The influence of type 2 diabetes on fibrin clot properties in patients with coronary artery disease

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Summary

Type 2 diabetes mellitus (T2DM) increases the risk of coronary thrombosis and both conditions are associated with altered fibrin clot properties. However, the influence of T2DM on fibrin clot properties in patients with coronary artery disease (CAD) remains unclear. We aimed to investigate the influence of T2DM on fibrin clot properties in patients with CAD. Fibrin clot structure and fibrinolysis were investigated in 581 CAD patients (148 with T2DM) using turbidimetric assays, confocal and scanning electron microscopy. Clots made from plasma and plasma-purified fibrinogen were studied, and plasma levels of inflammatory markers were analysed. T2DM patients had increased clot maximum absorbance compared with non-diabetic patients (0.36 ± 0.1 vs 0.33 ± 0.1 au; p=0.01), displayed longer lysis time (804 [618;1002] vs 750 [624;906] seconds; p=0.03) and showed more compact fibrin structure assessed by confocal and electron microscopy. Fibrinogen levels were elevated in T2DM (p<0.001), but clots made from purified fibrinogen showed no differences in fibrin properties in the two populations. Adjusting for fibrinogen levels, T2DM was associated with C-reactive protein and complement C3 plasma levels, with the former correlating with clot maximum absorbance (r=0.24, p<0.0001) and the latter with lysis time (r=0.30, p<0.0001). Independent of fibrinogen levels, females had more compact clots with prolonged lysis time compared with males (all p-values<0.001). In conclusion, T2DM is associated with prothrombotic changes in fibrin clot properties in patients with CAD. This is related to quantitative rather than qualitative changes in fibrinogen with a possible role for inflammatory proteins.

Keywords
Fibrin clot, fibrinolysis, coronary artery disease, type 2 diabetes mellitus, inflammation

Introduction

Coronary artery disease (CAD) is a leading cause of morbidity and mortality in the Western world (1). In particular, patients with type 2 diabetes mellitus (T2DM) have increased risk of CAD and cardiovascular events (2, 3). Despite advances in medical therapy, the prognosis following a cardiovascular event remains poor in these patients (4, 5). This is thought to be related to extensive vascular pathology and a pro-thrombotic environment (2). Moreover, recent evidence suggests that gender may contribute to the poor prognosis in diabetes following a cardiovascular event, with women faring less well than men (6).

Aspirin is a key anti thrombotic agent in the management of CAD, modulating both platelet function and the fibrinolytic process (7–9). However, the clinical efficacy of aspirin appears to be reduced in diabetes (9), and this may be related to a compromised antiplatelet and/or fibrinolytic effect (10–14).

Fibrin clot formation is the final step in the atherothrombotic process (15), and the structure of the fibrin network may predict a predisposition to cardiovascular events (12, 16, 17). Both CAD and T2DM are associated with altered fibrin clot properties (16, 18, 19) and increased resistance to fibrinolysis (16, 20, 21). We have recently shown that women with T2DM display more compact fibrin clots than men (22), which may contribute to the poor clinical prognosis in women with diabetes following cardiovascular events (6). Despite the various studies conducted to date, it remains unclear whether T2DM and female gender have additive effects on fibrin network properties in patients with CAD.

The mechanisms for altered clot structure are complex and may include quantitative as well as qualitative changes in coagulation and fibrinolytic factors (18, 20, 23, 24). Interactions between inflammatory and thrombotic pathways may also affect fibrin clot properties (25–27). Importantly, complement C3, a key inflammatory protein, has been shown to modulate fibrinolysis (27), particularly in patients with diabetes (28).
In the present study, we hypothesised that in patients with CAD, T2DM is associated with changes in fibrin clot properties compared with non-diabetic patients despite treatment with aspirin. Thus, we aimed to investigate 1) the influence of T2DM on fibrin clot properties in a cohort of aspirin-treated patients with CAD, 2) mechanisms influencing fibrin clot properties including quantitative and qualitative changes in fibrinogen and markers of inflammation, and 3) the role of gender on fibrin clot properties.

