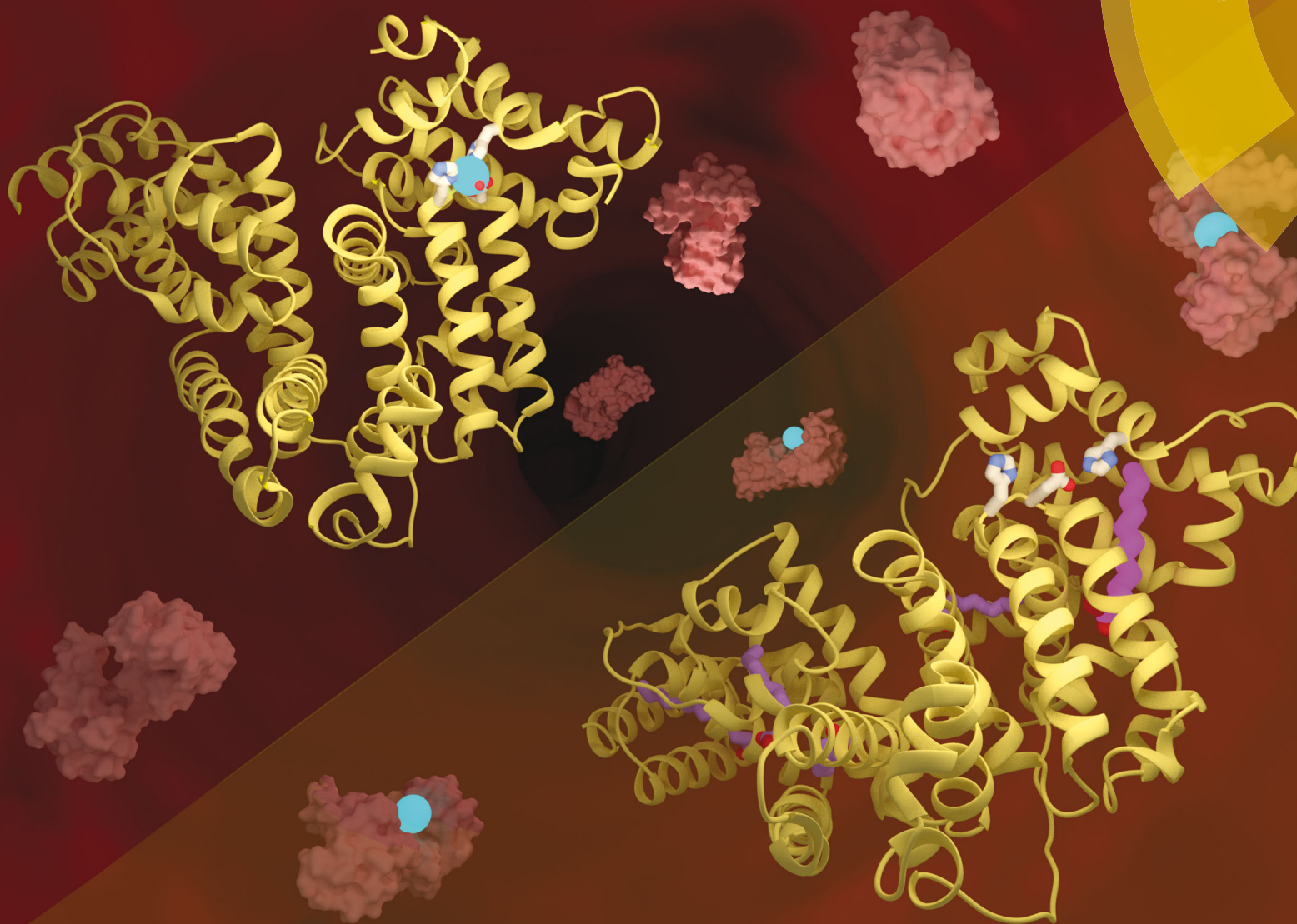


# Metallomics

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**PAPER**

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A metalloproteomic analysis of interactions between  
plasma proteins and zinc: elevated fatty acid levels affect  
zinc distribution

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## A metalloproteomic analysis of interactions between plasma proteins and zinc: elevated fatty acid levels affect zinc distribution†

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Serum albumin is a highly abundant plasma protein associated with the transport of metal ions, pharmaceuticals, fatty acids and a variety of small molecules in the blood. Once thought of as a molecular 'sponge', mounting evidence suggests that the albumin-facilitated transport of chemically diverse entities is not independent. One such example is the transport of  $Zn^{2+}$  ions and non-esterified 'free' fatty acids (FFAs) by albumin, both of which bind at high affinity sites located in close proximity. Our previous research suggests that their transport in blood plasma is linked via an allosteric mechanism on serum albumin. In direct competition, albumin-bound FFAs significantly decrease the binding capacity of albumin for  $Zn^{2+}$ , with one of the predicted consequences being a change in plasma/serum zinc speciation. Using liquid chromatography (LC), ICP-MS and fluorescence assays, our work provides a quantitative assessment of this phenomenon, and finds that in the presence of high FFA concentrations encountered in various physiological conditions, a significant proportion of albumin-bound  $Zn^{2+}$  is re-distributed amongst plasma/serum proteins. Using peptide mass fingerprinting and immunodetection, we identify candidate acceptor proteins for  $Zn^{2+}$  liberated from albumin. These include histidine-rich glycoprotein (HRG), a multifunctional protein associated with the regulation of blood coagulation, and members of the complement system involved in the innate immune response. Our findings highlight how FFA-mediated changes in extracellular metal speciation might contribute to the progression of certain pathological conditions.

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### Significance to metallomics

Building on previous evidence from single-protein model systems and computational simulations, this work provides a metalloproteomic assessment of serum/plasma zinc speciation, and identifies a significant re-distribution of  $Zn^{2+}$  amongst serum/plasma proteins, as a consequence of increased free fatty acid levels. Fluctuations in both serum free fatty acids and serum zinc have been independently associated with a plethora of diseases. This work now enhances our understanding of how metalloproteomic approaches are vital in order to understand the bio-inorganic mechanisms at play. Moreover, our work also suggests how metal ion speciation might even be affected in normal physiology, such as after the consumption of food or intense aerobic exercise.

## Introduction

The importance of zinc as an essential micronutrient for all life forms is well recognised. Increases of between 30–600% in crop yields have been achieved solely by zinc fertilisation,<sup>1</sup> as well as dietary zinc supplementation being identified as a top priority by the 2008 Copenhagen Consensus Conference for 80% of the

world's 140 million malnourished children.<sup>2</sup> As a Type II nutrient,<sup>3</sup> zinc directly affects multiple physiological processes with deficiency leading to growth retardation, skin lesions, diarrhoea, infertility, and compromised immune<sup>4</sup> and cognitive function.<sup>5</sup> Milder zinc deficiency is also a risk factor for cardiomyopathy and myocardial infarction.<sup>6</sup> The pervasive effects of inadequate zinc supply occur not only because of the essentiality of zinc for the function of hundreds of enzymes and thousands of transcription factors,<sup>7,8</sup> but also because zinc is a signalling agent, and mediates and modulates bio-molecular interactions.<sup>9</sup>

Considering these multiple effects, it is perhaps at first surprising that to plants and animals including humans, excess zinc is not particularly toxic. However, the picture of zinc's apparent biological harmlessness disintegrates once the effects

