Persistent endothelial dysfunction is related to elevated C-reactive protein (CRP) levels in Type II diabetic patients after acute myocardial infarction

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ABSTRACT

The atherosclerotic process is an ongoing dynamic and progressive state arising from endothelial dysfunction and inflammation. Although suffering from an acute coronary artery disease, patients with Type II diabetes have a poor outcome compared with non-diabetic patients, which may only partly be explained by traditional risk factors. Our purpose was to compare non-traditional risk factors, such as endothelial function, C-reactive protein (CRP) and adiponectin, in Type II diabetic and non-diabetic patients following AMI (acute myocardial infarction). Twenty Type II diabetic patients were compared with 25 non-diabetic patients at baseline (1–3 days from the onset of chest pain) and at 60 days follow-up after an AMI. Using high-resolution ultrasound, brachial artery responses to FMD (flow-mediated vasodilatation; endothelium-dependent vasodilatation) and NTG (nitroglycerine-induced vasodilatation; endothelium-independent vasodilatation) were measured. Plasma levels of CRP and adiponectin were measured by ELISA. At baseline, FMD (1.9 compared with 3.2%; \( P = 0.22 \)) and CRP levels (6.95 compared with 5.51 mg/l; \( P = 0.40 \)) did not differ between Type II diabetic and non-diabetic patients, whereas adiponectin levels were lower in Type II diabetic patients (2.8 compared with 5.0 ng/ml; \( P < 0.05 \)). At 60 days follow-up, there were significant differences in FMD (1.5 compared with 4.1%; \( P < 0.02 \)), CRP (4.23 compared with 1.46 mg/ml; \( P < 0.01 \)) and adiponectin (3.3 compared with 5.3 ng/ml; \( P < 0.05 \)) levels between Type II diabetic and non-diabetic patients. In contrast, NTG responses improved in both groups between baseline and follow-up (Type II diabetic patients, 9.7 compared with 13.2% respectively, \( P < 0.05 \); non-diabetic patients, 7.9 compared with 12.4% respectively, \( P < 0.01 \)). These results show a persistent endothelium-dependent dysfunction and inflammatory activity in patients with Type II diabetes, but not in non-diabetic patients, after AMI. These findings may, in part explain, the poor outcome in coronary artery disease seen in Type II diabetes.

INTRODUCTION

Type II diabetic patients have a poor outcome in suffering from CAD (coronary artery disease) [1]. Traditional risk factors, including hyperglycaemia, can explain no more than half of this increased mortality [2]. The atherosclerotic process is an ongoing dynamic and progressive state arising from endothelial dysfunction and inflammation [3]. Endothelial dysfunction is one of the earliest events identified in the pathogenesis of atherosclerosis and is

Key words: adiponectin, coronary artery disease, C-reactive protein (CRP), endothelial dysfunction, Type II diabetes. 
Abbreviations: ACE, angiotensin-converting enzyme; AMI, acute myocardial infarction; CAD, coronary artery disease; CK-MB, creatine kinase MB; CRP, C-reactive protein; CV, coefficient of variation; FMD, flow-mediated vasodilatation; HDL, high-density lipoprotein; IL-6, interleukin-6; LDL, low-density lipoprotein; NO, nitric oxide; eNOS, endothelial NO synthase; NS, not significant; NTG, nitroglycerine-induced vasodilatation; TNF-α, tumour necrosis factor-α.
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associated mainly with abnormalities in the metabolism of NO (nitric oxide) [4,5]. A loss of normal NO function not only causes impairment in vasodilatation, but also deficient anti-atherogenic effects, i.e. inhibition of platelet aggregation and prevention of adhesion of inflammatory cells to the endothelial surface [6,7]. Endothelial function can be assessed non-invasively using high-resolution ultrasonography measuring postischaemic FMD (flow-mediated vasodilatation) of peripheral conduit arteries, which correlates closely with endothelial function in coronary arteries [8]. This stimulus provokes the endothelium to release NO, quantified as an index of endothelial function [5]. Endothelial dysfunction in coronary and brachial arteries of humans has profound prognostic implications in that it predicts adverse cardiovascular events and long-term outcome [9,10]. Chronic low-grade inflammation, e.g. reflected by elevated levels of CRP (C-reactive protein), is involved in the pathogenesis of atherosclerosis, and CRP concentration correlates with the extent of CAD [11]. High levels of CRP also predict long-term mortality in CAD [12]. Adiponectin, a novel member of the adipocytokine family of peptide hormones, has significant insulin-sensitizing and anti-inflammatory properties [13]. It was also recently shown that adiponectin has a novel vascular action in as much as it directly promotes NO production in endothelial cells [14].