Material and methods

Study population

A total of 581 patients with stable CAD of whom 148 had T2DM were recruited from the Western Denmark Heart Registry from February 2009 to January 2011. We aimed to recruit a high risk cardiovascular population. Therefore patients were eligible for inclusion if they were 18 years or older and had significant CAD verified by coronary angiography showing at least 50% luminal narrowing in one or more coronary artery, previous percutaneous coronary intervention (PCI) or coronary artery bypass grafting. Patients were also required to have at least one additional risk factor of either: i) T2DM, ii) previous myocardial infarction (MI), iii) or both. All patients were treated with 75 mg non-enteric coated aspirin daily. All patients with diabetes were treated with glucose-lowering agents, and those who were on diet only were excluded from the study. The non-diabetic group had fasting plasma glucose < 7 mmol/l at the time of inclusion. Exclusion criteria were 1) platelet count < 120 × 10^9/l or > 450 × 10^9/l or 2) treatment with anticoagulants, non-steroidal anti-inflammatory agents or any antipla- telet drugs other than aspirin or 3) previous ischaemic vascular events or PCI within the previous 12 months or 4) coronary artery bypass grafting (CABG) within the previous three months.

The study was conducted in agreement with the Helsinki-II-declaration and approved by The National Committee on Health Research Ethics (M-20090110) and by the Danish Data Protection Agency. All participants gave written informed consent.

Compliance

To optimise compliance and uniform pharmacokinetics, each patient received a pill-dispensing box with seven tablets of 75 mg non-enteric coated aspirin (Hjerdyl, Sandoz, Denmark). All patients were instructed to ingest aspirin 1 hour (h) before blood sampling. Compliance was optimised by face-to-face interviews, pill-counting and was confirmed by serum thrombomodulin B2 (TXB2). Blood for measurement of serum TXB2 was allowed to clot at 37°C for 1 h in non-siliconised glass tubes before serum was separated by centrifugation at 2600 g for 10 minutes (min). The concentration of serum TXB2 was measured using a commercial enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer’s instructions.

Blood sampling

Blood samples were obtained from the antecubital vein with patients in supine position after 30 min of rest using vacuum tubes, a large bore needle (19 G), and a minimum of stasis. Non-siliconised glass tubes without anticoagulants were used for separation of serum and tubes containing 3.2% sodium citrate (Terumo, Leuven, Belgium) were used for separation of platelet-poor plasma for analyses of fibrin clot structure. Citrated plasma was centrifuged (25 min, 3300 g) within 30 min of platelet-poor plasma collection, immediately frozen and stored in aliquots at -80°C.

Turbidimetric analysis of fibrin clot structure and lysis

Fibrin clot structure and lysis were evaluated by turbidimetric assays in duplicate as previously described (29). The following parameters were recorded: a) Clot maximum absorbance (arbitrary units, au) indicating network density, b) Lysis time (seconds) corresponding to time from full clot formation to 50% lysis, indicating fibrinolytic potential; c) Lysis area (au), reflecting the balance between clot formation and lysis.

Measurement of fibrinogen and markers of inflammation

Fibrinogen levels were determined by the clotting method of Clauss using a KC 10™ coagulometer (Henrich Amelung GmbH, Lemgo, Germany) (30). High sensitivity C-reactive protein (CRP) was evaluated using KoneLab 30i (ILS Laboratories Scandinavia, Allerød, Denmark). Measurements of CRP were missing in 57 patients and CRP values above 10 mg/l were excluded from analyses, thus a total of 493 patients were included in high sensitive CRP analyses. Complement C3 were determined by ELISA according to manufacturer’s instructions (GenWay Biotech, Inc., San Diego, CA, USA) (31). Interleukin-6 was evaluated using cobas 6000 analyzer E-module (Roche, Manheim, Germany). Leucocyte counts were determined together with haemoglobin and platelet count using a XE-2100 hematology analyzer (Sysmex, Kobe, Japan).

Laser scanning confocal microscopy

Fibrin clots from pooled plasma samples were visualized using confocal microscopy. Clots from pooled plasma of 10 randomly chosen T2DM patients and 10 age- and gender-matched non-diabetes patients were studied. Fibrin clots were created by mixing 7.5 µl of plasma with 21 µl permeation buffer (composed of 50 mM Tris, 100 mM NaCl and pH 7.5), 1.5 µl fluorescent labelled fibrinogen (concentration at 0.5 mg/ml Fibrinogen, Alexa Fluor® 488 Conjugate, Life Technologies, Paisley, UK) and 5 µl activation mix (containing: 0.35 U/ml human thrombin (Calbiochem, Merck KGaA, Darmstadt, Germany) and 35 mmol/l calcium). Clots were visualised using a LSM 510 microscope with a 40 × immersion lens (Carl Zeiss, UK Ltd). All clots were made and visualised in triplicates and repeated on two different occasions.
A macro was developed to assess clot density in Image J 1.45 (National Institute of Health, Bethesda, MD, USA). The macro takes the z-stack confocal clot image in Image J and generates a single image that contains the total signal at each pixel location in the image. It then removes the background to identify the clot structures and generates a mask. This mask is then placed back onto the single image that contains the total signal at each pixel location and measures the total signal in the image. This gives an accurate estimate of how many fibre bundles are present in the image, and thus clot density.