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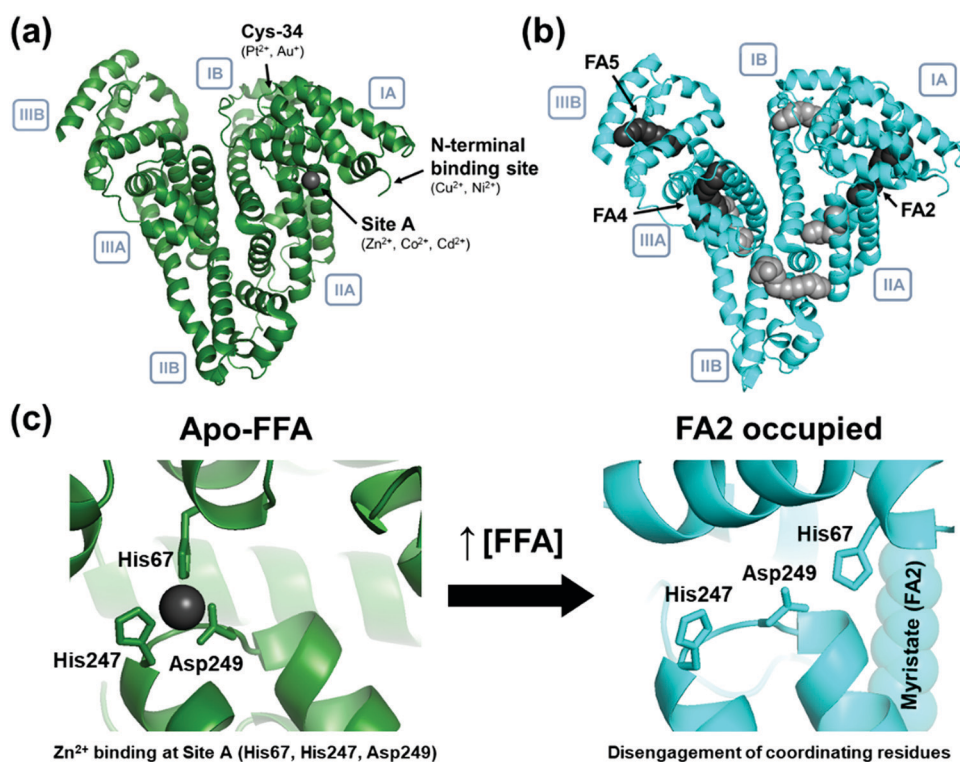


of elevated free  $Zn^{2+}$  concentrations on individual cells are considered, as low micromolar concentrations are sufficient to induce apoptotic or necrotic cell death pathways in a variety of cell types.<sup>4,10</sup> Many unicellular organisms are also highly sensitive to  $Zn^{2+}$ .<sup>11,12</sup> It follows that for higher eukaryotes, zinc toxicity is largely avoided by careful control of gastrointestinal tract absorption and – crucially – regulation of extracellular free  $Zn^{2+}$ ,<sup>13–15</sup> ensuring its concentrations remain below the levels at which it would begin to exert any cytotoxic effects.

It is well known that human blood plasma or serum contain significant amounts of zinc (9.6–31.6  $\mu M$ ),<sup>16,17</sup> alongside similar quantities of iron (6.05–26.96  $\mu M$ )<sup>18</sup> and copper (17  $\pm$  6  $\mu M$ ),<sup>19</sup> whilst only low-nanomolar amounts of manganese, cobalt and molybdenum are present.<sup>20–22</sup> Assuming a typical total amount of body zinc of 2.7 g, and a plasma volume of 2.5 L, it can be estimated that less than 0.2% of total body zinc is in the blood plasma. Despite this minor proportion, and although plasma/serum zinc levels are not reliable indicators of total organismal zinc status,<sup>23</sup> plasma zinc speciation and dynamics should not be disregarded, as it may not only influence overall organismal zinc distribution, but also modulate important bio-molecular interactions in the bloodstream.<sup>24,25</sup> It is also important to note that although the levels of total plasma zinc are usually maintained at fairly constant levels, there are a number of conditions where they are significantly decreased, including during the

acute phase response to infections and inflammation,<sup>26,27</sup> and in response to stress, for example during surgery.<sup>24</sup> There are also a number of chronic conditions that are associated with low plasma levels of zinc, including asthma and type 2 diabetes.<sup>6,25</sup> The molecular causes for the latter observations are not well understood.<sup>28</sup>

Along with a great many studies on total metal contents of plasma and sera,<sup>29</sup> research concerning  $Zn^{2+}$ –protein interactions that dominate plasma zinc speciation has been ongoing for several decades.<sup>30–34</sup> Serum albumin, a 66 kDa globular protein (Fig. 1), is the most abundant serum protein (0.6 mM), has high-nanomolar to low-micromolar affinity for  $Zn^{2+}$  and is the major carrier of plasma zinc.<sup>35,36</sup> Metal binding occurs at an inter-domain site involving tetrahedral metal coordination by His67, His247, Asp249 and a fourth non-protein ligand such as a water molecule (site A; Fig. 1c).<sup>37</sup> Under normal conditions, albumin is the dominant  $Zn^{2+}$  chelator, binding between 75–90% of the total plasma zinc.<sup>14</sup> Albumin-bound  $Zn^{2+}$  constitutes the largest part of the so-called exchangeable zinc pool (EZP), which comprises up to 90% of the total plasma zinc. Albumin is also known to transport various other small molecules, including pharmaceuticals,<sup>38,39</sup> various hormones, bilirubin, and free (non-esterified) fatty acids (FFAs).<sup>40</sup> The binding of long-chain FFAs to albumin (Fig. 1b) has been demonstrated to decrease the binding affinity of albumin for  $Zn^{2+}$ .<sup>41</sup> The underlying molecular mechanism (Fig. 1) has been



**Fig. 1** Serum albumin is a key transporter of metal ions and ‘free’ fatty acids (FFAs) in the blood plasma of all mammals. (a) Four prominent metal ion binding sites have been identified on albumin: site A, Cys34, the N-terminal binding site (NTS) formed by an ATCUN (amino-terminal copper- and nickel-binding) motif, and site B (location unknown); (b) seven free fatty acid (FA) binding sites have been identified by X-ray crystallographic studies of albumin complexed with palmitic (hexadecanoic) acid (PDB 1E7H). The three high affinity sites<sup>47</sup> are identified with arrows. (c) Coordination of a free fatty acid (FA, myristate; PDB 1BJ5) at site FA2 leads to disengagement of His67, His247 and Asp249 from metal binding at site A (PDB 5IJF), preventing coordination of  $Zn^{2+}$ .<sup>37,49</sup>





studied in depth using X-ray crystallography,<sup>37,42,43</sup> molecular modelling, NMR spectroscopy,<sup>44</sup> and isothermal titration calorimetry,<sup>41,45</sup> and is now well-understood in chemical model systems.<sup>46</sup> Interdependence of Zn<sup>2+</sup> and FFA binding is due to the proximal location of the primary Zn<sup>2+</sup> binding site (site A) and one (out of three;<sup>47</sup> Fig. 1b) high affinity fatty acid binding site (FA2), both situated at the interface of domains I and II of albumin. Upon fatty acid binding, providing the fatty acid is of sufficient chain length, a 'spring-lock' allosteric switching mechanism is invoked in albumin, disengaging amino acids involved in Zn<sup>2+</sup> coordination at site A, resulting in a significant decrease in metal binding affinity, and hence this otherwise major binding site is no longer available to Zn<sup>2+</sup>.<sup>48</sup> In FA2, FFAs bind with their carboxylate headgroup to Arg257 in domain II. The allosteric switch is elicited by FFAs with 10 or more carbon atoms,<sup>42</sup> whilst no analogous X-ray crystal structures with bound octanoate are available. Molecular modelling had suggested that octanoate may fit into the half-pocket in domain II, without the need to align the half-pocket in domain I.<sup>41</sup> The affinities of the three high-affinity sites cannot be distinguished; therefore, all three sites become occupied simultaneously.<sup>47</sup> Consistent with this finding, our previous work has shown that myristate affects the Zn<sup>2+</sup> affinity of both BSA<sup>41</sup> and HSA<sup>45</sup> already at 1 molar equivalent (mol. eq.). The latter concentration is within a normal physiological range (0.1–2 mol. eq.), but FFA levels can rise to 4–6 mol. eq. in a range of conditions (strenuous exercise, diabetes, cardiovascular disease, non-alcoholic fatty liver disease).<sup>47</sup>

As a result of this finding, it has been suggested that fluctuations in the levels of FFAs in the blood may impact zinc speciation, with several downstream effects predicted.<sup>14,46,48</sup> Although cellular zinc uptake and compartmentalisation is largely governed by membrane-bound transporters of the ZIP and ZnT families,<sup>50,51</sup> it is conceivable that plasma speciation could impact the uptake of Zn<sup>2+</sup> by endothelial cells and blood cells, including leukocytes involved in immune function.<sup>13</sup> In this regard, the effects of zinc on immune cells are of high relevance, since zinc is required for both innate and adaptive immune response, modulating cytokine secretion,<sup>52</sup> and playing roles in T cell maturation<sup>53</sup> and B cell response to vaccination.<sup>54</sup>

For these reasons, it is of interest to study the effect of FFAs on zinc speciation in plasma. The most basic and rapid separation method to address zinc speciation in serum or plasma is ultrafiltration,<sup>55–57</sup> which allows for distinction between protein-bound Zn<sup>2+</sup> (the high-molecular weight fraction, including serum albumin) from both 'free' (aquated) Zn<sup>2+</sup> and Zn<sup>2+</sup> bound to small ligands, such as free amino acids. The low-molecular weight fraction does usually not exceed 1% of total serum or plasma zinc, for which a typical free Zn<sup>2+</sup> concentration has been estimated at around 2–4 nM, using the Zn<sup>2+</sup>-responsive dye ZnAF-2.<sup>58</sup> The high-molecular weight fraction also consists of non-exchangeable zinc, bound firmly to proteins such as  $\alpha$ 2-macroglobulin (*ca.* 10–20% of total plasma zinc) and retinol-binding protein.<sup>59</sup> It is also worth noting that the concentrations of zinc-dependent enzymes (which are also expected to mostly contain non-exchangeable zinc) present in plasma are so low that so far, they have mostly escaped quantitation.

