 0.63 mm$^2$) was not significantly different as compared to non-diabetic controls (24 ± 3 per 0.63 mm$^2$) (Figure 2). However, after correction for age and other cardiovascular risk factors in a backward multiple linear regression model, the presence of type 1 diabetes (with non-diabetic controls as reference) was found to be associated with an increased capillary density ($\exp \beta = 2.4$, 95% CI 0.8-3.9; $P = 0.003$).

Capillary density in type 2 diabetes

The mean capillary density was significantly increased in patients with type 2 diabetes (27 ± 4 per 0.63 mm$^2$) as compared to non-diabetic controls ($P = 0.001$) (Figure 2). Importantly, also after correction for age and other cardiovascular risk factors in a backward multiple linear regression model, the presence of type 2 diabetes (with non-diabetic controls as reference) maintained a significant association with capillary density ($\exp \beta = 1.0$, 95% CI 0.3-1.6; $P = 0.01$).
Labial capillary tortuosity in patients with diabetes as compared to non-diabetic controls

Capillary tortuosity in type 1 diabetes
Whereas in non-diabetic controls a high proportion of subjects had a low tortuosity score of 1 (45%), with a lower proportion of subjects in the tortuosity score 2 and 3 category (20%) (Figure 3A); in type 1 diabetes, the patients were more evenly distributed among the tortuosity scores 1-3 (Figure 3B). In type 1 diabetes, a relatively higher proportion of patients were stratified as having a tortuosity score 2 (27%) and 3 (37%) (Figure 3B). Accordingly, after correction for age and other cardiovascular risk factors in a backward multiple linear regression model, presence of type 1 diabetes (with non-diabetic controls as reference) was found to be associated with capillary tortuosity (Exp β 0.6, 95% CI 0.1-1.0; \( P = 0.02 \)).

Capillary tortuosity in type 2 diabetes
In contrast with non-diabetic controls, a minor proportion of patients with type 2 diabetes were stratified as having a low tortuosity score of 0 (1%) or 1 (13%) (Figure 3C). Whereas, a relatively large proportion of these patients had a high tortuosity score of 4 (27%). Indeed, after correction for age and other cardiovascular risk factors in a backward multiple linear regression model, the presence of type 2 diabetes (with non-diabetic controls as reference) was found to be a predictor of capillary tortuosity (Exp β 0.5, 95% CI 0.2-0.8; \( P = 0.001 \)).

CAD as assessed by MSCT in patients with diabetes
In the total sub-population of patients with diabetes, the mean CAC score was 213 ± 451. Overall, 39 (30%) patients with diabetes had an elevated CAC score of >100. Using MSCT angiography, presence of obstructive CAD (luminal narrowing ≥50%) was revealed in 31 (24%) patients with diabetes.

Relation of labial microvascular parameters with CAD in diabetes
The mean capillary density was higher in diabetic patients with a CAC score >100 (27 ± 4 per 0.63 mm²) as compared to those with a CAC score in the range 0-100 (25 ± 4 per 0.63 mm²) (\( P = 0.04 \)) (Figure 4A). Similarly, after stratification according to MSCT angiography results, a higher mean capillary density was observed in diabetic patients with obstructive CAD (27 ± 4 per 0.63 mm²), than in those without obstructive CAD (25 ± 4 per 0.63 mm²) (\( P = 0.02 \)) (Figure 4B). As demonstrated in Figure 5A, none of the diabetic patients with a tortuosity score 0 (pinhead capillaries) had an elevated CAC score of >100. The prevalence of an elevated
Figure 4. Relation of mean capillary density and CAD in asymptomatic diabetic patients. Mean capillary density was higher in patients with an increased CAC score of >100 (4A). Similarly, mean capillary density was higher in diabetic patients with obstructive CAD, as compared to those with no obstructive CAD (4B).

Figure 5. Relation of capillary tortuosity and CAD in asymptomatic diabetic patients. Relatively low prevalence of increased CAC scores of >100 were observed in patients with low tortuosity scores. On the contrary, the majority of patients with a high tortuosity score of 4 were revealed to have an increased CAC score of >100 (5A). A similar relation was observed between tortuosity score and the presence of obstructive CAD (5B).
CAC score increased modestly to 11% and 14% with a tortuosity score of 1 and 2. The prevalence of an elevated CAC score increased further to 33% in those with a tortuosity score of 3. However, the most prominent increase in the prevalence of an elevated CAC score (74%) was observed in diabetic patients with highly tortuous capillaries (tortuosity score 4).

