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Endothelial dysfunction and inflammation in asymptomatic proteinuria

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Endothelial dysfunction and inflammation in asymptomatic proteinuria.

Background. Proteinuria is associated with vascular risk and a systemic increase in vascular permeability. Endothelial dysfunction occurs early in atherosclerosis and modulates vascular permeability. Vascular risk and chronic inflammation are associated. This study investigates whether the increased vascular permeability in proteinuria reflects systemic endothelial dysfunction and chronic inflammation.

Methods. Twenty-one patients with asymptomatic proteinuria (1.29 g/24 h; range 0.18 to 3.17) and 21 matched controls were studied. Microvascular endothelial function was assessed using acetylcholine iontophoresis. Maximum microvascular hyperemia (MMH) was assessed by flux response to local skin heating. Macrovascular endothelial function was assessed by flow-associated dilation (FAD) in the brachial artery using ultrasound. von Willebrand factor (vWF) was measured as a marker of endothelial activation. Low-grade inflammation was assessed by measurement of circulating C-reactive protein (CRP) values using a high sensitivity assay.

Results. FAD was impaired in proteinuric subjects (AP) compared to controls [1.8 (0.2 to 5.3) AP vs. 3.8 (1.5 to 6.2) C %; $P = 0.014$]. There was no significant difference between groups in MMH or in the response to acetylcholine iontophoresis. The AP group had a higher CRP [4.0 (0.5 to 39.0) AP vs. 0.2 (0.1 to 21.3) C mg/L; $P < 0.001$] and tendency to higher vWF [101.5 (67.0 to 197.0) AP vs. 77.5 (45.0 to 185.0) C IU/dL; $P = 0.046$] compared to controls. In the AP, but not control, group there was an inverse correlation between CRP and microvascular function as determined by acetylcholine iontophoresis ($r = -0.509$; $P = 0.018$).

Conclusions. In AP subjects there is evidence of macrovascular endothelial dysfunction remote from the kidney and of low-grade inflammation that is associated with microvascular endothelial dysfunction.

Key words: endothelial function, iontophoresis, brachial artery ultrasound, inflammation, C-reactive protein, von Willebrand factor, proteinuria.

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Proteinuria is associated with an increased prevalence of vascular disease in both diabetic [1] and non-diabetic populations [2, 3].

Evidence suggests that proteinuria does not solely reflect renal pathology but is also associated with a systemic increase in vascular permeability. In the normal physiological response to exercise or to passive ascent to altitude, for example, the development of proteinuria and a raised capillary fluid permeability in the calf are contemporaneous [4]. Capillary fluid permeability is also increased in otherwise healthy patients with dipstick positive proteinuria [abstract; Lewis DM, *XIV International Congress of Nephrology* 3(Suppl 1):S235, 1997] and in patients with nephrotic syndrome [5]. Even otherwise healthy patients with microalbuminuria demonstrate increased transvascular albumin leakage [6]. Given that increased transvascular leakage, which could allow entry of lipoproteins into the vessel wall [7], is an early event in atherogenesis, these demonstrations of an association between proteinuria and vascular permeability are interesting.

The mechanism of this increased vascular permeability in proteinuric subjects is unknown. One possibility is that it is due to abnormal endothelial function. There is now considerable evidence to suggest that contraction of endothelial cells may change intercellular cleft size [8], that transcellular holes influence fluid and macromolecular movement across the vascular wall [9], and that vesicular transport of albumin is controlled by endothelial function. Although the role of caveolae in endothelial uptake and transport is not fully defined, most [10–12] but not all [13] studies suggest that caveolae are important for albumin transport across microvessels. Recent evidence supports an association between isolated proteinuria and endothelial dysfunction, at least at the macrovascular level [14]. Studies investigating serum values of soluble markers of endothelial activation such as von Willebrand factor (vWF) in proteinuria have produced conflicting results [14–16]. To date there have been no studies in-

vestigating microvascular endothelial function in isolated proteinuria.

Endothelial dysfunction is a primary event in atherosclerosis [7, 17] and occurs in established atherosclerosis [18, 19] as well as in young asymptomatic patients at risk of atherosclerosis [20, 21]. Injury to the endothelium results in increased adhesiveness, increased permeability, altered production of vasoactive mediators and, importantly, a continued inflammatory response [22]. Recently the association between atherosclerosis and chronic low grade inflammation has become apparent. The transition from stable to unstable angina is associated with increased plasma values of C-reactive protein (CRP), serum amyloid A protein and interleukin-6 (IL-6) indicative of a systemic inflammatory response [23, 24]. Numerous studies have confirmed the independent prognostic relevance of CRP for the risk of coronary artery disease not only in patients with angina [25, 26], but also in apparently healthy men [27, 28].

This study aims to investigate whether the increased vascular permeability in asymptomatic proteinuric subjects reflects micro- and macrovascular endothelial dysfunction and/or chronic low grade inflammation, as assessed by circulating CRP values using a high sensitivity assay (HsCRP). Control of blood vessel and endothelial function is complex, with different mechanisms operating depending on vascular bed and vessel diameter [29, 30]. Therefore, we studied endothelial function not only in large conduit arteries, using a brachial artery wall-tracking technique, but also in the microcirculation, using iontophoresis. Circulating values of von Willebrand factor (vWF), a soluble marker of endothelial activation [31], were studied also.

METHODS

Patients and control subjects

Twenty-one patients (15 males, median age 39 years; range 19 to 59) with stable asymptomatic dipstick-positive proteinuria (AP) and 21 age, sex, body mass index (BMI), smoking habit and hormonal status matched control subjects were studied. Suitable patients were recruited consecutively from the renal outpatient clinic; all were otherwise well and were not nephrotic. Underlying renal pathologies were as follows: eight IgA nephropathy, two thin basement membrane disease, one focal segmental proliferative glomerulonephritis (GN), one Henoch-Schönlein purpura, and nine presumed chronic GN as no renal biopsy was performed. Control subjects were recruited from a panel of healthy volunteers. Exclusion criteria were: (*1*) hypertension (BP >160/95); (*2*) serum total cholesterol >7 mmol/L; (*3*) diabetes or fasting hyperglycemia; (*4*) evidence of macrovascular atherosclerotic disease (as determined by history, electrocardiogram, diminished lower limb pulses or ankle brachial pressure index <1.0); (*5*) Raynaud's disease or other condition known to influence vascular function; and (*6*) use of vasoactive medication (except hormonal; of the proteinuric patients, one was taking post-menopausal hormone replacement therapy and one was receiving depot contraception; both were matched with control subjects on the same type of medication).

Macrovascular function was measured in nine proteinuric subjects, the estimation not being possible in the remainder because of: inadequate image of artery ($N = 7$), movement artifact ($N = 2$), cuff inflation not tolerated ($N = 1$), and inability to schedule the examination ($N = 2$).