The aim of the present study was to compare Type II diabetic patients with non-diabetic patients following AMI (acute myocardial infarction) with regard to non-traditional markers of cardiovascular disease, such as endothelial function, CRP and adiponectin.

METHODS

Study group

The study group consisted of 20 Type II diabetic patients [duration of diabetes, 7 ± 1 years and HbA1c (glycated haemoglobin) levels of 6.6 ± 0.3 %] and 25 non-diabetic patients suffering AMI. Details of the study groups are given in Table 1. Patients suffering from an AMI were recruited consecutively from the Coronary Care Unit or Department of Cardiology. Patients with AMI were defined according to the European Society of Cardiology criteria [15], namely a typical rise and gradual fall of CK-MB (creatinine kinase MB), together with at least one of the following: ischaemic symptoms and development of pathological Q-waves or ECG changes indicative of ischaemia (ST segment elevation or depression). For the non-diabetic group, diabetes [diagnosed according to ADA (American Diabetes Association) criteria] was an exclusion criterion. For both groups, other exclusion criteria were Type I diabetes (as indicated by positive autoantibody titres), renal insufficiency, age > 80 years, more than 3 days after the onset of chest pain, any type of cancer, ongoing infection and inflammatory diseases. Medical treatment in the study groups is specified in Table 2. Subjects gave informed written consent, and the study protocol was approved by the local Ethics Committee.

Study protocol

Vascular responses of the brachial artery and fasting plasma levels of CRP, adiponectin, routine blood samples and glucose were studied in each patient on two separate occasions: (i) at baseline (acutely), with the investigation being performed within 1–3 days (from the onset of chest pain); and (ii) at follow-up 60 days after AMI. Patients were examined in a supine position after 30 min at rest in a quiet dark room with a temperature of 22 ± 1°C. Long-acting nitrates were withheld 24 h before the study, but no other drugs were withheld. Patients were asked to avoid coffee or tea and to refrain from smoking at least 12 h before the ultrasonogram. Patients were not given any specific instructions on lifestyle modification during the study nor did they take part in a special exercise programme. Between the two examinations, patients were treated by physicians who were not aware of the particular study. Questionnaires at follow-up did not reveal any history of infection or inflammatory disease between the two procedures.

Ultrasound scan for determination of endothelium-dependent and -independent vasodilatation

