Circulatory zinc “speciation” and its relevance to cardiovascular disease

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A thesis submitted for the degree of PhD
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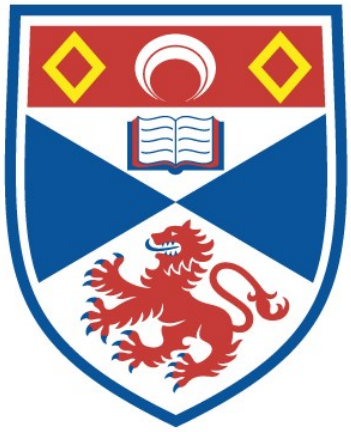

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Abstract

Zn^{2+} ions are released from activated platelets and are important regulators of coagulation. Human serum albumin (HSA) controls the concentration of available Zn^{2+}, but binding of free fatty acids (FFAs) to HSA disrupts Zn^{2+}-binding. Elevated plasma FFAs concentrations are associated with disease states (e.g. diabetes, obesity and cancer) characterised by increased thrombotic risk. It is therefore important to understand this dynamic and the roles Zn^{2+} plays in coagulation. The aims of this project were to investigate the interplay between binding of Zn^{2+} and different FFAs to HSA using isothermal titration calorimetry. Increasing the FFA concentration (0-5 mol. eq.) or chain length (C8:0-C18:0) decreased Zn^{2+}-binding to HSA. The effect of Zn^{2+} and FFAs upon fibrin clot formation and lysis was determined in a purified system, in pooled plasma and in plasma from subjects with type-1 and type-2 diabetes mellitus (T1DM and T2DM) using turbidimetric assays and scanning electron microscopy. Plasma concentrations of zinc, copper, magnesium and selenium in T1DM and T2DM were also measured. In both the purified system and pooled plasma, Zn^{2+} increased clot density, an effect further increased by the presence of FFAs. Clot density was found to be increased in T2DM subjects (compared to controls) and positively correlated with plasma FFA concentration. In T1DM subjects, clot lysis time was increased (compared to controls) and negatively correlated with magnesium concentration. Magnesium deficiency in T1DM likely contributes to increased thrombotic risk. Finally, the effects of Zn^{2+} on heparin neutralisation by histidine-rich glycoprotein (HRG), fibrinogen and fibronectin was assessed using surface plasmon resonance and anti-factor Xa and anti-thrombin activity assays. The abilities of HRG and fibrinogen to neutralise heparins were found to increase in the presence of Zn^{2+}, while neutralisation by fibronectin was unaffected. These results collectively support the hypothesis that FFAs impact on coagulation *in vivo* through mishandling of Zn^{2+}.

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Abbreviations

ANOVA	analysis of variance
AT	antithrombin
C	C-terminal domain of HRG
CI	confidence interval
DS	dermatan sulfate
EDA	extra domain A of fibronectin
EDB	extra domain B of fibronectin
ELISA	enzyme-linked immunosorbent assay
ESL	endothelium surface layer
FFA	free fatty acid
FXa	activated factor X
GAG	glycosaminoglycan
HbA1c	glycated haemoglobin
HCII	heparin cofactor II
HRG	histidine-rich glycoprotein
HRR	histidine rich region of HRG
HS	heparan sulfate
HSA	human serum albumin
IIICS	type III connecting segment of fibronectin
ITC	isothermal titration calorimetry
K_1	affinity of binding site 1 of HSA, site A
K_2	affinity of binding site 2 of HSA, site B
LMWH	low molecular weight heparin

MES	2-(*N*-morpholino)ethanesulfonic acid buffer
N_1	stoichiometry of binding site 1 of HSA, site A
N_2	stoichiometry of binding site 2 of HSA, site B
N1/N2	the two N-terminal domains of HRG
PRR1	proline rich region 1 of HRG
PRR2	proline rich region 2 of HRG
OR	odds ratios
Q	quartile
RU	resonance unit
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SPR	surface plasmon resonance
TBS	tris buffer saline
T1DM	type-I diabetes mellitus
T2DM	type-II diabetes mellitus
tPA	tissue plasminogen activator
Tris	tris(hydroxymethyl)aminomethane
UFH	unfractionated heparin
ΔH_1	energy of binding site 1 of HSA, site A
ΔH_2	energy of binding site 2 of HSA, site B

Chapter 1: Introduction

Coagulation is a physiological process in vertebrates that plays a dual role in both preventing excessive bleeding and participating in the capture of pathogens following injury. However, excessive coagulation is extremely dangerous and can result in death through restriction of blood flow, of oxygen delivery and through necrosis of tissue. Coronary heart diseases, heart attacks and strokes have become leading causes of death in the UK, with coronary diseases causing more than 66,000 deaths in the UK in 2016, which represents 14% of all deaths for men and 9% for women.[1-4] Many conditions, such as diabetes, obesity and malignant tumours, are linked with an alteration of plasma molecules that interfere with coagulation control.[5,6] This dysregulation of coagulation can lead to pathological clotting called thrombosis, which can block blood vessels and lead to heart attacks and strokes. Coagulation is a very complex process with many factors implicated in its regulation. A better understanding of those factors is necessary to prevent and treat thrombosis.

Overview of the mechanisms of coagulation

When a tissue injury occurs, the content of the damaged cells (including collagen and proteins) is exposed to the blood, which in turn initiates platelet activation and coagulation. Firstly, platelets are activated and release pro-thrombotic proteins as well as both calcium and zinc ions (Ca^{2+} and Zn^{2+}), two important cations involved in coagulation with Ca^{2+} an essential cofactor of several coagulation protein and Zn^{2+} an important regulator of their activity (Figure 1.1A). In addition, the coagulation cascade is triggered culminating with the activation of thrombin, thus enabling it to cleave fibrinogen into fibrin, the protein that polymerises to form the fibrin

clot. There are two primary mechanisms by which thrombin can become activated: the extrinsic or tissue factor pathway and the intrinsic or contact activation pathway. The tissue factor pathway is activated when tissue factor is released by damaged cells (Figure 1.1B), while the contact activation pathway is activated when the surface of the endothelium is damaged and collagen is exposed to the blood (Figure 1.1C). As most of the proteins involved in coagulation (including prothrombin and fibrinogen) are synthesised in the liver as zymogens (unactivated forms) both coagulation mechanisms function by activating one of these zymogens which can then go on to activate the next protein in the cascade. Finally, the tissue plasminogen activator (tPA) inside damaged cells is also exposed to the blood. Both tPA and plasminogen bind to fibrin and all three together result in the transformation of plasminogen into plasmin, the protein that lyses the fibrin clot. This process is called fibrinolysis (Figure 1.1D). Outside of the fibrin clot, the actions of both tPA and plasminogen are regulated by specific inhibitors.

Most coagulation factors can be activated by more than one protein or complex. In addition, many anticoagulation mechanisms exist. Some key anticoagulant proteins are: 1) antithrombin (AT), a serine protease inhibitor or serpin that inhibits mainly thrombin and activated factor X (FXa) but also activated factor IX, activated factor XI, activated factor XII, activated factor VII and plasmin (AT traps the proteins and the complex is cleared by the liver); 2) heparin cofactor II (HCII), a serpin that inhibits thrombin (the complex is cleared by the liver); 3) tissue factor pathway inhibitor which inhibits FXa and the tissue factor pathway inhibitor-FXa complex inhibits the tissue factor-activated factor VII complex (the quarternary complex is internalised and cleared by endothelial cells); 4) protein C which cleaves and inactivates activated factor V and activated factor VIII.

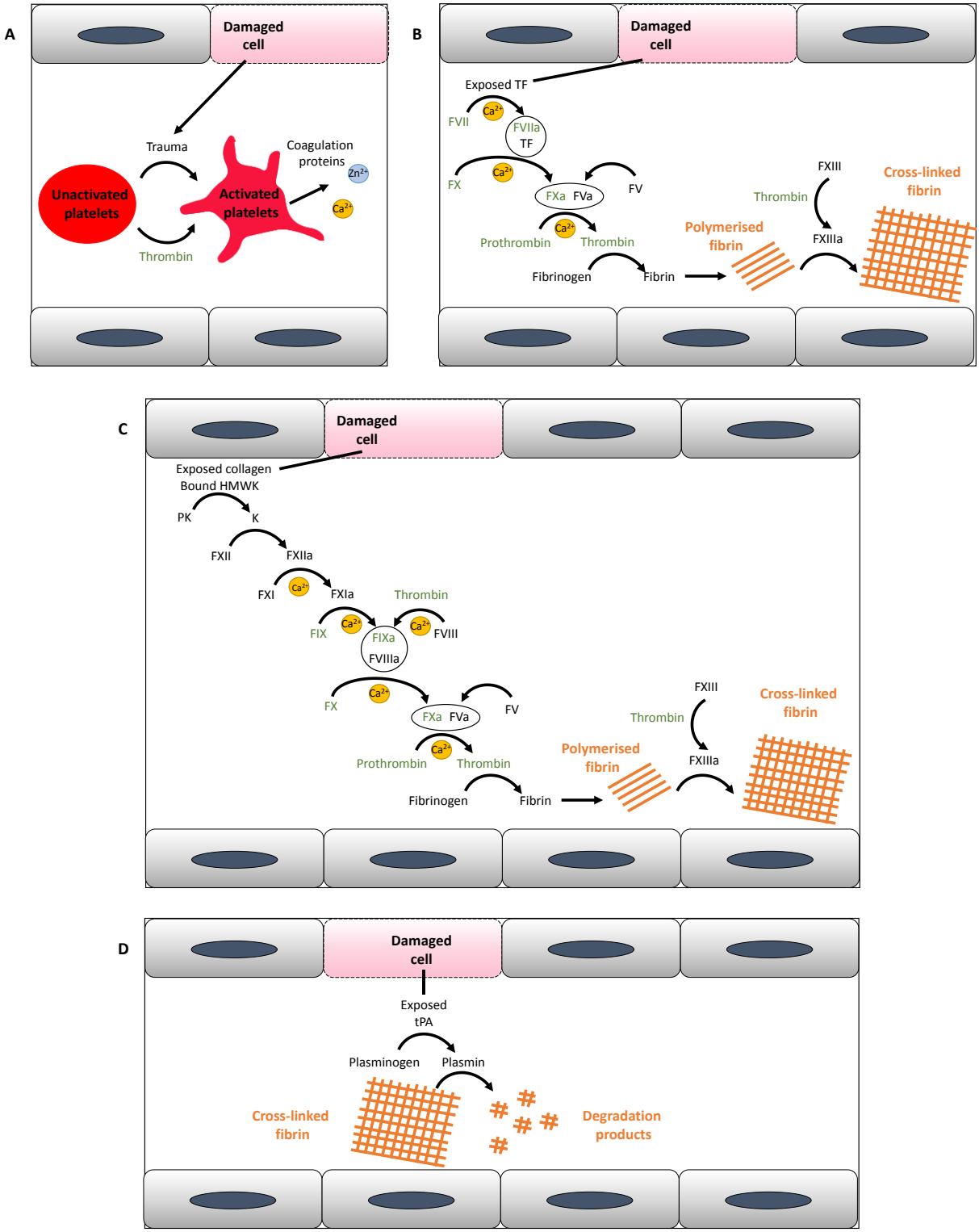


Figure 1.1. Simplified schema of coagulation-activation and fibrinolysis by various mechanisms.

A. Platelet activation. **B.** Extrinsic pathway. **C.** Intrinsic pathway. **D.** Fibrinolysis. Abbreviations used: F, coagulation factor; HMWK, high molecular weight kininogen; K, kallikrein; PK, prekallikrein; TF, tissue factor; tPA, tissue plasminogen activator. Activated coagulation factors are indicated with an "a".

Plasma zinc and coagulation

All aspects of coagulation are tightly regulated through several mechanisms and molecules, including zinc.[7] The fraction of zinc responsible for the effects on proteins and cells is the "free" aquo ion of zinc, Zn^{2+}. Zinc is the second most abundant transition metal in the body after iron and plays essential structural, catalytic and signalling roles in many physiological process including coagulation, but also cell replication, tissue growth and immune functioning.[7] Its importance in coagulation is best illustrated through the effects of zinc dietary deprivation (zinc deficiency being defined as having a total plasma zinc concentration below 0.7 mg/L i.e. 11 µM, while normal range is 12-20 µM) which can lead to increased bleeding and reduced platelet aggregation.[8-10] It has been shown that supplementation with zinc can quickly correct those effects.[8] The actions of Zn^{2+} during coagulation are complex as it can potentially regulate many pathways, including pro- and anti-coagulation and pro- and anti-fibrinolysis pathways.[11] This means that the global effect of an increase in Zn^{2+} concentrations is difficult to predict. However, experimentally it has been shown to generally enhance platelet activation and aggregation and to accelerate fibrin clot formation, increase clot density and stability as well as delay the lysis of the fibrin clots.[8,11-13]

In plasma, the total Zn^{2+} concentration is reported to be between 12-20 µM.[8-10] Extracellular Zn^{2+} is tightly regulated and well-buffered; this is because free Zn^{2+} is toxic at high nanomolar levels.[14,15] Around 25% of total zinc is tightly bound to proteins such as α-macroglobulin and is not exchangeable,[7,16] while around 75% of total zinc is bound to and buffered by human serum albumin (HSA) and is exchangeable/labile only under specific conditions.[17] The zinc bound to HSA represents >99% of the labile Zn^{2+} pool and most of the remaining labile Zn^{2+}

is bound to small molecules. This is thought to leave ca. 1-3 nM Zn^{2+} free in solution at rest.[16] However, this quantity is subject to highly dynamic spatio-temporal variations (Figure 1.2).

Platelets store around 35 μg/mL of Zn^{2+} in two pools; around 60% is in their cytosol and the remaining 40% in their α-granules where it is sequestered by proteins such as factor XIII and fibrinogen.[18,19] At the point where coagulation is initiated and platelets are activated, the α-granules fuse with the membrane of the platelets, increasing the platelet surface area and releasing their content into plasma.[12,20,21] It has been estimated that platelets could release around 5-10 μM Zn^{2+} (estimated by measuring the amount of Zn^{2+} released by activated platelet and extrapolating the corresponding plasma concentration), with local maxima at sites of injury having the potential to be much higher.[12,20] Some of this Zn^{2+} will immediately be buffered by HSA, but the rest will be available for interactions with other molecules, including numerous coagulation proteins. Thus, the release of Zn^{2+} participates in propagating several pro- and anti-coagulation and pro- and anti-fibrinolytic pathways.[11] Other cells present in the blood also contain elevated concentrations of Zn^{2+}; those include erythrocytes, neutrophils and lymphocytes which are reported to contain 41 μg of Zn^{2+} per gram of haemoglobin, 105 μg of Zn^{2+} per 10^{10} cells and 116 μg of Zn^{2+} per 10^{10} cells, respectively.[22] It is possible that those cells also release their Zn^{2+} ions in specific circumstances such as at when they are activated or damaged. In addition, epithelial cells contain around 60 μg of Zn^{2+} per gram of dry weight, and have been reported to release an unknown proportion of this when damaged.[23,24] Furthermore, plasma Zn^{2+} concentrations could also potentially increase during atherosclerotic plaque rupture as those plaques are known to contain up to six times more Zn^{2+} than healthy tissue.[25] This could explain part of the pro-thrombotic nature of those events.

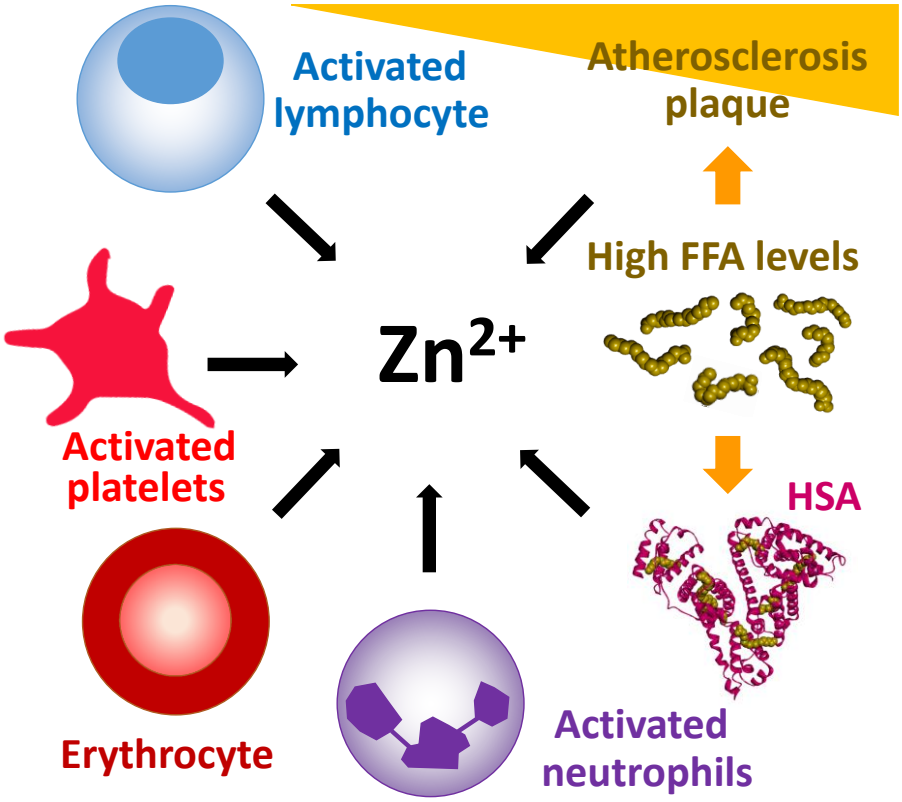


Figure 1.2. Cellular and molecular sources of plasma Zn^{2+}.[26]
Zn^{2+} is released by activated platelets during coagulation. It may also be released by erythrocytes, activated neutrophils and activated lymphocytes. In addition, when plasma FFA levels are elevated, FFA binds to HSA and disrupts Zn^{2+}-binding to the protein. Elevated plasma FFA levels also enhance the build-up of atherosclerosis plaques. Such plaques also contain Zn^{2+} that can be released during plaque rupture.

HSA is the most abundant protein in plasma; it binds metal ions in plasma, including Zn^{2+}.[27-30] Zn^{2+} ions can bind to potentially two sites on HSA. Those sites have been identified with $^{111/113}Cd^{2+}$ binding experiments and are called sites A and B.[31] Site A has been confirmed to bind Zn^{2+}, while binding of Zn^{2+} to site B has been presumed but is yet to be confirmed.[32] Another important function of HSA is the transport of small molecules including hormones, toxic metabolites, pharmaceutical drugs and free fatty acids (FFA).[33-35] HSA is the main transporter of FFA and in normal physiology the protein circulates in plasma with 0.1-2.0 mol. eq. of FFA bound.[36] This proportion can increase in disease states characterised by high plasma FFA levels and can, in some cases, exceed 5 mol. eq relative to HSA.[37] HSA can bind up to

seven medium to long chain FFAs (C10-C18) at a time, at seven binding sites which are called FA1-7.[38] Binding of FFA to those sites is known to change the conformation of the protein.[38] The site FA2 is of particular interest as it is a high affinity binding site (it can become populated at low plasma FFA levels) and is located close to Zn^{2+} binding site A.[32,39] Binding of myristate (C14:0) at the FA2 site can alter the conformation of site A and thus prevent Zn^{2+} from binding at this site.[40] Even 1 mol. eq. of myristate has been shown to have an effect on Zn^{2+} binding at site A, whilst 5 mol. eq. of myristate can completely disrupt Zn^{2+} binding to HSA at this site.[40] Thus, this allosteric switch links plasma FFA concentrations (energy status) with plasma Zn^{2+} availability.

The total plasma FFA concentration has been reported to be 475 ± 252 μM in healthy individuals at rest.[41] It increases after a meal and during exercise.[42] In addition, many pathological conditions are associated with elevated plasma FFA levels. Those include chronic liver and kidney diseases,[37,43] obesity,[44] cancers,[45] diabetes,[46] hypothyroidism,[47] intrauterine growth restriction,[48] chronic inflammation (rheumatoid arthritis and ankylosing spondylitis),[49,50] infection (sepsis and malaria),[51,52] trauma,[53] and acute coronary syndrome.[54,55] The FFAs levels found in those conditions are detailed in Table 1.1. Therefore, all such diseases may be linked with altered plasma Zn^{2+} speciation (the distribution of plasma Zn^{2+} among the different plasma proteins) and potentially dysregulated coagulation. This is supported by the fact that the second leading cause of death from malignant tumours is thrombosis and that non-alcoholic fatty liver disease, diabetes and obesity are also linked with a higher incidence of developing thrombotic complications.[5,6]

Table 1.1. Conditions associated with elevated plasma FFA levels represented as mean (± SD) or mean (range).

Conditions		Mean plasma FFA concentrations in controls	Mean plasma FFA concentrations in disease states	References
ACS	ST-segment elevated myocardial infarction	0.75 (± 0.28) mM	0.84 (± 0.32) mM	[54]
	Acute myocardial infarction	0.77 (± 0.34) mM	1.03 (± 0.45) mM	[55]
Acute ischemic stroke		0.24 (0.12–0.38) mM	0.53 (0.35–0.71) mM	[56]
Diabetes	Men	0.63 (0.59–0.68) mM	0.75 (0.69–0.82) mM	[46]
	Women	0.79 (0.74–0.83) mM	0.90 (0.82–0.98) mM	
Obesity		0.57 (0.28–0.89) mM	0.93 (0.56–1.15) mM	[44]
Rheumatoid arthritis		0.40 (0.35–0.50) mM	0.59 (0.47–0.65) mM	[49]
Ankylosing spondylitis		760.84 (± 31.40) µg/mL	883.89 (± 55.32) µg/mL	[50]
Non-alcoholic chronic liver disease		0.45 (0.11–0.90) mM	0.62 (0.12–3.40) mM	[37]
Chronic renal disease		0.30 (± 0.14) µM	0.49 (± 0.14) mM	[43]
Subclinical hypothyroidism		~0.33 mM	~0.68 mM	[47]
Malignant lymphoma		No data	0.92 (0.55–1.88) mM	[45]
Sepsis		No data	4-fold increase	[51]
Malaria		No data	2.17-fold increase	[52]
Trauma		No data	2.01 (± 0.19) mM	[53]
Polycystic ovarian syndrome		No data	Total levels unknown but increase in C16:0 and C18:1 c9	[57]
Intrauterine growth restriction		0.13 mM (in amniotic fluid)	0.36 mM (in amniotic fluid)	[48]
Exercise		< 0.60 mM	>2.00 mM	[42]

Glycosaminoglycans of the endothelium surface layer

Another important factor in regulating coagulation is the presence of anticoagulant glycosaminoglycans (GAGs) at the surface of the endothelium.[58] The surface of the endothelium is made of a layer of proteoglycans and glycoproteins called the endothelium

surface layer (ESL).[58] Proteoglycans are made of a core protein bound to one or several GAG chains.[58] GAGs are linear polysaccharides, heterogeneous in sulfation percentage, saccharide sequence and length because their synthesising enzymes are expressed in a tissue-specific manner.[59] The ability of the GAGs to bind many proteins and other molecules is essential for endothelial-associated functions, including coagulation, inflammation, lipid metabolism and cell attachment, migration, invasion, and differentiation.[58] These processes are exerted through the binding of enzymes and their substrates, and receptors and their ligands to GAGs. This binding localises these molecules at particular sites, constituting a pool that can be accessed by the cleavage of the GAGs when needed.[60] The composition of the ESL itself is dynamic: its proteins, the specific GAGs to which they are bound and the sulfation pattern of those GAGs vary over time depending on local chemokine stimuli directed at the endothelial cells or because of the effect of oxidative stress or inflammation.[61] In addition, the thickness of the ESL depends on its location (ranging from 0.5-3 μm in small arteries to up to 4.5 μm in carotid arteries).[61]

The ESL naturally possesses important anticoagulant properties that prevents clot formation under normal circumstances.[58] This action is exerted through anticoagulant GAGs which bind to and enhance the actions of specific serpins, the principal ones being AT and HCII but this also includes thrombomodulin and tissue factor pathway inhibitor. The endothelial anticoagulant GAGs normally exposed to blood are thought to be saturated with AT as the K_d for their interaction is 15 nM, while the plasma concentration of AT is 3.5 μM.[62] Both AT and HCII inhibit several coagulation proteins with the main target of AT being thrombin and FXa, while HCII mainly interact with thrombin and cannot inhibit FXa (Figure 1.3).[63,64] The binding of a GAG chain allows a conformational change to take place in the serpin that renders the

reactive centre loop more accessible and reactive.[63] In addition, thrombin and the other target proteins bind along the chain of the GAG and are then channelled toward the serpin, thus increasing the rate of the reaction.[63] Because of this, longer chain GAGs are required to inhibit thrombin (for AT-mediated and to a lesser degree HCII-mediated inhibition), however this is not the case for FXa, which presumably binds the serpin through a different mechanism.[63] Once the coagulation proteins are inhibited, the GAG is freed from the serpin and can participate in further inhibition reactions.[63]

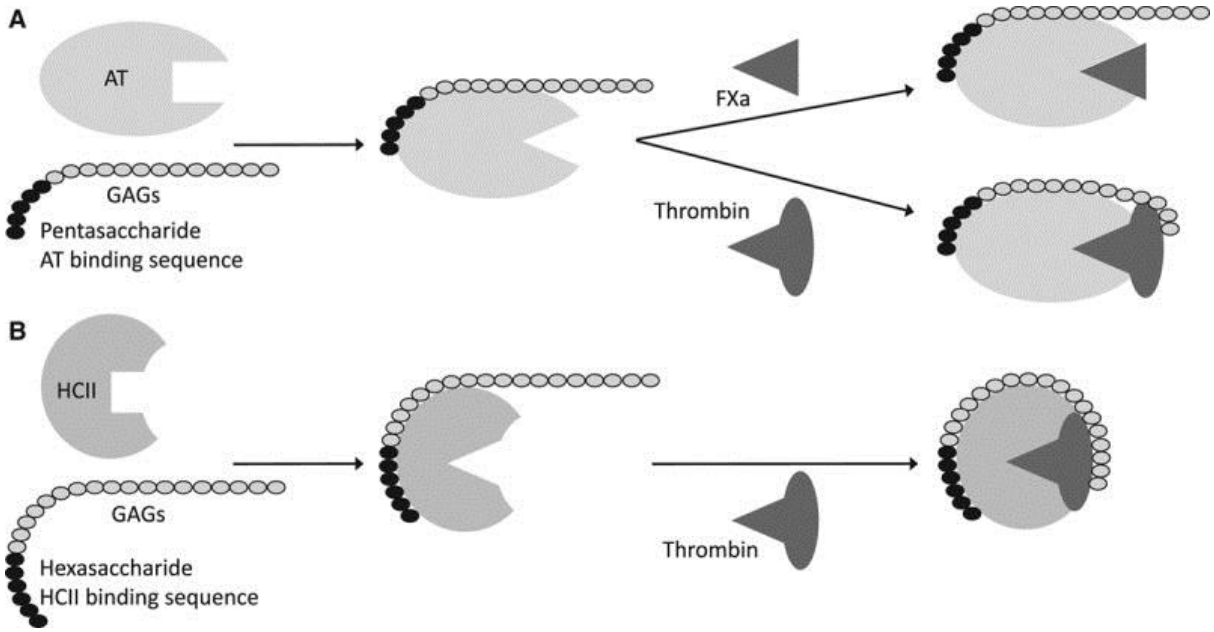


Figure 1.3. Inhibitory activity of anticoagulant GAGs.[58]
A. GAGs-mediated inhibition of FXa and thrombin by AT, with GAGs interacting with AT through its pentasaccharide AT-binding sequence. **B.** GAGs-mediated inhibition of thrombin by HCII, with GAGs interacting with HCII through its hexasaccharide HCII-binding sequence.

The principal GAG in the endothelium is heparan sulfate (HS, 50-90% of total GAG content), with chondroitin sulfate being second.[61] Dermatan sulfate (DS) is a subtype of chondroitin sulfate. The primary difference between HS and DS is the specific di-motifs that are repeated

within their polysaccharide sequences. HS are made of N-sulfated glucosamine linked to iduronic acid as a first motif and the other is N-acetylated glucosamine linked to glucuronic acid.[59] In DS, the first motif is N-acetylated galactosamine linked to iduronic acid and the second is N-acetylated galactosamine linked to glucuronic acid.[59] Those motifs are represented in Figure 1.4. All anticoagulant GAGs in the endothelium are either HS or DS. However, not all HS and DS GAGs are anticoagulants, as they need to possess an AT- or HCII-binding sequence in order to bind those proteins with high affinity under physiological conditions (Figure 1.5).[64-66] The main binding partner of HS is AT, however it can also bind HCII, while the main binding partner of DS is HCII.[58] Of all endothelial HS, only 0.5-10% possess the AT-binding sequence.[62,67] Of those, around 95% are present in the subendothelial matrix and are only exposed during injury.[62,67] The percentage of HS able to bind HCII with high affinity is unknown but it is probably lower than for AT, as a greater amount of HS is necessary for HCII-mediated thrombin inhibition.[64] Around 5% of all DS possesses the sequence of which three consecutive repeats are necessary to form the HCII- binding sequence (a partial sequence can bind with a lower affinity).[64] All DS are present in the subendothelial matrix.[64]

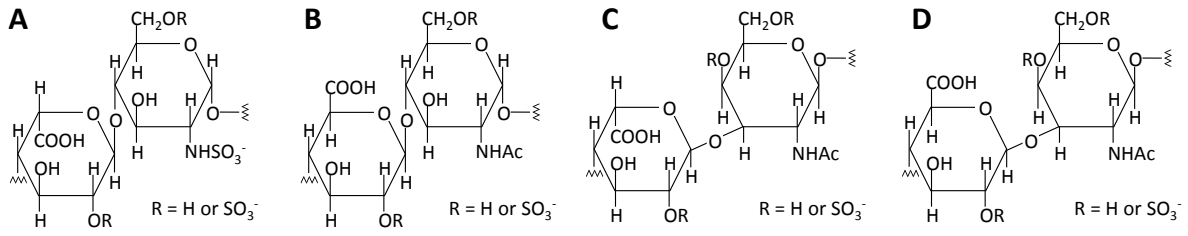
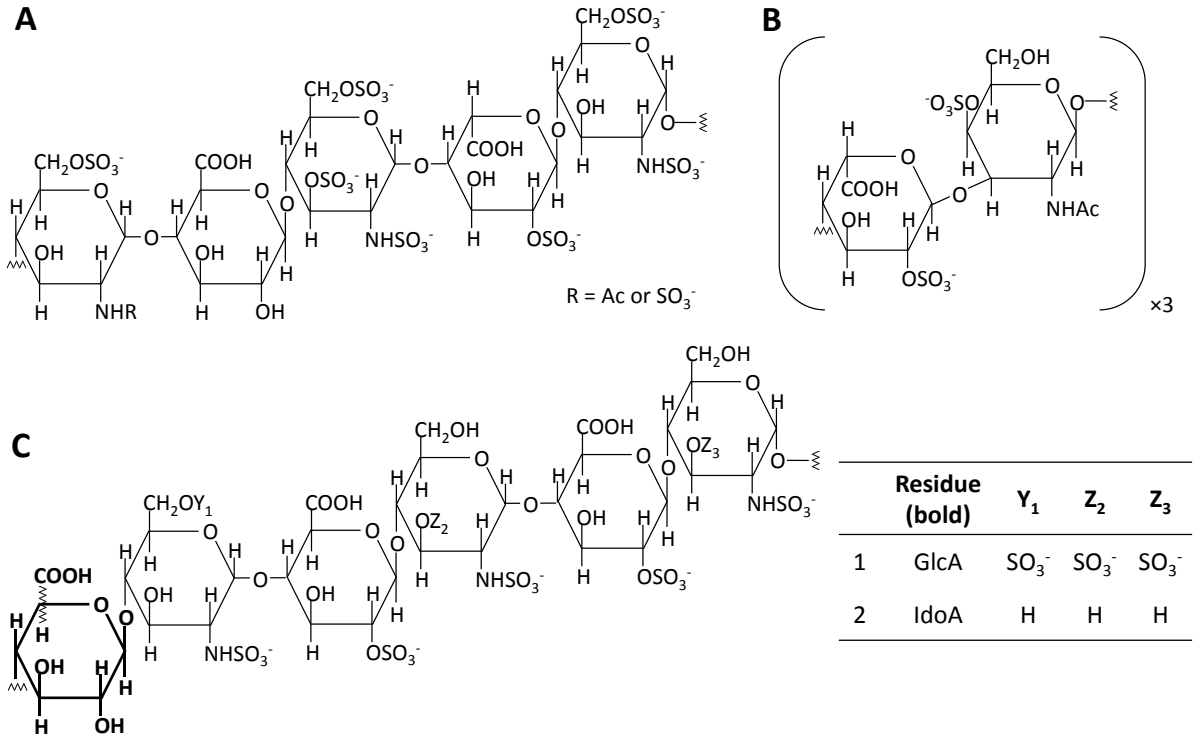


Figure 1.4. Principal disaccharide motifs in HS, DS and heparin.[58]
A. Iduronic acid-N-sulfated glucosamine, motif 1 in heparin and HS. **B.** Glucuronic acid-N-acetylated glucosamine, motif 2 in heparin and HS. **C.** Iduronic acid-N-acetylated galactosamine, motif 1 in DS. **D.** Glucuronic acid-N-acetylated galactosamine, motif 2 in DS.



	Residue (bold)	Y_1	Z_2	Z_3
1	GlcA	SO_3^-	SO_3^-	SO_3^-
2	IdoA	H	H	H

Figure 1.5. High affinity binding sequences in GAG for AT and HCII.[58]
A. Pentasacharide sequence in HS and heparin allowing high affinity binding to AT. **B.** Hexasacharide sequence in DS allowing high affinity binding to HCII. **C**. Two hexasacharide sequences in HS and heparin predicted *in silico* to bind HCII. The binding was confirmed *in vitro*.[58] Abbreviation used: GlcA for glucuronic acid; and IdoA for iduronic acid.

Heparin is structurally similar to HS, with HS being defined as being a GAG with a percentage of motif 1 (N-sulfated glucosamine linked to iduronic acid) < 70% and heparin with a percentage of motif 1 ≥ 70%.[68] The size of heparin molecules varies from 3 to 30 kDa, with an average of 15 kDa. Around 30% of heparin possess the high affinity AT-binding site.[69] Just as with HS, the exact proportion of heparin able to bind HCII with high affinity is unknown but is thought to be lower than AT.[64] Distinct from HS, heparin is only synthesised in mast cells.[70] Whether or not heparins are released during coagulation is still contested. A study examining coagulation in *NDST2*-knockout mice (which are unable to synthesise heparin but not HS) did not detect any bleeding abnormalities in these animals, although the authors of the study did

not specifically examine clot parameters.[71-73] In addition, several groups have failed to detect heparin in plasma,[71,74] while Engelberg and Dudley (1961) measured 1.0 to 2.4 mg/L of heparin in plasma (*ca*. 66–160 nM).[75] Whether the role of endogenous heparin in anticoagulation is physiologically relevant or not, heparin and heparin-based drugs are the main anticoagulants administered clinically in several conditions including venous thromboembolism, acute coronary syndrome, cardiopulmonary bypass, and haemodialysis.[76] When used as drugs, heparins are classified into two categories: unfractionated heparins (UFHs) and low molecular weight heparins (LMWHs). UFHs are naturally occurring heparins which can be neutralised by protamine sulfate when there is a risk of bleeding.[77] However, because of the numerous physiological function of GAGs, UFH drugs can cause many side effects such as osteoporosis and heparin-induced thrombocytopenia.[78] Because of these risks, UFH can be fractionated or depolymerised to obtain LMWHs, as their smaller size limits the risk of interactions with molecules other than serpins.[79] This also means that LMWHs have a more predictable dose-response than UFH and that, contrary to UFH, patients do not have to be closely monitored during administration of the drug.[78] However, LMWHs are more expensive, can only be partially neutralised by protamine sulfate and cannot be cleared by patients with renal insufficiency, in which case they will accumulate over time.[76,80] Despite these limitations, LMWHs have now replaced UFHs for many clinical applications in Europe, while UFHs are still used when rapid intervention is needed or for patients with an increased risk of bleeding or suffering from renal failure.[81,82] In the USA, the higher price of LMWHs have made them less attractive despite having lower downstream costs.[81] A list of approved heparin-based drugs is presented in Table 1.2.

Table 1.2. A list of US and EU-approved heparin-based drugs, as well as their major side effects, contraindications and main applications.[58]

Drug name	Average size (kDa)	Country where approved	GAG type	Side effects and contraindications	Applications
UFH	15	USA, Europe	UFH	Heparin-induced thrombocytopenia, osteoporosis.	Venous thromboembolism, acute coronary syndrome, cardiopulmonary bypass, haemodialysis, patients with underlying bleeding risk, or in those with renal insufficiency.
Enoxaparin	4.5	USA, Europe	LMWH		
Dalteparin	6	USA, Europe	LMWH		
Tinzaparin	6.5	USA, Europe	LMWH	Some heparin-induced thrombocytopenia, only partial neutralisation possible, risk of bleeding, renal insufficiency or disease.	Osteoporosis, heparin-induced thrombocytopenia.
Reviparin	3.9	Europe	LMWH		
Nadroparin	4.3	Europe	LMWH		
Bemiparin	3.6-3.8	Europe	LMWH		
Certoparin	3.8	Europe	LMWH		
Parnaparin	5	Europe	LMWH		
Danaparoid	5.5	Europe	Mixture of HS, DS and chondroitin sulfate	Some heparin-induced thrombocytopenia, risk of bleeding, renal insufficiency or disease.	Treatment of heparin-induced thrombocytopenia.
Fondaparinux	1.7	USA, Europe	Synthetic heparin analogue	Neutralisation not possible, risk of bleeding, renal insufficiency or disease.	Treatment of heparin-induced thrombocytopenia.

Anticoagulant GAG neutralisation during coagulation

When coagulation is required, such as during an injury, anticoagulant GAGs need to be neutralised to facilitate fibrin clotting.[58] Several proteins have this ability, many of which are present in relatively high concentrations in plasma.[58] Other proteins are released during certain circumstances, such as being released by damaged cells, by neutrophils during inflammation or by activated platelets during coagulation (Figure 1.6).[58] Among this last group are the proteins histidine-rich glycoprotein (HRG), fibrinogen and fibronectin.[40,83]

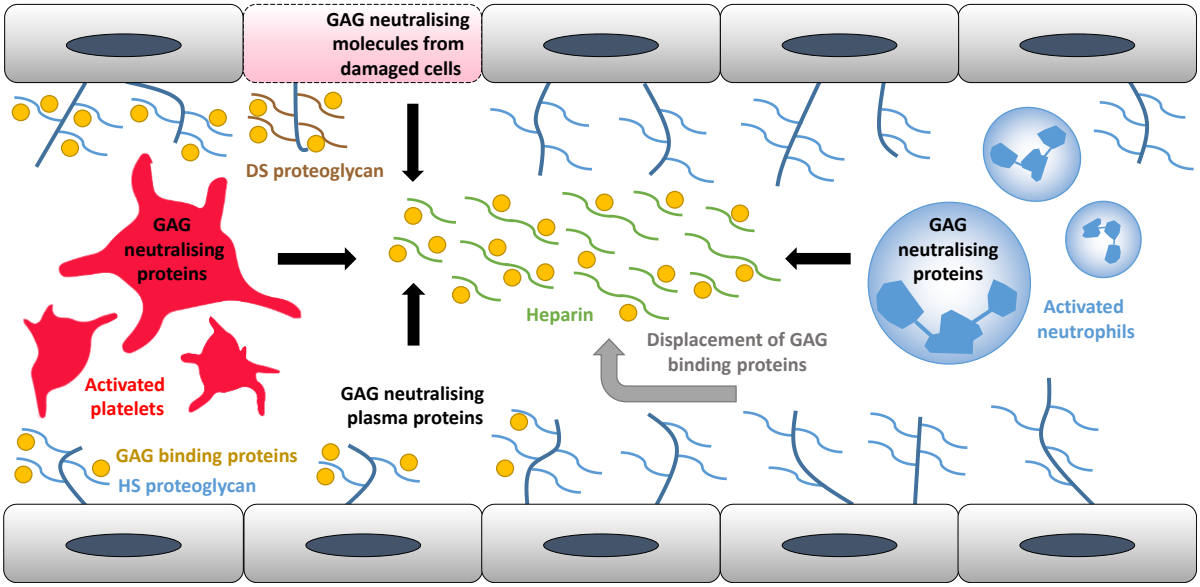


Figure 1.6. Schema representing the key locations of anticoagulant-GAG in the circulation and of the proteins and molecules neutralising them.[58]
Anticoagulant GAGs in the circulation are made up from HS proteoglycans at the surface of endothelial cells and the DS proteoglycans exposed when endothelial cells are damaged. Heparin can also be administered as an anticoagulant drug, thus displacing the numerous proteins normally bound to the endothelial GAGs. Molecules able to neutralise anticoagulant GAGs are released by activated platelets and activated neutrophils and they are released or exposed when endothelial cells are damaged; other neutralising molecules that circulate freely in plasma are also present.

GAG neutralisation by HRG

HRG is a 75 kDa linear protein synthesised in the liver and, in addition to being stored in platelets, it circulates with a basal plasma concentration of 1.3 to 2.0 μM.[84,85] It is an adaptor protein involved in the regulation of angiogenesis, immune functioning, and coagulation.[86] In this latter process, HRG inhibits plasminogen activation and so acts as an inhibitor of fibrinolysis.[86] It also binds activated factor XII, inhibiting its auto-activation, and inhibits the activation of factor XI.[86] Thus, it has been suggested to limit the propagation of the contact activation pathway of coagulation. In addition, HRG has been shown to neutralise heparins, including those with and without the AT-binding sequence.[40,83] HRG can bind to and neutralise LMWHs and UFHs, but the efficacy of this action depends on the size of the heparin, with LMWHs more difficult to neutralise. For example, an excess of HRG is not able to fully neutralise octasaccharide GAGs.[87] HRG can also prevent heparin-mediated inhibition of thrombin by HCII in plasma, but it is much weaker at neutralising DS-mediated thrombin inhibition by this mechanism.[88,89] Through the binding and neutralisation of GAGs, HRG regulates fibrin clot formation. It can also be incorporated into clots themselves affecting their lysis.[86] The binding of HRG to GAGs in the ESL also serves another function: it provides a tether site for other ligands of HRG such as plasminogen.[40,83] HRG can also compete with other GAG binding proteins such as fibroblast growth factors, which allows HRG to control their mitogenic activity.[86] HRG-GAG binding is also linked to the role HRG plays in immune responses, as the ability of the protein to bind polyanions is not limited to GAGs. Indeed, HRG can also bind DNA, pathogens and anionic phospholipids such as those exposed or released by dying cells.[86] In the case of binding to pathogens, this interaction can lead to the destabilisation of their membrane, leading to either cell death or their incorporation inside fibrin clots.

GAG neutralisation by fibrinogen

As with HRG, fibrinogen is synthesised in the liver and stored in platelets. It circulates as 340 kDa dimer with a basal concentration of 12 to 24 µM which makes it the second most abundant plasma protein after HSA.[90] Fibrinogen is a key protein in clotting as it is its cleavage into fibrin by thrombin (and the subsequent polymerisation) that forms the fibrin clots (Figure 1.1). Fibrinogen is known to bind and neutralise GAGs. Indeed, its plasma concentration is linked to heparin resistance in patients.[91] Direct study of GAG neutralisation by fibrinogen is complicated by the fact that thrombin cleaves fibrinogen. However, fibrinogen has been shown to neutralise heparin-mediated inhibition of thrombin by AT and DS-mediated inhibition of thrombin by HCII.[92] The neutralisation of DS (HCII activity) by fibrinogen is more effective than by HRG.[92] Neutralisation is also protein concentration-dependent, occurring at physiological fibrinogen concentrations and is not affected by the size or the degree of sulfation of DS.[92] The mechanism appears not to be through direct competition with HCII-thrombin for DS binding, but rather by modulating the rate of formation of the thrombin-HCII complex.[92] The physiological binding of GAGs by fibrinogen and the subsequent formation of ternary complexes (i.e. fibrinogen-GAG-thrombin) facilitates clot nucleation.[93] Once fibrinogen is cleaved into fibrin, it still participates in forming these complexes, to which AT is also incorporated, resulting in the reduction of the ability of AT to inhibit thrombin.[94,95]

GAG neutralisation by fibronectin

Fibronectin exists in many spliced forms. One of those is plasma fibronectin which is synthesised in the liver, stored in platelets and circulates in plasma with a basal concentration of 300 to 600 nM.[96] Other variants of the protein can also be synthesised by cells other than hepatocytes, notably endothelial cells.[97] This cellular fibronectin is present on cell surface and

is a major component of the ESL.[98] It is also released during wound healing.[97] In general, fibronectin is involved in many physiological processes such as cell–cell adhesion, platelet adherence, complement and coagulation system activation and wound healing.[98] In coagulation, plasma fibronectin is cross-linked to fibrin by activated factor XII and is incorporated into fibrin clots.[97] In addition, binding to endothelial GAG allows plasma fibronectin to form an extended conformation that then assembles into fibrils.[98] Those fibrils stabilise fibrin clots and promote platelet adhesion. It has also been shown that plasma fibronectin is able to prevent AT binding to immobilised LMWH as a function of heparin concentration, however AT is only completely displaced from heparin at fibronectin/AT ratios greater than are found physiologically.[96] Fibronectin possesses specific binding sites that are able to associate with heparin, HS or DS,[96,99] but the influence of the protein on HS and DS-dependent anticoagulant activities is not yet known.

The role of Zn^{2+} in GAG neutralisation

Part of the action of Zn^{2+} released from platelets during coagulation is to enhance anticoagulant GAG neutralisation (Figure 1.7). It is able to bind HRG, fibrinogen and fibronectin.[11,40] In particular, Zn^{2+} has been suggested to induce a conformational change in HRG and to influence GAG binding by HRG; however, it only affects binding to long chain GAGs, such as HS and UFH, but not LMWH.[40] In a similar fashion, Zn^{2+} binding to fibrinogen has been suggested to induce a conformational change in the protein, thus increasing the affinity of fibrinogen for GAGs.[100] It is not yet known if the chain length of the GAG affects the action of Zn^{2+} on GAG-fibrinogen binding. Fibronectin is also known to bind Zn^{2+} at several binding sites, although it is unknown whether this binding has any physiologically significance.[26,101,102] It has also been shown that binding of Zn^{2+} causes a conformational change in the protein but the effect those

changes have on GAG-binding is unknown.[103] GAGs themselves are extremely negatively-charged molecules and thus able to bind Zn^{2+} via their sulphate groups.[104] The binding of several cations to heparin has been shown to induce conformational changes in the molecule that have the potential to affect its interaction with various ligands.[105] Zn^{2+} was not among the cations studied but is likely to have the same effect. In addition to triggering conformational changes in GAGs, HRG, fibrinogen and fibronectin, Zn^{2+} also reduces the negative charges at the surface of the proteins by binding to exposed histidine residues.[106] This reduces the electrostatic repulsions between the proteins and GAGs, and serves as a potential additional ligand and thus also facilitates their binding.[106] Thus, through an increase in the neutralisation of anticoagulant GAGs in the ESL, high levels of Zn^{2+} have the potential to negate the anticoagulant properties of the endothelium.[26]

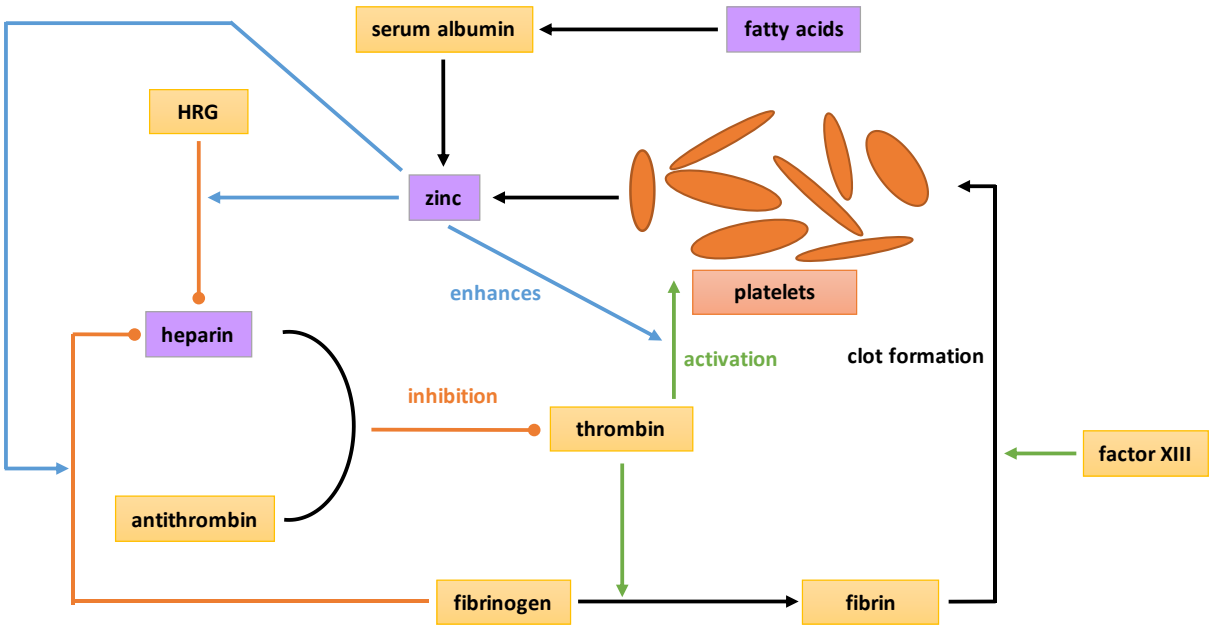


Figure 1.7. Simplified schema illustrating the roles of zinc in heparin-neutralisation during coagulation.
Inhibition reaction are indicated in red, activation in green, the enhancing activities of zinc in blue and binding, release or transformation in black.

Hypothesis and aims

Given that Zn^{2+} is an important regulator of coagulation, it is hypothesised that any change in the distribution of plasma Zn^{2+} among the different plasma proteins (Zn^{2+} speciation) has the potential to dysregulate this process and to cause thrombosis. Increased FFA binding to HSA in certain disease states (where elevated FFA levels are a feature) could reduce buffering of Zn^{2+}, leading, in part, to the observed increase in associated thrombotic risk.

In order to fully explore this hypothesis, this thesis focuses on three separate research strands:

1) Firstly, using isothermal titration calorimetry (ITC), the impact of different FFAs on Zn^{2+} binding by HSA is examined. The direct role of elevated plasma FFA and Zn^{2+} concentrations on fibrin clot formation and clot lysis is then considered. This is explored using turbidimetric fibrin clot formation and lysis assays in both purified protein and pooled plasma systems. Scanning electron microscopy (SEM) is used to examine the effect of elevated Zn^{2+} concentrations on fibrin fibre thickness in a purified fibrin clot system.

2) Secondly, the impact of the complex mixtures of FFAs present in type-I diabetes mellitus (T1DM) and type-II diabetes mellitus (T2DM) on fibrin clotting is assessed using the same turbidimetric fibrin clot assays and SEM experiments outlined above. Furthermore, as subjects with diabetes are known to possess altered plasma concentrations for different metal ions, including Zn^{2+}, variations in total plasma concentrations of zinc, copper, magnesium and selenium in plasma samples from subjects with T1DM and T2DM are measured and the impact of potential changes on fibrin clot parameters are examined.

3) Finally, given that alterations in plasma zinc speciation could increase anticoagulant GAG neutralisation (by HRG, fibrinogen and fibronectin), the extent to which Zn^{2+} affects heparin (as a proxy for HS and DS) and LMWH binding and neutralisation by those proteins is explored and the effect of this dynamic on FXa and thrombin activity is ascertained.

Chapter 2: Interplay between FFA and zinc binding to HSA

Introduction

HSA is the most abundant protein in plasma at around 600 µM, accounting for around 50% of total protein content in plasma and around 75% of the colloid osmotic pressure.[107] It is a single chain protein of 66 kDa and is made of three domains I, II and III, each divided into two subdomains A and B (Figure 2.1).[108] It is an important transporter of metal ions and in particular of Zn^{2+}.[109] It can bind Zn^{2+} through two sites, a high affinity binding site, site A and a putative secondary site with weaker affinity.[40] Site A is located between domain I and II of HSA.[32] An X-ray crystallographic structure of HSA has identified the residues involved in Zn^{2+} binding at site A. [32] Those are His67 (from domain I), His247 and Asp249 (both from domain II), with water as a fourth ligand. The affinity of this site for Zn^{2+} is relatively high, with an affinity of 2.73 x 10^5 M^{-1} as measured by ITC (i.e. a K_d of 3.66 µM).[40] The exact location of the potential second binding site is not yet known, but $^{111/113}$Cd NMR data suggests that only one nitrogen donor ligand is involved in Zn^{2+} binding, which would make this site harder (HSAB principle) than site A (as this nitrogen ligand would be replaced by an oxygen ligand) and thus with a reduced affinity for Zn^{2+}.[110] This is supported by ITC experiments, which have measured the affinity of a second binding site at 1.2 x 10^4 M^{-1} (i.e. a K_d of 83.3 µM).[40]

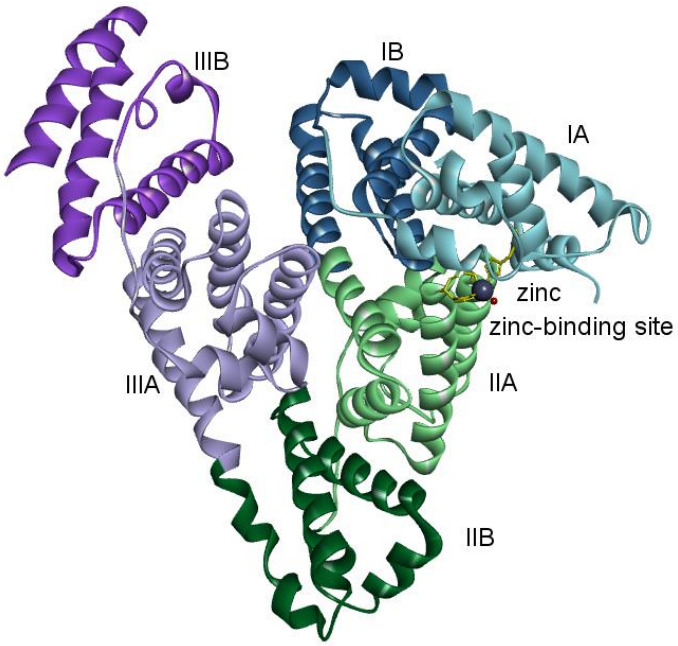


Figure 2.1. X-ray crystal structure of HSA with Zn^{2+} bound at site A (PDB 5IJF).[32] Domain I is represented in blue, II in green and III in purple. Subdomains A are in light colours, while subdomains B are in dark colours. The residues involved in binding Zn^{2+} are in yellow and the zinc atom in black.

HSA also transports FFAs in plasma and possesses several FFA-binding sites (Figure 2.2).[38] These include five high- to medium-affinity binding sites (FA2, FA4 and FA5 as high-affinity sites, FA1 and FA3 as medium- ones) and several lower-affinity ones (FA6, FA7 and possibly others) have been identified.[38,39,111] Those sites are distributed asymmetrically between the three domains of HSA.[38] For long-chain FFAs (C16:0 and C18:0), seven binding sites where observed: site FA1 situated in subdomain IB, site FA2 between IA and IIA, site FA3 and FA4 in IIIA, site FA5 in IIIB, site FA6 between IIA and IIB and site FA7 in IIA.[38,39,111] For medium-chain FFAs (C10:0, C14:0), 4 more sites are available, but they have a lower affinity for FFAs and are probably not physiologically relevant.[38]

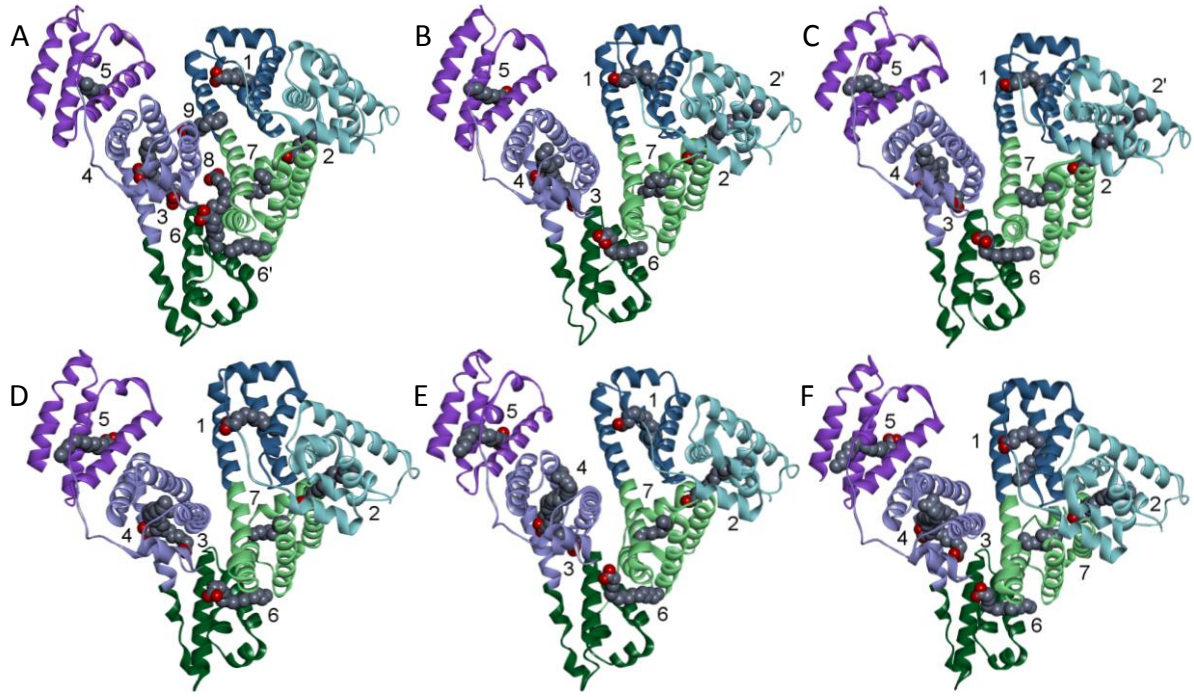


Figure 2.2. X-ray crystal structures of HSA binding FFAs of different chain lengths. **A.** decanoate (C10:0, PDB 1E7E),[38] **B.** laurate (C12:0, PDB 1E7F),[38] **C.** myristate (C14:0, PDB 1E7G),[38] **D.** palmitate (C16:0, PDB 1E7H),[38] **E.** stearate (C18:0, PDB 1E7I),[38] **F.** oleate (C18:1, PDB 1GNI).[111] The number of the binding sites is indicated next to each FFA. For decanoate, laurate and palmitate, two FFAs can fit in the binding site FA2 or FA6, the second one is then indicated as 2' or 6'.

Two kinds of interactions participate in FFA binding to HSA. The weakest interactions are electrostatic interactions between the carboxylate group of FFA and a cationic group located on HSA at or near the surface of the binding pocket.[112-114] The strongest interactions are hydrophobic interactions between hydrocarbon groups along the entire length of FFA and the non-polar amino acid chains in the binding pocket of HSA.[115] Thus, the strongest binding does not occur at the ω-terminus of the FFA or in the deepest point of penetration of the binding pocket, but rather along the chain of the FFA (but the FFAs is not rigidly bound as it exhibits rapid internal reorientation along its chain length).[113,115,116] In the binding pocket, the anchor point of the carboxylate groups remains the same whatever the chain length of FFA, only the depth to which the chain extends inside the pocket changes.[38] Those mechanisms explain why

saturated longer-chain FFAs generally have a higher binding affinity than saturated shorter-chain FFAs. Another important point about FFA binding to HSA is that, as the acyl chains of FFAs are not tightly bound to the protein (as there is no defined amino acid ligand in the binding pocket), the binding site can adapt in some measure to the incoming FFA by HSA changing its conformation.[113,117,118] This also means that binding of FFAs is cooperative (binding to one site alters the affinity of successive binding): when a FFA penetrates a binding pocket, the helices of the protein separate as the binding site adapts,[113,117,118] which induces small changes in the tertiary structure of the protein.

Site FA2 is a high affinity FFA binding site located between domain I and II.[38] To bind to this site, a FFA has to bind: 1) its carboxylate end to Arg-257 and Ser-287 from subdomain IIA and to Tyr-150 from subdomain IB and 2) its methyl end to subdomain IA.[38] To make this binding possible, some changes in the overall conformation of HSA have to occur; this is the rotation of domain I relative to domain II (Figure 2.3).[38] This rotation has also some important consequences on Zn^{2+}-binding site A: the Zn^{2+}-coordinating residue His67 moves around 8 Å from its original position in site A (measured in the overlay of the two structures as shown in Figure 2.3). This change locks the Zn^{2+}-binding site, preventing Zn^{2+} from binding to HSA when an FFA binds to site FA2.[31,38,119,120] A study of myristate binding to HSA has shown even 1 mol. eq. is sufficient to affect Zn^{2+}-binding and that 5 mol. eq. almost completely abolishes it.[40]

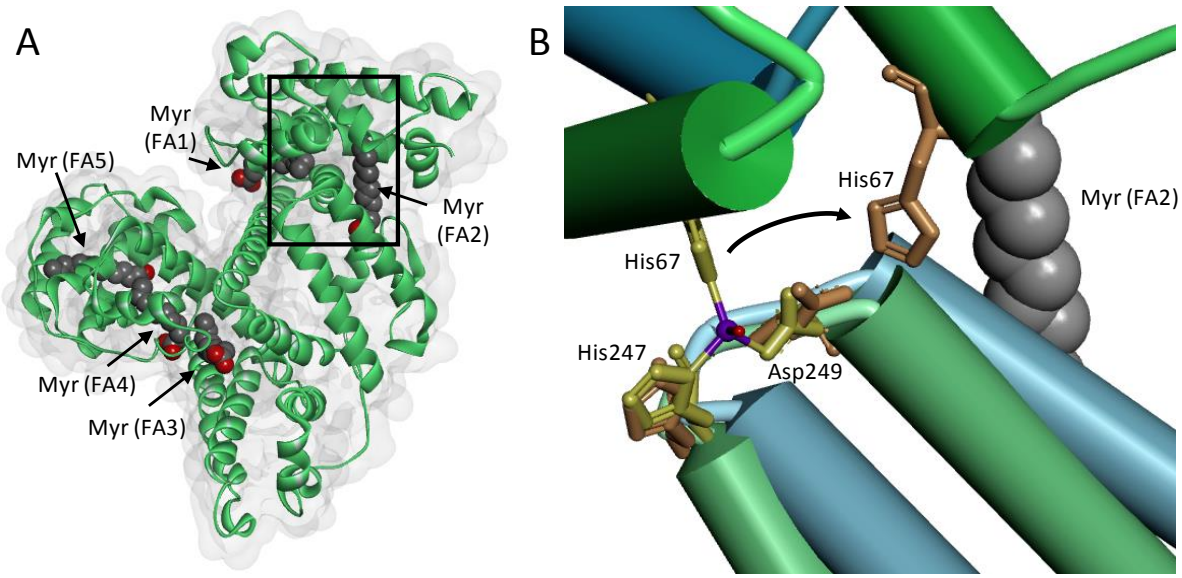


Figure 2.3. Myristate binding to HSA and its effect on Zn^{2+}-binding site A.
A. Five myristate molecules bound to HSA (PDB 1BJ5)[39] at its high affinity fatty acid binding sites (FA1-5). The surface of the protein is represented in grey. **B.** Overlay of the Zn^{2+}-binding site in a structure of HSA binding myristate (in green, PDB 1BJ5) with the structure of HSA binding a Zn^{2+} ion (in blue, PDB 5IJF).[32] The Zn^{2+} ion is shown in purple and the oxygen from the water participating in the binding site is in red. A domain-domain movement induced by the binding of myristate in the site FA2 results in the displacement of the His67 residues to ~8 Å from its original position.

Thus, the presence of an elevated FFA concentration in plasma has the potential to strongly increase the free Zn^{2+} concentration in plasma, as HSA binds up to 75% of total plasma zinc in healthy individuals, which could represent 9-15 µM zinc (for total zinc concentrations of 12-20 µM).[17] As the local concentrations of Zn^{2+} are thought to increase near activated platelets during normal coagulation,[12,20] the perturbations in Zn^{2+} speciation that would occur if HSA cannot bind the cation have the potential to strongly impact on the regulation of coagulation. In this chapter, the effect of FFAs of different chain lengths and saturation states on Zn^{2+}-binding by HSA is studied with ITC. Turbidimetric fibrin clot formation and lysis assays are also used to examine how those elevated FFA and Zn^{2+} plasma concentrations affect fibrin clot

formation and clot lysis, while the effect of Zn^{2+} on the thickness of fibrin fibres is examined with SEM.

Materials and methods

Materials

Chemicals were purchased at Sigma-Aldrich (Gillingham, UK) unless stated otherwise.

Isothermal Titration Calorimetry (ITC)

ITC experiments were performed using a MicroCal iTC200 instrument (Malvern, Malvern, UK) and a 50 mM Tris (tris(hydroxymethyl)aminomethane), 140 mM NaCl, pH 7.4 buffer. Two types of experiments were done:

1) For shorter chain saturated FFAs, 1.5 mM $ZnCl_2$ was titrated into 60 µM HSA in presence of 0-5 mol. eq. of different FFAs (octanoate, laurate, myristate and palmitate). The settings used were 25°C, reference power 5, stirring speed 750 rpm, 39 injections (first injection was of 0.4 µl, the remaining injections were of 1 µl), initial delay 60 s and spacing 120 s.

2) For less soluble FFAs, 0.75 mM $ZnCl_2$ was titrated into 25 µM HSA in presence of 0-5 mol. eq. of different FFAs (palmitate, palmitoleate, palmitelaidate and stearate). The settings used were 25°C, reference power 5, stirring speed 750 rpm, 19 injections (first injection was of 0.4 µl, the remaining injections were of 2 µl), initial delay 60 s and spacing 120 s.

FFAs were prepared as 150 mM stock solutions in alcohol (1% final alcohol concentration; methanol for octanoate, myristate and palmitate; ethanol for palmitoleate, palmitelaidate and stearate), before incubation with HSA for 2 h at 37°C. Heats of dilution were accounted for with blank titrations performed by injecting the ligand solution into the reaction buffer. The averaged heat of dilution was then subtracted from the main experiments. AFFINImeter software (Santiago de Compostela, Spain) was employed to fit the data using a "two sets-of sites" model. Initially fitting was performed on the isotherm data from the titration of Zn^{2+} into FFA-free HSA. The values obtained from this fitting were then used for the other titrations to fix the affinity of binding site A (K_1), the energy of binding site A (ΔH_1) and the stoichiometry of binding site B (N_2). The affinity of binding site B (K_2), the energy of binding site B (ΔH_2) and the stoichiometry of binding site A (N_1) were allowed to vary during fitting.

Some of those experiments were carried out by colleagues, Dr Ruitao Yu and Dr Siavash Khazaipoul, while I carried out the experiments for 2.5 mol. eq. palmitoleate and palmitealaidate, 4 mol. eq. palmitoleate and 5 mol. eq. palmitelaidate. I did all the fitting and analysis of the data.

Turbidimetric fibrin clot formation and lysis assays in a purified system with fibrinogen and HSA

Clot Assays were performed as previously described,[121,122] in a purified system containing fibrinogen and HSA and using permeation buffer (defined as 50 mM Tris, 100 mM NaCl, pH 7.4). Sodium myristate was mixed with HAS in the permeation buffer and the samples were incubated with shaking for 15 min at 37°C. $ZnCl_2$ was then added. Clotting was induced by the

addition of thrombin and $CaCl_2$ and lysis by the addition of t-PA and plasminogen. Final concentrations were: 0.5 mg/mL fibrinogen (plasminogen-depleted, Merck, Darmstadt, Germany), 100 µM HSA, 2.5 mM $CaCl_2$ (added in excess to compensate for the citrate), 0.05 U/mL thrombin (Calbiochem, San Diego, California, USA), 39 ng/mL tPA (Technoclone, Vienna, Austria) and 3.12 µg/mL human Glu-plasminogen (Enzyme Research Laboratories, Swansea, UK), 0-100 µM $ZnCl_2$ and either 0 or 400 µM sodium myristate (4 mol. eq. relative to HSA). Absorbance was read at 340 nm with a Multiskan FC plate reader (Thermo Scientific, Paisley, UK) every 12 s at 37 °C. Clot formation and lysis parameters (maximum absorbance, clot time defined as the time from induction of clotting to 50% clotting and lysis time defined as the time between maximum clotting and 50% lysis) were calculated from the resultant data.

Differences between groups were analysed using multiple Student's t-tests or analysis of variance (ANOVA) followed by Sidak's multiple comparisons tests. The significance threshold was set at $p \leq 0.05$. Statistical analyses were performed and graphs were generated with Prism 7.0 (GraphPad Software, La Jolla, California, USA). Data are shown as the mean ± the standard error of the mean.

Turbidimetric fibrin clot formation and lysis assays in pooled plasma

As the plasma was collected in citrated tubes, a compensation had to be made for the chemical neutralisation/chelation of Zn^{2+} by citrate. To this end, a calibration curve at 490 nm was made by adding 20 µM of the dye, 4-(2-pyridylazo) resorcinol into either permeation buffer or citrated plasma, both containing different $ZnCl_2$ concentrations. The amount of Zn^{2+} that should be added in citrated plasma in order to have the same amount of available Zn^{2+} as in the

buffer was then calculated (116 µM in citrated plasma was equivalent to 20 µM in buffer). Sodium myristate (either 0 or 4 mol. eq. final concentration) and $ZnCl_2$ (0-100 µM available Zn^{2+} final concentration) were added into pooled citrated plasma (First Link Ltd, Wolverhampton, UK). The experiments were performed as previously described for the purified system. Final concentrations were: plasma diluted 6-fold in permeation buffer, 7.5 mM $CaCl_2$, 0.03 U/mL thrombin and 20.8 µg/mL tPA.

Scanning electron microscopy (SEM)

Clots were formed in duplicate in the pierced lids of 0.6 mL centrifuge tubes in 45 µl volumes of permeation buffer containing 10 µM fibrinogen and 300 µM HSA in the presence and absence of 20 µM Zn^{2+}. Clotting was induced by addition of 5 µl of 25 mM $CaCl_2$ and 5 U/mL thrombin in permeation buffer. The clots were incubated for 2 h at 100 % humidity. For fixation, clots were given three 40 min washes in 67 mM sodium cacodylate, pH 7.4 and an overnight wash in 2% glutaraldehyde in sodium cacodylate buffer. The samples were dehydrated using a series of acetone washes and dried in a critical point drier. The clots were mounted on SEM stubs, coated with a 4 nm layer of iridium and viewed and photographed at ×10,000 magnification using a SUB230 scanning electron microscope (Hitachi, Maidenhead, UK) operating at 10 kV. Five images per samples were acquired and they were analysed with Adobe Photoshop (Adobe Systems, San Jose, CA); the diameters of 50 fibres per picture were measured.

