Fibrinogen interaction with complement C3: a potential therapeutic target to reduce thrombosis risk


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Fibrinogen interaction with complement C3: a potential therapeutic target to reduce thrombosis risk

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Running title: Hypofibrinolytic effects of complement C3 and its modulation

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Abstract

Complement C3 binds fibrinogen and compromises fibrin clot lysis thereby enhancing thrombosis risk. We investigated the role of fibrinogen-C3 interaction as a novel therapeutic target to reduce thrombosis risk by analysing: i) consistency in the fibrinolytic properties of C3, ii) binding sites between fibrinogen and C3 and iii) modulation of fibrin clot lysis by manipulating fibrinogen-C3 interactions. Purified fibrinogen and C3 from the same individuals (n=24) were used to assess inter-individual variability in the anti-fibrinolytic effects of C3. Microarray screening and molecular modelling evaluated C3 and fibrinogen interaction sites. Novel synthetic conformational proteins, termed Affimers, were used to modulate C3-fibrinogen interaction and fibrinolysis. C3 purified from patients with type 1 diabetes showed enhanced prolongation of fibrinolysis compared with healthy control protein [195±105 and 522±166 seconds, respectively (p=0.04)], with consistent effects but a wider range (5-51% and 5-18% lysis prolongation, respectively). Peptide microarray screening identified 2 potential C3-fibrinogen interactions sites within fibrinogen β chain (residues 424-433, 435-445). One fibrinogen-binding Affimer was isolated that displayed sequence identity with C3 in an exposed area of the protein. This Affimer abolished C3-induced prolongation of fibrinolysis (728±25.1 seconds to 632±23.7 seconds, p=0.005) and showed binding to fibrinogen in the same region that is involved in C3-fibrinogen interactions. Moreover, it shortened plasma clot lysis of patients with diabetes, cardiovascular disease or controls by 7-11%. C3 binds fibrinogen β-chain and disruption of fibrinogen-C3 interaction using Affimer proteins enhances fibrinolysis, which represents a potential novel target tool to reduce thrombosis in high risk individuals.
Introduction

Hypofibrinolysis is associated with increased risk of atherothrombotic events (1-3). Although most studies have only shown an association between hypofibrinolysis and cardiovascular (CV) disease, a more recent longitudinal study in a large population of patients with acute coronary syndrome has demonstrated that prolonged fibrin clot lysis is an independent predictor of CV mortality (4). Therefore, it was proposed that reducing residual thrombosis risk in patients with coronary artery disease requires targeting the fibrinolytic system in addition to anti-platelet therapies. Indeed the combination of anti-coagulant and anti-platelet therapies agents reduces vascular thrombotic events but at the expense of increased risk of bleeding (5). Therefore, a more targeted approach is required that improves hypofibrinolysis and reduces the risk of vascular thrombosis without increasing bleeding events.

Complement C3, which is incorporated into fibrin clots, has been shown to modulate fibrin clot lysis (6), an effect that may be exaggerated in higher vascular risk patients (7). C3 plasma levels have shown correlations with ex-vivo plasma clot lysis in individuals with diabetes as well as healthy controls (7;8). Moreover, plasma levels of this protein were independent predictors of resistance to fibrinolysis in 875 high vascular risk patients with type 2 diabetes (9). These data suggest that C3 represents a credible therapeutic target to shorten fibrin clot lysis and ultimately reduce thrombosis risk. However, these functional studies have been conducted using pooled C3 and studies assessing consistency of the response in different individuals, crucial to establish the therapeutic potential of C3, are lacking.

We have recently demonstrated that fibrin clot lysis can be modulated to stabilise the clot with the use of small conformational proteins, termed Affimers (10;11). This Affimer technology showed restoration of abnormal lysis of clots made from plasma
samples of individuals with haemophilia, indicating a potential therapeutic role for these proteins. However, it remains unknown whether Affimers can be used to enhance clot lysis, particularly in individuals at high vascular risk.

The aim of this work was to establish the role of C3 as a therapeutic target for enhancing fibrinolysis and reducing thrombosis risk. Therefore, we studied: i) Consistency in the fibrinolytic properties of C3 in healthy controls and high vascular risk patients, ii) potential binding sites between fibrinogen and C3 and iii) modulation of fibrin clot lysis by targeted interference of fibrinogen-C3 interactions.

Methods

Only a brief description is provided, full details can be found in the online supplement.

