

# Detection and characterisation of microcirculatory abnormalities in the skin of diabetic patients with microvascular complications

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## Abstract

**T**he aim of this study was to characterise microvascular blood flow in the skin and to compare it with biomarkers of endothelial dysfunction and tissue inflammation in patients with type 2 diabetes with ( $n=20$ ) or without ( $n=20$ ) microvascular complications and 20 control subjects. Microvascular function was measured by laser Doppler velocimetry in combination with iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP). Blood was collected for measurement of biomarkers including plasminogen activator inhibitor-1 (PAI-1), soluble intercellular adhesion molecule (sICAM), soluble vascular cell adhesion molecule (sVCAM) and high-sensitivity C-reactive protein (hsCRP).

Both ACh and SNP responses fall progressively with the development of diabetes and microvascular complications. For the total cohort, there was a significant overall correlation between ACh and SNP response ( $r=0.7$ ,  $p<0.0001$ ), and this relationship was particularly strong in those with microvascular complications. There was a trend towards higher hsCRP levels across the three groups, but no difference in other biomarkers.

Abnormalities of microvascular blood flow are evident in diabetes and become more marked with the develop-

ment of microvascular complications. This relationship was similar to that shown by the marker of inflammation (hsCRP), but stronger than that pertaining to biomarkers of endothelial function. As both ACh and SNP responses are attenuated, the disturbance is not characteristic of endothelial dysfunction alone.

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**Key words:** biomarker, complications, endothelium, microvascular blood flow.

## Introduction

Diabetic complications are the major cause of morbidity and mortality in diabetes. The increased prevalence and severity of macrovascular disease in diabetes has been extensively investigated. Endothelial dysfunction has been implicated as a key mechanism to explain this increase, along with other possibilities.<sup>1–5</sup> The role of similar microvascular abnormalities in complications specific to diabetes, such as diabetic retinopathy, nephropathy and neuropathy, has been less well studied. One of the difficulties in studying this area is the lack of a test that is unequivocally representative of microvascular health. In this regard, one of the most direct tests that can be used is the measurement of skin blood flow and its response to vasodilatory agents.<sup>6–8</sup> Less directly, certain circulating biomarkers have also been used as surrogate measures of endothelial function; some of these include plasminogen activator inhibitor-1 (PAI-1), soluble intercellular adhesion molecule (sICAM) and soluble vascular cell adhesion molecule (sVCAM).<sup>9–16</sup> From a broader perspective, inflammation has also been incriminated in the pathogenesis of diabetes and its complications and could be the common factor mediating various pathways leading to microvascular disease and complications.<sup>11,15–18</sup> Learning the extent to which these indices of endothelial dysfunction and inflammation correlate with diabetes-specific complications will assist our understanding of the pathophysiology underlying diabetic complications.

The aim of this study was, therefore, to study skin blood flow and biomarkers of endothelial dysfunction or inflammation in control subjects and in diabetic patients with or without microvascular complications. The technique of

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**Table 1. Demographic and clinical parameters of study participants**

	Controls	Diabetes -ve complications	Diabetes +ve complications	Test statistic and p value
Number of participants	20	20	20	-
Sex (M/F)	7/13	10/10	13/7	NS
Age (years)	56 (47–61)	58 (54–64)	55 (49–62)	NS
BMI (kg/m <sup>2</sup> )	27.4±4.0	28.6±4.8	32.6±6.6 <sup>a</sup>	F=5.3, p=0.008
Systolic BP (mmHg)	119±12	126±9	133±12 <sup>a</sup>	F=8.5, p=0.0006
Diastolic BP (mmHg)	75±8	77±9	77±7	NS
Duration of diabetes (years)	N/A	5.8±4.8	14.1±6.2 <sup>b</sup>	T=-4.7, p=0.00003
HbA <sub>1C</sub> (%)	N/A	6.4 (6.3–7.2)	8.2 (7.5–9.7) <sup>b</sup>	Z=-3.9, p=0.0001
Serum creatinine (μmol/L)	N/A	71.6±16.7	78.4±21.9	NS
Albuminuria (mg/L)	N/A	7.0 (3.1–12.4)	82.8 (20.1–278.6) <sup>b</sup>	Z=-4.3, p=0.00002
ACR (mg/mmol)	N/A	0.5 (0.3–1.5)	8.2 (2.5–26.3) <sup>b</sup>	Z=-4.5, p<0.00001
Vibration perception (volts)	N/A	15 (12–23)	39 (15–50) <sup>b</sup>	Z=-2.6, p=0.009
Albuminuria/neuropathy/ retinopathy (%)	N/A	N/A	60/55/60	

