Send Orders for Reprints to reprints@benthamscience.ae

Current Topics in Medicinal Chemistry, **2015,** *Volume* **1**

Fatty acid-mediated inhibition of metal binding to the multi-metal site on serum albumin: Implications for cardiovascular disease

Claudia A. Blindauer[*a], Siavash Khazaipoul[b], Ruitao Yu[b,c], and Alan J. Stewart[*b]

[a]*Department of Chemistry, University of Warwick, Coventry, CV4 7AL, United Kingdom;* [b]*School of Medicine, University of St Andrews, St Andrews, KY16 9TF, United Kingdom;* [c]*Northwest Institute of Plateau Biology, Chinese Academy of Sciences, 810008 Xining, China.*

Abstract: Human serum albumin (HSA) is the major protein in blood plasma and is responsible for circulatory transport of a range of small molecules including fatty acids, metal ions and drugs. We previously identified the major plasma Zn^{2+} transport site on HSA and revealed that fatty-acid binding (at a distinct site called the FA2 site) and Zn^{2+} binding are interdependent via an allosteric mechanism. Since binding affinities of long-chain fatty acids exceed those of plasma Zn^{2+}, this means that under certain circumstances the binding of fatty acid molecules to HSA is likely to diminish HSA Zn^{2+}-binding, and hence affects the control of circulatory and cellular Zn^{2+} dynamics. This relationship between circulatory fatty acid and Zn^{2+} dynamics is likely to have important physiological and pathological implications, especially since it has been recognised that Zn^{2+} acts as a signalling agent in many cell types. Fatty acid levels in the blood are dynamic, but most importantly, chronic elevation of plasma fatty acid levels is associated with some metabolic disorders and disease states – including myocardial infarction and other cardiovascular diseases. In this article, we briefly review the metal-binding properties of albumin and highlight the importance of their interplay with fatty acid binding. We also consider the impact of this dynamic link upon levels and speciation of plasma Zn^{2+}, its effect upon cellular Zn^{2+} homeostasis and its relevance to cardiovascular and circulatory processes in health and disease.

Keywords: Allostery, Circulation, Fatty acid, Protein-lipid interactions, Protein-metal interactions, Serum albumin

1. INTRODUCTION

Zinc is recognised as an important micronutrient [1,2]. At least 10% of all human proteins require a structural or catalytic zinc cofactor [3]. In addition, zinc acts as an intracellular and extracellular signalling agent [4]. In consequence, zinc is involved in virtually every conceivable biological process, and thus the organismal zinc status affects for example, growth and development, the immune system, mental health, as well as cardiac health. It must also be noted that zinc, as the free Zn^{2+} aquo-ion, is toxic to cells at very low concentrations [5]. Given its ubiquity, essentiality and potential cell toxicity, cellular zinc homeostasis [6,7] is usually well-controlled, involving no less than 24 membrane-bound transporters of the ZIP and ZnT families [8], and at least 13 expressed metallothioneins [9]. Alterations in zinc homeostasis are known to occur with increasing age [10], and in some of the most prevalent diseases [11], including neurodegenerative diseases, cancer, diabetes and cardiovascular disease (CVD) [12]. In many cases, it has remained obscure whether these alterations are cause or consequence, or both, of the respective disease state, and occasionally, it is even unclear whether the observed alterations are harmful or beneficial.

Mammalian zinc homeostasis on the organismal level also involves the circulatory system [13]. Although zinc plasma levels (reference values are around 16.6±6.2 µM [14]) are affected by a range of conditions ([15], also see section 4), they are not decreased by mild zinc deficiency [16]. This suggests that plasma zinc levels are also subject to tight control – and it is important to understand how this control works. Apart from the crucial importance of ZIP and ZnT transporters, it is conceivable that this is also influenced by Zn^{2+} binding to components of plasma – and chief amongst these is serum albumin [17]. Our studies have revealed that albumin directly links plasma zinc and free fatty acid (FFA) dynamics through allosteric binding. In the present review, we will explore how this allosteric link may impact on cardiovascular health and disease. Our findings also have direct implications for the diagnostics of myocardial ischemia through the biomarker "ischemia-modified albumin" (IMA), and since there is much confusion in the current literature regarding this, we will begin with a summary of the current state of the art on metal ion-albumin interactions.

2. The multi-metal binding site on serum albumin and its allosteric regulation by fatty acids

Albumin is not only the most abundant plasma protein (ca. 640 µM; 40 g/L), but under normal conditions also binds the vast majority of exchangeable plasma Zn^{2+} [18]. Metal binding to mammalian serum albumins has been studied for several decades [19], and it is now clear that most albumins harbour at least four distinct metal-binding sites [20,21]: best known is perhaps the N-terminal "ATCUN" (Amino-Terminal Copper and Nickel) binding motif [22], followed by two sites detectable by ^{113}Cd NMR spectroscopy, called site A and B [23-25], and lastly the free thiol of Cys34 [26].

XXX-XXX/14 $58.00+.00

The latter is only relevant for very thiophilic xenobiotic metal ions such as Au^{+} [27], Pt^{2+} [28] and Hg^{2+} [29], whereas the former three sites are capable of binding a range of 3d metal ions including Cu^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+} and Zn^{2+}.

These divalent metal ions and Cd^{2+} display different preferences for the ATCUN site, site A and site B. Taking advantage of the Jahn-Teller effect, the d^8 and d^9 ions Ni^{2+} and Cu^{2+} show a distinct preference for the square-planar geometry provided by the ATCUN motif [22,30,31]. This binding mode involves the unprotonated terminal amino group and the imidazole side-chain of His3, and requires the deprotonation of two backbone amides. Both Cu^{2+} and Ni^{2+} can elicit this deprotonation at physiological pH, but Zn^{2+} and Co^{2+} require a much higher pH to effect significant binding to model peptides [32]. Instead, the closed-shell Zn^{2+} ion prefers the distorted site A [23,24,33] (dissociation constant K_D = 0.3-40 μM [34-37]), and the highest affinity site for Co^{2+} (high-spin d^7) has been determined as site B (K_D = 11 μM [38]), followed by site A (90 μM) and, lastly, ATCUN (110 μM) [38]. Cu^{2+} binds with picomolar affinity to the ATCUN site [31], and with a K_D = 10 nM to site A [39]. The respective values for Ni^{2+} are 150 nM [40] and 12.5 μM [41].

Site A has also been termed the multi-metal binding site (MBS) [39,41]: whilst it is a primary site for Zn^{2+} and Cd^{2+}, it is a secondary site for Cu^{2+}, Ni^{2+} and also Co^{2+}. This means that whenever more than one equivalent of the latter ions are added to pure, ligand- and metal-free albumin, this site will become significantly occupied by the respective metal ion. It is also important to note that in human and bovine serum albumin (HSA and BSA), Cu^{2+} still forms stronger interactions with this site than either Cd^{2+}, Ni^{2+} Co^{2+}, or Zn^{2+} [40]. This is in keeping with expectations based on the Irving-Williams series, which describes the order of affinities of divalent high-spin complexes [42].

The location [33] and structure [43] of the major zinc site (and hence the secondary site for Co^{2+}, Ni^{2+}, and Cu^{2+}) have been determined using a range of spectroscopic techniques, although no X-ray crystallographic studies on any Zn-albumin complexes are as yet available. The most striking peculiarity of this site is its location at the domain interface between domain I and domain II of HSA (Figures 1a and b). It is formed by two residues from each domain: His67 and Asn99 from domain I and His247 and Asp249 from domain II. These four residues are conserved in albumins from mammals. A copious amount of X-ray crystallographic studies have been carried out on HSA [44-53], with structural information on other serum albumins having become available recently [54,55]. Inspection of these structures reveals that the MBS is consistently only present in fatty acid-free preparations [33] (Figure 1c). Fatty acids are the primary ligands for albumin, and albumin is the primary plasma transporter for long-chain unesterified (or "free") fatty acids (FFA), which are the primary source of energy for the myocardium. The origin of disruption of the MBS is traceable to the FFA binding site FA2 [46], which significantly also requires interacting residues from both domains I and II (Figure 1d). Although there is no overlap between the zinc- and fatty-acid binding residues, the domain-domain movement that is required to form the FFA binding pocket essentially leads to the destruction of the preformed zinc-binding site [33]. This observation raised the question whether zinc binding inhibited fatty acid binding to this site, or *vice versa*. Isothermal titration calorimetry (ITC) studies involving the ternary system BSA, Zn^{2+} and myristate clearly demonstrated that the C14 fatty acid inhibited zinc binding to albumin (Figure 2d) [37]. Recent studies confirmed that fatty acids inhibit zinc binding to the MBS also in HSA (Figure 2a) [56].

This allosteric switch is expected to have multiple physiological consequences, further described in sections 3 and 4. Under normal conditions, between 0.1 and 2 FFA molecules are carried per albumin molecule [19,57], but there a number of conditions, including fasting, extreme exercise [58], obesity, metabolic syndrome, diabetes mellitus, and cardiovascular disease [59] including atherosclerosis and myocardial infarction [60], under which up to four [19], or even more [61], FFA molecules can become bound. Importantly, many of these conditions are also associated with considerable shifts in plasma zinc levels, and these observations, together with our structural and thermodynamic studies, have led us to hypothesise that these correlations are mediated by HSA [15] - the main transporter for both zinc and FFAs in plasma. However, it should be stressed that FFA effects on zinc speciation and distribution are not expected to be restricted to such drastic conditions - we observed marked differences in zinc affinity and binding capacity even at just 1 molar equivalent of myristate for both BSA and HSA (Figures 2a and b) [37,56]. This is consistent with observations by the group of Hamilton that indicated that FA2 is one of the highest-affinity FFA binding sites on HSA [62,63].

One further momentous implication of this allosteric switch is based on the fact that MBS is, as mentioned earlier, also a secondary binding site for other M^{2+} metal ions. This simply means that the Co^{2+} (and Cu^{2+} and Ni^{2+}) binding capacity and affinity of albumin will also be affected by fatty acid levels, and for Co^{2+}, this has indeed been demonstrated (Figure 2e). Myocardial ischemia is one of the many conditions in which FFA levels are significantly elevated. Hence, the FFA-mediated allosteric switch also effortlessly explains the reduction in metal binding in diagnostic tests related to the Albumin-Cobalt-Binding (ACB) test [64,65], as well as the identity of the elusive biomarker "Ischemia-Modified Albumin" (IMA): IMA is simply albumin with a higher ratio of FFA bound. The case for this assertion, as well as ensuing implications, will be laid out in section 5.

