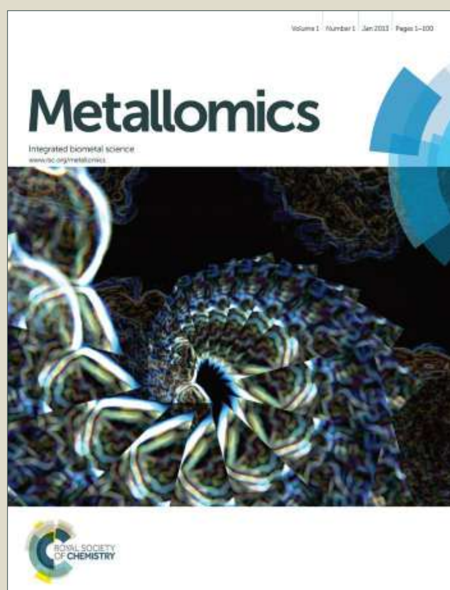
CrossMark
click for updates

View Article Online
View Journal

Metallomics

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: M. Linder, *Metallomics*, 2016, DOI: 10.1039/C6MT00103C.



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/metallomics

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

Ceruloplasmin and Other Copper Binding Components of Blood Plasma and Their Functions: An Update

M.C. Linder[a]

We know that the blood plasma contains many proteins and also other components that bind copper. The largest contributor to copper in the plasma is ceruloplasmin, which accounts for 40-70 percent. Apart from ceruloplasmin and albumin, most of these components have not been studied extensively, and even for ceruloplasmin and albumin, much remains to be discovered. New components with new functions, and new functions of known components are emerging, some warranting reconsideration of earlier findings. The author's laboratory has been actively involved in research on this topic. This review summarizes and updates our knowledge of the nature and functions of ceruloplasmin and the other known and emerging copper-containing molecules (principally proteins) in this fluid, to better understand how they contribute to copper homeostasis and consider their potential significance to health and disease.

Introduction: The roster of copper-containing components of blood plasma

In general, the copper concentrations of body organs and fluids are somewhat similar from one mammal to the next (with few exceptions) and within a given species these concentrations are kept quite constant. In adults, liver concentrations are in the 4-6 ug/g range; kidney concentrations are somewhat higher (7-12 ug/g); brain 3-5 ug/g, muscle about 1 ug/g, and so on [1]. Concentrations of copper in blood plasma and serum of normal adults in various mammals may be more variable and may fall into 3 groups that are 250-400 ng/ml (mice and dogs), about 1000 ng/ml (humans, rats, cows, sheep), and 1500-2000 ng/ml (swine). These differences suggest that specific copper containing plasma components have a greater importance in one species and less importance in another, which in turn may point to unique aspects of copper utilization or metabolism occurring to support the health and well being of that organism. Concentrations of individual plasma copper-binding components (and total plasma copper levels) do change in response to specific natural and un-natural circumstances. Thus for example, ceruloplasmin and total copper concentrations increase in pregnancy and cancer and decrease when there is dietary copper deficiency. The reasons for and mechanisms involved in these changes are described in this review.

Table 1 lists all the known copper-binding components in mammalian blood plasma, along with others that have not as yet been fully identified or confirmed, or for which evidence is preliminary. They are listed roughly in decreasing order of their established importance and the depth of knowledge we have about them, starting with ceruloplasmin (Cp) (generally recognized as the component contributing the most copper to blood fluid), then albumin and alpha$_2$-macroglobulin/transcuprein, followed by extracellular superoxide dismutase (SOD3) and other much less abundant copper containing molecules, including some as yet to be defined and/or that are very small (small copper carriers). There is quite a list, but also a great disparity between the copper contributed by Cp, albumin and the macroglobulin versus the others. What is known about the character and functions of these various proteins and other molecules will be detailed in the next section; but the list contains several enzymes, along with copper transporters that provide this element to cells via the blood and interstitial fluid, and factors involved in other functions, notably blood clotting.

[a]California State University, Fullerton, CA, U.S.A

Blood plasma proteins have been separated by many different techniques, ranging from native electrophoresis (which separates albumin from alpha, beta and gamma globulins), to ethanol and ammonium sulfate fractionation, and various kinds of chromatography. Figure 1 shows the relative abundance of albumin and the various globulins in human plasma separated by native (non-denaturing) electrophoresis on paper strips, and lists known copper binding proteins in a given group. As is well known, the copper binding protein albumin, accounts for most of the

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 03 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

protein present in plasma and serum (about 70% of the total, or 45 mg/ml). However, only a small fraction of albumin normally carries copper (see later). The two other major copper-binding proteins, ceruloplasmin and alpha-2-macroglobulin, are alpha-2 globulins. Most plasma proteins except the immune (gamma) globulins are produced by liver hepatocytes. Indeed, about 14 g of albumin are synthesized daily by the human adult, and this accounts for about a third of the total protein synthesized daily from dietary and recycled amino acids by the whole body e.g. this process accounts for about third of the daily protein requirement [2]. All three of the principle copper-binding proteins mentioned (Cp, albumin and $alpha_2$-macroglobulin/transciprein) have multiple functions (Table 1), to be described in the next section.

Figure 2 shows how the plasma proteins containing copper separate by size exclusion/gel chromatography (SEC) in various media. In all cases, the elution profiles show a major copper peak, with shoulders on either side, and traces in a shoulder at 45 kDa and low molecular weight components. Immuno- and enzyme activity assays for Cp indicate it is present in the main peak, as confirmed by comparing the Cu profiles of plasma from Cp-knockout mice (Figs. 2C and D). That albumin is responsible for at least part of the Cu in the first shoulder on the right of the Cp-containing peak was established by immunoassays, binding of bromcresol green, and immuno-precipitation of ^{67}Cu in a radioactive copper peak eluting in this position, after chromatography of whole plasma labeled *in vitro* with this copper radioisotope (Fig. 2E) [3,4]. The albumin copper peak in SEC was also greatly increased in mice given large doses of the heavy isotope, ^{65}Cu (Fig. 2F). The Cu “shoulder” to the left of the Cp peak is transcuprein/macroglobulin, and was first detected as a separate entity in plasma soon after injection of ^{67}Cu tracer into rats, and upon *in vitro* addition of radioactive copper ions (as in Fig. 2E), from which it was purified and identified in further studies. In general, only traces of copper elute with low molecular weight components, and if radiotracer is administered, these components are not radioactive even many hours after administration, as shown in rats, or after *in vitro* addition to whole plasma prior to chromatography (Fig. 2E).

At first glance, these findings suggest that there are not many different molecules in the plasma that contain copper, and that only two or three might still need to be discovered. Other research however indicates that the major peaks and shoulders seen in SEC contain additional copper binding components, though their relative contributions (to total plasma copper) in various physiological conditions requires further investigation.

Nature, function(s) and regulation of plasma copper components: Ceruloplasmin (Cp)

Cp structure and expression

Cp is encoded by a single gene. The main Cp form (in plasma, cerebrospinal fluid and milk) contains 1046 amino acids and has a total mass of about 132 kDa, 120 kDa of which is protein and 12 kDa is N-linked carbohydrate. X-ray crystallography of the holo (Cu-containing) protein shows a compact structure (about 214 x 85 Å) with 3 sequential homologous regions that each have two parts (thus 6 domains) (Fig. 3A). HoloCp has 6 tightly bound Cu atoms: one each in domains 2,4 and 6, the others in a trinuclear cluster between the first and last domains [1,5-8]. Copper also stabilizes the compact 3-D structure of holoCp [9], apoCp having a much looser conformation (see later). Like most blue multicopper oxidases, the Cu ligands are histidine or a mixture of his + cys/met residues. The Cu buried within holoCp does not come on and off the protein in plasma under physiological conditions, and so must interact with other proteins or cell surface components (or strong reducing agents) to be released. Plasma Cp is mainly produced by hepatocytes, where Cu is incorporated within the transGolgi network (and possibly endosomes), after delivery by the “chaperone” ATOX1 to the Cu “pump” ATP7B (the Wilson disease protein). Secretion is by exocytosis. Cells in several other secretory organs also produce Cp, including kidney, mammary gland, placenta, and the choroid plexus of brain [1,10-12]; also macrophages and mononuclear cells in the blood during inflammation [5,13,14]. At least two other forms of Cp are expressed. One is on the cell surface, linked to the membrane by glycophosphatidyl inositol (GPI). Here, alternative splicing of exons 19 and 20 at the C-terminus of the transcript results in addition of 30 alternative amino acids that substitute for the last 5 of the plasma form of Cp, marking it for GPI linkage [15,16]. GPI-linked Cp was first discovered in the brain (associated with astrocytes [15] and neuroglia [17]) but is quite widely expressed, though probably in relatively small amounts compared to that of soluble Cp in the extracellular fluids [18]. Prohaska’s group has demonstrated significant expression in

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C
Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.
Metallomics Accepted Manuscript

membranes of spleen and kidney, with lesser amounts in heart and liver. GPI-Cp is also on the surface of the Sertoli cells in the testis [19], leptomeningeal cells of the central nervous system [20]; and in immune cells (lymphocytes/monocytes and macrophages), where it is associated with lipid rafts and ferroportin [13,14]. The GPI-linked form is assumed to also contain 6 Cu atoms. An additional Cp form, 4 amino acids longer, is selectively expressed in placenta [21]. The usefulness of these alternative Cps remains to be fully explored. It is worth noting that Cp circulating in the blood is not only in the holo form (with 6 copper atoms). Indeed, immunoassays suggest that as much as 50% or more may be in the apo form [22-24]. Some apoCp appears to be secreted along with holoCp by (hepatocytes), and is the main form secreted in copper deficiency [25]. ApoCp is also produced during transfer of copper from holoCp to cells at the cell surface (see later). In the circulation, apoCp turns over more rapidly than holoCp, being removed probably mainly by hepatocytes via the galactose receptor [1].

Roles of Cp in mammalian metabolism

Cp is one of the best examples of a mulifunctional protein. Indeed, Bielli and Calabrese have dubbed it a "moonlighting protein", and for good reason [5]. All the established and documented functions of Cp are based on the fact that it contains and carries copper i.e. the known functions are of the holo form. Whether the almost equally abundant apo form in the plasma plays some kind of independent role in mammalian metabolism (or copper metabolism) has not been explored. Most of the roles of holoCp are enzymatic, involving the absorption and transfer of electrons from various substrates to oxygen. Several classes of substrates have distinct binding sites on the molecule [5-7].

Ferroxidase activity and iron metabolism

Cp is best known for its ability to oxidize Fe^{2+} to Fe^{3+} (ferro-oxidation), and it is the principal ferroxidase in the blood plasma [26,27]. It is generally accepted that the ability of Cp to oxidize iron facilities the release of this metal from cells and allows iron binding to transferrin in extracellular fluid and blood. A role of Cp in this process was originally indicated by studies with copper deficient pigs [28] and dogs [29], where as in other mammals, blood Cp-Cu levels were low and accumulation of iron occurred in the liver and some other tissues. In livers of these animals, excess iron was rapidly released upon perfusion of the organ with holoCp. Frieden's group then discovered that Cp has ferroxidase activity [1,30]; and it was later demonstrated that the main pathological feature of genetic Cp deficiency (aceruloplasminemia), detected in humans and replicated in mice, was excessive iron accumulation in cells of the liver, brain and pancreas [26,31,32]. Thus, there is good evidence that enzymatically-active Cp enhances the efflux of iron from liver, and perhaps also from cells in other tissues like those in brain and pancreas, as well as macrophages, the latter particularly under conditions of hypoxia [33]. The GPI-linked form of Cp is also thought to play this kind of role. Research on the effects of iron accumulation in the brain in aceruloplasminemia also support this concept [31,34], as do studies with macrophages, in which GPI-Cp has been co-localized with ferroportin in lipid rafts within the plasma membrane [13]. Moreover, expression of this form of Cp was increased upon exposure of macrophages (but not hepatocytes) to 50-200 uM concentrations of iron-nitrilotriacetate. As concerns the steps involved in mediating iron efflux, it is thought that iron released from cells through the exporter, ferroportin, is in the Fe^{2+} state; and that oxidation by Cp allows the iron to bind to its extracellular and plasma carrier protein, transferrin, which binds Fe^{3+}[35]. Indeed, a physical interaction between Cp and transferrin has been demonstrated *in vitro* and through modeling [36]. More recently, the steps involved in electron and iron transfer between them have been elucidated. Under anaerobic conditions *in vitro*, Eid et al. [37] mixed Cp, transferrin and Fe^{2+} and followed the electron flow (from Fe^{2+}) within the copper atoms in Cp using chemical relaxation and spectrophotometric techniques. This showed that the Fe is first oxidized and transferred to two "holding sites" on Cp in a di-transferrin-Cp complex, and that both Fe^{3+} atoms then move to the C-lobes of the two transferrins, resulting in conformational changes that then releases them as two monoferric transferrins. In line with this, Sarkar et al. [33] had reported that holoCp stimulated iron efflux from macrophage-like cells and binding to apotransferrin, but only under hypoxic conditions.

Although anaerobic conditions generally do not prevail in the blood plasma, these kinds of studies are convincing that this series of events can happen. At the same time, questions about the relative importance of this function of Cp remain to be answered. For example, why does the release of iron by Cp appear to be tissue selective? Cp mediation of iron efflux seems to be important for the liver [31,38] and probably macrophages [33] but not for enterocytes involved in dietary iron absorption [39], where the ferroxidase, hephaestin, appears to play that role

View Article Online
DOI: 10.1039/C6MT00103C

[40]. (Hephaestin is a close "cousin" to Cp and has a similar structure, except that it is tethered to the plasma membrane (and internal vesicles) by a transmembrane domain at the C-terminus of the Cp-like unit [40]. Inactivation of hephaestin results in retention of iron by the enterocyte and causes iron deficiency.) Also, it was reported long ago by Phil Aisen's group that transferrin itself has ferroxidase activity [41], so another ferroxidase (like Cp or hephaestin) might not be needed. Another potential anomaly is that increased levels of Cp do not correlate with a greater rate of iron efflux into the blood; in fact the opposite is true in inflammation, where levels of enzymatically-active Cp increase [1] but levels of iron being transported on transferrin decrease [42,43]. Increases in total plasma copper (mainly due to Cp) and decreases in iron (on transferrin) are hallmarks of the acute phase response to infection and inflammatory agents. An added puzzle is the slow speed at which excess iron accumulates in the absence of Cp expression, such as occur in aceruloplasminemia or severe copper deficiency. If Cp in extracellular fluid were essential for cellular Fe efflux, the rate of excess Fe accumulation in organs such as the liver would be rapid in its absence, which is not the case. Large amounts of iron cycle in and out of liver, spleen and bone marrow cells every day (18-22 mg) [38,44] as part of the genesis and removal of erythrocytes; but accumulation of toxic amounts of Fe in liver takes decades. Whether there also is accumulation of excess iron in spleen is either unknown or not mentioned in the literature, as far as this author can ascertain. Another observation is that mice and humans without circulating Cp (aceruloplasminemia) have hepatic iron overload but do not develop anemia despite lacking this iron efflux facilitator [45]. This however could be explained by the presence of additional ferroxidases in the blood (see section on other ferroxidases, below). Finally, one can wonder why, particularly in the oxygen-laden blood, Fe^{2+} would not oxidize spontaneously, as the studies of Sarkar et al suggest [33]. This possibility was addressed by Wong et al. [46], who took a kinetic approach, and compared relative rates of Fe^{2+} oxidation in biological polyanion-containing buffers at physiological pH (and not under anaerobic conditions) with those when Cp was present. Their findings indicated that rates of oxidation in such physiological buffers were adequate for iron binding to transferrin in the absence of Cp. However, they did not consider that transferrin itself might also have participated in the ferroxidation. Overall, these various findings suggest that perhaps Cp (and hephaestin) are only important for ferroxidation of iron under conditions of relative hypoxia, which undoubtedly do occur but are not normally present. In addition, the possibility exists that the mechanism of the Cp (and Hp) effect is primarily at the physical rather than enzymatic level; Fe^{2+} atoms coming out of ferroportin bind to the cation binding site on Cp, and Fe is delivered when Cp binds to transferrin, with or without oxidation by Cp (or transferrin). It should also be noted that it is an assumption rather than a proven fact that the iron efflux transporter, ferroportin, transports Fe^{2+}rather than Fe^{3+}. The strongest argument is that the cell cytosol has high levels of reductants like glutathione, which are likely to maintain iron in the reduced state; but we do not yet know in what form the iron that enters ferroportin is delivered from the cytosol or to what it is even bound in the cytosol. Additional even less easily explained complications contradicting the ferroxidase theory of Cp action have been reported by Fox and Mukhopadhyay, including that Cp does not enhance release of Fe from hepatocytes, and that Cp enhances uptake of non-transferrin-bound iron by those cells [47,48].