Patient recruitment

We recruited 12 patients with type 1 diabetes (T1DM) and 12 age and sex matched controls to purify fibrinogen and C3. We additionally tested 24 stored plasma samples from high vascular risk individuals. Ethical approval for the study was provided by the Leeds East, National Research Ethics Service committee and informed consent obtained from participants in accordance with the Declaration of Helsinki.

Protein purification and turbidimetric analysis

Fibrinogen and complement C3 were purified as described (7) and clots formed from purified fibrinogen in the presence/absence of corresponding C3 from the same individual. Time from full clot formation to 50% lysis was calculated and presented as clot lysis time throughout this work.
**Peptide Microarray**

A peptide microarray chip containing overlapping 15 aa peptides of the fibrinogen molecule (PEPperPRINT, Germany) was synthesised to screen against pooled C3 (0.8 to 8 µg/ml) purified from 6 healthy individuals.

**Non-antibody synthetic proteins (Affimers)**

Affimers are small proteins composed of a scaffold protein that constrains two variable conformational 9 amino acid loops (11;12).

**Panning and protein production**

A phage display library of Affimer proteins, comprising $1.3 \times 10^{10}$ random variable clones, was screened against human fibrinogen. In order to select specific Affimers capable of targeting fibrinogen–C3 interactions, competitive elution with C3 for 20 min was applied in the fourth panning.

**Affimers and fibrinolysis**

*Purified experiments*

Fibrinogen was incubated with Affimer A6 at room temperature for 30 min followed by the addition of C3 and Factor XIII and clot formation/lysis was triggered as described (13).

*Plasma experiments*

Plasma samples were mixed with increasing concentrations of Affimer A6 or control scaffold followed by turbidimetric experiments as described (13).

**Molecular modelling**

In order to identify potential sites of ligand binding on C3, the molecular modelling software Autoligand was employed to scan the whole protein (14). Molecular
modelling was used to predict binding sites of any peptide sequences identified from the microarray screening and Affimer work using the webserver Pepsite2® (http://pepsite2.russelllab.org). Images were viewed and produced using Pymol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) (15).

**Mass Spectrometry**

Purified C3 from 6 T1DM and 6 healthy controls was digested by trypsin and analysed by matrix-assisted-laser-desorption/ionisation-time-of-flight-mass spectrometry (MALDI-TOF/TOF) as described (16). To compare potential differences between in vivo and in vitro C3 glycation, we undertook in vitro glycation of the protein as previously described (17) and posttranslational modifications were assessed as above.

**Statistical analysis**

Between-group comparisons of normally and non-normally distributed variables were carried out by independent Student’s t and Mann–Whitney U tests, respectively. Pearson and Spearman coefficients were applied to assess correlations between normally and non-normally distributed variables respectively. Based on previous data (7) and assuming a common standard deviation of the variable studied of 225 seconds we calculated that the number of samples analysed (n=24 in total) would be enough to detect a difference of 270 seconds in C3-induced prolongation in clot lysis time (power 80% at p<0.05).

**Results**

**Patient Characteristics**
Twelve patients with type 1 diabetes and 12 healthy controls were recruited and their characteristics can be seen in table 1. Patients with T1DM were on no medications other than subcutaneous insulin injections and had no clinically significant microvascular or macrovascular complications of diabetes. The healthy controls were also not taking any prescribed medication.

**Effect of C3 on lysis time**

One control sample did not lyse within the allotted time and thus was excluded from further analysis. Mean lysis time of all clots made from fibrinogen in the absence and presence of corresponding C3 was 2691±105 and 3057±172 secs (p<0.01). Mean lysis time of clots made from purified fibrinogen of healthy controls and T1DM patients was 2510±132 seconds and 2857±151 seconds respectively (p=0.07). Following C3 addition, there was an increase in lysis time in both groups to 2705±180 seconds and 3379±259 seconds, respectively, with individual data provided in Figure 1A. The degree of C3 induced prolongation in lysis time was greater in the diabetes samples (179±101 and 522±166 seconds, p=0.04; Figure 1B and C), and showed a more diverse range of 144-1476 seconds (5-51%) in T1DM, compared with 108-480 seconds (5-18%) in controls. These data show that C3 consistently prolongs clot lysis time in both healthy controls and patients with diabetes, although the degree of prolongation shows wider inter-individual variability in the diabetes group.

