Diabetologia (2014) 57:1737–1741
DOI 10.1007/s00125-014-3267-z

SHORT COMMUNICATION

# Hypofibrinolysis in type 2 diabetes: the role of the inflammatory pathway and complement C3

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Received: 20 December 2013 / Accepted: 29 April 2014 / Published online: 17 May 2014


**Abstract**
*Aims/hypothesis* Plasminogen activator inhibitor-1 (PAI-1) has been regarded as the main antifibrinolytic protein in diabetes, but recent work indicates that complement C3 (C3), an inflammatory protein, directly compromises fibrinolysis in type 1 diabetes. The aim of the current project was to investigate associations between C3 and fibrinolysis in a large cohort of individuals with type 2 diabetes.
*Methods* Plasma levels of C3, C-reactive protein (CRP), PAI-1 and fibrinogen were analysed by ELISA in 837 patients enrolled in the Edinburgh Type 2 Diabetes Study. Fibrin clot lysis was analysed using a validated turbidimetric assay.
*Results* Clot lysis time correlated with C3 and PAI-1 plasma levels ($r$=0.24, $p$<0.001 and $r$=0.22, $p$<0.001, respectively). In a multivariable regression model involving age, sex, BMI, C3, PAI-1, CRP and fibrinogen, and using log-transformed data as appropriate, C3 was associated with clot lysis time (regression coefficient 0.227 [95% CI 0.161, 0.292], $p$<0.001), as was PAI-1 (regression coefficient 0.033 [95% CI 0.020, 0.064], $p$<0.05) but not fibrinogen (regression coefficient 0.003 [95% CI −0.046, 0.051], $p$=0.92) or CRP (regression coefficient 0.024 [95% CI −0.008, 0.056], $p$=0.14). No correlation was demonstrated between plasma levels of C3 and PAI-1 ($r$=−0.03, $p$=0.44), consistent with previous observations that the two proteins affect different pathways in the fibrinolytic system.
*Conclusions/interpretation* Similarly to PAI-1, C3 plasma levels are independently associated with fibrin clot lysis in individuals with type 2 diabetes. Therefore, future studies should analyse C3 plasma levels as a surrogate marker of fibrinolysis potential in this population.

**Keywords** Complement C3 · C-reactive protein · CRP · Diabetes · Fibrinolysis

**Abbreviations**

| | |
|---|---|
| C3 | Complement C3 |
| CRP | C-reactive protein |
| ET2DS | Edinburgh Type 2 Diabetes Study |
| PAI-1 | Plasminogen activator inhibitor-1 |

K. Hess and S. H. Alzahrani contributed equally to this work.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00125-014-3267-z) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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## Introduction

Hypofibrinolysis is a key abnormality in type 2 diabetes and contributes to increased atherothrombosis risk in this condition. Elevated plasma levels of plasminogen activator inhibitor-1 (PAI-1) have been traditionally regarded as the main cause of impaired fibrin clot lysis in diabetes [1]. However, we have recently described a novel mechanism for hypofibrinolysis in diabetes, secondary to interactions between the inflammatory and coagulation pathways, mediated by complement C3 (C3) [2]. C3 prolongation of clot lysis was more pronounced in individuals with type 1 diabetes compared with healthy controls, suggesting a diabetes-specific additional effect of this protein. Although plasma levels of C3 correlate with clot lysis in individuals with type 1 diabetes and in those without diabetes [2, 3], it is unclear whether this is the case in type 2 diabetes [4].

We hypothesise that C3 represents a key PAI-1-independent mechanism for hypofibrinolysis in type 2 diabetes. The aim of our work was therefore to analyse the cross-sectional associations between fibrinolytic potential and C3 plasma levels in a large cohort of individuals with type 2 diabetes and compare the results with the association between fibrin clot lysis and PAI-1 levels. Our secondary aims were to establish the associations between plasma levels of C3, metabolic measures and various therapies used in this population.

## Methods

*Study population and examination* Details of the Edinburgh Type 2 Diabetes Study (ET2DS) are described elsewhere [3, 5]. Blood samples were taken into citrated tubes without a tourniquet after a 4 h fast, usually at midday. Samples were spun down within 2 h of collection and plasma was stored at −80ºC until analysis. A total of 837 plasma samples were available for measurement of plasma protein levels and clot structure analysis. The study was approved by the Lothian Research Ethics Committee and written informed consent was obtained from all patients.