A different promotional role of Cp in cellular iron efflux has been suggested by data from Kono et al. [49] and others [35]. Functions of both surface GPI-linked Cp and soluble (plasma) Cp were studied in cultured glioma cells treated with the iron efflux suppressor, hepcidin [31]. Hepcidin is secreted by hepatocytes in response to high levels of iron or inflammatory cytokines, and causes the internalization and lysosomal destruction of ferroportin, thus reducing iron release [35]. In the glioma system, removal and degradation of ferroportin was antagonized by both forms (GPI-linked and soluble) of copper-containing (holo) Cp, as was the case with iron depletion induced by chelators, suggesting that Cp normally stabilizes ferroportin [31,35]. ApoCp did not have this hepcidin-antagonistic effect [49]. Other evidence supporting this Cp role comes from studies with neural cells and macrophages, where Cp depletion resulted in loss of cell surface ferroportin expression due to enhanced degradation (see Ward and Kaplan [31]). Human subjects with aceruloplasminemia have also been shown to have low levels of ferroportin, at least in their livers, but they also have low levels of hepcidin suggesting a more complicated mechanism.

Small hormone oxidation by Cp

Another well-known activity of Cp is the ability to oxidize various biogenic amines. Binding of these substrates occurs at a different site on the enzyme than that for iron. Measuring Cp enzyme activity by following oxidation of the synthetic amine-containing substrates, like p-phenylene diamine and o-dianisidine is a standard procedure for identifying and quantifying Cp, and at least for human Cp this activity (like ferroxidase activity) is completely

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.
Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

inhibited by azide (N_3-). *In vivo*, the most likely physiological amine substrates are dopamine, 5-OH tryptamine (serotonin), epinephrine and nor-epinephrine [50], but not histamine (although histamine does bind) [6,51]. These amines are hormones or hormone precursors, and oxidation would result in their inactivation. As all hormone signals must be rapidly eliminated to prevent prolonged unregulated effects, it seems likely that Cp contributes to the inactivation of these natural bioactive amines. However, Cp is not the only enzyme involved. There are other copper-dependent amine and diamine oxidases in the plasma, including one that oxidizes histamine (see later section).

Another signaling agent that appears also to be metabolized by Cp is NO, which is probably converted to NO+ and then hydrated to form nitrite [52,53]. Discovery of this Cp role stemmed from observations that in the absence of plasma, blood cells formed little nitrite from NO [52]. Removal of Cp from plasma by immunoprecipitation greatly decreased plasma NO oxidase activity, and Cp knockout mice were found to have lower levels of nitrite in their blood. These mice were also abnormally susceptible to liver infarction (following ischemia and reperfusion), which could be reversed by administration of nitrite. Thus, the role of Cp related to NO may be more complex than just the removal of the NO signal and Cp may also be a means of providing nitrite to the circulation that itself protects against blood vessel constriction. Although Cp can have these activities, it may not be a major player. For one, much of the NO formed is metabolized within cells rather than in the plasma and interstitial fluid [53]. Vrancken et al. [54] have thoroughly reviewed the evidence for Cp involvement and its relative importance under physiological conditions. They point out that most NO is converted by red blood cells to nitrate (rather than nitrite) upon reaction with oxygenated hemoglobin, and that Cp is not essential either to the rate of NO oxidation by plasma nor to production of nitrite. They also found no correlation between NO metabolism and levels of plasma nitrite relative to levels of Cp in fetal versus adult plasma (of humans and sheep), and could not explain the increased NO oxidation of plasma relative to aqueous buffers on the basis of Cp. Thus, although capable of inactivating NO and perhaps forming nitrite, the normal contribution of Cp to this process in the blood may be small.

Cp may however have still another connection to NO. As demonstrated for microglial cells of the brain, Cp may enhance the increase in NO synthase activity (iNOS) induced by inflammatory agents [55]. In rat primary microglial cell-enriched cultures, iNOS activity did not respond to Cp alone, but Cp enhanced the increase in activity induced by LPS, and raised the level of iNOS protein. Cp also enhanced microglial cell production of the pro-inflammatory cytokine, IL-6. These phenomena indicate that Cp may have several as yet unexplored roles related to the inflammatory response. (See section on Cp regulation for further information about the role of Cp in inflammation.)

Antioxidant defense

Cp is one of the main extracellular radical scavengers. Its ability to dampen or nullify the effects of reactive oxygen species was demonstrated long ago with purified Cp *in vitro* principally by the groups of Goldstein and Gutteridge [1,56-58]. Cp was shown to inhibit a variety of oxidative reactions, involving peroxide and superoxide, including the Fenton reaction that forms OH radicals from H_2O_2, and dismutation of superoxide [1]. Numerous studies also demonstrated specific inhibition by Cp of damage to biomolecules, such as formation of deoxyribose induced by hydroxyl radicals and superoxide [58], and many others. Thus already in the 1980's Cp was viewed as a scavenger and neutralizer of reactive oxygen species, capable of taking up extra electrons and conveying them to O_2 to form water. More recent studies of the capability of Cp to neutralize radicals are hard to find, partly because the role of Cp as a ferroxidase has overshadowed the specifics of its other functions. As already described under "ferroxidase activity and iron metabolism", a relative lack or absence of Cp (either in the circulation or bound to the surface through GPI) results in iron accumulation in some cells; iron abundance can increase formation of reactive oxygen species and cause damage to biomolecules and cell death; and the presence of Cp would most likely also prevent radical damage. Indeed, patients not expressing Cp (aceruloplasminemia) have increased lipid peroxidation in portions of the brain associated with iron accumulation (the caudate nucleus and patumen) [59], as well as in the cerebral and cerebellar cortices [60]. However, a second reason for the resulting damage in aceruloplasminemia would be due to lack of the antioxidant function of Cp to deal with the radicals and prevent cellular damage. Consistent with these ideas, low concentrations of Cp activity and protein in the cerebrospinal fluid are found in Parkinson's disease, where iron has accumulated in and damaged the substantia nigra [61], but without

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

aceruloplasminemia. Interestingly, Parkinson's patients also have lower levels of Cp in their blood plasma [62]. The reason for less Cp expression in this disease (both in brain and blood) remains to be elucidated.

Circulating source of copper for cells

Although the copper in holoCp of plasma and other fluids is buried in its structure and cannot be dialyzed away under normal physiological conditions, there has long been evidence that the copper in Cp is delivered to cells in many tissues [1,63-66], and indeed that some tissues (like placenta and heart) prefer Cp-Cu over that bound to albumin and transcuprein/macroglobulin [63,67]. Consistent with the possibility that Cp delivers copper directly to cells were reports from several different laboratories of specific binding of Cp to the surface of several kinds of cells and to plasma membrane fractions from several tissues [1]. Recent work from our laboratory has confirmed and extended these studies, using cultured human and mouse cells incubated with purified human and mouse ^{64}Cu-radiolabeled Cp, respectively [44]. These studies showed that transfer of copper from Cp to cells occurred at the cell surface, and that during incubation with cells (but not without cells), holoCp was converted to the apoform, with no change in the total amount of Cp (as determined by Western blotting). Figure 4 shows some examples of the findings in that article. Adding various endocytosis inhibitors failed to inhibit uptake of the radioactive copper delivered by Cp (Fig. 4A); holoCp disappeared when Cp was incubated with cells (and not without cells) while levels of apoCp increased (Fig. 4B). (It is noteworthy that in contrast to what occurs in the circulation of the whole organism, where apoCp is removed fairly rapidly, incubation of purified Cp in tissue culture medium with non-hepatic cells for 24 h did not lower total Cp levels and increased rather than decreased apoCp.) Other findings were that cell uptake of the copper in Cp was mediated by CTR1 but also by another as yet unidentified transporter (Fig. 4C). Addition of silver ions – which inhibit CTR1 Cu uptake – only reduced uptake by about half in normal mouse fibroblasts and HepG2 cells, and cells not expressing CTR1 took up copper from Cp. Uptake of Cp-Cu (which was radiolabeled with ^{64}Cu) was inhibited by higher concentrations of non-radioactive Cu^{2+} and Cu^{1+} ions (delivered as the nitrilotriacetate complex) (Fig. 4D). Inhibition by excess non-radioactive Cu^{1+} would be expected, since both CTR1 and the unknown copper transporter present in these mouse embryonic fibroblasts [68,69] appear to take up monovalent copper [69]. The copper in Cp must therefore (at least in part) have been reduced from the divalent form, and excess non-radioactive Cu^{2+} would have competed with radiolabeled $^{64}Cu^{2+}$ coming off Cp. Most likely, the reduction was mediated by one of the known cell surface reductases that also reduce Fe^{3+} [44,70], although this still needs to be established. Thus, although other proteins can deliver copper to cells [71] and substitute for Cp, since humans and animals with aceruloplasminemia do not suffer from obvious copper deficiencies [72], Cp is an important circulating source of this trace element for cells.

Regulation of Cp expression

The main determinants of Cp levels in the blood plasma are inflammatory cytokines, estrogens/progestegens, and copper deficiency [1]. Cp is very sensitive to copper deficiency. Its activity (dependent on copper) and amount decline when dietary intake of copper is low, and measurements of Cp activity have thus been helpful in diagnosing copper insufficiency. Anoxia may also play a role, at least in some abnormal conditions (see later). What regulates levels in other body fluids has not been examined. As an acute phase reactant in all species tested, levels of Cp activity and protein in blood plasma increase as the immune system responds to infections and inflammation. Inflammatory responses are largely mediated by cytokines, and specific responses of plasma Cp to IL-1, IL-6 and TNF were recorded in rabbits and cultured human hepatoma cells already long ago [73]. The liver is the main organ responsible for the changes in plasma proteins that accompany the acute phase response, it being the tissue of origin for most proteins circulating in the blood fluid. Cp is among the group of proteins responding positively to inflammation and infection (i.e. synthesis and secretion are increased). Other positive response proteins involved with copper and iron metabolism include serum ferritin, and coagulation Factor VIII. "Negative" acute phase copper and iron related plasma proteins include albumin (the most affected in terms of protein amounts) and transferrin. All these changes result in decreased transport of iron and increased levels of copper in the plasma during the acute phase response. As mentioned previously, acute-phase upregulation of Cp production thus occurs in conjunction with decreased (rather than increased) efflux of iron from cells principally in the liver, spleen and intestinal mucosa, which are mediated particularly by increased release of hepcidin from the liver. Hepcidin enhances degradation of ferroportin, reducing its deployment on the cell surface [74]. The resulting reduction in iron efflux from these main iron-holding tissues (and import from the diet) causes iron accumulation (see earlier section on ferroxidase activity and iron metabolism). For example, recent studies by Naz et al. [75] in

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 03 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

rats and mice given turpentine injections to induce the acute phase response, recorded increased liver mRNA and protein levels for hepcidin, transferrin and transferrin receptor (as well as DMT1) and decreased levels for ferroportin isoforms (as well as HFE, hemojuvelin and hephaestin), all of which contributed to or correlated with increased total, cytosolic and nuclear iron concentrations. The spleen also had increased expression of hepcidin and decreased ferroportin. Treatment of mice with IL-6, IL-1b and TNFa had similar effects, and these changes were blunted (though not absent) in IL-6 knockout mice. Not only is ferroportin activity decreased in the acute phase response, but cellular uptake of iron is enhanced, as shown for example in primary cultures of hepatocytes responding to the same cytokines, but particularly to IL-6 [76]. Indeed, IL-6 has emerged as the main cytokine responsible for changes affecting iron transport and regulation of Cp levels in the acute phase. There are 3 IL-6 response elements in the enhancer and promoter regions of the Cp gene [77], and the pathway for IL-6 response (which occurs through non receptor tyrosine kinase receptors) appears to involve FOXO1 at least in hepatocytes [78].

The details of the role(s) Cp plays in immunity remain to be worked out and are likely to be complex. Indeed, we now know that peripheral blood lymphocytes express both the soluble and GPI-linked forms of Cp constitutively [14], and that lymphocytes and monocytes as well as macrophages express the GPI form on their surfaces, and respond to iron by increased sequestration of GPI-Cp in lipid rafts, co-localizing with ferroportin [13]. At the same time, there is evidence that soluble (plasma) Cp forms complexes with lactoferrin and/or myeloperoxidase in the blood during severe inflammation in humans [79]. Specific regions in Cp and these proteins necessary for their complexation have been identified, and there is evidence that complexation influences the enzymatic activities of Cp and myeloperoxidase. As part of our innate immunity, lactoferrin – which binds iron more tightly than transferrin – is secreted by white blood cells, and helps to withhold this rate-limiting nutrient from invading organisms in the circulation [38]. Myeloperoxidase participates in the “respiratory burst” mounted by neutrophils and monocytes to kill bacteria or fungi through formation of reactive oxygen species. Myeloperoxidase is the main constituent of neutrophil granules, which are released during this defensive action, and also produce hypochlorous acid and tyrosine radicals from peroxide, chloride ions, and tyrosine. The role(s) of Cp in this complex remains to be investigated but might involve its ability to scavenge radicals. Indeed, the deployment of Cp on cell surfaces – either through specific binding to membrane receptors and/or GPI-linkage – is also likely to protect cells from damage by reactive oxygen species, the formation of which (by granulocytes in the blood) increases during inflammation and infection.