Brachial artery responses to vasodilatation were monitored with ultrasound/Doppler (Acuson 128 XP/7.0 MHz linear array transducer) [16]. The brachial artery was scanned longitudinally 4–10 cm above the elbow with the assistance of a mechanical arm. The transmit (focus) zone was set to optimize images of the lumen arterial wall interface. The B-mode images were magnified by a resolution box and obtained with gating from the R wave of the
ECG as the triggering mode. The condition of reactive hyperaemia was induced by inflation of a pneumatic tourniquet placed around the forearm to a pressure of 300 mmHg for 4.5 min, followed by release [endothelium-dependent vasodilatation (FMD)]. Measurements were made at baseline and at 45 and 60 s after cuff release. After 10 min rest, 0.4 mg of glyceryl trinitrate spray was applied and new images were obtained 4.5 min later to determine NTG (nitroglycerine-induced vasodilatation; endothelium-independent vasodilatation). Changes in the brachial artery diameter were measured and expressed as the percentage increase of FMD and NTG. Images were recorded on S-VHS videotape and, off-line, transformed to digitalized images and processed further by an automated computerized analysing system [17]. Measurements were performed off-line and in a blinded manner. In brief, the analysing program is a PC/Windows-based software with digitized ultrasound image. The starting point of the measurement area is set by the operator, and a 10 mm box is automatically drawn. The different echo interfaces are automatically outlined. If obvious errors are detected, it is possible to modify the measurement by marking a correct echo in the ultrasound image. The different echo points are automatically generated by this automated system. This automated system has proven superior to manual measurement systems, exhibiting a dramatically improved reproducibility both in inter- and intra-observer CVs (coefficients of variation) [17].

In our laboratory, according to the guidelines for FMD and NTG techniques as well as for repeated measurements [5,18], the RC (coefficient of repeatability) and CV between two determinations 1 week apart (based on 32 subjects) is 7.2 and 10.8 % for FMD and 10.7 and 14.1 % for NTG respectively.

**Blood chemistry**

Blood samples for glucose, total cholesterol, HDL (high-density lipoprotein)-cholesterol, LDL (low-density lipoprotein)-cholesterol, triacylglycerols (triglycerides), CRP and adiponectin were collected after a 12 h overnight fast. Aliquots were placed on ice, centrifuged within half an hour, and the separated plasma stored at −20 °C pending analysis. Levels of adiponectin (R&D Systems, Abingdon, Oxfordshire, U.K.) and CRP (Immunodiagnostik AG, Bensheim, Germany) were determined by use of ELISAs, according to the instructions provided by the manufacturers of the kits. Routine laboratory methods were used for the determination of plasma glucose, serum cholesterol and triacylglycerols.

**Statistical analysis**

Results are shown as means ± S.E.M. Because CRP, adiponectin, and triacylglycerols were skewed, these data were logarithmically transformed prior to analyses. For baseline characteristics, comparisons between groups were made using an unpaired Student’s t test. The percentage data were tested by χ² analysis. For follow-up data, one-way ANOVA with repeated measurement was used, followed by a post hoc Sheffe’s test. Furthermore, a Pearson’s correlation analysis of changes between baseline and 60 days follow-up was performed. Changes in the dependent variables FMD and NTG were tested against changes in brachial artery vessel diameter, systolic blood pressure, levels of CRP, adiponectin, total

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**Table 2** Pharmacological treatment at baseline and at 60 days follow-up after AMI

<table>
<thead>
<tr>
<th>Drug/intervention</th>
<th>Type II diabetic patients</th>
<th>Non-diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>60 days</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Aspirin</td>
<td>8 (40)</td>
<td>19 (100)</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>4 (20)</td>
<td>19 (100)</td>
</tr>
<tr>
<td>ACE-inhibitor/ARB</td>
<td>4 (20)</td>
<td>9 (47)</td>
</tr>
<tr>
<td>Statin</td>
<td>8 (40)</td>
<td>18 (95)</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>4 (20)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Fibrate</td>
<td>1 (5)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>Diet</td>
<td>4 (20)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Metformin</td>
<td>1 (5)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>Sulphonylurea</td>
<td>1 (5)</td>
<td>0</td>
</tr>
<tr>
<td>Metformin + sulphonylurea</td>
<td>7 (35)</td>
<td>5 (26)</td>
</tr>
<tr>
<td>Insulin</td>
<td>4 (20)</td>
<td>4 (21)</td>
</tr>
<tr>
<td>Insulin + metformin</td>
<td>1 (5)</td>
<td>4 (21)</td>
</tr>
<tr>
<td>Insulin + sulphonylurea</td>
<td>1 (5)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>Insulin + metformin + sulphonylurea</td>
<td>1 (5)</td>
<td>1 (5)</td>
</tr>
</tbody>
</table>
cholesterol, LDL-cholesterol, HDL-cholesterol, triacylglycerols and glucose. \( P < 0.05 \) was deemed statistically significant. All statistical analyses were performed with the Statistica 6.0 software package (Statsoft, Inc., Tulsa, OK, U.S.A.).