**Key:** BMI = body mass index; BP = blood pressure; HbA<sub>1C</sub> = haemoglobin A<sub>1C</sub>; ACR = albumin/creatinine ratio; M = male; F = female

<sup>a</sup> Different to controls; <sup>b</sup> different to diabetes -ve complications

laser Doppler velocimetry in combination with iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) was used to evaluate the relative contribution of endothelial and non-endothelial microvascular abnormalities to the observed changes.

### Research design and methods

#### Diabetic participants and control subjects

A total of 40 consecutively eligible and consenting patients (23 males and 17 females, mean age 57 years, range 42 to 73 years) with type 2 diabetes who attended the Diabetes Centre, Royal Prince Alfred Hospital (RPAH) were recruited to take part in this study. Patients were excluded if they had clinical evidence of macrovascular disease such as angina, myocardial infarction, coronary angioplasty, a history of stroke or an abnormal ECG. Five patients were on dietary treatment alone, 18 patients were on oral hypoglycaemic agents and 17 patients were on insulin or combined insulin/oral agent treatment.

A total of 20 control subjects (seven males and 13 females, mean age 53 years, range 37 to 66 years) were also recruited from the general community, or were staff members of the Hospital. All subjects gave no history of cardiac disease or diabetes; they had their blood glucose level checked to exclude the presence of diabetes.

The protocol was approved by the Sydney South West Area Health Service Ethics Review Committee (RPAH Zone), and all study participants gave their written informed consent.

#### Study design

This was a cross-sectional study performed at the Diabetes Centre, RPAH. All study participants attended the Diabetes Centre for laser Doppler velocimetry studies and collection of blood for measurement of biomarkers. Diabetic patients also attended the Diabetes Centre for a full metabolic and complications assessment.

#### Laser Doppler iontophoresis

Skin blood flow (SkBF) was measured using a single-point laser probe, a MICI iontophoresis system, and a DRT4 Laser Doppler Blood Flow Monitor (MOOR Instruments Millwey, Devon, UK). Laser Doppler velocimetry measures SkBF in terms of red cell flux (the product of the average speed and concentration of moving red blood cells in the tissue sample volume) through the capillary bed.

All participants were seated comfortably in a chair and allowed to rest for 20 minutes in a temperature-controlled room before studies were begun. A perspex chamber with a hole in its centre was attached to the volar surface of the forearm by double-sided adhesive tape. To measure the component of SkBF directly mediated by endothelial function, the laser probe was placed within the hole, which contained a solution of 1% ACh (Sigma-Aldrich, St Louis, MO, US) in deionised water. Basal SkBF was monitored for two minutes by the laser Doppler probe, and then ACh was iontophoresed (200 μA for 80 seconds, achieving a dose of 16 millicoulombs) into the subcutaneous space. Post-iontophoresis SkBF was measured over the next four

**Table 2. Skin blood flow and biomarker parameters of study participants**

	Controls	Diabetes -ve complications	Diabetes +ve complications	Test statistic and p value
Number of participants	20	20	20	-
SkBF (ACh)	11.4 (6.7–14.5)	9.1 (7.1–12.1)	6.0 (4.2–8.7) <sup>a</sup>	F=9.8, p<0.001 T <sub>trend</sub> =4.2, p<0.001
SkBF (SNP)	7.4±2.3 <sup>b</sup>	5.1±2.4	4.3±1.9	F=10.1, p<0.001 T <sub>trend</sub> =4.3, p<0.001
PAI-1 (ng/ml)	78.6±64.8	59.9±53.8	69.7±50.3	NS
sICAM (pg/ml)	1,451±459	1,434±585	1,745±627	NS
sVCAM (ng/ml)	2,084±614	2,061±784	2,477±838	NS
hsCRP (mg/L)	1.6 (0.4–2.9)	2.2 (0.8–3.0)	2.8 (1.5–7.8)	F=3.0, p=0.06; T <sub>trend</sub> =2.3, p<0.05