Likewise, a low tortuosity score of 0 excluded the presence of obstructive CAD in patients with diabetes (Figure 5B). A relatively low prevalence of obstructive CAD (11% and 9%) was observed in patients with a tortuosity score 1 and 2. In contrast, the prevalence of obstructive CAD more than doubled (26%) in patients with a tortuosity score 3. Importantly, a 57% majority of diabetic patients with a high tortuosity score of 4, were shown to have obstructive CAD.

**Predictors of an elevated CAC score in diabetes**

The results of binary logistic CAC regression analysis for the evaluation of the risk factors associated with an elevated CAC score of >100 are provided in Table 2. Age, micro-albuminuria, hypercholesterolemia, hypertension and both capillary density and tortuosity were identified as potential predictors of an elevated CAC score, in patients with diabetes. Of note, after correction for other cardiovascular risk factors in a backward

### Table 2. Predictors of a CAC score > 100, in patients with diabetes. Results of binary logistic regression analyses.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Univariate Analysis</th>
<th>Multivariate Analysis (Backward)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp ß (95% CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>Age</td>
<td>1.12 (1.1-1.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male sex</td>
<td>1.94 (0.9-4.2)</td>
<td>0.09</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>1.02 (1.0-1.1)</td>
<td>0.16</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>1.14 (0.5-2.4)</td>
<td>0.73</td>
</tr>
<tr>
<td>Smoking</td>
<td>1.58 (0.7-3.6)</td>
<td>0.27</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>1.00 (0.9-1.1)</td>
<td>0.99</td>
</tr>
<tr>
<td>HbA1c</td>
<td>1.14 (0.9-1.4)</td>
<td>0.26</td>
</tr>
<tr>
<td>Micro-albuminuria</td>
<td>6.3 (2.4-15.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>2.58 (1.1-6.3)</td>
<td>0.04</td>
</tr>
<tr>
<td>Hypertension</td>
<td>5.52 (2.2-13.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Type 2 diabetes (vs. Type 1 diabetes)</td>
<td>1.29 (0.6-2.7)</td>
<td>0.50</td>
</tr>
<tr>
<td>Capillary density (number/0.63mm²)</td>
<td>1.10 (1.0-1.2)</td>
<td>0.06</td>
</tr>
<tr>
<td>Capillary tortuosity (score 0-4)</td>
<td>2.65 (1.7-2.7)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* All risk factors were entered in the model. Results are displayed for risk factors with a P-value ≤0.25. CAD = Coronary artery disease; HbA1c = glycated haemoglobin.
multiple logistic regression model, capillary density (Exp β 1.2, 95% CI 1.0-1.4; P = 0.03) as well as capillary tortuosity (Exp β 2.6, 95% CI 1.3-5.2; P = 0.01), were shown to maintain a significant association with the presence of elevated CAC. Using receiver operating characteristic (ROC) analysis a cut-off value of 24.9 per 0.63 mm² was identified for capillary density. This cut-off value yielded a negative- and positive predictive value of respectively 84% and 39% for predicting a CAC score >100. Of note, the positive predictive value improved from 39% to 66% in presence of a high tortuosity score of 4 besides a capillary density of ≥24.9 per mm².

Predictors of obstructive CAD in diabetes

As illustrated in Table 3, age, micro-albuminuria, hypertension, capillary density and capillary tortuosity were found to be associated with obstructive CAD in patients with diabetes. Notably, analysis in a multivariate binary logistic model showed capillary density (Exp β 1.3, 95% CI 1.1-1.5; P = 0.01) and capillary tortuosity (Exp β 1.9, 95% CI 1.0-3.6; P = 0.04) to be independently associated with the presence of obstructive CAD (Table 3).

