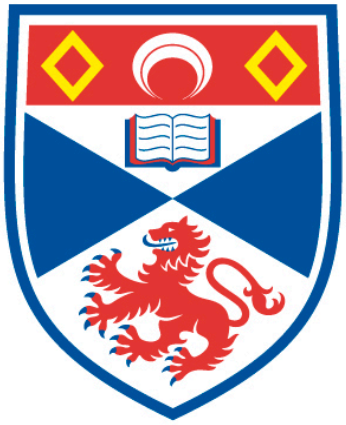
Influence of plasma fatty acids on serum albumin-metal interactions and blood clotting

Kondwani George Happy Katundu

A thesis submitted for the degree of PhD
at the
University of St Andrews



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ABSTRACT

Non-communicable diseases (NCDs) are a cause of high mortality and morbidity globally. HIV infection has complicated the burden of NCDs further by increasing its prevalence, mostly in low- and middle-income countries. NCDs and HIV are associated with higher plasma free fatty acids (FFA) in plasma and are risk factors for thromboembolic events.

Human serum albumin (HSA) is a carrier of many metal ions and is also the primary transporter of FFA in plasma. Higher concentrations of FFA in plasma and their binding to the fatty acid binding site FA2, may elicit conformational changes that may perturb metal binding at site A. The binding affinity of albumin to Co^{2+} and Zn^{2+} can be compromised in pathological conditions associated with higher FFA concentrations and can influence clinical diagnostic tests such as the albumin cobalt-binding (ACB) assay. Zn^{2+} is a key regulator of haemostasis, and the concentration of labile Zn^{2+} in plasma is tightly regulated through binding to HSA. Abnormally high FFA concentrations can thus allosterically disrupt the binding of Zn^{2+} to HSA and potentiate abnormal platelet aggregation. This thesis aimed to assess the influence of elevated FFA concentrations on the interaction between albumin and metal ions in the circulation, and how abnormal pathophysiological processes such as thromboembolic events may be precipitated in pathological conditions especially in NCDs and HIV infection due to the higher FFA levels.

The results of this study, using the albumin cobalt binding (ACB) assay, showed that long chain fatty acids perturbed the binding of Co^{2+} at the multimetal binding site A, which is also the primary binding site for Zn^{2+}, in a concentration-dependent manner. These results were also supported by isothermal titration calorimetry.

Platelet aggregation experimental results revealed that Zn^{2+}, in the absence of HSA, potentiated platelet aggregation by positively influencing the maximum and rate of aggregation responses. Higher (supraphysiological) concentrations of added myristate, but not octanoate, positively affected the maximum platelet aggregation responses, plausibly via allosteric Zn^{2+} switch from albumin. The in-silico analysis of albumin crystal structures examined in this project, exposed the disruption of the primary Zn^{2+} -binding site on albumin by long chain fatty acids, due to changes in the distances between the specific amino acid residues forming the site.

Lastly, a clinical study was conducted to investigate the FFA concentrations, albumin levels and coagulability in HIV – infected individuals compared to controls. The results indicated that participants with HIV infection and recently commenced on (antiretroviral therapy) ART had subnormal levels of albumin concentration than the controls. Also, the FFA concentrations were significantly higher in HIV – infected participants on ART for more than 6 months than the controls. Nevertheless, the coagulability tests assessed by international normalized ratio (INR) and the turbidity tests of plasma did not show any differences among the groups.

These study results contribute to the body of knowledge on how FFA can influence abnormal handling of Zn^{2+} by HSA in circulation, which may precipitate platelet aggregation and increase the risk for thromboembolic events, such as stroke, in populations at risk. Possible and relevant suggestions on how the mishandling of Zn^{2+} by HSA may be mitigated in populations at risk have been discussed.

DEDICATION

We can make food for our bodies, but eventually, the food makes our bodies.

We decide on what and how to think, but ultimately, we are a product of thoughts and decisions.

“Every day, in every way, I am getting better and better."

Émile Coué de la Châtaigneraie

"For we walk by faith, not by sight.”

Paul, the apostle

Science, in its depths, reveals but the plain truth that behind the magnificent order of the universe, even at a molecular level, there is a creator - God!

Dedicated to Precious and Splendour Katundu

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Firstly, I am forever grateful to the Lord Almighty for the excellent opportunity I have had to study at this level. The Lord is my strength!

The School of Medicine University of St Andrews and the College of Medicine University of Malawi

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My late dad George; you sent me forth like an arrow, and though you are gone, I will hit the target for sure. Thank you, dad, for believing in me - *"Zingalume, phula nditenga"*. Medium my mum, your prayers are always answered, and God never ceases to keep watch over me - *Zimatha Njazi*! My brothers and sisters; Mangani, Martha, Mputeni, Mervis and Rodwell; you have individually contributed to my academic work and success in various ways. Uncle Alex, Aunt Pauline and family, the Magombo and the Maseko families - your support is appreciated, and I love you all.

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ABBREVIATIONS

AA	Arachidonic acid
ABSU	Absorbance units
ACB	Albumin cobalt-binding
ACC	Acetyl-CoA carboxylase
ACD	Acid-citrate-dextrose
ACL	Adenosine triphosphate -citrate lyase
ADP	Adenosine diphosphate
AGPAT	Acylglycerol-3-phosphate acyltransferase
AM	Activation mix
ANOVA	Analysis of variance
APC	Activated protein C
ART	Antiretroviral therapy
ASVD	Atherosclerotic vascular disease
ATCUN	Amino terminal Cu^{2+} and Ni^{2+} binding motif
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
CA	California
CFU-EC	Colony forming unit endothelial cells
CHD	Coronary heart disease
CMOS	Complementary metal–oxide–semiconductor
COPD	Chronic obstructive pulmonary disease
COX-1	Cyclooxygenase 1
CRP	C-reactive protein
CVD	Cardiovascular disease
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNL	*De novo* lipogenesis
DOI	Digital Object Identifier
DTS	Dense tubular system
DTT	Dithiothreitol
EBV	Epstein-Barr virus

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
ERK	Extracellular-signal-regulated kinase
FAS	Fatty acid synthase
FFA	Free fatty acids
FMD	Flow-mediated dilation
G THROMB	Gamma Thrombin
GK	Glycerol kinase
GP Ib-IX-V	Glycoprotein Ib-IX-V
GP VI	Glycoprotein VI
GPAT	Glycerol-3-phosphate acyltransferase
GPIV	Glycoprotein IV
GPIX	Glycoprotein IX
HAS	Human serum albumin
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
HK	High-molecular-weight kininogen
HPV	Human papillomavirus
HRG	Histidine-rich glycoprotein
HSA	Human serum albumin
HSL	Hormone-sensitive lipase
ICAM-1	Intercellular Adhesion Molecule 1
IFN-α	Interferon alpha
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin 1
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IMA	Ischemia modified albumin
IQR	Interquartile range
IR	Insulin resistance
IS	insulin sensitive

ITC	Isothermal titration calorimetry
KPCB	Protein kinase C beta type
LCFA	Long-chain fatty acids
LDL	Low-density lipoprotein
LMIC	low and middle-income countries
LPL	lipoprotein lipase
LTA	Light transmission aggregometry
MA	Massachusetts
MCP-1	Monocyte chemoattractant protein 1
MGL	Monoacylglycerol lipase
MI	Myocardial infarction
MLCK	Myosin light-chain kinase
MQ	Milli-Q
NAP-2	Neutrophil Activating Peptide 2
NCX	Na+/Ca2+ exchanger
NEFA	Non-esterified fatty acids
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NL	Non-ligand bound
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NTS	N-terminal site
OCS	Open canalicular system
PA	Phosphatidic acid
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease-activated-receptor
PAR-1	Protease-activated-receptors 1
PAR-4	Protease-activated-receptors 4
PARS	Proteinase-activated receptors
PB	Permeation buffer
PC	Protein C
PDB	Protein Data Bank
PEP	Phosphoenolpyruvic acid

PEPCK	Phosphoenolpyruvate carboxykinase
PKC	Protein Kinase C
PLC	phospholipase C
PPP	Platelet poor plasma
PRP	Platelet-rich plasma
PSGL-1	P-selectin glycoprotein ligand-1
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
ROS	Reactive oxygen species
SAT	Subcutaneous adipose tissue
SD	Standard deviation
SFLLRN	Thrombin receptor activating peptide
SLAM	Signalling lymphocyte activation molecule
TAG	Triglyceride
TDF	Tenofovir
TF	Tissue Factor
TNFSF 14	Tumor necrosis factor superfamily 14
TNF-α	Tumor necrosis factor alpha
TPEN	N,N,N',N'-Tetrakis(2-pyridylmethyl) ethylenediamine
UK	United Kingdom
USA	United States of America
VAT	Visceral adipose tissue
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VWF	von Willebrand factor
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

1.1 The burden of noncommunicable diseases

Non-communicable diseases (NCDs) represent a group of medical conditions or diseases which are non-infectious and non-transmissible among individuals (Kim et al., 2013). NCDs contribute significantly to global morbidity and are the leading cause of mortality globally (Figure 1.1). According to the World Health Organisation (WHO), 41 million deaths in 2018 were attributed to NCDs, which translates to 71% of all global deaths each year (WHO, 2018). Sadly, the mortality due to NCDs is predicted to further increase by 15% by 2020 and up to 5 times the current 2018 number of deaths due to NCDs by the year 2030 (Mc Namara et al., 2019, World Health, 2018).

Of these deaths due to NCDs, the four major contributors accounting for more than 80% are cardiovascular diseases (CVD) which claim 17.9 million deaths annually, respiratory diseases (9 million), cancer (3.9 million) and diabetes (1.6 million) (Mc Namara et al., 2019, World Health, 2018) (Figure 1.2). However, the distribution of the mortality burden due to the NCDs is disproportionate across the different economic categories of the world whereby low- and middle-income countries (LMIs) bear more than 75% of the death-burden (Gowshall and Taylor-Robinson, 2018). In a country such as Malawi which is also already grappling with higher mortality due to infectious diseases, NCDs contribute not less than 28% of all deaths - a further challenge to the already overwhelmed health system (Gowshall and Taylor-Robinson, 2018, World Health, 2018). Further to this, it is a concern that 15 million of the global annual deaths due to NCDs deaths occur at a premature age (between 30 and 69 years), and 85% of which are in LMIs (Mikkelsen et al., 2019, World Health, 2018). Thus, in as much as prevention of NCDs is of vital importance, appropriate strategies towards ameliorating these premature deaths especially in the LMIs is desirable.

Antagonising the detrimental global effects of NCDs certainly requires evidence-based strategies derived from scientific evidence for effective interventions (Sturke et al., 2016). Furthermore, it is essential to develop local research capacity in LMIs to address the evidence needs as a necessary step towards combating the burden of NCDs (Sturke et al., 2016). The WHO recommended the need for such high-quality research within its endorsed Global Action Plan for the Prevention and Control of NCDs (World Health, 2013).

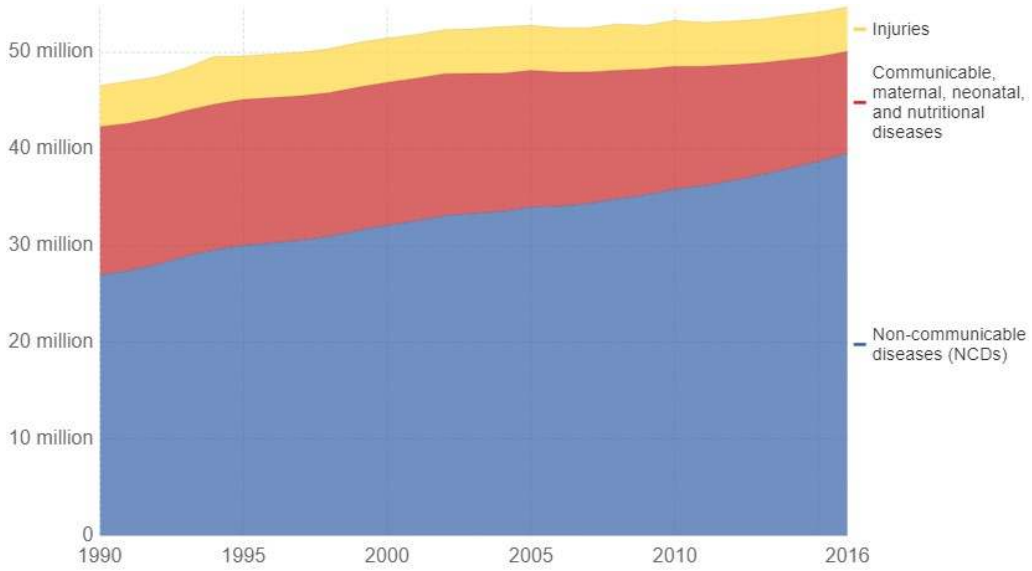


Figure 1.1 Total number of world deaths by cause from 1990 to 2016. Source (Institute for Health Metrics and Evaluation)

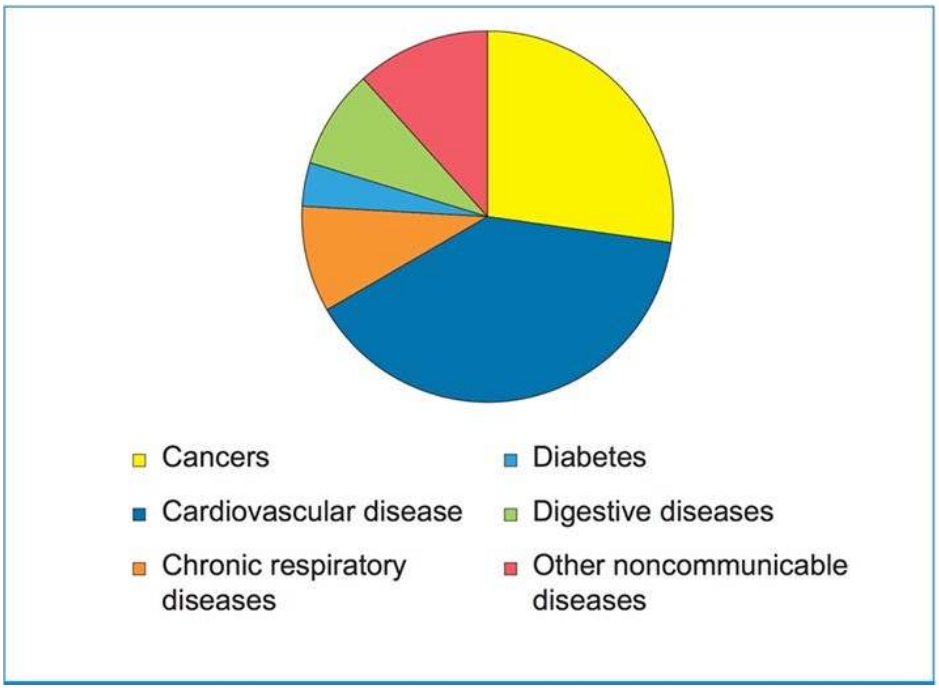


Figure 1.2 Proportion of deaths contributed by different NCDs. Cardiovascular diseases **(**CVD) are the leading cause of deaths contributing to 37% (World Health, 2014).

1.2 The pathophysiology of the major NCDs are interlinked

Most of the NCDs that contribute significantly to mortality namely CVDs, cancer, chronic respiratory diseases and type II diabetes are inter-related and have similar risk factors. A consensus of evidence indicates that these NCDs are associated with four lifestyle risk factors which are poor physical inactivity, poor diet, tobacco use and excessive alcohol use (Yeates et al., 2015). CVDs are indisputably the leading cause of most global deaths compared to other NCDs and are therefore of research importance (Yeates et al., 2015, Mendis and Banerjee, 2010). By definition, CVD can be any of the following; coronary heart disease, stroke, peripheral arterial disease, rheumatic and congenital heart disease and deep vein thrombosis (World Health, 2018). The two main contributors to CVD morbidity and mortality are stroke and coronary heart disease (Yeates et al., 2015). Each of the other three most significant NCDs seems to be associated with CVD or end up complicating with it.

Cancer and CVD, for example, have some similar pathophysiological processes and are tightly linked more than by chance (Opie, 2015). Inflammation is one of the common hallmarks for both cancer and cardiovascular disease (Tesfamariam and Cohen, 1992, Coussens and Werb, 2002, Libby, 2006). It is not surprising that both of these conditions are also associated with risks factors such as obesity, hyperglycemia, hypertension, smoking and hypertriglyceridemia all of which induce inflammation (Koene et al., 2016). These risk factors induce atherosclerosis through the expression of adhesion molecules produced by the endothelial cells, thereby promoting leukocyte attachment to the endothelial walls - a process which is resisted in normal physiological conditions (Libby, 2006). Atherosclerosis is one of the pathophysiological mechanisms that underlie these conditions and is mediated by inflammation from initiation, progression and consequently thrombosis (Koene et al., 2016). In cancer, there is a role of chronic inflammation in promoting carcinogenesis and tumour progression (Kamp et al., 2011). Evidence has also emerged in recent years that several cancer types are induced through chronic inflammatory processes attributed to infection evidenced by the interlink between diseases such as human papillomavirus (HPV) and cervical cancer, H-pylori and stomach cancer, Epstein-Barr virus (EBV) and lymphoma (Kamp et al., 2011). Malignant transformation of cells, carcinogenesis, and its progression is influenced by the inflammatory and immune signalling processes (Nelson et al., 2013).

Oxidative stress, an imbalance between pro-oxidants and antioxidants in the body, and its main consequence - lipid peroxidation is another pathophysiology associated with atherosclerosis and chronic inflammation in both CVD and cancer (Barrera, 2012). Comorbid conditions most common

in cancer and CVD include diabetes, hypertension and obesity which induce chronic inflammation exhibit oxidative stress (Thanan et al., 2014). Furthermore, hypertension a wellknown condition associated with CVD is evident to influence higher levels of plasma vascular endothelial growth factor (VEGF) which is central in inducing the generation of new bloodvessel in tumour growth in cancer (Ferrara, 2002, Felmeden et al., 2003). Compounded with the association with oxidative stress and arterial wall hardening, hypertension also is well related to the pathophysiology of both CVD and cancer (Koene et al., 2016).

Obesity interlinks cancer and CVD in pathways associated with diet, physical inactivity, body fat distribution, hormonal influences and oxidative stress (Koene et al., 2016). Inflammatory cytokines and markers such as interleukin (IL)-6, tumour necrosis factor-alpha (TNF-alpha), leptin, angiotensinogen, resistin and CRP exhibited in obesity have tumorigenic effects in other sites of the body and can also influence CVD (Hou and Luo, 2011). Leptin, for example, is on one hand, key to obesity-linked CVD, and critically regulates the progression of hepatocellular carcinoma through its effects on telomerase reverse transcriptase (Kamp et al., 2011). Similarly, overexpression of IL-6 induces hepatic production of C-reactive protein (CRP) is linked to CVD, and in cancer, IL-6 promotes tumour progression through the inhibition of cancer cell apoptosis and stimulation of angiogenesis increase tumour resistance to drugs (Ridker, 2000, Guo et al., 2012).

Diabetes, especially type II diabetes is closely associated with obesity and low-grade chronic inflammation (Duncan et al., 2003). Insulin resistance associated with these conditions induces the increased expression of inflammatory markers such as IL-1, IL-6, TNF-α, resistin, monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), fibrinogen and angiotensin (Shoelson et al., 2006). Overexpression of the inflammatory markers and CRP leads to endothelial dysfunction and poor endothelial nitric oxide (NO) secretion responsible for normal vascular wall compliance (Leon and Maddox, 2015). Higher levels of inflammatory cytokine IL-1 can also contribute to the destabilisation of atheromatous plaques and result in myocardial infarction (MI) (Vicenova et al., 2009). More importantly, insulin resistance is associated with elevated levels of plasma free fatty acids which lead to an increase in muscular triglycerides stores, hepatic glucose production, and increased insulin production in patients with type-2 diabetes mellitus (T2DM) (Leahy, 2005). Additionally, diabetes also elicits cardiomyocyte hypertrophy and hence increased left ventricular mass - a risk for cardiomyopathy (Galderisi et al., 1991, Barouch et al., 2003, Kim et al., 2008).

Another intriguing postulated mechanism for the development of myocardial infarction (MI) in diabetic patients, over and above complications of atherosclerosis is increased coagulability (Williams and Zaman, 2003). Overexpression of glycoprotein IIB/ IIIA receptors and von Willebrand factor (vWF) mediate platelet activation has been described in these patients which may potentiate thromboembolism (Vischer et al., 1998, Vinik et al., 2001). Furthermore, in diabetes, there is an increase in plasminogen activator inhibitor type 1 which decreases fibrinolysis (Sobel et al., 1998). Also, the decreased circulating levels of protein c and antithrombin III as anticoagulants, due to proteinuria (Ceriello et al., 1990) put these patients in a prothrombotic and procoagulant state which can be responsible for the increased rates of myocardial infarction. Hyperglycaemia in diabetes induces the insulin growth-like-factor-1 (IGF-1) which promotes the proliferation and migration of smooth muscle thereby promoting atherosclerosis (Guo et al., 2012). Hyperglycaemia also facilitates endothelial dysfunction through oxidative stress and promotes dyslipidaemia, and both these factors have damaging effects on the vasculature and may lead to CVD (Tesfamariam and Cohen, 1992, Giovannucci et al., 2010). Even though there is inconclusive evidence for other cancers, a consensus report from the American Diabetes Association and recent meta-analyses have reported that there is convincing evidence diabetes is associated with cancers such as colorectal, breast, endometrial, liver, pancreatic and bladder cancers (Giovannucci et al., 2010, Tsilidis et al., 2015).

Dyslipidaemia is another risk factor and complication that cuts across the major NCDs. Higher levels of serum lipoproteins such as low-density lipoproteins have been are known to be associated with CVD (Stamler et al., 1986). The cholesterol metabolite 27-hydroxycholesterol which simulates oestradiol in structure and function has been implicated in breast cancer (Warner and Gustafsson, 2014). Another modifiable factor which cut across the NCDs is smoking which generates pro-inflammatory stimuli, produces carcinogens and oxidising agents which arouse abnormal signalling pathways which promote cancer and CVD (Leikauf et al., 2002). Poor dietary habits with high consumption of processed meats and low fibre and low micronutrients such as folate and polyphenols in conjunction with genetic mutations can also predispose one to both CVD and cancers due to genotoxic substances including nitrosamines, which can directly act on DNA and cause point mutations, deletions, and insertions (English et al., 2004, Stampfer and Jahn, 2013, Wood et al., 2014, Baena Ruiz and Salinas Hernandez, 2014, Wang et al., 2017). Excessive alcohol intake, another risk factor which may increase the risk for CVD by increasing triglycerides is a risk for hypertension, atrial fibrillation and cardiomyopathy (Koene et al., 2016). Alcohol may also induce cancer through the genotoxic effect of the main alcohol metabolite acetylaldehyde and the release

of oxygen and nitrogen species (Sen and Bonita, 2000). Lack of adequate physical activity poses a risk to NCDs as it puts one at risk of obesity, dyslipidaemia, diabetes, poor vascular function and hence CVD (King et al., 2009). Furthermore, biochemically, poor physical activity promotes the accumulation of adipose tissue which mediates the increased circulation of sex hormones, leptin, insulin and markers of inflammation which may be carcinogenic (King et al., 2009).

Chronic obstructive pulmonary disease (COPD) is also closely associated and complicated with CVD (Morgan et al., 2018). Patients with COPD are more likely to die from CVD than respiratory complications (Berry and Wise, 2010). It has been discovered that apart from smoking as the common risk factor for both CVD and COPD, inflammation and endothelial dysfunction with atherosclerosis are also mutual pathophysiological events between these diseases (Fabbri, 2016, Rabe and Watz, 2017). Figure 1.3 shows the pathophysiological mechanisms which link COPD and CVD. Cigarette smoke and the noxious substances in it, destroy the small lung airways and the lung parenchyma thereby causing emphysema and hence COPD (Geovanini and Libby, 2018, Morgan et al., 2018). On the other hand, as previously described, smoking contributes to endothelial damage, the release of inflammatory markers and development of atherosclerosis up to the point of instability and rupture of the atherosclerotic plaque leading to thromboembolic disease, coronary heart disease and heart failure (Morgan et al., 2018, Geovanini and Libby, 2018). Interestingly, an exacerbation of COPD has been associated with increased inflammatory markers and during such an event, the risk of a stroke and acute coronary syndrome is highest (Marchetti et al., 2011). Accelerated ageing in COPD has been associated with shortened leukocyte telomere length and senescence of endothelial cells which eventually leads to endothelial dysfunction, arterial stiffness and atherogenesis which predispose patients to CVD (Benetos et al., 2001, Minamino et al., 2002).

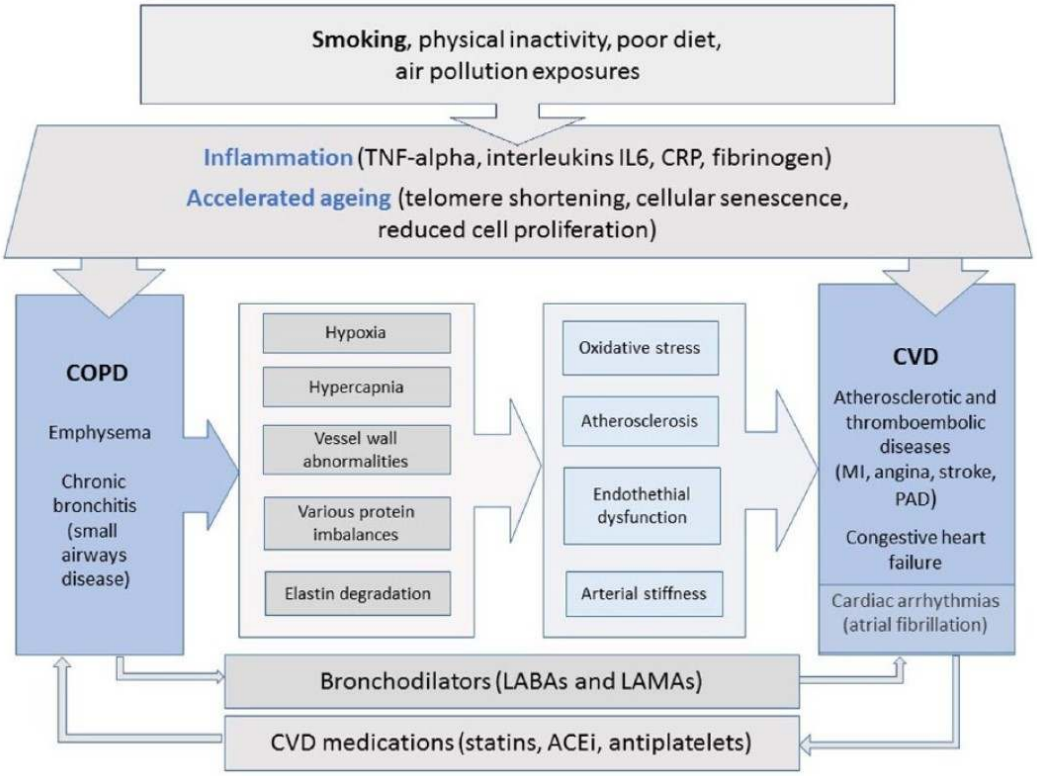


Figure 1.3 The risk factors and complications of COPD and CVD. Smoking, sedentary lifestyle and poor diet can elicit chronic inflammation leading to the release of inflammatory markers which causes endothelial damage and mediate atherosclerosis. These factors cause accelerated ageing characterised by telomere shortening, cellular senescence and reduced cell proliferation. Inflammation and accelerated ageing may potentiate both COPD and CVD. COPD may complicate or lead to CVD through hypoxic and vascular wall damage which potentiates atherosclerosis and endothelial dysfunction. Some medication for COPD can worsen or elicit CVD by promoting the sympathetic pathway. Adapted from (Morgan et al., 2018)

In COPD, hypoxia is another factor that potentiates the risk of CVD. Hypoxia which may either be intermittent or sustained in COPD elicits further systemic inflammation, oxidative stress and more endothelial dysfunction which promotes the progression of atherosclerosis and hence CVD (Morgan et al., 2018). Furthermore, gas trapping on exertion can lead to arterial compression owing to the dynamic hyperinflation, and this may ultimately lead to pulmonary hypertension, a further risk factor for a CVD (Chen et al., 2015). The risk for CVD can also occur from the medication for COPD mainly long-acting muscarinic antagonists (LAMAs) and long-acting beta agonists (LABAs) which may worsen existing underlying CVD or even increase the risk of developing CVD as they may increase activation of the sympathetic nervous system leading to cardiac rhythm disturbances (Heindl et al., 2001, Decramer and Janssens, 2013, Lahousse et al., 2016).

It follows therefore that CVD is the most important form of NCDs and a common complication of the other NCD including cancer, diabetes and chronic respiratory conditions causing increased morbidity and mortality. Furthermore, CVD events in the names of stroke and coronary heart disease are what contribute more to the weight of the CVD burden of disease (Yeates et al., 2015). It is also apparent that there are common risk factors such as smoking, obesity, poor diet and sedentary lifestyle which cut through these diseases. More importantly, there are underlying pathophysiological mechanisms which are shared among the major NCD. Of interest among the physiological derangements are the inflammation and release of inflammatory markers, endothelial dysfunction, oxidative stress, dyslipidaemia, atherosclerosis and abnormal hormonal stimulation. The risk for cute coronary syndromes and strokes is high in these patients due to the pathophysiological processes and increased coagulability (Ceriello et al., 2010, Vischer et al., 1998, Sobel et al., 1998, Vinik et al., 2001).

High free fatty acid concentration appears to be a prominent, yet not well-discussed factor observed in NCDs.

1.3 NCDs and plasma free fatty acids in the circulation

Free fatty acids (FFA) also known as non-esterified fatty acids (NEFA) act as a major source of energy for the cardiac and skeletal muscle and are also the main energy source during periods of fasting or when glucose is unavailable (Miller et al., 2012, Coverdale et al., 2018). The turnover rate for FFA is rapid with a half-life of 2-4 minutes (Eaton et al., 1969). FFA are released from adipose tissue through the catabolic process of lipolysis to mobilise metabolic fuel to peripheral tissues from the adipose tissue in response to appropriate energy demands (Saponaro et al., 2015). Figure 1.4 depicts the processes of lipolysis and lipogenesis. Lipolysis involves the hydrolysis of triacylglycerol (TAG) and subsequently yields the release of fatty acids (FA) and glycerol in plasma. Hydrolysis requires several lipases including adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) which yield the release of three FFA and one glycerol molecule (Yu and Ginsberg, 2005).

ATGL catalyses the first hydrolysis of TAG into DAG releasing one fatty acid (Figure 1.4). Ultimately, DAG is converted by the enzyme monoacylglycerol lipase (MGL) into monoacylglycerols (MAG) and releases one FFA. Alternatively, DAG is completely hydrolysed by hormone-sensitive lipase (HSL) to release two FFA and one glycerol (Saponaro et al., 2015). Lipolysis occurs mainly in the adipose tissue. Subcutaneous adipose tissue (SAT) amount is usually

greater than that of visceral adipose tissue (VAT) and hence SAT is the main contributor to plasma FFA (Mittendorfer et al., 2009, Gastaldelli, 2014). The amount of total fat in the body (and SAT mainly), is the major determinant of FFA release in the systemic circulation. Obesity, therefore, and a higher proportion of subcutaneous fat is a risk factor for higher FFA in the circulation, and, in a recent large observational study of more than 7000 participants, an increase in FFA concentrations of more than 30% was observed in obese individuals compared to normal controls (Arner and Ryden, 2015).

In normal physiology, lipolysis and the release of FFA from adipose tissue occurs when there is a demand for FFA as a source of fuel, or when the net caloric intake has exceeded the ability of the adipose tissue to store triglycerides as may be the case when there is impairment in the recruitment, proliferation, differentiation or hypertrophy of the adipose cells (Bays et al., 2006, Despres, 2006). The metabolism of FFA hinges mainly on balance between the intracellular adipocyte HSL, which is responsible for the hydrolysis of intra-adipocyte TAG and the lipoprotein lipase (LPL) secreted by the adipocytes which hydrolyse the extra-adipocyte TAG (Bays et al., 2006). Beta-adrenergic stimulation positively affects the activity of HSL in adipose tissue, but insulin inversely influences its activity, while on the contrary adipose tissue LPL activity is enhanced by insulin action and decreased with beta-adrenergic stimulation (Bays et al., 2006).

In normal physiological circumstances, following feeding, insulin levels are high which decrease HSL and, on the other hand, increase LPL activity in the adipose tissue leading to the trapping of about 90% of plasma-derived FFA in adipocytes and hence lower postprandial circulating FFA levels (Bays, Blonde & Rosenson, 2006). LPL in the capillaries of adipose tissue hydrolyses TAG mainly from the dietary fat carried in the chylomicrons and is usually trapped and stored by the adipocytes, but some may escape to join the plasma FFA pool and can constitute up to 40-50% of the FFA composition depending on the diet (Heimberg et al., 1974, Fielding et al., 1996, McQuaid et al., 2011). During fasting, however, insulin levels are lower, and the catalytic hormones come into play. The activity of HSL is thus increased and LPL activity downregulated (Bays et al., 2006).

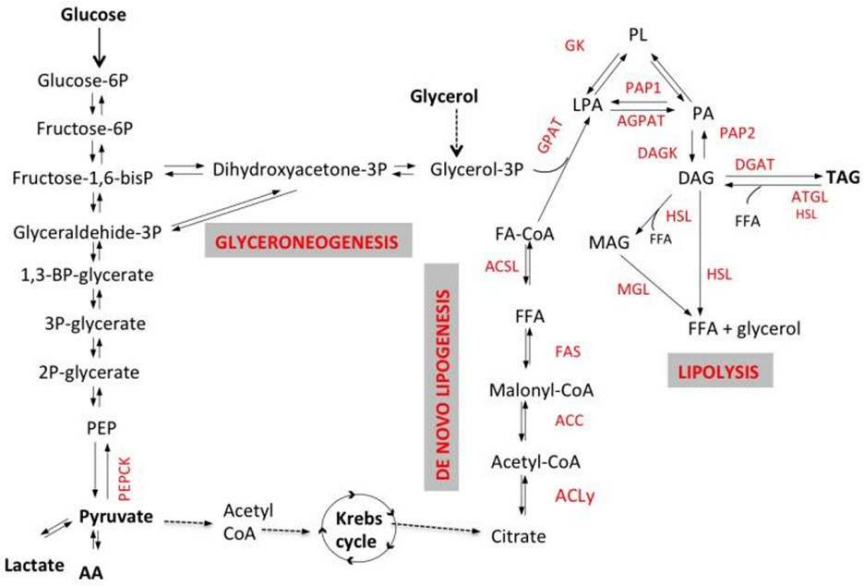


Figure 1.4 Diagrammatic representation of the lipolytic and lipogenic pathways. TAG requires the activation of FFA into Acyl-CoA by enzyme acyl-CoA synthetase. FFA-CoA and G3P are transformed via acylation, by glycerol-3-phosphate acyltransferase (GPAT) and acylCoA acylglycerol-3-phosphate acyltransferases (AGPAT), to phosphatidic acid (PA); then, after a dephosphorylation by phosphohydrolase (PAP2), diacylglycerols (DAG) are formed. Diacylglycerol acyltransferase (DGAT) catalyzes the conversion of DAG into TAG. In the adipocyte, G3P might come either from glycolysis or from non-carbohydrate substrates via the enzyme phosphoenolpyruvate carboxykinase (PEPCK), through a process named glyceroneogenesis. In the liver G3P can also be synthesized from plasma glycerol. De lipogenesis (DNL) occurs in the cytoplasm of various cells (e.g., adipocytes and hepatocytes) where citric acid is converted to acetyl-CoA by ATP-citrate lyase (ACL) and subsequently to malonyl-CoA by acetyl-CoA carboxylase (ACC). DNL occurs mainly in the liver, but it might occur in adipose tissue as well, although with low rates. This process requires the two enzymes ATP-citrate lyase (ACL), acetyl-CoA carboxylase (ACC) and the multi-enzymatic complex fatty acid synthase (FAS). G3P can be synthesized directly from non-carbohydrate substrates such as pyruvate, lactate or amino acids in oxaloacetate, that is converted to G3P either directly from phoenolpyruvate (PEP), via the key enzyme phosphoenolpyruvate carboxykinase (PEPCK), or through synthesis of dihydroxyacetone (DHA). TAG catabolism (i.e., lipolysis) involves several lipases, adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) and produces the release of three free fatty acids (FFA) and one glycerol molecule. GK = Glycerol kinase. From (Saponaro et al., 2015)

1.3.1 Insulin resistance, obesity, NCDs and their influence of FFA in circulation

Insulin resistance (IR) entails a condition whereby the cells are unable to respond normally to insulin (Arner and Ryden, 2015). Higher insulin levels are often observed in individuals with insulin resistance as the beta cells in the pancreas produce more insulin following increased demand to facilitate glucose uptake (Gastaldelli, 2011). Insulin resistance attenuates the effect of functions of insulin in most of the organs including the muscle, liver and adipose tissue (Saponaro et al., 2015). Fatty acid esterification and synthesis of TAG process are suppressed while the normal inhibitory action of insulin on adipose tissue against lipolysis and the release of FFA is attenuated and hence the conditions favour TAG hydrolysis and FFA release in IR (Saponaro et al., 2015).

Figure 1.5 illustrates the relationship between insulin release and circulating FFA in insulin sensitive (IS) and insulin resistance conditions. Normal FFA release against insulin secretion follows a hyperbolic curve in IS conditions while in IR, there is a shift towards the right in the curve with a higher concentration in FFA in circulation regardless of the insulin released (Groop et al., 1989). Insulin resistance in patients has been found to proportionally affect the visceral and hepatic fat more (Gaggini et al., 2013). Abdominal and ectopic fat accumulation is associated with more resistance to the antilipolytic action of (Fabbrini et al., 2015).

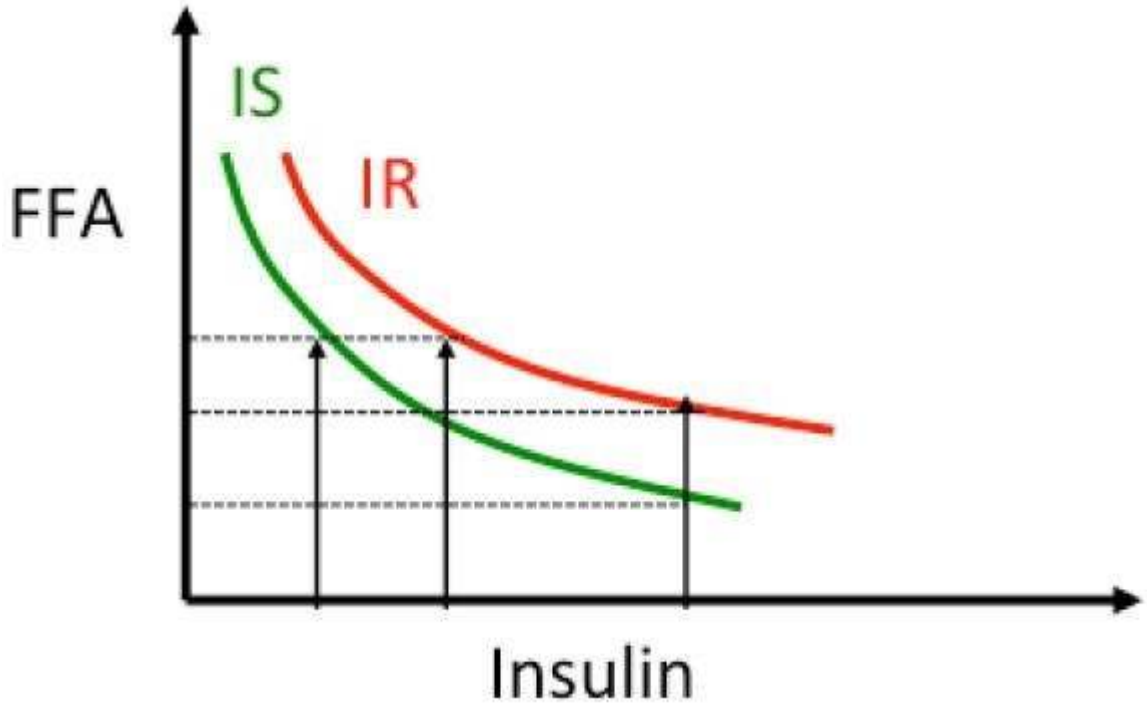


Figure 1.5. A graph on the relationship between insulin secretion and FFA release in insulin sensitive (IS) and Insulin resistance (IR) conditions. An increase in insulin concentration in IS conditions induce a suppression of adipose tissue lipolysis and decreased plasma FFA in a nonlinear curve. In IR, there is a shift in the curve to the right illustrating that lipolysis is less suppressed and circulating FFA levels are higher. Adapted from (Saponaro et al., 2015).

As discussed in the sections above, chronic low-grade inflammation has been associated with obesity-related metabolic diseases and the NCDs (Tateya et al., 2013). Animal and human studies have shown that chronic inflammation is characterised by macrophage infiltration evident in adipose tissue or liver with the activation of immune cells and is linked with insulin sensitivity (Weisberg et al., 2003, Xu et al., 2003). Macrophages are categorised as either proinflammatory M1 macrophages, which are associated with enhanced inflammatory cytokine production such as TNF-α, IL-6, and IL-1; or M2 macrophages, which generate high levels of anti-inflammatory cytokines such as IL-10 and IL-4 (Tateya et al., 2010). Regarding insulin sensitivity, M2 macrophages sustain insulin sensitivity through the secretion of IL-4 and IL-10, while M1 macrophages induce insulin resistance through the secretion of the proinflammatory cytokines (Tateya et al., 2013). Animal studies have indicated that chemokines and specifically monocyte chemoattractant protein-1 (MCP-

1) secreted in the adipose tissue attract circulating monocytes which to differentiate into M1 macrophages and bring about inflammatory characteristics of the adipose tissue and produce inflammatory cytokines which promote further inflammation (Tateya, Kim & Tamori, 2013a). An increase in MCP-1 concentration on its own has been associated with insulin resistance, and in fact, increased concentrations of MCP-1 have been described in type II diabetes and insulin resistant patients (Piemonti et al., 2003, Mine et al., 2006, Tateya et al., 2010). Figure 1.6 is a demonstration of obesity-induced macrophage infiltration into adipose tissue which induces insulin resistance.

Thus, studies have established that the major NCDs are associated with obesity and metabolic syndrome, underlying chronic inflammation, insulin resistance, pathophysiology associated with atherosclerosis and elevated FFA (Nyberg et al., 2018, Xin et al., 2019). Higher plasma concentrations of FFA are both known to promote insulin resistance, and risk of diabetes and insulin resistance itself promotes the release of higher FFA in circulation, hence a vicious cycle (Boden, 2008). Through a plethora of mechanisms, elevated FFA is one of the common characteristic pathologies for CVD, diabetes, cancer, COPD and other NCDs conditions such as fatty liver disease, chronic kidney disease, metabolic syndrome, and rheumatoid arthritis (Pirro et al., 2002, Kovacs and Stumvoll, 2005, Wu et al., 2010, Blindauer et al., 2016). The risk for cardiovascular events namely acute coronary syndromes and ischaemic strokes which are as a result of thromboembolic events are high in these patients with elevated FFA and are associated with increased risk of mortality (Ceriello et al., 2010, Vinik et al., 2001). Elevated FFA in plasma and insulin resistance-driven endothelial dysfunction with diminished endothelial nitric oxide production promote the development of hypertension and hence perpetuation of CVD (Inoguchi et al., 2000). Elevated plasma FFA has been shown as an independent predictor for recurrence and mortality following acute coronary syndrome (Zhang et al., 2017, Pilz et al., 2007, Huber et al., 2014).

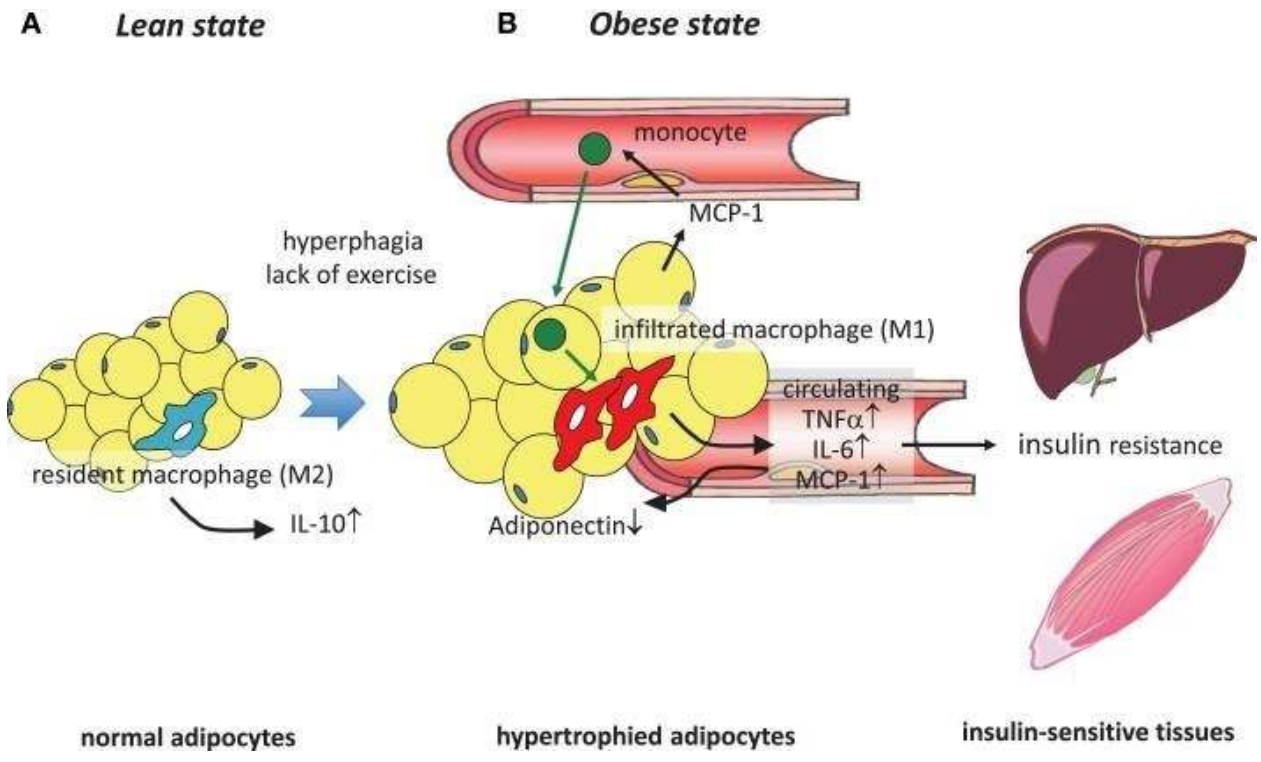


Figure 1.6. A demonstration of how obesity-induced macrophage infiltration induces insulin resistance. (A) The adipose tissue in lean conditions has an abundance of M2 macrophages population which promote insulin sensitivity through the secretion of IL-10. (B) Sedentary lifestyle however with unhealthy eating habits and lack of exercise facilitates hypertrophy of adipocytes and induces MCP-1 secretion to the circulation which ultimately leads to recruitment of circulating monocytes to adipose tissues. These infiltrated monocytes then differentiate into M1 macrophages, which secrete proinflammatory cytokines such as TNFα, IL-6, and MCP-1, thus contributing to low-grade inflammation in adipose tissue and a decrease of adiponectin. Furthermore, the secreted cytokines lead to insulin resistance in liver and skeletal muscle by acting as insulin resistance-inducing adipokines. The insulin resistance contributes to increased circulation of FFA. Adapted from (Tateya et al., 2013).

Elevated plasma FFA plays a critical role in the pathophysiology of cardiovascular events and investigating the mechanisms through which FFA influence such adverse outcomes are vital for generating possible mechanisms to ameliorate such burden of disease. As depicted in Figure 1.6 (A and B), FFA are central to the pathological processes of IR and inflammation, contribute to the development of type II diabetes, hypertension, atherogenic dyslipidemias and disorders of blood coagulation and fibrinolysis - all of which are independent risk factors for atherosclerotic vascular disease (ASVD) such as coronary artery syndrome, strokes and peripheral arterial disease (Bray, 2004, Tataranni and Ortega, 2005).

Having established that increased concentrations of FFA in plasma are central to the pathologies of obesity-related NCDs, elucidating other mechanisms through which FFA in circulation lead to cardiovascular and thrombo-embolic events which are common consequences of NCDs is essential. Understanding how FFA are handled in plasma and the molecular derangements they may cause once in circulation is of importance. FFA in plasma are mainly transported bound to human serum albumin.

1.4 Human Serum albumin (HSA)

HSA is the most abundant protein in human serum and lymph fluid (Fanali et al., 2012). The585 amino acid protein, which has a molecular weight of 66.5 kDa is produced by the liver and is found in the circulation (largely non-glycosylated) at normal concentrations of about 600 μM (Curry et al., 1998, Guizado, 2014). HSA is one of the most studied circulatory proteins (Fasano et al., 2005), and structurally it contains three domains namely domain I, II and III with each domain consisting of subunits A and B (He and Carter, 1992, Kawakami et al., 2006). The structure of HSA is stabilised by 17 disulphide bridges, with the protein possessing a single free thiol group at cysteine-34 (Kawakami et al., 2006). Figure 1.7 illustrates the structure of
HSA.

The general physiological functions of HSA are the maintenance of colloid osmotic pressure, free radical scavenging, antithrombotic effects and effects on vascular permeability, platelet function inhibition and importantly, binding and transport of circulating substances including metal ions (Margarson and Soni, 1998). HSA is the major protein providing 80% of the required oncotic pressure in plasma, and this function is what mainly regulates the synthesis of HSA (Fanali et al., 2012). HSA also contributes to the oncotic pressure of the interstitium thereby influencing the shift

of fluid from the capillaries into the interstitium (Margarson and Soni, 1998). HSA exerts oncotic pressure by both simple osmotic pressure and Donnan effect (Figge et al., 1991, Peters Jr, 1995). Apart from providing oncotic pressure, HSA also participates in antioxidant activities as a non-enzymatic antioxidant alongside other endogenous molecules such as glutathione and bilirubin, and micronutrients such as vitamin C, vitamin E and beta-carotene (Berger, 2005). HSA provides reduced sulfhydryl groups which may scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Fanali et al., 2012). HSA also binds Cu (II), a metal which is important in accelerating production of free radicals and in this way limit the production of these reactive species (Fanali et al., 2012). Further to these functions, HSA has antithrombotic effects. It is suggested that HSA binds nitric oxide (NO) in the circulation forming *S*-nitrosothiols thereby protecting it from inactivation and hence prolonging the active antithrombotic effects of NO (Marelli et al., 1989, Evans, 2002).

Although the net charge of HSA is negative, the protein can bind both cations and anions (Margarson and Soni, 1998, Oettl and Stauber, 2007). In fact, HSA among other proteins is well known for its binding ability of a wide range of molecules (Peters Jr, 1995). HSA binds fatty acids, metal ions such as Zn^{2+} and Cu^{2+}, bilirubin and even therapeutic agents in blood (Peters Jr, 1995). This ability of HSA to bind a wide range of substances of different characteristics and molecular weights has the implication that substances may compete for binding sites on the protein and other substances can allosterically perturb binding of others (Fasano et al., 2005, Lu et al., 2012a).

It has also been suggested, that some pathophysiological conditions that cause hypoxia, acidosis, oxidative stress, disruptions in active transport across the cell membrane, and free iron and copper exposure may cause *in vivo* alterations of HSA-metal binding capacity (BarOr et al., 2001). The effects of disturbances in the binding of particular ligands to HSA by other substances and pathological conditions may result in an abnormal increase in the unbound concentration of other substances. For tightly regulated substances in plasma such as zinc ions, this abnormal increase in plasma concentration may elicit abnormal complex formations and reactions leading to abnormal activation of mechanisms such as coagulation.

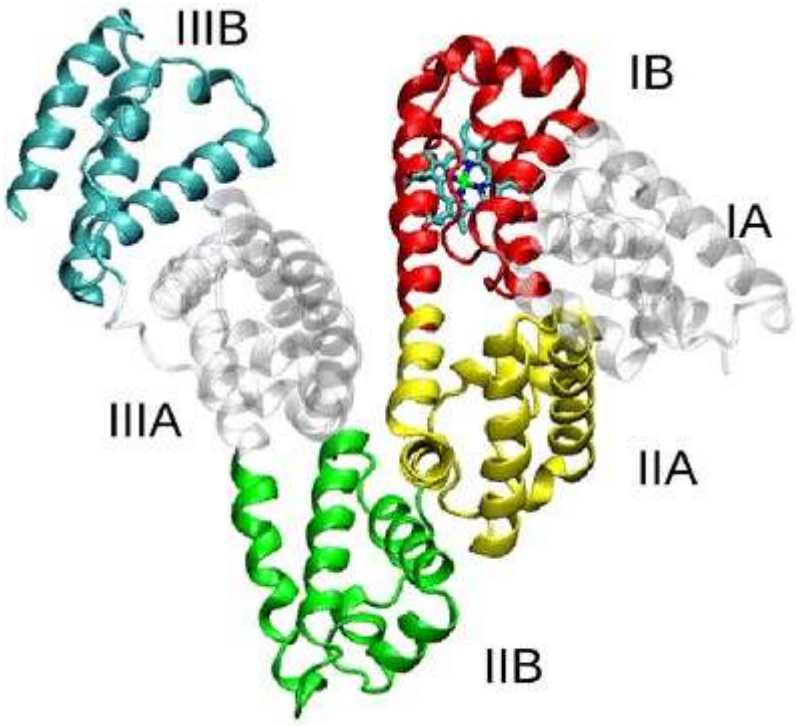


Figure 1.7 The general structure of HSA with the three domains (I – III) and two subunits (A and B) for each domain. Adapted from (Guizado, 2014).

Free fatty acids are transported in plasma bound to HSA. The putatively termed free fatty acids in plasma are not frankly free but are mostly bound to HSA leaving only a small concentration unbound (Apple et al., 2005). Obesity-related conditions such as diabetes, CVD, cancer and other NCDs seem to affect the Co^{2+} binding capacity of HSA (Bhagavan et al., 2003, Kaefer et al., 2010, Gurumurthy et al., 2014, Uslu et al., 2019). Interestingly, these disease conditions are also associated with increased plasma free fatty acid levels (Apple et al., 2005, Amirtharaj et al., 2008). Indeed, in conditions such as fatty liver disease, significant increases in saturated fatty acids (oleic, myristic and lauric acids) were found to be bound to HSA (Amirtharaj et al., 2008). It has hence been suggested that it is actually the increased plasma free fatty acids that influence the poor metal binding capacity of the HSA in cardiovascular-related disease conditions, hence a molecular mechanism that can explain some diagnostic assays such as the albumin cobalt-binding assay (ACB) (Lu et al., 2012b, Coverdale et al., 2018).

Seven fatty acid binding sites (FA1 – FA7) have been described in a high-resolution crystal structure of HSA (Bhattacharya et al., 2000, Simard et al., 2006). Figure 1.8 shows the albumin structure and the location of the seven fatty acid binding sites FA1 – FA7. Interestingly, one of the major fatty acid binding sites, FA2 is located between domains IA and IIA which is in proximity with the multi-metal binding site (site A), which happens to be the main binding site for zinc (Figure 1.8) (Fanali et al., 2012). Such a relationship between the two sites may lead to conformational changes that would affect the site A, especially in conditions where FFAs present at higher concentrations (as will be described in Chapter 2), and this may form a basis for some pathological conditions which may be perpetuated by abnormal handling of metal ions such as zinc (Lu et al., 2012a). Since FFA may perturb the binding and buffering of some metal ions and possibly potentiate pathological mechanisms, it is essential to review the metal-binding features of HSA.

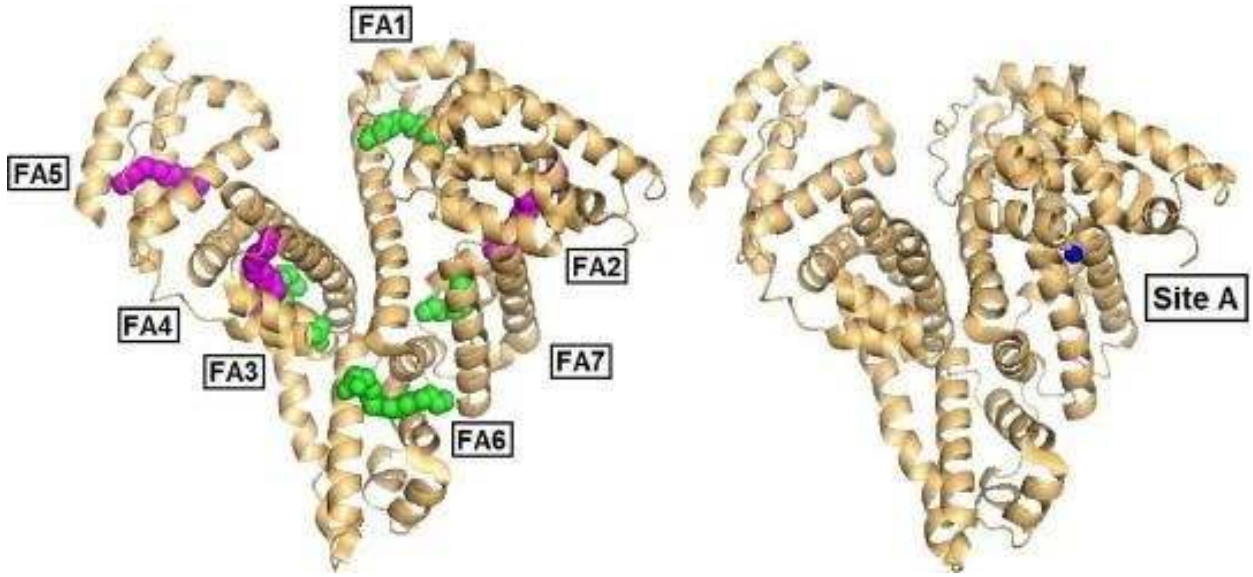


Figure 1.8 Structure of human albumin complexed with hexadecanoic (palmitic) acid showing the locations of fatty acid (FFA) binding sites FA1-7 (PDB: 1E7H). *Left*, In Magenta are FFAs bound to the high-affinity sites while the green colour represents FFAs bound at low-affinity binding sites. *Right*, the multi-metal binding site (Site A) (PDB: 5IJF) occupied by Zn^{2+} (blue) is shown on the right. FA2 is located near site A situated between subdomains IA-IIA. The inter-domain nature and the proximity of FA2 to site A allow for the allosteric switching of metal ion binding. Adapted from (Coverdale et al., 2018).

1.4.1 HSA and metal ion binding

Table 1 shows a list of transitional metals that can bind to HSA in circulation. Some metal binding sites have been described on HSA which are; site A, site B, the N-terminal site (NTS) and Cys34 (Figure 1.9). Using xray crystalography structure analysis techniques, equilibrium dialysis experiments and recently, isothermal titration calorimetry (ITC), the binding sites and affinity characteristics of the sites have been studied (Guthans and Morgan, 1982, Goumakos et al., 1991, Zhou et al., 1994, Masuoka and Saltman, 1994, Bou-Abdallah and Terpstra, 2012). For HSA, on site A the following ions can bind in this order from the strongest to the weakest affinity: VO^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+} while for Site B, it is Cd^{2+} first, then Zn^{2+}, and Co^{2+} (Bal et al., 2013). For the NTS, Cu^{2+} has more affinity, then VO^{2+}, Ni^{2+} and Co^{2+} (Bal et al., 2013). Zn^{2+} has more affinity therefore for site B and then site A, while the opposite holds for $Co^{2+.}$ Conditions that may affect sites A and B can affect the binding of such as the binding of Zn^{2+} and Co^{2+}.

Copper in biological systems exists in two main forms: Cu^{+} and Cu^{2+}. Cu^{2+} plays various roles in catalysis and in the transfer of electrons such as in the cytochrome oxidase involved in the respiratory chain (Rubino and Franz, 2012). Most of the copper in circulation is bound to ceruloplasmin, and only about 15% of the copper in the form of Cu^{2+} is bound to albumin which controls the distribution among the internal organs (Bal et al., 2013).

Table 1.1 A list of transition elements which interact with HSA and the stable physiological oxidation states in circulation. Adapted from (Bal et al., 2013)

Element			Oxidation state	Donor atom preferences	Kinetic properties
	Essential (e)/ toxic (t)/ medicinal (m) For human	**With HSA**			
Cu	e	e	Cu^{2+}	N > O	labile
Zn	e	e	Zn2+	S, N, O	Labile
Co	e	t	Co^{2+}	S, N, O	Intermediate
Fe	e	t	Fe^{3+}	O	Labile
Ni	t	t	Ni^{2+}	S, N > O	Labile/inert[1]
Cd	t	t	Cd^{2+}	S > N > O	Labile
Hg	t	t	Hg^{2+}	S	Labile
V	t,m	t,m	VO^{2+}	O > N	Labile
Au	t,m	t,m	Au^{+}	S, P	Intermediate
Pt	t,m	t,m	Pt^{2+}	N, S	Very inert

[1] Depending on the complex structure.

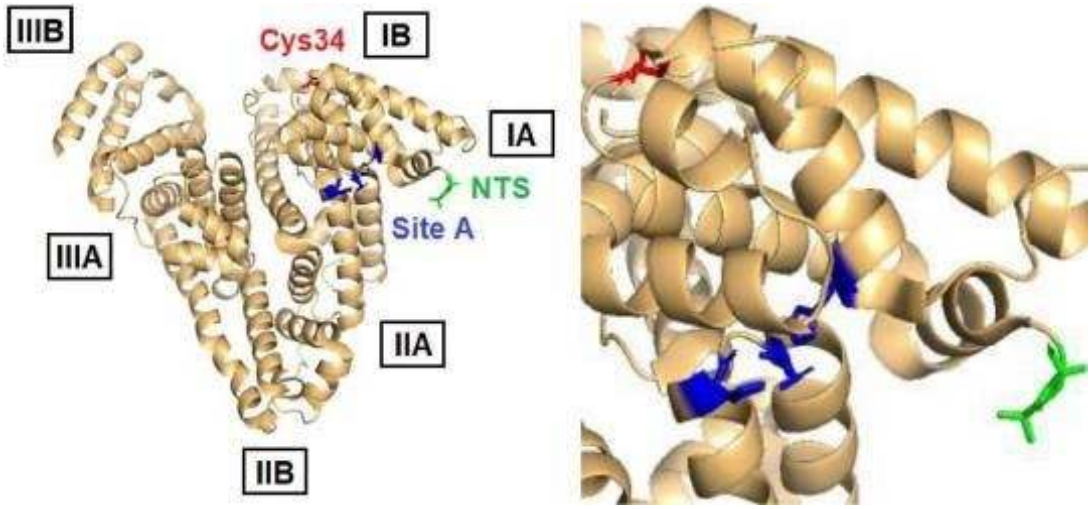


Figure 1.9 The metal binding sites on HAS and their locations. PDB: 5IJF. Site A (blue); NTS/ATCUN motif (green); Cys34 (red). The exact location for site B has not been yet delineated. The boxed labels indicate the six sub-domains of albumin. Labels in the box represent the domains and subunits of the HSA structure. Adapted from (Coverdale et al., 2018).

Nickel is a nonessential element in humans and important as a toxic compound which is bound to albumin in plasma in the form of Ni^{2+}(Sunderman, 1983, Patel and John, 1993, Kurowska and Bal, 2010). Cadmium is also a metal that is not essential in human physiology, and in fact, accumulation of the element from food or tobacco with chronic exposure may lead to cancer (Kurowska and Bal, 2010). Nevertheless, because of other favourable properties in addition to being isoelectric to Zn^{2+} (Martins and Drakenberg, 1982), Cd^{2+} is used as a substituent and probe in studies of Zn^{2+} binding by NMR and absorption spectroscopies (Martins & Drakenberg, 1982; Kopera et al., 2004). Other ions which bind to albumin include Hg^{2+} (Stefan Trümpler and Uwe, 2009), VO^{2+} (Rubino and Franz, 2012), Au^{+} (Messori et al., 2011) and Pt^{2+} which can interact with other pharmacological substances in plasma (Benedetti et al., 2011). Other metal ions are not elaboratively discussed here because they were not of research interest to this study.

1.5 Zinc

Zinc is an essential metal in the body and is an abundant transition metal ion, second only to iron (Vu et al., 2013). The total amount of zinc in the human body is about 2-4 g, and about 90% of this zinc is in the muscles and bones while the rest is found in trace amounts throughout the body (Bialek and Zyska, 2014). Zn^{2+} has many known physiological functions in the human body. It is important for essential cellular functions such as protein synthesis, signal transduction and gene transcription as well as immune functioning, antioxidant functions and participates in haemostasis (Gordon et al., 1982, Shankar and Prasad, 1998). Indeed, zinc deficiency has been associated with poor platelet aggregation. The characteristic of Zn^{2+} to participate in several metabolic processes results from its ability to bind and interact with proteins and influence their activities (Vallee and Falchuk, 1993, Maret, 2011). Zn^{2+} is known to act as a catalytic and a structural cofactor in approximately 3,000 human proteins (Maret, 2011, Sanna et al., 2018). It is not surprising, therefore, that the concentration of zinc in circulation is tightly regulated (Tubek et al., 2008).

In plasma, the concentration of Zn^{2+} ranges from 10 to 20 μM and most of it is bound to proteins (Vu et al., 2013). This mechanism ensures that the zinc is not unintentionally bound to the myriad of haemostatic proteins that it modulates. In effect, the concentration of the putative free zinc in plasma is 0.5 to 1μM (Foote and Delves, 1984, Tubek et al., 2008). Nonetheless, the concentration of free zinc in plasma may vary in certain metabolic and pathological conditions. Platelets, for instance, are known to accumulate 60 times more zinc in their cytoplasm and αgranules than plasma (Marx et al., 1993). During the platelet aggregation component of haemostasis, the platelets release the zinc in their immediate external milieu, increasing the free zinc concentration up to 10 μM (Mahdi et al., 2002).

Similarly, in certain conditions such as in the mounting of an immune response, white blood cells may also release zinc in plasma (Whitehouse et al., 1982). Binding of zinc to plasma proteins may also be disturbed in other pathological conditions associated with increased FFAs in blood and reduced plasma pH which may occur in disease conditions such as diabetes, cardiovascular disease, cancer and their related complications (Borza and Morgan, 1998, Allison et al., 2005, Vu et al., 2013). It is plausible, therefore, that the abnormal increase in the plasma zinc concentration predisposes individuals with such disease conditions to abnormal vascular events influenced by Zn^{2+} such as thromboembolism. In a normal physiological state, buffering of Zn^{2+} by the plasma proteins prevents these pathological events.