The baseline numbers given above, both in terms of total plasma or serum zinc as well as in terms of speciation, do not reflect the fairly dramatic changes in overall organismal zinc distribution, or the possibility for re-distribution between different plasma proteins under abnormal conditions. Speciation studies involving metal–protein complexes have recently been encompassed under the term "metalloproteomics", which aims to combine established proteomic techniques with complementary methods for analytical detection of metal ions. These typically comprise separation (most commonly chromatography or electrophoresis) and identification (often mass spectrometry) steps. Techniques such as SDS-PAGE, western blotting, and more recently native SDS-PAGE,<sup>60</sup> have been combined with elemental analysis, using sensitive techniques including inductively-coupled plasma mass spectrometry (ICP-MS) and ICP-OES (optical emission spectroscopy) to identify and quantify metal ions.<sup>61,62</sup> Further methodological refinements include the use of laser ablation (LA-ICP-MS) as well as isotope quantification in biological systems.<sup>63–66</sup> Hyphenated liquid chromatography methods, including capillary and nano-flow HPLC,<sup>63</sup> have attracted attention owing to the relative ease and speed of sample throughput in combination with low-nanomolar quantitation limits associated with ICP-MS. Serum metalloproteomes have been previously investigated using mass spectrometry to identify both metal ions (ICP-MS) and tens of metal-binding serum proteins (LC-MS/MS).<sup>67</sup> ICP-MS has also been used to inspect variations in the levels of free metal ions in serum obtained from patients with bipolar disorder undergoing different treatments.<sup>68</sup> Other researchers have employed size exclusion chromatography (SEC) paired with ICP-MS/AES to study metal binding to plasma proteins,<sup>69–73</sup> and the effect of chelating agents on plasma metal speciation.<sup>74,75</sup>

In this study, we have also utilised SEC in conjunction with offline ICP-MS analysis to investigate zinc speciation changes in foetal calf serum (FCS) and human blood plasma, upon addition of free fatty acids (FFAs). By administering controlled concentrations of fatty acids to human blood plasma (and a simple model systems), we show how FFAs are able to modulate extracellular zinc speciation in a quantitative manner. Immobilised metal affinity chromatography (IMAC) was employed as a complementary technique to identify key Zn<sup>2+</sup>-binding proteins in plasma samples which may be involved in such changes in zinc speciation.

## Methods

### Materials

Ammonium acetate (99.999% trace metals basis), ammonium hydroxide (28% NH<sub>3</sub> in H<sub>2</sub>O,  $\geq$ 99.99% trace metals basis), bovine serum albumin (low endotoxin, lyophilized powder, BioReagent, suitable for cell culture,  $\geq$ 98% by agarose gel electrophoresis), sodium myristate and sodium octanoate were purchased from Sigma Aldrich UK. Foetal calf serum (FCS, lot 70428, heat-inactivated, non-USA origin) was purchased from Labtech International UK, and a single lot was used throughout all experiments. Citrated human plasma was purchased from



TCS Biosciences UK. Inorganic Ventures ICP-MS standards for Zn (1000 ppm) and Er (1000 ppm) were purchased from Essex Scientific Supplies UK. Nitric acid (72% v/v) was freshly distilled before use. Water used for all experiments was doubly deionised (Type-I) Milli-Q water. All other reagents were purchased from Sigma Aldrich UK and used as received, unless specified otherwise.

### Size exclusion chromatography (FPLC)

Experiments were carried out using a GE Healthcare ÄKTA purifier 10 FPLC fitted with a Superdex G-75 16/60 size exclusion column (13  $\mu\text{m}$  average particle size, optimum separation range 3000–70 000, column volume 120 mL), Frac-950 fraction collector, pH/C probe 900, UV900 detector, and a P-900 pump, with ammonium acetate buffer (50 mM, pH 7.8  $\pm$  0.1, adjusted using ammonium hydroxide) mobile phase (277 K). A flow rate of 1 mL min<sup>-1</sup> and an injection volume of 0.5 mL was used. All samples were prepared at physiological concentrations (600  $\mu\text{M}$  BSA + 20  $\mu\text{M}$  Zn<sup>2+</sup> in 50 mM ammonium acetate buffer pH 7.8, or neat serum), incubated for a minimum of 18 h at 310 K before analysis, and diluted 2-fold in ammonium acetate buffer before filtration using a 0.22  $\mu\text{m}$  syringe filter. Samples were analysed in triplicate. FPLC data were acquired and analysed using UNICORN 5.11 for Windows. Experiments were repeated with the following modifications: (i) bovine serum albumin (BSA) was prepared with 0–5 mol. eq. fatty acid: octanoate (C8) or myristate (C14). Solutions of (ii) foetal calf serum and (iii) human citrated plasma were used in place of BSA, with and without FFAs, but no additional zinc. Natively present zinc was quantified separately using ICP-MS prior to use. Chemical concentrations in final samples were 300  $\mu\text{M}$  BSA (or 50% of FCS or human plasma, as appropriate), 10  $\mu\text{M}$  Zn<sup>2+</sup>, 0–1.5 mM (0–5 mol. eq.) FFA.

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

Zinc concentrations were determined using an Agilent 7900 series ICP-MS in He-gas mode with an internal standard of <sup>166</sup>Er (Agilent Technologies, USA). Calibration standards (1–1000 ppb) were freshly prepared in either ammonium acetate (50 mM) buffer or 3.6% ultrapure nitric acid, depending on the nature of the samples in order to match the sample matrix. Data were acquired and analysed using MassHunter 4.3 (Agilent Technologies, USA).

### Monitoring levels of non-protein bound Zn<sup>2+</sup> concentration using FluoZin-3

Briefly, a 96-well plate was washed thoroughly using 50  $\mu\text{L}$  of EDTA solution (50 mM) per well, followed by washing using Milli-Q (Type I) water (8  $\times$  200  $\mu\text{L}$ ) and thoroughly dried. For the experiments involving fatty acids, two BSA concentrations were employed (60 and 600  $\mu\text{M}$ ). Solutions of de-metalated BSA (pre-treated with Chelex-100 resin to remove any potential traces of Zn<sup>2+</sup>) were prepared (1.2 mM or 120  $\mu\text{M}$ , 2 $\times$  final concentration) in ammonium acetate buffer (50 mM, pH 7.8) in the presence and absence of sodium myristate or sodium octanoate (1, 3 or 5 mol. eq.). The pH was adjusted to 7.8 using

ammonium hydroxide. After 24 h incubation (310 K), 100  $\mu\text{L}$  aliquots were added per well to a 96-well plate. Separately, an 80  $\mu\text{M}$  (4 $\times$  final concentration) solution of Zn<sup>2+</sup> (from ZnSO<sub>4</sub>) was prepared in ammonium acetate buffer (Zn<sup>2+</sup> concentration determined by ICP-MS). To each BSA-FFA condition was added either 50  $\mu\text{L}$  of Zn<sup>2+</sup> (80  $\mu\text{M}$ ) or 50  $\mu\text{L}$  of buffer (0  $\mu\text{M}$  Zn<sup>2+</sup>). Calibration standards for Zn<sup>2+</sup> (0.001–40  $\mu\text{M}$ ) were separately prepared in 50 mM ammonium acetate buffer (150  $\mu\text{L}$ ), in the absence of BSA. To each well FluoZin-3 solution (50  $\mu\text{L}$ ; 0.1–2  $\mu\text{M}$ , final concentration) was then added, to give a final volume of 200  $\mu\text{L}$ . Final working concentrations of samples were 60–600  $\mu\text{M}$  BSA, 0–20  $\mu\text{M}$  Zn<sup>2+</sup> and 0–3 mM (up to 5 mol. eq.) FFA. Individual compositions are given in the respective Figure captions. All samples were prepared in triplicate. After 30 min incubation at 310 K, fluorescence was measured using a Promega GloMax fluorescence microplate reader (blue optical kit, reagent Ex/Em = 494/516 nm). Data were analysed and processed using Microsoft Excel. Arithmetic means and standard deviations were calculated, and statistical significance was evaluated using a two-tailed *t*-test assuming non-equal variances (Welch's *t*-test).