Results and discussion

ITC study on Zn^{2+}-binding by HSA in the presence of different FFAs

ITC experiments were performed in order to study how Zn^{2+} binding at site A of HSA is affected by the presence of FFA of different chain lengths and saturation state. Building on previous work,[40,120] a "two sets-of-sites" model was chosen, with the affinity and energy of site A and the stoichiometry of site B fixed, in order to monitor potential change in the stoichiometry of site A. The raw data are shown in Figures A1.1-25, the parameters used for the fitting are detailed in Table 2.1 and the fitting results in Table 2.2.

Table 2.1. The parameters used to fit the datasets from the ITC experiments examining Zn^{2+}-binding to HSA in the presence of different FFAs.
The values for K_1 and ΔH_1 from the fit "no FFA, 60 μM HSA" were used to fix those parameters in the other datasets; "v" indicates parameters that were left to vary.

Fit	Model	Fixed parameters					
		N_1	K_1 (M^{-1})	ΔH_1 (kcal mol^{-1})	N_2	K_2 (M^{-1})	ΔH_2 (kcal mol^{-1})
No FFA, 60 μM HSA	**Two sets of sites**	v	v	v	v	v	v
Others	**Two sets of sites**	v	405300	-6066	1.000	v	v

Table 2.2. The fitting results from the ITC experiments examining Zn^{2+}-binding to HSA in the presence of different FFAs.

The values for K_1 and ΔH_1 from the fit "no FFA, 60 μM HSA" were used to fix those parameters in the other datasets and the N_2 value was fixed at 1.000.

Fitted Parameter	Fit	0 FFA	2.5 FFA	3 FFA	4 FFA	5 FFA
N_1	No FFA, 60 μM HSA	1.000				
K_1 (M^{-1})		405300				
ΔH_1 (kcal mol^{-1})		-6066				
N_2		1.000				
K_2 (M^{-1})		8850				
ΔH_2 (kcal mol^{-1})		-11020				
N_1	Octanoate			0.843		0.866
	Laurate			0.869	0.244	4e-15
	Myristate			0.497	0.203	2e-14
	Palmitate, 60 μM HSA			0.485	8e-14	4e-18
	No FFA, 25 μM HSA	0.822				
	Palmitate, 25 μM HSA		0.788		0.056	2e-15
	Palmitoleate		1.000		0.395	6e-15
	Palmitelaidate		0.549		0.318	1e-15
	Stearate		0.447		0.079	5e-20
K_2 (M^{-1})	Octanoate			14730		13610
	Laurate			9310	10630	7820
	Myristate			11970	7430	6290
	Palmitate, 60 μM HSA			12170	7560	5830
	No FFA, 25 μM HSA	15120				
	Palmitate, 25 μM HSA		13200		8000	9660
	Palmitoleate		8523		17790	8180
	Palmitelaidate		6200		14430	10830
	Stearate		24920		12420	13860
ΔH_2 (kcal mol^{-1})	Octanoate			-7060		-5580
	Laurate			-11720	-15140	-16050
	Myristate			-9420	-1.990	-12000
	Palmitate, 60 μM HSA			-11390	-12740	-12830
	No FFA, 25 μM HSA	-13040				
	Palmitate, 25 μM HSA		-10600		-20320	-19790
	Palmitoleate		-19885		-14380	-15460
	Palmitelaidate		-28520		-17030	-16400
	Stearate		-12810		-20560	-15890

The ITC instrument measures the amount of energy necessary to maintain the temperature in the sample cell (containing HSA) after heat is absorbed or released when the sample protein interact with a titrated ligand (Zn^{2+}). Each peak in the raw data corresponds to an injection of the ligand. Zn^{2+} binding to HSA is an exothermic reaction. The curve obtained by fitting the raw data correspond to the progressive saturation of the Zn^{2+}-binding site on HSA. In the first set of experiments, 60 μM of HSA was used (Figure 2.4). The smallest FFA studied was octanoate (C8:0). No effect on Zn^{2+} binding could be seen when adding 3 or 5 mol. eq. octanoate. These results were to a certain extent expected, as molecular modelling previously revealed that the half pocket forming the FA2 site in domain II is sufficient to accommodate octanoate, which means that the domain I movement (relative to domain II) is not required for octanoate to bind at FA2 and so site A is not affected.[123] The presence of either laurate (C12:0), myristate (C14:0) and palmitate (C16:0) affected Zn^{2+} binding to site A (Figure 2.4B), with increasing FFA concentrations reducing the stoichiometry of site A in a concentration-dependent manner. Indeed, while 3 mol. eq. of laurate had little effect, 4 mol. eq. reduced site A availability by 76% and 5 mol. eq. completely abolished Zn^{2+}-binding at this site. Myristate and palmitate exerted similar effects, with site A availability reduced by approximately half with 3 mol. eq. of these FFAs. In the presence of 4 mol. eq. of myristate or palmitate site A availability was reduced by 80% and 100%, respectively. With 5 mol. eq. of myristate or palmitate Zn^{2+} binding at site A was abolished.

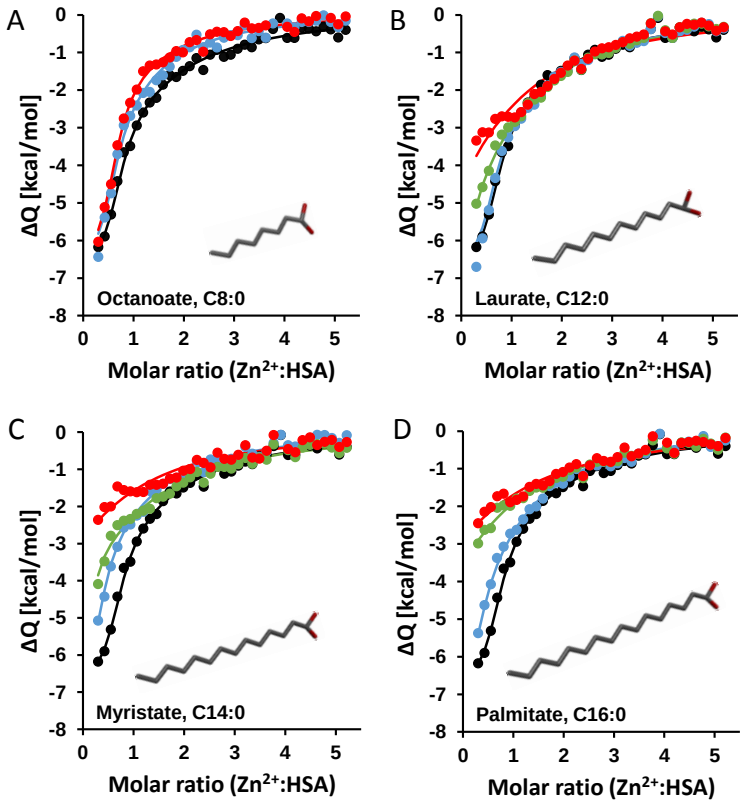


Figure 2.4. Isotherms of the titration of Zn^{2+} into HSA in presence of octanoate, laurate, myristate and palmitate.
HSA (60 µM) was loaded with 0 (black), 3 (blue) 4 (green) or 5 (red) mol. eq. FFA for 2 h at 37°C. 1500 µM Zn^{2+} was then titrated into HSA for 39 injections. Experiments were conducted in a buffer containing 50 mM Tris, 140 mM NaCl, pH 7.4. Each fit corresponds to a two-sets-of-sites model. Raw data can be found in Appendix 1. **A.** octanoate, **B.** laurate, **C.** myristate and **D.** palmitate.

Stearate, palmitoleate and palmitelaidate were not soluble under the same conditions as the FFAs assayed above, so a concentration of 25 µM HSA was used for this set of experiments (Figure 2.5). The experiments with palmitate were repeated to have a reference between the two sets of ITC titrations. Comparable results for Zn^{2+} binding in the presence of palmitate were obtained in the two sets of experiments, with the availability of site A reduced by 20% with 2.5 mol. eq., 90% with 4 mol. eq. and no Zn^{2+} binding observed with 5 mol. eq. The presence of stearate (C18:0) exerted a similar effect as palmitate, with availability of site A

reduced by 60% with 2.5 mol. eq., 90% with 4 mol. eq. and no binding with 5 mol. eq. The effect of the palmitelaidate (C16:1), a *trans* unsaturated FFA of the same chain length as palmitate, was then examined. It was found that palmitelaidate influenced Zn^{2+} binding, with the availability of site A reduced by half with 2.5 mol. eq., by 70% with 4 mol. eq. and 5 mol. eq. completely preventing Zn^{2+} binding. Palmitoleate (C16:1 *cis*), a *cis* unsaturated FFA of the same chain length, exerted no effect on Zn^{2+} binding at 2.5 mol. eq. with a reduction in Zn^{2+} binding only observable from 4 mol. eq. with the availability of site A reduced by 70% and no Zn^{2+} binding apparent with 5 mol. eq.

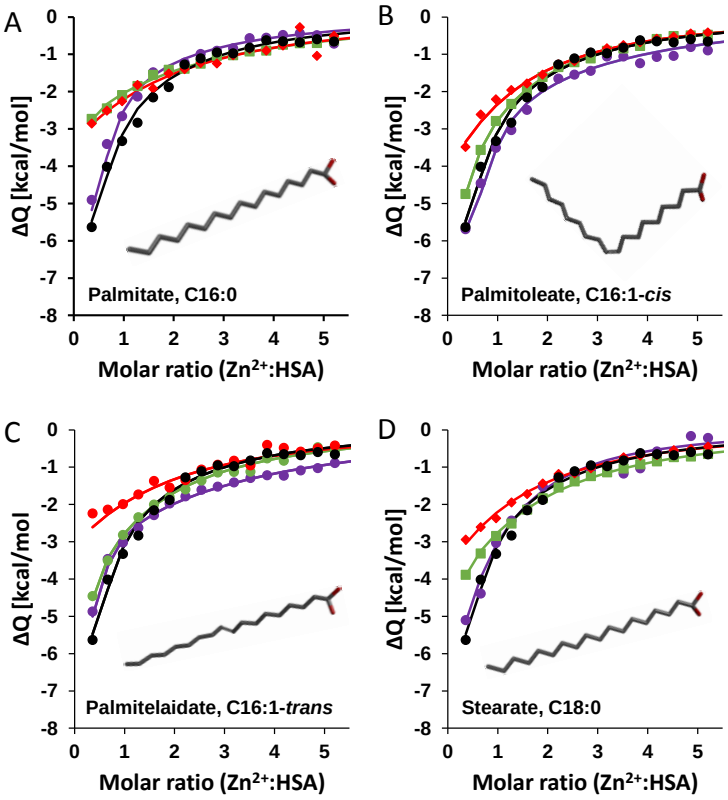


Figure 2.5. Isotherms of the titration of Zn^{2+} into HSA in presence of palmitate, palmitoleate and palmitelaidate and stearate.
HSA (25 µM) was loaded with 0 (black), 2.5 (purple), 4 (green) or 5 (red) mol. eq. FFA for 2 h at 37°C. 750 µM Zn^{2+} was then titrated into HSA for 19 injections. Experiments were conducted in a buffer containing 50 mM Tris, 140 mM NaCl, pH 7.4. Each fit corresponds to a two-sets-of-sites model. Raw data can be found in Appendix 1. **A.** palmitate. **B.** palmitoleate, **C.** palmitelaidate and **D.** stearate.

Thus, these results show that the reduction in Zn^{2+}-binding to HSA exerted by saturated FFAs increases with the chain length of FFA from 8 to 18 carbons. This can be explained by the importance of the interactions between HSA and the carbohydrate chain of the FFAs. Unsaturated FFAs however behave differently. The affinity of palmitoleate and palmitelaidate for HSA are unknown. However, it can be hypothesised that the affinity of palmitelaidate for HSA is only slightly lower than the affinity of palmitate, as *trans* unsaturated FFAs are structurally similar to saturated FFAs (in that the double bond does not provide a kink in the chain as with *cis* unsaturated FFAs). Palmitoleate would thus have a lower affinity for HSA than both palmitate and palmitelaidate as the kink in the hydrocarbon chain caused by the *cis* double-bond may well prevent the FFA from easily fitting in the binding pocket. However, factors other than the fitting of the FFA in the FA2 binding pocket and the interactions between the carbohydrate chain and the protein can also influence their affinity for this site and their effect on Zn^{2+}-binding by HSA. Indeed, as mentioned previously, the binding of FFA to the different binding sites of HSA is cooperative, with binding of FFA to one site triggering changes in the protein conformation that are going to affect the binding affinity of other sites. Binding of a FFA to the FA2 binding site is also affected by the competition between FA2 and the other high affinity FFA binding sites of HSA. Finally, as demonstrated with octanoate, not all FFAs that fit into the FA2 binding pocket will impact on the conformation of HSA in a manner that will results in a reduction of Zn^{2+}-binding by the protein.

The total plasma FFA concentration and the proportion of specific FFAs present vary between individuals depending on their sex, age and ethnicity and they are strongly influenced by diet and by disease states.[41] Fatty acids are ingested in the form of phospholipids and triglycerides (three esterified fatty acids bound to a glycerol molecule). During digestion, triglycerides are

broken down into mono- and di-glyceride as well as FFAs.[124] Only short and medium chain fatty acids (up to 12 carbons) are absorbed in the blood directly as FFAs and are then transported by HSA.[124] Long chain fatty acids (over 13 carbons) are absorbed as triglycerides and are transported in chylomicrons, a type of lipoprotein particle.[124] The lipoprotein lipase enzyme, present at the surface of endothelial cells, then cleaves those triglycerides to form FFAs that are taken up by the cells. This process occurs in most cells of the body but is particularly important in adipose tissue, where FFAs are re-esterified to form triglycerides that are stored in the fat droplet of the adipocytes. However, when triglycerides in the chylomicron are cleaved, the resulting FFAs are not always directly taken up by the nearby cells. In such cases, they instead bind to HSA and are transported through the circulation to other cells. Thus, levels of FFAs in the blood at least partially reflect dietary intake.

The ratio of saturated FFAs/unsaturated FFAs in the diet is important as high levels of saturated FFAs are known to be associated with an increased risk of developing cardiovascular diseases, including thrombosis.[125] The results presented here show that the saturated FFA palmitate (C16:0) has a stronger effect on Zn^{2+} binding to HSA than the unsaturated FFA of the same chain length, palmitoleate (C16:1). This could explain in part the higher thrombotic risk associated with high saturated FFAs intake. In addition, elevated dietary intake of saturated FFAs (in particular palmitate and stearate) is associated with T2DM through its effect on insulin sensitivity.[126,127] An elevated saturated FFAs/polyunsaturated FFAs ratio is also associated with metabolic syndrome.[128] Both of those diseases are associated with elevated thrombotic risks, which could partially be explained by the influence of saturated FFAs on Zn^{2+} binding to (and hence buffering by) HSA.[6] Furthermore, the FFAs that are present in blood are mainly long chain FFAs shown to have a greater effect on Zn^{2+}-binding by HSA (see Table

2.3). The most abundant FFAs in plasma are linoleate (C18:2 c9c12), palmitate (C16:0), oleate (C18:1 c9) and stearate (C18:0).[41] While the effect of linoleate and oleate are uncertain, the experiments presented here show that plasma concentrations of palmitate and stearate, two saturated FFAs, can potentially exert a strong effect on Zn^{2+} speciation in plasma. Elevated *trans* FFA levels in the diet are also known to be associated with cardiovascular diseases: a study has shown that for every 2% increase of energy derived from *trans* FFA, there is an associated 23% increase of cardiovascular risks.[129] In addition, elevated *trans* FFA levels in the diet are also associated with T2DM,[126] a disease that is itself linked to higher thrombotic risks. This may be due to the fact that they adversely affect lipid profiles (raising total cholesterol, low density lipoprotein cholesterol and triglyceride levels and reducing high density lipoprotein cholesterol levels), systemic inflammation, and endothelial function.[130] However, higher thrombotic risks may also be partially explained by the effect of *trans* FFAs on Zn^{2+}-binding by HSA, as palmitelaidate (C16:1 *trans*) has a similar effect on Zn^{2+} binding by HSA than palmitate, the saturated FFA of the same chain length, contrary to palmitoleate (C61:1 *cis*) which has less influence on Zn^{2+} binding.

Table 2.3. Abundance of fatty acids found in plasma total lipids (adapted from Abdelmagid, et al., 2015).[41]

Plasma samples were taken from an ethnically diverse population (including Caucasian, Asian, African, South Asian, Middle Eastern, Hispanic, Native American and Jewish) of young (20-29 years old) healthy Canadians consuming their usual diet. Extracted lipids were then analysed using gas chromatography. Subjects who were pregnant, breast-feeding, smoking taking a hormonal contraceptive or who had underlying health problems were excluded. The four most abundant FFAs are presented in bold.

Fatty acids concentrations in plasma total lipids (in µM)		Mean ± SD	Minimum	Maximum
Total amount of fatty acid		6947.6 ± 1816.2	1251.1	16225.3
Saturated	14:0 myristate	63.6 ± 37.1	16.2	325.7
	16:0 palmitate	**1631.1 ± 459.3**	**285.4**	**4064.5**
	18:0 stearate	**489.5 ± 124.3**	**110.2**	**1013.7**
Unsaturated *cis*	16:1 c9 palmitoleate	133.0 ± 67.2	27.7	555.9
	18:1 c9 oleate	**1285.5 ± 416.7**	**178.7**	**3210.5**
	18:1 c11 vaccenate	129.2 ± 59.5	11.4	562.9
	18:2 c9c12 linoleate	**2233.8 ± 622.6**	**279.7**	**4970.5**
	18:3 c6c9c12 γ-linoleate	23.5 ± 13.8	1.4	93.3
	18:3 c9c12c15 α-linolenate	54.4 ± 25.1	12.0	186.9
	20:3 c8c11c14 homo-γ-linolenate	74.3 ± 30.4	7.9	222.1
	20:4 c5c8c11c14 arachidonate	393.0 ± 119.1	42.7	882.8
	20:5 c5c8c11c14c17 eicosapentaenoate	40.3 ± 28.3	4.4	215.4
	22:5 c7c10c13c16c19 N-3 docosapentaenoate	23.9 ± 10.0	trace	88.5
	22:6 c4c7c10c13c16c19 docosahexaenoate	88.8 ± 36.8	7.2	237.5

Another source of plasma FFAs is via their endogenous synthesis from carbohydrates, which is called *de novo* lipogenesis. This process occurs in most cells but is particularly important in the liver, adipose tissue and the mammary glands. *De novo* lipogenesis is upregulated by insulin hormone in the blood and downregulated by high levels of epinephrine and glucagon hormones. FFA synthesis begins through the synthesis of palmitate (C16:0) which can then be elongated or desaturated to form other FFAs. *De novo* lipogenesis is upregulated in malignant tumours, which results in more FFA being synthesised from carbohydrates, which are released

into the blood.[131] Metabolic disturbances such as those arising from obesity are also known to cause a dysregulation of *de novo* lipogenesis.[132-134] This results in higher levels of several FFAs, including C16:0 and C18:0. Both malignant tumours and obesity are associated with higher risks of developing cardiovascular diseases and this may be partly explained by the actions of those higher plasma FFA levels on Zn^{2+} binding by HSA.[5,6] A third source of FFAs in the plasma is the cleavage of the triglycerides stored in adipose tissue during lipolysis. This occurs when plasma levels of insulin hormone are low and levels of catecholamines are high.[135] Lipolysis is upregulated in several disease states. In obesity for example, adipocytes undergo hypertrophy: the cell size increases to provide additional fat storage. This upsets the regulation of lipolysis and increase plasma levels of FFAs.[134,135] In diabetes, blood levels of insulin are low and this upregulates lipolysis.[135] In non-alcoholic fatty liver disease, insulin is unable to suppress lipolysis. This also results in an increase in plasma FFA levels.[135-137] Those increases in FFA blood levels have been linked with increased thrombotic risks in those diseases.[6] FFA levels in the blood have the potential to reach up to 5 mol. eq. in specific conditions (in particular in non-alcoholic fatty liver disease, see Chapter 1 for FFA levels).[37] As the study presented here has shown that 3 mol. eq. of myristate, palmitate and stearate can have strong effect on Zn^{2+} binding and 4 mol. eq. of myristate, palmitate and stearate can be sufficient to prevent most of Zn^{2+} binding, this provides a potential mechanism that could in part explain the higher thrombotic risks associated with those diseases.[5,6]

Finally, FFA concentrations are also affected by platelet activation. Indeed, phospholipase A2, is present in the platelet cytosol and secretary granules. This enzyme is responsible for the hydrolysis of platelet membrane phospholipids, with a high affinity for the fatty acid in the *sn2* position, resulting in the internal release of FFAs and lysophospholipids.[138] When platelets are

activated, the increase of platelet cytosolic Ca^{2+} concentration activates cytosolic phospholipase A2 which triggers a lipid flux inside the platelet.[139,140] It is likely that some of the resulting FFAs move across the platelet membrane (by passive diffusion alone or through facilitation and regulation by transport proteins such as CD36 which is ubiquitously expressed platelets), thus increasing their concentration in the proximity of the activated platelets.[140,141] In addition, both Ca^{2+} and phospholipase A2 are secreted during platelet activation. The enzyme can thus hydrolyse nearby phospholipids, further increasing local FFA concentrations.[142] Evidence for this comes from the observation that the FFA concentration of thrombi extracted from human patients has been observed to be in the millimolar range (the average concentration has not been reported).[143] In platelets, the most abundant phospholipid is lecithin.[144] The most prevalent fatty acids that constitute lecithin are long chain fatty acids: palmitate (C16:0) forming more than 30% of its total fatty acid content, oleate (18:1) forming around 27%, stearate (18:0) forming around 15% and arachidonate (C20:4) forming around 12.5%.[144] However, arachidonate is present in the *sn2* position in high proportion and thus the activity of phospholipase A2 has been shown to result principally in the hydrolysis of this FFA.[145] Nevertheless, a lipidomic study has confirmed that the cytosolic concentrations of palmitate, oleic and stearate also increase upon thrombin activation of platelets.[139] Thus, platelet activation may result in local accumulation of arachidonate, palmitate, oleate and stearate and, through their binding to HSA, in a further increase in local Zn^{2+} concentration, in addition to the Zn^{2+} released directly by platelets.

Fibrin clot formation and lysis assays and clot structure in the purified protein system and in pooled plasma

This study has shown that elevated total FFA concentration as well as elevated concentrations of specific FFAs reduce Zn^{2+} binding by HSA *in vitro* and are likely to strongly impact Zn^{2+} speciation in plasma. Given that elevated FFA levels are associated with disease states where thrombotic complications frequently arise, it was important to ascertain how altered Zn^{2+} speciation may impact on coagulation. To this end, turbidimetric fibrin clot formation and clot lysis assays were carried out in a purified system and in pooled plasma to examine the effects FFA and Zn^{2+} concentrations in blood have on fibrin clotting. First, a purified system containing fibrinogen and HSA was used (Figure 2.6A-C). Myristate concentration was chosen to represent total FFA concentration *in vivo*, as it behaves similarly to long chain FFA but is more soluble. Pathophysiological concentrations of up to 5 mol. eq. total FFA have been observed *in vivo* (seen Chapter 1); here 4 mol. eq. was assayed. The maximal local Zn^{2+} concentration can reached in proximity to activated platelets is unknown (but the concentration of Zn^{2+} available for binding with coagulation protein will be higher than the free Zn^{2+} concentration). Here, up to 100 µM Zn^{2+} was examined which is probably higher than would occur physiologically but was used in order to observe the effect of Zn^{2+} over a wide range of concentrations. Addition of 20-100 µM Zn^{2+} increased in a concentration-dependent manner both maximum absorbance and lysis time. Clot time increased with 20 and 40 µM Zn^{2+}, but then decreased with 60-100 µM Zn^{2+}. Addition of 4 mol. eq. myristate on its own increased maximum absorbance, but it did not significantly affect clot time or lysis time. In order to differentiate this effect of FFA alone from the effect of FFA on Zn^{2+}-buffering by HSA, parameter values relative to their values in the absence of Zn^{2+} were calculated for the samples without and with myristate (Figure 2.6D-F). This showed that addition of Zn^{2+} resulted in a higher increase of maximum absorbance in the presence of myristate than in its absence. The

decrease (at 40-100 μM Zn^{2+}) of clot time, was also more apparent in the presence of myristate. The increase of lysis time, however, was not significantly different in the presence or absence of myristate.

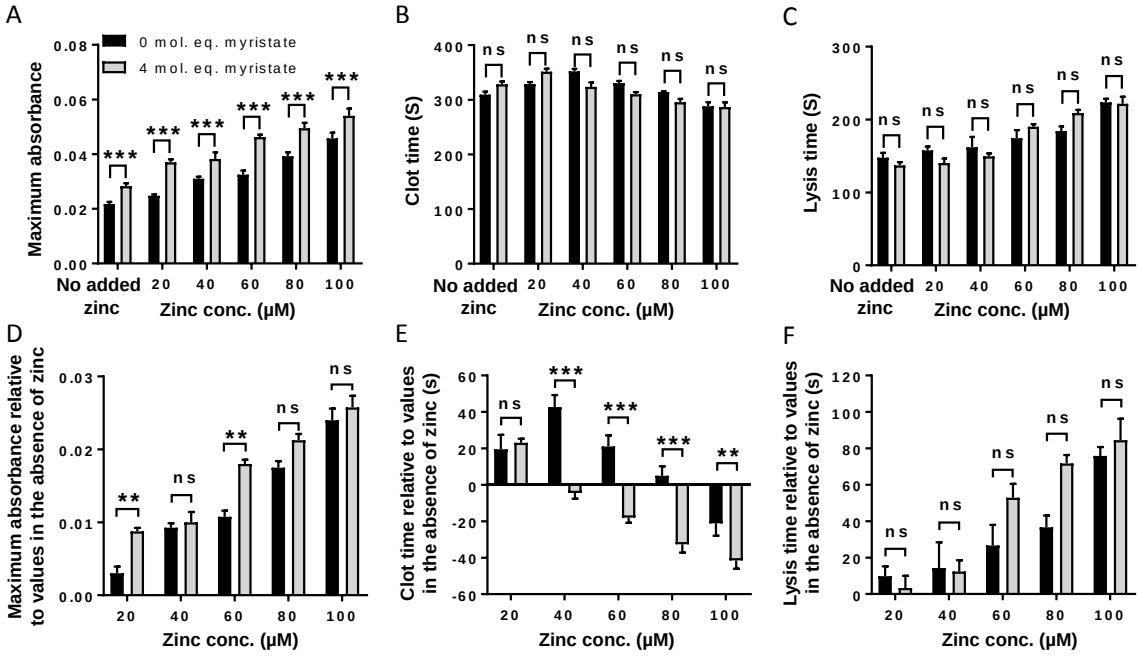


Figure 2.6. Effects of Zn^{2+} and myristate on fibrin clot formation and lysis parameters in a purified system and effects relative to the parameter values in the absence of Zn^{2+}. Turbidimetric fibrin clot formation and lysis assays were carried out in permeation buffer with final concentrations of 2.9 μM fibrinogen, 100 μM HSA, 2.5 mM $CaCl_2$, 0.05 U/mL thrombin, 39 ng/mL tPA, 3.12 μg/mL plasminogen, 0-100 μM $ZnCl_2$, and either 0 or 4 mol. eq. myristate. The experiments were repeated 4 times. The parameters measured were **A.** maximum absorbance, **B.** clot time and **C.** lysis time. All three parameters resulted in significant differences as measured by two-way ANOVA: for maximum absorbance $p < 0.0001$ and $p = 0.0242$ for Zn^{2+} and 4 mol. eq. myristate respectively, while for clot time and lysis time $p < 0.0001$ for Zn^{2+} and the differences were not significantly with myristate. Then the parameter values relative to their values in the absence of Zn^{2+} were calculated: **D.** maximum absorbance, **E.** clot time and **F.** lysis time. Addition of Zn^{2+} significantly increased all three parameters ($p < 0.0001$ for all three). Addition of 4 mol. eq. myristate increased maximum absorbance and reduced clot time at all Zn^{2+} concentrations ($p = 0.0160$ and $p = 0.0055$ respectively) but did not alter lysis time. Statistical significance from multiple comparison tests is indicated with ns (not significant) for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

In order to examine the effect of Zn^{2+} on clot ultrastructure, SEM experiments were then performed after inducing clotting with thrombin and Ca^{2+} in a purified system containing both fibrinogen and HSA. The fibrin fibre thickness was then measured (Figure 2.7). Addition of 20 μM Zn^{2+} in this system resulted in a significant increase in fibrin fibre diameter ($p < 0.0001$), explaining the increase in maximum absorbance seen by addition of Zn^{2+}.

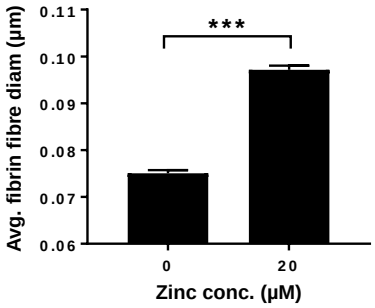


Figure 2.7. Effect of Zn^{2+} on fibrin fibre thickness as measured by SEM.
The experiments were carried out in a purified system with a 50 mM Tris, 100 mM NaCl, pH 7.4 buffer by mixing 45 μL of 10 μM fibrinogen, 300 μM HSA and either 0 or 20 μM $ZnCl_2$ with 5 μL of 25 mM $CaCl_2$ and 5 U/mL thrombin. The clots were formed in duplicate, with 5 images taken per duplicate and 50 fibres measured per images (final $n = 500$). Addition of 20 μM resulted in a significant increase in fibrin fibre thickness ($p < 0.0001$).

The turbidimetric fibrin clot formation and clot lysis experiments were then carried out in pooled citrated plasma (Figure 2.8A-C). To account for the Zn^{2+}-buffering capacity of citrate, a dye, 4-(2-pyridylazo) resorcinol, was used to measure the amount of Zn^{2+} required to be added into citrated plasma to obtain the desired concentrations of available Zn^{2+}. Addition of 20-100 μM Zn^{2+} increased maximum absorbance and clot time, while 20 μM Zn^{2+} increased lysis time and 40-100 μM Zn^{2+} decreased it. Addition of 4 mol. eq. myristate did not affect maximum absorbance but decreased clot time and increased lysis time. Calculation of the parameter values relative to the values in the absence of Zn^{2+} showed that the increase in maximum absorbance and clot time was higher in the presence of myristate, while the decrease in lysis time was also more apparent in the presence of myristate (Figure 2.8D-F). The effects

of Zn^{2+} in plasma were different to those observed in the experiments using the purified system; this can be explained by the different combination of proteins present in plasma while the purified system only contained a few components involved in the coagulation cascade.

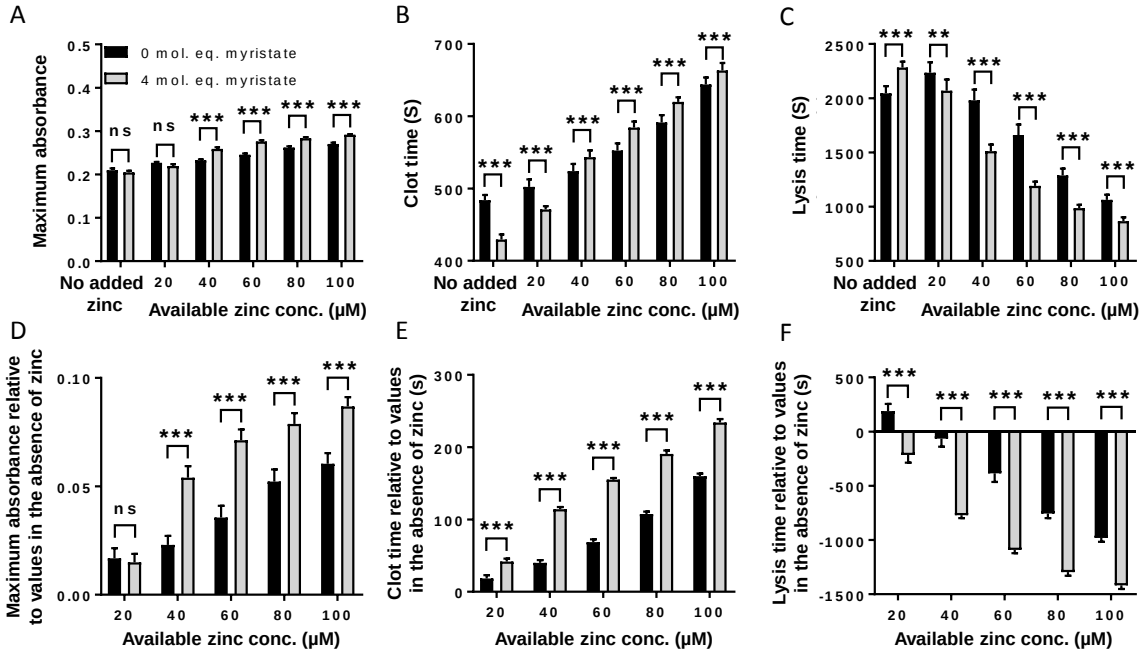


Figure 2.8. Effects of Zn^{2+} and myristate on fibrin clot formation and lysis parameters in pooled plasma and effects relative to the parameter values in the absence of Zn^{2+}.
Turbidimetric fibrin clot formation and lysis assays were carried out in pooled plasma diluted 6-fold in permeation buffer, with final concentrations of 7.5 mM $CaCl_2$, 0.03 U/mL thrombin, 20.8 ng/mL tPA, 0-100 μM available Zn^{2+} added as $ZnCl_2$ (with the concentrations calculated before the dilution of the plasma) and either 0 or 4 mol. eq. myristate. The experiments were repeated 12 times. The parameters measured were **A.** maximum absorbance, **B.** clot time and **C.** lysis time. All three parameters resulted in significant differences in the presence of Zn^{2+} and 4 mol. eq. myristate as measured by two-way ANOVA: for maximum absorbance $p < 0.0001$ for Zn^{2+} and $p < 0.0001$ for 4 mol. eq. myristate, for clot time $p < 0.0001$ for Zn^{2+} but myristate did not result in significant difference, for lysis time $p < 0.0001$ for Zn^{2+} and $p < 0.0001$ for myristate. Then the parameter values relative to their values in the absence of Zn^{2+} were calculated: **D.** maximum absorbance, **E.** clot time and **F.** lysis time. Addition of Zn^{2+} significantly altered all three parameters ($p < 0.0001$ for all three). Addition of 4 mol. eq. myristate increased maximum absorbance and clot time and decreased lysis time at all Zn^{2+} concentrations ($p = 0.0002$, $p < 0.0001$ and $p < 0.0001$ respectively). Statistical significance from multiple comparison tests is indicated with ns (not significant) for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

Zn^{2+} is known to be a strong regulator of coagulation. Notably, Henderson et al. have shown that high plasma Zn^{2+} levels are known to affect coagulation by accelerating clot formation in a purified system and in dialysed plasma.[13] An elevation in Zn^{2+} concentrations in plasma also: 1) increases the thickness of the fibrin fibres in clots by binding to the αC domain of fibrinogen; 2) increases the pore size and the porosity of the fibrin clots, which reduces the risk of clot rupture; 3) makes the clots more elastic by offsetting the increase in clot stiffness induced by factor XIII and so further reducing the risk of clot rupture.[13] Those modifications to the structure of the clot are thought to increase the flow of plasma components inside the clot and thus promoting clot maturation and fibrinolysis.[13] However, Zn^{2+} also reduces plasminogen activation and fibrin clot degradation by plasmin.[12] Those two mechanisms are in competition and it has been shown in both a purified system and in dialysed plasma that an increase in Zn^{2+} concentration results in delayed clot lysis.[12,13] This competition explains the dual effect seen when measuring the lysis time in pooled plasma in the experiments presented here, while the purified protein system did not contain all the proteins necessary to see this phenomenon.

The previous studies published by Henderson et al. were mainly conducted in a purified system similar to the one presented here.[12,13] Only the measurement of the speed of clot formation and clot lysis were carried out in dialysed plasma, a process that may have resulted in the loss of small molecules. To avoid this, the present study has used a dye to calculate the quantities of Zn^{2+} to add to plasma to obtain the desired concentrations of available Zn^{2+}. In addition, the previous studies have only examined the effect of a small range of Zn^{2+} concentrations: up to 5 µM for the clot formation experiments and up to either 6 or 15 µM for the lysis experiments. As the local maxima in Zn^{2+} concentrations that can be reached in proximity of activated platelets are still unknown, the current study has expanded on this by using a wider range of

Zn^{2+} concentrations: up to 100 µM Zn^{2+} (which is probably not physiological). Finally, while addition of FFAs in the absence of HSA has been shown to increase the maximum absorbance and the clot time of the fibrin clot,[140] the effect of FFA binding to HSA on fibrin clot formation and lysis had not been investigated before. The results presented here show that independently to their own effect, FFAs prevent Zn^{2+} from being buffered by HSA and that pathophysiological concentrations of FFAs are sufficient to affect plasma Zn^{2+} speciation. In addition, the results demonstrate that increased plasma concentrations of available Zn^{2+} have observable consequences on fibrin clot formation and lysis.

In summary, this chapter has demonstrated the impact of elevated plasma Zn^{2+} and FFA concentrations on fibrin clot formation in both a purified system and in pooled plasma, where FFA concentration positively correlates with maximum absorbance and clot time and negative correlates with lysis time. In the next chapter this potential disease mechanism is examined further and the relationship between FFAs and fibrin clot parameters are investigated in subjects with T1DM, T2DM and in control subjects.

Chapter 3: Impact of metal ion and FFA concentrations on fibrin clotting in type-I and type-II diabetes

Introduction

Diabetes is a disease characterised by the mishandling of glucose through defective insulin signalling. There exist two types of diabetes. In type-I diabetes mellitus (T1DM), the β-cells in the pancreas that secrete insulin are lost, typically destroyed by the immune system, causing an insulin deficiency in the body. In type-II diabetes mellitus (T2DM), insulin is still produced but the cells become insulin resistant (probably through a defect of insulin receptors) and fail to respond properly to the hormone. Thus, insulin levels are either unchanged or increased relative to blood glucose levels, but T2DM can also develop into insulin deficiency through a loss of the insulin storage function of β-cells. Blood glucose levels are important as they are directly or indirectly associated with many physiological processes including the control of glycogen and lipid and protein metabolism. They also participate in the control of food intake (satiety) and in the maintenance of body weight and they also indirectly regulate inflammation, vasodilatation and basic cell growth and replication. Thus, a dysregulation of glucose levels can have wide-ranging consequences. Among those is an alteration in the concentration of several molecules in plasma, including FFAs and metal ions,[46,146] which can increase the risk of developing thrombotic complications. In the UK, 3.8 million individuals have been diagnosed with diabetes and a further 0.8 million are estimated to be undiagnosed.[4] Those adults with diabetes are two to three times more likely to develop cardiovascular diseases compared to adults without diabetes and nearly twice as likely to die from a heart disease or

stroke.[4] In total, one third of the adults with diabetes in the UK die from some form of cardiovascular disease (compared to a quarter of all adults).[4]

In diabetes, it is interesting to consider the associated elevations in plasma FFA levels and how these may influence coagulation. Indeed, impaired insulin secretion, impaired insulin sensitivity and glucose intolerance are associated with elevated plasma FFA levels, especially of saturated FFAs (including palmitate and stearate).[126,127] In addition, levels of palmitate (C16:0) are positively correlated with levels of glycated haemoglobin (HbA1c) in T2DM subjects, while levels of oleate (C18:1) are only correlated to HbA1c levels in patients with inadequate diabetes control.[147] In chapter 2, it was shown that longer chain FFAs, especially those with 16 and 18 carbons, highly affect Zn^{2+}-binding by HSA. Thus, blood clotting in T2DM subjects have the potential to be highly affected compared to healthy controls. T1DM however is not associated with elevated FFA levels and therefore constitute an interesting comparison with T2DM. Thus, comparison between T1DM and T2DM subjects and healthy controls may be useful for examining the link between fibrin clotting, FFA levels and Zn^{2+} levels.

In addition to plasma FFA concentrations, metal ion homeostasis is known to be altered in both T1DM and T2DM subjects. This is due to several factors including an increase in fluid loss from the body, increased micronutrient demand caused by altered protein metabolism and defective metal transport caused by oxidative stress or inflammation.[146,148] Total plasma concentrations of metals (including zinc, copper, magnesium and selenium) have been measured in subjects with both types of diabetes (and controls), however the results have been

very heterogeneous. Several studies and meta-analyses have highlighted the importance of studying non-linear correlations between diabetes and plasma micronutrient concentrations (by looking at lower and higher concentrations separately), as well as of looking at factors such as sex and plasma HbA1c concentrations as a proxy of diabetes management.[149-154] As those metals are involved in the regulation of many physiological processes, with Zn^{2+} in particular regulating coagulation, any alteration in their plasma concentrations is bound to have a strong impact on coagulation and any correlation with fibrin clot parameters should be assessed. After calcium, zinc is the most important metal involved in the regulation of coagulation, however it is not the only one. Copper ions (Cu^{2+}/Cu^{+}) are known cofactors of several proteins involved in coagulation, including coagulation factors V and VIII.[155,156] This is also the case for magnesium ions (Mg^{2+}) which interact notably with coagulation factor IX.[157] In addition, the selenoprotein glutathione peroxidase is known to be important for the regulation of platelet aggregation (individuals deficient in selenium show hyperaggregation).[158] Thus, plasma concentrations of zinc, copper, magnesium and selenium have the potential to impact upon coagulation.

In this chapter, turbidimetric fibrin clot formation and lysis assays were carried out to examine any alteration in clot formation and lysis parameters in the plasma of subjects with T1DM or T2DM. Total plasma concentrations of zinc, copper, magnesium and selenium were analysed to ascertain whether there were any differences between T1DM, T2DM and controls and between the sexes. They were also assessed to determine whether there was any correlation between their respective levels and fibrin clot parameters.

Materials and methods

Clinical sample collection

A total of 45 patients with T1DM, 54 patients with T2DM and 62 controls (47 age-matched for T1DM and 18 for T2DM) were recruited from Leeds Teaching Hospital Trust following approval by the local Research Ethics Committee in Leeds. The exclusion criteria for the T1DM cohort were: a history of acute coronary syndrome or stroke within 3 months of enrolment, prior treatment with aspirin, clopidogrel, warfarin or non-steroidal anti-inflammatory drugs, current treatment with any drug other than insulin, a history of deep venous thrombosis or pulmonary embolism, previous or current history of upper gastrointestinal pathology, malignancy or coagulation disorders, abnormal liver function test (alanine amino transferase >3 fold upper limit of normal) or abnormal thyroid function test, proteinuria, advanced nephropathy and clinical signs of neuropathy or retinopathy (except for those with background changes). Written informed consent was obtained and baseline fasting blood samples were collected in trisodium citrate or lithium heparin coated tubes. Plasma was separated by centrifugation at 2,400 × g for 20 min at 4°C within 2 hours of collection. The samples were snap frozen in liquid nitrogen and stored at -40°C until analysis.

Turbidimetric fibrin clot formation and lysis assays in plasma from subjects with T1DM, T2DM and from controls

$ZnCl_2$ (0-20 µM available Zn^{2+}) was added into citrated plasma from subjects with T1DM or T2DM and from controls. Two types of experiments were then performed: 1) turbidimetric fibrin clotting assays, with the following final concentrations: plasma diluted 3-fold in permeation buffer, 7.5 mM $CaCl_2$ and 0.03 U/ml thrombin; 2) turbidimetric fibrin clotting and

lysis assays, with the following final concentrations: plasma diluted 6-fold in permeation buffer, 3.75 mM $CaCl_2$, 0.03 U/ml thrombin and 20.8 ng/ml tPA. The experiments were conducted as described in Chapter 2.

Scanning electron microscopy (SEM)

Clots were formed in duplicate in the pierced lids of 0.6 ml centrifuge tubes in 45 µl volumes of pooled plasma diluted 1-fold in permeation buffer in the presence and absence of 20 µM available Zn^{2+}. The pooled plasma samples were from 6 randomly chosen subjects with either T1DM or T2DM or from controls. The samples were then treating in the same manner as described in Chapter 2.

Measurement of plasma FFA, HSA, glycated haemoglobin and fibrinogen concentrations and of total plasma zinc, copper, magnesium and selenium concentrations

Dole's protocol was used to extract the FFAs from the citrated plasma samples from subjects with T1DM and T2DM and from controls.[159] The concentrations of the extracted FFAs were then measured using an FFA Assay Kit - Quantification (Abcam, Cambridge, UK). Total plasma zinc, copper, magnesium and selenium concentrations were measured by Dr Fiona Stefanowicz (Scottish Trace Element and Micronutrient Diagnostic and Research Laboratory, University of Glasgow, UK) using inductively coupled plasma-mass spectrometry on the lithium-heparin plasma samples (as described by Sobczak et al.).[160] The lithium-heparin plasma samples were also used by St James' University Hospital (Leeds, UK) to measure HSA levels

with the bromocresol purple method using an automated analyser (Architect; Abbot Diagnosis, Maidenhead, UK), to measure the plasma fibrinogen concentration using the Clauss assay and to measure the plasma glycated haemoglobin concentrations using a routine method.

Data analysis and representation

Differences between groups were analysed using multiple Student's t-tests or ANOVA followed by Dunnet's (for one-way ANOVA) or Sidak's (for two-way ANOVA) multiple comparisons tests or, for continuous parameters, using Pearson's correlation test. The metal concentration data was also separated into quartiles and odds ratios were calculated. Significance threshold was set at $p \leq 0.05$. Statistical analyses were performed and graphs were generated with Prism 7.0 (GraphPad Software, La Jolla, CA). Data are represented as mean ± standard error of the mean.

Results and discussion

Fibrin clot formation and lysis assays in T2DM

In order to assess whether the effects of FFAs on Zn^{2+}-handling and fibrin clot formation and lysis are relevant *in vivo*, plasma from controls and from subjects with T2DM, a disease associated with elevated plasma FFA levels, was examined. Demographics information on the individuals in the two groups as well as measures of plasma total FFA, total zinc, HSA and HbA1c concentrations are found in Table 3.1. The groups were matched in age but not in sex. No difference in plasma FFA concentrations could be seen between the sexes in the T2DM group (Figure 3.1). Plasma FFA, HSA and HbA1c concentrations and BMI were higher in the

T2DM group than in controls ($p = 0.0011$, $p < 0.0001$, $p < 0.0001$ and $p < 0.0001$ respectively), but there was no difference in total plasma zinc concentration.

Table 3.1. **Demographic information on the individuals in the control and the T2DM groups, as well as measures of plasma total FFA, total zinc, HSA and HbA1c concentrations.**

	Controls		T2DM subjects		Statistical significance
	Mean	**Standard deviation**	**Mean**	**Standard deviation**	**P values**
Age (years)	57.1	8.9	60.9	7.6	0.0757
Sex (% of male)	44	-	87	-	0.0001
Weight (kg)	70.2	12.9	96.2	17.6	<0.0001
BMI (kg/m^2)	25.0	3.2	32.6	5.3	<0.0001
FFA concentration (mM)	1.52	0.30	1.90	0.45	0.0011
HSA concentration (g/L)	41.3	3.5	45.1	2.2	<0.0001
Zinc concentration (µM)	12.4	1.7	12.7	1.4	0.4874
HbA1c concentration (mmol/mol)	37.6	4.2	71.6	22.2	<0.0001

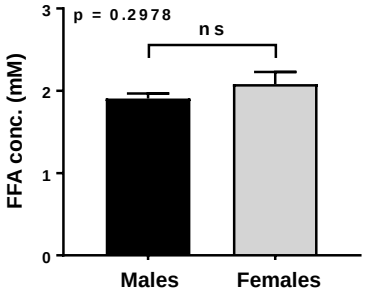


Figure 3.1. Comparison of total plasma FFA concentrations between sexes in plasma from individuals with T2DM.
There was no significant difference in plasma FFA concentration observed between the sexes.

Turbidimetric fibrin clot formation and lysis assays were performed on plasma from both groups, without adding Zn^{2+} and with 20 µM of available Zn^{2+} added. Maximum absorbance and lysis time were significantly increased in the T2DM group compared to the controls, as indicated by two-way ANOVA ($p < 0.0001$ and $p = 0.0448$ respectively), but clot time was unchanged (Figure 3.2). Addition of Zn^{2+} did not induce a significant effect on any of the parameters. When using multiple comparison tests, only maximum absorbance was increased in the T2DM group relative to the control group both in the presence and absence of 20 µM available Zn^{2+}. FFA concentration was also positively correlated with BMI. ($p = 0.042$) and also to maximum absorbance in the absence of Zn^{2+} ($p = 0.03010$, Figure 3.3), but not to either clot time or lysis time in the absence of Zn^{2+}. When comparing maximum absorbance, clot time and lysis time between the sexes, no difference could be observed (Figure 3.4). SEM experiments were then performed on pooled plasma from the T2DM and the control groups to measure the average diameter of fibrin fibres (Figure 3.5). Fibrin fibres were thicker in the T2DM group compared to the controls ($p < 0.0001$). Addition of 20 µM available Zn^{2+} also increased fibre thickness in both groups ($p < 0.0001$).

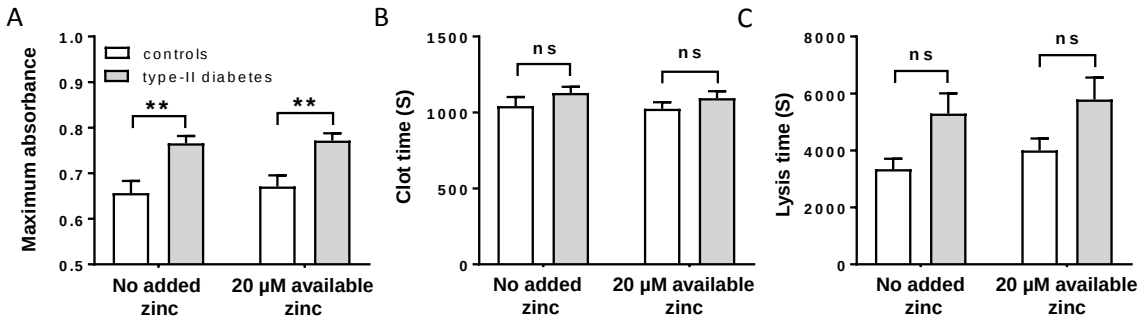


Figure 3.2. Comparison of clot formation and lysis parameters in plasma from individuals with T2DM and from controls.

Two types of experiments were carried out: clot formation only and clot formation and lysis (*n* = 54 for T2DM subjects and 18 for controls). Clot formation experiments were performed in plasma (diluted 3-fold with buffer) and the final concentrations were: 7.5 mM $CaCl_2$, 0.03 U/mL thrombin and either no zinc or 20 µM available Zn^{2+} added in the form of $ZnCl_2$. **A.** Maximum absorbance and **B.** clot time were obtained from these experiments. In clot formation and lysis experiments, plasma was diluted 6-folds in buffer and the final concentrations were: 7.5 mM $CaCl_2$, 0.03 U/mL thrombin, 20.8 ng/mL tPA and either no zinc or 20 µM available Zn^{2+} added in the form of $ZnCl_2$. **C.** Lysis time was obtained from these experiments. Maximum absorbance and lysis time all increased significantly in the T2DM group compared to the controls ($p < 0.0001$ and $p = 0.0448$ respectively), but clot time was unchanged. Addition of Zn^{2+}did not significantly altered any of the parameters. Statistical significance is indicated with ns (not significant) for $p > 0.05$ and ** for $p < 0.01$.

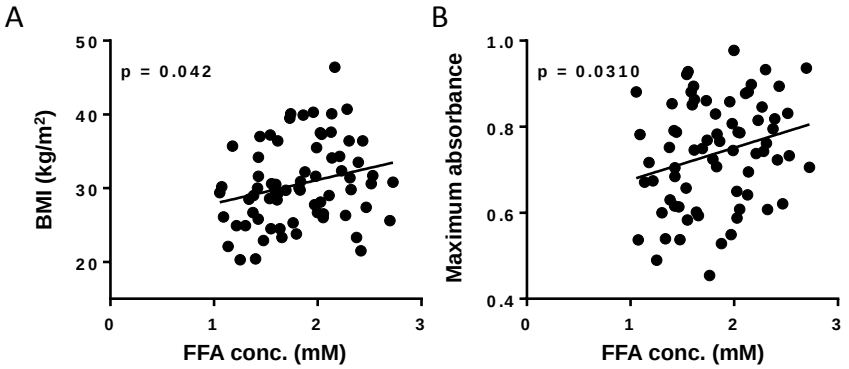


Figure 3.3. Correlation between total plasma FFA concentration and both BMI and maximum clot absorbance.

A positive correlation is observed between **A.** total plasma FFA levels and BMI ($p = 0.0420$) and **B.** total plasma FFA levels and maximum absorbance when no Zn^{2+} was added ($p = 0.03010$).

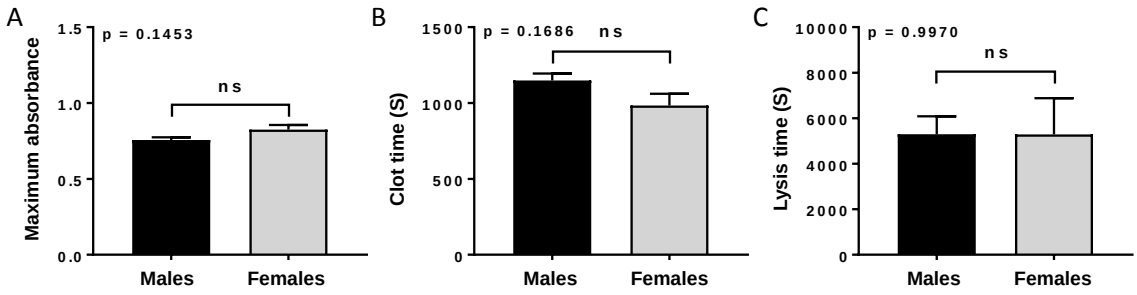


Figure 3.4. Comparison between the sexes of fibrin clot formation and lysis parameters in the T2DM group when no Zn^{2+} was added.

A. Maximum absorbance, **B.** clot time, **C.** lysis time. There was no difference between the two sexes. Statistical significance is indicated with ns (not significant) for $p > 0.05$.

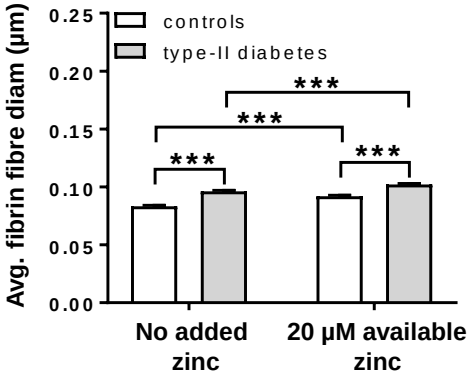


Figure 3.5. Comparison of the diameters of fibrin fibres in plasma from individuals with T2DM and from healthy controls measured with SEM.

Plasma was diluted two-fold in buffer with either no Zn^{2+} added or 20 µM of available Zn^{2+} added as $ZnCl_2$. The clots were formed in duplicate with 45 µL of plasma with Zn^{2+} and 5 µL of 25 mM $CaCl_2$ and 5 U/mL thrombin; 5 images were taken per clot and 50 fibres were measured per images for a final $n = 500$ per sample type. Fibrin fibres were thicker in the T2DM group compared to the controls ($p < 0.0001$). Addition of Zn^{2+} increased fibre diameter in both groups ($p < 0.0001$). Statistical significance is indicated with *** for $p < 0.001$.

The observation that plasma FFA levels are higher in T2DM subjects than in controls has been reported before.[46,161] However, contrary to the results presented here, the total zinc concentration in plasma has been reported to be lower in T2DM subjects.[150] The T2DM group also had a higher concentration of plasma HSA than the control group. This is in agreement with a previous study that found that the HSA concentration in subjects with pre-diabetes increases.[162] This was presumed to relate to the antioxidant properties of the protein (by binding metal cations, those are not free to form free radicals).[162] In subjects who develop diabetes, HSA concentrations have been shown to remain elevated.[162] While Zn^{2+} can cause an increase in fibrin fibre thickness (as shown in Chapter 2), the increase observed here in T2DM subjects may also be due in part to higher fibrinogen concentrations in plasma found in those subjects (average 2.64 ± 0.43 mg/mL in the T2DM subjects, the data was not available in the age-matched-controls for T2DM but was 2.28 ± 0.47 mg/mL in the controls age-matched for T1DM), an effect that has been shown to be caused by insulin increasing fibrinogen production in T2DM.[163] No significant difference in maximum absorbance, clot time and lysis time were found between males and females, however this is contrary to a previous study with a higher number of subjects that found maximum absorbance (following a turbidimetric analysis similar to that performed here) to be higher in females with T2DM than in males.[164]

The elevated FFA levels found in the T2DM cohort are hypothesised to alter plasma Zn^{2+} speciation and result in an elevated thrombotic risk. Another possible explanation for a reduction in Zn^{2+}-binding by HSA is increased non-enzymatic glycation when the protein is exposed to high glucose concentrations, as was shown in a recent study.[165] However, reduced Zn^{2+}-binding by glycated HSA has not yet been confirmed *in vivo* and this hypothesis needs to

be investigated further. Here, a direct correlation has been shown between maximum absorbance and plasma FFA concentrations in T2DM subjects. A previous study by Tanka-Salamon et al. has shown that addition of FFA to plasma increases the maximum absorbance when assessing fibrin clotting using a turbidimetric assay.[140] Thus, part of the increase of maximum absorbance seen here in the absence of any added Zn^{2+} might be caused by the direct effect of FFA alone. However, as shown in Chapter 2, the increase in maximum absorbance caused by the effect of FFA on Zn^{2+}-binding by HSA is more apparent than the effect of FFA alone and is therefore a more major contributor to the change in clot parameters observed here.

Fibrin clot formation and lysis in T1DM

To complement the experiments outlined above, fibrin clot parameters were examined using plasma from individuals with T1DM and controls. Demographic information and FFA, HSA, HbA1c and zinc plasma concentrations are presented in Table 3.2. Those two groups were matched in age and sex. The T1DM group had a higher BMI ($p = 0.0206$), but no difference was observed in total plasma FFA concentrations. Plasma HSA concentrations were lower in the T1DM group ($p = 0.0269$), while HbA1c concentrations were higher in this group ($p = 0.0072$) compared to controls.

Table 3.2. Demographic information on the individuals in the control and T1DM groups, as well as measures of plasma total FFA, total zinc and HSA and HbA1c concentrations.

	Controls		T1DM subjects		Statistical significance
	Mean	**Standard deviation**	**Mean**	**Standard deviation**	**P values**
Age (years)	24.3	6.2	26.3	6.8	0.1436
Sex (% of male)	55	-	58	-	0.8145
Height (m)	1.73	0.09	1.73	0.10	0.7594
BMI (kg/m^2)	23.0	2.9	24.6	3.6	0.0206
FFA concentration (mM)	1.75	0.32	1.77	0.29	0.7866
HSA concentration (g/L)	45.9	2.4	44.7	2.6	0.0269
Zinc concentration (µM)	13.5	2.3	12.8	1.5	0.0894
HbA1c concentration (mmol/mol)	33.5	0.7	66.8	18.0	0.0072

Fibrin clotting was delayed in T1DM, with a lower maximum absorbance and clot time as assessed using two-way ANOVA ($p < 0.0001$ and $p = 0.0002$ respectively, Figure 3.6A and B), while lysis time was delayed in the T1DM group ($p = 0.0412$, Figure 3.6C). Addition of 20 µM available Zn^{2+} did not significantly alter any of the measured parameters. Multiple comparison tests showed that maximum absorbance was lower and clot time was higher in the absence of added Zn^{2+} and in the presence of 20 µM available Zn^{2+}, while lysis time was not significantly different. FFAs concentration was not correlated to any of the parameters. No differences between the sexes were found for maximum absorbance, clot time or lysis time (Figure 3.7). SEM experiments on pooled plasma from the T1DM and the control groups were then performed to measure the average diameter of fibrin fibres (Figure 3.8). In the T1DM group, the diameter of the fibrin was reduced compared to the control group as measured by

two-way ANOVA ($p = 0.0021$). The fibres thickness was not altered in the T1DM group compared to the controls in the absence of Zn^{2+}. Addition of 20 µM available Zn^{2+} increased fibre thickness in both groups ($p < 0.0001$) and fibre thickness was slightly lower in the T1DM group compared to the controls.

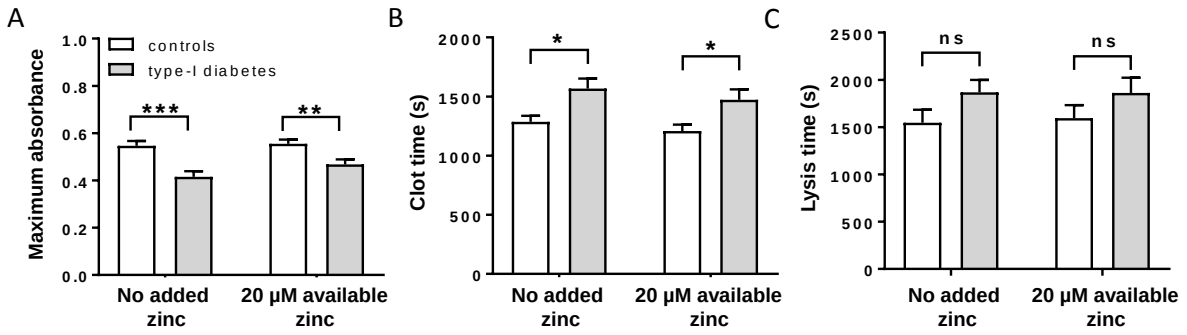


Figure 3.6. Comparison of clot formation and lysis parameters in plasma from individuals with T1DM and from controls.

Two types of experiments were carried out: clot formation only and clot formation and lysis (*n* = 47 for T1DM subjects and *n* = 45 for controls). Clot formation experiments were performed in plasma (diluted 3-fold with buffer) and the final concentrations were: 7.5 mM $CaCl_2$, 0.03 U/mL thrombin and either no zinc or 20 µM available Zn^{2+} added in the form of $ZnCl_2$. **A.** Maximum absorbance and **B.** clot time were obtained from these experiments. In clot formation and lysis experiments, plasma was diluted 6-folds in buffer and the final concentrations were: 7.5 mM $CaCl_2$, 0.03 U/mL thrombin, 20.8 ng/mL tPA and either 0 or 20 µM available Zn^{2+} added as $ZnCl_2$. **C.** Lysis time was obtained from these experiments. Maximum absorbance decreased and clot time and lysis time increased in the T1DM group compared to the controls ($p < 0.0001$, $p = 0.0002$ and $p = 0.0412$ respectively). Addition of Zn^{2+} did not significantly alter any of the parameters. Statistical significance is indicated with ns (not significant) for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

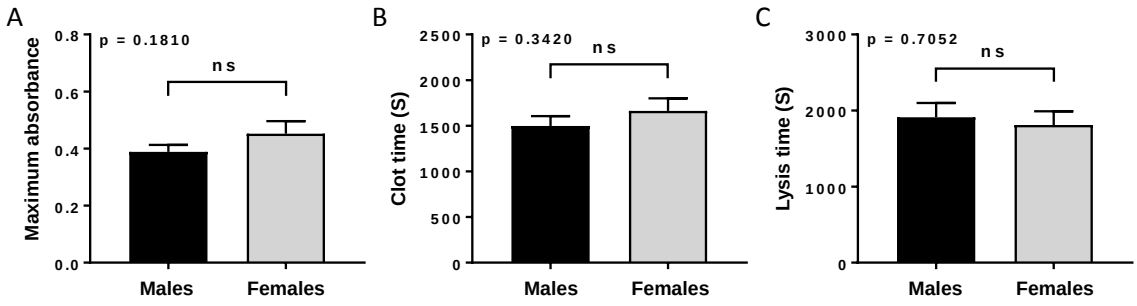


Figure 3.7. Comparison of fibrin clot formation and lysis parameters between the sexes in the T1DM group when no Zn^{2+} was added.

A. Maximum absorbance, **B.** clot time and **C.** lysis time. No statistically significant difference could be seen between the sexes. Statistical significance is indicated with ns (not significant) for $p > 0.05$.

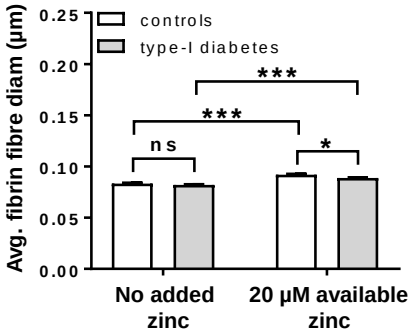


Figure 3.8. Comparison of the diameters of fibrin fibres in plasma from individuals with T1DM and from controls measured with SEM.

Plasma was diluted two-folds in buffer with either no Zn^{2+} added or 20 µM of available Zn^{2+} added as $ZnCl_2$. The clots were formed in duplicate with 45 µL of plasma with Zn^{2+} and 5 µL of 25 mM $CaCl_2$ and 5 U/mL thrombin; 5 images were taken per clot, 50 fibres were measured per images for a final $n = 500$ per sample type. A slight decrease in fibrin fibre thickness was observed in the T1DM group compared to the controls ($p = 0.0021$). Addition of Zn^{2+} increased fibre diameter in both groups ($p < 0.0001$). Statistical significance is indicated with ns (not significant) for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

Contrary to T2DM subjects, the plasma HSA concentration from T1DM subjects is reduced compared to controls, potentially due to insulin deficiency.[166] Insulin deficiency has also been shown to be associated with increased fibrinogen synthesis, probably as an acute-phase response due to metabolic stress, and indeed a slight but not significant increase in plasma fibrinogen concentration (2.39 ± 0.46 mg/mL in T1DM subjects for 2.28 ± 0.47 mg/mL in the controls) was measured.[166] Therefore fibrinogen concentration cannot be used to explain the slightly lower fibrin fibre thickness seen in this group compared to controls. A previous study has shown that no difference in fibrin clot formation and lysis parameters could be seen between males and females (aged 20 years or above) with T1DM.[167] However, the females aged less than 30 years had less permeable fibrin clots and prolonged lysis times than age-matched males.[167] This is not an effect that was observed with the cohort studied here. The fact that no correlation could be seen between FFA concentrations and fibrin clot parameters in T1DM is consistent with the hypothesis that they are at least partially responsible for the alterations of fibrin clot parameters in T2DM.

Alterations of total plasma concentrations of zinc, copper, magnesium and selenium in T1DM and T2DM subjects

Differences in plasma metal concentrations in T1DM or T2DM

In order to investigate whether the total plasma concentrations of zinc, copper, magnesium and selenium and the zinc/copper ratio contribute to the alterations in fibrin clot parameters found in diabetes subjects, these particular metals were measured in the plasma taken from T1DM and T2DM subjects and their respective age-matched controls using inductively coupled plasma-mass spectrometry (Table 3.3). The mean plasma concentrations of zinc, copper and selenium were not different between the T1DM or T2DM groups and their respective controls.

Mean plasma magnesium concentration was lower in the T1DM group compared to its control group but there was no difference between the T2DM group and its control group. The zinc/copper ratio was higher in the T2DM group compared to its control group, but not in the T1DM groups compared to its control group.

Table 3.3. Plasma HbA1c and metal cation concentrations in the T1DM and T2DM groups and their respective controls.
Significance is shown with ns (not significant) for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

Characteristics	T1DM subjects (*n* = 45)	T1DM age-matched controls (*n* = 47)	t-test between T1DM and age-matched controls	T2DM subjects (*n* = 54)	T2DM age-matched controls (*n* = 18)	t-test between T2DM and age-matched controls
HbA1c mean (mmol/mol ± SD)	69.8 ± 18.0	33.5 ± 0.7	**	72.4 ± 22.8	37.6 ± 4.2	***
Zinc mean (µM ± SD)	12.80 ± 1.50	13.50 ± 2.30	ns	12.70 ± 1.40	12.38 ± 1.69	ns
Copper mean (µM ± SD)	17.56 ± 6.60	16.26 ± 5.50	ns	15.49 ± 2.78	17.30 ± 4.70	ns
Magnesium mean (mM ± SD)	0.747 ± 0.057	0.806 ± 0.055	***	0.738 ± 0.075	0.765 ± 0.115	ns
Selenium mean (µM ± SD)	1.147 ± 0.145	1.129 ± 0.136	ns	1.142 ± 0.188	1.222 ± 0.261	ns
Zinc/copper (ratio ± SD)	0.813 ± 0.273	0.886 ± 0.246	ns	0.841 ± 0.152	0.751 ± 0.187	*

In order to check for possible non-linear relationships between incidences of diabetes and plasma metal concentrations, data from each metal concentration and from each subgroup were split into quartiles (defined as being delimited by the 25%, the median and the 75% highest values from each control group). The odds ratios (ORs) of a subject in each quartile having T1DM or T2DM were then calculated (Table 3.4). This did not lead to any significant difference in total plasma zinc concentrations between the T1DM group and its control group,

despite a small study having reported lower zinc concentrations.[168] Similarly, no difference could be seen when examining quartiles of copper concentrations in T1DM which was also contrary to a previous study.[149] Splitting of plasma magnesium concentrations from subjects with T1DM into quartiles showed that the ORs of having diabetes in the second (Q2), third (Q3) and fourth (Q4) magnesium quartile groups were 0.176 (95% confidence interval (CI) 0.058 - 0.628, $p < 0.01$), 0.1173 (95% CI 0.036 - 0.432, $p < 0.01$) and, 0.086 (95% CI 0.028 - 0.325, $p < 0.001$) compared with the first (Q1) quartile group. This is consistent with a previous study which showed that subjects with T1DM have lower plasma magnesium concentrations that control subjects.[169] No difference was observed when examining quartiles of selenium concentrations from subjects with T1DM, while lower selenium concentrations have previously been observed in T1DM subjects.[149] This was also the case when assessing quartiles of plasma zinc/copper ratio in T1DM, despite the lower zinc/copper ratio reported in the literature.[170]

In T2DM, no significant difference was observed when examining quartiles of plasma zinc concentrations, despite a previous meta-analysis (involving 20,183 T2DM subjects) showing lower plasma zinc concentrations in subjects with T2DM and other studies having detected both higher and lower zinc concentrations.[150] The ORs of having T2DM in the Q2, Q3 and Q4 copper groups were 0.031 (95% CI 0.080 - 1.275, $p > 0.05$), 0.193 (95% CI 0.051 - 0.966, $p < 0.05$) and 0.276 (95% CI 0.070 - 1.154, $p > 0.05$) respectively, compared with the Q1 group. The fact that only Q3 is associated with the absence of diabetes is due to the very low copper concentrations in the T2DM group coupled with high concentrations in the control group, the difference almost sufficient to be significant ($p = 0.0698$), despite a meta-analysis (with 20,183 T2DM subjects) having previously shown higher plasma copper concentrations in T2DM.[150]

No difference was seen when examining quartiles of magnesium concentrations in subjects with T2DM, contrary to a meta-analyses (with 286,668 T2DM subjects) that detected lower dietary magnesium levels and to another study (with 12,128 non-diabetic subjects) that showed an inverse relationship between serum magnesium concentrations and incidence of T2DM among white individuals followed over 6 years.[171,172] Assignment of plasma selenium concentrations from subjects with T2DM into quartiles showed that the ORs of having diabetes in the Q2, Q3, Q4 selenium groups were respectively 0.850 (95% CI 0.221 - 3.292, $p > 0.05$), 0.560 (95% CI 0.152 - 2.484, $p > 0.05$) and 0.100 (95% CI 0.017 - 0.682, $p < 0.01$) compared with the Q1 group. The fact that lower selenium concentrations associate with T2DM (as Q4 is associated with the absence of T2DM) is in accord with a previous meta-analysis (involving 13,460 T2DM patients).[151] However, in this study both Q1 and Q4 associated with T2DM.[151] No difference was observed when assessing quartiles of plasma zinc/copper ratio in T2DM, despite the higher mean zinc/copper ratio observed in the T2DM group compared to the controls group and the lower ratio reported in the literature.[154]

Thus, some of the relationships between plasma metal concentrations and T1DM or T2DM reported previously were not detected here. This is probably due to the difference in the numbers of subjects involved and the resultant effect on the statistical power available, allowing studies with large cohorts to detect subtle difference between subjects with T1DM or T2DM and controls. However, such differences may not be as relevant as those detected with smaller cohorts.