**C3-fibrinogen interaction sites**

Our data have so far shown that C3 represents a credible target to modulate fibrinolysis and therefore we investigated fibrinogen-C3 binding sites. Using peptide
microarray screening of the whole fibrinogen molecule, we identified complex spot patterns, based on peptides with the consensus motifs $A^{136}{\text{VSQTSSSFQYM}}{\text{L}}^{150}$ (peptide A), $Q^{423}{\text{CSDKEDGGGW}}^{434}$ (peptide B) and $Y^{434}{\text{NRCchaanPNGRYY}}^{447}$ (peptide C), which are all located within the $\beta$ chain of fibrinogen (Uniprot ID P02675). Two of these peptides were in close proximity in the N terminus of the $\beta$-chain (B and C) and shared one amino acid. One peptide was closer to the C-terminal region of the $\beta$-chain of fibrinogen as shown in Figure 2. These data indicate that the $\beta$ chain of fibrinogen contains key binding sites for C3.

**Use of Affimer for modulation of C3 induced prolongation of fibrinolysis**

**Isolation of Affimer A6**

Sixteen fibrinogen binding Affimers, that competed with C3 for fibrinogen binding, were sequenced and revealed 8 distinct sequences. Each Affimer is composed of a scaffold protein and two 9 amino acid loops. We identified one Affimer (A6), which showed sequence identity in one of its loops with residues Ser 38-His 47 of C3 (Uniprot ID P01024, Figure 3A). This site resides in an exposed area of C3 with the potential to interact with other plasma proteins as predicted by molecular modelling (Figure 3B).

**Effects of Affimer A6 on fibrinolysis**

*Purified protein experiments.* Using pooled fibrinogen from healthy controls, we investigated changes in lysis time after the addition of A6, C3 or combination of the two. A6 had no significant effect on clot lysis whereas C3 prolonged clot lysis, an effect that was completely abolished by the addition of A6 (Figure 4A). Affimer A6
had no effect on lysis time when C3 was substituted by plasmin inhibitor (PI), indicating a C3-specific effect (Figure 4A).

*Plasma experiments.* Given results of purified protein experiments, plasma samples were next analysed. A dose response curve, using pooled healthy control plasma sample, verified that 1:1 molar concentration of fibrinogen:Affimer A6 is optimal for modulation of clot lysis (Figure 4B). Using this molar ratio, Affimer A6 reduced lysis of clots made from pooled healthy control plasma from 593±17 to 542±10 sec (7%, p<0.05) with a decrease from 618±13 to 548±15 sec in clots made from diabetes plasma (11%, p<0.05; Figure 4C). Moreover, the effect of Affimer A6 was tested in individual samples and not only pooled plasma. Affimer A6 reduced plasma clot lysis in 24 high vascular risk patients (11 with coronary artery disease and 13 with type 2 diabetes) from 1237±150 to 1120±129 (10%, p<0.05; Figure 4D).

**Binding sites of Affimer A6 on fibrinogen**

Following the results of the microarray screening of C3 and fibrinogen, the sequences for the two random loops of A6 were individually entered into the Pepsite2 webserver to predict where A6 might be binding to the β-chain of fibrinogen [PDB code 3GHG; (18)]. The strongest binding prediction for the two loops on the β chain was adjacent to the peptide sequences (peptides B and C) identified by the microarray screening (Figure 4E).

**Mechanistic insight into potential enhancement of fibrinolysis prolongation by C3 from patients with diabetes**
We have previously shown that pooled C3 purified from patients with diabetes may have enhanced anti-fibrinolytic effects compared with protein from healthy controls (7). The current work, using C3 purified from individual plasma samples, demonstrates that diabetic C3 has an enhanced anti-fibrinolytic activity compared with control protein, at least in some samples. Therefore, we investigated whether increased protein glycation may be one of the mechanisms implicated in the enhanced anti-fibrinolytic effects of C3. MS identified similar glycation of amino acids serine, threonine, arginine, and asparagine in control and diabetes C3 samples. However, additional lysine residues (Lys) were noted to be glycated in all 6 diabetes samples but not in the control group, affecting six separate residues, with a mean of 3±0.9 modifications in each sample. Details of glycated Lys residues and positions within C3 are illustrated in Figure 5A and B. A characteristic MS/MS fragment mass-spectrum of a fragment of C3 with the molecular weight of 1206 Dalton after tryptic digestion is shown in Figure 5C together with the protein glycation score. We verified the molecular mass of 1206 as a characteristic post-translational glycation modification of the fragment with the molecular weight of 882 of C3. This molecular mass 1206 m/z shows glycation of Lysine, highlighted by an asterisk in the amino acid sequence $^{1203}$K*GPLLNK$^{1209}$. Although the additional glycation of C3 in patients with diabetes was associated with longer clot lysis time, Spearman coefficient did not demonstrate a significant correlation between number of glycated Lys residues within C3 and ex vivo plasma clot lysis time (Spearman r=0.19, p=0.73).