**Scanning electron microscopy**

Clots were prepared in duplicate from pooled plasma from 10 randomly chosen T2DM patients and 10 age- and gender-matched non-diabetic patients. Briefly, plasma pools were diluted 1:1 with permeation buffer and subsequently 45 μl diluted plasma was added to 5 μl of activation mix (containing 8.8 U/ml human thrombin (Calbiochem, Merck KGaA, Darmstadt, Germany) and 50 mmol/l calcium chloride). Further preparations of clots were made as previously described (32). All clots were prepared in duplicate and viewed and photographed at ×5,000, ×10,000 and ×30,000 magnifications using a field-emission scanning electron microscope (Quanta 200F FEG ESEM, FEI Company, Eindhoven, Netherlands) in four different areas of each clot. Fibre diameters of all clots were measured using Image J 1.45 (National Institutes of Health). To exclude bias, all clots were viewed by two operators blinded to the type of sample.

**Fibrinogen purification**

Fibrinogen was purified from pooled plasma from 10 randomly chosen T2DM patients and 10 age- and gender-matched non-diabetic patients by affinity chromatography using commercial IF-1 antibody (Kamiya Biomedical Company, Seattle, WA, USA), as previously described (33). Intactness of fibrinogen was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were standardised at a concentration 1 mg/ml for the analysis of clot structure using a turbidimetric assay, which was performed five times in each group on five different occasions.

**Statistical analyses**

All data were checked for normality and equality of variances. Continuous data are presented as mean and standard deviation (SD) if normally distributed, and as median and (25%; 75%) percentiles if not. A two-sided t-test was used to test the difference between two unpaired groups with log transformation as appropriate. For data that were not normally distributed, Mann-Whitney’s test was used for comparison of two unpaired groups. To evaluate differences in proportions between two or more groups, we used Fisher’s exact test or the Chi-square test. Pearson correlation coefficient was used to test for correlation between markers of inflammation and fibrin clot properties without and with adjustment for fibrinogen levels and log transformation as appropriate. Multiple regression analyses were used to adjust for variables when comparing groups and to identify independent determinants of fibrin clot maximum absorbance, lysis area and lysis time. A two-sided p-value <0.05 was considered statistically significant. Statistical analyses were performed using STATA® version 11 (StataCorp, College Station, TX, USA) and graphs were prepared using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

The primary outcome measure was the difference in clot lysis time. Mean (SD) for clot lysis time has previously been observed to be 583 (± 199) seconds (sec) (12). With a significance level (2α) at 5% and a power (1-β) of 90% and a chosen MIREDIF (minimal relevant difference) at 80 sec, we had to include at least 145 patients in each group. To have a representative cohort, we aimed to include three times as many non-diabetic patients as diabetic patients (34).

**Results**

**Clinical characteristics**

Clinical characteristics of the study population are shown in Table 1. Differences between the two groups included body mass index (BMI) and diastolic blood pressure. We aimed to include a population of high risk CAD patients and as per the inclusion criteria, all CAD patients without diabetes had previous MI with two thirds of T2DM having a previous MI. Furthermore, previous PCI was more frequent in patients without diabetes, whereas bypass surgery was more prevalent in patients with T2DM. A higher proportion of patients with T2DM received antihypertensive therapy, which may explain the lower diastolic blood pressure.

All patients claimed to be fully compliant with aspirin monotherapy in face-to-face interviews and by returning empty pill boxes. Compliance was confirmed by serum TXB₂ levels below 27 ng/ml in all patients, thus corresponding to a more than 95% inhibition of platelet cyclooxygenase-1 activity (35). Serum TXB₂ levels were higher in individuals with diabetes than in non-diabetic patients (median [25%; 75%]: 1.8 [0.9; 3.7] vs 0.9 [0.5; 1.5] ng/ml; p<0.0001).