*Plasma levels of C3, C-reactive protein, PAI-1 and fibrinogen* Plasma C3 was determined using a commercial ELISA Kit (GenWay Biotech, San Diego, CA, USA) and high-sensitivity C-reactive protein (CRP) was analysed as previously described [2]. PAI-1 was assessed by ELISA (Invitrogen, Paisley, UK). Fibrinogen levels were determined by the Clauss method.

*Analysis of fibrinolysis* This was conducted using turbidimetric assays as previously described [2]. Time from full clot formation to 50% lysis was subsequently calculated, termed lysis time.

*Statistical analysis* Pearson's correlations were used to estimate associations, after log-transformation of data that were not normally distributed. The R environment for statistical computing was used to fit regression models to determine independent association of clot lysis time and C3 plasma levels, with both univariate and multivariable models to study associations between the variables and the influence of age, BMI and sex, which are known to affect fibrinolysis [3]. All variables that were not normally distributed were log-transformed before inclusion in the model. The effect of each of the variables was characterised in a regression model using 95% CIs for the regression coefficients. Data are presented as mean ± SD or median (interquartile range) for normally or non-normally distributed data, respectively.

## Results

*C3 plasma levels are associated with prolonged clot lysis* Characteristics of the study population are summarised in Table 1. Clot lysis time correlated with C3 and PAI-1 plasma levels ($r$=0.24, $p$<0.001 and $r$=0.22, $p$<0.001, respectively). Lysis time showed correlations with fibrinogen and CRP levels ($r$=0.08, $p$=0.03 and $r$=0.14, $p$<0.001, respectively). There was no correlation between C3 and PAI-1 plasma levels in the whole group ($r$=−0.03, $p$=0.44), nor when analysed by tertiles of age or BMI (data not shown). Similar results were obtained when men and women were analysed separately (data not shown).

*C3 plasma levels predict clot lysis independently of PAI-1* In a regression model including C3, CRP, fibrinogen and PAI-1 (adjusted for age, sex and BMI), and after log-transformation of data as appropriate, C3 was associated with clot lysis time (regression coefficient 0.227 [95% CI 0.161, 0.292], $p$<0.001), as was PAI-1 (regression coefficient 0.033 [95% CI 0.020, 0.064], $p$<0.05), whereas fibrinogen and CRP showed no associations (regression coefficient 0.003 [95% CI −0.046, 0.051], $p$=0.92; and 0.024 [95% CI −0.008, 0.056], $p$=0.14, respectively). Results are summarised in Fig. 1a.

In a separate univariate analysis, results were largely similar for C3 (regression coefficient 0.234 [95% CI 0.169, 0.299], $p$<0.001) and PAI-1 (regression coefficient 0.052 [95% CI 0.022, 0.082], $p$=0.001), but fibrinogen showed an association (regression coefficient 0.046 [95% CI 0.004, 0.09], $p$<0.05), as did CRP (regression coefficient 0.055 [95% CI 0.028, 0.082], $p$<0.001). Results are summarised in electronic supplementary material (ESM) Table 1.

Associations of C3 and PAI-1 with clot lysis were not materially altered when analysed mutually adjusted or separately for each variable.

**Table 1** Clinical characteristics of the study population

| Variable | Value |
|---|---|
| Sex, *n* (male/female) | 431/406 |
| Age, years | 67.9±4.2 |
| Current smokers, *n* (%) | 104 (12) |
| Ex-smokers, *n* (%) | 392 (47) |
| Non-smokers, *n* (%) | 341 (41) |
| Inflammatory measures | |
| CRP, nmol/l | 17.33 (7.81–39.05) |
| C3, g/l | 1.35 (1.10–1.60) |
| Fibrinogen, μmol/l | 11±2 |
| PAI-1, pmol/l | 118 (54–230) |
| Vascular measures | |
| IHD, *n* (yes/no) | 253/584 |
| Systolic BP, mmHg | 133±16 |
| Diastolic BP, mmHg | 69±9 |
| Anthropometrics | |
| BMI, kg/m$^2$ | 30.7 (27.3–34.4) |
| Waist circumference, cm | 106.7±12.9 |
| Cardiometabolic factors | |
| $HbA_{1c}$, mmol/mol | 55.0 (50–61) |
| $HbA_{1c}$, % | 7.2 (6.7–7.8) |
| Plasma glucose, mmol/l | 7.2 (6.2–8.5) |
| Total cholesterol, mmol/l | 4.2 (3.7–4.8) |
| HDL-cholesterol, mmol/l | 1.3 (1.1–1.5) |
| Diabetes duration, years | 6 (3–11) |
| Clot lysis time, s | 618 (480–816) |
| Clot final turbidity, AU | 0.33 (0.24–0.40) |