Study design

Subjects were studied in the morning after an overnight fast and were asked to refrain from smoking on the day of the study. All studies were performed with the subject supine in a temperature-controlled room (21.5 to 22.5°C) after acclimatizing supine for 30 minutes. Brachial artery BP was determined from the mean of the last three of five readings using an automated BP recorder (Dynamap 845; Critikon Inc., Tampa, FL, USA). Skin temperature was measured close to site of study by thermocouple (Fluke 52; RS Components, Corby, UK).

Iontophoresis, wall tracking and maximum blood flow measurements were undertaken at separate times but under the same experimental conditions. At the end of the study a supine venous blood sample was taken. Plasma concentration of creatinine, glucose, albumin, fibrinogen, urate and lipids were measured by standard methods in the laboratory of the Royal Devon and Exeter (RD&E) Hospital. A sensitive, two-site enzyme-linked immunosorbent assay (ELISA) for the determination of CRP was set up using antibodies and human CRP calibrator (X0923) from Dako Diagnostics (Ely, Cambridgeshire, UK). The calibrator was prepared per the manufacturer's instructions to provide an assay range of 1.5 to 48 μg/L. Samples were diluted 1:100 prior to assay, allowing levels of 0.15 to 4.8 mg/L to be detected. The assay has a sensitivity of 0.15 mg/L and inter- and intra-assay coefficients of variation (CV) of <10%. All values under the limit of detection were assigned a value of 0.1 mg/L. von Willebrand factor antigen was assayed by an in-house ELISA, using a monoclonal antibody from Dako Diagnostics and the current British Standard (NIBSC, Potter's Bar, UK). The assay has a sensitivity of 5 to 250 IU/dL and inter- and intra-assay CVs of 6.0% and 7.7%, respectively.

Proteinuria was quantified by early morning urinary albumin/creatinine ratio and single 24-hour urine collection. Creatinine clearance was determined using 24-hour urine collection. Urinary infection was excluded by microscopy and culture. The study was approved by the local research ethics committee and participants gave written informed consent.

Measurement of microvascular endothelial function

Skin erythrocyte flux in the forearm microcirculation following iontophoretic application of acetylcholine (Ach; an endothelial-dependent vasodilator [32]) and sodium nitroprusside (SNP; a nitric oxide donor and endothelial-independent vasodilator acting via smooth muscle) was evaluated by laser Doppler perfusion imaging (LDPI) as described in detail previously [33].

An electrode chamber (Moor Instruments, Axminster, Devon, UK) was attached to the flexor aspect of the cleaned forearm, avoiding hair and freckles. An indifferent electrode was attached to the wrist to complete the circuit.

An iontophoresis controller (MIC 1; Moor Instruments) provided direct current for iontophoresis. Ach (1% Miochol; IOLAB, Bracknell, Berkshire, UK) and Ach vehicle (3% mannitol in water for injection; RD&E Hospital Pharmacy, Exeter, Devon, UK) were delivered using an anodal current [5 $\times$ 0.1 mA for 20 seconds with a 60 second interval between each dose; total charge 10 millicoulombs (mC)]. Erythrocyte flux was recorded immediately before the start of iontophoresis (baseline) and 60 seconds after each period of drug application. SNP (0.01% Nipride; Roche, Welwyn Garden City, Herts., UK) and SNP vehicle (sterile distilled water; Baxter Healthcare Ltd., Thetford, Norfolk, UK) were delivered using a cathodal current (1 $\times$ 0.2 mA for 60 seconds; total charge 12 mC). Erythrocyte flux was recorded at baseline and at 0, 60, 120, 180, 240 and 300 seconds after drug application. The responses to each vehicle and drug were sequentially measured at one and three sites on the forearm, respectively. The cathodal current employed in SNP iontophoresis causes non-specific skin vasodilation probably via local sensory nerve activation [34]. To minimize this charge effect, all SNP iontophoresis was performed within areas pre-treated with EMLA cream (2.5% lignocaine, 2.5% prilocaine; Astra Pharmaceuticals Ltd., Kings Langley, UK). The adequacy of sensory nerve blockade was assessed by pinprick testing immediately before and after each iontophoresis protocol. A site was rejected if anesthesia was $<$40%. At the end of each study, biological zero, that is, the flux value without arterial inflow, was measured at both an untreated and a vasodilated site during arterial occlusion of the upper arm by a cuff inflated to 220 mm Hg.

The laser Doppler perfusion imager (Lisca PIM 1.0; Lisca Development AB, Linköping, Sweden) generates a signal proportional to tissue perfusion (defined as concentration $\times$ average velocity of moving erythrocytes). The responses to drug application were calculated as the mean of perfusion at 81 points over a 0.78 cm^2 area and expressed in volts (V). This is important as there is significant heterogeneity of skin blood flow [35].

Results are expressed as absolute responses minus vehicle responses, which removes the biological zero component in addition to any vehicle effect. The flux response for each subject was taken as the mean response from the three forearm sites used for each drug.

The intrasubject day-to-day reproducibility for Ach iontophoresis, measured at maximal response, was 8.9 $\pm$ 1.8% and for SNP iontophoresis, measured 300 seconds after the end of the current, was 25.5 $\pm$ 2.1% (five times in each of two subjects).

Measurement of maximum microvascular hyperemia

This method is described in detail elsewhere [36]. Briefly, an area of skin on the dorsum of the foot was heated to 42°C for 30 minutes using a thermostatically controlled brass heater (Moor Instruments). Heating the skin to this temperature induces maximal vasodilation [36]. Maximum microvascular hyperemia (MMH) was measured non-invasively by laser Doppler fluximetry (3 mW, 640 nm red laser source; Periflux Pf2; Perimed, Stockholm, Sweden) and expressed in arbitrary units of volts. MMH was taken as the mean of flux in eight equidistant sites within the heated area. Measuring flow in multiple sites improves the reproducibility of this technique, as there is significant point-to-point variation in microvascular structure and function in the skin. The mean intrasubject CV for this technique is 5.8 $\pm$ 1.7%. As BP depends on cardiac output and total peripheral resistance, it is possible to estimate the resistance to flow in a maximally dilated microcirculatory bed by dividing mean arterial pressure (MAP) by MMH [37]. This minimum vascular resistance (MVR) is expressed in arbitrary units of mm Hg $\cdot$ V^{-1}.

Measurement of macrovascular endothelial function

Conduit artery endothelial-dependent function was determined by measuring flow-associated dilation (FAD) in the brachial artery using a high resolution ultrasound Wall Tracking System (Pie Medical, Maastricht, the Netherlands). The diameter of the brachial artery was measured at rest, during reactive hyperemia (endothelial-dependent FAD) [38, 39] and after sublingual glyceryl trinitrate (GTN; a nitro donor causing endothelial-independent dilation). This is a well-established, validated technique and has been previously described [20, 40, 41]. Briefly, the right arm was supported at heart level and scanned continuously above the elbow in longitudinal section using a 7.5 MHz linear array probe. Vessel wall movements were tracked allowing determination of end diastolic internal vessel diameter. Studies were rejected if satisfactory images of the artery could not be obtained or if there was a significant change in sampling position during the experiment. The mean end diastolic diameter was calculated from five cardiac cycles incident with the R wave on the electrocardiogram.