**Discussion**

There are a number of novel findings in this work that can be summarised as follows: i) C3 purified from individual patients consistently prolongs clot lysis, ii) the β-chain of
fibrinogen in the area of Cys424-Arg445 represents one region of interaction with complement C3, iii) Affimer proteins provide a tool for targeted modulating of fibrin clot lysis by interfering with fibrinogen-C3 interactions.

While C3 protein purified from individual plasma samples consistently prolonged clot lysis, an inter-individual variability in the response was noted, which may be related to the degree of incorporation of C3 into the clot (19). This was particularly pronounced in C3 purified from diabetes samples, which may be related to alternative post-translational modifications in the protein. We identified six lysine residues that were glycated in T1DM samples, but not controls. The variability in glycation sites comparing individuals with T1DM suggests that the ability of lysine residues on C3 to undergo glycation has a large inter-individual variability, which may modulate the anti-fibrinolytic effects of the protein. Interestingly, in vitro glycation of C3 showed largely similar patterns, favouring glycation of certain lysine residues, suggesting this consistency is related to a steric effect. Previous work has shown that glycation of proteins involved in coagulation and lysis alters clot structure and/or the efficiency of fibrinolysis. For example, fibrinogen glycation alters fibrin network characteristics and the degree of protein glycation correlates with glycaemic control measured as HbA1c (20;21). Proteins in the fibrinolytic system are also affected as plasminogen glycation in diabetes compromises conversion to plasmin and modulates enzyme activity (22). There was no clear correlation between number of lysine residues glycated and the antifibrinolytic effects of C3 but this may be due to the small number of samples analysed or it may simply indicate that some lysine residues are more important than others and extensive glycation of multiple residues is not required to observe an effect. Overall, however, our data suggest that
glycation of C3 increases its antifibrinolytic effect, although it remains unclear which lysine residues are important for the observed effect.

Our binding studies indicate that three areas on the β-chain of fibrinogen play a role in C3-fibrinogen interactions. In particular, two of these areas, located on the N-terminus, were in close proximity and separated by a single amino acid. We should acknowledge that the microarray technique only identifies linear interactions and it is possible that additional conformational interactions take place between the two proteins. However, we should not underestimate the importance of linear binding between proteins with previous work demonstrating the importance of such interactions between fibrinogen and FXIII (23).

A key finding of our work is a proof of concept for a novel methodology to modulate fibrin clot lysis, and hence thrombosis risk, in individuals with diabetes. We describe a new technique that identified a small binding protein with two variable loops (Affimer A6), with one of the loops sharing sequence identity with an exposed portion of C3, likely to be involved in protein-protein interactions. Interestingly, Affimer A6 was able to abolish C3-induced prolongation of lysis with high specificity, regardless as to whether C3 was purified from control or diabetes samples. Moreover, Affimer A6 was capable of reducing plasma clot lysis from both healthy controls and patients with diabetes. We and others have shown that changes in clot lysis by 6-18% are clinically significant (24-26) and therefore the observed 7-11% reduction in clot lysis by Affimer A6 is likely to be clinically meaningful. Although speculative, this targeted enhancement of clot lysis is unlikely to significantly increase bleeding risk, making this approach clinically promising.

The predicted binding sites of both loops of Affimer A6 were in close proximity within the β-chain of fibrinogen and close to the region of fibrinogen that interacts with C3
using microarray screening. Taken together, the N terminus of the β chain of fibrinogen represents a binding site for complement C3, which may be important for the development of agents that improve efficiency of fibrinolysis, particularly in high risk vascular patients. These findings open a new avenue for the identification of therapeutic targets to modulate the hypofibrinolytic environment in high risk patients, which may have future clinical implications.

In conclusion, our work shows that C3 represents a credible therapeutic target to reduce thrombosis risk. The β-chain of fibrinogen represents an interaction site with C3, and modulation of this interaction can abolish C3-induced prolongation of clot lysis in a specific manner. This targeted approach has the potential to develop novel therapeutic agents for the reduction of thrombotic vascular occlusion.