Even though several plasma proteins bind Zn^{2+}, HSA is the major zinc binding protein, complexing approximately 75% of plasma zinc. HSA acts as a Zn^{2+} reservoir and buffering protein (Foote and Delves, 1984, Vallee and Falchuk, 1993, Stewart et al., 2009). Thus, pathophysiological conditions that affect HSA indirectly affect zinc. Considering that one of the complications of NCDs and CVD are thromboembolic events such as ischaemic stroke, myocardial ischemia/infarction and peripheral venous thrombosis and that zinc has a known role in blood clotting, it is logical to hypothesise that poor handling of zinc in plasma by its major buffering protein HSA may have a role. The known roles of zinc in haemostasis are discussed below. The specific roles involved in platelet aggregation and the possible pathophysiological processes that can precipitate abnormal haemostasis via poor zinc handling will be discussed in chapter 3 of this thesis.

1.5.1 Haemostasis and the role of Zn^{2+}

Physiologically, haemostasis comprises the processes of contraction of the blood vessel at the site of injury, platelet adherence and the blood coagulation cascade (Gabriel, 2002). Zn^{2+} is important in the haemostasis cascade. Zn^{2+} modulates several protein activities in the coagulation, platelet aggregation, anticoagulation and fibrinolytic pathways (Vu et al., 2013). The importance of Zn^{2+} in haemostasis was initially described following abnormalities in blood clotting that was observed in men on a low zinc diet (Gordon et al., 1982). It was later established that poor zinc status from chronic diseases such as cancer might lead to poor handling of calcium in the haemostasis cascade, contributing to conditions such as cutaneous bleeding – a phenomenon that can be reversed by zinc supplementation (Emery and O'Dell, 1993). Figure 1.10 summarises the functions of Zn^{2+} in the regulation of haemostasis and thrombosis. The subsections that follow describe the specific roles of Zn^{2+} in the different components of the cascade.

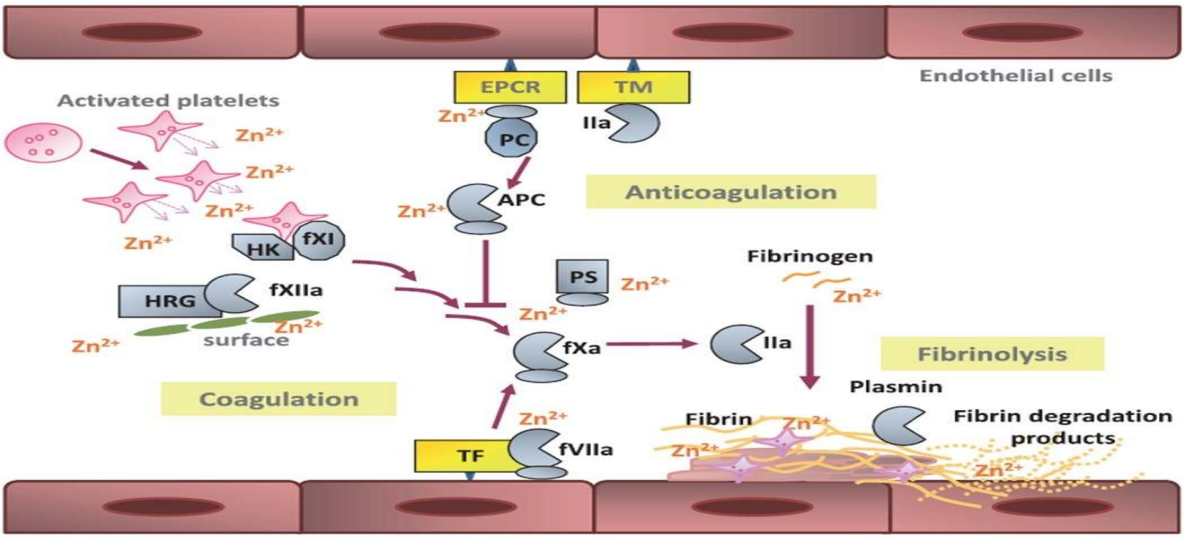


Figure 1.10 Role of the Zn^{2+} as a regulator of haemostasis and thrombosis. Zn^{2+} is secreted by activated platelets during haemostasis. The concentration of zinc in the vicinity of a forming thrombus increases and the Zn^{2+} modulates the activity of several proteins in both the intrinsic and extrinsic pathways of coagulation. APC = activated protein C; EPCR = Epithelial cell protein C receptor; fXa = activated factor X; fXI = factor XI; fXIIa = activated factor XII; fVIIa = activated factor VII; HRG = histidine-rich glycoprotein; HK = high-molecular-weight kininogen; IIa= activated factor II; PC = Protein C; PS = Protein S; TF = tissue factor; Zn^{2+} = Zn^{2+}. From (Vu et al., 2013)

1.5.2 Role of Zn^{2+} in coagulation

The blood coagulation theory, first proposed by Morwitz, involves thromboplastin (which is normally formed in response to blood vessel injury) converting prothrombin to thrombin in plasma, which in turn converts fibrinogen to fibrin - ultimately forming a fibrin clot which arrests bleeding (Gabriel, 2002). Factors in blood and tissues classically numbered from I to XIII participate in a series of reactions leading to the formation of thromboplastin and participate in the coagulation cascade (Gabriel, 2002, Vu et al., 2013). Thromboplastin is a complex comprising of activated factor X (fXa), factor V and platelet factor 3 (PF3) in the presence of calcium (Gabriel, 2002). The coagulation cascade has an intrinsic and an extrinsic pathway, and the former through the contact system is suggested to be very important in thrombus formation (Gailani and RennÉ, 2007, Müller et al., 2011). Zn^{2+} acts as a cofactor in the contact activation system of coagulation (Vu et al., 2013).

The contact system is initiated through the activation of FXII either by its contact with the polyanionic surface of the endothelium (surface-dependent), or by autoactivation once FXII is

activated (Surface-independent), and both processes are enhanced up to ten times by Zn^{2+} binding to FXII (Bernardo et al., 1993, Røjkjær and Schousboe, 1997). Zn^{2+} is suggested to potentiate the activation processes by eliciting conformational changes of FXII thereby priming it for proteolysis (Røjkjær and Schousboe, 1997). Zn^{2+} also enhances FXII activation by potentiation of kallikrein-mediated activation process which ultimately offers another positive feedback loop to the FXII activation (Vu et al., 2013). At the endothelial surface, Zn^{2+} binds to high-molecular-weight kininogen (HK) which facilitates the localisation of contact proteins to the endothelial cell polyanionic surfaces and hence promoting contact-mediated activation of coagulation (Thompson et al., 1977, Schmaier et al., 1988). Zn^{2+} is further known to modulate the binding of FXI to platelets in the propagation of coagulation either in the presence of HK or alone (Baglia et al., 2002, Baglia et al., 2004).

Zn^{2+} is also known to influence coagulation through potentiation of human histidine-rich glycoprotein (HRG), a single chain polypeptide protein with a molecular weight of approximately 75 kDa (Ranieri-Raggi et al., 2014, Allison et al., 2005). The protein was first characterised and isolated from human plasma in 1972 (Haupt and Heimburger, 1972, Heimburger et al., 1972). The plasma concentration of human HRG ranges between 100 and 150 ng/ml (1.3-2 μM) (Morgan, 1978, Corrigan et al., 1990). The multi-domain structure of HRG allows it to interact with several ligands including heparin, plasminogen, fibrinogen, phospholipids, immunoglobulins, haem and importantly Zn^{2+} (Poon et al., 2011). Due to this characteristic, HRG acts as an adapter protein that regulates various vital processes in the body such as coagulation, fibrinolysis, immune functions, angiogenesis and cell adhesion (Poon et al., 2011).

The coagulation function of HRG is in part due to its interaction with heparin. In both *in-vitro* and *in vivo* studies, potentiated by Zn^{2+}, HRG has been shown to strongly interact with heparin where heparin is neutralised, and its anticoagulant effect inhibited (Lijnen et al., 1983). From its primary structure, HRG has two cystanin-like domains at the N-terminus, a C-terminal and a histidine-rich region (HRR) (Kassaar et al., 2014, Koide et al., 1986). Zn^{2+} interacts with HRG at the HRR domain where it contains repeating GHHPH motifs (Allison et al., 2005, Barnett et al., 2013). The association of Zn^{2+} and HRG modifies the binding properties of HRG, increasing its affinity for molecules such as heparin and heparan sulphate (Borza and Morgan, 1998). Through this potentiation, HRG neutralises the anticoagulants and also inhibits the activity of antithrombin III - hence offering a prothrombotic effect (Mori et al., 2003, Patel et al., 2013). Of interest also is that it has been shown using enzyme-linked immunosorbent assay (ELISA)-based

studies that Zn^{2+} increases the affinity of HRG for unfractionated (3-30 kDa) heparins in a dose-dependent manner, but this effect is not evident with low molecular weight heparins (6850 Da) (Kassaar et al., 2015).

1.6 Research hypothesis and objectives

It has been shown, thus, that NCDs are a significant global challenge. HIV infection has complicated and worsened the burden of NCDs, mostly in LMIC. Both NCDs and HIV may be associated with higher plasma FFA concentrations, which may exert poor buffering of metal ions such as Zn^{2+} in circulation by HSA and lead to consequences such as abnormal blood clotting and thromboembolic events. Currently, there is paucity on clinical data coupled with relevant biochemical studies on elevated plasma FFA concentrations and how this influences the interaction between albumin and metal ions in circulation. Furthermore, the effects of poor Zn^{2+} handling on platelet aggregation in the presence of higher FFA requires further research attention. In addition to this, the FFA concentration, albumin levels and coagulability of HIV-infected individuals in comparison to healthy controls has not been well-evaluated in rural Malawi.

This PhD study hypothesizes that increased concentrations of FFA in plasma as may be observed in certain disease conditions negatively influences albumin-metal interactions, in particular, Zn^{2+} and Co^{2+}. Poor Zn^{2+} buffering by HSA due to higher FFA concentration may lead to abnormal blood clotting and hence potentiate cardiovascular and cerebrovascular disease. This PhD study was aimed at investigating the influence of plasma free fatty acids on serum albumin-metal interactions and blood clotting.

The specific objectives were to:

1. Investigate the influence of free fatty acids of different chain lengths on the binding of zinc to HSA using the ACB assay.
2. Optimise a platelet aggregation assay using a 96-well-plate and examine the influence of Zn^{2+} and FFA on platelet aggregation using platelet aggregation assays.
3. Examine different albumin crystal structures and how binding of different ligands affects the multi-metal binding site (Zn^{2+} -binding site).
4. Investigate the plasma free fatty acid concentration and blood coagulability of HIV-infected individuals at Neno district hospital in Malawi and compare with HIV-negative controls.

REFERENCES FOR CHAPTER 1

1. ALLISON, L. J., MARK, D. H. & PARISH, C. R. C. P. A. E. A. 2005. Histidine-rich glycoprotein: A novel adaptor protein in plasma that modulates the immune, vascular and coagulation systems. *Immunology & Cell Biology,* 83**,** 106.

2. AMIRTHARAJ, G. J., NATARAJAN, S. K., MUKHOPADHYA, A., ZACHARIAH, U. G., HEGDE, S. K., KURIAN, G., BALASUBRAMANIAN, K. A. & RAMACHANDRAN, A. 2008. Fatty acids influence binding of cobalt to serum albumin in patients with fatty liver. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease,* 1782**,** 349.

3. APPLE, F. S., WU, A. H. B., MAIR, J., RAVKILDE, J., PANTEGHINI, M., TATE, J., PAGANI, F., CHRISTENSON, R. H., MOCKEL, M., DANNE, O. & JAFFE, A. S. 2005. Future biomarkers for detection of ischemia and risk stratification in acute coronary syndrome. *Clinical chemistry,* 51**,** 810.

4. ARNER, P. & RYDEN, M. 2015. Fatty Acids, Obesity and Insulin Resistance. *Obesity facts,* 8**,** 147.

5. BAENA RUIZ, R. & SALINAS HERNANDEZ, P. 2014. Diet and cancer: risk factors and epidemiological evidence. *Maturitas,* 77**,** 202.

6. BAGLIA, F. A., BADELLINO, K. O., LI, C. Q., LOPEZ, J. A. & WALSH, P. N. 2002. Factor XI binding to the platelet glycoprotein Ib-IX-V complex promotes factor XI activation by thrombin. *The Journal Of Biological Chemistry,* 277**,** 1662.

7. BAGLIA, F. A., SHRIMPTON, C. N., EMSLEY, J., KITAGAWA, K., RUGGERI, Z. M., LOPEZ, J. A. & WALSH, P. N. 2004. Factor XI interacts with the leucine-rich repeats of glycoprotein Ibalpha on the activated platelet. *The Journal of biological chemistry,* 279**,** 49323.

8. BAL, W., SOKOLOWSKA, M., KUROWSKA, E. & FALLER, P. 2013. Binding of transition metal ions to albumin: sites, affinities and rates. *Biochimica et biophysica acta,* 1830**,** 5444.

9. BAR-OR, D., WINKLER, J. V., VANBENTHUYSEN, K., HARRIS, L., LAU, E. & HETZEL, F. W. 2001. Reduced albumin-cobalt binding with transient myocardial ischemia after elective percutaneous transluminal coronary angioplasty: A preliminary comparison to creatine kinase-MB, myoglobin, and troponin I. *American Heart Journal,* 141**,** 985.

10. BARNETT, J. P., BLINDAUER, C. A., KASSAAR, O., KHAZAIPOUL, S., MARTIN, E. M., SADLER, P. J. & STEWART, A. J. 2013. Allosteric modulation of zinc speciation by fatty acids. *Biochimica et biophysica acta,* 1830**,** 5456.

11. BAROUCH, L. A., BERKOWITZ, D. E., HARRISON, R. W., O'DONNELL, C. P. & HARE, J. M. 2003. Disruption of leptin signaling contributes to cardiac hypertrophy independently of body weight in mice. *Circulation,* 108**,** 754.

12. BARRERA, G. 2012. Oxidative stress and lipid peroxidation products in cancer progression and therapy. *ISRN oncology,* 2012**,** 137289.

13. BAYS, H., BLONDE, L. & ROSENSON, R. 2006. Adiposopathy: how do diet,

exercise and weight loss drug therapies improve metabolic disease in overweight patients? *Expert review of cardiovascular therapy,* 4**,** 871.

14. BENEDETTI, B. T., PETERSON, E. J., KABOLIZADEH, P., MARTINEZ, A., KIPPING, R. & FARRELL, N. P. 2011. Effects of noncovalent platinum drug-protein interactions on drug efficacy: use of fluorescent conjugates as probes for drug metabolism. *Molecular pharmaceutics,* 8**,** 940.

15. BENETOS, A., OKUDA, K., LAJEMI, M., KIMURA, M., THOMAS, F.,

SKURNICK, J., LABAT, C., BEAN, K. & AVIV, A. 2001. Telomere length as an indicator of biological aging: the gender effect and relation with pulse pressure and pulse wave velocity. *Hypertension (Dallas, Tex.: 1979),* 37**,** 381.

16. BERGER, M. M. 2005. Can oxidative damage be treated nutritionally? *Clinical*

Nutrition, 24**,** 172.

17. BERNARDO, M. M., DAY, D. E., OLSON, S. T. & SHORE, J. D. 1993. Surface-

independent acceleration of factor XII activation by zinc ions. I. Kinetic characterization of the metal ion rate enhancement. *The Journal Of Biological Chemistry,* 268**,** 12468.

18. BERRY, C. E. & WISE, R. A. 2010. Mortality in COPD: causes, risk factors, and prevention. *Copd,* 7**,** 375.

19. BHAGAVAN, N. V., LAI, E. M., RIOS, P. A., YANG, J., ORTEGA-LOPEZ, A. M., SHINODA, H., HONDA, S. A., RIOS, C. N., SUGIYAMA, C. E. & HA, C. E. 2003. Evaluation of human serum albumin cobalt binding assay for the assessment of myocardial ischemia and myocardial infarction. *Clinical chemistry,* 49**,** 581.

20. BHATTACHARYA, A. A., GRÜNE, T. & CURRY, S. 2000. Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin. *Journal of Molecular Biology,* 303**,** 721.

21. BIALEK, M. & ZYSKA, A. 2014. The Biomedical Role of Zinc in the Functioning of the Human Organism. *Polish Journal of Public Health,* 124**,** 160.

22. BLINDAUER, C. A., KHAZAIPOUL, S., YU, R. & STEWART, A. J. 2016. Fatty Acid-Mediated Inhibition of Metal Binding to the Multi-Metal Site on Serum Albumin: Implications for Cardiovascular Disease. *Current topics in medicinal chemistry,* 16**,** 3021.

23. BODEN, G. 2008. Obesity and free fatty acids. *Endocrinology & Metabolism Clinics of North America,* 37**,** 635.

24. BORZA, D. B. & MORGAN, W. T. 1998. Histidine-proline-rich glycoprotein as a plasma pH sensor. Modulation of its interaction with glycosaminoglycans by ph and metals. *The Journal Of Biological Chemistry,* 273**,** 5493.

25. BOU-ABDALLAH, F. & TERPSTRA, T. R. 2012. The thermodynamic and binding properties of the transferrins as studied by isothermal titration calorimetry. *Biochimica et biophysica acta,* 1820**,** 318.

26. BRAY, G. A. 2004. Medical consequences of obesity. *The Journal of clinical endocrinology and metabolism,* 89**,** 2583.

27. CERIELLO, A., ESPOSITO, K., IHNAT, M., THORPE, J. & GIUGLIANO, D. 2010. Effect of acute hyperglycaemia, long-term glycaemic control and insulin on endothelial dysfunction and inflammation in Type 1 diabetic patients with different characteristics.

Diabetic Medicine: A Journal Of The British Diabetic Association, 27**,** 911.

28. CERIELLO, A., GIUGLIANO, D., QUATRARO, A., MARCHI, E., BARBANTI, M.

& LEFEBVRE, P. 1990. Evidence for a hyperglycaemia-dependent decrease of antithrombin III-thrombin complex formation in humans. *Diabetologia,* 33**,** 163.

29. CHEN, R., LIN, L., TIAN, J. W., ZENG, B., ZHANG, L., CHEN, X. & YAN, H. Y. 2015. Predictors of dynamic hyperinflation during the 6-minute walk test in stable chronic obstructive pulmonary disease patients. *Journal of thoracic disease,* 7**,** 1142.

30. CORRIGAN, J. J. J., JETER, M. A., BRUCK, D. & FEINBERG, W. M. 1990.

Histidine-rich glycoprotein levels in children: the effect of age. *Thrombosis research,* 59**,** 681.

31. COUSSENS, L. M. & WERB, Z. 2002. Inflammation and cancer. *Nature,* 420**,** 860.

32. COVERDALE, J. P. C., KATUNDU, K. G. H., SOBCZAK, A. I. S., ARYA, S., BLINDAUER, C. A. & STEWART, A. J. 2018. Ischemia-modified albumin: Crosstalk

between fatty acid and cobalt binding. *Prostaglandins, leukotrienes, and essential fatty acids,* 135**,** 147.

33. CURRY, S., MANDELKOW, H., BRICK, P. & FRANKS, N. 1998. Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. *Nat Struct Biol,* 5**,** 827-35.

34. DECRAMER, M. & JANSSENS, W. 2013. Chronic obstructive pulmonary disease and comorbidities. *The Lancet.Respiratory medicine,* 1**,** 73.

35. DESPRES, J. P. 2006. Intra-abdominal obesity: an untreated risk factor for Type 2 diabetes and cardiovascular disease. *Journal of endocrinological investigation,* 29**,** 77.

36. DUNCAN, B. B., SCHMIDT, M. I., PANKOW, J. S., BALLANTYNE, C. M., COUPER, D., VIGO, A., HOOGEVEEN, R., FOLSOM, A. R., HEISS, G. & ATHEROSCLEROSIS RISK IN COMMUNITIES, S. 2003. Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study. *Diabetes,* 52**,** 1799.

37. EATON, R. P., BERMAN, M. & STEINBERG, D. 1969. Kinetic studies of plasma free fatty acid and triglyceride metabolism in man. *The Journal of clinical investigation,* 48**,** 1560.

38. EMERY, M. P. & O'DELL, B. L. 1993. Low zinc status in rats impairs calcium uptake and aggregation of platelets stimulated by fluoride. *Proceedings Of The Society For*

Experimental Biology And Medicine.Society For Experimental Biology And Medicine (New York, N.Y.), 203**,** 480.

39. ENGLISH, D. R., MACINNIS, R. J., HODGE, A. M., HOPPER, J. L., HAYDON, A. M. & GILES, G. G. 2004. Red meat, chicken, and fish consumption and risk of colorectal cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* 13**,** 1509.

40. EVANS, T. W. 2002. Review article: albumin as a drug--biological effects of albumin unrelated to oncotic pressure. *Alimentary Pharmacology & Therapeutics,* 16 Suppl 5**,** 6.

41. FABBRI, L. M. 2016. Smoking, Not COPD, as the Disease. *The New England journal of medicine,* 374**,** 1885.

42. FABBRINI, E., YOSHINO, J., YOSHINO, M., MAGKOS, F., TIEMANN

LUECKING, C., SAMOVSKI, D., FRATERRIGO, G., OKUNADE, A. L., PATTERSON, B. W. & KLEIN, S. 2015. Metabolically normal obese people are protected from adverse effects following weight gain. *The Journal of clinical investigation,* 125**,** 787.

43. FANALI, G., DI MASI, A., TREZZA, V., MARINO, M., FASANO, M. & ASCENZI,

P. 2012. Human serum albumin: From bench to bedside. *Molecular aspects of medicine,* 33**,** 209.

44. FASANO, M., CURRY, S., TERRENO, E., GALLIANO, M., FANALI, G.,

NARCISO, P., NOTARI, S. & ASCENZI, P. 2005. The extraordinary ligand binding properties of human serum albumin. *IUBMB life,* 57**,** 787.

45. FELMEDEN, D. C., SPENCER, C. G., BELGORE, F. M., BLANN, A. D., BEEVERS, D. G. & LIP, G. Y. 2003. Endothelial damage and angiogenesis in hypertensive patients: relationship to cardiovascular risk factors and risk factor management. *American journal of hypertension,* 16**,** 11.

46. FERRARA, N. 2002. VEGF and the quest for tumour angiogenesis factors. *Nature reviews.Cancer,* 2**,** 795.

47. FIELDING, B. A., CALLOW, J., OWEN, R. M., SAMRA, J. S., MATTHEWS, D. R. & FRAYN, K. N. 1996. Postprandial lipemia: the origin of an early peak studied by specific dietary fatty acid intake during sequential meals. *The American Journal of Clinical Nutrition,* 63**,** 36.

48. FIGGE, J., ROSSING, T. H. & FENCL, V. 1991. The role of serum proteins in acidbase equilibria. *The Journal of laboratory and clinical medicine,* 117**,** 453.

49. FOOTE, J. W. & DELVES, H. T. 1984. Albumin bound and alpha 2-macroglobulin

bound zinc concentrations in the sera of healthy adults. *Journal of clinical pathology,* 37**,** 1050. 50. GABRIEL, C. E. 2002. Haemostasis. *In:* OXFORD UNIVERSITY, P. (ed.) *Text Book of Physiology.* 1 ed.: Oxford University Press.

51. GAGGINI, M., MORELLI, M., BUZZIGOLI, E., DEFRONZO, R. A., BUGIANESI, E. & GASTALDELLI, A. 2013. Non-alcoholic fatty liver disease (NAFLD) and its connection with insulin resistance, dyslipidemia, atherosclerosis and coronary heart disease. *Nutrients,* 5**,** 1544.

52. GAILANI, D. & RENNÉ, T. 2007. The intrinsic pathway of coagulation: a target for treating thromboembolic disease? *Journal of Thrombosis & Haemostasis,* 5**,** 1106.

53. GALDERISI, M., ANDERSON, K. M., WILSON, P. W. & LEVY, D. 1991.

Echocardiographic evidence for the existence of a distinct diabetic cardiomyopathy (the Framingham Heart Study). *The American Journal of Cardiology,* 68**,** 85.

54. GASTALDELLI, A. 2011. Role of beta-cell dysfunction, ectopic fat accumulation and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *Diabetes research and clinical practice,* 93 Suppl 1**,** S60.

55. GASTALDELLI, A. 2014. Visceral adipose tissue and ectopic fat deposition. *In:* BRAY, G. A. B. C. E. (ed.) *Handbook of Obesity.* 1 ed. Boca Raton, FL: CRC Press:.

56. GEOVANINI, G. R. & LIBBY, P. 2018. Atherosclerosis and inflammation: overview and updates. *Clinical science (London, England : 1979),* 132**,** 1243.

57. GIOVANNUCCI, E., HARLAN, D. M., ARCHER, M. C., BERGENSTAL, R. M., GAPSTUR, S. M., HABEL, L. A., POLLAK, M., REGENSTEINER, J. G. & YEE, D. 2010.

Diabetes and cancer: a consensus report. *Diabetes care,* 33**,** 1674.

58. GORDON, P. R., WOODRUFF, C. W., ANDERSON, H. L. & O'DELL, B. L. 1982.

Effect of acute zinc deprivation on plasma zinc and platelet aggregation in adult males. *The American Journal of Clinical Nutrition,* 35**,** 113.

59. GOUMAKOS, W., LAUSSAC, J. P. & SARKAR, B. 1991. Binding of cadmium(II) and zinc(II) to human and dog serum albumins. An equilibrium dialysis and 113Cd-NMR study. *Biochemistry and cell biology = Biochimie et biologie cellulaire,* 69**,** 809.

60. GOWSHALL, M. & TAYLOR-ROBINSON, S. D. 2018. The increasing prevalence of non-communicable diseases in low-middle income countries: the view from Malawi. *International journal of general medicine,* 11**,** 255.

61. GROOP, L. C., BONADONNA, R. C., DELPRATO, S., RATHEISER, K., ZYCK, K., FERRANNINI, E. & DEFRONZO, R. A. 1989. Glucose and free fatty acid metabolism in noninsulin-dependent diabetes mellitus. Evidence for multiple sites of insulin resistance. *The Journal of clinical investigation,* 84**,** 205.

62. GUIZADO, T. R. 2014. Analysis of the structure and dynamics of human serum albumin. *Journal of molecular modeling,* 20**,** 2450.

63. GUO, Y., XU, F., LU, T., DUAN, Z. & ZHANG, Z. 2012. Interleukin-6 signaling pathway in targeted therapy for cancer. *Cancer treatment reviews,* 38**,** 904.

64. GURUMURTHY, P., BORRA, S. K., YERUVA, R. K., VICTOR, D., BABU, S. & CHERIAN, K. M. 2014. Estimation of Ischemia Modified Albumin (IMA) Levels in Patients with Acute Coronary Syndrome. *Indian journal of clinical biochemistry : IJCB,* 29**,** 367.

65. GUTHANS, S. L. & MORGAN, W. T. 1982. The interaction of zinc, nickel and cadmium with serum albumin and histidine-rich glycoprotein assessed by equilibrium dialysis and immunoadsorbent chromatography. *Archives of Biochemistry and Biophysics,* 218**,** 320.

66. HAUPT, H. & HEIMBURGER, N. 1972. Human serum proteins with high affinity for carboxymethylcellulose. I. Isolation of lysozyme, C1q and 2 hitherto unknown -globulins. *Hoppe-Seyler's Zeitschrift Für Physiologische Chemie,* 353**,** 1125.

67. HE, X. M. & CARTER, D. C. 1992. Atomic structure and chemistry of human serum albumin. *Nature,* 358**,** 209-15.

68. HEIMBERG, M., DUNN, G. D. & WILCOX, G. 1974. The derivation of plasma-free fatty acids from dietary neutral fat in man. *The Journal of laboratory and clinical medicine,* 83**,** 393.

69. HEIMBURGER, N., HAUPT, H., KRANZ, T. & BAUDNER, S. 1972. Human serum

proteins with high affinity to carboxymethylcellulose. II. Physico-chemical and immunological characterization of a histidine-rich 3,8S- 2 -glycoportein (CM-protein I). *Hoppe-Seyler's Zeitschrift Für Physiologische Chemie,* 353**,** 1133.

70. HEINDL, S., LEHNERT, M., CRIEE, C. P., HASENFUSS, G. & ANDREAS, S. 2001. Marked sympathetic activation in patients with chronic respiratory failure. *American journal of respiratory and critical care medicine,* 164**,** 597.

71. HOU, N. & LUO, J. D. 2011. Leptin and cardiovascular diseases. *Clinical and experimental pharmacology & physiology,* 38**,** 905.

72. HUBER, A. H., KAMPF, J. P., KWAN, T., ZHU, B., ADAMS, J., 3RD &

KLEINFELD, A. M. 2014. Usefulness of serum unbound free fatty acid levels to predict death early in patients with ST-segment elevation myocardial infarction (from the Thrombolysis In Myocardial Infarction TIMI II trial). *The American Journal of Cardiology,* 113**,** 279.

73. INOGUCHI, T., LI, P., UMEDA, F., YU, H. Y., KAKIMOTO, M., IMAMURA, M., AOKI, T., ETOH, T., HASHIMOTO, T., NARUSE, M., SANO, H., UTSUMI, H. &

NAWATA, H. 2000. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes,* 49**,** 1939.

74. KAEFER, M., PIVA, S. J., DE CARVALHO, J. A. M., DA SILVA, D. B., BECKER, A. M., COELHO, A. C., DUARTE, M. M. M. F. & MORESCO, R. N. 2010. Association between ischemia modified albumin, inflammation and hyperglycemia in type 2 diabetes mellitus. *Clinical biochemistry,* 43**,** 450.

75. KAMP, D. W., SHACTER, E. & WEITZMAN, S. A. 2011. Chronic inflammation and

cancer: the role of the mitochondria. *Oncology (Williston Park, N.Y.),* 25**,** 400.

76. KASSAAR, O., MCMAHON, S. A., THOMPSON, R., BOTTING, C. H., NAISMITH, J. H. & STEWART, A. J. 2014. Crystal structure of histidine-rich glycoprotein N2 domain reveals redox activity at an interdomain disulfide bridge: implications for angiogenic regulation. *Blood,* 123**,** 1948.

77. KASSAAR, O., SCHWARZ-LINEK, U., BLINDAUER, C. A. & STEWART, A. J.

2015. Plasma free fatty acid levels influence Zn2+-dependent histidine-rich glycoproteinheparin interactions via an allosteric switch on serum albumin. *Journal of Thrombosis & Haemostasis,* 13**,** 101.

78. KAWAKAMI, A., KUBOTA, K., YAMADA, N., TAGAMI, U., TAKEHANA, K., SONAKA, I., SUZUKI, E. & HIRAYAMA, K. 2006. Identification and characterization of

oxidized human serum albumin. A slight structural change impairs its ligand-binding and antioxidant functions. *The FEBS Journal,* 273**,** 3346.

79. KIM, M., OH, J. K., SAKATA, S., LIANG, I., PARK, W., HAJJAR, R. J. & LEBECHE, D. 2008. Role of resistin in cardiac contractility and hypertrophy. *Journal of Molecular and Cellular Cardiology,* 45**,** 270.

80. KIM, S. S., ROGERS, B. L., COATES, J., GILLIGAN, D. O. & SARRIOT, E. 2013. Building evidence for sustainability of food and nutrition intervention programs in developing countries. *Advances In Nutrition (Bethesda, Md.),* 4**,** 524.

81. KING, M., SMITH, A. & GRACEY, M. 2009. Indigenous health part 2: the underlying causes of the health gap. *Lancet (London, England),* 374**,** 76.

82. KOENE, R. J., PRIZMENT, A. E., BLAES, A. & KONETY, S. H. 2016. Shared Risk Factors in Cardiovascular Disease and Cancer. *Circulation,* 133**,** 1104.

83. KOIDE, T., FOSTER, D., YOSHITAKE, S. & DAVIE, E. W. 1986. Amino acid

sequence of human histidine-rich glycoprotein derived from the nucleotide sequence of its cDNA. *Biochemistry,* 25**,** 2220.

84. KOVACS, P. & STUMVOLL, M. 2005. Fatty acids and insulin resistance in muscle and liver. *Best practice & research.Clinical endocrinology & metabolism,* 19**,** 625.

85. KUROWSKA, E. & BAL, W. 2010. Chapter 3 - Recent Advances in Molecular Toxicology of Cadmium and Nickel. *Advances in Molecular Toxicology,* 4**,** 85.

86. LAHOUSSE, L., VERHAMME, K. M., STRICKER, B. H. & BRUSSELLE, G. G.

2016. Cardiac effects of current treatments of chronic obstructive pulmonary disease. *The Lancet.Respiratory medicine,* 4**,** 149.

87. LEAHY, J. L. 2005. Pathogenesis of type 2 diabetes mellitus. *Archives of Medical Research,* 36**,** 197.

88. LEIKAUF, G. D., BORCHERS, M. T., PROWS, D. R. & SIMPSON, L. G. 2002.

Mucin apoprotein expression in COPD. *Chest,* 121**,** 166S.

89. LEON, B. M. & MADDOX, T. M. 2015. Diabetes and cardiovascular disease: Epidemiology, biological mechanisms, treatment recommendations and future research. *World journal of diabetes,* 6**,** 1246.

90. LIBBY, P. 2006. Inflammation and cardiovascular disease mechanisms. *The American Journal of Clinical Nutrition,* 83**,** 456S.

91. LIJNEN, H. R., HOYLAERTS, M. & COLLEN, D. 1983. Heparin binding properties of human histidine-rich glycoprotein. Mechanism and role in the neutralization of heparin in plasma. *The Journal of Biological Chemistry,* 258**,** 3803. LOZANO, R., NAGHAVI, M., FOREMAN, K., LIM, S., SHIBUYA, K., ABOYANS, V., ABRAHAM, J., ADAIR, T., AGGARWAL, R., AHN, S. Y., ALVARADO, M., ANDERSON, H. R., ANDERSON, L. M., ANDREWS, K. G., ATKINSON, C., BADDOUR, L. M., BARKER-COLLO, S., BARTELS, D. H., BELL, M. L., BENJAMIN, E. J., BENNETT, D., BHALLA, K., BIKBOV, B., BIN ABDULHAK, A., BIRBECK, G., BLYTH, F., BOLLIGER, I., BOUFOUS, S., BUCELLO, C., BURCH, M., BURNEY, P., CARAPETIS, J., CHEN, H., CHOU, D., CHUGH, S. S., COFFENG, L. E., COLAN, S. D., COLQUHOUN, S., COLSON, K. E., CONDON, J., CONNOR, M. D., COOPER, L. T., CORRIERE, M., CORTINOVIS, M., DE VACCARO, K. C., COUSER, W., COWIE, B. C., CRIQUI, M. H., CROSS, M., DABHADKAR, K. C., DAHODWALA, N., DE LEO, D., DEGENHARDT, L., DELOSSANTOS, A., DENENBERG, J., DES JARLAIS, D. C., DHARMARATNE, S. D., DORSEY, E. R., DRISCOLL, T., DUBER, H., EBEL, B., ERWIN, P. J., ESPINDOLA, P., EZZATI, M., FEIGIN, V., FLAXMAN, A. D., FOROUZANFAR, M. H., FOWKES, F. G., FRANKLIN, R., FRANSEN, M., FREEMAN, M. K., GABRIEL, S. E., GAKIDOU, E., GASPARI, F., GILLUM, R. F., GONZALEZ-MEDINA, D., HALASA, Y. A., HARING, D., HARRISON, J. E., HAVMOELLER, R., HAY, R. J., HOEN, B., HOTEZ, P. J., HOY, D., JACOBSEN, K. H., JAMES, S. L., JASRASARIA, R., JAYARAMAN, S., JOHNS, N., KARTHIKEYAN, G., KASSEBAUM, N., KEREN, A., KHOO, J. P., KNOWLTON, L. M., KOBUSINGYE, O., KORANTENG, A., KRISHNAMURTHI, R., LIPNICK, M., LIPSHULTZ, S. E., OHNO, S. L., et al. 2012. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet (London, England),* 380**,** 2095.

93. LU, J., STEWART, A. J., SADLER, P. J., PINHEIRO, T. J. T. & BLINDAUER, C. A. 2012a. Allosteric inhibition of cobalt binding to albumin by fatty acids: implications for the detection of myocardial ischemia. *Journal of medicinal chemistry,* 55**,** 4425.

94. LU, J., STEWART, A. J., SLEEP, D., SADLER, P. J., PINHEIRO, T. J. & BLINDAUER, C. A. 2012b. A molecular mechanism for modulating plasma Zn speciation by fatty acids. *Journal of the American Chemical Society,* 134**,** 1454.

95. MAHDI, F., MADAR, Z. S., FIGUEROA, C. D. & SCHMAIER, A. H. 2002. Factor XII interacts with the multiprotein assembly of urokinase plasminogen activator receptor, gC1qR, and cytokeratin 1 on endothelial cell membranes. *Blood,* 99**,** 3585.

96. MARCHETTI, N., CICCOLELLA, D. E., JACOBS, M. R., CROOKSHANK, A., GAUGHAN, J. P., KASHEM, M. A., BOVE, A. A. & CRINER, G. J. 2011. Hospitalized acute exacerbation of COPD impairs flow and nitroglycerin-mediated peripheral vascular dilation. *Copd,* 8**,** 60.

97. MARELLI, D., PAUL, A., SAMSON, R., EDGELL, D., ANGOOD, P. & CHIU, R. C. 1989. Does the addition of albumin to the prime solution in cardiopulmonary bypass affect clinical outcome? A prospective randomized study. *The Journal of thoracic and cardiovascular surgery,* 98**,** 751.

98. MARET, W. 2011. Metals on the move: zinc ions in cellular regulation and in the coordination dynamics of zinc proteins. *Biometals: An International Journal On The Role Of Metal Ions In Biology, Biochemistry, And Medicine,* 24**,** 411.

99. MARGARSON, M. P. & SONI, N. 1998. Serum albumin: touchstone or totem?

Anaesthesia, 53**,** 789.

100. MARTINS, E. O. & DRAKENBERG, T. 1982. Cadmium(II), zinc(II), and copper(II) ions binding to bovine serum albumin. A 113Cd NMR study. *Inorganica Chimica Acta,* 67**,** 71.

101. MARX, G., KORNER, G., MOU, X. & GORODETSKY, R. 1993. Packaging zinc,

fibrinogen, and factor XIII in platelet alpha-granules. *Journal of cellular physiology,* 156**,** 437. 102. MASUOKA, J. & SALTMAN, P. 1994. Zinc(II) and copper(II) binding to serum

albumin. A comparative study of dog, bovine, and human albumin. *The Journal of biological chemistry,* 269**,** 25557.

103. MC NAMARA, K., ALZUBAIDI, H. & JACKSON, J. K. 2019. Cardiovascular disease as a leading cause of death: how are pharmacists getting involved? *Integrated pharmacy research & practice,* 8**,** 1.

104. MCQUAID, S. E., HODSON, L., NEVILLE, M. J., DENNIS, A. L., CHEESEMAN, J., HUMPHREYS, S. M., RUGE, T., GILBERT, M., FIELDING, B. A., FRAYN, K. N. & KARPE, F. 2011. Downregulation of adipose tissue fatty acid trafficking in obesity: a driver for ectopic fat deposition? *Diabetes,* 60**,** 47.

105. MENDIS, S. & BANERJEE, A. 2010. Cardiovascular disease: Equity and social determinants. *In:* BLAS, E. & KURUP, A. S. (eds.) *Equity, Social Determinants and Public Health Programmes.* Geneva, Switzerland: World Health Organisation.

106. MESSORI, L., BALERNA, A., ASCONE, I., CASTELLANO, C., GABBIANI, C., CASINI, A., MARCHIONI, C., JAOUEN, G. & CONGIU CASTELLANO, A. 2011. X-ray absorption spectroscopy studies of the adducts formed between cytotoxic gold compounds and two major serum proteins. *Journal of Biological Inorganic Chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry,* 16**,** 491.

107. MIKKELSEN, B., WILLIAMS, J., RAKOVAC, I., WICKRAMASINGHE, K., HENNIS, A., SHIN, H. R., FARMER, M., WEBER, M., BERDZULI, N., BORGES, C., HUBER, M. & BREDA, J. 2019. Life course approach to prevention and control of noncommunicable diseases. *BMJ (Clinical research ed.),* 364**,** l257.

108. MILLER, M. R., PEREIRA, R. I., LANGEFELD, C. D., LORENZO, C., ROTTER, J. I., CHEN, Y. D., BERGMAN, R. N., WAGENKNECHT, L. E., NORRIS, J. M. & FINGERLIN, T. E. 2012. Levels of free fatty acids (FFA) are associated with insulin resistance but do not explain the relationship between adiposity and insulin resistance in Hispanic Americans: the IRAS Family Study. *The Journal of clinical endocrinology and metabolism,* 97**,** 3285.

109. MINAMINO, T., MIYAUCHI, H., YOSHIDA, T., ISHIDA, Y., YOSHIDA, H. &

KOMURO, I. 2002. Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. *Circulation,* 105**,** 1541.

110. MINE, S., OKADA, Y., TANIKAWA, T., KAWAHARA, C., TABATA, T. & TANAKA, Y. 2006. Increased expression levels of monocyte CCR2 and monocyte chemoattractant protein-1 in patients with diabetes mellitus. *Biochemical and biophysical research communications,* 344**,** 780.

111. MITTENDORFER, B., MAGKOS, F., FABBRINI, E., MOHAMMED, B. S. &

KLEIN, S. 2009. Relationship between body fat mass and free fatty acid kinetics in men and women. *Obesity (Silver Spring, Md.),* 17**,** 1872.

112. MORGAN, A. D., ZAKERI, R. & QUINT, J. K. 2018. Defining the relationship between COPD and CVD: what are the implications for clinical practice? *Therapeutic advances in respiratory disease,* 12**,** 1753465817750524.

113. MORGAN, W. T. 1978. Human serum histidine-rich glycoprotein. I. Interactions with heme, metal ions and organic ligands. *Biochimica et biophysica acta,* 535**,** 319.

114. MORI, S., SHINOHATA, R., RENBUTSU, M., TAKAHASHI, H. K., FANG, Y.-I., YAMAOKA, K., OKAMOTO, M., YAMAMOTO, I. & NISHIBORI, M. 2003. Histidine-rich glycoprotein plus zinc reverses growth inhibition of vascular smooth muscle cells by heparin. Cell and tissue research, 312, 353.

115. MÜLLER, F., GAILANI, D. & RENNÉ, T. 2011. Factor XI and XII as antithrombotic targets. *Current opinion in hematology,* 18**,** 349.

116. MURRAY, C. J., VOS, T., LOZANO, R., NAGHAVI, M., FLAXMAN, A. D., MICHAUD, C., EZZATI, M., SHIBUYA, K., SALOMON, J. A., ABDALLA, S., ABOYANS, V., ABRAHAM, J., ACKERMAN, I., AGGARWAL, R., AHN, S. Y., ALI, M. K.,

ALVARADO, M., ANDERSON, H. R., ANDERSON, L. M., ANDREWS, K. G., ATKINSON, C., BADDOUR, L. M., BAHALIM, A. N., BARKER-COLLO, S., BARRERO, L. H., BARTELS, D. H., BASANEZ, M. G., BAXTER, A., BELL, M. L., BENJAMIN, E. J., BENNETT, D., BERNABE, E., BHALLA, K., BHANDARI, B.,

BIKBOV, B., BIN ABDULHAK, A., BIRBECK, G., BLACK, J. A., BLENCOWE, H., BLORE, J. D., BLYTH, F., BOLLIGER, I., BONAVENTURE, A., BOUFOUS, S., BOURNE, R., BOUSSINESQ, M., BRAITHWAITE, T., BRAYNE, C., BRIDGETT, L., BROOKER, S., BROOKS, P., BRUGHA, T. S., BRYAN-HANCOCK, C., BUCELLO, C., BUCHBINDER, R., BUCKLE, G., BUDKE, C. M., BURCH, M., BURNEY, P., BURSTEIN, R., CALABRIA, B., CAMPBELL, B., CANTER, C. E., CARABIN, H., CARAPETIS, J., CARMONA, L., CELLA, C., CHARLSON, F., CHEN, H., CHENG, A. T., CHOU, D., CHUGH, S. S., COFFENG, L. E., COLAN, S. D., COLQUHOUN, S., COLSON, K. E., CONDON, J., CONNOR, M. D., COOPER, L. T., CORRIERE, M., CORTINOVIS, M., DE VACCARO, K. C., COUSER, W., COWIE, B. C., CRIQUI, M. H., CROSS, M., DABHADKAR, K. C., DAHIYA, M., DAHODWALA, N., DAMSERE-DERRY, J., DANAEI, G., DAVIS, A., DE LEO, D., DEGENHARDT, L., DELLAVALLE, R., DELOSSANTOS, A., DENENBERG, J., DERRETT, S., DES JARLAIS, D. C., DHARMARATNE, S. D., et al. 2012. Disabilityadjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet (London, England),* 380**,** 2197.

117. NELSON, E. R., WARDELL, S. E., JASPER, J. S., PARK, S., SUCHINDRAN, S., HOWE, M. K., CARVER, N. J., PILLAI, R. V., SULLIVAN, P. M., SONDHI, V., UMETANI, M., GERADTS, J. & MCDONNELL, D. P. 2013. 27-Hydroxycholesterol links hypercholesterolemia and breast cancer pathophysiology. *Science (New York, N.Y.),* 342**,** 1094.

118. NYBERG, S. T., BATTY, G. D., PENTTI, J., VIRTANEN, M., ALFREDSSON, L., FRANSSON, E. I., GOLDBERG, M., HEIKKILA, K., JOKELA, M., KNUTSSON, A., KOSKENVUO, M., LALLUKKA, T., LEINEWEBER, C., LINDBOHM, J. V., MADSEN, I. E. H., MAGNUSSON HANSON, L. L., NORDIN, M., OKSANEN, T., PIETILAINEN, O., RAHKONEN, O., RUGULIES, R., SHIPLEY, M. J., STENHOLM, S., SUOMINEN, S., THEORELL, T., VAHTERA, J., WESTERHOLM, P. J. M., WESTERLUND, H., ZINS, M., HAMER, M., SINGH-MANOUX, A., BELL, J. A., FERRIE, J. E. & KIVIMAKI, M. 2018. Obesity and loss of disease-free years owing to major non-communicable diseases: a multicohort study. The Lancet.Public health, 3, e490.OETTL, K. & STAUBER, R. E. 2007. Physiological and pathological changes in the redox state of human serum albumin critically influence its binding properties. *British journal of pharmacology,* 151**,** 580.

119. OPIE, L. 2015. Cancer and cardiovascular disease: more tightly linked than by chance. *Lancet (London, England),* 385**,** 1182.

120. PATEL, K. K., POON, I. K. H., TALBO, G. H., PERUGINI, M. A., TAYLOR, N. L., RALPH, T. J., HOOGENRAAD, N. J. & HULETT, M. D. 2013. New method for purifying histidine-rich glycoprotein from human plasma redefines its functional properties. *IUBMB life,* 65**,** 550.

121. PETERS JR, T. 1995. 2 - The Albumin Molecule: Its Structure and Chemical Properties. *In:* PETERS, T. (ed.) *All About Albumin.* San Diego: Academic Press.

122. PIEMONTI, L., CALORI, G., MERCALLI, A., LATTUADA, G., MONTI, P., GARANCINI, M. P., COSTANTINO, F., RUOTOLO, G., LUZI, L. & PERSEGHIN, G. 2003. Fasting plasma leptin, tumor necrosis factor-alpha receptor 2, and monocyte chemoattracting protein 1 concentration in a population of glucose-tolerant and glucose-intolerant women: impact on cardiovascular mortality. *Diabetes care,* 26**,** 2883.

123. PILZ, S., SCHARNAGL, H., TIRAN, B., WELLNITZ, B., SEELHORST, U., BOEHM, B. O. & MARZ, W. 2007. Elevated plasma free fatty acids predict sudden cardiac death: a 6.85-year follow-up of 3315 patients after coronary angiography. *European heart journal,* 28**,** 2763.

124. PIRRO, M., MAURIÈGE, P., TCHERNOF, A., CANTIN, B., DAGENAIS, G. R., DESPRÉS, J.-P. & LAMARCHE, B. T. 2002. Plasma free fatty acid levels and the risk of ischemic heart disease in men: prospective results from the Québec Cardiovascular Study.

Atherosclerosis, 160**,** 377.

125. POON, I. K. H., PATEL, K. K., DAVIS, D. S., PARISH, C. R. & HULETT, M. D. 2011. Histidine-rich glycoprotein: the Swiss Army knife of mammalian plasma. *Blood,* 117**,** 2093.

126. RABE, K. F. & WATZ, H. 2017. Chronic obstructive pulmonary disease. *Lancet (London, England),* 389**,** 1931.

127. RANIERI-RAGGI, M., MOIR, A. J. G. & RAGGI, A. 2014. The Role of HistidineProline-Rich Glycoprotein as Zinc Chaperone for Skeletal Muscle AMP Deaminase. *Biomolecules (2218-273X),* 4**,** 474.

128. RIDKER, P. M. 2000. Novel risk factors and markers for coronary disease. *Advances in Internal Medicine,* 45**,** 391.

129. RØJKJÆR, R. & SCHOUSBOE, I. 1997. The surface-dependent autoactivation mechanism of factor XII. *European Journal of Biochemistry,* 243**,** 160.

130. RUBINO, J. T. & FRANZ, K. J. 2012. Coordination chemistry of copper proteins: how nature handles a toxic cargo for essential function. *Journal of inorganic biochemistry,* 107**,** 129.

131. SANNA, A., FIRINU, D., ZAVATTARI, P. & VALERA, P. 2018. Zinc Status and

Autoimmunity: A Systematic Review and Meta-Analysis. *Nutrients,* 10**,** 10.3390/nu10010068.

132. SAPONARO, C., GAGGINI, M., CARLI, F. & GASTALDELLI, A. 2015. The Subtle Balance between Lipolysis and Lipogenesis: A Critical Point in Metabolic Homeostasis.

Nutrients, 7**,** 9453.

133. SCHMAIER, A. H., KUO, A., LUNDBERG, D., MURRAY, S. & CINES, D. B. 1988. The expression of high molecular weight kininogen on human umbilical vein endothelial cells.

The Journal Of Biological Chemistry, 263**,** 16327.

134. SEN, K. & BONITA, R. 2000. Global health status: two steps forward, one step back. *Lancet (London, England),* 356**,** 577.

135. SHANKAR, A. H. & PRASAD, A. S. 1998. Zinc and immune function: the biological basis of altered resistance to infection. *The American Journal of Clinical Nutrition,* 68**,** 447S. 137. SHOELSON, S. E., LEE, J. & GOLDFINE, A. B. 2006. Inflammation and insulin

resistance. *The Journal of clinical investigation,* 116**,** 1793.

136. SIMARD, J. R., ZUNSZAIN, P. A., HAMILTON, J. A. & CURRY, S. 2006. Location of high and low affinity fatty acid binding sites on human serum albumin revealed by NMR drug-competition analysis. *Journal of Molecular Biology,* 361**,** 336.

137. SOBEL, B. E., WOODCOCK-MITCHELL, J., SCHNEIDER, D. J., HOLT, R. E., MARUTSUKA, K. & GOLD, H. 1998. Increased plasminogen activator inhibitor type 1 in coronary artery atherectomy specimens from type 2 diabetic compared with nondiabetic patients: a potential factor predisposing to thrombosis and its persistence. *Circulation,* 97**,** 2213.

138. STAMLER, J., WENTWORTH, D. & NEATON, J. D. 1986. Is relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and graded? Findings in 356,222 primary screenees of the Multiple Risk Factor Intervention Trial (MRFIT). *Jama,* 256**,** 2823.

139. STAMPFER, M. & JAHN, J. L. 2013. Partnerships for promoting prevention.

Circulation, 127**,** 1267.

140. STEFAN TRÜMPLER, A. W. L. A. B. M. W. B. M. S. & UWE, K. 2009. Interaction of thimerosal with proteins—ethylmercuryadduct formation of human serum albumin and βlactoglobulin A *Metallomics***,** 87.

141. STEWART, A. J., BLINDAUER, C. A. & SADLER, P. J. 2009. Plasma fatty acid levels may regulate the Zn2+-dependent activities of histidine-rich glycoprotein. *Biochimie,* 91**,** 1518. STURKE, R., VORKOPER, S., DUNCAN, K., LEVINTOVA, M. & PARASCONDOLA, M. 2016. Addressing NCDs through research and capacity building in LMICs: lessons learned from tobacco control. Global health action, 9, 32407.

142. SUNDERMAN, F. W., JR. 1983. Potential toxicity from nickel contamination of intravenous fluids. *Annals of Clinical and Laboratory Science,* 13**,** 1.

143. SUNIL U. PATEL, P. J. S. A. T. & JOHN, H. V. 1993. Direct detection of albumin in human blood plasma by proton NMR spectroscopy. Complexation of nickel2+ *Journal of the American Chemical Society,* 115**,** 9285.

144. TATARANNI, P. A. & ORTEGA, E. 2005. A burning question: does an adipokineinduced activation of the immune system mediate the effect of overnutrition on type 2 diabetes? *Diabetes,* 54**,** 917.

145. TATEYA, S., KIM, F. & TAMORI, Y. 2013. Recent advances in obesity-induced

inflammation and insulin resistance. *Frontiers in endocrinology,* 4**,** 93.

146. TATEYA, S., TAMORI, Y., KAWAGUCHI, T., KANDA, H. & KASUGA, M. 2010. An increase in the circulating concentration of monocyte chemoattractant protein-1 elicits systemic insulin resistance irrespective of adipose tissue inflammation in mice. *Endocrinology,* 151**,** 971.

147. TESFAMARIAM, B. & COHEN, R. A. 1992. Free radicals mediate endothelial cell dysfunction caused by elevated glucose. *The American Journal of Physiology,* 263**,** H321.

148. THANAN, R., OIKAWA, S., HIRAKU, Y., OHNISHI, S., MA, N., PINLAOR, S., YONGVANIT, P., KAWANISHI, S. & MURATA, M. 2014. Oxidative stress and its significant roles in neurodegenerative diseases and cancer. *International journal of molecular sciences,* 16**,** 193.

149. THOMPSON, R. E., MANDLE, R. J. & KAPLAN, A. P. 1977. Association of factor XI and high molecular weight kininogen in human plasma. *The Journal of clinical investigation,* 60**,** 1376.

152. TSILIDIS, K. K., KASIMIS, J. C., LOPEZ, D. S., NTZANI, E. E. & IOANNIDIS, J. P. 2015. Type 2 diabetes and cancer: umbrella review of meta-analyses of observational studies. *BMJ (Clinical research ed.),* 350**,** g7607.

153. TUBEK, S., GRZANKA, P. & TUBEK, I. 2008. Role of zinc in hemostasis: a review. *Biological trace element research,* 121**,** 1.

154. USLU, A. U., KUCUK, A., BALTA, S., OZTURK, C., ARSLAN, S., TEKIN, L., KUCUKSEN, S., TOKER, A. & KAYRAK, M. 2019. The relation between ischemia modified albumin levels and carotid intima media thickness in patients with rheumatoid arthritis. *International journal of rheumatic diseases,* 22**,** 32.

155. VALLEE, B. L. & FALCHUK, K. H. 1993. The biochemical basis of zinc physiology. *Physiological Reviews,* 73**,** 79.

156. VICENOVA, B., VOPALENSKY, V., BURYSEK, L. & POSPISEK, M. 2009. Emerging role of interleukin-1 in cardiovascular diseases. *Physiological research,* 58**,** 481.

157. VINIK, A. I., ERBAS, T., PARK, T. S., NOLAN, R. & PITTENGER, G. L. 2001. Platelet dysfunction in type 2 diabetes. *Diabetes care,* 24**,** 1476.

158. VISCHER, U. M., EMEIS, J. J., BILO, H. J., STEHOUWER, C. D., THOMSEN, C., RASMUSSEN, O., HERMANSEN, K., WOLLHEIM, C. B. & INGERSLEV, J. 1998. von Willebrand factor (vWf) as a plasma marker of endothelial activation in diabetes: improved reliability with parallel determination of the vWf propeptide (vWf:AgII). *Thrombosis and haemostasis,* 80**,** 1002.

159. VU, T. T., FREDENBURGH, J. C. & WEITZ, J. I. 2013. Zinc: an important cofactor in haemostasis and thrombosis. *Thrombosis and haemostasis,* 109**,** 421.

160. WANG, J., MA, J. J., LIU, J., ZENG, D. D., SONG, C. & CAO, Z. 2017. Prevalence and Risk Factors of Comorbidities among Hypertensive Patients in China. *International journal of medical sciences,* 14**,** 201-212.

161. WARNER, M. & GUSTAFSSON, J. A. 2014. On estrogen, cholesterol metabolism, and breast cancer. *The New England journal of medicine,* 370**,** 572.

162. WEISBERG, S. P., MCCANN, D., DESAI, M., ROSENBAUM, M., LEIBEL, R. L. & FERRANTE, A. W., JR. 2003. Obesity is associated with macrophage accumulation in adipose tissue. *The Journal of clinical investigation,* 112**,** 1796.

163. WHITEHOUSE, R. C., PRASAD, A. S., RABBANI, P. I. & COSSACK, Z. T. 1982. Zinc in plasma, neutrophils, lymphocytes, and erythrocytes as determined by flameless atomic absorption spectrophotometry. *Clinical chemistry,* 28**,** 475.

164. WHO. 2018. *Noncommunicable diseases and their risk factors* [Online]. Available: https://www.who.int/ncds/en/ [Accessed 03/15 2019].

165. WILLIAMS, I. L. N. B. & ZAMAN, A. G. 2003. Review: The management of acute myocardial infarction in patients with diabetes mellitus. *The British Journal of Diabetes & Vascular Disease,* 3**,** 319.

166. WOOD, A. D., STRACHAN, A. A., THIES, F., AUCOTT, L. S., REID, D. M., HARDCASTLE, A. C., MAVROEIDI, A., SIMPSON, W. G., DUTHIE, G. G. &

MACDONALD, H. M. 2014. Patterns of dietary intake and serum carotenoid and tocopherol status are associated with biomarkers of chronic low-grade systemic inflammation and cardiovascular risk. *The British journal of nutrition,* 112**,** 1341.

167. WORLD HEALTH, O. 2013. Global action plan for the prevention and control of noncommunicable diseases 2013-2020. Geneva, Switzerland: WHO.

168. WORLD HEALTH, O. 2014. Global status report on noncommunicable diseases 2014.

Geneva, Switzerland: World Health Organization Press.

169. WORLD HEALTH, O. 2018. *Noncommunicable Diseases* [Online]. Available: https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases [Accessed 03/11 2019].

170. WU, B. B., ZHANG, L. M., MEI, C. L., TANG, Q. & LU, Y. Z. 2010. Relationship between serum free fatty acid and cytokines, carotid atherosclerosis in chronic kidney disease.

Zhonghua nei ke za zhi, 49**,** 572.

171. XIN, Y., WANG, Y., CHI, J., ZHU, X., ZHAO, H., ZHAO, S. & WANG, Y. 2019.

Elevated free fatty acid level is associated with insulin-resistant state in nondiabetic Chinese people. *Diabetes, metabolic syndrome and obesity : targets and therapy,* 12**,** 139.

172. XU, H., BARNES, G. T., YANG, Q., TAN, G., YANG, D., CHOU, C. J., SOLE, J., NICHOLS, A., ROSS, J. S., TARTAGLIA, L. A. & CHEN, H. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *The Journal of clinical investigation,* 112**,** 1821.

173. YEATES, K., LOHFELD, L., SLEETH, J., MORALES, F., RAJKOTIA, Y. & OGEDEGBE, O. 2015. A Global Perspective on Cardiovascular Disease in Vulnerable Populations. *The Canadian journal of cardiology,* 31**,** 1081.

174. YU, Y. H. & GINSBERG, H. N. 2005. Adipocyte signaling and lipid homeostasis: sequelae of insulin-resistant adipose tissue. *Circulation research,* 96**,** 1042.

175. ZHANG, H. W., ZHAO, X., GUO, Y. L., ZHU, C. G., WU, N. Q., SUN, J., LIU, G., DONG, Q. & LI, J. J. 2017. Free fatty acids and cardiovascular outcome: a Chinese cohort study on stable coronary artery disease. *Nutrition & metabolism,* 14**,** 41.

176. ZHOU, Y., WANG, Y., HU, X., HUANG, J., HAO, Y., LIU, H. & SHEN, P. 1994. Equilibrium dialysis of metal-serum albumin. I. Successive stability constants of Zn(II)-serum albumin and the Zn(2+)-induced cross-linking self-association. *Biophysical chemistry,* 51**,** 81.

CHAPTER 2

INVESTIGATING THE INFLUENCE OF FREE FATTY ACIDS OF DIFFERENT CHAIN LENGTHS ON THE CAPACITY OF ALBUMIN TO BIND ZINC USING THE ALBUMIN COBALT BINDING (ACB) ASSAY

2.1 Introduction

2.1.1 Different types of free fatty acids in circulation

Fats are a vital part of the human diet and biologically, form parts of cellular components (e.g. membranes) and contribute as a source of energy for metabolism. Common dietary fatty acids have been classified into three major categories namely saturated (no double bonds), monounsaturated (one double bond) and polyunsaturated fatty acids which have two or more double bonds. Animal source foods from ruminants and other tropical foods such as palm and coconut are a source of saturated fatty acids common in most diets. Table 2.1 shows a list and examples of common dietary saturated fatty acids. Saturated fatty acids are categorised further into four groups depending on the length of the carbon chains (note that natural fatty acids always contain an even number of carbon atoms).

As elaborated in the introduction (Chapter 1) to this thesis, excessive intake of saturated fatty acids in combination with other risk factors such as sedentary lifestyle, genetic predisposition, and obesity may lead to metabolic syndrome - a term used to denote a group of disorders including cardiovascular disease and diabetes (Crowe, 2012). Specifically, individual longchain saturated FFA such as myristic, palmitic and stearic acids have been associated with metabolic syndrome and cardiovascular disease (Ebbesson et al., 2010). Metabolic syndrome is a well-known high-risk factor for cardiovascular events, including acute myocardial ischaemia (Nyberg et al., 2018).

Table 2.1 List and examples of the common dietary saturated fatty acids. Taken from (Food and Agricultural Organisation report, 2010)

Common Name	Systemic Name	Abbreviation	Common Source	Category based on carbon chain length
Butyric	Butanoic	C4:0	Dairy	Short chain fatty acids
Caproic	Hexanoic	C6:0	Dairy	
Caprylic	Octanoic	C8:0	Dairy, Coconut and palm kernel oils	Medium chain fatty acids
Capric	Decanoic	C10:0	Dairy, Coconut and palm kernel oils	
Lauric	Dodecanoic	C12:0	Coconut and palm kernel oils	
Mystiric	Tetradecanoic	C14:0	Dairy, Coconut and palm kernel oils	Long chain fatty acids
Palmitic	Hexadecanoic	C16:0	Most fats and oils	
Stearic	Octadecanoic	C18:0	Most fats and oils	
Arachidic	Eicosanoic	C20:0	Peanut oil	
Behenic	Docosanoic	C22:0	Peanut oil	Very long chain fatty acid
Lignoceric	Tetracosanoic	C24:0	Peanut oil	

Importantly, myocardial ischaemia is associated with higher levels of plasma FFA (Chen et al., 2015b, Zhang et al., 2017). Studies using ITC, suggest that free fatty acids such as myristic acid lead to a reduction in the binding of Zn^{2+} to albumin in a dose-dependent manner (Coverdale et al., 2019b, Kassaar et al., 2015). Increased FFA may, therefore, be responsible for the derangements in the buffering of metal ions by albumin and may affect and challenge the known mechanisms of biomarkers for some conditions. One of the biomarkers which may be affected by increased free fatty acid levels in serum is the ACB assay (Lu et al., 2012).

2.1.2 The ACB assay and influence of FFA

The ACB assay was initially developed in the early 1990s and certified by the Food and Drug Administration as a test used to exclude acute myocardial ischaemia in patients presenting with acute chest pains (Sbarouni et al., 2011, Bar-Or et al., 2000). The assay was initially developed on the observation that HSA in patients with acute myocardial ischaemia had reduced capacity to bind cobalt and was termed ischaemia modified albumin (IMA) (Apple et al., 2005). At that point, it was assumed that the primary site for Co^{2+} was the N-terminus (Bar-Or et al., 2008, Liang et al., 2001, Sadler et al., 1994). IMA was thought to occur due to the oxidation of and cleavage of the first two amino acid residues at the N-terminus Asp1 and Ala2 which together with His3 forms the N-terminus binding site (NTS) of HSA (Lakusta and Sarkar, 1979). Figure 2.1 shows the view of the proposed structure of the NTS which is also a binding site for Cu^{2+} and Ni^{2+} on HSA.

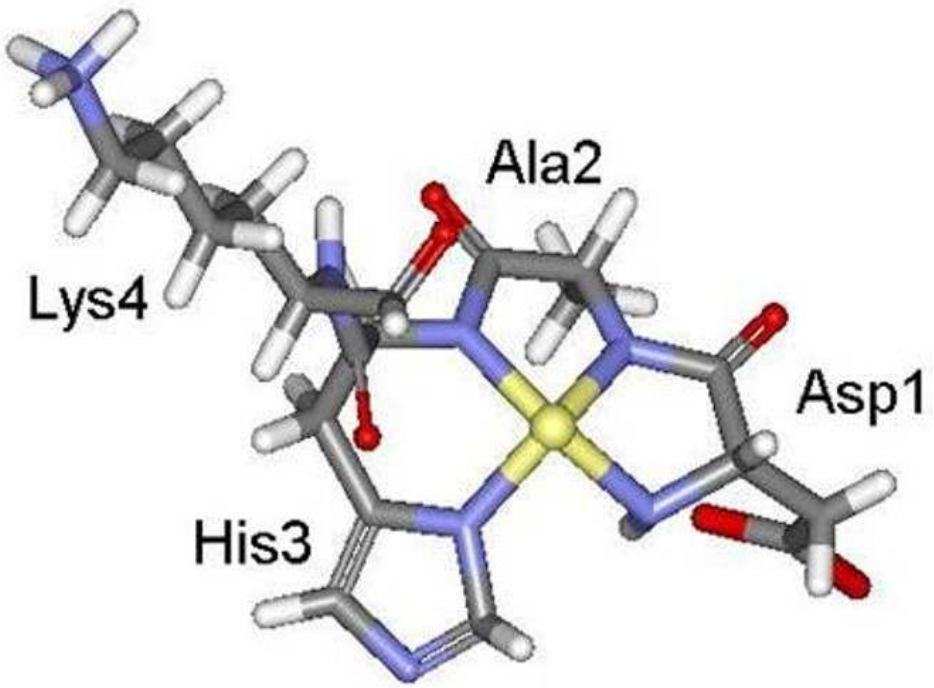


Figure 2.1 N-terminal binding site (NTS) on HSA. A view of the proposed structure of the NTS Cu^{2+} and Ni^{2+} binding site of human albumin. The NTS was previously thought to be the primary binding site for Co^{2+}. The site is 4-co-ordinate and thought to adopt a planar geometry. The metal ion is coloured yellow and residues Asp^1, Ala^2, His^3 and Lys^4 are shown. Taken from (Stewart, 2003).