Using a cut-off value of 24.9 per 0.63 mm² for capillary density yielded a negative- and positive predictive value of respectively 89% and 32% for predicting obstructive CAD in diabetes.
diabetes. The positive predictive value improved from 32% to 60% in presence of a high tortuosity score of 4 in addition to a capillary density of ≥24.9 per mm².

Video clip examples of labial microcirculation as assessed by SDF are provided in the supplementary files (online availability via Diab Vasc Dis Res). Supplementary file 1 shows an example of the well ordered labial capillaries in the healthy. In comparison, the more tortuous and malformed capillaries often observed in diabetic patients with CAD are shown in supplementary files 2 and 3.

DISCUSSION
The main findings of the current study were as follows: firstly, the inter-observer (regression coefficients per observer 0.75 and 0.72) and intraobserver (regression coefficients per observer 0.80 and 0.72) for the assessment of capillary density using SDF imaging were reasonable. Similarly, a good inter-observer (agreement per observer 88%) and intraobserver (agreement per observer 90% and 88%) was found for the assessment of capillary tortuosity using SDF imaging. Secondly, after correction for age, gender and other cardiovascular risk factors, the presence of both type 1- and type 2 diabetes was found to be associated with an increased capillary density and tortuosity. Most importantly, in the sub-population of asymptomatic patients with diabetes, the mean labial capillary density was significantly higher in the presence of an increased CAC of >100 (P = 0.04) and in obstructive CAD (P = 0.02). Moreover, after correction for other cardiovascular risk factors, mean capillary density was shown to be an independent predictor of increased CAC (P = 0.03) and obstructive CAD (P = 0.01), in diabetes. Likewise, the prevalence of increased CAC and obstructive CAD increased with capillary tortuosity. Indeed, the capillary tortuosity score was also found to be an independent predictor of increased CAC (P = 0.01) and obstructive CAD (P = 0.04) on MSCT of asymptomatic patients with diabetes.

Assessment of microcirculation by SDF
Past studies of vital microcirculation were restricted to the use of contrast microscopy and laser Doppler. Non-invasive imaging of the superficial skin and mucous microcirculation was initially implemented using the orthogonal polarization spectral (OPS) device. In OPS imaging the tissue embedding the microcirculation is illuminated with polarized green light. Illuminated light is absorbed by the haemoglobin in erythrocytes flowing through the tissue under investigation. As a result, the haemoglobin is used as the contrast agent, so that erythrocytes are imaged as dark globules in motion, against a white background. Consequently, the intravascular erythrocytes of perfused microvessels, rather than the microvessel walls are visualized. The imaging technique has been further modified in the SDF device to provide better visualization of the
microcirculation at capillary level. In SDF, stroboscopic imaging partially prevents smearing of moving features such as the flowing red blood cells due to short illumination intervals. The microcirculatory image is more restricted from contamination by tissue surface reflection. Also, as compared to OPS, imaging by SDF has a shallower focusing depth. Therefore, the structures underlying the microcirculatory image field interfere to a lower extent.

The OPS and SDF imaging devices have been previously validated and used to assess the functional anatomy of the sublingual, and nail fold microcirculation in critical care, and in patients with heart failure, sepsis and rheumatic diseases. In the present study the assessment of the quantity (capillary density) and structure (tortuosity) of the labial capillaries using the SDF was validated. Evaluation of the labial microvascular network in non-diabetic controls and patients with diabetes, showed the capillary density and tortuosity to increase with the presence of diabetes. The increased labial capillary density and tortuosity may be a maker of microvascular disease.

**Microvascular disease in diabetes**

In diabetes, abnormal microvascular patterns have been described in nephropathy, retinopathy, and the myocardial capillaries. Early morphological changes in the kidney of humans and animals with diabetic nephropathy include an increase in the number of glomerular capillaries as well as elongation and intermittent dilation and occlusion of the microvessels. Alternatively, diabetic retinopathy can be classified as the early non-proliferative stage with microaneurysms and haemorrhages, or the later proliferative stage with formation of neovessels. A study showed increased tortuosity of retinal vessels in presence of gestational diabetes. Less information is available on the architecture of the myocardial microvessels in humans. However, in animal models, higher spatial capillary density and tortuosity have been observed in the myocardium in presence of diabetes.