**Key:** SkBF = skin blood flow; ACh = acetylcholine; SNP = sodium nitroprusside; hsCRP = high-sensitivity C-reactive protein; sICAM = soluble intercellular adhesion molecule; sVCAM = soluble vascular cell adhesion molecule

<sup>a</sup> Different to controls and diabetes -ve complications; <sup>b</sup> different to diabetes -ve complications and diabetes +ve complications

minutes. Endothelial function was quantitated by calculating the ratio of blood flow post-iontophoresis and at baseline, and expressed as fold increase. Acetylcholine causes a localised endothelium-dependent vasodilatation, although the precise contribution of nitric oxide (NO), vasodilatory prostanoids, endothelium-dependent hyperpolarising factor and cutaneous sensory nerves in mediating the vasodilatation remains uncertain.

The increase in SkBF induced by a solution of 1% SNP (Sigma-Aldrich, St Louis, MO, US) in deionised water was measured using the same technique at a different site on the forearm. Sodium nitroprusside bypasses the endothelium to relax vascular smooth muscle directly, thus allowing measurement of endothelium-independent vasodilatation. Again, this was calculated as the ratio of blood flow post-iontophoresis and at baseline, and expressed as fold increase.

The day-to-day reproducibility of the technique was evaluated in six healthy people (aged between 27 and 59 years). The coefficient of variation (CV) for the fold increase from baseline was 22% for iontophoresis of ACh and 30% for SNP.

#### Measurement of biomarkers

Blood samples (20 ml) were collected from each participant and put into either a heparinised or plain tube and allowed to clot. Samples were then spun at 3,000 rpm for 10 minutes and the plasma or serum was then aliquoted and stored frozen at -20°C for assay. Plasma samples were used for the measurement of high-sensitivity C-reactive protein (hsCRP) by rate nephelometry on an automated image instrument (Beckman-Coulter, Ryde, Australia) and sVCAM and sICAM by ELISA (R&D Systems, MN, US). Serum samples were used for measurement of PAI-1 by

enzyme-linked immunosorbent assay (ELISA) (Biopool international, Ventura, CA, US). Haemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>) was determined by high-performance liquid chromatography (HPLC) (BioRad, Regents Park, Australia). All samples were assayed according to the manufacturers' instructions, with between-assay CVs less than 10%.

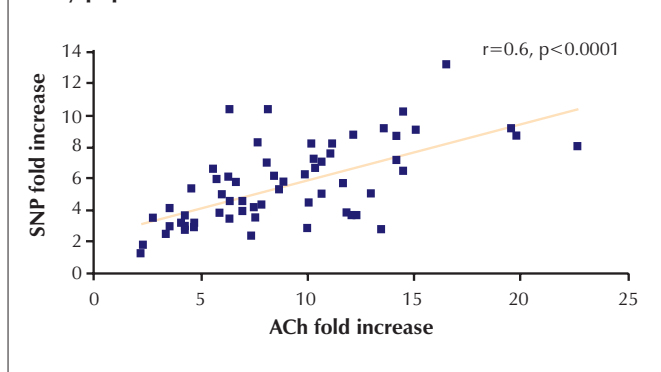
#### Diabetes metabolic and complications assessment

Glycaemic control of diabetic subjects was assessed by measurement of HbA<sub>1c</sub> level. Study participants were screened for the presence of microvascular complications. Direct funduscopy was performed, with the participant's pupils dilated, to screen for diabetic retinopathy. A spot urine collection was performed to screen for micro-albuminuria; sensory nerve fibre function was assessed by measuring vibration perception on the dorsal surface of the foot using a biothesiometer. Study participants were classified as having microvascular complications if they had evidence of retinopathy and/or albuminuria and/or peripheral neuropathy. Diabetic retinopathy was diagnosed if there was evidence of microaneurysms, haemorrhages, hard exudates and/or neovascularisation on funduscopy. Albuminuria was defined as an albumin/creatinine ratio > 2.5 mg/mmol for men and > 3.5 mg/mmol for women. Peripheral neuropathy was considered to be present if biothesiometer readings were > 40 volts in both feet. Patients with a history of previous vascular disease were excluded from the study.