When conducting the ACB assay, cobalt solution of approximately 1.5 mol equivalents per albumin molecule is added to a serum sample. Then, dithiothreitol (DTT) which is a cobalt chelator is added to a serum sample and produces a colour change to an ill-defined brown colour whose absorbance can be measured by spectrophotometry in comparison to blank serum without DTT (Bar-Or et al., 2000). Thus, a deeper colour change with corresponding higher absorbance reading due to more unbound cobalt would indicate IMA (Sarawut, 2012). The ACB test has been used more as a negative predictor of myocardial ischaemia due to its sensitivity, but the caveat is that it is less specific (Dusek et al., 2005). Figure 2.2. represents the originally thought basis of the ACB assay.

Over the past two decades since the establishment of the ACB assay, there has been a challenge regarding the mechanism for the assay and substantiation of the alteration of the N-terminus of HSA as the basis for IMA (Lu et al., 2012). More elaborate studies on human and bovine albumin using Cd^{2+} and Cu^{2+} monitored by electronic absorption spectroscopy strongly indicated that Co^{2+} preferentially binds to sites A and B (Sokolowska et al., 2009, Mothes and Faller, 2007, Oh et al., 2012). Further to these studies, ITC and spectroscopic studies have indicated that site B is the most potent binding site for cobalt (Sokolowska et al., 2009). Subsequent studies using ^{111}Cd nuclear magnetic resonance (NMR) spectroscopy and competition studies with Zn^{2+} using ITC confirmed that the sites A and B are the major binding sites for Co^{2+} (Lu et al., 2012) (Figure 2.3). Moreover, it was noted that blocking the NTS using Cu^{2+} insignificantly affects the binding of Co^{2+} to HSA, confirming that the NTS is not the primary binding site for Co^{2+} (Lu et al., 2012, Mothes and Faller, 2007). Of importance to note also, is that Co^{2+} has similar behavioural properties to Zn^{2+} in that they have a similar ionic radius (0.58 and 0.60 Å respectively) (Shannon, 1976). The binding constants of Co^{2+} to its strongest sites are (log *K*app = $4.6 \pm 0.3 \times 10^4$ M^{-1}; Fig. 2.3 b; and $9 \pm 5 \times 10^4$ M^{-1} for bovine albumin and HSA respectively, which is only one magnitude lower that of Zn^{2+} (Lu et al., 2012, Sokolowska et al., 2009). Due to the similarities, Co^{2+} has often been used as a spectroscopic probe for Zn^{2+} in protein studies (Vallee, 1993, Lu et al., 2012).

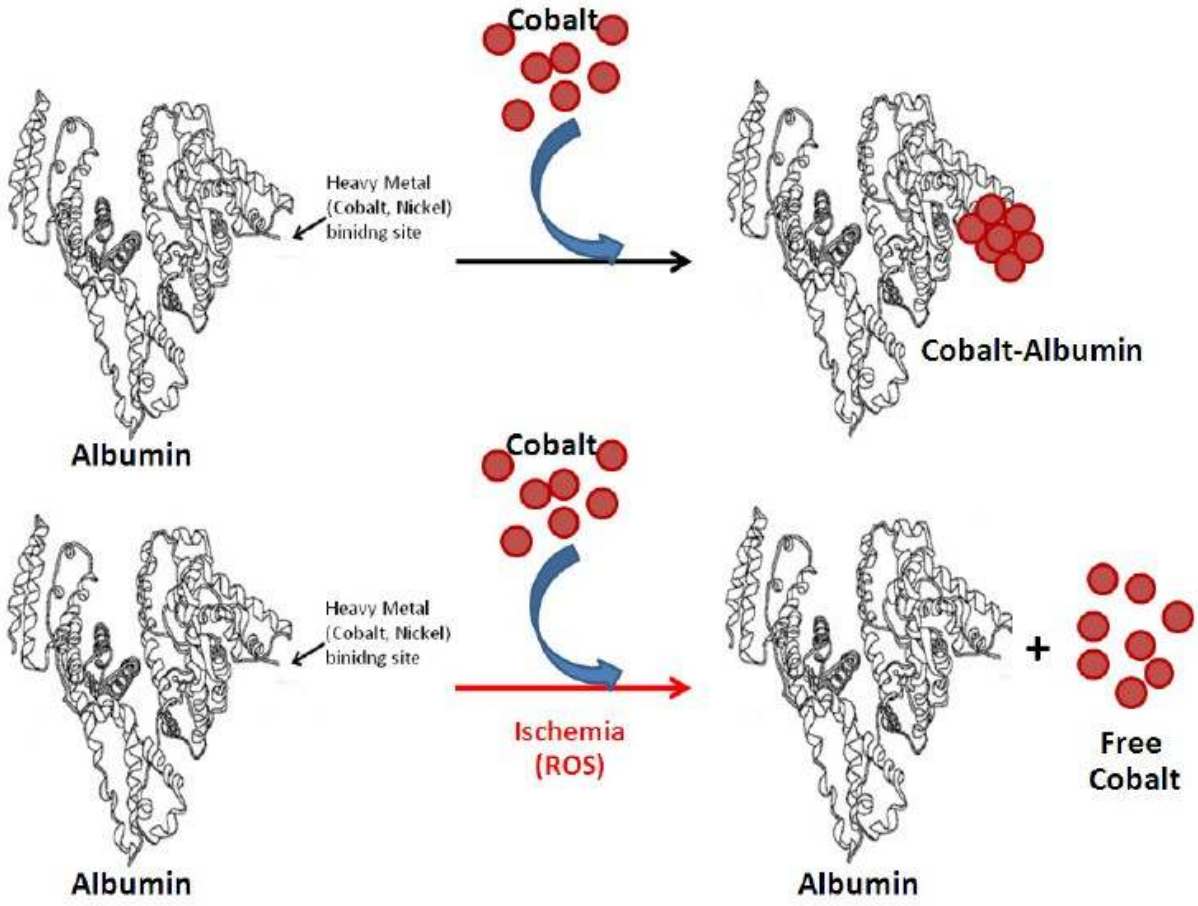


Figure 2.2 Diagrammatic presentation of the principle of the ACB assay. In normal conditions, albumin can bind metal ions including cobalt, and this was thought to be at the amino terminal end of the protein. During ischemia, the N-terminal portion of albumin is disrupted and results in the reduction in albumin-metal ions binding ability. Adapted from (Sarawut, 2012).

It was later shown that in fact, ischaemic modified HSA was associated with increased free fatty acid plasma levels (Apple et al., 2005, Lopaschuk et al., 2010). Indeed, acute coronary syndromes are well-known to be associated with increased levels of FFA in serum (Bhagavan et al., 2009, Pirro et al., 2002a). The pain and the stress associated with the syndrome triggers a sympathetic discharge, with the release of catecholamines which activate hormone-sensitive tissue lipase – an enzyme which hydrolyses triglycerides and hence liberating free fatty acids in circulation (Loria et al., 2008, Roy et al., 2013, Stich and Berlan, 2004).

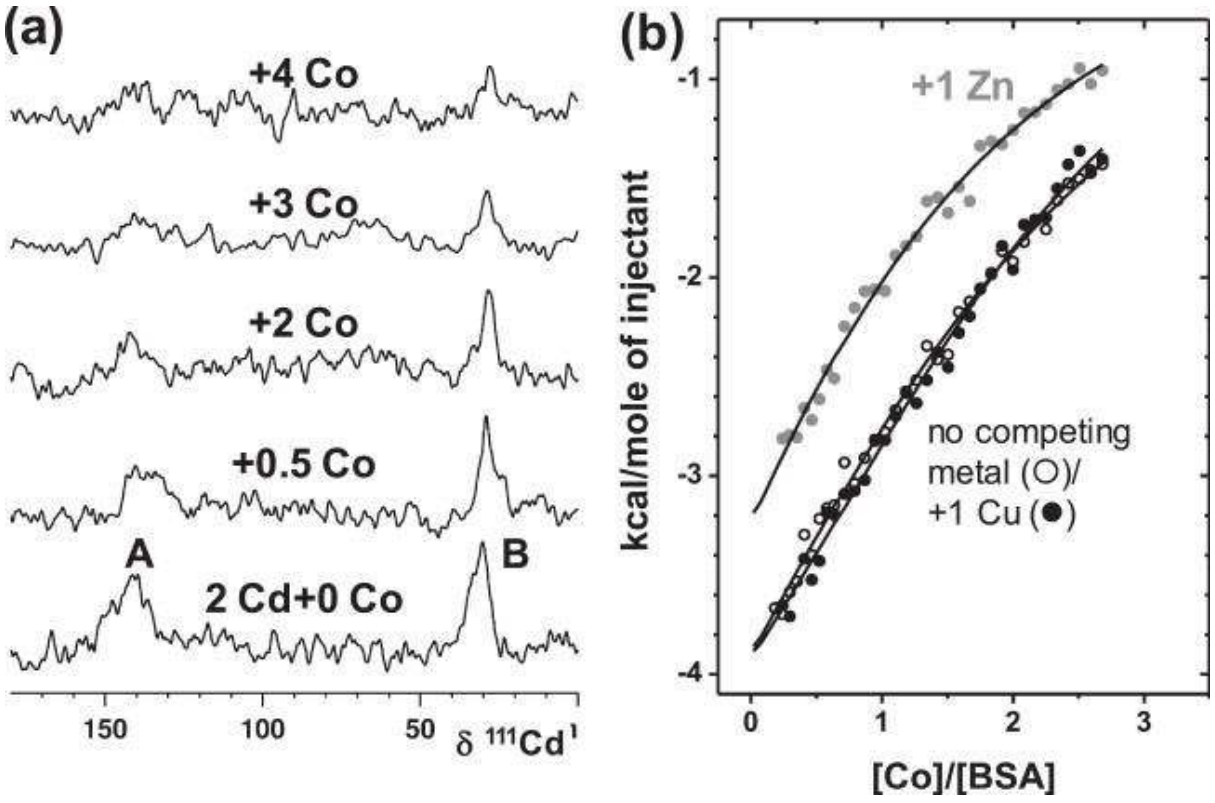


Figure 2.3 Co^{2+} competes with both Cd^{2+} and Zn^{2+} for albumin binding under physiological conditions (pH 7.4, 50 mM Tris-Cl, 50 mM NaCl) but not with Cu^{2+}. (a) ^{111}Cd NMR spectra of Cd_2BSA (1.5 mM) with increasing addition of Co^{2+}. ^{111}Cd resonances corresponding to both site A and B (~140 ppm and 35 ppm, respectively) are affected by Co^{2+} binding. (b) Isothermal calorimetry experiments performed in the presence of 1 molar equivalent of Cu^{2+} (●) or Zn^{2+} (●) demonstrate that addition of Zn^{2+} decreases albumin's affinity and capacity for Co^{2+}-binding, while the addition of Cu^{2+} has no significant effect.
Taken from (Coverdale et al., 2018).

The concentration of FFA in acute coronary syndrome within 1-2 hours from the onset positively correlate with serious ventricular arrhythmias (Oliver, 2011). Interestingly, ACB assay values are also positively related to the severity of the acute coronary syndrome condition (Bhagavan et al., 2003, Gurumurthy et al., 2014, Oliver, 2011). Moreover, the IMA levels can be detected after some minutes following an ischaemic event, remain high for 6 to 12 hours after, and return to normal within 24 hours (Coverdale et al., 2018a). These changes are similar to those observed for plasma FFA levels, which tend to rise and normalise within 24 to 48 hours following an episode of myocardial ischaemia (Lopaschuk et al., 1994). These observations are contrary to the principle that IMA results from the destruction of the N-terminus because in that case, IMA would be still detected

for days following onset of myocardial ischaemia because albumin has a half-life of 20 days (Cho et al., 2007, Sinha et al., 2004).

To date, the ACB assay has been tested and used in a plethora of other conditions such as chronic liver and kidney disease and cancer; infectious diseases such as malaria; exercise and trauma; and in pregnancy-related conditions such as pre-eclampsia (Basu et al., 1960, Bhagavan et al., 2009, Blindauer et al., 2016, Gupta et al., 2017). These conditions, therefore, should have a common feature other than the illusive truncated HSA N-terminus, which is detected by the ACB assay. Free fatty acids have been shown to independently and positively influence the ACB assay in the same ratio as an acute coronary syndrome, and other conditions do (Bhagavan et al., 2009, Lu et al., 2012). Table 2.1 shows a list of selected conditions which are positive for the ACB assay and are also associated with increased serum free fatty acids to elaborate on the co-existence of IMA and increased concentration of FFA.

As in acute coronary syndrome, higher free fatty acid concentrations have been observed in other non-communicable diseases, which are also present with a positive ACB assay (Blindauer et al., 2016). Fatty liver disease, for example, is associated with insulin resistance and hence the inhibition of dephosphorylation of hormone-sensitive lipase activity to reduce fat hydrolysis is withdrawn (Capurso and Capurso, 2012, Kovacs and Stumvoll, 2005, Vernon et al., 2011). Further to this, the capacity of the liver to utilise and export free fatty acids is impaired, leading to increased free fatty acids in circulation (Gaemers and Groen, 2006, Musso et al., 2009, Zhang et al., 2014). Similarly, chronic kidney disease is associated with raised free fatty acid concentration arising from TNF-α - induced adipose tissue lipolysis which is associated with systemic inflammation at play in the disease condition (Min-Tser Liao and Kuo-Cheng, 2012, Wu et al., 2010). In cancer, through idiopathic mechanisms, malignant cells induce adipocytes to undergo hydrolysis and release free fatty acids in circulation which are utilised by the cancer cells for growth (Liu et al., 2014, Merino Salvador et al., 2017). It is scientifically sound, therefore, that these conditions are associated with a positive ACB assay due to the elevated concentrations of free fatty acids as described previously in the case of acute coronary syndrome.

The ACB assay has also been tested and produced positive results in exercise, trauma, preeclampsia and infectious diseases such as malaria (Can et al., 2006, Ghosh et al., 2017, Vyakaranam et al., 2015). Virtually, all these conditions are also associated with high concentrations of free fatty acids

(Table 2.2) through various physiological and pathophysiological pathways (Basu et al., 1960, Gupta et al., 2017, Hubel et al., 1996, Rodahl et al., 1964, Vigne et al., 1997). IMA has hence been recently argued in recent reviews as crosstalk between fatty acids and cobalt binding (Coverdale et al., 2018b, Oran and Oran, 2017).

Effectively, an acceptable hypothesis would be that the ACB assay is positive and is influenced by increased FFA in serum, which perturbs the binding of cobalt to serum albumin. Thus, IMA would principally be albumin saturated with FFA and having reduced binding capacity for metal ions including Co^{2+} and Zn^{2+} which have more avidity to sites A and B (Lu et al., 2012). Figure 2. 4 depicts the more recent theory of the ACB assay, taking into consideration the effect of the FFA. Importantly, due to the similar binding properties of Co^{2+} and Zn^{2+}, the influence of FFA of the ACB assay can reveal potential binding interactions and allosteric inhibition of Zn^{2+} to HSA by FFA.

Table 2.2 Selected conditions associated with positive ischaemia modified albumin (IMA) test and increased free fatty acids (FFA)

Condition	Ischaemia modified albumin Absorbance units(ABSU), U/mL or ng/mL	Control	Reference	Plasma /serum free fatty acid Concentration	Control	Reference
Acute Coronary syndrome: ST-segment elevated myocardial infarction	92.1 (± 10.6) Abs units/mL	77.9 (± 6.69) Abs units/mL	(Gurumurthy et al., 2014)	840 µM (± 320)	750 µM (± 280)	(Pirro et al., 2002b)
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	(Bhagavan et al., 2009)	1030 µM (±450)	770 µM (± 340)	(Bhagavan et al., 2009)
Acute Ischaemic Stroke	1.180 (±0.223) Abs units/mL	0.820 (0.129)Abs units/mL	(Ertekin et al., 2013)	530 µM (350–710)	240 µM (120–380)	(Duan et al., 2017)
Obstructive Sleep apnoea syndrome	0.58 (±0.11) Abs units	0.43 (±0.09)	(Xu et al., 2017)	Proposed higher levels	No data	(Arnardottir et al., 2009, Jun et al., 2011)
Rheumatoid arthritis	0.495 (±0.01) Abs units	0.433 (±0.02) Abs units	(Leitemperguer et al., 2014)	0.59 (0.47–0.65) mM	0.40 (0.35–0.50) mM	(Tang et al., 2017)
Ankylosing spondylitis	0.44 (±0.17) Abs units	0.32 (±0.13) Abs units	(Kucuk et al., 2017)	883.89 (±55.32) µg/mL	760.84 (±31.40) µg/mL	(Chen et al., 2015a)
Psoriasis	0.85 (±0.15) Abs units	0.79 (±0.09) Abs units	(Işık et al., 2016)	No global increase but increases in C16:1n-7, C18:2n-6, C18:3n-3, C20:0	No data	(Myśliwiec et al., 2017)

Chronic liver disease		0.5320 (±0.1677) Abs units	0.3203 (±0.1257) Abs units	(Kumar and Subramanian, 2016)	620 µM (120–3400)	450 µM (110 – 900)	(Zhang et al., 2014)

Diabetes	Diabetes only Diabetic foot	0.478 (±0.095) Abs units 0.721 (±0.123) Abs units	0.395 (±0.054) Abs units	(Muhtaroglu et al., 2016)	>750 µM	<550 µM	(Reaven et al., 1988)
Chronic renal disease		0.357 (±0.083) Abs units	No data	(Kiyici et al., 2010)	492.63 µM (±143.59)	302.65 µM (±142.18)	(Wu et al., 2010)
Polycystic ovarian syndrome		0.52 (0.21–1.12) Abs units	0.35 (0.06–0.90) Abs units	(Beyazit et al., 2016)	Increase in C16:0 and C18:1n9cis	No data	(Niu et al., 2014)
Malaria		0.56 (± 0.13) Abs units	0.24 (± 0.04) Abs units	(Ghosh et al., 2017)	2.17 fold increase	No data	(Gupta et al., 2017)
Exercise		0.324 (± 0.039) Abs units	0.281 (± 0.052) Abs units	(Çolak et al., 2010)	>2000 µM	< 600 µM	(Wolfe et al., 1990)
Trauma		0.63 (±0.18) Abs units	0.39 (±0.05) Abs units	(Can et al., 2006)	2010 µM (± 190)	No data	(Svensson et al., 1990)
Sepsis		0.967 (±0.734) Abs units	0.007 (±0.009) Abs units	(Ashok Kumar and Anand, 2016)	4 fold increase	No data	(Nogueira et al., 2008)
Intrauterine growth restriction		78.7 (±6.9) Abs units/mL	74.4 (±7.8) Abs units/mL	(Karadeniz et al., 2015)	355 µM (in amniotic fluid)	125 µM (in amniotic fluid)	(Urban and Iwaszkiewicz-Pawłowska, 1986)

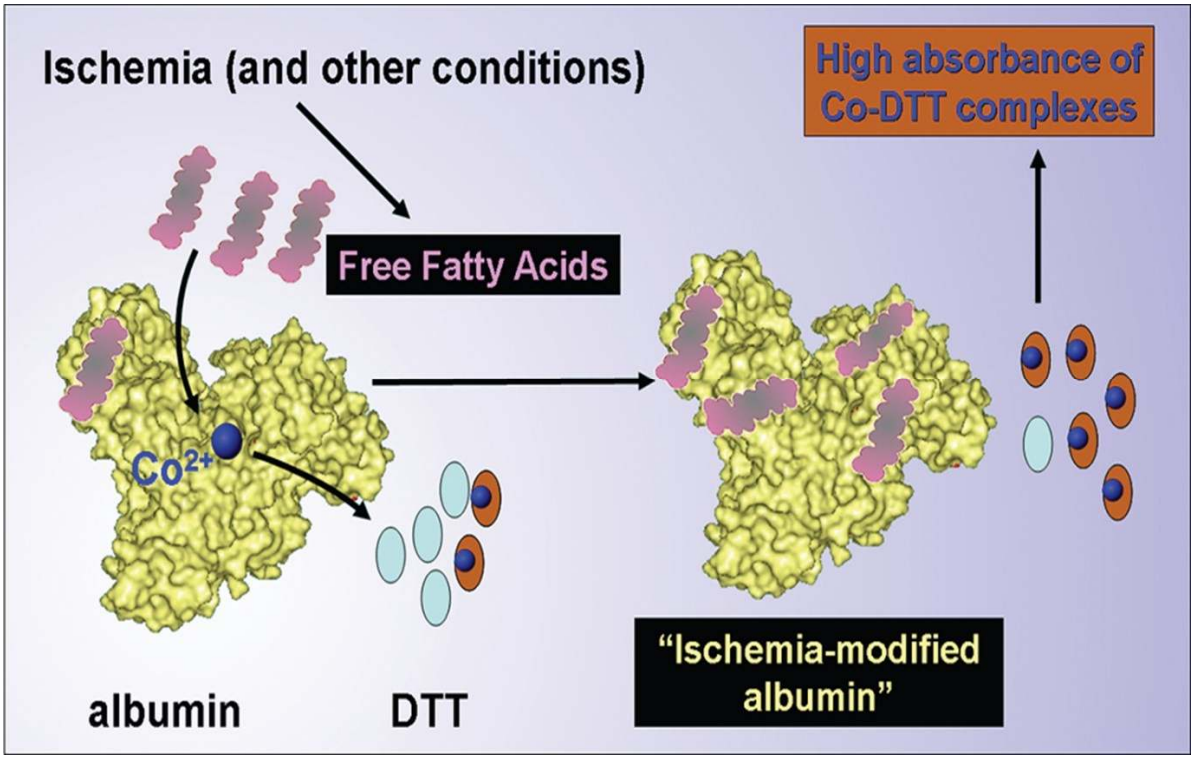


Figure 2.4 Schematic diagram showing how free fatty acids may influence the ACB assay. Increased concentrations of free fatty acids and their binding to albumin perturb the binding of cobalt. More free cobalt which binds to the added DTT forms a more profound colour change and hence giving a higher absorbance reading. Taken from (Lu et al., 2012).

2.2 Aim

The aim of this aspect of the study was to investigate the influence of free fatty acids of different chain lengths and concentrations on the ACB assay. Different saturated fatty acids of different carbon chain lengths (C:8 to C:18) and HSA molar equivalent concentrations (1 to 5) were used to assess their influence on Cobalt (Co^{+2}) -binding capacity of HSA. The approach used was adapted and modified from experiments as described by Lu *et al.,* 2012. Briefly, the procedure was as described below. Furthermore, ITC studies were conducted to elucidate the cobalt-switch via free fatty acid binding to HSA.

2.3 Methods

2.3.1 Materials

The fatty acid/salts used for the ACB experiments included; caprylate, caprate, laurate, myristate, palmitate and stearate. Essentially fatty acid-free lyophilised albumin from human serum was also used. Other reagents and aqueous solutions used included cobalt chloride, 1,4, dithiothreitol (DTT). These reagents were purchased from Sigma-Aldrich, Poole, UK. The buffer used in these experiments was NH_4HCO_3 and the dialysing solution was $(NH_4)_2CO_3$ – both of which were purchased from ACROS Organics, New Jersey, USA. NaCl was purchased from Fisher Scientific, Leicester, UK) solution.

The ITC experiments were performed to assess the effect of medium-chain and long-chain fatty acids on the binding of cobalt to HSA. A MicroCal iTC200 instrument (Malvern, Malvern, UK) was used for the ITC experiments. Cobalt chloride and the fatty acids, namely caprylate and myristate were used. Essentially fatty acid-free lyophilized albumin from human serum, which was extensively dialysed in buffer solution was used for the binding studies. The buffer used comprised of 50 mM Tris (tris(hydroxymethyl)aminomethane) and 140 mM NaCl buffer, pH 7.4.

2.3.2 Procedure for the ACB assay

Commercially purchased essentially fat-free HSA was extensively dialysed in 100 mM $(NH_4)_2CO_3$ to remove any bound divalent metal ions. A solution of 100 µM dialysed HSA was prepared in 10

mM NH_4HCO_3, pH 7.8. For each fatty acid studied, concentrations of 0, 1, 2, 3, 4, and 5 molar equivalents of HSA were prepared and added to the HSA solution. The samples were incubated for approximately 2 hours to allow for adequate interactions between the fatty acids and albumin. The samples were assayed in triplicate with a corresponding blank (200 µl each). Following this, 50 µl of the cobalt chloride (1.28 mM to make up final concentrations of 0.256 mM) was added to each sample and allowed to stand for 10 minutes. Then, 50 µl of DTT solution (1.62 mM to make up final concentrations of 0.27 mM) was added to the solutions except for the blank sample. After an incubation time of two minutes, the reaction was quenched by adding 1 ml of 0.9% NaCl solution. The results (colour change) was then read using a spectrophotometer at a wavelength of 470 nm and the absorbance was subtracted from the blank sample (without DTT).

The ITC experiments performed were assessing the binding activity of cobalt to HSA in the presence of caprylate and myristate. Basically, 1.5 mM $CoCl_2$ was added into 60 µM HSA in the presence of 0-5 molar equivalents (mol. eq.) of caprylate and myristate. ITC experiments were conducted with a rotatory speed of 750 rpm. A total of 19 injections were recorded for each experiment, with the first injection of 0.4 μl in volume over 0.8 s and spacing of 120 s with the second injection. Subsequent 18 further injections of 2 μl over 5.0 s with a spacing of 150 s were further performed. The equilibrium temperature of 25 ℃ was conserved between the cells. Heats of dilution were accounted for with blank titrations performed by injecting ligand solution into reaction buffer and subtracting the averaged heat of dilution from the main experiments.

2.3.3 Analysis of results

Results of the ACB assays were recorded into an excel database. The analysis was performed by plotting the mean absorbance (SD) against the fatty acid concentration. Graphs for the different fatty acids studied, and the different concentrations were compared to determine differences in their influence on the metal binding capacity of albumin. Statistical analysis was performed using STATA 12 and GraphPad Prizm software. One-way analysis of variance (ANOVA) was used to assess the difference in absorbance at repeated measurements at different fatty acid concentrations. The Pearson correlation test was used to measure the correlation between changes in absorbance with a change in fatty acid chain length, and changes in absorbance with a change in fatty acid concentration. The t-test was used to assess the differences in the mean absorbance values between

any two of the fatty acids or different concentrations of the same fatty acids. A p-value of <0.05 was considered statistically significant. Data analysis for the ITC studies was initially performed and fitted using Origin 7 software (OriginLab, MA, USA), then the data points were fitted and re-plotted using Microsoft Excel.

2.4 Results

2.4.1 Effects of fatty acids on the ACB assay

Using the ACB assay, the influence of the following fatty acids; caprylate, caprate, and laurate (medium chain fatty acids), myristate, palmitate and stearate (long chain fatty acids) on the ACB assay were assessed). The assays were done to assess the different effects, if any, of the different fatty acids chain lengths at different concentrations on the ACB assay. Figure 2.5 shows a series of bar graphs depicting the influence of the medium chain and long-chain saturated fatty acids on the ACB assay.

For the medium-chain saturated fatty acids assayed in the experiments caprylate, caprate and laurate, there was no statistically significant difference in the absorbance with an increase in the fatty acid concentration using ANOVA. Similarly, using the paired t-test, there was no statistical difference in absorbance between 5 molar equivalents and 1 molar equivalent of fatty acid concentration for caprylate, caprate and laurate.

On the contrary, the long fatty acids which are usually more prevalent in human plasma showed significant difference across the different molar equivalents. For myristate, there was a statistically significant difference in the mean milli absorbance (mA) at 1 molar equivalent (2 mA) and 5 molar equivalents (18.3 mA) ($p<0.01$). Nevertheless, the paired ANOVA test for change in absorbance across the different concentrations of myristate was not statistically significant. Palmitate, on the other hand, exhibited a dose-dependent increase in absorbance with an increase in free fatty acid concentration. There was a statistically significant increase in the mean absorbance using ANOVA test from 1 to 5 molar equivalents of palmitate ($p=0.03$), and a direct comparison between 1 molar and 5 molar equivalents also showed a statistical significance using a paired t-test ($p=0.01$). Similarly, for stearate, there was a significant positive change in mean absorbance with an increase in concentration ($p=0.02$), and the significant difference was also detected between 1 (8 mA units) and 5 molar equivalents (27.3 mA units) of stearate using the t-test ($p=0.04$) see Figure 2.5.

The next step for this experimental study was to assess the association between fatty acid chain length and change in absorbance, thereby assessing whether different fatty acids of different chain length affect the ACB assay differently. Figure 2.6 shows a bar chart of the change in absorbance against the fatty acid chain length. Absorbance at 5 molar equivalent values were used for each FFA used in the assay. As hypothesised, it was observed that there was a positive relationship between the fatty acid chain length and absorbance as detected by the ACB assay which was statistically significant ($p=0.03$). Thus, the longer chain fatty acids influenced the ACB assay more than short-chain fatty acids.

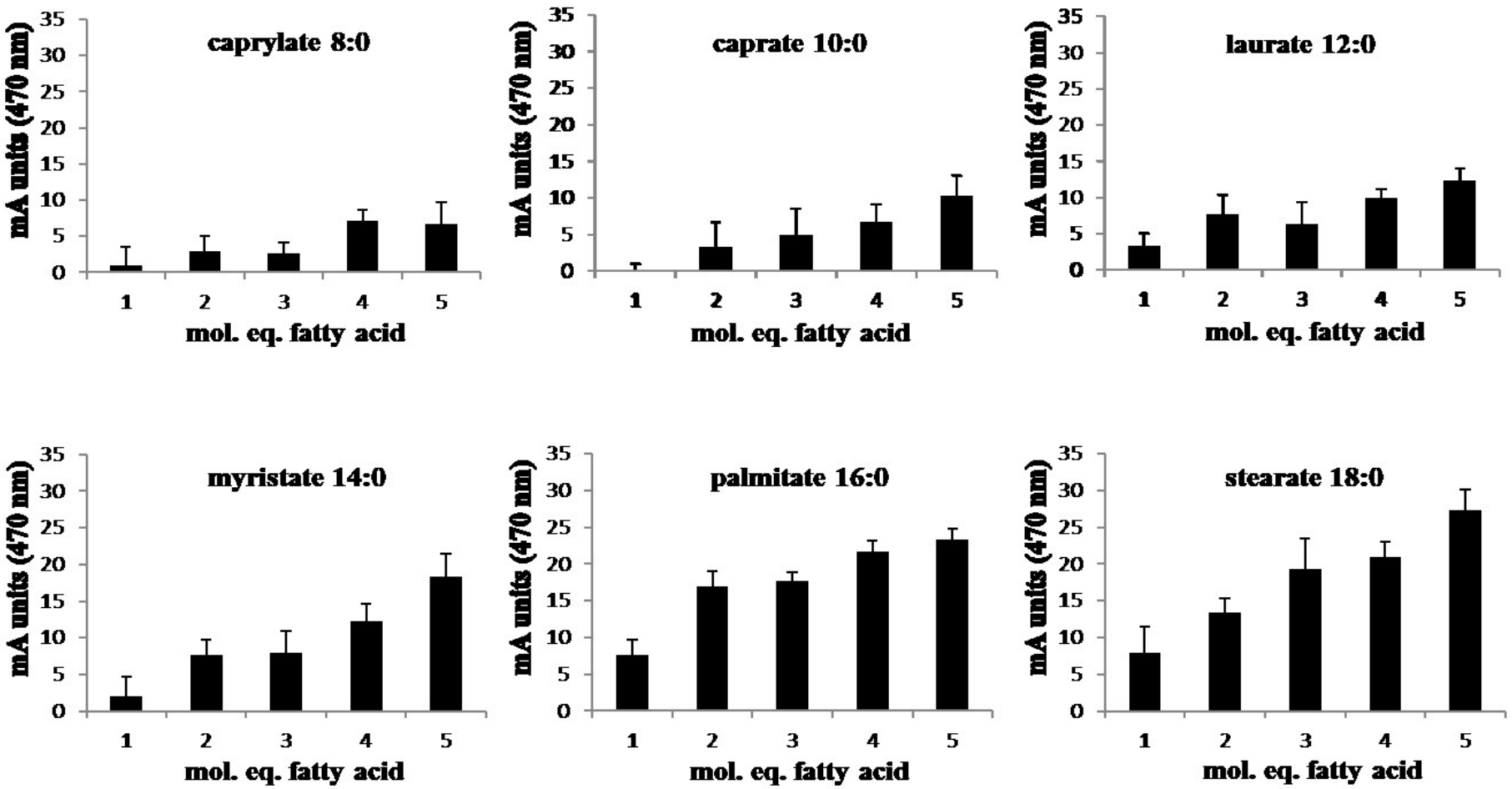


Figure 2.5 ACB assays carried out in the presence of 1-5 mol. eq. of various saturated FFAs. Results are expressed as changes in absorbance relative to a FFA-free control. Assays were performed in triplicate. Error bars represent S.E.M. (mol. Eq. = molar equivalent; mA = milli absorbance).

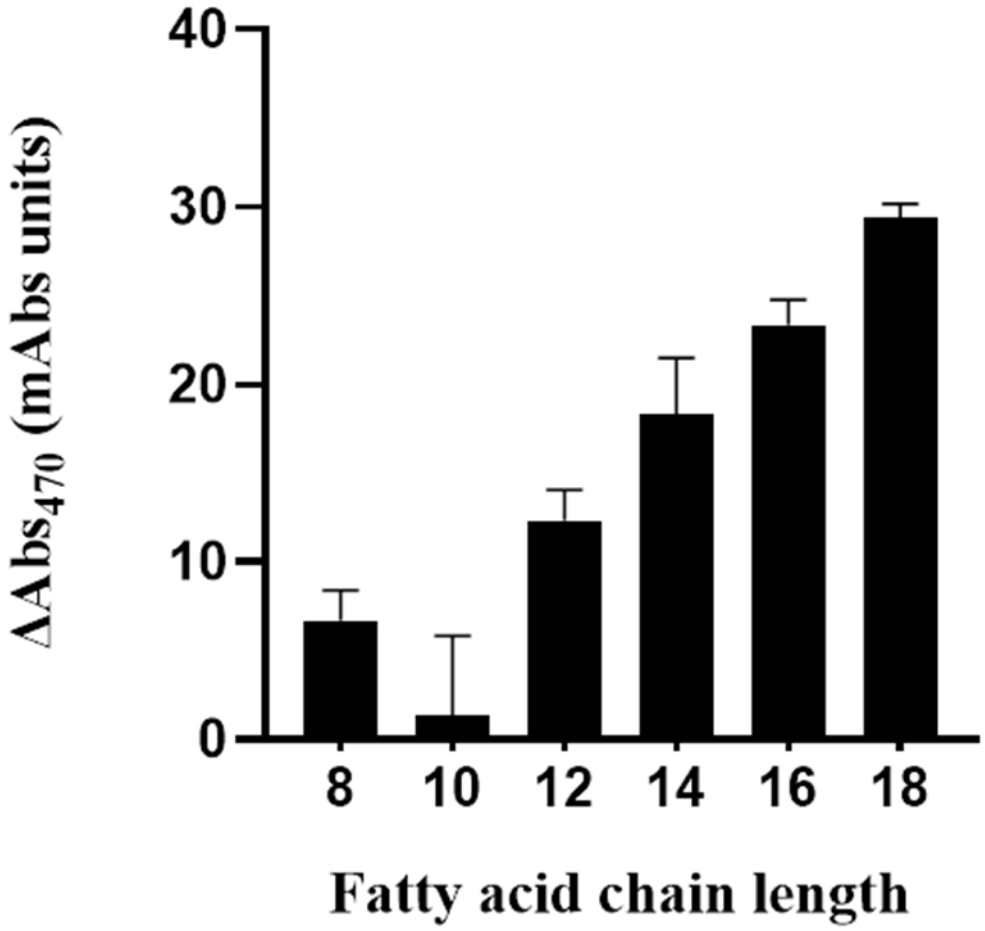


Figure 2.6 Association between fatty acid chain length at 5 molar equivalent concentration and change in absorbance in the ACB assay. Increase in fatty acid chain length was associated with a statistically significant increase in the absorbance using the Spearman's test for correlation ($p=0.03$).

To support the ACB assay results, ITC experiments were conducted to assess the effect of a medium chain length fatty acid (caprylate) and a long chain fatty acid (myristate) on the binding ability of Co^{2+} to sites B, A and the NTS motif on HSA. A "three sets-of-sites" model was selected to monitor potential changes to the stoichiometry. The fitted isotherms are shown in Figures 2.7 and 2.8 for caprylate and myristate, respectively.

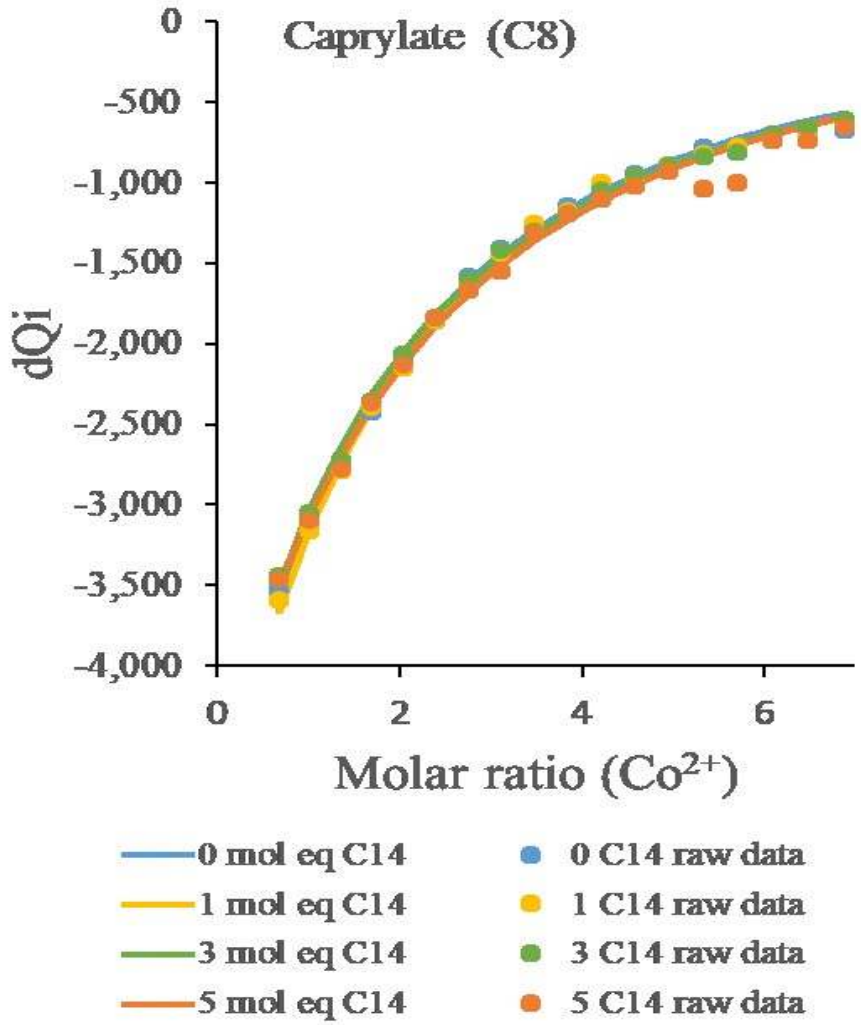


Figure 2.7 ITC data corresponding to the binding of Co^{2+} to HSA in the presence of caprylate. ITC experiments were conducted with 1.5 mM $CoCl_2$ titrated into 60 µM HSA, in the presence of either 0 (blue), 1 (yellow), 3 (green), or 5 (brown) molar equivalents (mol eq) of caprylate. There was no noticeable perturbation in the binding of the Co^{2+} with the different caprylate concentrations even at 5 molar equivalents. The binding constants at the three binding sites for 0 mol eq were $3.37 \times 10^4 \pm 1.28 \times 10^4$, $1.78 \times 10^4 \pm 4.36 \times 10^3$ and 737 ± 205; for 1 mol eq, they were $5.15 \times 10^4 \pm 1.62 \times 10^4$, $6.45 \times 10^3 \pm 2.15 \times 10^3$ and 870 ± 215; for 3 mol eq, the binding constants were $4.52 \times 10^4 \pm 1.04 \times 10^3$, $8.85 \times 10^3 \pm 1.02 \times 10^3$ and 902 ± 112; and for 5 mol eq, the binding constants were $5.86 \times 10^4 \pm 1.23 \times 10^4$, $6.86 \times 10^3 \pm 1.13 \times 10^3$ and 401 ± 88.7 respectively.

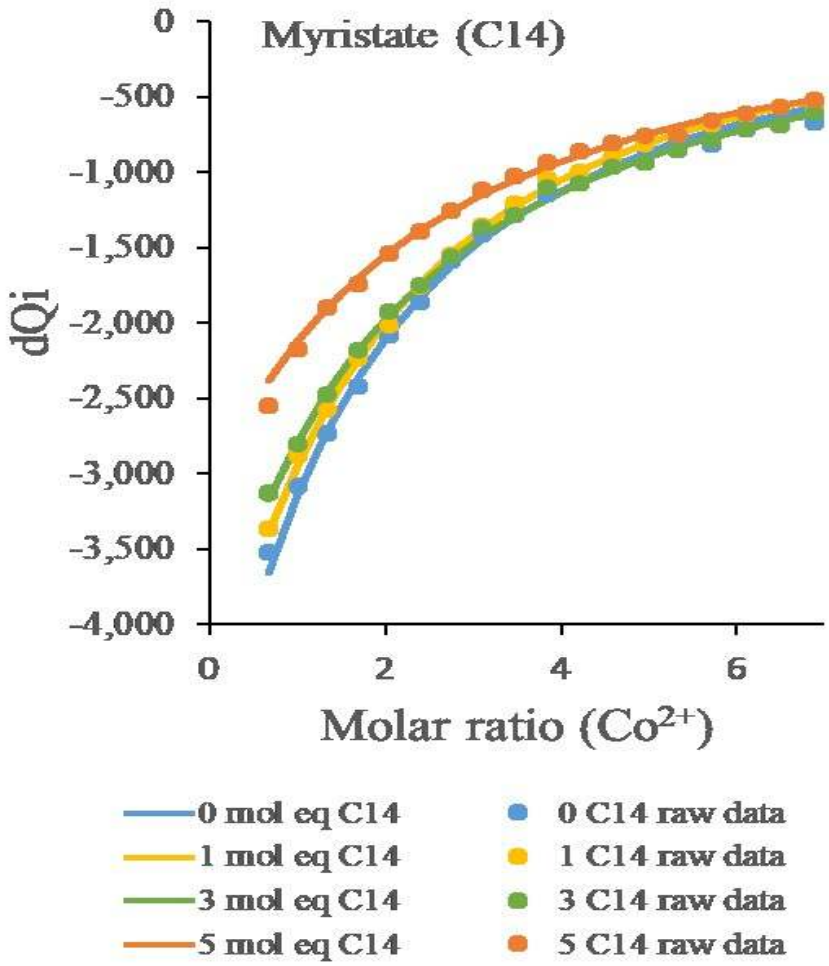


Figure 2.8 ITC data corresponding to the binding of Co^{2+} to HSA in the presence of Myristate. ITC experiments were conducted with 1.5 mM $CoCl_2$ titrated into 60 µM HSA, in the presence of either 0 (blue), 1 (yellow), 3 (green), or 5 (brown) molar equivalents of myristate. Co^{2+} binding to the HSA was perturbed with an increase in myristate concentration as indicated by the shift of the curves to the left. Maximal and significant perturbation was observed at 5 molar equivalents of myristate. The binding constants at the three binding sites for 0 mol eq were 4.77 x $10^4 \pm 1.02$ x 10^4, 5.16 x $10^4 \pm 1.13$ x 10^3 and 1.28 $10^3 \pm 2.72$ x 10^2; for 1 mol eq, they were 6.69 x $10^4 \pm 2.36$ x 10^4, 7.03 x $10^3 \pm 2.97$ x 10^3 and 1.61 x $10^3 \pm 495$; for 3 mol eq, the binding constants were 6.33 x $10^3 \pm 1.31$ x 10^3, 786 ± 263 and 200 ± 47.4; and for 5 mol eq, the binding constants were 9.01 x $10^3 \pm 3.37$ x 10^3, 157 ± 43.8 and 1.25 x $10^5 \pm 5.18$ x 10^4 respectively.

2.5 Discussion

The results on the effects of different FFA of different chain lengths and concentrations on ACB assays have indicated that long-chain fatty acids, unlike the medium-chain free fatty acids, exert a negative effect on the binding of Co^{2+} to HSA. Furthermore, the ACB assay appears to be influenced by the FFA in a concentration-dependent manner where higher concentrations affected the assay more than lower concentrations. Also, the ITC results in this study showed that caprylate, a medium-chain fatty acid, did not have a significant effect on the ability of HSA to bind Co^{2+}. Nevertheless, the ITC studies suggested that long-chain fatty acids, as in the case of myristate used in the experiments perturbed the binding of Co^{2+} to HSA with a significant and maximal effect observed at 5 molar equivalents.

Fatty acids are essential substrates for metabolism and are a significant source of energy for the heart and skeletal muscle (Coverdale et al., 2018a). Long-chain saturated fatty acids such as myristate, palmitate and stearate form a major proportion of free fatty acids in plasma in healthy adult plasma (Abdelmagid et al., 2015). In normal physiological states, an HSA molecule can bind 0.1 – 2 fatty acid molecules (Peters Jr, 1995). However, up to 6-7 fatty acids may bind to HSA during stressful and pathological conditions (Simard et al., 2006, Peters Jr, 1995, Fanali et al., 2012). Thus, the higher concentrations of free fatty acids exhibited in the different pathological and conditions described previously including CVD, diabetes, fatty liver disease, chronic kidney disease, metabolic syndrome and cancer (Kovacs and Stumvoll, 2005, Vernon et al., 2011, Wu et al., 2010, Pirro et al., 2002b, Blindauer et al., 2016, Liao et al., 2012) may saturate the FFA binding sites on albumin. Thus, the perturbation in the binding capacity of albumin for the metal ions is plausible, and the results of the *in vitro* experiments of this study may represent actual phenomena that occur *in vivo*.

The result of this study, which suggested that longer chain fatty acids have a more significant perturbing effect on Co^{2+} binding, is of interest. It confirms results of previous studies which used myristic acid and assessed its effect on the ACB assay using bovine serum albumin (Lu et al., 2012). In the present study, however, HSA was used, which is the type of albumin in humans and hence may provide a more clinically relevant representation. Furthermore, a comparison of the variety of medium and long chain fatty acids confirms the hypothesis that the long chain fatty acids have stronger effect in perturbing the binding of divalent metal ions including Co^{2+} and similar cations such as Zn^{2+} to HSA relatively more than the shorter chain fatty acids. More significantly,

both the ACB assays and the ITC experiments strongly support the current view regarding the mechanistic basis of the ACB assay that it reflects increased serum FFA (Oran and Oran, 2017, Coverdale et al., 2019a, Stewart and Blindauer, 2015).

One of the explanations to the stronger effect of the long chain fatty acids would be related to their relatively higher avidity for the FA2 binding site. It has been reported previously that the binding affinity of saturated fatty acids of carbon chain lengths 6 - 18 are positively related with their chain lengths (Spector, 1975). It is also well-known, as described earlier, that among the identified FA binding sites on HSA FA2 is considered a higher affinity fatty acid binding site together with FA4 and FA5 in comparison to FA1, FA3, FA6 and FA7 (Simard et al., 2006, Rizzuti et al., 2015). FA2 and metal binding site A both exist at the interface between domain I and II of the HSA (Lu et al., 2012). The binding of FFA to the FA2 can causes rotatory changes to domain I of HSA in relation to domain II and disturb the normal physiological alignment of the amino acid residues His247, N99, D249, H67 and their relative distance from each other may be widened (Fanali et al., 2012). Thus, due to the avidity for FA2 and the geographical positioning of site A, longer chain FFA may perturb the binding of metal ions such as Co^{2+} and as Zn^{2+} at this site. Since long-chain saturated fatty acids such as myristate, palmitate and stearate form a significant proportion of free fatty acids in plasma in healthy adult plasma (Abdelmagid et al., 2015), an increase in the concentration of these fatty acids can potentially exert unsolicited disturbances in the metal ion buffering by HSA.

Unlike the long-chain fatty acids, short-chain fatty acids (<C8) were previously thought to be too short to exert a similar effect as that of the long-chain fatty acids and (Bhattacharya et al., 2000). More recent studies, however, and molecular modelling suggest that the half-pocket in domain II of HSA is sufficient to accommodate octanoate and hence does not result in the disruption of the site A as it does not cause interdomain distance changes (Lu et al., 2012).

The results of the present study also suggested a FFA concentration-dependent relationship to the disturbance in metal ion binding to HSA. In normal physiological conditions, the longchain FFA concentrations in plasma may be as low as 16 μM but may be elevated in pathological conditions (Abdelmagid et al., 2015). In pathological conditions such cancer, diabetes, cardiovascular diseases and chronic renal diseases, and even during infection such as sepsis, FFA plasma concentrations can increase due to different mechanisms (Coverdale et al., 2018a). The

concentrations of up to 5 molar equivalents used in the experimental ACB assays of this study reflect what can occur in pathological conditions. Often, patients may have multiple co-morbidities, whereby patients with diabetes may also have cardiovascular diseases, and patients with HIV infection are known to be predisposed to NCDs - hence the risk for higher level of plasma FFA (Myerson et al., 2015, van Heerden et al., 2017, Srinivasa and Grinspoon, 2014). In such cases, there may be an amplified risk for poor buffering of metal ions such as Zn^{2+} and propensity for prothrombotic activity (Vu et al., 2013,
Lu et al., 2012). Patients may, therefore, be predisposed an increased risk of consequences such as cardiovascular and cerebrovascular events (Kelesidis and Currier, 2014, Benjamin et al., 2016, Feigin et al., 2014).

As alluded to previously, the preferential binding site for Co^{2+} is site B, and unlike Zn^{2+}, can also bind to the NTS (Lu et al., 2012). It is not surprising that FFA at a concentration of 5 molar equivalents of myristate (Figure 2.5), as depicted in the present study, the FFA did not severely or entirely inhibit the binding of Co^{2+} to albumin. However, 5 molar equivalents of myristate have been shown to almost completely inhibit the binding of Zn^{2+} whose primary binding site is site A and hence much affected by FFA binding to FA2 (Lu et al., 2012, Kassaar et al., 2015). Thus, unlike Co^{2+}-binding, Zn^{2+}-binding is be more severely affected by increased FFA concentrations in plasma, and hence more likely to influence potential pathophysiological effects (Coverdale et al., 2018a). The results of this study, therefore, points towards the importance of further research aimed at elucidating the potential adverse effects of allosteric switch of important physiological metal ions such as Zn^{2+} in disease states.

REFERENCES FOR CHAPTER 2

1. ABDELMAGID, S. A., CLARKE, S. E., NIELSEN, D. E., BADAWI, A., ELSOHEMY, A., MUTCH, D. M. & MA, D. W. L. 2015. Comprehensive Profiling of Plasma Fatty Acid Concentrations in Young Healthy Canadian Adults. *PLoS ONE,* 10**,** 1.

2. APPLE, F. S., WU, A. H. B., MAIR, J., RAVKILDE, J., PANTEGHINI, M., TATE, J., PAGANI, F., CHRISTENSON, R. H., MOCKEL, M., DANNE, O. & JAFFE, A. S. 2005. Future biomarkers for detection of ischemia and risk stratification in acute coronary syndrome. *Clinical chemistry,* 51**,** 810.

3. ARNARDOTTIR, E. S., MACKIEWICZ, M., GISLASON, T., TEFF, K. L. & PACK, A. I. 2009. Molecular Signatures of Obstructive Sleep Apnea in Adults: A Review and Perspective. *Sleep,* 32**,** 447-470.

4. ASHOK KUMAR, P. & ANAND, U. 2016. Multiple Biomarkers to Assess the Pathophysiological State in Critically Ill Patients with Sepsis. *Indian Journal of Clinical Biochemistry,* 31**,** 310-314.

5. BAR-OR, D., LAU, E. & WINKLER, J. V. 2000. A novel assay for cobalt-albumin binding and its potential as a marker for myocardial ischemia-a preliminary report. *The Journal of emergency medicine,* 19**,** 311.

6. BAR-OR, D., RAEL, L. T., BAR-OR, R., SLONE, D. S., MAINS, C. W., RAO, N. K. & CURTIS, C. G. 2008. The cobalt-albumin binding assay: insights into its mode of action. *Clinica chimica acta; international journal of clinical chemistry,* 387**,** 120.

7. BASU, A., PASSMORE, R. & STRONG, J. A. 1960. The effect of exercise on the level of non-esterified fatty acids in the blood. *Quarterly journal of experimental physiology and cognate medical sciences,* 45**,** 312.

8. BENJAMIN, L. A., CORBETT, E. L., CONNOR, M. D., MZINGANJIRA, H., KAMPONDENI, S., CHOKO, A., HOPKINS, M., EMSLEY, H. C., BRYER, A., FARAGHER, B., HEYDERMAN, R. S., ALLAIN, T. J. & SOLOMON, T. 2016. HIV,

   antiretroviral treatment, hypertension, and stroke in Malawian adults: A case-control study. *Neurology,* 86**,** 324.

9. BEYAZIT, F., YILMAZ, N., BALCI, O., ADAM, M. & YAMAN, S. T. 2016. Evaluation of Oxidative Stress in Women with Polycystic Ovarian Syndrome as Represented by Serum Ischemia Modified Albumin and Its Correlation with Testosterone and Insulin Resistance. *Internal Medicine,* 55**,** 2359-2364.

10. BHAGAVAN, N. V., HA, J. S., PARK, J. H., HONDA, S. A., RIOS, C. N., SUGIYAMA, C., FUJITANI, G. K., TAKEUCHI, I. K. & HA, C. E. 2009. Utility of serum Fatty Acid concentrations as a marker for acute myocardial infarction and their potential role in the formation of ischemia-modified albumin: a pilot study. *Clinical chemistry,* 55**,** 1588.

11. BHAGAVAN, N. V., LAI, E. M., RIOS, P. A., YANG, J., ORTEGA-LOPEZ, A. M., SHINODA, H., HONDA, S. A., RIOS, C. N., SUGIYAMA, C. E. & HA, C. E. 2003. Evaluation of human serum albumin cobalt binding assay for the assessment of myocardial ischemia and myocardial infarction. *Clinical chemistry,* 49**,** 581.

12. BHATTACHARYA, A. A., GRÜNE, T. & CURRY, S. 2000. Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin. *Journal of Molecular Biology,* 303**,** 721.

13. BLINDAUER, C. A., KHAZAIPOUL, S., YU, R. & STEWART, A. J. 2016. Fatty Acid-Mediated Inhibition of Metal Binding to the Multi-Metal Site on Serum Albumin:

    Implications for Cardiovascular Disease. *Current topics in medicinal chemistry,* 16**,** 3021.

14. CAN, M., DEMIRTAS, S., POLAT, O. & YILDIZ, A. 2006. Evaluation of effects of ischaemia on the albumin cobalt binding (ACB) assay in patients exposed to trauma. *Emergency medicine journal : EMJ,* 23**,** 537.

15. CAPURSO, C. & CAPURSO, A. 2012. From excess adiposity to insulin resistance: the role of free fatty acids. *Vascular pharmacology,* 57**,** 91.

16. CHEN, R., HAN, S., DONG, D., WANG, Y., LIU, Q., XIE, W., LI, M. & YAO, M. 2015a. Serum fatty acid profiles and potential biomarkers of ankylosing spondylitis determined by gas chromatography–mass spectrometry and multivariate statistical analysis. *Biomedical Chromatography,* 29**,** 604-611.

17. CHEN, R., LIN, L., TIAN, J. W., ZENG, B., ZHANG, L., CHEN, X. & YAN, H. Y. 2015b. Predictors of dynamic hyperinflation during the 6-minute walk test in stable chronic obstructive pulmonary disease patients. *Journal of thoracic disease,* 7**,** 1142.

18. CHO, D. K., CHOI, J.-H., KIM, S. H., CHOI, J., RHEE, I., KI, C.-S., LEE, S.-C. & GWON, H.-C. 2007. Ischemia-modified albumin is a highly sensitive serum marker of transient myocardial ischemia induced by coronary vasospasm. *Coronary Artery Disease,* 18**,** 83-87.

19. ÇOLAK, T., BAMAÇ, B., ÇOLAK, S., DUMAN, C., BAYAZIT, B., ÖZTÜRK, S., MERIÇ, B., ÖZBEK, A. & YILDIZ, F. 2010. The Influence of a Single Bout of Wrestling Exercise on Serum Levels of Ischemia-modified Albumin. *Journal of Exercise Science & Fitness,* 8**,** 67.

20. COVERDALE, J. P. C., KATUNDU, K., SOBCZAK, A. I. S., ARYA, S., BLINDAUER, C. A. & STEWART, A. J. 2018a. Ischemia-modified albumin: crosstalk between fatty acid and cobalt binding. *Prostaglandins, Leukotrienes and Essential Fatty Acids*.

21. COVERDALE, J. P. C., KATUNDU, K. G. H., SOBCZAK, A. I. S., ARYA, S., BLINDAUER, C. A. & STEWART, A. J. 2018b. Ischemia-modified albumin: Crosstalk between fatty acid and cobalt binding. *Prostaglandins, leukotrienes, and essential fatty acids,* 135**,** 147.

22. COVERDALE, J. P. C., KHAZAIPOUL, S., ARYA, S., STEWART, A. J. & BLINDAUER, C. A. 2019a. Crosstalk between zinc and free fatty acids in plasma. *Biochimica et biophysica acta.Molecular and cell biology of lipids,* 1864**,** 532.

23. COVERDALE, J. P. C., KHAZAIPOUL, S., ARYA, S., STEWART, A. J. & BLINDAUER, C. A. 2019b. Crosstalk between zinc and free fatty acids in plasma. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* 1864**,** 532-542.

24. CROWE, T. 2012. Oxford Handbook of Nutrition and Dietetics - By J Webster-Gandy, A Madden and M Holdsworth. *Nutrition & Dietetics,* 69**,** 316.

25. DUAN, X. X., ZHANG, G. P., WANG, X. B., YU, H., WU, J. L., LIU, K. Z., WANG, L. & LONG, X. 2017. Elevated Serum and Cerebrospinal Fluid Free Fatty Acid Levels Are Associated with Unfavorable Functional Outcome in Subjects with Acute Ischemic Stroke. *Molecular neurobiology,* 54**,** 1677.

26. DUSEK, J., TICHY, M., STASEK, J., BIS, J., GREGOR, J., PUDIL, R. & VOJACEK, J. 2005. Ischemia-modified albumin: new marker of myocardial ischemia? *Casopis lekaru ceskych,* 144**,** 295.

27. EBBESSON, S. O. E., TEJERO, M. E., LÓPEZ-ALVARENGA, J. C., HARRIS, W. S., EBBESSON, L. O. E., DEVEREUX, R. B., MACCLUER, J. W., WENGER, C., LASTON, S., FABSITZ, R. R., HOWARD, B. V. & COMUZZIE, A. G. 2010. Individual saturated fatty acids are associated with different components of insulin resistance and glucose metabolism: the GOCADAN study. *International journal of circumpolar health,* 69**,** 344.

28. ERTEKIN, B., KOCAK, S., DEFNE DUNDAR, Z., GIRISGIN, S., CANDER, B., GUL, M., DOSEYICI, S., MEHMETOGLU, I. & KEMAL SAHIN, T. 2013. Diagnostic value of ischemia-modified albumin in acute coronary syndrome and acute ischemic stroke. *Pakistan journal of medical sciences,* 29**,** 1003.

29. FANALI, G., DI MASI, A., TREZZA, V., MARINO, M., FASANO, M. & ASCENZI, P. 2012. Human serum albumin: From bench to bedside. *Molecular aspects of medicine,* 33**,** 209.

30. FEIGIN, V. L., FOROUZANFAR, M. H., KRISHNAMURTHI, R., MENSAH, G. A., CONNOR, M., BENNETT, D. A., MORAN, A. E., SACCO, R. L., ANDERSON, L.,

    TRUELSEN, T., O'DONNELL, M., VENKETASUBRAMANIAN, N., BARKERCOLLO,S., LAWES, C. M., WANG, W., SHINOHARA, Y., WITT, E., EZZATI, M., NAGHAVI, M., MURRAY, C., GLOBAL BURDEN OF DISEASES, I., RISK

    FACTORS, S. & THE, G. B. D. S. E. G. 2014. Global and regional burden of stroke during 1990-2010: findings from the Global Burden of Disease Study 2010. *Lancet (London, England),* 383**,** 245.

31. GAEMERS, I. C. & GROEN, A. K. 2006. New insights in the pathogenesis of nonalcoholic fatty liver disease. *Current opinion in lipidology,* 17**,** 268.

32. GHOSH, K., MUDDESHWAR, M. G., LOKHANDE, M. & GHOSH, K. 2017. Albumin Cobalt Binding or Ischaemia Modified Albumin: a Test of Great Prognostic Value in Malaria. *Mediterranean journal of hematology and infectious diseases,* 9**,** e2017041.

33. GUPTA, S., SEYDEL, K., MIRANDA-ROMAN, M. A., FEINTUCH, C. M., SAIDI, A., KIM, R. S., BIRBECK, G. L., TAYLOR, T. & DAILY, J. P. 2017. Extensive alterations of blood metabolites in pediatric cerebral malaria. *PloS one,* 12**,** e0175686.

34. GURUMURTHY, P., BORRA, S. K., YERUVA, R. K., VICTOR, D., BABU, S. & CHERIAN, K. M. 2014. Estimation of Ischemia Modified Albumin (IMA) Levels in Patients with Acute Coronary Syndrome. *Indian journal of clinical biochemistry: IJCB,* 29**,** 367.

35. HUBEL, C. A., MCLAUGHLIN, M. K., EVANS, R. W., HAUTH, B. A., SIMS, C. J. & ROBERTS, J. M. 1996. Fasting serum triglycerides, free fatty acids, and malondialdehyde are increased in preeclampsia, are positively correlated, and decrease within 48 hours postpartum. *American Journal of Obstetrics and Gynecology,* 174**,** 975.

36. IŞıK, S., KıLıÇ, S., ÖĞRETMEN, Z., ÇAKıR, D. Ü., TÜRKÖN, H., CEVIZCI, S. & HIZ, M. M. 2016. The correlation between the psoriasis area severity index and ischemia-modified albumin, mean platelet volume levels in patients with psoriasis. *Advances in Dermatology and Allergology/Postępy Dermatologii i Alergologii,* 33**,** 290-293.

37. JUN, J. C., DRAGER, L. F., NAJJAR, S. S., GOTTLIEB, S. S., BROWN, C. D., SMITH, P. L., SCHWARTZ, A. R. & POLOTSKY, V. Y. 2011. Effects of Sleep Apnea on Nocturnal Free Fatty Acids in Subjects with Heart Failure. *Sleep,* 34**,** 1207-1213.

38. KARADENIZ, O., MENDILCIOGLU, I., OZDEM, S., OZEKINCI, M., SANHAL, C. Y., UZUN, G., SAKINCI, M. & SIMSEK, M. 2015. The association between ischaemia-modified albumin levels in umbilical vein and intrauterine growth restriction. *Journal of Obstetrics and Gynaecology,* 35**,** 9-12.

39. KASSAAR, O., SCHWARZ-LINEK, U., BLINDAUER, C. A. & STEWART, A. J. 2015. Plasma free fatty acid levels influence Zn2+-dependent histidine-rich glycoprotein-heparin interactions via an allosteric switch on serum albumin. *Journal of Thrombosis & Haemostasis,* 13**,** 101.

40. KELESIDIS, T. & CURRIER, J. S. 2014. Dyslipidemia and cardiovascular risk in human immunodeficiency virus infection. *Endocrinology and metabolism clinics of North America,* 43**,** 665.

41. KIYICI, A., MEHMETOGLU, I., KARAOGLAN, H., ATALAY, H., SOLAK, Y. & TURK, S. 2010. Ischemia-modified albumin levels in patients with end-stage renal disease patients on hemodialysis: does albumin analysis method affect albuminadjusted ischemia-modified albumin levels? *Journal of clinical laboratory analysis,* 24**,** 273.

42. KOVACS, P. & STUMVOLL, M. 2005. Fatty acids and insulin resistance in muscle and liver. *Best practice & research.Clinical endocrinology & metabolism,* 19**,** 625.

43. KUCUK, A., UĞUR USLU, A., ICLI, A., CURE, E., ARSLAN, S., TURKMEN, K., TOKER, A. & KAYRAK, M. 2017. The LDL/HDL ratio and atherosclerosis in ankylosing spondylitis. *Zeitschrift für Rheumatologie,* 76**,** 58-63.

44. KUMAR, P. A. & SUBRAMANIAN, K. 2016. The Role of Ischemia Modified Albumin as a Biomarker in Patients with Chronic Liver Disease. *Journal of clinical and diagnostic research: JCDR,* 10**,** BC09.

45. LAKUSTA, H. & SARKAR, B. 1979. Equilibrium studies of zinc(II) and cobalt(II) binding to tripeptide analogues of the amino terminus of human serum albumin. *Journal of Inorganic Biochemistry,* 11**,** 303-315.

46. LEITEMPERGUER, M. R., TATSCH, E., KOBER, H., DE CARVALHO, J. A. M., MORESCO, R. N. & DA SILVA, J. E. P. 2014. Assessment of ischemia-modified albumin levels in patients with rheumatoid arthritis. *Clinical Laboratory,* 60**,** 10651070.

47. LIANG, H., HUANG, J., TU, C. Q., ZHANG, M., ZHOU, Y. Q. & SHEN, P. W. 2001. The subsequent effect of interaction between Co(2+) and human serum albumin or bovine serum albumin. *Journal of inorganic biochemistry,* 85**,** 167.

48. LIAO, M. T., SUNG, C. C., HUNG, K. C., WU, C. C., LO, L. & LU, K. C. 2012. Insulin resistance in patients with chronic kidney disease. *Journal of biomedicine & biotechnology,* 2012**,** 691369.