The Cp gene promoter also contains a HIF1-alpha response element, which again connects it with iron metabolism. During hypoxia (as for example when moving to higher elevations with lower oxygen pressure), there is stimulation of erythropoiesis, which increases levels of circulating red blood cells that can bind more of the limited oxygen available. A similar response is generated by iron deficiency. Increased expression of Cp in hypoxia and iron deficiency makes sense in view of the already described role of Cp to enhance the efflux of iron from storage sites into the blood so that it may be used for additional red blood cell production (see earlier section on ferroxidase). Iron deprivation – induced by administration of synthetic chelators that sequester it and prevent utilization by cells – has the same effect as that induced by bleeding (loss of iron) or treatment with erythropoetin, all of which stimulate efflux of storage iron from liver and spleen to support red cell production by the bone marrow (if sufficient iron is available). An iron depletion response resulting in increased Cp expression has even been demonstrated in rats given i.v. apolactoferrin, which as already mentioned binds iron much more tightly than transferrin [80]. However, most of the studies demonstrating this kind of Cp response have measured changes only in terms of cellular mRNA [81], and whether this translates into more Cp protein and enzyme activity in the blood remains to be demonstrated. Marked increases in the expression of Cp mRNA have been measured in the uterus of women experiencing pre-eclampsia (an anoxic response), and in syncytiotrophoblasts grown under low oxygen conditions [82]. However, changes in placental and circulating Cp may be more modest. In studies of humans adjusting to the hypoxia of high altitudes, increases in circulating Cp developed very slowly. As detailed in the review by Chepelev and Willmore [81], there was even a slight decrease at 3 months, and after a year the increase was only 37% above levels originally observed at sea level.

The opposite, namely hyperbaric oxygen exposure, may also increase Cp expression in plasma, at least in rats [83]. Exposure to high oxygen has the potential of generating more reactive oxygen species, and if so, having more

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

circulating Cp should be useful. (This effect is of course not likely to be mediated by HIF-1alpha.) Much earlier work also implicated glucocorticosteroids in the maintenance of normal levels of Cp protein in the blood plasma [1], although this has not been followed up. These early studies reported that adrenalectomy and hypophysectomy almost doubled plasma Cp levels and activity, while administration of hydrocortisone reversed the effect [1].

Estrogen and progesterone, however, have well established enhancing effects on the expression of Cp circulating in the blood plasma, made by the liver. Many years ago it was shown in rats that ovariectomy reduces plasma Cp activity by about half, and that levels of Cp increase during pregnancy and in women taking oral contraceptives that contain estrogenic and progestegenic steroids [1,24,64]. Studies from our laboratory used rats to demonstrate that daily treatment with estrogen for two weeks doubled levels of Cp activity and protein in the circulation, and increased the rate of synthesis of Cp [24] – with no effect on rate of turnover. Cp is also expressed by other cell types associated with gestation, and these hormones (and hypoxia) may be involved. Cp mRNA is expressed by the epithelial lining of the uterus [84,85]. Uterine secretions contain copper [1], and these secretions most likely contain Cp released by cells in the uterine endometrium [85]. At least in rats, maximal expression of uterine Cp mRNA occurs at estrus [86], suggesting a role in implantation. Cp is also expressed by the placenta, and its mRNA and protein expression have been localized to the syncytiotrophoblasts (and not villous fibroblasts) in human placental tissue from the third trimester, where Cp appears to be released into the intervillous space. As already noted, placental expression of the protein is markedly enhanced in the anoxic condition of pre-eclampsia [82], resulting not only in increased soluble intervillus Cp but marked immunostaining on the surface of villous cells (probably GPI-linked Cp). (Something similar has been suggested by studies of normal embryos [87], in which it was reported that two forms of Cp are produced by the yolk sac in early embryogenesis, one flowing towards the embryo the other towards the maternal decidual membrane.) Guller et al. have proposed that in pre-eclampsia, the Cp response serves to mitigate against oxidative injury that may subsequently occur when oxygen-adequate blood re-perfuses the placenta [82] – the radical scavenger function of Cp. Roles of Cp in implantation and placental function most likely are to promote iron flow towards the fetus from the mother, lack of adequate copper being associated not just with reduced transfer of copper but also of iron to the fetus [88]. On the other hand, plasma Cp levels almost double during pregnancy, and we had shown in rats that the ^{67}Cu of purified Cp administered i.v. to pregnant rats entered the placenta and fetus 7 times more rapidly than ^{67}Cu administered as the ion – which would bind to albumin and transcuprein before entering the liver and incorporating into endogenously-synthesized Cp [63]. Studies by McArdle and colleagues confirmed the ability of Cp to deliver copper to placental cells (cultured trophoblasts) [89], and they isolated Cp “receptors” from human placental vesicles, which could be involved in this uptake process [66]. Coupled with our work showing that Cp-Cu is delivered directly to the cell surface with formation of apoCp [44], these observations indicate that Cp delivers copper to the placenta preferentially over other plasma protein copper carriers (albumin and transcuprein), which is also consistent with Cp levels being elevated in the maternal blood during fetal development [11]. Following parturition, in lactation, Cp is again involved but in a different way, being synthesized by mammary epithelial cells for secretion into the milk – where it accounts for about 25% of the total copper [11]. Elevated levels of estrogen and progesterone in gestation help to prepare breast tissue for this process; on the other hand, lactation itself is precipitated by a drop in progesterone at the end of gestation, during which estrogen levels are also declining [1,11]. In lactation as in gestation, Cp is probably providing copper for the nutrition of the developing infant, since liver copper stores are limited, and a great deal of copper is needed in support of the rapid growth that continues after birth [11]. Indeed, we found that CpKO dams had pups with half the normal liver copper stores [90].

Nature, function(s) and regulation of plasma copper components: Additional ferroxidases

Cp is not the only ferroxidase present in mammalian blood plasma. Recent studies from our laboratory have documented that Cp accounts for only a portion of the total ferroxidase activity of blood plasma, in humans and rodents, which was measured with three different assays [8]. Figure 5 shows the results just for activity measured with the most traditional assay that follows the production of the Fe^{3+}-transferrin complex over time. Since at least in humans, Cp enzyme activity is inhibited by azide, ferroxidase activity remaining in the presence of azide is also shown. First it may be noted that levels of total plasma ferroxidase activity measured by this assay were much

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

higher in humans than in the rodents. Humans also had higher activity using the other assays, but the differences were not as great. In the case of human plasma, the effect of azide was also much greater, e.g. probably a larger proportion of the enzyme activity was due to Cp, compared to that in the rodents. Most importantly perhaps, knocking out Cp only decreased the ferroxidase activity of mouse plasma by half, and the same response was observed using the two other ferroxidase assays [8]. For the CpKO mice, additional findings were that upon size exclusion chromatography of whole plasma, a major peak of ferroxidase activity was still present, eluting near the position of Cp , and a substantial peak of Cu eluted in roughly the same position (Fig. 2D), suggesting that the additional ferroxidase might also be a copper protein.

An additional very large ferroxidase (containing Cu) had previously been reported in human, rat and rabbit serum by Topham and Frieden and dubbed ferroxidase II [1]. [This ferroxidase II is too large to be the main copper peak seen in Cp knockout mice (Fig. 2D).] In human plasma it accounted for about 6% of total ferroxidase activity [30]. It was distinguished from Cp not only by its size but in not being inhibited by azide, and was calculated to contribute about 15 ng Cu per ml to the blood plasma [1]. The purified protein had an apparent molecular weight of 620 kDa and was composed of two subunits (Mr 380 and 220 kDa), the smaller with one tightly bound Cu atom and catalytic activity [30,91]. The smaller subunit also contained phospholipid and cholesterol, which appeared to be necessary for catalytic activity. It is noteworthy that the Frieden and Topham groups were not the only ones to successfully isolate this Cu-containing lipoprotein and verify its composition and activity [91], and that the Garnier group identified the spectroscopic signature of carotinoids in the purified protein. (This would explain the yellow color noted by Topham and Frieden in their first publication [30].) Strangely, it appears that ferroxidase II activity is inhibited by albumin, which begs the question of its physiological significance [92]. Purification always began with Cohn fractionation, which would have eliminated albumin. However and as previously noted, Topham and his group also provided evidence that i.v. administration to copper deficient rabbits (with low total ferroxidase activity and tissue iron accumulation) resulted in rapid and sustained increases in serum iron levels [93]. (The same response was obtained with infusion of Cp in these rabbits.) Despite numerous publications about this enzyme from the Frieden and Topham groups, no further reports on isolated ferroxidase II have appeared since Topham died in the 1980's, although we ourselves did detect ferroxidase activity in a large component obtained by fractionation of plasma from Cp knockout mice [8]. The identity of this large Cu-containing lipoprotein and its subunits thus remains to be determined. With new evidence of the involvement of copper in lipid metabolism [94,95], the data surrounding this plasma copper protein may be worth reviewing.

Nature, function(s) and regulation of plasma copper components: Other amine oxidases

As already mentioned, Cp is not the only copper-containing amine oxidase in the blood plasma. Small amounts of mono and diamine oxidases are also present, also produced by hepatocytes [1]. Plasma mono and diamine oxidases participate in the inactivation of bioactive amines that differ somewhat from those targeted by Cp, but include dopamine, tyramine, tryptamine, benzylamine, and notably histamine, methyl histamine, and polyamines like putrescine [96]. For catalysis, these amine oxidases utilize Cu plus an interesting and peculiar Cu-generated cofactor, topoquinone (2,4,5-trihydroxyphenylalanine quinone), oxidatively deaminating their substrates to form aldehydes and ammonia [97-99]. The aldehydes generated are in themselves potentially toxic (reacting with lysine amino groups on proteins) and also may lead to formation of peroxides and subsequent damage of blood cells and cells lining the veins and arteries [96,100]. Not only endogenous but exogenous (food-derived) amines find their way into the blood and become substrates for these enzymes. Indeed, oxidative deamination by these enzymes in the blood may be first step in metabolism of many xenobiotic amines, which enter the blood after ingestion of food and drugs. Many drugs and pesticides that contaminate food and the environment are amines. In addition, amines are generated during processing and production of various animal and vegetable foods and beverages, where intracellular polyamines are released, and amino acids are decarboxylated.

The production of potentially toxic metabolites from exogenous amines obtained from food and drugs is of increasing concern [96]. These include exogenous histamine, an important endogenous signaling agent, prevalent in certain foods, ingestion of which is problematic for individuals with histamine sensitivity and intolerance. Histamine needed for signaling is produced endogenously by decarboxylation of histidine and mediates numerous

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.
Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Published on 03 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

normal and pathological processes in mammals, ranging from those of the immune response to infection and inflammation (that includes increased capillary permeability), to stimulation of stomach acid secretion [101]. The first step in histamine catabolism is inactivation either through non-circulating (cellular) histamine N-methyl transferases, or oxidation to imidazole acetaldehyde by circulating diamine oxidase (DAO). [DAO is also known as amiloride-binding protein 1 (ABP1).] Products from both reactions are further metabolized by circulating mono- and diamine oxidases (and other enzymes). Many common foods and drinks, notably scombroid (dark meat) fishes like tuna, mackerel, marlin, as well as non-scombroids (sardines, herring, bluefish), most cheeses and processed dairy products, along with wines and processed (smoked) meats, beans pulses and nuts [102] have significant concentrations of histamine (>1000 ppm) and other bioamines (like tryptamine and polyamines), and there are many individuals with histamine sensitivity. Studies so far suggest that these individuals have lower than normal circulating levels of DAO [103,104], and that measuring serum DAO activity may aid in diagnosis of histamine sensitivity [104]. Thus, switching to low histamine diets, or oral intake of DAO-containing capsules may be beneficial [103-104]. Variants in diamine oxidase have been associated with abnormal conditions that include risk of migraine [101] and hypersensitivity to non-steroidal anti-inflammatory drugs [105]. In a small study of heat stroke patients, higher plasma DAO activities were associated with a (beneficial) greater resistance to loss of barrier function of the intestinal mucosa [106]. (Plasma DAO levels in heat stroke patients, however, were much higher than normal.) Possibly, dietary copper status may influence levels of DAO activity [107]: serum DAO activity in 8 young healthy women supplemented with 2 mg copper per day for 8 weeks was almost twice as high as in that of the unsupplemented group.

Nature, function(s) and regulation of plasma copper components: Superoxide dismutase 3 (SOD3)

SOD3, also known as extracellular (ec) SOD, is a Cu and Zn-containing enzyme in the blood circulation and extracellular fluids. However, most of it is bound to collagen, proteoglycans and fibulin-5 in the extracellular matrix of most organs, but particularly those of the lung, kidney, and uterus, with lower levels in the heart [108]. It is a 135 kDa glycated homotetramer, consisting of two disulfide-linked dimers, and its catalytic domain has considerable homology to intracellular Cu/Zn enzyme (SOD1), with the same critical active-site amino acid residues. It is produced mainly by fibroblasts and smooth muscle cells of the vasculature, and receives its copper through the same delivery system as other secreted Cu proteins, namely via passage from the intracellular Cu chaperone ATOX1 to one of the P-types ATPases in the transGolgi network, in this case ATP7A. (This contrasts with cytosolic SOD1, which receives it from another chaperone, CCS.) Interestingly for SOD3, ATOX1 serves not only as the source of copper but as a transcription factor that enhances expression of the enzyme [109]. Moreover, copper stimulates movement of the chaperone to the nucleus (as the copper complex). Thus, transcription of SOD3 is regulated through the Cu-induced migration of ATOX1 to the nucleus, where it binds to a specific Cu-ATOX1 response element, promoting transcription and formation of SOD3 mRNA. As part of the superoxide dismutase family it is a significant player in antioxidant defense, in this case extracellularly (with ceruloplasmin). As such, it plays a role in modulating blood pressure by lowering levels of superoxide that arise and can decompose the vascular relaxant, nitrogen oxide [110].

In line with recent interest in the relationship between oxidative stress, reactive oxygen species and cancer, there are also some studies suggesting an inverse relationship of SOD3 expression to the development of more aggressive tumors and metastasis [111,112]. It seems that cancers tend to have low levels of SOD3 (i.e. extracellular levels of the enzyme in the cancer tissue), and that this correlates with greater malignancy and poor prognosis. Implantation of cancer cells in which SOD3 overexpression was induced resulted in decreased growth rates and invasiveness of the resulting tumors, as shown in breast and lung cancer models [112] and pancreatic ductal carcinoma [113]. Normal breast tissue expresses significant levels of SOD3 protein and mRNA, and the same is the case when normal mammary epithelial cells are cultured in the presence (but not in the absence) of laminin-rich extracellular matrix (ECM) and form polarized acinar structures [112]. The response to ECM may be mediated by decreased methylation of the SOD3 promoter [112]. Increased levels of SOD3 correlating with normal versus malignant status is not always observed however. Thus for example, in highly aggressive (triple negative)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

breast cancer cells with high VEGF production, the latter growth factor increased SOD3 expression (via its receptor and receptor-associated Nrp2), and this increased tumor survival and metastasis in the face of oxidative stress [114]. Thus SOD3 may play both inhibiting and enhancing roles in the development and evolution of malignant cells and tumors.