#### Statistical methods

Statistical analysis was performed using the NCSS (Number Cruncher Statistical System, Kaysville, UT, US) statistical package. Data are expressed as proportions, percentages, mean ± standard deviation (SD), or median and interquartile

**Figure 1. Relationship between ACh-dependent and SNP-dependent skin microvascular blood flow in the total study population**



range, depending on whether the data are normally distributed. A two sample *t*-test or Wilcoxon's rank sum test was used to analyse data for continuous variables and the  $\chi^2$  test was used for categorical variables. Analysis of variance, including analysis for linear trend, was used to examine the relationship between endothelial dysfunction and biomarkers between the three groups. Linear regression was used to examine the relationship between ACh and SNP response. If required, variables were normalised by log transformation (log 10). Statistical significance was inferred at a two-tailed *p* value < 0.05.

## Results

Diabetic patients and control subjects were well matched in terms of age, although diabetic patients had a higher body mass index (BMI) and systolic blood pressure (table 1). Diabetic patients with microvascular complications had longer duration of diabetes and worse glycaemic control than diabetic patients without complications (table 1).

### *Relationship between endothelial dysfunction, biomarkers and diabetic complications*

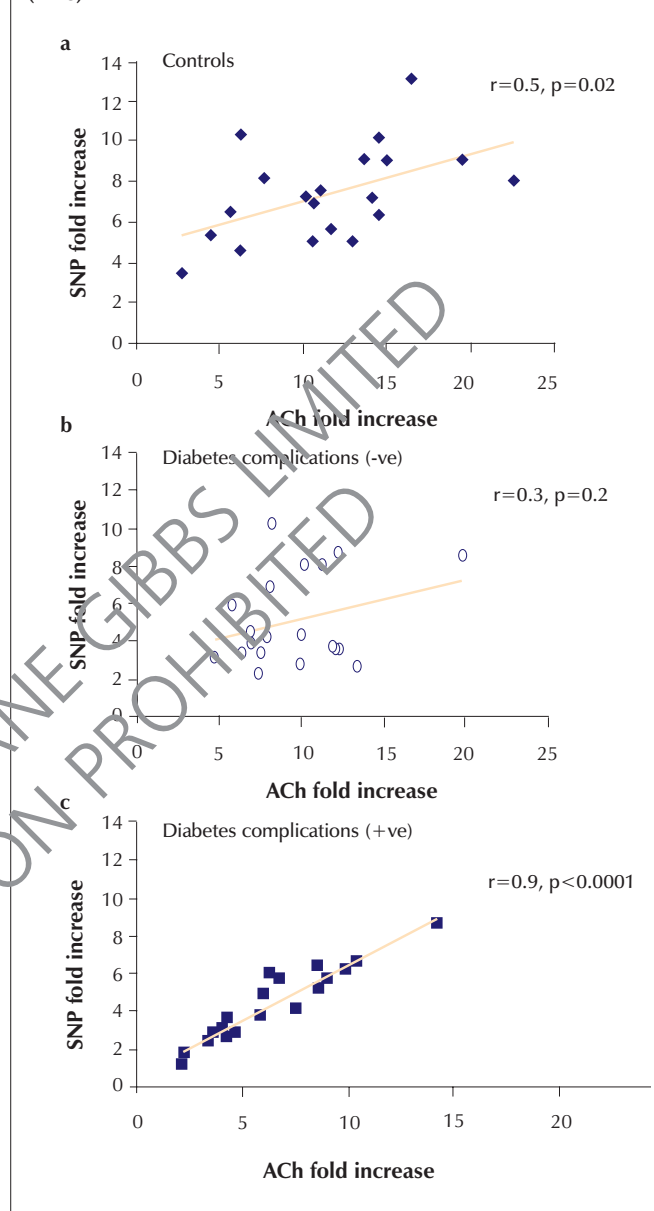
Both ACh and SNP responses fall progressively with the development of diabetes and microvascular complications (table 2). There was a significant overall correlation between ACh and SNP responses ( $r=0.6$ ,  $p<0.0001$ , figure 1), and this relationship was particularly evident in those with complications (figure 2).

There was a trend towards higher CRP levels across the three groups, but no difference between groups for the levels determined for PAI-1, sICAM or sVCAM (table 2).