### RESULTS

#### Patient characteristics

Fifty-one patients were eligible for the study, but six patients (two diabetic and four non-diabetic patients) had to be excluded for technical reasons (poor ultrasound images). The baseline characteristics of the remaining 45 patients are shown in Table 1. Distribution of gender, age, smoking and other established cardiovascular risk factors were well matched between the two groups. In the Type II diabetic group, more patients were taking aspirin but, at follow-up, there were no significant differences between the study groups in the use of cardiac medication (Table 2). In the coronary unit, all patients received heparin or low-molecular-mass heparin. No differences were seen between groups in use of thrombolytic therapy [35% in the Type II diabetic patients compared with 40% in the non-diabetic patients; \( P = \text{NS} \) (not significant)], in levels of the myocardial necrosis marker CK-MB (128 ± 28 μg/l in Type II diabetic patients compared with 123 ± 18 μg/l in non-diabetic patients; \( P = \text{NS} \)) or left ventricular ejection fraction (44 ± 2% in Type II diabetic patients compared with 47 ± 2% in non-diabetic patients; \( P = \text{NS} \)). In the Type II diabetic group, another four patients had been prescribed insulin at follow-up (Table 2). In addition, one patient in the Type II diabetic group died from a complication of AMI (ventricular septum defect) prior to follow-up.

#### Vascular response data

At onset before inflation of the tourniquet, brachial artery diameter did not change within or between groups at baseline or at 60 days follow-up (Table 3). In addition, brachial artery diameter did not change at onset before treatment with the glyceryl trinitrate spray within or between groups at baseline or at 60 days follow-up (Table 3). In the Type II diabetic group, FMD did not change during the 2 months following AMI, in contrast with a slight, but non-significant, increase in the non-diabetic group (Figure 1a). However, at 2 months after the AMI, a significant difference in FMD between the Type II diabetic and non-diabetic groups was observed (Figure 1a). There were significant improvements in NTG in both the Type II diabetic and non-diabetic groups at 60 days after AMI, but no differences between the groups (Figure 1b). Blood pressure did not change within or between groups at baseline or at 60 days follow-up (Table 3).

#### Biochemical characteristics

At baseline, plasma HDL-cholesterol, total cholesterol and LDL-cholesterol levels were higher in the non-diabetic group compared with the Type II diabetic group (Table 3). At follow-up, differences were still significant, except for HDL-cholesterol (Table 3). Surprisingly, there was only a minor reduction in lipids at follow-up, even though almost every patient received statins. One reason for this could be that cholesterol levels were initially low for this could be that cholesterol levels were initially low.
remained elevated in the Type II diabetic group indicative of a persisting proinflammatory state (Figure 2a). Interestingly, temporal changes in CRP showed an inverse pattern to that seen with FMD, with no difference observed between groups at baseline, but a significant lowering at follow-up in the non-diabetic group. Plasma adiponectin levels were already significantly lower in the Type II diabetic group than in the non-diabetic group at onset, a pattern that did not change over 60 days (Figure 2b).

### Correlation between baseline and 60 days follow-up

Between baseline and 60 days follow-up, changes in FMD and CRP concentrations correlated significantly in non-diabetic patients, with a borderline significance ($P = 0.057$) in Type II diabetic patients (Figure 3a). In contrast, no correlation was seen between temporal changes in FMD and adiponectin concentrations (Figure 3b). Changes in FMD did not correlate with total cholesterol, LDL-cholesterol, HDL-cholesterol, triacylglycerols or glucose concentrations in any group (Table 4). Furthermore, no inverse correlation was noted between CRP and adiponectin concentrations in the Type II diabetic group ($r = 0.13; P = \text{NS}$) or the non-diabetic group ($r = 0.08, P = \text{NS}$). Finally, changes in NTG between baseline and 60 days follow-up also did not reveal any significant correlation with the above variables in any group (results not shown).