Brachial artery blood velocity was measured simulta-

Table 1. Patient characteristics

Variable	All subjects		*P* value
	Proteinuric subjects	Controls	
Age *years*	39 (19–59)	41 (20–59)	0.782
Sex *male/female*	15/6	15/6	
BMI kg/m^2	26.3 (19.8–42.7)	24.6 (18.0–39.3)	0.421
Smoking *current/ex/never*	4/7/10	4/7/10	
SBP *mm Hg*	128 (110–160)	122 (106–154)	0.078
DBP *mm Hg*	77 (63–94)	73 (54–91)	0.113
MAP *mm Hg*	95 (79–114)	89 (73–112)	0.107
Serum creatinine *μmol/L*	85 (60–237)	89 (57–100)	0.940
Creatinine clearance *mL/min*	113 (40–223)	127.5 (89–177)	0.355
Urinary protein[a] *g/24 h*	1.29 (0.18–3.17)	0.09 (0.01–0.23)	<0.001
Urinary Alb/Cr ratio[a] *mg/mmol*	44.5 (0.3–184.4)	0.2 (0.1–3.0)	<0.001
Albumin[a] *g/L*	37 (32–41)	39 (35–43)	0.002
Fasting glucose *mmol/L*	4.7 (4.0–5.4)	4.6 (3.6–6.0)	0.536
Cholesterol *mmol/L*	5.0 (3.5–7.4)	4.6 (2.7–6.9)	0.246
LDL *mmol/L*	3.3 (1.3–5.2)	3.0 (1.0–4.6)	0.589
Triglyceride *mmol/L*	1.56 (0.54–2.61)	0.94 (0.52–2.49)	0.148

Data are shown as median (range). Abbreviations are: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic BP; MAP, mean arterial pressure; Alb/Cr, albumin/creatinine; LDL, low density lipoprotein.

[a] $P < 0.05$

neously with an 8 MHz continuous wave Doppler probe (Huntleigh, Cardiff, Wales, UK) positioned just distal to the 7.5 MHz transducer. Since velocity was taken from the center of the artery absolute values may be overestimated, but relative values before and after cuff inflation are accurate [42].

For each study baseline brachial artery diameter was measured at rest and then reactive hyperemia was induced by inflation of a forearm pneumatic cuff to a pressure of 220 mm Hg for five minutes. After the cuff was released, the brachial artery diameter was measured at 1, 2, 3, 5, 10 and 15 minutes. Once all hemodynamic measurements had returned to baseline, sublingual GTN (400 μg) was administered and diameter measured after 3, 5, 10 and 15 minutes. Vessel diameter data are expressed as percentage change compared to baseline and blood velocity as forward and reverse velocity and peak time average velocity.

The CV for baseline brachial artery diameter was 2.5 ± 1.7%. Intrasubject day-to-day CV for FAD at one minute was 20.9 ± 8.9% (2 subjects studied 4 times each) and for measurements following administration of GTN 20.0%, 21.5%, 15.4% and 20.5% at 3, 5, 10 and 15 minutes, respectively.

Statistics

Power calculations suggest that our sample size provided a 90% chance of detecting a 21.6% difference in MMH, a 25.4% difference in microvascular endothelial-dependent vasodilation (Ach), and a 51.3% difference in macrovascular FAD at 5% level of significance. A previous study of vascular permeability in AP subjects showed a 63% increase compared to controls [abstract; Lewis DM, *XIV International Congress of Nephrology* 3 (Suppl 1):S235, 1997]. Unless otherwise stated, results are expressed as median and range. Normality was checked. Comparisons between groups are made by the Mann-Whitney test. Spearman's rank-correlation coefficients were calculated where appropriate. Pearson correlation coefficients were calculated for CRP data after normality was achieved with appropriate transformation. Assessment of iontophoresis responses are made using two-way analysis of variance for repeated measures (ANOVA); sphericity was not assumed. Results were considered to be statistically significant with a P value <0.05.

RESULTS

Characteristics for the two groups are shown in Table 1.

Microvascular endothelial function

Basal forearm skin erythrocyte flux was not significantly different in the AP subjects compared with control subjects [0.17 (0.08 to 0.40) AP vs. 0.16 (0.09 to 0.33) C volts; $P = 0.283$]. The responses of erythrocyte flux to the iontophoresis of Ach and SNP in AP and C subjects are shown in Figure 1. Erythrocyte flux significantly increased following iontophoresis of Ach or SNP in both groups ($P < 0.001$). The vasodilation to ACh ($P = 0.534$ at the sixth time point, time 400 seconds) or to SNP ($P = 0.568$ at the seventh time point, time 360 seconds) was not significantly different between groups.

Maximum microvascular hyperemia

Maximum microvascular hyperemia was not significantly different between the two groups [1.67 (0.94 to 2.67) AP vs. 1.47 (1.18 to 2.12) C volts; $P = 0.571$; Fig. 2].

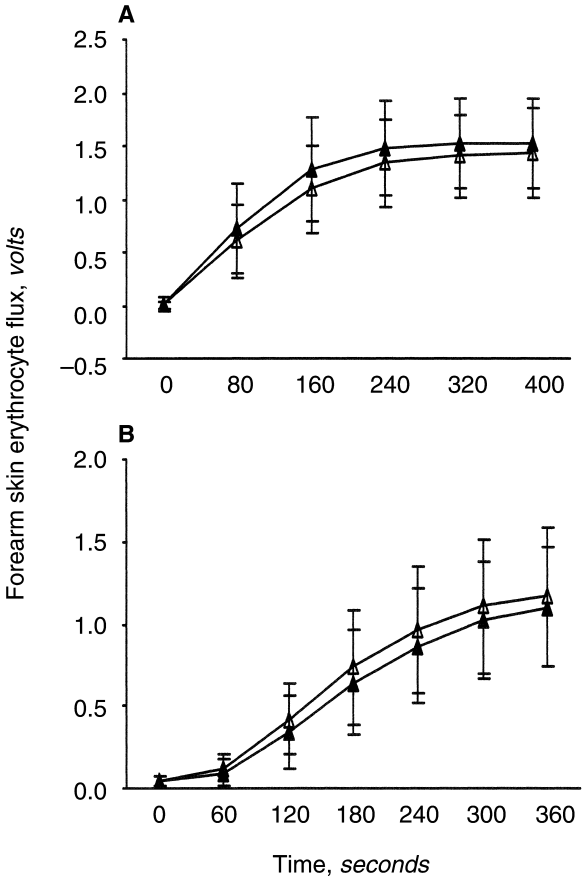

Fig. 1. Response of forearm skin erythrocyte flux (volts, mean ± SD, $N = 21$) following iontophoresis of acetylcholine (Ach; *A*) and sodium nitroprusside (SNP; *B*) in proteinuric subjects (▲) and controls (△). $P = 0.534$ in Ach, $P = 0.538$ in SNP.