Future work should concentrate on investigating the in vivo role of Affimer A6 in limiting vascular occlusion, using animal models of thrombosis. Additionally, our data suggest that targeting a specific site of the β-chain of fibrinogen, which may be amenable to small molecule intervention, represents a possible new therapeutic target to improve fibrinolysis and reduce thrombosis in high risk conditions.

**Acknowledgment**

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Foundation for constant support, along with help from the Biomedical Health Research Centre for the BioScreening Technology Group and the Stratified Medicine Hub at the University of Leeds who funded the initial NABP screens. We would also like to thank AVACTA Life Sciences for their support.

**Contribution statement**

RJK and KS researched and analyzed data and wrote the manuscript. CT, VJ, KS researched and analyzed data and revised the manuscript. VJ, AT researched data and revised the manuscript. SP, MJM, RFS revised the manuscript. CWGF, DT, designed experiments and revised the manuscript. RAA conceived the study, designed experiments, analyzed data and wrote the manuscript.

**Duality of interest**

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

**Reference List**


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**Table 1:** Clinical variables of controls and patients with type 1 diabetes. Data are presented as mean±SEM.
Figure 1. C3 modulation of clot lysis in individuals with diabetes and healthy controls. Fibrinogen and C3 were purified from matched samples of 12 patients with diabetes and 12 healthy controls. Turbidimetric experiments were conducted on individual fibrinogen samples after the addition of thrombin, calcium, tissue plasminogen activator and plasminogen, in the presence and absence of C3. A) Clot lysis time was increased following the addition of C3 when all samples (n=24) were analysed together. B) Lysis time of individual samples before and after the addition of C3 from healthy control and diabetes samples (n=12 in each group). C) Prolongation in lysis time by C3 in healthy control and diabetes samples (n=12 in each group). Data are presented as mean±SEM.

Figure 2. Fibrinogen peptide binding to C3 and location within the fibrinogen molecule. A. Intensity plot of fibrinogen peptide binding to C3 showing mainly three binding sites (visualised as 3 distinct intensity peaks, red arrows), all located on fibrinogen β-chain (A, B and C). B. The peptide sequences identified by the microarray screening are illustrated within the crystal structure of β-chain of fibrinogen. The red spheres indicate peptide A (136AVSQTSSSFQYMYL150), pink spheres indicate peptide B (423QCSKEDGGGWY434) and orange spheres indicate peptide C (434YNRPCAANPNGR445).

Figure 3. Affimer A6 sequence identity with C3 and potential protein-protein interaction sites on C3. A) Loop 2 of Affimer A6 showing sequence identity with the area labelled in red spheres on the C3 molecule. B) Enlarged area of potential
protein-protein interaction sites on complement C3 (labelled in different colours) identified as the best scoring site using the SiteMap module of Glide (Schrodinger Inc).

**Figure 4.** *Modulation of C3 induced prolongation of fibrinolysis by Affimer A6 and potential interaction sites on fibrinogen.* A) Pre-incubation of fibrinogen with Affimer A6 completely abolished C3-mediated prolongation of lysis with no effect observed on prolongation of lysis by plasmin inhibitor (PI) indicating a C3 specific effect. B) Dose response curve using different molar ratios of Affimer A6:fibrinogen (or scaffold only control protein), conducted on pooled plasma samples from 12 individuals. C) Effects of Affimer A6 on plasma clot lysis using pooled healthy control plasma (Control, n=12) or plasma from patients with type 1 diabetes (Patients, n=12). Results represent the mean±SEM of 3 independent experiments each performed in duplicate, unpaired t-test. D) The crystal structure of fibrinogen with predicted binding sites of loop 1 (red) and loop 2 (blue) of Affimer A6 within the β chain (shown in green), lying in close proximity to peptide motifs B and C identified from microarray screening (coloured spheres).