### Albumin quantitation using bromocresol green

Albumin quantitation of FCS and human citrated plasma was carried out using a commercial bromocresol green (BCG) colorimetric assay kit (Abcam, ab235628) according to the manufacturer's instructions. Samples were diluted 1:100 in assay buffer before analysis and read using a Promega GloMax microplate reader (absorbance at 620 nm).

### Determination of fatty acids by GC-MS

Fatty acids were extracted from FCS using chloroform : methanol (2:1 v/v, 1 mL reagent per 100  $\mu\text{L}$  serum) with vortexing, as described by the Folch method.<sup>76</sup> The layers were separated using centrifugation, and the chloroform layer was evaporated to dryness. Lipid samples were dissolved using 90  $\mu\text{L}$  of chloroform : hexane (1:1, Folch extractant) and derivatized with 10  $\mu\text{L}$  Meth-Prep II (methanolic *m*-trifluoromethyl-phenyltrimethylammonium hydroxide) at room temperature for 30 min. Samples were analysed using an Agilent Technologies GC-MS (7890B GC – 5977B MS), operated in EI mode (70 eV), fitted with an Agilent 19091S-433, HP-5MS (5% phenyl methyl siloxane) 30 m  $\times$  250  $\mu\text{m}$   $\times$  0.25  $\mu\text{m}$  column with helium carrier gas. Temperature gradient: initial: 50  $^{\circ}\text{C}$  (1 min), increased to 280  $^{\circ}\text{C}$  at a rate of 20  $^{\circ}\text{C}$  min<sup>-1</sup>, then 7.5 min hold time. Total run time: 20 min.

### Immobilized metal affinity chromatography (IMAC)

1 mL IMAC FF columns (packed with IMAC Sepharose Fast Flow resin; GE Healthcare, UK) were prepared as described by the manufacturer and either charged with Zn<sup>2+</sup> or left un-charged. Columns were equilibrated with 10 mM HEPES (pH 7.2, 0.5 M NaCl) ready for application of plasma. Human citrated plasma was clarified by centrifugation at 12 000  $\times$  *g* for 5 minutes and the supernatant was passed through a 0.22  $\mu\text{m}$  filter. 1 mL of clarified plasma was applied to each column and the flow-through (FT) fraction was collected. Unbound protein was washed through the column using 6  $\times$  1 mL of buffer containing 2 mM imidazole,



before bound proteins were eluted using  $2 \times 1$  mL of 20 mM imidazole buffer and  $2 \times 1$  mL of 200 mM imidazole buffer.

### SDS-polyacrylamide gel electrophoresis

Samples from SEC and IMAC fractions were mixed with an equal volume of (2 $\times$ ) Laemmli buffer (Sigma-Aldrich) and heated to 80 °C for 5 minutes before loading onto mini-Protean<sup>®</sup> TGX<sup>™</sup> precast gels (4–15% resolving gel percentage; 20–250 kDa separation range; Bio-Rad, UK),<sup>77</sup> which were run in standard Tris-glycine buffer following the manufacturer's instructions. Gels were stained using Coomassie brilliant blue R-250 (National Diagnostics, USA).

### Western blotting

Proteins were transferred onto a nitrocellulose membrane using a semidry blotting assembly (90 min, 150 mA). Non-fatty milk solution (2.5 g of non-fatty milk powder in 50 mL of Tris-buffered saline with 0.1% Tween 20; TBST) was used as blocking reagent (1 h at 277 K). The membrane was washed with TBST buffer before adding Anti-HRG primary antibody (produced in rabbit, Sigma-Aldrich, 10  $\mu\text{g mL}^{-1}$  in 20 mL TBST buffer, 1 hour). After further washing with TBST buffer, 20 mL solution of anti-rabbit IgG alkaline phosphatase conjugate secondary antibody (Sigma-Aldrich) was added and incubated for 1 h. The membrane was washed three times with TBST buffer after every 5 min of rocking. Finally, 2 mL of Novex<sup>®</sup> AP chromogenic substrate (Invitrogen) was added and incubated for 15 min. After rinsing with water, gels were imaged using an ImageQuant LAS4000 Blot Imaging System (GE Healthcare Life Sciences, Pittsburgh, USA).

### Peptide mass fingerprinting

Protein bands of interest were cut from SDS-PAGE gels with a scalpel blade and were subjected to in-gel tryptic digestion using a commercially produced kit (Pierce, Thermo Scientific, UK). The masses of the generated peptides were determined by MALDI-TOF MS analysis. Briefly, 2  $\mu\text{L}$  of sample matrix (10 mg  $\text{mL}^{-1}$   $\alpha$ -cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid) was mixed with an equal volume of sample and pipetted onto a steel MALDI-target plate. A Bruker Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, UK) with a 337 nm laser and operated in reflectron mode was used to measure the peptide masses. Mass calibration was performed with a PEG2000 MALDI-MS standard, and mass accuracy was verified using Bradykinin and Substance P peptide standards. Internal mass accuracy was further confirmed with the presence of the autolytic trypsin peaks of 845.2 and 2211.1 Da. Peptide masses were acquired over the range of 800–3500 Da, and mass lists were generated using Bruker Flex-analysis software with default parameters, and searched against the NCBI database using Mascot (Matrix Science, UK). The following search criteria were selected: fixed modification of carbamidomethyl on cysteine, variable modification of oxidation of methionine, maximum of 1 missed cleavage, <50 ppm mass accuracy, “*Homo sapiens*” was selected for taxonomy.

Only searches giving significant MOWSE (MOlecular Weight SEarch) scores were recorded.

## Results and discussion

### Zinc speciation in serum and plasma is affected by free fatty acids (FFAs)

Zinc speciation methodology was first optimised using a simplified single-protein system containing bovine serum albumin (BSA), in the absence of FFAs. A Superdex G-75 16/60 FPLC-SEC column was employed for size exclusion chromatography, with the selection of a mobile phase which was compatible with both SEC under near-native conditions and direct elemental analysis by ICP-MS (50 mM ammonium acetate, pH 7.8). Physiological concentrations of BSA (600  $\mu\text{M}$ ) and  $\text{Zn}^{2+}$  (20  $\mu\text{M}$ ) were analysed after 2-fold dilution to ensure sufficiently high concentrations of metal ions were present in the eluent for quantitation by ICP-MS whilst minimizing deposition of non-volatile salts on the sampling cone in the subsequent experiments with serum and plasma. The method was shown to have high reproducibility in both FPLC-SEC ( $A_{280\text{nm}}$ ) and ICP-MS ( $^{66}\text{Zn}$ ) datasets (Fig. S1 and S2, ESI<sup>†</sup>), with 109.9% recovery of protein-bound  $\text{Zn}^{2+}$  from the original 20  $\mu\text{M}$  sample. Data were then acquired in the presence of two different FFAs, octanoate (C8) and myristate (C14). In both instances, the chromatography traces for protein ( $A_{280\text{nm}}$ ) did not statistically differ from the FFA-free control, suggesting that BSA elution (or later, serum protein elution) was not significantly affected by the presence of FFAs (Fig. S3, ESI<sup>†</sup>). However, ICP-MS analysis of SEC fractions determined that 5 mol. eq. of myristate significantly decreased the amount of albumin-bound  $\text{Zn}^{2+}$  by 69.9% (from 20  $\mu\text{M}$  to 6.02  $\mu\text{M}$ ) relative to the FFA-free control (Fig. 2a). In contrast, 5 mol. eq. of octanoate had no significant effect on the concentration of  $\text{Zn}^{2+}$  in albumin-containing fractions (Fig. S4, ESI<sup>†</sup>). This is in agreement with previous crystallographic<sup>72</sup> and molecular modelling studies,<sup>41</sup> which suggested that FFAs with a chain length  $\leq\text{C}8$  are too short to elicit the allosteric switch on albumin. Previous ITC experiments had confirmed that octanoate indeed did not affect the  $\text{Zn}^{2+}$ -binding capacity of BSA.<sup>41</sup> In contrast, site A in both BSA and HSA<sup>45</sup> was demonstrated to be essentially absent in the presence of 5 mol. eq. of myristate. Residual weak  $\text{Zn}^{2+}$ -binding capacity was still observed from a secondary  $\text{Zn}^{2+}$ -binding site on both bovine ( $K_{2(\text{ITC})} \approx 1.4 \times 10^4 \text{ M}^{-1}$ ; ionic strength = 95 mM) and human albumin ( $K_{2(\text{ITC})} \approx 3 \times 10^3 \text{ M}^{-1}$ ; ionic strength = 183 mM), assumed to be site B based on previous <sup>111</sup>Cd NMR competition studies.<sup>41,78</sup> A possible secondary  $\text{Zn}^{2+}$  binding site has been observed by crystallography of equine serum albumin, involving His<sub>9</sub>, Asp<sub>13</sub> and Asp<sub>254</sub>, all of which are fully conserved in HSA and BSA. This, in principle, justifies the observation of a ca. 70% reduction in albumin-bound  $\text{Zn}^{2+}$ . It must also be considered that the composition of our buffer (50 mM ammonium acetate) has significantly lower ionic strength (<50 mM) than either previously employed experimental conditions or physiological media (>100 mM), which is expected to increase complex stability and hence the proportion of  $\text{Zn}^{2+}$  bound to BSA in our model system used for offline LC-ICP-MS.