49. LIU, J., MAZZONE, P. J., CATA, J. P., KURZ, A., BAUER, M., MASCHA, E. J. & SESSLER, D. I. 2014. Serum free fatty acid biomarkers of lung cancer. *Chest,* 146**,** 670.

50. LOPASCHUK, G. D., COLLINS-NAKAI, R., OLLEY, P. M., MONTAGUE, T. J., MCNEIL, G., GAYLE, M., PENKOSKE, P. & FINEGAN, B. A. 1994. Plasma fatty acid levels in infants and adults after myocardial ischemia. *American Heart Journal,* 128**,** 61-67.

51. LOPASCHUK, G. D., USSHER, J. R., FOLMES, C. D. L., JASWAL, J. S. & STANLEY, W. C. 2010. Myocardial Fatty Acid Metabolism in Health and Disease. *Physiological Reviews,* 90**,** 207.

52. LORIA, V., LEO, M., BIASILLO, G., DATO, I. & BIASUCCI, L. M. 2008. Biomarkers in Acute Coronary Syndrome. *Biomarker insights,* 3**,** 453.

53. LU, J., STEWART, A. J., SADLER, P. J., PINHEIRO, T. J. T. & BLINDAUER, C. A. 2012. Allosteric inhibition of cobalt binding to albumin by fatty acids: implications for the detection of myocardial ischemia. *Journal of medicinal chemistry,* 55**,** 4425.

54. MERINO SALVADOR, M., GÓMEZ DE CEDRÓN, M., MORENO RUBIO, J., FALAGÁN MARTÍNEZ, S., SÁNCHEZ MARTÍNEZ, R., CASADO, E., RAMÍREZ DE MOLINA, A. & SERENO, M. 2017. Lipid metabolism and lung cancer. *Critical Reviews in Oncology/Hematology,* 112**,** 31.

55. MIN-TSER LIAO, C.-C. S. K.-C. H. C.-C. W. L. L. & KUO-CHENG, L. 2012. Insulin Resistance in Patients with Chronic Kidney Disease. *Journal of Biomedicine and Biotechnology,* 2012.

56. MOTHES, E. & FALLER, P. 2007. Evidence that the principal CoII-binding site in human serum albumin is not at the N-terminus: implication on the albumin cobalt binding test for detecting myocardial ischemia. *Biochemistry,* 46**,** 2267.

57. MUHTAROGLU, S., BARLAK KETI, D. & UNLUHIZARCI, K. 2016. Investigation of ischemia-modified albumin levels and some atherosclerosis-related serum parameters in patients with diabetic foot. *Turkish journal of medical sciences,* 46**,** 126.

58. MUSSO, G., GAMBINO, R. & CASSADER, M. 2009. Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD). *Progress in lipid research,* 48**,** 1.

59. MYERSON, M., MALVESTUTTO, C. & ABERG, J. A. 2015. Management of lipid

disorders in patients living with HIV. *Journal of clinical pharmacology,* 55**,** 957.

60. MYŚLIWIEC, H., BARAN, A., HARASIM-SYMBOR, E., MYŚLIWIEC, P., MILEWSKA, A. J., CHABOWSKI, A. & FLISIAK, I. 2017. Serum fatty acid profile in psoriasis and its comorbidity. *Archives of Dermatological Research,* 309**,** 371-380.

61. NIU, Z., LIN, N., GU, R., SUN, Y. & FENG, Y. 2014. Associations Between Insulin Resistance, Free Fatty Acids, and Oocyte Quality in Polycystic Ovary Syndrome During In Vitro Fertilization. *The Journal of Clinical Endocrinology & Metabolism,* 99**,** E2269-E2276.

62. NOGUEIRA, A. C., KAWABATA, V., BISELLI, P., LINS, M. H., VALERI, C., SECKLER, M., HOSHINO, W., JÚNIOR, L. G., BERNIK, M. M. S., DE ANDRADE MACHADO, J. B., MARTINEZ, M. B., LOTUFO, P. A., CALDINI, E. G., MARTINS, E., CURI, R. & SORIANO, F. G. 2008. CHANGES IN PLASMA FREE FATTY ACID LEVELS IN SEPTIC PATIENTS ARE ASSOCIATED WITH CARDIAC DAMAGE AND REDUCTION IN HEART RATE VARIABILITY. *Shock,* 29**,** 342-348.

63. NYBERG, S. T., BATTY, G. D., PENTTI, J., VIRTANEN, M., ALFREDSSON, L., FRANSSON, E. I., GOLDBERG, M., HEIKKILA, K., JOKELA, M., KNUTSSON, A., KOSKENVUO, M., LALLUKKA, T., LEINEWEBER, C., LINDBOHM, J. V., MADSEN, I. E. H., MAGNUSSON HANSON, L. L., NORDIN, M., OKSANEN, T., PIETILAINEN, O., RAHKONEN, O., RUGULIES, R., SHIPLEY, M. J., STENHOLM, S., SUOMINEN, S., THEORELL, T., VAHTERA, J., WESTERHOLM, P. J. M., WESTERLUND, H., ZINS, M., HAMER, M., SINGH-MANOUX, A., BELL, J. A., FERRIE, J. E. & KIVIMAKI, M. 2018. Obesity and loss of disease-free years owing to major non-communicable diseases: a multicohort study. *The Lancet.Public health,* 3**,** e490.

64. OH, B. J., SEO, M. H. & KIM, H. S. 2012. Insignificant role of the N-terminal cobaltbinding site of albumin in the assessment of acute coronary syndrome: discrepancy between the albumin cobalt-binding assay and N-terminal-targeted immunoassay. *Biomarkers,* 17**,** 394-401.

65. OLIVER, M. F. 2011. Free fatty acids and acute coronary syndromes--the history. *QJM : monthly journal of the Association of Physicians,* 104**,** 625.

66. ORAN, I. & ORAN, B. 2017. Ischemia-Modified Albumin as a Marker of Acute Coronary Syndrome: The Case for Revising the Concept of "N-Terminal Modification" to "Fatty Acid Occupation" of Albumin. *Disease markers,* 2017**,** 5692583.

67. PETERS JR, T. 1995. 2 - The Albumin Molecule: Its Structure and Chemical Properties. *In:* PETERS, T. (ed.) *All About Albumin.* San Diego: Academic Press.

68. PIRRO, M., MAURIÈGE, P., TCHERNOF, A., CANTIN, B., DAGENAIS, G. R., DESPRÉS, J.-P. & LAMARCHE, B. T. 2002a. Plasma free fatty acid levels and the risk of ischemic heart disease in men: prospective results from the Québec Cardiovascular Study. *Atherosclerosis,* 160**,** 377.

69. PIRRO, M., MAURIEGE, P., TCHERNOF, A., CANTIN, B., DAGENAIS, G. R., DESPRES, J. P. & LAMARCHE, B. 2002b. Plasma free fatty acid levels and the risk of ischemic heart disease in men: prospective results from the Quebec Cardiovascular Study. *Atherosclerosis,* 160**,** 377.

70. REAVEN, G. M., HOLLENBECK, C., JENG, C. Y., WU, M. S. & CHEN, Y. D. 1988. Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. *Diabetes,* 37**,** 1020.

71. REPORT, E. 2010. Fats and fatty acids in human nutrition. Report of an expert consultation. *FAO food and nutrition paper.*

72. RIZZUTI, B., BARTUCCI, R., SPORTELLI, L. & GUZZI, R. 2015. Fatty acid binding into the highest affinity site of human serum albumin observed in molecular dynamics simulation. *Archives of Biochemistry & Biophysics,* 579**,** 18.

73. RODAHL, K., MILLER, H. I. & ISSEKUTZ, B., JR. 1964. Plasma Free Fatty Acids in Exercise. *Journal of applied physiology,* 19**,** 489.

74. ROY, V. K., KUMAR, A., JOSHI, P., ARORA, J. & AHANGER, A. M. 2013. Plasma free Fatty Acid concentrations as a marker for acute myocardial infarction. *Journal of clinical and diagnostic research: JCDR,* 7**,** 2432.

75. SADLER, P. J., TUCKER, A. & VILES, J. H. 1994. Involvement of a lysine residue in the N-terminal Ni2+ and Cu2+ binding site of serum albumins. *European Journal of Biochemistry,* 220**,** 193-200.

76. SARAWUT, K. 2012. - Oxidatively Modified Biomolecules: An Early Biomarker for Acute Coronary Artery Disease N2 *IntechOpen.* 1 - Volodymyr I. Lushchak 2 - Dmytro V. Gospodaryov Y1 - 2012-04-25 ed.

77. SBAROUNI, E., GEORGIADOU, P. & VOUDRIS, V. 2011. Ischemia modified albumin changes - review and clinical implications. *Clin Chem Lab Med,* 49**,** 177-84.

78. SHANNON, R. 1976. Revised effective ionic radii and systematic studies of interatomic distances in halides and chalcogenides. *Acta Crystallographica Section A,* 32**,** 751-767.

79. SIMARD, J. R., ZUNSZAIN, P. A., HAMILTON, J. A. & CURRY, S. 2006. Location of high and low affinity fatty acid binding sites on human serum albumin revealed by NMR drug-competition analysis. *Journal of Molecular Biology,* 361**,** 336.

80. SINHA, M. K., ROY, D., GAZE, D. C., COLLINSON, P. O. & KASKI, J. C. 2004. Role of "Ischemia Modified Albumin", a new biochemical marker of myocardial ischaemia, in the early diagnosis of acute coronary syndromes. *Emergency Medicine Journal,* 21**,** 29.

81. SOKOLOWSKA, M., WSZELAKA-RYLIK, M., POZNANSKI, J. & BAL, W. 2009.

Spectroscopic and thermodynamic determination of three distinct binding sites for Co(II) ions in human serum albumin. *Journal of inorganic biochemistry,* 103**,** 1005.

82. SPECTOR, A. A. 1975. Fatty acid binding to plasma albumin. *Journal of lipid research,* 16**,** 165.

83. SRINIVASA, S. & GRINSPOON, S. K. 2014. Metabolic and body composition effects of newer antiretrovirals in HIV-infected patients. *European journal of endocrinology,* 170**,** R185.

84. STEWART, A. J. 2003. *Metal binding and redox properties of human albumin.* PhD, University of Edinburgh.

85. STEWART, A. J. & BLINDAUER, C. A. 2015. The reduced Co(2+) -binding ability of ischaemia-modified albumin is unlikely to be because of oxidative modification of the N-terminus. *Liver international : official journal of the International Association for the Study of the Liver,* 35**,** 2622.

86. STICH, V. & BERLAN, M. 2004. Physiological regulation of NEFA availability: lipolysis pathway. *The Proceedings of the Nutrition Society,* 63**,** 369.

87. SVENSSON, S., SVEDJEHOLM, R., EKROTH, R., MILOCCO, I., NILSSON, F., SABEL, K. G. & WILLIAM-OLSSON, G. 1990. Trauma metabolism and the heart. Uptake of substrates and effects of insulin early after cardiac operations. *The Journal of thoracic and cardiovascular surgery,* 99**,** 1063.

88. TANG, M. W., KOOPMAN, F. A., VISSCHER, J. P. M., DE HAIR, M. J., GERLAG, D. M. & TAK, P. P. 2017. Hormone, metabolic peptide, and nutrient levels in the earliest phases of rheumatoid arthritis—contribution of free fatty acids to an increased cardiovascular risk during very early disease. *Clinical Rheumatology,* 36**,** 269-278.

89. URBAN, J. & IWASZKIEWICZ-PAWŁOWSKA, A. 1986. Concentration of free fatty acids (FFA) in amniotic fluid and maternal and cord serum in cases of intrauterine growth retardation. *Journal of Perinatal Medicine - Official Journal of the WAPM.*

90. VALLEE, W. M. B. L. 1993. [3] Cobalt as probe and label of proteins. *Methods in Enzymology.* Academic Press.

91. VAN HEERDEN, A., BARNABAS, R. V., NORRIS, S. A., MICKLESFIELD, L. K., VAN ROOYEN, H. & CELUM, C. 2017. High prevalence of HIV and non-communicable disease (NCD) risk factors in rural KwaZulu-Natal, South Africa. *Journal of the International AIDS Society,* 20**,** 10.1002/jia2.25012.

92. VERNON, G., BARANOVA, A. & YOUNOSSI, Z. M. 2011. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Alimentary Pharmacology & Therapeutics,* 34**,** 274.

93. VIGNE, J. L., MURAI, J. T., ARBOGAST, B. W., JIA, W., FISHER, S. J. & TAYLOR, R. N. 1997. Elevated nonesterified fatty acid concentrations in severe preeclampsia shift the

isoelectric characteristics of plasma albumin. *The Journal of clinical endocrinology and metabolism,* 82**,** 3786.

94. VU, T. T., FREDENBURGH, J. C. & WEITZ, J. I. 2013. Zinc: an important cofactor in haemostasis and thrombosis. *Thrombosis and haemostasis,* 109**,** 421.

95. VYAKARANAM, S., BHONGIR, A. V., PATLOLLA, D. & CHINTAPALLY, R. 2015. Maternal serum ischemia modified albumin as a marker for hypertensive disorders of pregnancy: a pilot study. *International journal of reproduction, contraception, obstetrics and gynecology,* 4**,** 611.

96. WOLFE, R. R., KLEIN, S., CARRARO, F. & WEBER, J. M. 1990. Role of triglyceride-fatty acid cycle in controlling fat metabolism in humans during and after exercise. *The American Journal of Physiology,* 258**,** E382.

97. WU, B. B., ZHANG, L. M., MEI, C. L., TANG, Q. & LU, Y. Z. 2010. Relationship between serum free fatty acid and cytokines, carotid atherosclerosis in chronic kidney disease. *Zhonghua nei ke za zhi,* 49**,** 572.

98. XU, Q., DU, J., LING, X. & LU, Y. 2017. Evaluation of MIh Scoring System in Diagnosis of Obstructive Sleep Apnea Syndrome. *Medical science monitor : international medical journal of experimental and clinical research,* 23**,** 4715-4722.

99. ZHANG, H. W., ZHAO, X., GUO, Y. L., ZHU, C. G., WU, N. Q., SUN, J., LIU, G., DONG, Q. & LI, J. J. 2017. Free fatty acids and cardiovascular outcome: a Chinese cohort study on stable coronary artery disease. *Nutrition & metabolism,* 14**,** 41. 101. ZHANG, J., ZHAO, Y., XU, C., HONG, Y., LU, H., WU, J. & CHEN, Y. 2014. Association between serum free fatty acid levels and nonalcoholic fatty liver disease: a cross-sectional study. *Scientific reports,* 4**,** 5832.

CHAPTER 3

Zn^{2+} AND HAEMOSTASIS: INVESTIGATING THE INFLUENCE OF FREE FATTY ACIDS AND POOR Zn^{2+} BUFFERING BY ALBUMIN ON PLATELET FUNCTION

3.1 Introduction

3.1.1 Platelet aggregation and the role of Zn^{2+}

Platelets are anuclear blood cells that are approximately 2-3 μm in diameter and are the major cellular determinants of haemostasis (Rahman and De Ley, 2008, Taylor and Pugh, 2016). Figure 3.1 shows the structure of a human platelet observed under transmission electron microscopy. Under resting conditions, platelets are discoid in shape, but their shape and ultrastructure change when they are activated (Rumbaut and Thiagarajan, 2010). Platelets have important functional constituents on their cell membrane and in the cytoplasm, especially within the granules that participate in thrombosis. On the cell membrane are receptorglycoproteins which interact with different types of ligands. These include those found in the circulation and those fixed to the vessel wall, which allow adherence of platelets (Rumbaut and Thiagarajan, 2010). On the interior of the cell wall, platelets have a network of invaginations called the open canalicular system (OCS), which communicates with the exterior through small pores (White et al., 1999). Another organelle system within the platelet is the dense tubular system (DTS), which is not associated with the cell membrane but contains calcium and an assortment of enzymes that participate in platelet activation (Ebbeling et al., 1992, Rendu and Brohard-Bohn, 2001). The DTS has recently been proposed to be a potential repository area for Zn^{2+}, which is released together with calcium during platelet aggregation, but this hypothesis requires further research to be verified (Watson et al., 2016).

Two types of granules have been described in platelets, and these are alpha (α)- granules and dense granules. The α-granules are the largest, most numerous (50-60 granules per cell) and heterogeneous type of platelet granules (Blair and Flaumenhaft, 2009).

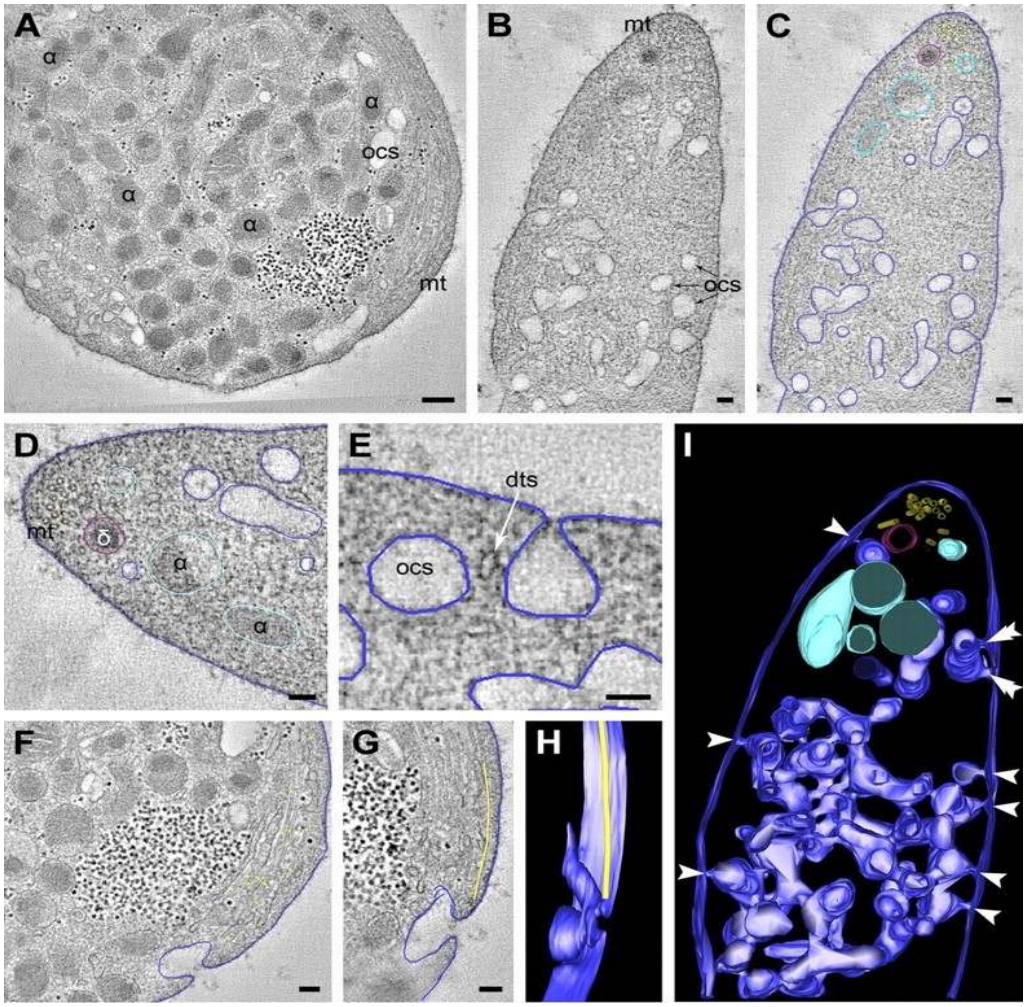


Figure 3.1 Electron microscopy tomography of the intracellular membrane organization in nonstimulated platelet. (A-C) Tomographic slices of directly fixed platelet. Dark blue represents OCS; light blue, platelet secretory α-granule; and red, dense granule. (D) Microtubules are located at the platelet periphery. The OCS membranes are continuous with the cell surface at multiple sites (panel E and arrowheads in panel I) and span the platelet cell surface from one side to the other, revealing numerous branching areas. (F-H) Manual tracking of the peripheral microtubular coil (yellow dots at the cell periphery) reveal a microtubule ending at an OCS invagination. α = α-granule; δ = dense granule; mt = microtubules; dts = dense tubular system. (A) Bars represent 200 nm. (B-G) Bars represent 100 nm. From (van Nispen tot Pannerden et al., 2010).

Contained in α-granules are most of the platelet factors which participate in haemostasis and thrombosis such as thrombospondin, P-selectin, platelet factor 4 and beta thromboglobulins as well as coagulation factors V, XI, XIII, fibrinogen, von Willebrand factor (VWF) and high molecular weight kininogens (Rumbaut and Thiagarajan, 2010).

3.1.2 Platelet granules and their contents

The α-granules also house adhesion molecules such as fibronectin and vitronectin for platelet-vessel wall interactions (Rumbaut and Thiagarajan, 2010). Zn^{2+} is known to be stored within the α-granules and cytoplasm in platelets in higher concentration than plasma (Marx et al., 1993). The accumulation of the Zn^{2+} within these compartments is thought to be facilitated by the presence of metallothioneins, which are transferred from the nucleated megakaryocytes during differentiation (Sugiura and Nakamura, 1994, Rahman and De Ley, 2008). Megakaryocytes have been shown to express TRPC6 and TRPM7, which are non-specific cation channels and four times permeable to Zn^{2+} than Ca^{2+} (Monteilh-Zoller et al., 2003). During platelet activation, the release of the contents from the α-granules is preceded by coalescence of the granules in the centre of the platelets and fusion with the OCS and among themselves (Stenberg et al., 1984, Flaumenhaft, 2003, Blair and Flaumenhaft, 2009). Some of the released substances from the α-granules such as P-selectin exert their effect once incorporated within the cell membrane, while others such as Zn^{2+}, exert their effects once released from the granules. Dense granules are the smallest of the platelet granules, numbering 3-8 per platelet but contain a high concentration of calcium and phosphate, as well as adenine nucleotides and serotonin (Rendu and Brohard-Bohn, 2001, Rumbaut and Thiagarajan, 2010).

During platelet activation, the dense granules fuse with the cell membrane of the platelet and release their contents to the external environment (Rumbaut and Thiagarajan, 2010). The constituents of the dense granules include ADP, which is essential for platelet aggregation (recruitment of other platelets) and serotonin, which regulates local vasoconstriction (Rumbaut and Thiagarajan, 2010). Lysosomes are organelles of intermediate size between α- and dense granules and contain hydrolytic enzymes (Ciferri et al., 2000, Rendu and Brohard-Bohn, 2001). The release of lysosome contents requires stronger stimulation than that required for the release of the other granules (Rumbaut and Thiagarajan, 2010). The hydrolytic proteins from the lysosomes are known to be important in the regulation and modelling of the forming thrombus (Ciferri et al., 2000, Rendu and Brohard-Bohn, 2001).

3.1.3 Platelet adhesion molecules and receptors

Platelets have adhesion molecules on the cell membrane and inside their granules that are essential for haemostasis and thrombosis. Ligands exert their effect on platelets through a variety of receptors located on the platelet cell membrane. Figure 3.2 is a schematic diagram showing the major platelet adhesion molecules and G-protein-coupled protein receptors with their signalling pathways. P-selectin is one of the adhesion molecules in platelets and is found primarily on α-granules but has also been described on dense granules (Israels et al., 1992). The expression of P-selectin on the platelet surface is more evident once the platelets are activated and is maximal between 30 seconds and 10 minutes following stimulation by an agonist (Whiss et al., 1997, Merten and Thiagarajan, 2000). Ligands for P-selectin include P-selectin glycoprotein ligand-1 (PSGL-1), VWF, glycoprotein Ibα and sulfatides. Interactions of P-selectin with its ligands contribute to haemostasis and thrombosis (Merten et al., 2005).

Glycoprotein Ib/IX/V (GPIb/IX/V) is a receptor complex and the main platelet receptor for

VWF which is composed of GPIbα, GPIbβ, GPIX and GPIV (Rumbaut and Thiagarajan, 2010). When VWF binds to GPIb, signal transduction is initiated which leads to the activation of integrin GPIIb/IIa (α_{IIb}/β_3), which becomes primed for the binding of VWF or fibrinogen to mediate platelet aggregation (Rumbaut and Thiagarajan, 2010). GPIb may also bind other ligands including thrombin, coagulation factors XII, XI and VIIa thrombospondin and kininogen (Harmon and Jamieson, 1986, Weeterings et al., 2008, Bradford et al., 2000).

Another important and well-studied adhesion molecule on platelets is platelet GP IIb/IIIa ($\alpha_{IIb}\beta_3$), which plays an essential role in platelet aggregation (Rumbaut and Thiagarajan, 2010). There are about 80,000 GP IIb/IIIa molecules on the surface of platelets, and some are also found on the surface of granules which translocate to the membrane surface when released (Niiya et al., 1987). Ligands for platelet GPIIb/IIIa include fibrinogen, VWF, fibronectin and vitronectin (Bennett, 2005). Collagen receptors include $\alpha_2\beta_1$ integrin and glycoprotein VI (GP VI) and these are the primary receptors that play an important role in haemostasis (Rumbaut and Thiagarajan, 2010). Integrin $\alpha_2\beta_1$ induces activation of platelet GPIIb/IIIa via phospholipase C (PLC)-dependent stimulation. Platelet GPVI is expressed on both the plasma membrane and on the membrane of α-granules (Guidetti et al., 2009). During platelet activation, expression of GPVI is increased on the platelet surface as the α-granules contents released, are incorporated into the plasma membrane (Suzuki et al., 2003).

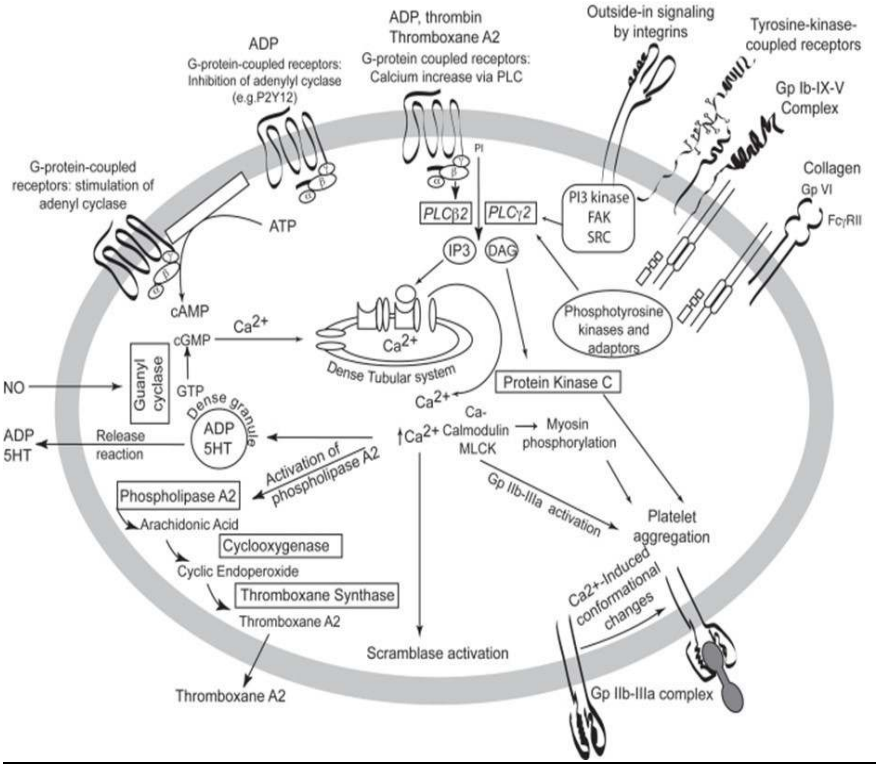


Figure 3.2 A Schematic diagram of the major platelet adhesion molecules and G-proteincoupled receptors and their signalling pathways. ADP = Adenosine diphosphate; 5HT = 5hydroxytryptamine (serotonin); PLCβ2 = Phospholipase C beta 2; PLCγ2 = Phospholipase C gamma 2; GP VI = Glycoprotein VI; FcγRII = Fc gamma receptor II ; GP IIb-IIIa complex = Glycoprotein IIb-IIIa complex; MLCK = Myosin light-chain kinase; cAMP = Cyclic adenosine monophosphate; cGMP = Cyclic guanosine monophosphate; NO = Nitric oxide; GP Ib-IX-V complex = Glycoprotein Ib-IX-V complex; PI3 kinase = Phosphoinositide 3 kinase; FAK = Focal adhesion kinase; SRC = Tyrosine-protein kinase; DAG = Diacylglycerol; PLC = Phospholipase C. From (Rumbaut and Thiagarajan, 2010).

A set of G-protein-coupled receptors exists that mediate the effects of thrombin, adenosine diphosphate (ADP) and adenosine triphosphate (ATP). The effects of thrombin are mediated by protease-activated-receptors (PAR) to which thrombin induces proteolytic cleavage and unmasking a specific ligand (Coughlin, 2001). Binding of thrombin to PAR-1 and PAR-4 which form a new amino acid terminus with its tethered ligand activates the receptor and signalling is induced (Rumbaut and Thiagarajan, 2010). Once activated, PAR-1 and PAR-4 couple to G_q and

$G_{12/13}$ G-proteins, thereby activating phospholipase C and causing calcium mobilisation as well as protein kinase C activation (Woulfe, 2005).

ADP, another stimulant for platelet adhesion and aggregation, acts through the P2Y receptor family of G protein-coupled, seven transmembrane domain receptors (P2Y1 and P2Y12) (Rumbaut and Thiagarajan, 2010). Coupling of P2Y12 to G_q causes mobilisation of intracellular calcium ions which mediate platelet shape change and aggregation (Rumbaut and Thiagarajan, 2010). ATP is a less potent platelet agonist and induces signalling via ligandgated ion channels (P2X1), which result in platelet shape change and enhances other agonists such as collagen (Murugappan et al., 2004).

Thromboxane A_2 (TXA_2) is one of the prostanoids whose receptors are expressed on the platelet surface. TPα and TPβ are the two isoforms of thromboxane receptors, and TPα is the predominant isoform (Habib et al., 1999, Murugappan et al., 2004). When TP receptors couple with G_q and G_{13}, activation of phospholipase C and RhoGEF occurs respectively (Nakahata, 2008). TXA_2 elicits platelet shape change, aggregation, degranulation and enhancement of response to other agonists, hence amplifying platelet activation (Rumbaut and Thiagarajan, 2010). Platelets also express receptors for prostacyclin and prostaglandin E_2.

3.1.4 The process of platelet aggregation

Platelet aggregation is a process of platelet-to-platelet adhesion which follows adhesion of platelets to the site of injury *in vivo*. Platelets are activated to aggregate by agonists such as thrombin, ADP and collagen which act by binding to specific receptors on the platelet surface as discussed above (Rumbaut and Thiagarajan, 2010). The ultimate result of occupancy of the receptors is an increase in cytoplasmic Ca^{2+} released from intracellular stores and influx through the plasma membrane (Varga-Szabo et al., 2008, Mahaut-Smith, 2012).

The increase in platelet free calcium concentration leads to morphological and functional changes in the platelet. Morphologically, in a process called shape change, platelets change from discoid shapes to spiny-sphere shapes (Rumbaut and Thiagarajan, 2010). The change in the platelet shape is facilitated by the cytoskeleton which is linked to platelet signalling molecules (Fox, 2001). The reorganisation of the cytoskeleton, polymerisation of actin and phosphorylation of the light chain of myosin occur when platelets change shape (Fox, 2001,

Escolar et al., 1986, Daniel et al., 1984). The platelet projections formed during shape change enable platelets to interact and form aggregates (Rumbaut and Thiagarajan, 2010). Increased intracellular calcium in platelets also causes granules within the platelet to become centrally located and their contents to be discharged into the lumen of the OCS, and after that released to the exterior, in a process termed "the release reaction" (Rumbaut and Thiagarajan, 2010). The increased calcium concentrations in the platelets also result in stimulation of membrane phospholipase A_2 activity, which leads to the liberation of arachidonic acid from the membrane phospholipids. Cyclooxygenase-1 (COX-1) converts the arachidonic acid to prostaglandin H_2 (PGH_2) which is further metabolised by thromboxane synthase to TXA_2 - a potent platelet activator (Gryglewski, 2008).

The membrane protein GPIIb/IIIa complex is the major adhesion molecule involved in platelet aggregation (Rumbaut and Thiagarajan, 2010). The result of platelet activation by almost all agonists is conformational changes in GPIIb/IIIa, which becomes primed to bind soluble plasma fibrinogen, and the receptor-bound fibrinogen between two GPIIb/IIIa molecules on adjacent platelets acts as a bridge (Varga-Szabo et al., 2008). The above process is the ultimate common pathway of platelet aggregation following stimulation by chemical agonists (Rumbaut and Thiagarajan, 2010). Other proteins involved in platelet aggregation are junctional adhesion molecules (JAMs), signalling lymphocyte activation molecule (SLAM), and CD40 ligand but these have not been well studied (Andre et al., 2002, Sobocka et al., 2004, Nanda et al., 2005). Activated platelets also stimulate and recruit other platelets to the growing haemostatic plug through the release of ADP and serotonin stored in the α-granules, as well as the *de novo* synthesis of pro-aggregatory TXA2 (Rumbaut and Thiagarajan, 2010). These released agonists consolidate the initial haemostatic plug at sites of vascular injury.

3.1.5 The role and influence of Zn^{2+} on platelet activation and aggregation

There is clear evidence that Zn^{2+} plays an important role in haemostasis, but its specific functions on platelet activity have not yet been well delineated (Taylor and Pugh, 2016). Figure 3.3 shows a theoretical model of the influence of Zn^{2+} on platelet mechanisms during activation.

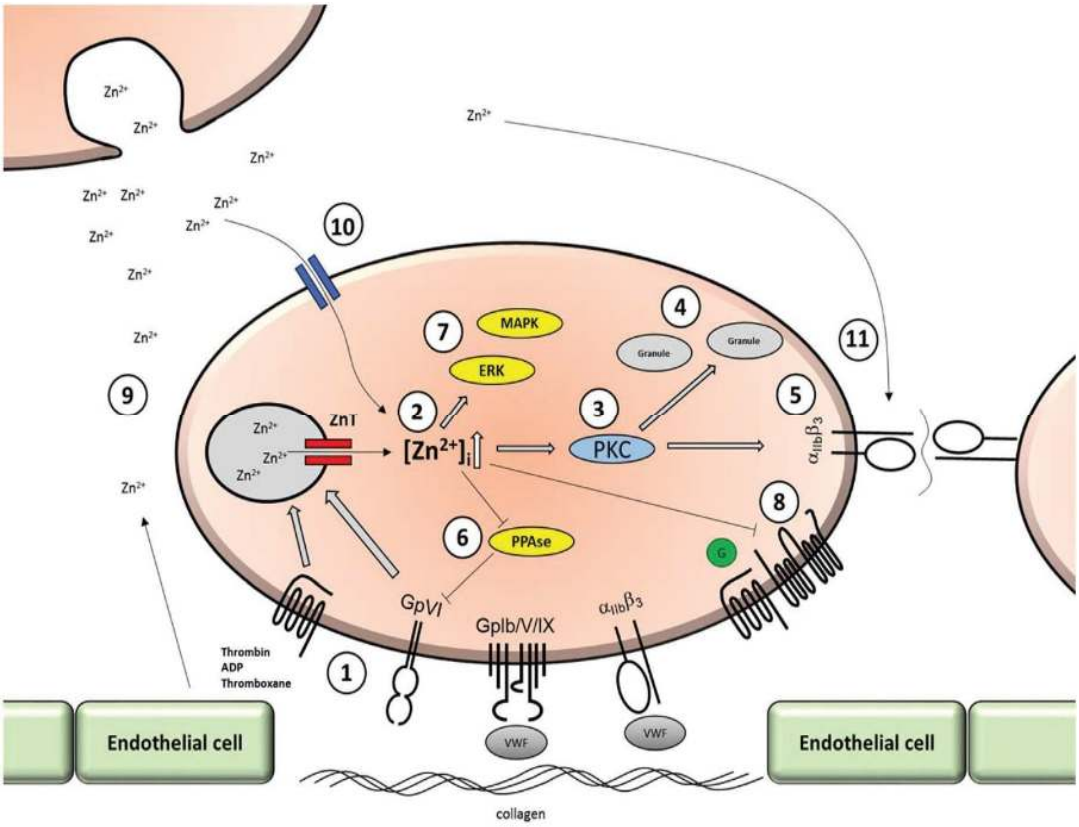


Figure 3.3 A theoretical model of the mechanisms that are influenced by Zn^{2+} during platelet activation. (1) Platelets interact with subendothelial matrix proteins (i.e. collagen and VWF) following vascular injury, and by agonists (ADP, thrombin and thromboxane A2). (2) Platelet activation leads to the release of Zn^{2+} from intracellular stores into the cytoplasm, through channels or ZnT transporters, resulting in an increased cytoplasmic Zn^{2+} concentration ($[Zn^{2+}]_i$). (3) $[Zn^{2+}]_i$ interacts with PKC, potentiating enzyme activity. PKC-mediated phosphorylation promotes granule release (4) and activation of integrin aIIbβ3 (5), which crosslinks platelets via binding of fibrinogen, mediating platelet aggregation. (6) Inhibition of cytosolic protein phosphatases by $[Zn^{2+}]_i$ enhances tyrosine phosphorylation of platelet signalling proteins. (7) $[Zn^{2+}]_i$ activates protein tyrosine kinases (e.g. ERK), which regulate tyrosine phosphorylation events downstream of platelet activation. (8) $[Zn^{2+}]_i$ inhibits adenylate cyclase, hence reducing cAMP levels and promoting platelet activation. (9) Zn^{2+} is released from damaged endothelial cells, sub-endothelial matrix and platelet granules, contributing to a localised increase in labile $[Zn^{2+}]_o$. (10) Extracellular Zn^{2+} gains access the cytosol via non-selective transporters and cation channels. (11) Zn^{2+} may also interact directly with integrin aIIbβ3 altering the activity and regulating platelet/platelet interactions leading to thrombus formation. From (Taylor and Pugh, 2016).

Thus, research has been able to show that poor zinc status is associated with poor platelet aggregation responses in both humans and animals, a factor that can be reversed by Zn^{2+} supplementation (O'Dell et al., 1977, Gordon et al., 1982, Stefanini, 1999). The literature also suggests that Zn^{2+} modulates secondary platelet activation by modulation of PKC activity, but primary platelet activation does not intrinsically depend on Zn^{2+} (Gordon et al., 1982, Taylor and Pugh, 2016). Zn^{2+} also interacts with HRG and increases its affinity for heparin and heparan sulphate, thereby neutralising these anticoagulants; and it also inhibits the activity of antithrombin III - hence offering a prothrombotic effect (Borza and Morgan, 1998, Mori et al., 2003, Jones et al., 2004). Supra-physiological Zn^{2+} concentrations of up to 0.5 mM have been shown to impact on platelet aggregation directly in a concentration-dependent manner (Heyns Adu et al., 1985, Trybulec et al., 1993, Watson et al., 2016). Zn^{2+}-induced platelet activation has been associated with tyrosine phosphorylation of platelet proteins, granule release and secondary activation that results in activation of the GPIIb/IIIa complex (Watson et al., 2016). It has also been shown that Zn^{2+} augments the effect of platelet agonists. In the presence of Zn^{2+}, stimulation of platelets with ADP has been associated with a 2-fold increase in fibrinogen receptors on the platelet surface (Trybulec et al., 1993, Kowalska et al., 1994). Though not well elucidated, Zn^{2+} also participates in promoting platelet activation by modulating calcium influx into platelets (O'Dell, 2000). Zn^{2+} regulates the rate of fibrin clot formation by enhancing the rate of fibrin formation induced by thrombin and on the other hand, inhibiting the activity of thrombin on fibrinogen to fibrin conversion in a dose-dependent manner (Marx and Hopmeier, 1986, Marx et al., 1987, Marx, 1988). How exactly Zn^{2+} modulates both these processes is still of research interest, but it is clear that Zn^{2+}, unlike other metal ions, regulates these processes (Marx and Eldor, 1985, Vu et al., 2013).

These pieces of evidence strongly suggest that Zn^{2+} can influence the pathways associated with platelet aggregation by either potentiating other agonists, acting as a second messenger or in extreme conditions, directly stimulating aggregation. It is important to note that pathophysiological increases in available Zn^{2+} concentration may occur not only in the vicinity of a forming thrombus, but also in disease states associated with increased FFAs in plasma such as diabetes, cancer, and cardiovascular disease conditions (Lu et al., 2012, Watson et al., 2016). How Zn^{2+} is transported across the platelet cell membrane is still under investigation (Taylor and Pugh, 2016). Nevertheless, non-specific channels such as the Na+/Ca^{2+} exchanger (NCX) (isoforms NCX1.3, NCX3.2, NCX3.4), and Zn^{2+} transporter ZIP7 appear to be the possible

transporter candidates for the transport of Zn^{2+} across the platelet membrane (Roberts et al., 2012, Taylor and Pugh, 2016).

3.1.6 Conditions that may lead to higher concentrations of Zn^{2+}

If higher concentrations of Zn^{2+} may precipitate coagulation, the source and potential causes of the increase and pathophysiological states which may cause such become important. In the extracellular matrix of dermal and epidermal tissue, there are Zn^{2+} pools which are released during an injury and are essential for normal wound healing (Michaelsson et al., 1980, Lansdown et al., 2007). Acutely increased concentrations of Zn^{2+} are therefore expected in areas surrounding an injury. Platelets are also known to release Zn^{2+} during platelet aggregation, and this is another source of free Zn^{2+} within a forming thrombus which may potentiate other platelet agonists that act through PARS and GpVI (Mahdi et al., 2002, Watson et al., 2016). Furthermore, in cardiovascular disease states, atherosclerotic plaques have six times higher Zn^{2+} concentration than normal tissues and the plaques can release the Zn^{2+} around the affected areas when they rupture (Sadler et al., 1994). Importantly, pathophysiological conditions which are associated with increased concentrations of FFAs in circulation can potentially cause the allosteric release of Zn^{2+} into plasma – hence another potential source of labile Zn^{2+} (Lu et al., 2012). Even though the abnormal increase in Zn^{2+} concentration may rarely lead to undesired platelet aggregation in healthy individuals, it becomes important in patients who have other prothrombotic risks in whom inappropriate platelet activation – the major precipitating factor in cardiovascular disease is more likely to ensue (Michelson, 2013).

Collectively, having considered the different ways in which Zn^{2+} may influence haemostasis, thrombosis and platelet aggregation, it is logical to consider that abnormal increases in Zn^{2+} concentration or poor buffering of Zn^{2+} by HSA may positively influence prothrombotic activity. This is of concern because such conditions may occur due to increased circulating FFA in pathological conditions associated with cancer, cardiovascular disease, diabetes, metabolic syndrome and HIV infection (Charles et al., 2001, Boden, 2008, Benjamin et al., 2012). Importantly, these stated conditions are associated with abnormal coagulation and thrombotic events which contribute to increased morbidity and mortality (Colwell and Nesto, 2003, Piketty

et al., 2005). It is also clearly evident that the prevalence of non-communicable diseases which are associated with these risks are on the increase, not just globally but even in resource-poor settings; hence the need to focus on interventions that may reduce the risk of morbidity and deaths (Feigin et al., 2014, Marrero and Adashi, 2015). It is imperative, therefore, that further studies be done on this subject to provide further information on whether maintenance of plasma FFA at specific ranges may provide a preventative mechanism for thromboembolic events in these patient populations at risk.

3.1.7 FFA and platelet aggregation

FFA have been suggested to influence blood clotting. In 1967, Hook et al. described that FFA, especially longer chain saturated FFA such as stearate, induced platelet aggregation in human washed platelets and that the effect was mitigated by the addition of albumin (John C. Hook and William E, 1967). Of note in this study is that the FFA concentrations used were 3.5 mM, which would usually be expected in pathological conditions (Table 2.2, chapter 2 of this thesis). In 1977, Brurstein et al., also reported a positive correlation between increased FFA and intensity of platelet aggregation using turbidimetric assays and microscopic studies (Burstein et al., 1978). Further studies by Zentner et al. on the influence of fatty acids on platelet aggregation in washed porcine blood indicated that oleic, palmitic, and myristic acids were able to induce platelet aggregation on their own but not when solubilised in albumin – indicating the role of the fatty acids in the free form (Zentner et al., 1981). Importantly, in the study by Zentner et al., the FFA they used were in the concentration in the range 70 and 600 μM, which is below the ranges usually observed in pathological conditions (Zentner et al., 1981). Recently, other studies have strengthened the idea that FFA induces or promote a prothrombotic state in patients at risk and how low albumin levels would aggravate the condition (Dhindsa et al., 2015). Figure 3.4 illustrates the potential roles of FFA in platelet aggregation and the potential mechanisms in the model of type 2 diabetes (Dhindsa et al., 2015). Nevertheless, the particular role of the FFA, in the presence of albumin on metal ion buffering and the potential effect on platelet aggregation, has not been elucidated.

Further studies have shown that high intake of saturated fatty acids promotes the activation of clotting factor VII and hence renders one at risk of both atherosclerosis and thrombosis (Miller,

1997). It has recently further been suggested that FFA such as stearate and oleate reduce the stability of fibrin generated through thrombin – a mechanism that may enhance thrombus embolism from the source to other organs such as the brain, causing strokes (Tanka-Salamon et al., 2016). Thus, increased concentrations of FFA such as seen in NCDs increases the risk for thrombo-embolism by inducing platelet aggregation, rendering the blood vessels prothrombotic and through the formation of nonstable thrombus. However, the influence of the FFA on Zn^{2+} buffering and the potential effects on platelet aggregation has not been well studied.

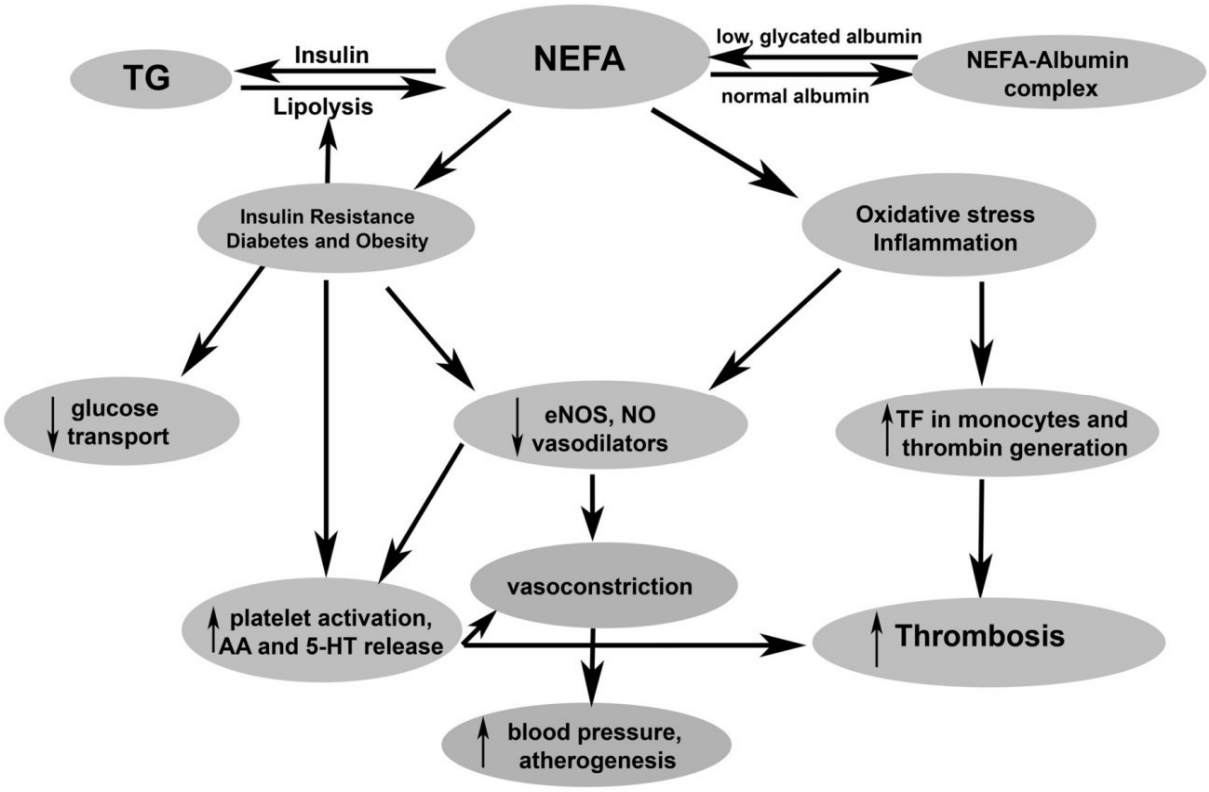


Figure 3.4 The role of FFA in a prothrombotic state as in the case of insulin resistance and diabetes. eNOS = endothelial nitric oxide synthase; NO = Nitric oxide; AA = Arachidonic acid, TF = Tissue factor; 5 hydroxytryptamin. From (Dhindsa et al., 2015).

3.1.8 Principle of Light Transmission Aggregometry (LTA)

LTA is a technique commonly used for assessing platelet functioning in both research and clinical settings (Cattaneo et al., 2009). The test measures platelet aggregation in stirred platelet-rich plasma (PRP) at 37°C, by assessing the changes in light transmission (optic density) as platelets aggregate following the addition of an agonist (Cattaneo et al., 2007). At baseline, PRP sample represents 0% platelet aggregation while a platelet poor plasma (PPP) sample from the same individual sample, placed in another well during the test represent 100% platelet aggregation. A chart reflecting platelet aggregation is hence plotted from 0% to 100% (Zhou and Schmaier, 2005). The following parameters can be measured from an LTA curve; lag phase/ time to onset of response following addition of an agonist, maximal amplitude, aggregation rate, and disaggregation if it occurs (Harrison et al., 2011) (see Figure 3.5).

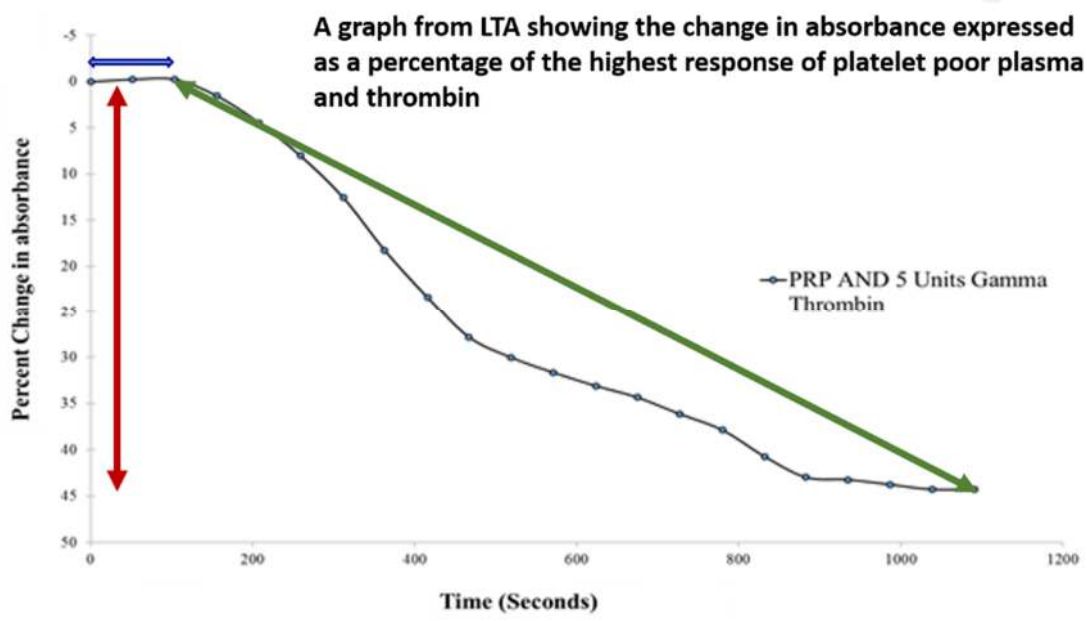


Figure 3.5 A diagram showing a typical platelet aggregation curve. At baseline, PRP sample represents 0% platelet aggregation while a platelet poor plasma (PPP) sample from the same individual sample represents 100% platelet aggregation. The dotted line is the curve. The blue arrow on top of the onset of the curve represents the time to onset of response, the red double-arrowed represents the maximum aggregation response, and the green arrow represents the rate of aggregation response.

Preparation of PRP for LTA

Preparation of PRP involves a process termed as differential centrifugation whereby centrifugation of whole blood at a particular acceleration force results in separation and sedimentation of the different cellular components (Dhurat and Sukesh, 2014). PRP may be prepared either by a one spin method or a double spin method. The first spin method involves venepuncture and blood collection in a tube containing an anticoagulant and spinning the blood at room temperature. The result of the spinning are three layers; a bottom layer containing red blood cells, a middle layer (buffy coat) rich in white blood cells, and an upper layer containing PRP. The PRP may then be carefully transferred into a clean empty tube using a Pasteur pipette and the sample may be used for the assay.

The double spinning method involves initial blood collection and spinning in the same way as the single spin method. Unlike the single spin method, however, after the first spin, the PRP collected is spun again at a higher speed to form three layers; a platelet pellet at the bottom, a lower 1/3 of PRP just above the pellet and an upper 2/3 containing PPP (Dhurat and Sukesh, 2014). The upper PPP layer is removed and the platelet pellet, then homogenised in the lower

PRP layer and ready to be used for the test. Figure 3.5 is a flow diagram indicating the steps in PRP preparation and the spinning steps involved in the two types of PRP preparation.

Choice of anticoagulant for blood collection and commonly used platelet agonists

When examining platelet functioning it is important to consider the method of blood collection given that constituents in the blood collection tubes (anticoagulants) can interact with biochemical components of blood and alter test results (Bowen and Remaley, 2014). The correct choice of an anticoagulant used in the collection of a blood sample for platelet function tests is of particular importance to preserve essential components of blood in physiological states. There are several anticoagulants commonly used for clinical and research purposes. Commonly used anticoagulants include heparin, ethylenediaminetetraacetic acid (EDTA) and citrate (Narayanan, 2000, Kumura et al., 2000). Another, although less commonly used anticoagulant, is the thrombin inhibitor, hirudin (Kumura et al., 2000).

Heparin disrupts coagulation by its interaction with antithrombin III molecule, causing conformational changes to the molecule and hence accelerates inhibition of coagulation factors Xa, IXa, and thrombin (Narayanan and Hamasaki, 1998). The challenge with heparin as regards to platelet aggregation tests is that it may interfere with ionised calcium and magnesium by binding these electrolytes (Toffaletti and Wildermann, 2001), thereby disturbing the physiological environment for platelet aggregation in which calcium plays a critical role. The other caveat in this study is that we seek to investigate the interaction of Zn^{2+} and its interaction with HRG, which acts by partly neutralising the effects of heparin and hence the use of heparin as an anticoagulant may influence the results.

EDTA is another anticoagulant which acts by chelating calcium ions in plasma (Tate and Ward, 2004), hence not preferable for platelet aggregation studies where the biochemical status is to be preserved. EDTA can also chelate Zn^{2+} and magnesium ions hence interfering with the enzymatic and / or protein processes that these electrolytes participate in (Tate and Ward, 2004). Citrate, in the form of trisodium citrate 3.2% (109 mmol/L) or 3.8% (129 mmol/L) concentrations is said to be the preferred anticoagulant for coagulation, unlike acid citrate dextrose (ACD) and citrate theophylline adenosine dipyridamole which are known to inhibit platelet activation (Narayanan, 2000). Nevertheless, it should be noted that trisodium citrate forms soluble complexes with calcium and Zn^{2+} and may limit their availability in plasma, but unlike ACD, factor V is stable in trisodium citrate (Narayanan, 2000, Narayanan and Hamasaki, 1998).

The anticoagulant hirudin may also be used in other haematological tests. Hirudin belongs to a group of highly homologous polypeptides that originate from medicinal leech (*Hirudo medicinalis*) (Haruyama and Wüthrich, 1989). Hirudin exhibits its anticoagulant properties through its high affinity for thrombin thereby inhibiting its activities (Kumura et al., 2000, Rydel et al., 1990). It may be noted here that hirudin, unlike the other anticoagulants has less if any recorded interactions with electrolytes in plasma and hence offers more advantage in preserving the physiological electrolyte state for platelet aggregation. The challenge in its use, however, lies in its interference with thrombin especially where the thrombin is the choice of inducer for platelet activation.

Once the sample has been processed and PRP extracted, the next step is to carry out the required LTA experiments where an agonist is required to cause platelet activation and elicit aggregation. There are several types and examples of platelet agonists that may be used in LTA experiments. Commonly used agonists include adenosine diphosphate (ADP), collagen, epinephrine, arachidonic acid, ristocetin, thromboxane analogue U46619, platelet activating factor, thrombin receptor activating peptide (SFLLRN), alpha-thrombin and gamma-thrombin (Cattaneo et al., 2009). Thrombin is known to be the most potent platelet agonist, and in this study, gamma-thrombin was opted because unlike alpha-thrombin, it does not bind fibrinogen and therefore does not clot the plasma in PRP (Zhou and Schmaier, 2005). Gamma thrombin causes platelet activation by activating protease-activated receptors 1 and 4 (PAR1 and PAR 4) on the platelet cell membrane (Zhou and Schmaier, 2005).

3.2 Study aim and objectives

The main aim of this study was to investigate the effects of higher concentrations of Zn^{2+} and FFA on platelet aggregation using platelet aggregation assays.

The specific objectives were:

1. To optimise a platelet aggregation assay using a 96-well-plate

2. To investigate the effect of higher Zn^{2+} concentrations on the maximum aggregation and rate of aggregation in washed platelets resuspended in platelet buffer and platelets resuspended in plasma

3. Investigate the influence of increased concentration of FFA in plasma on the maximum platelet aggregation and rate of aggregation responses

3.3 Methods

3.3.1 Preparation of platelets

Platelets were prepared either using the single-spin or the double-spin method. For the singlespin method of PRP preparation, a venous blood samples of about 30 ml was collected from a donor in heparinised tubes. The sample was centrifuged at 400 x g for 10 minutes. Then, 75% of the supernatant (platelet rich plasma) was then pipetted using a Pasteur pipette into a new tube, without disturbing the buffy coat. The platelet count was done and samples the platelet count was at least 200×10^9 cells / L. The rest of the blood was spun again, now at 4000 × g for 5 min and platelet poor plasma (PPP) which was collected from the upper supernatant. Following this, platelet aggregation tests were performed using the PRP and gamma-thrombin (G. thrombin) as an agonist.

For the double-spin method, platelets were isolated from whole blood collected in acid citrate dextrose collecting tubes from healthy donors. The blood was spun twice at 23 °C, once at 700 × g for 8 min to isolate platelet-rich-plasma (PRP) and once at 400 × g for 20 min to pellet the platelets. The platelets were washed and re-suspended in buffer solution (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose, pH 7.4) for washedplatelet experiments, or re-suspended in platelet-poor plasma prepared from hirudin-coated tubes for platelet experiments requiring whole plasma. Hirudin-coated collection tubes were used to avoid chelation of Zn^{2+} by other agents (e.g. citrate or EDTA). Platelet aggregation experiments were performed to assess the effect of Zn^{2+} and FFA (octanoate or myristate) on platelet aggregation. Solutions of $ZnCl_2$ (10, 50 or 100 μM), sodium octanoate or sodium myristate (2 or 4 mol. eqs.) and N,N,N′,N′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN, diluted in ethanol) were added to washed platelets or PRP. Vehicle control experiments were also performed. Platelet aggregation was elicited with 5 U/ml of γ-thrombin (G. thrombin; Merck, Watford, UK; final volume 200 μL). The absorbance was read at 430 and was monitored every 55 s for 35 min using an Optima plate-reader (BMG Labtech, Ortenberg, Germany) while incubating the plate at 37 ºC and shaking it in orbital mode. Data were recorded as a negative change in absorbance from baseline (0%) and expressed as a percentage of the maximum response (100%). Calibration of 100 % aggregation was achieved using platelet-poor plasma.

From the recorded responses, a maximum aggregation response was obtained, and the rate was calculated between 10% and 90% of maximum aggregation.

3.3.2 Data analysis and presentation for the platelet aggregation assays

Data was expressed as mean ± standard error of mean. Graphs were generated and statistical analysis was performed using Prism 7.0 (GraphPad, La Jolla, CA). Comparisons were analysed using multiple Student's t-tests or analysis of variance (ANOVA).

3.3.3 Fluozin-3 fluorescence imaging of washed human platelets

Platelets in PRP were loaded with FluoZin3 by incubation with FluoZin3-AM in the presence of 20 % (w/v) pluronic F127 in DMSO at 37 ºC for 30 minutes. During the incubation period, platelets were inverted every 10 mins. The PRP was then centrifuged at 350 x g for 20 min and the pellet resuspended in nominally calcium free saline. Platelets were allowed to adhere to a collagen coated 8 well chamber slide prior to imaging. Fluorescent images were captured at 515 nm using a Leica DMi8 equipped with a 100x oil objective using a Hamamatsu 5 ORCAflash v3 Digital CMOS camera using a single image capture. Images were recorded under the control of Leica LAX software. The images were processed using ImageJ software

3.4 Results

3.4.1 Optimisation of platelet aggregation experiments

Platelet aggregation results using the single-spin method for the preparation of PRP

No aggregation responses were observed from PRP prepared from blood collected in sodium citrate containing tubes from 3 different donors. However, PRP prepared from blood collected in heparin-containing tubes showed some platelet aggregation response. The responses were observed with the addition of 5 U/ml of G. thrombin (see Figure 3.6). The LTA curve parameters were measured and recorded.

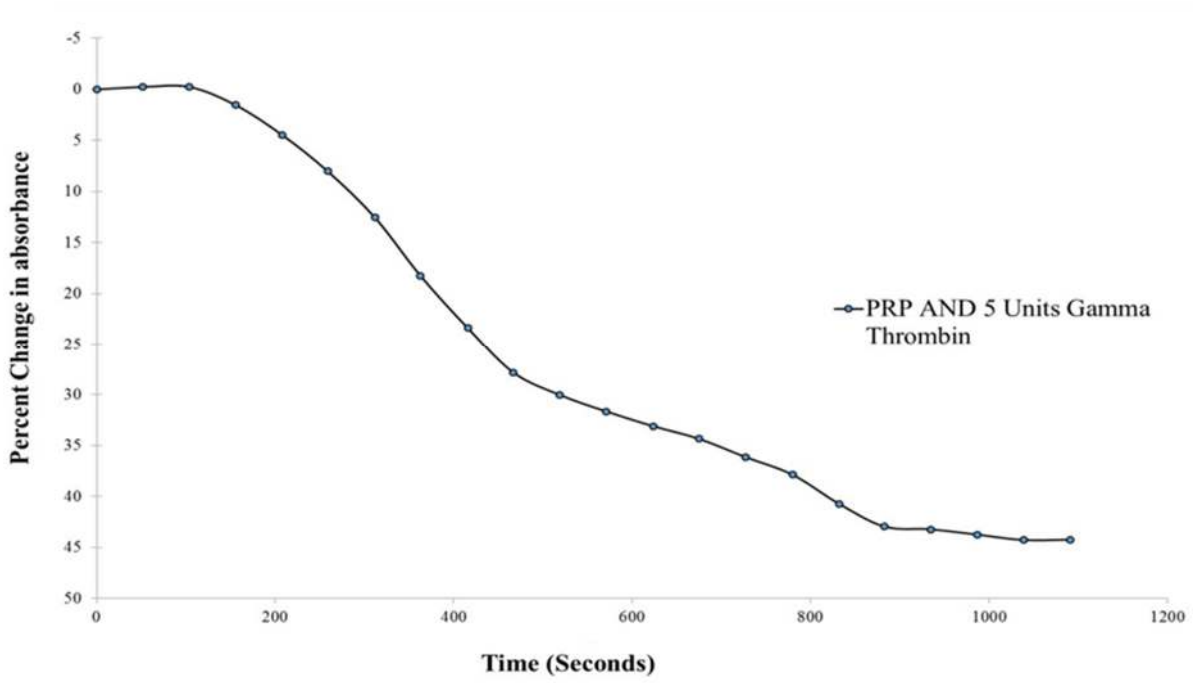


Figure 3.6 A platelet aggregation curve from PRP prepared from blood collected in heparin-containing tubes. The change in absorbance was expressed as a percentage of the highest response of platelet poor plasma and thrombin. For this test, the maximum amplitude 44.3 %, the aggregation rate was 0.06 % per second, and there was no disaggregation observed.

Using the same technique, the influence of 10 µM Zn^{2+} and 200 µM of myristate in ethanol on platelet aggregation was investigated. Figures 3.7 (A-D) are the aggregation responses observed

in the experiments and Table 3.1 shows the parameters calculated in the responses. Of note is that the presence of 10 µM Zn^{2+} was associated with higher maximum amplitude of aggregation than PRP alone (Figure 3.7 D). The vehicle ethanol in which myristate was dissolved mitigated the platelet aggregation (Figure 3.7 C). The addition of 200 µM myristate dissolved in ethanol was associated with higher maximum amplitude than that of PRP and ethanol (Figure 3.7 B), but lower than that of PRP alone, possibly due to the effect of ethanol. standard PRP response, but better than that of ethanol which was the vehicle. Ethanol, which was the vehicle had the least maximum amplitude of aggregation of all test samples analysed.

The results of these experiments suggested that Zn^{2+} positively influenced platelet aggregation, as shown by the high maximum amplitude of aggregation in the PRP which had 10 µM added and is in line with results from other studies (Taylor and Pugh, 2016, Watson et al., 2016). Zn^{2+} can influence the platelet aggregation by potentiating other agonists, act as a second messenger and in supra-physiological concentrations directly stimulate aggregation (Trybulec et al., 1993, Taylor and Pugh, 2016, Watson et al., 2016). The addition of 200 µM myristate did not lead to a higher platelet aggregation response as expected than that of normal PRP alone. It should be considered, however, that ethanol was used as a vehicle through which the myristate was dissolved in. The literature clearly shows that alcohol significantly reduces platelet function both in vitro and *in vivo* (Ruf, 2004, Schramko and Niemi, 2014). It is highly likely, therefore, that the influence of myristate was masked by the inhibition effect of ethanol in this experiment as seen in Figure 3.7 C, where the addition of ethanol alone produced the lowest maximum amplitude of platelet aggregation. Still more, the addition of myristate led to a platelet aggregation response which was higher than that of ethanol alone - demonstrating a possible influence on the aggregation, likely attributed to an allosteric disturbance of Zn^{2+} binding to albumin.