**Influence of diabetes on fibrin clot properties using plasma**

**Turbidimetric analysis**

Patients with T2DM had altered clot structure and prolonged lysis time compared with non-diabetic patients. Clot maximum absorbance was significantly higher, lysis time longer and lysis area larger in diabetic compared with non-diabetic patients assessed by turbidity-lysis as shown in Table 2. Similar findings applied to fibrinogen levels with increased levels in diabetic (2.9 [2.6; 3.4] compared with non-diabetic patients 2.8 [2.6; 3.1] mg/ml, p<0.001). When adjusting for age, gender and fibrinogen levels, the effect of T2DM remained significant for lysis time (p=0.04),
Influence of diabetes on fibrin clot properties using plasma purified fibrinogen

The integrity of purified fibrinogen was confirmed by running on SDS-PAGE gel (Figure 2A). Using purified fibrinogen levels at 1 mg/ml, no differences in clot structure were observed between patients with and without T2DM evaluated by maximum absorbance (0.15 ± 0.02 vs 0.13 ± 0.03 au, p=0.38) or lysis time (605 ± 163 vs 490 ± 99 seconds, p=0.21). Results are summarised in Figure 2B.
Figure 1: Visualisation of fibrin clots from age- and sex-matched non-diabetic controls and patients with type 2 diabetes mellitus using pooled plasma (n=10 in each group). A) Confocal microscopy with clots visualised using 20-µm Z-stack in 3D. B) Scanning electron microscopy with clots visualised at ×30,000 magnification. C) Fibre thickness in a total of 240 fibrin fibres (30 fibres were measured in eight different clot areas from each group from scanning electron microscopy pictures).

Figure 2: Fibrin clot properties using purified fibrinogen from pooled plasma (n = 10 in each group). A) SDS-PAGE image showing dimers of α, β and γ chains, thus demonstrating the integrity of purified fibrinogen used in this experiment. B) Fibrin clot structure evaluated by turbidimetric assays using purified fibrinogen at 1 mg/ml from pooled plasma on five separate occasions.
Inflammatory proteins and fibrin clot properties

Levels of fibrinogen, CRP and complement C3 were significantly increased in patients with T2DM compared with non-diabetic patients, whereas only a trend was evident for interleukin-6. Results are summarised in Table 2.

Correlations between fibrin clot properties and markers of inflammation are shown in Table 3. CRP, complement C3 and interleukin-6 correlated positively with all clot structure parameters. These correlations remained significant for all clot properties after adjustment for fibrinogen levels, except for the relationship between maximum absorbance and complement C3 levels (Table 3). Furthermore, fibrinogen levels correlated with CRP (r=0.41, p<0.0001) and interleukin-6 (r=0.32, p<0.0001). Additionally, fibrinogen and CRP correlated positively with clot structure parameters. Furthermore, fibrinogen levels correlated with CRP (r=0.41, p<0.0001) and interleukin-6 (r=0.32, p<0.0001). These correlations remained significant for all clot properties after adjustment for fibrinogen levels, except for the relationship between maximum absorbance and complement C3 levels (Table 3). Furthermore, fibrinogen levels correlated with CRP (r=0.41, p<0.0001) and interleukin-6 (r=0.32, p<0.0001) to a lesser extent with complement C3 (r=0.18, p<0.0001) and interleukin-6 (r=0.18, p<0.0001).

Influence of gender on fibrin clot parameters

A significant difference in fibrin clot properties was observed between female (n=120) and male patients (n=458). Females and males did not differ in clinical characteristics in relation to age, BMI, smoking, presence of T2DM, previous MI, stroke or type of vascular intervention (all p-values > 0.10; data not shown). Female patients had altered fibrin clots, characterised by increased maximum absorbance (0.37 ±0.1 vs 0.33 ±0.1 au, p=0.0001), prolonged lysis time [894 (705; 1047) vs 738 (603; 888) sec, p<0.0001] and larger lysis area [522 (378; 678) vs 414 (300; 558) au, p<0.0001]. This was also evident when analysing gender differences in all patients without diabetes (all p-values < 0.001). There was no difference in fibrinogen levels between female and male patients [2.9 (2.6; 3.1) vs 2.8 (2.6; 3.2) mg/ml, p=0.13] and levels of inflammatory markers, including CRP, C3 and interleukin-6 were also similar (all p-values ≥0.25). When adjusted for age, fibrinogen and T2DM, the importance of gender remained significant for maximum absorbance, lysis area and lysis time (all p-values < 0.001).