Table 3.4. Quartiles of plasma concentrations of zinc, copper, magnesium and selenium and their association with T1DM and T2DM.
Significance is shown as * for p < 0.05, ** for p < 0.01 and *** for p < 0.001.

	T1DM				T2DM			
	Q1	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**	**Q4**
Zinc (µM)	***n*=23**	***n*=23**	***n*=30**	***n*=16**	***n*=15**	***n*=16**	***n*=20**	***n*=20**
	<11.95	**11.95-12.91**	**12.91-14.46**	**≥14.46**	**<11.33**	**11.33-12.15**	**12.15-13.35**	**≥13.35**
% diabetes	52	48	60	25	73	75	75	80
OR (95% CI)	1	0.840 (0.285-2.873)	1.375 (0.457-4.234)	0.306 (0.090-1.204)	1	1.091 (0.264-4.490)	1.091 (0.281-5.312)	1.455 (0.357-5.822)
Copper (µM)	***n*=22**	***n*=17**	***n*=23**	***n*=26**	***n*=33**	***n*=13**	***n*=12**	***n*=12**
	<13.99	**13.99-14.47**	**14.47-16.79**	**≥16.79**	**<15.06**	**15.06-16.40**	**16.40-17.78**	**≥17.78**
% diabetes	50	29	48	64	88	69	58	67
OR (95% CI)	1	0.417 (0.111-1.541)	0.9167 (0.310-2.790)	1.800 (0.559-5.139)	1	0.310 (0.080-1.275)	0.193 (0.051-0.966)*	0.276 (0.070-1.154)
Magnesium (mM)	***n*=41**	***n*=17**	***n*=15**	***n*=19**	***n*=23**	***n*=25**	***n*=13**	***n*=9**
	<0.78	**0.78-0.81**	**0.81-0.83**	**≥0.83**	**<0.72**	**0.72-0.79**	**0.79-0.84**	**≥0.84**
% diabetes	75	35	27	21	83	84	69	44
OR (95% CI)	1	0.176 (0.058-0.628)**	0.117 (0.036-0.432)**	0.086 (0.028-0.325)***	1	1.105 (0.288-4.220)	0.474 (0.118-1.969)	0.168 (0.031-1.064)
Selenium (µM)	***n*=21**	***n*=20**	***n*=19**	***n*=32**	***n*=24**	***n*=21**	***n*=19**	***n*=6**
	<1.04	**1.04-1.12**	**1.12-1.18**	**≥1.18**	**<1.09**	**1.09-1.24**	**1.24-1.42**	**≥1.42**
% diabetes	52	40	37	59	83	80	74	33
OR (95% CI)	1	0.606 (0.172-1.958)	0.530 (0.136-1.784)	1.329 (0.424-4.188)	1	0.850 (0.221-3.292)	0.560 (0.152-2.484)	0.100 (0.017-0.682)*
Zinc/copper ratio	***n*=29**	***n*=23**	***n*=19**	***n*=20**	***n*=9**	***n*=35**	***n*=25**	***n*=27**
	<0.734	**0.734-0.893**	**0.893-1.070**	**≥1.070**	**<0.667**	**0.667-0.732**	**0.732-0.865**	**≥0.865**
% diabetes	62	48	37	45	56	86	80	85
OR (95% CI)	1	0.560 (0.172-1.695)	0.357 (0.104-1.233)	0.500 (0.169-1.660)	1	4.800 (1.110-19.940)	3.200 (0.732-13.560)	4.600 (1.001-22.260)

Influence of sex on plasma metal concentrations in subjects with T1DM or T2DM

Next, potential differences in plasma metal concentrations between males and females were examined (Table 3.5). When looking at the control group age-matched with the T1DM group, females had lower zinc concentrations and higher copper concentrations than males. In the T1DM group, females had lower plasma zinc and magnesium concentrations and higher copper concentrations than males. The number of female subjects in the T2DM group was low, making potential sexual differences harder to study in this group. Nevertheless, a difference in copper concentrations was observed, with lower concentrations in males than in females and so a higher zinc/copper ratio. When comparing the T1DM and T2DM groups with their respective age-matched control groups, lower magnesium concentrations were observed in males with T1DM compared to male controls and in females with T1DM compared to female controls, while males with T2DM but not females had lower magnesium concentrations than their respective controls. In T2DM, the zinc/copper ratio was also higher in males with diabetes than in male controls.

Table 3.5. Sexual differences in mean plasma concentrations of zinc, copper, magnesium and selenium in subjects with T1DM or T2DM.

Significance is shown as ns (not significant) for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

Parameters	Controls age-matched with T1DM			T1DM			Differences between T1DM and age-matched controls	
	Males (*n*=24), mean ± SD	Females (*n*=23), mean ± SD	t-test between male and female controls	Males (*n*=26), *mean* ± *SD*	Females (*n*=19), mean ± SD	t-test between males and females with T1DM	t-test between T1DM males and control males	t-test between T1DM females and control females
Zinc (µM)	14.34 ± 2.72	12.62 ± 1.33	**	13.22 ± 1.54	12.22 ± 1.24	*	ns	ns
Copper (µM)	13.70 ± 1.44	18.81 ± 6.79	***	14.88 ± 3.73	21.23 ± 7.91	***	ns	ns
Magnesium (mM)	0.807 ± 0.056	0.804 ± 0.054	ns	0.763 ± 0.056	0.726 ± 0.052	*	**	***
Selenium (µM)	1.151 ± 0.117	1.106 ± 0.152	ns	1.120 ± 0.143	1.184 ± 0.145	ns	ns	ns
Zinc/copper ratio	1.025 ± 0.139	0.747 ± 0.253	***	0.930 ± 0.220	0.651 ± 0.260	***	ns	ns
Parameters	**Controls age-matched with T2DM**			**T2DM**			**Differences between T2DM and age-matched controls**	
	Males (*n*=9), mean ± SD	**Females (*n*=8), mean ± SD**	**t-test between male and female controls**	**Males (*n*=46), mean ± *SD***	**Females (*n*=7), mean ± SD**	**t-test between males and females with T2DM**	**t-test between T2DM males and control males**	**t-test between T2DM females and control females**
Zinc (µM)	12.33 ± 1.35	12.43 ± 2.11	ns	12.72 ± 1.45	12.32 ± 1.46	ns	ns	ns
Copper (µM)	16.44 ± 1.53	18.27 ± 6.76	ns	15.12 ± 2.28	18.67 ± 1.31	**	ns	ns
Magnesium (mM)	0.812 ± 0.083	0.713 ± 0.128	ns	0.737 ± 0.078	0.757 ± 0.047	ns	*	ns
Selenium (µM)	1.273 ± 0.149	1.165 ± 0.351	ns	1.153 ± 0.179	1.070 ± 0.229	ns	ns	ns
Zinc/copper ratio	0.757 ± 0.108	0.744 ± 0.257	ns	0.865 ± 0.140	0.683 ± 0.141	**	*	ns

All quartiles of plasma metal concentrations calculated previously were then assigned according to sex (Table 3.6). In T1DM, no difference was observed in male or female copper quartiles, in male or female selenium quartiles or in male or female zinc/copper quartiles. The effect of sex on plasma copper concentration in subjects with T1DM has been reported previously, with one study reporting higher copper concentrations in females with T1DM compared to (female) controls but not with males.[149] Another small study (involving only 18 subjects with either T1DM or T2DM) did not report any sexual difference in plasma copper concentrations.[173] A sex difference was observed when analysing quartiles of magnesium concentrations. Indeed, no difference was observed in male magnesium quartiles (despite males with T1DM having a lower mean plasma magnesium concentration than male controls), while the ORs of having T1DM in the Q2, Q3 and Q4 female magnesium quartile groups were 0.000 (95% CI 0 - 0.480, $p < 0.01$), 0.059 (95% CI 0.005 - 0.482, $p < 0.05$) and 0.033 (95% CI 0.003 - 0.287, $p < 0.001$) respectively, compared to the Q1 group. No differences between sexes in plasma magnesium concentrations in subjects with T1DM have previously been reported.

Table 3.6. Sex differences in quartiles of plasma concentrations of zinc, copper, magnesium and selenium and their association with T1DM and T2DM.
Significance is shown as * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

		T1DM				T2DM			
		Q1	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**	**Q4**
Zinc (µM)		**11.95**	**11.95-12.91**	**12.91-14.46**	**≥14.46**	**<11.33**	**11.33-12.15**	**12.15-13.35**	**≥13.35**
Males	*n*	10	9	17	14	11	11	16	16
	OR (95% CI)	1	0.533 (0.078-2.843)	1.600 (0.370-7.145)	0.267 (0.047-1.600)	1	2.222 (0.221-35.010)	0.667 (0.110-3.837)	3.333 (0.334-51.000)
Females	*n*	13	14	13	2	3	5	4	4
	OR (95% CI)	1	1.167 (0.261-5.488)	1.000 (0.203-4.927)	0.000 (0.000-3.184)	1	1.333 (0.091-28.020)	4.000 (0.175-83.060)	0.667 (0.027-18.510)
Copper (µM)		**<13.99**	**13.99-14.47**	**14.47-16.79**	**≥16.79**	**<15.06**	**15.06-16.40**	**16.40-17.78**	**≥17.78**
Males	*n*	18	14	11	6	28	11	9	6
	OR (95% CI)	1	0.320 (0.088-1.558)	1.400 (0.315-5.436)	4.000 (0.514-52.23)	1	0.167 (0.011-1.649)	0.074 (0.006-0.625)*	0.074 (0.005-0.842)
Females	*n*	4	3	12	23	5	2	3	6
	OR (95% CI)	1	1.500 (0.054-37.030)	1.500 (0.165-23.840)	3.900 (0.490-53.940)	1	0.000 (0.000-5.724)	0.750 (0.036-11.030)	3.000 (0.321-26.480)
Magnesium (mM)		**<0.78**	**0.78-0.81**	**0.81-0.83**	**≥0.83**	**<0.72**	**0.72-0.79**	**0.79-0.84**	**≥0.84**
Males	*n*	19	13	9	9	19	18	9	8
	OR (95% CI)	1	0.306 (0.074-1.226)	0.179 (0.040 to 1.033)	0.179 (0.040-1.033)	1	0.944 (0.047-18.900)	0.194 (0.013-2.000)	0.056 (0.004-0.582)*
Females	*n*	22	4	6	10	4	7	4	1
	OR (95% CI)	1	0.000 (0.000-0.480)**	0.059 (0.005-0.482)*	0.033 (0.003-0.287)***	1	4.000 (0.359-63.030)	3.000 (0.194-56.040)	0.000 (0.000-36.000)
Selenium (µM)		**<1.04**	**1.04-1.12**	**1.12-1.18**	**≥1.18**	**<1.09**	**1.09-1.24**	**1.24-1.42**	**≥1.42**
Males	*n*	12	10	12	16	17	16	18	3
	OR (95% CI)	1	0.750 (0.144-3.807)	0.250 (0.045-1.383)	0.500 (0.129-2.201)	1	0.938 (0.047-18.890)	0.2188 (0.017-1.703)	0.031 (0.002-0.601)*
Females	*n*	9	10	7	16	7	5	1	3
	OR (95% CI)	1	0.500 (0.074-3.211)	1.500 (0.240-9.071)	4.400 (0.722-20.100)	1	0.500 (0.061-4.077)	0.000 (0.000-9.000)	0.375 (0.021-4.924)
Zinc/copper		**<0.734**	**0.734-0.893**	**0.893-1.070**	**≥1.070**	**<0.667**	**0.667-0.732**	**0.732-0.865**	**≥0.865**
Males	*n*	4	14	14	17	4	7	21	24
	OR (95% CI)	1	0.000 (0.000-2.397)	0.000 (0.000-1.316)	0.000 (0.000-1.408)	1	1.333 (0.138-12.360)	6.000 (0.662-45.600)	11.000 (1.069-89.650)
Females	*n*	25	9	5	3	5	3	4	3
	OR (95% CI)	1	0.393 (0.094-1.827)	0.196 (0.015-1.616)	0.393 (0.026-3.839)	1	0.333 (0.018-5.167)	0.667 (0.062-7.580)	0.333 (0.018-5.166)

In the T2DM cohort, no statistically significant differences were detected when assessing quartiles of metal concentrations among female subjects and controls. This may be explained by the low number of females in this cohort. Among males, examining quartiles of zinc concentrations and zinc/copper ratio also did not reveal any significant differences. The ORs of males having T2DM in the Q2, Q3 and Q4 copper quartile groups were 0.167 (95% CI 0.011 - 1.649, $p > 0.05$), 0.074 (95% CI 0.006 - 0.625, $p < 0.05$) and 0.074 (95% CI 0.005 - 0.842, $p > 0.05$) respectively, compared to the Q1 group. This is different from a meta-analysis that did not observe any sex difference in plasma copper concentrations in subjects with T2DM.[150] The ORs of males having T2DM in the Q2, Q3 and Q4 magnesium quartile groups were 0.944 (95% CI 0.047 - 18.900, $p > 0.05$), 0.194 (95% CI 0.013 - 2.000, $p > 0.05$) and 0.056 (95% CI 0.004 - 0.582, $p < 0.05$) respectively compared to the Q1 group. Contrary to this, it has been reported before that females with T2DM were more likely than males with T2DM to have lower plasma concentrations of magnesium.[172] The ORs of males having T2DM in the Q2, Q3 and Q4 selenium quartile groups for selenium were 0.938 (95% CI 0.047 - 18.890, $p > 0.05$), 0.219 (95% CI 0.017 - 1.703, $p > 0.05$), 0.031 (95% CI 0.002 - 0.601, $p < 0.05$) respectively, compared to the Q1 group. A correlation has been previously observed between occurrences of T2DM and a subject having too high or too low plasma selenium concentrations in males but not females.[174-176]

Association between HbA1c concentration and plasma metal concentration

Next, potential associations between the plasma concentrations of zinc, copper, magnesium and selenium and the concentration of HbA1c in subjects with T1DM (Figure 3.9) or T2DM (Figure 3.10). Such associations may be due to the dysregulation of metal homeostasis through

changes in insulin,[177,178] to modifications of the proteins transporting metal ions through glycation or oxidation,[165] or to the formation of glycocholates (the biliary anions created from the conjugation of glycine with cholic acid) that would form complexes with metals and cause their excretion in the bile.[154] In T1DM, no correlation was found between HbA1c concentration and plasma zinc concentration, which is in agreement with another study.[179] Plasma copper concentration did not correlate with HbA1c concentration in subjects with T1DM when looking at both sexes together (which is in accord with a previous study)[149] or in females only, but they positively correlated in males ($p = 0.042$, Figure 3.9E). In T1DM, plasma magnesium concentration negatively correlated with HbA1c concentration when looking at males and females together ($p = 0.004$, Figure 3.9G) and in males only ($p = 0.007$, Figure 3.9H), however it did not correlate in females despite the mean magnesium concentration being lower in females with T1DM than in males with T1DM. Nevertheless, this is in agreement with previous studies that found a negative correlation with magnesium excretion with females with T1DM but not in males.[169,180] In subjects with T1DM, plasma selenium concentration was negatively correlated with HbA1c concentration when looking at males and females together ($p = 0.031$, Figure 3.9J), which is in agreement with a previous study,[149] but it was not correlated in males or females only. The plasma zinc/copper ratio negatively correlated with HbA1c concentration in subjects with T1DM when looking at males and females together ($p = 0.0258$, Figure 3.9M), which again is consistent with a previous report,[170] but not when looking at males or females individually.

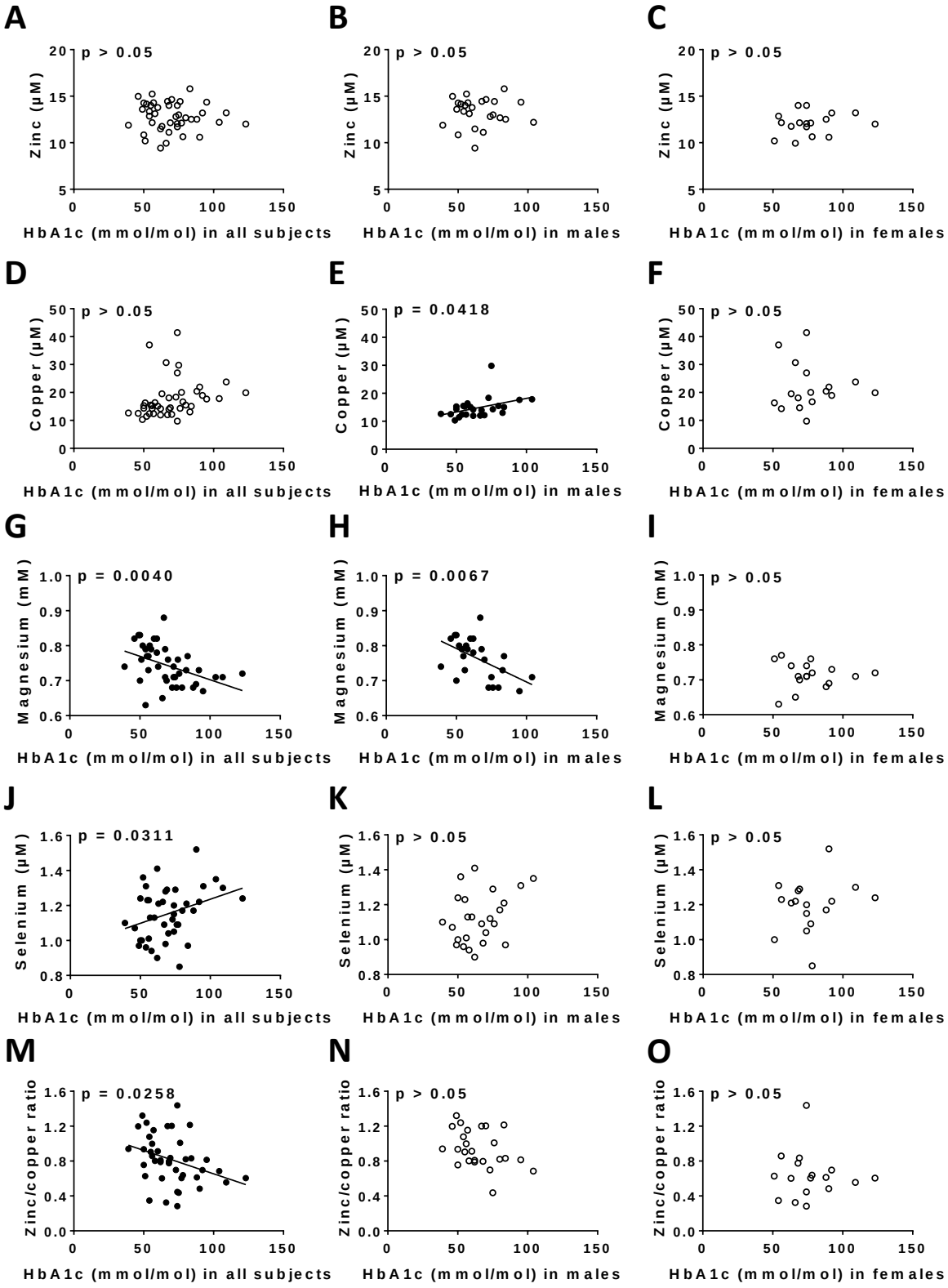


Figure 3.9. Association between plasma HbA1c concentration and plasma zinc, copper, magnesium and selenium concentrations or zinc/copper ratio in males and females with T1DM.

Relationships between HbA1c concentration and **A-C.** zinc concentration, **D-F.** copper concentration, **G-I.** magnesium concentration, **J-L.** selenium concentration and **M-O.**

zinc/copper ratio, in **A,D,G,J,M.** males and females, **B,E,H,K,N.** males or **C,F,I,L,O.** females. White circles represent data that did not correlate with HbA1c concentration while black circles represent significant correlations. Positive correlations were found between HbA1c concentration and plasma selenium concentration ($p = 0.0311$) in males and females together, and between Hba1c concentration and plasma copper concentration in males ($p = 0.0418$). Negative correlations were found between HbA1c concentration and the zinc/copper ratio in males and females together ($p = 0.0258$), and between HbA1c concentration and plasma magnesium concentration in males and females together ($p = 0.0040$) and in males ($p = 0.0067$).

In T2DM, no correlation was found between plasma zinc concentration and HbA1c concentration, which is contrary to a previous study that observed a negative correlation between the two.[181] Plasma copper concentration positively correlated with HbA1c concentration in subjects with T2DM when looking at males and females together ($p = 0.003$, Figure 3.10D), which is in accord with a previous study.[154] However, there was no correlation when looking at males and females separately. No correlation was found in T2DM between plasma magnesium concentration and HbA1c concentration, while a negative correlation has been found previously.[152] Plasma selenium concentration positively correlated with HbA1c when looking at males and females together ($p = 0.039$, Figure 3.10J) and in females alone ($p = 0.015$, Figure 3.10L) but not in males. A previous publication had observed a negative correlation between selenium concentration and dysregulation of glucose in males but not females.[176] The difference can be explained by the non-linear relationship between plasma selenium concentration and T2DM reported before.[151] The zinc/copper ratio in plasma was correlated when looking at males and females with T2DM together ($p = 0.0428$, Figure 3.10M) as shown in a previous publication,[154] but not when looking at males or females separately.

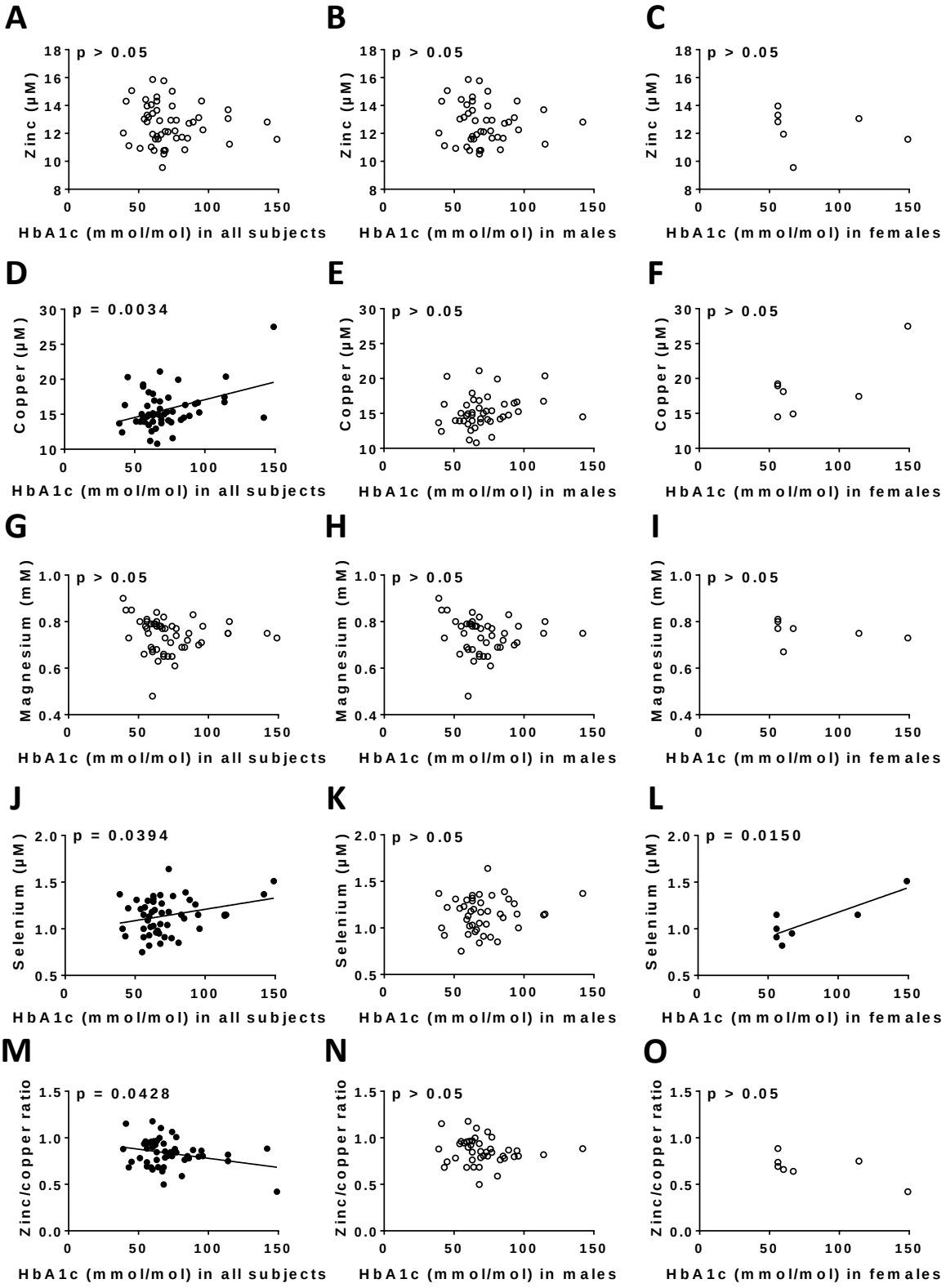


Figure 3.10. Association between plasma HbA1c concentration and plasma zinc, copper, magnesium and selenium concentrations or zinc/copper ratio in males and females with T2DM.

Relationships between HbA1c concentration and **A-C.** zinc concentration, **D-F.** copper concentration, **G-I.** magnesium concentration, **J-L.** selenium concentration and **M-O.**

zinc/copper ratio, in **A,D,G,J,M.** males and females, **B,E,H,K,N.** males or **C,F,I,L,O.** females. White circles represent data that did not correlate with HbA1c concentration while black circles represent significant correlations. Positive correlations were found between HbA1c concentration and plasma selenium concentration in males and females together ($p = 0.0394$) and in females ($p = 0.0150$), and between Hba1c concentration and plasma copper concentration in males and females together ($p = 0.0034$). A negative correlation was found between HbA1c concentration and the zinc/copper ratio in males and females together ($p = 0.0428$).

Causes and consequences of the dysregulation of metal handling in T1DM an T2DM

As described above, changes in plasma glucose concentration can influence metal homeostasis such as to impact upon plasma metal concentrations. In addition, the plasma metal concentrations can themselves further affect metabolism and glucose handling. Indeed, zinc for example is essential for the synthesis, storage and release of insulin: in the pancreatic β-cell, zinc plays a role in the crystallisation and thus the storage of insulin, causing both zinc and insulin to be released together from β-cells.[182,183] Thus, a low concentration of zinc can potentially results in a perturbation of insulin storage and a reduced secretion of insulin.[184] As zinc also participates in the stimulation of lipogenesis and glucose uptake and in the reduction of lipolysis in adipocytes, a deficiency in zinc would also affect those processes.[185,186]

The homeostasis of zinc and copper are closely associated. Thus, it is well established that a deficiency in zinc or copper can provoke an over-absorption of the other metal, while over-supplementation in one would lead to a deficiency in the other.[187,188] Zinc and copper are both necessary for the antioxidant enzyme superoxide dismutase to be active, thus a deficiency in either zinc or copper will affect the zinc/copper ratio and increase sensitivity to oxidative damage.[189] This explains why T2DM is associated with a reduction in superoxide dismutase activity and why an abnormal zinc/copper ratio is linked to the development of diabetes and

associated complications.[189] Furthermore, an elevated plasma glucose concentrations results in the glycation of superoxide dismutase and its fragmentation, causing the release of copper from the protein and, as copper is pro-oxidant, potentially further increasing the formation of reactive oxygen species.[190] This is confirmed by the fact that their formation correlates with the higher copper concentrations found in T2DM.[191] Finally, insulin participate in the control of copper concentrations in the liver as it regulates ATP7B, an ATPase transporting copper.[192] Lower insulin concentrations could lead to copper accumulation in the liver.[192]

Mg^{2+} homeostasis is also closely linked to glucose and insulin concentrations. Physiologically, binding of Mg^{2+} to the insulin receptor increases its sensitivity to ATP (by increasing the binding affinity) and is essential for insulin receptor autophosphorylation and tyrosine kinase activity.[178,193,194] Thus, Mg^{2+} increases cell sensitivity to insulin signals, while chronically low concentrations of Mg^{2+} result in insulin resistance.[178,193,194] In addition, Mg^{2+} plays a role in insulin release, glucose transport across the cell membrane and carbohydrate oxidation as a cofactor for enzymes such as phosphotransferases and phosphohydrolases (e.g. ATPases).[194,195] Moreover, Mg^{2+} regulates Ca^{2+} entry into adipocyte by blocking their passage through L-type calcium channels. Thus, low Mg^{2+} concentrations lead to an increase of Ca^{2+} in adipocytes that can cause inflammation and oxidation, resulting in an elevated risk of developing insulin resistance.[178,194,196] Insulin itself also affects Mg^{2+} concentration by reducing tubular reabsorption of Mg^{2+}. Thus, elevated insulin concentrations such as those seen in T2DM can lead to a reduction in plasma Mg^{2+} concentration,[177,178] resulting in a higher risk of developing T1DM or T2DM (and poor glycaemic control).[152,180]

Plasma selenium is also affected by plasma glucose/insulin concentrations. Selenium is present in the amino acid selenomethionine, which is found in several proteins, the selenoproteins. Several of those proteins are important antioxidants and are therefore upregulated during inflammation and oxidative stress, thus increasing the total selenium content of plasma.[197] This is the case of selenoprotein P, which is present in higher concentrations in the plasma of individuals with T2DM (with diabetes itself associated with high levels of oxidative stress), as well as being linked with insulin resistance.[198,199] On the other hand, during inflammation expression of selenoprotein P is down-regulated,[200,201] while expression of the selenoprotein plasma glutathione peroxidase and of HSA, the transporter of selenomethionine in plasma, are both lower in individuals with diabetes.[202] This results in a decrease in total plasma selenium concentration during systemic inflammation responses, with which diabetes is also associated.[203] Whether individuals with T2DM have an early stage of impaired glucose tolerance or a well-established T2DM has been suggested to explain how both high and low plasma selenium concentrations can be associated with the disease through variations of oxidative stress level.[204]

As low plasma concentrations of zinc, magnesium and selenium have been linked with elevated incidences of complications associated with diabetes, potential beneficial effect of administering zinc, magnesium and selenium supplements to individuals with diabetes or at risk of developing the disease has been investigated. While selenium supplementation did not have any beneficial effect (likely because selenium concentration does not have a linear relationship with the incidence of diabetes and elevated selenium concentrations may increase the risk of developing T2DM),[199] both zinc and magnesium supplements reduced the risk of developing T2DM and improved diabetes management.[205-210]

This study is the first to assess plasma zinc, copper, magnesium and selenium concentrations in both T1DM, T2DM and controls, as well as their association with diabetes management (through HbA1c concentrations) and sex-specific differences. Uncontrolled concentrations of zinc/copper, magnesium and selenium affect energy metabolism and the regulation of insulin concentrations and are associated with the development of diabetic complications. Abnormal glucose and insulin concentrations then further affect the regulation of the concentrations of those metals. This work has shown that, in T1DM, it is plasma magnesium concentration that is the most affected, while in T2DM both plasma copper and selenium concentrations are altered.

Relationship between total plasma concentrations of zinc, copper, magnesium and selenium and clot formation and lysis parameters in T1DM and T2DM subjects.

An analysis was carried out to examine whether there were any relationships between total plasma concentrations of either zinc, copper, magnesium or selenium with fibrin clot formation and lysis parameters in T1DM and T2DM subjects. In T2DM subjects, the only relationship found was a negative correlation between the plasma zinc/copper ratio and lysis time ($p = 0.0468$, Figure 3.11). In T1DM subjects, positive correlations were found between the total plasma copper concentration and maximum absorbance ($p = 0.0005$, Figure 3.12A) and total plasma magnesium concentration and maximum absorbance ($p = 0.0459$, Figure 3.12C). A negative correlation was observed between plasma zinc/copper ratio and maximum absorbance ($p = 0.0043$, Figure 3.12B) and between total plasma magnesium concentration and lysis time ($p = 0.384$, Figure 3.12D).

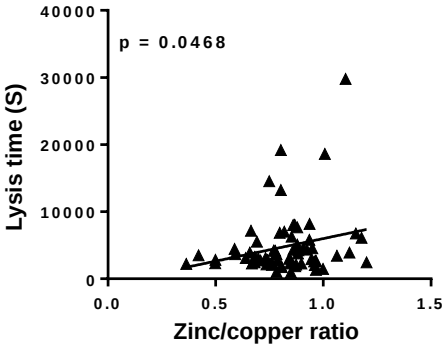


Figure 3.11. Correlation between the plasma zinc/copper ratio and lysis time in T2DM subjects when no Zn^{2+} was added.
A negative correlation between zinc/copper ratio in plasma and maximum absorbance was observed (p = 0.0468).

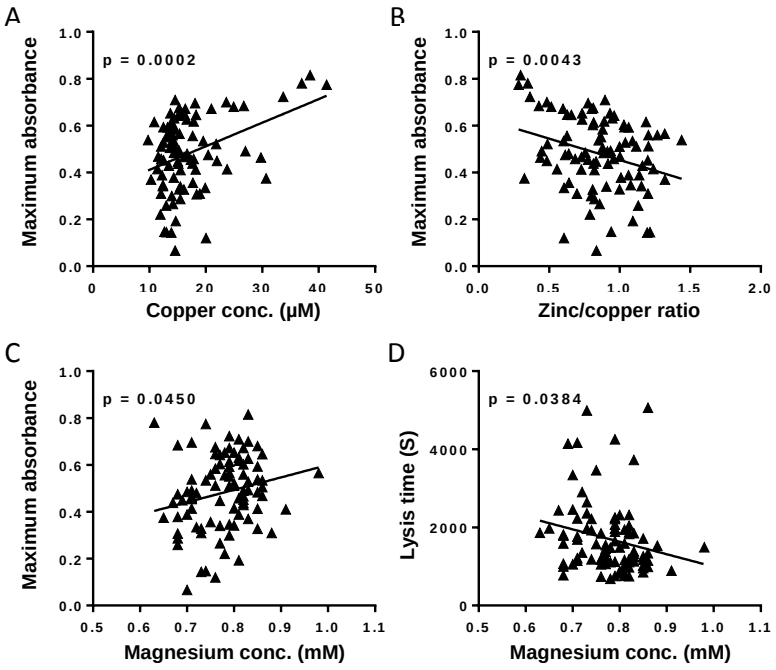


Figure 3.12. Correlation between total plasma concentrations of micronutrients and fibrin clot formation and lysis parameters in T1DM subjects when no Zn^{2+} was added.
A. Positive correlation between total plasma copper concentration and maximum absorbance (p = 0.0005). **B.** Negative correlation between zinc/copper ratio in plasma and maximum absorbance (p = 0.0043). **C.** Positive correlation between total plasma magnesium concentrations and maximum absorbance (p = 0.0459). **D.** Negative correlation between total plasma magnesium concentrations and lysis time (p = 0.384).

The correlation with magnesium in T1DM can potentially be explained by a potentiation of the coagulatory cascade. It is known that Mg^{2+} accelerates the activation of factor X by activated factor IX (in the presence of activated factor VIII, phospholipids and Ca^{2+}) as well as the activation of factor IX by the activated factor VIIa-tissue factor complex.[157] Mg^{2+} also potentiates the inactivation of activated factor V by activated protein C.[211] Furthermore, addition of Mg^{2+} in plasma from healthy subjects has been shown to increase clot time when clotting was induced by addition of Ca^{2+} or thrombin, but to reduce clot time when clotting was induced by tissue factor.[212-214] Also, increasing Mg^{2+} concentrations have been shown to reduce fibrin lysis time due to its inhibition of plasminogen activator inhibitor-I in the presence of thrombin and vitronectin (thus reducing tPA inhibition and decreasing lysis time).[214] This could explain why low Mg^{2+} concentrations are correlated with increased lysis time. Those effects of Mg^{2+} are consistent with the observation that total plasma magnesium concentrations were the most altered among those measured in the T1DM cohort and this could at least partially explain the low maximum absorbance and elevated lysis time found in those subjects (as during those experiments clotting was induced by addition of Ca^{2+} and thrombin).

The potential effect of copper on coagulation is not well known, despite Cu^{+} and Cu^{2+} being essential co-factors of several coagulation proteins (e.g. coagulation factors V and VIII).[155,156] Elevated dietary levels of copper in rats (which were reflected in copper concentrations in the liver) were found not to affect clot time when clotting was induced by thromboplastin and Ca^{2+} (when assaying for factor X, thrombin and fibrinogen activation) or Ca^{2+} and phospholipids (testing the whole extrinsic pathway of coagulation).[215] However, high blood concentrations of ceruloplasmin, a copper-binding plasma protein, has been shown to bind to activated protein

C, which could reduce the anticoagulant activity of activated protein C and induce acquired activated protein C resistance, a state associated with a higher risk for venous thrombosis.[216]

Thus, elevated plasma FFA concentrations have been shown to be correlated with increased clot density (maximum clot absorbance) in T2DM subjects, causing an alteration in plasma zinc speciation (rather than an increase in total plasma zinc) and at least partially explaining the elevated thrombotic risk associated with T2DM. It would be interesting to investigate whether a similar effect can be observed in other disease states associated with both elevated FFA levels and a higher thrombotic risk. In T1DM subjects, total plasma concentrations of magnesium and copper have been found to be positively correlated with clot density. However, as magnesium concentrations are significantly lower in T1DM subjects and copper concentrations are not significantly different, this results in lower clot density overall in those subjects. Total plasma concentrations of magnesium are also negatively correlated with lysis time, explaining the increase in lysis time observed in T1DM subjects. As T1DM is also associated with elevated thrombotic risks, it appears that this effect is exerted through an increase in the lysis time of the fibrin clot rather than an increase in density. Magnesium supplementation in individuals with T1DM has been shown to decrease the risk of developing complications.[217] The results presented here show that this beneficial effect could probably extend to reducing thrombotic risk.

Chapter 4: Characterisation of heparin binding to HRG and fibrinogen

Introduction

At the initiation of coagulation, anticoagulant GAGs need to be neutralised so that thrombin, FXa and the other proteins of the coagulation cascade can be activated to allow a fibrin clot to be formed (Figure 4.1).[58] Activated platelets release several proteins, including HRG, fibrinogen and fibronectin, alongside Zn^{2+} and Ca^{2+} ions. HRG, fibrinogen and fibronectin can all bind anticoagulant GAGs and thus prevent their interactions with AT and HCII.[26] The interactions of HRG and fibrinogen with GAGs are both enhanced by the co-binding of Zn^{2+}. Given that fibronectin can also bind Zn^{2+}, there is a possibility for its interaction with GAGs to also be affected by it but this is yet to be demonstrated experimentally.[26] Negatively-charged GAGs interact with protein though binding to basic (positively-charged) amino acids, mainly lysines and arginines.[106] Zn^{2+} does not co-ordinate to those amino acids, but by binding to histidines exposed on the surface of the proteins (HRG, fibrinogen and fibronectin), it reduces the degree of negative charges at their surface and in-turn the electrostatic repulsion between the negatively-charged GAGs and the protein.[106] Zn^{2+} ions could then also serve as a potential additional ligand and thus also facilitate binding.[106]

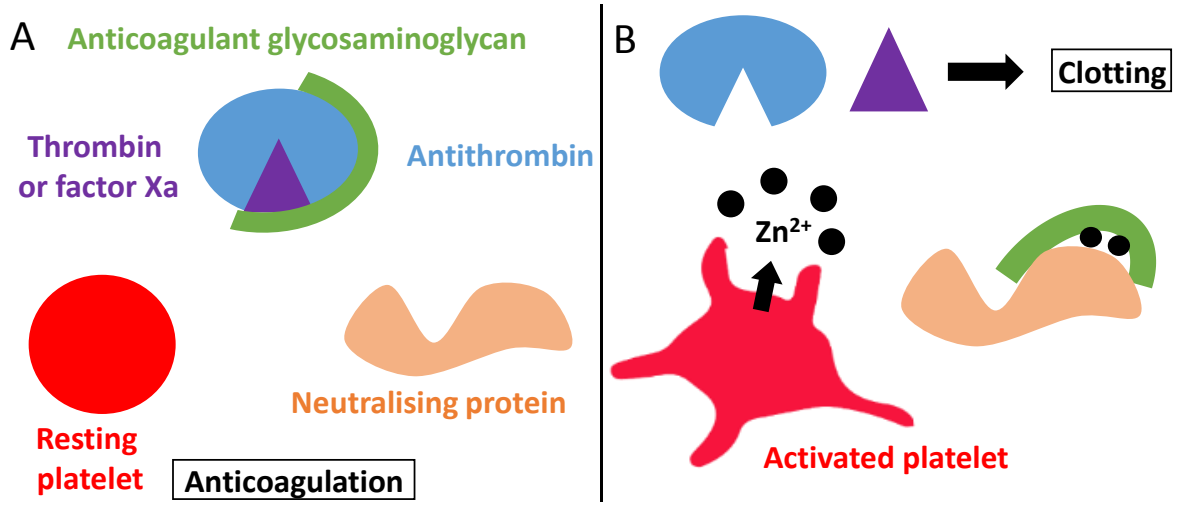


Figure 4.1. The neutralisation of anticoagulant GAG allows fibrin clotting to occur.[26] Proteins that neutralise GAGs such as HRG, fibrinogen and fibronectin are necessary in order to prevent the inhibition of thrombin and FXa so that fibrin clotting can occur. HRG and fibrinogen binding to GAGs is known to increase in the presence of Zn^{2+}, which is released by platelets.

Histidine-rich glycoprotein (HRG)

HRG is a single-chain 75 kDa protein composed of several domains: two N-terminal domains forming the N1/N2 region, a first proline rich region PRR1, a histidine rich region HRR, a second proline rich region PRR2 and a C-terminal domain C (Figure 4.2).[218] The domains are linked together by several disulfide bridges. HRG can bind Zn^{2+} in the HRR as Zn^{2+} binds to histidine residues.[84,219] HRG has been shown to bind up to 10 Zn^{2+} ions with an average K_d of 6.13 µM.[40,84] The exact structure of the HRR is not yet known but current evidence suggests that there is no clearly defined preferential binding site for Zn^{2+}.[220] The overall charge of HRG can become positive by binding to metal ions such as Zn^{2+} or through a change in pH (as happens during hypoxia). When this occurs, the conformation of the protein is thought to change such as to influence the binding of HRG to its ligands.[83,84,220-224]

HRG can bind GAGs through two modes of binding; one dependent on Zn^{2+}-binding and the other independent of Zn^{2+} binding.[40] The exact locations of the binding sites are unknown, but the Zn^{2+}-independent binding is thought to occur in the N1/N2 region, while Zn^{2+}-dependent binding has been suggested to occur in the HRR. In the latter case Zn^{2+} could serve to potentially crosslink the protein to heparin by co-ordinating to both.[83,225] However, synthetic peptides based on sequences found within the HRR are unable to bind to HS on the cell surface.[226] Thus, binding of Zn^{2+} ions to the HRR may trigger a change in HRG conformation that increases the affinity of the N1/N2 region for heparin. This is supported by the fact that the affinity of the whole protein for heparin increase in the presence of Zn^{2+}, but not that of a peptide corresponding to the N1/N2 domain.[226] Without Zn^{2+}, HRG binds UFH with a K_d of 32.9 nM and the affinity of this binding is not affected by changes in heparin chain length.[40] In this case, HRG and heparin form 1:1 complexes.[225] In the presence of 1 μM Zn^{2+}, the K_d of the binding of long chain heparin molecules (≥10 kDa) to HRG decreases to 5.1 nM.[40,225] Zn^{2+} does not affect the affinity of shorter-chain heparins to HRG.[40,225] In the presence of 1 μM Zn^{2+}, HRG and long-chain heparins can form 1:2 complexes.[225] Thus, HRG is an important anticoagulant GAG neutraliser in plasma and this neutralisation is dependent upon the plasma Zn^{2+} concentration.

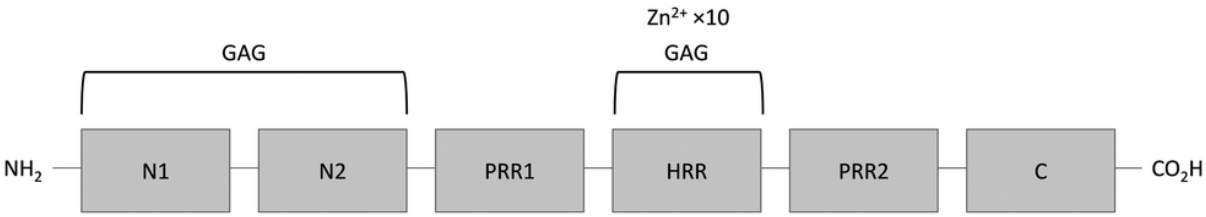


Figure 4.2. Schematic structure of HRG.[26]
The protein is composed of the N-terminal region N1/N2, a first proline rich region PRR1, a histidine rich region HRR, a second proline rich region PRR2 and a C-terminal domain. The N1/N2 region can bind GAGs while the HRR is thought to bind both GAG and up to 10 Zn^{2+} ions.

Fibrinogen

Fibrinogen is a homodimeric protein of 340 kDa. Each monomer is composed of three different chains, Aα, Bβ and γ.[227] The N-termini of all six chains form a central globular domain, the E domain (Figure 4.3). Progressing along the protein chains, each dimer separates into two coil-coil regions, then two globular regions, known as the D domains, where the C-termini of the β and γ chains are located. The α chains are longer and possess an additional region that extends from the D domains to the E domains to form two small globular domains, the αC regions. The αC regions bind to the central E domain. The structure of fibrinogen has been almost solved in its entirety (PDB: 3GHG) with the exception of the αC region which is too highly variable in structure to allow crystallisation.[227] Fibrinogen has two sets of binding sites for Zn^{2+}; the binding of Zn^{2+} to the protein has an overall K_d of 1-18 μM, but the individual contributions of each set of sites are unknown.[12,100,228,229] One set is located in the D domains where six ions (three per D domain) can bind.[228] The amino acid residues involved in these sites are mainly histidines located on the γ chain, potentially including His-γ217 and His-γ234 (Figure 4.3 insert 1).[228] It is not known if binding of Zn^{2+} at this location induces any conformational change in the protein. The other set of sites is located in the αC regions and residues His-α544 and His-α545 are known to be involved.[100] The stoichiometry of the binding to this region is not clear, but it is probable that it binds either one or two Zn^{2+} ions per αC region.[100] Binding of Zn^{2+} there is thought to induce a conformational change in the protein.[100]

Fibrinogen, like HRG, can bind heparin through two different binding modes; one dependent on Zn^{2+}-binding and the other independent of Zn^{2+}-binding. The Zn^{2+}-independent binding involves binding at the E domain and involves the residues Bβ1-57 (Figure 4.3, insert 2). A peptide of this region binds heparin with a K_d of 16.5 μM.[230] A dimer of this peptide, (Bβ1-

66)$_2$ binds with a K_d of 210 nM, which is an affinity almost two orders of magnitude higher and is comparable to the K_d of the whole protein (228 nM), potentially highlighting the importance of the dimeric structure of fibrinogen for heparin binding.[230] The global K_d of UFH binding to fibrinogen decreases to 60 nM in the presence of 12.5 µM Zn^{2+}.[100] This represents a 4-fold increase in affinity. This has been shown to occur through binding of Zn^{2+} to the αC regions. A synthetic peptide corresponding to the αC region has been shown to bind heparin in a Zn^{2+}-dependent manner.[100] Alternatively, Zn^{2+} binding to αC could potentially cause a conformational change in the nearby heparin binding site of the E domain, increasing its affinity for heparin. Note that fibrinogen that lacks the αC region has an average K_d for UFH of 539 nM.[100] Whether Zn^{2+}-binding affects the binding of fibrinogen to heparins of all chain lengths or if it only affects binding to high molecular weight heparins is not yet known. It is also unclear whether Zn^{2+} and heparin preferentially bind HRG or fibrinogen.

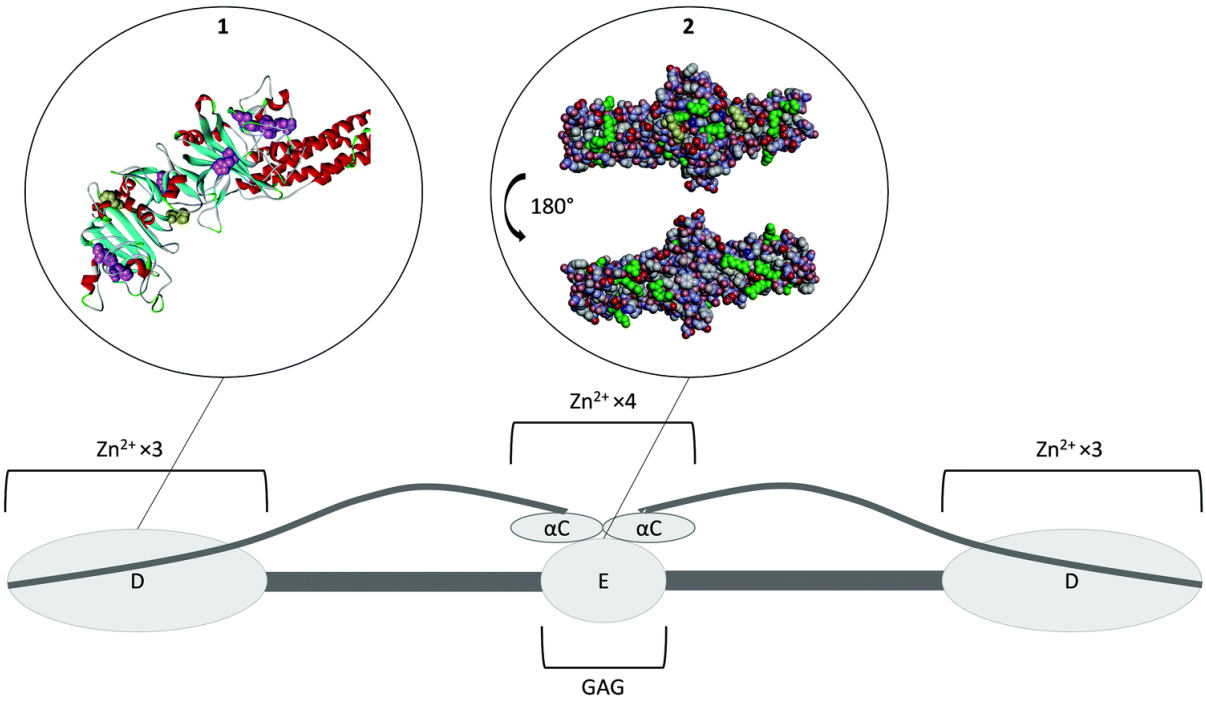


Figure 4.3. Schematic structure of fibrinogen.[26]
The protein is a hexamer formed from three different strands: $(A\alpha\ B\beta\ \gamma)_2$. The N-termini of the six chains are located in the E domain, which is a heparin binding domain. Outside of the E domain, each version of the three strands coil together. At the opposite ends of the coils, they reach two globular domains, the D domains (Zn^{2+}-binding domains), where the C-termini of the β and the γ strands are located. The α chains are longer and possess an additional region that extends from the D domain to the E domain to form two other small globular domains, the αC regions, that bind to the central E domain. **Insert 1.** The crystal structure of the D domain of fibrinogen (which is one of the Zn^{2+}-binding domains) and part of the coil–coil region of the protein (PDB: 3GHG).[227] Zn^{2+} binding mostly occurs through histidines. Residues His217 and His234, which are known to be involved in Zn^{2+}-binding, are represented in yellow. Other histidine residues (which also have the potential to be involved in Zn^{2+}-binding) are represented in pink. Most of those histidines are located beneath the surface of the protein. **Insert 2.** The crystal structure of the E domain of fibrinogen and part of the two coil-coil regions of the protein (PDB structure 3GHG).[227] The positive and negative charges are represented in blue and red respectively. GAGs usually bind to the lysines and arginines of proteins. Those residues are represented in green here. The first residues of the Bβ chain that can be observed here is β58 (represented in yellow), as residues β1-57 were mobile and thus could not be observed in the crystal structure. β58 is a Lys that protrudes from the surface of the protein and is thus fully available for heparin-binding. The exposure of this residue may be due to the absence of the β1-57 residues, as the affinity of the protein for heparin is known to increase when the peptides A and B are cleaved form the Aα and Bβ strands, as occurs during the conversion of fibrinogen in fibrin. No crystal structure of the αC domains exist as they are too mobile. The binding of Zn^{2+} ions to residues His α544 and His α545 of the αC domain is thought to change the conformation of this domain and possibly of the nearby E domain and thus to enhance heparin-binding in the E domain.

Fibronectin

Plasma and cellular fibronectins are dimeric modular proteins with each monomer composed of a repetition of three types of homologous sequences termed I, II and III.[103,231] These repeats assemble to form specific domains. Extra domain A (EDA), extra domain B (EDB) and the type III connecting segment (IIICS) can also be found in multiple spliced variants forming a total of 20 different fibronectins (Figure 4.4).[103] In plasma, fibronectin is present as a heterodimer of similar but not identical chains with only one chain bearing the IIICS connecting segment region and neither chain bearing EDA or EDB.[103] Because of its size, the full fibronectin molecule has not yet been crystallised, but most of its domains have been crystallised as fragments. Zn^{2+}-binding by fibronectin has not yet been fully characterised, however several regions have been shown to bind Zn^{2+} *in vitro*. These include the collagen/gelatin binding domain (made of repeats type II_{1-2} and type I_{7-9}),[232,233] the cell binding domain (III_{8-10})[232] and the alternatively spliced IIICS region.[101] Binding of Zn^{2+} to the gelatin domain is known to involve two histidine residues (His535 and His539) and a fragment of this region has been crystallised bound to 7 Zn^{2+} ions (Figure 4.4, insert 1).[103,234] The affinity and overall stoichiometry of Zn^{2+}-binding to this domain are not yet known, but binding of Zn^{2+} has been shown to elicit a conformational change in this region.[103] Zn^{2+}-binding to the cell-binding region has not yet been characterised but this region contains several histidine residues that may potentially be involved (Figure 4.4, insert 2). In the IIICS region, Zn^{2+}-binding occurs through coordination of a single zinc atom to four histidine residues.[101] Binding to a fragment of this region occurs with a K_d of 18 µM, however, only a small portion of plasma fibronectin contains the full IIICS sequence.[101] It is still unclear whether plasma fibronectin binds Zn^{2+} physiologically or whether this only occurs with cellular fibronectin.[101,102] Considering that Zn^{2+} induces a conformational change in the protein and may thus influence GAG binding, this is an important question to answer.

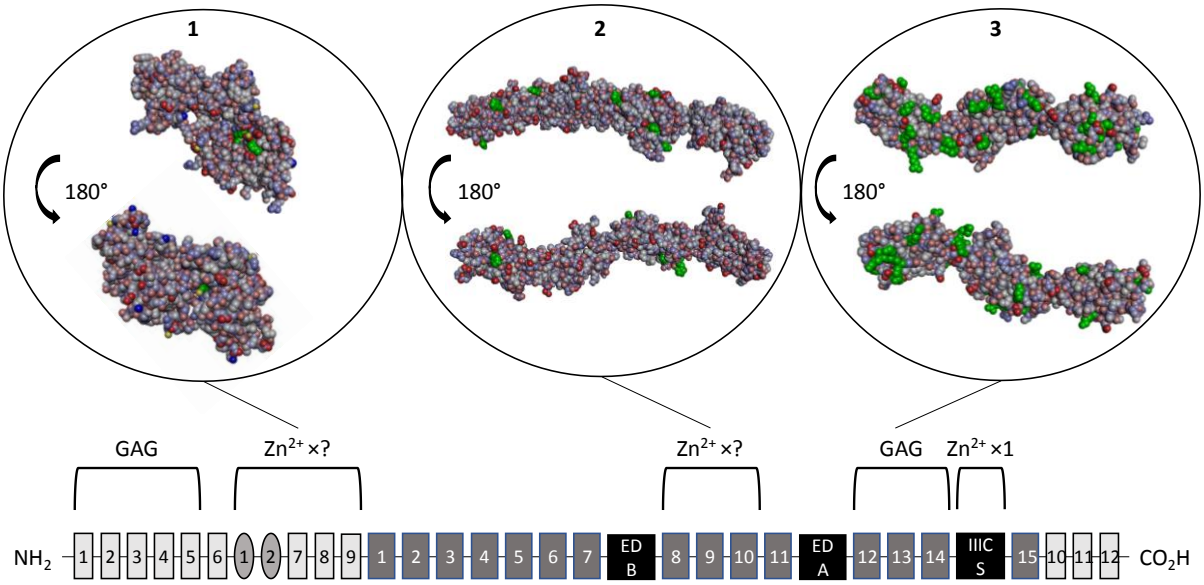


Figure 4.4. Schematic structure of fibronectin.
The protein is made of three types of repeated domains, the small pale rectangles are type I, the grey ovals are type II and the bigger darker grey rectangles are type III. The black rectangles are alternatively spliced regions, EDB, EDA and IIICS, the last one being the connective segment region. The binding domains are: $I_{5\text{-}6}$ and III_{15}-$I_{10\text{-}12}$ for fibrin, $I_{1\text{-}5}$ and $III_{12\text{-}14}$ for heparin, $II_{1\text{-}2}$-$I_{7\text{-}9}$ for gelatin/collagen and $III_{8\text{-}10}$ for cells. Zn^{2+} binds to the gelatin/collagen- and the cell-binding domains and to the IIICS region. **Insert 1.** Crystal structure of fibronectin domains I_6-$II_{1\text{-}2}$-$I_{7\text{-}9}$ binding 7 Zn^{2+} ions (PDB 3M7P).[103] The positive charges are represented in blue and the negative charges in red. His535 and His539, two residues known to be involved in Zn^{2+}-binding are represented in green. Zn^{2+} ions are in yellow. Binding of Zn^{2+} induces a conformational change in the region;[103] this may affect GAG binding to nearby $I_{1\text{-}6}$ region. **Insert 2.** Crystal structure of fibronectin domains $III_{7\text{-}10}$, a Zn^{2+} binding region (PDB 1FNF).[235] The positive charges are represented in blue and the negative charges in red. The histidine residues that could form potential Zn^{2+} binding sites are represented in green. **Insert 3.** Crystal structure of fibronectin domains $III_{12\text{-}14}$, a binding region with two heparin binding sites (PDB 1FNH).[236] The positive charges are represented in blue and the negative charges in red. The region has a net positive charge with a pI of 8.7-9.2.[237] The lysine and arginine residues which usually constitutes the main binding partners of GAGs are represented in green. GAG-binding to this region may be affected by Zn^{2+}-binding to the nearby IIICS or $III_{8\text{-}11}$ regions.

The binding of plasma fibronectin to GAG depends on the charge density and chain length of the GAG and requires a minimum of 1.9-2.4 mol of sulphated content per disaccharide to be able to bind heparin.[99] Fibronectin shares binding sites for heparin, HS and DS and has been found to possess 5-6 ionic GAG-binding sites.[96,99] The first binding site (often termed Hep1)

is found in the N-terminal region and is composed of 5 repeats of type I modules (I_{1-5}).[237,238] It has a net positive charge which should facilitate GAG-binding (pI = 8.2-8.4). As Zn^{2+} binding to the neighbouring II_{1-2}-I_{7-9} region induces a conformation change in the protein, it is probable that this could impact on GAG-binding in Hep1.[103] The second GAG-binding region (Hep2) is made of type III repeats (III_{12-14}) and also has a net positive charge (pI = 8.7-9.2) (Figure 4.4, insert 3).[237] This region has two distinct GAG-binding sites and constitutes the high affinity GAG-binding region.[237] This region is found near the IIICS domain.[237] As the region is flanked by two Zn^{2+}-binding regions, III_{8-10} and IIICS, Zn^{2+}-binding may also impact on GAG-binding at this site. Two additional weaker GAG-binding sites have been identified but are unlikely to be physiologically relevant, as binding at these sites only occurs at low ionic strength.[237] The global K_d of heparin binding to fibronectin is 18 µM for a 6-saccharide heparin (molecular weight 1,950 Da) and at 0.9 µM for a 18 to 20-saccharide heparin (molecular weight 6,000 Da).[237] Longer chain heparins were not tested and the affinity of UFH is also not yet known. The main site responsible for this affinity is Hep2 (K_d 12 and 15 µM for the two binding sites in Hep2), Hep1 only makes a minor contribution (K_d around 300 µM).[237] A decrease in pH or in the ionic strength of the buffer results in an increase in the binding affinity.[237] Those are not attributed to the presence of histidine residues (which are absent from the Hep2 region) but rather to intramolecular changes in the interactions between the different domains of fibronectin.[237] Thus, as plasma fibronectin may bind Zn^{2+} and it is certain that the fibronectin released from endothelial cells does so, plasma Zn^{2+} levels have (at least in theory) the potential to influence GAG-binding and neutralisation by fibronectin.

The goal of this study was to learn more about the role of Zn^{2+} in GAG-binding and neutralisation by HRG, fibrinogen and fibronectin, in particular to investigate whether Zn^{2+}

influences the binding of the proteins to heparins of different chain lengths. This is of particular interest given that LMWH molecules are clinically used as anticoagulants. To this end, surface plasmon resonance (SPR) and kits measuring anti-FXa and anti-thrombin activities were used.

Materials and methods

Protein and heparins

Heparins of different molecular weights were bought from Iduron (Cheshire, United Kingdom): UFH with an average molecular weight of 15,700 Da and heparins purified from fractionation with molecular weights of 22,210 Da, 14,9580 Da, 10,980 Da, 4,850 Da and 4,650 Da. Fibrinogen purified from human plasma and depleted in plasminogen was bought from Merck and fibronectin purified from human plasma was bought from Sigma. HRG was purified from human plasma using the method described below.

Purification of HRG from human plasma

HRG was purified from human plasma. Plasma was centrifuged at 4,000 × g for 30 min at 4°C; imidazole was added to the plasma to a final concentration of 5 mM, before filtering the plasma through a 0.45 µm filter (Sartorius, Epsom, UK). Immobilized Ni^{2+}-affinity chromatography using a HisTrap column (GE Healthcare Life Sciences, Little Chalfont, UK) was performed with an ÄKTA Purifier FPLC system (GE Healthcare) by increasing the imidazole content of the elution buffer. This method exploited the affinity of histidine residues for nickel and the structural similarity of imidazole (both are diazole rings) to provide competitions for binding to nickel. The column was equilibrated with 10 column volumes of 5 mM sodium phosphate, 50 mM NaCl, 5 mM imidazole, pH 7.4 buffer. After loading the column with 50 mL of plasma,

the column was washed with 10 column volumes of 20 mM sodium phosphate, 50 mM NaCl, 20 mM imidazole, pH 7.4 buffer. Elution was performed with 20%, 40%, 60%, 80% and 100% of elution buffer (500 mM imidazole, 50 mM NaCl, 5 mM sodium phosphate, pH 7.4) with each elution step carried out with 10 column volumes of elution buffer/equilibration buffer mix. The fractions containing HRG were concentrated to 2.5 mL using a Vivaspin centrifugal concentrator with 50 kDa molecular weight cut-off (Sartorius). The protein was then further purified with size exclusion chromatography using a HiLoad Superdex 26/600 200 pg column (GE Healthcare). The column was prepared for purification with 1 column volume of 100 mM NaOH at 2.6 mL/min, 2 column volumes of water and 1.5 column volume of a 10 mM Tris, 150 mM NaCL, pH 7.4 buffer. After loading the column with of 2.5 mL of the concentrated protein mixture, elution was performed at 0.6 mL/min with 1.5 column volume of 10 mM Tris, 150 mM NaCL, pH 7.4.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under denaturing conditions to assess the purity of the protein, using NuPAGE 4-12% Bis-Tris gel 1.0 mmm × 12 wells (Invitrogen, ThermoFisher Scientific). HRG was diluted in NuPAGE LDS Sample buffer 4× concentrate (protein sample final concentration 33% v/v, Invitrogen, ThermoFisher Scientific) containing β-mercaptoethanol (final concentration 2.5% v/v). Electrophoresis was conducted for 50 min at 150 V in a 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer. Protein markers were used to determine the sizes of resultant bands (PageRuler Plus Prestained protein ladder, ThermoFisher Scientific). The gel was stained with coomassie blue (0.025% coomassie brillant blue R250, 40% methanol, 10% acetic acid), destained (by microwaving the gel placed in H_2O) and visualised using a ChemiDoc XRS+ Imaging System (Bio-Rad, Hercules, California, USA). The concentration of the protein was determined by

measuring the absorbance at 280 nm using an extinction coefficient of 26,900 $M^{-1}cm^{-1}$ for human HRG (estimated using the formula: extinction coefficient = number of tryptophan × 5,500 + number of tyrosine × 1,490 + number of cysteine × 62.5).[239]

Anti-FXa assays

Heparins bind AT in a manner which enhances its inhibition of thrombin and FXa (see Chapter 1). The Anti-Xa Heparin Kit (Iduron) was used to assess the degree to which FXa activity is reduced by heparin (thus measuring the anti-FXa activity of heparin) and to determine how the presence of different proteins (fibrinogen, HRG and fibronectin) and the Zn^{2+} concentration neutralise heparin activity. The assay was carried out as follows. All samples and reagents were pre-incubated at 37°C. AT was added to heparin and they were incubated 2 min at 37°C to form a AT-heparin complex. A known excess of FXa was then added and the solution incubated 2 min at 37°C. FXa bond to AT-heparin, forming an inactive complex and leaving some residual FXa. A colourless FXa substrate (Cbo-D-Arg-Gly-Arg-pNA•2HCl) was added and they were incubated 2 min at 37°C. The substrate reacted with the residual FXa to form the chromophore molecule pNA (detectable at 405 nm) and a peptide.

The assays were carried out in triplicate in 50 mM Tris, 150 mM NaCl, pH 7.4 buffer (tris buffer saline, TBS) containing different concentrations (0 – 10 µg/mL) of heparins ranging in size (4,650 – 22,210 Da). To each well in a 96-well plate, 50 µL of each heparin sample was added and incubated 2 min at 37°C. Following this, 50 µL of AT (0.5 IU/mL in TBS), 50 µL of FXa (2.5 µg/mL in TBS) and 50 µL of FXa substrate (0.5 mg/mL in H_2O) were added in succession and after each step the wells were mixed 4 times (by pipetting up and down with a

multichannel pipette) and the plate was incubated for 2 min at 37°C. The reaction was stopped by addition of 50 µL of 20% (v/v) acetic acid. The final solutions were mixed four times and the absorbance was read immediately at 405 nm. The absorbance was plotted against the heparin concentrations. In order to obtain maximum sensitivity in the next assays, the heparin concentration needed to obtain an absorbance of 0.3 AU was calculated for each heparin. Heparins at these corresponding concentrations were then mixed with either 0.4 or 1 µM HRG, fibrinogen or fibronectin and 0 or 10 µM Zn^{2+} diluted in TBS. Assays were performed with those samples in triplicate with each individual assay repeated at least twice. Differences between the samples were analysed using two-way ANOVA followed by Sidak's multiple comparisons tests. Data are shown as the mean ± the standard error of the mean. The significance threshold was set at $p \leq 0.05$. Statistical analyses were performed and graphs were generated using GraphPad Prism 7.0.

Anti-thrombin assays

A similar kit, an Anti-IIa Heparin Kit (Iduron), was used to assess the degree to which thrombin activity is reduced by heparin (thus measuring the anti-thrombin activity of heparin) and to determine how the presence of different proteins (fibrinogen, HRG and fibronectin) and the Zn^{2+} concentration neutralise heparin activity. The assay was carried out as follow. All samples and reagents were pre-incubated at 37°C. AT was added to heparin to form a AT-heparin complex and the solution was incubated 2 min at 37°C. A known excess of thrombin was then added and the solution was incubated 2 min at 37°C. Thrombin bound to AT-heparin, forming an inactive complex and leaving some residual thrombin. A colourless thrombin substrate (H-D-Phe-Pip-Arg-pNA•2HCl) was added and the solution was incubated 2 min at 37°C. The

substrate reacted with the residual thrombin to form the chromophore molecule pNA (detectable at 405 nm) and a peptide.

The assays were carried out in duplicate in TBS containing different concentrations (0 – 5 µg/mL) of heparins ranging in size (4,650 – 22,210 Da). To each well in a 96-well plate, 50 µL of each heparin sample was added and incubated for 2 min at 37°C. Following this, 50 µL of AT (0.25 IU/mL in TBS), 50 µL of thrombin (4 IU/mL in TBS) and 50 µL of thrombin substrate (0.625 mg/mL in purified H_2O) were added in succession and after each step the wells were mixed 4 times (by pipetting up and down with a multichannel pipette) and the plate was incubated 2 min at 37°C. The reaction was stopped by addition of 50 µL of 20% (v/v) acetic acid. The final solutions were mixed four times and the absorbance was read immediately at 405 nm. The absorbance was plotted against the heparin concentrations. UFH (at the same concentration as determined during the anti-FXa experiments) was mixed with 0.4 µM HRG, fibrinogen or fibronectin and 0 or 10 µM Zn^{2+}. Assays were performed with those samples in 6 separate replicates. Differences between the samples were analysed with two-way ANOVA followed by Sidak's multiple comparisons tests. Data are shown as the mean ± the standard error of the mean. The significance threshold was set at $p \leq 0.05$. Statistical analyses were performed and graphs were generated using GraphPad Prism 7.0.

Surface plasmon resonance (SPR)

SPR is a technique which can be used to measure the binding of a molecule (here fibrinogen) to a ligand immobilized on the surface of the flow cell (here heparin) over time. Collective oscillations of free electrons are called "surface plasmons". During experiments, a polarized light hits a metal film (generally gold, which is rich in free electrons) placed at the interface of

two media with different refractive indices. When the surface plasmons of the metal film resonate with the incident light (a phenomenon called "surface plasmon resonance"), the light is absorbed at a specific angle. This create a dark line among the reflective light. Any change in the angle of this dark line is used to detect any change occurring near the metal film, such as a molecule binding to or near the metal film or when a conformation change occurs to a molecule bound to the film (through a change in the refractive index of the medium).