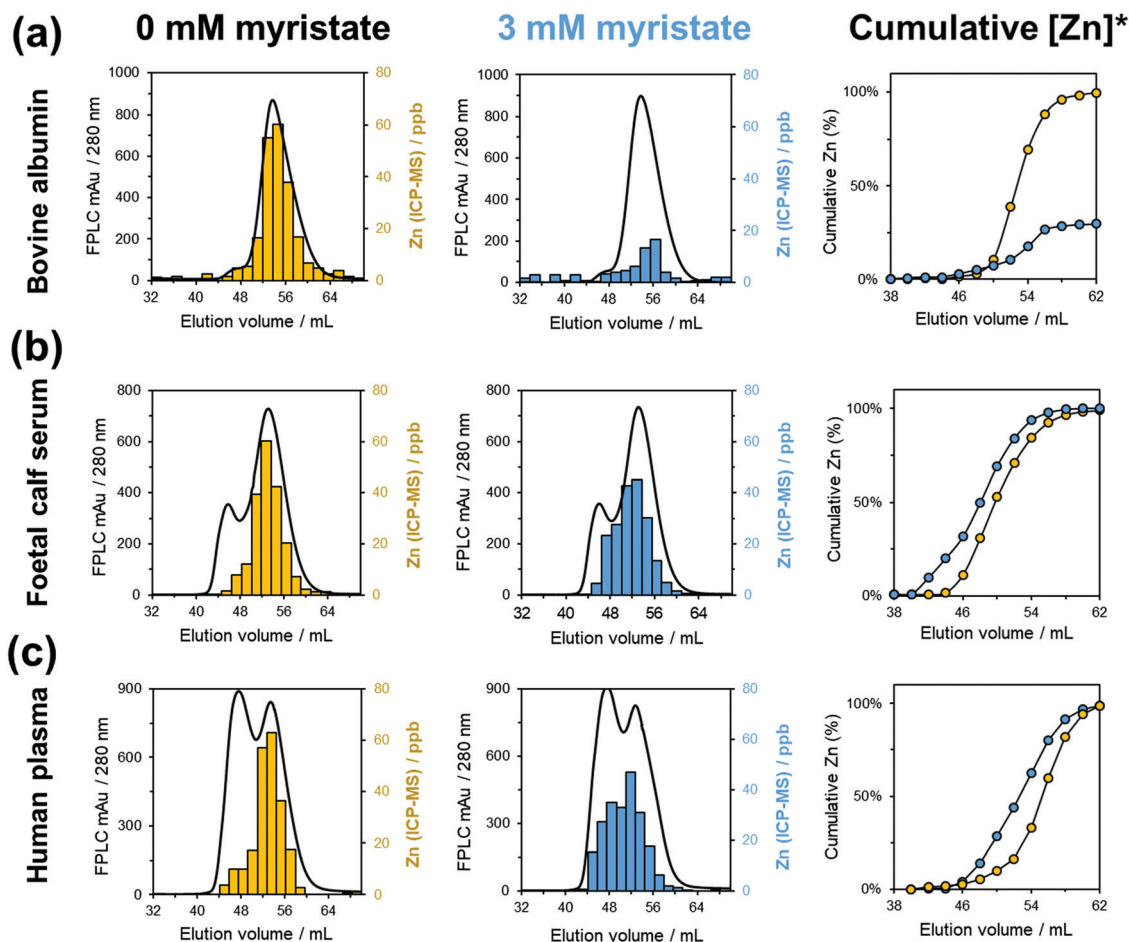


Fig. 2 Offline SEC-ICP-MS (Superdex G-75 16/60 FPLC) analysis demonstrates that sodium myristate (3 mM, 5 mol. eq.) impacted zinc speciation in: (a) bovine serum albumin, where the  $Zn^{2+}$  binding capacity of BSA is significantly reduced, resulting in decreased cumulative protein-bound zinc. For both foetal calf serum (b) and human citrated plasma (c), cumulative protein-bound zinc was not significantly affected, but instead, zinc was re-distributed amongst other plasma/serum proteins with  $Zn^{2+}$  binding capability. \*Protein-bound zinc only. The black lines in the chromatograms refer to protein elution, monitored by UV absorption at 280 nm, whilst the yellow and blue bars refer to zinc concentrations measured by ICP-MS. In the right-hand plots, black lines connecting the experimental data are drawn to guide the eye.

Foetal calf serum (FCS) is a common component of extra-cellular media for the culture of *in vitro* cell lines, and provides a more complex system for chromatographic analysis. First, the albumin content of FCS was quantified using bromocresol green, ( $345 \pm 65 \mu\text{M}$ ). This is lower than physiological albumin levels in adult humans ( $\sim 600 \mu\text{M}$ ); however the comparable total zinc concentrations of FCS and plasma ( $25.8 \pm 0.3 \mu\text{M}$  and  $21.4 \pm 0.4 \mu\text{M}$ , respectively) mean that albumin is in excess to zinc in both instances. Size exclusion chromatography experiments were repeated using FCS in place of bovine albumin. In this complex medium, five mol. eq. of myristate did not impact the cumulative recovery of zinc relative to the initial sample (*i.e.* no 'free'  $Zn^{2+}$  released, unlike in the BSA model system). Rather, the presence of myristate invoked a re-distribution of zinc, predominantly to higher molecular weight (lower elution volume) species (Fig. 2b). Data obtained in the presence of sodium octanoate (C8) in FCS confirm that octanoate did not impact either the distribution of serum zinc or cumulative zinc recovery (Fig. S4, ESI<sup>+</sup>). Thus, for both short-chain and

long-chain fatty acids, the system BSA + fatty acid +  $Zn^{2+}$  behaves in a similar way in this complex medium (FCS) as in the pure ternary mixture, with the main difference being the destination of the  $Zn^{2+}$  liberated from albumin.

Direct comparison of data acquired for BSA and FCS in the absence of myristate indicates that  $Zn^{2+}$  in FCS is not solely bound by albumin, with *ca.* 12.6% eluting together with higher molecular weight proteins. This is in line with expectations, as the known  $Zn^{2+}$ -binding protein  $\alpha 2$ -macroglobulin has been identified in commercial preparations of foetal calf serum.<sup>79</sup> It is also important to note that FCS already contains native FFAs. After chloroform:methanol extraction and derivitisation of FFAs using a methanolic solution of *m*-trifluoromethylphenyltrimethylammonium hydroxide, GC-MS analysis principally identified palmitic (C16:0), stearic (C18:0) and oleic acids (C18:1) in FCS (Fig. S5, ESI<sup>+</sup>), all of which are known to bind to FA2.<sup>42,80</sup> One must therefore consider that the fatty acid binding sites of albumin are likely to be already partially occupied, and this may further contribute to the wider distribution of





$\text{Zn}^{2+}$  relative to the pure binary albumin +  $\text{Zn}^{2+}$  system. As a further consequence, the redistribution observed after *in vitro* FFA supplementation may be less dramatic, compared to the model system involving (FFA-free) BSA. However, in FCS,  $\text{Zn}^{2+}$  is likely to be bound to other proteins that happen to also elute in the same fractions as albumin, which could not be resolved by the SEC column.