Determinants of fibrin clot properties

Independent determinants of fibrin clot properties were investigated using multiple regression analyses including age, gender, previous MI, T2DM, BMI, smoking and fibrinogen. As shown in Table 4, fibrinogen was an independent determinant of clot maximum absorbance and lysis area, whereas age only determined maximum absorbance. Gender was an independent determinant of all three parameters of clot structure, and BMI determined lysis maximum absorbance (0.37 ±0.1 vs 0.33 ±0.1 au, p=0.0001), prolonged lysis time [894 (705; 1047) vs 738 (603; 888) sec, p<0.0001] and larger lysis area [522 (378; 678) vs 414 (300; 558) au, p<0.0001]. This was also evident when analysing gender differences in all patients without diabetes (all p-values < 0.001). There was no difference in fibrinogen levels between female and male patients [2.9 (2.6; 3.1) vs 2.8 (2.6; 3.2) mg/ml, p=0.13] and levels of inflammatory markers, including CRP, C3 and interleukin-6 were also similar (all p-values ≥0.25). When adjusted for age, fibrinogen and T2DM, the importance of gender remained significant for maximum absorbance, lysis area and lysis time (all p-values < 0.001).

### Table 3: Correlation between markers of inflammation and fibrin clot properties in coronary artery disease patients with and without type 2 diabetes mellitus. Correlations evaluated using Pearson correlation coefficient and partial correlation.

<table>
<thead>
<tr>
<th>Parameters of fibrin clot structure</th>
<th>High sensitive CRP</th>
<th>Complement C3</th>
<th>Interleukin-6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r; P-value</td>
<td>r; P-value</td>
<td>r; P-value</td>
</tr>
<tr>
<td>Maximum absorbance</td>
<td>0.37; &lt;0.0001</td>
<td>0.14; &lt;0.001</td>
<td>0.36; &lt;0.001</td>
</tr>
<tr>
<td>adjusted for fibrinogen</td>
<td>0.24; &lt;0.0001</td>
<td>0.07; 0.10</td>
<td>0.26; &lt;0.001</td>
</tr>
<tr>
<td>Lysis time</td>
<td>0.16; &lt;0.001</td>
<td>0.31; &lt;0.001</td>
<td>0.13; &lt;0.01</td>
</tr>
<tr>
<td>adjusted for fibrinogen</td>
<td>0.15; &lt;0.001</td>
<td>0.30; &lt;0.001</td>
<td>0.11; &lt;0.01</td>
</tr>
<tr>
<td>Lysis area</td>
<td>0.31; &lt;0.0001</td>
<td>0.28; &lt;0.0001</td>
<td>0.27; &lt;0.0001</td>
</tr>
<tr>
<td>adjusted for fibrinogen</td>
<td>0.22; &lt;0.0001</td>
<td>0.24; &lt;0.0001</td>
<td>0.18; &lt;0.0001</td>
</tr>
</tbody>
</table>

1hs-CRP 88 values missing. CRP: C-reactive protein.

### Table 4: Multiple regression analyses in CAD patients with and without diabetes (n=575). Significant predictors of fibrin clot properties are highlighted in bold.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Parameters of fibrin clot structure</th>
<th>Maximum absorbance, au</th>
<th>Lysis time, seconds</th>
<th>Lysis area, au*seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beta; 95 % CI</td>
<td>P-value</td>
<td>Beta; 95 % CI</td>
<td>P-value</td>
</tr>
<tr>
<td>Age, years</td>
<td>0.001; 0.0004–0.0002</td>
<td>0.01</td>
<td>-0.002; -0.004–0.001</td>
<td>0.23</td>
</tr>
<tr>
<td>Gender</td>
<td>0.032; 0.014–0.050</td>
<td>0.00</td>
<td>0.149; 0.091–0.207</td>
<td>0.00</td>
</tr>
<tr>
<td>Previous MI</td>
<td>-0.020; 0.050–0.010</td>
<td>0.18</td>
<td>-0.051; -0.149–0.047</td>
<td>0.31</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>-0.003; -0.022–0.017</td>
<td>0.79</td>
<td>0.008; -0.058–0.074</td>
<td>0.81</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>0.001; -0.001–0.003</td>
<td>0.23</td>
<td>0.009; 0.004–0.015</td>
<td>0.00</td>
</tr>
<tr>
<td>Smoking</td>
<td>-0.001; -0.019–0.017</td>
<td>0.91</td>
<td>-0.21; -0.080–0.038</td>
<td>0.48</td>
</tr>
<tr>
<td>Fibrinogen, mg/ml</td>
<td>0.070; 0.058–0.082</td>
<td>0.00</td>
<td>0.015; -0.024–0.055</td>
<td>0.44</td>
</tr>
</tbody>
</table>