The SPR experiments were performed following a previously described protocol.[100] UFH and a LMWH of a molecular weight of 4,850 Da were biotinylated by incubating for 2.5 h in a shaker at 23°C 14 mg/mL heparin diluted in H_2O with (+)-biotinamidohexanoic acid hydrazide (13 mg/mL diluted in 0.1 M sodium acetate pH 5.5 buffer). The heparins were then dialysed for 3 days in three 5 L buffer of 50 mM HEPES, 150 mM NaCl, 0.2% Tween 20, pH 7.4. Biotinylated heparin was then immobilised on a SA chip (with streptavidin bound at the surface of the flow cells, Biacore, GE Healthcare) following the manufacturer instructions and using a Biacore T200 instrument (GE Healthcare). The immobilisation target was set at 640 resonance units (RU) and the buffer used was 50 mM HEPES, 150 mM NaCl, 0.2% Tween 20, pH 7.4. Aliquots of 250 nM fibrinogen were dialysed overnight at room temperature against 5 L of the same buffer containing 0-20 µM Zn^{2+}. They were then injected for 700 s at 30 µL/min over the flow cells in 5 repeats. The flow cells were regenerated with a solution containing 0.5% SDS and 1 mM EDTA. The RU of reference flow cells were subtracted from the RU of the flow cells where heparin was bound. The RU values at stability were then calculated and plotted against Zn^{2+} concentrations. Graphs were generated using GraphPad Prism 7.0.

Results and discussion

HRG purification

In order to study HRG, fibrinogen and fibronectin binding to and neutralisation of heparins, fibrinogen and fibronectin proteins purified from human plasma were obtained commercially, while HRG was purified from human plasma using Ni-NTA chromatography (Figure 4.5A). An SDS-PAGE (not shown) indicated that HRG was eluted between 120 and 140 mL of eluted volume. After this purification step, HRG was concentrated using a 50 kDa molecular weight cut-off concentrator and further purified using size exclusion chromatography (Figure 4.5B). An SDS-PAGE gel showed that the protein was eluted between 190 and 210 mL of eluted volume and that it was of high purity (Figure 4.5C).

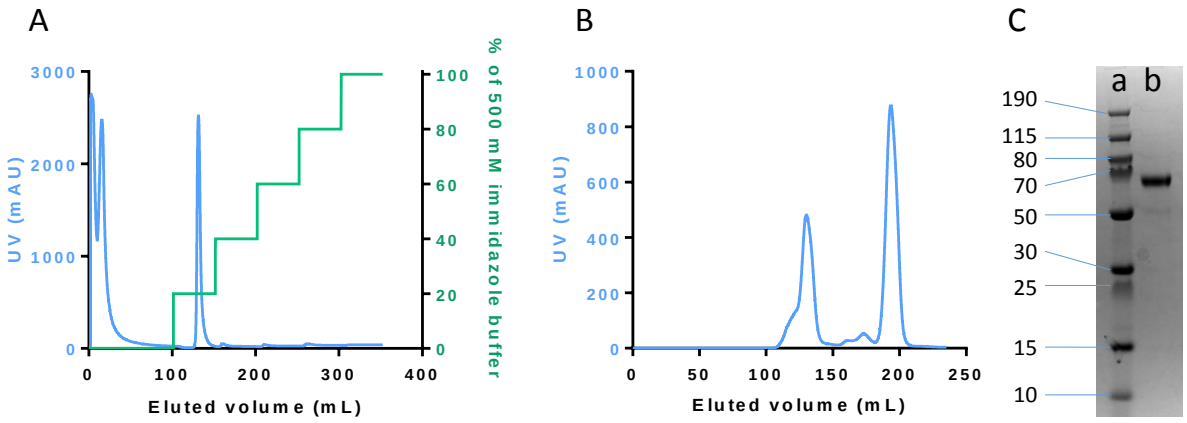


Figure 4.5. Chromatograms of HRG purification from plasma and SDS-PAGE of the purified protein.
A. Chromatogram of HRG purification from plasma with a Ni-NTA column displaying the UV of the eluted solution and percentage of 500 mM imidazole, 10 mM Tris, 150 mM NaCl, pH 7.4 buffer in the elution solution as a function of the eluted volume; HRG was eluted between 120 and 140 mL of eluted volume. **B.** Chromatogram of HRG purification by size exclusion displaying the UV of the eluted solution in function of the eluted volume. HRG was eluted between 190 and 210 mL of eluted volume. **C.** SDS-PAGE gel of HRG after purification, with a. a protein ladder (in kDa), b. the purified HRG.

Anti-FXa and anti-thrombin activities of heparins

HRG, fibrinogen and fibronectin are known to neutralise heparin in a manner which prevents its inhibition of FXa. Neutralisation of UFH by HRG is increased in the presence of Zn^{2+}, however this is not the case of LMWH.[40] Binding of fibrinogen to UFH has also been shown to increase in the presence of Zn^{2+},[100] however it has not yet been demonstrated that this translates to a direct increase in neutralisation. In addition, the effect of Zn^{2+} on fibrinogen binding to and neutralisation of LMWHs is not known. The effect of Zn^{2+} on binding and neutralisation of UFH by fibronectin has also not been studied before. In order to answer these questions, the inhibition of FXa by heparins of different molecular weight (anti-FXa activity) was measured in the presence of HRG, fibrinogen or fibronectin and Zn^{2+}.

Firstly, the anti-FXa activity of different heparins as a function of their concentrations was measured (Figure 4.6). In the absence of heparin, the rate of FXa inhibition by AT is negligible and so FXa is free to cleave the chromogenic substrate. Thus an increase in FXa activity is translated by an increase in absorbance, while inhibition of FXa by heparin-AT is reflected by a decrease in absorbance. Increasing inhibition of FXa was observed for a given heparin concentration (both mass concentration, reflecting the number of saccharides and molar concentration) as the size of the heparins increased (from 4,650 Da to 22,210 Da). UFH is a mixture of heparins of different sizes with an average molecular weight of 15,700 Da, yet it behaved in a similar manner to the purified heparin closest in molecular weight, the 14,580 Da heparin. Linear regression was performed to calculate the concentrations needed for each heparin to correspond to an absorbance of 0.3 AU. The resultant concentrations obtained were 5.0 nM for UFH, 2.5 nM for the 22,210 Da heparin, 5.1 nM for the 14,580 Da heparin, 19.0 nM for the 10,980 Da heparin and 76.0 nM for the 4,650 Da heparin.

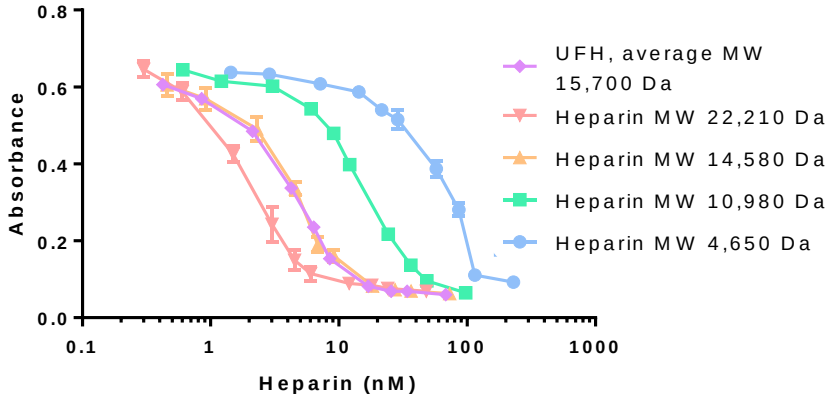


Figure 4.6. Anti-FXa activity of heparins of different molecular weights (4,650 Da - 22,210 Da) as measured with a chromogenic assay.
Inhibition of FXa (measured as a change in absorbance) as a function of heparin concentration. The experiments were performed in triplicate. For the same heparin concentration, inhibition of FXa increases with the molecular weight of the heparins. Data is represented as mean ± standard error of the mean.

Neutralisation of UFH by HRG, fibrinogen and fibronectin and the influence of this process on inhibition of FXa was examined in the presence or absence of 10 µM Zn^{2+} and compared (Figure 4.7). An increase in absorbance translated as an increase in FXa activity and thus increased neutralisation of heparin. The three proteins neutralised the anti-FXa activity of UFH, with HRG having the biggest effect, followed by fibronectin then fibrinogen. Addition of 10 µM Zn^{2+} did not affect the anti-FXa activity of UFH in the absence of protein (so Zn^{2+} does not have any background effect) or in the presence of fibronectin. However, 10 µM Zn^{2+} increased neutralisation of UFH by HRG and fibrinogen, having a greater effect on fibrinogen than on HRG, so that both proteins neutralised UFH to similar degrees when in the presence of Zn^{2+}. The heparin concentration was previously calculated so that the absorbance on the standard curve in the absence of protein and Zn^{2+} was 0.3 AU (Figure 4.6). However, the effect observed in this series of experiments was greater (Figure 4.7). This is likely due to the inter-

assay variability affecting the precision of the calculation of heparin concentration with the standard curve.

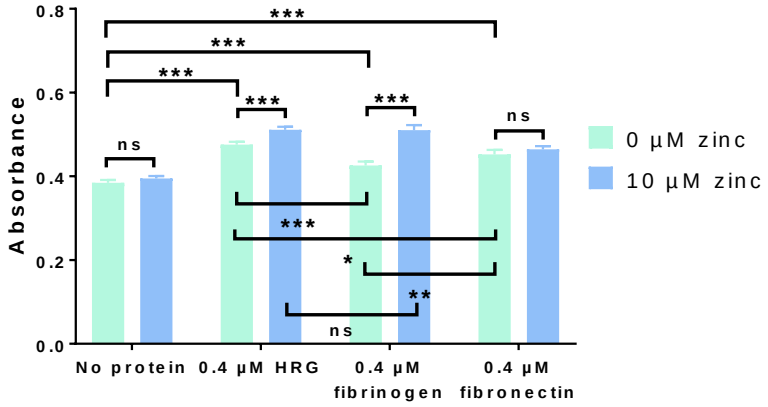


Figure 4.7. Comparison of the neutralisation of the anti-FXa activity of UFH by 0.4 µM HRG, fibrinogen or fibronectin in the presence and absence of 10 µM Zn^{2+}, as measured with a chromogenic assay.
The experiments were performed three times in duplicate. Addition of Zn^{2+} increased UFH neutralisation by HRG and fibrinogen but not by fibronectin. Data is represented as mean ± standard error of the mean. Statistical significance is indicated by ns (not significant) for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

The ability of HRG and fibrinogen to neutralise heparins of different molecular weights (and thus their impact on its anti-FXa activity) in the presence and absence of Zn^{2+} was then assessed (Figure 4.8). With 22,210 Da and 14,580 Da heparins both 0.4 µM HRG and 0.4 µM fibrinogen were able to partially neutralise heparin as evidenced by measuring its anti-FXa activity, with HRG having a greater effect than fibrinogen (Figure 4.8A and B). For 10,980 Da heparin both 0.4 µM HRG and 0.4 µM fibrinogen were able to partially neutralise the anti-FXa activity of heparin to a similar degree (Figure 4.8C). Neither 0.4 µM HRG or 0.4 µM fibrinogen were able to neutralise a LMWH of 4,650 Da heparin. To examine whether a higher concentration of HRG and fibrinogen could exert an effect on this LMWH, the assay was repeated with the

concentration of each protein increased to 1 μM and the heparin concentration reduced by half (down to 38.0 nM, Figure 4.8D). Under these conditions, some degree of neutralisation was seen with HRG, but the degree of neutralisation by fibrinogen was not sufficient to see a significant change in anti-FXa activity. The smaller degree of LMWH neutralisation occasioned by the presence of either protein was due in part to the different concentrations that had to be used in the assay. However, heparin neutralisation by HRG is known to be less efficient when the molecular weight of the heparin is reduced and this may also be the case with fibrinogen.[87] Addition of 10 μM Zn^{2+} did not significantly affect the anti-FXa activity of heparin in the absence of fibrinogen or HRG. Zn^{2+} increased neutralisation of heparin by fibrinogen with heparins of all molecular weights examined. However, Zn^{2+} only increased the neutralisation of heparin by HRG with heparins of 22,210 Da, 14,580 Da and 10,980 Da and did not affect the neutralisation of the LMWH (4,650 Da). Thus, Zn^{2+} can increase the neutralisation of LMWHs by fibrinogen but not by HRG. Addition of Zn^{2+} increased neutralisation of heparins of all molecular weight by fibrinogen more than it increased the neutralisation of heparins by HRG. HRG had a greater effect on the anti-FXa activity of the heparin of 22,210 Da than fibrinogen, even when both proteins were in the presence of Zn^{2+}. However, in the presence of Zn^{2+} fibrinogen neutralised the heparin of 14,580 Da to a greater extent than HRG in the presence of Zn^{2+}, while the neutralisations of the heparin of 10,980 Da and 4,650 Da in the presence of Zn^{2+} were similar for both proteins.

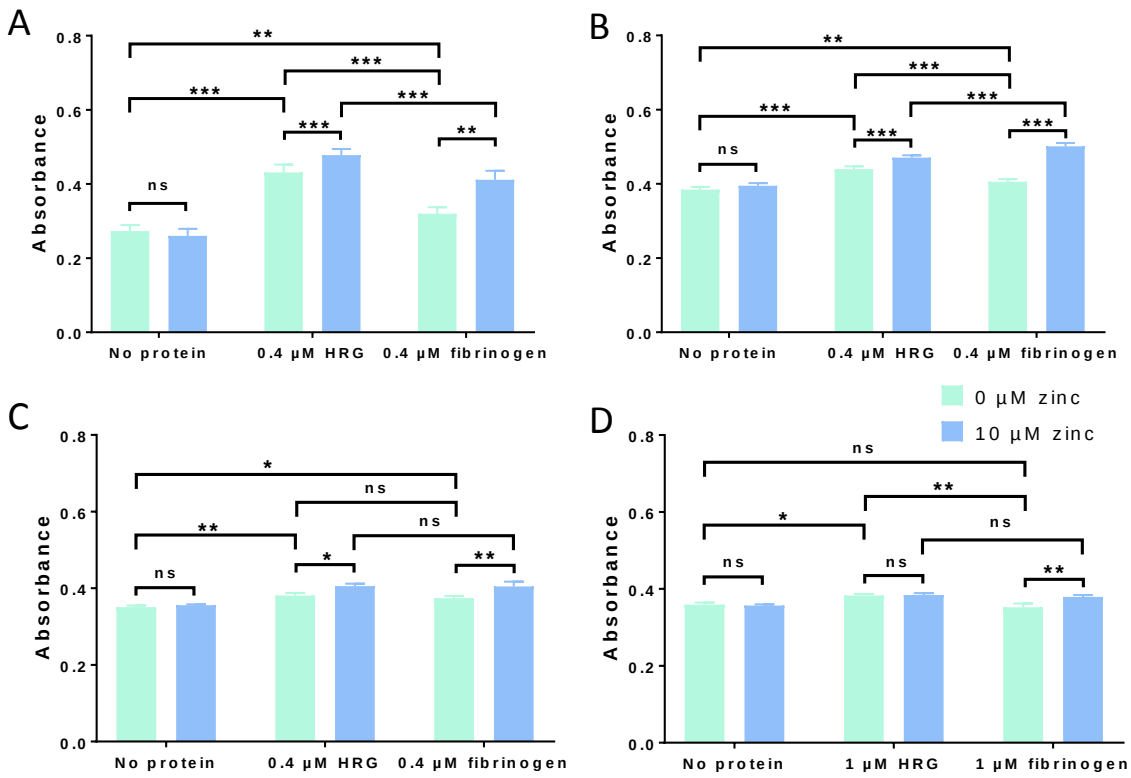


Figure 4.8. Comparison of the neutralisation of the anti-FXa activity of heparins of different molecular weights by 0.4 μM or 1 μM HRG or fibrinogen in the presence and absence of 10 μM Zn^{2+}, as measured with a chromogenic assay.
A. Heparin of 22,210 Da (2.5 nM), **B.** heparin of 14,580 Da (5.1 nM), **C.** heparin of 10,980 Da (19.0 nM) and **D.** heparin of 4,650 Da (76.0 nM). The experiments were repeated three times in triplicate. Zn^{2+} increased the neutralisation of all heparins including LMWH (< 5 kDa heparins) by fibrinogen but it only increased the neutralisation of longer chain heparins by HRG. Data is represented as mean ± standard error of the mean. Statistical significance is indicated by ns (not significant) for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

Next, the anti-thrombin activity of heparins of different molecular weights was compared (Figure 4.9). As reported before, LMWHs (here 4,650 Da) cannot inhibit thrombin as they are too short to possess the full AT-thrombin binding site.[58] Larger heparins (10,980 – 22,210 Da) were all able to inhibit thrombin, with inhibition at the same heparin concentration (mass or molar) increasing with the molecular weight of the heparins.

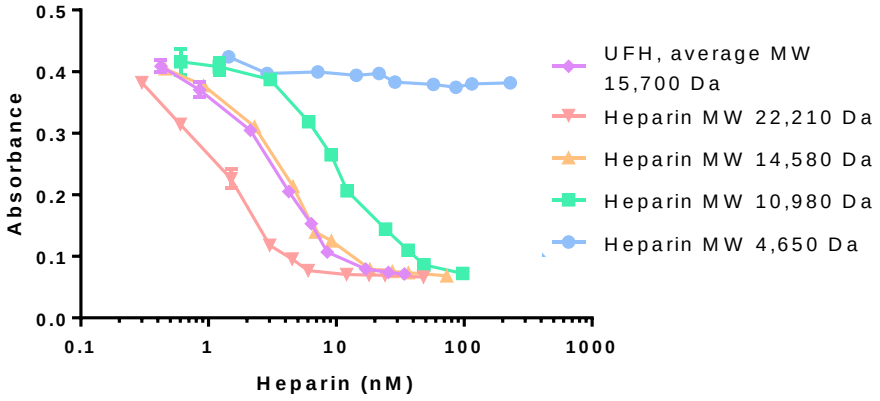


Figure 4.9. Measurement of the anti-thrombin activity of heparins of different molecular weights (4,650 Da - 22,210 Da), as measured with a chromogenic assay.
Inhibition of thrombin (measured as an increase in absorbance) as a function of heparin molar concentration. The experiments were performed in duplicate. The heparin of 4,650 Da did not neutralise the anti-thrombin activity of UFH, while the other heparins neutralised UFH increasingly with increasing molecular weight of the heparins. Data is represented as mean ± standard error of the mean.

The anti-thrombin activity of UFH was then measured in order to compare the degree to which HRG, fibrinogen and fibronectin neutralise heparins and to assess the influence of Zn^{2+} on this neutralisation (Figure 4.10). All three proteins (0.4 µM) reversed thrombin inhibition by UFH to some degree, with HRG having the greatest effect, followed by fibronectin and fibrinogen (fibronectin had a similar effect to fibrinogen). Addition of 10 µM Zn^{2+} did not affect heparin activity in the absence of neutralising proteins. Zn^{2+} increased UFH neutralisation by HRG and fibrinogen, with the effect on fibrinogen more pronounced. However, overall neutralisation by HRG in the presence of Zn^{2+} was still greater than by fibrinogen in the presence of Zn^{2+}. Neutralisation of UFH by fibronectin was not affected by addition of Zn^{2+}. Those results showed that Zn^{2+} has similar effect on the neutralisation of thrombin and FXa inhibition by UFH. The difference between the effect of Zn^{2+} on HRG and fibrinogen binding to LMWHs is not relevant to thrombin inhibition as LMWHs do not have this ability. The presence of thrombin in the assay means that fibrinogen is going to be transformed into fibrin. However,

this does not result in a decrease in heparin neutralisation as fibrin has been shown to form a complex with heparin and thrombin, resulting in thrombin protection from inactivation by AT.[94] The formation of the fibrin-heparin-thrombin complex is also enhanced by Zn^{2+}.[95]

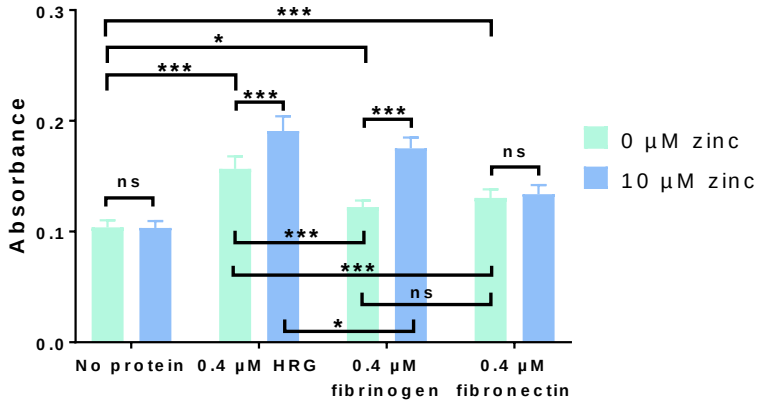


Figure 4.10. Comparison of the neutralisation of the anti-thrombin activity of UFH by 0.4 µM HRG, fibrinogen or fibronectin in the presence and absence of 10 µM Zn^{2+}.
The experiments were performed in 6 separate replicates. HRG, fibrinogen and fibronectin all neutralise the inhibition of thrombin by UFH, but only HRG and fibrinogen neutralisation is increased by addition of Zn^{2+}. Data is represented as mean ± standard error of the mean. Statistical significance is indicated by ns (not significant) for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

Zn^{2+}-dependent heparin-binding by fibrinogen

Further experiments were then performed in order to better understand the effect of Zn^{2+} on the neutralisation of heparins of different sizes by fibrinogen. ITC and enzyme-linked immunosorbent assay (ELISA)-based approaches have previously been used to examine the effect of Zn^{2+} on heparin neutralisation by HRG,[40] similar approaches were attempted with fibrinogen. However, those experiments were not successful due to the fact that fibrinogen easily precipitated at relatively low concentrations and Zn^{2+}-binding further increased

precipitation. Thus, concentrations sufficiently high to detect heparin binding with ITC could not been reached without the protein precipitating. In addition, during ELISA experiments with immobilised heparin, fibrinogen bound to the plastic of several types of ELISA plates in the absence of heparin, complicating the assay. Experiments with anti-fibrinogen antibodies (IgG purified from rabbit) immobilised by binding to plates coated in protein A (Pierce, ThermoFisher) were also not successful, as the anti-heparin antibodies (IgG purified from mouse) also bound to the plate (despite the manufacturer's indications that the plate would bind to IgG from rabbit but not from mouse) and no other commercial anti-heparin antibodies were available. Therefore, an SPR-based approach was performed to assess whether Zn^{2+} increased the affinity of fibrinogen for heparins of different molecular weights. This technique was previously used by Fredenburgh et al. to study fibrinogen binding to heparin.[100]

Fibrinogen was first immobilised (up to 1002 RU) on a CM5 chip (Biacore) by amination and heparin (up to 10 µM) was injected over the flow cell in the presence of 0 or 20 µM Zn^{2+}. However, only limited heparin-binding to fibrinogen could be observed and no increase in heparin-binding could be seen with addition of Zn^{2+}. This is probably due to the fact that an amination site is present in the E domain of fibrinogen and may interfere with heparin binding nearby to the E domain or αC regions. Therefore, UFH and a LMWH of 4,850 Da were biotinylated and immobilised on a streptavidin chip. Despite the same immobilisation target (640 RU), the degrees of immobilisation obtained were different for the two heparins: 635 RU for UFH and 848.4 RU for the LMWH. Fibrinogen samples (250 nM) containing 0, 2.5, 10 or 20 µM Zn^{2+} were injected over the flow cells for 700 s. At the end of the injection, the flow cells were washed and the RU was immediately measured (point at stabilisation). For each experiment, the data obtained from a blank cell was subtracted from the response measured in

the heparin flow cells. The values obtained in the absence of Zn^{2+} were subtracted from those obtained in its presence and the data (Rmax) was plotted against Zn^{2+} concentration (Figure 4.11). The binding rate of both UFH and the LMWH to fibrinogen increased with addition of Zn^{2+} in a concentration-dependent manner up to 20 µM Zn^{2+}. The Rmax obtained with LMWH at 20 µM Zn^{2+} was lower than with UFH, however the difference in the degree of immobilisation of both heparins made a direct comparison of the amount each heparin bound to fibrinogen impossible.

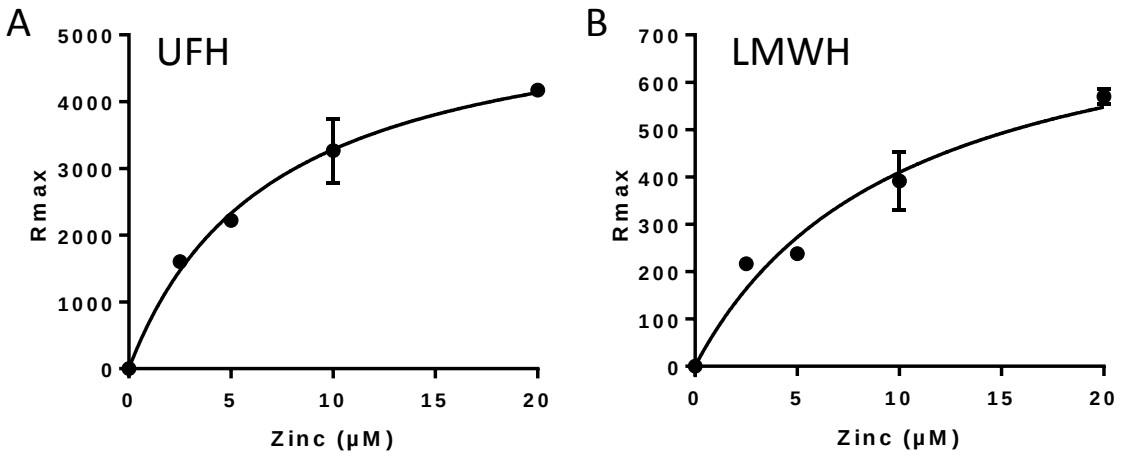


Figure 4.11. Effect of Zn^{2+} on the binding of fibrinogen to immobilised UFH and LMWH (4,850 Da) as measured by SPR.
Solutions of 250 nM fibrinogen containing 0-20 µM Zn^{2+} were injected into flow cells containing immobilised UFH or LMWH, followed by a wash with the buffer. The RU values at stability obtained from the sensorgrams were corrected for binding to blank flow cells as well as for binding in the absence of Zn^{2+}. Each experiment was repeated 5 times. Data is represented as mean ± standard error of the mean while the line plotted was obtained with nonlinear regression.

HRG, fibrinogen and fibronectin neutralise the ability of heparins to inhibit thrombin and FXa when bound to AT. Because of the structural similarity between heparin and HS, this means

that those proteins are also probably able to neutralise the anticoagulant activity of the HS on the ESL, thus negating the natural anticoagulant properties of the endothelium. In addition, during coagulation platelets are activated and release Zn^{2+}. As described before,[40] this increase in the local plasma Zn^{2+} concentration is likely sufficient to increase the affinity of HRG for heparins (but not for LMWHs). The results presented here show that Zn^{2+} also increases the binding rate (and so possibly the affinity) of fibrinogen for heparins, including LMWHs. Furthermore, this effect of Zn^{2+} on heparin-binding also leads to an increase neutralisation of heparin (excluding LMWHs for HRG) and so to an increase in FXa and thrombin activity (pro-coagulant activity). While the effect of Zn^{2+} on heparin binding by fibronectin was not studied directly, it was shown not to increase UFH neutralisation by this protein. Thus, Zn^{2+} can be presumed not to affect heparin binding by fibronectin.

The neutralisation of the anticoagulant properties of the endothelium by HRG, fibrinogen and fibronectin is particularly relevant, as those three proteins are released by activated platelets alongside Zn^{2+}. This neutralisation helps to enable coagulation to take place, an effect that is enhanced by the local increase in Zn^{2+} concentration (for HRG and fibrinogen). This action is desirable in normal circumstances when coagulation is required. However, when Zn^{2+} concentration is dysregulated, as is the case in the presence of pathologically elevated plasma FFA concentrations, this negates an important endothelial protection pathway safeguarding against undesired coagulation. In addition to the effect of Zn^{2+} on fibrin clotting, this provides another mechanism by which elevated Zn^{2+} concentrations can result in an increase in thrombotic risk in diseases associated with elevated plasma FFA concentrations. Finally, it is important to note that fibrinogen is better able to neutralise LMWHs than HRG. In addition, contrary to HRG, the neutralisation of LMWH by fibrinogen is enhanced by Zn^{2+}. As the

plasma concentration of fibrinogen is ten times greater than HRG (with an average of around 12-24 μM against 1.3-2.0 μM),[84,85,90] this makes fibrinogen the most relevant protein of the two for LMWH neutralisation, even if HRG is an important neutraliser of high molecular weight heparins. LMWHs are considered to be a more favourable drug than UFH as their smaller size leads to less interactions with other molecules in the plasma, reducing secondary effects.[58] However, the results presented here show that some neutralisation still occurs. Therefore, it could be important to consider the plasma concentrations of HRG, fibrinogen and fibronectin of a subject during administration of UFH and of fibrinogen during administration of LMWHs in order to adjust the dose to obtain the desire anticoagulant effect.

In summary, this chapter has shown another facet of the effects that an elevation in plasma Zn^{2+} concentration can have on coagulation. The presented results reveal that an increase in Zn^{2+} concentration can increase anticoagulant GAG neutralisation by HRG and fibrinogen, thus changing the natural anticoagulant properties of plasma. They also show that altered Zn^{2+}-handling caused by elevated FFA levels can impact upon heparin-based drugs neutralisation, potentially impacting upon its dose-response profile.

Chapter 5: Conclusion

The principal aims of this project were to study:

1. The interplay between Zn^{2+} and FFAs of different chain lengths and saturation state binding to HSA.
2. The effect of this dynamic upon fibrin clot formation and lysis in a purified protein system, in pooled plasma and in plasma from subjects with T1DM and T2DM and from healthy controls.
3. The plasma concentrations of zinc, copper, magnesium and selenium from the T1DM, T2DM and healthy controls.
4. The consequences of defective Zn^{2+} handling (caused by elevated FFA concentrations) on heparin neutralisation by HRG, fibrinogen and fibronectin.

The impact of FFAs of different length and saturation degree on Zn^{2+}-buffering by HSA was studied using ITC. The results presented here revealed that palmitate, palmitoleate and stearate (which are among the most abundant FFAs in the blood) strongly affect Zn^{2+}-binding by HSA, with 4 mol. eq. of palmitate and stearate sufficient to prevent Zn^{2+}-binding (Figure 5.1A). The total plasma FFA concentrations are increased in several disease states including diabetes, obesity and fatty liver disease, while the individual concentrations of palmitate and stearate are themselves also increased in some conditions. This is likely to impact on plasma zinc speciation through FFA binding to HSA. The effect of increased plasma FFA (myristate) and Zn^{2+} concentrations on fibrin clot formation was then assessed in both a purified protein system and in pooled plasma from healthy subjects using a validated turbidimetric assays (Figure 5.1A). These studies showed that, in the purified system, addition of up to 100 μM Zn^{2+} increased the

density of the clot (increased maximum absorbance), clot time and lysis time. Addition of 4 mol. eq. myristate correlated with an increase in the density of the clot and clot time, while lysis time was not affected. In the plasma system, addition of Zn^{2+} increased all three parameters while addition of myristate correlated with an increase in the density of the clot and clot time and a decrease in lysis time. Furthermore, SEM experiments showed that addition of Zn^{2+} to a purified protein system leads to an increase in the thickness of fibrin fibres, thus explaining the increased density of the clots.

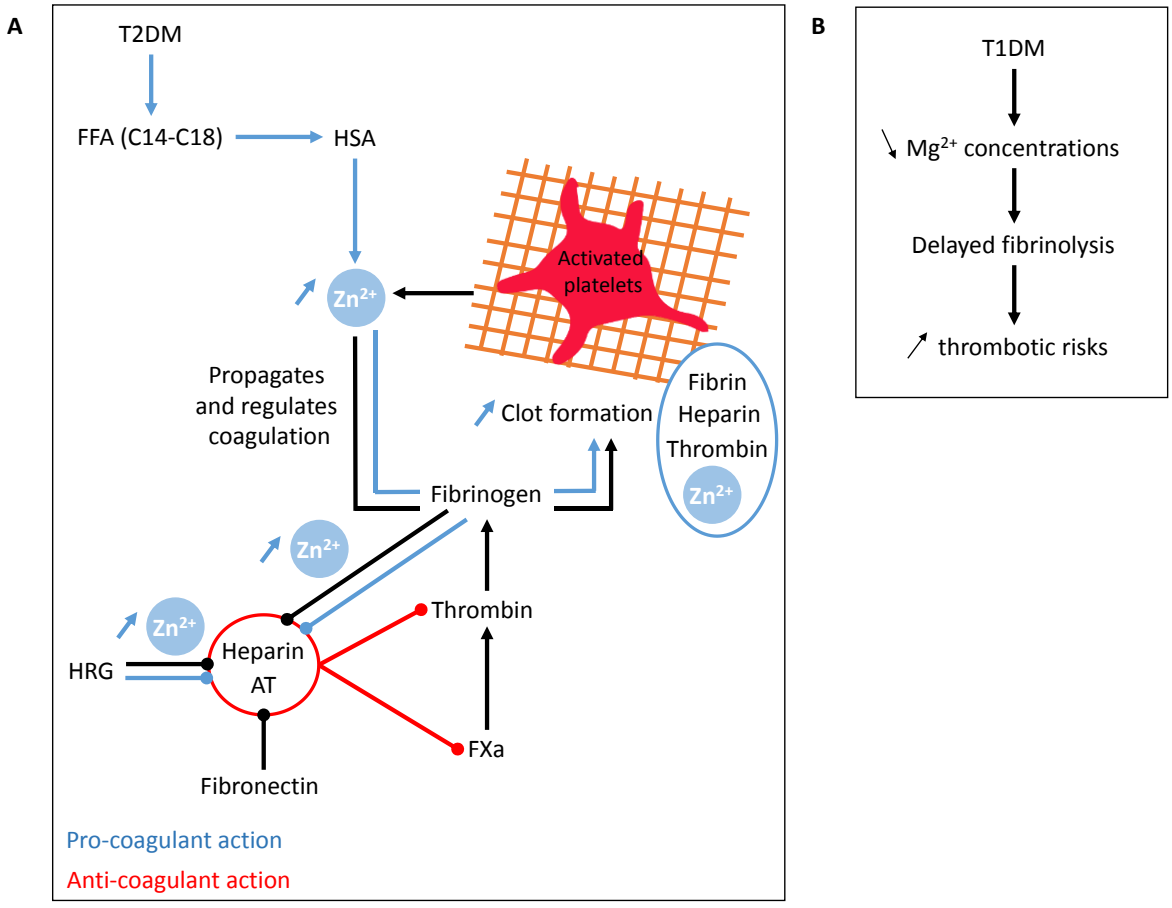


Figure 5.1. Summary of the main findings.
A. The high plasma FFA concentrations found in T2DM prevent Zn^{2+} from binding to HSA and those higher plasma Zn^{2+} concentrations lead to an increase in clot formation. In addition, fibrinogen, HRG and fibronectin neutralise heparins, preventing the heparin-AT complex from inhibiting thrombin and FXa, thus having a pro-coagulant action. Neutralisation by HRG and fibrinogen is increased by Zn^{2+}. Zn^{2+} also enhances the formation of a complex between fibrin, heparin and thrombin, thus protecting thrombin from inhibition. **B.** The deficit in Mg^{2+} found in T1DM leads to delayed fibrinolysis which increases thrombotic risk.

In order to assess whether the effect on fibrin clotting exerted by myristate occurred in the presence of a complex mixture of FFAs, those experiments were repeated with plasma from individuals with T1DM and T2DM as well as with plasma from healthy controls. It was found that T2DM subjects have a higher clot density, lysis time and fibrin fibre thickness and that clot density correlates with plasma FFA concentration. This is in support of the hypothesis that the elevated total plasma FFA concentrations found in T2DM participate in the increased thrombotic risks observed in this disease through a Zn^{2+}-dependent mechanism (Figure 5.1A). T1DM subjects do not have elevated FFA concentrations and their plasma showed a decreased clot density, delayed clotting and delayed fibrinolysis, while the fibrin fibres were on average thinner. Clot density was positively correlated with both plasma magnesium and copper concentrations, while lysis time was negatively correlated with magnesium concentrations (Figure 5.1B). The effect of elevated plasma glucose concentrations on the regulation of different metals (including zinc) was then studied in T1DM and T2DM, as metal homeostasis is known to be dysregulated in diabetes. The T1DM cohort was marked by a deficit in magnesium while copper concentration was not significantly altered. Thus, the higher thrombotic risks found in T1DM subjects appears to be due to an increase in lysis time associated with a deficit in magnesium, rather than to an increase in clot density. Magnesium supplementation has been shown to reduce the risk of developing complications. In T2DM, subjects had lower plasma selenium concentrations and higher copper concentrations than in controls, however no significant correlation were found between plasma zinc, copper, magnesium or selenium concentrations and fibrin clot parameters. Only the zinc/copper ratio was positively correlated with clot density. This showed that the effect of zinc on fibrin clot is due to an altered plasma speciation of zinc rather than to a change in total plasma zinc concentration.

Finally, the effect of an increase in plasma Zn^{2+} on heparin neutralisation by fibrinogen, HRG and fibronectin was assessed by measuring the anti-FXa and anti-thrombin activities of heparins and by measuring the affinity of fibrinogen for heparins by SPR. It was shown that Zn^{2+} increased the ability of HRG and fibrinogen to neutralise UFH leading to increased thrombin and FXa activities, while Zn^{2+} had no effect on UFH neutralisation by fibronectin (Figure 5.1A). Furthermore, Zn^{2+} did not increase LMWH neutralisation by HRG but it did increase both the binding affinity for LMWH and the neutralisation of LMWH by fibrinogen. This implies that the release of Zn^{2+}, HRG, fibrinogen and fibronectin by activated platelets work together to neutralise the anticoagulant activity of the HS which lines the endothelium. This is a desirable effect when clotting is required, but when plasma Zn^{2+} speciation is dysregulated, as is the case in the presence of high plasma FFA concentrations, this likely participates in increasing the risk of thrombosis. In addition, this also means that while HRG will only have a minor effect on clinically administered LMWHs, fibrinogen is an important neutraliser of such molecules and that fibrinogen plasma concentrations should be taken into account in order to obtain a better dose-response predictability.

As a next step, it would be interesting to confirm the role of plasma Mg^{2+} concentration in fibrin clotting through carrying out turbidimetric assays in plasma in the presence of different Mg^{2+} concentrations. If the increased lysis time observed in subjects with T1DM can be replicated, this would highlight the importance of Mg^{2+} in increasing thrombotic risks in this disease. The effect of magnesium supplementation in reducing this risk could then be assessed in a clinical context. Such a study would be important in the treatment of T1DM subjects, as magnesium supplementation is not currently systematically prescribed.[240] In order to further differentiate between the individual effect of FFAs on fibrin clotting and the effect it exerts through the

change in Zn^{2+} speciation, the turbidimetric assays presented here could be replicated in the presence of a specific zinc chelator. In addition, those assays were performed by adding myristate in plasma. Replicating them with addition of octanoate may be useful, as this FFA has been shown here not to affect Zn^{2+} binding by HSA.

The examination here of fibrin clotting with plasma from individuals with diabetes has further clarified the role of Zn^{2+} and FFAs in causing hypercoagulability in T2DM. It would be interesting to quantitatively examine individual lipid concentrations in the plasma of the T1DM, T2DM and healthy control cohorts examined here in order to assess whether increased coagulability is associated with altered concentrations of particular FFAs. In addition, the study performed here could be extended to other diseases associated with elevated plasma FFA concentrations or altered plasma zinc speciation (such as obesity or fatty liver disease) to see if the same effects as in T2DM are observed. Furthermore, the ITC experiments may also be replicated with different FFAs, in particular long-chain unsaturated FFAs which are abundant in the blood and therefore very relevant, such as linoleate, oleate and arachidonate.

In addition, the effect of HRG, fibrinogen and Zn^{2+} on heparin neutralisation could be further examined by assessing whether *in vivo* heparin (UFH) resistance correlates with fibrinogen and HRG concentrations and whether LMWH resistance is correlated to fibrinogen but not HRG concentrations. Furthermore, a study could be realised on the ability of fibrinogen, HRG, fibronectin and other heparin neutralising molecules to neutralise synthetic heparin analogues. Indeed, those molecules are much shorter than UFH and LMWH (e.g. Fondaparinux only possesses the thrombin-binding site of heparin) and so, contrary to heparins, those molecules

are potentially less likely to have complex interactions with other molecules. However, molecules that neutralise heparins can do so through different mechanisms including direct competition for the thrombin binding site and non-specific electrostatic interactions, both of which are relevant for synthetic heparin analogues. Potential effects of Zn^{2+} on such neutralisation should also be investigated. Knowing which proteins and molecules neutralise which drugs could be important in order to allow for a better and personalised choice of anti-thrombotic treatment based on an individual's plasma protein composition.

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Appendix 1: ITC raw data

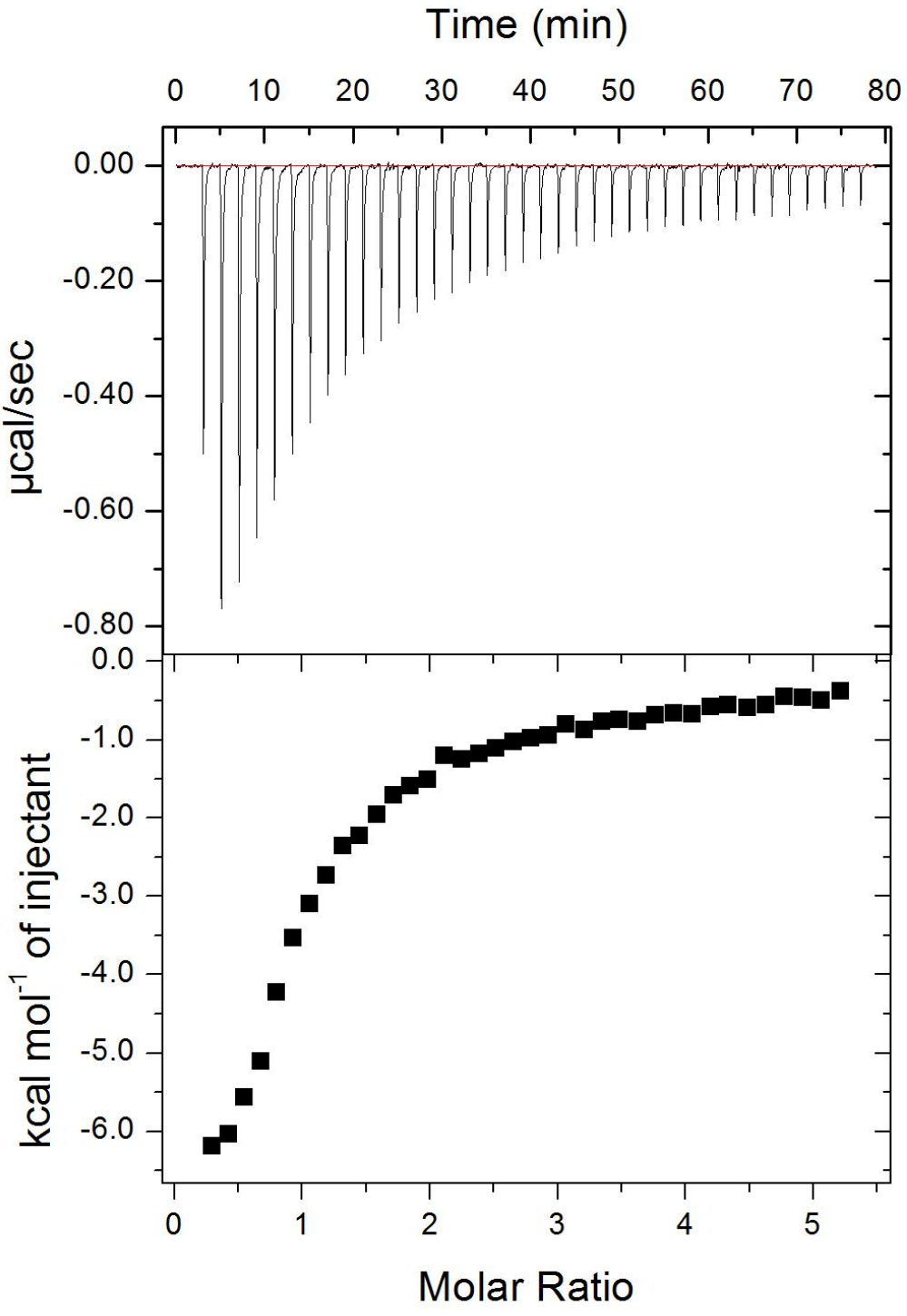

Figure A1.1. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the absence of FFA, corresponding to data shown in Figure 2.4A-D.

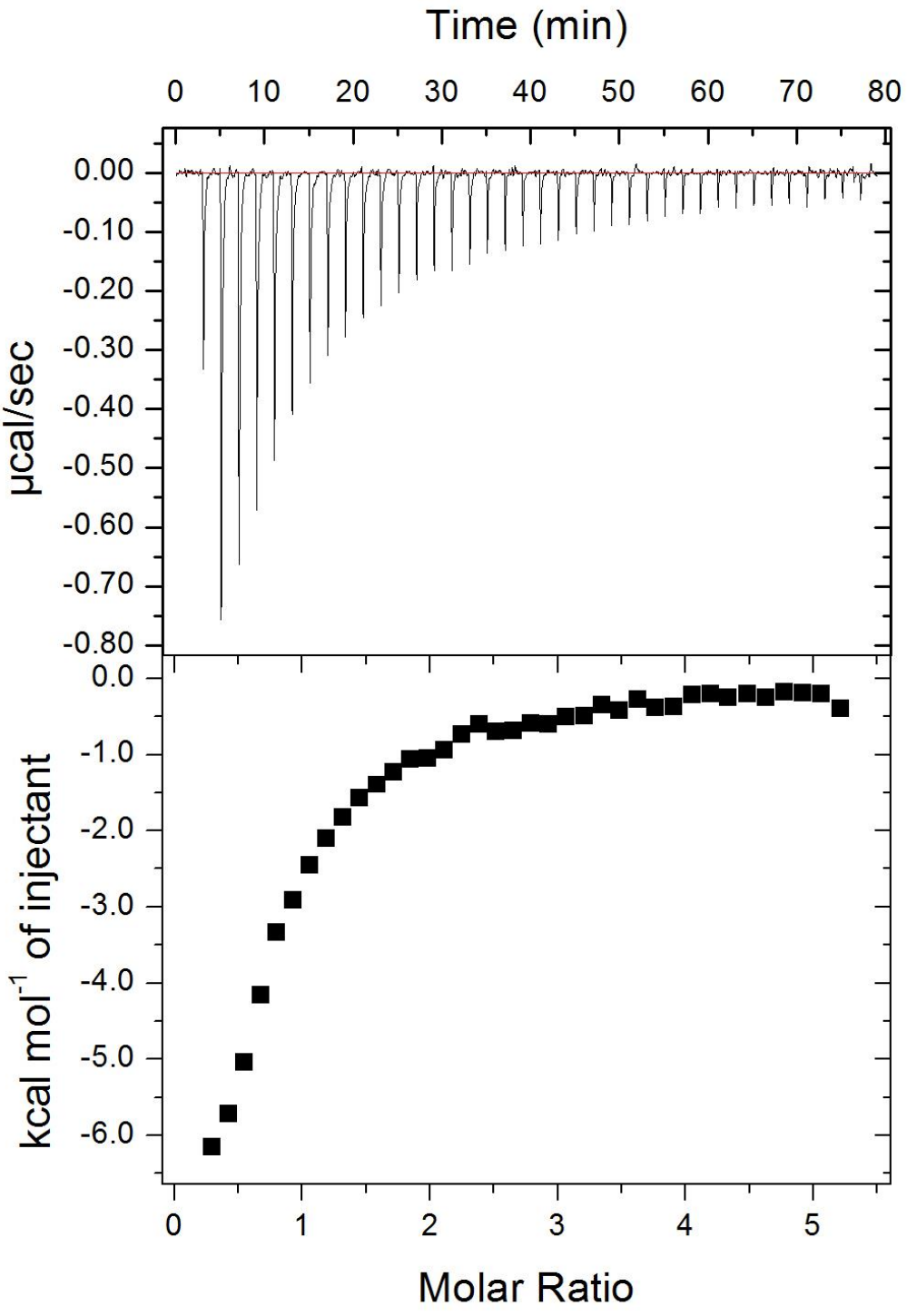

Figure A1.2. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 3 mol. eq. of octanoate, corresponding to data shown in Figure 2.4A.

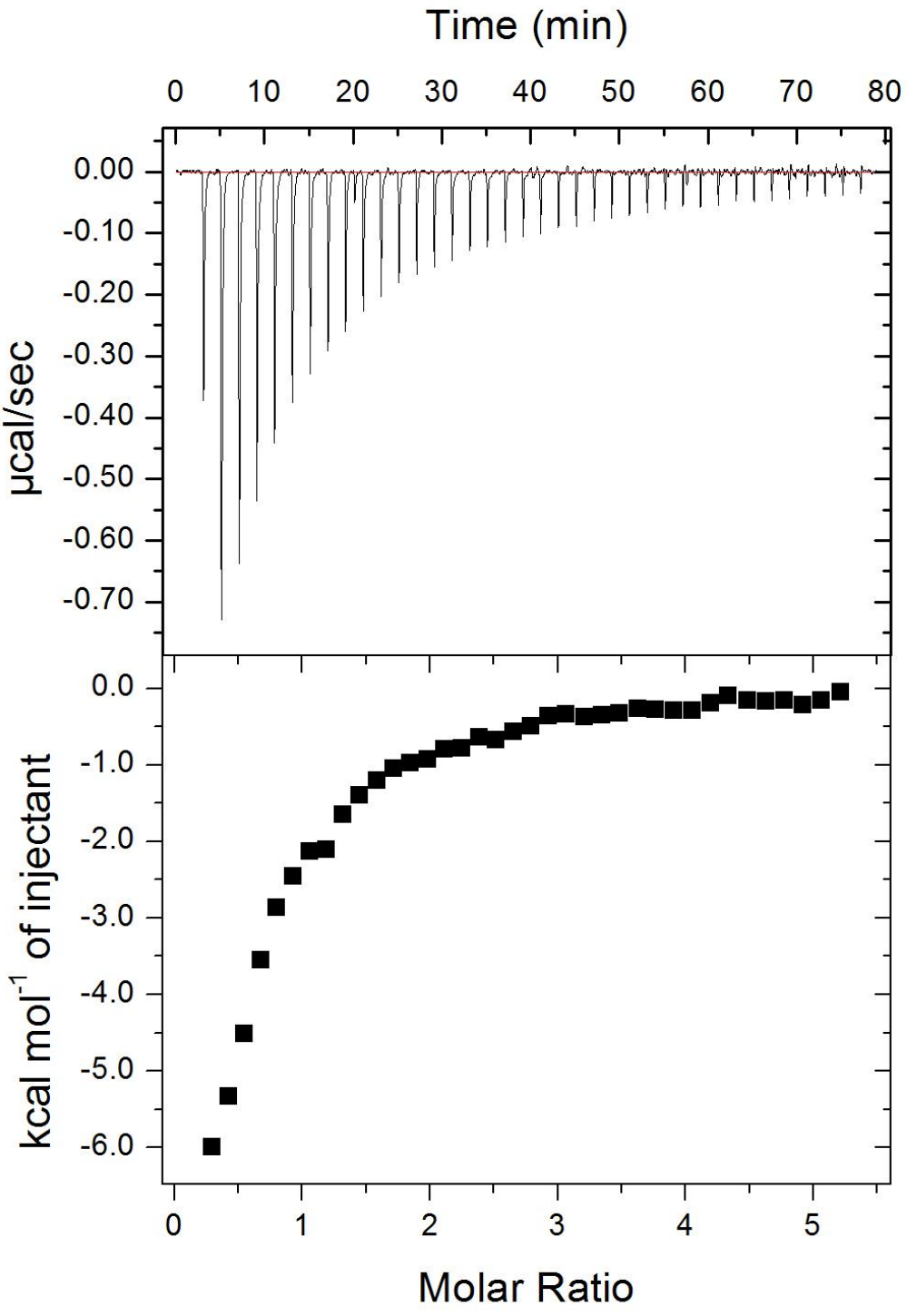

Figure A1.3. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 5 mol. eq. of octanoate, corresponding to data shown in Figure 2.4A.

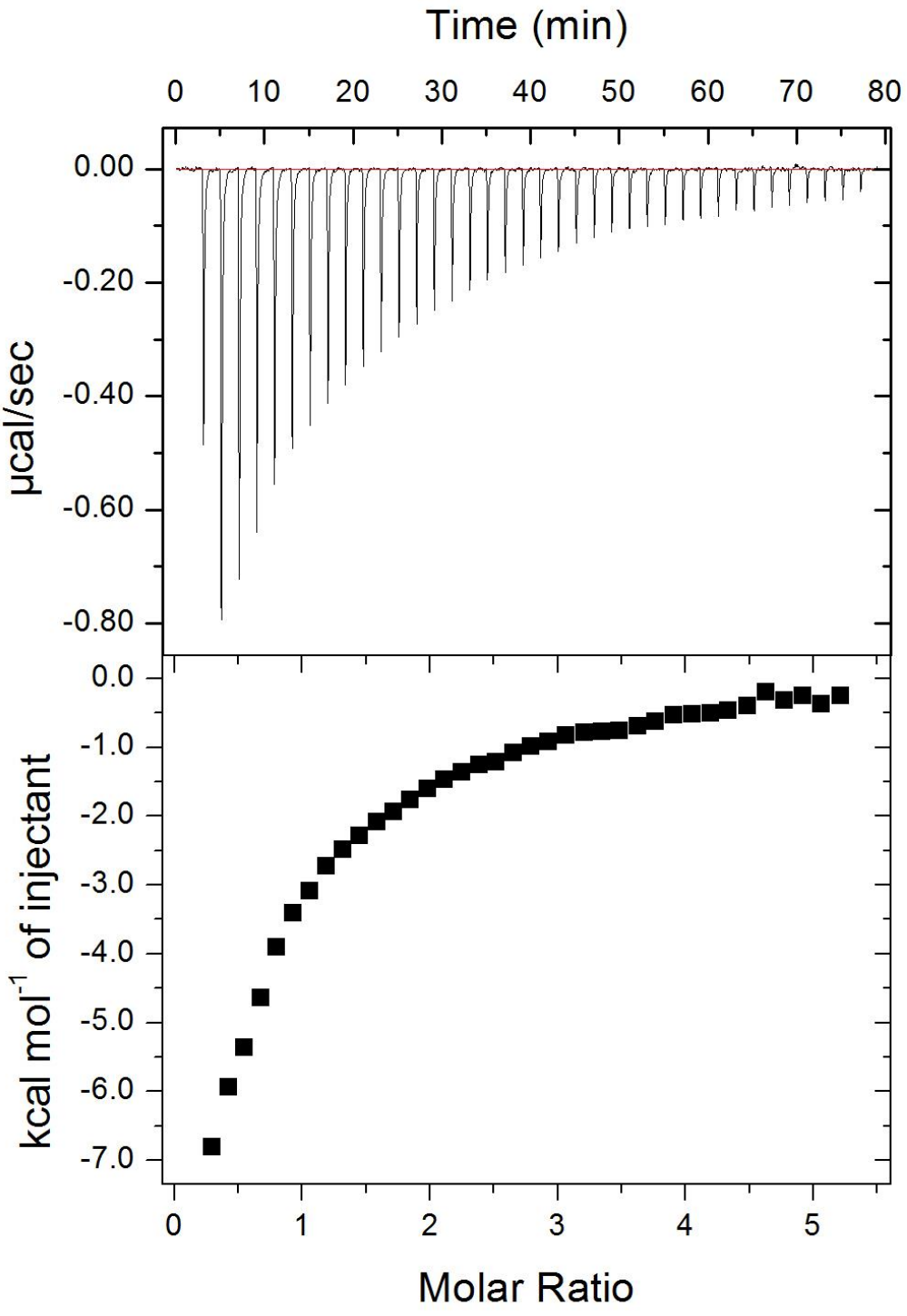

Figure A1.4. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 3 mol. eq. of laurate, corresponding to data shown in Figure 2.4B.

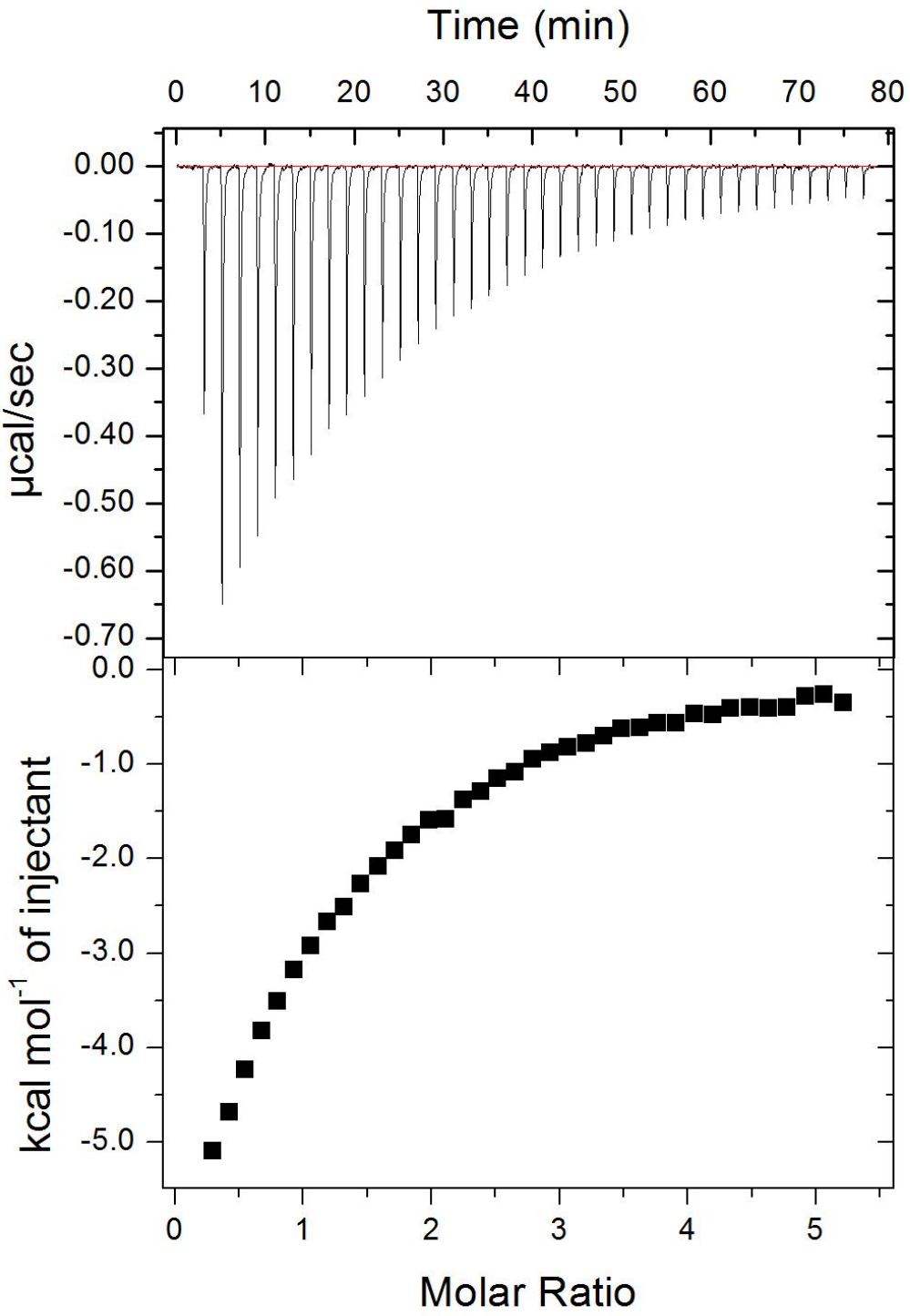

Figure A1.5. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 4 mol. eq. of laurate, corresponding to data shown in Figure 2.4B.

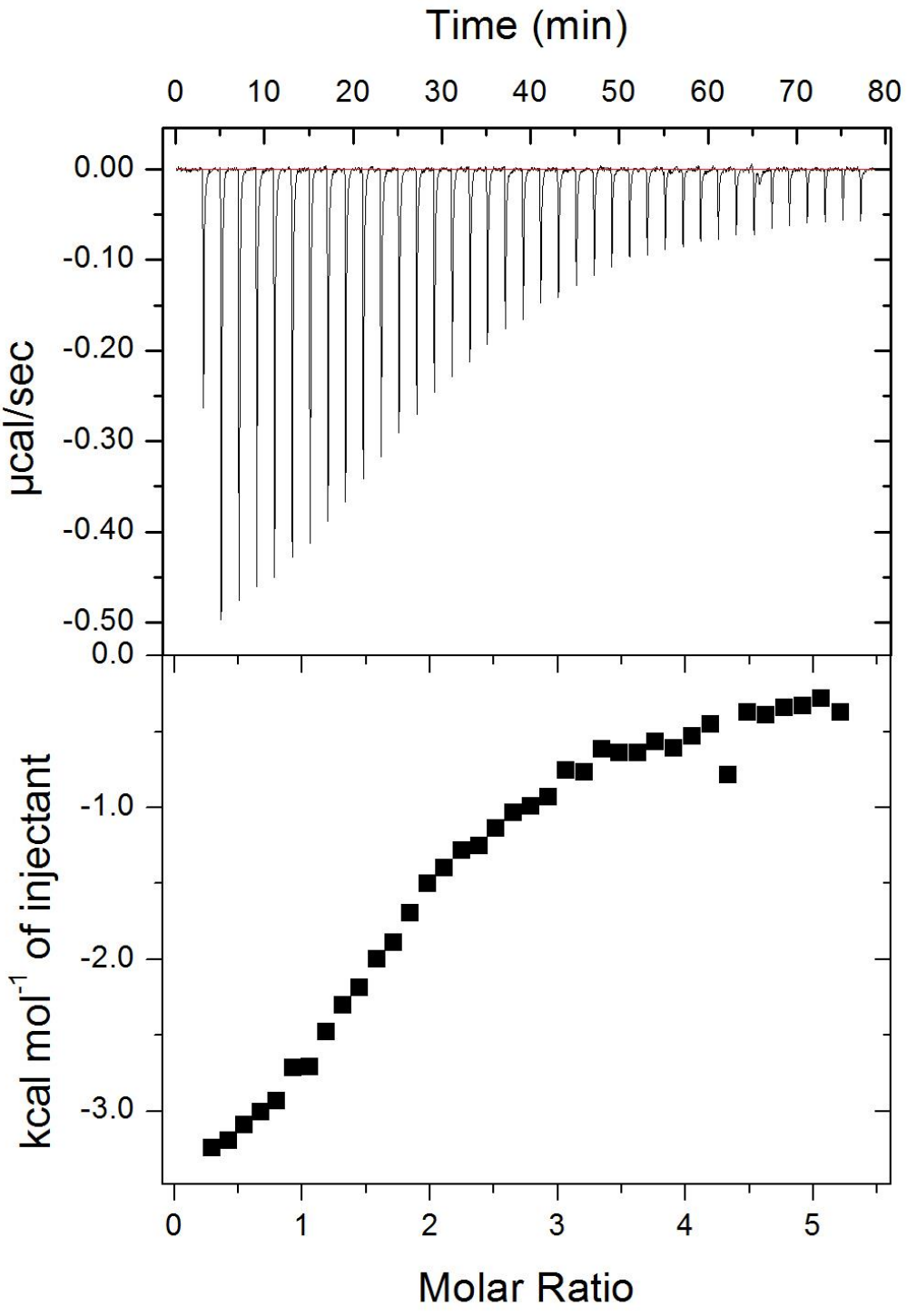

Figure A1.6. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 5 mol. eq. of laurate, corresponding to data shown in Figure 2.4B.

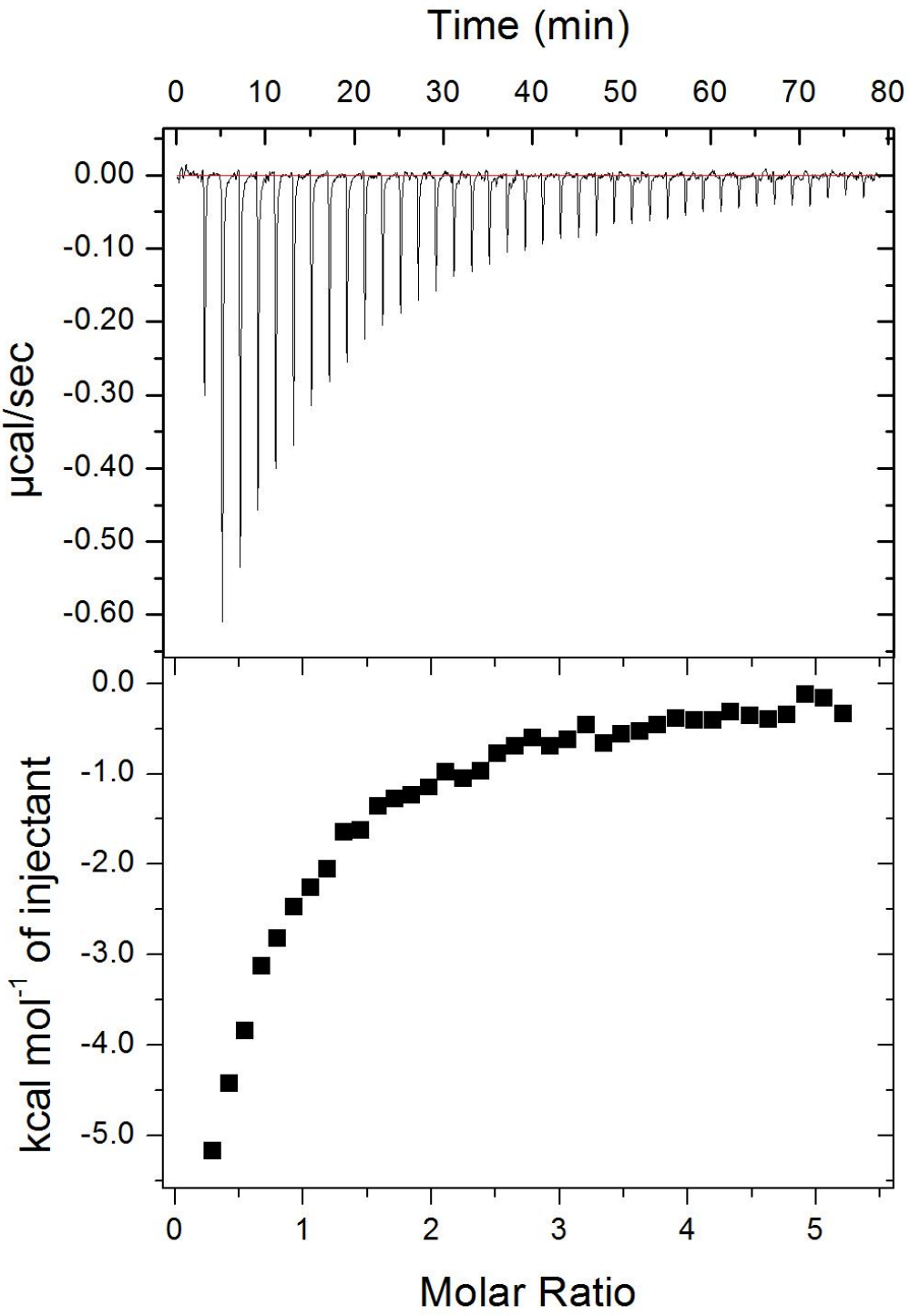

Figure A1.7. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 3 mol. eq. of myristate, corresponding to data shown in Figure 2.4C.

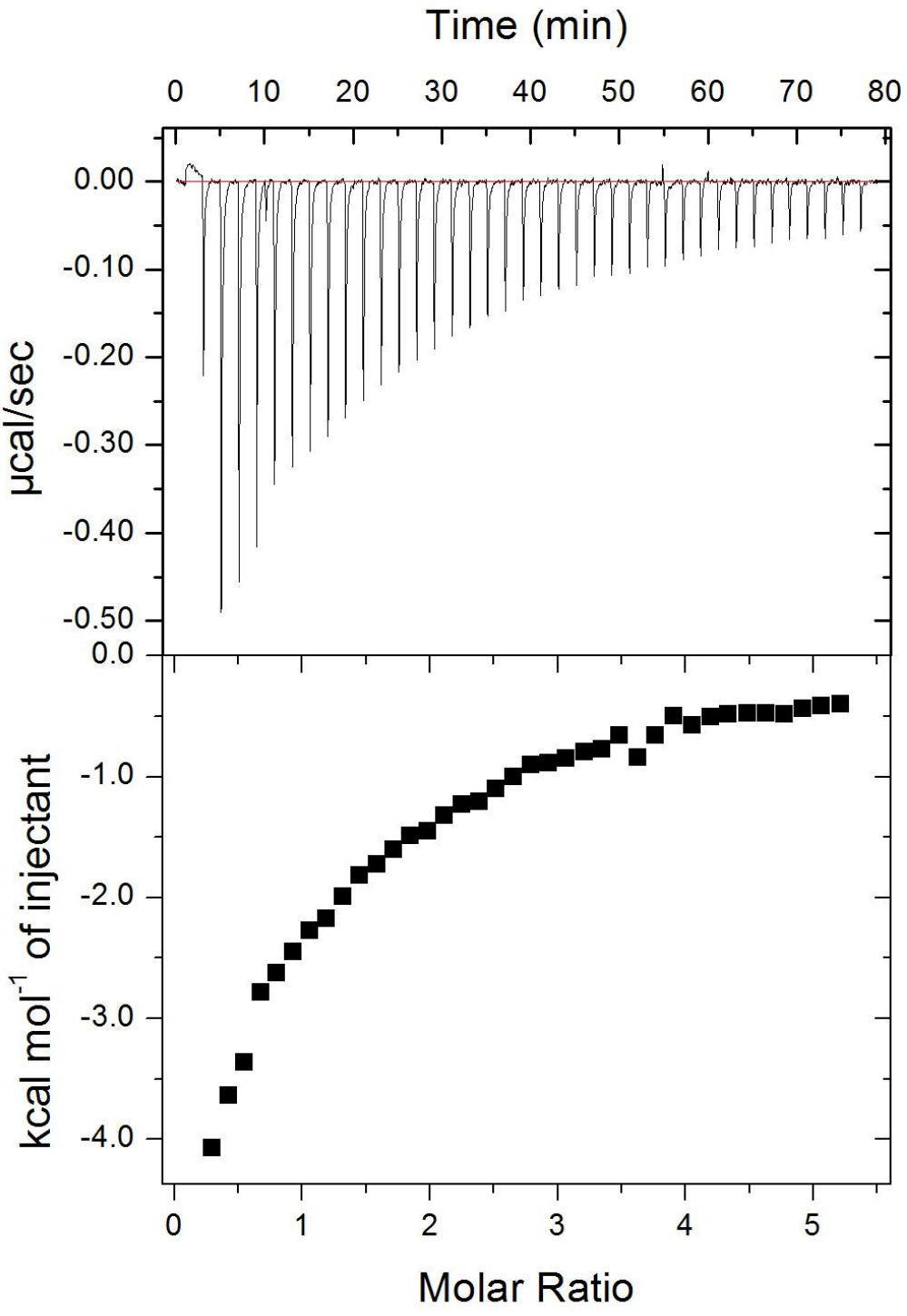

Figure A1.8. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 4 mol. eq. of myristate, corresponding to data shown in Figure 2.4C.