The concentration of non-albumin bound  $\text{Zn}^{2+}$  in eluates from the simple BSA + myristate model system was not quantifiable by SEC-ICP-MS because the released  $\text{Zn}^{2+}$  did not elute in the small-molecule fraction. However, repeated elution of the Superdex column using EDTA (20 mM) demonstrated significant stationary-phase retention of  $\text{Zn}^{2+}$  (Fig. S6, ESI<sup>†</sup>). The data shown in Fig. 2 were obtained after rigorous pre-cleaning of the column with 20 mM EDTA, which enabled reasonable recovery (110%) of zinc in the absence of myristate. Ideally, speciation analysis should provide quantitation across all species, hence an alternative method to capture non-protein-bound zinc in the various systems was desirable.

### Monitoring $\text{Zn}^{2+}$ release from albumin by FluoZin-3

Attempts were made to quantify the proportion of non-albumin bound zinc in presence and absence of myristate using a fluorescent  $\text{Zn}^{2+}$  sensing reagent (FluoZin-3; FZ3 in the following discussion). It was hoped that this approach would allow a measure of fatty-acid induced release of  $\text{Zn}^{2+}$  with minimal perturbation of the system. FZ3 was chosen as its affinity is relatively close to that of albumin (the conditional dissociation constant of FZ3 at pH 7.4 has recently been refined,  $K_d = (9.1 \pm 0.4) \times 10^{-9}$  M),<sup>81</sup> and FZ3 has high selectivity for  $\text{Zn}^{2+}$  over other divalent metal ions such as  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ .<sup>82</sup> Since fluorescent sensors are typically employed at concentrations that are much lower than those of surrounding proteins,<sup>83–85</sup> the influence of the sensor on the original equilibrium should be relatively small. Other  $\text{Zn}^{2+}$  sensors were considered, but rejected based on their documented tendency to form ternary complexes with  $\text{Zn}^{2+}$  and proteins.<sup>85–87</sup>

The response of either 0.1 or 2  $\mu\text{M}$  FZ3 to  $[\text{Zn}^{2+}]$  was recorded in ammonium acetate buffer using a range of defined  $\text{Zn}^{2+}$  concentrations (Fig. S7, ESI<sup>†</sup>), before exploring the effect of BSA concentration on FZ3 fluorescence (Fig. S8, ESI<sup>†</sup>). We found that BSA promoted FZ3 fluorescence in the absence of

$\text{Zn}^{2+}$ ; this suggested that BSA had the ability to bind FZ3. Indeed, fluorescein and related xanthene derivatives have previously been shown to bind to albumin;<sup>88–90</sup> thus analogous interactions with the structurally similar fluorescent moiety in FZ3 are unsurprising. In the presence of  $\text{Zn}^{2+}$ , the fluorescence decreased with increasing BSA concentration. This trend was expected, as increasing [BSA] should decrease the amount of free  $[\text{Zn}^{2+}]$ . However, the fluorescence at 600  $\mu\text{M}$  BSA and 2  $\mu\text{M}$  FZ3 in presence and absence of 20  $\mu\text{M}$   $\text{Zn}^{2+}$  was identical (Table S1, ESI<sup>†</sup>), indicating that under these conditions, FZ3 was not able to report on  $\text{Zn}^{2+}$ , free or protein-bound. At lower BSA concentrations, readings also deviated significantly from estimates based on published equilibrium constants, most likely due to the formation of ternary complexes and also FZ3 abstracting BSA-bound zinc, which becomes significant at lower [BSA] (Fig. S9, ESI<sup>†</sup>). Hence, due to the number and complexity of the various equilibria and processes in operation, the recorded fluorescence response could not be linked in a systematic manner to free  $[\text{Zn}^{2+}]$ . Our observations are consistent with those of others who have highlighted the pitfalls in quantitation of free  $[\text{Zn}^{2+}]$  using fluorescent dyes such as Zinquin,<sup>86,87</sup> TSQ,<sup>86</sup> ZnAF dyes,<sup>91</sup> and indeed FluoZin-3.<sup>92</sup> Problems have been attributed to ternary complex formation and other interactions with proteins<sup>86,87</sup> and low-molecular weight components of physiological media.<sup>91,92</sup> Our observations and the latter study suggest that the previously cited low nanomolar free  $[\text{Zn}^{2+}]$  determined for plasma<sup>58</sup> should be revisited.

Although the presence of BSA thus hindered the use of FZ3 to quantify non-protein bound  $\text{Zn}^{2+}$  in absolute terms, the effect of fatty acids was studied at lower BSA (60  $\mu\text{M}$ ) or FCS (10%) concentrations, employing the higher FZ3 concentration (2  $\mu\text{M}$ ), which was found to leave sufficient free FZ3 to respond to liberated  $\text{Zn}^{2+}$  (Fig. 3). FFAs were also confirmed to not affect background FZ3 fluorescence (Fig. S10, ESI<sup>†</sup>). Most importantly, there was a clear trend for myristate-induced  $\text{Zn}^{2+}$  release in the simple systems with BSA that was not observed with octanoate, essentially mirroring the observations from offline SEC-ICP-MS (Fig. 2). Myristate-induced  $\text{Zn}^{2+}$  release was also observed for the FCS systems. This indicates that FZ3 is able to also compete with the ' $\text{Zn}^{2+}$  acceptor' proteins apparent in the SEC data. Given that the FZ3 concentration (2  $\mu\text{M}$ ) and affinity for  $\text{Zn}^{2+}$  are both

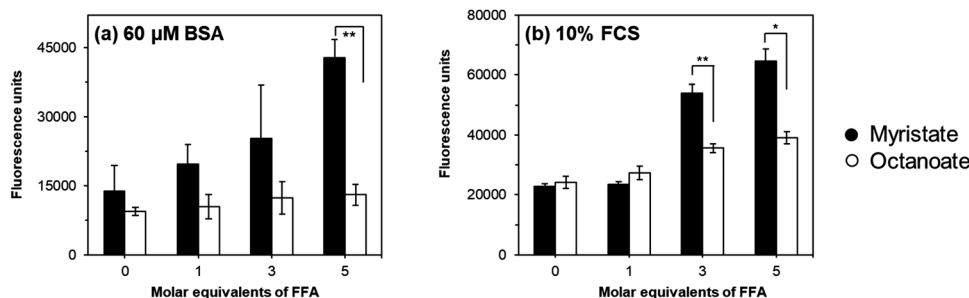


Fig. 3 Measurement of FluoZin-3 (2  $\mu\text{M}$ ) fluorescence in the presence of either: (a) 60  $\mu\text{M}$  BSA + 20  $\mu\text{M}$   $\text{Zn}^{2+}$  or (b) 10% FCS; with additional free fatty acids (FFAs). Myristate (black) increases the fluorescence of FluoZin-3, in contrast to the short-chain fatty acid octanoate (white), which had no significant effect on fluorescence. Statistics carried out using a two-tailed *t*-test assuming unequal sample variance (Welch's *t*-test), \**P* < 0.05, \*\**P* < 0.01. See ESI<sup>†</sup> for full statistical analysis.





expected to be higher than those for most of these proteins, this observation is qualitatively in line with expectations. In FCS, unlike for BSA alone, fluorescence was also increased by the addition of octanoate (Fig. 3). We suggest that this is likely due to the presence of intrinsic FFAs in serum (Fig. S5, ESI<sup>†</sup>), which may be re-distributed amongst the 7 principal FFA binding sites of albumin, upon addition of further equivalents of FFA. This, in turn, may lead to the (partial) population of site FA2 by longer-chain intrinsic FFAs, leading to the release of Zn<sup>2+</sup> from site A. We also note that fluorescence readings for the 10% FCS systems are higher than for the 60 μM BSA systems throughout, in line with the significantly lower BSA concentration in FCS (*ca.* 35 μM, according to quantitation with bromocresol green; *vide supra*).