MI: myocardial infarction; BMI: body mass index.
Independent determinants of clot structure were also investigated using multiple regression analyses with above-mentioned variables (age, gender, previous MI, T2DM, BMI, smoking and fibrinogen) and in the presence of CRP, complement C3 and interleukin-6. Complement C3 was an independent determinant of all three clot parameters (maximum absorbance, lysis time and lysis area, all p-values < 0.01), whereas CRP only determined maximum absorbance and lysis area (both p-values < 0.001). Further analysis by adding statins, antihypertensive medications and insulin to the model (including age, gender, previous MI, T2DM, BMI, smoking and fibrinogen) failed to demonstrate an association between type of treatment and any of the fibrin clot parameters studied.

Discussion

This is the largest study to investigate the influence of T2DM on the fibrin network and function in patients with CAD. A number of novel findings emerge from this work: 1) CAD patients with T2DM have more compact clots with impaired fibrinolysis compared with CAD patients without diabetes. 2) The changes in fibrin clot properties were related to quantitative rather than qualitative changes in fibrinogen, with an additional possible influence of inflammatory proteins. 3) Independent of fibrinogen levels, female CAD patients had more compact fibrin networks with resistance to lysis compared with male CAD patients.

Until now, only a limited number of studies have investigated clot structure in patients with T2DM. These studies demonstrated altered fibrin networks, characterised by a denser, less porous structure with prolonged lysis time in this population (18, 20, 36, 37). CAD has also been associated with more compact fibrin networks with resistance to fibrinolysis (16, 38). However, so far it has remained unclear whether diabetes has an additional effect on clot structure in the presence of established vascular disease, since only a single and relatively small study involving 132 CAD patients has previously investigated this (21). Patients in the study represented a selected cohort with all scheduled for CABG surgery and indirect measures of clot characteristics were conducted. Our data showed that in a large population of CAD patients, confirmed by coronary angiography, the presence of T2DM was associated with additional changes in clot structure properties. Diabetic clots were characterised by a more prothrombotic phenotype evaluated by dynamic analyses and confirmed visually by both confocal and scanning electron microscopy. These changes may partly contribute to the increased thrombotic risk and adverse clinical outcome following cardiovascular events in T2DM patients.

The underlying mechanisms of altered clot structure are complex and likely multifactorial. We have studied some of the potential pathways involved. Platelet count and mean platelet volume did not differ between groups and thus are unlikely to explain the observed differences in fibrin clot properties between groups (39). Our data confirm that CAD patients with T2DM have elevated levels of plasma fibrinogen. This, together with the absence of a difference in maximum absorbance between study groups after adjusting for fibrinogen levels, suggest that raised fibrinogen levels were responsible for increased clot maximum absorbance in diabetes (15). This was further supported by the absence of differences in clots made from plasma-purified fibrinogen comparing T2DM and non-diabetes patients. Other than quantitative changes in fibrinogen, it is possible that other plasma proteins may have contributed to the difference observed in clot maximum absorbance. For example, FXIII is responsible for fibrin α- and γ-chain crosslinking and results in a small increase in fibrin clot density in a purified system (40). Studies have reported changes in α-chain cross-linking in diabetes, including an increase and a decrease (41, 18), whereas others show no differences (37). It is possible that addition of FXIII in our purified experiments may have resulted in a difference between diabetes and non-diabetes samples due to alternative α-chain cross-linking of fibrinogen. However, more recent work suggests the modest increase in fibrin network density by FXIII is related to fibrinogen γ-chain cross-linking, which is not believed to be affected in diabetes (42). Moreover, the main reason for undertaking the purified experiments was to clarify whether quantitative changes in fibrinogen are responsible per se for the observed differences in plasma clot properties, which does not appear to be the case.