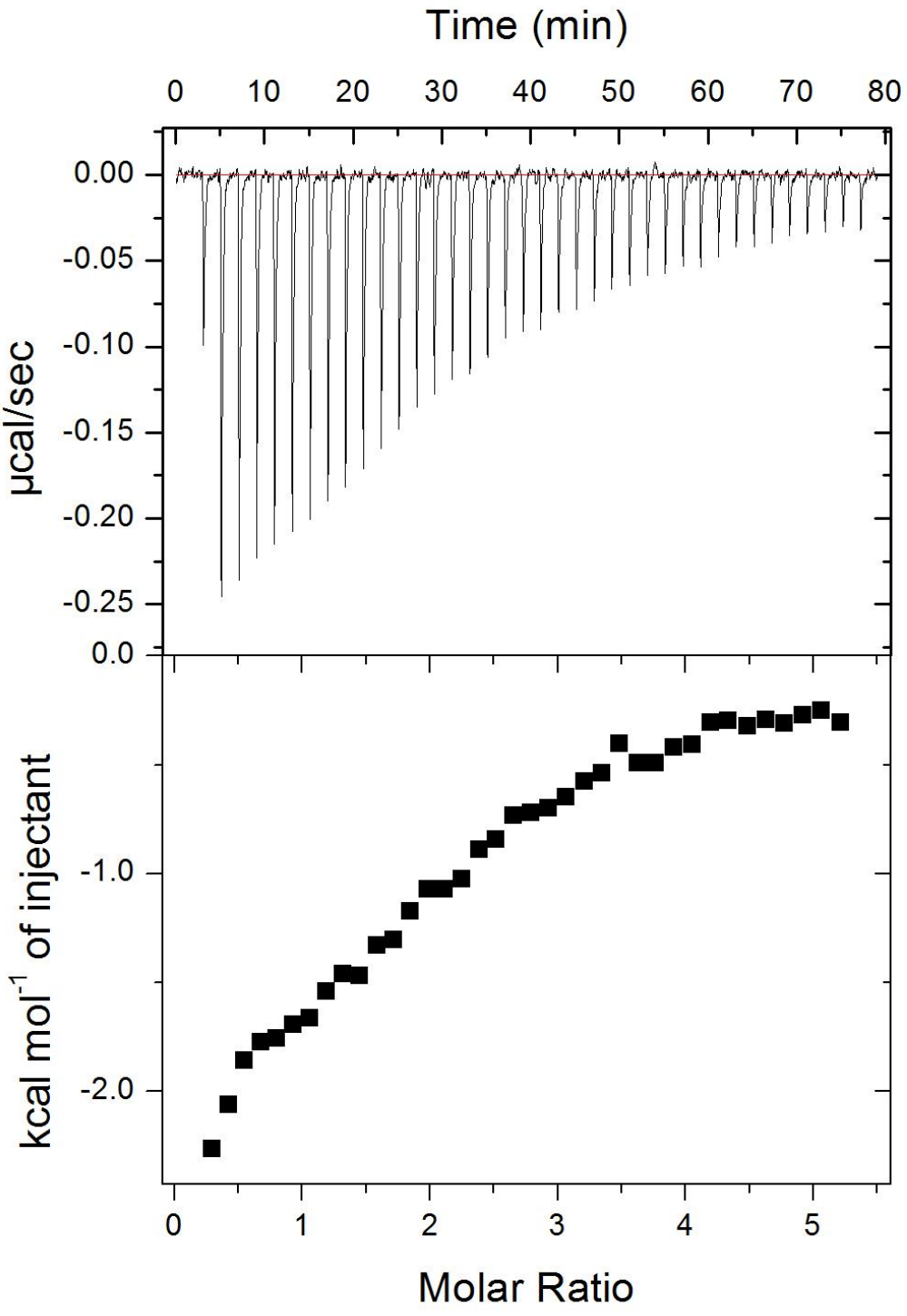

Figure A1.9. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 5 mol. eq. of myristate, corresponding to data shown in Figure 2.4C.

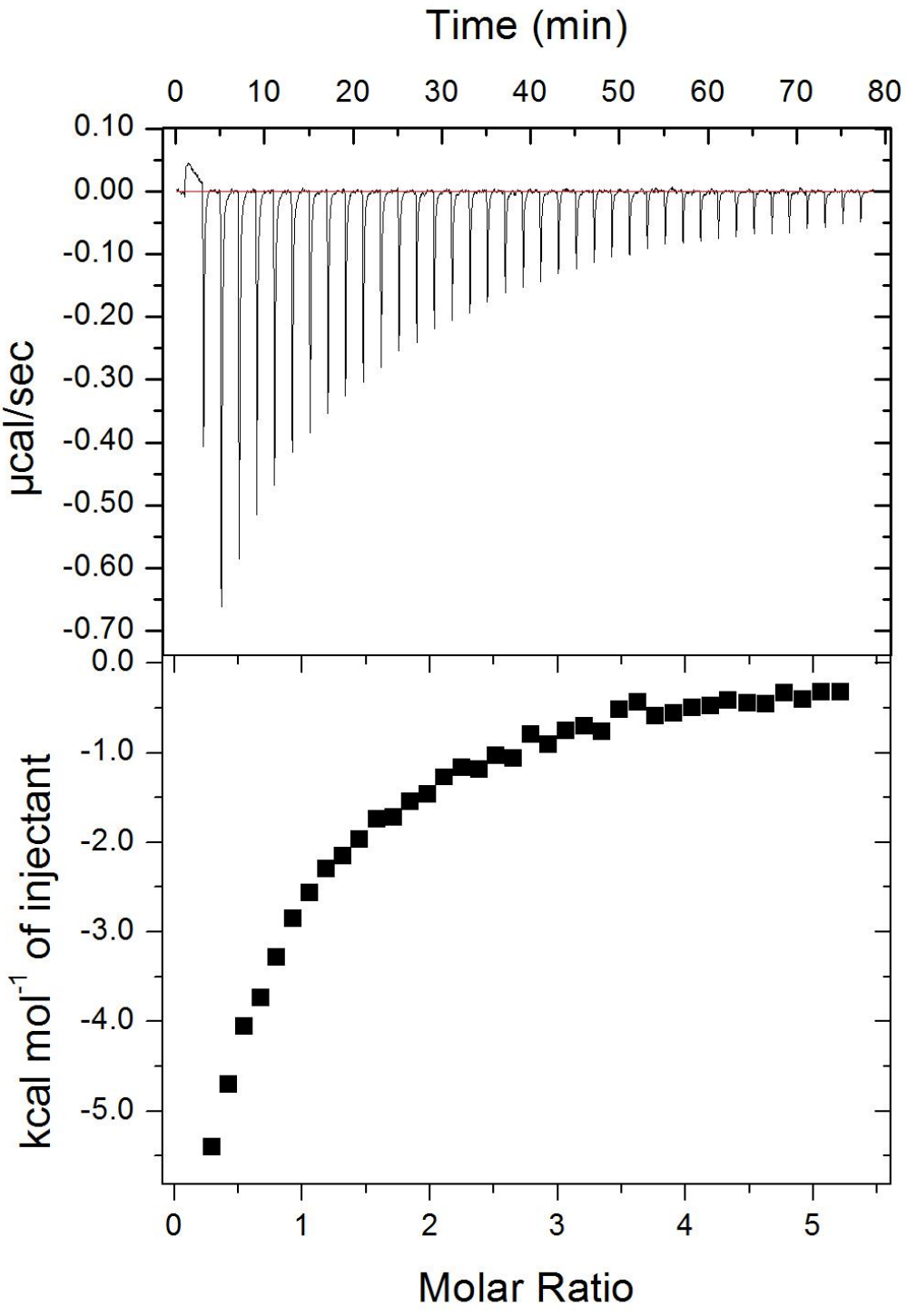

Figure A1.10. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 3 mol. eq. of palmitate, corresponding to data shown in Figure 2.4D.

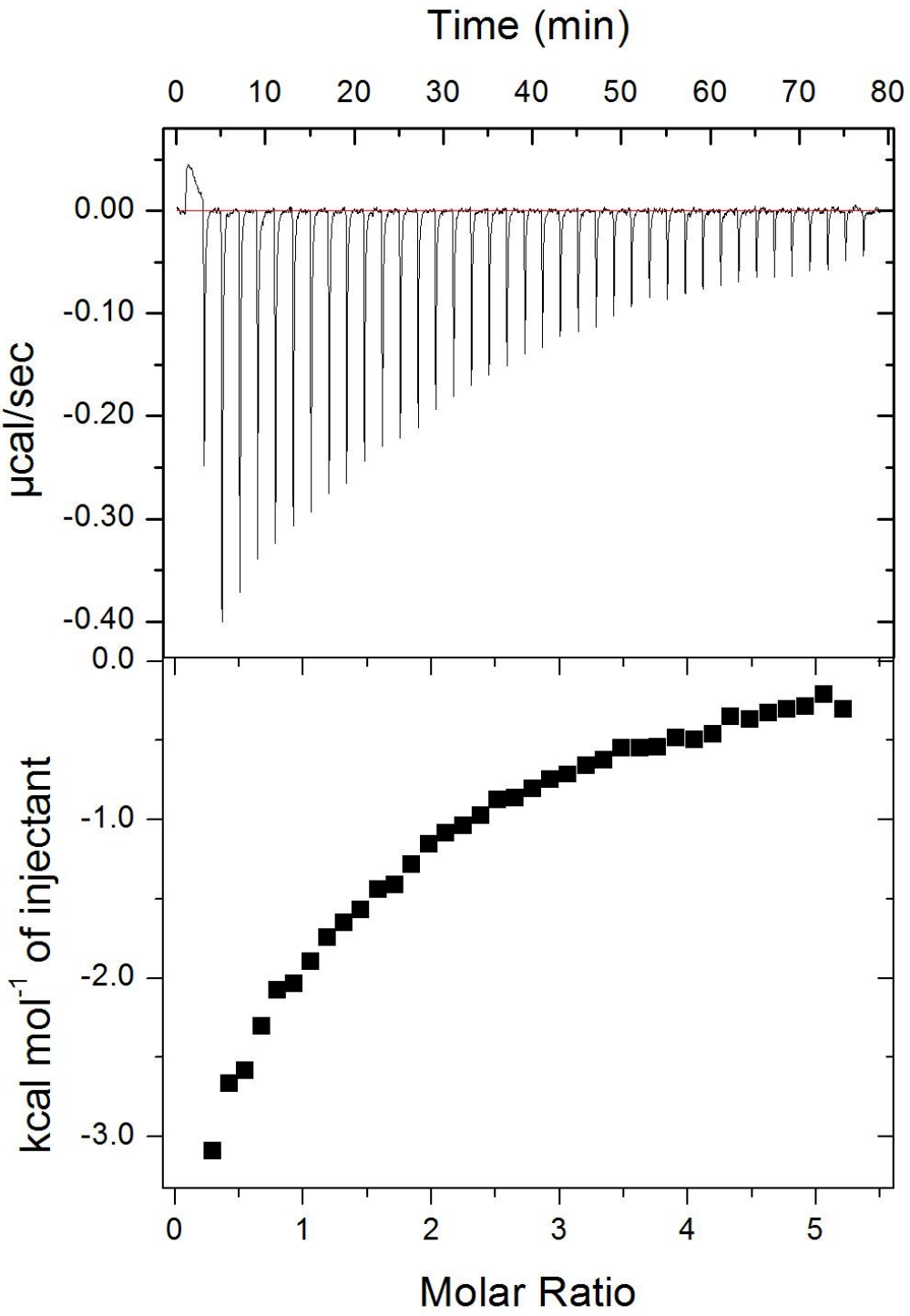

Figure A1.11. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 4 mol. eq. of palmitate, corresponding to data shown in Figure 2.4D.

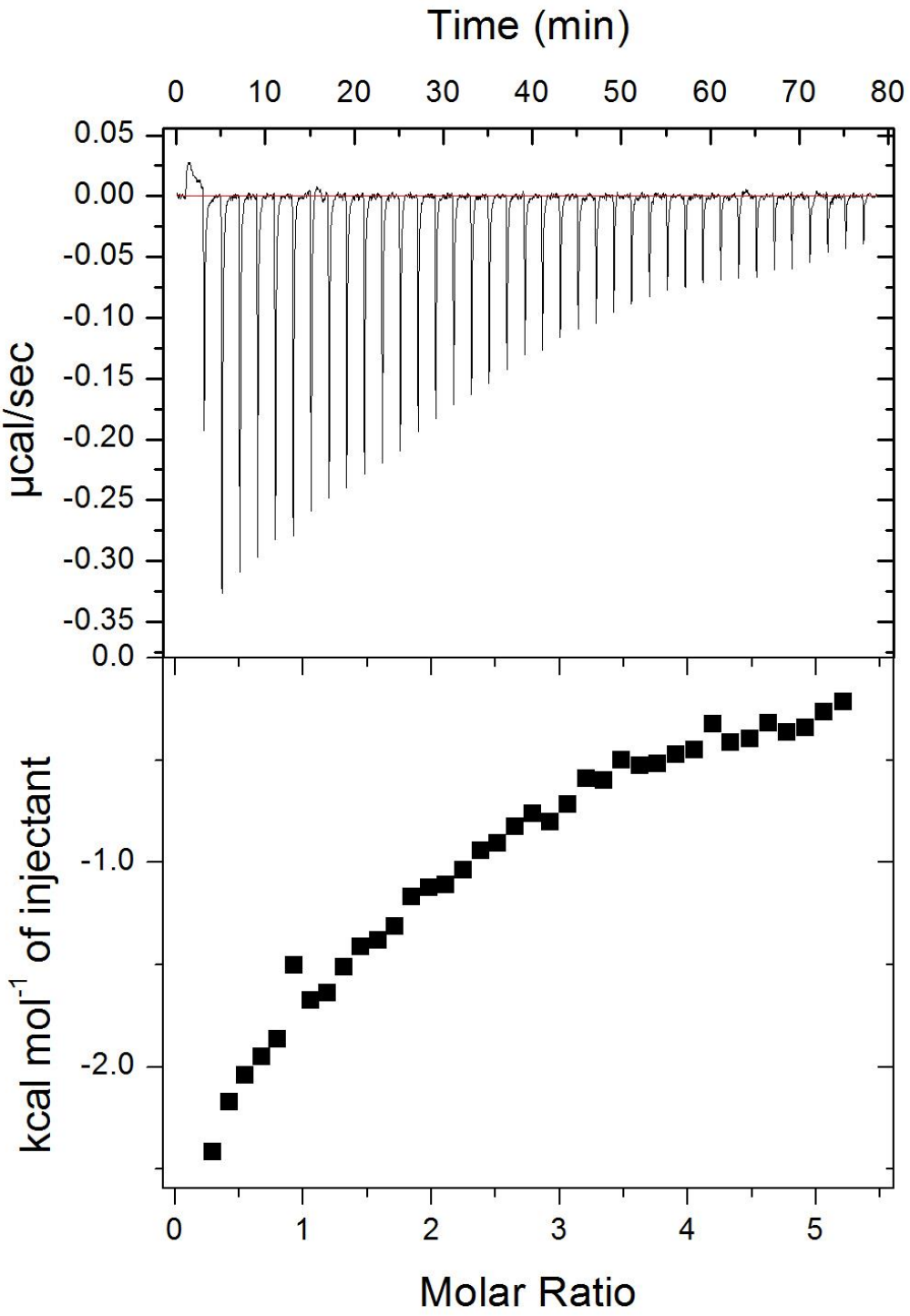

Figure A1.12. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 5 mol. eq. of palmitate, corresponding to data shown in Figure 2.4D.

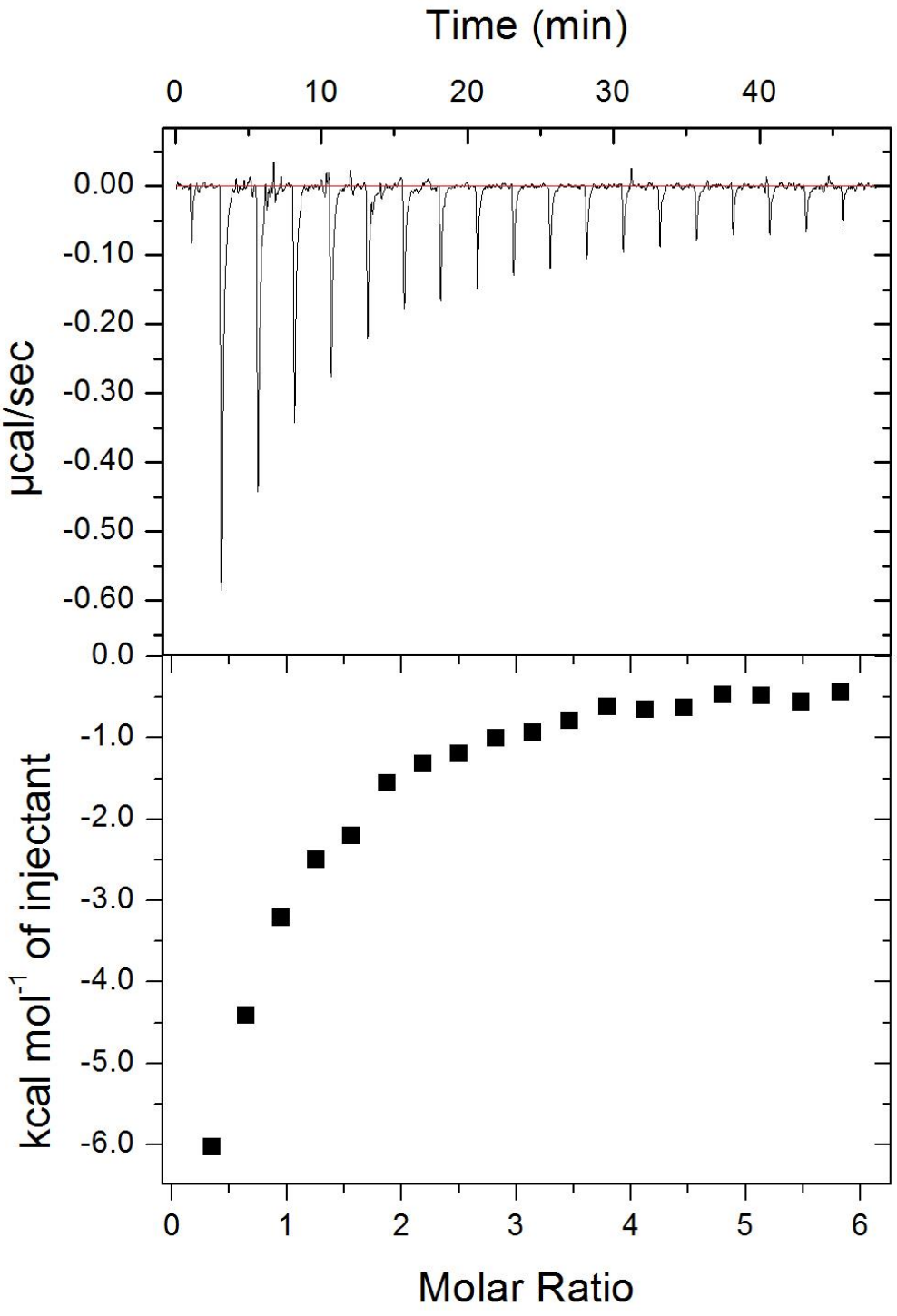

Figure A1.13. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the absence of FFA, corresponding to data shown in Figure 2.5A-D.

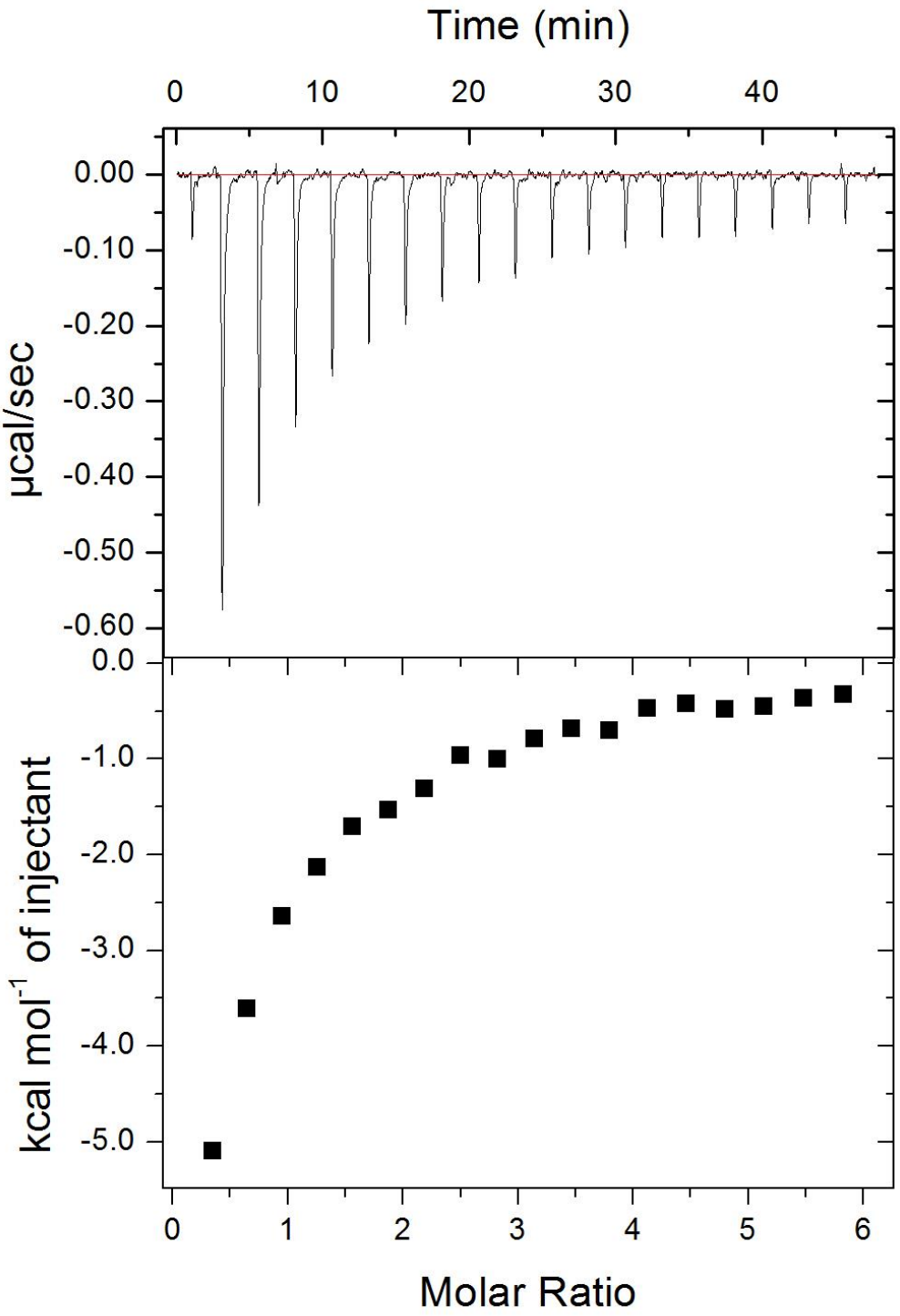

Figure A1.14. **Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 2.5 mol. eq. of palmitate, corresponding to data shown in Figure 2.5A.**

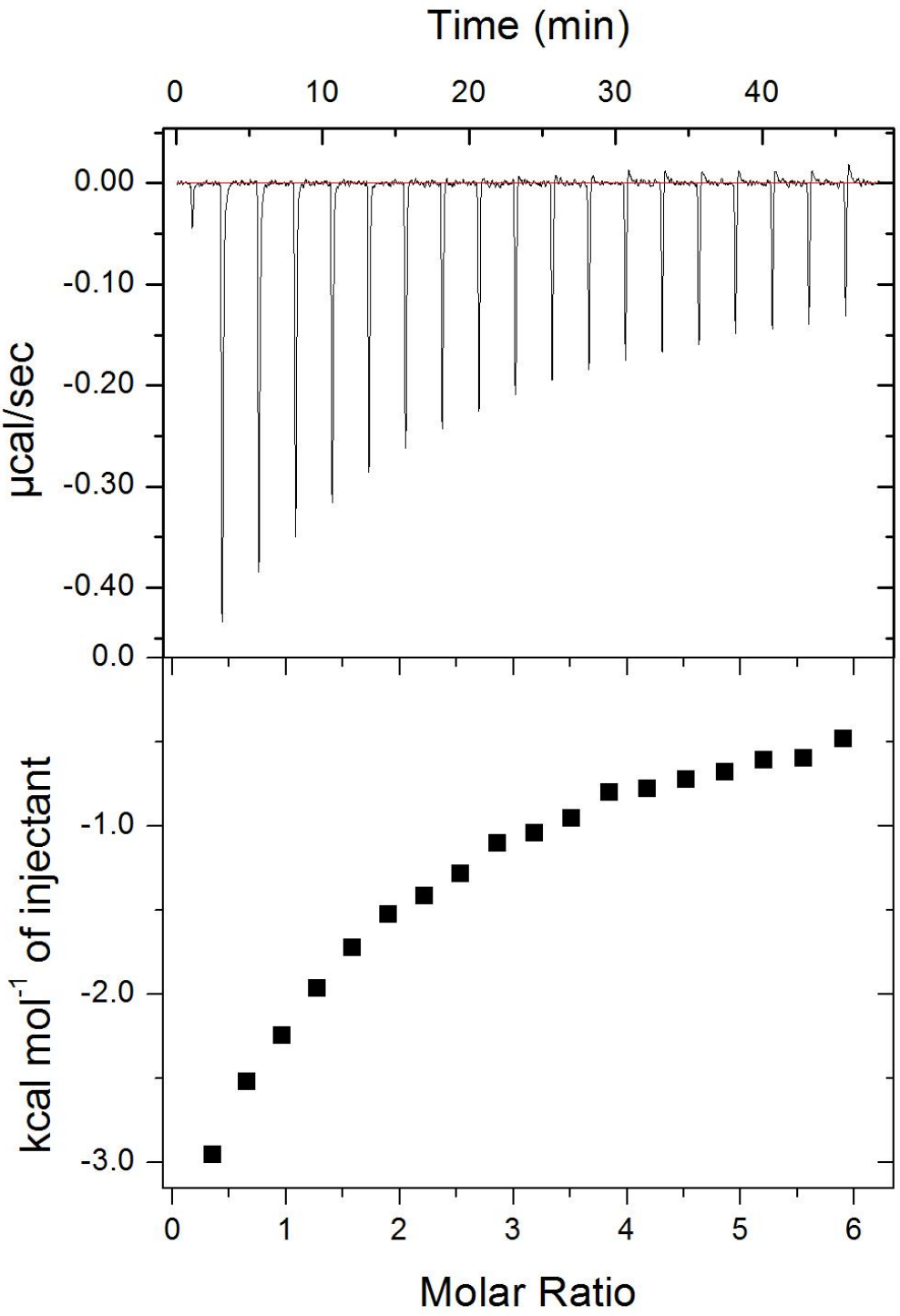

Figure A1.15. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 4 mol. eq. of palmitate, corresponding to data shown in Figure 2.5A.

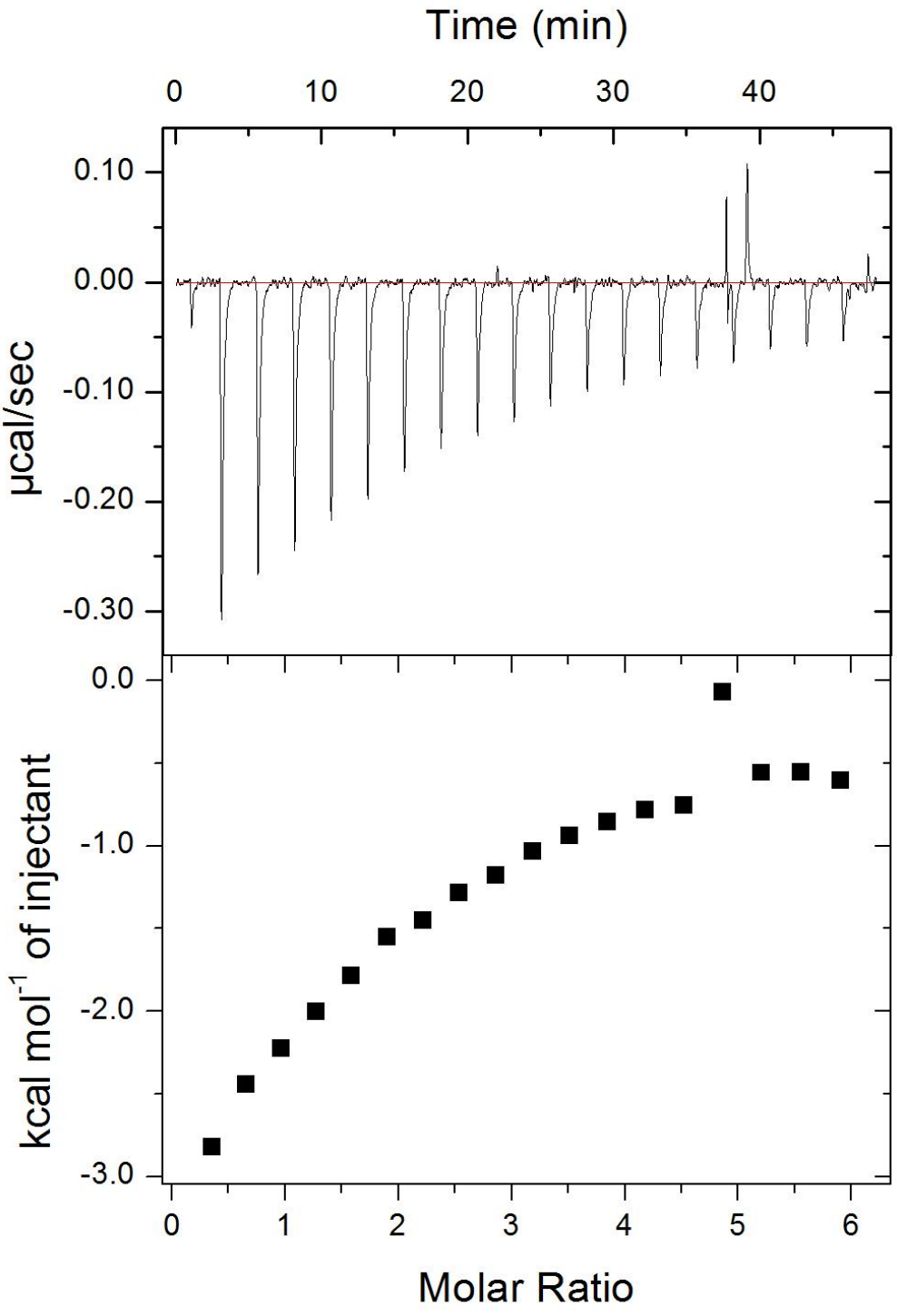

Figure A1.16. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 5 mol. eq. of palmitate, corresponding to data shown in Figure 2.5A.

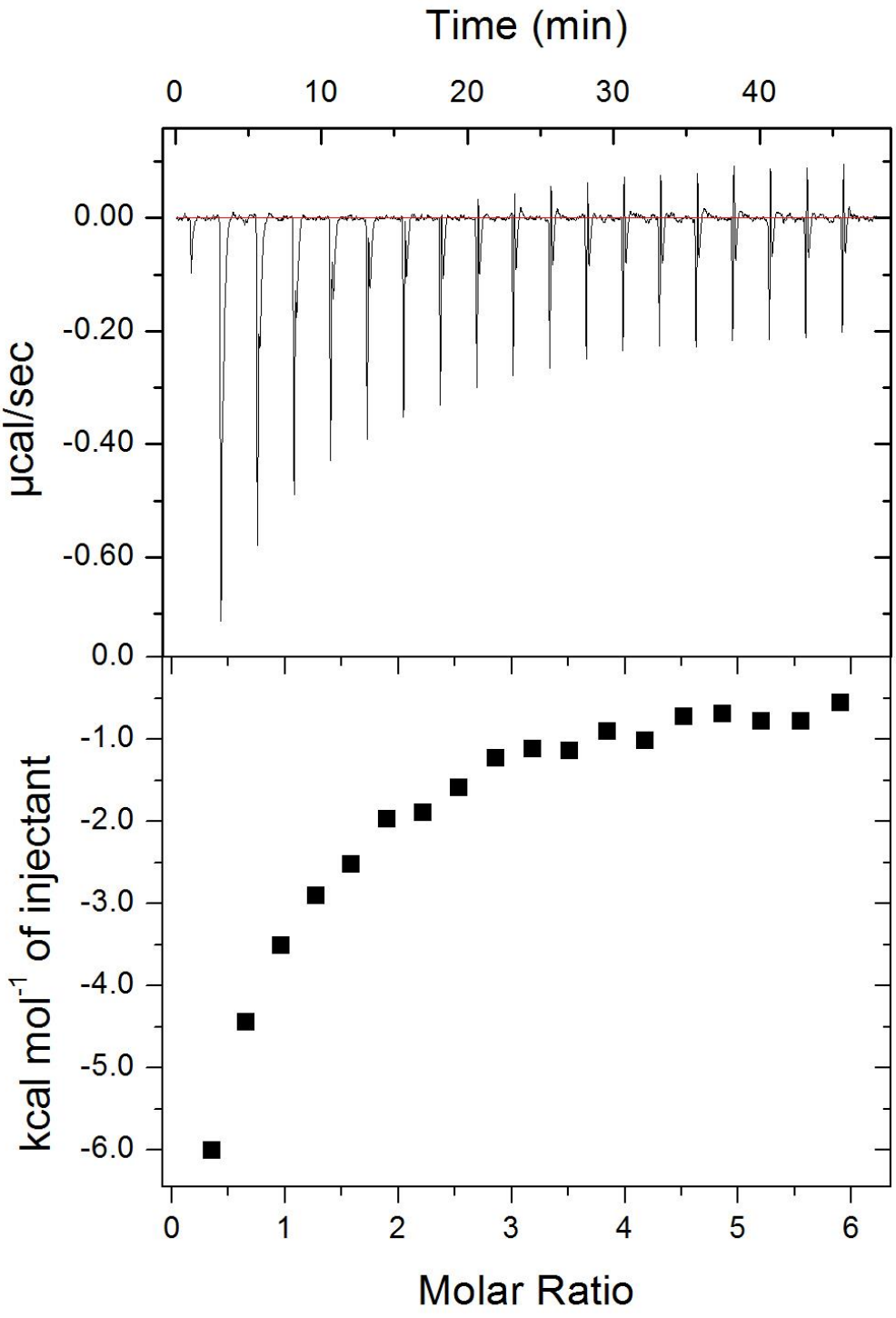

Figure A1.17. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 2.5 mol. eq. of palmitoleate, corresponding to data shown in Figure 2.5B.

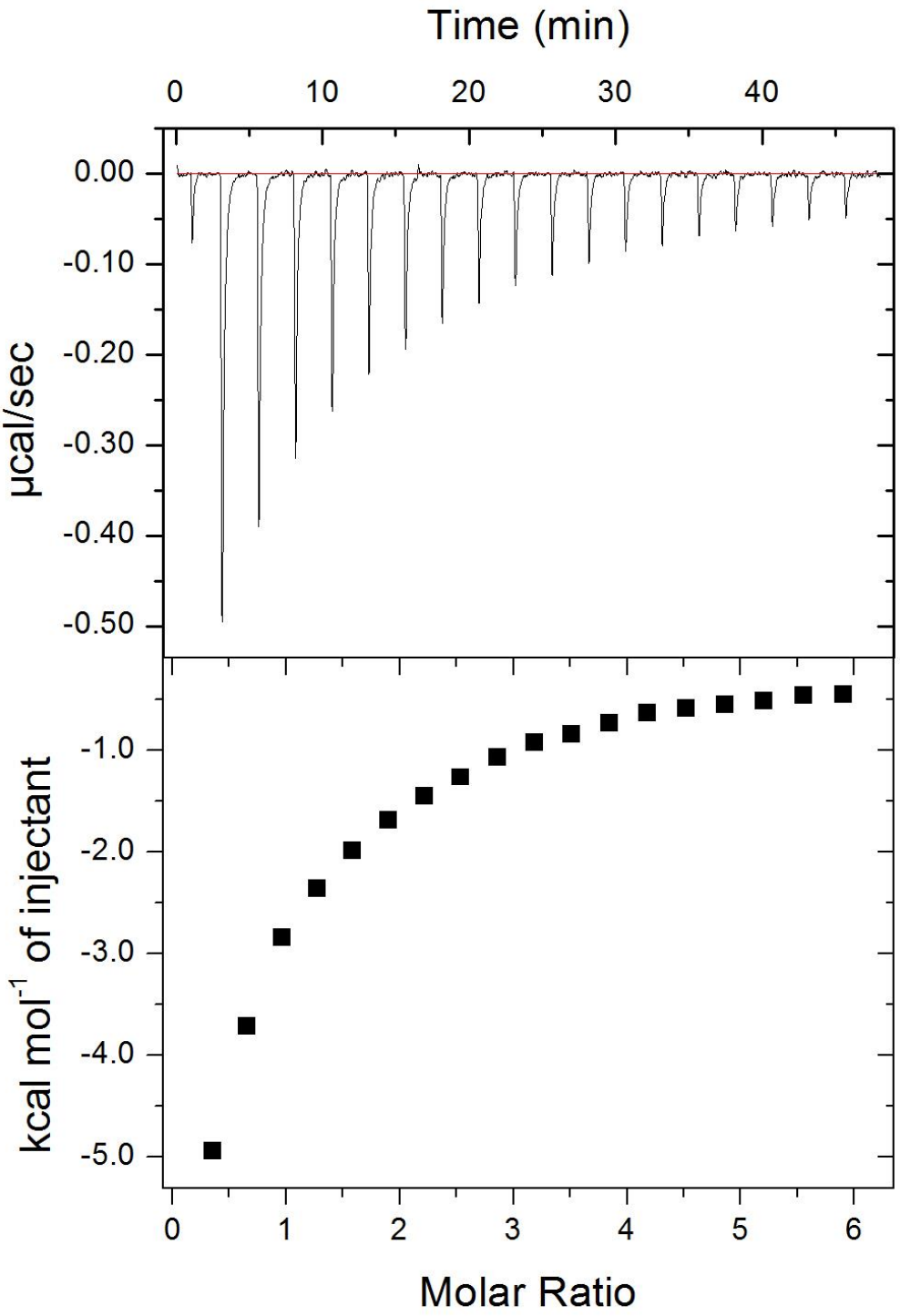

Figure A1.18. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 4 mol. eq. of palmitoleate, corresponding to data shown in Figure 2.5B.

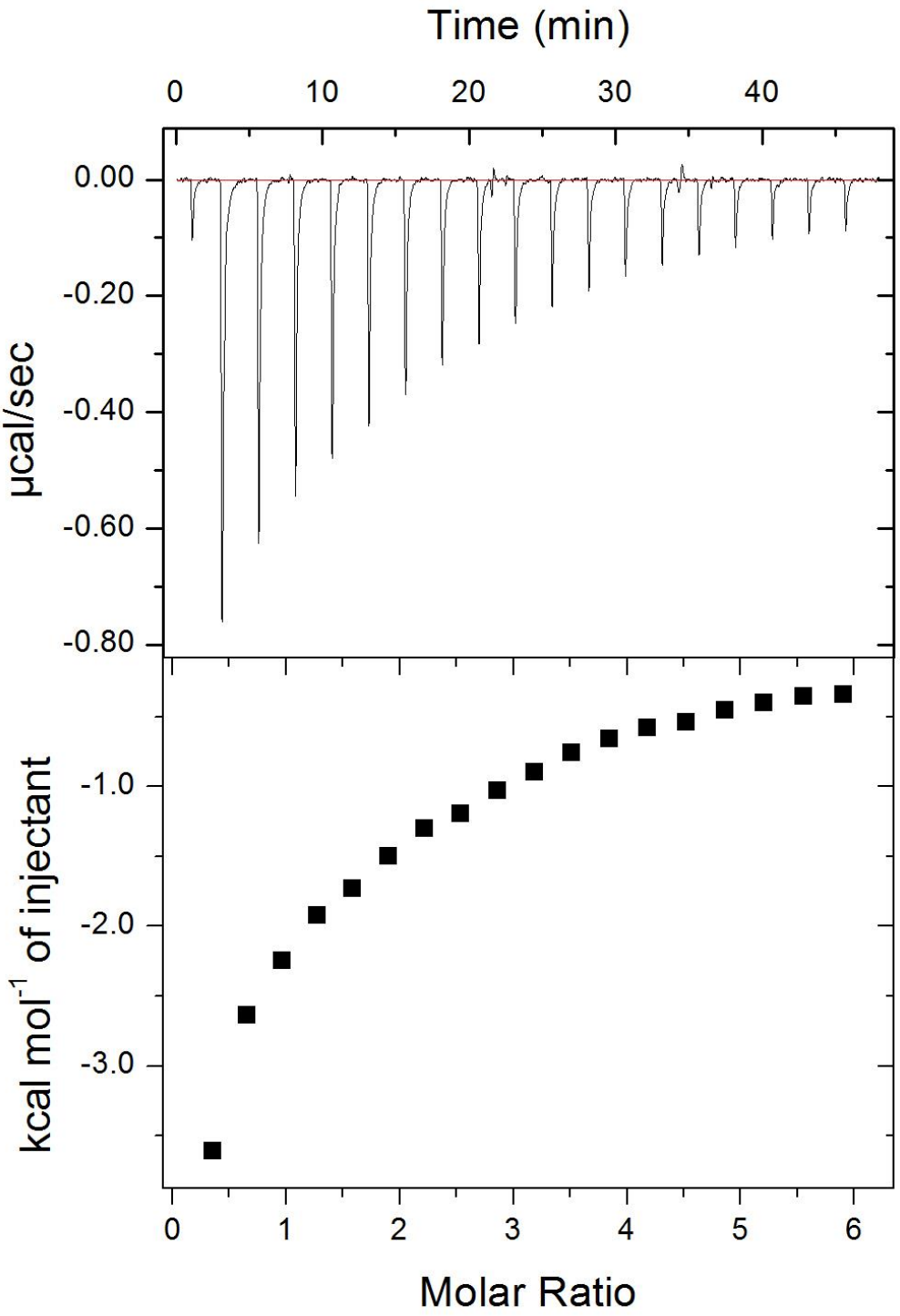

Figure A1.19. **Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 5 mol. eq. of palmitoleate, corresponding to data shown in Figure 2.5B.**

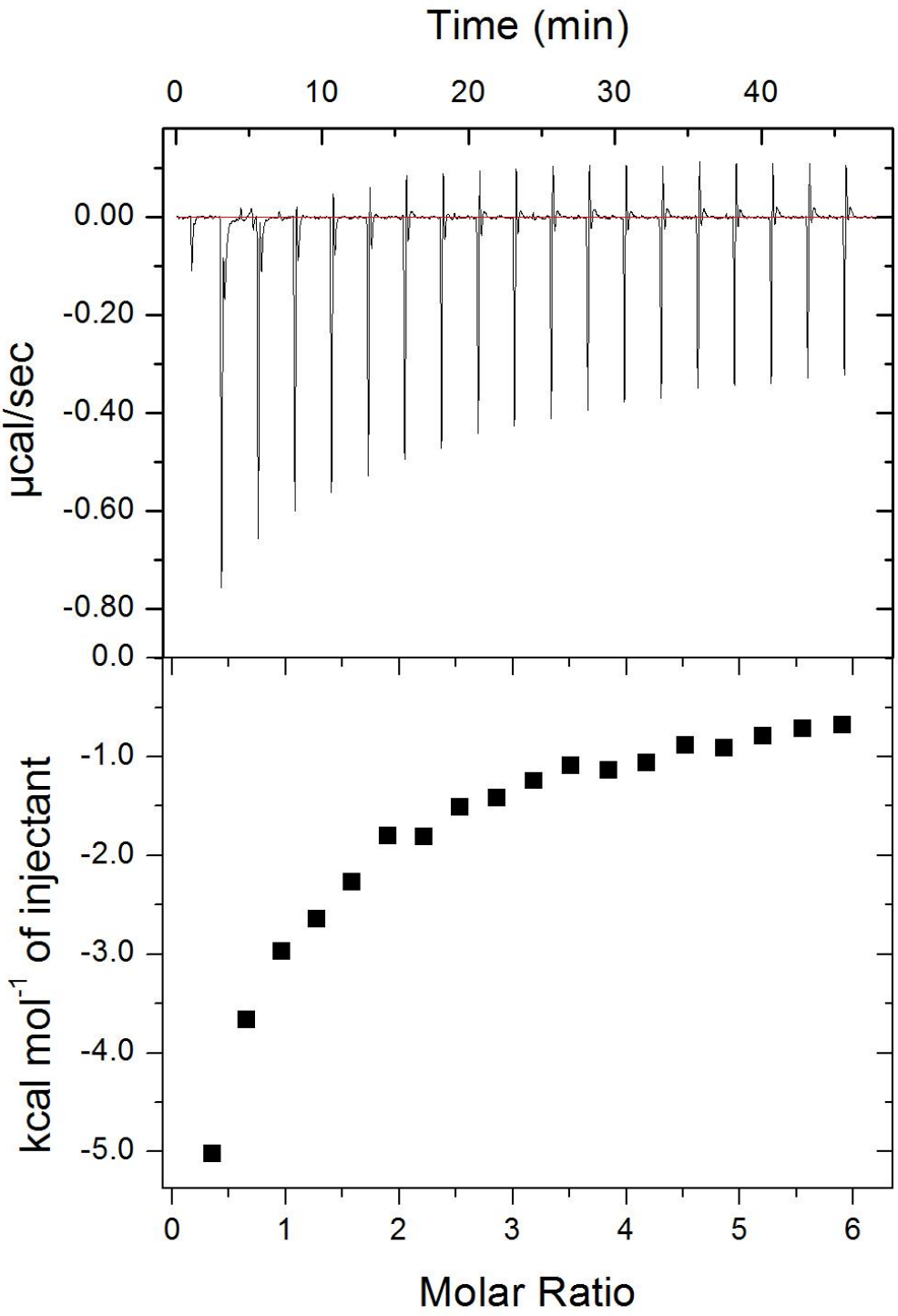

Figure A1.20. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 2.5 mol. eq. of palmitelaidate, corresponding to data shown in Figure 2.5C.

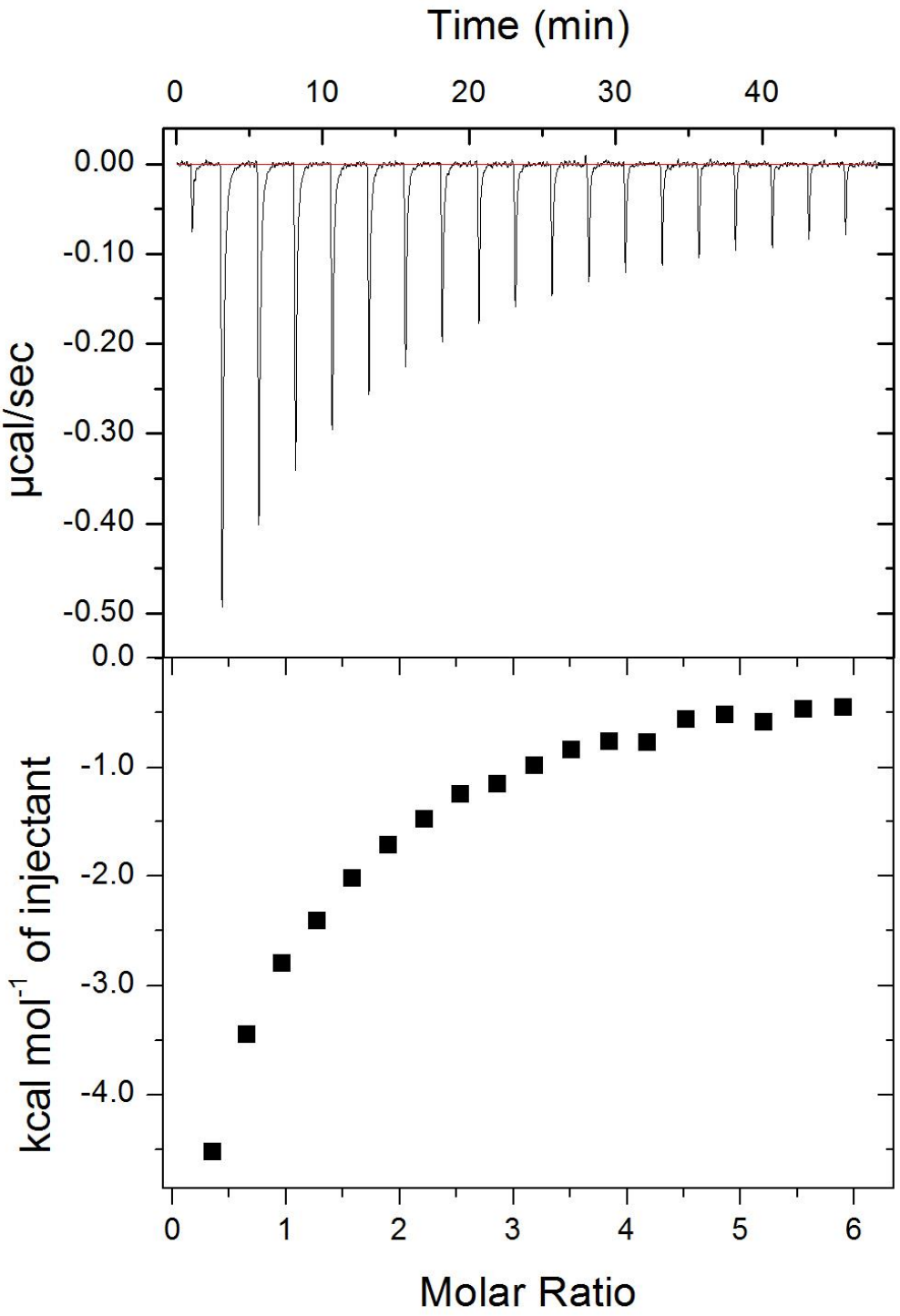

Figure A1.21. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 4 mol. eq. of palmitelaidate, corresponding to data shown in Figure 2.5C.

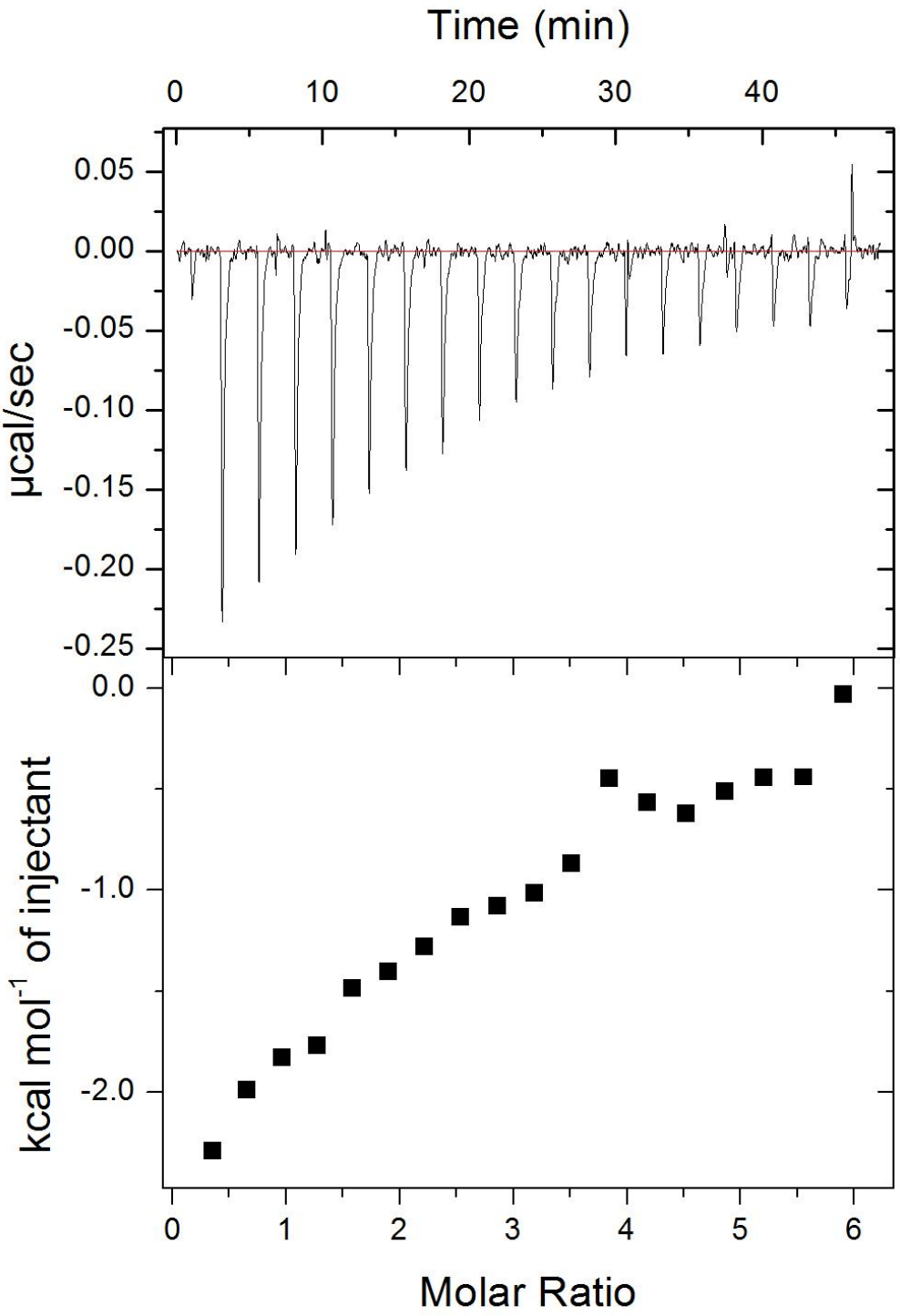

Figure A1.22. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 5 mol. eq. of palmitelaidate, corresponding to data shown in Figure 2.5C.

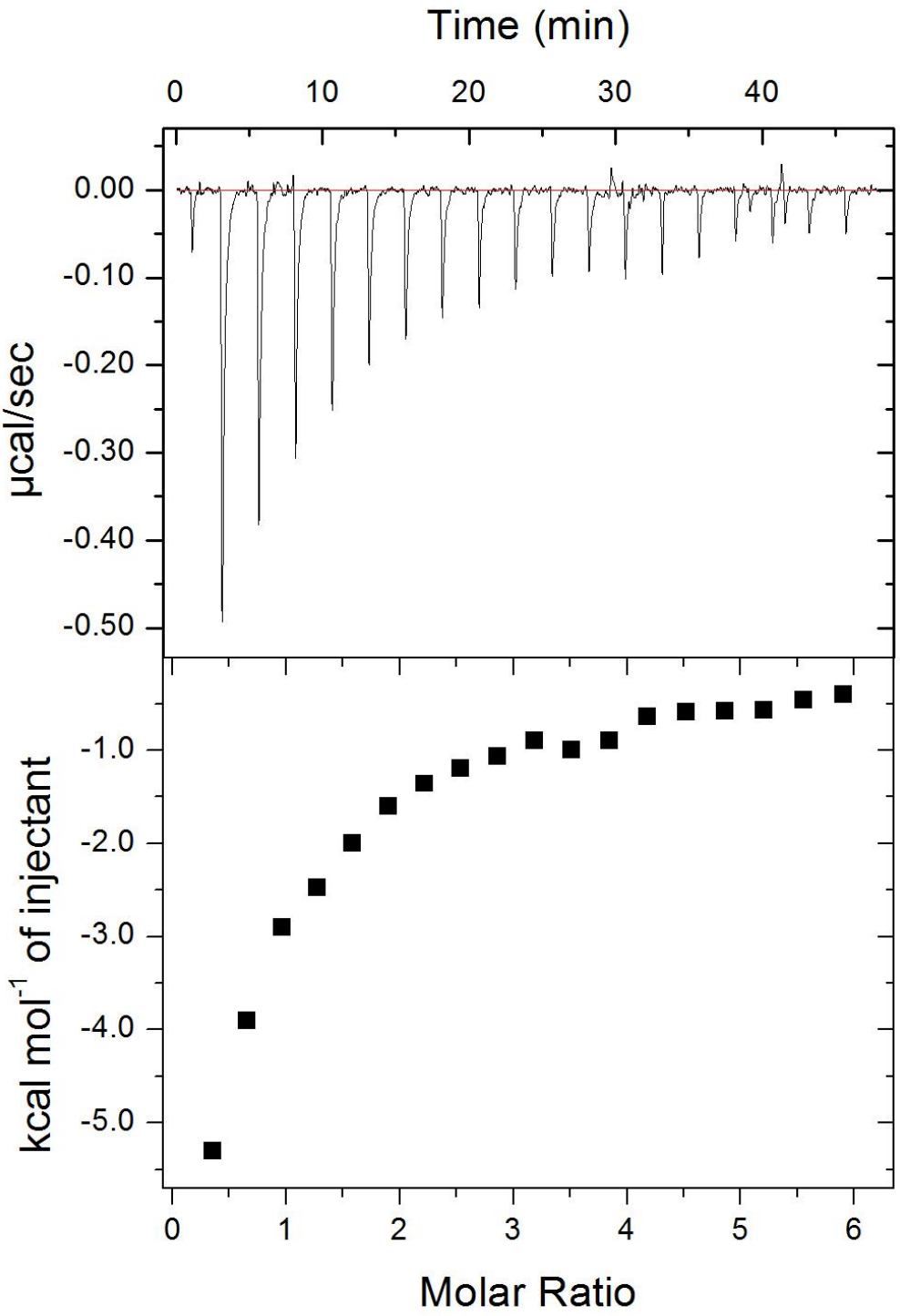

Figure A1.23. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 2.5 mol. eq. of stearate, corresponding to data shown in Figure 2.5D.

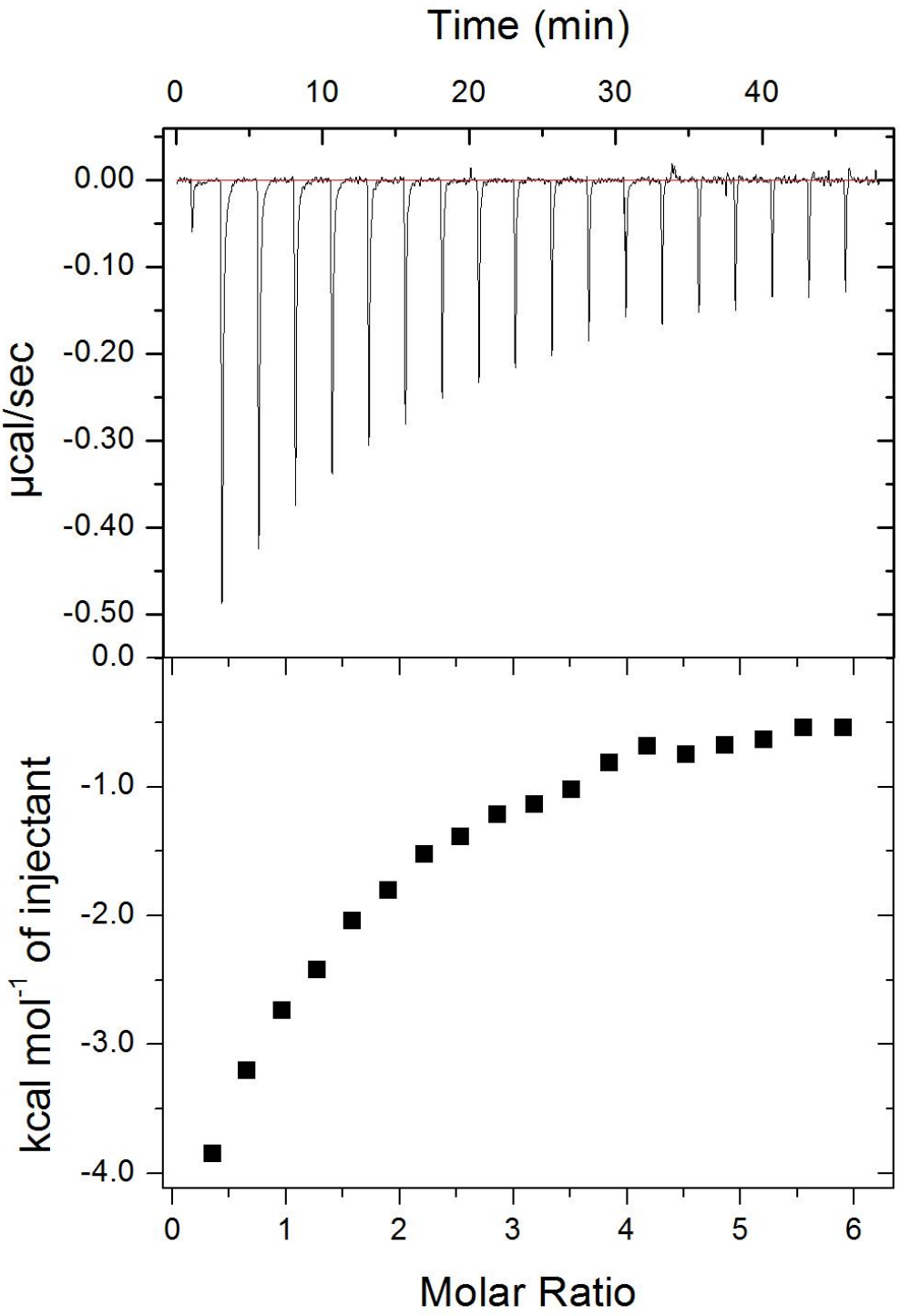

Figure A1.24. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 4 mol. eq. of stearate, corresponding to data shown in Figure 2.5D.

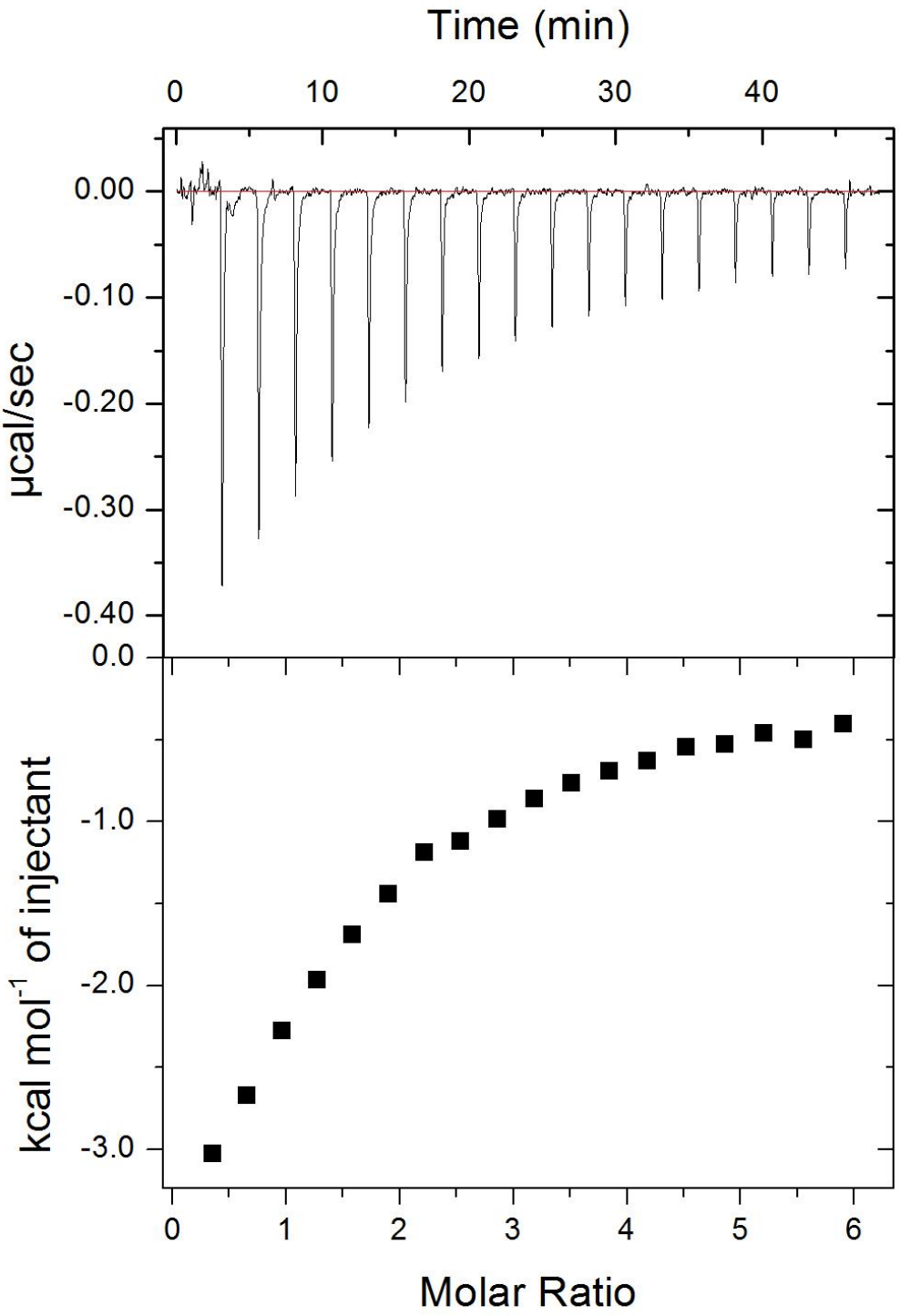

Figure A1.25. Full ITC data (including raw data) for Zn^{2+} binding to HSA in the presence of 5 mol. eq. of stearate, corresponding to data shown in Figure 2.5D.

Appendix 2: Representative SEM images

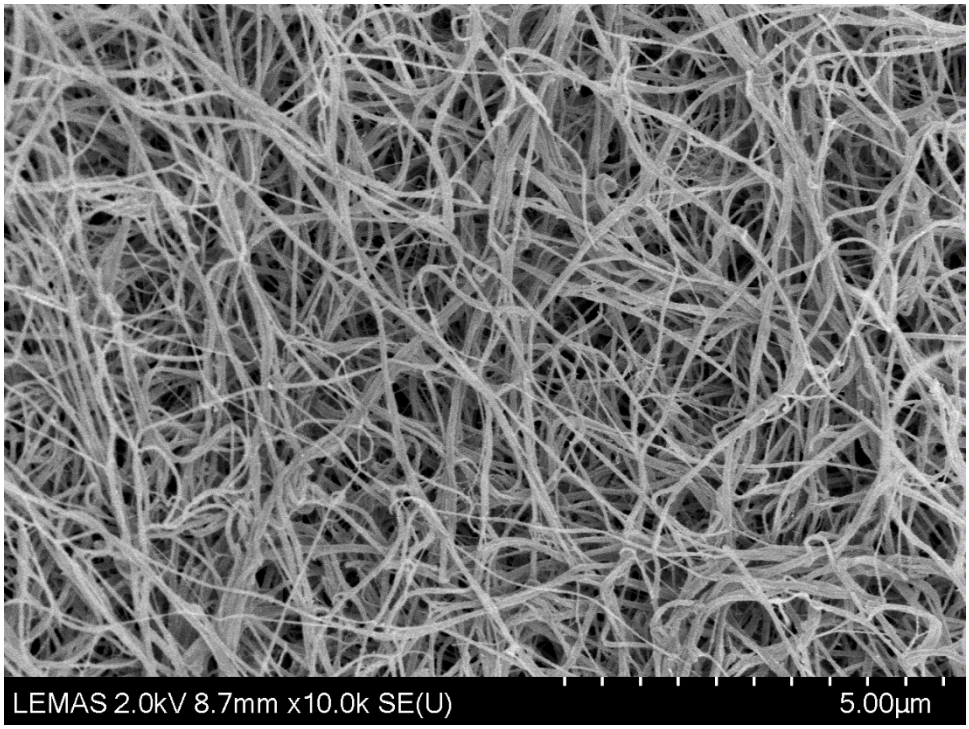

Figure A2.1. Representative image from the SEM experiments corresponding to the purified system in the absence of Zn^{2+}.
The image was viewed and photographed at ×10,000 magnification using a SU8230 scanning electron microscope.

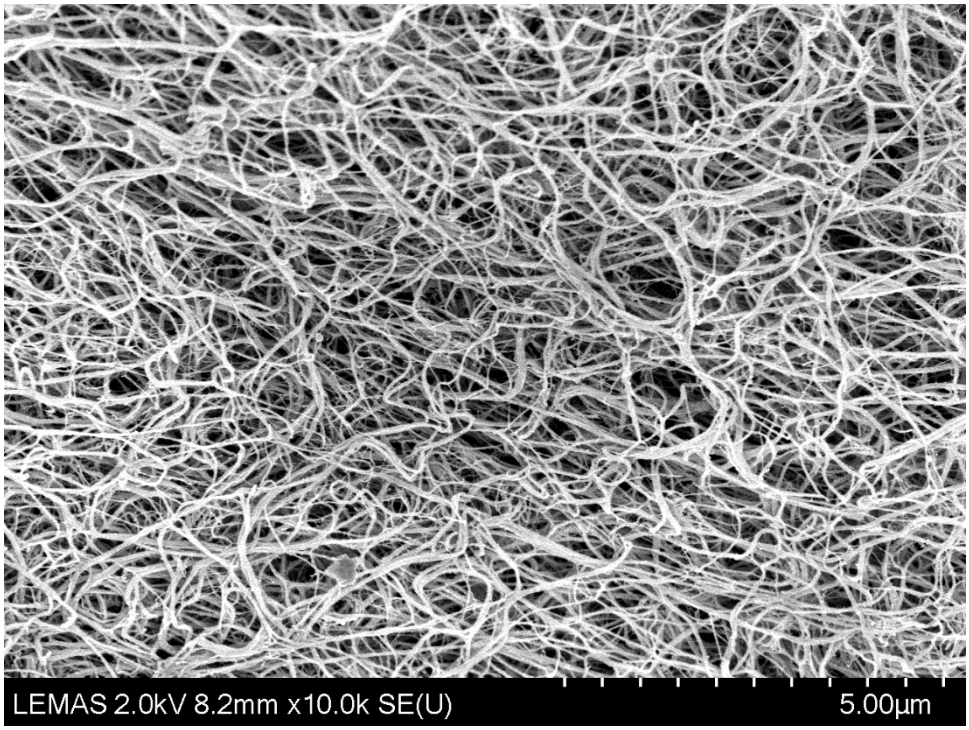

Figure A2.2. Representative image from the SEM experiments corresponding to the purified system in the presence of 20 µM Zn^{2+}.
The image was viewed and photographed at ×10,000 magnification using a SU8230 scanning electron microscope.