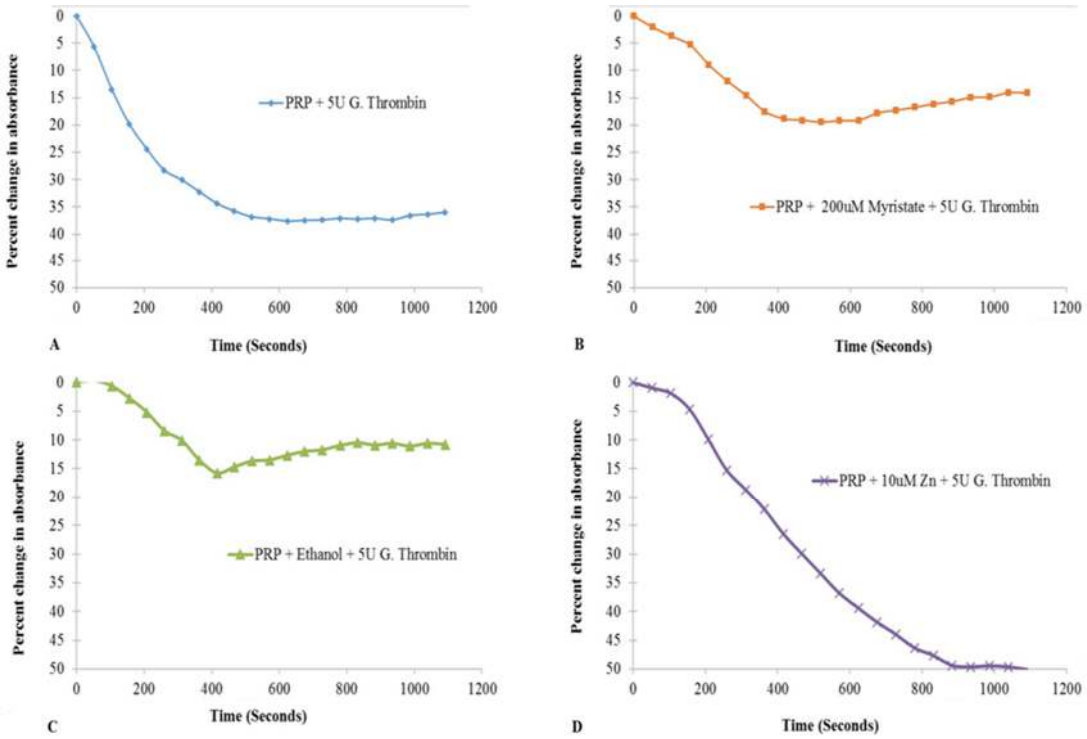


Figure 3.7 Platelet aggregation curves for simultaneously performed experiments.

(A) PRP with 5 U/ ml G, thrombin; (B) 200 µM Myristate in PRP and 5 U/ml G. thrombin; (C)Ethanol in PRP and 5 units G. thrombin; (D) 10 µM Zn^{2+} in PRP and 5 U/ml thrombin.

Table 3.1 Parameters for platelet aggregation curves for PRP, PRP with myristate, PRP and ethanol and PRP with Zn^{2+}: all induced by 5 U/ml of G. thrombin

Experiment	Parameter	
	Maximum amplitude (%)	**Primary Slope (% change / Second)**
PRP	37.6	0.09
200 µM Myristate in PRP	19.5	0.06
Ethanol in PRP	15.8	0.05
10 µM Zn^{2+} in PRP	50.1	0.07

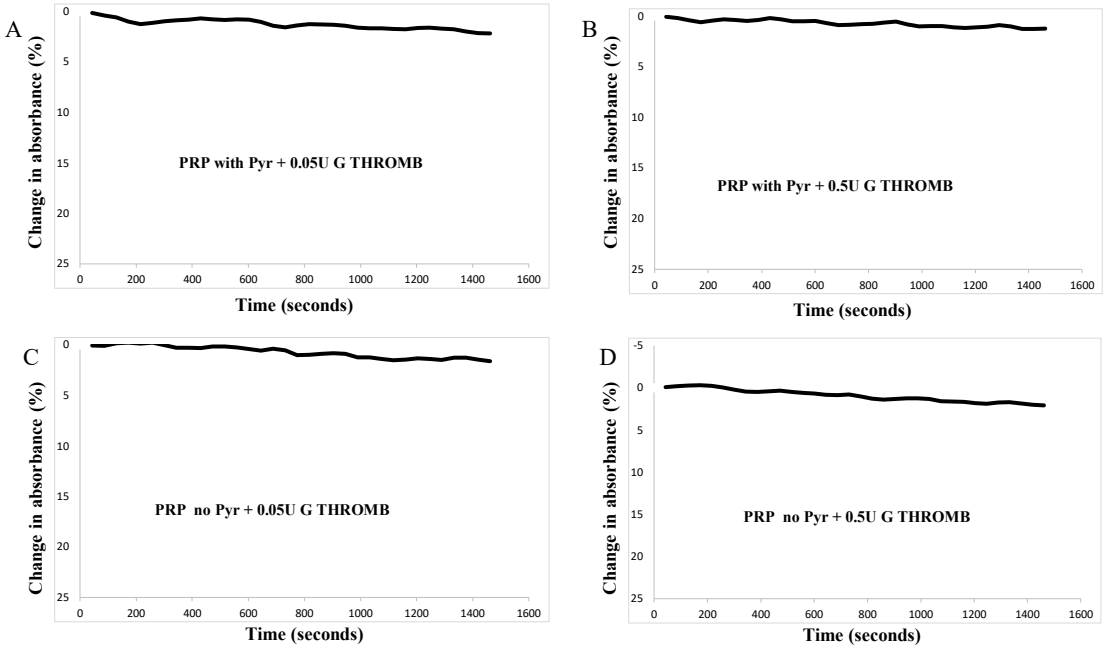
The challenge associated with the approach used in the above experiments was that the blood collection was done using tubes containing heparin as the anticoagulant. As described in the introductory section of this chapter, heparin may interfere with electrolytes in plasma such as calcium and magnesium – disrupting the physiological state; but also, heparin neutralisation by HRG following Zn^{2+} potentiation is one of the pathways in which Zn^{2+} may elicit coagulation under investigation in this study. Given these facts, heparin was considered an inappropriate anticoagulant to use for the platelet aggregation experiments.

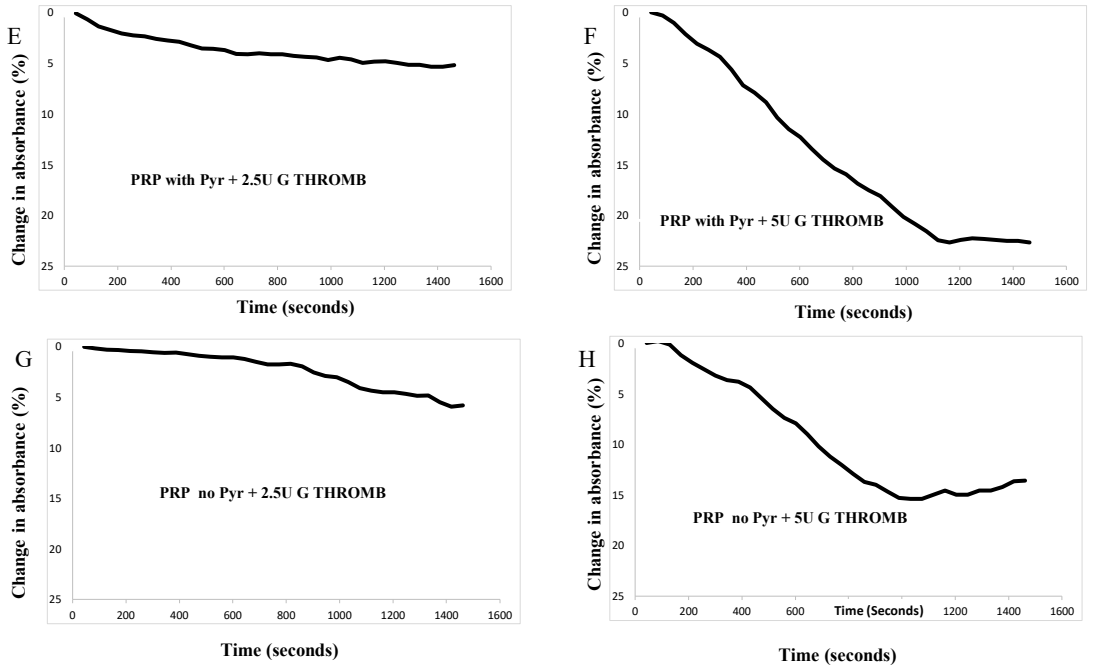
The next anticoagulant of choice was hirudin. Using the single spin method of PRP preparation, an attempt was made to elicit platelet aggregation using hirudin as an anticoagulant. However, this approach resulted in no response observed. An expert opinion was then sought from Dr Samantha Pitt, who has experience with these experiments, and it was recommended that the double-spin method be used and anticoagulants to be used should be hirudin and ACD.

3.4.2 Platelet aggregation using the double-spin method for the preparation of platelets

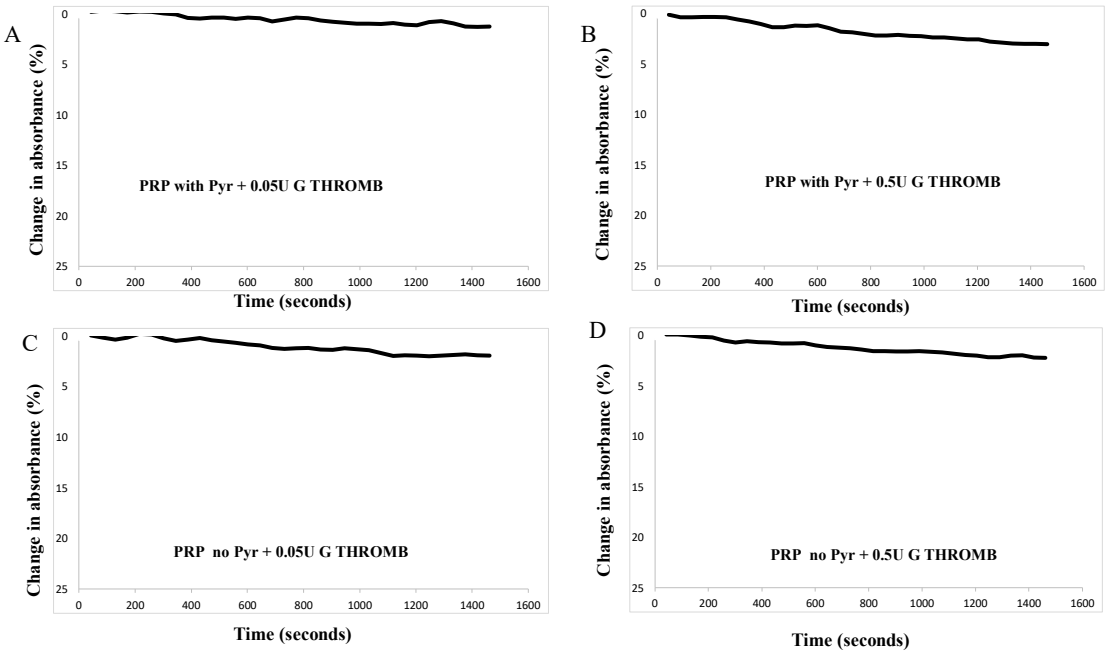
Experiments were performed to assess and consider the best concentration of G. thrombin to use for the platelet aggregation assays. The concentrations assessed were 0.05 U/ml, 0.5 U/ml, 2.5 U/ml and 5 U/ml. Furthermore, the effect of the use of apyrase in addition to aspirin, to prevent premature platelet aggregation, was investigated. Blood samples were collected, and platelets were prepared from three independent healthy volunteers. Figure 3.8 shows the platelet aggregation curves observed in the experiments in the three donors (I, II and III). It was noted that the use of 5 U/ml, with apyrase, produced better curves. Nonetheless, the maximum aggregation responses were lower than that of PRP from the single-spin method. Still, the double-spin method was preferred to achieve the desired investigations, as discussed previously.

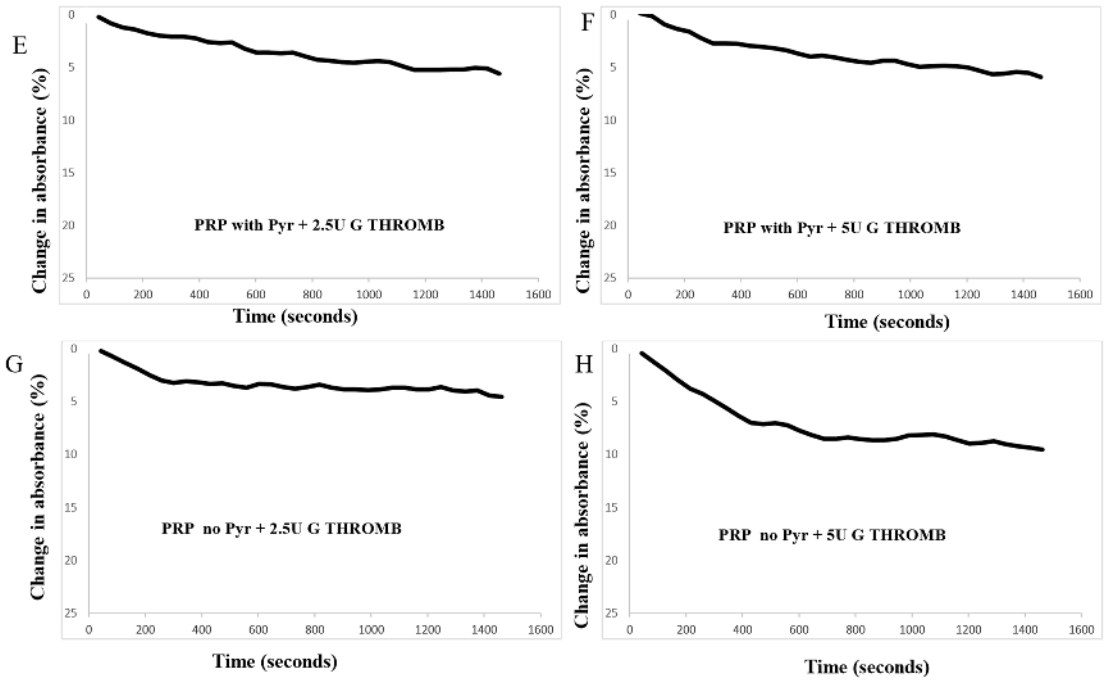
Next, the difference between 5 U/ml and 10 U/ml concentrations of G. thrombin and the effect of resuspending platelets either in hirudin-prepared PPP or in Tyrode's platelet buffer were assessed. Table 3.2 is a record of the aggregation parameters calculated from the experiments.


A
Change in absorbance (%)
PRP with Pyr + 0.05U G THROMB
Time (seconds)
B
Change in absorbance (%)
PRP with Pyr + 0.5U G THROMB
Time (seconds)
C
Change in absorbance (%)
PRP no Pyr + 0.05U G THROMB
Time (seconds)
D
Change in absorbance (%)
PRP no Pyr + 0.5U G THROMB
Time (seconds)

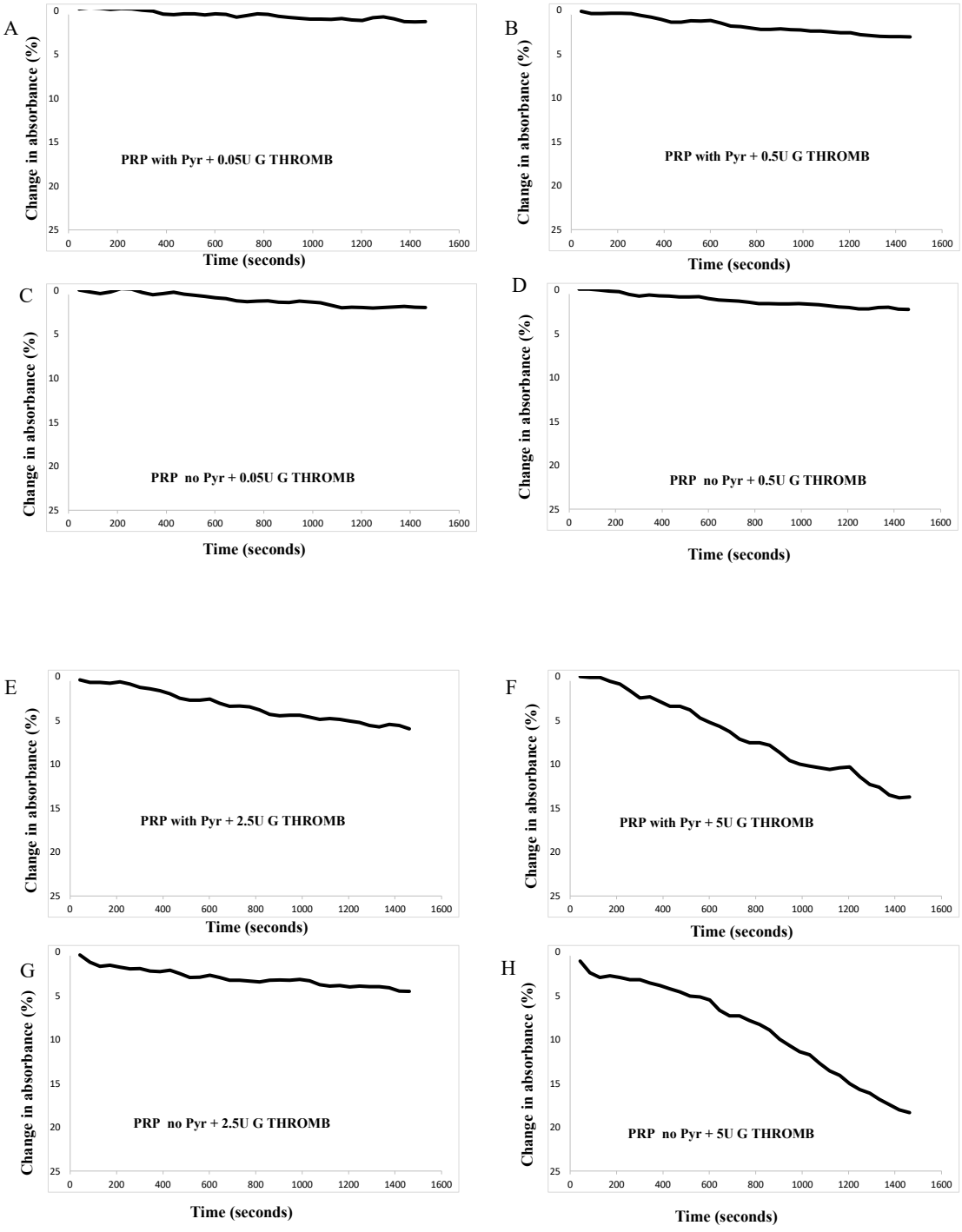

E
Change in absorbance (%)
PRP with Pyr + 2.5U G THROMB
Time (seconds)
F
Change in absorbance (%)
PRP with Pyr + 5U G THROMB
Time (seconds)
G
Change in absorbance (%)
PRP no Pyr + 2.5U G THROMB
Time (seconds)
H
Change in absorbance (%)
PRP no Pyr + 5U G THROMB
Time (Seconds)
Time (seconds)

(I)


A
Change in absorbance (%)
PRP with Pyr + 0.05U G THROMB
Time (seconds)
B
Change in absorbance (%)
PRP with Pyr + 0.5U G THROMB
Time (seconds)
C
Change in absorbance (%)
PRP no Pyr + 0.05U G THROMB
Time (seconds)
D
Change in absorbance (%)
PRP no Pyr + 0.5U G THROMB
Time (seconds)


E
Change in absorbance (%)
PRP with Pyr + 2.5U G THROMB
Time (seconds)
F
Change in absorbance (%)
PRP with Pyr + 5U G THROMB
Time (seconds)
G
Change in absorbance (%)
PRP no Pyr + 2.5U G THROMB
Time (seconds)
H
Change in absorbance (%)
PRP no Pyr + 5U G THROMB
Time (seconds)

(II)



(III)

Figure 3.8 Investigating the optimal G. thrombin dose for the platelet aggregation experiments. Concentrations assessed were 0.05 U/ml, 0.5 U/ml, 2.5 U/ml and 5 U/ml. (I) = First volunteer; (II) = Second volunteer; (III) = Third volunteer; Pyr = added Apryrase; G THROMB = Gamma thrombin.

Table 3.2 Comparison between 5 U/ml and 10U/ml G thrombin on platelet aggregation maximum response and rate in platelets resuspended in buffer and hirudin PRP

CURVE	DONOR	PARAMETER	
		Maximum response (%)	Rate of response % change per second
Platelets suspended in Hirudin PPP + 5U G. Thrombin	A	13.82	0.022
	B	5.28	0.009
Platelets suspended in Hirudin PPP + 10U G. Thrombin	A	5.4	0.015
	B	2.98	0.004
Washed platelets resuspended in Buffer + 5U G. thrombin	A	13.33	0.013
	B	15.36	0.023
Platelets suspended in Buffer + 10U G. thrombin	A	48.32	0.093
	B	14.08	0.027

Notably, there was some variability in the parameters between the two donors. Also, it was observed that the maximum aggregation responses were higher in platelets re-suspended in 1:9 Hirudin PPP and Buffer. Variability in platelet aggregation experiments and tests according to literature is common, and reproducibility is usually a challenge even in the same individual (Harrison et al., 2011). One crucial variable to check is the platelet count, and in this study, they

were all within the reference range of 200 to 600 x 10^9cells cells/ L, within which normal aggregation is expected (Cattaneo et al., 2007). Individual variability can, therefore, occur in platelet aggregation experiments, but this is of less concern if the platelet cell counts are within normal ranges. On the other hand, to ensure that the results of platelet experiments are credible, it is also essential that comparative experiments are conducted concurrently and simultaneously.

From the results of the experiments, a decision was made to use 5 U/ml of Gamma Thrombin and that the double-spin method for the preparation of platelets was to be used. The approach then used to assess the effects of octanoate as a medium-chain fatty acid and myristate as a long-chain fatty acid on platelet aggregation in washed platelets and platelets resuspended in PPP.

3.4.3 Effect of Zn^{2+} and FFA on platelet aggregation

Firstly, the effect of added Zn^{2+} on platelet aggregation in washed platelets and platelets resuspended in plasma was assessed. Figure 3.9 Shows the platelet aggregation representative curves with and without the addition of Zn^{2+} for washed platelets (A) and platelets resuspended in PPP (B). The data were summarised, and Figure 3.10 illustrates the maximum aggregation responses in the experiments (A) and the rates of aggregation (B).

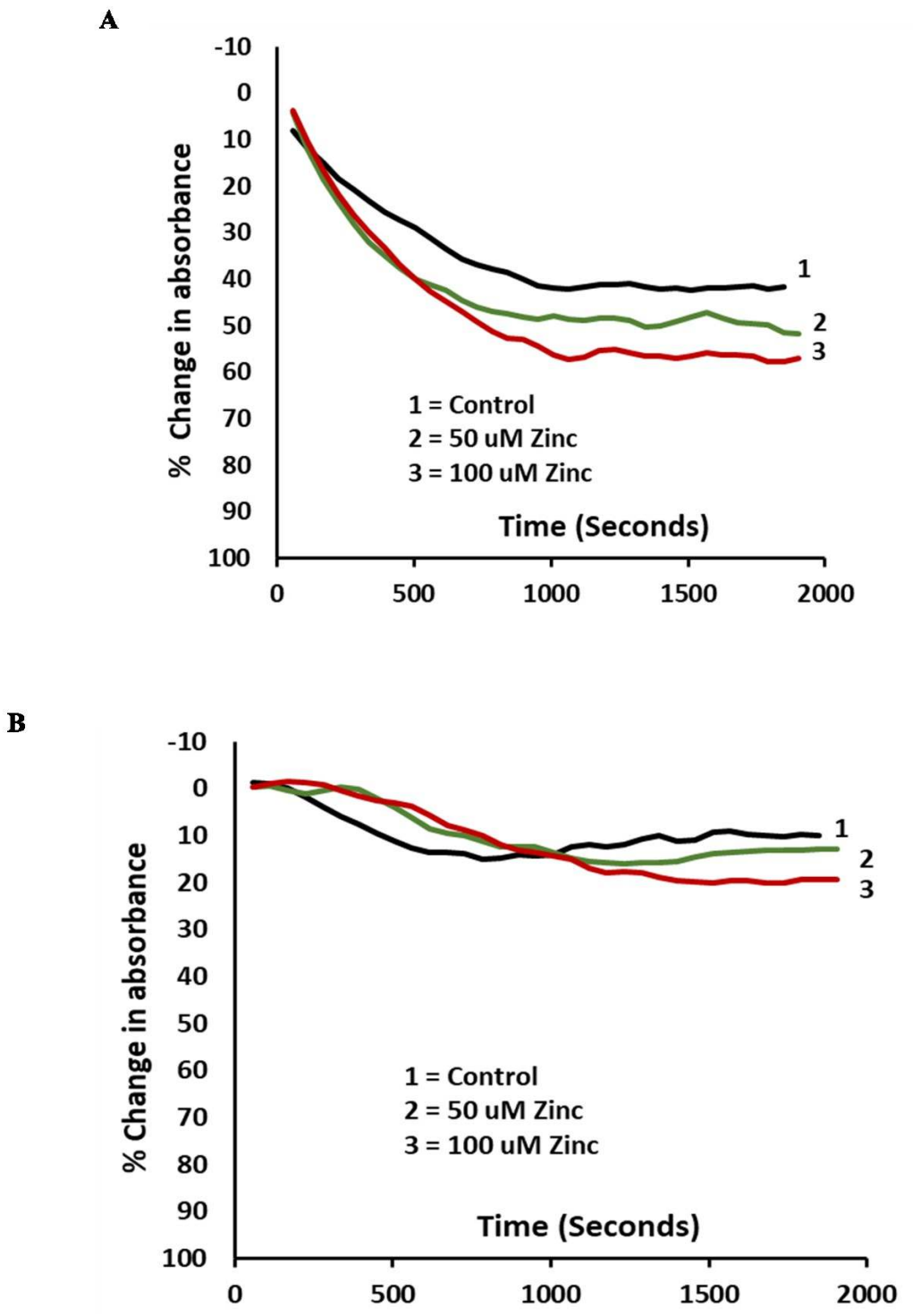


Figure 3.9 Platelet aggregation representative curves for platelet aggregation responses with and without the addition of Zn^{2+} for washed platelets (A) and platelets resuspended in PPP (B). The curves for washed platelets resuspended in buffer were had more pronounced aggregation responses recorded than curves of platelets resuspended in PPP. Control = 0 µM zinc.

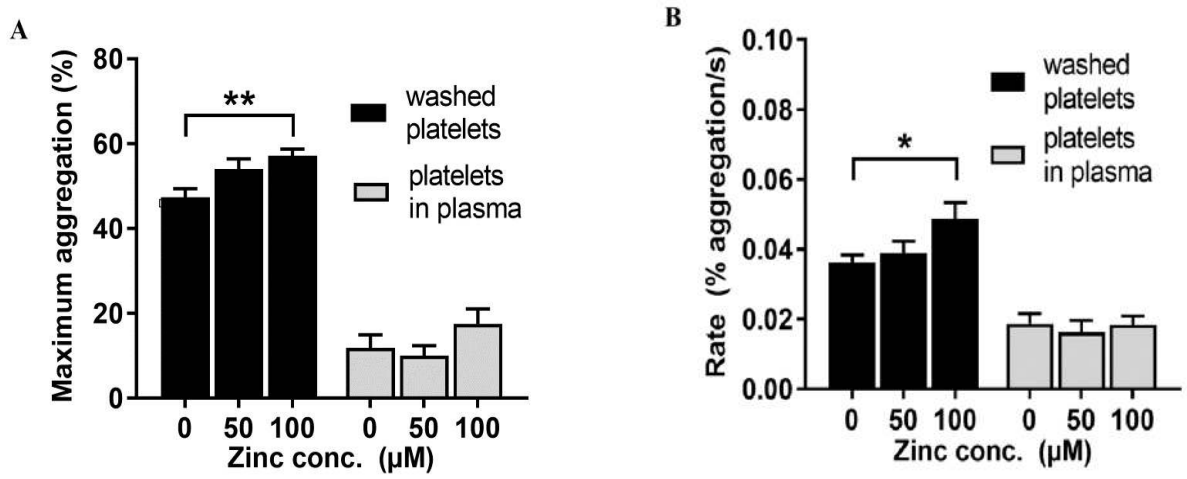


Figure 3.10 The effect of Zn^{2+} on platelet aggregation parameters in washed-platelets and platelets-in-plasma (n=11). In washed-platelets, the addition of 100 μM Zn^{2+} increased the maximum aggregation ($p<0.01$), with a trend towards significance observed at 50 μM ($p=0.054$), and the aggregation rate increased at both concentrations ($p<0.05$). In platelets re-suspended in plasma, no difference was observed with the addition of Zn^{2+}, presumably due to Zn^{2+} buffering by HSA. Notably, maximum aggregation was higher in washed-platelets than in platelets re-suspended in plasma and the addition of Zn^{2+} also increased the maximum aggregation response in washed-platelets ($p=0.008$). For platelets re-suspended in plasma, however, no statistically significant difference was observed with the addition of Zn^{2+} in platelets 0 to 100 μM ($p=0.2$). On the rate of aggregation, the addition of Zn^{2+} in washed platelets achieved an increased rate with the increase in Zn^{2+} concentration from 0 to 50 μM and 100 μM ($p<0.05$), with a statistically significant difference observed between 0 and 100 μM ($p<0.03$). Nevertheless, no statistically significant changes in the rate of platelet aggregation were detected with the addition of Zn^{2+} in platelets re-suspended in plasma ($p=0.8$).

The effect of both octanoate and myristate on platelet aggregation was examined to compare effects of FFA that perturbs Zn^{2+} binding to HSA with one that does not (while aware that Zn^{2+} is already present in plasma). Also, TPEN, which is a Zn^{2+} -selective chelator, was used to assess if it could mitigate any effect exerted by the FFA. Figure 3.11 illustrates the effects of the fatty acids on the platelet aggregation parameters maximum aggregation (A) and rate of aggregation (B). The addition of octanoate did not affect the maximum aggregation or aggregation rate. Contrary to the effect of octanoate, the addition of 4 mol. eq. of myristate

increased both the maximum aggregation and aggregation rate, but the increase in the rate was not statistically significant. TPEN reversed the effects of myristate. Finally, 100 μM Zn^{2+} in combination with 4 mol. eq. myristate further increased maximum aggregation (compared to 4 mol. eq. myristate alone) but not aggregation rate.

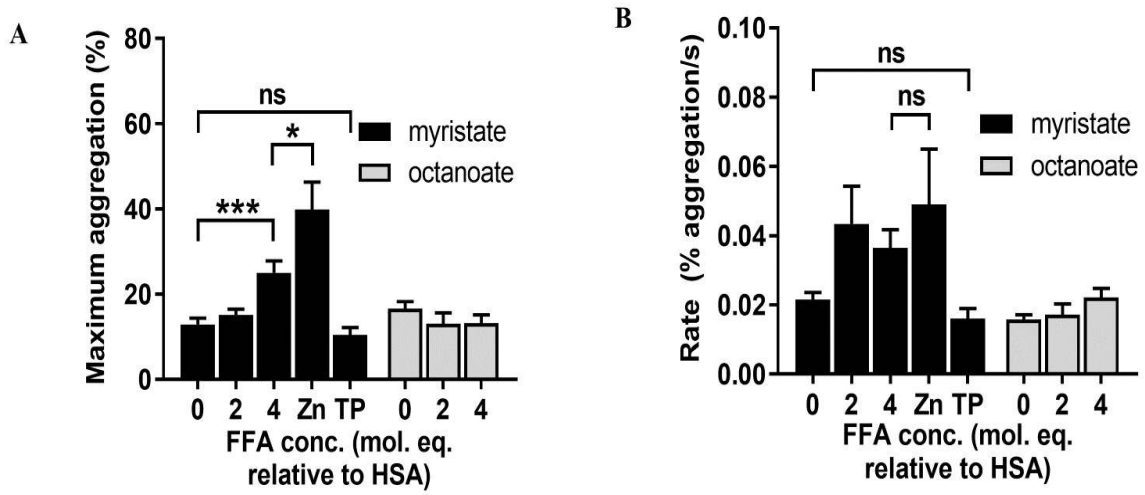


Figure 3.11 The effect of added sodium octanoate and sodium myristate in plasma on platelet aggregation (n=12). (A) Platelet maximum aggregation response significantly increased with the addition of 4 molar equivalents of sodium myristate ($p<0.001$). An addition of 100 μM Zn^{2+} in addition to the 4 molar equivalents of sodium myristate (Zn) further increased the maximum aggregation response ($p=0.02$). The addition of TPEN, however, as a Zn^{2+} chelator, in the presence of 4 molar equivalents sodium myristate (TPEN) produced maximum platelet aggregation responses comparable to the controls (no Zn^{2+}) indicating that the effect of sodium myristate was mediated by Zn^{2+}. No statistically significant changes were observed, nevertheless, with the addition of sodium octanoate at the same concentrations of 0, 2 and 4 molar equivalents (even with the addition of 100 μM of Zn^{2+} in the presence of 4 molar equivalents of sodium octanoate ($p=0.16$). (B)The intervention with sodium myristate but not sodium octanoate resulted in a statistically significant increase in the rate of platelet aggregation rate at 4 molar equivalents ($p<0.02$). With the addition of 100 μM Zn^{2+} to 4 molar equivalents of sodium myristate, the effect was sustained, but no further significant change in the aggregation rate was registered.

3.4.4 Fluorescent Zn^{2+} imaging in washed human platelets

The FluoZin-3 is a fluorescence Zn^{2+}-selective indicator with a structure which exhibits high Zn^{2+}-binding affinity. Fluozin-3 fluorescence imaging was performed on washed platelets to ascertain the presence of Zn^{2+} accumulation in the platelets, which is released and taken up from the internal milieu during the process of platelet aggregation. Collagen-adhered Fluozin3-loaded platelets were imaged by single image capture using a Leica DMi8 inverted fluorescent microscope. Images were collected using a 100 x oil objective. The platelet preparation and imaging were facilitated in collaboration with Dr Samantha Pitt and Dr Juan Varela from the Schools of Medicine and Biology respectively, University of St Andrews. Figure 3.12 shows the collagen-adhered fluozin-3-loaded platelets imaged by single image capture using a Leica DMi8 inverted fluorescent microscope. Images were collected using a 100× oil objective.

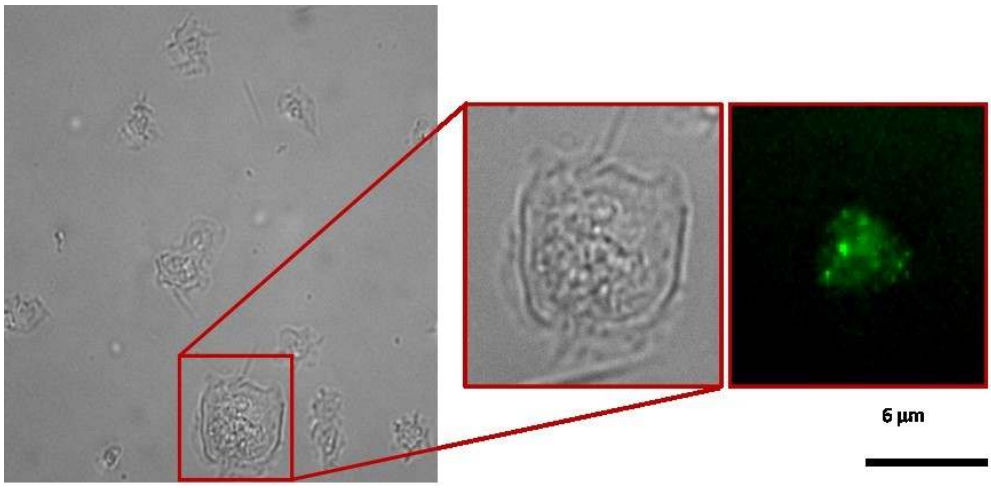


Figure 3.12 Representative image of Fluozin-3 fluorescence of washed human platelets. Collagen-adhered Fluozin-3-loaded platelets were imaged by single image capture using a Leica DMi8 inverted fluorescent microscope. Images were collected using a 100 x oil objective. Zoomed out panels (red box) show bright field image of a single platelet (left) and punctate staining of Zn^{2+} rich subcellular structures within the platelet (right).

3.5 Discussion

Platelet aggregation following their adherence is a vital process in coagulation and formation of a thrombus (Rumbaut and Thiagarajan, 2010). Factors that promote platelet aggregation, therefore, can influence coagulation and thrombosis. Platelet aggregation experiments can be used to elucidate factors that influence coagulation via their effect on platelet aggregation (Heyns Adu et al., 1985, Taylor et al., 2016, Watson et al., 2016). The present study used platelet aggregation assays adapted and modified from the methods described by Vial *et al.,* (2003) to assess the effects of increased Zn^{2+} concentration and different fatty acids on the magnitude and rate of platelet aggregation.

The results of the present study showed that the aggregation responses were heightened in the washed platelets. The results also suggested that increased Zn^{2+} in the absence or poor handling of a buffering protein such as albumin potentiates platelet aggregation by positively influencing the maximum aggregation response and the rate of aggregation. The influence of Zn^{2+} was exhibited in washed platelets resuspended in platelet buffer but not in platelets resuspended in PPP, likely due to the presence of albumin in the PPP - confirming the important buffering role of Zn^{2+} by albumin (Barnett et al., 2013). The results of the study also suggested that long chain FFA such as myristate, unlike medium chain FFA such as octanoate potentiate platelet aggregation by increasing the intensity of platelet aggregation also influencing the rate of aggregation.

The findings of this study regarding the positive effect of Zn^{2+} on platelet aggregation response agree with previous studies. Other research studies have suggested that once the concentration of plasma Zn^{2+} increases, it can potentiate platelet aggregation through several ways as it gains access into the platelet cytosol (Taylor and Pugh, 2016). The fluozin-3 fluorescence image of washed human platelets performed in this study confirms the presence and likely participation of Zn^{2+} in platelet aggregation. Zn^{2+} can interact with PKC, potentiating enzyme activity, thereby facilitating PKC-mediated phosphorylation which ultimately results in the activation of integrin $\alpha IIb\beta_3$ which cross-links platelets via binding of fibrinogen, mediating platelet aggregation (Gordon and O'Dell, 1980, Taylor and Pugh, 2016). Zn^{2+} also enhances tyrosine phosphorylation of platelet signalling proteins, activates protein tyrosine kinases such as ERK which regulate tyrosine phosphorylation events downstream of platelet activation and inhibits adenylate cyclase, hence reducing cAMP levels and promoting platelet activation (Taylor and

Pugh, 2016). Zn^{2+} may also interact directly with integrin aIIbb3 altering the activity and regulating platelet-to-platelet interactions leading to thrombus formation.

The positive effect of exogenous Zn^{2+}on the rate of platelet aggregation in the washed platelets in the present study can be explained. Zn^{2+} is known to augment platelet agonists and exerts a double effect on fibrinogen receptors on the platelet surface (Kowalska et al., 1994, Trybulec et al., 1993). The results of the present study, therefore, suggest that Zn^{2+} can precipitate the intensity and rate of platelet aggregation, especially in the absence of proper buffering of Zn^{2+}. The buffering effect of Zn^{2+} by HSA was observed in this study by the non-significant observable effect of additional Zn^{2+} on platelet aggregation in the platelets resuspended in PPP containing HSA. To a lesser extent, the absence of or low concentrations of plasma proteins in the washed platelets justifies the heightened responses in platelet aggregation due to minimal interaction of the G. thrombin with plasma proteins and hence exerting its effects more (Taylor and Pugh, 2016).

The effect of Zn^{2+}, especially in the washed platelets, was positively associated with the concentration of the Zn^{2+}, where 100 µM exerted more effect than 50 µM. This result is similar to findings from other studies where supraphysiological concentrations of Zn^{2+} of up to 0.5 mM ultimately induced platelet aggregation in a concentration-dependent manner (Trybulec et al., 1993, Heyns Adu et al., 1985, Watson et al., 2016). It should also be recalled, as eluded to in the introductory section of this chapter, that Zn^{2+} promotes Ca^{2+} influx, which is essential for facilitating platelet activation and hence promotes platelet aggregation (O'Dell, 2000). Thus, in pathological conditions of poor Zn^{2+} handling, Zn^{2+} can potentiate platelet aggregation by increasing both the intensity and rate of aggregation through the discussed ways, and in extreme conditions can solely induce platelet aggregation.

The results from the experiments with the intervention of FFA and their effect on platelet aggregation provide additional insights on their effect on platelet aggregation and are of clinical importance. Addition of 4 mol. eq. myristate to PRP increased both maximum aggregation and rate of aggregation, while the addition of Zn^{2+} in combination with myristate further increased maximum aggregation. These results support the hypothesis that long-chain fatty acids induce an allosteric switch of Zn^{2+}, causing compromised buffering of the ions by HSA (Lu et al., 2012). The perturbed Zn^{2+} would then exert pro-aggregation effect on the platelets. The

hypothesis that myristate exerted the pro-platelet-aggregation effects by disrupting the Zn^{2+} buffering capacity of HSA was supported by the observation that TPEN reversed the effect.

On the other hand, the addition of up to 4 mol. eq. octanoate (a medium chain FFA) to PRP had no observable effect on platelet aggregation. Unlike long chain FFA, medium chain FFA such as octanoate are unlikely to disrupt the amino acid conformation and the binding of Zn^{2+} to site A on HSA because it requires only a half-pocket in domain II of HSA for its binding (Lu et al., 2012).

Clinical application of the platelet aggregation experiments

During a normal physiological coagulation process, plasma Zn^{2+} concentrations may acutely increase, arising from multiple sources, and when released, the Zn^{2+} potentiate platelet aggregation. Extracellular Zn^{2+} from the matrix of dermal and epidermal tissues is released following an injury (Michaelsson et al., 1980, Lansdown et al., 2007). Platelets themselves also secrete Zn^{2+} during platelet aggregation within a forming thrombus (Mahdi et al., 2002, Watson et al., 2016). Furthermore, and of importance to this study are pathological states such as cardiovascular diseases associated with atherosclerosis pose risk for further acute increased Zn^{2+} concentrations in the vicinity of an atherosclerotic plaque lesion (Stadler et al., 2008). Considering that atherosclerotic plaques contain up to six times higher Zn^{2+} concentration than healthy tissues; the case of the rapture of these lesions may release higher concentration of Zn^{2+} in the immediate milieu which would positively relate to its size (Stadler et al., 2008). The concentrations of Zn^{2+} used in the platelet aggregation experiments, therefore, do mimic what would actually occur in pathological conditions such as disseminated intravascular coagulation, following an atherosclerotic plaque rupture and in the presence of increased concentrations of FFA in circulation (Taylor and Pugh, 2016, Marx and Eldor, 1985, Stadler et al., 2008).

Patients with NCDs are known to have a higher risk for thrombo-embolic events with complications of cardiovascular and cerebrovascular events (Williams and Zaman, 2003, Grinspoon and Carr, 2005, Vallee and Falchuk, 1993). From the results of the present study, it can be deduced that a combination of both increased FFA associated with the NCDs (Coverdale et al., 2018) and an acute increase in plasma Zn^{2+} as in the rupture of an atherosclerotic plaque

(Stadler et al., 2008) can strongly precipitate platelet aggregation and promote coagulation. In such a case, the formation of a thrombus would further potentiate platelet aggregation leading to a vicious cycle (Taylor and Pugh, 2016). Crucially, the presence of higher concentrations of long-chain FFA in the plasma such as stearate can reduce the stability of fibrin generated through thrombin and enhance thrombus embolism from the source to other organs leading to cardiovascular and cerebrovascular events (Tanka-Salamon et al., 2016). Thus, in this way, the synergic effect of higher FFA and Zn^{2+} concentrations, which may occur in NCDs pose a higher risk increases the risk for thromboembolism and its consequences.

The results of the current study further motivate for the consideration of management strategies aimed at the amelioration of risk for prothrombotic events in populations at risk, given the suggested pathological mechanisms of Zn^{2+} dysregulation and higher plasma FFA. A preventative strategy would include lifestyle modification which includes healthy diet not high in saturated long chain fatty acids and exercise to reduce the effect of metabolic syndrome which is associated with dyslipidaemia (Nyberg et al., 2018, Xin et al., 2019). Treatment approaches would include those aimed at reducing the FFA when sustainably high to reduce the burden on albumin and curb the allosteric Zn^{2+} switch. A recent systemic review and meta-analysis of controlled clinical trials reported that statin therapy of at least two weeks could lower plasma FFA concentrations (Sahebkar et al., 2016). Another approach would be the use of fenofibrates, but the use of these drugs for lowering FFA requires further research (Vega et al., 2003). In diseases such as cancer where there is a *de novo* synthesis of FFA, targeting the fatty acid synthase in order to reduce the synthesis of FFA would be useful (Menendez and Lupu, 2007).

On the other hand, chelating agents for excess Zn^{2+} can be another potential strategy. The use of ethylenediaminetetraacetic acid (EDTA), for example, would be one approach. EDTA is a heavy metal chelator, which is already used in clinical practice to chelate Ca^{2+} in the treatment of atherosclerosis and can also chelate Zn^{2+} (Ferrero, 2016, Lamas et al., 2013). Another mechanism would be to find or design a pharmacological substance that can bind to albumin in a manner that prevents FFA binding at the FA2 site, thereby preventing the perturbing effect of Zn^{2+} binding to HSA.

REFERENCES FOR CHAPTER 3

1. ANDRE, P., PRASAD, K. S., DENIS, C. V., HE, M., PAPALIA, J. M., HYNES, R. O., PHILLIPS, D. R. & WAGNER, D. D. 2002. CD40L stabilizes arterial thrombi by a beta3 integrin--dependent mechanism. *Nature medicine,* 8**,** 247.

2. BARNETT, J. P., BLINDAUER, C. A., KASSAAR, O., KHAZAIPOUL, S., MARTIN, E. M., SADLER, P. J. & STEWART, A. J. 2013. Allosteric modulation of Zinc speciation by fatty acids. *Biochimica et biophysica acta,* 1830**,** 5456.

3. BENJAMIN, L. A., BRYER, A., EMSLEY, H. C. A., KHOO, S., SOLOMON, T. & CONNOR, M. D. 2012. HIV infection and stroke: current perspectives and future directions. *Lancet Neurology,* 11**,** 878.

4. BENNETT, J. S. 2005. Structure and function of the platelet integrin alphaIIbbeta3. *The ournal of clinical investigation,* 115**,** 3363.

5. BLAIR, P. & FLAUMENHAFT, R. 2009. Platelet alpha-granules: basic biology and clinical correlates. *Blood reviews,* 23**,** 177.

6. BODEN, G. 2008. Obesity and free fatty acids. *Endocrinology & Metabolism Clinics of North America,* 37**,** 635.

7. BORZA, D. B. & MORGAN, W. T. 1998. Histidine-proline-rich glycoprotein as a plasma pH sensor. Modulation of its interaction with glycosaminoglycans by ph and metals. *The Journal Of Biological Chemistry,* 273**,** 5493.

8. BOWEN, R. A. & REMALEY, A. T. 2014. Interferences from blood collection tube components on clinical chemistry assays. *Biochemia medica,* 24**,** 31.

9. BRADFORD, H. N., PIXLEY, R. A. & COLMAN, R. W. 2000. Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. *The Journal of biological chemistry,* 275**,** 22756.

10. BURSTEIN, Y., BERNS, L., HELDENBERG, D., KAHN, Y., WERBIN, B. Z. & TAMIR, I. 1978. Increase in platelet aggregation following a rise in plasma free fatty acids. *American Journal of Hematology,* 4**,** 17.

11. CATTANEO, M., HAYWARD, C. P. M., MOFFAT, K. A., PUGLIANO, M. T., LIU, Y. & MICHELSON, A. D. 2009. Results of a worldwide survey on the assessment of platelet function by light transmission aggregometry: a report from the platelet physiology subcommittee of the SSC of the ISTH. *Journal of Thrombosis & Haemostasis,* 7**,** 1029.

12. CATTANEO, M., LECCHI, A., ZIGHETTI, M. L. & LUSSANA, F. 2007. Platelet aggregation studies: autologous platelet-poor plasma inhibits platelet aggregation when added to platelet-rich plasma to normalize platelet count. *Haematologica,* 92**,** 694.

13. CHARLES, M. A., FONTBONNE, A., THIBULT, N., CLAUDE, J. R., WARNET, J. M., ROSSELIN, G., DUCIMETIÈRE, P. & ESCHWÈGE, E. 2001. High plasma nonesterified fatty acids are predictive of cancer mortality but not of coronary heart disease mortality: results from the Paris Prospective Study. *American Journal of Epidemiology,* 153**,** 292.

14. CIFERRI, S., EMILIANI, C., GUGLIELMINI, G., ORLACCHIO, A., NENCI, G. G. & GRESELE, P. 2000. Platelets release their lysosomal content in vivo in humans upon activation. *Thrombosis and haemostasis,* 83**,** 157.

15. COLWELL, J. A. & NESTO, R. W. 2003. The platelet in diabetes: focus on prevention of ischemic events. *Diabetes care,* 26**,** 2181.

16. COUGHLIN, S. R. 2001. Protease-activated receptors in vascular biology. *Thrombosis and haemostasis,* 86**,** 298.

17. COVERDALE, J. P. C., KATUNDU, K., SOBCZAK, A. I. S., ARYA, S., BLINDAUER, C. A. & STEWART, A. J. 2018. Ischemia-modified albumin: crosstalk between fatty acid and cobalt binding. *Prostaglandins, Leukotrienes and Essential Fatty Acids*.

18. DANIEL, J. L., MOLISH, I. R., RIGMAIDEN, M. & STEWART, G. 1984. Evidence for a role of myosin phosphorylation in the initiation of the platelet shape change response. *The Journal of biological chemistry,* 259**,** 9826.

19. DHINDSA, S., GHANIM, H. & DANDONA, P. 2015. Nonesterified fatty acids, albumin, and platelet aggregation. *Diabetes,* 64**,** 703.

20. DHURAT, R. & SUKESH, M. 2014. Principles and Methods of Preparation of PlateletRich Plasma: A Review and Author's Perspective. *Journal of cutaneous and aesthetic surgery,* 7**,** 189.

21. EBBELING, L., ROBERTSON, C., MCNICOL, A. & GERRARD, J. M. 1992. Rapid ultrastructural changes in the dense tubular system following platelet activation. *Blood,* 80**,** 718.

22. ESCOLAR, G., KRUMWIEDE, M. & WHITE, J. G. 1986. Organization of the actin cytoskeleton of resting and activated platelets in suspension. *The American journal of pathology,* 123**,** 86.

23. FEIGIN, V. L., FOROUZANFAR, M. H., KRISHNAMURTHI, R., MENSAH, G. A., CONNOR, M., BENNETT, D. A., MORAN, A. E., SACCO, R. L., ANDERSON, L., TRUELSEN, T., O'DONNELL, M., VENKETASUBRAMANIAN, N., BARKERCOLLO, S., LAWES, C. M., WANG, W., SHINOHARA, Y., WITT, E., EZZATI, M., NAGHAVI, M., MURRAY, C., GLOBAL BURDEN OF DISEASES, I., RISK FACTORS, S. & THE, G. B. D. S. E. G. 2014. Global and regional burden of stroke during 1990-2010: findings from the Global Burden of Disease Study 2010. *Lancet (London, England),* 383**,** 245.

24. FERRERO, M. E. 2016. Rationale for the Successful Management of EDTA Chelation Therapy in Human Burden by Toxic Metals. *BioMed research international,* 2016**,** 8274504.

25. FLAUMENHAFT, R. 2003. Molecular basis of platelet granule secretion. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 23**,** 1152.

26. FOX, J. E. 2001. Cytoskeletal proteins and platelet signaling. *Thrombosis and haemostasis,* 86**,** 198.

27. GORDON, P. R. & O'DELL, B. L. 1980. Rat platelet aggregation impaired by short-term Zinc deficiency. *The Journal of nutrition,* 110**,** 2125.

28. GORDON, P. R., WOODRUFF, C. W., ANDERSON, H. L. & O'DELL, B. L. 1982. Effect of acute Zinc deprivation on plasma Zinc and platelet aggregation in adult males. *The American Journal of Clinical Nutrition,* 35**,** 113.

29. GRINSPOON, S. & CARR, A. 2005. Cardiovascular risk and body-fat abnormalities in HIV-infected adults. *The New England journal of medicine,* 352**,** 48.

30. GRYGLEWSKI, R. J. 2008. Prostacyclin among prostanoids. *Pharmacological reports : PR,* 60**,** 3.

31. GUIDETTI, G. F., BERNARDI, B., CONSONNI, A., RIZZO, P., GRUPPI, C., BALDUINI, C. & TORTI, M. 2009. Integrin alpha2beta1 induces phosphorylationdependent and phosphorylation-independent activation of phospholipase Cgamma2 in platelets: role of Src kinase and Rac GTPase. *Journal of thrombosis and haemostasis : JTH,* 7**,** 1200.

32. HABIB, A., FITZGERALD, G. A. & MACLOUF, J. 1999. Phosphorylation of the thromboxane receptor alpha, the predominant isoform expressed in human platelets. *The Journal of biological chemistry,* 274**,** 2645.

33. HARMON, J. T. & JAMIESON, G. A. 1986. The glycocalicin portion of platelet glycoprotein Ib expresses both high and moderate affinity receptor sites for thrombin. A soluble radioreceptor assay for the interaction of thrombin with platelets. *The Journal of biological chemistry,* 261**,** 13224.

34. HARRISON, P., MACKIE, I., MUMFORD, A., BRIGGS, C., LIESNER, R., WINTER, M. & MACHIN, S. 2011. Guidelines for the laboratory investigation of heritable disorders of platelet function. *British journal of haematology,* 155**,** 30.

35. HARUYAMA, H. & WÜTHRICH, K. 1989. Conformation of recombinant

desulfatohirudin in aqueous solution determined by nuclear magnetic resonance. *Biochemistry,* 28**,** 4301.

36. HEYNS ADU, P., ELDOR, A., YAROM, R. & MARX, G. 1985. Zinc -induced platelet aggregation is mediated by the fibrinogen receptor and is not accompanied by release or by thromboxane synthesis. *Blood,* 66**,** 213.

37. ISRAELS, S. J., GERRARD, J. M., JACQUES, Y. V., MCNICOL, A., CHAM, B., NISHIBORI, M. & BAINTON, D. F. 1992. Platelet dense granule membranes contain both granulophysin and P-selectin (GMP-140). *Blood,* 80**,** 143.

38. JOHN C. HOOK, E. D. W. & WILLIAM E, C. 1967. Platelets, Fatty Acids and Thrombosis. *Circulation Research,* 20**,** 11.

39. JONES, A. L., HULETT, M. D. & PARISH, C. R. 2004. Histidine-rich glycoprotein binds to cell-surface heparan sulfate via its N-terminal domain following Zinc chelation. *The Journal Of Biological Chemistry,* 279**,** 30114.

40. KOWALSKA, M. A., JULIANO, D., TRYBULEC, M., LU, W. & NIEWIAROWSKI, S. 1994. Zinc ions potentiate adenosine diphosphate-induced platelet aggregation by activation of protein kinase C. *The Journal of laboratory and clinical medicine,* 123**,** 102.

41. KUMURA, T., HINO, M., YAMANE, T. & TATSUMI, N. 2000. Hirudin as an anticoagulant for both haematology and chemistry tests. *Journal of Automated Methods & Management in Chemistry,* 22**,** 109.

42. LAMAS, G. A., GOERTZ, C., BOINEAU, R., MARK, D. B., ROZEMA, T., NAHIN, R. L., LINDBLAD, L., LEWIS, E. F., DRISKO, J., LEE, K. L. & INVESTIGATORS, T. 2013. Effect of disodium EDTA chelation regimen on cardiovascular events in patients with previous myocardial infarction: the TACT randomized trial. *Jama,* 309**,** 1241.

43. LANSDOWN, A. B., MIRASTSCHIJSKI, U., STUBBS, N., SCANLON, E. & AGREN, S. 2007. Zinc in wound healing: theoretical, experimental, and clinical aspects. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society,* 15**,** 2.

44. LU, J., STEWART, A. J., SLEEP, D., SADLER, P. J., PINHEIRO, T. J. & BLINDAUER, C. A. 2012. A molecular mechanism for modulating plasma Zn speciation by fatty acids. *Journal of the American Chemical Society,* 134**,** 1454.

45. MAHAUT-SMITH, M. P. 2012. The unique contribution of ion channels to platelet and megakaryocyte function. *Journal of thrombosis and haemostasis : JTH,* 10**,** 1722.

46. MAHDI, F., MADAR, Z. S., FIGUEROA, C. D. & SCHMAIER, A. H. 2002. Factor XII interacts with the multiprotein assembly of urokinase plasminogen activator receptor, gC1qR, and cytokeratin 1 on endothelial cell membranes. *Blood,* 99**,** 3585.

47. MARRERO, S. & ADASHI, E. Y. 2015. Noncommunicable Diseases. *Seminars in reproductive medicine,* 33**,** 35.

48. MARX, G. 1988. Zinc binding to fibrinogen and fibrin. *Archives of Biochemistry and Biophysics,* 266**,** 285.

49. MARX, G. & ELDOR, A. 1985. The procoagulant effect of Zinc on fibrin clot formation. *American Journal of Hematology,* 19**,** 151.

50. MARX, G. & HOPMEIER, P. 1986. Zinc inhibits FPA release and increases fibrin turbidity. *American Journal of Hematology,* 22**,** 347.

51. MARX, G., HOPMEIER, P. & GURFEL, D. 1987. Zinc alters fibrin ultrastructure. *Thrombosis and haemostasis,* 57**,** 73.

52. MARX, G., KORNER, G., MOU, X. & GORODETSKY, R. 1993. Packaging Zinc , fibrinogen, and factor XIII in platelet alpha-granules. *Journal of cellular physiology,* 156**,** 437.

53. MENENDEZ, J. A. & LUPU, R. 2007. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nature Reviews Cancer,* 7**,** 763.

54. MERTEN, M., BEYTHIEN, C., GUTENSOHN, K., KUHNL, P., MEINERTZ, T. & THIAGARAJAN, P. 2005. Sulfatides activate platelets through P-selectin and enhance platelet and platelet-leukocyte aggregation. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 25**,** 258.

55. MERTEN, M. & THIAGARAJAN, P. 2000. P-selectin expression on platelets determines size and stability of platelet aggregates. *Circulation,* 102**,** 1931.

56. MICHAELSSON, G., LJUNGHALL, K. & DANIELSON, B. G. 1980. Zinc in epidermis and dermis in healthy subjects. *Acta Dermato-Venereologica,* 60**,** 295.

57. MICHELSON, A. D. (ed.) 2013. *Platelets,* Amsterdam: Amsterdam : Academic Press 2013.

58. MILLER, G. J. 1997. Dietary fatty acids and blood coagulation. *Prostaglandins, Leukotrienes and Essential Fatty Acids,* 57**,** 389.

59. MONTEILH-ZOLLER, M. K., HERMOSURA, M. C., NADLER, M. J., SCHARENBERG, A. M., PENNER, R. & FLEIG, A. 2003. TRPM7 provides an ion channel mechanism for cellular entry of trace metal ions. *The Journal of general physiology,* 121**,** 49.

60. MORI, S., SHINOHATA, R., RENBUTSU, M., TAKAHASHI, H. K., FANG, Y.-I., YAMAOKA, K., OKAMOTO, M., YAMAMOTO, I. & NISHIBORI, M. 2003. Histidine-rich glycoprotein plus Zinc reverses growth inhibition of vascular smooth muscle cells by heparin. *Cell and tissue research,* 312**,** 353.

61. MURUGAPPAN, S., SHANKAR, H. & KUNAPULI, S. P. 2004. Platelet receptors for adenine nucleotides and thromboxane A2. *Seminars in thrombosis and hemostasis,* 30**,** 411.

62. NAKAHATA, N. 2008. Thromboxane A2: physiology/pathophysiology, cellular signal transduction and pharmacology. *Pharmacology & therapeutics,* 118**,** 18.

63. NANDA, N., ANDRE, P., BAO, M., CLAUSER, K., DEGUZMAN, F., HOWIE, D., CONLEY, P. B., TERHORST, C. & PHILLIPS, D. R. 2005. Platelet aggregation induces platelet aggregate stability via SLAM family receptor signaling. *Blood,* 106**,** 3028.

64. NARAYANAN, S. 2000. The preanalytic phase. An important component of laboratory medicine. *American Journal of Clinical Pathology,* 113**,** 429.

65. NARAYANAN, S. & HAMASAKI, N. 1998. Current concepts of coagulation and fibrinolysis. *Advances in Clinical Chemistry,* 33**,** 133.

66. NIIYA, K., HODSON, E., BADER, R., BYERS-WARD, V., KOZIOL, J. A., PLOW, E. F. & RUGGERI, Z. M. 1987. Increased surface expression of the membrane glycoprotein IIb/IIIa complex induced by platelet activation. Relationship to the binding of fibrinogen and platelet aggregation. *Blood,* 70**,** 475.

67. NYBERG, S. T., BATTY, G. D., PENTTI, J., VIRTANEN, M., ALFREDSSON, L., FRANSSON, E. I., GOLDBERG, M., HEIKKILA, K., JOKELA, M., KNUTSSON, A., KOSKENVUO, M., LALLUKKA, T., LEINEWEBER, C., LINDBOHM, J. V., MADSEN, I. E. H., MAGNUSSON HANSON, L. L., NORDIN, M., OKSANEN, T., PIETILAINEN, O., RAHKONEN, O., RUGULIES, R., SHIPLEY, M. J., STENHOLM, S., SUOMINEN, S., THEORELL, T., VAHTERA, J., WESTERHOLM, P. J. M., WESTERLUND, H., ZINS, M., HAMER, M., SINGH-MANOUX, A., BELL, J. A., FERRIE, J. E. & KIVIMAKI, M. 2018. Obesity and loss of disease-free years owing to major non-communicable diseases: a multicohort study. *The Lancet.Public health,* 3**,** e490.

68. O'DELL, B. L. 2000. Role of Zinc in plasma membrane function. *The Journal of nutrition,* 130**,** 1432S.

69. O'DELL, B. L., REYNOLDS, G. & REEVES, P. G. 1977. Analogous effects of Zinc deficiency and aspirin toxicity in the pregnant rat. *The Journal of nutrition,* 107**,** 1222.

70. PIKETTY, A. C., FLÉCHON, A., LAPLANCHE, A., NOUYRIGAT, E., DROZ, J. P., THÉODORE, C. & FIZAZI, K. 2005. The risk of thrombo-embolic events is increased in patients with germ-cell tumours and can be predicted by serum lactate dehydrogenase and body surface area. *British journal of cancer,* 93**,** 909.

71. RAHMAN, M. T. & DE LEY, M. 2008. Metallothionein in human thrombocyte precursors, CD61+ megakaryocytes. *Cell biology and toxicology,* 24**,** 19.

72. RENDU, F. & BROHARD-BOHN, B. 2001. The platelet release reaction: granules' constituents, secretion and functions. *Platelets,* 12**,** 261.

73. ROBERTS, D. E., MATSUDA, T. & BOSE, R. 2012. Molecular and functional characterization of the human platelet Na(+) Ca(2+) exchangers. *British journal of pharmacology,* 165**,** 922.

74. RUF, J. C. 2004. Alcohol, wine and platelet function. *Biological research,* 37**,** 209.

75. RUMBAUT, R. E. & THIAGARAJAN, P. 2010. Platelet-Vessel Wall Interactions in Hemostasis and Thrombosis. San Rafael (CA): by Morgan & Claypool Life Sciences.

76. RYDEL, T. J., RAVICHANDRAN, K. G., TULINSKY, A., BODE, W., HUBER, R., ROITSCH, C. & FENTON, J. W. 1990. The structure of a complex of recombinant hirudin and human alpha-thrombin. *Science (New York, N.Y.),* 249**,** 277.

77. SADLER, P. J., TUCKER, A. & VILES, J. H. 1994. Involvement of a lysine residue in the N-terminal Ni2+ and Cu2+ binding site of serum albumins. *European Journal of Biochemistry,* 220**,** 193-200.

78. SAHEBKAR, A., SIMENTAL-MENDIA, L. E., PEDONE, C., FERRETTI, G., NACHTIGAL, P., BO, S., DEROSA, G., MAFFIOLI, P. & WATTS, G. F. 2016. Statin therapy and plasma free fatty acids: a systematic review and meta-analysis of controlled clinical trials. *British journal of clinical pharmacology,* 81**,** 807.

79. SCHRAMKO, A. & NIEMI, T. 2014. Alcohol induces profuse reversible decrease in platelet function and delayed initiation of co-agulation. 2**,** 1.

80. SOBOCKA, M. B., SOBOCKI, T., BABINSKA, A., HARTWIG, J. H., LI, M., EHRLICH, Y. H. & KORNECKI, E. 2004. Signaling pathways of the F11 receptor (F11R; a.k.a. JAM1, JAM-A) in human platelets: F11R dimerization, phosphorylation and complex formation with the integrin GPIIIa. *Journal of receptor and signal transduction research,* 24**,** 85.

81. STADLER, N., STANLEY, N., HEENEMAN, S., VACATA, V., DAEMEN, M. J., BANNON, P. G., WALTENBERGER, J. & DAVIES, M. J. 2008. Accumulation of Zinc in human atherosclerotic lesions correlates with calcium levels but does not protect against protein oxidation. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 28**,** 1024.

82. STEFANINI, M. 1999. Cutaneous bleeding related to Zinc deficiency in two cases of advanced cancer. *Cancer,* 86**,** 866.

83. STENBERG, P. E., SHUMAN, M. A., LEVINE, S. P. & BAINTON, D. F. 1984.

Redistribution of alpha-granules and their contents in thrombin-stimulated platelets. *The Journal of cell biology,* 98**,** 748.

84. SUGIURA, T. & NAKAMURA, H. 1994. Metallothionein in platelets. *International archives of allergy and immunology,* 103**,** 341.

85. SUZUKI, H., MURASAKI, K., KODAMA, K. & TAKAYAMA, H. 2003. Intracellular localization of glycoprotein VI in human platelets and its surface expression upon activation. *British journal of haematology,* 121**,** 904.

86. TANKA-SALAMON, A., KOMOROWICZ, E., SZABO, L., TENEKEDJIEV, K. & KOLEV, K. 2016. Free Fatty Acids Modulate Thrombin Mediated Fibrin Generation Resulting in Less Stable Clots. *PloS one,* 11**,** e0167806.

87. TATE, J. & WARD, G. 2004. Interferences in immunoassay. *The Clinical biochemist.Reviews / Australian Association of Clinical Biochemists,* 25**,** 105.