### DISCUSSION

The main findings in the present study are a persistent endothelial dysfunction, along with a concomitant high plasma CRP and low adiponectin concentration, in Type II diabetic patients, but not in non-diabetic controls, following AMI. Interestingly, endothelial function recovered with a reciprocal lowering in plasma CRP upon follow-up 60 days after AMI. The correlation coefficient (Figure 3), and in particular the $r^2$ value, suggests that 16% of the change in FMD can be explained or accounted for by changes in CRP in Type II diabetes, whereas 18% of the change in FMD can be explained or accounted for by changes in CRP in non-diabetic patients. This reciprocal association between endothelial dysfunction and CRP is consistent with a previous study [19], but these data also indicate that persistent inflammation, as represented by CRP, plays a relatively small role in determining FMD. It cannot be entirely excluded that putative subtle differences in drug therapy between groups may also have influenced FMD and plasma levels of CRP or adiponectin. The effects of statins, ACE
Figure 3 Correlations between temporal changes in FMD and CRP or adiponectin
The scatter diagram shows correlation between changes in FMD (as the dependent variable) between baseline and 60 days follow up and (a) differences in log CRP concentrations and (b) differences in log adiponectin concentrations. T2DM, Type II diabetic patients; non-DM, non-diabetic patients.

Table 4 Univariate analysis with differences in FMD between baseline and 60 days follow-up as the dependent variable
Values are correlation coefficients (r). No significant correlations were observed between any of the variables.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Type II diabetic patients</th>
<th>Non-diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachial artery diameter</td>
<td>-0.28</td>
<td>-0.17</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>-0.10</td>
<td>-0.11</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>-0.32</td>
<td>-0.03</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>-0.20</td>
<td>-0.14</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>0.18</td>
<td>0.02</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>-0.24</td>
<td>-0.12</td>
</tr>
<tr>
<td>Glucose</td>
<td>-0.29</td>
<td>-0.18</td>
</tr>
</tbody>
</table>

The intact endothelium plays a pivotal role in the regulation of vascular tone through local release of NO. A disturbance in the endothelium may participate in the course of the acute coronary syndrome and predicts adverse cardiovascular events and long-term outcome in CAD patients [9,10,20]. Also, there is abundant evidence that a chronic inflammatory process promotes the progress of atherosclerosis as well as endothelial dysfunction [3]. Atherosclerosis is now considered to be a consequence of a chronic low-grade inflammatory disease and there is evidence that Type II diabetes mellitus and atherosclerosis may share a concomitant inflammatory basis [21]. A positive relationship between the inflammatory marker CRP and CAD exists [22]. CRP levels are elevated in patients with Type II diabetes and also predict the development of Type II diabetes, suggesting a chronic inflammatory process is also involved in the aetiology of this disease [23]. Therefore the persistent chronic inflammatory activity and impaired FMD in diabetic patients observed in our present study are in agreement with this hypothesis. Furthermore, investigators have suggested that CRP may be a causal part of the atherosclerotic process and not merely a biomarker [24]. Interestingly, Verma et al. [25] showed that CRP at pathophysiologically relevant concentrations caused a marked, sustained and dose-dependent decrease in NO production in endothelial cells, suggesting that CRP may directly aggravate or promote the development of adverse cardiovascular events. Because FMD is NO-dependent, the endothelial dysfunction observed in the diabetic group may thus, in part, be caused by the elevated level of CRP decreasing NO production.