### The potential Zn<sup>2+</sup> acceptor protein HRG is present in SEC fractions with FFA-promoted increases in [Zn]

Albumins from different species are known to bind Zn<sup>2+</sup> at site A with subtle variance in their affinities, and published values concerning the differences in the Zn<sup>2+</sup> affinity of human and bovine albumins are often conflicting, though both are generally accepted to be in the sub-micromolar range.<sup>93,94</sup> Given the wide-ranging involvement of both zinc and fatty acids in various pathological diseases in humans, not limited to Type 2 diabetes,<sup>25</sup> coronary heart disease,<sup>6</sup> thrombosis,<sup>45</sup> and Alzheimer's disease,<sup>95,96</sup> we also investigated the impact of FFAs on zinc speciation in human citrated plasma, containing human serum albumin (HSA), using the previously described methodology. Using SEC and ICP-MS, a similar re-distribution of zinc to larger human plasma proteins with Zn<sup>2+</sup>-binding capability (smaller elution volume) was clearly apparent, analogous to our findings in FCS (Fig. 2c). It was therefore of interest to identify such proteins, and identify target molecules which (a) display significant Zn<sup>2+</sup>-binding ability under physiological conditions, and (b) are larger than serum albumin (*i.e.* have shorter retention times in size exclusion chromatography). Plasma is known to contain tens of thousands of proteins,<sup>97</sup> and while the resolution of our SEC ICP-MS method is sufficient to clearly demonstrate a re-distribution of zinc to protein(s) of higher molecular weight, it is not sufficient to directly identify individual (zinc-binding) proteins, as seen by two broad bands in the chromatograms of both FCS and human plasma (Fig. 2). Complementary techniques (SDS-PAGE, western blotting) were therefore employed as a further step of protein identification in the eluent fractions.

Previous work from our team has suggested that such re-distribution may involve human histidine-rich glycoprotein (HRG), which has the capacity to bind up to ten Zn<sup>2+</sup> ions ( $K = 1.63 \times 10^5$ ).<sup>45</sup> In human plasma, HRG is present at low micromolar (1.3–2.0 μM) concentrations.<sup>98</sup> The *ca.* 72 kDa serum protein exists as a native dimer and was successfully identified in elution volumes 43–53 mL of human plasma from size exclusion fractions by western blotting and immunodetection using HRG-specific antibodies (Fig. 4; 66 kDa albumin eluted predominantly between 49 and 61 mL). HRG is principally involved with the regulation of blood coagulation, but is also associated with angiogenesis and cell proliferation.<sup>98</sup> The histidine-rich region (HRR) of

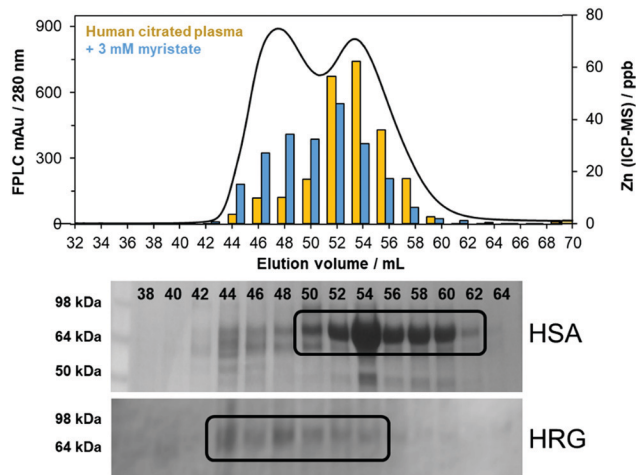


Fig. 4 Human serum albumin (HSA, 66 kDa) identified in fractions 49–61 mL by SDS-PAGE (Coomassie staining), and histidine-rich glycoprotein (HRG, 72 kDa) in fractions 43–53 mL, identified by western blotting and immunodetection using specific HRG antibodies. Data for protein content ( $A_{280}$ ) and zinc distribution (in the presence and absence of myristate) obtained using SEC-ICP-MS have been overlaid using data from Fig. 2c.

HRG is known to complex Zn<sup>2+</sup>, and upon Zn<sup>2+</sup> coordination, the affinity of the HRG-Zn<sup>2+</sup> complex for heparin (a well-known anticoagulant) is known to increase.<sup>99</sup> For this reason, we suggest that zinc speciation may be the ‘missing link’ between blood clotting disorders and elevated levels of fatty acids (leading to the re-distribution of Zn<sup>2+</sup> from albumin to proteins associated with blood clotting, affecting their function).<sup>100</sup> The greater affinity of the Zn<sup>2+</sup>-HRG complex for heparin prevents heparin from inhibiting clot formation,<sup>101</sup> and hence Zn<sup>2+</sup> is an important regulator of heparin neutralisation.<sup>102</sup> Though HRG detection does coincide with fractions associated with increased [Zn<sup>2+</sup>] after redistribution of plasma zinc, we emphasise that HRG is only one of potentially hundreds of possible acceptor proteins in the aforementioned fractions. It is quite likely that many other proteins in these fractions also have affinity for Zn<sup>2+</sup>. Summarily, it is evident that in human plasma, like in FCS, Zn<sup>2+</sup> ions liberated from albumin remain largely protein-bound, rather than occurring as ‘free’ aquated ions or in complexes with small molecules such as histidine or glutathione.

### Immobilized metal ion affinity chromatography to identify Zn<sup>2+</sup> binding proteins

As indicated, 1D-size exclusion chromatography cannot resolve and identify single proteins which may participate in the re-distribution of Zn<sup>2+</sup> from albumin. We therefore looked to a complementary chromatographic method: immobilized metal ion affinity chromatography (IMAC). This technique requires surface-exposed metal sites, and so should be ideally suited to capture proteins with sites that are involved in the “exchangeable zinc pool”. Proteins from unfractionated human plasma were captured on a Zn-IMAC column, eluted with imidazole, further separated by SDS-PAGE (Fig. 5a) and identified by peptide mass fingerprinting (Table 1). Plasma was also applied to a column



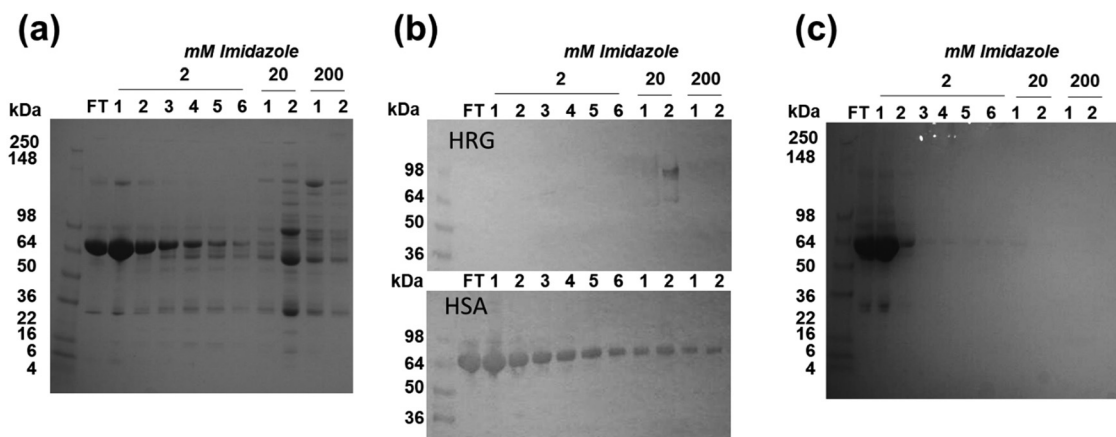


Fig. 5 Fractions from IMAC chromatography on a Zn-charged column (a) analysed using western blotting and immunodetection (b) with antibodies against HSA and HRG. For comparison, fractions from a metal-free IMAC column are shown in (c). FT = flow-through.

Table 1 *Homo sapiens* plasma proteins binding to Zn-IMAC columns, identified by peptide mass fingerprinting using Mascot to search results against the NCBI database. Only searches with significant MOWSE (MOlecular Weight SEarch) scores were recorded

Protein	Mass/kDa	Peptides matched	Sequence coverage (%)	MOWSE score
Haemoglobin subunit $\beta$	16.1	9	76	90
Immunoglobulin light chain	28.1	10	63	81
Haptoglobin	38.7	14	35	113
Chain B IgG1 Fc fragment	23.9	5	37	75
Immunoglobulin heavy chain	42.4	12	30	72
Albumin	70.6	16	31	119
Complement C3b	71.2	26	47	240
Chain A transferrin	77.3	34	48	228
Chain B complement C4	84.8	15	23	67
Plasminogen type II	91.1	22	34	136
Complement C3	114.2	30	33	210
Ceruloplasmin	116.2	14	16	103
$\alpha$ 2-Macroglobulin (Zn)	164.6	44	39	279
Inter-alpha inhibitor H1	99.8	13	21	81

that had not been charged with any metal ions to check for any non-specific binding to the resin (Fig. 5c), and in this case no proteins were found to be retained on the column.