3. FFA-mediated alterations in plasma zinc speciation: Implications for neutralisation of heparins

Zinc is an important regulator of coagulation following its release from activated platelets [66]. The resultant local increases in Zn^{2+} concentration are sensed by a range of haemostatic proteins to stimulate a pro-coagulatory response [67]; this represents an essential transition phase that promotes the propagation of coagulation. Plasma proteins that bind Zn^{2+} under these conditions include histidine-rich glycoprotein (HRG), a key effector molecule upon which Zn^{2+} acts to regulate coagulation [68,69]. HRG is a 75 kDa glycoprotein present at a concentration of 1.3-2.0 μM in adult blood [69,70].

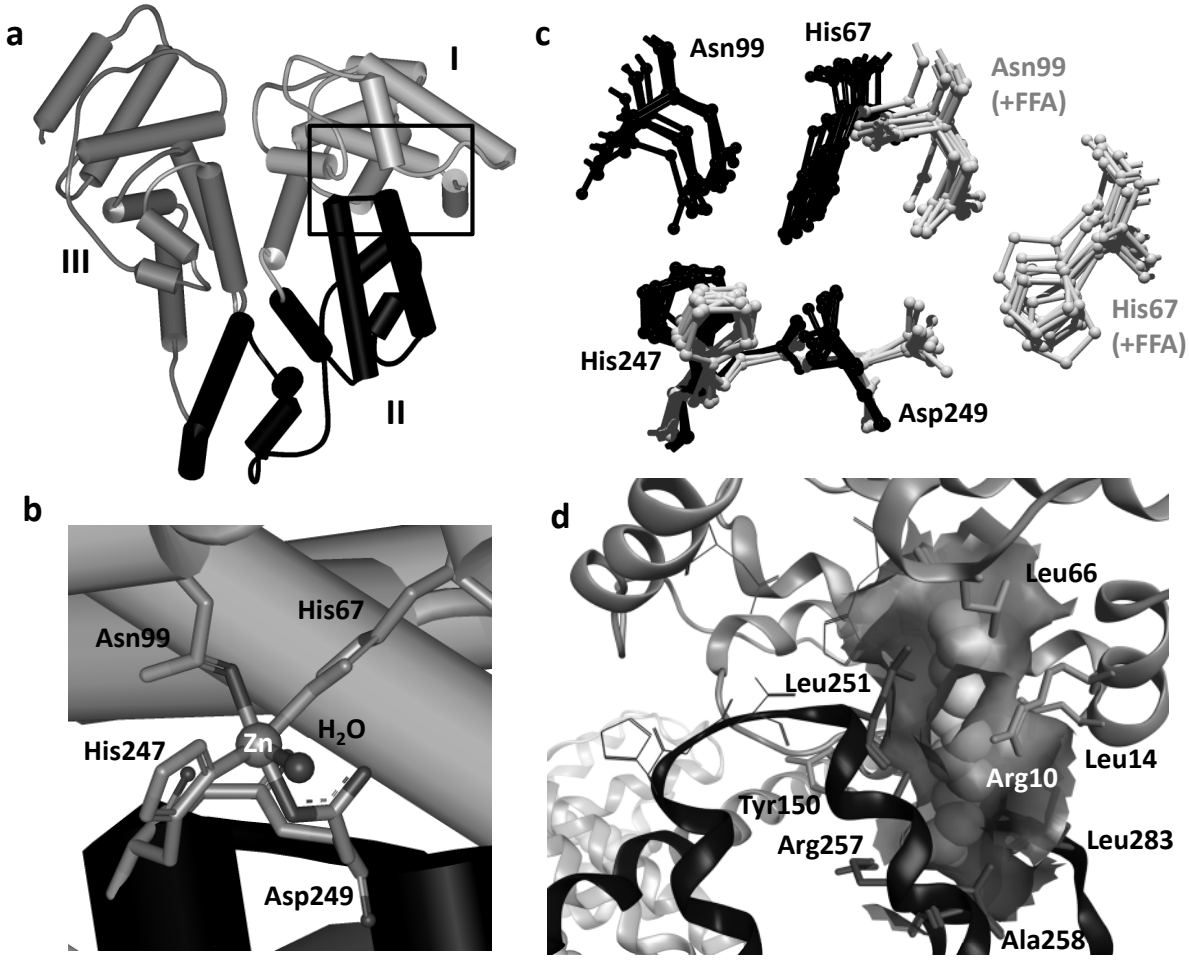

Figure 1. Structural basis of allosteric binding of zinc and fatty acids to serum albumin. (a) Location of inter-domain zinc site A (MBS) (b) [43] and fatty-acid binding site FA2 (d) [46]. The three domains of albumin are shown in grey (domains I and III) and black (domain II). (b) shows a close-up of the distorted 5+1 metal binding site; the backbone carbonyl oxygen of His247 also forms a long bond to the Zn^{2+} ion. In (d), the four zinc-binding residues are shown with thin lines, and some residues forming the two halves of the FA2 binding pocket are shown as sticks, with the resolved part of a myristate molecule shown in space-filling mode. (c) MBS residues in a selection of X-ray structures with and without (pdb 4G03, 2BXH, 4F5V (rabbit SA), 3V09 (rabbit SA), 3JRY, 1E78, 1AO6, 4K71 (HSA in complex with the Fc receptor) [45,50,51,53,54,55]) bound fatty acids (decanoate (1E7E), dodecanoate (1E7F), myristate (3SQJ, 1E7G), palmitate (1E7H), stearate (1E7I), oleate (1GNI), arachidonate (1GNJ) [47,48]). The overlay was generated by aligning the backbone atoms of residues His247, Gly248, and Asp249 in Swiss pdb viewer v. 3.7.

We recently demonstrated that HRG possesses ten Zn^{2+} sites (with an average apparent K_d of ~6 μM) and that Zn^{2+}-binding enhances the ability of HRG to bind and neutralize long-chain heparins [56]. Under normal conditions heparin binds to and stabilizes the anticoagulatory antithrombin III/thrombin complex. During clotting, thrombin cleaves fibrinogen into insoluble strands (fibrin) that are cross-linked by factor XIII to form a blood clot [71]. Zn^{2+}-dependent neutralization of heparin by HRG provides increased competition for heparin binding to this complex, which may result in release of thrombin and an increase in its activity, which will ultimately promote clotting.

Speciation modelling experiments based upon affinity and stoichiometry data for Zn^{2+}-binding to HRG and to albumin (in the absence and presence of various concentrations of myristate) suggested that the FFA-mediated reduction in availability of the MBS increases the proportion of plasma Zn^{2+} bound to HRG (Figure 2c), such that its ability to neutralize heparins will be increased [56]. However, it is important to point out that this simple model

only considered serum albumin and HRG and did not take into account other circulatory proteins that could bind displaced Zn^{2+}. These include other components of the blood-clotting cascade [67], as well as numerous components of the immune system [72]. It is also important to point out that HRG can also associate with molecules other than heparin (such as tropomyosin, factor XIIa and soluble IgG) in a Zn^{2+}-dependent manner [69,73], thus the actions of increased Zn^{2+}-binding to HRG are likely to be complex.

Interestingly, a recent study by Fredenburgh *et al*. has revealed that fibrinogen, like HRG, also binds heparin with high affinity in a Zn^{2+}-dependent manner [74]. Fibrinogen is a glycoprotein present in plasma at a concentration of around 6-12 μM [71,75]. The study by Fredenburgh *et al.* revealed that Zn^{2+} binds fibrinogen with a K_d in the low micromolar range; values of 9.4 μM and 4.5 μM were measured using surface plasmon resonance and a fluorescence-based assay, respectively. Furthermore, they found that Zn^{2+} promotes a 4-fold increase in heparin binding to fibrinogen. This study highlights the fact that Zn^{2+} can potentially influence heparin neutralization by fibrinogen as well as by HRG. Also a further reduction of heparin activity will occur when fibrinogen is clotted because heparin promotes thrombin binding to fibrin and induces the formation of a complex that limits inhibition by antithrombin [76,77]. Zinc also stimulates platelet aggregation by promoting fibrinogen binding to $\alpha_{IIb}\beta_3$, its cognate receptor on the platelet surface [66,78].

The observations above are particularly interesting as disorders characterized by chronically elevated levels of plasma fatty acids are associated with an increased risk of thrombosis [79,80]. These include obesity [81,82], diabetes [83], fatty liver disease [84], and cancer [85]. For example, thromboembolism (caused by obstructive blood clots) is the second leading cause of death associated with malignancy [79]. Collectively, these observations suggest that under conditions where FFA levels are elevated, Zn^{2+} displaced from serum albumin could bind HRG or fibrinogen to enhance their respective interactions with heparins and induce a pro-coagulatory effect [86], as summarized in Figure 3. In older adults, this may be further compounded as plasma albumin levels are known to decrease with age [87]. An age-related decrease in plasma albumin also correlates with an observable increase in the ratio of FFAs per albumin molecule [88]. It is important to point out that the degree to which each of the respective proteins, HRG and fibrinogen, contribute to Zn^{2+}-dependent neutralization of heparins remains to be determined. However, evidence for a direct link between defective plasma Zn^{2+}-handling, mediated by FFA-displacement of Zn^{2+} from albumin, and thrombotic risk is compelling.