Another explanation for the persistent endothelial dysfunction in diabetes observed in the present study could be hyperglycaemia itself, because plasma glucose was elevated in Type II diabetic patients. Marfella et al. [26] showed that acute hyperglycaemia induces vascular oxidative stress in healthy subjects, possibly related to increased formation of peroxynitrite and vascular damage. Recent in vitro studies show that hyperglycaemia, through the hexosamine pathway, impairs activation of the insulin receptor resulting in degradation of eNOS (endothelial NO synthase) [27,28]. Moreover, hyperglycaemia is also associated with increased levels of inflammatory markers [29]. Therefore, in the present study, hyperglycaemia may be one reason for the disturbance in endothelial function and the low-grade inflammation seen in Type II diabetic patients.

We observed a significantly decreased concentration of adiponectin in the diabetic group that did not change over time. Plasma levels of adiponectin are reportedly reduced in Type II diabetes, and experimental studies have indicated anti-inflammatory and anti-atherogenic (angiotensin-converting enzyme) inhibitors and insulin have been described in the literature to be mediated directly or indirectly by NO signalling pathways.
properties of this adipocytokine [13,30]. An inverse correlation between CRP and adiponectin concentrations has been reported in other settings [31], although this did not apply in the present study. Very recently it was shown that high plasma levels of adiponectin were associated with a lower risk of myocardial infarction without any relationship with CRP [32]. This suggests that adiponectin may not correlate with CRP but with other inflammatory markers. Adiponectin correlates negatively with TNF-α (tumour necrosis factor-α) and IL-6 (interleukin-6), [13,33], two proinflammatory cytokines whose levels rise in diabetes and impact negatively on the endothelium (for review, see [34]). Since we did not measure these cytokines, we cannot rule out that adiponectin deficiency may have exacerbated the inflammatory activity and affected endothelial function seen in the diabetic group, secondary to increases in IL-6 and TNF-α. Furthermore, Chen et al. [14] showed in vitro that adiponectin stimulates production of NO in endothelial cells in a different way to insulin. They concluded that this novel vascular action directly stimulates production of NO independent of insulin, involving phosphorylation of eNOS [14]. Taken together, the persistent endothelial dysfunction seen in the diabetic group reported in the present study could therefore be a consequence of reduced concentrations of adiponectin, elevated levels of NO independent of insulin, involving phosphorylation of eNOS [14].

At baseline, 1–3 days from the onset of chest pain, we found a mild impairment in NTG in both groups, but a significant recovery upon follow up after 2 months. Impaired vasodilatation responses to both nitroglycerine and acetylcholine have been described in epicardial arteries in humans with CAD [35]. It has also been suggested that smooth muscle dysfunction occurs independently of impaired endothelium-dependent vasodilation in adults at risk of atherosclerosis [36]. In contrast, it appears that the subjects in this study retained much of their ability to react to organic NO and that responses were improved during 2 months of follow-up, both in diabetic and in non-diabetic patients. The reason for this improvement in NTG is not clear. In oxidative stress, there is an exaggerated generation of free radicals, e.g., superoxide anions, normally scavenged by multiple intra- and extra-cellular mechanisms. At high concentrations, NO may react rapidly with superoxide anions to form peroxynitrite, which can impair the activity of eNOS [37]. However this mechanism does not appear to be operating in the present study, because nitroglycerine is converted into NO inside the muscle cell [38]. As the response to the direct NO donor improves significantly in both Type II diabetic and non-diabetic patients at 60 days, an alternative explanation of the results is feasible. The non-diabetic patients may simply produce the same amount of endothelium-derived NO at day 60 and day 0, but the smooth muscle is more responsive to the endothelium-derived NO at day 60.

In conclusion, we show in the present study that Type II diabetic patients, but not non-diabetic patients, exhibit a persistent endothelial dysfunction that coincides with elevated plasma CRP concentrations after AMI. We suggest that this persistent proinflammatory state and endothelial dysfunction may contribute to the high incidence of morbidity and mortality in CAD-characterizing diabetes.

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