Nature, function(s) and regulation of plasma copper components: Albumin and transcuprein

Albumin

Albumin is the most abundant protein in mammalian blood plasma, accounting for about 4.5 of the 7 g of protein in 100 ml of plasma; but it also occurs in other body fluids such as the cerebrospinal fluid and milk. A truly multifunctional protein with many roles, it can carry a plethora of different molecules or ions ranging from fatty acids, bilirubin and bile acids, to hepcidin and other signaling agents (like thyroxine, some lipophilic hormones and many lipophilic xenobiotics, including drugs), the amino acid, tryptophan, and metal ions that include copper. Not only is it a carrier of binding components, but as an amino acid polymer it itself is a means of carrying amino acids to other parts of the organism, supplying amino acids to cells without causing large changes in blood osmolarity. One third of the daily amino acid/protein requirements of the body are funneled into albumin synthesis by the liver upon entry into the hepatic portal circulation after eating. The large amounts of albumin produced by hepatocytes every day (about 14 g/70 kg adult human) are also essential to maintain the oncotic pressure of the blood plasma, so that metabolites can return from the extracellular fluid to the blood capillaries. A recent review on this protein in health and disease is that of Fanali et al. [115].

Albumin in the product of a single gene, which encodes for a precursor protein of 609 amino acids that contains an 18 amino acids signal recognition peptide. The latter shifts albumin mRNA translation from the free to the endoplasmic reticulum (ER)-bound polyribosomes [116]. There the signal sequence and a further 6 amino acids are cleaved, leaving a final product of 66.5 kDa, containing 585 amino acids. Unlike most other blood plasma proteins, carbohydrate chains are not added to its structure along the way through the smooth ER to the transGolgi network and into the blood. Synthesis of albumin is mainly controlled at the translational level through shifts in its mRNA from the non-translating mRNP pool (mRNA not bound to ribosomes and not being translated) to that of the polyribosomes that are translating the message. The shift towards more synthesis occurs in response to the increased influx of amino acids from the diet following a meal [116]. The reverse shift (into the mRNP pool) occurs when dietary amino acids have been assimilated. These shifts are not accompanied by significant changes in total albumin mRNA. It seems likely that a shift of albumin mRNA from active translation to the inactive mRNP pool may also be occurring during the acute phase reaction, although this has not been studied. In this condition, there are substantial increases in rates of production of other plasma proteins (like Cp), in response to the development of inflammation and/or infection, and less albumin is synthesized. This reflects the fact that albumin synthesis is sensitive to changes in the oncotic pressure of extracellular fluid and blood [117], and a mechanism to maintain constant blood osmolarity. The presence of a cysteine residue (in position 34) provides not only an additional nucleus for metal ion binding, but (along with some other residues) a potential means of contributing to antioxidant defense [118,119].

Almost all mammalian albumins investigated (except that of canines) have a high affinity copper binding site at the otherwise unstructured N terminus of the protein [1]. Tight binding involves a critical histidine residue in position 3 as well as the N-terminal nitrogen atom and peptide nitrogens between the first three amino acids, as indicated by studies of the tripeptide complex [120]. The dissociation constant for Cu^{2+} is 10^{-12} or lower [120,121], and Ni^{2+} also binds tightly to this site. This site is key to the roles albumin plays in copper transport and metabolism. Despite its tight binding, the copper bound to albumin is exchangeable with that on chelators or other proteins in the surrounding fluid (such as transcuprein; see later) i.e. it is dialyzable, and considered one of two major components of the exchangeable Cu pool of blood plasma [1,11].

Albumin's main role in copper metabolism is to aid in transport and delivery of copper to cells via the blood, and presumably also in the other fluids where it is found. In addition, it provides an extracellular defense against the existence of free copper ions that might otherwise promote the production of reactive oxygen species. Due to its

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.
Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

great abundance, albumin provides high affinity binding sites for about 600 ug Cu per ml of blood plasma, but only about 0.03% of those sites are normally occupied. Thus, when faced with excess copper entering the blood, albumin offers a large amount of empty Cu binding sites, and indeed, those sites become occupied under those kinds of conditions. This was demonstrated when we administered a total of more than 141 ug the stable isotope of copper, ^{65}Cu, to mice in several injections over 15 hours, and measured the abundance of the isotope associated with albumin 30 min after the last injection and later on [122]. (The dose of copper injected was more than 3 times the total estimated to be present in a mouse.) Fig. 2F shows the distribution of ^{65}Cu isotope soon after the last injection for plasma separated by size exclusion chromatography. The huge peak of this isotope is eluting in the position of albumin, which is just after elution of the main Cu peak (seen in the form of ^{63}Cu, dotted line) attributable to Cp. As concerns its function as a source of copper for cellular uptake, we and others have shown that radiolabeled Cu (provided as a 1:1 complex with pure albumin to cultured cells) readily enters those cells [69]. Kinetic studies indicate that uptake occurs at concentrations of copper normally found with albumin in the plasma, and that the Km for the uptake systems involved is also in the physiological range, and lower than that for the ionic copper provided as the (non-physiological) Cu^{2+}-dihistidine complex. Uptake is mediated by CTR1 as well as by an as yet unidentified Cu uptake transporter. Uptake probably requires prior reduction by a cell surface reductase (like dCytB or Steaps 2 and 4), since both CTR1 and the unknown transporter most likely only take up the cuprous ion; ferric iron (also a substrate for these reductases) inhibited copper uptake from albumin-Cu^{2+} [69], as it does for copper from Cp (Fig. 4D). As already mentioned, canine albumin is missing the crucial histidine necessary for tight copper binding at the N-terminus. Dogs have another peculiarity in copper metabolism, namely that throughout their lives, they have much higher liver copper concentrations than other mammals [1, 123], which is partly but not fully explained by a reduced ability to excrete copper in the bile. Whether there could be a link between this and the inability of albumin to bind copper is unknown and remains to be explored.

Transcuprein

Transcuprein was first discovered when high specific activity radioactive ^{67}Cu became available for experimentation, and administration of this radioisotope to rats (or to blood plasma *in vitro* in ionic form) resulted in the immediate labeling of two proteins in the blood, one being albumin and the other much larger and initially unknown (see also Fig. 2E) [1,3,124]. The unknown was then isolated and identified as alpha-1-inhibitor 3, the main macroglobulin in rodents. Its homolog in humans – alpha-2-macroglobulin (a2M), was shown to bind copper tightly even in the presence of high concentrations of albumin, indicating an even greater affinity for copper. The two proteins also were shown to exchange radiocopper rapidly, making transcuprein/macroglobulin part of the exchangeable blood plasma copper pool [3,4]. More recently, it was shown that like albumin, the Cu^{2+}-a2M complex readily delivers the metal ion to cultured human cells at physiologically relevant concentrations and by a mechanism that does not involve endocytosis [69]. A_2M is mainly a homo-tetramer with glycosylated subunits of 179 kDa and 1451 amino acids (after removal of a 23 amino acid signal sequence) [125]. The subunits assemble in pairs, each pair forming an opening into a large cavity. We have found that this native or “open” form of a2M can tightly bind two Cu^{2+} atoms [69]. Exactly where copper binds is unknown, but it is likely to be within the cavity of each dimer (see below).

Apart from being a copper carrier, a2M/transcuprein is best known for its ability to scavenge and trap all manner of proteases [125]. A protease enters into the cavity and cleaves a thioester bond at the far end, which closes the opening (to the cavity). The transformed or “closed” form of a2M is then removed from the plasma mostly by the liver, and via LRP receptor-mediated endocytosis. Copper binding eliminates this important trapping activity [4]. A2M is however also a carrier of many different kinds of factors, including some involved in immune system signaling (such as alpha-1-microglobulin [126]), pro and anti-inflammatory cytokines [127], growth factors [128], leptin, and even apolipoprotein E. It also is the major carrier of plasma zinc, although Zn does not bind to the same site as Cu [1,3]. More recently, a2M has also been identified as the carrier of the small peptide, hepcidin, an important regulator of iron metabolism [129], binding it with cooperative kinetics and high affinity (initial binding Kd 177 nM). A2M effectively delivers hepcidin to ferroportin on cell surfaces [129]. This induces ferroportin destruction, thus reducing iron efflux into the blood and lowering levels of circulating iron [11,130,131]. Hepcidin is secreted in response to the acute phase reaction and when there is excess iron in the organism.

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

Metallomics Accepted Manuscript

1
2
3 In rats but not in humans, where alpha-1-inhibitor3 is the main macroglobulin and a2M is expressed in very low amounts, a2M itself is an acute phase reactant, increased expression being induced by pro-inflammatory cytokines (mainly !L-6) and signaling cascades involving STAT3 [132]. Expression is however suppressed by the cytokine IL-1beta, working through activation of Nf-kappaB. Also in the rat, the expression of alpha-1-inhibitor 3 appears to be modulated by dietary iron and copper status. Copper deficiency or treatment with additional iron were found to increase levels of mRNA 60-100% [4]. However, this was accompanied by only modest changes in circulating levels of the protein [4]. The possible regulation of both a2M and alpha-1-inhibitor3 macroglobulins by these metals in rats and other mammals remains to be further examined.

Nature, function(s) and regulation of plasma copper components: Small copper carriers

Ultrafiltration of blood plasma indicates that in humans and some other mammals there are traces of copper associated with components of very low molecular weight [133]. This can also be seen by size exclusion chromatography, where copper elution is monitored by ICP-MS or furnace atomic absorption (see for example Fig. 2). At least in humans and rats, however, addition of trace amounts of radioactive copper ions to plasma prior to chromatography does not label these low molecular weight fractions (Fig. 2E). Instead, it binds to albumin and transcuprein/macroglobulins. We do know that small quantities of metallothionein (MT) can be present in certain circumstances, notably when the toxic element cadmium accumulates in liver and kidney [134,135]. However, in that case it is in the form of Cd-MT and not likely to be carrying copper. Whether traces of Cu-MT are present in plasma an some conditions is unknown. If so, that might explain the Mr 15 kDa copper peak sometimes detected in the plasma size exclusion chromatographic profiles (such as Fig. 2B).

The nature of the small molecules containing copper (small copper carriers, or SCCs) that can appear in the plasma is still unknown, but there has been a recent breakthrough that may lead to elucidation of their structure, origins and function. In humans with Wilson disease, genetic abnormalities lead to inactivation of the copper “pump” ATP7B in hepatocytes [136]. This results in poor excretion of copper through the bile, the main normal route for excretion of this metal from the mammalian organism. Consequently, there is a large accumulation of copper in the liver that can “bleed” into other organs as well, causing severe pathology. Only very small amounts of copper are normally lost in the urine. In the adult human, about 1 mg Cu is absorbed from the diet and excreted in the feces on a daily basis, but only about 50 ug of Cu are lost in the urine [11]. During studies of the mouse model of Wilson disease in Lutsenko’s laboratory, it was determined that as in humans, Atp7-/- mice accumulated massive amounts of copper in their livers and excreted larger amounts of copper in the urine [137]. In the case of these mice an interesting adaptation occurred over time however, and led to marked drop in liver copper concentrations. The drop was accompanied by a marked increase in the levels of copper in the urine. This inverse relationship suggested that SCCs might offer an alternative means of excreting copper. Preliminary analysis of these urinary SCCs indicated they were in the size range of 1 kDa, thus clearly much larger than nutrients like amino acids and glucose. The question was then whether SCCs might also be present in large quantities in the blood plasma of these Atp7b-/- mice, and this has been verified [Linder and Lutsenko, unpublished]. The origins and nature of these SCCs is currently under investigation and may lead to new insights into mechanisms by which copper homeostasis may be manipulated and maintained by the mammalian organism.

Nature, function(s) and regulation of plasma copper components: Blood coagulation proteins

Two factors involved in blood coagulation (factors V and VIII) also contain copper [11]. A major portion of the structure of both proteins appears to have a close resemblance to that of ceruloplasmin (Cp) [138,139]. The structure of Factor VIII modeled on the crystal structure of human Cp is shown in Fig. 3B, where it is clear that domains A1 through A3 form a molecule shaped like Cp. Factor V is homologous to both factor VIII and Cp [139]. The copper sites in domains A1 and 3 of factor VIII (again modeled after those in Cp) have identical or similar amino acid residues, and there are similarities as well as differences in the case of factor V. Factor VIII was first identified in the 1980’s as the entity associated with familial hemophilia i.e. as “antihemophilic factor” (or factor VIII). This form of hemophilia affects 1 out of 5000 male humans [140]. An excellent review of the history of this factor, its biosynthesis, processing and role in coagulation is that of Lenting et al. [140]. Like most plasma proteins,
58
59
60

View Article Online
DOI: 10.1039/C6MT00103C
Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

factor VIII (and factor V) are produced by hepatocytes in liver, but can also be produced by cells of the kidney and spleen. When released into the blood, factor VIII has already undergone a great deal of processing, involving proteolytic cleavages and N as well as O-linked glycosylation. Presumably, copper is incorporated during transit through the transGolgi network. Factor VIII enters the plasma in a form not yet activated to participate in coagulation, and binds to von Willibrand factor (vWF), produced by endothelial cells of the vasculature. When coagulation is triggered, serine proteases initiate structural changes in factor VIII that result in its dissociation from vWF and binding to Factor IX, with formation of a "coagulation activating complex" composed of factors IX, X, Ca^{2+} and the remaining portions of factor VIII. The copper in factor VIII (and presumably also in factor V) is thought to play a role in allowing the A1 and A3 portions of the molecule to assume a particular conformation in the activated state, one that also requires Ca^{2+} but is enhanced by Cu^{2+} [140].

Conclusions

The information presented in these pages shows the enormous variety and range of roles that copper-containing proteins and other substances in the blood plasma play not only in copper homeostasis but in support of critical metabolic processes underlying health and well being. Despite continued and continuing active research, many questions remain about the nature and contributions of these important components circulating in the blood that need copper for their activities, or transport it in support of metabolic processes and overall copper homeostasis.