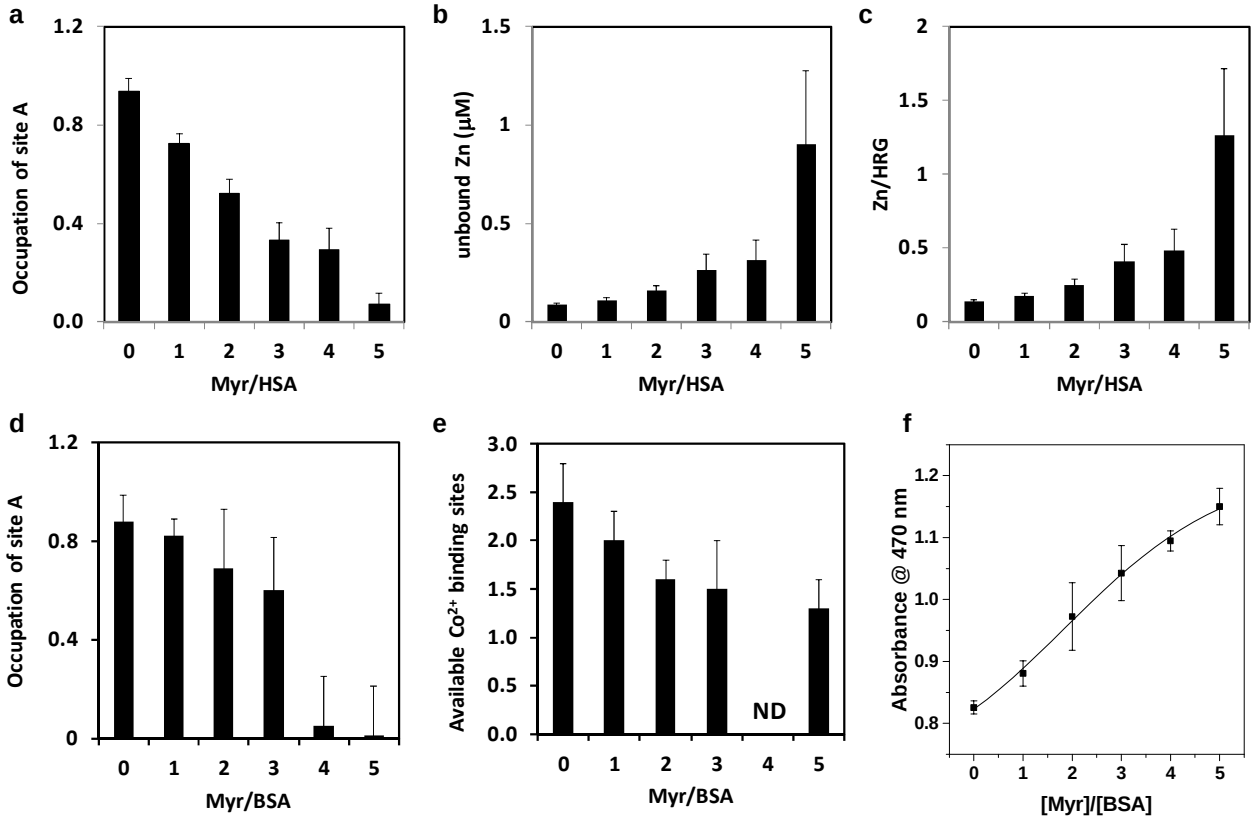

Figure 2. Impact of fatty acids on binding site availability on albumin for Zn^{2+} and Co^{2+} and consequences thereof. (a) and (d) Myristate-induced reduction in availability of site A (MBS) for HSA and BSA, as determined by isothermal titration calorimetry [37,56]. (b) and (c) Plasma speciation modelling suggested that the reduction in binding site availability may result in an increase of free Zn^{2+} and zinc bound to HRG [56]. (e) ITC experiments revealed a similar reduction of overall binding site availability for Co^{2+} [149]. One out of three possible binding sites is lost in the presence of 5 mol. equiv. of myristate. ND indicates that no data is available. (f) Myristate binding to BSA leads to increased Co-DTT complex formation in the ACB assay [149]. The line is drawn to guide the eye and does not represent a fit.

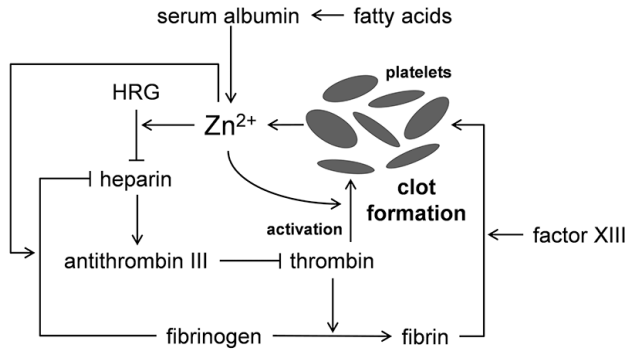

Figure 3. Diagram illustrating the mechanisms by which Zn^{2+} may influence coagulation through stimulation of HRG- and fibrinogen-dependent pathways.

4. Cellular effects of altered plasma zinc speciation and cardiovascular disease

A number of studies have examined the association of zinc with coronary diseases, cardiomyopathies and arrhythmias, and links between etiopathogenesis of CVD have been suggested [89-91]. Aberrant Zn^{2+}-homeostasis is associated with cardiomyopathy including chronic heart failure and myocardial damage [11,89], as well as primary arterial hypertension [92,93]. Many intracellular enzymes and other proteins involved in regulating cardiac function are Zn^{2+}-dependent [89]; furthermore, zinc homeostasis and intracellular redox balance are intimately linked [6,94]. Therefore, the impact of zinc on cardiovascular health is most probably multifactorial; some potential mechanisms and pathways are discussed in this section.

4.1. Potential impact of albumin on cellular zinc homeostasis

Intracellular zinc concentrations are known to be regulated largely by zinc transporters, zinc binding proteins including metallothioneins, and zinc sensors [6,7], but the extracellular space also participates in zinc dynamics. Indeed, various studies have suggested that serum albumin helps to regulate the uptake of Zn^{2+} by a range of cell types. For example, serum albumin facilitates the uptake of Zn^{2+} into endothelial cells [95] and erythrocytes [96]. Also receptor-mediated vesicular co-transport of Zn^{2+} with albumin across the endothelium has been demonstrated *in vitro* [97]. Despite this, any direct role for albumin in mediating delivery of Zn^{2+} to cells via endocytosis is likely to be relatively minor as only ~2% of circulating albumin molecules carry Zn^{2+} at any one time. The primary function of albumin in mediating Zn^{2+} uptake is through maintenance of the plasma exchangeable zinc pool. Ultimately, Zn^{2+} will cross cell membranes predominantly through ZIP (transport from plasma into cells) and ZnT (transport into plasma) transporters. Crucially, the activity of these transporters depends on availability of free Zn^{2+} – and this in turn is regulated by the assortment of molecules that can reversibly bind Zn^{2+} in plasma. If the zinc-binding capacity of plasma drops, this may lead to an increase in free Zn^{2+} (Figure 2b), which should, in the first instance, result in increased cellular uptake. This may manifest in decreased levels of plasma zinc. Intriguingly, the same conditions that are associated with high FFA levels, including congestive heart failure, myocardial ischemia and infarction, are also known to result in decreased plasma zinc [98,99] – more precisely, exchangeable, albumin-bound zinc [100,101]. This is consistent with the idea that in these conditions, the increased FFA levels lead to lower zinc-binding capacity by albumin, and an increase in free Zn^{2+} available for cellular uptake. We hypothesise that conditions that lead to elevated plasma FFA might cause significant changes to cellular zinc homeostasis, at the very least in cells in direct contact with plasma, including erythrocytes, platelets, macrophages, B- and T-cells, endothelial cells, but potentially also vascular smooth muscle cells (VSMC) and cardiomyocytes. Based on the facts that both a lack of zinc [102] as well as excessive extracellular free Zn^{2+} [5] has deleterious effects on cells in general, any large perturbation of plasma zinc homeostasis may be expected to have direct or indirect detrimental consequences for cells. Some possible effects are described below.

4.2. Effects of zinc on intracellular calcium signalling in cardiomyocytes

Ca^{2+} is crucially involved in the function of cardiomyocytes and VSMCs [103], with intracellular Ca^{2+} release from the sarcoplasmatic reticulum through ryanodine receptors triggering the contraction of these muscle cells [104]. CVD has been linked to dysregulated intracellular Ca^{2+} release, reduced cardiac contractility and prolonged rises of systolic Ca^{2+} [105]. There is cross-talk between the fluxes of Ca^{2+} and Zn^{2+} [106]; hence, it is thought that zinc dyshomeostasis will impact on Ca^{2+} trafficking in cardiomyocytes (as recently reviewed in [107]). Various studies suggest that defective Zn^{2+} handling contributes to cardiomyopathies including altered contractility and heart failure [89,108-110]. Indeed, it has been hypothesised that increased cytosolic Zn^{2+} can influence cardiac excitation-contraction coupling through regulation of the ryanodine receptor in cardiomyocytes [111,112]. The combined effects of Zn^{2+} and Ca^{2+} dyshomeostasis are also thought to generate oxidative stress, lead to cardiac myocyte necrosis and replacement fibrosis [113].

4.3. Blood pressure effects of zinc

Changes of both intracellular and extracellular zinc contents have been documented for primary arterial hypertension [92,93]. Serum zinc levels are decreased in arterial hypertension, as are those in lymphocytes and bone, whereas zinc levels in erythrocytes, heart, liver, kidney and various glands are increased; overall, the organism becomes zinc-deficient. It has remained unclear whether this systemic zinc dyshomeostasis is cause or consequence of arterial hypertension. Lower plasma zinc levels are suggested to impact on Ca^{2+} fluxes, and the activities of angiotensin-converting enzyme (ACE), endothelin-converting enzyme, and neutral endopeptidase. Increased erythrocyte zinc is associated with increased carbonic anhydrase activity, which may result in increased blood pH. Angiotensins are vasoconstrictors; thus a decrease in ACE activity leads to increased levels of angiotensins and hence increased blood pressure. Also endothelial NO-synthase (eNOS) is involved in the generation of the vasodilator NO, hence important for

the regulation of blood pressure. eNOS contains a structural zinc site that is essential for enzyme function; its disruption by reactive oxygen and nitrogen species has been demonstrated [114]. Thus, zinc dyshomeostasis may either directly or indirectly affect its activity, with a lack of zinc expected to lead to decreased NO levels and hence increased blood pressure.

4.4. Zinc dyshomeostasis in atherosclerosis

The major underlying mechanism in the development of CVD is the formation of atherosclerotic plaques in the wall of blood vessel which eventually leads to stroke, heart attack and lower limb disease [115,116]. There is much evidence that zinc deficiency is pro-atherogenic [117]. A number of pathways have been invoked to explain this correlation, as summarized in Figure 4. For example, inflammation is known to play a major role in the development of atherosclerosis [118]. Zinc has been shown to impact on several of the molecules that have been implicated in this etiology. For instance, the zinc-dependent [119] transcription factor NF-κB, a key element in regulation of inflammatory responses [120] may in many cells be regulated by Zn^{2+} status [121]. NF-κB regulates amongst others the expression of vascular cell adhesion molecule (VCAM)-1, ICAM-1, E-selectin, cytokines of the interleukin family, plasminogen activator inhibitor (PAI)-1, and inducible nitric oxide synthase (iNOS). Cytokine transcription is also directly regulated by zinc [122], and several cytokines (IL-β, IL8 and TNF-α) themselves induce expression of E-selectin, ICAM and VCAM. This results in trapping of monocytes, platelets, neutrophils and erythrocytes, which subsequently block blood vessels.