We have previously shown that aspirin facilitates fibrinolysis, which is related to acetylation of fibrinogen (7). In the present study, all patients were treated with aspirin 75 mg/day and no other antithrombotic drugs. The elevated levels of serum thromboxane B2 indicated a compromised effect of aspirin in patients with T2DM. Despite aspirin, patients with CAD and concomitant T2DM had impaired fibrinolysis using plasma samples, but no difference in fibrinolysis time using pooled samples of purified fibrinogen. Thus, our data did not support an impaired acetylation of fibrinogen by aspirin in diabetes, although no firm conclusions can be made, since the analyses of clots using purified fibrinogen were made using a limited number of pooled samples.

What is known about this topic?

- Patients with coronary artery disease (CAD) and type 2 diabetes (T2DM) have increased risk of cardiovascular events.
- Altered fibrin clot properties have been linked to premature cardiovascular events.
- Both CAD and T2DM are independently associated with altered fibrin networks and impaired fibrinolysis.

What does this paper add?

- T2DM is associated with additional prothrombotic changes in fibrin clot properties in patients with established CAD.
- The observed changes in fibrin clot properties appear to be related to quantitative changes in fibrinogen with an additional influence of the inflammatory protein, complement C3.
- Amongst patients with CAD, women have more compact fibrin networks with increased resistance to fibrinolysis compared with men.
Low-grade inflammation may contribute to an altered fibrin clot structure (25, 26). As expected, patients with T2DM had increased levels of CRP and complement C3. CRP showed good correlation with clot maximum absorbance, which may be explained by the close association between plasma levels of fibrinogen and CRP, given that their transcription is mediated by similar factors (15). Yet, the difference in plasma clot lysis time was still evident after adjusting for fibrinogen levels. This, together with the absence of a difference in lysis time using plasma-purified fibrinogen, suggests that the difference in fibrinolysis was not related to quantitative changes in fibrinogen but owing to influence of other plasma proteins. A candidate protein is complement C3, which is incorporated into the fibrin clot and modulates fibrinolysis (27, 28). Indeed, C3 plasma levels showed positive correlation with clot lysis time, suggesting that this protein is involved in impaired fibrinolysis in CAD patients with T2DM. Furthermore, C3 was an independent determinant of all parameters of clot structure, supporting its importance in fibrin clot networks. Our findings suggest, that an enhanced inflammatory milieu in CAD patients with T2DM further contributes to altered fibrin clot properties, emphasising the importance of links between inflammatory and coagulation pathways.

Female gender has been associated with increased cardiovascular risk and poor prognosis following MI (43), particularly in women with diabetes (6). In the present study, clot structure in female patients was characterised by increased maximum absorbance, larger lysis area and prolonged lysis time compared with male patients. These changes were evident despite no differences in fibrinogen levels and remained significant after adjusting for age, fibrinogen and diabetes. In addition, gender was the only independent determinant of all three dynamic parameters of clot structure in multiple regression analyses. This supports a role of gender in determining fibrin clot characteristics (22), and the observed prothrombotic phenotype in women may be one reason for their adverse prognosis following MI (44, 45). The mechanisms explaining these gender differences in clot properties remain unclear and warrant further investigation.

Limitations

Although all patients had CAD verified by coronary angiography, one-third of patients with T2DM did not have previous MI, in contrast to the non-diabetic group, where all patients had a history of MI as per the inclusion criteria. However, one may argue that this makes our findings even stronger and emphasises the additional effect of T2DM on the fibrin network (12). Also, our data suggest a role for fibrinogen and elevated markers of inflammation in determining clot structure in CAD patients with diabetes, however contribution from other plasma proteins such as (pro)thrombin, α2-antiplasmin and plasminogen activator inhibitor were not investigated. Moreover, criticisms can be directed at the failure to evaluate clot structure before aspirin administration, yet this was not possible since discontinuation of aspirin would be unethical in these high-risk CAD patients. Finally, the cross-sectional nature of the work does not allow for causal relationships to be explored, which require future longitudinal studies to fully understand the role of the fibrin network in predisposition to cardiovascular events in high-risk patients, including patients with diabetes.

Conclusion

The present study is the largest to evaluate fibrin clot structure properties in a population of CAD patients with and without concomitant T2DM. Our results showed that patients with diabetes had a more prothrombotic clot phenotype, which may be explained by elevated levels of fibrinogen and inflammatory proteins. We also demonstrate that female gender was associated with a prothrombotic fibrin clot phenotype. Future longitudinal studies are warranted to assess the predictive role of fibrin clot properties on clinical outcome in CAD patients with and without diabetes.

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Conflicts of interest

None declared.

References


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