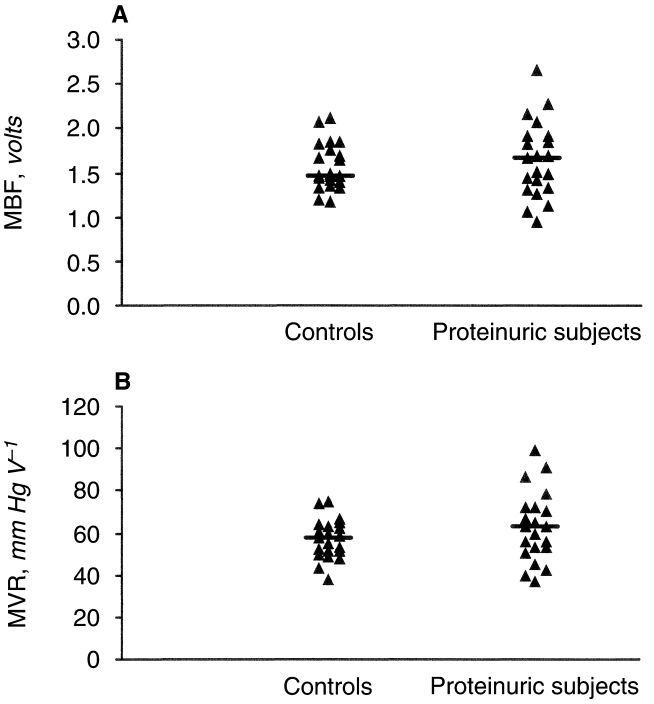

Fig. 2. Maximum microvascular hyperemia (MMH, volts, $N = 21$; $P = 0.571$; *A*) and minimum vascular resistance (MVR, mm Hg · V^{-1}, $N = 21$; $P = 0.252$; *B*) in response to local heating of skin in proteinuric subjects and controls (horizontal bars represent median values).

This remained the case when results were expressed as MVR [63.16 (36.55 to 99.27) AP vs. 58.06 (37.89 to 74.85) C mm Hg · V^{-1}; $P = 0.252$], which took into account any changes in BP that could influence the MMH response.

Macrovascular endothelial function

The demographic data for the nine matched pairs who underwent brachial artery wall tracking are shown in Table 2. Serum albumin was significantly lower in proteinuric subjects compared to controls [39 (32 to 41) AP vs. 42 (37 to 43) C g/L; $P = 0.031$]. BP was not significantly different in the two sub-groups [mean arterial pressure (MAP) 86 (79 to 102) AP vs. 88 (78 to 112) C mm Hg; $P = 0.546$]. FAD in the brachial artery after one minute of hyperemia was significantly impaired in AP subjects compared to controls [1.8 (0.2 to 5.3) AP vs. 3.8 (1.5 to 6.2) C %; $P = 0.014$; Fig. 3]. In contrast, dilation in response to GTN was not significantly different between the two groups at any time point over the 15-minute observation period [increase in diameter 3 min after GTN, 6.3 (1.8 to 12.4) AP vs. 8.7 (1.7 to 17.1) C %; $P = 0.546$] (Fig. 3). Baseline brachial artery diameter was not significantly different in the two groups [4.62 (3.71 to 6.05) AP vs. 4.42 (3.90 to 5.90) C mm; $P = 0.931$] and was inversely correlated with flow-associated dilation at one minute in the AP group ($r = -0.7$; $P = 0.036$). The hyperemic stimulus was quantified as the percentage change in net blood velocity between baseline and one minute after the cuff release and was not significantly different between groups [192.0 (18.9 to 312.9) AP vs. 224.9 (7.76 to 437.3) C %; $P = 0.937$]. Indeed, the control and AP groups showed no significant difference in blood velocity at any time point throughout the experiments.

HsCRP and soluble markers of endothelial function

C-reactive protein, measured by a high sensitivity assay (HsCRP), was significantly elevated in the AP group compared to controls [4.0 (0.5 to 39.0) AP vs. 0.2 (0.1 to 21.3) C mg/L; $P < 0.001$; Fig. 4]. In the AP, but not control, group there was an inverse correlation between CRP and microvascular endothelial function as determined by ACh iontophoresis ($\log_e$CRP vs. Ach at the sixth time point, $r = -0.509$; $P = 0.018$; Pearson's correlation; Fig. 5). CRP was not related to FAD in either AP ($r = 0.261$; $P = 0.467$) or control ($r = 0.271$; $P = 0.293$) groups. Nor was CRP related to MMH or MVR in either AP (MMH: $r = -0.243$, $P = 0.301$; MVR: $r =$

Table 2. Demographics of patients who underwent brachial wall tracking

Variable	Macrovascular function		*P* value
	Proteinuric subjects	Controls	
Age *years*	33 (19–51)	35 (20–51)	0.965
Sex *male/female*	5/4	5/4	
BMI kg/m^2	22.6 (19.8–32.0)	22.7 (18.0–30.5)	0.605
Smoking *current/ex/never*	2/2/5	2/2/5	
SBP *mm Hg*	120 (110–137)	122 (106–154)	0.796
DBP *mm Hg*	71 (63–84)	71 (64–91)	0.666
MAP *mm Hg*	86 (79–102)	88 (78–112)	0.546
Serum creatinine *μmol/L*	72 (67–95)	82 (61–100)	0.796
Creatinine clearance *mL/min*	106 (74–223)	128 (109–161)	0.878
Urinary protein[a] *g/24 h*	0.98 (0.18–2.22)	0.10 (0.05–0.14)	<0.001
Urinary Alb/Cr ratio[a] *mg/mmol*	33.6 (0.3–123.0)	0.6 (0.1–3.0)	0.001
Albumin[a] *g/L*	39 (32–41)	42 (37–43)	0.031
Fasting glucose *mmol/L*	4.8 (4.0–5.1)	4.6 (4.2–4.8)	0.436
Cholesterol *mmol/L*	4.9 (3.5–6.9)	4.6 (3.0–6.9)	0.863
LDL *mmol/L*	3.1 (1.3–5.0)	3.0 (1.0–4.6)	1.000
Triglyceride *mmol/L*	0.82 (0.54–2.04)	0.85 (0.52–2.49)	1.000