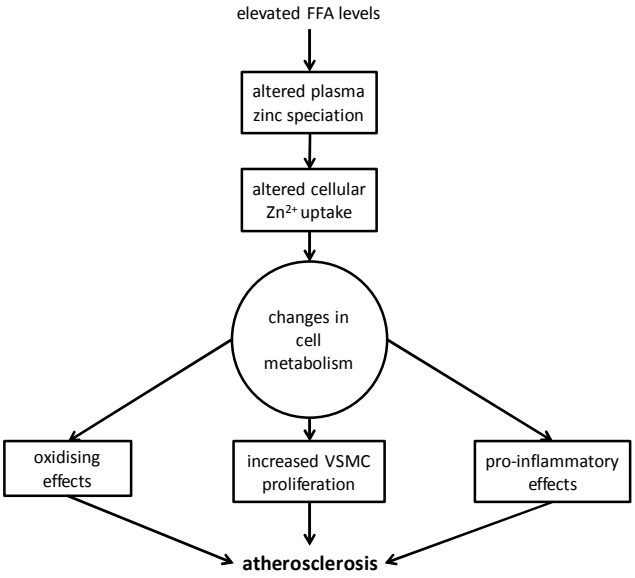

Figure 4. Schematic illustration showing how elevated plasma FFA levels may influence cell metabolism such as to impact upon pro-atherosclerotic pathways.

Zinc also impacts on various other signalling networks, including those involving protein kinase C (PKC), which plays a central role in many signalling pathways in endothelial and vascular smooth muscle cells [123]. Thrombin, angiotensin and endothelin all trigger the activation of PKC, via signalling cascades involving G-protein-coupled receptors and phospholipase C. The latter hydrolyses phospholipids, and the resulting diacylglycerols activate PKC [89,124]. PKC contains a redox-sensitive zinc-binding site, and it is thought that through this site, the cellular redox state regulates the activity of PKC [89]. The link between zinc and cellular redox state is well established [6]. PKC plays an important role in proteoglycan synthesis. Proteoglycans are components of the extracellular matrix. Their composition has been shown to be critical for lipid retention, and hence, atherosclerosis [125]. Thus, modulating PKC activity by Zn^{2+} will in turn regulate various vasoactive factors that ultimately impact on the development of atherosclerosis. Another group of zinc-dependent enzymes critically involved in the extracellular matrix are the matrix metalloproteinases. They are implicated in the response to inflammation [126], and regulate the bioavailability and activity of cytokines, chemokines, and growth factors. They degrade the extracellular matrix, cell surface components as well as intracellular proteins, and are therefore particularly important for vascular and cardiac tissue remodelling, both in normal conditions but also, e.g., during the development of atherosclerosis [127] and the adverse structural remodelling of the myocardium [128]. In atherosclerosis, they may either stabilise or promote rupture of plaques, and hence contribute to acute myocardial infarction [129]. Since they require a zinc cofactor, their activity may be modulated by zinc dyshomeostasis, although no detailed studies on this hypothesis are available.

In addition, a lack of zinc is associated with apoptosis in many cell types [130]. Endothelial cell injury and apoptosis is a defining event in atherosclerosis [131], and it is thought that oxidative stress resulting from lipid peroxidation and the action of inflammatory cytokines is a major contributor to these processes [11]. Zinc has both anti-inflammatory and anti-oxidant effects [132], and it has been suggested that Zn^{2+} performs a crucial role in maintaining the integrity of endothelial cells [121]. Thus it is possible that chronically lower plasma levels contribute to endothelial cell apoptosis. In contrast, long-term zinc deprivation has also been shown to accelerate rat VSMC proliferation via down-regulation of the JNK1/2 pathway [133]. Accelerated VSMC proliferation is a contributing factor in development of atherosclerosis through thickening of the vascular wall [134].

Finally, one may also consider the effects of increased free extracellular Zn^{2+}. Although the anti-inflammatory function of dietary zinc has been mentioned, the direct action of Zn^{2+} on vascular endothelial cells induces inflammation [135]. Induction of senescence by Zn^{2+} of VSMCs has also been reported, and it is thought that this is mediated through angiotensin [136]. More drastically, cell survival of primary cultures of cardiomyocytes was negligible at only 0.6 μM free Zn^{2+} [5].

5. FFA-loading of albumin as the molecular basis of ischemia-modified albumin

Ischemia-modified albumin (IMA) is a biochemical marker of myocardial ischemia (MI) for the detection and the early diagnosis of acute coronary syndromes [137]. IMA can be measured using the albumin-cobalt-binding (ACB) assay, which was first established by Bar-Or *et al.* [138]. The ACB assay has a high negative prognostic value, which is useful in rapidly excluding ischemia as a cause of chest pain

[65,139]. Despite this, the specificity of the test is low, with increased IMA values also characteristic of various other conditions including fatty liver, cancer, infections, renal disease, preeclampsia, diabetes, stroke, and even sustained exercise [140-145].

The basic principle of this test is such that following an ischemic event, serum albumin is somehow chemically modified such that its ability to bind Co^{2+} is compromised. As a quantitative analytical method, the ACB test is performed by the addition of $CoCl_2$ to a patient plasma sample, followed by the addition of dithiothreitol (DTT) to complex the portion of Co^{2+} that has remained unbound to serum albumin, and finally detection of a brown color corresponding to formation of the Co-DTT complex [138].

The molecular basis for IMA and what it exactly is has remained controversial. For some time it was incorrectly assumed that the primary Co^{2+} binding site on albumin is at the N-terminus and that ischemia leads to the cleavage of the first two residues or some other oxidative modification such that this metal binding site is effectively removed [64,146]. However, several efforts to prove a correlation between N-terminal modification and positive ACB assay have failed repeatedly [139,146,147]. Moreover, several *in-vitro* metal binding studies since 2007 concluded that the N-terminal ATCUN site is not the primary Co^{2+}-binding site [38,148,149].

Based on our identification of the location of MBS and its allosteric regulation by fatty acids [33], Mothes and Faller hypothesised in 2007 that the reduced Co^{2+} binding of IMA may be related to fatty-acid inhibition of Co^{2+} binding to the MBS [148]. Soon after, based on the observation that plasma concentrations of FFAs rise during MI [150,151], a relationship between FFAs and the reversible generation of IMA was suggested [144], and Bhagavan *et al*. found a "plausible but not causative relationship" between IMA levels and FFA levels in the sera of patients with acute myocardial infarction relative to controls, whereby an increase in FFA levels resulted in a lower Co^{2+}-binding capacity, and hence an increase in IMA [60]. This explains why positive ACB tests are also found in a plethora disorders unrelated to MI, but which are associated with increased plasma concentrations of FFAs [141,142,144,145].

Crucially, plasma FFAs are predominantly bound to albumin in the circulation [152]. Based on our previous work where we demonstrated interactive binding of FFAs and Zn^{2+} at the MBS [33,37], we examined the effects of FFAs on Co^{2+} binding to albumin *in vitro* [149]. The results of this study revealed that physiologically relevant concentrations of the long chain FFA myristate were sufficient to reduce the Co^{2+}-binding affinity and capacity of bovine serum albumin [149], in a manner broadly similar to that of Zn^{2+} [37]. Myristate binding to BSA was shown to inhibit metal binding to both sites A (MBS) and B [37,149] (with the latter being the primary Co^{2+} binding site [38]), resulting in a decrease in total Co^{2+}-binding site availability and a corresponding increase in Co-DTT complex formation in the ACB assay [149] (Figures 2e and 2f). The results of this work, taken together with the already established link between elevated plasma FFA levels and MI [60,144,150,151], strongly suggest that IMA corresponds to albumin with increased levels of bound FFAs and not N-terminally modified albumin. These observations have important implications for the detection of MI as they suggest that "IMA" is strictly speaking neither a marker for ischemia nor oxidative stress, but a proxy measurement of plasma FFA levels.

Even though hypotheses based on permanent covalent modifications are incompatible with the rapid clearance of IMA within hours of the ischemic event - serum albumin has a circulating half-life of 19 days! - and even though a 2012 study by Oh *et al.* categorically stated an "Insignificant role of the N-terminal cobalt-binding site of albumin in the assessment of acute coronary syndrome" - and even though our own 2012 study had offered experimental evidence supporting a coherent molecular mechanism that is compatible with all clinical observations [149], many researchers working in this area appear ignorant to this collective evidence, with a host of recent publications perpetuating the myth of N-terminal modification of albumin being the basis for the assay [153-155]. Very recently Eom *et al.*, acknowledging that the N-terminus is not the primary Co^{2+} binding site but still convinced that the N-terminus of IMA is modified, even attempted to improve the ACB assay through use of Cu^{2+} (rather than Co^{2+}) [153], which has been known for many years to bind strongly to albumin at the N-terminus [20,22,38,39]. This new test was employed with success, as was a previous attempt by others to use binding of Ni^{2+} (the first equivalent of which also binds at the N-terminus) as the basis of the assay [156]. In each case the metal ion (either Cu^{2+} or Ni^{2+}) bound, like Co^{2+}, less effectively to IMA than control albumin [153,156]. However, the concept of Cu^{2+} and Ni^{2+}-binding to albumin also being influenced by FFA-loading is not surprising as ^{113}Cd-NMR and other spectroscopic studies previously revealed the multi-metal site to be a strong secondary binding site also for these metal ions following saturation of the N-terminal site [20,39,40]. Therefore, as long as the metal ion is added in excess over albumin, MBS will act as a sensor for plasma FFA for all three M^{2+} mentioned. Indeed, in the original ACB assay, 140 μM Co^{2+} and 90-100 μM albumin are present [138]. In the nickel-based test, there was a 3-fold excess of Ni over albumin [156], whereas we were unable to establish the Cu:albumin ratio in the copper-based test. We also note that site B, the primary Co^{2+} binding site, is also FFA-responsive in BSA [37,149]; whether this is also the case for HSA has not yet been established conclusively.

These observations are not meant to bring these important diagnostic tests into disrepute; our main motivation for this discussion is the desire that the molecular basis for the test be well and truly understood. IMA can be detected within minutes of an ischemic event, and remains the only marker for the early detection of MI. We strongly believe that metal-based tests detecting IMA are valuable and should have their place in modern clinical diagnostics - not only in MI, but other diseases related to elevated plasma FFA. Indeed, a recent study has established that IMA is a better prognostic marker than other biomarkers for peripheral arterial occlusive disease [157].