**Figure 5.** *In-vivo glycation of C3 and lysine residues involved.* Six individual C3 samples from diabetes and healthy controls were analysed by Matrix-Assisted Laser Desorption/Ionisation Mass-spectrometry (MALDI–MS) for post-translational glycation. A) Six lysine residues have shown additional glycation in diabetes samples. B) Spatial position of glycated residues within C3 are shown. C) Detailed analysis of the MS/MS fragment from C3 with the molecular mass of 1,206 Dalton
and the interpretation by the Mascot search engine is shown together with a protein score of 125 for post-translational glycation modification.
A. Lysis time (secs)

Without C3  With C3

B. Lysis time (secs)

Co  Co+C3  DM  DM+C3

p = 0.06  p = 0.02

C. Prolongation in lysis time (secs)

Co  DM

p = 0.04
A

B
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![Diagram of a protein structure with labeled lysine residues: K 1526, K 1209, K 1203, K 1139, K 685, K 502.](image)
Methods

Patient recruitment

We recruited 12 patients with type 1 diabetes (T1DM) and 12 age and sex matched controls with enough blood samples collected to allow purification of fibrinogen and C3 from each individual sample. Ethical approval for the study was provided by the Leeds East, National Research Ethics Service (NRES) committee and informed consent was obtained from participants in accordance with the Declaration of Helsinki.

We additionally tested a total of 24 stored plasma samples from high vascular risk individuals, including 11 with a history of ischaemic heart disease and 13 with a history of type 2 diabetes. All these samples were collected after appropriate ethical approval.

Protein purification and turbidimetric analysis

Fibrinogen and complement C3 were purified from each plasma sample as previously described (7). Briefly fibrinogen was purified by affinity chromatography using a calcium dependant IF-1 monoclonal antibody and C3 was purified by precipitation with polyethylene glycol 4000, anion exchange chromatography using Mono Q 5/50 GL (GE Healthcare, Bucks, UK) and finally gel filtration using Superose 12 10/300 GL (GE Healthcare).

Clots were formed from each purified fibrinogen (0.22 mg/ml [0.65 µM]) in the presence/absence of C3 (0.11 mg/ml [0.6 µM]) from the same individual following the addition of a lysis mixture containing 1.25 µg/ml plasminogen (Enzyme Research...
Laboratories, Swansea, UK) and 2.2 µg/ml FXIII in tris buffered saline (TBS), pH 7.4 and subsequently an activation mix containing tPA (Tecnoclone, Vienna, Austria), at 50 ng/ml, CaCl$_2$ 2.5 mM and thrombin (Calbiochem, Germany), 0.12 U/ml (all final concentrations). Measurements were taken at 340nm every 24 seconds for 6 hours in a plate reader as previously described (7). Time from full clot formation to 50% lysis was calculated and presented as clot lysis time throughout this work.

**Peptide Microarray**

A peptide microarray chip containing overlapping 15 aa peptides of the entire fibrinogen molecule (PEPperPRINT, Germany) was synthesised and used to screen against pooled C3 (0.8 to 8 µg/ml) purified from 6 healthy individuals. Fibrinogen peptide chips were incubated with C3 and, after extensive washing, bound protein was detected using anti-C3 antibody (LifeSpan Biosciences Inc, USA). Control peptides framing the peptide arrays were stained as internal quality control to confirm peptide microarray integrity.

Quantification of spot intensities and peptide annotation were performed with PepSlide® Analyzer (PEPperPRINT). Using a validated software algorithm, intensity maps were generated for each assay and averaged spot intensities were plotted against the linked fibrinogen sequence from the N- to the C-terminus. The intensity plots were correlated with peptide and intensity maps as well as with visual inspection of the microarray scan to identify peptides and consensus motifs that interacted with C3.

**Non-antibody synthetic proteins (Affimers)**
Affimers are small proteins developed locally and are composed of a scaffold protein that constrains two variable conformational 9 amino acid loops. The variable region has great diversity (>3x10^{10} random peptides) and have the capability of binding “target” protein as previously described (11;12).