Data are shown as median (range).
[a] $P < 0.05$

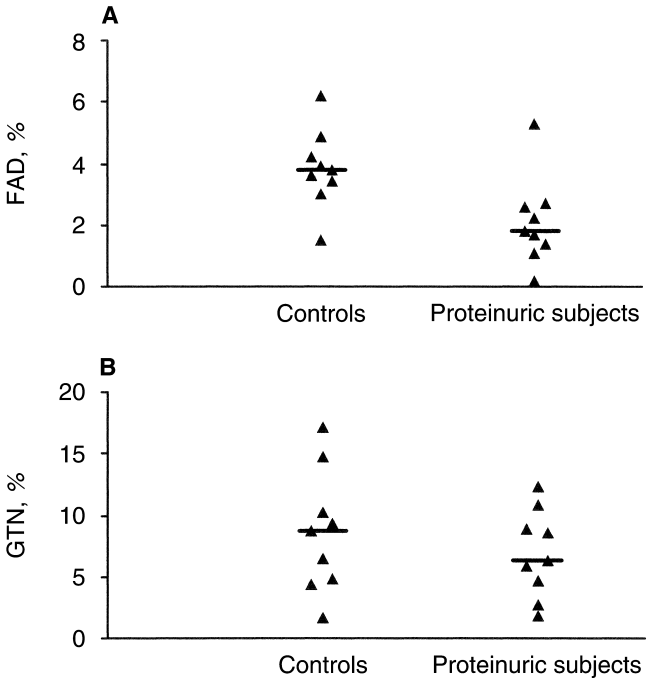

Fig. 3. Flow associated dilation (FAD) at one minute after cuff release (%, $N = 9$; $P = 0.014$; *A*) and glyceryl trinitrate (GTN)-induced dilation at 3 minutes after 400 μg GTN (%, $N = 9$; $P = 0.546$; *B*) in proteinuric subjects and controls (horizontal bars represent median values).

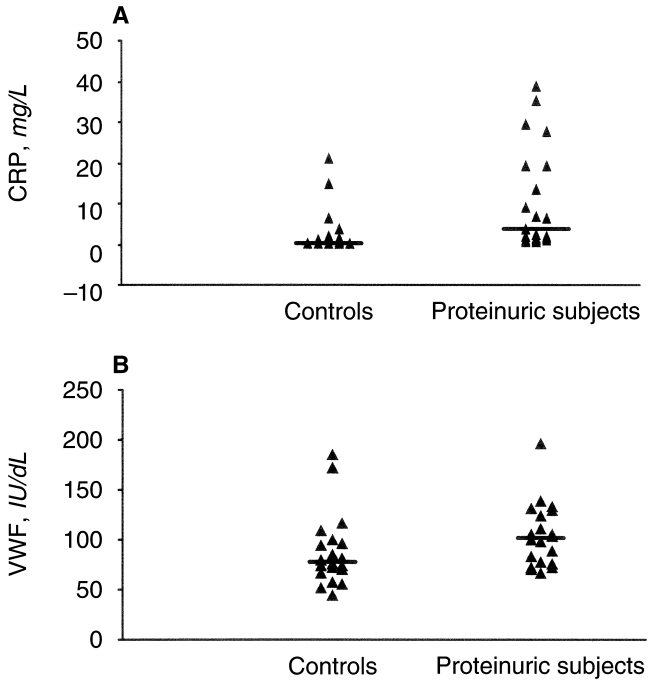

Fig. 4. Serum C-reactive protein (CRP, mg/L, $N = 21$; $P < 0.001$; *A*) and von Willebrand factor (vWF, IU/dL, $N = 20$; $P = 0.046$; *B*) in proteinuric subjects and controls (horizontal bars represent median values).

0.255, $P = 0.278$) or control (MMH: $r = -0.032$, $P = 0.898$; MVR: $r = 0.004$, $P = 0.988$) groups.

Plasma vWF was elevated in the AP group compared to controls [101.5 (67.0 to 197.0) AP vs. 77.5 (45.0 to 185.0) C IU/dL; $P = 0.046$; Fig. 4], the difference only just reaching conventional levels of significance. There was no significant difference in serum urate [350 (198 to 456) AP vs. 347 (202 to 494) C μmol/L; $P = 0.796$] or fibrinogen [3.1 (1.9 to 4.7) AP vs. 2.4 (1.3 to 4.6) C g/L; $P = 0.190$] between the two groups. In the AP group, but not in the control group, there was a very definite trend suggesting a correlation between vWF and CRP ($r = 0.424$; $P = 0.049$); this did reach conventionally accepted levels of significance (Fig. 5). In the AP, but not control, group vWF correlated with the degree of proteinuria (g/L; $r = 0.502$; $P = 0.024$) and just failed

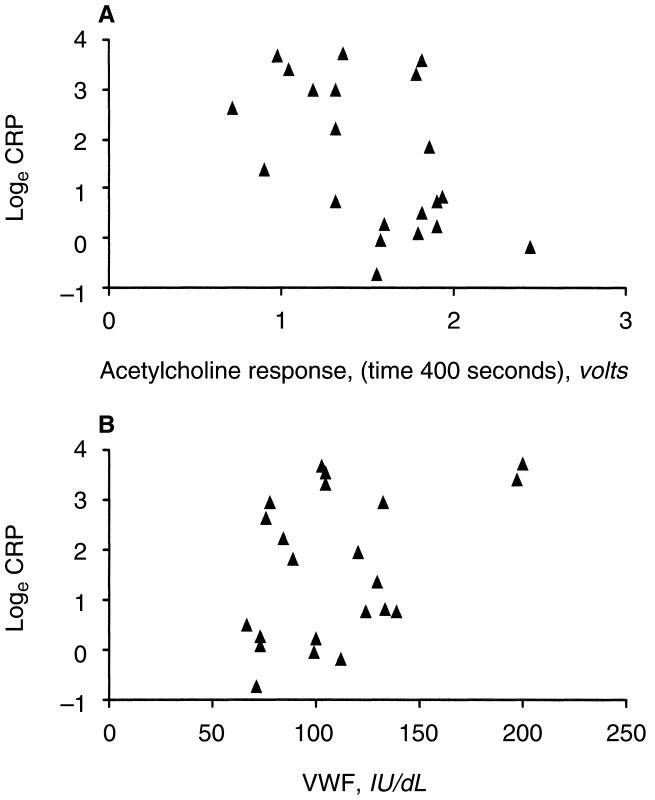

Fig. 5. Relationship between log$_e$ C-reactive protein (CRP) and response of skin erythrocyte flux to acetylcholine (6th time point, time 400 seconds, volts; $r = -0.509$, $P = 0.018$; *A*) and serum von Willebrand factor (vWF, IU/dL; $r = +0.424$, $P = 0.049$; *B*) in proteinuric subjects.

to reach conventional levels of significance with serum albumin ($r = -0.421$; $P = 0.051$), fibrinogen ($r = 0.388$; $P = 0.074$) and urate ($r = 0.407$; $P = 0.06$). CRP did not correlate with fibrinogen, urate or degree of proteinuria.