References

1. M.C. Linder, Biochemistry of Copper, New York, Plenum Press, 1991.
2. M.C. Linder, Nutritional Biochemistry and Metabolism, 2nd ed., Norwalk CN, Appleton-Lange, 1991.
3. K. Weiss, M. Linder, Copper transport in rats involving a new transport protein, *Am. J. Physiol.*, 1985, **249,** E77-E88
4. N.M. Liu, L.S.L. Lo, S.H. Askary, L.T. Jones, J. Goforth, E. Vivas, M.T. Tsai, T. Westbrook, M.C. Linder, Transcuprein is a macroglobulin regulated by copper availability, *J. Nutr. Biochem.*, 2007, **18**, 597-608.
5. P. Bielli, L. Calabrese, Structure and function relationships in ceruloplasmin: a 'moonlighting' protein, *Cell Mol. Life Sci.* **59**, 2002, 1413-1427.
6. I. Zaitseva, V. Zaitsev, G. Card, K. Moshkov, B. Bax, A. Ralph, P. Lindley, Nature of the copper centres in human ceruloplasmin, *J. Biol. Inorg. Chem*., 1996, **1,** 15-23.
7. V.N. Zaitsev, I. Zaitseva, M. Papiz, P. Lindley, An X-ray crystallographc study of the binding sites of the azide inhibitor and organic substrates to ceruloplasmin, a multicopper oxidase in the plasma, *J. Biol. Inorg. Chem*., 1999, **4,** 579-587.
8. Gray LW, Kidane TZ, Nguyen A, et al. Copper proteins and ferroxidases in human plasma and that of wild-type and ceruloplasmin knockout mice. *Biochem. J*. 2009, **419**, 237-45.
9. E. Sedlak, G. Zoldak, P. Wittung-Stafshede, Role of copper in thermal stability of human ceruloplasmin, *Biophys. J.* 2008, **94,** 1384-1391.
10. S.A. Donley, B.J. Ilagan, H. Rim, M.C. Linder, Copper transport to mammary gland and milk during lactation in rats, *Am. J. Physiol. Endocrinol. Metab*. 2002, **283**, E667-675.
11. M.C. Linder, Nutritional biochemistry of copper, with emphasis on the perinatal period, In: L. Avigliano, L. Rossi, ed., *Biochemical Aspects of Human Nutrition*, Trivandrum, Kerala, India: Research Signpost, 2010, 143-179.
12. L. Wooten, R. Shulze, R. Lancey, M. Lietzow, M.C. Linder, Ceruloplasmin is found in milk and amniotic fluid and may have a nutritional role, *J. Nutr. Biochem*., 1996, **7**, 632-639.
13. L. Marques, A. Auriac, A. Willemetz, J. Banha, B. Silva, F. Canonne-Hergaux, L. Costa, Immune cells and hepatocytes express glycophosphatidylinositol-anchored ceruloplasmin at their cell surface. *Blood Cells, Molecules Disease,* 2012, **48**, 110-120.
14. J. Banha, I. Marques, R. Oliveira, M. deF. Martins, E. Paixao, D. Pereira, R. Malho, D. Penque, L. Costa. Ceruloplasmin expression by human peripheral blood lymphocytes; a new link between immunity and iron metabolism. *Free Rad. Biol. Med.* 2008, **44**, 483-492.
15. B.N. Patel, S. David, A novel glycosylphosphatidylinositol-anchored form of ceruloplasmin is expressed by mammalian astrocytes, *J. Biol. Chem*., 1997, **272**, 20185-90.

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

16. B.N. Patel, R.J. Dunn, S. David, Alternative RNA splicing generates a glycosylphosphatidylinositol-anchored form of ceruloplasmin in mammalian brain, *J. Biol. Chem*., 2000, **275**, 4305-4310.
17. J.L. Salzer, L. Lovejoy, M.C. Linder, C. Rosen, Ran-2, a glial lineage marker, is a GPI-anchored form of ceruloplasmin, *J. Neurosci. Res*. 1998, **54**, 147-57.
18. E.J. Mostad, J.R. Prohaska, Glycophosphatidylinositol-linked ceruloplasmin is expressed in multiple rodent organs and is lower following dietary copper deficiency, *Exp. Biol. Med*. 2011, **236,** 298-308.
19. Fortna RR, Watson HA, Nyquist SE. Glycosyl phosphatidylinositol-anchored ceruloplasmin is expressed by rat Sertoli cells and is concentrated in detergent-insoluble membrane fractions, *Biol. Reprod.* 1999, **61**, 1042-1049.
20. B. Mittal, M.M. Doroudchi, S.Y. Jeong, B.N. Patel, S. David, Expression of a membrane-bound form of the ferroxidase ceruloplasmin by leptomeningeal cells, Glia, 2003, **41**, 337-46.
21. F.M. Yang, W.E. Friedrichs, R.L. Cupples, M.J. Bonifacio, J.A. Sanford, W.A. Horton, B.H. Bowman, Human ceruloplasmin, Tissue-specific expression of transcripts produced by alternative splicing, *J. Biol. Chem.*, 1990, **265**, 10780-10785.
22. M. Sato, J.D. Gitlin JD, Mechanisms of copper incorporation during the biosynthesis of human ceruloplasmin. *J. Biol. Chem.,* 1991, **266**, 5128-5134.
23. Hirano K, Ogihara T, Ogihara H, Hiroi M, Hasegawa M, Tamai H. Identification of apo- and holo-forms of ceruloplasmin in patients with Wilson's disease using native polyacrylamide gel electrophoresis. *Clin. Biochem.*, 2005, **38**, 9-12.
24. R.B. Middleton, M.C. Linder, Synthesis and turnover of ceruloplasmin in rats treated with 17 beta-estradiol, *Arch. Biochem. Biophys*., 1993, **302**, 362-368.
25. N.A. Holtzman, B.M. Gaumnitz, Studies on the rate of release and turnover of ceruloplasmin and apoceruloplasmin in rat plasma, *J. Biol. Chem*. 1970, **245**, 2354-2358.
26. Z.L. Harris, A.P. Durley, T.M. Man, J.D. Gitlin, Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux, *Proc. Natl. Acad. Sci. USA*, 1999, **96**,10812-10817.
27. G. Vashchenko, R.T.A. MacGillivray, Multi-copper oxidases and iron metabollism, *Nutrients,* 2013, **5**, 2289-2313.
28. H.A. Ragan, S. Nacht, G.R.. Lee, C.R. Bishop, G.E. Cartwright, Effect of ceruloplasmin on plasma iron in copper deficient swine, Am. J. Physiol., 1969,217, 1320-1323.
29. S. Osaki, D.A. Johnson, Mobilization of liver iron by ferroxidase (ceruloplasmin), J. Biol. Chem., 1969, 244, 5757-5761.
30. R.W. Topham, E. Frieden E, Identification and purification of a non-ceruloplasmin ferroxidase of human serum. *J. Biol. Chem*., 1970, **245,** 6698-6705.
31. S. Kono, Aceruloplasminemia: an update*, Int. Rev. Neurobiol*. 2013, **110**, 125–51.
32. J.F. Collins, J.R. Prohaska, M.D. Knutson, Metabolic crossroads of iron and copper, *Nutrition Reviews*. 2010, **68**, 133-147.
33. J. Sarkar, V. Seshadri, N.A. Tripoulas, M.E. Ketterer, P.L. Fox, Role of ceruloplasmin in macrophage iron efflux during hypoxia, *J. Biol. Chem*. 2003, **278**, 44018-44024.
34. S.Y. Jeong, S. David, Glycosylphosphatidylinositol-anchored ceruloplasmin is required for iron efflux from cells in the central nervous system, *J. Biol. Chem*., 2003, **278**, 27144-27148.
35. D.M. Ward, J. Kaplan. Ferroportin-mediated iron transport: Expression and regulation. *Biochim. Biophys. Acta,* 2012, **1823**, 1426-1433.
36. K.N. White, C. Conesa, L. Sanchez, M. Amini, S. Farnaud, C. Lorvoralak, R.W. Evans, The transfer of iron between ceruloplasmin and transferrins. *Biochim. Biophys. Acta*, 2012, **1920**, 411-416.
37. C. Eid, M. Hemadi, H. J.-M. Ha-Duong, J.-M., J.M. Chahine J-M EH. Iron uptake and transfer from ceruloplasmin to transferrin. *Biochim. Biophys. Acta*, 2014, **840**, 1771-1781.
38. M.C. Linder, Mobilization of stored iron in mammals: a review, *Nutrients*, 2013, **5**, 4022-4050.
39. N.R. Zerounian, M.C. Linder, Effects of copper and ceruloplasmin on iron transport in the Caco2 cell intestinal model*, J. Nutr. Biochem.* 2002, **13**, 138-148.
40. G.J. Anderson, D.M. Frazer, A.T. McKie, C.D. Vulpe, The ceruloplasmin homolog hephaestin and the control of intestinal iron absorption, *Blood Cells Mol. Dis*., 2002, **29**, 367-75.
41. D.C. Harris, P. Aisen, Facilitation of Fe(II) autooxidation by Fe(3) complexing agents, *Biochim. Biophys. Acta*,

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 03 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

1973, **329**, 156-158.

42. A.U. Steinbicker, M.U. Muckenthaler, Out of balance - systemic iron homeostasis in iron-related disorders, *Nutrients*, 2013, **5,** 3034-3061.

43. E. Poggiali, M. Migone De Amicis, I. Motta, Anemia of chronic disease: a unique defect of iron recycling for many different chronic diseases, *Eur. J. Intern. Med*., 2014, **25**, 12-17.

44. D. Ramos, D. Mar, M. Ishida, R. Vargas, M. Gaite, A. Mlntgomery, M.C. Linder, Mechanism of copper uptake from blood plasma ceruloplasmin by mammalian cells, *PLoS ONE*, 2016, **11**(3): e0149516.

45. J.R. Prohaska, Impact of copper limitation on expression and function of multicopper oxidases (ferroxidases), *Adv. Nutr*., 2011, **2**, 89-95.

46. B.X. Wong, S. Ayton, L.Q. Lam, P.A. Adlard, A.I. Bush, J.A. Duce. A comparison of ceruloplasmin to biologicalpolyanions in promoting the oxidation of Fe2+ under physiologically relevant conditions. *Biochim. Biophys. Acta*, 2014, **1840**, 3299-3310.

47. Z.K. Attieh, C.K. Mukhopadhyay, V. Seshadri, N.A. Tripoulas, P.L. Fox, Ceruloplasmin ferroxidase activity stimulates cellular iron uptake by a trivalent cation-specific transport mechanism, *J. Biol. Chem*. 1999, **274**, 1116-1123.

48. C.K. Mukhopadhyay, Z.K. Attieh, P.L. Fox, Role of ceruloplasmin in cellular iron uptake, *Science*, 1998, **279**, 714-717.

49. S. Kono, K. Yoshida, N. Tomosugi, T. Terada, Y. Hamaya, S. Kanaoka, H. Miyajima, Biological effects of mutant ceruloplasmn on hepcidin-mediated internalization of ferroportin, *Biochim. Biophys. Acta*, 2010, 1802, 968-975.

50. D.A. Richards, Use of high-performance liquid chromatography to study the caeruloplasmin-catalysed oxidation of biogenic amines. I. Single substrate systems, *J. Chromatogr*., 1983, 256, 71-79.

51. T. Bieganski, M.Z. Blasinska, J. Kusche, Determination of histaminase (diamine oxidase) activity by o-dianisidine test: interference of ceruloplasmin, *Agents Actions*, 1977, **7**, 85-92.

52. S. Shiva, X. Wang, L.A. Ringwood, X. Xu, S. Yuditskaya, V. Annavajihala, H. Miyamjima, N. Hogg, Z.L. Harris, M.T. Gladwin, Ceruloplasmin is a NO oxidase and nitrate synthase that determines endocrine NO homeostasis, *Nature Chem. Biol*., 2006, **2**, 486-493.

53. K. Vrancken, H.J. Schroeder, L.D. Loongo, G.G. Power, A.B. Blood. Role of cerulolasmin in nitric oxide metabolism in plasma of humans and sheep: a comparison of adults and fetuses. *Am. J. Physiol. Regul. Integr. Comp. Physiol*., 2013, **305**, R1401-R1410.

54. X.L. Zhao, L. Wang, S. Shiva, J. Tejero, M.M. Myerburg, J. Wang, S. Frizzell, M.T. Gladwin, Mechanisms for cellular NO and nitrite formation in lung epithelial cells, *Free Radic. Biol. Med*., 2013, **61**, 428-437.

55. M. Lazzaro, B. Gettegazzi, F. Codazzi, D. Zacchetti, M. Alessio. Ceruloplasin potentiates nitric oxide synthase activity and cytokine secretion in activated microglia. *J. Neuroinflammation*, 2014, **11**, 164.

56. I.M. Goldstein, H.B. Kaplan, H.S. Edelson, G. Weissman, Ceruloplasmin: A scavenger of superoxide anion radicals, *J. Biol. Chem.,* 1979, **254**, 4040-4045.

57. I.M. Goldstein, I.F. Charo, Ceruloplasmin: an acute phase reactant and anti-oxidant, *Lymphokines*, **8**, 373-411.

58. J.M.C. Gutteridge, P.G. Winyard, D.R. Blake, J. Lunec, S. Brailsford, B. Halliwell, the behavior of caeruloplasmin in stored human extracellular fluids in relation to ferroxidase II acativity, lipid peroxidation and phenanthroline-detectable copper, *Biochem. J*., 1985, **230**, 517-523.

59. K. Yoshida, K. Kaneko, H. Miyajima, T. Tokuda, A. Nakamura, M. Kato, S. Ikeda, Increased lipid peroxidation in the brains of aceruloplasminemic patients*. J. Neurolog. Sci*., 2000, **175**, 91-95.

60. H. Miyajima,S. Kono, Y. Takahashi, M. Sugimoto, Increased lipid peroxidation and mitochondrial dysfunction in aceruloplasminemia, *Blood Cells Molecules Diseases*, 2002, **29**, 433-438.

61. G. Grolez, C. Moreau, B. Sablonniere, G. Garcon, J.C. Devedjian, S. Meguig, P. Gele, C. Delmaire, R. Bordet, L. Defevre, I.Z. Cabantchik, D. Devos. Ceruloplasmin activity and iron chelation treatment of patients with Parkinson’s Disease, *B.M.C. Neurol*., 2015, **15**, 74. doi: 10.1186/s12883-015-0331-3

62. G. Torsdottir, J. Kirstinsson, S. Sveinbjornsdottir, J. Snaedal, T. Johannesson. Copper, ceruloplasmin, superoxide dismutase and iron parameters in Parkinson’s disease. *Pharmacol. Toxicol*. 1999, **85,** 239-243.

63. S.H. Lee, R. Lancey, A. Montaser, N. Madani, M.C. Linder, Ceruloplasmin and copper transport during the latter part of gestation in the rat, *Proc. Soc. Exp. Biol. Med*., 1993, *203*, 428-39.

64. L. Gambling, R. Danzeisen, C. Fosset, H.S. Anderson, S. Dunford, S.K.S. Srai, H.J. McArdle, Iron and copper

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

interactions in development and the effect on pregnancy outcome, *J. Nutr.*, 2003, **133**, 1554S-1556S.

65. S.S. Percival, E.D. Harris, Copper transport from ceruloplasmin: characterization of the cellular uptake mechanism, *Am. J. Physiol*., 1990, **258,** 140-146.

66. Hilton M, Spenser DC, Ross P, Ramsey A, McArdle HJ. Characterisation of the copper uptake mechanism and isolation of the ceruloplasmin receptor/copper transporter in human placental vesicles. *Biochim. Biophys. Acta*,1995, **1245**,153-60.

67. C.H. Campbell, R. Brown, M.C. Linder, Circulating ceruloplasmin is an important source of copper for normal and malignant animal cells, *Biochim.. Biophys Acta* 1981, **678**, 27-38.

68. J. Lee, M. Petris, D.J. Thiele, Characterization of mouse embryonic cells deficient in the Ctr1 high affinity transporter. Identification of a Ctr1-independent copper transport system, *J. Biol. Chem*., 2002; **277**, 4380-4387.