Panning and protein production

A phage display library of these Affimer proteins, comprising 1.3 x10^{10} random variable clones, was screened against human fibrinogen to identify high affinity binders as follows: Commercial human fibrinogen (Calbiochem) was further purified using IF-1 monoclonal antibody (13), biotinylated using EZ-link NHS-SS-biotin (Pierce Biotechnology, Rockford, Illinois, USA) and successful biotinylation was confirmed by ELISA using streptavidin-conjugated horseradish peroxidase (HRP). Biotinylated fibrinogen was then added to streptavidin coated wells (ThermoFisher, UK) for 1 hour followed by addition of the Affimer phage display library with repeated panning steps undertaken as described (12). Briefly, Affimers were incubated with fibrinogen for 2.5 h, after which wells were washed 10 times, followed by elution of the bound phage and expression in Escherichia coli ER2738 electrocompetent cells (Lucigen, USA). Colonies were grown on LB agar plates overnight followed by inoculation with M13K07 helper phage in order to generate a new library for the second round of panning. In order to select specific Affimers capable of targeting fibrinogen–C3 interactions, competitive elution with C3 (0.5mg/ml) for 20 min was applied in the fourth round of panning. Individual ER2738 colonies were selected and confirmed to be specific binders to fibrinogen by phage ELISA as previously described (12).
To rule out interference from the phage and to generate high quantity of the protein, Affirmers of interest were produced by subcloning the coding regions into pET11 vectors. Affimers of interest were then expressed in *E. coli* BL21 (DE3) with subsequent purification as previously described (12).

**Affimers and fibrinolysis**

**Purified experiments**

A total of 0.65uM μM fibrinogen (Calbiochem) was incubated with the relevant Affimer (0.163 mg/ml or 5:1 molar concentration of Affimer:fibrinogen) at room temperature for 30 min followed by the addition of C3 at 0.6 μM and Factor XIII 2.2 μg/ml. A lysis mix containing plasminogen (final concentration 1.25 μg/ml) and TBS was added followed by an activation mix containing tPA at 50 ng/ml, CaCl₂ 2.5 mM and thrombin, 0.12 U/ml (all final concentrations). Measurements were taken at 340nm every 12 seconds for 1 hour as described (7). In all experiments, an Affimer scaffold with no insert was used as a control.

**Plasma experiments**

Plasma samples were mixed with increasing concentrations of Affimer A6 and control (ranging from 0:10 – 50:10 Affimer:fibrinogen molar ratio). Turbidimetric experiments using plasma conditions were subsequently conducted as previously described (14).
**Molecular modelling**

In order to identify potential sites of ligand binding on C3, the molecular modelling software Autoligand was employed to scan the whole protein (15). Molecular modelling was used to predict binding sites of any peptide sequences identified from the microarray screening and Affimer work used the webserver Pepsite2® (http://pepsite2.russelllab.org). Pepsite2 predicts the binding site of each residue in a peptide (up to 10 aa in length) to a known protein surface using pre-determined preferred binding environments for each residue. The result is an approximation of the peptide structure bound to the protein surface. Images were viewed and produced using Pymol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) (16).

**Mass Spectrometry**

This was performed to investigate potential mechanisms for the difference in the anti-fibrinolytic activity of C3 from patients with diabetes compared with healthy controls. Purified C3 from 6 T1DM and 6 healthy controls was digested by trypsin and analysed by matrix-assisted-laser-desorption/ionisation-time-of-flight-mass spectrometry (MALDI-TOF/TOF) as previously described (17). Briefly, C3 was incubated with aqueous ammonium bicarbonate (50 mmol l⁻¹) and 0.2% w/c trypsin for 24 h at 37°C. The resulting tryptic peptides were desalted and concentrated utilizing the ZipTip<sub>C18</sub> technology (Millipore, USA) using 0.1% trifluoroacetic acid (TFA) and 80% acetonitrile (ACN). The eluate was spread onto the (MALDI) target plate (MTP-AnchorChip 400/384; Bruker-Daltonic, Germany) using α-cyano-4-
hydroxycinamic acid (205 mg ml\(^{-1}\)) as matrix with mass spectrometric analyses carried out using MALDI-TOF/TOF mass spectrometer (Ultraflex III; Bruker-Daltonic, Germany). MS/MS fragments were analysed using Lift-option of the mass-spectrometer. Calibrated and annotated spectra were subjected to the database search Swiss-Prot (http://www.expasy.org/) utilizing the software tool “Bruker Bio-Tool 3.2 and the “Mascot 2.2 search engine” (Matrix Science Ltd, London, UK). Results were entered into the database and subsequently used empirically determined factors to assign a statistical weight to each individual peptide match.

To compare potential differences between \textit{in vivo} and \textit{in vitro} C3 glycation, we undertook \textit{in vitro} glycation of the protein as previously described (18). Briefly, 1 ng/ml C3 solution was mixed with 0.5 mol/l D-glucose solution to reach a final concentration of 1.5 nmol/l. After an incubation period of 4 h, the reaction mixture was diluted with water and dialyzed against PBS. Afterwards, the post-translational modifications of C3 were validated by mass-spectrometry as described above.