DISCUSSION

This study explored potential mechanisms responsible for the increased vascular permeability observed in otherwise healthy patients with proteinuria and has demonstrated impaired endothelial-dependent conduit artery dilatory capacity in such subjects compared to controls. As far as we are aware, this is the first study to demonstrate impaired endothelial-dependent vasodilation in non-nephrotic GN. In contrast, skin microvascular function, as assessed by vasodilatory response to iontophoresis of Ach, was similar in the two groups. However, serum vWF, a soluble marker of endothelial function [31] and serum CRP, a marker of the acute inflammatory response, were elevated in the AP group compared to controls. Furthermore, there was an inverse correlation between the inflammatory marker CRP and the endothelial-dependent vasodilatory response to Ach iontophoresis, an association that, to our knowledge, has not been described previously.

The population studied was highly selected and carefully matched to limit confounding factors. Only otherwise healthy patients with dipstick-positive proteinuria and with no evidence of co-existing disease were included. This is important as abnormalities of vascular function have been demonstrated in hyperlipidemia [43], diabetes [44], hypertension [45] and chronic renal failure, both pre-dialysis [46] and end stage [47].

The difference in conduit artery dilatory capacity cannot be explained by differences in baseline vessel diameter, extent of the hyperemic stimulus or by differences in classic cardiovascular risk factors, such as age, BMI, smoking habits, blood pressure, cholesterol or blood glucose. Only FAD was impaired; there was no significant difference in endothelial-independent GTN-induced dilation. FAD is endothelial-dependent [18, 48] and is the result of shear dependent NO release [49]. NO has a number of favorable vascular actions, including vasodilatory, anti-inflammatory and anti-coagulant effects, supporting its role as an anti-atherogenic molecule [50, 51]. Loss of the vascular effects of NO may favor the development of atherosclerosis [52]. Therefore, impaired NO-dependent FAD in proteinuric subjects may reflect the increased risk of atherosclerosis in this group. Animal studies suggest that NO is a regulator of microvascular permeability [53], although there is conflicting evidence as to whether it has an inhibitory [54] or stimulatory role [55].

The findings of the present study are interesting in the light of the recent observation that FAD is impaired in clinically healthy subjects with microalbuminuria [14]. Is the vascular abnormality an intrinsic abnormality in those destined to develop proteinuria of any degree or is it specific to glomerulonephritis? Examination of resistance rather than conduit arteries in glomerulonephritis failed to reveal any abnormalities at levels of proteinuria lower than those of the present study [56].

We observed a small but significant reduction in serum albumin in the asymptomatic proteinuric group compared to controls, whereas there was no difference in the active GN group of the Stroes study [56]. It has been suggested that hypoalbuminemia in nephrotic syndrome contributes to a disturbed endothelial function by its effect on lysophosphatidylcholine binding [57], and it may be that a similar mechanism occurs in non-nephrotic proteinuria.

Microvascular endothelial function, as assessed by iontophoresis of Ach, to our knowledge has not been studied previously in otherwise healthy proteinuric subjects. It was unexpected that there was no impairment in response compared to controls. Such an impairment has been demonstrated in subjects with other cardiovascular risk factors, such as diabetes [33], hypertension [58] and hypercholesterolemia [59]. Some [60, 61] but not all studies [62, 63] suggest that a prostaglandin-dependent mechanism accounts for approximately 50% dermal vasodilation fol-

lowing Ach iontophoresis in the forearm, but there is general agreement that control mechanisms clearly differ depending on the type of vascular bed. L-NMMA has no effect on the Ach response in the forearm nutritive dermal vasculature [60, 61] but does impair basal flux in finger pulp thermoregulatory vasculature [29]. AP subjects may have an abnormality in NO-dependent processes, supported by impaired brachial artery wall tracking, but not a significant abnormality in prostanoid or endothelial-derived hyperpolarizing factor (EDHF) dependent processes in the dermal vasculature. The elevated vWF, a soluble marker of endothelial activation, further supports the existence of impaired endothelial function in this proteinuric group.

Recently evidence has emerged linking atherosclerosis and vascular risk with chronic low grade inflammation. We found that serum values of highly sensitive CRP were elevated in the AP group compared to controls and were inversely correlated with endothelial-dependent microvascular function. Raised CRP values previously have been reported in glomerulonephritis. As far as we are aware, however, no studies have investigated CRP values specifically in glomerulonephritis with hematuria but not proteinuria. Therefore, it is impossible to say whether the elevated CRP seen in this study is related to glomerulonephritis or to proteinuria per se. The association of CRP with microvascular endothelial function to our knowledge has not been investigated previously in non-diabetic proteinuria. CRP is raised in type 1 diabetic patients with or without macrovascular disease and correlates with vWF [64]. Analysis of data from the large cohort in the Insulin Resistance Atherosclerosis Study showed an association of CRP with urinary albumin excretion in the microalbuminuric range in type 1 diabetic and non-diabetic individuals [65]. The present study provides further evidence for the association between activation of the endothelium and low-grade inflammation.

We did not find an association between CRP and macrovascular endothelial function, as measured by FAD in conduit artery, in either the AP or control groups. This is in contrast to previous studies showing an inverse association between CRP and forearm blood flow as measured by venous occlusion plethysmography (VOP) [66, 67]. However, the technique of VOP examines resistance, not conduit, artery function. Cleland et al investigated basal, not stimulated, NO release, and of the subjects with coronary artery disease studied by Fichtscherer et al, a significant number had diabetes or hypertension and all were taking vasoactive medication.

What are the limitations of this study? We studied those AP subjects who reached a nephrology clinic with a diagnosis of GN, and who consented to participate. This may introduce a selection bias. Brachial artery wall tracking studies were performed on only 39% of subjects for reasons outlined previously. Subjects were excluded at the time of study by the operator who was blinded to their status. Nevertheless, this may reflect a difference between groups and could introduce bias.

The degree of flow-associated and GTN-induced dilation observed was relatively low compared to other studies. This may be explained by the higher baseline vessel diameter in this study as it is well documented that this is inversely related to vasodilatory response [20, 41]. It may be that the brachial artery was scanned more proximally in the current study.

The sample size was small. The study had the power to detect a 21.6% difference in MMH and a 25.4% difference in vasodilatory response to Ach iontophoresis and, therefore, smaller differences cannot be excluded. However, no trends suggesting impaired microvascular function were observed. A previous study found a 63% increase in capillary fluid permeability in asymptomatic proteinuric subjects (abstract; Lewis, *ibid*). The current study should be able to detect a difference in microvascular function of this size.

The full group, but not the wall tracking subgroup, included two subjects with abnormal renal function. Endothelial function, as assessed by brachial artery wall tracking and by plasma vWF, is impaired in patients with pre-dialysis chronic renal failure [46, 68], albeit at a greater impairment of renal function than in the present study. Results remain the same if the two subjects with impaired renal function are excluded.