69. T.Z. Kidane, R. Farhad, K. J. Lee, A. Santos, E. Russo, M.C. Linder, Uptake of copper from plasma proteins in cells where expression of CTR1 has been modulated, *Biometals,* 2012, **25**, 697-709.

70. M.D. Knutson, Steap proteins: Implications for iron and copper metabolism, *Nutr. Rev.*, 2007, **65**, 335-340.

71. M. Moriya, Y.H. Ho, A. Grana, L. Nguyen, A. Alvarez, R. Jamil, M.L. Ackland, A. Michalczyk, P. Hamer, D. Ramos, S. Kim, J.F. Mercer, M.C. Linder, Copper is taken up efficiently from albumin and alpha-2-macroglobulin by cultured human cells by more than one mechanism, *Am. J. Physiol. Cell Physiol*., 2008, **295**, C708-21.

72. L.A. Meyer, A.P. Durley, J.R. Prohaska, Z.L. Harris, Copper transport and metabolism are normal in aceruloplasminemia, *J. Biol. Chem*., 2001, **276**, 36857-36861.

73. M.K. Mackiewicz, D. Ganapathi, D. Schultz, D. Samols, J. Reese, I. Kushner, Regulation of rabbit acute phase protein biosynthesis, *Biochem. J*., 1988, **253**, 851-857.

74. T. Ganz, E. Nemeth. Iron homeostasis in host defense and inflammation. *Nat. Rev. Immunol*., 2015, **15**, 500-510.

75. N. Naz, I.A. Malik, N. Sheikh, S. Ahmad, S. Khan, M. Blaschke, F.M. Schultze, G. Ramadon. Ferroportin-1 is a'nuclear'-negative acture-phase protein in rat liver; a comparison with other iron-transport proteins. *Lab. Invest*., 2012, **92**, 842-856.

76. S. Ahmad, S. Sultan, N. Naz, G. Ahmad, S.M. Alwahsh, S. Cameron, F. Moriconi, G. Ramadori, I.A. Malik, Regulation of iron uptake in primary culture rate hepatocytes: the role of acute-phase cytokines. *Shock*, 2014, **41**, 337-345.

77. L. Conley, T.L. Geurs, L.A. Levi, Transcriptional regulation of ceruloplasmin by an IL-6 response element pathway. *Brain Res. Mol. Brain Res*. 2005, **139,** 235-241.

78. A. Sidhu, P.J. Miller, A.D. Hollenbach, FOXO1 stimulates ceruloplasmin promoter activity in human hepatoma cells treated with IL-6, *Biochem. Biophys, Res. Comm*., 2011, **404**, 963-967.

79. V.B. Vasilyev, Interactions of caeruloplasmin with other proteins participating in inflammation, *Bioch. Soc. Trans*., 2010, **38**, 947-951.

80. E.T. Zhakarova, V.A. Kostevich, A.V. Sokolov, V.B. Vasilyev, Human apo-lactoferrin as a physiological mimetic of hypoxia stabilizes hypoxia-inducible factor-1 alpha, *Biometals*, 2012, **25**, 1247-1259.

81. N.L. Chepelev, W.G. Willmore, Regulation of iron pathways in response to hypoxia, *Free Rad. Biol. Med.,* 2011, **50**, 645-666.

82. S. Guller, C.S. Buhimschi, Y.Y. Ma, S.T.J. Huang, L. Yang, E. Kuczynski, E. Zambrano, C.J. Lockwood, I.A. Buhimschi, Placental expression of ceruloplasmin in pregnancies complicated by severe preeclampsia, *Lab. Invest*., 2008, **88**, 1057-1067.

83. B.A. Hart, G.W. Voss, J.S. Garvey, Induction of pulmonary metallothionein following oxygen exposure, *Environ. Res*.,1989, **50**, 269-278.

84. R.E. Fleming, J.D. Gitlin, Primary structure of rat ceruloplasmin and analysis of tissue-specific gene expression during development, *J. Biol. Chem*., 1990, **265**, 7701-7707.

85. M.L. Schilsky, R.J. Stockert, J.W. Pollard, Caeruloplasmin biosynthesis by the human uterus, *Biochem. J*., 1992, 288, 657-661.

86. T. Thomas, A. Macpherson, P. Rogers, Ceruloplasmin gene expression in the rat uterus, *Biochim. Biophys. Acta*, 1995, **1261**, 77-82.

87. L.V. Puchkova, C.A. Zhiguleva, S.V. Mokshina, N.K. Bichevaia, N.A. Platonova, N.N. Skvortsova, L.K. Sasina, N.V. Tsymbalenko, N.A. Chebotar, V.S. Gaitshoki, The role of the yolk sac in copper metabolism during rat

Published on 03 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

embryogenesis, *Ontogenez*, 2001, **32**, 204-211.

88. H.S. Anderson, L. Gambling, G. Holtrop, H.J. McArdle, Effect of dietary copper deficiency on iron metabolism in the pregnant rat, *Br. J. Nutr.*, 2007, **97**, 239-246.

89. K.K. Tong, H.J. McArdle, Copper uptake by cultured trophoblast cells isolated from human term placenta, *Biochim. Biophys. Acta*, 1995, VOL 1269, 233-236.

90. Y.-L. Chu, E.N. Sauble, A. Cabrera, A. Roth, M.L. Ackland, J.F.B. Mercer, M.C. Linder, Lack of ceruloplasmin expression alters aspects of copper transport to the fetus and newborn, as determined in mice, *Biometals*, 2012, **25**, 373-382.

91. A. Garnier, L. Tosi, M. Steinbuch. Ferroxidase II. The essential role of copper in enzymatic activity. *Biochem. Biophys. Res. Comm*., 1981, **98**, 66-71.

92. M.P. Calisch, R.W. Topham. Purification and characterization of the serum ferroxidase inhibitor. *Int. J. Biochem*., 1984, **16**,1127-1134.

93. R.W. Topham, J.H. Woodruff, G.P. Neatrour, M.P. Calisch, R.B. Russo, M.R. Jackson, Role of ferroxidase-II and a ferroxidase inhibitor in iron mobilization from tissue stores, *Biochem. Biophys. Res. Comm*., 1980, **96**, 1532-1539.

94. N.M. Hasan, S. Lutsenko. Regulation of copper transporters in human cells. *Curr. Top Membr*., 2012, **69**, 137-161.

95. Gaetke LM, Chow-Johnson HS, Chow CK, Copper: Toxicological relevance and mechanisms. *Arch. Toxicol*., 2014, **88**, 1929-1938.

96. H.E.-M. Mahmoud, N. Kishikawa, K. Ohyama, T. Imazato, Y. Ueki, N. Kuroda, Determination of human serum semicarbazide=sensitive amine oxidase activity via flow injection analysis with fluorescence detection after online derivitization of the enzymatically produced benzaldehyde with 1,2-diaminoanthraquinone, *Analyt. Chim. Acta*, 2015, **881**,139-147.

97. E.I. Solomon, D.E. Heppner, E.M. Johnston, J.W. Ginsbach, J. Cirera, M. Qayyum, M.T. Kieber-Emmons, C.H. Kjaergaard, R.G. Hadt, L. Tian, Copper active sites in biology, *Chem. Rev*., 2014, **114**, 3659-3853.

98. E.M. Shepard, D.M. Dooley, Inhibition and oxygen activation in copper amine oxidases. *Acc. Chem. Res*. 2015, **48**, 1218-1226.

99. T. Murakawa, A. Hamaguchi, S. Nakanishi, M. Kataoka, T. Nakai, Y. Kawano, H. Yamaguchi, H. Hayashi, K. Tanizawa, Probing the catalytic mechanism of copper amine oxidase from arthrobacter globiformis with halide ions, *J .Biol. Chem*. 2015, **290**, 23094-23109.

100. M. Largeron, Amine oxidases of the quinoproteins family: their implication in the metabolic oxidation of xenobiotics*, Ann. Pharm. Fr*. 2011, **69**, 53-61.

101. E. Garcia-Martin, C. Martinez, M. Serrador, H. Alonzo-Navarro, P. Ayuso, F. Navacerrada, J.A. Agundez, F.J. Jimenez-Jimenez. Diamine oxidase rs10156191 and rs2052129 variants are associated with their risk for migraine, *Headache,* 2015, **55**, 276-286.

102. S. Bodmer, C. Imark, M. Kneubuhl. Biogenic amines in foods: Histamine and food processing, *Inflamm. Res*., 1999, **48**, 296-300.

103. G. Manzotti, D. Breda, M. Di Gioacchino, S.E. Burastero, Serum diamine oxidase activity in patients with histamine intolerance, *Int. J. Immunopathol. Pharmacol.,* 2016, **29**, 105-111.

104. E. Music, P. Korosec, M. Silar, K. Adamic, M. Kosnik, M. Rijavec, Serum diamine oxidase activity as a diagnostic test for histamine intolerance. *Wien, Klin. Wochenschr.*, 2013, **125**, 239-243.

105. J.A. Agundez, P. Ayuso, J.A. Cornejo-Garcia, M. Binaca, M.J. Torres, I. Dona, M. Salas, N. Blanca-Lopez, G. Canto, C. Rondon, P. Campo, J.J. Laguna, J. Fernandez, C. Martinez, E. Garcia-Martin. The diamine oxidase gene is associated with hypersensitivity response to non-steroidal anti-inflammatory drugs. *PLoS One,* 2012, **7**: e47571.

106. L. Zhang, X. Fan, A. Zhong, G. Xu, J. Shen, Association of plasma diamine oxidase and intestinal fatty acid-binding protein with severity of disease in patient with heat stroke, *Am. J. Emergency Med*., 2015, **33**, 867-871.

107. R.A. DiSilvestro, J. Selsby, K. Siefker. A pilot study of copper supplementation effects on plasma F2a isoprostanes and urinary collagen crosslinks in young adult women. *J. Trace Elements Med. Biol*. 2010, **24**, 165-168.

108. T. Fukai, M. Ushio-Fukai, Superoxide dismutases: role in redox signaling, vascular function, and diseases, Antioxid. Redox Signal. 2011, **15**, 1583–1606.

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 03 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

109. S. Itoh, K. Ozumi, H.W. Kim, O. Nakagawa, R.D. McKinney, R.J. Folz, I.N. Zelko, M. Ushio-Fukai, T. Fukai, Novel mechanism for regulation of extracellular SOD transcription and activity by copper: role of antioxidant-1, *Free Rad. Biol. Med.,* 2009, **46**, 95-104.

110. Z. Qin, M.C. Gongora, K. Ozumi, S. Itoh, K. Akram, M. Ushio-Fukai, T. Fukai, T, Role of Menkes ATPase in angiotensin II-induced hypertensioin: a key modulator for extracellular superoxide dismutase function, Hypertension, 2008, **52**, 945-951.

111. M. Che, R. Wang, X. Li, H.-Y. Wang, X.F.S. Zheng, Expanding roles of superoxide dismutases in cell regulation and cancer, *Drug Discovery Today*, 2016, 21, 143-149.

112. M.L. Teoh-Fitzgerald, M.P. Fitzgerald, W. Zhong, R.W. Askeland, F.E. Domann, Epigenetic reprogramming governs ecSOD expression during human mammary epithelial cell differentiation, tumorigenesis and metastasis, *Oncogene*, 2014, **33**, 358-368.

113. B.R. O'Leary, M.A. Fath, A.M. Bellizzi, J.E. Hrabe, A.M. Button, B.G. Allen, A.J. Case, S. Altekruse, B.A. Wegner, G.R. Buettner, C.F. Lynch et al, Loss of SOD3 (ecSOD) expression promotes an aggressive phenotype in human pancreatic ductal adenocarcinoma, *Clin. Cancer Res*., 2015, **21**, 1741. doi: 10.1158/1078-0432.CCR-14-1959

114. C.A. Wang, J.C. Harrell, R. Iwanaga, P. Jedlicka, H.L. Ford, Vascular endothelial growth factor C promotes breast cancer progression via a novel antioxidant mechanism that involves regulation of superoxide dismutase 3, *Breast Cancer Res*., 2014, 16(5):462. doi: 10.1186/s13058-014-0462-2.

115. G. Fanali, A. di Masi, V. Trezza, M. Marino, M. Fasano, P. Ascenzi, Human serum albumin: from bench to bedside, *Mol. Aspects Med*., 2012, **33**, 209-290.

116. S.H. Yap, R.K. Strair, D.A. Shafritz, Effect of a short term fast on the distribution of cytoplasmic albumin messenger ribonucleic acid in rat liver, *J. Biol. Chem*., 1978, **253,** 4944-4950.

117. A. Pietrangelo, A. Panduro, J. R. Chowdhury, D.A. Shafritz, Albumin gene expression is down-regulated by albumin or macromolecular infusion in the rat, *J. Clin. Invest*., 1992, **89**: 1755-1760.

118. M. Anraku, V.T, Chuang, T. Maruyama, M. Otagiri, Redox properties of serum albumin, *Biochim. Biophys. Acta,* 2013, **1830**, 5465-5472.

119. M.E. Sitar, S. Avdin, U. Cakatay, Human serum albumin and its relation with oxidative stress, *Clin. Lab*. 2013, **59,** 945-952.

120. S. Lau, B. Sarkar, Ternary coordination complex between human serum albumin, copper(II) and L-histidine, *J. Biol. Chem.,* 1971, **246,** 5938-5943.

121. J. Masuoka, J. Hegenauer, B.R. Van Dyke, P. Saltman, 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.

122. A. Cabrera, E. Alonzo, E. Sauble, Y.-L. Chu, M.C. Linder, D.S. Sato, A.Z. Mason, Copper binding components of plasma and cytoplasm, copper turnover and excretion, as determined in the mouse with large doses of ^{65}Cu, *Biometals* **21**, 2008, 525-543.

123. H. Fieten, P.A.J. Leegwater, A.L. Watson, J. Rothuizen. Canine models of copper toxicosis for understanding mammalian copper metabolism, *Mamm. Genome,* 2012, **23**, 62-75.

124. M. Linder, M. Hazegh-Azam, The biochemistry and molecular biology of copper, *Am. J. Clin. Nutr. Supplement* 1996, **63**, 797S-811S.

125. A.A. Rehman, H. Ahsan, F.H. Khan. Alpha-2-macroglobulin: A physiological guardian. J. Cell Physiol., 2013, **220,** 1665-1675.

126. C. Falkenberg, A. Grubb, B. Akerstrom, Isolation of rat serum alpha 1-microglobulin. Identification of a complex with alpha 1-inhibitor-3, a rat alpha 2-macroglobulin homologue, *J. Biol. Chem*., 1990, **265**, 16150-16157.

127. A.V. Gourine, V.N. Gourine, Y. Tesfaigzi, N. Caluwaerts, F. van Leuven, M.J. Kluger, Role of alpha(2)-macroglobulin in fever and cytokine responses induced by lipopolysaccharide in mice, *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* 2002, **283**, R218-R226.

128. S.L. Gonias, A. Carmichael, J.M. Mettenburg, D.W. Roadcap, W.P. Irvin, D.J. Webb, Identical or overlapping sequences in the primary structure of human a2-macroglobulin are responsible for the binding of nerve growth factor-beta, platelet-derived growth factor-BB, and transforming growth factor-beta. *J. Biol. Chem*., 2000, **275**, 5826-5831.