Sensitivity is a clear limitation of a 1D SDS-PAGE approach, however comparison with the uncharged gel demonstrates that several proteins were selectively enriched in the fractions eluted with 20 and 200 mM imidazole. Molecular weights ranged between 16 and 165 kDa (Table 1). Identified proteins also included haemoglobin, likely due to partial lysis of erythrocytes during the plasma manufacturing process. The remaining entries mostly refer to known high-abundance plasma proteins.

A large quantity of the most abundant serum protein, human serum albumin (HSA) was observed in all fractions, evidenced by western blotting and immunodetection using HSA antibodies (Fig. 5b). Interestingly, comparison of the gels for Zn-loaded and Zn-free IMAC demonstrates that the occurrence of HSA across all fractions is not a mere consequence of its high abundance, since the albumin-related bands are very faint in either wash or elution fractions from the metal-free IMAC column (Fig. 5c). In contrast, albumin was present in both wash and elution fractions of the Zn-charged column (Fig. 5a). Nonetheless, most albumin was found in either flow-through or

wash fractions, indicating a rather low affinity of HSA for a Zn-IMAC column. This finding can be understood when the locations and structures of the known metal binding sites in HSA are considered: site A (the major  $\text{Zn}^{2+}$ -binding site) is not a surface site, but is partially buried between domains IA and IIA (Fig. 1). The interaction of this site with immobilised  $\text{Zn}^{2+}$  is therefore sterically unlikely. It might be envisioned that partial unfolding of albumin might lead to exposure of the two half-sites, but since these are only composed of either one (His67) or two (His247 and Asp249) residues, this would not generate a strong interaction with the column either. Another known binding site is the N-terminal ATCUN motif, which is the primary binding site for  $\text{Cu}^{2+}$  and  $\text{Ni}^{2+}$ . Although  $\text{Zn}^{2+}$ -binding has been demonstrated for short peptide mimics of this motif, the affinity of this site for  $\text{Zn}^{2+}$  at neutral pH is rather weak.<sup>37,103</sup> Furthermore, calorimetric and crystallographic evidence of a secondary  $\text{Zn}^{2+}$  site of equine albumin, involving His<sub>9</sub>, Asp<sub>13</sub> and Asp<sub>254</sub> (fully conserved in HSA),<sup>37</sup> might present a possible candidate for the secondary  $\text{Zn}^{2+}$  binding site (site B). This site is more exposed than site A, therefore its contribution to the IMAC affinity of albumin cannot be ruled out. However, its affinity for  $\text{Zn}^{2+}$  is almost



2 orders of magnitude weaker than site A.<sup>45</sup> In conclusion, neither of the well-defined metal sites in HSA are expected to display strong interaction with a Zn-IMAC column, although Cu- and Ni-IMAC columns may display stronger HSA binding.<sup>67</sup> The two most abundant Fe- and Cu-binding plasma proteins, respectively transferrin and ceruloplasmin, were also amongst the captured proteins. It is unknown whether their capture on the Zn-IMAC column involves their known metal-binding sites.

Amongst the strongly enriched proteins were immunoglobulin light and heavy chains (Table 1). Several components of the complement system were also detected, including complement C3 and C4. These proteins, as well as further complement components, are in fact already known to interact with Zn<sup>2+</sup>. In particular, Zn<sup>2+</sup> inhibits factors I and H, and promotes protein–protein interactions between C3 and factor H.<sup>104,105</sup> Many of these proteins are expected to elute in the SEC fractions with fatty-acid mediated increases in zinc. Another protein with higher molecular weight,  $\alpha$ 2-macroglobulin, is partially homologous to complement proteins.<sup>106</sup> As previously explained,  $\alpha$ 2-macroglobulin harbours the largest proportion of non-exchangeable zinc in plasma,<sup>48</sup> but the site(s) responsible for this fact are likely to differ from those responsible for the affinity to the IMAC column, as metal sites with reasonably fast exchange kinetics are prerequisite for binding to an IMAC column. Furthermore, isotope-exchange experiments using <sup>65</sup>Zn indicated that  $\alpha$ 2-macroglobulin does not exchange, or bind extra Zn<sup>2+</sup> under relevant conditions,<sup>107</sup> rendering it an unlikely candidate in the re-distribution of Zn<sup>2+</sup> from albumin.

Though histidine-rich glycoprotein (HRG) was not amongst the proteins identified by peptide mass fingerprinting of SDS-PAGE bands, western blotting and immunodetection revealed the presence of HRG in the second IMAC elution fraction (20 mM imidazole), confirming the capacity of this protein for binding (partially complexed) Zn<sup>2+</sup> (Fig. 5b). This has been reported previously,<sup>108–110</sup> and as mentioned, many HRG activities are accepted to be Zn<sup>2+</sup>-dependent.<sup>110,111</sup> This is in agreement with our earlier detection of HRG in size exclusion chromatography (SEC) fractions with increased Zn<sup>2+</sup> after metal re-distribution in the presence of FFAs.

Given the association between elevated plasma fatty acid levels and the incidence of thrombotic disease,<sup>112</sup> a possible Zn<sup>2+</sup> re-distribution from albumin to an important coagulation-regulating protein suggests that the described allosteric switch, linking plasma FFA levels and Zn<sup>2+</sup> distribution, may have significant implications in the progression of various cardiovascular disorders. Physiologically-relevant long-chain saturated FFAs such as palmitate (C16) and stearate (C18) have even higher affinity for albumin,<sup>45,113</sup> and so it is likely that such an effect would be at least as apparent, if not more pronounced, *in vivo*. Furthermore, HRG has a comparatively high abundance (1–2  $\mu$ M), relative to many other plasma proteins. Considering the affinities of HSA ( $K_{1(\text{ITC})} = 1.35 \times 10^5 \text{ M}$  and  $K_{2(\text{ITC})} = 3 \times 10^3 \text{ M}$  for site B) and HRG ( $K_{\text{ITC}} 8.06 \times 10^4 \text{ M}$ ) for Zn<sup>2+</sup>,<sup>45</sup> we suggest that HRG might be a likely candidate acceptor protein during serum zinc re-distribution, with abundant proteins of the complement system (C3, C3b, C4, factor H1) also being likely candidates. It is well-known that zinc dynamics play roles in coagulation and

immune response, and therefore there must be conditions under which associated proteins become populated with Zn<sup>2+</sup>. Since albumin (the major Zn<sup>2+</sup> binding plasma protein) is always present, it too must be involved in such dynamic networks. Furthermore, by virtue of the allosteric switch, changes in FFA levels are hence predicted to impact on both blood coagulation and the immune system, as a result of Zn<sup>2+</sup> speciation changes in blood plasma.

## Conclusions

SEC combined offline with ICP-MS for the analysis of extracellular fluids has provided insight into changes in Zn<sup>2+</sup> speciation, induced by the presence of myristate, a long-chain (C14:0) non-esterified fatty acid. This is a consequence of a now well-established allosteric mechanism on serum albumin, the key Zn<sup>2+</sup> carrier protein in the bloodstream. The operation of this molecular mechanism and its immediate consequences were for the first time observed in complex biological systems by chromatographic techniques, with complementary evidence provided by the use of a Zn<sup>2+</sup>-sensing fluorescent reagent, FluoZin-3. In pure albumin systems (*i.e.* in the absence of any other Zn<sup>2+</sup> acceptors), any Zn<sup>2+</sup> displaced from albumin was scavenged by the SEC resin. In foetal calf serum and human plasma, Zn<sup>2+</sup> liberated from albumin was overwhelmingly re-distributed to other plasma proteins that were largely of higher molecular weight than albumin. One highly likely candidate Zn<sup>2+</sup> acceptor protein is HRG, a multifunctional protein whose activities are modulated by zinc binding. Further potential Zn<sup>2+</sup> acceptor plasma proteins were identified by capture on an IMAC column, followed by SDS-PAGE and peptide mass fingerprinting, and include several proteins of the complement system.

From this, we infer that the decreased Zn<sup>2+</sup> binding capacity of albumin in the presence of FFAs may have significant implications, for both normal and disease-altered physiology. Whilst numerous pathological diseases are associated with elevated levels of free fatty acids, not limited to cardiovascular diseases (including thrombosis),<sup>114–116</sup> diabetes,<sup>117–119</sup> and neurodegenerative diseases,<sup>120–122</sup> FFA levels also fluctuate during periods of intense exercise, fasting and after the consumption of food.<sup>123–125</sup> A deeper understanding of the downstream consequences of FFA-mediated Zn<sup>2+</sup> re-distribution is now required to comprehend the wider impact this may have on organismal physiology.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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