CONCLUSIONS

CVD represents colossal challenges due to its huge health burden on society. The importance of metals as dietary factors and essential inorganic micronutrients capable of modifying CVD has come to prominence only recently; hence, guidelines from the national heart associations at

present are mostly silent on the relation between CVD and metal ions [89]. When the levels of circulatory trace metals in blood become depleted, nutrient metals absorbed from the diet are actively transported into blood and from there to cells as needed. However, abnormalities in the ratios and levels of trace metals will likely develop when this system fails. Although albumin chemistry is of undoubted importance in controlling the circulatory exchange of such metals (and other ligands), many features of its chemistry are yet to be fully understood. Here we have attempted to illustrate this by examining the interplay between the MBS and FFA binding to albumin. It is apparent that the interactive binding of Zn^{2+} (and other metals that bind at this site) and long-chain fatty acids has potential physiological and clinical consequences. This is highlighted in the discovery that IMA represents FFA-loaded albumin and that the ACB assay is a proxy measurement of the FFA content of albumin. However despite this, further work is necessary to fully understand the physiological and pathological importance of elevated FFA levels and their impact on circulatory metal ion speciation. We envision that the work highlighted here will motivate others to examine causes and potential downstream effects of altered plasma metal ion speciation. It is hoped that such studies will enable us to better understand the molecular relationships that exist between the transport of metabolites and metal ions in plasma and their relevance to physiological and pathological processes.

LIST OF ABBREVIATIONS

ACB, albumin-cobalt binding; ATCUN, amino-terminal copper and nickel; BSA, bovine serum albumin; CVD, cardiovascular disease; DTT, dithiothreitol; FFA, free fatty acid; HRG, histidine-rich glycoprotein; HSA, human serum albumin; IMA, ischemia-modified albumin; ITC, isothermal titration calorimetry; LDL, low density lipoprotein; MI, myocardial ischemia; MBS, multi-metal binding site; PKC, protein kinase C; VSMC, vascular smooth muscle cell.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

ACKNOWLEDGEMENTS

We wish to thank the Biotechnology and Biological Sciences Research Council (grant no. BB/J006467/1; to C.A.B and A.J.S.) and the Chinese Academy of Sciences (Travel Fellowship to R.Y.) for funding.

*Address correspondence to Dr Claudia A. Blindauer, Department of Chemistry, University of Warwick, Gibbet Hill Road, Coventry, CV4 7AL, United Kingdom.; Tel: +44-2476-528264; Fax: +44-2476-524112; E-mail: C.Blindauer@warwick.ac.uk or Dr Alan J. Stewart, School of Medicine, University of St Andrews, St Andrews, KY16 9TF, United Kingdom; Tel: +44-1334-463546; Fax: +44-1334-463482; E-mail: ajs21@st-andrews.ac.uk.

REFERENCES

[1] King, J.C. Zinc: an essential but elusive nutrient. *Am. J. Clin. Nutr.*, **2011**, *94*, 679S-684S.

[2] Prasad, A. S. Discovery of human zinc deficiency: its impact on human health and disease. *Adv. Nutr.*, **2013**, *4*, 176-190.

[3] Bertini, I.; Decaria, L.; Rosato, A. The annotation of full zinc proteomes. *J. Biol. Inorg. Chem.*, **2010**, *15*, 1071-1078.

[4] Fukada, T.; Yamasaki, S.; Nishida, K.; Murakami, M.; Hirano T. Zinc homeostasis and signaling in health and diseases: Zinc signaling. *J. Biol. Inorg. Chem.*, **2011**, *16*, 1123-1134.

[5] Bozym, R.A.; Chimienti, F.; Giblin, L.J.; Gross, G.W.; Korichneva, I.; Li, Y.A.; Libert, S.; Maret, W.; Parviz, M.; Frederickson, C.J.; Thompson, R.B. Free zinc ions outside a narrow concentration range are toxic to a variety of cells in vitro. *Exp. Biol. Med.*, **2010**, *235*, 741-750.

[6] Maret, W. Molecular aspects of human cellular zinc homeostasis: redox control of zinc potentials and zinc signals, *Biometals* **2009**; *22*, 149-157.

[7] Blindauer, C.A. Advances in the molecular understanding of biological zinc transport. *Chem. Commun.*, **2015**; 51; 4544-4563.

[8] Kambe, T.; Hashimoto, A.; Fujimoto, S. Current understanding of ZIP and ZnT zinc transporters in human health and diseases. *Cell. Mol. Life Sci.*, **2014**, *71*, 3281-3295.

[9] Li, Y.; Maret, W. Human metallothionein metallomics, *J. Anal. Atom. Spectrom.*, **2008**, *23*, 1055-1062.

[10] Mocchegiani, E.; Costarelli, L.; Giacconi, R.; Cipriano, C.; Muti, E.; Rink, L.; Malavolta, M. Zinc homeostasis in aging: Two elusive faces of the same "metal", *Rejuv. Res.*, **2006**, *9*, 351-354.

[11] Jurowski, K.; Szewczyk, B.; Nowak, G.; Piekoszewski, W. Biological consequences of zinc deficiency in the pathomechanisms of selected diseases. *J. Biol. Inorg. Chem.*, **2014**, *19*, 1069-1079.

[12] Foster, M.; Samman, S. Zinc and redox signaling: Perturbations associated with cardiovascular disease and Diabetes Mellitus. *Antioxid. Redox Signal.*, **2010**, *13*, 1549-1573.

[13] Pullen, R.G.; Franklin, P.A.; Hall, G.H. ^{65}Zinc uptake from blood into brain and other tissues in the rat. *Neurochem. Res.*, **1990**, *15*, 1003-1008.

[14] Rukgauer, M.; Klein, J.; KruseJarres, J. D., Reference values for the trace elements copper, manganese, selenium, and zinc in the serum/plasma of children, adolescents, and adults. *J. Trace Elem. Med. Biol.*, **1997**, *11*, 92-98.

[15] Barnett, J.P.; Blindauer, C.A.; Kassaar, O.; Khazaipoul, S.; Martin, E.M.; Sadler, P.J.; Stewart, A.J. Allosteric modulation of zinc speciation by fatty acids. *Biochim. Biophys. Acta*, **2013**, *1830*, 5456-5464.

[16] Ou, O.; Allen-Redpath, K.; Urgast, D.; Gordon, M.J.; Campbell, G.; Feldmann, J.; Nixon, G. F.; Mayer, C.D.; Kwun, I.S.; Beattie, J.H. Plasma zinc's alter ego is a low-molecular-weight humoral factor. *FASEB J.*, **2013**, *27*, 3672-3682.

[17] Smith, K. T.; Failla, M. L.; Cousins, R. J., Identification of albumin as the plasma carrier for zinc absorption by perfused rat intestine. *Biochem. J.*, **1979**, *184*, 627-633.

[18] Chilvers, D. C.; Dawson, J. B.; Bahreynitoosi, M. H.; Hodgkinson, A., Identification and determination of copper-protein and zinc-protein complexes in blood-plasma after chromatographic separation on DEAE-Sepharose CL-6B. *Analyst*, **1984**, *109*, 871-876.

[19] Peters, T., Jr. All about Albumin: Biochemistry, Genetics, and Medical Applications Academic Press: San Diego, London, 1996.

[20] Bal, W.; Sokolowska, M.; Kurowska, E.; Faller, P. Binding of transition metal ions to albumin: Sites, affinities and rates. *Biochim. Biophys. Acta*, **2013**, *1830*, 5444-5455.

[21] Lu, J.; Stewart, A.J.; Sadler, P.J.; Pinheiro, T.J.T.; Blindauer, C.A. Albumin as a zinc carrier: properties of its high-affinity zinc-binding site. *Biochem. Soc. Trans.*, **2008**, *36*, 1317-1321.

[22] Harford, C.; Sarkar, B. Amino terminal Cu(II)- and Ni(II)-binding (ATCUN) motif of proteins and peptides: Metal binding, DNA cleavage, and other properties. *Acc. Chem. Res.*, **1997**, *30*, 123-130.

[23] Martin, E.O.; Drakenberg, T. Cadmium(II), zinc(II), and copper(II) ions binding to bovine serum albumin. A ^{113}Cd NMR study. *Inorg. Chim. Acta*, **1982**, *67*, 71-74.

[24] Goumakos, W.; Laussac, J.P.; Sarkar, B. Binding of cadmium(II) and zinc(II) to human and dog serum albumins - an equilibrium dialysis and Cd-113-NMR study. *Biochem. Cell Biol.*, **1991**, *69*, 809-820.

[25] Sadler, P.J.; Viles, J.H. ^{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.*, **1996**, *35*, 4490-4496.

[26] Esposito, B. P.; Najjar, R. Interactions of antitumoral platinum-group metallodrugs with albumin. *Coord. Chem. Rev.*, **2002**, *232*, 137-149.

[27] Christodoulou, J.; Sadler, P.J.; Tucker, A. A new structural transition of serum albumin dependent on the state of Cys34. Detection by ^{1}H-NMR spectroscopy. *Eur. J. Biochem.*, **1994**, *225*, 363-368.

[28] Ivanov, A.I.; Christodoulou, J.; Parkinson, J.A.; Barnham, K.J.; Tucker, A.; Woodrow, J.; Sadler, P.J. Cisplatin binding sites on human albumin. *J. Biol. Chem.,* **1998**, *273*, 14721-14730.

[29] Lau, S.; Sarkar, B. Inorganic mercury(II)-binding components in normal human-blood serum. *J. Toxicol. Environ. Health,* **1979**, *5*, 907-916.

[30] Kozlowski, H.; Bal, W.; Dyba, M.; Kowalik-Jankowska, T. Specific structure-stability relations in metallopeptides. *Coord. Chem. Rev.,* **1999**, *184*, 319-346.

[31] Rozga, M.; Sokolowska, M.; Protas, A. M.; Bal, W. Human serum albumin coordinates Cu(II) at its N-terminal binding site with 1 pM affinity. *J. Biol. Inorg. Chem.,* **2007**, *12*, 913-918.

[32] Lakusta, H.; Sarkar, B. Equilibrium studies of zinc(II) and cobalt(II) binding to tripeptide analogs of the amino terminus of human serum albumin. *J. Inorg. Biochem.,* **1979**, *11*, 303-315.