129. G. Peslova, J. Petrak, K. Kuzelova, I. Hrdy, P. Halada, P.W. Kuchel, S. Soe-Lin, P. Ponka, R. Sutak, E. Becker,

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

M. L.-H. Huang, Y.S. Rahmanto, D.R. Richardson, Hepcidin, the hormone of iron metabolism, is bound specifically to alpha-2-macrogllobulin in blood, *Blood*, 2009, **113**, 6225-6236.

130. E. Nemeth, M.S. Tuttle, J. Powelson, M.B. Vaughn, A. Donovan, D.M. Ward, T. Ganz, J. Kaplan, Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization, *Science*, 2004, **306**, 2090- 2093.

131. G. Ramey, J.C. Deschemin, B. Durel, F. Canonne-Hergaux, G. Nicolas, S. Vaulont, Hepcidin targets ferroportin for degradation in hepatocytes, *Haematologica*, 2009, 95, 501-504.

132. Uskokovic A, Dkinic S, Mihailovic M, Grigorov I, Ivanovic-Matic S, Bogojevic D, Grdovic N, Arambasic J, Vidakovic M, Martinovic V, Petrovic M, Poznanovic G. STAT3/NFkB interplay in the regulation of a2-macroglobulin gene expression during rat liver development and the acute phase response, *Life*, 2007, **59,** 170-178.

133. A. Pal, M. Siotto, R. Prasad, R. Squitti, Towards a unified vision of copper involvement in Alzheimer's disease: A review connecting basic, experimental, and clinical research, *J. Alzheimers Dis*., 2015, **44**, 343-354.

134. A. Onodera, M. Tani, T. Michigami, M. Yamagata, K.S. Min, K. Tanaka, T. Nakanishi, T. Kimura, N. Itoh, Role of megalin and the soluble form of its ligand RAP in Cd-metallothionein endocytosis and Cd-metallothionein-onduced nephrotoxicity in vivo, *Toxicol. Lett*., 2012, **212**, 91-96.

135. C. Langelueddecke, W.K. Lee, F. Thevenod, Differential transcytosis and toxicity of the hNGAL receptor ligands metallothionein and cadmium-phytochelatin in colon-like Caco2 cells: implications for in vivo cadmium toxicity, *Toxicol. Lett*., 2014, **226**, 228-235.

136. S. Lutsenko, Human copper homeostasis: a network of interconnected pathways, Curr. Opin. Chem. Biol., 2010, **14**, 211–217.

137. L.W. Gray, F. Peng, S.A. Molloy, V.S. Pendyala, A. Muchenditsi, O. Muzik, J. Lee, J.H. Kaplan, S. Lutsenko, Urinary copper elevation in a mouse model of Wilson's disease is a regulated process to specifically decrease the hepatic copper load, *PLoS ONE*, 2012, **7**(6): e38327. doi:10.1371/journal.pone.0038327

138. S. Pemberton, P. Lindley, V. Zaitzev, G. Card, E.G.D. Tuddenham, G. Kemball-Clark, A molecular model for the triplicated A domains of human factor VIII based on the crustal structure of human ceruloplasmin, *Blood*, 1997, **89**, 2413-2421.

139. B.W. Shen, P.C. Spiegel, C.-H. Chang, J.-W. Huh, J.-S. Lee, J. Kim, Y.-H. Kim, B.L. Stoddard, The tertiary structure and domain organization of coagulation factor VIII, *Blood*, 2008, **111**, 1240-1247.

140. P.J. Lenting, J.A. van Mourik, K. Mertens, The life cycle of coagulation factor VIII in view of its structure and function, *Blood*, 1998, **92**, 3983-996.

Figure Legends

Fig. 1 Separation of plasma proteins via electrophoresis. Example of the separation of human plasma in non-denaturing electrophoresis, as commonly used in clinical chemistry to assess potential changes related to disease processes, such as the acute phase reaction, or multiple myeloma. This clinical assay used paper strips stained for protein (below), and the densitometric scan is shown above. The largest peak (most acidic protein) is albumin, followed by alpha 1 and alpha-2 globulins, then beta globulin and immunoglobulins (gamma globulins), going from the largest negative charge at neutral pH to the slightly basic IgGs (right to left). The three main copper binding proteins are albumin, and two alpha-2-globulins – ceruloplasmin and α_2-macroglobulin.

Fig. 2 Elution of blood plasma copper binding components in size exclusion chromatography (plasma "Cu profiles"). (A), (B) - Normal adult blood plasma from a mouse and human, respectively, separated on a Biosep 4000 large pore HPLC column, with ^{63}Cu detection by coupled ICP-MS (ion intensity, y axis). One main peak and several "shoulders" are detected, which other data indicate correspond to the three main copper binding proteins, transcuprein/macroglobulin (Mr 180/720 kDa), ceruloplasmin (Mr 132 kDa), and albumin (66 kDa) – which elutes in a position similar to that of hemoglobin (Mr 64 kDa; dotted line in (A)). (B) shows the elutions of 5 separate human samples. Modified and printed with permission from Cabrera et al., *Biometals* 21, 2008, 525-543 [122]. (C), (D) – Elution of plasma from WT and ceruloplasmin knockout (CpKO) mice in Superdex200 FPLC (with furnace atomic absorption analysis of Cu) or Biosep 4000 FPLC, respectively, showing the average result for several samples. (D) is reproduced from Gray et al., *Biochem. J.,* 2009, 419, 237-245 [8]. (E) – Elution of rat plasma non-radioactive copper (X and dotted line) and radioactive copper (solid dots and line) after *in vitro* addition of tracer ^{64}Cu in size exclusion chromatography on Sephacryl S300. The thin line with no symbols is A_{280}. Reprinted with permission from Liu et al., *J Nutr.*

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 03 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

Biochem., 2007, 18, 597-608 [4]. (F) – Cu profile of the blood plasma of mice receiving massive doses of the stable isotope ^{65}Cu, separated by size exclusion chromatography in Biosep 4000 HPLC coupled with tandem ICP-MS. The solid line shows distribution of newly injected ^{65}Cu associated with the elution of albumin; the dotted line shows the normally dominant stable isotope, ^{63}Cu, where Cp is the major peak. Reprinted with permission from Cabrera et al. [122].

Fig. 3 Structures of ceruloplasmin and coagulation factor VIII. (A) – Crystal structure of copper-containing (holo) ceruloplasmin (Cp) based on studies of Zaitseva et al. [6,7], showing the overall three lobe structure each with two domains. Copper atoms are shown in blue and are buried within the molecule associated with domains 2, 4 and 6, and the trinuclear cluster between the N and C-terminal domains, likely to open to release the copper for uptake by cells (see text). Structure reprinted with permission from ACS C & E News (8533scicon_cp) was annotated. (B) – Structure of factor VIII modeled on the Cp crystal structure, showing the similarities of the A1-A3 domains to Cp, and copper atoms in domains A1 and A3, which have ligands identical or similar to those for the single Cu ions in Cp. Reprinted with permission from Shen et al., *Blood*, 2008, 111, 1240-1247 [139], with annotations added.

Fig. 4 Mechanism of uptake of copper from purified ceruloplasmin (Cp) during interaction with mouse embryonic fibroblasts in culture. Demonstration that this occurs at the cell surface with conversion of holo to apo Cp, can be mediated by copper transter 1 (CTR1), and is likely to involve a cell surface reductase. (A) – Uptake of the copper in Cp is not inhibited by 4 different inhibitors of endocytosis (Noc = nocodazole; FSBA = fluorosulfonyl benzoyladenosine ; Dyna = DynaSore©; Pit = Pitstop 2©). (B) – Western blot of native PAGE showing that incubation of pure Cp (containing apo as well as holoCp) with cells but not without cells, results in loss of holoCp, and no change when incubated without cells. (As there was a no change in total Cp detected in the medium, this means holo- was converted to apo-Cp.) Reprinted from Ramos et al. [44]. (C) – CTR1 can mediate uptake of copper from Cp: Cu uptake from Cp by mouse embryonic fibroblasts expressing (WT) and not expressing (Null) Ctr1 (top); effect of Ag^{1+} (which inhibits Cu uptake through Ctr1) on uptake of copper delivered on albumin (bottom). [Albumin has a high affinity copper-binding site (see text).] Differences between WT and null cells, and between cells treated and not treated with silver ions were statistically significant ($p<0.001$). Data for the bar graph are from the text of Ramos et al. [44]; kinetic data are reprinted with permission from Kidane et al., *Biometals*, 2012, 25, 697-709 [69]. (D) – Inhibition of uptake of radiolabeled Cu in Cp by mouse embryonic fibroblasts in the presence of a large excess of non-radioactive Cu^{2+}, Fe^{3+}, or Cu^{1+}. Effects were statistically significant ($p <0.001$). Replotted using data from Ramos et al. [44].

Fig. 5 Ferroxidase activity of blood plasma from humans and rodents and effect of azide and of knocking out ceruloplasmin (Cp). Ferroxidase activity was measured by three different methods (dark bars), and only the results for the transferrin assay are shown. The effect of azide, thought to specifically inhibit Cp enzyme activity, is also shown (light bars). Reprinted from Gray et al., *Biochem. J.,* 2009, 419, 237-245 [8]. Differences between levels of ferroxidase activity in humans versus rodents, and effects of azide and knocking out Cp were statistically significant $p<0.01$ or lower.