In summary, in a well-matched group of otherwise healthy patients with dipstick proteinuria there is evidence of abnormality in NO-dependent macrovascular endothelial function remote from the kidney and of low-grade chronic inflammation that is associated with microvascular endothelial dysfunction. This provides further support for the link between proteinuria per se, systemic endothelial dysfunction and atherosclerosis. Large-scale longitudinal studies are needed to determine whether those subjects with impaired endothelial function do express increased rates of atherosclerosis.

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REFERENCES

1. Mogensen CE: Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. *N Engl J Med* 310:356–360, 1984
2. Kannel WB, Stampfer MJ, Castelli WP, Verter J: The prognostic significance of proteinuria: The Framingham study. *Am Heart J* 108:1347–1352, 1984

3. Yudkin JS, Forrest RD, Jackson CA: Microalbuminuria as predictor of vascular disease in non-diabetic subjects. Islington Diabetes Survey. *Lancet* 2:530–533, 1988
4. Lewis DM, Bradwell AR, Shore AC, *et al*: Capillary filtration coefficient and urinary albumin leak at altitude. *Eur J Clin Invest* 27: 64–68, 1997
5. Lewis DM, Tooke JE, Beaman M, *et al*: Peripheral microvascular parameters in nephrotic syndrome. *Kidney Int* 54:1261–1266, 1998
6. Jensen JS, Borch-Johnsen K, Jensen G, Feldt-Rasmussen B: Microalbuminuria reflects a generalised transvascular albumin leakiness in clinically healthy subjects. *Clin Sci Lond* 88:629–633, 1995
7. Ross R: The pathogenesis of atherosclerosis - An update. *N Engl J Med* 8:488–500, 1986
8. Blum MS, Toninelli E, Anderson JM, *et al*: Cytoskeletal rearrangement mediates human microvascular endothelial tight junction modulation by cytokines. *Am J Physiol* 273:H286–H294, 1997
9. Michel CC: Transport of macromolecules through microvascular walls. *Cardiovasc Res* 32:644–653, 1996
10. Razani B, Engelman JA, Wang XB, *et al*: Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *J Biol Chem* 276:38121–38138, 2001
11. Schubert W, Frank PG, Razani B, *et al*: Caveolae-deficient endothelial cells show defects in the uptake and transport of albumin in vivo. *J Biol Chem* 276:48619–48622, 2001
12. Vogel SM, Easington CR, Minshall RD, *et al*: Evidence of transcellular permeability pathway in microvessels. *Microvasc Res* 61: 87–101, 2001
13. Drab M, Verkade P, Elger M, *et al*: Loss of caveolae, vascular dysfunction and pulmonary defects in caveolin-1 gene-disrupted mice. *Science* 293:2404–2405, 2001
14. Clausen P, Jensen JS, Jensen G, *et al*: Elevated urinary albumin excretion is associated with impaired arterial dilatory capacity in clinically healthy subjects. *Circulation* 103:1869–1874, 2001
15. Hernandez E, Toledo T, Alamo C, *et al*: Elevation of von Willebrand factor levels in patients with IgA nephropathy: Effect of ACE inhibition. *Am J Kidney Dis* 30:397–403, 1997
16. Jensen JS, Myrup B, Borch-Johnsen K, *et al*: Aspects of haemostatic function in healthy subjects with microalbuminuria—A potential atherosclerotic risk factor. *Thromb Res* 77:423–430, 1995
17. Fuster V, Badimon L, Badimon JJ, Chesebro JH: The pathogenesis of coronary artery disease and the acute coronary syndromes (1). *N Engl J Med* 326:242–250, 1992
18. Nabel EG, Selwyn AP, Ganz P: Large coronary arteries in humans are responsive to changing blood flow: An endothelium-dependent mechanism that fails in patients with atherosclerosis. *J Am Coll Cardiol* 16:349–356, 1990
19. Ludmer PL, Selwyn AP, Shook TL, *et al*: Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N Engl J Med* 315:1046–1051, 1986
20. Celermajer DS, Sorensen KE, Gooch VM, *et al*: Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* 340:1111–1115, 1992
21. Adams MR, Celermajer DS: Detection of presymptomatic atherosclerosis: A current perspective. *Clin Sci Lond* 97:615–624, 1999
22. Ross R: Atherosclerosis - An inflammatory disease. *N Engl J Med* 340:115–126, 1999
23. Liuzzo G, Biasucci LM, Gallimore JR, *et al*: The prognostic value of C-reactive protein and serum amyloid a protein in severe unstable angina. *N Engl J Med* 331:417–424, 1994
24. Biasucci LM, Vitelli A, Liuzzo G, *et al*: Elevated levels of interleukin-6 in unstable angina. *Circulation* 94:874–877, 1996
25. Haverkate F, Thompson SG, Pyke SD, *et al*: Production of C-reactive protein and risk of coronary events in stable and unstable angina: European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. *Lancet* 349:462–466, 1997
26. Morrow DA, Rifai N, Antman EM, *et al*: C-reactive protein is a potent predictor of mortality independently of and in combination with troponin T in acute coronary syndromes: A TIMI 11A substudy: Thrombolysis in Myocardial Infarction. *J Am Coll Cardiol* 31:1460–1465, 1998
27. Ridker PM, Cushman M, Stampfer MJ, *et al*: Inflammation, aspirin and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* 336:973–979, 1997
28. Koenig W, Sund M, Frohlich M, *et al*: C-reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men: Results from the MONICA (Monitoring Trends and Determinants in Cardiovascular Disease) Ausburg Cohort Study, 1984 to 1992. *Circulation* 99: 237–242, 1999
29. Noon JP, Haynes WG, Webb DJ, Shore AC: Local inhibition of nitric oxide generation in man reduces blood flow in finger pulp but not in hand dorsum skin. *J Physiol* 490:501–508, 1996
30. Luscher TF, Barton M: Biology of the endothelium. *Clin Cardiol* 20(11 Suppl 2):II-3–II-10, 1997
31. Lip GY, Blann A: von Willebrand factor: a marker of endothelial dysfunction in vascular disorders? *Cardiovasc Res* 34:255–265, 1997
32. Furchgott RF, Zawadzki JV: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288:373–376, 1980
33. Morris SJ, Shore AC, Tooke JE: Responses of the skin microcirculation to acetylcholine and sodium nitroprusside in patients with NIDDM. *Diabetologia* 38:1337–1344, 1995
34. Grossmann M, Jamieson MJ, Kellogg DLJ, *et al*: The effect of iontophoresis on the cutaneous vasculature: Evidence for current-induced hyperemia. *Microvasc Res* 50:444–452, 1995
35. Tenland T, Salerud EG, Nilsson GE, Oberg PA: Spatial and temporal variations in human skin blood flow. *Int J Microcirc Clin Exp* 2:81–90, 1983
36. Rayman G, Williams SA, Spencer PD, *et al*: Impaired microvascular hyperaemic response to minor skin trauma in type I diabetes. *Br Med J (Clin Res Ed)* 292:1295–1298, 1986
37. Williams SA, Tooke JE: Noninvasive estimation of increased structurally-based resistance to blood flow in the skin of subjects with essential hypertension. *Int J Microcirc Clin Exp* 11:109–116, 1992
38. Rubanyi GM, Romero JC, Vanhoutte PM: Flow-induced release of endothelium-derived relaxing factor. *Am J Physiol* 250:H1145–H1149, 1986
39. Laurent S, Lacolley P, Brunel P, *et al*: Flow-dependent vasodilation of brachial artery in essential hypertension. *Am J Physiol* 258: H1004–H1011, 1990
40. Sorensen KE, Celermajer DS, Spiegelhalter DJ, *et al*: Non-invasive measurement of human endothelium dependent arterial responses: Accuracy and reproducibility. *Br Heart J* 74:247–253, 1995
41. Adams MR, Robinson J, Sorensen KE, *et al*: Normal ranges for brachial artery flow-mediated dilatation: A non-invasive ultrasound test of arterial endothelial function. *J Vasc Invest* 2:146–150, 1996
42. Chauveau M, Levy B, Dessanges JF, *et al*: Quantitative Doppler blood flow measurement method and in vivo calibration. *Cardiovasc Res* 19:700–706, 1985
43. Casino PR, Kilcoyne CM, Cannon RO Jr, *et al*: Impaired endothelium-dependent vascular relaxation in patients with hypercholesterolaemia extends beyond the muscarinic receptor. *Am J Cardiol* 75:40–44, 1995
44. Tooke JE, Morris SJ, Shore AC: Microvascular functional abnormalities in diabetes: The role of the endothelium. *Diabetes Res Clin Pract* 31(Suppl):S127–S132, 1996
45. Shore AC, Tooke JE: Microvascular function in human essential hypertension. *J Hypertens* 12:717–728, 1994
46. Thambyrajah J, Landray MJ, McGlynn FJ, *et al*: Abnormalities of endothelial function in patients with predialysis renal failure. *Heart* 83:205–209, 2000
47. van Guldener C, Janssen MJ, Lambert J, *et al*: Endothelial-dependent vasodilatation is impaired in peritoneal dialysis patients. *Nephrol Dial Transplant* 13:1782–1786, 1998
48. Pohl U, Holtz J, Busse R, Bassenge E: Crucial role of endothelium in the vasodilator response to increased flow in vivo. *Hypertension* 8:37–44, 1986
49. Doshi SN, Naka KK, Payne N, *et al*: Flow-mediated dilatation following wrist and upper arm occlusion in humans: The contribution of nitric oxide. *Clin Sci Lond* 101:629–635, 2001
50. Moncada S, Palmer RM, Higgs EA: Nitric oxide: Physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43:109–142, 1991
51. Cooke JP, Tsao PS: Is NO an endogenous antiatherogenic molecule? *Arterioscler Thromb* 14:653–655, 1994
52. Quyyumi AA, Dakak N, Mulcahy D, *et al*: Nitric oxide activity in