Data are presented as mean ± SD for normally distributed data, median with interquartile range for non-normally distributed data, or as a percentage for categorical data

AU, arbitrary units; IHD, ischaemic heart disease (defined as validated angina or myocardial infarction at baseline)

We also analysed associations between C3 and clot final turbidity, a measure of clot density. C3 showed associations with clot final turbidity, using both univariable and multivariable analysis (ESM Table 2), as did fibrinogen levels. However, PAI-1 failed to show any association with clot final turbidity, whereas associations with CRP were only evident in the univariate analysis. Results are summarised in ESM Table 2.

*Inflammatory markers, metabolic risk factors and sex* Given sex differences in fibrin clot lysis in diabetes [3], C3 plasma levels were analysed separately in men and women with no difference detected (regression coefficient 0.207 [95% CI 0.165, 0.249] and 0.203 [95% CI 0.157, 0.248], respectively, $p=0.89$), even after correcting for BMI (data not shown). CRP levels, however, were lower in men compared with women (regression coefficient 0.434 [95% CI 0.333, 0.534] and 0.928 [95% CI 0.831, 1.026], respectively, $p<0.001$).

In a multivariable regression analysis including BMI, $HbA_{1c}$, glucose levels, duration of diabetes, total cholesterol and HDL-cholesterol, adjusted for age and sex, BMI was associated with C3 plasma levels (regression coefficient 0.206 [95% CI 0.013, 0.399], $p<0.05$; Fig. 1b) as well as CRP levels (regression coefficient 1.35 [95% CI 0.88, 1.81], $p<0.001$). Plasma cholesterol showed a positive association with CRP levels (regression coefficient 0.83 [95% CI 0.42, 1.23], $p<0.001$), but HDL-cholesterol demonstrated a negative association (regression coefficient −0.57 [95% CI −0.88, −0.26], $p<0.001$) (details of various analyses on C3 and CRP are provided in ESM Tables 3 and 4, respectively).

*Inflammatory markers and medications* In multivariate regression analysis including self-reported use of statins, antiplatelet therapy, oral hypoglycaemic agents (thiazolidinediones, metformin and sulfonylureas), insulins

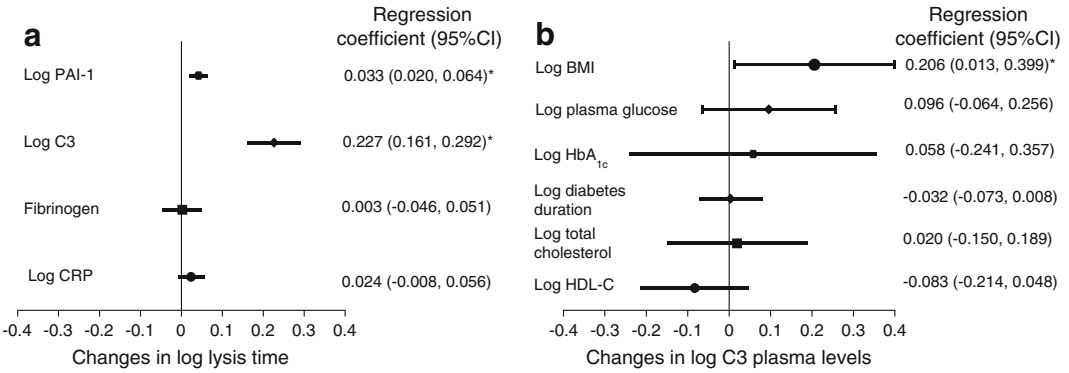


**Fig. 1** Fibrin clot lysis, C3 and determinants of plasma levels in individuals with diabetes. (**a**) Associations between clot lysis time and PAI-1, C3, fibrinogen and CRP, adjusted for age, sex and BMI using a multivariable regression model. Results are presented as regression coefficients with 95% CIs and show both C3 and PAI-1 to be associated with clot lysis time (*$p<0.05$), whereas fibrinogen and CRP fail to demonstrate an association. (**b**) Metabolic determinants of C3 plasma levels. A multivariate regression model was constructed including BMI, plasma glucose, $HbA_{1c}$, diabetes duration, total cholesterol and HDL-cholesterol (HDL-C) and results are presented as regression coefficients with 95% CIs. *$p<0.05$ for the association between log BMI and changes in log C3 plasma levels. Clot lysis time, C3, CRP and PAI-1 plasma levels, BMI, $HbA_{1c}$, diabetes duration, total cholesterol and HDL-cholesterol were all $\log_e$-transformed before analysis