[33] Stewart, A.J.; Blindauer, C.A.; Berezenko, S.; Sleep, D.; Sadler, P.J. Interdomain zinc site on human albumin. *Proc. Natl. Acad. Sci. U.S.A.,* **2003**, *100*, 3701-3706.

[34] Masuoka, J.; Hegenauer, J.; Van Dyke, B.R.; Saltman, P. Intrinsic stoichiometric equilibrium constants for the binding of zinc(II) and copper(II) to the high affinity site of serum albumin. *J. Biol. Chem.,* **1993**, *268*, 21533-21537.

[35] Masuoka, J.; Saltman, P. Zinc(II) and copper(II) binding to serum-albumin - a comparative study of dog, bovine, and human albumin. *J. Biol. Chem.,* **1994**, *269*, 25557-25561.

[36] Ohyoshi, E.; Hamada, Y.; Nakata, K.; Kohata, S. The interaction between human and bovine serum albumin and zinc studied by a competitive spectrophotometry. *J. Inorg. Biochem.,* **1999**, *75*, 213-218.

[37] Lu, J.; Stewart, A.J.; Sleep, D.; Sadler, P.J.; Pinheiro, T.J.T.; Blindauer, C.A. A molecular mechanism for modulating plasma Zn speciation by fatty acids. *J. Am. Chem. Soc.,* **2012**, *134*, 1454-1457.

[38] Sokolowska, M.; Wszelaka-Rylik, M.; Poznanski, J.; Bal, W. Spectroscopic and thermodynamic determination of three distinct binding sites for Co(II) ions in human serum albumin. *J. Inorg. Biochem.,* **2009**, *103*, 1005-1013.

[39] Sokolowska, M.; Pawlas, K.; Bal, W. 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.

[40] Sokolowska, M.; Krezel, A.; Dyba, M.; Szewczuk, Z.; Bal, W., Short peptides are not reliable models of thermodynamic and kinetic properties of the N-terminal metal binding site in serum albumin. *Eur J Biochem,* **2002**, *269*, 1323-133.

[41] Bal, W.; Christodoulou, J.; Sadler, P.J.; Tucker, A. Multi-metal binding site of serum albumin. *J. Inorg. Biochem.,* **1998**, *70*, 33-39.

[42] Irving, H.; Williams, R.J.P. The stability of transition-metal complexes. *J. Chem. Soc.,* **1953**, 3192-3210.

[43] Blindauer, C.A.; Harvey, I.; Bunyan, K.E.; Stewart, A.J.; Sleep, D.; Harrison, D.J.; Berezenko, S.; Sadler, P.J. Structure, properties, and engineering of the major zinc binding site on human albumin. *J. Biol. Chem.,* **2009**, *284*, 23116-23124.

[44] Carter, D.C.; He, X.M.; Munson, S.H.; Twigg, P.D.; Gernert, K.M.; Broom, M.B.; Miller, T. Y. Three-dimensional structure of human serum albumin. *Science,* **1989**, *244*, 1195-1198.

[45] Sugio, S.; Kashima, A.; Mochizuki, S.; Noda, M.; Kobayashi, K. Crystal structure of human serum albumin at 2.5 angstrom resolution. *Protein Eng.,* **1999**, *12*, 439-446.

[46] Curry, S.; Mandelkow, H.; Brick, P.; Franks, N. Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. *Nat. Struct. Biol.,* **1998**, *5*, 827-835.

[46] Bhattacharya, A.A.; Grüne, T.; Curry, S. Crystallographic analysis reveals common modes of binding of medium and long-chain fatty acids to human serum albumin. *J. Mol. Biol.,* **2000**, *303*, 721-732.

[48] Petitpas, I.; Grune, T.; Bhattacharya, A. A.; Curry, S., Crystal structures of human serum albumin complexed with monounsaturated and polyunsaturated fatty acids. *J. Mol. Biol.,* **2001**, *314*, 955-960.

[48] Wardell, M.; Wang, Z.; Ho, J. X.; Robert, J.; Ruker, F.; Ruble, J.; Carter, D. C., The atomic structure of human methemalbumin at 1.9 A. *Biochem. Biophys. Res. Commun.,* **2002**, *291*, 813-819.

[50] Ghuman, J.; Zunszain, P. A.; Petitpas, I.; Bhattacharya, A. A.; Otagiri, M.; Curry, S., Structural basis of the drug-binding specificity of human serum albumin. *J. Mol. Biol.,* **2005**, *353*, 38-52.

[51] Hein, K. L.; Kragh-Hansen, U.; Morth, J. P.; Jeppesen, M. D.; Otzen, D.; Moller, J. V.; Nissen, P., Crystallographic analysis reveals a unique lidocaine binding site on human serum albumin. *J. Struct. Biol.,* **2010**, *171*, 353-360.

[52] 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., Large-scale production of functional human serum albumin from transgenic rice seeds. *Proc. Natl. Acad. Sci. U. S. A.,* **2011**, *108*, 19078-19083.

[53] Schmidt, M. M.; Townson, S. A.; Andreucci, A. J.; King, B. M.; Schirmer, E. B.; Murillo, A. J.; Dombrowski, C.; Tisdale, A. W.; Lowden, P. A.; Masci, A. L.; Kovalchin, J. T.; Erbe, D. V.; Wittrup, K. D.; Furfine, E. S.; Barnes, T. M., Crystal structure of an HSA/FcRn complex reveals recycling by competitive mimicry of HSA ligands at a pH-dependent hydrophobic interface. *Struct. Fold. Des.,* **2013**, *21*, 1966-1978.

[54] Majorek, K.A.; Porebski, P.J.; Dayal, A.; Zimmerman, M.D.; Jablonska, K.; Stewart, A.J.; Chruszcz, M.; Minor, W. Structural and immunologic characterization of bovine, horse, and rabbit serum albumins. *Mol. Immunol.,* **2012**, *52*, 174-182.

[55] Bujacz, A. Structures of bovine, equine and leporine serum albumin. *Acta Crystallogr. D Biol. Crystallogr.,* **2012**, *68*, 1278-1289.

[56] Kassaar, O.; Schwarz-Linek, U.; Blindauer, C.A.; Stewart, A.J. Plasma free fatty acid levels influence Zn^{2+}-dependent histidine-rich glycoprotein-heparin interactions via an allosteric switch on serum albumin. *J. Thromb. Haemost.,* **2015**, *13*, 101-110.

[57] van der Vusse, G.J. Albumin as fatty acid transporter. *Drug Metab. Pharmacokinet.,* **2009**, *24*, 300-307.

[58] Pedersen, A.O.; Honore, B.; Brodersen, R. Thermodynamic parameters for binding of fatty acids to human serum albumin. *Eur. J. Biochem.,* **1990**, *190*, 497-502.

[59] Boden, G. Obesity and Free Fatty Acids. *Endocrinol. Metabol. Clin. North Amer.,* **2008**, *37*, 635-646.

[60] 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. 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.,* **2009**, *55*, 1588-1590.

[61] Simard, J.R.; Zunszain, P.A.; Hamilton, J.A.; Curry, S. Location of high and low affinity fatty acid binding sites on human serum albumin revealed by NMR drug-competition analysis. *J. Mol. Biol.,* **2006**, *361*, 336-351.

[62] Simard, J.R.; Zunszain, P.A.; Ha, C.E.; Yang, J.S.; Bhagavan, N.V.; Petitpas, I.; Curry, S.; Hamilton, J.A. Locating high-affinity fatty acid-binding sites on albumin by x-ray crystallography and NMR spectroscopy. *Proc Natl Acad Sci U S A,* **2005**, *102*, 17958-17963.

[63] Hamilton, J.A. NMR reveals molecular interactions and dynamics of fatty acid binding to albumin. *Biochim. Biophys. Acta,* **2013**, *1830*, 5418-5426.

[64] Bar-Or, D.; Curtis, G.; Rao, N.; Bampos, N.; Lau, E. Characterization of the Co^{2+} and Ni^{2+} binding amino-acid residues of the N-terminus of human albumin: an insight into the mechanism of a new assay for myocardial ischemia. *Eur. J. Biochem.,* **2001**, *268*, 42-47.

[65] Christenson, R.H.; Duh, S.H.; Sanhai, W.R.; Wu, A.H.B.; Holtman, V.; Painter, P.; Branham, E.; Apple, F.S.; Murakami, M.; Morris, D.L. Characteristics of an albumin cobalt binding test for assessment of acute coronary syndrome patients: A multicentre study. *Clin. Chem.,* **2001**, *47*, 464-470.

[66] Heyns, A. du P.; Eldor, A.; Yarom R.; Marx, G. Zinc-induced platelet aggregation is mediated by the fibrinogen receptor and is not accompanied by release or by thromboxane synthesis. *Blood,* **1985**, *66*, 213-219.

[67] Vu, T.T.; Fredenburgh, J.C.; Weitz, J.I. Zinc: An important cofactor in haemostasis and thrombosis. *Thromb, Haemost,* **2013**, *109*, 421-430.

[68] Kluszynski, B.A.; Kim, C.; Faulk, W.P. Zinc as a cofactor for heparin neutralization by histidine-rich glycoprotein. *J. Biol. Chem.,* **1997**, *272*, 13541-13547.

[69] Jones, A.L.; Hulett, M.D.; Parish, C.R. Histidine-rich glycoprotein: A novel adaptor protein in plasma that modulates the immune, vascular and coagulation systems. *Immunol. Cell. Biol.* **2005**, *83*, 106-118.

[70] Corrigan, J.J.; Jeter, M.A.; Bruck, D.; Feinberg, W.M. Histidine-rich glycoprotein levels in children: the effect of age. *Thromb. Res.*, **1990**, *59*, 681-686.

[71] Lord, S.T. Molecular mechanisms affecting fibrin structure and stability. *Arterioscler. Thromb. Vasc. Biol.*, **2011**, *31*, 494-499.

[72] Shankar, A.H.; Prasad, A.S. Zinc and immune function: the biological basis of altered resistance to infection. *Am. J. Clin. Nutr.*, **1998**, *68*, 447S-463S.

[73] MacQuarrie, J.L.; Stafford A.R.; Yau J.W.; Leslie B.A.; Vu, T.T.; Fredenburgh, J.C.; Weitz, J.I. Histidine-rich glycoprotein binds factor XIIa with high affinity and inhibits contact-initiated coagulation. *Blood*, **2011**, *117*, 4134-4141.