88. TAYLOR, B. E., MCCLAVE, S. A., MARTINDALE, R. G., WARREN, M. M., JOHNSON, D. R., BRAUNSCHWEIG, C., MCCARTHY, M. S., DAVANOS, E., RICE, T. W., CRESCI, G. A., GERVASIO, J. M., SACKS, G. S., ROBERTS, P. R., COMPHER, C., SOCIETY OF CRITICAL CARE, M., AMERICAN SOCIETY OF, P. & ENTERAL, 2016. Guidelines for the Provision and Assessment of Nutrition Support Therapy in the Adult Critically Ill Patient: Society of Critical Care Medicine (SCCM) and American Society for Parenteral and Enteral Nutrition (A.S.P.E.N.). *Critical Care Medicine,* 44**,** 390.

89. TAYLOR, K. A. & PUGH, N. 2016. The contribution of Zinc to platelet behaviour during haemostasis and thrombosis. *Metallomics : integrated biometal science,* 8**,** 144.

90. TOFFALETTI, J. G. & WILDERMANN, R. F. 2001. The effects of heparin anticoagulants and fill volume in blood gas syringes on ionized calcium and magnesium measurements. *Clinica chimica acta; international journal of clinical chemistry,* 304**,** 147.

91. TRYBULEC, M., KOWALSKA, M. A., MCLANE, M. A., SILVER, L., LU, W. &

NIEWIAROWSKI, S. 1993. Exposure of platelet fibrinogen receptors by Zinc ions: role of protein kinase C. *Proceedings Of The Society For Experimental Biology And Medicine.Society For Experimental Biology And Medicine (New York, N.Y.),* 203**,** 108.

92. VALLEE, B. L. & FALCHUK, K. H. 1993. The biochemical basis of Zinc physiology. *Physiological Reviews,* 73**,** 79.

93. VAN NISPEN TOT PANNERDEN, H., DE HAAS, F., GEERTS, W., POSTHUMA, G., VAN DIJK, S. & HEIJNEN, H. F. 2010. The platelet interior revisited: electron tomography reveals tubular alpha-granule subtypes. *Blood,* 116**,** 1147-56.

94. VARGA-SZABO, D., PLEINES, I. & NIESWANDT, B. 2008. Cell adhesion mechanisms in platelets. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 28**,** 403.

95. VEGA, G. L., CATER, N. B., HADIZADEH, D. R., 3RD, MEGURO, S. & GRUNDY, S. M. 2003. Free fatty acid metabolism during fenofibrate treatment of the metabolic syndrome. *Clinical pharmacology and therapeutics,* 74**,** 236.

96. VU, T. T., FREDENBURGH, J. C. & WEITZ, J. I. 2013. Zinc: an important cofactor in haemostasis and thrombosis. *Thrombosis and haemostasis,* 109**,** 421.

97. WATSON, B. R., WHITE, N. A., TAYLOR, K. A., HOWES, J.-M., MALCOR, J.-D. M., BIHAN, D., SAGE, S. O., FARNDALE, R. W. & PUGH, N. 2016. Zinc is a

transmembrane agonist that induces platelet activation in a tyrosine phosphorylationdependent manner. *Metallomics: Integrated Biometal Science,* 8**,** 91.

98. WEETERINGS, C., DE GROOT, P. G., ADELMEIJER, J. & LISMAN, T. 2008. The glycoprotein Ib-IX-V complex contributes to tissue factor-independent thrombin generation by recombinant factor VIIa on the activated platelet surface. *Blood,* 112**,** 3227.

99. WHISS, P. A., ANDERSSON, R. G. & SRINIVAS, U. 1997. Modulation of P-selectin expression on isolated human platelets by an NO donor assessed by a novel ELISA application. *Journal of immunological methods,* 200**,** 135.

100. WHITE, J. G., KRUMWIEDE, M. D. & ESCOLAR, G. 1999. Glycoprotein Ib is homogeneously distributed on external and internal membranes of resting platelets. *The American journal of pathology,* 155**,** 2127.

101. WILLIAMS, I. L. N. B. & ZAMAN, A. G. 2003. Review: The management of acute myocardial infarction in patients with diabetes mellitus. *The British Journal of Diabetes & Vascular Disease,* 3**,** 319.

102. WOULFE, D. S. 2005. Platelet G protein-coupled receptors in hemostasis and thrombosis. *Journal of thrombosis and haemostasis : JTH,* 3**,** 2193.

103. XIN, Y., WANG, Y., CHI, J., ZHU, X., ZHAO, H., ZHAO, S. & WANG, Y. 2019. Elevated free fatty acid level is associated with insulin-resistant state in nondiabetic Chinese people. *Diabetes, metabolic syndrome and obesity : targets and therapy,* 12**,** 139.

104. ZENTNER, G. M., CARDINAL, J. R. & KIM, S. W. 1981. Free fatty acid-induced platelet aggregation: studies with solubilized and nonsolubilized fatty acids. *Journal of pharmaceutical sciences,* 70**,** 975.

105. ZHOU, L. & SCHMAIER, A. H. 2005. Platelet aggregation testing in platelet-rich plasma: description of procedures with the aim to develop standards in the field. *American Journal of Clinical Pathology,* 123**,** 172.

CHAPTER 4

IN SILICO ANALYSIS OF ALBUMIN CRYSTAL STRUCTURES AND HOW THE BINDING OF OTHER LIGANDS AFFECT THE ZINC-BINDING SITE A

4.1 Introduction

X-ray crystallography is one of the most favoured techniques for structure determination of proteins and biological macromolecules (Smyth and Martin, 2000). Protein crystallisation is a technique that was discovered and developed in the late 19th century as a powerful purification tool and as a demonstration of chemical purity (McPherson and Gavira, 2013). The crystallisation process requires supersaturation of the molecules by the addition of precipitating agents such as neutral salts or polymers and through the manipulation of various parameters such as temperature, ionic strength and pH (McPherson and Gavira, 2013). Other vital factors for crystallisation process include the presence of metal ions, inhibitors, cofactors or other conventional small molecules (McPherson and Gavira, 2013). Approaches most widely used for crystallisation include vapour diffusion, dialysis, batch and liquid-liquid diffusion (McPherson and Gavira, 2013). The ultimate aim of X-ray crystallography is to obtain a three-dimensional molecular structure from a crystal, and this is achieved by X-ray diffraction analysis (Smyth and Martin, 2000).

X-ray crystallography is essential for the characterisation of macromolecules, such as proteins and nucleic acids (Ennifar, 2013). The technique has aided in the elucidation of biochemical processes and the discovery of pharmacological substances and how the substances interact with carrier proteins such as albumin (Ennifar, 2013, Wang et al., 2013b). He and Carter published the first X-ray crystal structure for HSA in 1992 (He and Carter, 1992). Following this publication, more HSA crystal structures have been published and assisted in characterising the binding properties of the protein for several ligands, including drugs.

Crystallography is useful and critical for determining how ligand binding can affect the structure and properties of a molecule such as HSA. This technique allows for the analysis of ligand bound x-ray structures and can reveal information regarding the binding sites of ligands. The technique has been used to explore the binding sites for metal ions such as zinc on albumin

(Handing et al., 2016a). Crystallography can also be used to study the effects of bound ligands on the binding sites of other molecules on a protein, a relevant example in this respect being the effect of FFAs bound on FA2 on the primary Zn^{2+} binding site.

4.2 Aim and objectives

This chapter provides an examination of albumin crystal structures from the protein data bank (PDB) and specifically assesses the potential effects of different ligands bound to the protein on the zinc binding site-A.

The specific objectives were to;

I. To summarise relevant albumin crystal structures from the PDB with different ligands bound.
II. Measure the distances between the amino acid side-chains forming zinc binding site A for the structures recorded.
III. Perform a comparative statistical analysis between the HSA crystal structures with LCFA bound and those without a ligand or with Zn^{2+} bound and how the distances between the relevant amino acid side-chains differ.

4.3 Methods

The albumin crystal structures were obtained from the PDB website (http://www.rcsb.org/). The various protein structures were downloaded in the PDB format and analysed using the BIOVIA Discovery Studio Visualizer 2017 program (CA, USA). The amino acids forming the zinc binding site A namely; HIS67, ASN99, HIS247 and ASP249 were selected from the rest, and the distances between them were measured in the different structures. Recording of the different albumin crystal structures were done in a Microsoft Excel sheet, where the PDB code and the distances between the different amino acids were recorded. The analysed crystal structure diagrams for the different albumins analysed were captured. Statistical analysis was performed using GraphPad Prizm software (CA, USA). Student's t-test was used to compare the distances assessed in the LCFA-bound HSA and non-ligand bound or zinc-bound HSA. A p-value of <0.05 was regarded as statistically significant.

4.4 RESULTS

A total of 119 albumin crystal structures were analysed from the PDB. Of these structures, 92 (73.3%) were human, 17 (14.3%) were equine, 4 (3.4%) were bovine, 1 (0.8%) was canine, 2 (1.7%) ovine, 3 (2.5) were leporine. A proportion of 87.4% of the structures had their resolution of less than 3 Å, which indicated more reliable structures for analysis (PDB, 2018). The molecular structures were analysed to determine the changes, if any, in the distance between the following pairs of amino acid side-chain atoms following binding of ligands; H67 Nε2 and H247 Nδ1, H67 Nε2 and D249 Oδ2, H247 Nδ1 and D249 Oδ2, H67 Nε2 and H247 Hε1, H67 Nε2 and H249 Hα, H67 Hδ2 and N99 Oδ1, H67 Hδ1 and N99 Oδ1, H67 Hδ1 and D249 Oδ2, H67 Hε1 and D249 Oδ1, H67 Hε1 and D249 Oδ2, H67 Hε1 and N99 Oδ1, H67 Hε1 and H247 O.

Table 4.1 shows a list of the albumin structures analysed and the respective parameters investigated. A total of 96 albumin structures had their analysed structures captured successfully. Figure 4.1 depicts the crystal structures which were analysed and distance measured between the amino acids forming the zinc binding site A measured.

Table 4.1 List of albumin crystal structures with and without ligands bound and their effect on distances of the amino acid residue forming zinc binding site A

PDB code	Species	Resolution (Å)	Ligands	Distance (Å)			Reference or DOI
				H67 Nε2 - H247 Nδ1	H67 Nε2 - D249 Oδ2	H247 Nδ1 - D249 Oδ2	
1AO6	Human	2.5	None	4.39	4.66	2.94	(Simard et al., 2005)
5IFO	Human	3.2	Indazolium trans-[Tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019)	11.1	3.34	7.91	(Bijelic et al., 2016)
5ID7	Human	2.26	Phosphorodithioate derivative of myristoyl cyclic phosphatidic acid (cPA)	14.81	3.14	11.99	(Madej et al., 2014)
5IJF	Human	2.65	0.5 mM zinc at pH 9.0	3.63	3.53	3.18	(Handing et al., 2016a)
4Z69	Human	2.19	Palmitic acid and diclofenac	9.03	3.15	6.07	(Zhang et al., 2015)
4S1Y	Human	3.16	Cisplatin	3.83	3.49	2.94	(Ferraro et al., 2015)
4BKE	Human	2.35	Palmitic acid	8.51	2.79	5.75	(Sivertsen et al., 2014)
4N0F	Human	3.02	Human FcRn complexed with human serum albumin	7.53	2.8	7.86	(Oganesyan et al., 2014)
4L8U	Human	2.01	9 amino camptothecin	8.67	3.14	8.81	(Wang et al., 2013b)
3UIV	Human	2.2	Myristic acid and Amantadine	8.91	2.98	6.04	DOI: 10.2210/pdb3uiv/pdb
4IW2	Human	2.41	Glucose	4.09	4.16	4.17	23592780

4IW1	Human	2.56	Fructose, D-fructose, Phosphate ion	8.55	4.46	5.93	DOI: 10.2210/pdb4iw1/pdb
4N0U	Human	3.8	Neonatal Fc receptor, serum albumin and Fc	7.75	2.83	7.91	DOI: 10.2210/pdb4n0u/pdb
4K71	Human	2.4	sulfate Ion	4.51	4.11	2.48	DOI: 10.2210/pdb4k71/pdb
4K2C	Human	3.23	Ligand-free	4.57	3.85	4	(Wang et al., 2013a)
4G04	Human	2.3	None	6.03	3.98	5.51	DOI: 10.2210/pdb4g04/pdb
4G03	Human	2.22	None	5.9	4.3	5.23	DOI: 10.2210/pdb4g03/pdb
4LB9	Human	2.7	Etoposide, Myristic acid	10.42	3.43	8.07	(Wang et al., 2013b)
4LB2	Human	2.8	Idarubicin	5.048	4.89	2.97	(Wang et al., 2013b)
4LA0	Human	2.4	R-Bicalutamide	4.76	4.55	3.182	(Wang et al., 2013b)
4L9Q	Human	2.7	Teniposide	5.06	4.87	3.07	(Wang et al., 2013b)
5FUO	human	3.6	None	6.01	4.61	5.17	(Adams et al., 2016)
3B9L	human	2.6	Myristic acid and Zidovudine	8.95	2.85	6.74	(Zhu et al., 2008)
2VUE	human	2.42	Biliverdine IX Alpha	4.19	4.79	3.46	(Zunszain et al., 2008)
2VUF	human	3.05	Fusidic acid	6.24	4.87	5.61	(Zunszain et al., 2008)
3CX9	Human	2.8	Myristic acid and lysophosphatidylethanolamine	9.29	4.33	6.01	(Guo et al., 2009)
3A73	human	2.19	delta12-prostaglandine J2 and Myristic acid	9.31	3.53	5.95	(Yamaguchi et al., 2010)
3JRY	human	2.3	Sulfate ion	6.71	4.56	5.21	(Hein et al., 2010)

3JQZ	human	3.3	2-(diethylamino)-N-(2,6-dimethylphenyl)ethanamide	4.17	4.3	4.13	(Hein et al., 2010)
3LU8	human	2.6	$C_{16}H_{16}FN_5O_3S_3$	4.39	4.27	2.87	(Buttar et al., 2010)
3LU7	human	2.8	$C_{22}H_{21}FN_2O_3$ and phosphate ion	3.88	3.32	2.78	(Buttar et al., 2010)
3LU6	human	2.7	$C_{20}H_{17}FN_2O_3$	3.47	6	2.69	(Buttar et al., 2010)
2XW1	human	2.5	Dansyl-l-norvaline	4.37	4.58	3.69	(Ryan et al., 2011b)
2XW0	human	2.4	Dansyl-l-phenylalanine	3.97	4.5	3.35	(Ryan et al., 2011b)
2XVW	human	2.65	Dansyl-l-arginine and myristic acid	8.95	2.76	6.3	(Ryan et al., 2011b)
2XVV	human	2.4	Dansyl-l-asparagine and myristic acid	10.49	3.3	no	(Ryan et al., 2011b)
2XVU	human	2.6	Dansyl-l-asparagine	5.76	4.51	5.06	(Ryan et al., 2011b)
2XVQ	human	2.9	Dansyl-l-sarcosine	6.6	4.51	5.7	(Ryan et al., 2011b)
2XSI	human	2.7	Dansyl-l-glutamate and myristic acid	8.77	2.81	6.49	(Ryan et al., 2011b)
2YDF	human	2.75	Iophenoxic acid	9.04	4.52	5.56	(Ryan et al., 2011a)
3SQJ	human	2.05	Myristic acid	8.72	2.3	6.71	(He et al., 2011)
4EMX	human	2.3	Chloride ion	5.49	4.32	4.76	DOI: 10.2210/pdb4emx/pdb
4E99	human	2.3	Perfluorooctane sulfonate	3.55	3.8	2.79	(Luo et al., 2012)
3TDL	human	2.6	11-(Dansylamino)undecanoic acid and myristic acid	11.8	3.99	8.98	(Wang et al., 2011)
1YSX	human	not provided	$C_{29}H_{26}FN_3O_5S_2$	not provided	not provided	not provided	(Oltersdorf et al., 2005)

2BXQ	human	2.6	Indomethacin, 4-butyl-1,2-diphenyl-pyrazolidine-3,5-dione and myristic acid	9.15	2.54	7.01	(Ghuman et al., 2005)
2BXP	human	2.3	4-butyl-1,2-diphenyl-pyrazolidine-3,5-dione and myristic acid	8.79	3.13	5.8	(Ghuman et al., 2005)
2BXO	human	2.6	Oxyphenbutazone and myristic acid	8.98	3.02	6.1	(Ghuman et al., 2005)
2BXN	human	2.65	C_{20} H_{14} I_6 N_2 O_6 and myristic acid	8.84	3.53	5.59	(Ghuman et al., 2005)
2BXM	human	2.5	Indomethacin and myristic acid	8.82	2.73	6.18	(Ghuman et al., 2005)
2BXL	human	2.6	2-hydroxy-3,5-diiodo-benzoic acid and myristic acid	8.92	2.8	6.19	(Ghuman et al., 2005)
2BXK	human	2.4	Azapropazone, indomethacin and myristic acid	9	2.56	6.59	(Ghuman et al., 2005)
2BXI	human	2.5	Azapropazone and myristic acid	9	3.07	5.98	(Ghuman et al., 2005)
2BXH	human	2.25	3-Sulfooxy-1h-indole	5.84	4.25	5.46	(Ghuman et al., 2005)
2BXG	human	2.7	Ibuprofen	6.17	5.09	6.16	(Ghuman et al., 2005)
2BXF	human	2.95	7-chloro-1-methyl-5-phenyl-1,3-dihydro-2h-1,4-benzodiazepin-2-one	6.68	5.03	5.3	(Ghuman et al., 2005)
2BXE	human	2.95	Diflunisal	6.32	4.49	5.23	(Ghuman et al., 2005)
2BXD	human	3.05	R-warfarin	6.14	4.75	4.98	(Ghuman et al., 2005)
2BXC	human	3.1	4-Butyl-1,2-diphenyl-pyrazolidine-3,5-dione	6.22	4.83	5.2	(Ghuman et al., 2005)
2BXB	human	3.2	Oxyphenbutazone	6	4.98	5.1	(Ghuman et al., 2005)

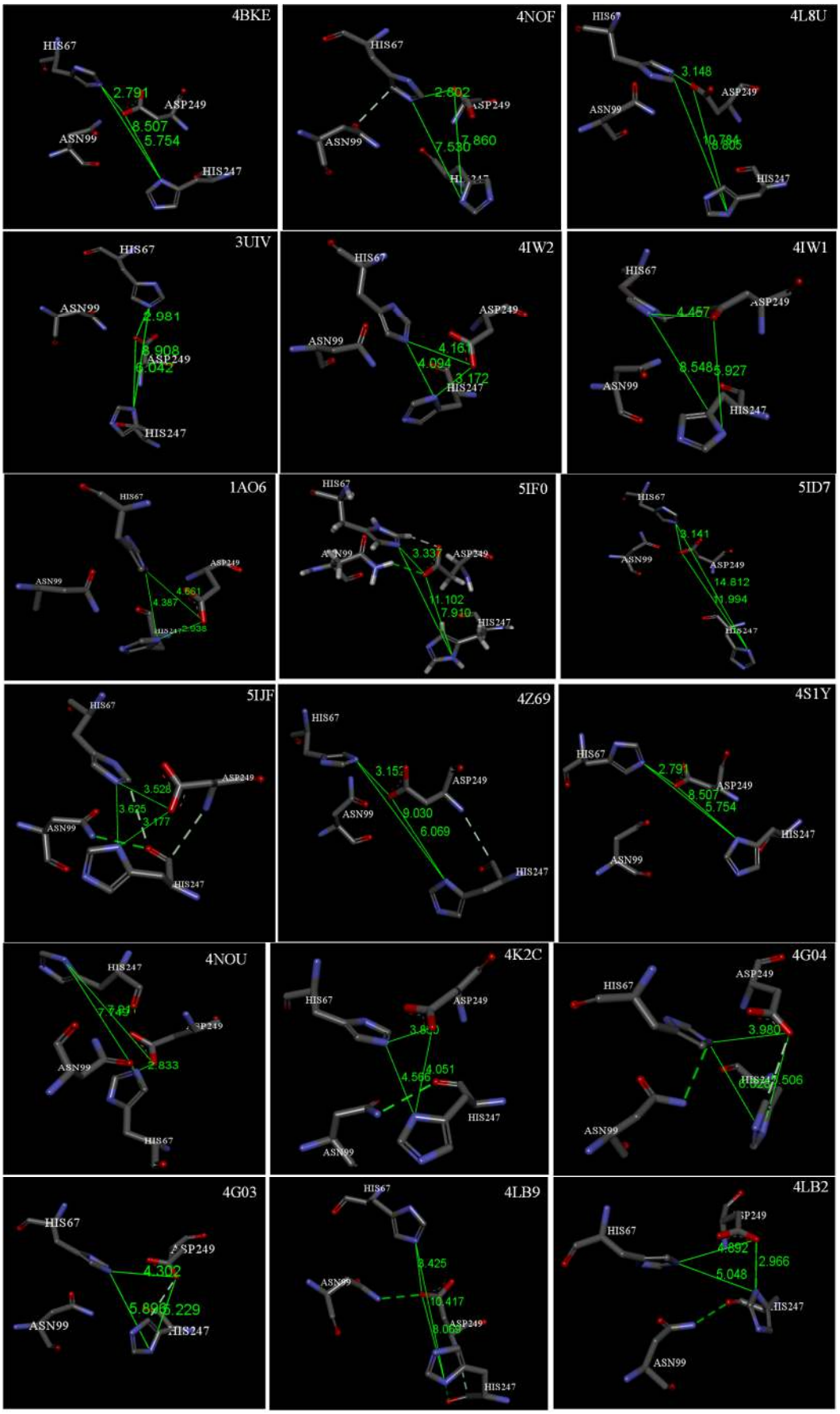
2BXA	human	2.35	3-carboxy-4-methyl-5-propyl-2-furanpropionic	6.12	4.32	5.36	(Ghuman et al., 2005)
2BX8	human	2.7	Azapropazone	6.09	4.42	5.05	(Ghuman et al., 2005)
2I30	human	2.9	Myristic acid and salicylic acid	8.71	2.59	6.21	(Yang et al., 2007)
2I2Z	human	2.7	Myristic acid and salicylic acid	8.87	3.05	5.84	(Yang et al., 2007)
2VDB	human	2.52	Naproxen and decanoic acid	12.56	6.15	8.26	(Lejon et al., 2008)
3B9M	human	2.7	Zidovudine, myristic acid and salicylic acid	8.39	2.62	5.92	(Lejon et al., 2008)
1E7C	human	2.4	Myristic acid and 2-bromo-2-chloro-1,1,1-trifluoroethane	8.7	2.77	6.1	(Bhattacharya et al., 2000a)
1E7B	human	2.38	2-bromo-2-chloro-1,1,1-trifluoroethane	4.27	4.6	4.43	(Bhattacharya et al., 2000a)
1E7A	human	2.2	Propofol	5.58	4.3	4.9	(Bhattacharya et al., 2000a)
1E78	human	2.6	None	6.21	4.47	5.05	(Bhattacharya et al., 2000a)
1HA2	human	2.5	S-warfarin and myristic acid	8.86	3.04	5.89	(Petitpas et al., 2001a)
1H9Z	human	2.5	R-warfarin and myristic acid	8.86	3.26	8.86	(Petitpas et al., 2001a)
1GNJ	human	2.6	Arachidonic acid	8.91	2.88	6.12	(Petitpas et al., 2001b)
1O9X	human	3.2	Heme and myristic acid	9.2	3.9	5.38	(Zunszain et al., 2003)
1HK5	human	2.7	3,5,3',5'-tetraiodo-l-thyronine and myristic acid	8.87	3.16	5.93	(Petitpas et al., 2003)
1HK4	human	2.4	3,5,3',5'-tetraiodo-l-thyronine and myristic acid	8.6	2.93	5.73	(Petitpas et al., 2003)

1HK3	human	2.8	3,5,3',5'-tetraiodo-l-thyronine	3.87	4.36	2.5	(Petitpas et al., 2003)
1HK2	human	2.8	3,5,3',5'-tetraiodo-l-thyronine	8.29	4.31	5.88	(Petitpas et al., 2003)
1HK1	human	2.65	3,5,3',5'-tetraiodo-l-thyronine	3.83	4.11	2.36	(Petitpas et al., 2003)
1N5U	human	1.9	Heme and myristic acid	8.7	4.03	6.55	(Lejon et al., 2004)
1TF0	human	2.7	Citric acid and decanoic acid	5.48	4.83	3.89	(Lejon et al., 2004)
1E7G	human	2.5	Myristic acid	8.84	3.3	5.6	(Bhattacharya et al., 2000b)
1E7I	human	2.7	Stearic acid	8.66	2.89	5.88	(Bhattacharya et al., 2000b)
1BM0	human	2.5	None	4.5	4.4	2.8	(Sugio et al., 1999)
1BKE	human	3.15	2,3,5-triiodobenzoic acid and myristic acid	8.6	3	5.66	(Curry et al., 1998)
1BJ5	human	2.5	Myristic acid	8.62	3.27	5.4	(Curry et al., 1998)
1GNI	Human	2.4	Oleic acid	8.99	3.51	5.56	(Petitpas et al., 2001b)
1E7H	Human	2.43	Palmitic acid	8.74	3.01	5.75	(Bhattacharya et al., 2000b)
1E7F	Human	2.43	Lauric acid	8.5	2.63	6.1	11061971 (Bhattacharya et al., 2000c)
1E7E	Human	2.5	Decanoic acid	8.53	2.87	5.72	11061971 (Bhattacharya et al., 2000b)
1UOR	human	2.8	None	8.65	4.82	6.98	(He and Carter, 1992)
4HGK	Human SA and shark V-NAR antibody	3.04	None	7.63	3.22	6.47	(Kovalenko et al., 2013)
5V0V	Equine	2.45	(S)-Etodolac, (r)-etodolac, tris buffer and sulfate ion	7.61	4	6.14	DOI: 10.2210/pdb5V0V/pdb

5GHK	Canine	3.2	None	5.6	3.35	5.65	(Yamada et al., 2016)
5ID9	Equine	2.48	(4S)-2-sulfanylidene-4-[(tetradecanoyloxy)methyl]-1,3,2lambda~5~-dioxaphospholane-2-thiolate, malonate ion and formic acid	6.06	4.47	5.29	(Sekula et al., 2016)
5IJE	Equine	2.4	Sulfate ion and zinc ion	7.1	3.96	5.18	(Handing et al., 2016a)
5IIU	Equine	2.3	Sulfate ion and zinc ion	3.44	3.54	3.33	(Handing et al., 2016a)
5IIH	Equine	2.4	Sulfate ion and zinc ion	3.48	3.61	3.27	(Handing et al., 2016a)
5IIX	Equine	2.2	Sulfate ion and zinc ion, unknown ligand	8	3.45	6.29	(Handing et al., 2016a)
5IJ5	Equine	2.55	Sulfate ion and zinc ion	6.86	3.55	5.33	(Handing et al., 2016a)
5HOZ	Equine	2.15	None	6.85	3.86	5.78	DOI: 10.2210/pdb5hoz/pdb
4ZBQ	Equine	1.92	Diclofenac, l(+)-tartaric acid, l-malate, succinic acid and acetate ion	7.83	3.79	5.79	(Sekula and Bujacz, 2016)
4ZBR	Equine	2.19	Diclofenac, naproxen, l-malate, succinic acid, malonate ion, acetate ion and formic acid	8.29	4.41	5.81	(Sekula and Bujacz, 2016)
5DBY	Equine	2.35	Diclofenac, naproxen, l-malate, succinic acid, malonate ion, acetate ion and formic acid	6.98	4.17	5.78	(Sekula and Bujacz, 2016)
5DQF	Equine	2.15	(S)-dextrocetirizine, R-levocetirizine, tetraethylene glycol, sulfate ion, chloride ion	7.46	3.36	5.61	(Handing et al., 2016b)

4LUF	Ovine	2.3	(2R)-2-([(2R)-2-([(2S)-2-([(2R)-2-hydroxypropyl]oxy)propyl]oxy)propyl]oxy)propan- 1-ol, L-Malate, succinic acid, malonate ion, acetate ion and formic acid	8.29	2.38	5.2	DOI: 10.2210/pdb4luf/pdb
4LUH	Ovine	2.2	2-hydroxy-3,5-diiodobenzoic acid, (2R)-2-([(2R)-2-([(2S)-2-([(2R)-2-hydroxypropyl]oxy)propyl]oxy}propyl]oxy)propan- 1-ol, L-Malate, succinic acid, malonate ion, acetate ion and formic acid	8.33	5.55	5.1	DOI: 10.2210/pdb4luh/pdb
4OR0	Bovine	2.58	Naproxen, triethylene glycol, di(hydroxyethyl)ether	4.42	4.06	3.24	(Bujacz et al., 2014)
4OT2	Equine	2.42	Naproxen, succinic acid, malonate ion, acetate ion	5.24	4.33	3.4	(Bujacz et al., 2014)
4PO0	Leporine	2.73	Naproxen	4.4	4.25	3.04	(Bujacz et al., 2014)
4J2V	Equine	2.12	2-hydroxy-3,5-diiodobenzoic acid, malonate ion, acetate ion and formic acid	4.53	4.45	3.4	(Sekula et al., 2013)
4JK4	Bovine	2.65	2-hydroxy-3,5-diiodo-benzoic acid, pentaethylene glycol, triethylene glycol, di(hydroxyethyl)ether and calcium ion	6.76	5.05	8.38	(Sekula et al., 2013)
4F5S	Bovine	2.47	Triethylene glycol	4.23	4.28	2.92	(Bujacz, 2012)
4F5T	Equine	2.32	Sulfate ion, glycerol, acetate ion	4.84	4.45	3.26	(Bujacz, 2012)
4F5U	Equine	2.04	L-malate, succinic acid, malonate ion	7.31	4.56	5.76	(Bujacz, 2012)

4F5V	Leporine	2.27	Tetraethylene glycol, triethylene glycol and acetate ion	4.42	4.11	3.29	(Bujacz, 2012)
3V09	Rabbit	2.27	2-(N-morpholino)-ethanesulfonic acid, tetraethylene glycol, C_4 H_9 N O_2, ethylene glycol, chloride ion	4.58	4.12	3.08	(Majorek et al., 2012)
3V08	Equine	2.45	Tetraethylene glycol, sulfate ion, bromide ion, ethylene glycol and unknown atom or ion	6.37	2.85	5.32	(Majorek et al., 2012)
3V03	Bovine	2.7	Acetate ion, calcium ion	6	4.18	8.87	(Majorek et al., 2012)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ASN99
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3.148
ASP249
ASN99
HIS247
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HIS67
ASN99
2.981
8.908
ASP249
6.042
HIS247
4IW2
HIS67
ASP249
ASN99
4.161
4.094
3.172
HIS247
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ASP249
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ASN99
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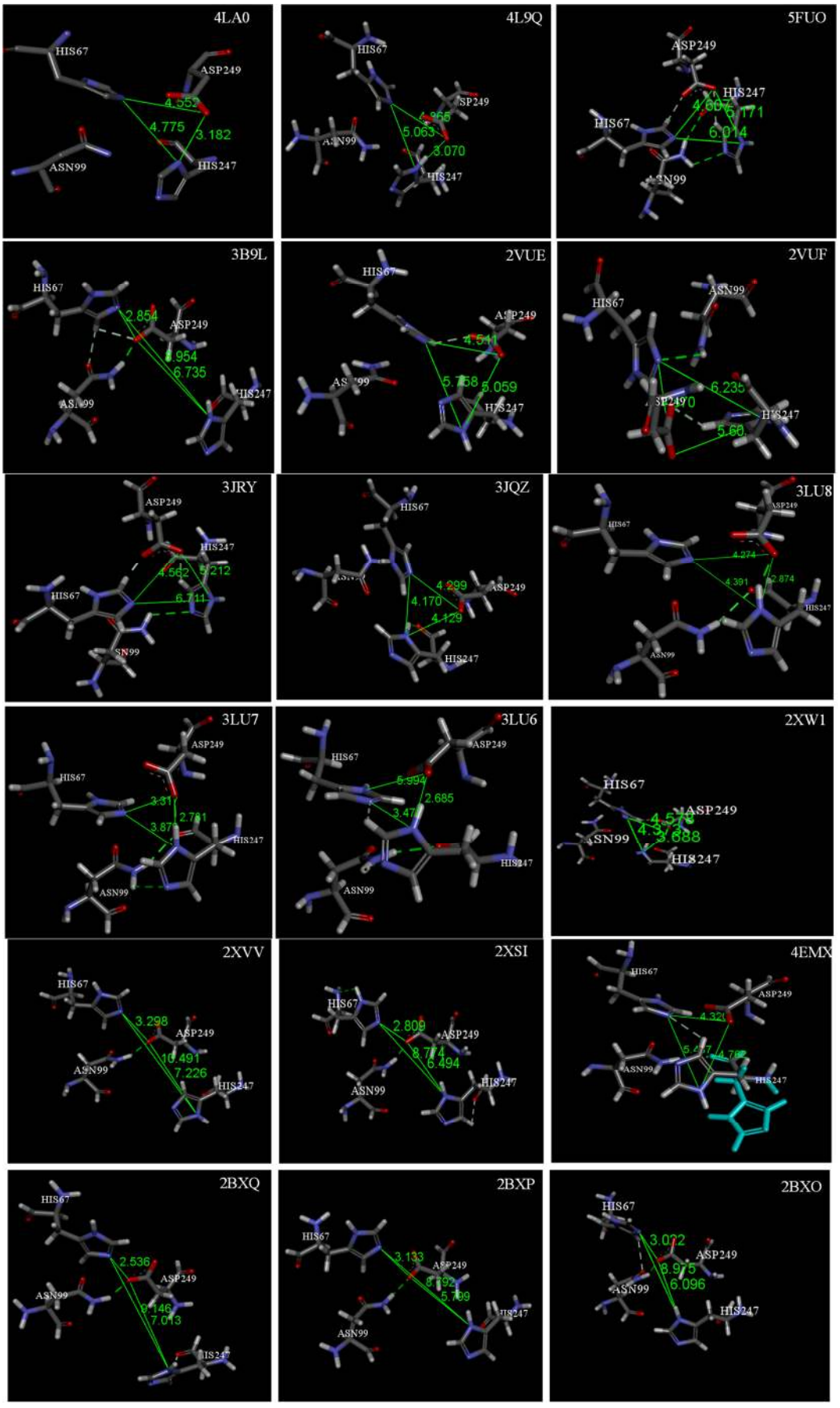
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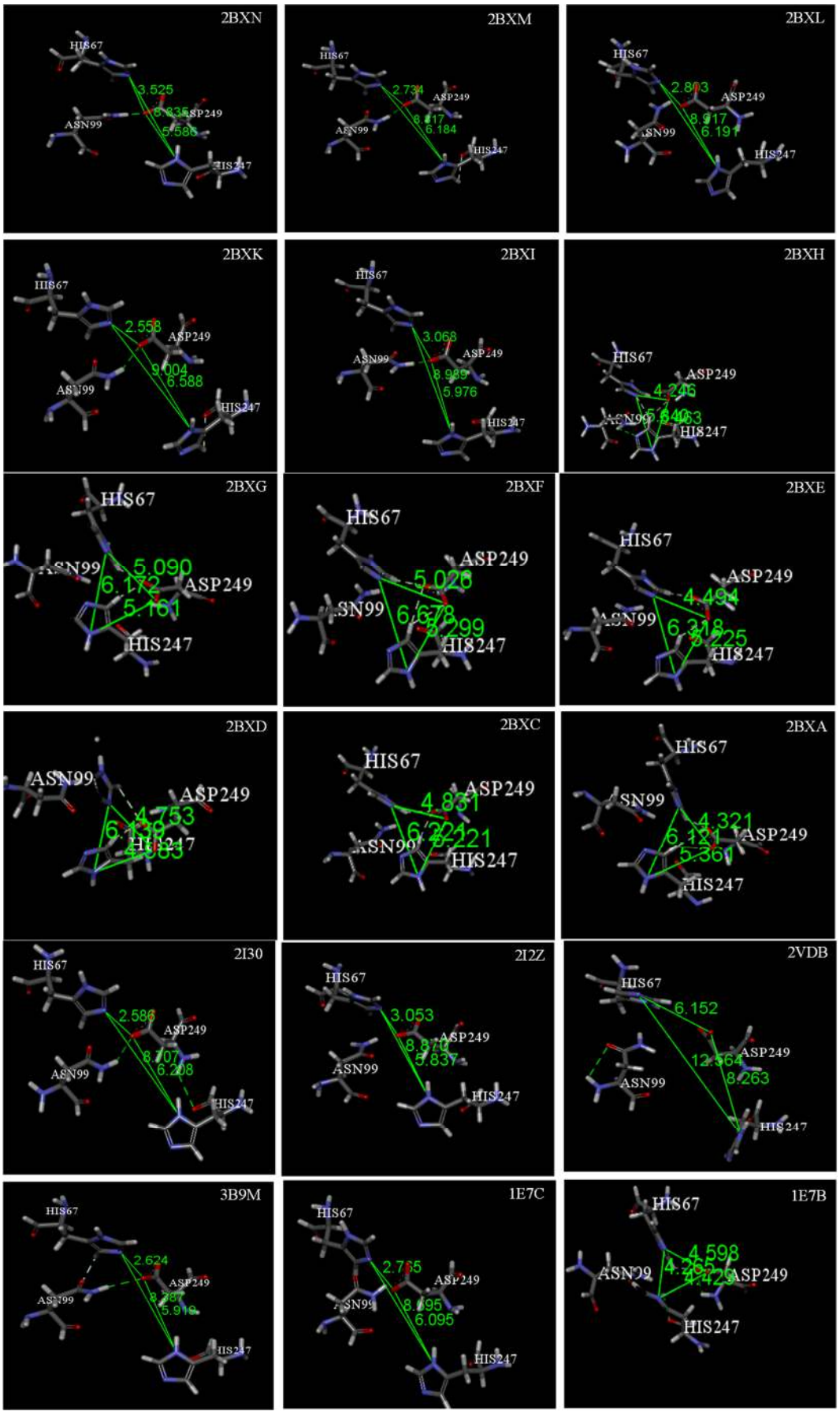
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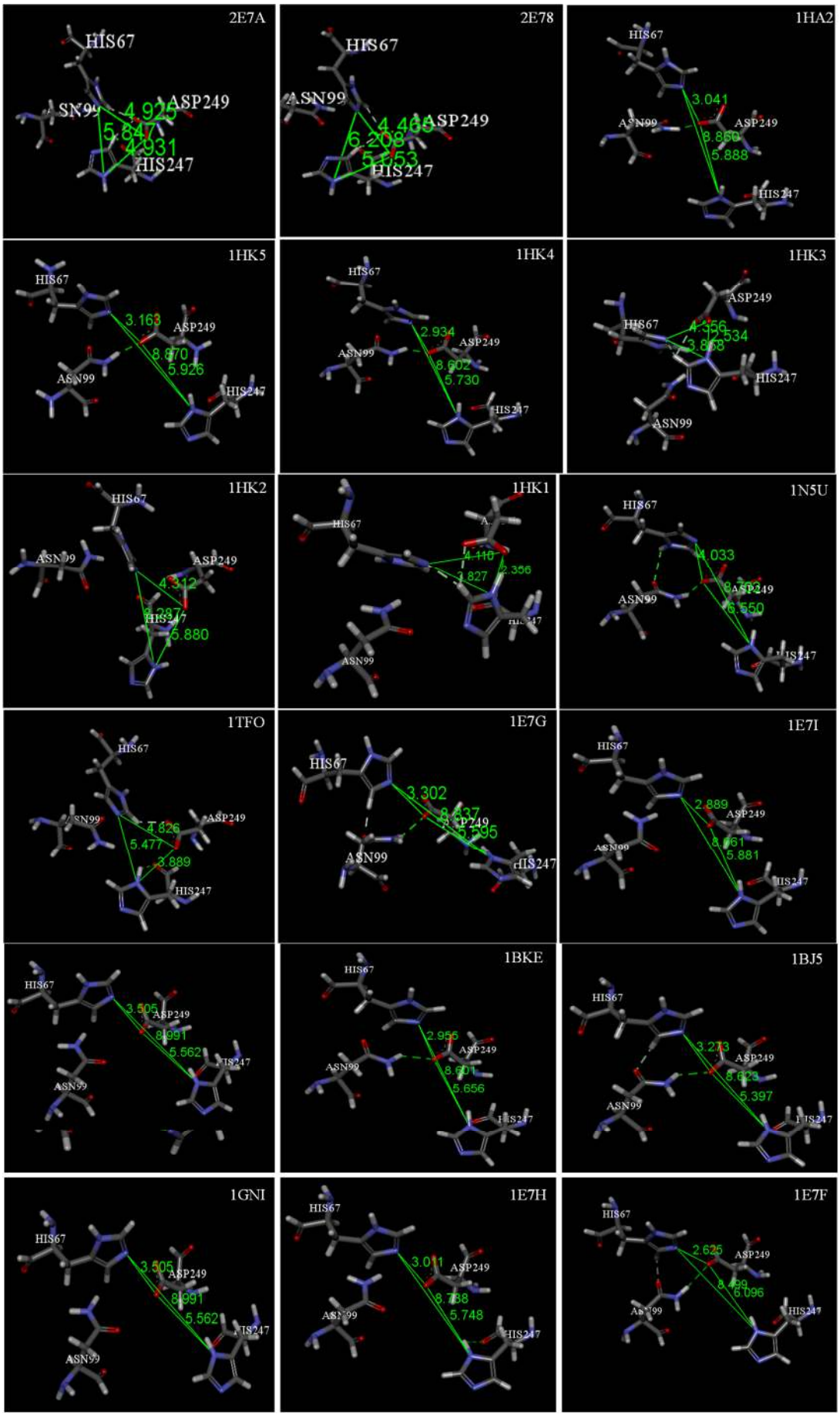
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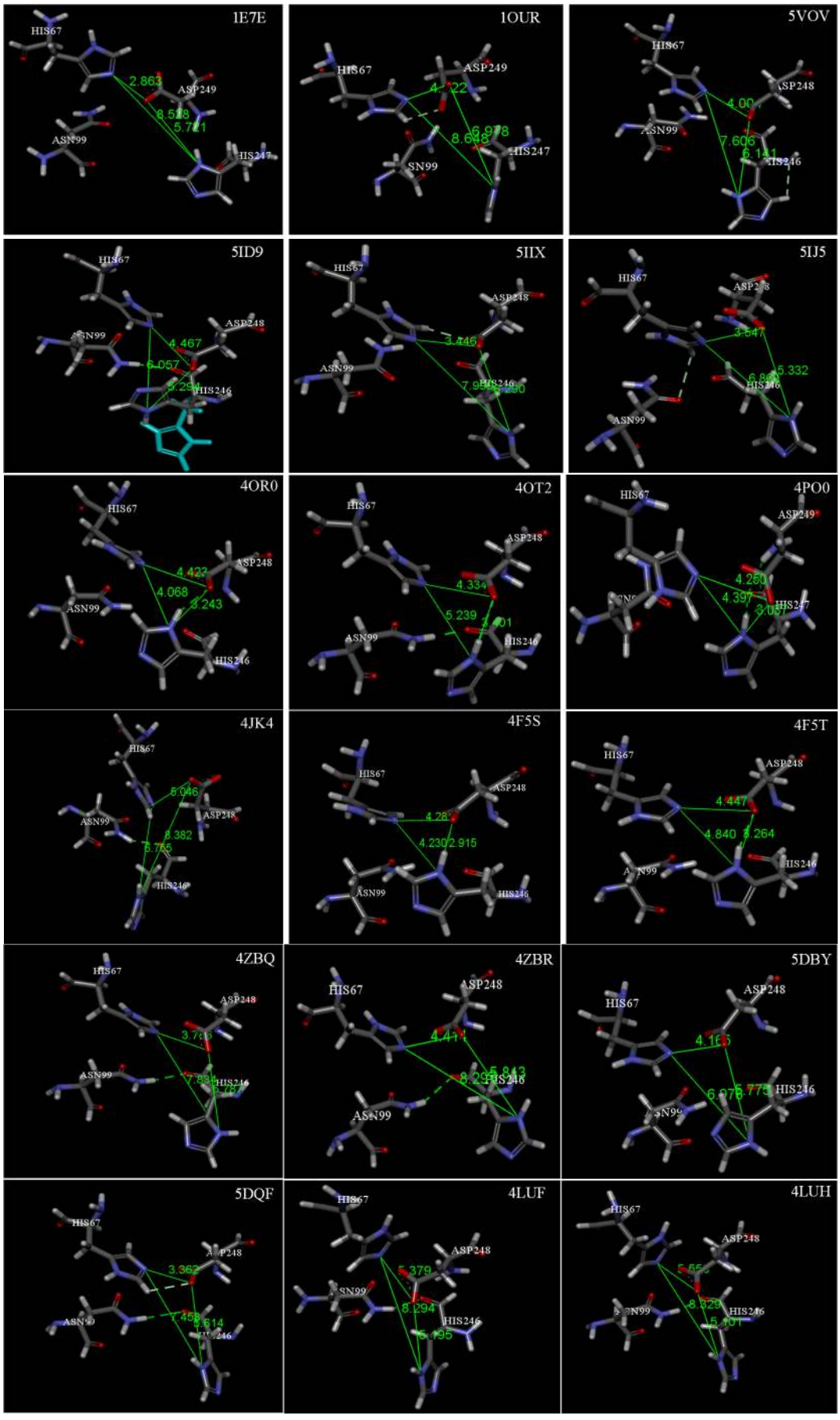
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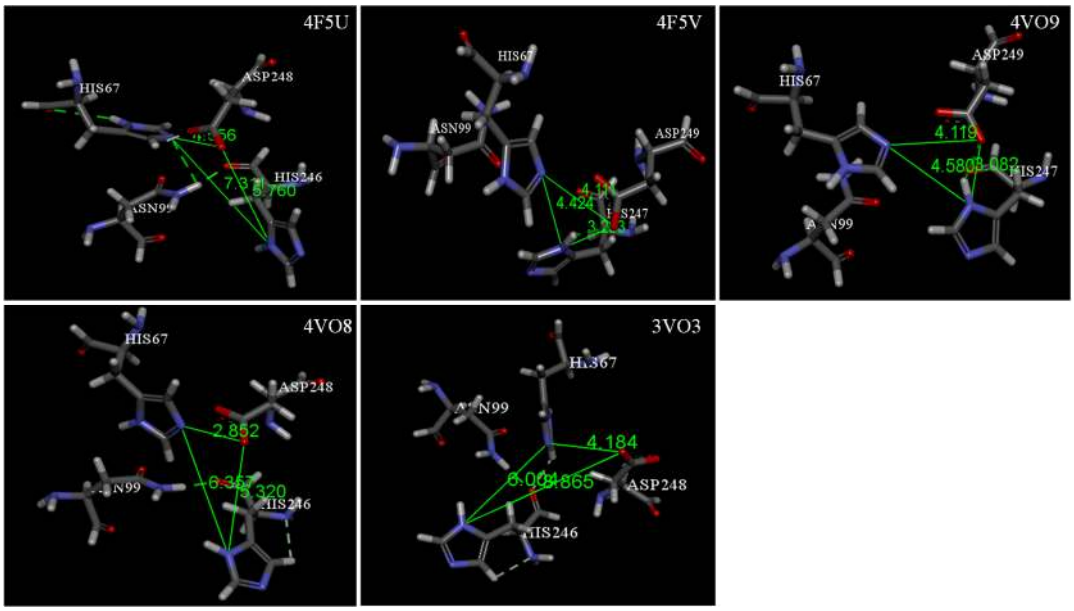

Figure 4.1 In silico analyses of the albumin crystal structures focusing on the zinc binding site A and how the amino acid alignment and their distances at the site are affected by the binding of different ligands on albumin. The primary amino acids forming the site A are His67, HIS247 and ASP249 for HSA; and HIS67, HIS246 and ASP248 for other animal albumins. The distances measured on the amino acid motifs were between H67 Nε2 and H247 Nδ1; H67 Nε2 and D249 Oδ2; and H247 Nδ1 and D249 Oδ2. ASN99 is in proximity to the site but does not form an essential part of the binding site. The code on the top-right-corner for each structure is the PDB code for the respective crystal structures. The white dotted lines indicate presence of hydrogen bonds.

To test the hypothesis that the binding of LCFA can disrupt the zinc binding site A by changing the distances between the amino acids forming the site, HSA crystal structures with LCFA as ligands were analysed and statistically tested for any significant changes in comparison with HSA with no ligand bound or with zinc bound to site A. Figure 4.2 shows the changes realised at the distances between HIS67 and HIS247, HIS67 and ASP249, HIS247 and ASP249 from 14 HSA crystal structures. Crystal structures used for the analysis included 4BKE, 3SQJ, 1E7G, 1E7I, 1BJ5, 1GNI and 1E7H for the HSA structures with LCFA bound and 1AO6, 5IJF, 4K2C, 1E7G, 4GO4, 5FUO and 1BM0 for the non-ligand bound or zinc bound HSA crystal structures. Notably, the mean distances from HIS67 to HIS247 increased significantly with the binding of LCFA and (8.726 Å vs 5.004 Å, $p<0.0001$). A similar

increase in the distance was noted between HIS247 and ASP249 (5.807 Å vs 4.119 Å, $p<0.01$). Conversely, the distance decreased significantly at the interval between HIS67 and ASP249 decreased with the binding of LCFA (3.010 Å vs 4.190 Å, $p<0.001$).

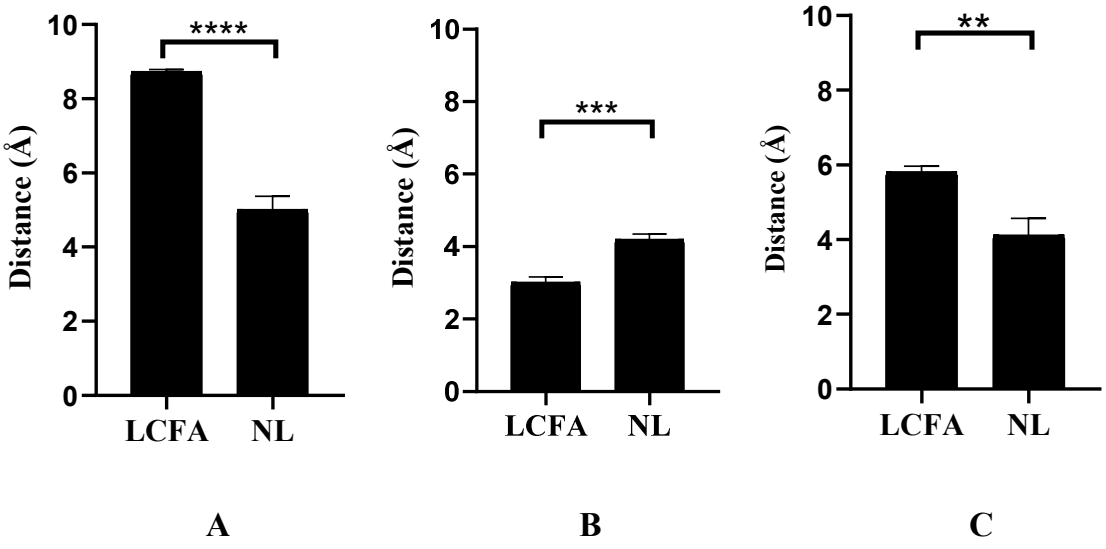

Figure 4.2 Comparison in the distances from HIS67 and HIS247 (A), HIS67 and ASP249 (B), HIS247 and ASP249 (C) between HSA crystal structures with long-chain fatty acid-bound (LCFA) and non-ligand or zinc-bound (NL) HSA. Importantly, the mean distances from HIS67 to HIS247 and between HIS247 and ASP249 increased significantly with the binding of LCFA ($p<0.0001$ and $p<0.01$ respectively), but decreased at the interval between HIS67 and ASP249 ($p<0.001$).

Interesting, it was also noted that HSA structures with other ligands in addition to LCFAs were observed to have a disrupted/altered zinc binding site A, further as noted in structures 4Z69 (ligands; palmitic acid and diclofenac), 3UIV (ligands; myristic acid and amantadine), 3B9L (ligands; myristic acid and zidovudine) 3TDL (ligands; 11-dansylamino undecanoic acid and myristic acid), 2I30 (ligands; myristic acid and salicylic acid) and 1BKE (ligands; 2,3,5-triiodobenzoic acid and myristic acid). However, it was unclear whether the non-LCFA ligands contributed to this or whether the effect was due to the LCFA molecule alone.

4.5 Discussion

This study provides some insights from in silico analysis of albumin crystal structures focusing on how the zinc binding site A can be affected by different ligands binding to the protein. The results of this analysis confirm the hypothesis that some ligands on albumin can disrupt the zinc binding site A. More specifically, LCFA were shown to exert statistically significant disruption of the zinc binding site A by increasing the distance between HIS67 and HIS247, HIS67 and ASP249 but caused a reduction in the distance between HIS247 and ASP249. The results also suggest that the addition of other ligands such as pharmacological substances in addition to the LCFA may disrupt the multimetal binding site further, but this hypothesis requires further research attention.

The results of this study regarding the perturbation effect of the LCFA on the zinc binding site A are in agreement with previous literature. The results support the hypothesis that LCFA, which has FA2 as one of the potent binding sites disrupts the multimetal binding site due to their proximity (Blindauer et al., 2016). LCFA, unlike short-chain fatty acids (SCFA), can fill the FA2 pocket and affect the zinc binding site A (Lu et al., 2012). The results on the perturbing effects of LCFA of the zinc binding site A also confirms previous findings using ITC approaches on the effect of LCFA as allosteric inhibitors of metal ions such as Zn^{2+} on their binding to albumin (Blindauer et al., 2016, Kassaar et al., 2015, Lu et al., 2012). Furthermore, these results support the other studies detailed within this thesis such as the observation that myristate perturbs the binding of cobalt to HSA (See Chapter 2).

It can be perceived, thus that at a molecular level, LCFA may indeed have a negative binding effect on metal ions binding to the zinc binding site A such as Zn^{2+} and Co^{2+}. It is therefore not surprising that it was shown in Chapter 2 of this PhD study that LCFA have a positive influence on the ACB assay also supported by recent literature (Coverdale et al., 2018). The current results also support the results observed in the platelet aggregation experiments observed in Chapter 3 where myristate potentiated platelet aggregation, possibly via the allosteric zinc switch on HSA (Lu et al., 2012).

This study also points at the potential added effect of other ligands on HSA in addition to LCFA, which may affect the multimetal binding site further. Albumin structures such as 4Z69, 3B9L and 2I30, where other ligands such as diclofenac, zidovudine and salicylic acid were added on top of the LCFA appeared to have had further disrupting effect to zinc binding site A. Crucially, as also eluded to in other chapters of this thesis, pathological conditions such as NCDs, which are associated with increased free fatty, are usually associated with other comorbidities which require the taking of other medication by the patients (Wang et al., 2017, Roberts et al., 2015). It is therefore expected that such patients may be on pain relief medications, antiviral medication or other pharmacological substances, for example, which may also have further negative effects on the zinc binding site A. The effects of these pharmacological substances on their own and in combination with LCFA on the binding of metal ions on HSA require further investigation. This in silico study, therefore, provides strong support to the hypothesis of that LCFAs allosterically inhibit binding of metal ions to the multimetal binding site (site A) but also sheds light on the need for further studies focusing on particular pharmacological substances which may potentiate or have the same negative effect on some metal binding capacity.

REFERENCES FOR CHAPTER 4

1. ADAMS, R., GRIFFIN, L., COMPSON, J. E., JAIRAJ, M., BAKER, T., CESKA, T., WEST, S., ZACCHEO, O., DAVE, E., LAWSON, A. D., HUMPHREYS, D. P. & HEYWOOD, S. 2016. Extending the half-life of a fab fragment through generation of a humanized anti-human serum albumin Fv domain: An investigation into the correlation between affinity and serum half-life. *MAbs,* 8**,** 1336-1346.

2. BHATTACHARYA, A. A., CURRY, S. & FRANKS, N. P. 2000a. Binding of the general anesthetics propofol and halothane to human serum albumin. High resolution crystal structures. *J Biol Chem,* 275**,** 38731-8.

3. BHATTACHARYA, A. A., GRUNE, T. & CURRY, S. 2000b. Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin. *J Mol Biol,* 303**,** 721-32.

4. BHATTACHARYA, A. A., GRÜNE, T. & CURRY, S. 2000c. Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin. *Journal of Molecular Biology,* 303**,** 721.

5. BIJELIC, A., THEINER, S., KEPPLER, B. K. & ROMPEL, A. 2016. X-ray Structure Analysis of Indazolium trans-[Tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019) Bound to Human Serum Albumin Reveals Two Ruthenium Binding Sites and Provides Insights into the Drug Binding Mechanism. *Journal of medicinal chemistry,* 59**,** 5894-5903.

6. BLINDAUER, C. A., KHAZAIPOUL, S., YU, R. & STEWART, A. J. 2016. Fatty Acid-Mediated Inhibition of Metal Binding to the Multi-Metal Site on Serum Albumin: Implications for Cardiovascular Disease. *Current topics in medicinal chemistry,* 16**,** 3021.

7. BUJACZ, A. 2012. Structures of bovine, equine and leporine serum albumin. *Acta Crystallogr D Biol Crystallogr,* 68**,** 1278-89.

8. BUJACZ, A., ZIELINSKI, K. & SEKULA, B. 2014. Structural studies of bovine, equine, and leporine serum albumin complexes with naproxen. *Proteins,* 82**,** 2199-208.

9. BUTTAR, D., COLCLOUGH, N., GERHARDT, S., MACFAUL, P. A., PHILLIPS, S. D., PLOWRIGHT, A., WHITTAMORE, P., TAM, K., MASKOS, K., STEINBACHER, S. & STEUBER, H. 2010. A combined spectroscopic and crystallographic approach to probing drug-human serum albumin interactions. *Bioorg Med Chem,* 18**,** 7486-96.

10. COVERDALE, J. P. C., KATUNDU, K. G. H., SOBCZAK, A. I. S., ARYA, S., BLINDAUER, C. A. & STEWART, A. J. 2018. Ischemia-modified albumin: Crosstalk between fatty acid and cobalt binding. *Prostaglandins, leukotrienes, and essential fatty acids,* 135**,** 147.

11. CURRY, S., MANDELKOW, H., BRICK, P. & FRANKS, N. 1998. Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. *Nat Struct Biol,* 5**,** 827-35.

12. ENNIFAR, E. 2013. X-ray crystallography as a tool for mechanism-of-action studies and drug discovery. *Curr Pharm Biotechnol,* 14**,** 537-50.

13. FERRARO, G., MASSAI, L., MESSORI, L. & MERLINO, A. 2015. Cisplatin binding to human serum albumin: a structural study. *Chem Commun (Camb),* 51**,** 9436-9.

14. GHUMAN, J., ZUNSZAIN, P. A., PETITPAS, I., BHATTACHARYA, A. A., OTAGIRI, M. & CURRY, S. 2005. Structural basis of the drug-binding specificity of human serum albumin. *J Mol Biol,* 353**,** 38-52.

15. GUO, S., SHI, X., YANG, F., CHEN, L., MEEHAN, E. J., BIAN, C. & HUANG, M. 2009. Structural basis of transport of lysophospholipids by human serum albumin. *Biochem J,* 423**,** 23-30.

16. HANDING, K. B., SHABALIN, I. G., KASSAAR, O., KHAZAIPOUL, S., BLINDAUER, C. A., STEWART, A. J., CHRUSZCZ, M. & MINOR, W. 2016a. Circulatory zinc transport is controlled by distinct interdomain sites on mammalian albumins. *Chem Sci,* 7**,** 6635-6648.

17. HANDING, K. B., SHABALIN, I. G., SZLACHTA, K., MAJOREK, K. A. & MINOR, W. 2016b. Crystal structure of equine serum albumin in complex with cetirizine reveals a novel drug binding site. *Mol Immunol,* 71**,** 143-151.

18. HE, X. M. & CARTER, D. C. 1992. Atomic structure and chemistry of human serum albumin. *Nature,* 358**,** 209-15.

19. HE, Y., NING, T., XIE, T., QIU, Q., ZHANG, L., SUN, Y., JIANG, D., FU, K., YIN, F., ZHANG, W., SHEN, L., WANG, H., LI, J., LIN, Q., SUN, Y., LI, H., ZHU, Y. & YANG, D. 2011. Large-scale production of functional human serum albumin from transgenic rice seeds. *Proc Natl Acad Sci U S A,* 108**,** 19078-83.

20. HEIN, K. L., KRAGH-HANSEN, U., MORTH, J. P., JEPPESEN, M. D., OTZEN, D., MOLLER, J. V. & NISSEN, P. 2010. Crystallographic analysis reveals a unique lidocaine binding site on human serum albumin. *J Struct Biol,* 171**,** 353-60.

21. KASSAAR, O., SCHWARZ-LINEK, U., BLINDAUER, C. A. & STEWART, A. J. 2015. Plasma free fatty acid levels influence Zn2+-dependent histidine-rich glycoprotein-heparin interactions via an allosteric switch on serum albumin. *Journal of Thrombosis & Haemostasis,* 13**,** 101.

22. KOVALENKO, O. V., OLLAND, A., PICHE-NICHOLAS, N., GODBOLE, A., KING, D., SVENSON, K., CALABRO, V., MULLER, M. R., BARELLE, C. J., SOMERS, W., GILL, D. S., MOSYAK, L. & TCHISTIAKOVA, L. 2013. Atypical antigen recognition mode of a shark immunoglobulin new antigen receptor (IgNAR) variable domain characterized by humanization and structural analysis. *J Biol Chem,* 288**,** 17408-19.

23. LEJON, S., CRAMER, J. F. & NORDBERG, P. 2008. Structural basis for the binding of naproxen to human serum albumin in the presence of fatty acids and the GA module. *Acta Crystallogr Sect F Struct Biol Cryst Commun,* 64**,** 64-9.

24. LEJON, S., FRICK, I. M., BJORCK, L., WIKSTROM, M. & SVENSSON, S. 2004. Crystal structure and biological implications of a bacterial albumin binding module in complex with human serum albumin. *J Biol Chem,* 279**,** 42924-8.

25. LU, J., STEWART, A. J., SLEEP, D., SADLER, P. J., PINHEIRO, T. J. & BLINDAUER, C. A. 2012. A molecular mechanism for modulating plasma Zn speciation by fatty acids. *Journal of the American Chemical Society,* 134**,** 1454.

26. LUO, Z., SHI, X., HU, Q., ZHAO, B. & HUANG, M. 2012. Structural evidence of perfluorooctane sulfonate transport by human serum albumin. *Chem Res Toxicol,* 25**,** 990-2.

27. MADEJ, T., LANCZYCKI, C. J., ZHANG, D., THIESSEN, P. A., GEER, R. C., MARCHLER-BAUER, A. & BRYANT, S. H. 2014. MMDB and VAST+: tracking structural similarities between macromolecular complexes. *Nucleic Acids Res,* 42**,** D297-303.

28. MAJOREK, K. A., POREBSKI, P. J., DAYAL, A., ZIMMERMAN, M. D., JABLONSKA, K., STEWART, A. J., CHRUSZCZ, M. & MINOR, W. 2012. Structural and immunologic characterization of bovine, horse, and rabbit serum albumins. *Mol Immunol,* 52**,** 174-82.

29. MCPHERSON, A. & GAVIRA, J. A. 2013. Introduction to protein crystallization. *Acta crystallographica. Section F, Structural biology communications,* 70**,** 2-20.

30. OGANESYAN, V., DAMSCHRODER, M. M., COOK, K. E., LI, Q., GAO, C., WU, H. & DALL'ACQUA, W. F. 2014. Structural insights into neonatal Fc receptor-based recycling mechanisms. *J Biol Chem,* 289**,** 7812-24.

31. OLTERSDORF, T., ELMORE, S. W., SHOEMAKER, A. R., ARMSTRONG, R. C., AUGERI, D. J., BELLI, B. A., BRUNCKO, M., DECKWERTH, T. L., DINGES, J., HAJDUK, P. J., JOSEPH, M. K., KITADA, S., KORSMEYER, S. J., KUNZER, A. R., LETAI, A., LI, C., MITTEN, M. J., NETTESHEIM, D. G., NG, S., NIMMER, P. M., O'CONNOR, J. M., OLEKSIJEW, A., PETROS, A. M., REED, J. C., SHEN, W., TAHIR, S. K., THOMPSON, C. B., TOMASELLI, K. J., WANG, B., WENDT, M. D., ZHANG, H., FESIK, S. W. & ROSENBERG, S. H. 2005. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature,* 435**,** 677-81.

32. PDB 2018. Guide to Understanding PDB Data; Resolution. Protein Datat Bank.

33. PETITPAS, I., BHATTACHARYA, A. A., TWINE, S., EAST, M. & CURRY, S. 2001a. Crystal structure analysis of warfarin binding to human serum albumin: anatomy of drug site I. *J Biol Chem,* 276**,** 22804-9.

34. PETITPAS, I., GRUNE, T., BHATTACHARYA, A. A. & CURRY, S. 2001b. Crystal structures of human serum albumin complexed with monounsaturated and polyunsaturated fatty acids. *J Mol Biol,* 314**,** 955-60.

35. PETITPAS, I., PETERSEN, C. E., HA, C. E., BHATTACHARYA, A. A., ZUNSZAIN, P. A., GHUMAN, J., BHAGAVAN, N. V. & CURRY, S. 2003. Structural basis of albumin-thyroxine interactions and familial dysalbuminemic hyperthyroxinemia. *Proc Natl Acad Sci U S A,* 100**,** 6440-5.

36. ROBERTS, K. C., RAO, D. P., BENNETT, T. L., LOUKINE, L. & JAYARAMAN, G. C. 2015. Prevalence and patterns of chronic disease multimorbidity and associated determinants in Canada. *Health promotion and chronic disease prevention in Canada : research, policy and practice,* 35**,** 87-94.

37. RYAN, A. J., CHUNG, C. W. & CURRY, S. 2011a. Crystallographic analysis reveals the structural basis of the high-affinity binding of iophenoxic acid to human serum albumin. *BMC Struct Biol,* 11**,** 18.

38. RYAN, A. J., GHUMAN, J., ZUNSZAIN, P. A., CHUNG, C. W. & CURRY, S. 2011b. Structural basis of binding of fluorescent, site-specific dansylated amino acids to human serum albumin. *J Struct Biol,* 174**,** 84-91.

39. SEKULA, B. & BUJACZ, A. 2016. Structural Insights into the Competitive Binding of Diclofenac and Naproxen by Equine Serum Albumin. *J Med Chem,* 59**,** 82-9.