Various mechanisms have been proposed for the distortion of the microvascular network and the subsequent microvascular complications in patients with diabetes. Hyperglycaemia is shown to promote exposure of endothelial cells to AGES, resulting in protein kinase C activation, abnormal endothelial nitric oxide synthase expression and induction of Angiotensin-2 and vascular endothelial growth factor (VEGF). Experimental studies suggest that VEGF may in turn stimulate the expression of adhesion molecules by endothelial cells and promote vascular inflammation, causing more adverse endothelial perturbations. The overall molecular and functional changes result in the final sequela of increased permeability of the microvessels and finally ischemia that drives unregulated angiogenesis.
Relation of microvascular disease and CAD
Micro- and macrovascular complications of diabetes share a number of pathogenic mechanisms. Primarily, both processes include components of endothelial dysfunction and inflammation. In addition, hypoxia induced angiogenesis is also increased in the vasa vasorum of the coronary arteries of patients with diabetes. The corresponding neovascularization microangiopathy is suggested to accelerate atherosclerosis and predispose plaque rupture. Thus, microvascular disease and CAD may be interconnected, with microvascular disease prompting atherosclerosis through hypoxia and changes in the vasa vasorum.

Accordingly, the majority of follow-up studies in patients with diabetes have found the presence of microvascular disease to increase risk of CAD irrespective of traditional cardiovascular risk factors. During 5 years follow-up, Gall and colleagues found albuminuria to be a strong predictor cardiovascular mortality in patients with diabetes type 2 diabetes (HR 2.5 (1.1-5.8)). Similarly, in the EURODIAB Prospective Cohort Study of 2,787 patients with type 1 diabetes, both albuminuria and peripheral neuropathy were shown to predict cardiovascular mortality, whereas retinopathy did not. In contrast, in the Atherosclerosis Risk in Communities Study of patients with type 2 diabetes, the presence of diabetic retinopathy was found to be associated with a twofold risk of incident CAD and a threefold risk of fatal CAD, during an average follow-up of 7.8 years. In line with these findings, we found a significant and independent relation between the labial parameters of microvascular disease, comprising of the capillary density and tortuosity, with increased CAC and obstructive CAD in patients with diabetes. In particular, a low capillary density of <24.9 per 0.63 mm² yielded a good negative predictive value for an increased CAC (84%) and obstructive CAD (89%). Also, labial capillary tortuosity score of 0 to 2, was associated with a low prevalence of an increased CAC (0 -14%) or obstructive CAD (0-11%). In contrast, in diabetic patients with a capillary tortuosity score of 4, a relatively high prevalence of increased CAC (74%) and obstructive CAD (57%) was observed.

Study limitations
A number of limitations must be acknowledged. First, the parameters of the microcirculation as assessed by SDF imaging only reflect characteristics of perfused capillaries. In the current study, influence of external factors on capillary recruitment was limited by standardizing the study environment. Also, flow alterations in the microcirculation due to external pressure were prevented by minimizing probe contact with the labial tissue during image acquisition. Herewith a good inter-session reproducibility of parameters of the microcirculation was observed in healthy non-diabetic controls. However, perfusion of capillaries may be less consistent in diabetes.
as a consequence of functional and morphological changes. Secondly, the relation of labial capillary density and tortuosity with CAD could not be verified in the non-diabetic control group. As MSCT coronary angiography is accompanied with radiation exposure, it is not feasible to perform a similar assessment in asymptomatic subjects free of cardiovascular risk. Finally, the analysis was restricted to evaluation of the association between labial capillary density and tortuosity as assessed by SDF with traditional risk factors, as well as with the presence of CAD. However, the proatherogenic process which relates these microvascular parameters with CAD was not investigated.

**Conclusion and future perspectives**

The assessment of the labial capillary density and tortuosity, as markers of microvascular disease, is feasible and reproducible using the SDF imaging device. The labial capillary density and tortuosity increased with several traditional cardiovascular risk factors, micro-albuminuria and the presence of diabetes. A yet further increase in the labial capillary density and tortuosity was observed in diabetic patients with CAD. Assessment of the labial microvascular parameters using the non-invasive SDF handheld device may convey the potential to estimate the degree of vascular morbidity in patients with diabetes at bedside.
REFERENCES