[74] Fredenburgh, J.C.; Leslie, B.A.; Stafford, A.R.; Lim, T.; Chan, H.H.; Weitz, J.I. Zn^{2+} mediates high affinity binding of heparin to the αC domain of fibrinogen. *J. Biol. Chem.*, **2013**, *288*, 29394-29402.

[75] Tennent, G.A.; Brennan, S.O.; Stangou, A.J.; O'Grady, J.; Hawkins, P.N.; Pepys, M.B. Human plasma fibrinogen is synthesized in the liver. *Blood*, **2007**, *109*, 1971-1974.

[76] Hogg, P.J.; Jackson, C.M. Fibrin monomer protects thrombin from inactivation by heparin-antithrombin III: implications for heparin efficacy. *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 3619-3623.

[77] Becker, D.L.; Fredenburgh, J.C.; Stafford, A.R.; Weitz, J.I. Exosites 1 and 2 are essential for protection of fibrin-bound thrombin from heparin-catalyzed inhibition by antithrombin and heparin cofactor II. *J. Biol. Chem.*, 1999, 274, 6226-6233.

[78] Trybulec, M.; Kowalska, M.A.; McLane, M.A.; Silver, L.; Lu, W.; Niewiarowski, S. Exposure of platelet fibrinogen receptors by zinc ions: role of protein kinase C. *Proc. Soc. Exp. Biol. Med.*, **1993**, *203*, 108-116.

[79] Previtali, E.; Bucciarelli, P.; Pasamonit, S.M.; Martinelli, I. Risk factors for venous and arterial thrombosis. *Blood. Transfus.*, **2011**, *9*, 120-138.

[80] Connolly, G.C.; Khorana, A.A. Risk stratification for cancer-associated venous thromboembolism. *Best. Pract. Res. Clin. Haematol.*, **2009**, *22*, 35-47.

[81] Bjorntorp, P.; Bergman, H.; Varnauskas, E. Plasma free fatty acid turnover rate in obesity. *Acta Med. Scand.*, **1969**, *185*, 351-356.

[82] Koutsari, C.; Jensen, M.D. Thematic review series: patient-oriented research. Free fatty acid metabolism in human obesity. *J. Lipid Res.*, **2006**, *47*, 1643-1650.

[83] Reaven, G.M.; Hollenbeck, C.; Jeng, C.Y.; Wu, M.S.; Chen, Y.D.I. Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM. *Diabetes*, **1988**, *37*, 1020-1024.

[84] Donnelly, K.L.; Smith, C.I.; Schwarzenberg, S.J.; Jessurun, J.; Boldt, M.D.; Parks, E.J. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J. Clin. Invest.*, **2005**, *115*, 1343-1351.

[85] Charles, M.A.; Fontbonne, A.; Thibult, N.; Claude, J.R.; Warnet, J.M.; Rosselin, G.; Ducimetière, P.; Eschwège, E. High plasma non-esterified fatty acids are predictive of cancer mortality but not of coronary heart disease mortality: results from the Paris Prospective Study. *Am. J. Epidemiol.*, **2001**, *153*, 292-298.

[86] Stewart, A.J.; Blindauer, C.A.; Sadler, P.J. Plasma fatty acid levels may regulate the Zn^{2+}-dependent activities of histidine-rich glycoprotein. *Biochimie*, **2009**, *91*, 1518-1522.

[87] Veering, B.T.; Burm, A.G.; Souverin, J.H.; Serree, J.M.; Spierdijk, J. The effect of age on serum concentrations of albumin and alpha 1-acid glycoprotein. *Br. J. Clin. Pharmacol.*, **1990**, *29*, 201-206.

[88] Pickart, L. Increased ratio of plasma free fatty acids to albumin during normal aging and in patients with coronary heart disease. *Atherosclerosis*, **1983**, *46*, 21-28.

[89] Little, P.J.; Bhattacharya, R.; Moreyra, A.E.; Korichneva, I.L. Zinc and cardiovascular disease. *Nutrition*, **2010**, *26*, 1050-1057.

[90] Xu, Z.; Zhou, J. Zinc and myocardial ischemia/reperfusion injury. *Biometals*, **2013**, *26*, 863-878.

[91] Tomat, A.L.; Costa, M.D.; Arranz, C.T, Zinc restriction during different periods of life: Influence in renal and cardiovascular diseases. *Nutrition*, **2011**, *27*, 392-398.

[92] Tubek, S. Role of zinc in regulation of arterial blood pressure and in the etiopathogenesis of arterial hypertension. *Biol. Trace Elem. Res.*, **2007**, *117*, 39-51.

[93] Tubek, S. Zinc balance normalization: an important mechanism of angiotensin-converting enzyme inhibitors and other drugs decreasing the activity of the rennin-angiotensin-aldosterone system. *Biol. Trace Elem. Res.*, **2007**, *115*, 223-226.

[94] Korichneva, I., Zinc dynamics in the myocardial redox signaling network. *Antioxid. Redox Signal.*, **2006**, *8*, 1707-1721.

[95] Rowe, D.J.; Bobilya, D.J. Albumin facilitates zinc acquisition by endothelial cells. *Proc. Soc. Exp. Biol. Med.*, **2000**, *224*, 178-186.

[96] Gálvez, M.; Moreno, J.A.; Elósequi, L.M.; Escanero, J.F. Zinc uptake by human erythrocytes with and without serum albumins in the medium. *Biol. Trace Elem. Res.*, **2001**, *84*, 45-56.

[97] Tibaduiza, E.C.; Bobilya, D.J. Zinc transport across an endothelium includes vesicular cotransport with albumin. *J. Cell. Physiol.*, **1996**, *167*, 539-547.

[98] Powell, S.R.; Aiuto, L.; Hall, D.; Tortolani, A.J. Zinc supplementation enhances the effectiveness of St. Thomas' Hospital No. 2 cardioplegic solution in an in vitro model of hypothermic cardiac arrest. *J. Thorac. Cardiovasc. Surg.*, **1995**, *110*, 1642-1648.

[99] Coudray, C.; Charlon, V.; de Leiris, J.; Favier, A. Effect of zinc deficiency on lipid peroxidation status and infarct size in rat hearts. *Int. J. Cardiol.*, **1993**, *41*, 109-113.

[100] Arnaud, J.; Faure, H.; Bourlard, P.; Denis, B.; Favier, A. E., Longitudinal changes in serum zinc concentration and distribution after acute myocardial infarction. *Clin. Chim. Acta*, **1994**, *230*, 147-156.

[101] Gomez, E.; del Diego, C.; Orden, I.; Elosegui, L. M.; Borque, L.; Escanero, J. F., Longitudinal study of serum copper and zinc levels and their distribution in blood proteins after acute myocardial infarction. *J. Trace Elem. Med. Biol.*, **2000**, *14*, 65-70.

[102] Clegg, M.S.; Hanna, L.A.; Niles, B.J.; Momma, T.Y.; Keen, C.L. Zinc deficiency-induced cell death. *IUBMB Life*, **2005**, *57*, 661-669.

[103] Prestle, J.; Quinn, F.R.; Smith, G.L. Ca^{2+}-handling proteins and heart failure: novel molecular targets? *Curr. Med. Chem.*, **2003**, *10*, 967-981.

[104] Gollasch, M.; Löhn, M.; Furstenau, M.; Nelson, M.T.; Luft, F.C.; Haller, H. Ca^{2+} channels, 'quantized' Ca^{2+} release, and differentiation of myocytes in the cardiovascular system. *J. Hypertens.*, **2000**, *18*, 989-998.

[105] Reuter, H.; Grönke, S.; Adam, C.; Ribati, M.; Brabender, J.; Zobel, C.; Frank, K.F.; Wippermann, J.; Schwinger, R.H.; Brixius, K.; Müller-Ehmsen, J. Sarcoplasmic Ca^{2+} release is prolonged in nonfailing myocardium of diabetic patients. *Mol. Cell. Biochem.*, **2008**, *308*, 141-149.

[106] Maret, W. Crosstalk of the group IIa and IIb metals calcium and zinc in cellular signaling. *Proc. Natl. Acad. Sci. USA*, **2001**, *98*, 12325-12327

[107] Pitt, S.J; Stewart, A.J. Examining a new role for zinc in regulating calcium release in cardiac muscle. *Biochem. Soc. Trans.*, **2015**, *43*, 359-363.

[108] Kleinfeld, M.; Stein E. Action of divalent cations on membrane potentials and contractility in rat atrium. *Am. J. Physiol.*, **1968**, *215*, 593-599.

[109] Ciofalo, F.R.; Thomas L.J. The effects of zinc on contractility, membrane potentials, and cation content of rat atria. *J. Gen. Physiol.* **1965**, *48*, 825-839.

[110] Kalfakakou, V.; Evangelou, A.; Benveniste, J.; Arnoux, B. The effects of Zn^{2+} on guinea pig isolated heart preparations. *Biol. Trace Elem. Res.*, **1993**, *38*, 289-299.

[111] Yi, T.; Vick, J.S.; Vecchio, M.J.H.; Begin, K.J.; Bell, S.P.; Delay, R.J.; Palmer, B.M. Identifying cellular mechanisms of zinc-induced relaxation in isolated cardiomyocytes. *Am. J. Physiol.*, **2013**, *305*, H706-H715.

[112] Woodier, J.; Rainbow, R.D.; Stewart A.J.; Pitt, S.J. Intracellular zinc modulates cardiac ryanodine receptor-mediated calcium release. *J. Biol. Chem.*, **2015**, *290*, 17599-17610.

[113] Zia, A.A.; Kamalov, G.; Newman, K.P.; McGee, J.E.; Bhattacharya, S.K.; Ahokas, R.A.; Sun, Y.; Gerling, I.C.; Weber, K.T. From aldosteronism to oxidative stress: the role of excessive intracellular calcium accumulation. *Hypertens. Res.*, **2010**, *33*, 1091-1101.

[114] Zou, M.H.; Shi, C.; Cohen, R.A. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J. Clin. Invest.*, **2002**, *109*, 817-826.

[115] Ross, R. Atherosclerosis - an inflammatory disease. *N. Engl. J. Med.*, **1999**, *340*, 115-126.

[116] Glass, C.K.; Witztum, J.L. Atherosclerosis: the road ahead. *Cell*, **2001**, *104*, 503-516.

[117] Beattie, J.H.; Gordon, M.J.; Duthie, S.J.; McNeil, C.J.; Horgan, G.W.; Nixon, G.F.; Feldmann, J.; Kwun, I.S. Suboptimal dietary

zinc intake promotes vascular inflammation and atherogenesis in a mouse model of atherosclerosis. *Mol. Nutr. Food Res.*, **2012**, *56*, 1097-1105.