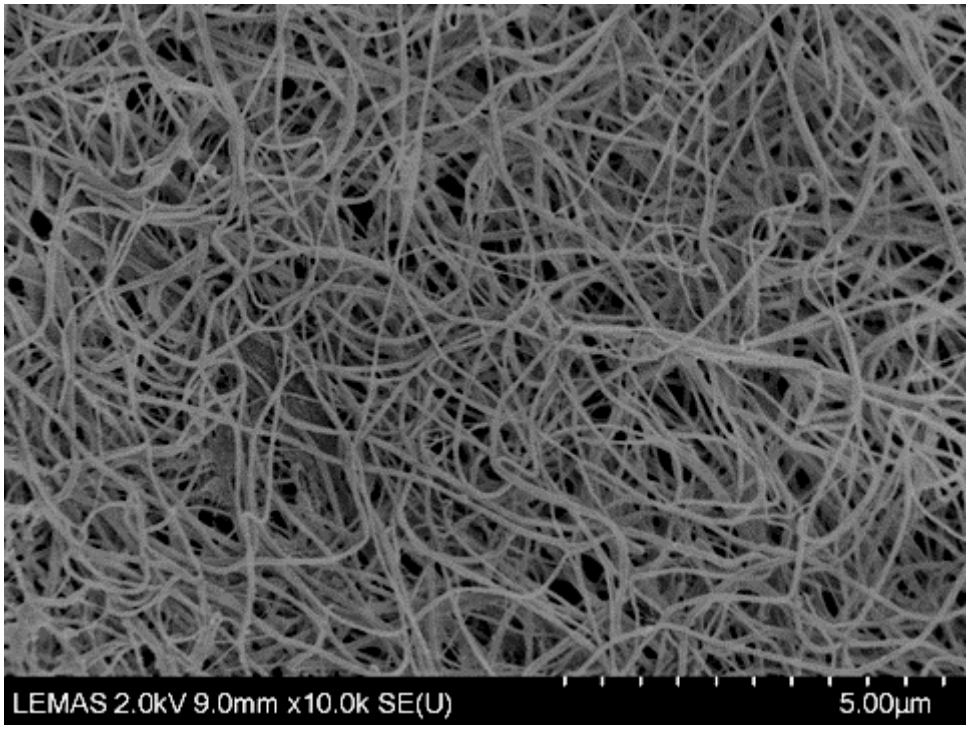

Figure A2.3. Representative image from the SEM experiments corresponding to the samples from healthy controls in the absence of Zn^{2+}.
The image was viewed and photographed at ×10,000 magnification using a SU8230 scanning electron microscope.

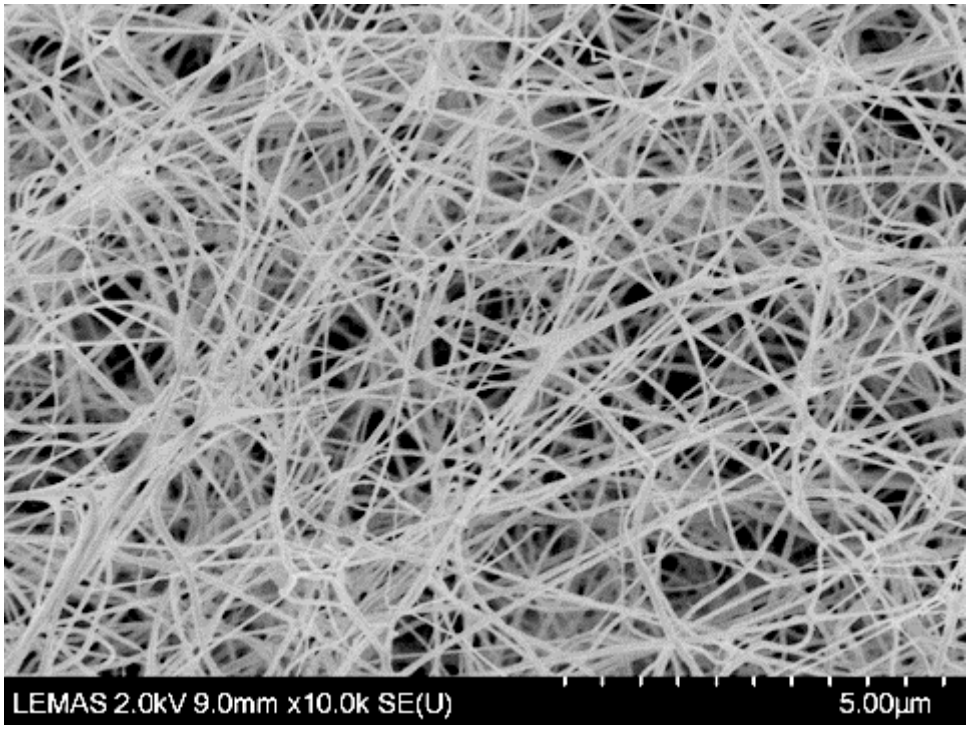

Figure A2.4. Representative image from the SEM experiments corresponding to the samples from healthy controls in the presence of 20 µM Zn^{2+}.
The image was viewed and photographed at ×10,000 magnification using a SU8230 scanning electron microscope.

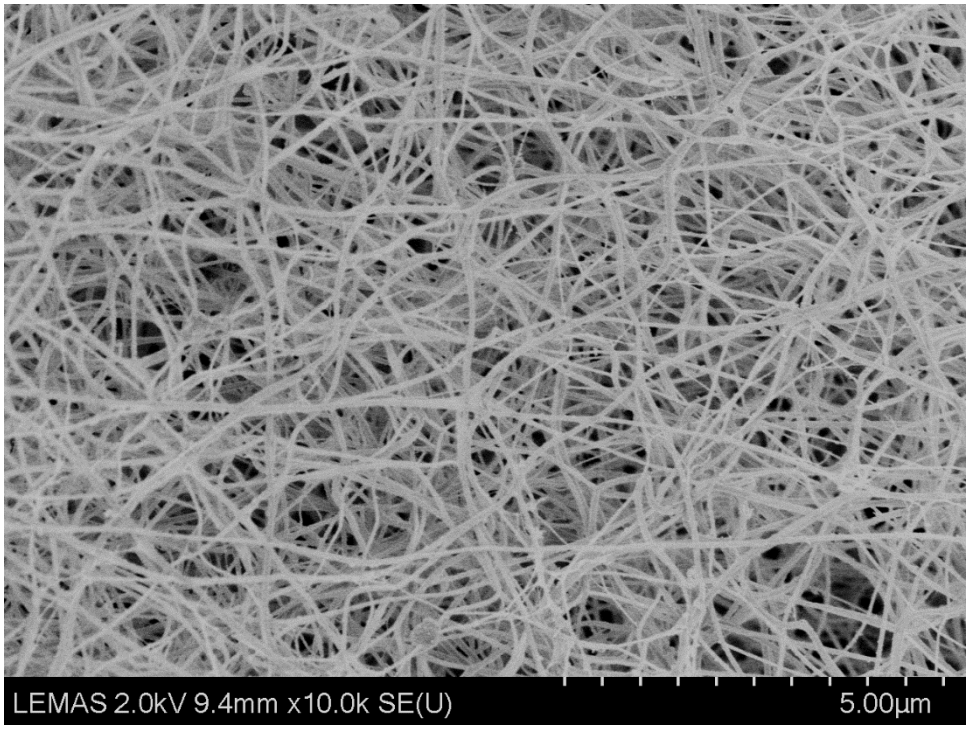

Figure A2.5. Representative image from the SEM experiments corresponding to the samples from subjects with T2DM in the absence of Zn^{2+}.
The image was viewed and photographed at ×10,000 magnification using a SU8230 scanning electron microscope.

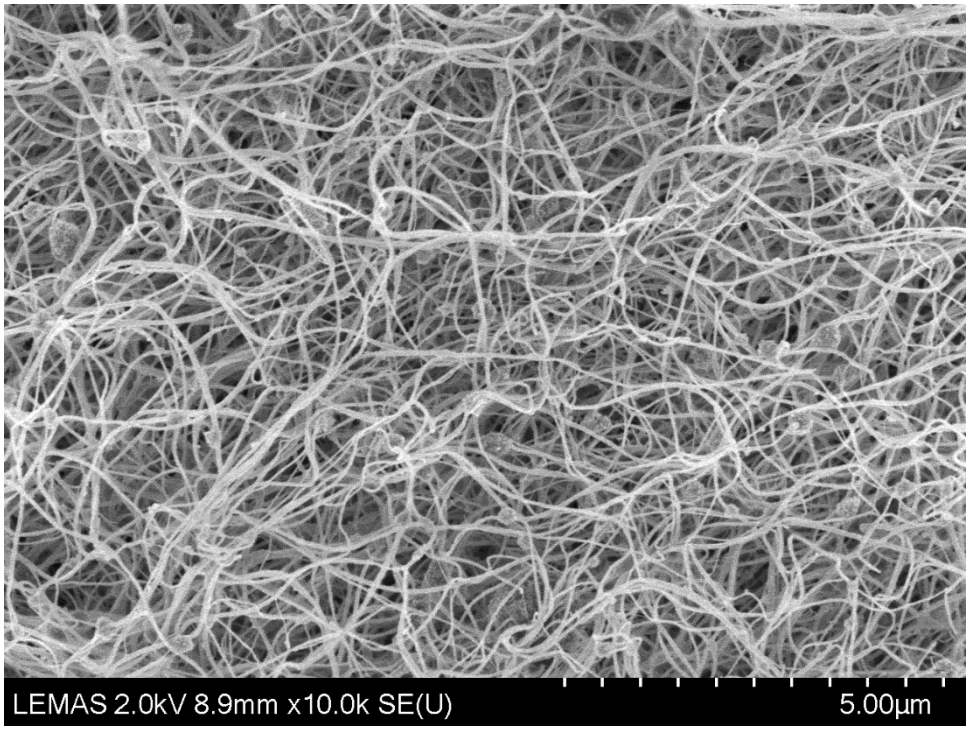

Figure A2.6. Representative image from the SEM experiments corresponding to the samples from subjects with T2DM in the presence of 20 µM Zn^{2+}.
The image was viewed and photographed at ×10,000 magnification using a SU8230 scanning electron microscope.

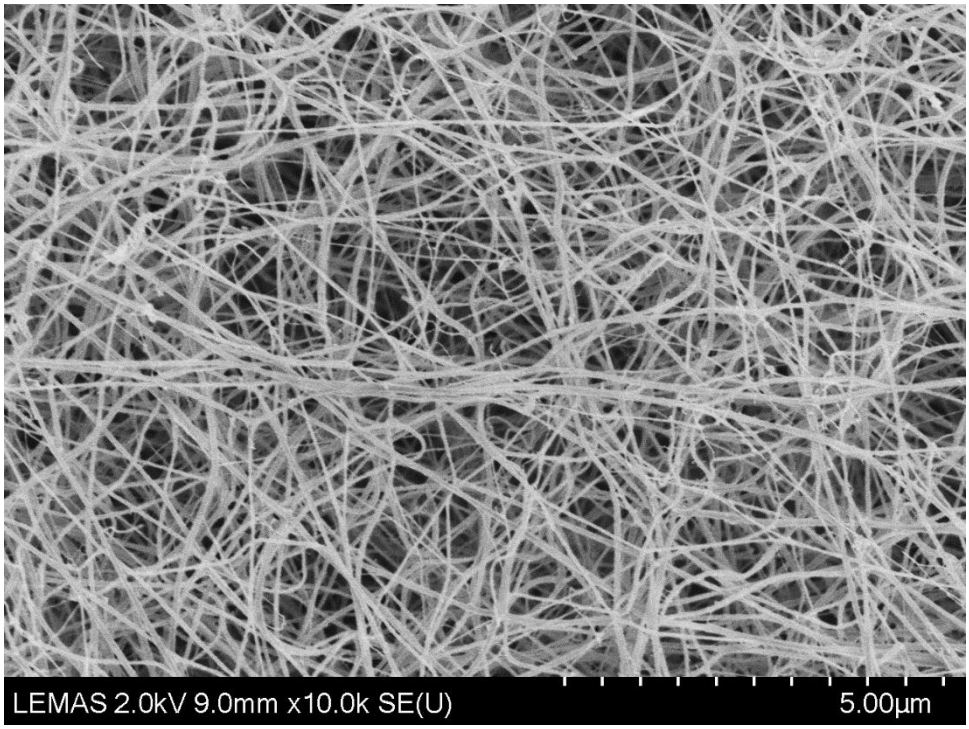

Figure A2.7. Representative image from the SEM experiments corresponding to the samples from subjects with T1DM in the absence of Zn^{2+}.
The image was viewed and photographed at ×10,000 magnification using a SU8230 scanning electron microscope.

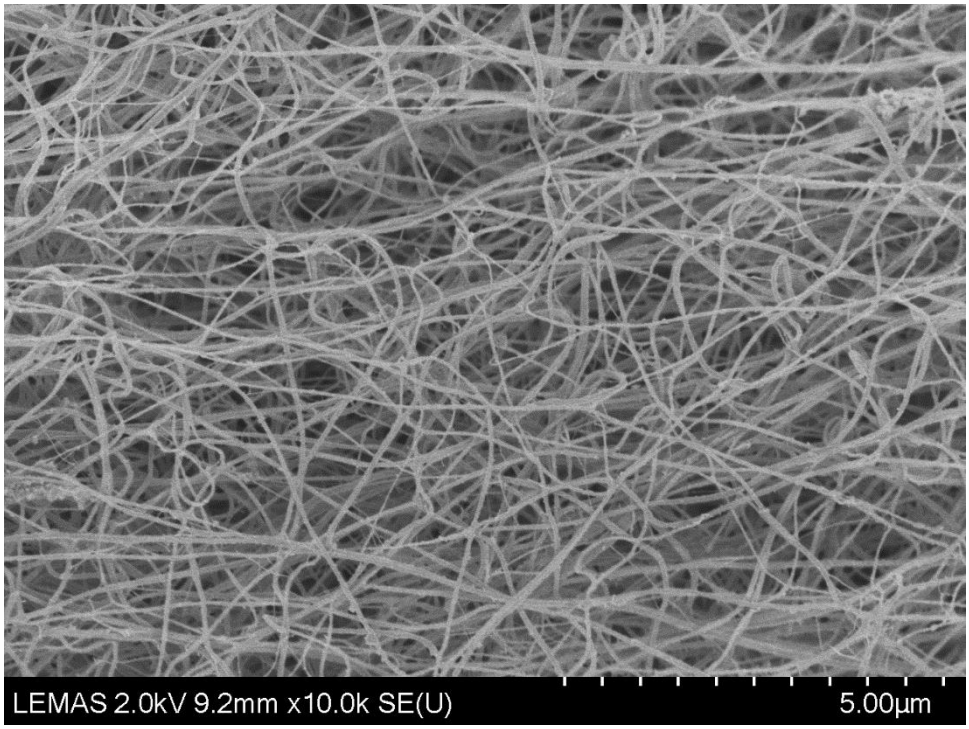

Figure A2.8. Representative image from the SEM experiments corresponding to the samples from subjects with T2DM in the presence of 20 µM Zn^{2+}.
The image was viewed and photographed at ×10,000 magnification using a SU8230 scanning electron microscope.

Appendix 3: SPR raw data

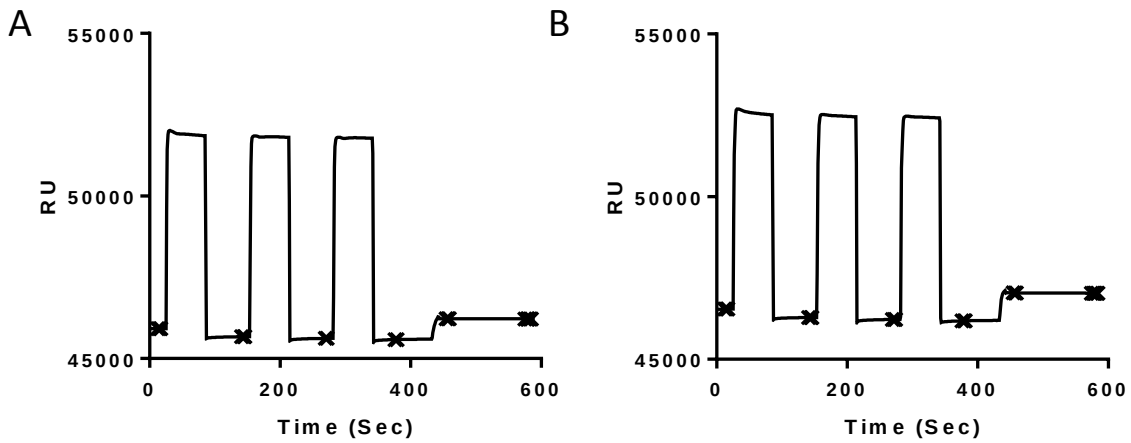

Figure A3.1. SPR raw data corresponding to the immobilisation of biotinylated-heparin on a SA chip.

A. Immobilisation of UFH. **B.** Immobilisation of a LMWH of 4,850 Da**.** The first three peaks correspond to a conditioning of the flow cell surface with three 1 min injections of a 1 M NaCl, 50 mM NAOH solution, while the last peak correspond to the injection of the biotinylated heparin. The immobilisation target was 640 RU, while the immobilisation obtained was 635 RU for UFH and 848.4 RU for the LMWH.

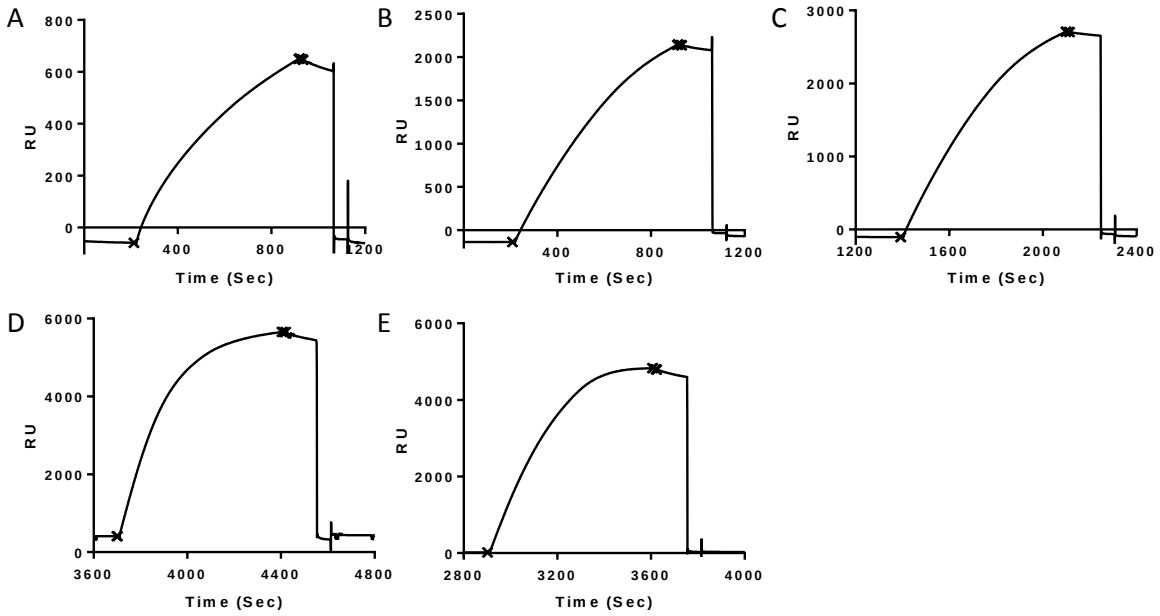

Figure A3.2. Representative set of SPR raw data corresponding to fibrinogen binding to immobilised UFH in the presence of 0-20 µM Zn^{2+}.

Binding in the presence of **A.** 0 µM Zn^{2+}, **B.** 2.5 µM Zn^{2+}, **C.** 5 µM Zn^{2+}, **D.** 10 µM $Zn^{2+}$and **E.** 20 µM Zn^{2+}. These raw data correspond to the data presented in Figure 4.9A.

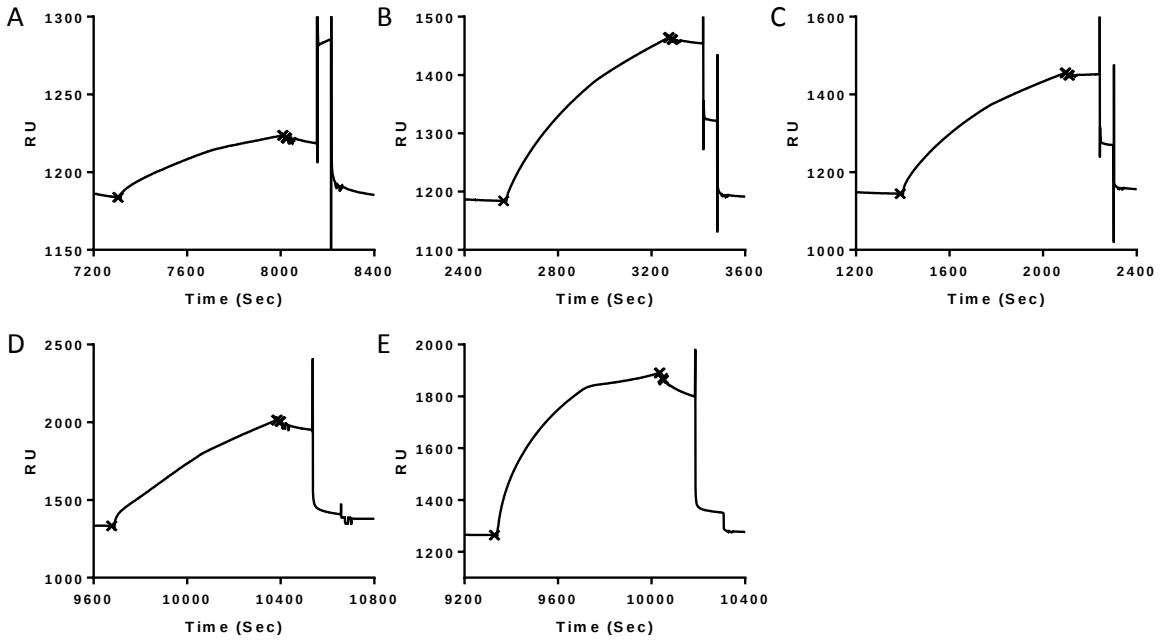

Figure A3.3. Representative set of SPR raw data corresponding to fibrinogen binding to immobilised LMWH (4,850 Da) in the presence of 0-20 µM Zn^{2+}.
Binding in the presence of **A.** 0 µM Zn^{2+}, **B.** 2.5 µM Zn^{2+}, **C.** 5 µM Zn^{2+}, **D.** 10 µM $Zn^{2+}$and **E.** 20 µM Zn^{2+}. These raw data correspond to the data presented in Figure 4.9B.

Appendix 4: Ethical approval

University of St Andrews | FOUNDED 1413

University Teaching and Research Ethics Committee

16th May 2018

Dr Alan Stewart
School of Medicine

Dear Alan

Thank you for submitting your ethical application which was considered by the School of Medicine Ethics Committee meeting on 16th May 2018 when the following documents were reviewed:

1. Ethical Application Form
2. NIIS Ethical approval letter

The School of Medicine Ethics Committee has been delegated to act on behalf of the University Teaching and Research Ethics Committee (UTREC) and has granted this application ethical approval. The particulars relating to the approved project are as follows -

Approval Code:	MD13932	**Approved on:**	16/5/18	**Approval Expiry:**	16/05/23
Term of Approval	5 YEARS				
Project Title:	Plasma fatty acid and zinc dynamics in diabetes: Relevance to fibrin clot formation				
Researcher(s):	Dr Alan Stewart, Dr Samantha Pitt, Dr Ramzi Aijan (University of Leeds), Amelie Sobczak, Dr Sarah Dack (NHS Fife)				
Supervisor(s):	Dr Alan Stewart				

Approval is given for the term granted above. Projects which have not commenced within two years of approval must be re-submitted for review by your School Ethics Committee. If you are unable to complete your research within the approval period, you are required to write to your School Ethics Committee Convener to request a discretionary extension of no greater than 6 months or to re-apply if directed to do so, and you should inform your School Ethics Committee when your project reaches completion.

If you make any changes to the project outlined in your approved ethical application form, you should inform your supervisor and seek advice on the ethical implications of those changes from the School Ethics Convener who may advise you to complete and submit an ethical amendment form for review.

Any adverse incident which occurs during the course of conducting your research must be reported immediately to the School Ethics Committee who will advise you on the appropriate action to be taken.

Approval is given on the understanding that you conduct your research as outlined in your application and in compliance with UTREC Guidelines and Policies (http://www.st-andrews.ac.uk/utrec/guidelinespolicies/). You are also advised to ensure that you procure and handle your research data within the provisions of the Data Provision Act 1998 and in accordance with any conditions of funding incumbent upon you.

Yours sincerely

Dr Robert Humphreys
Convener of the School of Medicine Ethics Committee

School of Medicine Ethics Committee
Medical and Biological Sciences Building, North Haugh, St Andrews, Fife, KY16 9TF, Scotland, UK
Email: medethic@st-andrews.ac.uk Tel No: 01334 463585
The University of St Andrews is a charity registered in Scotland: No SC013532

NHS
National Research Ethics Service
NRES Committee Yorkshire & The Humber - Leeds East
Yorkshire and Humber REC Office
First Floor, Millside
Mill Pond Lane
Meanwood
Leeds
LS6 4RA

Telephone: 0113 3050108
Facsimile:

21 November 2011

Dr Ramzi Ajjan
Senior Lecturer and Consultant in Diabetes and Endocrinology
University of Leeds
LIGHT Laboratories
University of Leeds
Clarendon Way, Leeds
LS2 9JT

Dear Dr Ajjan

Study title: **A new paradigm for impaired fibrinolysis in diabetes: the role of complement C3**

REC reference: **11/YH/0327**

Thank you for your letter of 10 November 2011, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		04 August 2011
Evidence of insurance or indemnity		10 September 2010
Other: Checklist		
Other: Email Circulation Text	1	08 August 2011
Other: Letter from Funder		10 February 2010
Participant Consent Form	1	29 July 2011
Participant Consent Form: complement C3 and inflammation in diabetes	2	26 September 2011
Participant Information Sheet	1	29 July 2011
Participant Information Sheet: Complement C3 in diabetes	2	29 September 2011
Participant Information Sheet	3	24 October 2011
Protocol	1	29 July 2011
REC application		11 August 2011
Response to Request for Further Information		29 September 2011
Response to Request for Further Information		24 October 2011
Response to Request for Further Information		10 November 2011

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

11/YH/0327	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Dr Carol Chu
Chair

Email: jade.thorpe@nhs.net

Copy to: *Mrs Rachel E de Souza, University of Leeds*
Ms Anne Gowing, University of Leeds/Leeds Teaching Hospitals NHS Trust

Appendix 5: Publications

List of publications:

1) Total plasma magnesium, zinc, copper and selenium concentrations in type-I and type-II diabetes.
2) Glycosaminoglycan Neutralization in Coagulation Control.
3) Influence of zinc on glycosaminoglycan neutralisation during coagulation.
4) Ischemia-modified albumin: Crosstalk between fatty acid and cobalt binding.

Biometals (2019) 32:123–138
https://doi.org/10.1007/s10534-018-00167-z

Total plasma magnesium, zinc, copper and selenium concentrations in type-I and type-II diabetes

Amélie I. S. Sobczak · Fiona Stefanowicz · Samantha J. Pitt · Ramzi A. Ajjan · Alan J. Stewart

Received: 19 December 2018 / Accepted: 29 December 2018 / Published online: 22 January 2019

Abstract Glycemia and insulin resistance are important regulators of multiple physiological processes and their dysregulation has wide-ranging consequences, including alterations in plasma concentrations of metal micronutrients. Here, magnesium, zinc, copper, selenium and glycated albumin (HbA1c) concentrations and quartile differences were examined in 45 subjects with type-I diabetes (T1DM), 54 subjects with type-II diabetes (T2DM) and 62 control subjects in order to assess potential differences between sexes and between T1DM and T2DM. Plasma magnesium concentration was decreased in T1DM subjects, with the second, third and fourth quartiles of magnesium concentrations associated with the absence of T1DM. This effect was observed in females but not males. In T2DM, the highest quartile of selenium concentrations and the third quartile of copper concentrations associated with the absence of diabetes in males. The highest quartile of magnesium concentrations was associated with the absence of T2DM in males but not females. HbA1c correlated with plasma concentrations of magnesium (negatively, in both sexes together in T1DM and T1DM males), copper (positively, in T1DM males and in both sexes together in T2DM), selenium (positively, in both sexes together in T1DM and T2DM, and T2DM females) and with zinc/copper ratio (negatively, in both sexes together in T1DM and T2DM). This study shows that plasma magnesium concentration is altered to the highest degree in T1DM, while in T2DM, plasma selenium and copper concentrations are significantly affected. This work increases our understanding of how T1DM and T2DM affects plasma metal concentrations and may have future implications for diabetes management.

Keywords Diabetes · HbA1c · ICP-MS · Metal homeostasis · Zinc/copper ratio

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Scottish Trace Element and Micronutrient Diagnostic and Research Laboratory, Department of Clinical Biochemistry, NHS Greater Glasgow and Clyde, Glasgow, UK

R. A. Ajjan
Leeds Institute of Cardiovascular and Metabolic Medicine, University of Leeds, Leeds, UK

Abbreviations

CI	Confidence interval
HbA1c	Glycated haemoglobin
ICP-MS	Inductively-coupled plasma-mass spectrometry
OR	Odds ratio
Q	Quartile
T1DM	Type-I diabetes mellitus
T2DM	Type-II diabetes mellitus

Introduction

Diabetes is mainly divided into two types. In type-I diabetes (T1DM), the β-cells of the islets of Langerhans in the pancreas responsible for producing insulin are lost, typically from an attack from the immune system, causing an insulin deficiency in the body. In type-II diabetes (T2DM), cells become resistant to insulin signalling, failing to respond to it sufficiently. This is combined with either normal or increased insulin levels but can also develop into insulin deficiency through a relative loss of the insulin storage function of β-cells. Glucose levels in the body are important, as directly or indirectly they regulate many physiological processes, including glucose, glycogen metabolism. It also acts to control food intake (satiety) and maintaining long-term body weight but also regulates inflammation, vasodilatation and basic cell growth and replication. Thus, mishandling of glucose has wide-ranging consequences in the body. Among them is an alteration in the total plasma concentrations of micronutrients including some metal ions (Kaur and Henry 2014). This is thought to be caused by numerous factors, which include increased fluid loss from the body, an increased demand for micronutrients due to altered protein metabolism as well as defective metal transport due to oxidative stress (Kaur and Henry 2014; Maret 2017).

Variations in plasma metal concentrations in diabetes have been extensively studied in different populations. However there is a great heterogeneity in the data, with both higher and lower levels of the same metals having been reported (e.g. zinc and selenium) (Wang et al. 2016; Sanjeevi et al. 2018). Several meta-analyses have been performed in order to find a consensus on whether levels of some metals are higher or lower in diabetes (Wang et al. 2016; Sanjeevi et al. 2018). Analyses of quartiles of selenium concentrations yielded interesting results, with both the lowest and highest quartiles being associated with T2DM (Wang et al. 2016). Sex differences have also been shown to be of some importance with higher levels of copper reported in females with T1DM but not males (Ruiz et al. 1998). In addition, glycated haemoglobin (HbA1c) level as an indication of diabetes management was found to correlate with several plasma metal concentrations, including zinc, copper and magnesium and also to the zinc/copper ratio (Naka et al. 2013; Ramadass et al. 2015; Atari-Hajipirloo et al. 2016).

Here, we have examined the total plasma concentrations of zinc, copper, magnesium and selenium in patients with T1DM and T2DM and in controls, assessing the correlation between quartiles of metal cations concentrations and diabetes, as well as the influence of sex and diabetes management through blood HbA1c levels. Plasma metal concentrations and potential sex differences in those concentrations have not been thoroughly examined both in T1DM and in T2DM in the same study before. The results indicate that magnesium deficiency is particularly important in T1DM while elevated copper and selenium deficiency in females are more important in T2DM. A better understanding of those effects could lead to a better understanding of metal micronutrient handling and to better management and treatment of T1DM and T2DM.

Methods

Samples collection and treatment

A total of 45 patients with T1DM, 54 patients with T2DM and 62 controls were recruited from Leeds Teaching Hospital Trust. Plasma samples from subjects with either T1DM or T2DM and controls were collected following approval by the local Research Ethics Committee in Leeds and after obtaining written informed consent. For all subjects, baseline fasting blood samples were collected in lithium heparin coated tubes. Plasma was then separated within 2 h of collection by centrifugation at $2400\times g$ for 20 min at 4 °C. Samples were snap frozen in liquid nitrogen and stored at − 40 °C until analysis.

Inductively-coupled plasma-mass spectrometry (ICP-MS)

Single element stock solutions (Centripure, Merck, UK) traceable to the United States National Institute of Standards and Technology (NIST) were used for calibration standards and internal standards. All calibration standards were prepared using a solution of 2% v/v butan-1-ol (Sigma Aldrich, USA), 0.05% w/v EDTA (Sigma Aldrich), 0.05% v/v Triton-X-100 (Sigma Aldrich), 1% v/v ammonia (Romil, UK) in

18.2 Mohm reverse osmosis deionised water (Elga Maxima High Wicombe, UK). Lithium heparin plasma samples were diluted 1 in 10 with the same solution used to prepare calibration standards with 25 ppb germanium. ClinChek® 1 & 2 (RECIPE, Germany) and Seronorm™ 1 (SERO, Norway) human serum certified reference materials were used to demonstrate accuracy. ClinChek®1 and 2 are materials with consensus values while Seronorm™ is traceable to a National Institute of Standard Technology (NIST) certified reference material.

Copper, zinc, selenium and magnesium analyses were performed simultaneously using an Agilent 7900 ORS-ICP-MS (Agilent Technologies, Santa Clara, California, USA). The instrument was controlled using Mass Hunter software (version 4.1, Agilent Technologies). Argon was used to form the plasma (CryoServices Ltd, purirty: 99.9%). Polyatomic interferences for copper, zinc and magnesium were removed through collision induced dissociation and kinetic energy discrimination using helium gas (Air Products and Chemicals, Inc, purity 99.9%). Polyatomic and doubly charged interferences for selenium were removed using a charge exchange reaction by using the collision cell with hydrogen. The concentration of all elements was measured three times within a single run using the central 0.05 m/z of the peak. The ICP-MS was equipped with an CETAC ASX-500 series autosampler (Teledyne CETAC, Omaha, USA), an Integrated Sample Introduction System and Discrete Sampler—3 (ISIS-DS, Agilent Technologies) and a G32992A re-circulating chiller (Polyscience, Illinois, USA). The ISIS-DS was fitted with a Quartz Scott-Type, Double-Pass Spray Chamber (Agilent Technologies), a glass micromist nebuliser (Agilent Technologies) and a sample loop. The sample loop was prepared in-house using 0.8 mm internal diameter and PTFE sample tubing (Agilent Technologies). A quartz torch with 2.5 mm internal diameter was used (Agilent Technologies). Nickel sampling and skimmer cones were used at all times (Agilent Technologies). All instrument parameters were optimised daily by performing an auto-tune while aspirating a tuning solution containing 10 ppb lithium, yttrium, cobalt, cerium and thallium. Typical instrument parameters are shown in Table 1. Three certified reference materials (Seronorm 1 and ClinCheck 1 and 2) with assigned values and ranges were used to demonstrate the accuracy of the method used to determine the concentrations of Mg, Cu, Zn and Se in human plasma (Table 2). The mean for all measurands were within the assigned range for all materials and had satisfactory recoveries ranging between 96.4 and 107%. The precision of the method, represented by coefficient of variation, was also satisfactory with values less than or equal to 3%. All metal concentrations in plasma were compared to the glycated haemoglobin concentrations which were measured in the samples as a routine test.

Statistical analysis and representation

Data are presented as mean ± standard error of the mean (SEM). Graphs were generated and statistical analysis was performed using Prism 7.0 (GraphPad Software, La Jolla, CA). Differences between groups were analysed using multiple Student's t-tests or analysis of variance, while correlations between linear variants were analysed with Pearson's correlation. The data was also separated into quartiles and odd ratios were calculated. Significance threshold was set at $p \leq 0.05$.

Results and discussion

Total plasma zinc, copper, magnesium, selenium concentrations and zinc/copper ratios in T1DM and T2DM

ICP-MS was used to determine the total plasma concentrations of zinc, copper, magnesium and selenium in subjects with T1DM or T2DM and in their respective age-matched controls. The zinc/copper ratio was also calculated in all samples. Table 3 summarises the demographic characteristics of our population as well as the mean plasma HbA1c and metal concentrations values in each group. As expected, t-tests indicate that subjects with T1DM or T2DM have higher plasma concentrations of HbA1c than controls. Both diabetes groups also had higher mean BMI values than controls, while the T2DM group also has a higher percentage of male subject than the controls. Mean plasma zinc, copper and selenium concentrations were not significantly altered in subjects with either T1DM or T2DM compared to controls. Whereas the mean plasma magnesium concentration was significantly lower in subjects with

Table 1 ICP-MS instrument parameters

Parameter	Setting
Isotopes monitored (m/z)	Cu 63, Zn 66, Se 78 and Mg 24
RF power (W)	1550
RF matching	1.70
Sampling depth (mm)	10
Carrier gas (L/min)	1.05
Make up gas L/min)	0.0
Spray chamber temperature (°C)	2
He octopole reaction system flow (mL/min)	5.0
Nebuliser pump (rps)	0.1

Table 2 Accuracy of the ICP-MS method and details of the concentration of trace elements in each material (coefficient of variation and recovery)

Measurand	Specimen	Assigned value	Range	Mean	CV (%)	Recovery (%)
Plasma Mg (mM)	ClinCheck 1	0.64	0.58–0.71	0.62	1.8	97.6
	ClinCheck 2	1.21	1.09–1.33	1.17	1.5	96.4
Plasma Cu (μM)	Seronorm1	17.1	15.7–18.7	17.0	1.8	99.6
	ClinCheck 1	10.9	8.7–13.1	10.7	1.3	97.8
	ClinCheck 2	19.1	15.4–23.0	19.0	1.4	98.8
Plasma Zn (μM)	Seronorm1	16.8	14.6–19.0	17.8	3.0	107
	ClinCheck 1	17.7	14.2–21.3	17.6	0.8	99.2
	ClinCheck 2	23.2	18.8–28.3	24.2	1.3	104.2
Plasma Se (μM)	Seronorm1	1.10	0.96–1.25	1.09	0.8	98.8
	ClinCheck 1	1.03	0.82–1.24	1.06	0.7	103
	ClinCheck 2	1.52	1.22–1.82	1.57	1.2	104

T1DM than in controls but not with T2DM, while the plasma zinc/copper ratio was significantly higher in T2DM subjects than in controls, but this was not the case in T1DM.

The data from each of the two diabetes groups, (T1DM and T2DM subjects; each with their respective age-matched controls) were split into quartiles for each metal, defined as four groups delimited by 25%, median and 75% of highest values in each of the groups. The odd ratios (ORs) of a subject in a specific quartile having diabetes were calculated (Table 4). No significant difference in total plasma zinc concentrations was observed between either the T1DM or the T2DM groups and their age-matched controls. This is contrary to two previous reports—a meta-analysis accumulating 20,183 T2DM subjects and a study with 51 T1DM subjects both showing that zinc concentration is lower in diabetes (Li et al. 2018; Sanjeevi et al. 2018). Despite previous reports of both higher and lower zinc concentrations being associated with T2DM, no significance could be gained by examining quartiles of zinc concentrations (Sanjeevi et al. 2018). In T2DM, the ORs of having diabetes in the second (Q2), third (Q3) and fourth (Q4) copper quartile groups were respectively 0.031 [95% confidence interval (CI) 0.080–1.275, $p > 0.05$], 0.193 (95% CI 0.051–0.966, $p < 0.05$) and 0.276 (95% CI 0.070–1.154, $p > 0.05$) compared with the first (Q1) quartile group. The absence of a clear difference between T2DM subjects and controls (with only Q3 being significantly associated with the absence of diabetes) can be explained by the mean plasma copper concentration being very low in our T2DM group and high in our age-matched controls, almost enough to be significantly higher in the controls ($p = 0.0698$), whereas a meta-analysis involving 20,183 T2DM

Table 3 Demographic characteristics of the studied population and mean plasma HbA1c and metal concentrations in each group

Characteristics	T1DM subjects (n = 45)	T1DM age-matched controls (n = 47)	t test between T1DM and age-matched controls	T2DM subjects (n = 54)	T2DM age-matched controls (n = 18)	t-test between T2DM and age-matched controls
Age (years ± SD)	26.3 ± 6.8	24.3 ± 6.2	ns	61.1 ± 7.6	57.1 ± 8.9	ns
Males, n (%)	26 (58)	24 (51)	ns	47 (87)	9 (50)	***
BMI						
BMI (kg/m^2 ± SD)	24.6 ± 3.6	23.0 ± 3.0	*	32.6 ± 5.3	25.0 ± 3.2	***
n (%) for BMI < 25	24 (53)	38 (81)	–	2 (4)	10 (59)	–
n (%) for BMI 25 to < 30	18 (40)	8 (17)	–	17 (31)	6 (35)	–
n (%) for BMI ≥ 30	3 (7)	1 (2)	–	35 (65)	1 (6)	–
HbA1c conc. mean (mM ± SD)	69.8 ± 18.0	33.5 ± 0.7	**	72.4 ± 22.8	37.6 ± 4.2	***
Zinc conc. mean (μM ± SD)	12.80 ± 1.50	13.50 ± 2.30	ns	12.70 ± 1.40	12.38 ± 1.69	ns
Copper conc. mean (μM ± SD)	17.56 ± 6.60	16.26 ± 5.50	ns	15.49 ± 2.78	17.30 ± 4.70	ns
Magnesium conc. mean (mM ± SD)	0.747 ± 0.057	0.806 ± 0.054	***	0.738 ± 0.075	0.765 ± 0.115	ns
Selenium conc. mean (μM ± SD)	1.147 ± 0.145	1.129 ± 0.136	ns	1.142 ± 0.188	1.222 ± 0.261	ns
Zinc/copper (ratio ± SD)	0.813 ± 0.273	0.886 ± 0.246	ns	0.841 ± 0.152	0.751 ± 0.187	*

Significance is indicated as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

individuals has shown that plasma copper is higher in T2DM (Sanjeevi et al. 2018). There were no differences in plasma copper concentrations between quartiles in the T1DM group, contrary to a previous study (Ruiz et al. 1998). The ORs of having T1DM in the Q2, Q3, Q4 magnesium groups were 0.176 (95% CI 0.058–0.628, $p < 0.01$), 0.1173 (95% CI 0.036–0.432, $p < 0.01$) and, 0.086 (95% CI 0.028–0.325, $p < 0.001$) compared with the Q1 group. Thus, T1DM is associated with lower plasma magnesium concentrations (Q2–Q4), as supported by a previous study (Brown et al. 1999). In T2DM, no differences in magnesium concentration were observed between quartiles. This is despite a meta-analysis involving 286,668 T2DM subjects having found an association between low dietary magnesium levels and T2DM, while another study following 12,128 non-diabetic subjects over 6 years reported an inverse relationship between serum magnesium concentrations and incidence of T2DM amongst white (but not black) participants (Kao et al. 1999; Larsson and Wolk 2007). For plasma selenium concentrations, the ORs of having T2DM in the Q2, Q3 and Q4 selenium groups were respectively 0.850 (95% CI 0.221–3.292, $p > 0.05$), 0.560 (95% CI 0.152–2.484, $p > 0.05$) and 0.100 (95% CI 0.017–0.682, $p < 0.01$) compared with

Table 4 Prevalences of diabetes in quartiles of total plasma concentrations of zinc, copper, magnesium and selenium and zinc/copper ratio in T1DM and T2DM study groups (including age-matched controls)

	T1DM and age-matched controls				T2DM and age-matched controls			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Zinc (μM)	n = 23	n = 23	n = 30	n = 16	n = 15	n = 16	n = 20	n = 20
	< 11.95	11.95–12.91	12.91–14.46	≥ 14.46	< 11.33	11.33–12.15	12.15–13.35	≥ 13.35
% diabetes	52	48	60	25	73	75	75	80
OR (95% CI)	1	0.840 (0.285–2.873)	1.375 (0.457–4.234)	0.306 (0.090–1.204)	1	1.091 (0.264–4.490)	1.091 (0.281–5.312)	1.455 (0.357–5.822)
Copper (μM)	n = 22	n = 17	n = 23	n = 26	n = 33	n = 13	n = 12	n = 12
	< 13.99	13.99–14.47	14.47–16.79	≥ 16.79	< 15.06	15.06–16.40	16.40–17.78	≥ 17.78
% diabetes	50	29	48	64	88	69	58	67
OR (95% CI)	1	0.417 (0.111–1.541)	0.9167 (0.310–2.790)	1.800 (0.559–5.139)	1	0.310 (0.080–1.275)	0.193 (0.051–0.966)*	0.276 (0.070–1.154)
Magnesium (mM)	n = 41	n = 17	n = 15	n = 19	n = 23	n = 25	n = 13	n = 9
	< 0.78	0.78–0.81	0.81–0.83	≥ 0.83	< 0.72	0.72–0.79	0.79–0.84	≥ 0.84
% diabetes	75	35	27	21	83	84	69	44
OR (95% CI)	1	0.176 (0.058–0.628)**	0.117 (0.036–0.432)**	0.086 (0.028–0.325)***	1	1.105 (0.288–4.220)	0.474 (0.118–1.969)	0.168 (0.031–1.064)
Selenium (μM)	n = 21	n = 20	n = 19	n = 32	n = 24	n = 21	n = 19	n = 6
	< 1.04	1.04–1.12	1.12–1.18	≥ 1.18	< 1.085	1.09–1.24	1.24–1.42	≥ 1.42
% diabetes	52	40	37	59	83	80	74	33
OR (95% CI)	1	0.606 (0.172–1.958)	0.530 (0.136–1.784)	1.329 (0.424–4.188)	1	0.850 (0.221–3.292)	0.560 (0.152–2.484)	0.100 (0.017–0.682)*
Zinc/copper	n = 29	n = 23	n = 19	n = 20	n = 9	n = 35	n = 25	n = 27
	< 0.734	0.734–0.893	0.893–1.070	≥ 1.070	< 0.667	0.667–0.732	0.732–0.865	≥ 0.865
% diabetes	62	48	37	45	56	86	80	85
OR (95% CI)	1	0.560 (0.172–1.695)	0.357 (0.104–1.233)	0.500 (0.169–1.660)	1	4.800 (1.110–19.940)	3.200 (0.732–13.560)	4.600 (1.001–22.260)

Data is presented as OR (95% CI)

Significance is indicated as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

the Q1 group. Thus, low selenium concentrations were linked with T2DM. The fact that subjects in Q4 did not associate with T2DM is contrary to the findings of previously published meta-analysis based on 13,460 T2DM patients, where both Q1 and Q4 were associated with T2DM (Wang et al. 2016). No differences in plasma selenium concentrations were observed between quartiles in T1DM, although a previous study indicated that lower plasma selenium concentrations have been found in T1DM (Ruiz et al. 1998). Here we were unable to see any differences between zinc/copper ratio quartiles in either T1DM or T2DM, contrary to the higher mean ratios we measured in T2DM. Some previous publications also indicated that lower plasma zinc/copper ratios have been observed in T1DM (Lin et al. 2014) and in T2DM (Atari-Hajipirloo et al. 2016). The failure to detect some of these previously reported relationships in our study may be due to the number of subjects (and the resultant effect on statistical power). Thus larger cohort studies enable relatively subtle differences in plasma metal concentrations to be observed between individuals with diabetes and controls. However, those differences may not be as relevant as those that can be detected in smaller cohorts.

Differences in plasma metal concentrations between males and females with T1DM or T2DM

The influence of the sex of the subjects on plasma metal concentrations between the various groups was examined (Table 5). In our age-matched controls for the T1DM group, males had significantly higher zinc concentrations and lower copper concentrations than females, while in the T1DM group males had significantly higher zinc and magnesium concentrations and lower copper concentrations than females. The T2DM group was harder to study as there were few females in the group. However, copper concentrations were significantly lower and the zinc/copper ratio higher in males than in females. The T1DM and T2DM groups were then compared with their respective age-matched controls. Magnesium concentrations were lower in males and females with T1DM than in the respective controls, while only males with T2DM had a lower magnesium concentration than their respective controls. In addition, the zinc/copper ratio was higher in males with T2DM than in the male controls.

The plasma metal concentration quartile groups described above were split according to sex (Table 6). The ORs of having T1DM in the Q2, Q3 and Q4 female magnesium quartile groups were respectively 0.000 (95% CI 0–0.480, $p < 0.01$), 0.059 (95% CI 0.005–0.482, $p < 0.05$) and 0.033 (95% CI 0.003–0.287, $p < 0.001$) compared to the Q1 group. There were no differences between magnesium quartiles in T1DM males despite a lower mean having been observed compared to male controls. To our knowledge, no sex differences in mean plasma magnesium concentrations in T1DM subjects have been reported before. No significance differences in male copper quartiles or in female copper quartiles were observed in T1DM. This is contrary to a report, which found that plasma copper concentrations were higher in females with T1DM than in females controls but that this was not the case in males (Ruiz et al. 1998). However another study (with only 18 individuals and both T1DM and T2DM analysed together) reported the absence of any difference in plasma copper concentration between males and females with diabetes (Terres-Martos et al. 1998). In addition, no significant differences in male or in female zinc or zinc/copper quartiles were identified in T1DM.

The low number of females among our T2DM cohort made accurate comparison between the sexes more difficult to perform and no statistically significant differences were found with any metal when examining female subjects in the quartiles. However, when looking at males only the copper Q3, magnesium Q4 and selenium Q4 groups were associated with the absence of T2DM. The ORs of having T2DM in the Q2, Q3 and Q4 male copper quartile groups were respectively 0.167 (95% CI 0.011–1.649, $p > 0.05$), 0.074 (95% CI 0.006–0.625, $p < 0.05$) and 0.074 (95% CI 0.005–0.842, $p > 0.05$) compared to the Q1 group. In a meta-analysis of copper concentrations in T2DM, no sex differences were observed (Sanjeevi et al. 2018). The ORs of having T2DM in the Q2, Q3 and Q4 male magnesium quartile groups were respectively 0.9444 (95% CI 0.04734–18.9, $p > 0.05$), 0.1944 (95% CI 0.01285–2, $p > 0.05$) and 0.056 (95% CI 0.004–0.582, $p < 0.05$) compared to the Q1 group. Contrary to this, a previous study has found that females with T2DM were more likely to have lower magnesium concentrations than males (Kao et al. 1999). The ORs of having T2DM in the Q2, Q3 and Q4 male selenium quartile groups were respectively for

Table 5 Differences in total plasma concentrations of zinc, copper, magnesium and selenium and zinc/copper ratio in males and females with T1DM and T2DM (and respective age-matched controls)

Parameters (mean ± SD)	Controls age-match with T1DM			T1DM			Differences between T1DM and age-matched controls	
	Males (n = 24), mean ± SD	Females (n = 23), mean ± SD	t-test between male and female controls	Males (n = 26), mean ± SD	Females (n = 19), mean ± SD	t-test between males and females with T1DM	t-test between T1DM males and control males	t-test between T1DM females and control females
Zinc (μM)	14.34 ± 2.72	12.62 ± 1.33	**	13.22 ± 1.538	12.22 ± 1.236	*	ns	ns
Copper (μM)	13.70 ± 1.44	18.81 ± 6.79	***	14.88 ± 3.727	21.23 ± 7.911	***	ns	ns
Magnesium (mM)	0.807 ± 0.056	0.804 ± 0.054	ns	0.763 ± 0.056	0.726 ± 0.052	*	**	***
Selenium (μM)	1.151 ± 0.117	1.106 ± 0.152	ns	1.120 ± 0.143	1.184 ± 0.145	ns	ns	ns
Zinc/copper ratio	1.025 ± 0.139	0.747 ± 0.253	***	0.930 ± 0.220	0.651 ± 0.260	***	ns	ns
Parameters (mean ± SD)	Controls age-match with T2DM			T2DM			Differences between T2DM and age-matched controls	
	Males (n = 9), mean ± SD	Females (n = 8), mean ± SD	t-test between male and female controls	Males (n = 46), mean ± SD	Females (n = 7), mean ± SD	t-test between males and females with T2DM	t-test between T2DM males and control males	t-test between T2DM females and control females
Zinc (μM)	12.33 ± 1.35	12.43 ± 2.11	ns	12.72 ± 1.45	12.32 ± 1.46	ns	ns	ns
Copper (μM)	16.44 ± 1.53	18.27 ± 6.76	ns	15.12 ± 2.28	18.67 ± 1.31	**	ns	ns
Magnesium (mM)	0.812 ± 0.083	0.712 ± 0.128	ns	0.737 ± 0.078	0.757 ± 0.047	ns	*	ns
Selenium (μM)	1.273 ± 0.149	1.165 ± 0.351	ns	1.153 ± 0.179	1.070 ± 0.229	ns	ns	ns
Zinc/copper ratio	0.757 ± 0.108	0.744 ± 0.257	ns	0.865 ± 0.140	0.683 ± 0.141	**	*	ns

Significance is indicated as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

Table 6 Prevalences of diabetes in quartiles of total plasma zinc, copper, magnesium and selenium concentration and zinc/copper ratio seperated by sex in T1DM and T2DM study groups (including age-matched controls)

		T1DM and age-matched controls				T2DM and age-matched controls			
		Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Zinc (μM)		11.95	11.95–12.91	12.91–14.46	≥ 14.46	< 11.33	11.33–12.15	12.15–13.35	≥ 13.35
Males	n	10	9	17	14	11	11	16	16
	OR (95% CI)	1	0.533 (0.078–2.843)	1.600 (0.370–7.145)	0.267 (0.047–1.600)	1	2.222 (0.221–35.010)	0.667 (0.110–3.837)	3.333 (0.334–51.000)
Females	n	13	14	13	2	3	5	4	4
	OR (95% CI)	1	1.167 (0.261–5.488)	1.000 (0.203–4.927)	0.000 (0.000–3.184)	1	1.333 (0.091–28.020)	4.000 (0.175–83.060)	0.667 (0.027–18.510)
Copper (μM)		< 13.99	13.99–14.47	14.47–16.79	≥ 16.79	< 15.06	15.06–16.40	16.40–17.78	≥ 17.78
Males	n	18	14	11	6	28	11	9	6
	OR (95% CI)	1	0.320 (0.088–1.558)	1.400 (0.315–5.436)	4.000 (0.514–52.23)	1	0.167 (0.011–1.649)	0.074 (0.006–0.625)*	0.074 (0.005–0.842)
Females	n	4	3	12	23	5	2	3	6
	OR (95% CI)	1	1.500 (0.054–37.03)	1.500 (0.165–23.84)	3.900 (0.490–53.94)	1	0.000 (0.000–5.724)	0.750 (0.036–11.030)	3.000 (0.321–26.480)
Magnesium (mM)		< 0.78	0.78–0.81	0.81–0.83	≥ 0.83	< 0.72	0.72–0.79	0.79–0.84	≥ 0.84
Males	n	19	13	9	9	19	18	9	8
	OR (95% CI)	1	0.306 (0.074–1.226)	0.179 (0.040 to 1.033)	0.179 (0.040–1.033)	1	0.944 (0.047–18.900)	0.194 (0.013–2.000)	0.056 (0.004–0.582)*
Females	n	22	4	6	10	4	7	4	1
	OR (95% CI)	1	0.000 (0.000–0.480)**	0.059 (0.005–0.482)*	0.033 (0.003–0.287)***	1	4.000 (0.359–63.030)	3.000 (0.194–56.040)	0.000 (0.000–36.000)
Selenium (μM)		< 1.04	1.04–1.12	1.12–1.18	≥ 1.18	< 1.085	1.09–1.24	1.24–1.42	≥ 1.42
Males	n	12	10	12	16	17	16	18	3
	OR (95% CI)	1	0.750 (0.144–3.807)	0.250 (0.045–1.383)	0.500 (0.129–2.201)	1	0.938 (0.047–18.890)	0.2188 (0.01698–1.703)	0.031 (0.002–0.601)*
Females	n	9	10	7	16	7	5	1	3
	OR (95% CI)	1	0.500 (0.074–3.211)	1.500 (0.240–9.071)	4.400 (0.722–20.100)	1	0.500 (0.061–4.077)	0.000 (0.000–9.000)	0.375 (0.021–4.924)
Zinc/copper		< 0.734	0.734–0.893	0.893–1.070	≥ 1.070	< 0.667	0.667–0.732	0.732–0.865	≥ 0.865

Table 6 continued

		T1DM and age-matched controls				T2DM and age-matched controls			
		Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Males	n	4	14	14	17	4	7	21	24
	OR (95% CI)	1	0.000 (0.000–2.397)	0.000 (0.000–1.316)	0.000 (0.000–1.408)	1	1.333 (0.138–12.360)	6 (0.662–45.600)	11 (1.069–89.650)
Females	n	25	9	5	3	5	3	4	3
	OR (95% CI)	1	0.393 (0.094–1.827)	0.196 (0.015–1.616)	0.393 (0.026–3.839)	1	0.333 (0.018–5.167)	0.667 (0.062–7.580)	0.333 (0.018–5.166)

Data is presented as OR (95% CI)

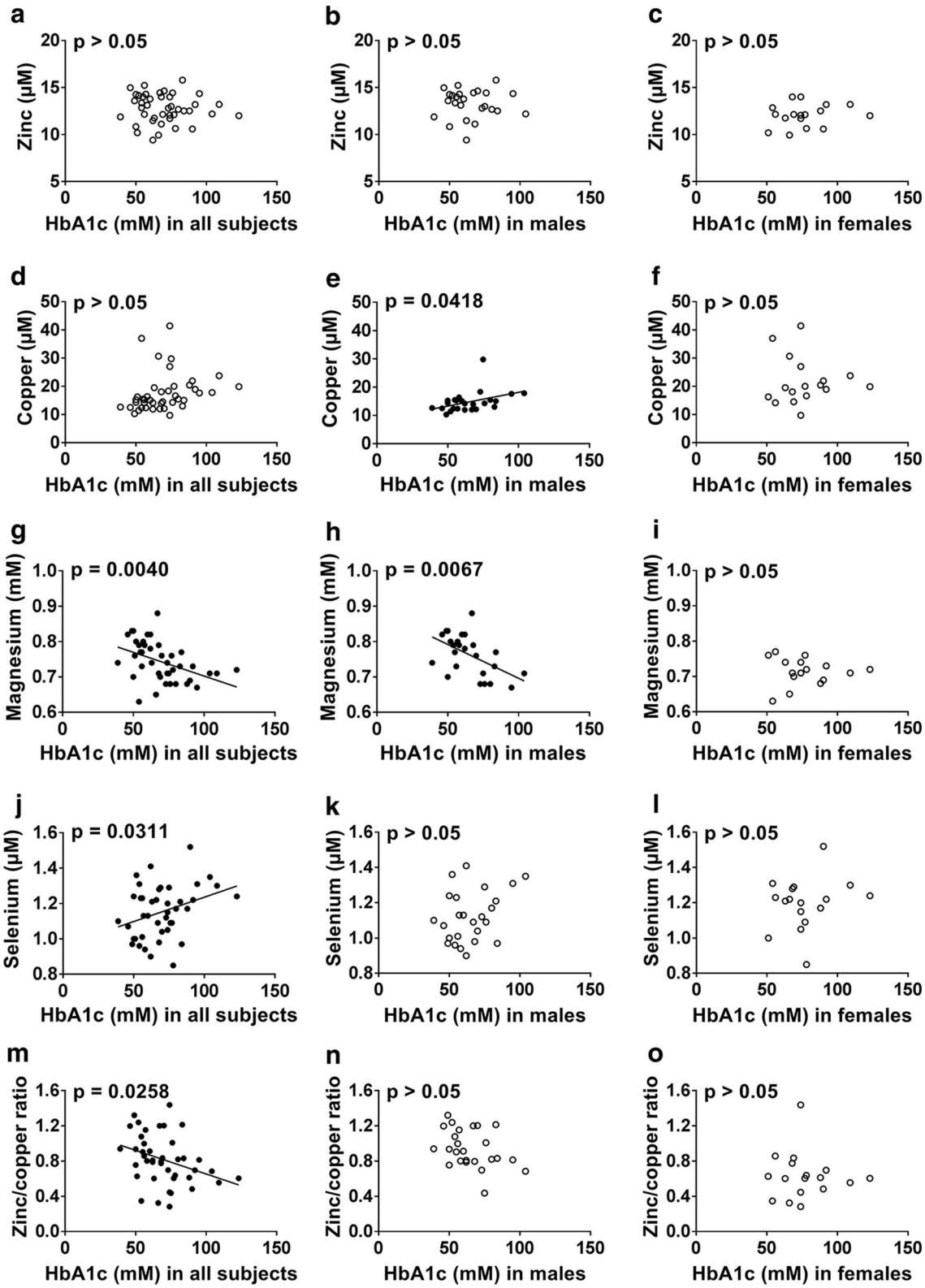
Significance is indicated as *p < 0.05, **p < 0.01 and ***p < 0.001

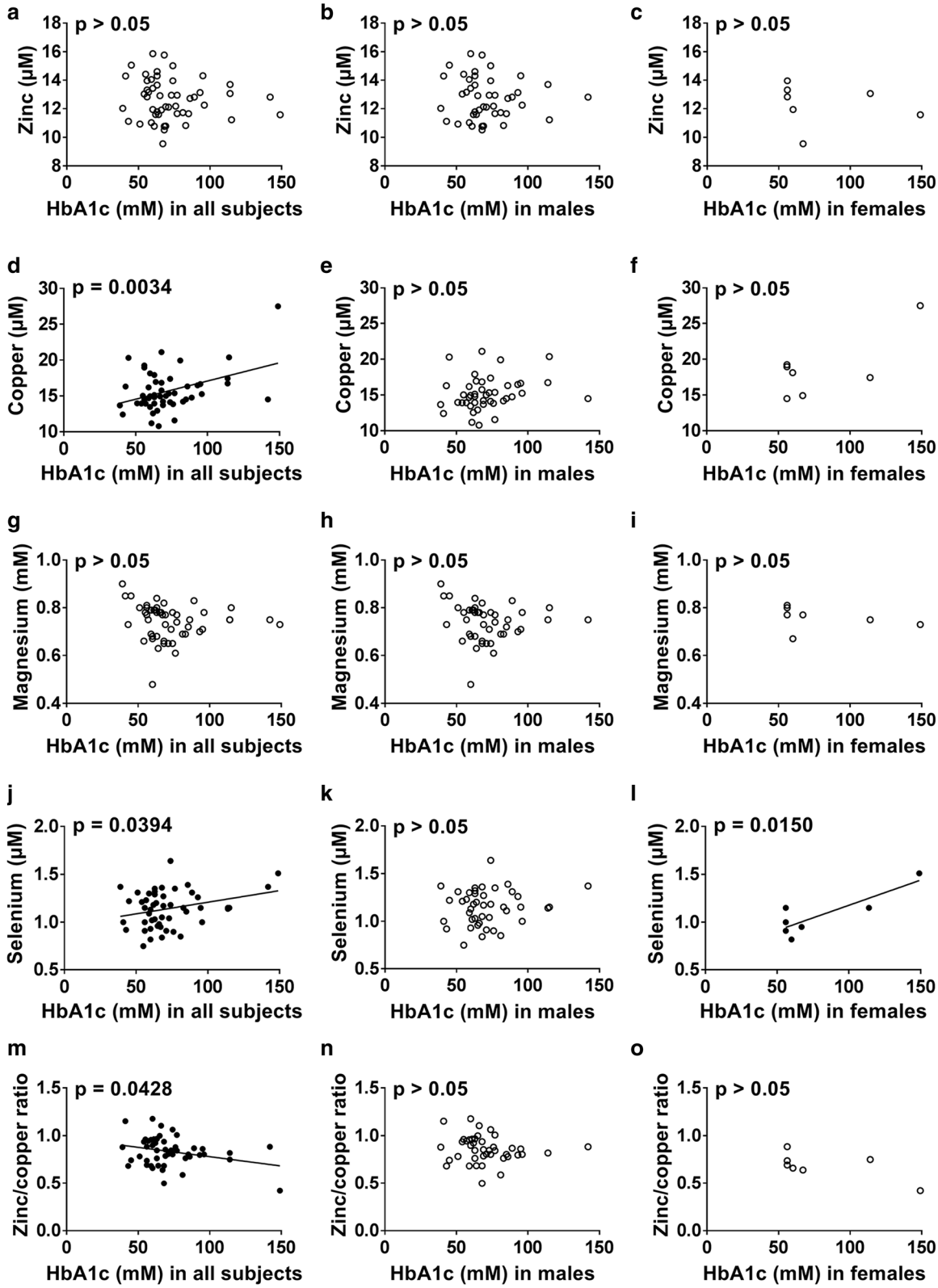
Fig. 1 Relationship between HbA1c concentration and plasma zinc, copper, magnesium and selenium concentrations as well as zinc/copper ratio in all T1DM subjects and male and female T1DM subjects. **a**–**c** HbA1c concentration vs zinc concentration, **d**–**f** HbA1c concentration vs copper concentration, **g**–**i** HbA1c concentration vs magnesium concentration, **j**–**l** HbA1c concentration vs selenium concentration and **m**–**o** HbA1c concentration vs zinc/copper ratio. **a**, **d**, **g**, **j**, **m** both sexes together, **b**, **e**, **h**, **k**, **n** male subjects and **c**, **f**, **i**, **l**, **o** female subjects. Black circles were used for the data that were correlated to HbA1c concentration, while white circles were used when the relationship was not significant. HbA1c concentration was positively correlated with plasma copper concentration in males (p = 0.0418) and with plasma selenium concentration in both sexes together (p = 0.0311), while it was negatively correlated with plasma magnesium concentration in both sexes together (p = 0.0040) and in males (p = 0.0067) and with the zinc/copper ratio in both sexes together (p = 0.0258)

selenium 0.938 (95% CI 0.047–18.89, $p > 0.05$), 0.219 (95% CI 0.017–1.703, $p > 0.05$), 0.031 (95% CI 0.002–0.601, $p < 0.05$) compared to the Q1 group. Association between T2DM and selenium concentrations (too low or too high) in males but not females have been reported before (Bleys et al. 2007; Laclaustra et al. 2009; Akbaraly et al. 2010).

Relationship between plasma metal concentrations and HbA1c concentration

Relationships between HbA1c concentration, as a proxy for glycemic control, and metal concentrations in subjects with T1DM (Fig. 1) or T2DM (Fig. 2) were examined. Plasma zinc concentration did not correlate with HbA1c concentration in either T1DM or T2DM, which is in accord with a previous finding in T1DM but contrary to a previous publication that has found a negative correlation (Luo et al. 2015; Lin et al. 2016). In T1DM, plasma copper concentration positively correlated with HbA1c concentration in males (p = 0.042, Fig. 1e) but not with females or with both sexes together. In T2DM, the plasma copper concentration correlated with HbA1c concentration only when considering both sexes together (p = 0.003, Fig. 2d) but not in males or females only. Previous studies have found that plasma copper concentration do not correlate with HbA1c concentration in T1DM but positively correlates in T2DM (Ruiz et al. 1998; Atari-Hajipirloo et al. 2016). Plasma magnesium concentration negatively correlated with HbA1c concentration in T1DM when looking at both sexes


a
p > 0.05
Zinc (μM)
HbA1c (mM) in all subjects
b
p > 0.05
Zinc (μM)
HbA1c (mM) in males
c
p > 0.05
Zinc (μM)
HbA1c (mM) in females
d
p > 0.05
Copper (μM)
HbA1c (mM) in all subjects
e
p = 0.0418
Copper (μM)
HbA1c (mM) in males
f
p > 0.05
Copper (μM)
HbA1c (mM) in females
g
p = 0.0040
Magnesium (mM)
HbA1c (mM) in all subjects
h
p = 0.0067
Magnesium (mM)
HbA1c (mM) in males
i
p > 0.05
Magnesium (mM)
HbA1c (mM) in females
j
p = 0.0311
Selenium (μM)
HbA1c (mM) in all subjects
k
p > 0.05
Selenium (μM)
HbA1c (mM) in males
l
p > 0.05
Selenium (μM)
HbA1c (mM) in females
m
p = 0.0258
Zinc/copper ratio
HbA1c (mM) in all subjects
n
p > 0.05
Zinc/copper ratio
HbA1c (mM) in males
o
p > 0.05
Zinc/copper ratio
HbA1c (mM) in females


a
p > 0.05
Zinc (μM)
HbA1c (mM) in all subjects
b
p > 0.05
Zinc (μM)
HbA1c (mM) in males
c
p > 0.05
Zinc (μM)
HbA1c (mM) in females
d
p = 0.0034
Copper (μM)
HbA1c (mM) in all subjects
e
p > 0.05
Copper (μM)
HbA1c (mM) in males
f
p > 0.05
Copper (μM)
HbA1c (mM) in females
g
p > 0.05
Magnesium (mM)
HbA1c (mM) in all subjects
h
p > 0.05
Magnesium (mM)
HbA1c (mM) in males
i
p > 0.05
Magnesium (mM)
HbA1c (mM) in females
j
p = 0.0394
Selenium (μM)
HbA1c (mM) in all subjects
k
p > 0.05
Selenium (μM)
HbA1c (mM) in males
l
p = 0.0150
Selenium (μM)
HbA1c (mM) in females
m
p = 0.0428
Zinc/copper ratio
HbA1c (mM) in all subjects
n
p > 0.05
Zinc/copper ratio
HbA1c (mM) in males
o
p > 0.05
Zinc/copper ratio
HbA1c (mM) in females

◀**Fig. 2** Relationship between HbA1c concentration and plasma zinc, copper, magnesium and selenium concentrations as well as zinc/copper ratio in all T2DM subjects and male and female T2DM subjects. **a–c** HbA1c concentration vs zinc concentration, **d–f** HbA1c concentration vs copper concentration, **g–i** HbA1c concentration vs magnesium concentration, **j–l** HbA1c concentration vs selenium concentration and **m–o** HbA1c concentration vs zinc/copper ratio. **a**, **d**, **g**, **j**, **m** both sexes together, **b**, **e**, **h**, **k**, **n** male subjects and **c**, **f**, **i**, **l**, **o** female subjects. Black circles were used for the data that were correlated to HbA1c concentration, while white circles were used when the relationship was not significant. HbA1c concentration was positively correlated with plasma copper concentration in both sexes together ($p = 0.0034$) and with plasma selenium concentration in both sexes together ($p = 0.0394$) and in females ($p = 0.0150$), while it was negatively correlated with the zinc/copper ratio in both sexes together ($p = 0.0428$)

together ($p = 0.004$, Fig. 1g) and in males ($p = 0.007$, Fig. 1h), but not in females and it did not correlate with HbA1c concentration in T2DM. The lack of an observable relationship between plasma magnesium concentration and HbA1c concentration in females with T1DM is surprising considering that magnesium concentration was lower in females with T1DM compared to males with T1DM (while no difference between the sexes could be seen in controls). Previous studies have shown that HbA1c levels correlate with magnesium excretion in females but not males (Brown et al. 1999; Lin and Huang 2015). A negative correlation between plasma magnesium concentration and HbA1c concentration has also been reported in T2DM (Ramadass et al. 2015). Plasma selenium concentration correlated negatively with HbA1c in T1DM when looking at both sexes together ($p = 0.031$, Fig. 1j), as in a previous study (Ruiz et al. 1998), but there was no correlation when looking individually at males or females. In T2DM, plasma selenium concentration was correlated with HbA1c concentration when looking at both sexes together ($p = 0.039$, Fig. 2j) and in females ($p = 0.015$, Fig. 2l), contrary to a previous study that found a negative correlation with dysregulation of glucose and selenium concentration in males but not in females (Akbaraly et al. 2010). The plasma zinc/copper ratio was negatively correlated with HbA1c in T1DM and T2DM when looking at both sexes together ($p = 0.0258$, Fig. 1m and $p = 0.0428$, Fig. 2m respectively) but not in males or females only. In previous publications, the zinc/copper ratio was also negatively correlated in T1DM and T2DM (Lin et al. 2014; Atari-Hajipirloo et al. 2016). Correlation of HbA1c concentration with total plasma metal concentrations could potentially be explained by glycation or oxidation-associated modifications of metal ion transport proteins (Abdelmagid et al. 2015), formation of glycocholate (anion of the bile acid glycholic acid) that can complex and excrete metals in bile (Atari-Hajipirloo et al. 2016) or abnormal insulin signalling leading to dysregulated metal homeostasis (McNair et al. 1982; Gommers et al. 2016).