40. SEKULA, B., CIESIELSKA, A., RYTCZAK, P., KOZIOLKIEWICZ, M. & BUJACZ, A. 2016. Structural evidence of the species-dependent albumin binding of the modified cyclic phosphatidic acid with cytotoxic properties. *Biosci Rep,* 36.

41. SEKULA, B., ZIELINSKI, K. & BUJACZ, A. 2013. Crystallographic studies of the complexes of bovine and equine serum albumin with 3,5-diiodosalicylic acid. *Int J Biol Macromol,* 60**,** 316-24.

42. SIMARD, J. R., ZUNSZAIN, P. A., HA, C. E., YANG, J. S., BHAGAVAN, N. V., PETITPAS, I., CURRY, S. & HAMILTON, J. A. 2005. Locating high-affinity fatty acid-binding sites on albumin by x-ray crystallography and NMR spectroscopy.

Proceedings of the National Academy of Sciences of the United States of America, 102**,** 17958.

43. SIVERTSEN, A., ISAKSSON, J., LEIROS, H. K., SVENSON, J., SVENDSEN, J. S. & BRANDSDAL, B. O. 2014. Synthetic cationic antimicrobial peptides bind with their hydrophobic parts to drug site II of human serum albumin. *BMC Struct Biol,* 14**,** 4.

44. SMYTH, M. S. & MARTIN, J. H. 2000. x ray crystallography. *Molecular pathology : MP,* 53**,** 8-14.

45. SUGIO, S., KASHIMA, A., MOCHIZUKI, S., NODA, M. & KOBAYASHI, K. 1999. Crystal structure of human serum albumin at 2.5 A resolution. *Protein Eng,* 12**,** 439-46.

46. WANG, J., MA, J. J., LIU, J., ZENG, D. D., SONG, C. & CAO, Z. 2017. Prevalence and Risk Factors of Comorbidities among Hypertensive Patients in China. *International journal of medical sciences,* 14**,** 201-212.

47. WANG, Y., LUO, Z., SHI, X., WANG, H., NIE, L. & HUANG, M. 2011. A fluorescent fatty acid probe, DAUDA, selectively displaces two myristates bound in human serum albumin. *Protein Sci,* 20**,** 2095-101.

48. WANG, Y., YU, H., SHI, X., LUO, Z., LIN, D. & HUANG, M. 2013a. Structural mechanism of ring-opening reaction of glucose by human serum albumin. *J Biol Chem,* 288**,** 15980-7.

49. WANG, Z. M., HO, J. X., RUBLE, J. R., ROSE, J., RUKER, F., ELLENBURG, M., MURPHY, R., CLICK, J., SOISTMAN, E., WILKERSON, L. & CARTER, D. C. 2013b. Structural studies of several clinically important oncology drugs in complex with human serum albumin. *Biochim Biophys Acta,* 1830**,** 5356-74.

50. YAMADA, K., YOKOMAKU, K., KUREISHI, M., AKIYAMA, M., KIHIRA, K. & KOMATSU, T. 2016. Artificial Blood for Dogs. *Sci Rep,* 6**,** 36782.

51. YAMAGUCHI, S., ALDINI, G., ITO, S., MORISHITA, N., SHIBATA, T., VISTOLI, G., CARINI, M. & UCHIDA, K. 2010. Delta12-prostaglandin J2 as a product and ligand of human serum albumin: formation of an unusual covalent adduct at His146. *J Am Chem Soc,* 132**,** 824-32.

52. YANG, F., BIAN, C., ZHU, L., ZHAO, G., HUANG, Z. & HUANG, M. 2007. Effect of human serum albumin on drug metabolism: structural evidence of esterase activity of human serum albumin. *J Struct Biol,* 157**,** 348-55.

53. ZHANG, Y., LEE, P., LIANG, S., ZHOU, Z., WU, X., YANG, F. & LIANG, H. 2015. Structural basis of non-steroidal anti-inflammatory drug diclofenac binding to human serum albumin. *Chem Biol Drug Des,* 86**,** 1178-84.

54. ZHU, L., YANG, F., CHEN, L., MEEHAN, E. J. & HUANG, M. 2008. A new drug binding subsite on human serum albumin and drug-drug interaction studied by X-ray crystallography. *J Struct Biol,* 162**,** 40-9.

55. ZUNSZAIN, P. A., GHUMAN, J., KOMATSU, T., TSUCHIDA, E. & CURRY, S. 2003. Crystal structural analysis of human serum albumin complexed with hemin and fatty acid. *BMC Struct Biol,* 3**,** 6.

56. ZUNSZAIN, P. A., GHUMAN, J., MCDONAGH, A. F. & CURRY, S. 2008. Crystallographic analysis of human serum albumin complexed with 4Z,15E-bilirubin-IXalpha. *J Mol Biol,* 381**,** 394-406.

CHAPTER 5

INVESTIGATING THE PLASMA FREE FATTY ACID CONCENTRATION, ALBUMIN LEVELS AND BLOOD COAGULABILITY OF HIV-INFECTED AND NON-INFECTED CONTROL INDIVIDUALS AT NENO DISTRICT HOSPITAL IN MALAWI

5.1. Introduction

Human immunodeficiency virus (HIV) is a virus that attacks the immune system of an infected individual and if left untreated can lead to the disease AIDS (acquired immunodeficiency syndrome). There is currently no cure for the infection but antiretroviral therapy (ART) can supress the replication of the virus and prevent the deleterious effects of the infection (Polizzotto et al., 2015). The battle with HIV has changed from only concentrating on reducing the viral load and boosting the immune system to reducing and managing NCDs in the patients. Currently, HIV infection and NCDs are syndemic, where a higher proportion of HIV-infected individuals also face the burden of NCDs including CVD, diabetes (DM), metabolic syndrome, cancer and renal diseases to name a few (Srinivasa and Grinspoon, 2014, Myerson et al., 2015, van Heerden et al., 2017). Traditional risk factors for CVD, diabetes and dyslipidaemia have been described to be significantly more prevalent in HIV infected individuals than matched controls (Currier et al., 2008).

5.1.1 HIV and prolonged ART influence cardiovascular disease

Compared to the general population, the incidence of CVD in HIV infected individuals is currently three times higher (Nkambule et al., 2017). Furthermore, a meta-analysis of 20 studies reported the development of hypertriglyceridemia, hypercholesterolemia, and hyperglycaemia to be as twice as likely to occur in HIV-infected patients on ART than those not on treatment (Islam et al., 2012).

Coronary artery disease, in particular, has been described to be among the leading causes of mortality among patients with HIV infection (Sackoff et al., 2006, Kelesidis and Currier, 2014). In a study conducted in California for example, coronary heart disease (CHD) was

higher in HIV infected young men and women of up to age 44 than those without the disease (Currier et al., 2003). Furthermore, in the same study, patients with HIV infection and on ART had a more than double relative risk of CHD than those not on ART (Currier et al., 2003). Cerebrovascular diseases such as ischaemic strokes have also been observed to be prevalently high in HIV infected populations. In Malawi and South Africa, there has been an increase in the prevalence of ischaemic strokes in young adults with HIV than those without (Feigin et al., 2014, Benjamin et al., 2016).

HIV infection and its treatment with ART, thus, contribute to the pathophysiology and risk factors for NCDs and dyslipidemia-related pathologies which put the patients to a higher risk of CVD and cerebrovascular events (Srinivasa and Grinspoon, 2014). Figure 5.1 displays the interaction between HIV, ART use and how these together with systemic inflammation lead to the pathologies of dyslipidaemia and insulin resistance (IR)-driven CVD and lipodystrophy (Srinivasa and Grinspoon, 2014).

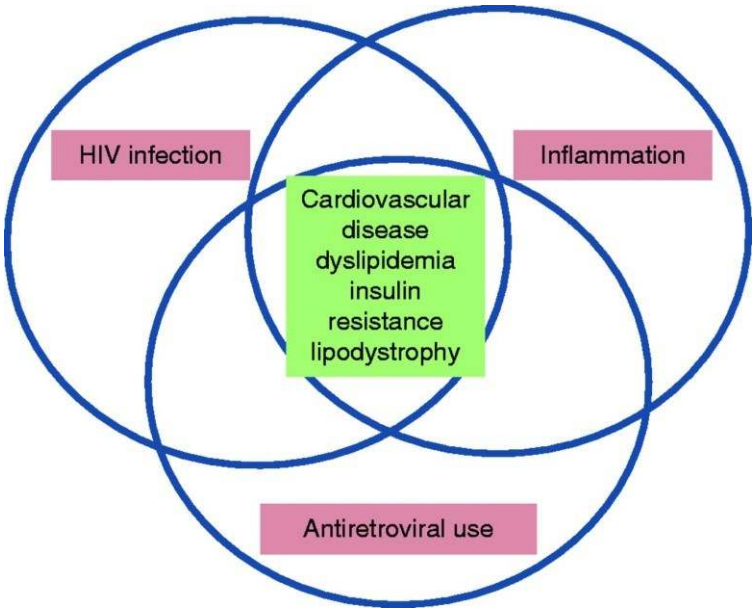

Figure 5.1 Overlapping contribution of HIV infection, inflammation, and ART use to metabolic disease and CVD. Adapted from (Srinivasa and Grinspoon, 2014).

Specifically, HIV and ART exposure affects the host and bring about systemic effects which promote the development of atherosclerosis (Kelesidis and Currier, 2014). Replication of HIV itself has been associated with chronic inflammation as an immune response to the virus (Kelesidis and Currier, 2014). HIV infection evokes immune activation and chronic inflammation, which is only corrected partially with the use of ART (Gresele et al., 2012). HIV infection characteristically reduces the production of IL-2 while the production of IL-4, IL-10, proinflammatory cytokines IL-1, IL-6, IL-8 and TNF-α, is increased, and is directly correlated with viral load (Gresele et al., 2012). HIV is said to activate the inflammatory pathways through the action of HIV protein trans-activator of transcription (Tat), which induces the production of TNF-α and NF-κB by mononuclear cells (Willerson and Ridker, 2004). Tat also induces the increased expression of NF-κB-regulated adhesion molecules such as E-selectin, VCAM-1 and ICAM-1 which affect the permeability of the endothelium, and also influences the proliferation of vascular smooth muscle cells to become more pro-coagulant (Gresele et al., 2012). These processes bring about endothelial dysfunction, which is a linking factor between infection, inflammation, and atherosclerosis (Epstein et al., 1999). HIV itself can also damage the endothelium by infecting the endothelial cells, evidenced by marked depletion of circulating colony forming unit endothelial cells (CFU-EC), and residual CFU-EC which are required for endothelial damage repair (Teofili et al., 2010). HIV has also been associated with impaired endothelial-derived NO production, as shown by brachial artery flow-mediated vasodilation studies (Solages et al., 2006). Furthermore, arterial stiffness, which is a predictor of CV events, has been described in HIV infected individuals not yet on treatment compared to controls (Schillaci et al., 2008).

ART use has shown temporary mitigation in HIV-mediated endothelial damage and dysfunction with a reduction of ICAM, VCAM, MCP-1 and VWF (Gresele et al., 2012). Nevertheless, studies have also reported that prolonged ART use can induce activation of endothelial cells and induce endothelial dysfunction (Torriani et al., 2008, de Gaetano Donati et al., 2003). Furthermore, ritonavir and indinavir have been reported to directly induce endothelial dysfunction, with mitochondrial DNA damage and cell death (Zhong et al., 2002). The type of ART also seems to influence endothelial damage. A research study with over five years of follow-up of patients with HIV infection revealed that endothelial dysfunction was more prevalent in those treated with protease inhibitors (PIs) unlike those treated with nonnucleoside reverse-transcriptase inhibitors NNRTIs (Stein et al., 2001). Much attention has

given to the nucleoside analogue reverse transcriptase inhibitor (NRTI) abacavir on its potential damage on the endothelium due to some epidemiological evidence associating it with an increased incidence of myocardial infarction (Gresele et al., 2012). ART is thought to induce endothelial dysfunction through a reduction in NO production with a possible increase in the production of ROS and impairment of cholesterol efflux associated with accelerated foam cell formation (Chan et al., 2009).

The production of C-reactive protein by the liver is increased during HIV infection, and the elevated levels of CRP is an independent risk of myocardial infarction (MI) in (Troll, 2011). Higher levels of CRP, interleukin 6, and d-dimer have been shown to have an independent association with CVD events in patients with HIV - indicating atherosclerotic pathophysiology (Duprez et al., 2012, Troll, 2011). The above mechanisms, are therefore, responsible for accelerated atherosclerosis and enhanced cardiovascular events (Kelesidis and Currier, 2014).

5.1.2 HIV infection pathophysiology and platelet activation

Platelets can internalise the HIV particles in a phagocytic-like manner, and this may affect coagulation (Zucker-Franklin et al., 1990, Youssefian et al., 2002). Firstly, HIV infects platelets, and this has been shown to activate or potentiate them such that they respond to low doses of agonists (Gresele et al., 2008). Platelets have been observed to circulate in HIV-infected individuals in an activated form, and with altered morphology - having 80% agranular cells and 31% giant cells with vacuoles as evidence of previous activation (Holme et al., 1998, Pretorius et al., 2008). The activated platelets have been shown to produce large amounts of proinflammatory cytokines such as IL-1 β and IL-18, cytokines found to be increased in HIVinfected patients (Ahmad et al., 2006). Higher plasma levels of a plethora of platelet-derived inflammatory molecules including P-selectin have been reported to persist and even increase further while a patient is on ART (Strategies for Management of Anti-Retroviral Therapy and Groups, 2008, Landro et al., 2011).

Platelet activation *in vivo* can be contributed by the endothelial dysfunction owing to the loss of platelet-inhibiting mediators such as NO and PGI_2 (Gresele et al., 2012). Besides, in vitro experiments have shown that abacavir, which is a guanosine analogue, competitively inhibits soluble guanylyl cyclase which negatively regulates platelet function and hence increases

platelet reactivity (Baum et al., 2011). A prospective study which evaluated platelet activation in HIV infection compared with patients treated with either abacavir or tenofovir (TDF) before and after 28–34 months of therapy, reported significantly higher markers of platelet activation in untreated HIV-infected patients than in healthy controls. At 6 to 12 months of follow up in the same study, platelet activation markers increased significantly in plasma of patients on abacavir-treated patients but not in TDF-treated or in untreated, and the hyper-responsiveness was confirmed with platelet aggregation experiments (Baum et al., 2011). Figure 5.2 illustrates how HIV can influence the endothelium leading to endothelial dysfunction and characteristics of platelet activation.

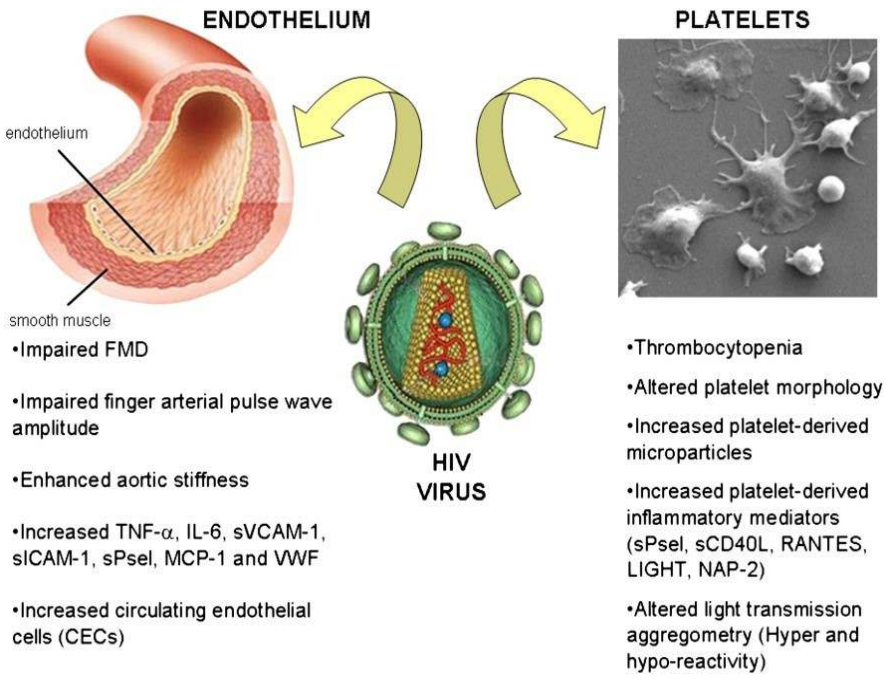

Figure 5.2 Diagrammatic illustration of the plausible interplay between HIV, the endothelium and platelets. FMD = Flow-mediated dilation; sPsel = Soluble P-selectin; sCD40L = soluble CD40Ligand; NAP-2 = Neutrophil Activating Peptide 2; RANTES = Regulated upon activation normal T cell expressed and presumably secreted; LIGHT (TNFSF 14) = tumor necrosis factor superfamily 14. Adapted from (Gresele et al., 2012).

5.1.3 HIV, free fatty acids and Dyslipidaemia

Another important sequela of HIV infection and ART has been associated with dysmetabolism characterised by dyslipidaemia, adipose tissue redistribution, lipodystrophy, insulin resistance and hyperglycaemia (Penzak and Chuck, 2000, Rasheed et al., 2008, Calza et al., 2016). A study which investigated genome-wide changes in the proteomes of a human T-cell line before and at different points up to 3 months after HIV infection discovered that in human T-cells, without interference with ART, can induce production and upregulation of cellular enzymes and proteins whose interaction pathways enhance FFA synthesis, increase low density lipoproteins (triglycerides), dysregulate lipid transport, oxidize lipids, and alter cellular lipid metabolism (Rasheed et al., 2008). Figure 5.3 Shows the putative biological processes involved in lipid metabolism that is influenced by HIV infection (Rasheed et al., 2008). Of interest is that in the mentioned study, 18 differentially expressed proteins were identified in HIVinfected T-cells and six of them namely complement C3 precursor (CO3/C3), phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing beta polypeptide (P3C2B/PIK3C2B), Fatty acid synthase (FAS/FASN), Long-chain-fatty-acid-CoA ligase 1 (ACSL1), protein kinase C beta type (KPCB/PRKCB1) and glutathione peroxidase GPX1 were exclusively expressed in HIV infection while protein disulfide isomerase A3 precursor (PDIA3) was downregulated in chronic HIV infection (Rasheed et al., 2008). The proteins and enzymes are involved in FFA synthesis and lipid metabolism, and none of them were detected in non-HIV infected cells - illustrating the influence of HIV infection on the abnormal synthesis of fatty acids (Rasheed et al., 2008). FAS, in particular, has been implicated in the synthesis of long-chain fatty acids and is key for the final steps in the *de novo* synthesis of FFA (Beach and Flick, 1982, Moon et al., 2002). Further studies have described FAS to be significantly associated with the enhanced *de novo* synthesis of myristate and stearic acid (Rasheed et al., 2008).

Additionally, HIV-1 negatively affects and reduces the concentration of HDL-cholesterol through the impairment of ATP-binding cassette transporter A1-dependent cholesterol efflux from macrophages and stimulates endothelial lipase and phospholipase A2 (Mujawar et al., 2006, Oh and Hegele, 2007). Also, because of the higher concentrations of inflammatory cytokines such as IFN-α and TNF-α prevalent in advanced HIV infection, there is usually poor clearance of triglyceride-rich lipoproteins; alteration of FFA metabolism and free fatty acid

metabolism and lipid oxidation and reduced insulin-mediated suppression of lipolysis which promotes dyslipidaemia (Shinohara et al., 1997, Haugaard et al., 2006, Calza et al., 2016).
The use of ART worsens the dysmetabolism already caused by the HIV infection itself. Several metabolic complications, most of which are lipid metabolism alterations, have been attributed to the use of ART (Calza et al., 2016). Dyslipidaemia associated with ART use is mainly characterised by hypertriglyceridaemia with a lower concentration of HDL-cholesterol and higher levels of total cholesterol and LDL-cholesterol (Calza et al., 2016). Often, dyslipidaemia is associated with lipodystrophy and IR, but it can also occur without these abnormalities (Penzak and Chuck, 2000, Oh and Hegele, 2007).

Protease inhibitors (PI), for example, have been shown to have a direct effect on human adipose and induce IR which contributes to overall adipose imbalance, development of lipodystrophy and metabolic syndrome in HIV-positive individuals (Carr et al., 1998, Flint et al., 2009). Furthermore, NRTIs can induce mitochondrial dysfunction which affects the adipose tissue and promote other abnormalities including lactic acidosis, myopathy, peripheral neuropathy, hepatic steatosis and pancreatitis (Cote, 2007, Maagaard and Kvale, 2009, Selvaraj et al., 2014). NNRTIs, though at a lesser extent, have been associated with dyslipidemia, and efavirenz has been one of the drugs in this group to be associated with such (Cote, 2007, Carr et al., 1998). Metabolic derangements and mitochondrial toxicity are potentially higher risk factors for cardiovascular events and mortality (Friis-Moller et al., 2003, Group et al., 2008, Grinspoon and Carr, 2005).

Several studies have reported a higher frequency of coronary heart disease and cerebrovascular disease among HIV-infected subjects receiving ART, and these seem to occur earlier in life than the general population (Holmberg et al., 2002, Friis-Moller et al., 2003, Obel et al., 2007). LMI countries, especially in sub-Saharan Africa, are being affected by the increased burden of NCDs induced and complicated by HIV infection including cardiovascular and cerebrovascular diseases (Nyirenda et al., 2015, van Heerden et al., 2017, Gowshall and Taylor-Robinson, 2018, Zungu et al., 2019).

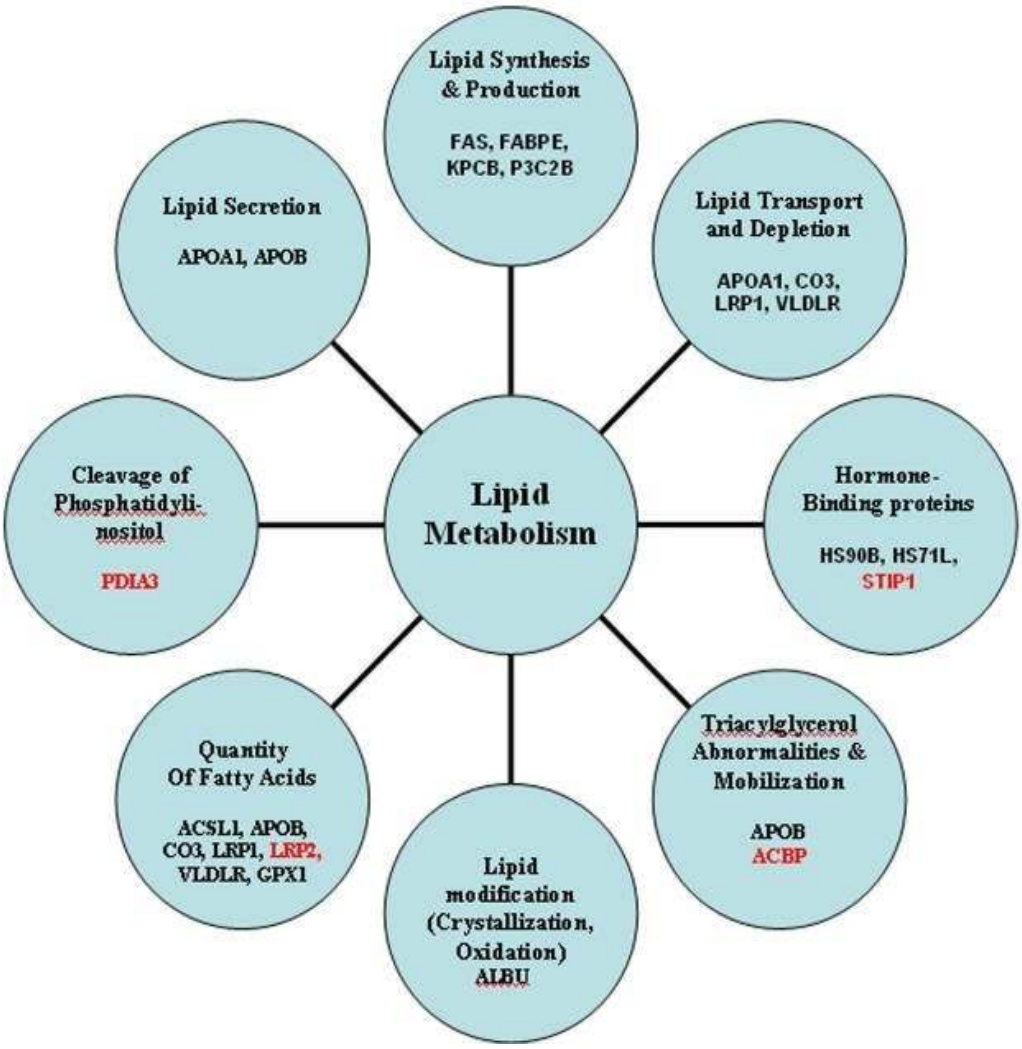

Figure 5.3 Processes and proteins associated with lipid metabolism in HIV infection. Each circle represents proteins associated with the respective functions. The proteins in red are downregulated post-HIV infection while the other proteins are either expressed exclusively in HIV or upregulated after virus infection. ACSL1 = Longchain-fatty-acid-CoA ligase 1 (synonym: Acyl-CoA synthetase 1); CO3 = Complement C3 precursor (C3); FAS = Fatty acid synthase; GPX1 = Glutathione peroxidase; KPCB (PRKCB1) = Protein kinase C beta type; P3C2B (PIK3C2B) = Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing beta polypeptide; PDIA3 = Protein disulfide isomerase A3 precursor. Adapted from (Rasheed et al., 2008).

Thus, the prevalence of NCDs is on the rise globally, and has been complicated further in HIV - demanding the need not only to reduce the prevalence, but also to reduce the risks of complications associated with them (Kavishe et al., 2015, Marrero and Adashi, 2015, Zungu et al., 2019). One notable NCD and a consequence of CVD is stroke. It is worrying that the prevalence of stroke is increasing in Sub-Saharan Africa (Feigin et al., 2014). HIV infection has emerged as a prominent risk factor for stroke and affecting young adults who happen to have a lower risk for predisposing factors such as hypertension (Tipping et al., 2007, Benjamin et al., 2016b, Muhammad et al., 2013). Causes of stroke in HIV patients include coagulopathy, cardioembolism, opportunistic infections and HIV-associated vasculopathy (Benjamin et al., 2012).

Coagulopathy in HIV infection is plausibly linked to dysregulation of zinc in plasma, associated with increased free fatty acid concentration (Blindauer et al., 2016). The increased concentration of FFA in the plasma may influence platelet aggregation through the perturbation of Zn^{2+} buffering by HSA (Lu et al., 2012). The disturbance in Zn^{2+} buffering by HSA may cause an abnormal increase in zinc concentration and elicit platelet aggregation and hence, thromboembolic events (Watson et al., 2016). Since HIV infection and use of ART is associated with both dyslipidaemia and increased risk of stroke (Benjamin et al., 2016b, Calza et al., 2016), assessment of plasma FFA and blood coagulability in such a population at risk is critical and warrants further research attention.

Studies from the United States revealed that FFA concentrations in the HIV-infected population may be above the upper normal limit of 0.6 mmol/L in up to 71% of individuals (Meininger et al., 2002). Nevertheless, this data cannot be translated to Sub-Sahara African countries such as Malawi, due to the differences in the social-demographic status and dietary patterns. Further to this, blood coagulability was not assessed in the above study.

There is a paucity of data on the free fatty acid concentration as well as coagulability in HIV infected patients from Malawi. Additionally, since albumin is the major zinc-buffering protein, it is important to assess its concentration in the individuals compared to normal controls. The results of these studies, in combination with the *in vitro* study results of this PhD project, would inform on whether increase FFA in plasma are associated with potential thromboembolic risk in such a population.

5.2 Aim and objectives

The aim of this study was to investigate the plasma free fatty acid concentration and blood coagulability of HIV-infected individuals at Neno District Hospital in Malawi and compare with HIV-negative controls.

The specific objectives are to;

1. Measure the plasma free fatty acid concentration of HIV-infected individuals at Neno district hospital and HIV-negative controls
2. Measure the blood coagulability of the HIV-infected individuals and HIV-negative controls included in the study
3. Measure the plasma albumin levels of the HIV-infected individuals and HIV-negative controls included in the study
4. Assess the nutritional status of the study participants
5. Compare the variables assessed above between HIV-infected individuals and HIVnegative controls; and among HIV-infected individuals not yet on ART, recently commenced on ART (within six months) and those on chronic ART treatment (more than six months)

The outcome measures of this study were the following;

Primary

1. Mean plasma free fatty acid concentration
2. The proportion of participants with high FFA concentrations above range
3. The mean international normalised ratio (INR)
4. The proportion of participants with INR above reference limit

Secondary

1. Plasma albumin concentration
2. Nutritional status as assessed by body mass index (BMI)
3. Differences in the plasma free fatty acid concentration, INR, plasma albumin concentration and BMI between HIV-infected individuals and HIV-negative controls; and among HIV-infected individuals not yet on ART, recently commenced on ART (within six months) and those on chronic ART treatment (more than six months)

5.3 Methodology

5.3.1 Study Design, place, population and study period

This was an observational cross-sectional study conducted at Neno district hospital at the ART clinic. The study population were adult HIV-infected patients (age 18 to 65 years) attending or referred to the ART clinic at Neno District Hospital, Malawi. The participants were HIVinfected individuals not yet on ART, recently commenced on ART (within six months) and on chronic ART (more than six months) at Neno district hospital. The controls were individuals with documented evidence of being HIV-negative from within the catchment area of the hospital who were guardians of patients at the hospital, caretakers coming with children at the under-five children's clinic, or people presenting to the voluntary HIV testing clinic at the hospital. The controls had a hospital-documented negative test within the past 12 months.

The entire period of the study including preparation of the proposal, submission and approval, training, pretesting (of the questionnaire), data collection, laboratory and data analysis and generation of results was between January 2017 and June 2019. The Data collection for the study occurred over the period between 1st August 2017 and 31st December 2018.

5.3.2 Inclusion and exclusion criteria

The study included HIV-infected adult individuals aged 18 to 65 years who were not yet on ART, recently commenced on ART (within six months) and on chronic ART treatment (more than six months) referred to and attending the ART clinic at Neno district hospital. The controls were HIV-negative adult individuals aged 18 to 65 years from within the catchment area of the hospital who were either guardians of patients at the hospital, caretakers coming with children at the under-five children's clinic, people presenting to the voluntary HIV testing clinic at the hospital or volunteers with documented evidence of a negative HIV test within the past 12 months.

A participant was excluded from the study due to any of the following;

- History of previously known clotting disorders
- Patients on anticoagulants and antiplatelet agents such as warfarin and aspirin respectively
- Known cancer and diabetic patients due to their known increased risk of thromboembolic events
- Individuals with active infections such as tuberculosis and pneumonia
- Pregnant individuals
- Individuals who did not provide consent to participate in the study

Participants were also withdrawn from the study;

- If they wish to do so at any time during the study, freely without prejudice
- Inability to provide blood samples for the study purposes

5.3.3 Sample Size

Previous literature from a study in the United States of America reported elevated FFA concentrations than the reference range in 71% of HIV-infected individuals and 41% in HIV-negative controls (Meininger et al., 2002). Nevertheless, FFA concentrations in a Malawian rural population may be low due to different dietary patterns and socio-economic status. A sample size of 102 HIV-infected participants and 34 control participants was decided to detect at least 62% and 30% proportions of high FFA concentrations than normal in these groups, respectively. The power was 80%, alpha of 0.05 at a sample ratio of 3:1 of the HIV -infected group to controls. A 5% possible attrition was accounted for in calculating the sample size.

Initially, it was planned that 34 control and 102 HIV-infected participants would be recruited, preferably and depending on the availability, 34 participants respectively will be recruited into the three strata;

a. those not yet commenced on ART

b. those recently commenced on ART (less than six months)

c. those on chronic ART treatment (more than six months)

Convenient sampling was used to recruit the participants for the study.

Eventually, it was not possible to recruit patients who were HIV-infected but not yet commenced on ART because of a recent test and treat-guideline which was affected in Malawi. Consequently, 40 controls, 35 HIV-infected participants on ART for less than 6 months and 64 HIV-infected participants on ART for more than 6 months, were recruited for the study.

5.3.4 Data Collection

Information regarding the study was provided verbally to potential participants at Neno District Hospital. Recruitment involved direct approaches by research staff. The research staff were responsible for ensuring that the participants understood the information of the study entirely. Participants were requested to return to the facility the next morning, after an overnight fast as a requirement for the FFA samples. Informed consent was sought from the participants before any data or sample collection occurred.

Data collected included demographic information of the participants, anthropometric measurements (weight, height, mid-upper arm circumference, and calculated BMI) and length of time on ART in months for HIV-infected patients. A blood sample of approximately 20 ml was collected from each participant in serum tubes and Ethylenediaminetetraacetic acid (EDTA) containing tubes. An instant INR test was done on-site, and the rest of the samples was centrifuged and transferred to a storage tube and frozen immediately in a dry shipper transport device: (http://www.mgscientific.com/products/arctic-expresssuptm/sup-dry-shippers-barnsteadthermolyne/) which maintained the samples at a minimum of -80 degrees Celsius. The samples were returned to the College of Medicine, University of Malawi where the specimens were stored in a freezer of at least -80 degrees Celsius until analysis. Ethical approval for the study was obtained from the College of Medicine Research Ethics Committee, the University of Malawi. A copy of the approval document can be found in the appendices.

5.3.5 Laboratory Investigations

Free fatty acid analysis

Free fatty acid extraction from plasma samples was performed according to Dole's method, where 35 µL plasma was added to 0.35 mL of MQ water and vortexed. 1.75 mL of Dole's buffer (80/20 isopropanol/n-hexane, and 0.1% H_2SO_4) was added, and the mixture was also vortexed. Following this, 1.05 mL hexane was added and vortexed again. A volume of 0.7 mL water was then added and vortexed. Finally, the upper organic phase was isolated and put in a new tube and left to evaporate overnight at room temperature. All the reagents and solutions were purchased from Sigma Aldrich (CA, USA).

The commercial colourimetric/fluorometric kit (Abcam, UK) was used for the free fatty acid quantification in the collected serum samples. This is a sensitive enzyme-based method that is used for detecting long-chain FFA in various biological samples, such as serum, plasma and other body fluids. During the assay procedure, fatty acids are converted to their coenzyme A (CoA) derivatives and subsequently oxidised, resulting in the development of a coloured fluorescent product. All standards and samples with 50 µl volumes were placed in a 96-well plate in duplicates. For acyl-CoA synthesis, 2 µl of the ACS reagent was added to all standard and sample wells. The mixture was gently stirred, and the reaction was incubated at 37 °C for 30 min. Following incubation, a 50 µl of reaction mix (containing assay buffer, 44 µl; fatty acid probe, 2 µl; enzyme mix, 2 µl and enhancer, 2 µl) was added to all the wells. The samples were then incubated for 30 minutes at 37 °C. Fatty acids are then quantified at a wavelength of 570 nm. A normal reference range for the test is 100 to 600 µM of FFA (Meininger et al., 2002).

Coagulation and albumin assessment

INR measurements were performed using CoaguChek Xs (Roche Diagnostics, USA). INR is a standardised way of assessing blood coagulability. The CoaguChek Xs is a portable point of care laser photometer. It measures the INR from capillary blood and uses an electrochemical method to determine the prothrombin time (PT) after activation of coagulation with the recombinant human thromboplastin located within the test strip. The PT is then converted to

an INR using an international sensitivity index of 1.01. According to the manufacturer manual, the normal INR reference range in the adult population is between 0.9 and 1.1. A high INR above the upper normal limit indicates an increased risk of bleeding while a low INR below the lower normal limit will indicate increased risk for clotting.

A validated turbidimetric assay was also used to measure coagulability. Plasma samples were micro-centrifuged at 3,000 rpm for 30 seconds. For each sample, 25 µL of the plasma was added in duplicate to a 96 well-plate for the turbidity assay. A volume of 75µl of permeation buffer (PB) made up of 50 mM Tris, 100 mM NaCl, pH 7.4 was added to each sample, then mixed gently. Then, 100 µM $ZnCl_2$ was added, after which 50 µL activation mix (AM) made up of 11,676 µL PB, 270 µL $CaCl_2$ and 54 µL of diluted 1:10 thrombin/ water was added and mixed. The activation mix was added to each sample and the plate was placed in a plate reader which was read at 340 nm at 37 °C using a Rayto 650 plate reader for 100 cycles. Albumin concentrations from the blood samples were analysed at the Division of Biochemistry, Department of Biomedical Sciences, College of Medicine, University of Malawi.

Data Analysis

Data was entered in Microsoft Excel spreadsheets. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., USA). Descriptive statistics for numerical continuous variables were expressed either as means with standard deviation (SD) or medians with interquartile range (IQR). The HIV-infected groups were compared with the control group for baseline characteristics using the student t-test for numerical. For categorical data, the Chisquared test was used for independent variables. For hypothesis testing, Student's t-test was used to analyse the differences in mean FFA concentration, INR values and albumin concentrations between the HIV-infected and control groups. Further to this, a one-way analysis of variance (ANOVA) was used to test the differences in the numeric variables among the three HIV-infected population strata as well as the control group. Separate pairwise comparisons using *t*-tests were performed for those variables that will be significant by ANOVA ($P \leq 0.05$). Correlations between the variables were tested using Spearman's test of correlation. In all cases, a *p-value* ≤ 0.05 was considered significant.

5.4 Results

A total of 154 participants were recruited for the study. Table 1 shows the primary demographic data of the study participants.

Table 5.1 Characteristics of the study population

Characteristic		All participants (n=154)	Statistical comments
Gender, n (%)			No significant differences compared to controls
Controls			
	Males	18 (40.9%)	
	Female	26	
<6 months on ART			
	Males	20(52.6%)	
	Fe male	18	
>6 months on ART			
	Males	20(27.8)	
	Female	52	
Age mean (SD)			
Controls		32.45(8.7)	
<6 months on ART		35.49(13.3)	p<0.001 (compared to controls)
>6 months on ART		39.02(10.2)	
BMI mean (SD)			
Controls		22.24(5.9)	No differences among the groups
<6 months on ART		21.16(2.3)	
>6 months on ART		21.73(3.1)	
Time on ART in months, mean (SD)			
<6 months on ART		3.027(1.641)	p<0.0001
>6 months on ART		80.51(37.5)	

BMI: Body Mass Index; SD: Standard deviation

Notably, the mean age of the participants in the group with participants on ART for more than 6 months (group C) was significantly higher than controls (group C) but similar to the group on ART for less than 6 months (group B). The higher age in group C is acceptable considering that the mean time on the ART was more than 7 years since the commencement of the treatment, and the period on the treatment was significantly higher than group B. Regarding plasma HSA concentration, it was noted that the mean concentrations were lower in the HIV infected groups than the controls (ANOVA p=0.02). Specifically, group B had a lower level (584.9 ±75.65) than the controls (627.4 ±58.05) ($p<0.01$), and the levels were not statistically different from those of group C, which, however, had normal levels (614.7 ±72.05). Figure 5. 4 shows a graph depicting the HSA levels among the groups.

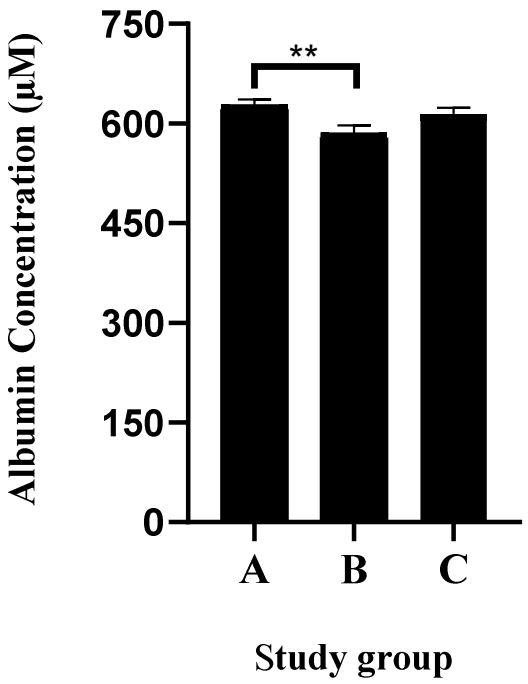

Figure 5. 4 Mean HSA concentration among control participants (A), participants on ART for less than 6 months and those on ART for more than 6 months (C). Notably, Group B subnormal levels (584.9 ±75.65) than the controls (627.4 ±58.05) ($p<0.01$).

INR was measured for each study participant during data collection as the on-site tool for coagulability. Figure 5.5 depicts the INR readings for the three groups. Of note, the INR values were not statistically different among all the groups (group A 1.070 ±0.07726, group B 1.057 ±0.0739 and group C 1.0671 ± 0.1049) using ANOVA (p=NS).

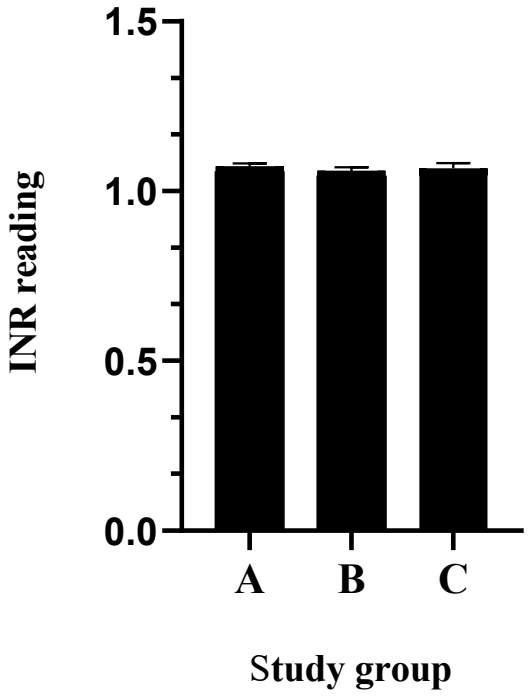

Figure 5.5 Bar graph of the INR readings for the three groups. No significant difference was detected among the groups. A = control participants, B = participants on ART for less than 6 months, C = participants on ART for more than 6 months.

The FFA concentration was then assessed in the participants' samples, as shown in Figure 5.6 below. As hypothesised, it was found that the median (IQR) FFA concentration was higher in the HIV infected groups (2649 μM (2445-5725) and 3720 μM (2463-5723) in groups B and C respectively than the controls (2433 μM (2272-4987) (ANOVA $p<0.05$).

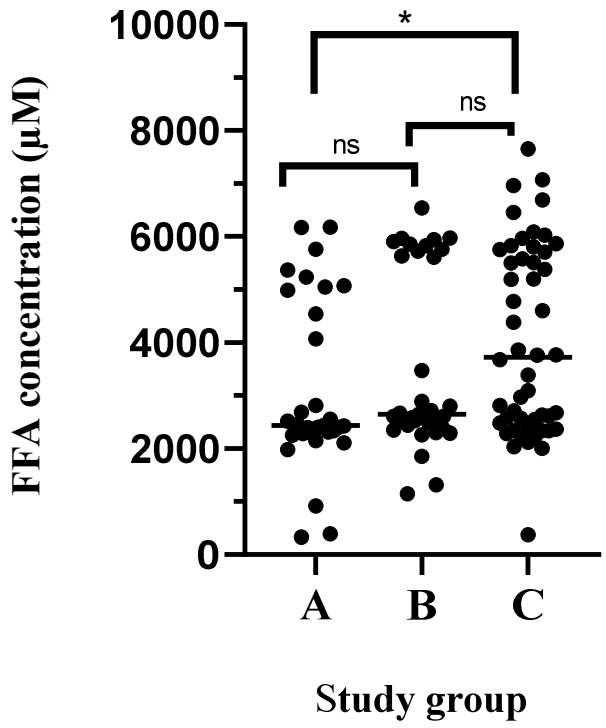

Figure 5.6 Scatter-plots for the FFA concentrations among the three study groups. The lines across the points in the groups represent median values. There was a noted significant increase in the FFA concentration across the groups (ANOVA $p<0.05$) and the t-test was significant between group C and A ($p<0.05$). A = control participants, B = participants on ART for less than 6 months, C = participants on ART for more than 6 months.

Further to this, the turbidity assay was used to assess the coagulability of the plasma from the collected samples. Unlike the expectation that groups B and C would have higher readings for coagulability than controls, as measured by changes positive changes in absorbance, there was no difference noted in the median (IQR) changes in absorbance across all the groups (group A 0.1996 Abs units (0.08290-0.2667); group B 0.1193 Abs units (0.08715-0.1031); group C 0.1357 Abs units (0.1939-0.2211). Figure 5.7 depicts the coagulability status of the plasma samples for the three groups assessed.

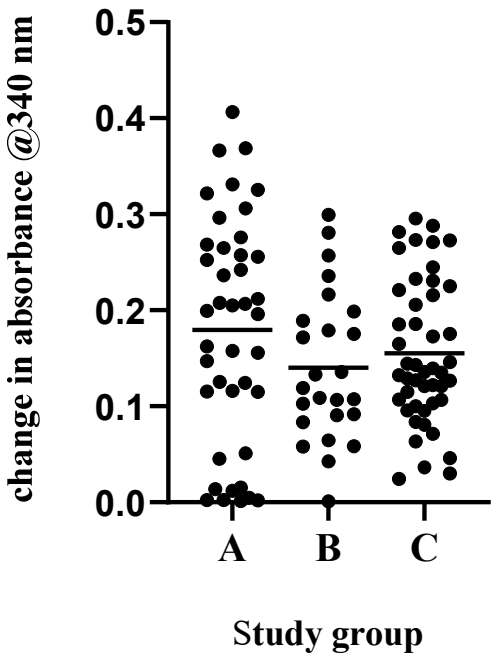

Figure 5.7 Scatter-plot of the turbidity assay results among the three groups of the study. The lines across the points in the groups represent median values. No significant differences were detected among the three groups of participants**.** A = control participants, B = participants on ART for less than 6 months, C = participants on ART for more than 6 months.

Lastly, considering that higher FFA levels in the presence of lower albumin concentrations may influence abnormal zinc handling and can also influence coagulability, correlation tests were performed of these variables with all the groups combined. No significant correlations were neither detected between FFA concentrations and albumin concentration nor between FFA concentration and the coagulability tests. Figure 5.8 depicts the scatterplots for the correlation tests.

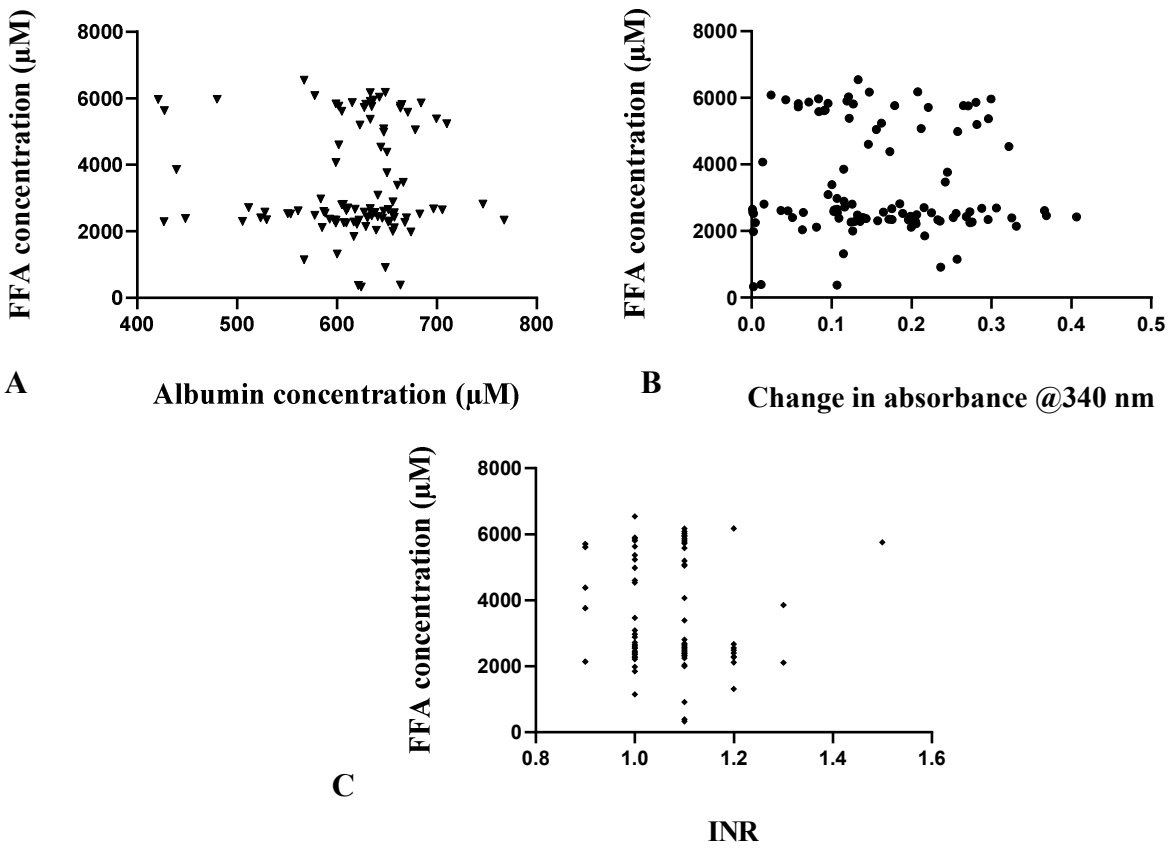

Figure 5.8 Correlation between FFA concentration and albumin concentration (A), FFA concentration, (B) turbidity and (C) FFA concentration and INR in all the three groups combined. No significant relationships were detected among the variables tested.

5.5 Discussion

The present research study has for the first time, to the investigator's knowledge, explored the potential dynamic between FFA concentrations, HSA concentrations and coagulability in HIV patients in rural Malawi and compared with HIV negative controls. The study groups were of comparable characteristics and normal BMI, with no differences in the three study groups. The age in group C, however, was significantly higher than the controls, but this was expected since most of the participants with HIV in Malawi are adults (National Statistical Office and ICF, 2017), and most of these participants were living with HIV for more than 7 years already. The general Malawian population, however, consists of mostly young adults, from which the controls were recruited.

The results of the current study have reported subnormal albumin levels in group B, and the levels in group C were not significantly different compared to the control group A. This result is of clinical importance. It has been previously reported in Malawi that in a population of HIV infected adults, most stroke cases occur in young adults, within 6 months following commencement of ART (Benjamin et al., 2016b). The period following the commencement of ART is critical, as it also a window for immune reconstitution inflammatory syndrome (IRIS), where there may be a paradoxical clinical worsening of a known condition or the appearance of new infections after initiating ART (Bosamiya, 2011). Infections and the inflammatory response can influence the release and increased concentrations of FFA in plasma, which can exert a negative-buffering effect on metal ions such as zinc by HSA (Lu et al., 2012). Concurrently, infections can induce a decrease in the HSA concentration, as HSA is a negative acute-phase protein (Jain et al., 2011). Considering that stroke can be contributed to by several factors including infections, the reduced albumin levels would reduce the zinc buffering capacity which may potentiate platelet activity in plasma, thereby adding to the risk of cardiovascular and cerebrovascular events such as stroke (Benjamin et al., 2012, Watson et al., 2016).

The free fatty acids were elevated in the HIV groups, especially in the study group C. This result is in keeping with the literature. HIV infection alone and chronic ART is known to induce *de novo* synthesis of FFA and dyslipidaemia (Rasheed et al., 2008, Gresele et al., 2012). Importantly, higher FFA concentrations may induce and reflect the development of

atherosclerosis and metabolic syndrome in HIV (Srinivasa and Grinspoon, 2014). Atherosclerosis contributes to CVD, a risk for cerebrovascular and cardiovascular events such as stroke (Kelesidis and Currier, 2014, Nkambule et al., 2017). Additionally, the induced atherosclerosis is associated with endothelial dysfunction, which promotes the abnormal activation of platelets and induces abnormal clotting (Gresele et al., 2012). In prolonged ART use and long-stay atherosclerosis, a rupture of an atherosclerotic plaque may occur, and induction of platelet aggregation may ensue in these patients - owing to the higher zinc concentrations in the immediate milieu released from the plaque (Stadler et al., 2008, Taylor and Pugh, 2016). It is critical, therefore, that higher levels of FFA in patients on long term ART be monitored and addressed to prevent adverse sequelae.

The coagulability of the blood samples was assessed by INR and the turbidity assay. No differences were observed among the different groups of the study participants. INR is usually in use for the monitoring of patients on anticoagulants and may not be very sensitive for the pro-coagulability states (Ts'ao and Neofotistos, 1994). The turbidity test, on the other hand, uses plasma and does not assess the influence of platelets on the coagulation. Platelet aggregation tests were not feasible in this study, as they require the use of fresh isolated platelets and availability of the respective instrumentation. Nevertheless, the INR and turbidity tests used in this the confirmed that as in the control group, the HIV-infected groups have functional coagulation systems capable of being induced in pathophysiological conditions using less invasive ways. It should also be noted that abnormal coagulation in HIV infection involves other sophisticated mechanisms involving the interplay between the platelets, inflammatory markers and the endothelium which could not be assessed in the present study (Dhindsa et al., 2015, Taylor and Pugh, 2016, Gresele et al., 2012).

Thus, this clinical study has shown that HIV infection, in a rural Malawian population was associated with lower albumin concentrations in the presence of higher levels of FFA, a synergism that can contribute to cardiovascular and cerebrovascular events considering the potential presence of other pathophysiological processes associated with HIV infection.

REFERENCES FOR CHAPTER 5

1. BAUM, P. D., SULLAM, P. M., STODDART, C. A. & MCCUNE, J. M. 2011. Abacavir increases platelet reactivity via competitive inhibition of soluble guanylyl cyclase. *AIDS (London, England),* 25**,** 2243.

2. BEACH, D. C. & FLICK, P. K. 1982. Early effect of myo-inositol deficiency on fatty acid synthetic enzymes of rat liver. *Biochimica et biophysica acta,* 711**,** 452.

3. BENJAMIN, L. A., BRYER, A., EMSLEY, H. C. A., KHOO, S., SOLOMON, T. & CONNOR, M. D. 2012. HIV infection and stroke: current perspectives and future directions. *Lancet Neurology,* 11**,** 878.

4. BENJAMIN, L. A., CORBETT, E. L., CONNOR, M. D., MZINGANJIRA, H., KAMPONDENI, S., CHOKO, A., HOPKINS, M., EMSLEY, H. C., BRYER, A., FARAGHER, B., HEYDERMAN, R. S., ALLAIN, T. J. & SOLOMON, T. 2016a. HIV, antiretroviral treatment, hypertension, and stroke in Malawian adults: A case-control study. *Neurology,* 86**,** 324.

5. BENJAMIN, L. A., CORBETT, E. L., CONNOR, M. D., MZINGANJIRA, H., KAMPONDENI, S., CHOKO, A., HOPKINS, M., EMSLEY, H. C. A., BRYER, A., FARAGHER, B., HEYDERMAN, R. S., ALLAIN, T. J. & SOLOMON, T. 2016b. HIV, antiretroviral treatment, hypertension, and stroke in Malawian adults: A case-control study. *Neurology,* 86**,** 324.

6. BLINDAUER, C. A., KHAZAIPOUL, S., YU, R. & STEWART, A. J. 2016. Fatty AcidMediated Inhibition of Metal Binding to the Multi-Metal Site on Serum Albumin: Implications for Cardiovascular Disease. *Current topics in medicinal chemistry,* 16**,** 3021.

7. BOSAMIYA, S. S. 2011. The immune reconstitution inflammatory syndrome. *Indian journal of dermatology,* 56**,** 476-479.

8. CALZA, L., COLANGELI, V., MANFREDI, R., BON, I., RE, M. C. & VIALE, P. 2016. Clinical management of dyslipidaemia associated with combination antiretroviral therapy in HIV-infected patients. *The Journal of antimicrobial chemotherapy,* 71**,** 1451.

9. CARR, A., SAMARAS, K., BURTON, S., LAW, M., FREUND, J., CHISHOLM, D. J. & COOPER, D. A. 1998. A syndrome of peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving HIV protease inhibitors. *AIDS (London, England),* 12**,** F51.

10. CHAN, W., SVIRIDOV, D. & DART, A. M. 2009. HIV, atherosclerosis and inflammation: implications for treatment. *Journal of HIV therapy,* 14**,** 61.

11. COTE, H. C. 2007. Mechanisms of antiretroviral therapy-induced mitochondrial dysfunction. *Current opinion in HIV and AIDS,* 2**,** 253.

12. CURRIER, J. S., LUNDGREN, J. D., CARR, A., KLEIN, D., SABIN, C. A., SAX, P. E., SCHOUTEN, J. T., SMIEJA, M. & WORKING, G. 2008. Epidemiological evidence for cardiovascular disease in HIV-infected patients and relationship to highly active antiretroviral therapy. *Circulation,* 118**,** e29.

13. CURRIER, J. S., TAYLOR, A., BOYD, F., DEZII, C. M., KAWABATA, H., BURTCEL, B., MAA, J. F. & HODDER, S. 2003. Coronary heart disease in HIV-infected individuals. *Journal of acquired immune deficiency syndromes (1999),* 33**,** 506.

14. DE GAETANO DONATI, K., RABAGLIATI, R., TUMBARELLO, M., TACCONELLI, E., AMORE, C., CAUDA, R. & LACOVIELLO, L. 2003. Increased soluble markers of endothelial dysfunction in HIV-positive patients under highly active antiretroviral therapy. *AIDS (London, England),* 17**,** 765.

15. DHINDSA, S., GHANIM, H. & DANDONA, P. 2015. Nonesterified fatty acids, albumin, and platelet aggregation. *Diabetes,* 64**,** 703.

16. DUPREZ, D. A., NEUHAUS, J., KULLER, L. H., TRACY, R., BELLOSO, W., DE WIT, S., DRUMMOND, F., LANE, H. C., LEDERGERBER, B., LUNDGREN, J., NIXON, D., PATON, N. I., PRINEAS, R. J., NEATON, J. D. & GROUP, I. S. S. 2012. Inflammation, coagulation and cardiovascular disease in HIV-infected individuals. *PloS one,* 7**,** e44454.

17. EPSTEIN, S. E., ZHOU, Y. F. & ZHU, J. 1999. Infection and atherosclerosis: emerging mechanistic paradigms. *Circulation,* 100**,** e20.

18. FEIGIN, V. L., FOROUZANFAR, M. H., KRISHNAMURTHI, R., MENSAH, G. A., CONNOR, M., BENNETT, D. A., MORAN, A. E., SACCO, R. L., ANDERSON, L., TRUELSEN, T., O'DONNELL, M., VENKETASUBRAMANIAN, N., BARKER-OLLO, S., LAWES, C. M. M., WANG, W., SHINOHARA, Y., WITT, E., EZZATI, M., NAGHAVI, M. & MURRAY, C. 2014. Global and regional burden of stroke during 19902010: findings from the Global Burden of Disease Study 2010. *Lancet,* 383**,** 245.

19. FLINT, O. P., NOOR, M. A., HRUZ, P. W., HYLEMON, P. B., YARASHESKI, K., KOTLER, D. P., PARKER, R. A. & BELLAMINE, A. 2009. The role of protease inhibitors in the pathogenesis of HIV-associated lipodystrophy: cellular mechanisms and clinical implications. *Toxicologic pathology,* 37**,** 65.

20. FRIIS-MOLLER, N., WEBER, R., REISS, P., THIEBAUT, R., KIRK, O., D'ARMINIO MONFORTE, A., PRADIER, C., MORFELDT, L., MATEU, S., LAW, M., EL-SADR, W., DE WIT, S., SABIN, C. A., PHILLIPS, A. N., LUNDGREN, J. D. & GROUP, D. A. S. 2003. Cardiovascular disease risk factors in HIV patients--association with antiretroviral therapy. Results from the DAD study. *AIDS (London, England),* 17**,** 1179.

21. GOWSHALL, M. & TAYLOR-ROBINSON, S. D. 2018. The increasing prevalence of non-communicable diseases in low-middle income countries: the view from Malawi. *International journal of general medicine,* 11**,** 255.

22. GRESELE, P., FALCINELLI, E. & MOMI, S. 2008. Potentiation and priming of platelet activation: a potential target for antiplatelet therapy. *Trends in pharmacological sciences,* 29**,** 352.

23. GRESELE, P., FALCINELLI, E., SEBASTIANO, M. & BALDELLI, F. 2012. Endothelial and platelet function alterations in HIV-infected patients. *Thrombosis research,* 129**,** 301.

24. GRINSPOON, S. & CARR, A. 2005. Cardiovascular risk and body-fat abnormalities in HIV-infected adults. *The New England journal of medicine,* 352**,** 48.

25. GROUP, D. A. D. S., SABIN, C. A., WORM, S. W., WEBER, R., REISS, P., EL-SADR, W., DABIS, F., DE WIT, S., LAW, M., D'ARMINIO MONFORTE, A., FRIIS-

MOLLER, N., KIRK, O., PRADIER, C., WELLER, I., PHILLIPS, A. N. & LUNDGREN, J. D. 2008. Use of nucleoside reverse transcriptase inhibitors and risk of myocardial infarction in HIVinfected patients enrolled in the D:A:D study: a multi-cohort collaboration. *Lancet (London, England),* 371**,** 1417.

26. HAUGAARD, S. B., ANDERSEN, O., PEDERSEN, S. B., DELA, F., FENGER, M., RICHELSEN, B., MADSBAD, S. & IVERSEN, J. 2006. Tumor necrosis factor alpha is associated with insulin-mediated suppression of free fatty acids and net lipid oxidation in HIV-infected patients with lipodystrophy. *Metabolism: clinical and experimental,* 55**,** 175.

D. HOLMBERG, S. D., MOORMAN, A. C., WILLIAMSON, J. M., TONG, T. C., WARD, J., WOOD, K. C., GREENBERG, A. E., JANSSEN, R. S. & INVESTIGATORS, H. I. V. O. S. 2002. Protease inhibitors and cardiovascular outcomes in patients with HIV-1. *Lancet (London, England),* 360**,** 1747.

27. HOLME, P. A., MULLER, F., SOLUM, N. O., BROSSTAD, F., FROLAND, S. S. & AUKRUST, P. 1998. Enhanced activation of platelets with abnormal release of RANTES in human immunodeficiency virus type 1 infection. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 12**,** 79.

28. ISLAM, F. M., WU, J., JANSSON, J. & WILSON, D. P. 2012. Relative risk of cardiovascular disease among people living with HIV: a systematic review and metaanalysis. *HIV medicine,* 13**,** 453.

29. JAIN, S., GAUTAM, V. & NASEEM, S. 2011. Acute-phase proteins: As diagnostic tool. *Journal of pharmacy & bioallied sciences,* 3**,** 118-127.

30. KAVISHE, B., BIRARO, S., BAISLEY, K., VANOBBERGHEN, F., KAPIGA, S., MUNDERI, P., SMEETH, L., PECK, R., MGHAMBA, J., MUTUNGI, G., IKOONA, E., LEVIN, J., BOU MONCLÚS, M. A., KATENDE, D., KISANGA, E., HAYES, R. & GROSSKURTH, H. 2015. High prevalence of hypertension and of risk factors for noncommunicable diseases (NCDs): a population based cross-sectional survey of NCDS and HIV infection in Northwestern Tanzania and Southern Uganda. *BMC Medicine,* 13**,** 1.

31. KELESIDIS, T. & CURRIER, J. S. 2014. Dyslipidemia and cardiovascular risk in human immunodeficiency virus infection. *Endocrinology and metabolism clinics of North America,* 43**,** 665.

32. LANDRO, L., UELAND, T., OTTERDAL, K., FROLAND, S. S. & AUKRUST, P. 2011. Persistently raised plasma levels of platelet-derived inflammatory mediators in HIVinfected patients during highly active anti-retroviral therapy. *Journal of thrombosis and haemostasis : JTH,* 9**,** 1075.

33. LU, J., STEWART, A. J., SLEEP, D., SADLER, P. J., PINHEIRO, T. J. & BLINDAUER, C. A. 2012. A molecular mechanism for modulating plasma Zn speciation by fatty acids. *Journal of the American Chemical Society,* 134**,** 1454.

34. MAAGAARD, A. & KVALE, D. 2009. Mitochondrial toxicity in HIV-infected patients both off and on antiretroviral treatment: a continuum or distinct underlying mechanisms? *The Journal of antimicrobial chemotherapy,* 64**,** 901.

35. MARRERO, S. & ADASHI, E. Y. 2015. Noncommunicable Diseases. *Seminars in reproductive medicine,* 33**,** 35.

36. MEININGER, G., HADIGAN, C., LAPOSATA, M., BROWN, J., RABE, J., LOUCA, J., ALIABADI, N. & GRINSPOON, S. 2002. Elevated concentrations of free fatty acids are associated with increased insulin response to standard glucose challenge in human immunodeficiency virus-infected subjects with fat redistribution. *Metabolism: clinical and experimental,* 51**,** 260.

37. MOON, Y. S., LATASA, M. J., GRIFFIN, M. J. & SUL, H. S. 2002. Suppression of fatty acid synthase promoter by polyunsaturated fatty acids. *Journal of lipid research,* 43**,** 691.

38. MUHAMMAD, S., SANI, M. U. & OKEAHIALAM, B. N. 2013. Cardiovascular disease risk factors among HIV-infected Nigerians receiving highly active antiretroviral therapy. *Nigerian Medical Journal,* 54**,** 185.

39. MUJAWAR, Z., ROSE, H., MORROW, M. P., PUSHKARSKY, T., DUBROVSKY, L., MUKHAMEDOVA, N., FU, Y., DART, A., ORENSTEIN, J. M., BOBRYSHEV, Y. V., BUKRINSKY, M. & SVIRIDOV, D. 2006. Human immunodeficiency virus impairs reverse cholesterol transport from macrophages. *PLoS biology,* 4**,** e365.

40. MYERSON, M., MALVESTUTTO, C. & ABERG, J. A. 2015. Management of lipid disorders in patients living with HIV. *Journal of clinical pharmacology,* 55**,** 957.

41. NATIONAL STATISTICAL OFFICE, M. & ICF 2017. Malawi Demographic and Health Survey 2015-2016. Zomba, Malawi, and Rockville, Maryland . NSO and ICF.

42. NKAMBULE, B. B., MKANDLA, Z., MUTIZE, T. & DLUDLA, P. V. 2017. Platelet function and cardiovascular risk in adult HIV-infected patients on HAART: a protocol for a systematic review and meta-analysis. *BMJ open,* 7**,** e019468.