[118] Libby, P. Inflammation and cardiovascular disease mechanisms. *Am. J. Clin. Nutr.*, **2006**, *83*, 456S-460S.

[119] Zabel, U.; Schreck, R.; Baeuerle, P.A. DNA binding of purified transcription factor NF-kappa B. Affinity, specificity, Zn^{2+} dependence, and differential half-site recognition. *J. Biol. Chem.*, **1991**, *266*, 252-260.

[120] De Martin, R.; Hoeth, M.; Hofer-Warbinek, R.; Schmid, J.A. The transcription factor NF-kappa B and the regulation of vascular cell function. *Arterioscler. Thromb. Vasc. Biol.*, **2000**, *20*, E83-E88.

[121] Hennig, B.; Meerarani, P.; Toborek, M.; McClain, C.J. Antioxidant-like properties of zinc in activated endothelial cells. *J. Am. Coll. Nutr.*, **1999**, *18*, 152-158.

[122] Bao, B.; Prasad, A.S.; Beck, F.W.; Godmere, M. Zinc modulates mRNA levels of cytokines. *Am. J. Physiol.*, **2003**, *285*, E1095-E1102.

[123] Newton, A.C. Protein kinase C - Structure, function, and regulation. *J. Biol. Chem.*, **1995**, *270*, 28495-28498.

[124] Ivey, M.E.; Little, P.J. Thrombin regulates vascular smooth muscle cell proteoglycan synthesis via PAR-1 and multiple downstream signalling pathways. *Thromb. Res.*, **2008**, *123*, 288-297.

[125] Little, P.J.; Osman, N.; O'Brien, K.D. Hyperelongated biglycan: the surreptitious initiator of atherosclerosis. *Curr. Opin. Lipidol.*, **2008**, *19*, 448-454.

[126] Nissinen, L.; Kähäri, V.M. Matrix metalloproteinases in inflammation. *Biochim. Biophys. Acta*, **2014**, *1840*, 2571-2580.

[127] Arroyo, A. G.; Andres, V., ADAMTS7 in cardiovascular disease: From bedside to bench and back again? *Circulation*, **2015**, *131*, 1156-1159.

[128] Weber, K.T.; Weglicki, W.B.; Simpson, R.U., Macro- and micronutrient dyshomeostasis in the adverse structural remodelling of myocardium. *Cardiovasc. Res.*, **2009**, *81*, 500-508.

[129] Newby, A.C. Metalloproteinases promote plaque rupture and myocardial infarction: A persuasive concept waiting for clinical translation. *Matrix Biol.*, **2015**, *44-46C*, 157-166.

[130] Clegg, M.S.; Hanna, L.A.; Niles, B.J.; Momma, T.Y.; Keen C.L. Zinc deficiency-induced cell death. *IUBMB Life*, **2005**, *57*, 661-669.

[131] Hennig, B.; Meerarani, P.; Ramadass, P.; Toborek, M.; Malecki, A.; Slim, R.; McClain, C.J. Zinc nutrition and apoptosis of vascular endothelial cells: implications in atherosclerosis. *Nutrition*, **1999**, *15*, 744-748.

[132] Prasad, A.S. Clinical, immunological, anti-inflammatory and antioxidant roles of zinc. *Exp. Gerontol.*, **2008**, *43*, 370-377.

[133] Alcantara, E.H.; Shin, M.Y.; Feldmann, J.; Nixon, G.F.; Beattie, J.H.; Kwun, I.S. Long-term zinc deprivation accelerates rat vascular smooth muscle cell proliferation involving the down-regulation of JNK1/2 expression in MAPK signaling. *Atherosclerosis*, **2013**, *228*, 46-52.

[134] Fuster, J.J.; Fernandez, P.; Gonzalez-Navarro, H.; Silvestre, C.; Nabah, Y.N.; Andres, V. Control of cell proliferation in atherosclerosis: insights from animal models and human studies. *Cardiovasc. Res.*, **2010**, *86*, 254-264.

[135] Tsou, T.C.; Liou, S.H.; Yeh, S.C.; Tsai, F.Y.; Chao, H.R. Crucial role of Toll-like receptors in the zinc/nickel-induced inflammatory response in vascular endothelial cells. *Toxicol. Appl. Pharmacol.*, **2013**, *273*, 492-499.

[136] Patrushev, N.; Seidel-Rogol, B.; Salazar, G. Angiotensin II requires zinc and downregulation of the zinc transporters ZnT3 and ZnT10 to induce senescence of vascular smooth muscle cells. *PLoS One*, **2012**, *7*, e33211.

[137] Sinha, M.K.; Roy, D.; Gaze, D.C.; Collinson, P.O.; Kaski, J.-C. Role of ''Ischemia Modified Albumin'', a new biochemical marker of myocardial ischaemia in the early diagnosis of acute coronary syndromes. *J. Emerg. Med.*, **2004**, *21*, 29–34.

[138] Bar-Or, D.; Lau, E.; Winkler, J.V. A novel assay for cobalt-albumin binding and its potential as a marker for myocardial ischemia-a preliminary report. *J. Emerg. Med.*, **2000**, *19*, 311-315.

[139] Bhagavan, N.V.; Lai, E.M.; Rios, P.A.; Yang, J.S.; Ortega-Lopez, A.M.; Shinoda, H.; Honda S.A.; Rios, C.N.; Sugiyama, C.E.; Ha, C.E. Evaluation of human serum albumin cobalt binding assay for the assessment of myocardial ischemia and myocardial infarction. *Clin. Chem.* **2003**, *49*, 581-585.

[140] Collinson, P.O.; Gaze, D.C. Ischaemia-modified albumin: clinical utility and pitfalls in measurement. *J. Clin. Pathol.*, **2008**, *6*, 1025-1028.

[141] Sbarouni, E.; Georgiadou, P.; Voudris, V. Ischemia modified albumin changes - review and clinical implications. *Clin. Chem. Lab. Med.*, **2011**, *49*, 177-184.

[142] 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.; Committee on Standardization of Markers of Cardiac Damage of the IFCC. Future biomarkers for detection of ischemia and risk stratification in acute coronary syndrome. *Clin. Chem.*, **2005**, *51*, 810-824.

[143] Dominguez-Rodriguez, A.; Abreu-Gonzalez, P. Current role of ischemia-modified albumin in routine clinical practice. *Biomarkers*, **2010**, *15*, 655-662.

[144] Amirtharaj, G.J.; Natarajan, S.K.; Mukhopadhya, A.; Zachariah, U.G.; Hegde, S.K.; Kurian, G.; Balasubramanian, K.A.; Ramachandran, A. Fatty acids influence binding of cobalt to serum albumin in patients with fatty liver. *Biochim. Biophys. Acta*, **2008**, *1782*, 349-354.

[145] Kaefer, M.; Piva, S.J.; De Carvalho, J.A.M.; Da Silva, D.B.; Becker, A.M.; Coelho, A.C.; Duarte, M.M.; Moresco, R.N. Association between ischemia modified albumin, inflammation and hyperglycemia in type 2 diabetes mellitus. *Clin. Biochem.*, **2010**, *43*, 450-454.

[146] Bar-Or, D.; Rael, L. T.; Bar-Or, R.; Slone, D. S.; Mains, C. W.; Rao, N. K.; Curtis, C. G. The cobalt-albumin binding assay: insights into its mode of action. *Clin. Chim. Acta*, **2008**, *387*, 120-127.

[147] Oh, B.J.; Seo, M.H.; Kim, H.S. 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*, **2012**, *17*, 394-401.

[148] Mothes, E.; Faller, P. Evidence that the principal Co-II-binding site in human serum albumin is not at the n-terminus: Implication on the albumin cobalt binding test for detecting myocardial ischemia. *Biochemistry*, **2007**, *46*, 2267-2274.

[149] Lu, J.; Stewart, A. J.; Sadler, P. J.; Pinheiro, T. J. T.; Blindauer, C. A. Allosteric inhibition of cobalt binding to albumin by fatty acids: Implications for the detection of myocardial ischemia. *J. Med. Chem.*, **2012**, *55*, 4425-4430.

[150] Kurien, V. A.; Oliver, M. F. Free fatty-acids during acute myocardial infarction. *Prog. Cardiovasc. Dis.*, **1971**, *13*, 361-373.

[151] Lopaschuk, G.D.; Ussher, J.R.; Folmes, C.D.L.; Jaswal, J.S.; Stanley, W.C. Myocardial fatty acid metabolism in health and disease. *Physiol. Rev.*, **2010**, *90*, 207-258.

[152] Ha, C. E.; Bhagavan, N. V., Novel insights into the pleiotropic effects of human serum albumin in health and disease. *Biochim. Biophys. Acta*, **2013**, *1830*, 5486-5493.

[153] Eom, J. E.; Lee, E.; Jeon, K. H.; Sim, J.; Suh, M.; Jhon, G. J.; Kwon, Y., Development of an albumin copper binding (ACuB) assay to detect ischemia modified albumin. *Anal. Sci.*, **2014**, *30*, 985-990.

[154] Oncel, M.; Kıyıcı, A.; Onen, S. Evaluation of the relationship between ischemia-modified albumin levels and thyroid hormone levels. *J. Clin. Lab. Anal.*, **2014**, doi: 10.1002/jcla.21789.

[155] Lee, E.; Eom, J.E.; Jeon, K.H.; Kim, T.H.; Kim, E.; Jhon, G.J.; Kwon, Y. Evaluation of albumin structural modifications through cobalt-albumin binding (CAB) assay. *J. Pharm. Biomed. Anal.*, **2014**, *91*, 17-23.

[156] da Silva, S.H.; Hausen Bdos, S.; da Silva, D.B.; Becker, A.M.; de Campos, M.M., Duarte, M.M.; Moresco, R.N. Characteristics of a nickel-albumin binding assay for assessment of myocardial ischaemia. *Biomarkers*, **2010**, *15*, 353-357.

[157] Falkensammer, J.; Frech, A.; Duschek, N.; Gasteiger, S.; Stojakovic, T.; Scharnagl, H.; Huber, K.; Fraedrich, G.; Greiner, A. Prognostic relevance of ischemia-modified albumin and NT-proBNP in patients with peripheral arterial occlusive disease. *Clin. Chim. Acta*, **2015**, *438*, 255-260.

Received: 7th March 2015 Accepted: 6th July 2015