Implications for dysregulated metal homeostasis in diabetes

Changes in total plasma metal concentrations in subjects with diabetes have the potential to negatively affect metabolic processes in the body. Low magnesium concentrations have been associated with an increased incidence of T1DM and T2DM and poor glycaemic control (Lin and Huang 2015; Ramadass et al. 2015). This may be due to the fact that magnesium ions (Mg^{2+}) are an essential cofactor in several processes. Mg^{2+} increases the affinity of insulin receptors for ATP and is thus essential for their auto-phosphorylation and tyrosine kinase activity, which results in Mg^{2+} sensitising cells to insulin (Guerrero-Romero and Rodriguez-Moran 2011; Gommers et al. 2016; Al Alawi et al. 2018). Thus chronically low Mg^{2+} concentrations can lead to insulin resistance (Guerrero-Romero and Rodriguez-Moran 2011; Gommers et al. 2016; Al Alawi et al. 2018). Furthermore, Mg^{2+} can also block the entry of Ca^{2+} into adipocytes through the L-type calcium channel. Therefore, when Mg^{2+} concentrations are insufficient, increased Ca^{2+} entry in adipocyte leads to inflammation, oxidative stress and increase insulin resistance (Nielsen et al. 2007; Gommers et al. 2016; Al Alawi et al. 2018). Mg^{2+} is also involved in the transport of glucose across membranes, it is a cofactor of several enzymes essential for carbohydrate oxidation (e.g. phosphotransferases and phosphohydrolases such as ATPases) and also plays a role in the release of insulin (Saris et al. 2000; Al Alawi et al. 2018). On the other hand, insulin decreases tubular reabsorption of Mg^{2+} and so high insulin levels (as in T2DM) could lead to reduced Mg^{2+} concentrations (McNair et al. 1982; Gommers et al. 2016).

Selenium is mostly found in selenoproteins (as selenomethionine), of which many are important antioxidants (Roman et al. 2014). Therefore, high plasma concentrations of selenium may be due to oxidative stress mobilising high levels of selenoproteins. For example, the plasma concentration of selenoprotein P is known to be higher in T2DM subjects, a disease state associate with high levels of oxidative stress, and to be associated with insulin resistance (Misu et al. 2010; Ogawa-Wong et al. 2016). However, expression of plasma glutathione peroxidase (another selenoprotein) as well as serum albumin (which transports selenomethionine) are reduced in diabetes (Roman et al. 2010). Expression of selenoprotein P is known to be reduced during inflammation (Nichol et al. 1998; Hesse-Bahr et al. 2000). Thus blood levels of selenium decrease during systemic inflammatory responses, which occur in T2DM (Wang et al. 2013). It has been hypothesised that the stages of the disease (impaired glucose tolerance or well-established T2DM) and the associated level of oxidative stress influences plasma selenium concentration (Rayman and Stranges 2013).

Zinc participates in the synthesis, storage and release of insulin. Notably, zinc is present in secretory vesicles within β-cells of the pancreas where it participates in the crystallisation/storage of insulin and is thus released alongside insulin into the plasma (Scott 1934; Chabosseau and Rutter 2016). Deficiency of zinc disrupts insulin homeostasis, resulting in a reduction of insulin secretion by β cells (Fung et al. 2015). Zinc also stimulates lipogenesis and glucose uptake and reduced lipolysis in adipocytes (Coulston and Dandona 1980; Nishide et al. 2008). As zinc and copper homeostasis are closely linked, notably through competition during intestinal absorption and through shared transporter proteins such as serum albumin, a deficiency in one can affect the other (Osredkar and Sustar 2011). For example, zinc over-supplementation can lead to copper deficiency (Duncan et al. 2015). Impaired metabolism of zinc and copper are associated with a higher sensitivity to oxidative damage, as both zinc and copper are needed for the activity of the antioxidant enzyme superoxide dismutase, whose activity is reduced in T2DM (Sundaram et al. 1996). Thus, changes in the ratio of zinc/copper will affect enzyme catalysis and potentially further increase levels of free radicals, which are already increased in diabetes (Giacco and Brownlee 2010). In addition, copper is released from superoxide dismutase at high glucose concentrations due to fragmentation of the protein during glycation (Ookawara et al. 1992). Elevated copper levels in T2DM correlate with formation of reactive oxygen species (Masad et al. 2007). Insulin also reduces copper concentration in the liver through the regulation of at least one copper-transporting ATPase, ATP7B (Hilario-Souza et al. 2016). A reduction in insulin concentrations may thus result in an accumulation of copper in the liver.

Deficiency in magnesium, selenium and zinc have been shown to increase the risks of developing complicates associated with diabetes. Therefore, the effect of supplementation of these metals has been assessed. Magnesium supplementation was found to both improve diabetes management and to reduce the development of T2DM in individuals at risk (Rodriguez-Moran and Guerrero-Romero 2003; Hruby et al. 2014; Guerrero-Romero et al. 2015). Zinc supplementation has been shown to improve insulin and glucose levels in diabetes subjects and decrease the risk of developing T2DM (Jayawardena et al. 2012; Islam et al. 2016; Ranasinghe et al. 2018). However, selenium supplementation was not found to improve the risk of developing T2DM or the risk of developing complications in individuals with diabetes (Ogawa-Wong et al. 2016). This could be because the association between selenium concentrations and diabetes is not linear and that only individuals with selenium deficiency would benefit from supplementation, while individuals with sufficient selenium could increase their risk of developing T2DM if given an excess of this metal (Ogawa-Wong et al. 2016). Another explanation may be that the form of selenium used for supplementation in some studies offer variable bioavailability (Ogawa-Wong et al. 2016).

Conclusion

In conclusion, the study reported here is the first to examine zinc, copper, magnesium, selenium and HbA1c concentrations and sex-specific differences together in both T1DM, T2DM and controls. The homeostatic control of circulatory metal concentrations, in particular zinc, copper, magnesium and selenium, is essential for insulin regulation and energy metabolism. If plasma metal concentrations are altered in an individual with diabetes, the management of their condition will be more complex and they are more

likely to develop complications. In addition, plasma metal concentrations are also influenced by insulin and glucose plasma levels. Thus, if those are not properly controlled, plasma metal concentrations are likely to become further dysregulated. Here we show that plasma magnesium concentration is altered to the highest degree in T1DM. In T2DM, plasma selenium and copper concentrations were significantly affected. This work increases our understanding of T1DM and T2DM pathogenesis and may have future implications for the management of diabetes.

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Brief Review

OPEN

Glycosaminoglycan Neutralization in Coagulation Control

Amélie I.S. Sobczak, Samantha J. Pitt, Alan J. Stewart

Abstract—The glycosaminoglycans (GAGs) heparan sulfate, dermatan sulfate, and heparin are important anticoagulants that inhibit clot formation through interactions with antithrombin and heparin cofactor II. Unfractionated heparin, low-molecular-weight heparin, and heparin-derived drugs are often the main treatments used clinically to handle coagulatory disorders. A wide range of proteins have been reported to bind and neutralize these GAGs to promote clot formation. Such neutralizing proteins are involved in a variety of other physiological processes, including inflammation, transport, and signaling. It is clear that these interactions are important for the control of normal coagulation and influence the efficacy of heparin and heparin-based therapeutics. In addition to neutralization, the anticoagulant activities of GAGs may also be regulated through reduced synthesis or by degradation. In this review, we describe GAG neutralization, the proteins involved, and the molecular processes that contribute to the regulation of anticoagulant GAG activity.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2018;38: 1258-1270. DOI: 10.1161/ATVBAHA.118.311102.)**

Key Words: dermatan sulfate ■ glycosaminoglycan ■ heparan sulfate ■ heparin ■ thrombosis

Heparan sulfate (HS), dermatan sulfate (DS), and heparin are natural glycosaminoglycans (GAG), which are linear polysaccharides, heterogeneous in both sequence and length.[1] GAGs carry out many functions in the body and can influence numerous physiological processes. The most notable is control of coagulation, but GAGs also affect lipid metabolism, inflammation, cell attachment, migration, invasion, and differentiation.[1] GAGs play a key role as anticoagulants, preventing coagulation from occurring when it is not required. GAGs are responsible for interacting with and enhancing the actions of several serpins; HS and heparin primarily bind to antithrombin and DS to heparin cofactor II (HCII).[2] However, HS and heparin can also interact with HCII.[3] The principal activities of antithrombin are to inhibit both thrombin and activated factor X, 2 important proteins of the coagulation cascade.[2] HCII inhibits thrombin but not activated factor X.[3]

When coagulation is necessary, for example, after tissue injury, GAGs need to be neutralized to enable clot formation.[4] This includes endogenous GAGs during normal clotting and heparin-based drugs, which are used clinically to treat a range of thrombotic disorders, including venous thromboembolism and acute coronary syndrome.[5] This review will describe current knowledge concerning the principal properties of key proteins involved in GAG neutralization, the mechanisms by which they interact with GAGs, and how this affects the coagulation process.

Synthesis of GAGs

The saccharide sequences of HS and heparin consist predominantly of 2 trisulfated disaccharide motifs. The first motif represents *N*-sulfated glucosamine linked to iduronic acid (IdoA) and the other *N*-acetylated glucosamine (GlcNAc) linked to glucuronic acid (GlcA).[1] The sequence in which these motifs occur results from enzyme-catalyzed modifications.[6–8] Heparin and HS differ in the ratio by which these 2 motifs are present within the GAG; heparin is defined as containing at least 70% of the first motif.[8] The sequences of DS consist of 2 different motifs. These are *N*-acetylated galactosamine (GalNAc) linked to IdoA and GalNAc linked to GlcA.[1] The sequences of the saccharide groups that form HS, heparin, and DS are shown in Figure 1.[1] Some GAGs carry specific binding sequences for antithrombin and HCII, and those can greatly enhance the efficiency of the binding to those serpins.[3,9,10]

The length of the GAGs, sulfation percentage, and saccharide sequence vary depending on the tissue in which they are generated because of cell type–specific expression of GAG-synthesizing enzymes (as summarized in Refs. Carlsson and Kjellen[9] and Silbert and Sugumaran[11]). Synthesis begins by formation of a tetrasaccharide, GlcA–galactose–galactose–xylose, which forms the linkage region. For HS and heparin, the next saccharide to attach is GlcNAc, which is performed by a unique GlcNAc transferase-I enzyme that plays no further part in the synthesis. For DS, it is a GalNAc transferred by a GalNAc transferase enzyme. The chain is then further elongated by transferase enzymes, which add alternating GlcA and either GlcNAc or GalNAc. As the GAG chain grows, it is modified by the action of various enzymes, which include (1) the *N*-sulfation of GlcNAc to *N*-sulfated glucosamines by *N*-deacetylase/*N*-sulfotransferase; (2) the epimerization of

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Nonstandard Abbreviations and Acronyms	
DS	dermatan sulfate
ESL	endothelial surface layer
FGF	fibroblast growth factors
GAG	glycosaminoglycan
GalNAc	*N*-acetylated galactosamine
GlcA	glucuronic acid
GlcNAc	*N*-acetylated glucosamine
HCII	heparin cofactor II
HMWK	high-molecular-weight kininogen
HRG	histidine-rich glycoprotein
HS	heparan sulfate
IdoA	iduronic acid
LMWH	low-molecular-weight heparin
PF4	platelet factor 4
UFH	unfractionated heparin

D-GlcA saccharides, adjacent to *N*-sulfated glucosamines or GalNAc 4-S, to L-IdoA by C5-epimerases; (3) the 6-*O*-sulfation of GlcNAc and GalNAc by 6-*O*-sulfotransferases; (4) the 4-*O*-sulfation of GalNAc by 4-*O*-sulfotransferases; and (5) the 2-*O*-sulfation of IdoA (and in to a lesser extent GlcA) by 2-*O*-sulfotransferases.

In addition, a less frequent but important modification is the sulfation in the C3 position of GlcNAc by 3-*O*-sulfotransferase, which forms part of the antithrombin-binding site.[9] This antithrombin-binding site on HS and heparin is a pentasaccharide sequence. On DS, the HCII-binding sequence is an hexasaccharide sequence consisting of a repeat of IdoA(2-OSO_3^-)-GalNAc(4-OSO_3^-).[3] The natural binding sequences for HCII on HS and heparin have not yet been confirmed, but 2 possible HS hexasaccharide structures have been predicted based on an in silico study, and enhancement of HCII inhibition of thrombin has been confirmed after synthesis of the hexasaccharides.[10] The serpin-binding sites on HS, DS, and heparin are shown in Figure 2.

Distribution of HS, DS, and Heparin and Their Anticoagulant Actions

HS and DS are synthesized by many cell types and tissues, whereas heparin is only synthesized in mast cells.[12] HS is mainly localized at the surface of cells and the endothelium,[1] while DS is present in the extracellular matrix of several types of tissue including skin, bone, cartilage, and the vasculature.[1] The surface of the endothelium is made of a layer of glycoproteins and proteoglycans called the endothelial surface layer (ESL), which is made of a core protein bound to one or several GAG chains. The ESL varies in thickness depending on its location: ranging from around 0.5 to 3 μm in small arteries ≤4.5 μmol/L in carotid arteries.[13] ESL thickness is also influenced by oxidative stress and atherosclerosis.[13] The composition of the ESL is dynamic; ESL proteins undergo a high rate of turnover, and the specific GAGs that are present (and their sulfation pattern) also change over time. Turnover and GAG-binding specificity depends on the activation of the endothelial cells by local chemokine stimuli.[13] A summary of proteoglycans present in the ESL and their GAG-binding properties are provided in Table 1.

An important aspect of the ESL is its anticoagulant properties. Indeed, ESL GAGs bind several anticoagulant proteins, antithrombin, HCII but also thrombomodulin and tissue factor pathway inhibitor. The endothelial anticoagulant HS, for example, is saturated with antithrombin (the K_d for this interaction is 15 nmol/L, while antithrombin plasma concentration is 3.5 μmol/L).[14] GAG binding to serpins induces a change in the conformation of the reactive center loop of the serpin, thus enhancing the inhibitory activity of the protein.[2] Longer chain GAGs are specifically required to enhance the binding of antithrombin to thrombin but not to activated factor X.[2] This is also the case for HCII and thrombin, although some thrombin inhibition still occurs in the presence of shorter chain GAGs (Figure 3).[3]

The main GAG in the vasculature is HS, which represents 50% to 90% of the total GAG content.[13] Next is chondroitin sulfate, of which DS is a subtype.[13] Although all membrane-associated HS can bind antithrombin, only a small fraction of these molecules (0.5%–10%) possess the antithrombin-binding sequence necessary to bind with high specificity under physiological conditions.[14,15] Approximately 95% of anticoagulant HS is present in the subendothelial matrix and is only in contact with blood when injury occurs.[14,15] In the case of heparin, ≈30% of molecules possess the antithrombin-binding sequence.[16] The exact proportion of HS and heparin that can bind HCII is unknown, but a much greater quantity of the GAG is required to overcome HCII-mediated inhibition than with antithrombin.[3] Vascular DS is located in the deeper layer of the vessel walls and in the subendothelium and is only able to interact with blood proteins during injury.[3] Roughly 5% of

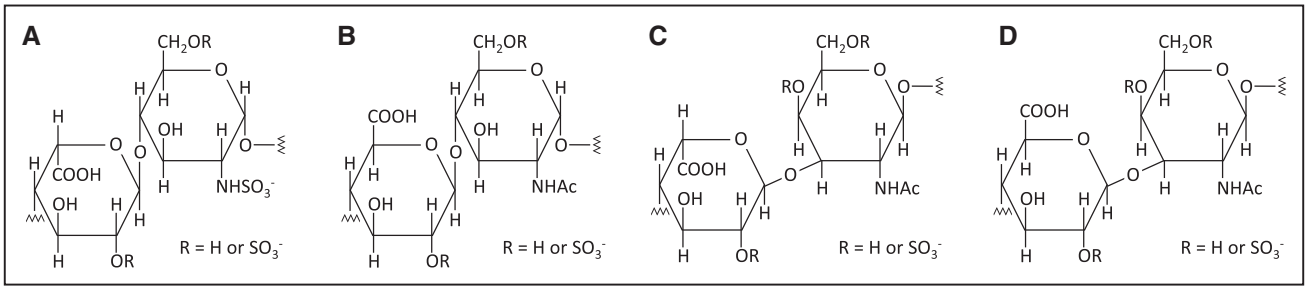

Figure 1. The principal disaccharide motifs that constitute heparin, heparan sulfate, and dermatan sulfate. **A**, Heparin and heparan sulfate motif 1, iduronic acid-*N*-sulfated glucosamine. **B**, Heparin and heparan sulfate motif 2, glucuronic acid-*N*-acetylated glucosamine. **C**, Dermatan sulfate motif 1, iduronic acid-*N*-acetylated galactosamine. **D**, Dermatan sulfate motif 2, glucuronic acid-*N*-acetylated galactosamine.

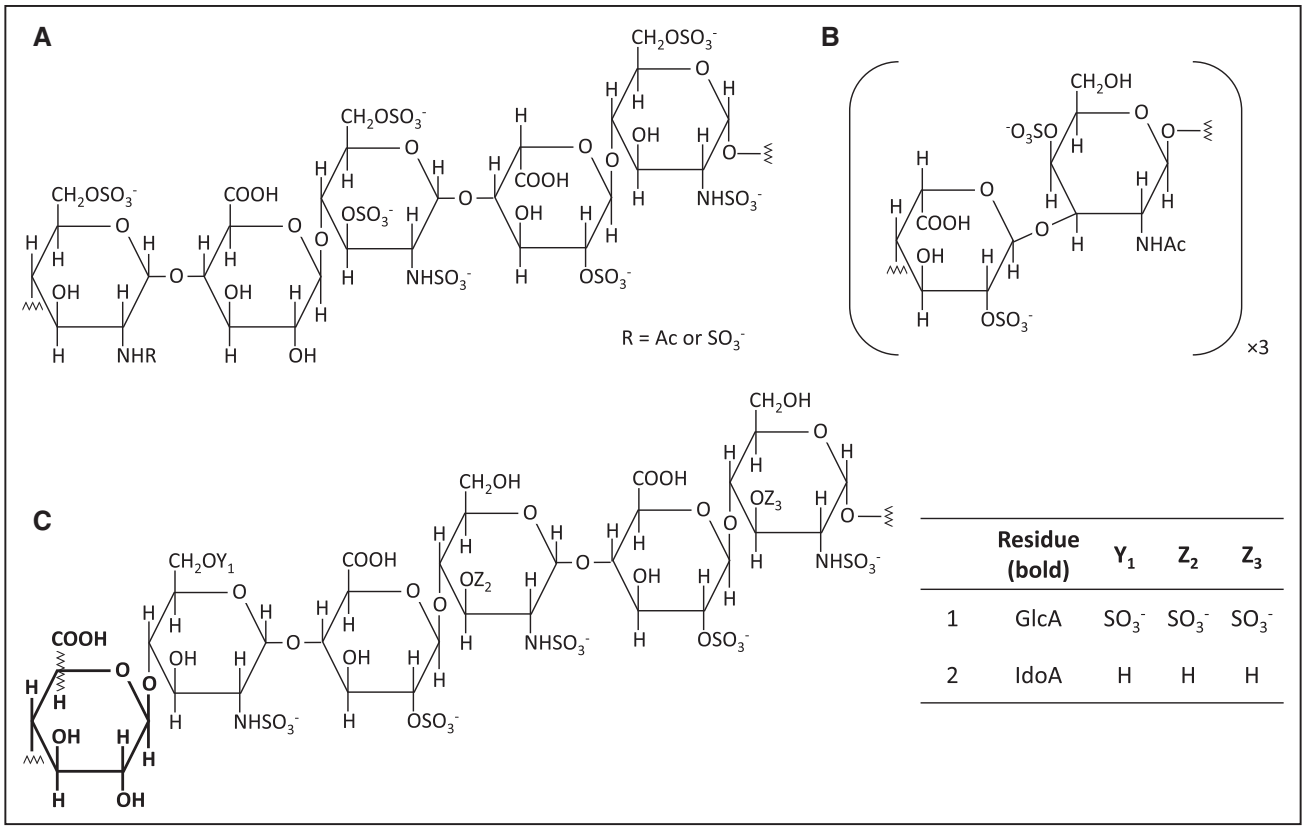

Figure 2. Anticoagulant glycosaminoglycans-binding sequence for antithrombin and heparin cofactor II. **A**, The main heparin and heparan sulfate sequence for binding to antithrombin. **B**, The main dermatan sulfate sequence for binding to heparin cofactor II. **C**, Two heparin and heparan sulfate sequences for binding to heparin cofactor II. The sequences were predicted in silico, and their ability to neutralize those two glycosaminoglycans was confirmed in vitro.[10] GlcA indicates glucuronic acid; and IdoA, iduronic acid.

DS disaccharides are the IdoA(2-OSO_3^-)-GalNAc(4-OSO_3^-) disaccharide unit, of which 3 consecutive repeats are required to form the HCII high-affinity-binding sequence. Chains containing only part of the total sequence still bind HCII but with a lower affinity.[3]

The importance of heparin release from mast cells at sites of injury is controversial. Some studies have been unable to detect heparin in plasma,[17,18] while Engelberg and Dudley[19] (1961) reported that each liter of plasma contains 1.0 to 2.4 mg of heparin (*ca.* 66–160 nmol/L). To ascertain the importance of endogenous anticoagulant GAG in vivo, 2 knockout mice models have been studied. Hemostasis in mice lacking 3-*O*-sulfotransferase-1 (the enzyme involved antithrombin-binding site formation) was not greatly affected by removal

Table 1. Proteoglycans Present in the ESL and Their GAG-Binding Properties[13]

Core Protein Name	Number of Subtypes	Type of GAG Chains Bound	Number of GAG Chains Bound	Attached to the ESL or Secreted Into the Plasma	Notes
Syndecan	4	HS and chondroitin sulfate	5	Attached	
Glypican	6	HS and chondroitin sulfate	3	Attached	Main anticoagulant proteoglycan
Mimecan	1	Keratan sulfate	2–3	Secreted	
Perlecan	1	HS and chondroitin sulfate	3	Secreted	
Biglycan	1	Chondroitin sulfate and DS	2	Secreted	
Versican	1	Chondroitin sulfate and DS	10–30	Secreted	
Decorin	1	Chondroitin sulfate and DS	1	Secreted	

DS indicates dermatan sulfate; ESL, endothelial surface layer; GAG, glycosaminoglycans; and HS, heparan sulfate.

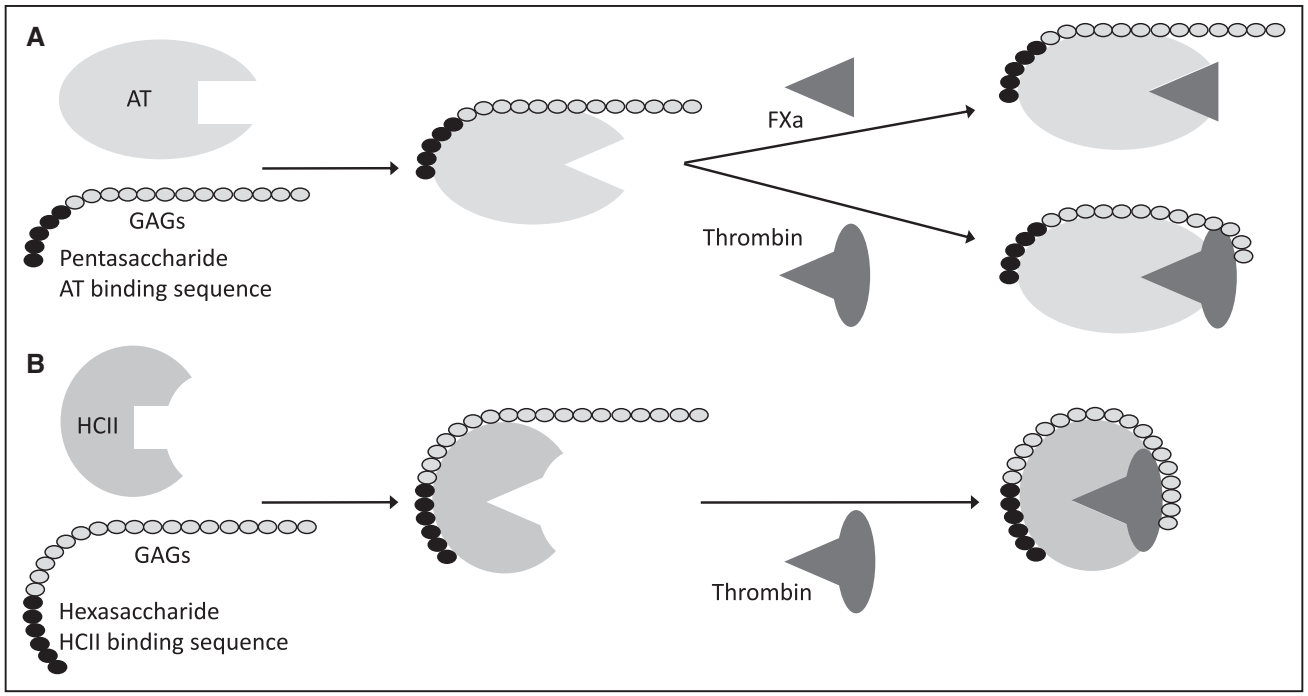

Figure 3. Interactions of glycosaminoglycans (GAGs) with (**A**) antithrombin (AT), thrombin, and activated coagulation factor X (FXa) and (**B**) heparin cofactor II (HCII) and thrombin. The GAGs bind AT and HCII via specific binding sequences, a pentasaccharide or a hexasaccharide sequence, respectively. This binding induces a conformational change in the serpins, which makes the reactive center loop more accessible for their substrates. A simultaneous binding to AT and thrombin requires a chain length of minimum 18 saccharides while binding to HCII, and thrombin requires 24 saccharides.

of the gene encoding this enzyme, where a strong procoagulant challenge failed to reveal a latent procoagulant state.[14] This suggests that either the anticoagulant activity of HS is not essential for normal homeostasis in vivo or that there is redundancy between sulfotransferase-1 and one of its isotypes (eg, 3-*O*-sulfotransferase-5). It is also possible that antithrombin expression is increased in these animals to compensate for the loss of the enzyme.[14,20] In addition, HS lacking the canonical antithrombin-binding sequence can still bind antithrombin but exhibits reduced affinity compared with HS with the binding sequence present. This HS has antithrombin activity but not anti–activated factor X activity.[14] Mice engineered to lack the *N*-deacetylase/*N*-sulfotransferase 2 enzyme (encoded by the *NDST2* gene), which is involved in heparin, but not HS, synthesis have also been studied.[21,22] No coagulatory defects were reported in these animals. However, these studies did not specifically examine clot parameters.[18,21,22]

Use of Heparin-Based Drugs

Clinically, heparins are the main anticoagulants administered for several conditions, including venous thromboembolism, acute coronary syndrome, cardiopulmonary bypass, and hemodialysis.[7] Naturally occurring heparins vary in size from 3 to 30 kDa, with an average of 15 kDa and when used therapeutically are termed unfractionated heparins (UFH). UFH can be fully neutralized by protamine sulfate when there is a risk of bleeding.[23] Low-molecular-weight heparins (LMWH) are artificially derived from UFH by depolymerization or fractionation,[6] and their use has now replaced that of UFH for many clinical applications. LMWHs are associated with fewer side effects (notably reduced prevalence/severity of osteoporosis and heparin-induced thrombocytopenia) and have a more predictable dose–response profile because they associate less with plasma proteins.[5] They also have a longer half-life and thus require less frequent administration. It should be noted, however, that LMWHs are more expensive, can only be partially neutralized by protamine sulfate, and cannot be cleared from patients having renal insufficiency (and in such cases they will accumulate over time).[4,7] A summary of US- and EU-approved heparin-based drugs are provided in Table 2. These also include the synthetic analogue of heparin, Fondaparinux—which is based on the antithrombin-binding sequence. This drug is thought to exhibit fewer side effects than LMWH drugs because of having a higher specificity for antithrombin. Importantly, Fondaparinux cannot be neutralized (although injections of recombinant factor VII may be effective to stop bleeding) and can only be cleared by the renal system. As with LMWH, it is therefore contraindicated in patients having renal disease.[5] Another drug, danaparoid is a mixture of HS, DS, and chondroitin sulfate and is used in some countries for the treatment of heparin-induced thrombocytopenia.[24]

The binding of endogenous GAGs to proteins is influenced by various factors, including the degree of sulfation, which facilitates electrostatic interactions between the GAG and the respective protein. Heparin is generally more sulfated than HS, and DS less so.[25] A possible clinical consequence of such interactions is that administration of heparin-based drugs can potentially displace many of the proteins bound to GAGs in the

Table 2. US- and EU-Approved Heparin-Based Drugs, Their Major Side Effects, Contraindications, and Main Applications[5–7,24]

Drug Name	Average Size, kDa	Country Where Approved	GAG Type	Side Effects and Contraindications	Applications
UFH	15	USA, Europe	UFH	Heparin-induced thrombocytopenia, osteoporosis	Venous thromboembolism, acute coronary syndrome, cardiopulmonary bypass, hemodialysis, patients with underlying bleeding risk, or in those with renal insufficiency
Enoxaparin	4.5	USA, Europe	LMWH	Some heparin-induced thrombocytopenia, only partial neutralization needed, risk of bleeding, renal insufficiency or disease	Osteoporosis, heparin-induced thrombocytopenia
Dalteparin	6	USA, Europe	LMWH		
Tinzaparin	6.5	USA, Europe	LMWH		
Reviparin	3.9	Europe	LMWH		
Nadroparin	4.3	Europe	LMWH		
Bemiparin	3.6–3.8	Europe	LMWH		
Certoparin	3.8	Europe	LMWH		
Parnaparin	5	Europe	LMWH		
Danaparoid	5.5	Europe	Mixture of HS, DS, and chondroitin sulfate	Some heparin-induced thrombocytopenia, risk of bleeding, renal insufficiency, or disease	Treatment of heparin-induced thrombocytopenia
Fondaparinux	1.7	USA, Europe	Synthetic heparin analogue	Neutralization not possible, risk of bleeding, renal insufficiency, or disease	Treatment of heparin-induced thrombocytopenia

DS indicates dermatan sulfate; EU, European Union; GAG, glycosaminoglycans; HS, heparan sulfate; LMWH, low-molecular-weight heparin; UFH, unfractionated heparin; and US, United States.

ESL. This may result in an increase in the availability of GAGs for interaction with clot-regulating proteins (see Figure 4).[26] Such a mechanism would alter the half-life and kinetic properties of these agents. The associated difficulties in predicting such events could result in excessive bleeding in patients.

Heparin-based drugs are generally best for acute management and prophylaxis of deep vein thrombosis and pulmonary embolism and for prophylaxis during and after orthopedic and general surgeries.[5,27,28] Current NICE guidelines from the United Kingdom suggest that, when prophylaxis is required, offering patients either Fondaparinux or LMWH (no distinction is made between the different LMWHs) is usually best practice. However, UFH, despite its associated side effects, can be advised when rapid intervention is needed, in individuals with an increased risk of bleeding or those who are having renal failure.[28] Whether LMWH or UFH are administered for venous thromboembolism prophylaxis is highly variable, and patients have not always received the recommended treatment. The 2007 study—IMPROVE (the International Medical Prevention Registry on Venous Thromboembolism), which examined hospitalized patients at risk of venous thromboembolism across 12 countries—found that out of all patients who should have received prophylaxis (in accordance to the guideline recommendations at the time from the American College of Chest Physicians), 14% of patients in the United States received LMWH and 21% UFH. This preference is because of UFH being a lower cost drug. Across the other participating countries, where downstream costs were more likely to be considered, 40% of patients on average received LMWH and 9% UFH.[29] From this, it is clear that practices for administration of prophylaxis are suboptimal, and stricter evidence-based guidelines in hospitals urgently need to be implemented.

GAG-Neutralizing Proteins Released During Injury and Coagulation

The ESL is a heterogeneous surface that can bind proteins and other molecules and is essential for the function of the endothelium.[13] GAGs associated with the ESL are involved in numerous physiological processes: coagulation, lipid metabolism, inflammation, cell attachment, migration, invasion, and differentiation.[1] Receptors, enzymes, and their respective ligands/substrates can bind to vascular GAGs, causing a localized rise in their concentration to impact on signaling or enzymatic modification. Fibroblast growth factors (FGF) notably need to bind to endothelial HS for functioning as this helps mediate FGF oligomerization, binding of FGFs to their cognate receptors, and transport of FGF between cells and can act as an FGF reservoir.[30] Proteins involved in regulating a variety of physiological processes have the ability to neutralize the anticoagulant activity of certain GAGs. A range of these proteins and their specific properties are listed in Table 3. Neutralization can be accomplished via different mechanisms, and so particular neutralizing proteins may affect only certain GAG–serpin combinations (as described in Table 4). Some of these neutralizing proteins are present at high levels in blood plasma, but other common sources include activated platelets, activated neutrophils, and damaged cells (Figure 4). As important binding partners of GAGs, some growth factors have GAG-neutralizing properties. This is the case for FGF7, heparin affin regulatory peptide and, to a lesser extent,

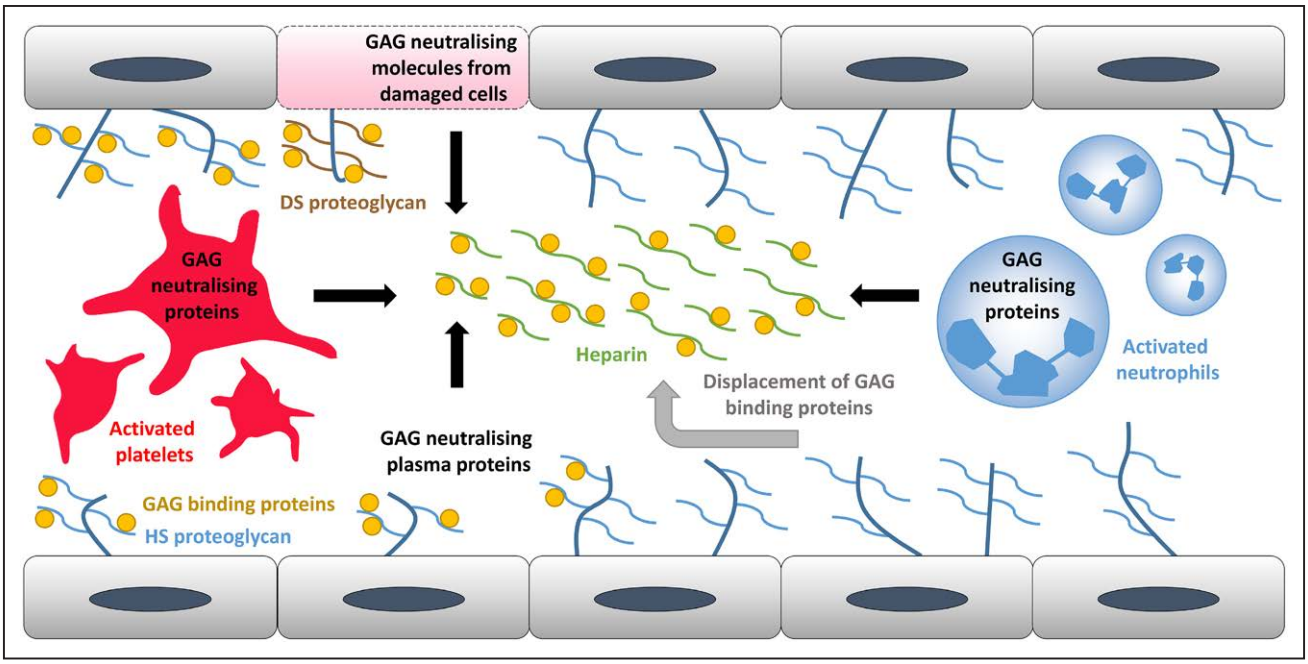

Figure 4. Representation of the key locations where circulatory glycosaminoglycans (GAG)-neutralizing proteins/molecules are derived. Numerous proteins normally bind to endothelial GAGs. They are displaced by injection of heparin. Several key neutralizing molecules are also found in the plasma. Activated platelets and neutrophils can also release such proteins through exocytosis of granular vesicles. Damaged cells expressing dermatan sulfate–containing proteoglycans also release GAG-neutralizing molecules.

FGF1.[31,32] FGF7 and heparin affin regulatory peptide are also upregulated during injury, thus giving GAGs a dual role in coagulation mediation and wound healing.[32,33]

Similar to FGF, chemokine and cytokine activity is directly linked to their ability to bind endothelial GAGs which can direct and enhance their actions. For example, GAGs can modulate the inflammatory response by binding cytokines and so preventing them from binding to cell surface receptors. Cleavage of those GAGs during inflammation releases cytokines which in turn increases endothelial cells activation.[13] Such chemokines also reside in the α-granules of platelets and are released when these cells are activated. Their GAG-binding ability has been linked to a dual role of GAG in wound healing. Among those is platelet factor 4 (PF4), an important GAG-neutralizing protein. It has been proposed that, when released, PF4 neutralizes the negative charge of GAGs at the surface of endothelial cells. This allows platelets (which possess a net negative charge at their surface) to associate with the endothelium to enhance thrombus formation.[52] In addition, PF4 binds to nucleic acid (another type of polyanion) exposed by damaged cells. Heparin-induced thrombocytopenia, one of the significant secondary effects caused by heparin administration, is induced by antibodies reacting to the presence of PF4–nucleic acid complexes.[75]

Another family of GAG-neutralizing proteins are proteins involved in coagulation that are also secreted by activated platelets at site of injury. Among those are vitronectin, fibronectin, and fibrinogen. Vitronectin is an abundant plasma protein, and it is released from platelets during the acute-phase response.[38] It is involved in the regulation of the coagulation, complement, and fibrinolytic systems, as well as that of cell differentiation, proliferation, and morphogenesis.[76] Vitronectin is also an anticoagulant GAG neutralizer.[77] The main consequence of GAG–vitronectin association is to allow the binding of a ternary complex composed of vitronectin, thrombin, and antithrombin to cell surface proteoglycans to facilitate internalization and degradation of the complex.[77] In disease, the increased concentration of vitronectin would increase this degradation and thus decrease thrombin and antithrombin availability.

As with vitronectin, fibronectin is abundant in plasma and is a major component of the ESL.[78] It is involved in numerous cellular processes, including development, organogenesis, cell adhesion and migration, hemostasis, angiogenesis, and vascular remodeling.[78] Plasma fibronectin circulates in the blood in a compact conformation until it binds to endothelial GAGs.[78] It then alters its structure to form an extended conformation that subsequently assembles into fibrils.[78] A consequence of this binding is that plasma fibronectin interferes with antithrombin binding to immobilized LMWH; however, antithrombin is only completely displaced from heparin at fibronectin/antithrombin ratios greater than those found physiologically.[39,79] On the other hand, the binding of injected heparin to endothelium-bound fibronectin fibers induces a conformational change in fibronectin that increases its affinity for vascular endothelial growth factor.[80]

Fibrinogen is another abundant plasma protein and is the main protein responsible for the creation of blood clots. Fibrinogen binds to endothelial cells through surface proteoglycans, which facilitates clot formation.[81] Heparin binding to this bound fibrinogen (to which they have a higher affinity than to free fibrinogen) then mitigates clot nucleation through the formation of a fibrinogen–heparin–thrombin ternary complex.[81] As a consequence of this

Table 3. General Properties of Important GAG-Binding Proteins Including Their Functions, Where They Are Synthesized and Stored, When They Are Released, Their Plasma Concentration, and Specific Information on the Abilities of These Proteins to Bind Particular GAGs

Protein	Size, kDa	Function	Cell/Tissue Expression	Localization	Normal Plasma Conc.	GAG-Binding Specificity
FGF7	28	Growth factor (epithelial cells)	Mesenchymal cells of parenchymal organs	Plasma (upregulated in response to injury)	643 pmol/L[34]	Heparin; HS; DS
FGF1	17.46	Growth factor (endothelial cells), angiogenesis	Brain, kidney and heart	Plasma	28–48 pmol/L[35]	Heparin; HS; DS
Heparin affin regulatory peptide	15.3	Growth factor (neurite outgrowth in embryos and tissue development)	At the surface of cells	Plasma (released locally on injury)	33 pmol/L[32]	Heparin; HS; DS
PF4	7.8	Coagulation	Megakaryocytes	Platelet α-granules; plasma (secreted during coagulation)	0.256–1.28 nmol/L,[36] can exceed 2 μmol/L near injury site[37]	Heparin (±AT sequence); HS; DS
Vitronectin	75	Coagulation, fibrinolysis	Liver	Megakaryocyte and platelet α-granules, plasma (increased during acute-phase response)	2.67–5.33 μmol/L[38]	Heparin (+AT sequence or + cleavage sequence); not DS
Fibronectin	500	Cell–cell adhesion, platelets adherence, complement and coagulation system activation and wound healing	Liver	Platelet α-granules; plasma (secreted during coagulation)	300–600 nmol/L[39]	Heparin (+AT sequence); HS; DS
Fibrinogen	340	Coagulation (form fibrin clot)	Liver	Platelet α-granules; plasma (secreted during coagulation)	12–24 μmol/L[40]	Heparin; DS
HRG	75	Angiogenesis, immunity, coagulation, fibrinolysis	Liver	Platelet α-granules; plasma (secreted during coagulation)	1.3–2.0 μmol/L[41,42]	Heparin (±AT sequence); HS; weakly to DS
High-molecular-weight kininogen	120	Coagulation (intrinsic pathway of coagulation cascade)	Mostly in the liver but also endothelial cells and neutrophils	Platelet α-granules; plasma (secreted during coagulation)	1–2 μmol/L[43]	Heparin (±AT sequence); HS
Serum amyloid P protein	25	Not fully known, clear cellular debris, innate immunity, neurodegeneration (amyloid binding)	Liver	Plasma	1.6 μmol/L[44]	Heparin; HS; DS
Kallistatin	58	Coagulation (serpin, inhibits kallikrein)	Mostly in the liver but also in the kidneys, pancreas, and blood cells	Plasma (decreases during sepsis[45,46])	380 nmol/L[45]	Heparin; not HS or DS
Lactoferrin	80	Inflammation	Most mucosal secretions such as uterine fluid, vaginal secretion, seminal fluid, saliva, bile, pancreatic juice, small intestine secretions, nasal secretion, and tears; milk; myeloid tissue	Secondary granules of neutrophils, plasma (increases ≤3-fold during pregnancy, menstrual cycle, infection, inflammation, and excessive iron intake)	0.25–19 nmol/L[47]	Heparin; HS; not DS
Alpha-1-acid glycoprotein	37–54 (glycosylation)	Inflammation (modulate immune response), coagulation (inhibit platelet aggregation), drug transport (heparin)	Liver on signaling by proinflammatory cytokines, lymphocytes, monocytes, and neutrophils	Plasma (upregulated during acute-phase response and ≤10 fold in response to injury, infection, pregnancy)	10–33 μmol/L[48]	Does not bind GAG but thrombin and FXa

(*continued*)

Table 3. Continued

Protein	Size, kDa	Function	Cell/Tissue Expression	Localization	Normal Plasma Conc.	GAG-Binding Specificity
Histones	10–21	Inflammation, coagulation (platelet activation and aggregation)	All cells	Cell nuclei (released from cells after death; secreted by activated inflammatory cells)	nmol/L range; increases during septic shock (eg, histone H3 increases from 13 to 756 nmol/L)[49]	Heparin
Low-density lipoproteins		Cholesterol, triglycerides, fat-soluble vitamins transport. Some proteins involved in acute-phase response, coagulation and hemostasis	Liver	Plasma (upregulated in athletes)	2.81 mmol/L[50]	Heparin; HS; DS
Tissue factor	33	Coagulation (extrinsic pathway of the coagulation cascade)	Subendothelial cells and fibroblast	Platelet α-granules; mononuclear, smooth muscle or endothelial cells (secreted and exposed during coagulation)		Heparin
Activated factor VII	50	Coagulation (extrinsic pathway of the coagulation cascade)	Liver (zymogen), vitamin K-dependent	Plasma	16 nmol/L[51]	Heparin
Slit3	167.6	Axon guidance, cell migration and proliferation, angiogenesis	Endothelial cells	Plasma (secreted by endothelial cells)	unknown	Heparin; HS

AT indicates antithrombin; DS, dermatan sulfate; FGF, fibroblast growth factors; GAG, glycosaminoglycans; HRG, histidine-rich glycoprotein; HS, heparan sulfate; and PF4, platelet factor 4.

binding, fibrinogen is also involved in anticoagulant GAG neutralization. The direct study of fibrinogen-mediated GAG neutralization is complicated by the fact that thrombin cleaves fibrinogen into fibrin. Yet, fibrinogen is known to be more effective at neutralizing DS than both PF4 and histidine-rich glycoprotein (HRG).[58] This neutralization occurs at physiological fibrinogen concentrations, and the mechanism seems not to be through direct competition for DS binding but by modulating the rate of formation of the thrombin–HCII complex.[58] Fibrin can form complexes with heparin, antithrombin, and thrombin to reduce thrombin inhibition by antithrombin.[59,60] Because of those interactions, plasma fibrinogen levels are linked to heparin resistance in patients.[82]

In addition to those proteins, both Ca^{2+} and Zn^{2+} are released from activated platelets, and they can also affect the activity of GAGs.[83] The role of Zn^{2+} in the neutralization is particularly interesting as the concentration of labile Zn^{2+} in plasma can be directly influenced by free fatty acid levels in plasma through a switch on human serum albumin, the main plasma transporter for both Zn^{2+} and free fatty acids.[84,85] This dynamic may be important for individuals with diabetes mellitus,[86] obesity,[87] and cancer[88] who typically associate with higher plasma free fatty acids levels and have a higher incidence of developing thrombotic complications.[89] This is further supported by a study suggesting that higher doses of UFH are required in diabetic versus nondiabetic individuals.[90]

GAG Neutralization During Inflammation

Inflammation and coagulation are processes that are closely linked because such inflammatory proteins often come into contact with GAGs and can influence their anticoagulant activity. Some proteins even play a dual role in both processes, as is the case of HRG, a key adaptor protein released by platelets that regulates angiogenesis, immune functioning, and coagulation.[91,92] HRG is the second most abundant HS-binding protein in plasma after antithrombin and binds endothelial HS in a Zn^{2+}-dependent manner. HRG–GAG binding is thus enhanced at injury sites where platelets release Zn^{2+}.[62] This allows the protein to both neutralize anticoagulant GAGs and to provide a tether site on the ESL to facilitate interaction with ligands such as plasminogen.[85,93] In addition, HRG can compete with FGF for binding to HS and thus mediate the mitogenic activity of growth factors.[94] High-molecular-weight kininogen (HMWK) and serum amyloid P protein share similar heparin-neutralizing functions (and ligands-binding properties) to HRG and are present in plasma in similarly high concentrations.[64,65] HMWK is involved in coagulation through the activation of factor XII. During this action, HMWK is cleaved by kallikrein into the peptide bradykinin, which plays a role in vasodilation.[64] Serum amyloid P protein is involved in the innate immune system and in clearing cellular debris but also contributes to the progression of neurodegeneration through its interaction with amyloid fibers.[44,95] HRG, HMWK, and serum amyloid P protein can all bind to polyanions such

Table 4. Combinations of GAGs and Serpins Neutralized by Specific Anticoagulant GAG-Neutralizing Proteins

Neutralizing Protein	Heparin-AT Activity Neutralization	HS-AT activity Neutralization	Heparin-HCII Activity Neutralization	DS-HCII Activity Neutralization
FGF7	Yes*[31]	Unknown	Unknown	Unknown
FGF1	Yes*[31]	Unknown	Unknown	Unknown
Heparin affin regulatory peptide	Yes[32]	Yes[32]	Unknown	Unknown
Platelet factor 4	Yes*[36,52,53]	Yes[54]	Yes[55]	Yes[55]
Vitronectin	Yes*[56,57]	Unknown	No[58]	No[58]
Fibronectin	Yes*†[39]	Unknown	Unknown	Unknown
Fibrinogen	Yes[59,60]	Unknown	Unknown	Yes[58]
HRG	Yes*[61]	Yes†‡[61,62]	Yes[55,58,63]	Yes‡[55,58,63]
High-molecular-weight kininogen	Yes*[64]	Unknown	Unknown	Unknown
Serum amyloid P protein	Yes[65]	Unknown	Yes[65]	Yes[65]
Lactoferrin	Yes[66]	Unknown	Unknown	Unknown
Alpha-1-acid glycoprotein	Yes[67,68]	Unknown	Unknown	Unknown
Histones	Yes*[69]	Unknown	Unknown	Unknown
Low-density lipoprotein	Yes[70]	Unknown	Unknown	Unknown
Tissue factor	Yes[71]	Unknown	Unknown	Unknown
Activated factor VII	Yes*[72,73]	Unknown	Unknown	Unknown
Slit3	Yes[74]	Unknown	Unknown	Unknown

AT indicates antithrombin; FGF, fibroblast growth factors; HCII, heparin cofactor II; HRG, histidine-rich glycoprotein; HS, heparan sulfate; and LMWH, low-molecular-weight heparin.

*Full or partial neutralization of LMWHs.

†Excess protein is needed for full neutralization.

‡Weak neutralization.

as GAGs but also pathogens, anionic phospholipids (such as those exposed by dying cells), and DNA.[94] For certain pathogens, this interaction can destabilize the membrane, leading to cell death or can reduce their pathogenicity through incorporation of the organisms inside fibrin clots.[94] Similarly, HRG can also tether IgG to necrotic cells through binding to specific phospholipids at their surface and thus facilitate their phagocytosis.[96] Binding of HMWK to polyanionic surface exposed by damaged cells also activates the kallikrein coagulation pathway.[94]

Lactoferrin is a low abundance (<19 nmol/L) iron-binding plasma protein stored in neutrophils.[97,98] It can increase in concentration up to 3-fold during severe infection, autoimmune disease, or pregnancy, in addition to a local increase at the site of infection.[47] It has been shown to bind and neutralize heparin in a dose-dependent manner, and its activity is comparable to that of PF4.[66,99] However, this interaction can outcompete the binding of pathogens at the cell surface, preventing cell entry and stopping infection at an early stage.[100] Thus, lactoferrin displaced by exogenous heparin administration has the potential to negate this activity and leave the organism more vulnerable to infection. Another inflammation-associated protein is alpha-1-acid glycoprotein, an acute-phase protein responsible for modulating the immune response. It inhibits platelet aggregation and is an important plasma drug carrier involved in transporting heparin.[48] Alpha-1-acid glycoprotein is abundant in plasma and is upregulated in certain disease states (liver cancer, HIV infection), drug use, or pregnancy. In addition to its basal concentration in plasma, it is also secreted locally by activated neutrophils.[48] Within the ESL, it plays an important role in maintaining capillary permeability.[101] Alpha-1-acid glycoprotein can neutralize heparin but only when present at high concentrations, such as those that occur during inflammation.[67,68] An injection of heparin could potentially saturate the transport site on the protein and prevent it from carrying other drugs or molecules.

Histones are usually associated with DNA inside cell nuclei but are released into plasma by activated inflammatory cells (to form neutrophil extracellular traps) and after cell death.[49] They are mediators of cytotoxicity and sepsis during which their plasma concentration increases significantly.[102] Histones have various procoagulatory activities (activation of platelets, stimulation of thrombin generation, and promotion of von Willebrand factor release), which include the ability to neutralize heparin.[69] As a consequence, heparin–histone binding interferes with formation of neutrophil extracellular traps and perturbs venous thrombosis.[103] Histones, however, bind more readily to other polyanions. Its interaction with polysialic acid, for example, is important in the development and regeneration of the nervous system.[104,105]

Although not strictly a GAG-neutralizing protein, kallistatin is a GAG-binding serpin. The binding of GAG prevents kallistatin from binding and inhibiting kallikrein, thus allowing activation of factor XII and cleavage of HMWK into bradykinin. Both events result in antiangiogenic and

procoagulatory effects.[45,106] An important consequence of this is that competition between kallistatin and vascular endothelial growth factor and bFGF for endothelial GAG binding reduces the angiogenic effects associated with these molecules.[106]

Other Molecules That Can Neutralize GAGs

Lipoproteins are macromolecular complexes made up of lipids and protein. Depending on their size, they are classified as chylomicrons, very-low-density lipoproteins, intermediate-density lipoproteins, low-density lipoproteins, and high-density lipoproteins.[107] In the blood, they are involved in the transport of cholesterol, triglycerides, and fat-soluble vitamins.[107] They are therefore present in the blood and interact easily with GAGs. Low-density lipoproteins form insoluble complexes with heparins.[108,109] Very-low-density lipoproteins have a similar but reduced effect on heparin while high-density lipoproteins do not neutralize heparin.[70] The binding of lipoproteins to endothelial proteoglycans and the subsequent inflammatory responses could potentially have important consequences in the initiation and progression of the atherosclerotic process.[13] Other proteins have also been shown to neutralize heparin (tissue factor,[71] factor VII,[72,73] and the axon guidance protein, Slit3[74]); however, their low concentrations make them unlikely to play a major role in endogenous neutralization.

Breakdown or Downregulation of GAGs

In addition to their neutralization, heparin, HS, and DS can be prevented from exercising their anticoagulant actions through either a reduction in synthesis or through targeted degradation. Homocysteine, a compound generated during amino acid synthesis, is an important regulator of GAG synthesis.[110] Its main action is to inhibit the protein C anticoagulant pathway by decreasing the thrombomodulin pool at the surface of endothelial cells and reducing protein C activation.[111] In addition, homocysteine has been shown to diminish the synthesis of anticoagulant HS at the surface of endothelial cells, thus also reducing the antithrombin-binding HS pool.[111] This process occurs at a slower rate than the inhibition of protein C and does not directly influence HS already present at the surface of the cells. To enable inhibition of HS synthesis, the homocysteine concentration needs to be around 100 μmol/L. This concentration can be achieved in vivo but only in certain disease states (eg, genetic polymorphisms in *MTHFR*—the gene encoding methylenetetrahydrofolate reductase, which is required for homocysteine synthesis) and in severe nutritional deficiency.[110,111] However, a concentration of 10 μmol/L may be sufficient if the redox potential of the cell is influenced by other factors, for example, by the presence of certain cations such as Cu^{+}, Cu^{2+}, Fe^{2+}, or Fe^{3+}.[110,111] Such elevated homocysteine levels are associated with cardiovascular diseases, disorders associated with abnormal renal function, administration of certain lipid-lowering drugs, and also caffeine or alcohol consumption.[110,111]

More directly, anticoagulant GAGs can be degraded by lyases (heparinases) or hydrolases (heparanases and elastases). Such enzymes are released during inflammation from macrophages after they are activated. These include cathepsin S, which can directly hydrolyse GAGs.[112] Antithrombin and HCII can also be cleaved by neutrophil elastase or by cathepsin G. These enzymes are present in the primary granules of neutrophils and are released during inflammation. Their cleavage of antithrombin and HCII is enhanced by the presence of GAGs.[113–115]

Summary and Clinical Impacts

There are multiple proteins that impact on coagulation via GAG neutralization to a degree which is not fully appreciated. These include proteins involved in control of inflammation, lipid transport, and cellular communication. In addition, most of the molecules that bind GAGs (many of which were not highlighted in this review) in vitro are unlikely to do so under physiological conditions where protein interactions are more complex (ie, presence of multiple interacting partners, formation of ternary complexes). Clarification of which GAG-binding proteins are relevant in vivo is thus still required. Details of the neutralization mechanisms involving HS and DS are lacking. Binding and neutralization of cell-associated HS and DS by proteins are more complex to study than with heparin, and this difficulty has likely limited the information available on their interactions with proteins. Furthermore, many studies examining GAG–protein interactions have focused on the resultant impact on other physiological processes and not coagulation, and so further studies are required to uncover specific roles in neutralization of anticoagulant GAGs. Finally, the relevance of endogenous heparin to physiological coagulation control is still controversial as genetic studies supporting its lack of importance (*NDST2* knockout mice) did not specifically analyze clot parameters.[21,22]

It is clear that the wide range of proteins that influence the anticoagulant properties of GAGs will affect a patients' response to particular forms of heparin or heparin-based drugs. Indeed, levels of GAG-neutralizing proteins are influenced by an individual's genetics, age, diet, and disease state. In a clinical context, this makes the dose–response profiles of heparins and heparin-based drugs difficult to predict. Knowing more about endogenous molecules that bind to GAGs and those that regulate their turnover will enable a better understanding of clotting disorders and treatment choices. Personalized treatments taking into consideration the plasma levels of a particular neutralizing protein may also be considered. In addition, it is important to take those information into account when dealing with specific disorders such as heparin-induced thrombocytopenia or pathologically high zinc plasma levels. More trials are needed in this area to better understand the advantages and drawbacks of each GAG when given to particular subsets of patients. Current guidelines also vary widely between regions. Consistency and better application of these guidelines is required by hospitals to provide a better care to patients. New knowledge gained by studying GAG neutralization will also aid the development and application of new clinical heparin neutralizers. Indeed, as protamine sulfate treatment is known to cause several adverse effects, including anaphylaxis, hypertension, nausea/fatigue, and

back pain,[27] other proteins or molecules including heparin-binding synthetic peptides are already being trialed as potential replacements.[4]

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None.

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Highlights

- The glycosaminoglycans, heparan sulfate, dermatan sulfate, and heparin are important anticoagulants that inhibit clot formation through interactions with antithrombin and heparin cofactor II.
- Unfractionated heparin, low-molecular-weight heparin, and heparin-derived drugs are used clinically to treat coagulatory disorders.
- A wide range of proteins have been reported to bind and neutralize glycosaminoglycans, as reviewed here.
- The anticoagulant activity of glycosaminoglycans may also be regulated through inhibition of synthesis or by degradative enzymes during inflammatory processes.

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Influence of zinc on glycosaminoglycan neutralisation during coagulation

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Heparan sulfate (HS), dermatan sulfate (DS) and heparin are glycosaminoglycans (GAGs) that serve as key natural and pharmacological anticoagulants. During normal clotting such agents require to be inactivated or neutralised. Several proteins have been reported to facilitate their neutralisation, which reside in platelet α-granules and are released following platelet activation. These include histidine-rich-glycoprotein (HRG), fibrinogen and high-molecular-weight kininogen (HMWK). Zinc ions (Zn^{2+}) are also present in α-granules at a high concentration and participate in the propagation of coagulation by influencing the binding of neutralising proteins to GAGs. Zn^{2+} in many cases increases the affinity of these proteins to GAGs, and is thus an important regulator of GAG neutralisation and haemostasis. Binding of Zn^{2+} to HRG, HMWK and fibrinogen is mediated predominantly through coordination to histidine residues but the mechanisms by which Zn^{2+} increases the affinity of the proteins for GAGs are not yet completely clear. Here we will review current knowledge of how Zn^{2+} binds to and influences the neutralisation of GAGs and describe the importance of this process in both normal and pathogenic clotting.

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Introduction

Glycosaminoglycans (GAGs) including heparin sulfate (HS), dermatan sulfate (DS) and heparins are key molecules involved in several biological processes, including coagulation where they play an important anticoagulant role.[1] HS is mostly synthesised by endothelial cells, where it lines the endothelium and participates in its intrinsic anticoagulant properties.[2] DS is mostly synthesised in the sub-endothelium and is exposed to plasma proteins during injury.[3] Heparins are synthesised by mast cells and may also be secreted following tissue injury,[4,5] however, this has been disputed by some.[6–8] The main relevance of heparins are in clinical settings, where they and heparin-based drugs are important agents used in the clinical treatment of coagulatory disorders.[9] As GAGs are physiologically found in a variety of sizes, heparin drugs are administered clinically as unfractionated heparin (UFH), which have not been cleaved or separated by size or as low molecular weight heparin (LMWH), which are generally <8000 Da.[10] All GAGs exercise their anticoagulant activity through their binding to serpins.[1] The main partners for HS and heparin are antithrombin (AT) and heparin cofactor II (HCII) while DS can only bind to HCII.[1] When bound together, the GAGs can change the conformation of the reactive centre loop of the serpin to increase the inhibitory activity of the molecule.[11] During normal coagulation, when clotting is required, anticoagulant GAGs are neutralised by several proteins, including histidine-rich-glycoproteins (HRG), high-molecular-weight kininogen (HMWK) and fibrinogen.[10,12]

After iron, zinc is the most abundant transition metal in the human body. Zinc is an important element in the body, playing key structural and catalytic roles as well as functioning as an extracellular and intracellular signalling molecule. Ionic zinc (Zn^{2+}) is essential for physiological processes such as cell replication, tissue growth, immune functioning and coagulation.[13–15] The importance of Zn^{2+} is best illustrated by the cases of zinc deficiency, which is defined as having a total plasma zinc concentration below 0.7 mg L^{-1} (normal range is 0.8–1.0 mg L^{-1}).[16–18] Zinc deficiency is associated with coagulatory abnormalities including a reduced ability for platelets to aggregate and longer bleeding times, which in most cases can be quickly corrected by zinc supplementation without secondary effects.[16–18] In addition to the resting plasma Zn^{2+} level, during coagulation platelets release Zn^{2+} stored in their α-granules, thus initiating a signalling process.[19–21] During this process Zn^{2+} acts to propagate several anticoagulation pathways as well as both pro- and anti-fibrinolytic pathways.[13] In addition to Zn^{2+}, platelet α-granules also release numerous proteins that impact on coagulation, among which are HRG, HMWK and fibrinogen.[10,22] These proteins have both the ability to bind Zn^{2+} and to bind and neutralise anticoagulant GAGs.[23–30] The mechanisms and impact by which Zn^{2+} influences GAG binding and neutralisation by these proteins is reviewed here.

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Zn^{2+} repartition in plasma

Despite its requirement for various physiological processes, Zn^{2+} is toxic at mid-high micromolar levels,[31,32] therefore its free/labile concentration is tightly regulated. The total concentration of Zn^{2+} in the plasma is approximately 20 μM.[33] Those ions are mostly bound to serum albumin (75% of the total Zn^{2+} concentration in the body, *i.e.* around 15 μM).[33] The remaining Zn^{2+} (around 5–6 μM) is bound to other proteins such as α_2-macroglobulin.[15,34] This fraction is regarded as non-exchangeable as the binding is very tight.[15,34] The remaining Zn^{2+} is bound to small ligands and is considered "free" or "labile" because those ligands can easily be exchanged for proteins or other ligands (more easily than when Zn^{2+} is bound to serum albumin).[15,34] The free Zn^{2+} concentration in plasma is generally thought to be in the micromolar range, between 0.5 to 1 μM.[15,34]

The proportion of free/labile Zn^{2+} in plasma is dynamic (Fig. 1). For example, during coagulation, Zn^{2+} is released from platelets.[35,36] Healthy platelets accumulate around 35 g L^{-1} of Zn^{2+} that they sequester into two pools, the cytoplasm (around 60% that is used to regulate platelet function)[21,37] and the α-granules (around 40%).[21] Variations of the total amount of Zn^{2+} present in the plasma affect the quantity present in the platelets, as well as the distribution of the two pools.[37] There is still some uncertainty as to how Zn^{2+} is incorporated into the platelets. Some Zn^{2+} may be incorporated when the Zn^{2+}-bound fibrinogen-coagulation factor XIII(a_2) complex enters the platelet through binding to fibrinogen receptors.[37] However the main mechanism for Zn^{2+} entry into platelets is likely to be through Zn^{2+} transporters (as reviewed by Taylor and Pugh);[38] the exact mechanism however remains to be elucidated. When platelets are activated, up to half of the α-granule Zn^{2+} pool is released.[35,36] This action has been reported to increase the labile/free plasma Zn^{2+} concentration to 7–10 μM in the proximities of activated platelets.[35,36] The resultant increase in Zn^{2+} concentration can then facilitate its binding to coagulatory proteins and in-turn alter their affinity for other proteins or ligands to influence the coagulation process.[19,20] Platelets are not the only cells in the blood that store Zn^{2+}. Indeed, neutrophils, lymphocytes and erythrocytes all contain Zn^{2+} (reported levels of total zinc are 105 μg/1 × 10^{10} cells, 116 μg/1 × 10^{10} cells and 41 μg g^{-1} haemoglobin, respectively)[39] and may therefore release Zn^{2+} under certain circumstances in a manner similar to platelets (such as at sites of injury, although this has yet to be confirmed). In addition, the epithelium contains around 60 μg Zn^{2+}/g of dry weight,[40] and epithelial cells release some of this when damaged (the exact amount is not known).[41]

Relevance of Zn^{2+} in coagulation control

For a long time, the role of Zn^{2+} in coagulation had been ignored and was likely often masked by the use of citrate as an anti-coagulant during blood collection (with citrate forming complexes with metallic cations). However, in more recent years, the importance of Zn^{2+} in coagulation and regulation of platelet function has started to emerge.[13,38,42] A variety of blood proteins involved in coagulatory processes have been identified as Zn^{2+}-binding proteins. In many cases the ability to bind Zn^{2+} has the potential to influence their activities and impact

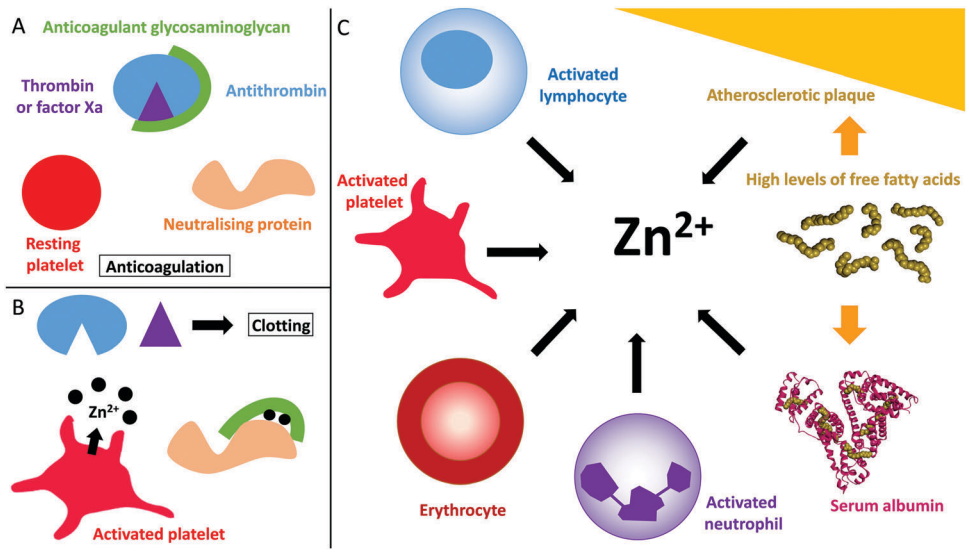

Fig. 1 Coagulation control by glycosaminoglycans and Zn^{2+}. (A) Anticoagulant glycosaminoglycans bind to antithrombin and enhance its neutralisation of thrombin (and/or factor Xa). (B) When platelets become activated, the Zn^{2+} released from the α-granules of platelets bind to the GAG neutralising proteins, increasing their affinity for GAGs and allowing them to neutralise them. Once neutralised, the GAG cannot promote the inhibition of thrombin and clotting occurs. (C) Sources of Zn^{2+} in plasma. During coagulation, Zn^{2+} is released by activated platelets. However, erythrocytes, lymphocytes and neutrophils contain Zn^{2+} which may be released under certain conditions. In some disease states, elevated levels of free fatty acids may also influence available Zn^{2+} levels through release from serum albumin. Atherosclerotic plaques contain up to six time more Zn^{2+} than healthy tissue and could potentially release Zn^{2+} when they rupture. The structure of human serum albumin (with stearate bound) was taken from PDB 1E7I.[109]

upon haemostasis. When looking at specific interactions, Zn^{2+} has been marked as an initiator of the contact activation pathway of coagulation through enhancing the interactions of contact proteins with polyanionic surfaces and their assembly on endothelial cells and platelets.[13] Zn^{2+} enhances platelet aggregation and activation by increasing internal platelet signalling and external binding of platelets to their ligands.[13] It also enhances fibrin formation while also attenuating thrombin activity.[13] Simultaneously, Zn^{2+} is also a regulator of the anticoagulant and fibrinolytic pathways. It can inhibit platelet activation by increasing HMWK and factor XII competition with thrombin to bind GPIb on platelet surface. Zn^{2+} also attenuates FXa generation by FVIIa, both increases and reduces fibrinolysis and modulates the activities of protein C, protein S and heparin-mediated anticoagulant pathways and the pro- and anti-coagulation activities of HRG.[13]

Many of the studies examining the impact of Zn^{2+} on coagulation have utilised purified protein systems where zinc-buffering or binding molecules normally found in plasma are absent. Therefore, it is not clear in some cases whether the labile Zn^{2+} concentrations used are physiologically (or pathophysiologically) attainable. With the involvement of Zn^{2+} in so many aspects of the clot process, it is difficult to tease out in which Zn^{2+} may be most involved. Dietary Zn^{2+} has been shown to exert a pronounced effect on platelet aggregation in humans and rats.[16–18] Several studies have also investigated correlations between Zn^{2+} concentrations and clot formation and lysis. Generally, Zn^{2+} enhances clotting but reduces lysis – specific effects include an increase in fibrin diameter and clot porosity and reductions in clot stiffness.[36,43] Yet, those studies were realised after dialysing the plasma and adding back Zn^{2+}, a process which may have altered the concentration of certain (likely smaller) molecules that influence clotting.

Zn^{2+} binding by anticoagulant GAGs

HS, DS and heparin are highly negatively-charged; thus their binding to other proteins mainly occurs through electrostatic interactions.[44] This type of interaction will increase with the degree of sulfation of the GAGs. HS is generally less sulfated than heparin but more so than DS. Metal ions are important binding partners of GAGs and, in plasma, both Ca^{2+} and Zn^{2+} have been shown to coordinate to them.[21] Seo, Schenauer and Leary revealed that the binding of metal ions to a heparin octasaccharide, including Ca^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+}, triggers conformational changes that have the potential to affect their interactions with their ligands.[45] The effect of Zn^{2+} was not examined in their study but is likely to mimic the effects of these other metal ions. As both Ca^{2+} and Zn^{2+} are released from platelets during coagulation and participate in the regulation of coagulation, this is of particular interest as it is likely that this mechanism alters the anticoagulant activities of heparin and HS following platelet activation.[21] UFH has been shown to bind Zn^{2+} *via* two different mechanisms: the first represents a high-affinity form of binding (the equilibrium constant is 976 M^{-1}) whilst the second is a low-affinity form of binding that only occurs at high Zn^{2+} concentrations (the equilibrium constant is 241 M^{-1}).[46] Both binding events are entropy driven and both involve sulfated side chains on the GAGs. The exact stoichiometry of these binding events has not yet been precisely defined but it is assumed that the first mode of binding involves one zinc ion binding per disaccharide and that the second mode intervenes only after saturation is reached for the first one.[46] GAGs interact with basic amino acids on proteins, generally lysine and arginine side-chains that are not normally affected by the presence of cations.[47] Those cations however often bind to exposed histidines, which are positively charged, and this binding may then facilitate the binding of the protein to GAGs by reducing the electrostatic repulsion between the two of them.[47] As Zn^{2+} is released at the beginning of the coagulation process, its effect on GAG neutralisation reduces anticoagulation and promotes clotting.