## Discussion

This study shows that abnormalities of microvascular blood flow are clearly demonstrable in diabetic subjects and that they become more marked with the development of microvascular diabetic complications. This trend is comparable to that of hsCRP, the marker for inflammation, but much stronger than the trend for other commonly measured markers of endothelial dysfunction measured in this study. In the literature, sICAM and sVCAM tend to be elevated when albuminuria is severe,<sup>19-21</sup> whereas in our

**Figure 2. Relationship between ACh-dependent and SNP-dependent skin microvascular blood flow: (a) controls; (b) diabetes complications (-ve); (c) diabetes complications (+ve)**



cohort, even in those patients with complications, the albumin/creatinine ratio was only about twice the upper limit of normal. This may explain the absence of a significant increase in sICAM and sVCAM in our study.

The measurement of factors in the circulation is limited by a number of potential confounders, especially the multiple sources of tissue production commonly affecting appearance of a particular factor in the bloodstream. Renal function is often a rate-limiting step regulating clearance and thus steady state levels may be difficult to measure. While our study participants did not have significant renal impairment, specificity of any circulating marker to the microvasculature remains an issue. For example, circulating PAI-1 is produced not only by vascular endothelial cells<sup>14</sup> but also robustly by adipose tissue.<sup>22</sup> In addition,

circulating markers may not necessarily reflect tissue function. With these considerations, it is not surprising that the dynamic assessment of microvessel function by laser Doppler velocimetry co-segregates best in this study with diabetic microvascular complications. The cross-sectional nature of our study does not allow us to attribute causality but our observations do suggest that chronic inflammation and microvascular blood flow abnormalities are related to development of diabetic microvascular complications.

Traditionally, increase in blood flow in response to ACh is regarded as an index of endothelial function. However, this would only be true when the response to SNP is normal. In our study, as both ACh and SNP responses were attenuated in diabetes, the microvascular disturbance we observed is not characteristic of endothelial dysfunction alone, but indicates that there must also be an impairment in the ability of the microvessels to respond directly to a vasodilator. This latter phenomenon could be due to a functional defect of vascular smooth muscle to respond to NO, excessive quenching of NO after it is either produced endogenously by the endothelium or administered exogenously, or to changes of the microvascular wall resulting in increased structural stiffness. Amongst many possible mechanisms, factors that may have contributed include excess production of reactive oxygen species, increased formation of advanced glycation end-products or increased cross-linking of vascular wall components. Further studies would be required to identify which of these factor(s) is playing a significant role.

Overall, there is a strong relationship between responsiveness of the ACh-dependent and SNP-dependent components of skin microvascular blood flow in the study cohort. Interestingly, different behaviours were observed when this association was stratified by subject group. The positive relationship between ACh-dependent and SNP-dependent blood flow was observed in control subjects and was particularly strong in diabetic patients with microvascular complications, but was less strong in the diabetes group without complications. This indicates that during the relatively early stage in the natural history of diabetes (in this case over a mean diabetes duration of about six years), blood flow response to ACh and SNP becomes abnormal at different rates. The falls in these two parameters are therefore not always in parallel in diabetic individuals. By the stage of diabetes when clinically significant microvascular complications have emerged, the correlation between ACh and SNP responsiveness was exceptionally strong. Our study does not provide a ready explanation for these observations. However, our hypothesis is that in the earlier phase of diabetes, microvascular blood flow abnormalities are the result of a number of functional changes in the cellular or extracellular biochemical milieu, of which only some are NO-related. This would explain the dissociation of ACh and SNP responses.

Consistent with this line of thinking is that ACh responsiveness of the skin microcirculation is thought to be affected by a number of factors, including NO, vasodilatory prostanoids, endothelium-dependent hyperpolarising factor and sensory cutaneous nerves; these can be affected to

a different extent at different stages of diabetes. By contrast, in the phase of diabetes with microvascular complications, some change(s) related to the NO responsiveness could become the dominant factor. Conceptually, this would affect the ACh and SNP responsiveness equally, accounting for their excellent correlation. The fact that skin microvascular blood flow is affected by endothelial and non-endothelial factors might also explain why it appears to be a more sensitive indicator of diabetes and its microvascular complications than the conventional markers of endothelial function tested in our study.

Although various dermatological diseases are described in diabetes, the skin is not usually considered to be an organ significantly affected by diabetic complications. It may be argued that microvascular changes in the skin are not of great consequence in the development of diabetic complications. However, our study showed that the microvasculature of the skin is affected by diabetes, and to a degree reflective of the severity and duration of diabetes, and may serve as a convenient conduit for studies of microvascular diabetic complications.

#### Conflict of interest statement

None declared.

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