(both long- and short-acting), anti-anginal and antihypertensive therapy (nitrates, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, beta blockers and calcium channel blockers), and adjusted for age, sex and BMI, none of the medication was associated with C3 plasma levels (ESM Table 5). Plasma CRP, however, was associated with insulin use (regression coefficient 0.49 [95% CI 0.24, 0.75], $p<0.001$), oral hypoglycaemia agents and statin therapy (regression coefficient −0.27 [95% CI −0.44, −0.11] and −0.38 [95% CI −0.58, −0.17], both $p<0.001$). Similar results were obtained for univariate analysis (ESM Table 6).

## Discussion

The complement system is involved in the early stages of atherothrombosis [6]. Recent work suggests that C3 also contributes to later stages of the process by compromising fibrinolysis [2]. The involvement of C3 in the pathogenesis of vascular thrombosis may explain the association between plasma levels of this protein and cardiovascular disease [7]. High plasma levels of C3 can be found in younger individuals with pathological conditions, including those with type 1 diabetes, and in children with a predisposition to future cardiovascular events [2, 8], suggesting C3 is part of the vascular risk factor cluster at an early age. An association between fibrinolysis and C3 plasma levels has been demonstrated in type 1 diabetes, and the current work shows a relationship between C3 levels and fibrin clot lysis in type 2 diabetes, which is at least as pronounced as the association between PAI-1 and fibrinolysis in this population. Furthermore, we show no correlation between C3 and PAI-1 plasma levels, consistent with the observations that the two molecules affect separate pathways in the fibrinolytic system [2]. PAI-1 inhibits fibrinolysis by interfering with plasmin generation, whereas C3 modulates clot lysis through incorporation into the clot. This increases mechanical resistance to lysis and may compromise the fibrinolytic activity of plasmin [2, 9]. By contrast, CRP levels did not show an independent association with fibrin clot lysis, suggesting that the interaction between the inflammatory and coagulation pathway is C3-specific. C3 levels were not influenced by $HbA_{1c}$ in the population studied, in contrast to individuals with type 1 diabetes, indicating that other factors have stronger effects on plasma protein levels in those with type 2 diabetes.

Clinically, this work suggests that measuring PAI-1 plasma levels only addresses part of the fibrinolytic potential in diabetes and C3 offers an additional assessment tool which investigates a different antifibrinolytic pathway. Moreover, interference with C3–fibrin interactions may offer a novel therapeutic target to improve hypofibrinolysis in diabetes. It is unclear at present whether C3 plasma levels can predict predisposition to or severity of atherothrombotic events in diabetes, and future longitudinal work is warranted to fully assess the role of C3 in vascular thrombosis in this population.

In summary, we demonstrate that C3 plasma levels are associated with fibrin clot lysis in type 2 diabetes independently of PAI-1. Our data indicate that C3 plasma levels should be measured when assessing fibrinolytic potential in diabetes. Future prospective studies are needed to elucidate the role of C3 in predisposition to atherothrombotic events in diabetes.

**Acknowledgements** J. F. Price and M. W. Strachan are principal investigators for the ET2DS and would like to thank staff and participants at the Wellcome Trust Clinical Research Facility in Edinburgh.

Some of the data were presented as an abstract at the XXIV Congress of the International Society on Thrombosis and Haemostasis in 2013 and the European Society of Cardiology in 2012.

**Funding** KH is supported by the Deutsche Forschungsgemeinschaft HE 5666/1-2 and by the Stiftung der Herzkranke Diabetiker. SHA is supported by Diabetes Centre at King Fahad Medical City, Riyadh, Kingdom of Saudi Arabia. We also wish to thank the UK's National Institute for Health Research, British Heart Foundation and Bayer HealthCare Pharmaceuticals for their generous support.

The Edinburgh Type 2 Diabetes Study (ET2DS) was funded by the Medical Research Council and Pfizer.

**Duality of interest** The authors declare that there is no duality of interest associated with this manuscript.

**Contribution statement** KH contributed to the concept and design, data acquisition, analysis and interpretation, and drafting of the article. JFP and MWS contributed to the concept and design, data acquisition and revision of the article. SHA, PDB, NO, RK, TG and VS contributed to the data acquisition and analysis, and revision of the article. RAA contributed to the concept and design, data acquisition, analysis and interpretation data, and revision of the article. RAA is the guarantor of the work. All authors approved the final version.

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