Impact of Zn^{2+} on protein–GAG interactions

Numerous proteins in plasma have the ability to neutralise anticoagulant GAGs, as reviewed previously.[10] Among them, three are known to bind Zn^{2+}: HRG,[28] HMWK[29] and fibrinogen.[27] However, the Zn^{2+} binding properties of all GAG-neutralising proteins have not been examined and there are probably more that possess this ability. This ability to bind Zn^{2+} is important as Zn^{2+} has the potential to influence GAG binding and neutralisation by those proteins (Fig. 1). HRG, HMWK and fibrinogen are stored in platelet α-granules alongside Zn^{2+} and are therefore released together during coagulation. All three proteins are synthesised in the liver and, in addition to being stored in platelets, they are present at high nanomolar to low micromolar concentrations in plasma (1.3–2.0 μM for HRG,[48,49] 1–2 μM for HMWK[50] and 12–24 μM for fibrinogen).[51] Their specific roles in coagulation are diverse. Fibrinogen plays a prominent role as when it is cleaved, it polymerises to form fibrin clots.[52] HRG plays a regulatory role by inhibiting fibrinolysis in addition to being incorporated in blood clots,[53] while HMWK is a key activator of the contact activation pathway of the coagulation cascade.[54] Thus all three proteins are in contact with endothelial GAGs and are likely to be important for GAGs neutralisation during coagulation. This section will examine the Zn^{2+} and GAG binding properties of these three proteins and how Zn^{2+} can influence GAG neutralisation.

Histidine-rich glycoprotein (HRG)

HRG is a single chain protein composed of several structural domains that include a histidine-rich region (HRR). This region is important in both proteins, as neutrally-charged histidine residues bind Zn^{2+} *via* their imidazole side chains.[28,48] HRG also contains two cystatin-like domains at its N-terminus (N1 and N2) and possesses a C-terminal domain, whilst its histidine-rich region is flanked by two proline-rich regions (Fig. 2).[55] The structure of HRG has not yet been fully resolved. A crystal structure of the N2 domain has been reported (PDB: 4CCV),[56]

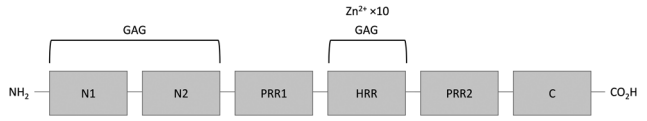

Fig. 2 Structure of histidine-rich glycoprotein. N1 and N2 are N-terminal domain 1 and 2, they have a GAG binding activity; PRR1 and PRR2 are proline-rich regions; HRR is the histidine rich region that binds Zn^{2+} and GAG; C is the C-terminal domain.

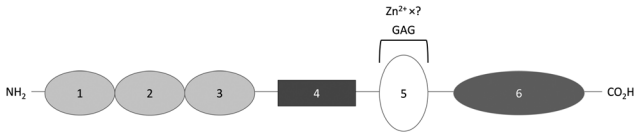

Fig. 3 Structure of high-molecular-weight kininogen. Domains 1, 2 and 3 are cystatin-like domains, with 2 and 3 having a cysteine protease inhibitor activity; domain 4 is bradykinin and another peptide; domain 5 is the surface-binding domain containing the histidine-rich region that binds GAGs and Zn^{2+}; domain 6 is the domain binding prekallikrein and activated coagulation factor XI.

but structural information relating to the other domains (including the Zn^{2+}-binding HRR) is lacking. Nevertheless, Human HRG has been demonstrated to bind up to 10 Zn^{2+} ions with an average K_d of 6.13 μM.[48,57] Current evidences suggest that there are no clearly defined preferential binding sites for Zn^{2+} on HRG.[58] When the net charge of HRG becomes positive, either through a change in protonation of the histidine residues (through a change in pH) or through binding of those residues to metal cations, the conformation of the molecule changes, influencing its affinity for binding its ligands.[19,48,58–62]

HRG binds heparin with a K_d of 32.9 nM in the absence of Zn^{2+} and 5.1 nM in the presence of 1 μM Zn^{2+}.[57] Isothermal titration calorimetry studies have shown that there are two different modes of heparin binding, which are thought to occur at different binding sites.[57] The first mode is Zn^{2+}-dependent and thus most likely involves binding at the HRR.[57,63] As Zn^{2+} only influences the binding of long chain heparins,[57] this first binding site only involves long chain heparins (≥10 kDa).[57,64] The second mode of binding is not dependent on chain length and is thought to occur at the N1 and N2 domains,[20] although the exact location of this site is still unknown. The affinity of the second mode of binding is not directly affected by the presence of Zn^{2+}, but Zn^{2+} binding to the HRR may induce conformation changes in HRG that would make this site more accessible to heparins. HRG forms mainly 1 : 1 complexes with heparin, but it can form 2 : 1 complexes with longer chain heparins in the presence of Zn^{2+}.[64] HRG has been shown to neutralise heparin in plasma with this ability (like binding) also dependent on the size of the heparin; with longer-chain heparins having a higher affinity for HRG.[65] For example, even excess ratio of 500 : 1 HRG : heparin octasaccharide can neutralise less than half of the ability of heparin to accelerate the inactivation of factor Xa by AT.[65] Zn^{2+}-Dependent heparin binding by HRG only occurs when Zn^{2+} is released from activated platelets; otherwise, the metal concentration is too low and heparin preferentially binds the AT-thrombin complex.[66] HRG can neutralise heparin-mediated thrombin inhibition by both AT and HCII, but it is much weaker in neutralising DS-mediated thrombin inhibition by HCII.[67–69] HRG can also bind and neutralise HS in a Zn^{2+}-dependent-manner.[70] Thus, HRG is an important anticoagulant GAG neutraliser in plasma and this neutralisation is dependent upon the plasma Zn^{2+} concentration.

High-molecular weight Kininogen (HMWK)

HMWK is a single chain protein that consists of 6 domains, one of which, domain 5, contains a HRR (Fig. 3).[54] Kallikrein cleaves domain 4 of HMWK, releasing bradykinin and another peptide, while the rest of the protein forms a two chain HMWK, with the heavy chain being the N-terminal section composed of domain 1, 2 and 3 and the light chain being domain 5 and 6. Both chains are then linked together by a single disulfide bond.[54] Like HRG, HMWK has not yet been fully crystallised and so the Zn^{2+} binding domain is also not fully characterised. It is known that Zn^{2+} binds mainly to histidine residues of the HRR located between the residues Gly440 and Lys458 of domain 5. Zn^{2+} binding is known to induce a conformational change in this domain.[71] The affinity and stoichiometry of Zn^{2+} binding by HMWK have not yet been determined despite its potential to influence the binding of HMWK to its ligands.

Like HRG, HMWK binds heparin in a Zn^{2+}-dependent manner.[25] The intact form of HMWK binds heparin with higher affinity than the cleaved form of the protein (the K_d values of intact and cleaved forms are 2.1 nM and 14.2 nM, respectively).[29] The presence of 50 μM Zn^{2+} increases the binding affinity even further (K_d = 0.30 nM for intact HMWK).[29] Heparin binds to the light chain portion of HMWK, at the HRR, in domain 5, which is known to mediate HMWK-binding to negatively-charged surfaces.[25,29] Within this domain the binding of GAGs occurs at a combination of different sites, some of which are sensitive to Zn^{2+}.[29,47] In addition to histidines, this region is rich in lysine residues, which are involved in heparin binding.[29,47] The heparin binding affinity of HMWK increases when the pH decreases and the histidine residues become protonated, regardless of the presence of cations.[25] UFH and LMWH bind with similar affinity to HMWK in the absence of Zn^{2+} but the influence of the heparin chain length on the binding affinity in the presence of Zn^{2+} is not yet known.[25] HMWK competes with AT, thrombin or the AT-thrombin complex for heparin-binding and can neutralise the anticoagulant effect of heparin in plasma.[25] However, this binding may not be specific, as HMWK can bind all heparins regardless of whether or not they possess the saccharide sequence used to bind AT with high affinity, and thus several HMWK molecules may be required to fully neutralise one heparin molecule.[25] Maximal neutralisation has been shown to occur in the presence of 10 μM Zn^{2+}.[25] In addition, HS proteoglycans located at the cell surface can bind HMWK in a Zn^{2+}-dependent manner but the effect of this binding on the anticoagulant activity of HS has not been investigated directly.[50] Thus HMWK appears to neutralise anticoagulant GAG in a similar manner to HRG. As HMWK is present in plasma at similar levels to HRG and binds heparin with similar affinity (K_d in low nanomolar range),

these proteins may be of equal importance in anticoagulant GAG neutralisation.

Fibrinogen

Fibrinogen is a homodimer composed of two sets of three different polypeptides chains, Aα, Bβ and γ (Fig. 4).[72] Most of the protein has been crystallised to some extent, with the exception of the highly variable αC domain (PDB 3GHG).[73] Fibrinogen binds Zn^{2+} at two different regions. The first set of binding sites is located in the D-domains (Fig. 4, insert 1) and has a stoichiometry of six (three ions per D-domain).[74] The sites predominantly consist of histidine residues located on the γ chain, with His-γ217 and His-γ234 thought to be involved,[74] however the effect of Zn^{2+} binding at this region on the conformation of the protein is unknown. Another Zn^{2+}-binding region has been identified in the αC-domain and also involves histidine residues (His-α544 and His-α545).[75] Binding of Zn^{2+} to this region is thought to induce a change in the conformation of the protein.[75] Based on several studies, Zn^{2+} binding to fibrinogen has an average K_d of ~1–18 μM, but the individual contributions of the two groups of sites are unknown.[27,36,75]

Two different heparin binding modes have been identified on fibrinogen. The first occurs at a site located on the β chain in the E domain around the Bβ1-57 region (Fig. 4, insert 2). It has been shown that a synthetic peptide corresponding to this exact region binds heparin with a K_d of 16.5 μM,[76] whilst a dimer of the peptide, $(B\beta 1\text{-}66)_2$ exhibits an almost two orders of magnitude higher affinity for heparin (K_d of 210 nM), compared to the monomer.[76] As this K_d value is close to the K_d of intact fibrinogen (228 nM), this suggests that the binding of heparin to fibrinogen occurs predominantly *via* this binding mode and that the dimeric structure of fibrinogen plays an essential role in this binding.[76] A second heparin-binding mode occurs when Zn^{2+} binds to the αC domains of fibrinogen.[75] Binding of Zn^{2+} is thought to induce a change in the conformation of the protein promoting heparin-binding to the nearby E domain.[75] In presence of 12.5 μM Zn^{2+}, the average K_d for heparin binding to fibrinogen is 60 nM.[75] This represents a 4-fold increase in affinity, contrasting to a lower affinity (K_d of 539 nM) for fibrinogen without the αC domain in the presence of Zn^{2+}.[75] The link between heparin chain length and binding affinity in the presence and absence of Zn^{2+} is not yet known. The binding of fibrinogen to heparin participates in the neutralisation of its anticoagulant activity. The direct study of fibrinogen-mediated GAG neutralisation has been complicated by the fact that thrombin cleaves fibrinogen. However, fibrinogen has been shown to be more effective at neutralising DS than HRG (and platelet factor 4).[68] This neutralisation occurs at physiological fibrinogen concentrations and is not affected by the size or the degree of sulfation of DS.[68] The mechanism of neutralisation appears not to occur through direct competition with

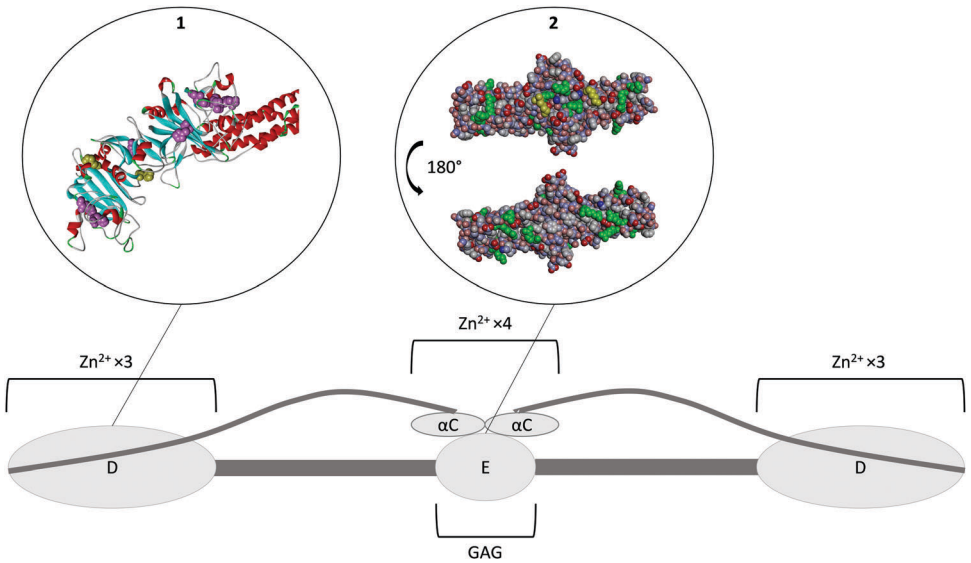

Fig. 4 Structure of fibrinogen. The protein forms hexamer made of three different strands $(A\alpha\ B\beta\ \gamma)_2$. All of the N-terminals are in the E domain which is the heparin binding domain. The three strands then coil together until they reach the D domains where the C-terminal of the β and γ strands are located. This domain is a Zn^{2+}-binding domain. The α strand goes back toward the E domain where its C-terminal forms the αC domain, another Zn^{2+} binding domain. Insert 1. Crystal structure of fibrinogen D domain (one of the Zn^{2+} binding domain) and part of the coil–coil region (PDB structure 3GHG).[73] The histidine residues which have the potential to be involved in Zn^{2+} binding are represented in pink with the residues His217 and His234 known to be involved represented in yellow. Most of those residues are hidden beneath the surface of the protein. Insert 2. Crystal structure of fibrinogen E domain (the heparin binding domain) and part of the coil–coil regions, (PDB structure 3GHG).[73] The positive charges are represented in blue and the negative charges in red. The Lys and Arg residues which usually constitutes the main binding partners of GAGs are represented in green. The first few residues of the Bβ chain are mobile and so they are not visible in the crystal structure, with β58 the first residue that can be observed (represented in yellow). This residue is a protruding Lys that is believed to be important for GAG binding. As the GAG binding affinity of fibrinogen is enhanced when the protein is converted into fibrin by cleavage of the A and B peptides, the absence in the crystal structure of the first few residues of the Bβ chain may be the reason for exposure of the β58 residue. The αC domain is attached to the E domain; binding of Zn^{2+} ions to its His α544 and His α545 is thought to change the conformation of the protein and thus facilitate GAG binding to the E domain.

the thrombin–HCII complex for DS binding but by controlling the rate of formation of this complex.[68] In addition, fibrin can also form complexes with heparin, AT and thrombin to reduce thrombin inhibition by AT in a Zn^{2+}-dependent manner.[52,77] Thus, fibrinogen is an important anticoagulant GAG neutraliser, with the plasma concentration of fibrinogen being linked to heparin resistance in patients.[78] The sensitivity of fibrinogen toward plasma Zn^{2+} levels relative to HRG and HMWK is not yet known.

Other anticoagulant GAG neutralising proteins

Zn^{2+} can also potentially affect the neutralisation of anticoagulant GAGs through, fibronectin, fibroblast growth factors (FGF), FGF-1 and FGF-7 and activated coagulation factor VII (FVIIa).[30,79,80] Fibronectin can be found as the alternative spliced forms of cellular or plasma fibronectin. Plasma fibronectin is present in plasma at a concentration of 300–600 nM and is also stored in platelet α-granules and released during coagulation.[81] Cellular fibronectin is synthesised by endothelial cells and can be released into plasma during wound healing.[82] Zn^{2+} binding by fibronectin has not yet been fully characterised, however several regions have been shown to bind Zn^{2+} *in vitro.* These include the collagen/gelatin binding domain (binding to which has been shown to elicit a conformational change in this region),[30,83,84] the cell binding domain[30] and the alternatively spliced type III connecting segment (IIICS) which is only fully present in cellular fibronectin and a small fraction of plasma fibronectin.[85] It is still unclear whether plasma fibronectin binds Zn^{2+} physiologically or whether this only occurs with cellular fibronectin.[85,86] Considering that Zn^{2+} induces a conformational change in the protein and may thus influence GAG binding, this is an important question to answer. Fibronectin possess 5–6 ionic GAG binding sites.[26,81] The first binding site (often termed Hep1) is found in the N-terminal region. As Zn^{2+} binding to the neighbouring gleatin-binding region induces a conformation change in the protein, it is probable that this could impact on GAG binding in Hep1.[83] The second GAG binding region (Hep2) has two distinct GAG binding sites and constitutes the high affinity GAG binding region.[87] As the region is flanked by two Zn^{2+}-binding regions, the cell-binding region and the IIICS, Zn^{2+} binding may also impact on GAG binding at this site. The global K_d of heparin binding to fibronectin is 0.9 μM for a 18–20 saccharide heparin (molecular weight 6000 Da).[87] The affinity for UFH is not yet known. Fibronectin does interfere with AT binding to immobilised heparin as a function of heparin concentration. However AT is only completely displaced from heparin at fibronectin/AT ratios higher than are found physiologically.[81] Fibronectin also binds HS and DS but its effect on their neutralisation is not known.[88,89] Thus plasma Zn^{2+} levels have (at least in theory) the potential to influence GAG binding and neutralisation by fibronectin. This means that fibronectin has the potential to strongly react with the Zn^{2+} released during coagulation and to be major GAG-neutralisers during this time.

Fibroblast growth factors are present at very low concentrations in plasma (*ca.* 28–48 pM for FGF-1 and 643 pM for FGF-7),[90,91] nevertheless they are normally attached to GAGs of the endothelial surface layer and depend on this binding to exert their functions (including oligomerisation, binding to their cognate receptors and transport between cells).[92] They are therefore important binding partners for GAGs. FGF-1 and FGF-7 bind UFH with K_d values of 29 nM and 140 nM, respectively.[93] DS also interacts with FGF-7 and FGF-1 but with lower affinity than heparin and HS. FGF-7 and to a lesser extent FGF-1 have been shown to neutralise UFH.[94] Unlike with HRG, HMWK and fibrinogen, the affinity of these interactions are reduced by the presence of metal ions (Na^+, K^+, Ca^{2+}, Cu^{2+} and Zn^{2+}).[93] However, it is unknown whether metal ion binding influences the neutralisation of the GAG. It is possible that binding of metal ions by these FGFs is a mechanism to facilitate their release from the endothelium when the plasma concentrations of those ions are elevated. Nevertheless, FGF-1 and FGF-7 are unlikely to be important GAG neutralisers *in vivo* due to their low plasma levels.

FVIIa is a coagulatory protein involved in the contact activation pathway.[79,80] It binds UFH with a K_d value of 3.38 μM in a Ca^{2+}-dependent manner.[95] FVIIa possesses two Zn^{2+} binding sites but it is not yet known if binding of Zn^{2+} plays any role in heparin binding.[96] FVIIa can neutralise both UFH and LMWH, but its effects on HS and DS have not yet been studied. However, like FGF-1 and FGF-7, FVIIa is only present at a low concentrations in plasma (*ca.* 16 nM)[97] and therefore it is not likely to be as relevant in anticoagulant GAG neutralisation as the proteins listed above. Nevertheless, the interaction of Zn^{2+} with HRG, HMWK and fibrinogen shows that Zn^{2+} is an important regulator of GAG binding and neutralisation by plasma protein and it is therefore important to investigate whether Zn^{2+} has the same effect on other GAG neutralising proteins.

Clinical significance of Zn^{2+}-induced GAG neutralisation

In addition to being released during injury by epithelial cells and platelets, plasma Zn^{2+} levels are also increased in certain disease states (Fig. 1). Indeed, atherosclerotic plaques are also known to contain up to six-times more Zn^{2+} than healthy tissue.[98] However, only total Zn^{2+} concentration has been measured and so the concentration of labile Zn^{2+} is not clear. The increase in Zn^{2+} concentration in atherosclerotic plaques correlates with an increase in Ca^{2+} concentration.[98] In addition, both metal ions are present at high levels in areas of plaque mineralisation.[99] This may signify that the accumulation in both metals occurs through a common mechanism that has not yet been identified. Accumulated Zn^{2+} could be released into the blood during plaque rupture, thus participating in the pro-thrombotic nature of these events.

The concentration of available Zn^{2+} is also directly influenced by the plasma free fatty acid (FFA) levels. Serum albumin is the main plasma carrier for both Zn^{2+} and FFAs.[57,100] When a FFA molecule binds at a high affinity binding site (called the FA2 site) adjacent to the main Zn^{2+} binding site, an allosteric interaction leading to perturbation of the Zn^{2+} binding site occurs. In healthy individuals around 75% of total plasma Zn^{2+}

(around 15 μM) is bound to serum albumin and so this mechanism may result in the release of up to 15 μM Zn^{2+} in plasma in individuals with elevated FFA levels (when those levels are elevated enough to completely prevent Zn^{2+} binding by serum albumin). In certain conditions, the concentration of FFA can increase by up to six times: in diabetes FFA concentrations have been reported to be 0.62–0.82 mM in men and 0.82–0.98 mM in women (compared to controls of 0.59–0.68 mM for men and 0.74–0.83 mM for women).[101] In non-alcoholic fatty liver disease, the corresponding concentrations are 0.12–3.4 mM (compared to controls of 0.11–0.9 mM).[102] In obesity, FFA concentrations are 0.56–1.15 mM (compared to controls of 0.28–0.89 mM).[103] Elevated FFA levels are also associated with some cancers; in malignant lymphoma FFA concentrations were found to be 0.55–1.8 mM (no controls);[104] All of these conditions are associated with a higher incidence of developing thrombotic complications.[105,106] In addition, under hyperglycemic conditions, serum albumin can undergo non-enzymatic glycation reactions which can disrupt the protein conformation and also directly affect its main Zn^{2+}-binding site.[107] This is another mechanism by which diabetic state can affect Zn^{2+} transport and speciation.

Another condition that may be associated with altered plasma Zn^{2+} homeostasis is analbuminemia (albumin deficiency), which is defined as having a plasma albumin level of <1 g L^{-1}. In total, 78 cases have been reported in the analbuminemia register (www.albumin.org) and the prevalence of the disease is estimated to be less than 1 in 1 million.[108] Individuals with analbuminemia have been reported to have elevated levels of other plasma proteins, including coagulation factors, as a compensatory mechanism.[108] It is thought that those proteins take up most of the functions of albumin and therefore it is unclear if Zn^{2+} transport in plasma is affected or not. However, it has been shown that Ca^{2+} and Fe^{2+} have an altered protein-binding profile in these individuals.[108] The transport of FFA is taken up by apolipoprotein B-100 and so FFA levels are close to normal (but dyslipidaemia is present).[108] Follow-up studies of patients with analbuminemia have been limited, which limits the report of complications. Nevertheless, such follow-up studies have included reports of atherosclerosis and hypercoagulability in these individuals,[108] but it is not clear if this may be due to alterations in plasma Zn^{2+} level/speciation or changes in plasma coagulatory protein levels.

The impact of Zn^{2+} on anticoagulant GAG-neutralisation is useful to consider, specifically as this process impacts on thrombin activation, an event that directly affects both platelet aggregation (through binding to cell surface receptors) and fibrin clot formation (through cleavage of fibrinogen). Indeed, by enhancing the neutralisation of the GAG present in the endothelium surface layer, Zn^{2+} induces a change in the natural anticoagulant properties of the endothelium.[10] When coagulation is needed, this participates in the promotion of clotting. However, if Zn^{2+} speciation is chronically altered, such as is likely in certain diseases states (atherosclerosis, diabetes, obesity and cancer), then this could affect anticoagulant processes in the endothelium resulting in a pro- or hyper-thrombotic state through enhanced GAG neutralistaion.[10] This phenomenon could also directly affect the efficiency of heparin-based treatments, which are widely used during surgeries and to manage thrombotic complications.[10] In order to confirm this, it would be interesting to measure plasma Zn^{2+} levels in patients undergoing heparin treatment and to compare it to their response to this treatment. If such a relationship is confirmed then an option may be to control Zn^{2+} levels in those patients rather than to switch to another anticoagulant treatment whose efficiency could also be potentially affected by Zn^{2+}.

Conclusion

Zn^{2+} plays a major role in the regulation of coagulation that is only starting to be understood. Because of this role, Zn^{2+} homeostasis in platelets and its speciation in plasma are especially relevant to the understanding and treatment of blood diseases. In particular, they can affect GAG binding and neutralisation by the platelet-stored proteins HRG, HMWK and fibrinogen. This mechanism is relevant to healthy coagulation processes through the natural anticoagulation properties of the endothelium, but also in anti-thrombotic treatments. Indeed, it may partly explain the observed variability in dose response to heparins and heparin-based drugs. This implies that free plasma Zn^{2+} levels need to be monitored in individuals with coagulatory disorders or at-risk of thrombotic events. The control of free plasma Zn^{2+} levels may also be a treatment lead for individuals suffering from high levels of plasma FFA who are at high risk of thrombotic disorders.

Abbreviations

AT	Antithrombin
DS	Dermatan sulfate
FGF	Fibroblast growth factor
FVIIa	Activated coagulation factor VII
GAG	Glycosaminoglycan
HCII	Heparin cofactor II
HMWK	High-molecular weight kininogen
HRG	Histidine-rich glycoprotein
HRR	Histidine-rich region
HS	Heparan sulfate
IIICS	Type III connecting segment of fibronectin
LMWH	Low molecular weight heparin
UFH	Unfractionated heparin

Conflicts of interest

There are no conflicts to declare.

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Ischemia-modified albumin: Crosstalk between fatty acid and cobalt binding

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ABSTRACT

Myocardial ischemia is difficult to diagnose effectively with still few well-defined biochemical markers for identification in advance, or in the absence of myocardial necrosis. "Ischemia-modified albumin" (IMA), a form of albumin displaying reduced cobalt-binding affinity, is significantly elevated in ischemic patients, and the albumin cobalt-binding (ACB) assay can measure its level indirectly. Elucidating the molecular mechanism underlying the identity of IMA and the ACB assay hinges on understanding metal-binding properties of albumin. Albumin binds most metal ions and harbours four primary metal binding sites: site A, site B, the N-terminal site (NTS), and the free thiol at Cys34. Previous efforts to clarify the identity of IMA and the causes for its reduced cobalt-binding capacity were focused on the NTS site, but the degree of N-terminal modification could not be correlated to the presence of ischemia. More recent work suggested that Co^{2+} ions as used in the ACB assay bind preferentially to site B, then to site A, and finally to the NTS. This insight paved the way for a new consistent molecular basis of the ACB assay: albumin is also the main plasma carrier for free fatty acids (FFAs), and binding of a fatty acid to the high-affinity site FA2 results in conformational changes in albumin which prevent metal binding at site A and partially at site B. Thus, this review advances the hypothesis that high IMA levels in myocardial ischemia and many other conditions originate from high plasma FFA levels hampering the binding of Co^{2+} to sites A and/or B. This is supported by biophysical studies and the co-association of a range of pathological conditions with positive ACB assays and high plasma FFA levels.

1. Introduction

Myocardial ischemia occurs due to restricted blood supply to the muscular tissue of the heart (myocardium) resulting in insufficient oxygen supply. The main cause of this can be the partial or complete blockage of a coronary artery, and a critical depletion of myocardial oxygen leads to cell death, or infarction. Diagnosis of myocardial ischemia typically includes exercise-electrocardiography stress tests, coronary angiography, and imaging stress-echo tests [1]. While a plethora of cardiac biomarkers have been described for detecting the development of other acute coronary syndromes (ACS) [2,3], there are still few well-defined biochemical markers for identification of myocardial ischemia in advance, or in the absence of myocardial necrosis. One of these biomarkers is based on albumin, the most abundant protein in blood plasma. So-called "ischemia-modified albumin" (IMA) is found to be significantly elevated in ischemic patients [2,4–7], and serves as a biomarker for early detection of myocardial ischemia before the onset of irreversible cardiac injury [6]. IMA is solely characterised by its reduced cobalt-binding affinity, which can be measured indirectly by the Food and Drug Administration-approved albumin cobalt-binding (ACB) assay [8,9].

In the commercially available ACB test, cobalt(II) chloride (approximately 1.5 mol equivalents per albumin molecule) is added to a serum sample, to allow albumin-cobalt binding. Dithiothreitol (DTT), a metal chelator that forms a coloured complex with Co^{2+}, is then added. The resulting ill-defined brown DTT-Co^{2+} product is measured by absorption spectrophotometry at 470 nm and compared to a serum-cobalt blank without DTT present. The reduced cobalt-binding capacity of IMA

Abbreviations: ACB, albumin cobalt-binding; ACS, acute coronary syndromes; ATCUN, amino terminal Cu(II) and Ni(II) binding motif; DTT, dithiothreitol; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure spectroscopy; FFAs, free fatty acids; HRG, histidine-rich glycoprotein; IMA, ischemia-modified albumin; ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; NTS, N-terminal binding site on albumin

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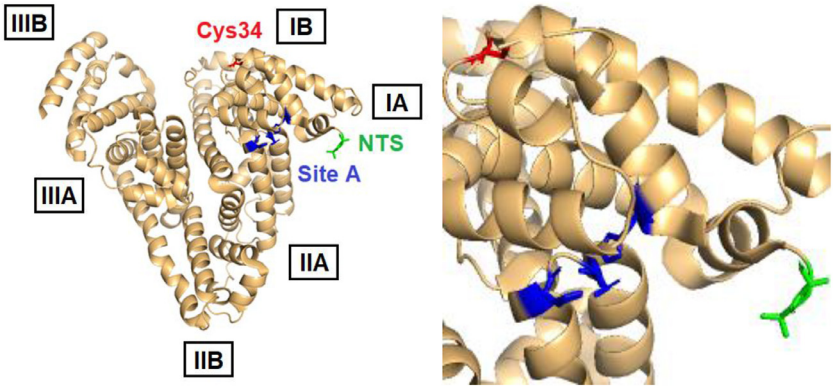

Fig. 1. Location of the three metal binding sites that have been successfully identified on human serum albumin,PDB: 5IJF [60]. Site A, the multi-metal binding site (MBS) (blue); NTS/ATCUN motif (green); Cys34 (red). The precise location of site B is not yet known. The boxed labels indicate the six sub-domains of albumin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

leaves more unbound Co^{2+} to complex with DTT, resulting in higher absorbance readings [10]. The ACB test has an excellent negative predictive value, *i.e.* low IMA readings correspond well to the absence of myocardial ischemia. However, a severe shortcoming is the high incidence of false positives, *i.e.* high readings in the absence of ischemia.

After its first description [8], the molecular identity of IMA remained elusive. Based on the general assumption that Co^{2+} would preferentially bind to an N-terminal site [11–13], efforts to elucidate the molecular causes of reduced cobalt binding concentrated on this site. It was hypothesized that ischemia causes the N-terminal end of the albumin protein to undergo structural modifications, hence that IMA corresponded to N-terminally modified albumin [13]. The structural modifications proposed and investigated included cleavage of the first two residues and oxidation [11], which were suggested to result from free radical damage, exposure to free iron and copper, or disruption of ion pumps [8,14].

However, in-depth studies could not reveal a correlation between N-terminal modifications and ACB readings [13,15]; more recently, no correlation was found between the ACB assay and an enzyme-linked immunosorbent assay that specifically detects N-terminal modification of albumin in patients with either acute coronary syndrome or non-ischemic chest pain [16]. Similarly, patients suffering from acute-on-chronic liver failure have significantly elevated ACB assay readings, but the same proportion of N-terminally modified albumin as healthy individuals [17,18]. In the light of such findings, low plasma pH as a result of acidosis, and altered plasma cysteine/cystine ratio as a consequence of hypoxia or oxidative stress have also been suspected as molecular causes of reduced cobalt binding [19]. The need to consider the contribution of other plasma components to the Co-DTT complex formation was also highlighted [19]. Indeed, a positive correlation has been identified between the highly elevated serum levels of free fatty acids (FFAs) in patients with acute ischemic myocardia and high levels of IMA [20]. Following our discovery of FFA-mediated inhibition of zinc binding to albumin [21–24], we have demonstrated that the conformational changes that FFA-binding to albumin elicits in the protein is sufficient to cause reduced cobalt binding capacity [22,25]. This review will present essential background information on metal ion-albumin interactions and discuss the molecular basis of FFA-mediated inhibition of metal (in particular Co^{2+}) binding. It will also provide a clinical perspective to highlight how conclusions from biochemical/bioinorganic investigations are reflected in patient data.

2. Albumin – a carrier of essential and xenobiotic metal ions in plasma

Albumin is a ~66 kDa protein containing 585 amino acids, contributing to around 50% of the total protein concentration in blood plasma, and up to 75% of the colloidal activity [26]. Albumin comprises three homologous but structurally distinct domains, each divided into two sub-domains [27]. One of its key roles in the body is to transport a variety of small molecules, including cholesterol [28], fatty acids [29], and pharmaceutical drugs [30]. Importantly, albumin also serves as an important carrier of inorganic ions, including those required for regular physiological function (Ca^{2+}, Cu^{2+} Zn^{2+}) [31], toxic metal ions (Cd^{2+} and Ni^{2+}) [32,33], as well as metal-based therapeutics (Au^{+} and Pt^{2+}) [34,35]. Before considering cobalt binding in depth, we will briefly summarise the interactions of albumin with other d-block metal ions, with the exception of Cr^{3+}, Fe^{3+}, and Mn^{2+}, which are preferentially transported by transferrin, another important metal ion transporter in blood plasma. Whilst Fe^{3+} can, in principle, also bind to albumin, this only occurs in cases of severe iron overload [34].

2.1. Metal binding sites in serum albumins

Though originally albumin was thought to transport ions in a non-specific 'sponge-like' manner [30], four partially selective metal binding sites have been identified, namely the N-terminal site (NTS), sites A and B, and Cys34 (Fig. 1) [34]. Metal binding to such sites can be studied using a variety of techniques. Stability constants for the binding of d-block metals, including Zn^{2+}, Cu^{2+}, Ni^{2+} and Cd^{2+}, were originally derived from equilibrium dialysis experiments [36-39]; more recently, isothermal titration calorimetry (ITC) has provided valuable thermodynamic data for metal ion binding [40]. Nevertheless, both of these techniques only provide global binding constants [34] and need to be complemented by techniques that address structural features. For true transition metal ions such as Cu^{2+} and Co^{2+}, electronic spectroscopic methods such as circular dichroism allow metal binding to albumin to be studied via transfer of chirality from metal-binding amino acid residues to the d-d/charge-transfer bands of complexed metal ions, providing insight into the geometry of metal-protein interactions [41,42]. The same ions have unpaired electrons, and can also be investigated using electron paramagnetic resonance (EPR) spectroscopy, which provides insight into the chemical environment surrounding the metal ion [43,44]. To obtain structural information on the binding of diamagnetic d^{10} ions, such as Zn^{2+} and Cd^{2+}, that are largely silent in the aforementioned spectroscopies, nuclear magnetic resonance (NMR) methods have been employed, making use of either partially-characterised ^{1}H-resonances of metal-binding residues, or NMR-active nuclei such as the ^{111}Cd or ^{113}Cd isotopes of cadmium [39,45–47]. Further information on the coordination mode, geometry and identification of likely donor ligands has been gained using extended X-ray absorption fine structure spectroscopy (EXAFS) [47]. In addition, mass spectrometry has been used as a tool to detect crosslinking of His67 and His247 by platinum in site A [48].

2.1.1. The N-terminal binding site (NTS)

One of the first metal binding sites to be identified on albumin was the N-terminal binding site (NTS), which arises from the first triplet amino acid motif of human albumin: Asp1–Ala2–His3 (Figs. 1 and 2) [49]. It involves the N-terminal amino group, the N(delta) of His3, and

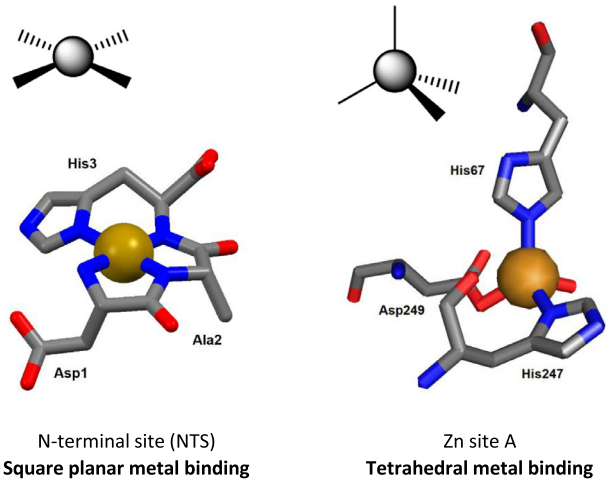

Fig. 2. Contrasting geometries of metal binding sites on albumin. Left: square planar coordination of Cu^{2+} or Ni^{2+} at the NTS site; the structure shown is derived from molecular modelling. The N-terminal amino group, two deprotonated backbone amide N atoms and the N(delta) of the imidazole ring of His3 form a square plane around the central metal ion. Right: tetrahedral coordination of Zn^{2+} at site A in human serum albumin (pdb 5ijf). His67 uses its N(epsilon) N atom, whilst His247 binds via N(delta). Asp249 binds in monodentate fashion, with the second carboxylate O at *ca.* 2.6 Å distance, too long for a metal-ligand bond. Typically for zinc sites in proteins, angles between ligands deviate substantially from the ideal tetrahedral angle (109.5°) and vary between 95° and 125°. Metal ions are rendered in gold, N atoms in blue, O atoms in red, carbon atoms in grey. No H atoms are shown.

two deprotonated backbone amide nitrogen atoms. This square planar configuration of N-donor atoms (Fig. 2) is particularly suitable for Cu^{2+} and Ni^{2+}, which has led to the NTS being referred to by the acronym 'ATCUN', for the Amino Terminal Cu(II) and Ni(II) binding motif [42,50]. The ATCUN motif is present in the majority of albumins from different mammalian species, though porcine and canine albumins are notable exceptions, as they lack His3 [34]. Oligopeptide models of the native ATCUN motif have been investigated extensively [34]. The NTS motif is thought to have high conformational flexibility in the absence of bound metal, reflected in the crystal structures of albumin, all of which lack defined structures of the first few N-terminal residues [12]. Interestingly, the N-terminal X-X-His motif is not unique to albumin – many other proteins, such as the peptide hormone Hepcidin, can also bind Ni^{2+} and Cu^{2+} ions via an ATCUN motif [51].

Cu^{2+} binds preferentially to the NTS in albumin, occupying approximately 1–2% of the available NTS – equating to around 15% of total copper in blood plasma [34,52]. Owing to the d^9 electronic configuration of Cu^{2+}, preference to form square planar complexes, and high stability in the Irving-Williams series, Cu^{2+} is coordinated at the NTS with 1 pM affinity [52], and binds preferentially over other metal ions [22]. Cu^{2+} can also bind at other metal binding sites with comparable or even higher affinities to those of Ni^{2+} and Zn^{2+} [41], however its low relative concentration (10–20 μM total Cu^{2+}, and submicromolar 'free' Cu^{2+} in plasma) [53] compared to albumin means that, in practice, only the NTS is ever occupied by Cu^{2+} [52]. Like Cu^{2+}, Ni^{2+} binds to albumin preferentially at the NTS site [33], with micromolar affinity [34]. Ni^{2+} is only present at nanomolar concentrations in plasma, however levels may be elevated under certain pathological conditions (*e.g.* stroke) [54]. Nearly all of plasma Ni^{2+} is albumin-bound [12,34]. Binding of Ni^{2+} and Cu^{2+} can be modulated by the redox state of Cys34 [43] with higher metal affinity in the reduced (free thiol) state.

Site A – the multi-metal binding site

Metal binding site A is located at the interface of domains I and II [34] (Fig. 1), and has been identified and characterised using ^{1}H and $^{111/113}Cd$ NMR spectroscopy [39,45,46,55,56], circular dichroism, site-directed mutagenesis [56], EXAFS [47] and recently X-ray crystallography [57]. As well as having a high nanomolar affinity [23,24,38,39,47] for the d^{10} divalent cations Zn^{2+} and Cd^{2+}, site A can also bind Cu^{2+}, Ni^{2+} and Co^{2+} – hence it is also referred to as the 'multi-metal' binding site [34,41,56]. In fact, up to 90% of the total zinc present in plasma (11.5–36.7 μM total Zn^{2+} in adults [58]) is bound to albumin [59,60]; this amounts to *ca.* 98% of exchangeable plasma zinc.

EXAFS, site-directed mutagenesis and molecular modelling initially suggested that site A is formed by His67, His247, Asp249, and Asn99 [47], and so distorted trigonal bipyramidal coordination of Zn^{2+} was proposed, with water (or chloride) as the fifth ligand completing the inner coordination sphere. However, the recent X-ray crystallographic structures of human and equine albumins discounted participation of Asn99 and showed site A to be essentially tetrahedral (Fig. 2), with the fourth ligand being a water molecule [57]. Cu^{2+} coordination at site A had also been suggested to be tetrahedral in geometry, as determined by EPR and CD experiments [61]. The combination of amino acid residues bearing intermediate-to-hard N/O-donors [60] (HSAB principle) provide a good coordination environment for metal ions with a small ionic radius and moderate charge (*e.g.* 2+ cations). Notably though, the affinity of site A for Cu^{2+} is 4 orders of magnitude lower than that of the NTS [34], thus site A only becomes populated by Cu^{2+} when more than one molar equivalent of Cu^{2+} is present. Finally, the comparison of apo- and Zn-bound crystal structures of albumin has revealed high structural similarity at site A. Thus, in marked contrast to the flexible NTS, site A is essentially 'pre-formed' for metal binding [57,60]. It is important to note that site A is an inter-domain site, with His67 from domain I, and His247 and Asp249 from domain II.

Site B

The other Cd^{2+} binding site (site B), which is distinct from site A and the NTS and readily identifiable using ^{111}Cd or ^{113}Cd NMR (Fig. 3a), appears to bind Cd^{2+} with similar affinity to the multi-metal binding site A [45,46]. In contrast, site B's affinity for zinc is markedly less than that of site A. Based on NMR data, it is likely that only one nitrogen donor ligand is involved at site B, suggesting this site to be harder (HSAB principle) than site A. The location of site B has remained elusive, but site-directed mutagenesis of His39Leu excluded His39 from involvement in either site A or B [47].

Cysteine-34

Albumin contains 17 disulfide bonds, which contribute to the structural stability of the protein. One free thiol residue (Cys34) is located between helices 2 and 3 of subdomain IA (Fig. 1) [34]. Cys34 is not involved in any intramolecular bridging, however it often forms intermolecular disulfides with small sulfur-containing molecules such as cysteine and glutathione [34]. Under normal physiological conditions, approximately 40% of albumin contains 'reduced' Cys34 (free thiol) [34]. The restricted location of Cys34 in a crevice of albumin helps to improve its specificity for binding metal ions that favour linear coordination, including Hg^{2+}, Au^{+}, Ag^{+} and Pt^{2+} [34,35], but not Cd^{2+} or Zn^{2+} [56].

Calcium binding sites

Albumin is an important transporter of Ca^{2+} in blood plasma. Many reports suggest that this occurs in a non-specific fashion, involving various carboxylate side chains on the surface of albumin [42,62], while work by Majorek et al. detected three defined Ca^{2+} binding sites on bovine albumin [63]. It may be significant that one of the Ca^{2+} sites detected by crystallography involves the key site A ligand, Asp248 (corresponding to Asp249 in human albumin) and indeed, Ca^{2+} ions were found to interfere with the ^{113}Cd signals for both sites A and B of human albumin [46]. However, the affinity of albumin for Ca^{2+} binding is relatively weak (K_d of 0.67 mM), with only around 45% of

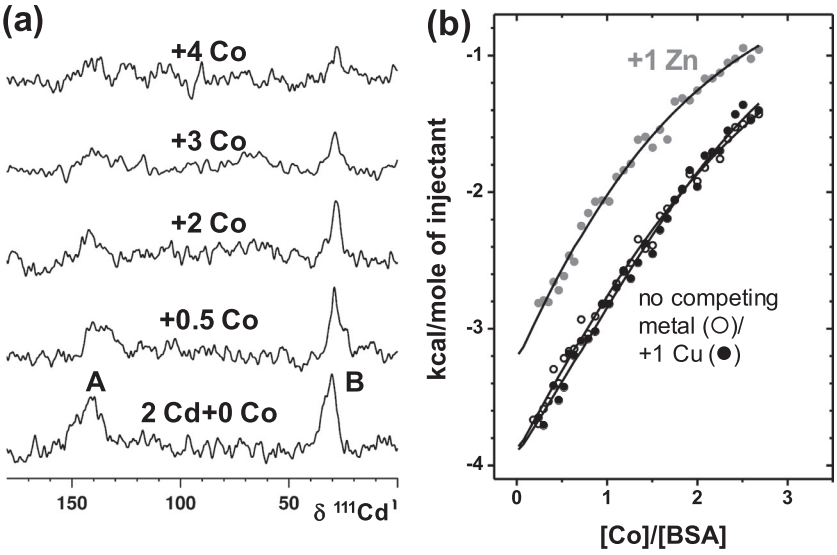

Fig. 3. Co^{2+} competes with both Cd^{2+} and Zn^{2+} for albumin binding under physiological conditions (pH 7.4, 50 mM Tris-Cl, 50 mM NaCl) but not with Cu^{2+}. (a) 111 Cd NMR spectra of Cd_2BSA (1.5 mM) with increasing addition of Co^{2+}. 111-Cd resonances corresponding to both site A and B (~140 ppm and 35 ppm, respectively) are affected by Co^{2+}. (b) Isothermal calorimetry experiments performed in the presence of 1 mol. equiv. of Cu^{2+} (●) or Zn^{2+} (●) demonstrate that addition of Zn^{2+} decreases albumin's affinity and capacity for Co^{2+}-binding, while addition of Cu^{2+} has no significant effect.

the 2.4 mM of circulating Ca^{2+} bound to albumin [63,64]. Mg^{2+}, which is also carried by albumin, is thought to bind to the same binding sites as Ca^{2+} but with an even lower affinity (K_d of 10 mM) [65].

2.2. Cobalt binding to albumin

Cobalt circulates in the blood as Co^{2+} and albumin is its principal transporter in plasma [34]. While it is widely assumed that its binding resembles that of Ni^{2+} and Cu^{2+} (d^8 and d^9 metal ions, respectively, with preference for the formation of square planar/tetragonal complexes), Co^{2+} (d^7) behaves in fact more like Zn^{2+} (d^{10}), has a similar ionic radius (0.58 vs. 0.60 Å for Zn^{2+} [66]), and shares a preference for tetrahedral, penta-coordinate or octahedral geometry [67]. For precisely this reason, Co^{2+} has been used extensively as a spectroscopic probe for Zn^{2+}in proteins [68,69].

In total, three significant Co^{2+} binding sites have been identified on albumins – the NTS, site A and site B [42]. Based on Co^{2+} perturbing ^{1}H NMR resonances for the three N-terminal residues [11] and Co^{2+} binding to an ATCUN peptide mimic [13,49], it was assumed that the primary cobalt-binding site was the NTS motif [11,19,70]. More recent comprehensive studies on human albumin by Mothes and Faller [71] and Sokolowska et al. [42], and on bovine albumin by our labs [25] have since rejected this claim. Competition with Cd^{2+} and Cu^{2+} monitored by electronic absorption spectroscopy strongly suggested that sites A and B are the preferred Co^{2+} binding sites [16,42,71]. Subsequently, ITC and spectroscopic studies identified site B as the strongest cobalt binding site [42]. Co^{2+} binding to sites A and B was also confirmed by ^{111}Cd NMR spectroscopy for bovine albumin (Fig. 3a), and competition with Zn^{2+} was evident from ITC (Fig. 3b) [25]. In contrast, blocking the NTS site with Cu^{2+} did not impart any significant effect on Co^{2+} binding [25,71]. It is important to note however, that even though Co^{2+} and Zn^{2+}may be regarded as metal ions with similar properties, the apparent binding constant for Co^{2+} binding to its strongest site on bovine albumin (log $K_{app} = 4.6 \pm 0.3 \times 10^4\ M^{-1}$; Fig. 5b; [25]) and $9 \pm 5 \times 10^4\ M^{-1}$ for human albumin [42]) were around one order of magnitude lower than those determined for Zn^{2+} [25].

In summary, even though all three apparent binding constants for Co^{2+} binding to human albumin lie between $9 \pm 5 \times 10^4\ M^{-1}$ and $0.9 \pm 0.3 \times 10^4\ M^{-1}$ [42], and hence the respective equilibria do overlap, the NTS site is now known to have the weakest affinity for Co^{2+} [42]. Most importantly, this weaker than anticipated binding of Co^{2+} to the NTS means that the initially proposed molecular basis of the ACB assay to assess the likelihood of myocardial infarction required revision, since the original studies assumed that Co^{2+} binds exclusively to the NTS [8,19].

3. Free fatty acid binding to albumin and allosteric inhibition of metal ion binding

Albumin has an unparalleled capacity to bind and transports a range of organic molecules under physiological conditions [72]. Notable among those transported are FFAs, important substrates in organismal metabolism for which albumin is the main carrier [73–76]. FFAs are the main source of energy for heart and skeletal muscle. Disturbances of the levels and/or distribution of fatty acids in the body have been linked to a spectrum of pathological disorders, including diabetes, cardiovascular and neurological diseases, and cancer [77]. Owing to the abundance of albumin in plasma, and the importance of fatty acids in metabolism and disease progression, binding of FFAs to albumin has been studied intensively in the past four decades [73], in particular by X-ray crystallography [75,78,79] and ^{13}C NMR spectroscopy [80,81].

Up to seven medium-to-long chain (C10-C18) fatty acid binding sites (FA1-7) have been identified on albumin, spread over the three domains (see Table 1 and Fig. 4) [75]. The binding affinities depend on both the site and the FFA chain length. Four additional binding locations have been described for short-to-medium chain fatty acids [82], however for the purposes of this review we will focus on FA1-7 (Fig. 4). In a normal physiological state, albumin circulates with between 0.1–2 equivalents of FFAs bound, however it pathologically can bind in excess of 6 equivalents [83]. The seven identified binding sites can be broadly split into two categories: the high-affinity sites (FA2, FA4 and FA5) and the low-affinity sites (FA1, FA3, FA6 and FA7) [83]. The high-affinity site FA2 is close to metal-binding site A and therefore of particular interest. This relatively hydrophobic site is, like metal site A, an inter-domain site and is located between sub-domains IA and IIA (Fig. 4) [82]. Compared to FFA-free albumin, accommodation of a fatty acid molecule in site FA2 requires a change in the mutual arrangement of domains I and II. While short-chain FFAs (<C8) were originally thought to be too short to successfully dock in the FA2 site [82], more recent ^{1}H and ^{111}Cd NMR studies indicated that octanoate can bind to this site. Molecular modelling suggested that the half-pocket in domain II is sufficient to accommodate octanoate, and therefore does not require the domain-domain movement [25].

While metal site A is essentially 'pre-formed' for metal (physiologically Zn^{2+}) binding in FFA-free albumin [60], this is not the case when FA2 is occupied by a longer chain FFA (*e.g.* myristic acid, C14), as the distance between the metal-coordinating residues is too large after

Table 1
The location and characteristics of fatty acid binding sites FA1-7 of albumin. Particular attention is drawn to binding site FA2, since occupation of this site by FFAs causes an allosteric switch in metal binding at site A, owing to its close proximity; both are located between subdomains IA and IIA.

Site	Affinity	Physiological[a]	Subdomain	Comments	Reference
FA1	Low	–	IB	Site is relatively accessible to solvent	[79,82]
FA2	High	Yes	IA-IIA	Allosteric switch affecting site A	[21,82,83]
FA3	Low	–	IIB-IIIA	Chain distorted in longer FFAs	[78,82]
FA4	High	Yes	IIIA	Inverted configuration for C18 FFAs	[82,83,143]
FA5	High	Yes	IIIB	C18 FFAs accommodated	[82,83]
FA6	Low	–	IIA-IIB	Absence of ligands for carboxylate	[78,79,82]
FA7	Low	–	IIA	Preference for shorter-chain FFAs	[78,82]

[a] (Partially) Occupied under basal physiological conditions (pH 7.4, 0.5–2 mol. equiv. of FFA).

the conformational change [21,24,56]. This crucial discovery suggested that FFA and zinc concentration(s) in blood plasma may be correlated through an allosteric mechanism based on albumin [21,22]. Competition experiments monitored by ITC demonstrated that the zinc-binding capacity of both bovine and human albumin for site A was dramatically reduced [23,25]. Five equivalents of myristate were sufficient to completely inhibit Zn^{2+} coordination to site A in bovine albumin (Fig. 5a), with site B also affected more or less severely [25]. Importantly, FA2 is one of the high affinity sites, and will become significantly populated already at 1 molar equivalent [83,84]. Indeed, the data in Fig. 5a indicate that the largest effect is seen between 0 and 2 molar equivalents. The downstream implications of this allosteric switch for the fate of plasma zinc are discussed elsewhere [21–23].

Crucially, although the binding preferences of Zn^{2+} and Co^{2+} are not identical, the presence of myristate also clearly reduced the binding capacity of bovine albumin for Co^{2+} (Fig. 5b) [25]. The effect on Co^{2+} binding is less severe than that on Zn^{2+} binding, with 5 molar equivalents of myristate reducing binding by *ca.* 50% [25]. This is in agreement with the fact that Co^{2+} does not bind preferentially to site A, but site B (which in BSA is affected by FFA binding, but less severely) [25], and can also bind to the NTS motif which is not expected to be adversely affected by the presence of FFA. Similarly to Zn^{2+}, it appears that the bound metal ion must first be removed from site A before fatty acid binding can occur at FA2, identified by a reduction in the exothermicity of the FFA-binding reaction (Fig. 5c) [25]. The number of apparent Co^{2+} binding sites in these experiments was in broad agreement with other experimental data, although we note that the selected experimental conditions did not allow to fully saturate all three binding sites. The apparent number of binding sites in the absence of myristate amounted to 2.4, and reduced to *ca.* 1.3 sites, implying that (at least) one binding site became non-functional (Fig. 6a). This is consistent with an inhibition of cobalt-binding to site A, as a result of FFA binding to the nearby FA2 site [25]. Most importantly, increasing the levels of FFA in a mock ACB assay is sufficient to lead to increased formation of the Co-DTT product, with concomitant higher absorbance readings (Fig. 6b). The magnitude of the changes in absorbance at 470 nm is broadly in line with effects seen in clinical studies [25]. We next explore whether this molecular mechanism may be reflected in clinical data.

4. Ischemia-modified albumin in disease states

As indicated previously, the diagnostic specificity of the ACB assay is very low, resulting in a high proportion of false positives, *i.e.* high readings despite the absence of ischemia [15,85]. This realisation has motivated a large number of studies which found positive ACB readings for a wide range of disease conditions including ACS [20, 86], chronic liver and kidney diseases [87,88], infectious diseases such as malaria [89], and pregnancy-related conditions such as pre-eclampsia [90]. In addition, elevated IMA levels have been measured in metabolic syndrome [91,92], diabetes [93] and obesity [94,95] while exercise and trauma have also been investigated [96,97]. These conditions, therefore, should have a common feature that can explain elevated IMA levels, and we propose that this common feature is elevated plasma FFAs. The latter have been shown to independently influence the ACB assay to the same extent as ACS and other conditions [20,25]. Together with the biochemical and biophysical studies detailed in Section 3, it is compelling to suggest that IMA corresponds to albumins in which FA2 is occupied. To further explore this hypothesis, Table 2 compiles selected conditions which are positive for the ACB assay and reports quantitative data on serum FFAs drawn from the literature. Cobalt binding to albumin is both specific and proportional to the total serum albumin concentration, and so many studies adjust for the total albumin level [10].

ACSs are well-known to be associated with increased serum FFA concentrations [20,98]. The pain and the stress associated with such syndromes is thought to trigger a sympathetic discharge, with the release of catecholamines which activate hormone-sensitive tissue lipase – the enzyme which hydrolyses triglycerides and hence liberates FFAs into the circulation [99–101]. This leads to elevated serum free fatty acid concentrations within 1–2 hours from the onset of ACS, and the degree of increase in FFA concentration has been positively associated with serious ventricular arrhythmias [102]. Significantly, the ACB assay values are also positively correlated to the severity of the ACS condition [20,86,102]. In addition, the IMA levels detected via the ACB assay

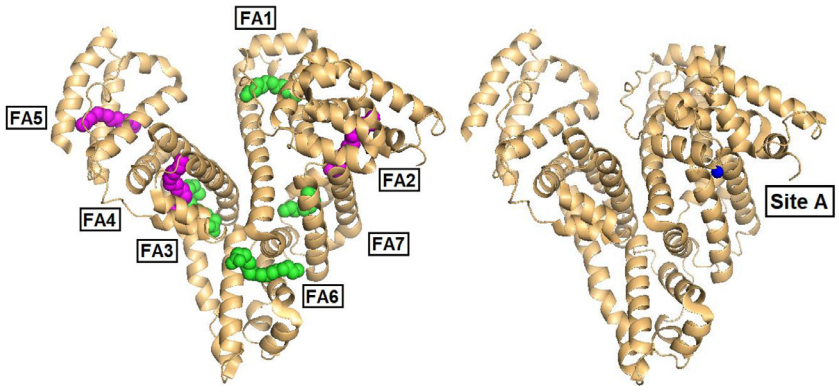

Fig. 4. (a) Location of fatty acid (FFA) binding sites FA1-7 on human serum albumin (PDB: 1E7H), complexed with hexadecanoic (palmitic) acid [82]. High (magenta) and low (green) affinity sites are shown. (b) Location of site A, the multi-metal binding site (PDB: 5IJF), occupied by Zn^{2+} (blue) [57]. Site A and FA2 are both located between subdomains IA-IIA. The inter-domain nature and the proximity of FA2 to site A allows for the allosteric switching of metal ion binding [21].

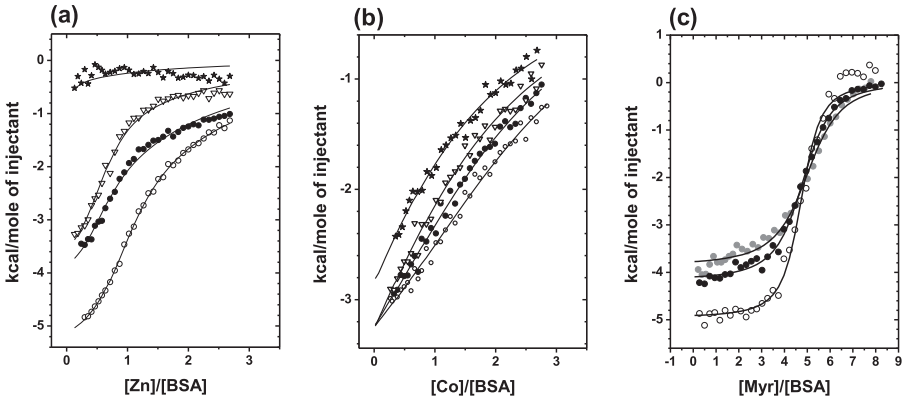

Fig. 5. Isothermal titration calorimetry experiments demonstrate the mutual modulation of metal and fatty acid binding to bovine albumin. The presence of the C14:0 fatty acid myristate (○, 0 mol. equiv.; ●, 1 mol. equiv.; ▽, 3 mol. equiv.; and ★ 5 mol. equiv.) affects the binding capacity of albumin for Zn^{2+} (a) and Co^{2+} (b) under near-physiological conditions (pH 7.4, 50 mM Tris-Cl, 50 mM NaCl). Co^{2+} binding to albumin is not only weaker than that of Zn^{2+}, but the effect of FFAs on Zn^{2+} binding is also much more pronounced than that of Co^{2+}. (c) The presence of 1 mol. equiv. of Zn^{2+} (◉) or Co^{2+} (●) affects the energetics of fatty acid binding relative to the metal-free experiment (○), likely due to the need to remove the metal before the FFA can bind. Notably, again the effect for Zn^{2+} is larger than that for Co^{2+}.

increase within minutes of the onset of ischemia, stay high for 6 to 12 hours before returning to normal level within 24 hours. This correlates to similar changes in FFA levels, which return to normal after 24 to 48 hours after myocardial ischemia [103], but is in contrast to explanations invoking N-terminally modified albumin, as albumin has a half-life of *ca.* 20 days and so IMA should be detected for several days following ischemia [104,105].

Higher FFA concentrations in plasma have been observed in several non-communicable diseases which also result in a positive ACB assay [22]. In fatty liver disease for example, there is insulin resistance which causes a withdrawal of the inhibition of dephosphorylation of hormone-sensitive lipase activity to reduce fat hydrolysis [106–108]. Further to this, the capacity of the liver to utilise and export FFAs is impaired, leading to increased FFAs in the circulation [87,109,110]. Similarly, chronic kidney disease is associated with raised FFA concentrations arising from TNF-α-induced adipose tissue lipolysis as a consequence of systemic inflammation [111,112]. In addition, when patients suffering from metabolic syndrome are given high-fat diets, a significant increase of their IMA/albumin ratio occurs [113]. It is therefore consistent with our hypothesis that those disease states are associated with positive ACB assays. Further conditions with positive ACB readings include diabetes [114], hypothyroidism [115], intrauterine growth restriction [116], chronic inflammation (rheumatoid arthritis [117] and ankylosing spondylitis [118]), infection (sepsis [119] and malaria [89]), exercise [96] and trauma [97]. All of these are also associated with high FFA concentrations (see Table 2) through various physiological and pathophysiological pathways [120–125].

However, for some other conditions associated with high IMA levels (psoriasis [126] and polycystic ovarian syndrome [127]) no variation in FFA levels compared to healthy controls have been found. Yet some specific long-chain FFAs were measured at higher concentrations and their increased binding affinity for albumin may explain the observed changes in albumin metal-binding capacities [82]. For other conditions (obstructive sleep apnoea syndrome [128], ovarian torsion [129], mothers bearing small-for-gestational-age foetuses [116] and preterm babies with respiratory distress syndrome [130]), FFA levels have not yet been measured. Several other studies detected higher IMA levels in yet more conditions (hyperemesis gravidarum [131], perinatal asphyxia [132], mild cognitive impairment [133], pre-eclampsia [90]), however they were not included in our analysis as “IMA levels” were measured with an immunoassay (see next section) instead of the ACB assay.

5. Proposed alternatives to the ACB assay

An enzyme-linked immunosorbent assay has been developed as an alternative to the ACB assay to specifically detect N-terminal modification of albumin. However, no correlation has been found between the results of this assay and IMA levels measured via the ACB assay in patients with either acute coronary syndrome or non-ischemic chest pain [16]. This is consistent with metal binding sites A and B playing a more important role in cobalt binding than the N-terminus.

Other studies on human serum albumin have utilised Cu^{2+} and Ni^{2+} instead of Co^{2+} to assess reduced metal binding. In some cases, these studies were inspired by the originally proposed mechanism involving binding to the NTS [134–136]. Even though Cu^{2+} and Ni^{2+} do indeed preferentially bind to the N-terminus, these studies were successful in demonstrating poor binding capacity of albumin for these ions in coronary artery syndromes – similar to the ACB assay [134–136]. It should however be considered that site A is a potent secondary binding site for these metal ions once the NTS is saturated, as

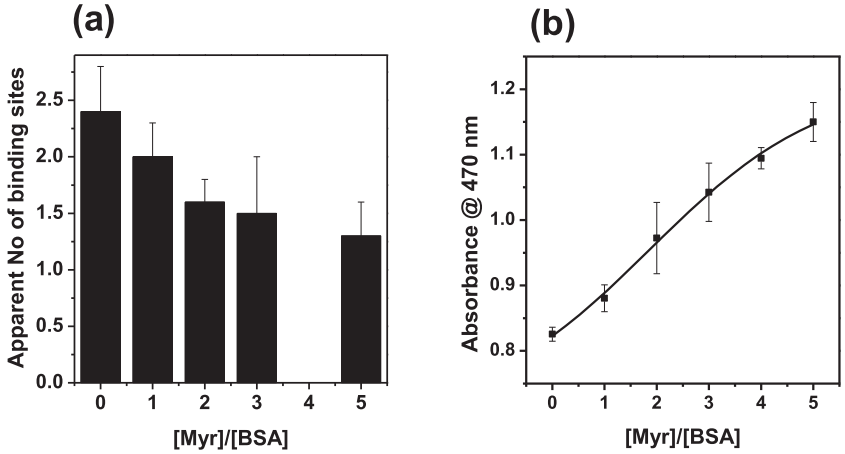

Fig. 6. Increasing FFA (myristate, C14:0) decreases the total Co^{2+} binding capacity of BSA, (a) reflected in the number of apparent binding sites of albumin for Co^{2+} (No data for 4 mol. eq. Myr). (b) In turn, this affects the formation of the Co-DTT complex as part of the ACB assay (b), used for the detection of myocardial ischemia.

Table 2
Selected conditions associated with a positive ischemia modified albumin (IMA) test and increased free fatty acids (FFAs) with the corresponding IMA and FFA levels.

Condition		IMA levels	Controls	References (IMA levels)	Plasma /serum FFA levels	Controls	References (FFA levels)
ACS	ST-segment elevated myocardial infarction	92.1 (± 10.6) Abs units/mL	77.9 (± 6.69) Abs units/mL	[86]	840 (± 320) μM	750 (± 280) μM	[98]
	Non-ST-segment elevated myocardial infarction	87.3 (± 5.95) Abs units/mL					
	Unstable angina	88.9 (± 6.16) Abs units/mL					
	Acute myocardial infarction	119 (± 37.3) Abs units/mL	88.6 (± 19.3) Abs units/mL	[20]	1030 (± 450) μM	770 (± 340) μM	[20]
Acute ischemic stroke		1.180 (± 0.223) Abs units	0.820 (± 0.129) Abs units	[144]	530 (350–710) μM	240 (120–380) μM	[145]
Obstructive sleep apnea syndrome		0.58 (± 0.11) Abs units	0.43 (± 0.09) Abs units	[128]	Proposed higher levels, increase with period of oxygen desaturation	no data	[146, 147]
Diabetes	Diabetes only	0.478 (± 0.095) Abs units	0.395 (± 0.054) Abs units	[114]	>750 μM	<550 μM	[148]
	Diabetic foot	0.721 (± 0.123) Abs units					
Rheumatoid arthritis		0.495 (± 0.01) Abs units	0.433 (± 0.02) Abs units	[117]	(0.59 (0.47–0.65) mM	0.40 (0.35–0.50) mM	[149]
Ankylosing spondylitis		0.44 (± 0.17) Abs units	0.32 (± 0.13) Abs units	[118]	883.89 (± 55.32) μg/mL	760.84 (± 31.40) μg/mL	[150]
Psoriasis		0.85 (± 0.15) Abs units	0.79 (± 0.09) Abs units	[126]	No global increase but increases in C16:1n-7, C18:2n-6, C18:3n-3, C20:0	no data	[151]
Chronic liver disease		0.532 (± 0.168) Abs units	0.320 (± 0.126) Abs units	[152]	620 (120–3400) μM	450 (110 – 900) μM	[87]
Chronic renal disease		0.357 (± 0.083) Abs units	no data	[88]	492.63 (± 143.59) μM	302.65 (± 142.18) μM	[111]
Subclinical hypothyroidism		no data	0.41 (± 0.06) Abs units	[115]	~675 (±) μM	~325 (±) μM	[153]
Sepsis		0.967 (± 0.734) Abs units	0.007 (± 0.009) Abs units	[119]	4 fold increase	no data	[154]
Malaria		0.56 (± 0.13) Abs units	0.24 (± 0.04) Abs units	[89]	2.17 fold increase	no data	[120]
Trauma		0.63 (± 0.18) Abs units	0.39 (± 0.05) Abs units	[97]	2010 (± 190) μM	no data	[124]
Ovarian torsion		0.704 (± 0.059) Abs units	0.667 (± 0.052) Abs units	[129]	no data	no data	no data
Polycystic ovarian syndrome		0.52 (0.21–1.12) Abs units	0.35 (0.06–0.90) Abs units	[127]	Total levels unknown but increase in C16:0 and C18:1n9cis	no data	[155]
Mothers bearing small-for-gestational-age foetuses		IMA/albumin: 1.28 (± 0.17) g/dLin 1st semester	IMA/albumin: 1.16 (± 0.21) g/dL in 1st semester	[116]	no data	no data	no data
Intrauterine growth restriction		78.7 (± 6.9) Abs units/mL	74.4 (± 7.8) Abs units/mL	[156]	355 μM (in amniotic fluid)	125 μM (in amniotic fluid)	[157]
Preterm babies with respiratory distress syndrome		0.91 (± 0.15) Abs units	0.63 (± 0.12) Abs units	[130]	no data	no data	no data
Exercise		0.324 (± 0.039) Abs units	0.281 (± 0.052) Abs units	[96]	>2000 μM	< 600 μM	[158]

explained in Section 2 [34,137,138]. Therefore, providing that such tests employ an appropriate metal: albumin molar ratio (≥ 2), FFAs can affect the binding capacity of albumin for Cu^{2+} and Ni^{2+} binding to site A (and site B) like for Zn^{2+} or Co^{2+} [8,22,135]. Most recently, a ^{13}C NMR-based protocol using ^{13}C-methyl-labeled oleic acid (OA) as a reporter molecule has also been developed to measure the amount of long chain FFAs bound to albumin as an alternative to the ACB assay that is not dependent on total albumin concentrations [139].

6. Conclusion

Use of the ACB assay to measure IMA levels in multiple pathological conditions has gained traction in recent years. The diagnostic value of this test critically depends on understanding its molecular basis. In the light of compelling evidence, there is now increasing recognition of the fact that N-terminal modification is not a plausible explanation for reduced cobalt binding by albumin [16,139,140]. Nonetheless, the FFA-based mechanism is not yet widely accepted either, with many recent studies claiming that IMA corresponds to a marker for “oxidative stress”. In principle, an altered redox balance may well affect the ill-defined chemistry of complex formation between Co^{2+} and DTT, as both agents are prone to oxidation. This alternative hypothesis which does not require covalent modification of albumin may also be more compatible with the timescales of increased and returned to normal ACB readings. At present, corresponding quantitative data and experiments to demonstrate the viability of this hypothesis are scarce, and it leaves unclear the role of albumin in the readout, although the possibility of ternary complex formation was raised [19]. The correlation between ACB assay readings and FFA levels is clear (Fig. 6b), provides a coherent explanation of the chemical identity of IMA, and is consistent with all clinical observations. Serum FFA, in particular unbound FFA, concentrations are useful biomarkers for early diagnosis of ACS [141]. We suggest that the ACB asay – or indeed one of its variants using other metal ions – may be re-purposed as a test for increased serum FFAs [22,25,140,142]. A comprehensive understanding of the chemical species contributing to the overall readouts, including effects of pH and redox chemistry, should enable the design of a test with much better specificity and diagnostic value.

Conflict of interest

There are no financial or other relationships that might lead to a conflict of interest for the authors.

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