43. NYIRENDA, C. K., KABAGAMBE, E. K., KOETHE, J. R., KIAGE, J. N., CHI, B. H., MUSONDA, P., BLEVINS, M., BOSIRE, C. N., TSAI, M. Y. & HEIMBURGER, D. C. 2015. Plasma Fatty Acids in Zambian Adults with HIV/AIDS: Relation to Dietary Intake and Cardiovascular Risk Factors. *Journal of nutrition and metabolism,* 2015**,** 635817.

44. OBEL, N., THOMSEN, H. F., KRONBORG, G., LARSEN, C. S., HILDEBRANDT, P. R., SORENSEN, H. T. & GERSTOFT, J. 2007. Ischemic heart disease in HIV-infected and HIV-uninfected individuals: a population-based cohort study. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America,* 44**,** 1625.

45. OH, J. & HEGELE, R. A. 2007. HIV-associated dyslipidaemia: pathogenesis and treatment. *The Lancet.Infectious diseases,* 7**,** 787.

46. PENZAK, S. R. & CHUCK, S. K. 2000. Hyperlipidemia associated with HIV protease inhibitor use: pathophysiology, prevalence, risk factors and treatment. *Scandinavian Journal of Infectious Diseases,* 32**,** 111.

47. POLIZZOTTO, M. N., CHEN, G., TRESSLER, R. L. & GODFREY, C. 2015. Leveraging Cancer Therapeutics for the HIV Cure Agenda: Current Status and Future Directions. *Drugs,* 75**,** 1447-1459.

48. PRETORIUS, E., OBERHOLZER, H. M., SMIT, E., STEYN, E., BRIEDENHANN, S. & FRANZ, C. R. 2008. Ultrastructural changes in platelet aggregates of HIV patients: a scanning electron microscopy study. *Ultrastructural pathology,* 32**,** 75.

49. RASHEED, S., YAN, J. S., LAU, A. & CHAN, A. S. 2008. HIV replication enhances production of free fatty acids, low density lipoproteins and many key proteins involved in lipid metabolism: a proteomics study. *PloS one,* 3**,** e3003.

50. SACKOFF, J. E., HANNA, D. B., PFEIFFER, M. R. & TORIAN, L. V. 2006. Causes of death among persons with AIDS in the era of highly active antiretroviral therapy: New York City. *Annals of Internal Medicine,* 145**,** 397.

51. SCHILLACI, G., DE SOCIO, G. V., PUCCI, G., MANNARINO, M. R., HELOU, J., PIRRO, M. & MANNARINO, E. 2008. Aortic stiffness in untreated adult patients with human immunodeficiency virus infection. *Hypertension (Dallas, Tex.: 1979),* 52**,** 308.

52. SELVARAJ, S., GHEBREMICHAEL, M., LI, M., FOLI, Y., LANGS-BARLOW, A., OGBUAGU, A., BARAKAT, L., TUBRIDY, E., EDIFOR, R., LAM, W., CHENG, Y. C. & PAINTSIL, E. 2014. Antiretroviral therapy-induced mitochondrial toxicity: potential mechanisms beyond polymerase-gamma inhibition. *Clinical pharmacology and therapeutics,* 96**,** 110.

53. SHINOHARA, E., YAMASHITA, S., KIHARA, S., HIRANO, K., ISHIGAMI, M., ARAI, T., NOZAKI, S., KAMEDA-TAKEMURA, K., KAWATA, S. & MATSUZAWA, Y. 1997. Interferon alpha induces disorder of lipid metabolism by lowering postheparin lipases and cholesteryl ester transfer protein activities in patients with chronic hepatitis C. *Hepatology (Baltimore, Md.),* 25**,** 1502.

54. SOLAGES, A., VITA, J. A., THORNTON, D. J., MURRAY, J., HEEREN, T., CRAVEN, D. E. & HORSBURGH, C. R., JR. 2006. Endothelial function in HIV-infected persons. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America,* 42**,** 1325.

55. SRINIVASA, S. & GRINSPOON, S. K. 2014. Metabolic and body composition effects of newer antiretrovirals in HIV-infected patients. *European journal of endocrinology,* 170**,** R185.

56. STADLER, N., STANLEY, N., HEENEMAN, S., VACATA, V., DAEMEN, M. J., BANNON, P. G., WALTENBERGER, J. & DAVIES, M. J. 2008. Accumulation of zinc

in human atherosclerotic lesions correlates with calcium levels but does not protect against protein oxidation. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 28**,** 1024.

57. STEIN, J. H., KLEIN, M. A., BELLEHUMEUR, J. L., MCBRIDE, P. E., WIEBE, D. A., OTVOS, J. D. & SOSMAN, J. M. 2001. Use of human immunodeficiency virus-1 protease inhibitors is associated with atherogenic lipoprotein changes and endothelial dysfunction. *Circulation,* 104**,** 257.

58. STRATEGIES FOR MANAGEMENT OF ANTI-RETROVIRAL THERAPY, I. & GROUPS, D. A. D. S. 2008. Use of nucleoside reverse transcriptase inhibitors and risk of myocardial infarction in HIV-infected patients. *AIDS (London, England),* 22**,** F17.

59. TAYLOR, K. A. & PUGH, N. 2016. The contribution of zinc to platelet behaviour during haemostasis and thrombosis. *Metallomics : integrated biometal science,* 8**,** 144.

60. TEOFILI, L., IACHININOTO, M. G., CAPODIMONTI, S., UCCIFERRI, C., NUZZOLO, E. R., MARTINI, M., TORTI, L., FALASCA, K., VECCHIET, J., LEONE, G. & LAROCCA, L. M. 2010. Endothelial progenitor cell trafficking in human immunodeficiency virus-infected persons. *AIDS (London, England),* 24**,** 2443.

61. TIPPING, B., DE VILLIERS, L., WAINWRIGHT, H., CANDY, S. & BRYER, A. 2007. Stroke in patients with human immunodeficiency virus infection. *Journal of Neurology, Neurosurgery & Psychiatry,* 78**,** 1320.

62. TORRIANI, F. J., KOMAROW, L., PARKER, R. A., COTTER, B. R., CURRIER, J. S., DUBE, M. P., FICHTENBAUM, C. J., GERSCHENSON, M., MITCHELL, C. K., MURPHY, R. L., SQUIRES, K., STEIN, J. H. & TEAM, A. S. S. 2008. Endothelial function in human immunodeficiency virus-infected antiretroviral-naive subjects before and after starting potent antiretroviral therapy: The ACTG (AIDS Clinical Trials Group) Study 5152s. *Journal of the American College of Cardiology,* 52**,** 569.

63. TROLL, J. G. 2011. Approach to dyslipidemia, lipodystrophy, and cardiovascular risk in patients with HIV infection. *Current atherosclerosis reports,* 13**,** 51.

64. TS'AO, C. & NEOFOTISTOS, D. 1994. The use and limitations of the INR system. *Am J Hematol,* 47**,** 21-6.

65. VAN HEERDEN, A., BARNABAS, R. V., NORRIS, S. A., MICKLESFIELD, L. K., VAN ROOYEN, H. & CELUM, C. 2017. High prevalence of HIV and non-communicable disease (NCD) risk factors in rural KwaZulu-Natal, South Africa. *Journal of the International AIDS Society,* 20**,** 10.1002/jia2.25012.

66. WATSON, B. R., WHITE, N. A., TAYLOR, K. A., HOWES, J.-M., MALCOR, J.-D. M., BIHAN, D., SAGE, S. O., FARNDALE, R. W. & PUGH, N. 2016. Zinc is a transmembrane agonist that induces platelet activation in a tyrosine phosphorylationdependent manner. *Metallomics: Integrated Biometal Science,* 8**,** 91.

67. WILLERSON, J. T. & RIDKER, P. M. 2004. Inflammation as a cardiovascular risk factor. *Circulation,* 109**,** II2.

68. YOUSSEFIAN, T., DROUIN, A., MASSE, J. M., GUICHARD, J. & CRAMER, E. M. 2002. Host defense role of platelets: engulfment of HIV and Staphylococcus aureus occurs in a specific subcellular compartment and is enhanced by platelet activation. *Blood,* 99**,** 4021.

69. ZHONG, D. S., LU, X. H., CONKLIN, B. S., LIN, P. H., LUMSDEN, A. B., YAO, Q. & CHEN, C. 2002. HIV protease inhibitor ritonavir induces cytotoxicity of human endothelial cells. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 22**,** 1560.

70. ZUCKER-FRANKLIN, D., SEREMETIS, S. & ZHENG, Z. Y. 1990. Internalization of human immunodeficiency virus type I and other retroviruses by megakaryocytes and platelets. *Blood,* 75**,** 1920.

71. ZUNGU, N. P., MABASO, M. L., KUMALO, F., SIGIDA, S., MLANGENI, L., WABIRI, N. & CHASELA, C. 2019. Prevalence of non-communicable diseases (NCDs) and associated factors among HIV positive educators: Findings from the 2015/6 survey of Health of Educators in Public Schools in South Africa. *PloS one,* 14**,** e0209756.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

This thesis aimed to assess the influence of elevated FFA concentrations on the interaction between albumin and metal ions in circulation, and how abnormal pathophysiological processes such as thromboembolic events may be precipitated in pathological conditions especially NCDs and HIV infection due to the higher FFA levels. Indisputably, NCDs are a significant cause of high mortality and morbidity worldwide. HIV infection has complicated and worsened the burden of NCDs, mostly in LMIC. Both NCDs and HIV may be associated with higher plasma FFA concentrations which may bring about poor buffering of metal ions such as Zn^{2+} in circulation and influence undesirable consequences such as abnormal blood clotting and hence thromboembolic events. The study of these interactions and the potential mechanisms leading to the thromboembolic events influenced by the elevated FFA is of high clinical value.

ACB assay studies reported in this thesis have provided further evidence that the basis of the test is the detection of acute changes in FFA levels, which bind to albumin (and not oxidative modification of HSA). They also confirm that long-chain fatty acids, unlike the medium-chain free fatty acids, exert a negative allosteric effect on the binding of metal ions to HSA in a concentration-dependent manner where higher concentrations affected the assay more than lower concentrations, with maximum effects observed at 5 molar equivalents of HSA. Further to this, the ITC studies confirmed that that medium-chain fatty acids, unlike long-chain fatty acids, do not have a significant effect on the ability of HSA to bind metal ions such as Co^{2+} and Zn^{2+}.

The dysregulated metal ions such as Zn^{2+} may contribute to undesired physiological processes. This thesis has elucidated how Zn^{2+} may potentiate platelet aggregation. Higher Zn^{2+} concentrations with a defected or absent HSA buffering system was shown to potentiates platelet aggregation by positively influencing the maximum aggregation response and the rate of platelet aggregation. The platelet aggregation studies also suggested that long chain fatty acids such as myristate, unlike medium chain fatty acids such as octanoate, potentiated platelet aggregation by increasing the intensity of platelet aggregation and the rate of aggregation

responses. Thus, it can be deduced that pathological conditions associated with elevated FFA concentrations and diminished buffering of Zn^{2+} by HSA can potentiate platelet aggregation leading to thromboembolic mechanisms; and plausibly this forms a potential mechanism for the thromboembolic events prevalent in NCDs and HIV.

The in-silico analysis of albumin crystal structures performed and reported in this thesis substantiates and delineates how at a molecular level, ligands such as long-chain FFA significantly disrupt the zinc binding site A as evidenced by the changes in the distances between the amino acids residues forming the site.

Finally, the clinical study which formed part of this PhD thesis investigated the FFA and albumin concentrations, evaluated the coagulability of the blood samples and examined the relationship among these variables in participants with and without HIV infection in a rural Malawian population. Importantly, HIV infection was associated with lower albumin concentrations in participants who had been recently commenced on ART, and this was coupled with higher levels of FFA in the HIV infected participants on long-term ART. Even though there was no evidence of increased coagulability as shown by the INR and the turbidity tests conducted in the study, the higher FFA concentrations and hypoalbuminaemia can potentially contribute to cardiovascular and cerebrovascular events, *in vivo*, via poor buffering of Zn^{2+} and and potentiation of platelets associated with vascular pathophysiological processes evident in HIV infection.

The knowledge from this work can be extended, and the evidence can be improved. ACB assays can be conducted on the human serum of the HIV participants and compared with controls to substantiate the metal-binding perturbation in the study population. Further research work can also be performed on the other pharmacological substances that showed potential to disrupt the primary Zn^{2+}-binding site, as suggested in Chapter 4 of this thesis on the in-silico analysis of albumin crystal structures.

It is recommended that platelet aggregation experiments be conducted to establish the platelet activities between HIV-infected individuals and negative controls to provide further insights on the subject. It would also be essential to examine the endothelial dysfunction markers such as soluble P-selectin in the HIV-infected individuals and relate the levels with the platelet function and coagulability markers in the population. In addition, the zinc status of the HIV

infected population in comparison with controls would be worth assessing. Markers of endothelial dysfunction and atherosclerosis from the participants' plasma samples would also require further investigation to provide more insight on the pathophysiology.

Considering that the present clinical aspect of the study was conducted in rural settings of Malawi, whose population is typical of lower socio-economic status, it would be interesting to repeat the study, in an urban setting. Urban and city populations in Malawi are exposed to fast foods and sedentary lifestyle than the rural population. Coupled with HIV infection, one would expect higher levels of FFA and plausibly, which may influence coagulability. A comparison between the HIV populations between the two socio-economic groups would be informative.

APPENDIX

Provided in this section are a copy of the published journal article based on Chapter 2 of this PhD thesis, and Research Ethics approval certificates from the School of Medicine University of St Andrews and the College of Medicine University of Malawi respectively.

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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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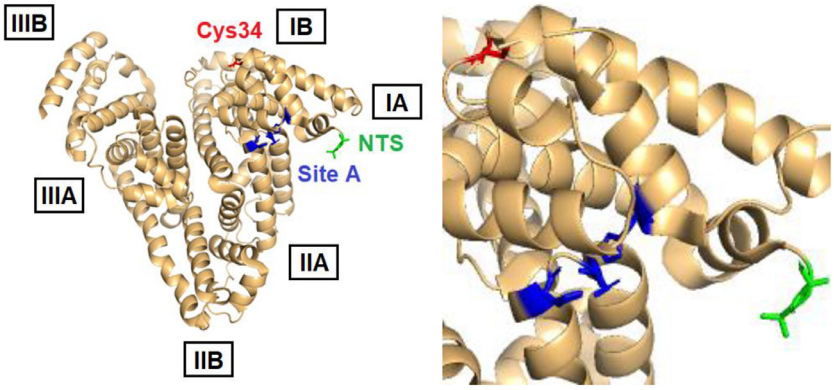

Fig. 1. Location of the three metal binding sites that have been successfully identified on human serum albumin,PDB: 5IJF [60]. Site A, the multi-metal binding site (MBS) (blue); NTS/ATCUN motif (green); Cys34 (red). The precise location of site B is not yet known. The boxed labels indicate the six sub-domains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

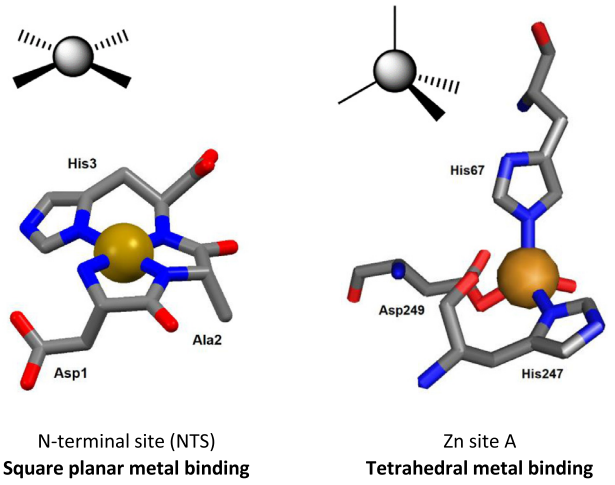

Fig. 2. Contrasting geometries of metal binding sites on albumin. Left: square planar coordination of Cu^{2+} or Ni^{2+} at the NTS site; the structure shown is derived from molecular modelling. The N-terminal amino group, two deprotonated backbone amide N atoms and the N(delta) of the imidazole ring 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

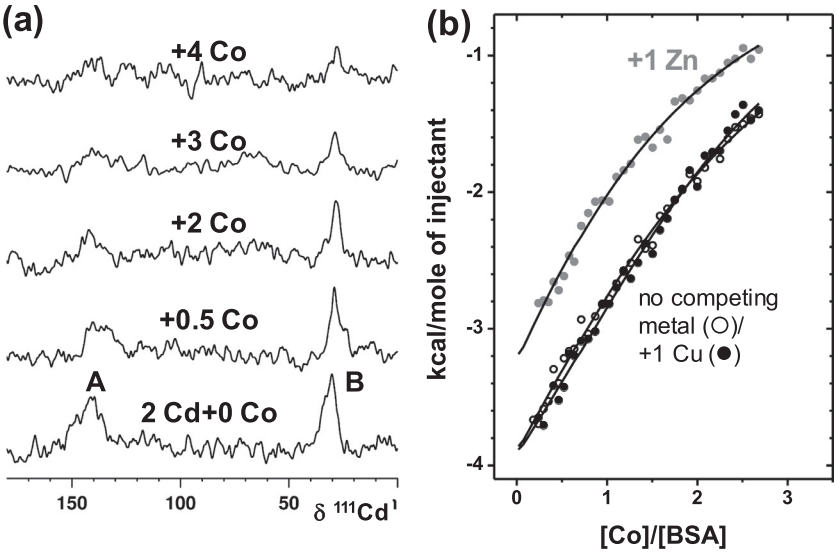

Fig. 3. Co^{2+} competes with both Cd^{2+} and Zn^{2+} for albumin binding under physiological conditions (pH 7.4, 50 mM Tris-Cl, 50 mM NaCl) but not with Cu^{2+}. (a) 111 Cd NMR spectra of Cd_2BSA (1.5 mM) with increasing addition of Co^{2+}. 111-Cd resonances corresponding to both site A and B (~140 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

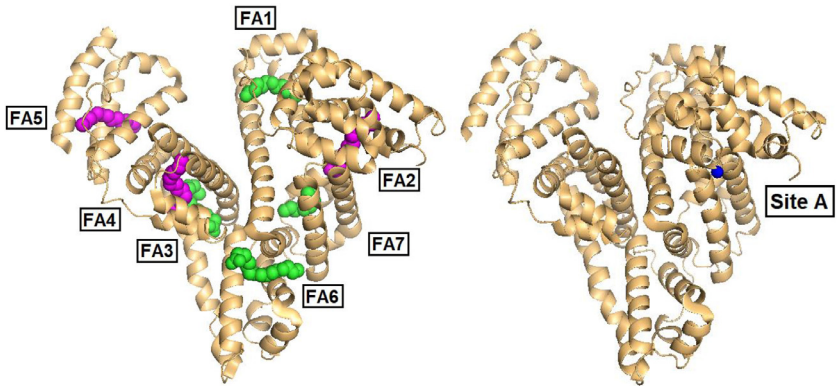

Fig. 4. (a) Location of fatty acid (FFA) binding sites FA1-7 on human serum albumin (PDB: 1E7H), complexed with hexadecanoic (palmitic) acid [82]. High (magenta) and low (green) affinity sites are shown. (b) Location of site A, the multi-metal binding site (PDB: 5IJF), occupied by Zn^{2+} (blue) [57]. 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].

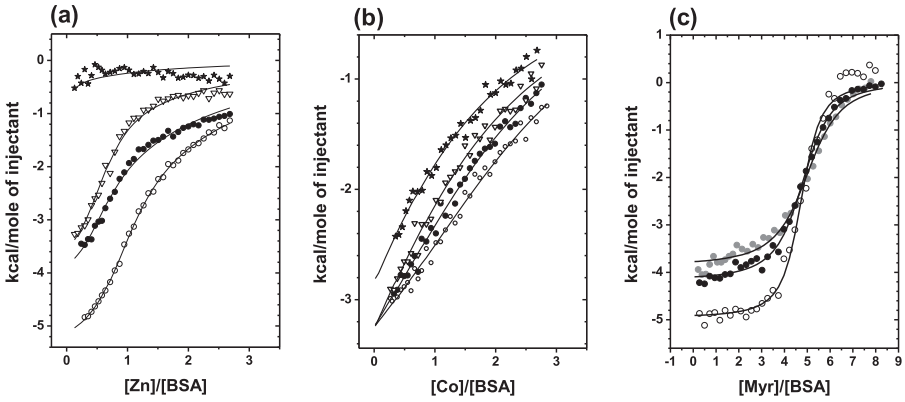

Fig. 5. Isothermal titration calorimetry experiments demonstrate the mutual modulation of metal and fatty acid binding to bovine albumin. The presence of the C14:0 fatty acid myristate (○, 0 mol. equiv.; ●, 1 mol. equiv.; ▽, 3 mol. equiv.; and ★ 5 mol. equiv.) affects the binding capacity of albumin 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

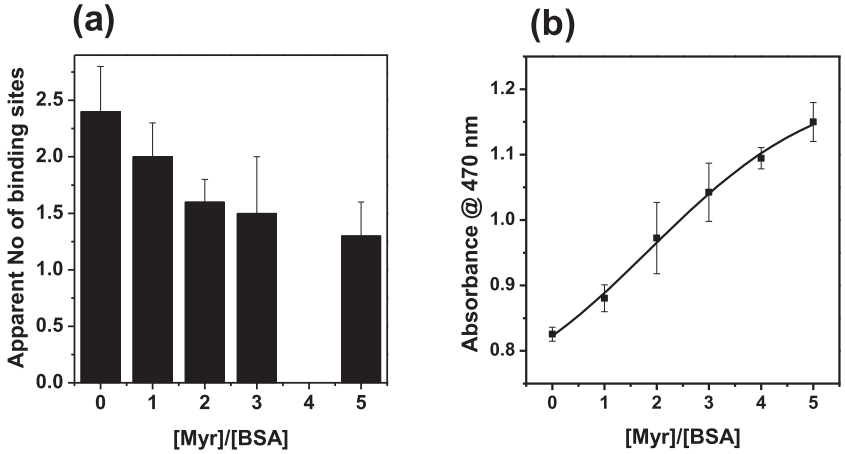

Fig. 6. Increasing FFA (myristate, C14:0) decreases the total Co^{2+} binding capacity of BSA, (a) reflected in the number of apparent binding sites of albumin for Co^{2+} (No data for 4 mol. eq. Myr). (b) In turn, this affects the formation of the Co-DTT complex as part of the ACB assay (b), used for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

[1] E. Picano, A. Palinkas, R. Amyot, Diagnosis of myocardial ischemia in hypertensive patients, J. Hypertens. 19 (2001) 1177–1183.
[2] F.S. Apple, A.H. Wu, J. Mair, J. Ravkilde, M. Panteghini, J. Tate, F. Pagani, R.H. Christenson, M. Mockel, O. Danne, A.S. Jaffe, I. Committee on standardization of markers of cardiac damage of the, future biomarkers for detection of ischemia and risk stratification in acute coronary syndrome, Clin. Chem. 51 (2005) 810–824.
[3] P.O. Collinson, D.C. Gaze, Biomarkers of cardiovascular damage and dysfunction–an overview, Heart Lung Circ. 16 (Suppl 3) (2007) S71–S82.
[4] P.O. Collinson, D.C. Gaze, Ischaemia-modified albumin: clinical utility and pitfalls in measurement, J. Clin. Pathol. 61 (2008) 1025–1028.
[5] D.C. Gaze, Ischemia modified albumin: a novel biomarker for the detection of cardiac ischemia, Drug Metab. Pharmacokinet. 24 (2009) 333–341.
[6] E. Sbarouni, P. Georgiadou, V. Voudris, Ischemia modified albumin changes - review and clinical implications, Clin. Chem. Lab. Med. 49 (2011) 177–184.
[7] A.H. Wu, The ischemia-modified albumin biomarker for myocardial ischemia, MLO Med. Lab. Obs. 35 (2003) 36–38 40.
[8] D. Bar-Or, E. Lau, J.V. Winkler, A novel assay for cobalt-albumin binding and its potential as a marker for myocardial ischemia-a preliminary report, J. Emerg. Med. 19 (2000) 311–315.
[9] https://www.accessdata.fda.gov/cdrh_docs/pdf2/k023824.pdf, accessed 12/07/2018.
[10] I.M.I. Inc., Albumin cobalt binding (ACB) test (package insert). Reagent pack for Beckman Coulter Synchron LX-20.
[11] P.J. Sadler, A. Tucker, J.H. Viles, Involvement of a lysine residue in the N-terminal Ni^{2+} and Cu^{2+} binding site of serum albumins comparison with Co^{2+}, Cd^{2+} and Al^{3+}, Eur. J. Biochem. 220 (1994) 193–200.
[12] C. Harford, B. Sarkar, Amino terminal Cu(II)- and Ni(II)-binding (ATCUN) motif of proteins and peptides: metal binding, DNA cleavage, and other properties, Acc. Chem. Res. 30 (1997) 123–130.
[13] D. Bar-Or, G. Curtis, N. Rao, N. Bampos, E. Lau, Characterization of the Co^{2+} and Ni^{2+} binding amino-acid residues of the N-terminus of human albumin, Eur. J. Biochem. 268 (2001) 42–48.
[14] D. Roy, J. Quiles, D.C. Gaze, P. Collinson, J.C. Kaski, G.F. Baxter, Role of reactive oxygen species on the formation of the novel diagnostic marker ischaemia modified albumin, Heart 92 (2006) 113–114.
[15] N.V. Bhagavan, E.M. Lai, P.A. Rios, J. Yang, A.M. Ortega-Lopez, H. Shinoda, S.A. Honda, C.N. Rios, C.E. Sugiyama, C.E. Ha, Evaluation of human serum albumin cobalt binding assay for the assessment of myocardial ischemia and myocardial infarction, Clin. Chem. 49 (2003) 581–585.
[16] B.J. Oh, M.H. Seo, H.S. Kim, Insignificant role of the N-terminal cobalt-binding site of albumin in the assessment of acute coronary syndrome: discrepancy between the albumin cobalt-binding assay and N-terminal-targeted immunoassay, Biomarkers 17 (2012) 394–401.
[17] M. Domenicali, M. Baldassarre, F.A. Giannone, M. Naldi, M. Mastroroberto, M. Biselli, M. Laggetta, D. Patrono, C. Bertucci, M. Bernardi, P. Caraceni, Posttranscriptional changes of serum albumin: clinical and prognostic significance in hospitalized patients with cirrhosis, Hepatology 60 (2014) 1851–1860.
[18] R. Jalan, K. Schnurr, R.P. Mookerjee, S. Sen, L. Cheshire, S. Hodges, V. Muravsky, R. Williams, G. Matthes, N.A. Davies, Alterations in the functional capacity of albumin in patients with decompensated cirrhosis is associated with increased mortality, Hepatology 50 (2009) 555–564.
[19] D. Bar-Or, L.T. Rael, R. Bar-Or, D.S. Slone, C.W. Mains, N.K.R. Rao, C.G. Curtis, The cobalt–albumin binding assay: Insights into its mode of action, Clin. Chim. Acta 387 (2008) 120–127.
[20] N.V. Bhagavan, J.S. Ha, J.H. Park, S.A. Honda, C.N. Rios, C. Sugiyama, G.K. Fujitani, I.K. Takeuchi, C.E. Ha, Utility of serum Fatty Acid concentrations as a marker for acute myocardial infarction and their potential role in the formation of ischemia-modified albumin: a pilot study, Clin. Chem. 55 (2009) 1588–1590.
[21] J.P. Barnett, C.A. Blindauer, O. Kassaar, S. Khazaipoul, E.M. Martin, P.J. Sadler, A.J. Stewart, Allosteric modulation of zinc speciation by fatty acids, Biochim. Biophys. Acta Gen. Subj. 1830 (2013) 5456–5464.
[22] C.A. Blindauer, S. Khazaipoul, R. Yu, A.J. Stewart, Fatty acid-mediated inhibition of metal binding to the multi-metal site on serum albumin: implications for cardiovascular disease, Curr. Top. Med. Chem. 16 (2016) 3021–3032.
[23] O. Kassaar, U. Schwarz-Linek, C.A. Blindauer, A.J. Stewart, Plasma free fatty acid levels influence Zn^{2+}-dependent histidine-rich glycoprotein–heparin interactions via an allosteric switch on serum albumin, J. Thromb. Haemost. 13 (2015) 101–110.
[24] J. Lu, A.J. Stewart, D. Sleep, P.J. Sadler, T.J.T. Pinheiro, C.A. Blindauer, A molecular mechanism for modulating plasma Zn speciation by fatty acids, J. Am. Chem. Soc. 134 (2012) 1454–1457.
[25] J. Lu, A.J. Stewart, P.J. Sadler, T.J.T. Pinheiro, C.A. Blindauer, Allosteric inhibition of cobalt binding to albumin by fatty acids: implications for the detection of myocardial ischemia, J. Med. Chem. 55 (2012) 4425–4430.
[26] L.D. Lehman-McKeeman, Chapter 1 - biochemical and molecular basis of toxicity A2, in: W. Haschek, C.G. Rousseaux, M.A. Wallig (Eds.), Haschek and Rousseaux's Handbook of Toxicologic Pathology, Third ed., Academic Press, Boston, 2013, pp. 15–38.
[27] T. Topală, A. Bodoki, L. Oprean, R. Oprean, Bovine serum albumin interactions with metal complexes, Clujul Medical 87 (2014) 215–219.
[28] S. Sankaranarayanan, M.dela Llera-Moya, D. Drazul-Schrader, M.C. Phillips, G. Kellner-Weibel, G.H. Rothblat, Serum albumin acts as a shuttle to enhance cholesterol efflux from cells, J. Lipid Res. 54 (2013) 671–676.
[29] G.J. van der Vusse, Albumin as fatty acid transporter, Drug Metab. Pharmacokinet. 24 (2009) 300–307.
[30] M. Roche, P. Rondeau, N.R. Singh, E. Tarnus, E. Bourdon, The antioxidant properties of serum albumin, FEBS Lett. 582 (2008) 1783–1787.
[31] S.H. Laurie, Transport and storage of metals, J. Inherit. Metab. Dis. 6 (1983) 9–14.
[32] M. Nordberg, Cadmium toxicology, in: B. Caballero, P. Finglas, F. Toldra (Eds.), Encyclopedia of Food Sciences and Nutrition, Second ed., Academic Press, Oxford, 2003, pp. 739–745.
[33] J.D. Glennon, B. Sarkar, Nickel(II) transport in human blood serum. Studies of nickel(II) binding to human albumin and to native-sequence peptide, and ternary-complex formation with L-histidine, Biochem. J. 203 (1982) 15–23.
[34] W. Bal, M. Sokołowska, E. Kurowska, P. Faller, Binding of transition metal ions to albumin: sites, affinities and rates, Biochim. Biophys. Acta Gen. Subj. 1830 (2013) 5444–5455.
[35] L. Messori, A. Merlino, Protein metalation by metal-based drugs: X-ray crystallography and mass spectrometry studies, Chem. Commun. 53 (2017) 11622–11633.
[36] S.L. Guthans, W.T. Morgan, The interaction of zinc, nickel and cadmium with serum albumin and histidine-rich glycoprotein assessed by equilibrium dialysis and immunoadsorbent chromatography, Arch. Biochem. Biophys. 218 (1982)

320–328.
[37] Y. Zhou, Y. Wang, X. Hu, J. Huang, Y. Hao, H. Liu, P. Shen, Equilibrium dialysis of metal-serum albumin. I. Successive stability constants of Zn(II)-serum albumin and the Zn^{2+}-induced cross-linking self-association, Biophys. Chem. 51 (1994) 81–87.
[38] J. Masuoka, P. Saltman, Zinc(II) and Copper(II) binding to serum-albumin - a comparative-study of dog, bovine, and human albumin, J. Biol. Chem. 269 (1994) 25557–25561.
[39] W. Goumakos, J.P. Laussac, B. Sarkar, Binding of cadmium(II) and zinc(II) to human and dog serum albumins. An equilibrium dialysis and ^{113}Cd-NMR study, Biochem. Cell Biol. 69 (1991) 809–820.
[40] D.E. Wilcox, Isothermal titration calorimetry of metal ions binding to proteins: an overview of recent studies, Inorg. Chim. Acta 361 (2008) 857–867.
[41] W. Bal, J. Christodoulou, P.J. Sadler, A. Tucker, Multi-metal binding site of serum albumin, J. Inorg. Biochem. 70 (1998) 33–39.
[42] M. Sokołowska, M. Wszelaka-Rylik, J. Poznański, W. Bal, Spectroscopic and thermodynamic determination of three distinct binding sites for Co(II) ions in human serum albumin, J. Inorg. Biochem. 103 (2009) 1005–1013.
[43] Y. Zhang, D.E. Wilcox, Thermodynamic and spectroscopic study of Cu(II) and Ni (II) binding to bovine serum albumin, J. Biol. Inorg. Chem. 7 (2002) 327–337.
[44] L. Quintanar, L. Rivillas-Acevedo, Studying metal ion–protein interactions: electronic absorption, circular dichroism, and electron paramagnetic resonance, in: M.A. Williams, T. Daviter (Eds.), Protein-Ligand Interactions: Methods and Applications, Humana Press, Totowa, NJ, 2013, pp. 267–297.
[45] E.O. Martins, T. Drakenberg, Cadmium(II), zinc(II),and copper(II) ions binding to bovine serum albumin - a Cd-113 NMR study, Inorg. Chim. Acta 67 (1982) 71–74.
[46] P.J. Sadler, J.H. Viles, ^{1}H and ^{113}Cd NMR investigations of Cd^{2+} and Zn^{2+} binding sites on serum albumin: competition with Ca^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}, Inorg. Chem. 35 (1996) 4490–4496.
[47] C.A. Blindauer, I. Harvey, K.E. Bunyan, A.J. Stewart, D. Sleep, D.J. Harrison, S. Berezenko, P.J. Sadler, Structure, properties, and engineering of the major zinc binding site on human albumin, J. Biol. Chem. 284 (2009) 23116–23124.
[48] W. Hu, Q. Luo, K. Wu, X. Li, F. Wang, Y. Chen, X. Ma, J. Wang, J. Liu, S. Xiong, P.J. Sadler, The anticancer drug cisplatin can cross-link the interdomain zinc site on human albumin, Chem. Commun. 47 (2011) 6006–6008.
[49] H. Lakusta, B. Sarkar, Equilibrium studies of zinc(II) and cobalt(II) binding to tripeptide analogues of the amino terminus of human serum albumin, J. Inorg. Biochem. 11 (1979) 303–315.
[50] C. Harford, B. Sarkar, Amino terminal Cu(II)- and Ni(II)-binding (ATCUN) motif of proteins and peptides: Metal binding, DNA cleavage, and other properties, Accounts Chem. Res. 30 (1997) 123–130.
[51] K. Kulprachakarn, Y.-L. Chen, X. Kong, M.C. Arno, R.C. Hider, S. Srichairatanakool, S.S. Bansal, Copper(II) binding properties of hepcidin, J. Biol. Inorg. Chem. 21 (2016) 329–338.
[52] M. Rózga, M. Sokołowska, A.M. Protas, W. Bal, Human serum albumin coordinates Cu(II) at its N-terminal binding site with 1 pM affinity, J. Biol. Inorg. Chem. 12 (2007) 913–918.
[53] G.A. McMillin, J.J. Travis, J.W. Hunt, Direct measurement of free copper in serum or plasma ultrafiltrate, Am. J. Clin. Pathol. 131 (2009) 160–165.
[54] B.L. O'Dell, R.A. Sunde, Handbook of Nutritionally Essential Mineral Elements, CRC Press, 1997.
[55] I.M. Armitage, T. Drakenberg, B. Reilly, Use of ^{113}Cd NMR to probe the native metal binding sites in metalloproteins: an overview, Met. Ions Life Sci. 11 (2013) 117–144.
[56] A.J. Stewart, C.A. Blindauer, S. Berezenko, D. Sleep, P.J. Sadler, Interdomain zinc site on human albumin, Proc. Natl. Acad. Sci. USA 100 (2003) 3701–3706.
[57] K.B. Handing, I.G. Shabalin, O. Kassaar, S. Khazaipoul, C.A. Blindauer, A.J. Stewart, M. Chruszcz, W. Minor, Circulatory zinc transport is controlled by distinct interdomain sites on mammalian albumins, Chem. Sci. 7 (2016) 6635–6648.
[58] W. Hussain, A. Mumtaz, F. Yasmeen, S.Q. Khan, T. Butt, Reference range of zinc in adult population (20-29 years) of Lahore, Pakistan, Pak. J. Med. Sci. 30 (2014) 545–548.
[59] D.C. Chilvers, J.B. Dawson, M.-H. Bahreyni-Toosi, A. Hodgkinson, Identification and determination of copper-and zinc-protein complexes in blood plasma after chromatographic separation on DEAE-Sepharose CL-6B, Analyst 109 (1984) 871–876.
[60] J. Lu, AlanJ. Stewart, PeterJ. Sadler, TeresaJ.T. Pinheiro, ClaudiaA. Blindauer, Albumin as a zinc carrier: properties of its high-affinity zinc-binding site, Biochem. Soc. Trans. 36 (2008) 1317–1321.
[61] M. Valko, H. Morris, M. Mazúr, J. Telser, E.J.L. McInnes, F.E. Mabbs, High-affinity binding site for copper(II) in human and dog serum albumins (an EPR study), J. Phys. Chem. B. 103 (1999) 5591–5597.
[62] D.J. Eatough, T.E. Jensen, L.D. Hansen, H.F. Loken, S.J. Rehfeld, The binding of Ca^{2+} and Mg^{2+} to human serum albumin: a calorimetric study, Thermochim. Acta 25 (1978) 289–297.
[63] K.A. Majorek, P.J. Porebski, A. Dayal, M.D. Zimmerman, K. Jablonska, A.J. Stewart, M. Chruszcz, W. Minor, Structural and immunologic characterization of bovine, horse, and rabbit serum albumins, Mol. Immunol. 52 (2012) 174–182.
[64] U. Kragh-Hansen, H. Vorum, Quantitative analyses of the interaction between calcium ions and human serum albumin, Clin. Chem. 39 (1993) 202–208.
[65] K.O. Pedersen, Binding of calcium to serum albumin. III. Influence of ionic strength and ionic medium, Scand. J. Clin. Lab. Invest. 29 (1972) 427–432.
[66] R. Shannon, Revised effective ionic radii and systematic studies of interatomic distances in halides and chalcogenides, Acta Cryst. 32 (1976) 751–767.
[67] F.A. Cotton, D.M.L. Goodgame, M. Goodgame, The electronic structures of tetrahedral cobalt(II) complexes, J. Am. Chem. Soc. 83 (1961) 4690–4699.
[68] W. Maret, B.L. Vallee, Cobalt as probe and label of proteins, Methods Enzymol. 226 (1993) 52–71.
[69] B. Bennett, EPR of cobalt-substituted zinc enzymes, in: G. Hanson, L. Berliner (Eds.), Metals in Biology: Applications of High-Resolution EPR to Metalloenzymes, Springer New York, New York, NY, 2010, pp. 345–370.
[70] H. Liang, J. Huang, C.-Q. Tu, M. Zhang, Y.-Q. Zhou, P.-W. Shen, The subsequent effect of interaction between Co^{2+} and human serum albumin or bovine serum albumin, J. Inorg. Biochem. 85 (2001) 167–171.
[71] E. Mothes, P. Faller, Evidence that the principal CoII-binding site in human serum albumin is not at the N-terminus: implication on the albumin cobalt binding test for detecting myocardial ischemia, Biochemistry 46 (2007) 2267–2274.
[72] M. Fasano, S. Curry, E. Terreno, M. Galliano, G. Fanali, P. Narciso, S. Notari, P. Ascenzi, The extraordinary ligand binding properties of human serum albumin, IUBMB Life 57 (2005) 787–796.
[73] A.A. Spector, Fatty acid binding to plasma albumin, J. Lipid Res. 16 (1975) 165–179.
[74] A. Stahl, R.E. Gimeno, L.A. Tartaglia, H.F. Lodish, Fatty acid transport proteins: a current view of a growing family, Trends Endocrinol. Metab. 12 (2001) 266–273.
[75] S. Curry, Plasma albumin as a fatty acid carrier, Advances in Molecular and Cell Biology, Elsevier, 2003, pp. 29–46.
[76] C.E. Ha, N.V. Bhagavan, Novel insights into the pleiotropic effects of human serum albumin in health and disease, Biochim. Biophys. Acta 1830 (2013) 5486–5493.
[77] N. Kaur, V. Chugh, A.K. Gupta, Essential fatty acids as functional components of foods- a review, J. Food Sci. Technol. 51 (2014) 2289–2303.
[78] S. Curry, H. Mandelkow, P. Brick, N. Franks, Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites, Nat. Struct. Biol. 5 (1998) 827.
[79] S. Curry, P. Brick, N.P. Franks, Fatty acid binding to human serum albumin: new insights from crystallographic studies, Biochim. Biophys. Acta Mol. Cell. Biol. Lipids 1441 (1999) 131–140.
[80] J.A. Hamilton, NMR reveals molecular interactions and dynamics of fatty acid binding to albumin, Biochim. Biophys. Acta 1830 (2013) 5418–5426.
[81] D.P. Cistola, D.M. Small, J.A. Hamilton, C-13 NMR-studies of saturated fatty-acids bound to bovine serum albumin. 1. The filling of individual fatty-acid binding sites, J. Biol. Chem. 262 (1987) 10971–10979.
[82] A.A. Bhattacharya, T. Grüne, S. Curry, Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin, J. Mol. Biol. 303 (2000) 721–732.
[83] J.R. Simard, P.A. Zunszain, C.-E. Ha, J.S. Yang, N.V. Bhagavan, I. Petitpas, S. Curry, J.A. Hamilton, Locating high-affinity fatty acid-binding sites on albumin by x-ray crystallography and NMR spectroscopy, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 17958–17963.
[84] J.R. Simard, P.A. Zunszain, J.A. Hamilton, S. Curry, Location of high and low affinity fatty acid binding sites on human serum albumin revealed by nmr drug-competition analysis, J. Mol. Biol. 361 (2006) 336–351.
[85] R.H. Christenson, S.H. Duh, W.R. Sanhai, A.H. Wu, V. Holtman, P. Painter, E. Branham, F.S. Apple, M. Murakami, D.L. Morris, Characteristics of an Albumin Cobalt Binding Test for assessment of acute coronary syndrome patients: a multicenter study, Clin. Chem. 47 (2001) 464–470.
[86] P. Gurumurthy, S.K. Borra, R.K. Yeruva, D. Victor, S. Babu, K.M. Cherian, Estimation of ischemia modified albumin (IMA) levels in patients with acute coronary syndrome, Indian J. Clin. Biochem. 29 (2014) 367–371.
[87] J. Zhang, Y. Zhao, C. Xu, Y. Hong, H. Lu, J. Wu, Y. Chen, Association between serum free fatty acid levels and nonalcoholic fatty liver disease: a cross-sectional study, Sci. Rep. 4 (2014) 5832.
[88] A. Kiyici, I. Mehmetoglu, H. Karaoglan, H. Atalay, Y. Solak, S. Turk, Ischemia-modified albumin levels in patients with end-stage renal disease patients on hemodialysis: does albumin analysis method affect albumin-adjusted ischemia-modified albumin levels? J. Clin. Lab. Anal. 24 (2010) 273–277.
[89] K. Ghosh, M.G. Muddeshwar, M. Lokhande, K. Ghosh, Albumin cobalt binding or ischaemia modified albumin: a test of great prognostic value in malaria, Mediterr. J. Hematol. Infect. Dis. 9 (2017) e2017041.
[90] S. Vyakaranam, A.V. Bhongir, D. Patlolla, R. Chintapally, Maternal serum ischemia modified albumin as a marker for hypertensive disorders of pregnancy: a pilot study, Int. J. Reprod. Contracept. Obstet. Gynecol. 4 (2015) 611–616.
[91] E. Zurawska-Plaksej, E. Grzebyk, D. Marciniak, A. Szymanska-Chabowska, A. Piwowar, Oxidatively modified forms of albumin in patients with risk factors of metabolic syndrome, J. Endocrinol. Invest. 37 (2014) 819–827.
[92] M.G.V. Gottlieb, I.B. da Cruz, M.M. Duarte, R.N. Moresco, M. Wiehe, C.H. Schwanke, L.C. Bodanese, Associations among metabolic syndrome, ischemia, inflammatory, oxidatives, and lipids biomarkers, J. Clin. Endocrinol. Metab. 95 (2010) 586–591.
[93] A. Piwowar, M. Knapik-Kordecka, M. Warwas, Ischemia-modified albumin level in type 2 diabetes mellitus - Preliminary report, Disease markers 24 (2008) 311–317.
[94] I. Mehmetoglu, S. Kurban, F.H. Yerlikaya, H. Polat, Obesity is an independent determinant of ischemia-modified albumin, Obesity facts 5 (2012) 700–709.
[95] S.J. Piva, M.M. Duarte, I.B. Da Cruz, A.C. Coelho, A.P. Moreira, R. Tonello, S.C. Garcia, R.N. Moresco, Ischemia-modified albumin as an oxidative stress biomarker in obesity, Clin. Biochem. 44 (2011) 345–347.
[96] T. Çolak, B. Bamaç, S. Çolak, C. Duman, B. Bayazit, S. Öztürk, B. Meriç, A. Özbek, F. Yildiz, The influence of a single bout of wrestling exercise on serum levels of ischemia-modified albumin, J. Exerc. Sci. Fit. 8 (2010) 67–72.
[97] M. Can, S. Demirtas, O. Polat, A. Yildiz, Evaluation of effects of ischaemia on the albumin cobalt binding (ACB) assay in patients exposed to trauma, Emerg. Med. J. 23 (2006) 537–539.
[98] M. Pirro, P. Mauriege, A. Tchernof, B. Cantin, G.R. Dagenais, J.P. Despres,

B. Lamarche, Plasma free fatty acid levels and the risk of ischemic heart disease in men: prospective results from the Quebec Cardiovascular Study, Atherosclerosis 160 (2002) 377–384.
[99] V. Loria, M. Leo, G. Biasillo, I. Dato, L.M. Biasucci, Biomarkers in acute coronary syndrome, Biomark. Insights 3 (2008) 453–468.
[100] V.K. Roy, A. Kumar, P. Joshi, J. Arora, A.M. Ahanger, Plasma free fatty acid concentrations as a marker for acute myocardial infarction, J. Clin. Diagn. Res. 7 (2013) 2432–2434.
[101] V. Stich, M. Berlan, Physiological regulation of NEFA availability: lipolysis pathway, Proc. Nutr. Soc. 63 (2004) 369–374.
[102] M.F. Oliver, Free fatty acids and acute coronary syndromes-the history, QJM 104 (2011) 625–627.
[103] G.D. Lopaschuk, R. Collins-Nakai, P.M. Olley, T.J. Montague, G. McNeil, M. Gayle, P. Penkoske, B.A. Finegan, Plasma fatty acid levels in infants and adults after myocardial ischemia, Am. Heart J. 128 (1994) 61–67.
[104] M.K. Sinha, D. Roy, D.C. Gaze, P.O. Collinson, J.C. Kaski, Role of "Ischemia modified albumin", a new biochemical marker of myocardial ischaemia, in the early diagnosis of acute coronary syndromes, Emerg. Med. J. 21 (2004) 29–34.
[105] D.K. Cho, J.O. Choi, S.H. Kim, J. Choi, I. Rhee, C.S. Ki, S.C. Lee, H.C. Gwon, Ischemia-modified albumin is a highly sensitive serum marker of transient myocardial ischemia induced by coronary vasospasm, Coron. Artery Dis. 18 (2007) 83–87.
[106] P. Kovacs, M. Stumvoll, Fatty acids and insulin resistance in muscle and liver, Best Pract. Res. Clin. Endocrinol. Metab. 19 (2005) 625–635.
[107] G. Vernon, A. Baranova, Z.M. Younossi, Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults, Aliment. Pharmacol. Ther. 34 (2011) 274–285.
[108] C. Capurso, A. Capurso, From excess adiposity to insulin resistance: the role of free fatty acids, Vascul. Pharmacol. 57 (2012) 91–97.
[109] G. Musso, R. Gambino, M. Cassader, Recent insights into hepatic lipid metabolism in non-alcoholic fatty liver disease (NAFLD), Prog. Lipid Res. 48 (2009) 1–26.
[110] I.C. Gaemers, A.K. Groen, New insights in the pathogenesis of non-alcoholic fatty liver disease, Curr. Opin. Lipidol. 17 (2006) 268–273.
[111] B.B. Wu, L.M. Zhang, C.L. Mei, Q. Tang, Y.Z. Lu, Relationship between serum free fatty acid and cytokines, carotid atherosclerosis in chronic kidney disease [Abstract], Zhonghua Nei Ke Za Zhi 49 (2010) 572–576.
[112] M.T. Liao, C.C. Sung, K.C. Hung, C.C. Wu, L. Lo, K.C. Lu, Insulin resistance in patients with chronic kidney disease, J. Biomed. Biotechnol. 2012 (2012) 691369.
[113] J. Hartwich, I. Leszczynska-Golabek, B. Kiec-Wilk, D. Siedlecka, P. Perez-Martinez, C. Marin, J. Lopez-Miranda, A. Tierney, J.M. Monagle, H.M. Roche, C. Defoort, P. Wolkow, A. Dembinska-Kiec, Lipoprotein profile, plasma ischemia modified albumin and LDL density change in the course of postprandial lipemia. Insights from the LIPGENE study, Scand. J. Clin. Lab. Invest. 70 (2010) 201–208.
[114] S. Muhtaroglu, D. Barlak Keti, K. Unluhizarci, Investigation of ischemia-modified albumin levels and some atherosclerosis-related serum parameters in patients with diabetic foot, Turk. J. Med. Sci. 46 (2016) 126–132.
[115] S.V. Reddy, M.M. Suchitra, V. Pradeep, S. Alok, V. Suresh, A.R. Bitla, P.V. Srinivasa Rao, Ischemia-modified albumin levels in overt and subclinical hypothyroidism, J. Endocrinol. Invest. 38 (2015) 885–890.
[116] A. Rossi, N. Bortolotti, S. Vescovo, I. Romanello, L. Forzano, A.P. Londero, G. Ambrosini, D. Marchesoni, F. Curcio, Ischemia-modified albumin in pregnancy, Eur. J. Obstet. Gynecol. Reprod. Biol. 170 (2013) 348–351.
[117] M.R. Leitemperguer, E. Tatsch, H. Kober, J.A. De Carvalho, R.N. Moresco, J.E. Da Silva, Assessment of ischemia-modified albumin levels in patients with rheumatoid arthritis, Clin. Lab. 60 (2014) 1065–1070.
[118] A. Kucuk, A. Ugur Uslu, A. Icli, E. Cure, S. Arslan, K. Turkmen, A. Toker, M. Kayrak, The LDL/HDL ratio and atherosclerosis in ankylosing spondylitis, Z. Rheumatol. 76 (2017) 58–63.
[119] P. Ashok Kumar, U. Anand, Multiple biomarkers to assess the pathophysiological state in critically Ill patients with sepsis, Indian J. Clin. Biochem. 31 (2016) 310–314.
[120] S. Gupta, K. Seydel, M.A. Miranda-Roman, C.M. Feintuch, A. Saidi, R.S. Kim, G.L. Birbeck, T. Taylor, J.P. Daily, Extensive alterations of blood metabolites in pediatric cerebral malaria, PLoS One 12 (2017) e0175686.
[121] A. Basu, R. Passmore, J.A. Strong, The effect of exercise on the level of non-esterified fatty acids in the blood, Q. J. Exp. Physiol. Cogn. Med. Sci. 45 (1960) 312–317.
[122] C.A. Hubel, M.K. McLaughlin, R.W. Evans, B.A. Hauth, C.J. Sims, J.M. Roberts, Fasting serum triglycerides, free fatty acids, and malondialdehyde are increased in preeclampsia, are positively correlated, and decrease within 48 hours post partum, Am. J. Obstet. Gynecol. 174 (1996) 975–982.
[123] K. Rodahl, H.I. Miller, B. Issekutz Jr., Plasma free fatty acids in exercise, J. Appl. Physiol. 19 (1964) 489–492.
[124] S. Svensson, R. Svedjeholm, R. Ekroth, I. Milocco, F. Nilsson, K.G. Sabel, G. William-Olsson, Trauma metabolism and the heart. Uptake of substrates and effects of insulin early after cardiac operations, J. Thorac. Cardiovasc. Surg. 99 (1990) 1063–1073.
[125] J.L. Vigne, J.T. Murai, B.W. Arbogast, W. Jia, S.J. Fisher, R.N. Taylor, Elevated nonesterified fatty acid concentrations in severe preeclampsia shift the isoelectric characteristics of plasma albumin, J. Clin. Endocrinol. Metab. 82 (1997) 3786–3792.
[126] S. Isik, S. Kilic, Z. Ogretmen, D.U. Cakir, H. Turkon, S. Cevizci, M.M. Hiz, The correlation between the psoriasis area severity index and ischemia-modified albumin, mean platelet volume levels in patients with psoriasis, Postepy Dermatol. Alergol. 33 (2016) 290–293.
[127] F. Beyazit, N. Yilmaz, O. Balci, M. Adam, S.T. Yaman, Evaluation of oxidative stress in women with polycystic ovarian syndrome as represented by serum ischemia modified albumin and its correlation with testosterone and insulin resistance, Intern. Med. 55 (2016) 2359–2364.
[128] Q. Xu, J. Du, X. Ling, Y. Lu, Evaluation of MIh scoring system in diagnosis of obstructive sleep apnea syndrome, Med. Sci. Monit. 23 (2017) 4715–4722.
[129] S. Guven, C. Kart, E.S. Guvendag Guven, E.C. Cetin, A. Mentese, Is the measurement of serum ischemia-modified albumin the best test to diagnose ovarian torsion? Gynecol. Obstet. Invest. 79 (2015) 269–275.
[130] H. Kahveci, C. Tayman, F. Laoglu, H.T. Celik, N. Kavas, O. Kilic, S. Aydemir, Serum ischemia-modified albumin in preterm babies with respiratory distress syndrome, Indian J. Clin. Biochem. 31 (2016) 38–42.
[131] N. Sari, H. Ede, Y. Engin-Ustun, A.Y. Gocmen, E.K. Caglayan, Hyperemesis gravidarum is associated with increased maternal serum ischemia-modified albumin, J. Perinat. Med. 45 (2017) 421–425.
[132] A. Kumral, E. Okyay, S. Guclu, P. Gencpinar, G.H. Islekel, S.S. Oguz, M. Kant, G. Demirel, N. Duman, H. Ozkan, Cord blood ischemia-modified albumin: is it associated with abnormal Doppler findings in complicated pregnancies and predictive of perinatal asphyxia, J. Obstet. Gynaecol. Res. 39 (2013) 663–671.
[133] Y. He, R. Chen, J. Wang, W. Pan, Y. Sun, F. Han, Q. Wang, C. Liu, Neurocognitive impairment is correlated with oxidative stress in patients with moderate-to-severe obstructive sleep apnea hypopnea syndrome, Respir. Med. 120 (2016) 25–30.
[134] J.E. Eom, E. Lee, K.H. Jeon, J. Sim, M. Suh, G.J. Jhon, Y. Kwon, Development of an albumin copper binding (ACuB) assay to detect ischemia modified albumin, Anal. Sci. 30 (2014) 985–990.
[135] S.H. da Silva, S. Hausen Bdos, D.B. da Silva, A.M. Becker, M.M. de Campos, M.M. Duarte, R.N. Moresco, Characteristics of a nickel-albumin binding assay for assessment of myocardial ischaemia, Biomarkers 15 (2010) 353–357.
[136] S.H. da Silva, S. Pereira Rda, S. Hausen Bdos, C. Signor, P. Gomes, M.M. de Campos, R.N. Moresco, Assessment of the nickel-albumin binding assay for diagnosis of acute coronary syndrome, Clin. Chem. Lab. Med. 49 (2011) 541–546.
[137] M. Sokolowska, A. Krezel, M. Dyba, Z. Szewczuk, W. Bal, Short peptides are not reliable models of thermodynamic and kinetic properties of the N-terminal metal binding site in serum albumin, Eur. J. Biochem. 269 (2002) 1323–1331.
[138] M. Sokolowska, K. Pawlas, W. Bal, Effect of common buffers and heterocyclic ligands on the binding of Cu(II) at the multimetal binding site in human serum albumin, Bioinorg. Chem. Appl. (2010) 725153.
[139] N. Jafari, R. Ahmed, M. Gloyd, J. Bloomfield, P. Britz-McKibbin, G. Melacini, Allosteric sensing of fatty acid binding by NMR: application to human serum albumin, J. Med. Chem. 59 (2016) 7457–7465.
[140] I. Oran, B. Oran, Ischemia-modified albumin as a marker of acute coronary syndrome: The case for revising the concept of "N-terminal modification" to "fatty acid occupation" of albumin, Disease markers 2017 (2017) 5692583.
[141] A. Bhardwaj, Q. Truong, W. Peacock, K.-T. Yeo, A. Storrow, S. Thomas, K.M Curtis, R.S Foote, H.K Lee, K. Miller, J. Januzzi, A multicenter comparison of established and emerging cardiac biomarkers for the diagnostic evaluation of chest pain in the emergency department, A. Heart J. 162 (2011) 276–282.
[142] A.J. Stewart, C.A. Blindauer, The reduced Co^{2+}-binding ability of ischaemia-modified albumin is unlikely to be because of oxidative modification of the N-terminus, Liver Int. 35 (2015) 2622–2623.
[143] J.H. Tonsgard, S.C. Meredith, Characterization of the binding sites for dicarboxylic acids on bovine serum albumin, Biochem. J. 276 (1991) 569–575.
[144] B. Ertekin, S. Kocak, Z. Defne Dundar, S. Girisgin, B. Cander, M. Gul, S. Doseyici, I. Mehmetoglu, T. Kemal Sahin, Diagnostic value of ischemia-modified albumin in acute coronary syndrome and acute ischemic stroke, Pak. J. Med. Sci. 29 (2013) 1003–1007.
[145] X.X. Duan, G.P. Zhang, X.B. Wang, H. Yu, J.L. Wu, K.Z. Liu, L. Wang, X. Long, Elevated serum and cerebrospinal fluid free fatty acid levels are associated with unfavorable functional outcome in subjects with acute ischemic stroke, Mol. Neurobiol. 54 (2017) 1677–1683.
[146] E.S. Arnardottir, M. Mackiewicz, T. Gislason, K.L. Teff, A.I. Pack, Molecular signatures of obstructive sleep apnea in adults: a review and perspective, Sleep 32 (2009) 447–470.
[147] J.C. Jun, L.F. Drager, S.S. Najjar, S.S. Gottlieb, C.D. Brown, P.L. Smith, A.R. Schwartz, V.Y. Polotsky, Effects of sleep apnea on nocturnal free fatty acids in subjects with heart failure, Sleep 34 (2011) 1207–1213.
[148] G.M. Reaven, C. Hollenbeck, C.Y. Jeng, M.S. Wu, Y.D. Chen, Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24h in patients with NIDDM, Diabetes 37 (1988) 1020–1024.
[149] M.W. Tang, F.A. Koopman, J.P. Visscher, M.J. de Hair, D.M. Gerlag, P.P. Tak, Hormone, metabolic peptide, and nutrient levels in the earliest phases of rheumatoid arthritis-contribution of free fatty acids to an increased cardiovascular risk during very early disease, Clin. Rheumatol. 36 (2017) 269–278.
[150] R. Chen, S. Han, D. Dong, Y. Wang, Q. Liu, W. Xie, M. Li, M. Yao, Serum fatty acid profiles and potential biomarkers of ankylosing spondylitis determined by gas chromatography-mass spectrometry and multivariate statistical analysis, Biomed. Chromatogr. 29 (2015) 604–611.
[151] H. Mysliwiec, A. Baran, E. Harasim-Symbor, P. Mysliwiec, A.J. Milewska, A. Chabowski, I. Flisiak, Serum fatty acid profile in psoriasis and its comorbidity, Arch. Dermatol. Res. 309 (2017) 371–380.
[152] P.A. Kumar, K. Subramanian, The role of ischemia modified albumin as a biomarker in patients with chronic liver disease, J. Clin. Diagn. Res. 10 (2016) BC09–BC12.
[153] N. Caraccio, A. Natali, A. Sironi, S. Baldi, S. Frascerra, A. Dardano, F. Monzani, E. Ferrannini, Muscle metabolism and exercise tolerance in subclinical hypothyroidism: a controlled trial of levothyroxine, J. Clin. Endocrinol. Metab. 90 (2005) 4057–4062.

[154] A.C. Nogueira, V. Kawabata, P. Biselli, M.H. Lins, C. Valeri, M. Seckler, W. Hoshino, L.G. Junior, M.M. Bernik, J.B. de Andrade Machado, M.B. Martinez, P.A. Lotufo, E.G. Caldini, E. Martins, R. Curi, F.G. Soriano, Changes in plasma free fatty acid levels in septic patients are associated with cardiac damage and reduction in heart rate variability, Shock 29 (2008) 342–348.

[155] Z. Niu, N. Lin, R. Gu, Y. Sun, Y. Feng, Associations between insulin resistance, free fatty acids, and oocyte quality in polycystic ovary syndrome during *in vitro* fertilization, J. Clin. Endocrinol. Metab. 99 (2014) E2269–E2276.

[156] O. Karadeniz, I. Mendilcioglu, S. Ozdem, M. Ozekinci, C.Y. Sanhal, G. Uzun, M. Sakinci, M. Simsek, The association between ischaemia-modified albumin levels in umbilical vein and intrauterine growth restriction, J. Obstet. Gynaecol. 35 (2015) 9–12.

[157] J. Urban, A. Iwaszkiewicz-Pawlowska, Concentration of free fatty acids (FFA) in amniotic fluid and maternal and cord serum in cases of intrauterine growth retardation, J. Perinat. Med. 14 (1986) 259–262.

[158] R.R. Wolfe, S. Klein, F. Carraro, J.M. Weber, Role of triglyceride-fatty acid cycle in controlling fat metabolism in humans during and after exercise, Am. J. Physiol. 258 (1990) E382–E389.

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University Teaching and Research Ethics Committee

15 November 2016

Dr Alan Stewart
School of Medicine

Dear Alan

Thank you for submitting your amendment application which comprised the following documents:

1. Ethical Amendment Application Form

The School of Medicine Ethics Committee is delegated to act on behalf of the University Teaching and Research Ethics Committee (UTREC) and has approved this ethical amendment application. The particulars of this approval are as follows –

Original Approval Code:	MD11160	**Approved on:**	25th September 2014
Amendment 1 Approval Date:	13th October 2016	**Approval Expiry Date:**	25th September 2017
Term of Approval	3 YEARS		
Project Title:	Role of zinc in controlling histidine-rich glycoprotein-heparin interactions and its implications for platelet aggregation.		
Researcher(s):	Dr Alan Stewart, Dr Samantha Pitt, Dr Siavash Khazaipoul, Kondwani Katundu, Amelie Sobczak	**Supervisor(s):**	Dr Alan Stewart & Dr Samantha Pitt

Ethical amendment approval does not extend the originally granted approval period, rather it validates the changes you have made to the originally approved ethical application. If you are unable to complete your research within the original validation 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.

Any serious adverse events or significant change which occurs in connection with this study and/or which may alter its ethical consideration, must be reported immediately to the School Ethics Committee, and an Ethical Amendment Form submitted where appropriate.

Approval is given on the understanding that you adhere to the 'Guidelines for Ethical Research Practice' (http://www.st-andrews.ac.uk/media/UTRECguidelines%20Feb%2008.pdf).

Yours sincerely

Dr Morven Shearer
Convenor School of Medicine Ethics Committee

University of St Andrews | FOUNDED 1413

University Teaching and Research Ethics Committee

20 November 2017

Dr Alan Stewart
School of Medicine

Dear Alan

Thank you for submitting your ethics extension request which comprised the following documents:

1. Extension Request

The School of Medicine Ethics Committee is delegated to act on behalf of the University Teaching and Research Ethics Committee (UTREC) and has approved this ethical amendment application. The particulars of this approval are as follows –

Original Approval Code:	MD11160	**Approved on:**	25th September 2014
Amendment 2 Approval Date:	13th November 2017	**Approval Expiry Date:**	25th September 2019
Term of Approval	5 YEARS		
Project Title:	Role of zinc in controlling histidine-rich glycoprotein-heparin interactions and its implications for platelet aggregation.		
Researcher(s):	Dr Alan Stewart, Dr Samantha Pitt, Dr Siavash Khazaipoul, Kondwani Katundu, Amelie Sobczak	**Supervisor(s):**	Dr Alan Stewart & Dr Samantha Pitt

Ethical amendment approval does not extend the originally granted approval period, rather it validates the changes you have made to the originally approved ethical application. If you are unable to complete your research within the original validation 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.

Any serious adverse events or significant change which occurs in connection with this study and/or which may alter its ethical consideration, must be reported immediately to the School Ethics Committee, and an Ethical Amendment Form submitted where appropriate.

Approval is given on the understanding that you adhere to the 'Guidelines for Ethical Research Practice' (http://www.st-andrews.ac.uk/media/UTRECguidelines%20Feb%2008.pdf).

Yours sincerely

Dr Morven Shearer
Convenor School of Medicine Ethics Committee

CERTIFICATE OF ETHICS APPROVAL

This is to certify that the College of Medicine Research and Ethics Committee (COMREC) has reviewed and approved a study entitled:

P.09/17/2289 - Influence of plasma fatty acids on zinc dynamics and blood clotting: investigating the blood coagulability and plasma free fatty acid concentration of HIV-infected individuals in Neno district, Malawi by Dr. K. Katundu

On 17-Dec-17

As you proceed with the implementation of your study, we would like you to adhere to international ethical guidelines, national guidelines and all requirements by COMREC as indicated on the next page

17-Dec-17

Dr. YB. Mlombe - Chairperson (COMREC)

Date

REQUIREMENTS FOR ALL COMREC APPROVED RESEARCH PROTOCOLS

1. Pay the research overhead fees as required by the College of Medicine for all approved studies.

2. You should note that the COMREC Sub-Committee on Research Participants' Safety will monitor the conduct of the approved protocol and any deviation from the approved protocol may result in your study being stopped.

3. You will provide an interim report in the course of the study and an end of study report.

4. All COMREC approvals of new applications and progress reports are valid for one year only. Therefore all approved studies running for more than one year are subject to continuing review annually. You are required to submit a progress report to COMREC within 90-30 days before the expiration date. Your current expiration date is 17-Dec-18. Studies shall be considered lapsed and inactive if continuing review application is not received one month after the expiry of the previous approval. In that case, all study related operations should cease immediately except those that are necessary for the welfare of subjects.

5. All investigators who are Medical Practitioners must be fully registered with the Medical Council of Malawi.