the atherosclerotic human coronary circulation. *J Am Coll Cardiol* 29:308–317, 1997
53. Kubes P, Granger DN: Nitric oxide modulates microvascular permeability. *Am J Physiol* 262:H611–H615, 1992
54. Johnston B, Gaboury JP, Suematsu M, Kubes P: Nitric oxide inhibits microvascular protein leakage induced by leucocyte adhesion-independent and adhesion-dependent inflammatory mediators. *Microcirculation* 6:153–162, 1999
55. Yuan Y, Granger HJ, Zawieja DC, *et al*: Histamine increases venular permeability via a phospholipase C-NO synthase-guanylate cyclase cascade. *Am J Physiol* 264:H1734–H1739, 1993
56. Stroes ES, Joles JA, Chang PC, *et al*: Impaired endothelial function in patients with nephrotic range proteinuria. *Kidney Int* 48: 544–550, 1995
57. Joles JA, Stroes ES, Rabelink TJ: Endothelial function in proteinuric renal disease. *Kidney Int* 71(Suppl):S57–S61, 1999
58. Serne EH, Gans RO, ter Maaten JC, *et al*: Capillary recruitment is impaired in essential hypertension and relates to insulin's metabolic and vascular actions. *Cardiovasc Res* 49:161–168, 2001
59. Khan F, Litchfield SJ, Stonebridge PA, Belch JJ: Lipid-lowering and skin vascular responses in patients with hypercholesterolaemia and peripheral arterial obstructive disease. *Vasc Med* 4:233–238, 1999
60. Khan F, Davidson NC, Littleford RC, *et al*: Cutaneous vascular responses to acetylcholine are mediated by a prostanoid-dependent mechanism in man. *Vasc Med* 2:82–86, 1997
61. Noon JP, Walker BR, Hand MF, Webb DJ: Studies with iontophoretic administration of drugs to human dermal vessels in vivo: Cholinergic vasodilatation is mediated by dilator prostanoids rather than nitric oxide. *Br J Clin Pharmacol* 45:545–550, 1998
62. Dietz NM, Rivera JM, Eggener SE, *et al*: Nitric oxide contributes to the rise in forearm blood flow during mental stress in humans. *J Physiol* 480:361–368, 1994
63. Morris SJ, Shore AC: Skin blood flow responses to the iontophoresis of acetylcholine and sodium nitroprusside in man: Possible mechanisms. *J Physiol* 496:531–542, 1996
64. Schalkwijk CG, Poland DC, van Dijk W, *et al*: Plasma concentration of C-reactive protein is increased in type 1 diabetic patients without clinical macroangiopathy and correlates with markers of endothelial dysfunction: Evidence for chronic inflammation. *Diabetologia* 42:351–357, 1999
65. Festa A, D'Agostino R, Howard G, *et al*: Inflammation and microalbuminuria in nondiabetic and type 2 diabetic subjects: The Insulin Resistance Atherosclerosis Study. *Kidney Int* 58:1703–1710, 2000
66. Fichtlscherer S, Rosenberger G, Walter DH, *et al*: Elevated C-reactive protein levels and impaired endothelial vasoreactivity in patients with coronary artery disease. *Circulation* 102:1000–1006, 2000
67. Cleland SJ, Sattar N, Petrie JR, *et al*: Endothelial dysfunction as a possible link between C-reactive protein levels and cardiovascular disease. *Clin Sci* 98:531–535, 2000
68. Haaber AB, Eidemak I, Jensen T, *et al*: Vascular endothelial cell function and cardiovascular risk factors in patients with renal failure. *J Am Soc Nephrol* 5:1581–1584, 1995