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

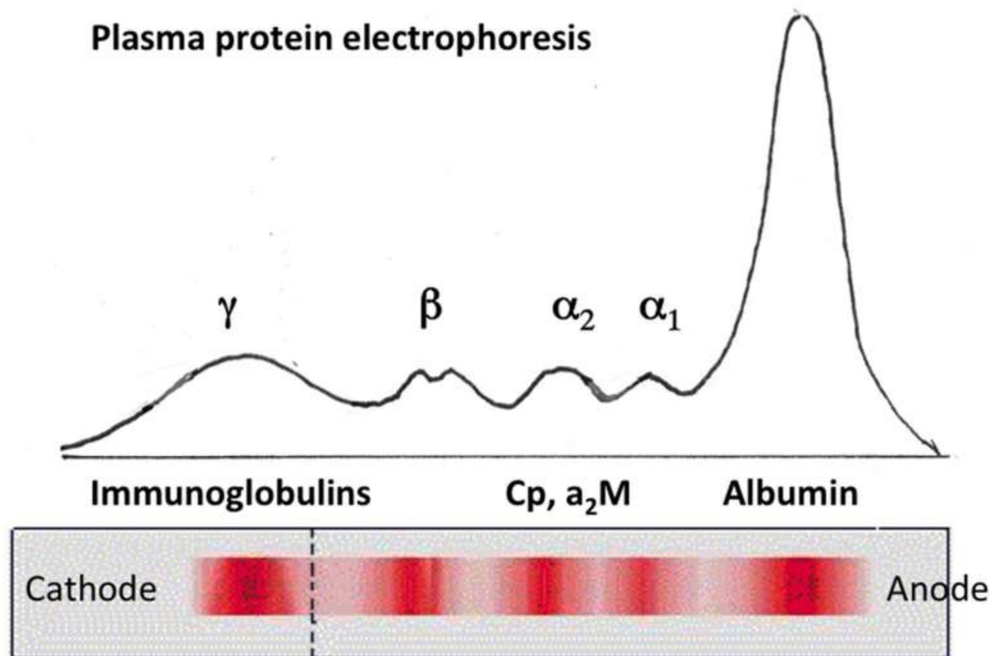


Fig. 1

254x190mm (72 x 72 DPI)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

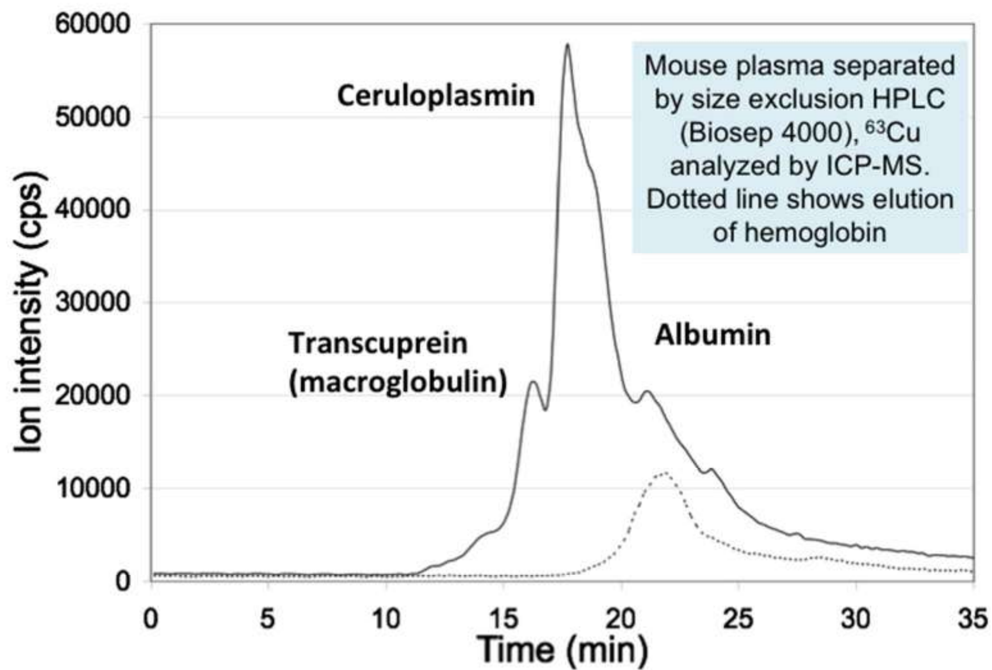


Fig. 2A

254x190mm (72 x 72 DPI)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

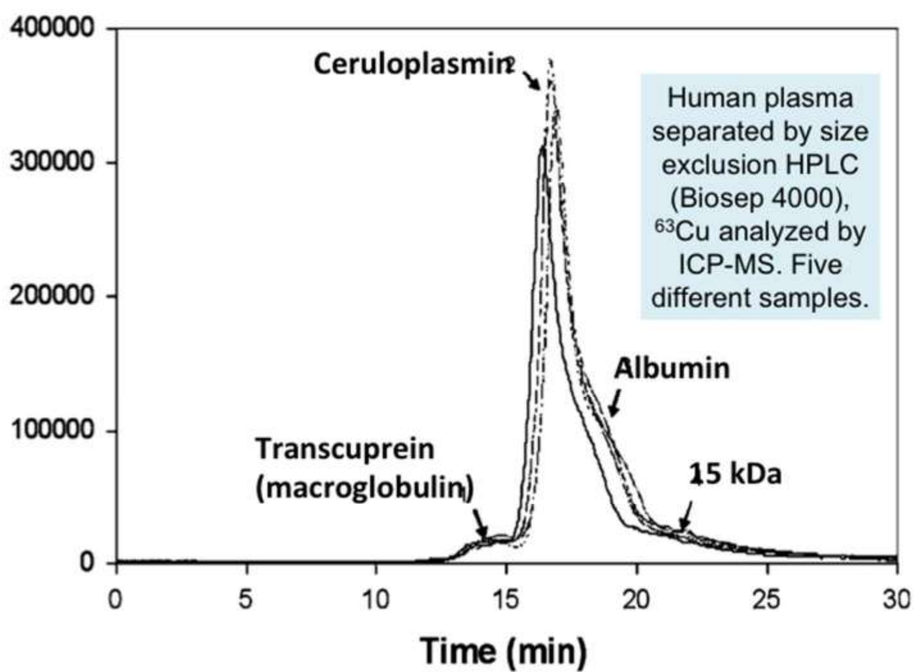


Fig. 2B

254x190mm (72 x 72 DPI)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

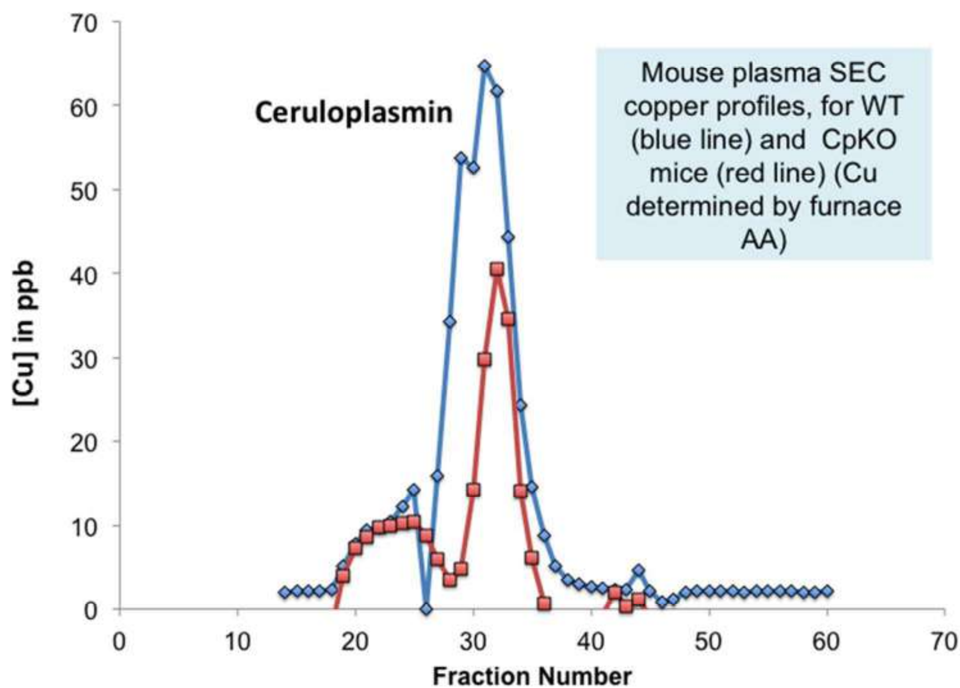


Fig. 2C

254x190mm (72 x 72 DPI)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

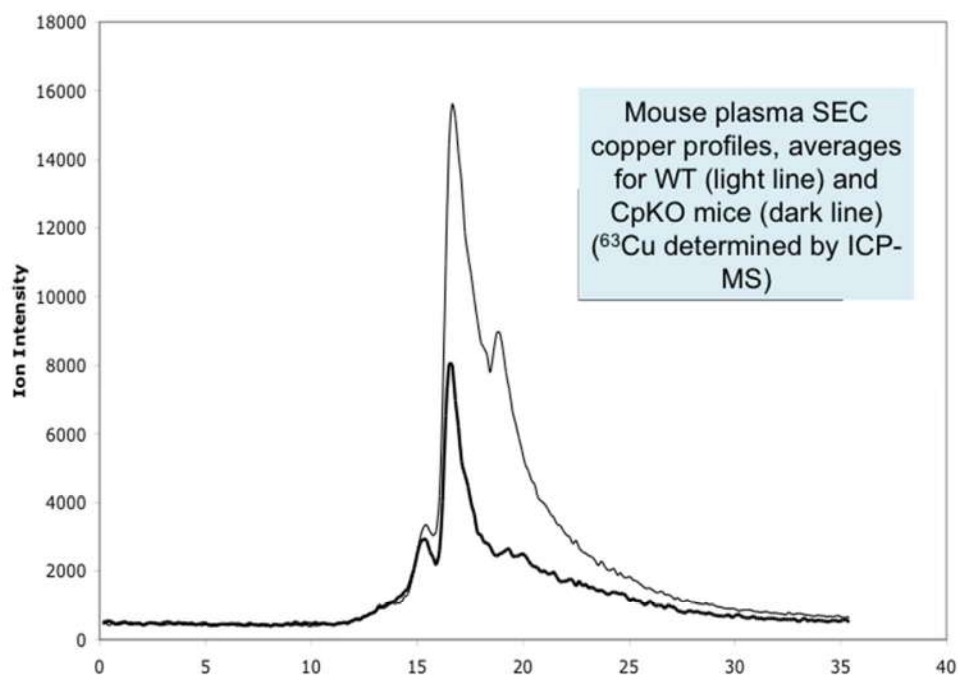


Fig. 2D

254x190mm (72 x 72 DPI)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

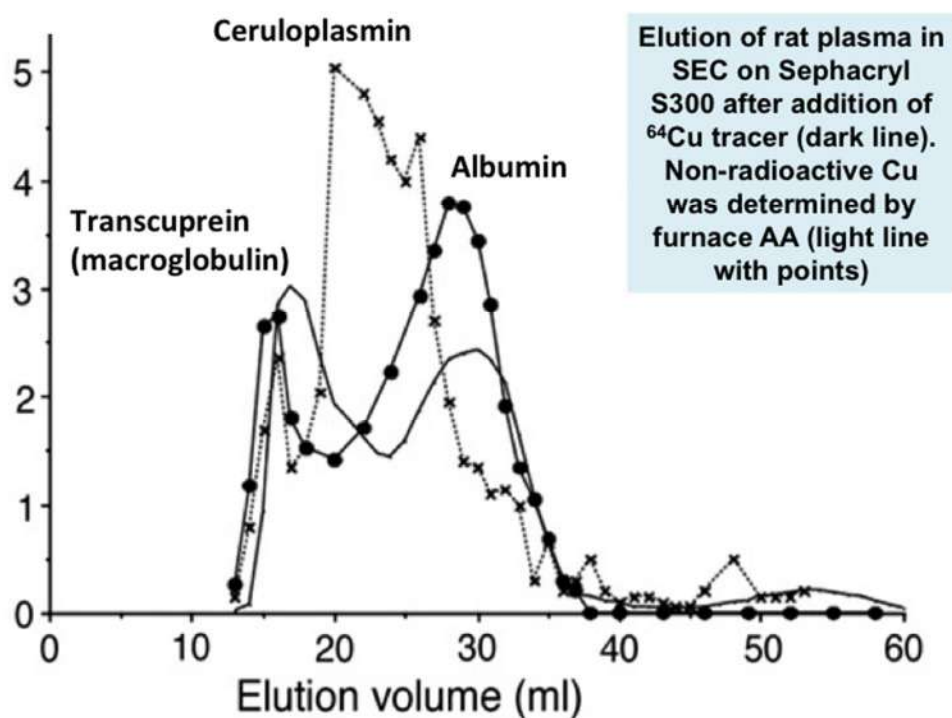


Fig. 2E

254x190mm (72 x 72 DPI)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

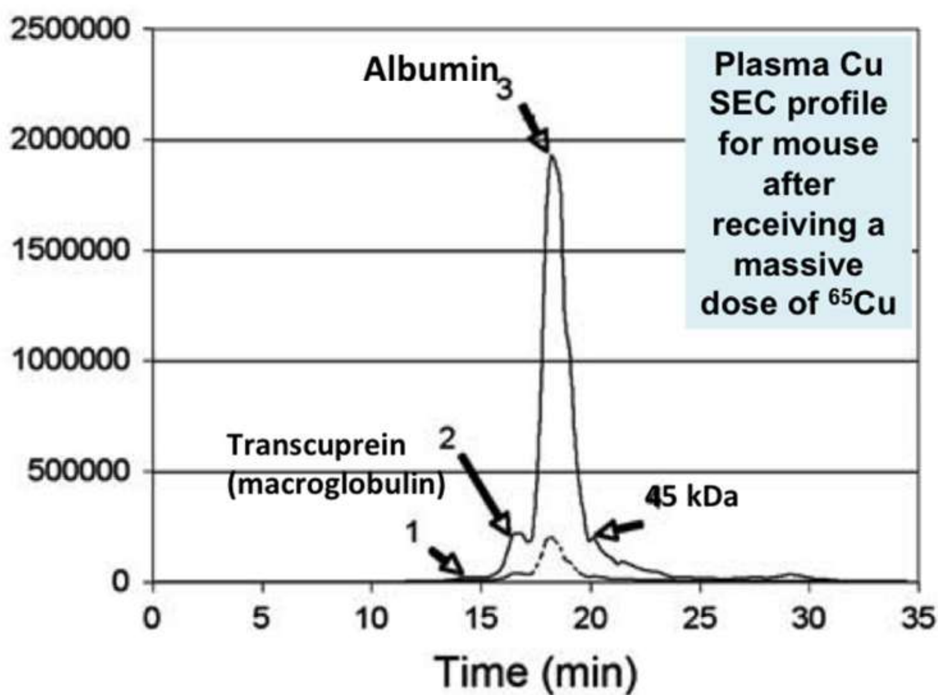


Fig. 2F

254x190mm (72 x 72 DPI)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

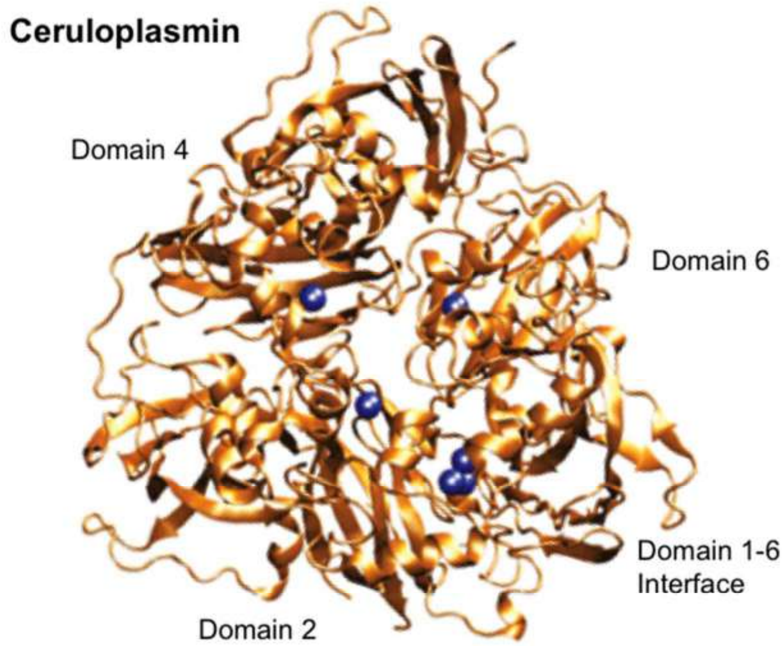


Fig. 3A

254x190mm (72 x 72 DPI)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

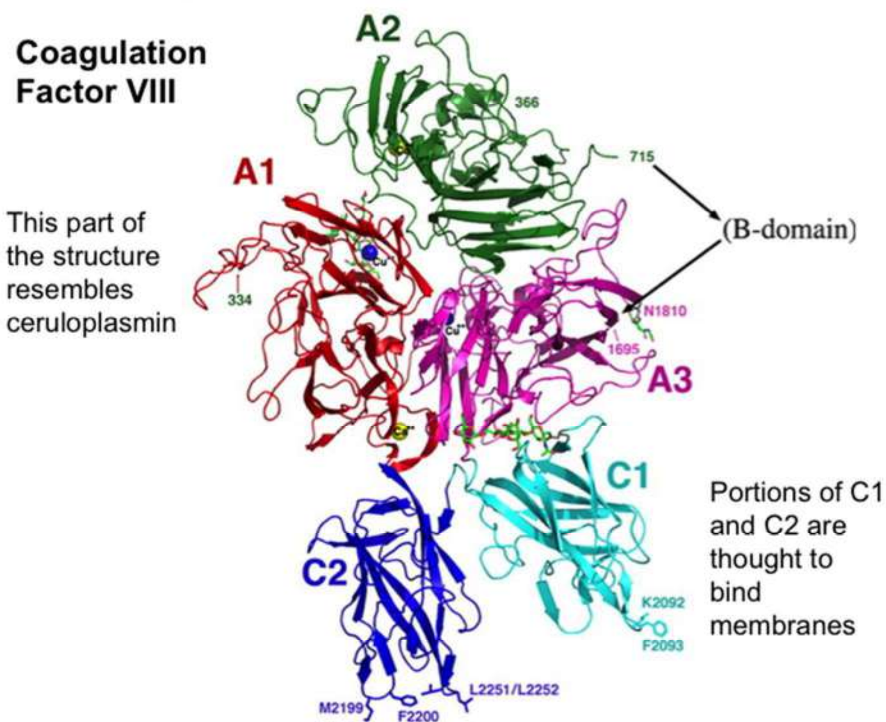


Fig. 3B

254x190mm (72 x 72 DPI)

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

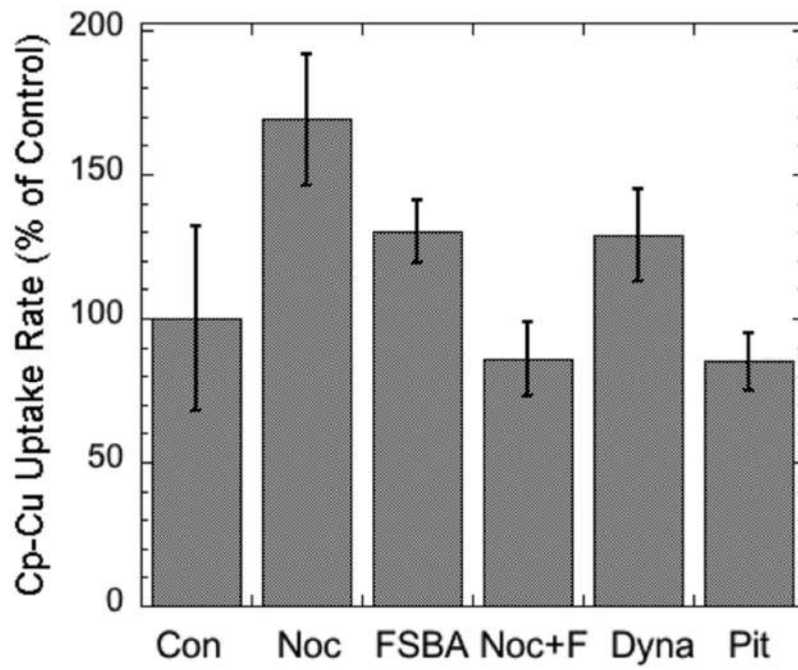


Fig. 4A

254x190mm (72 x 72 DPI)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

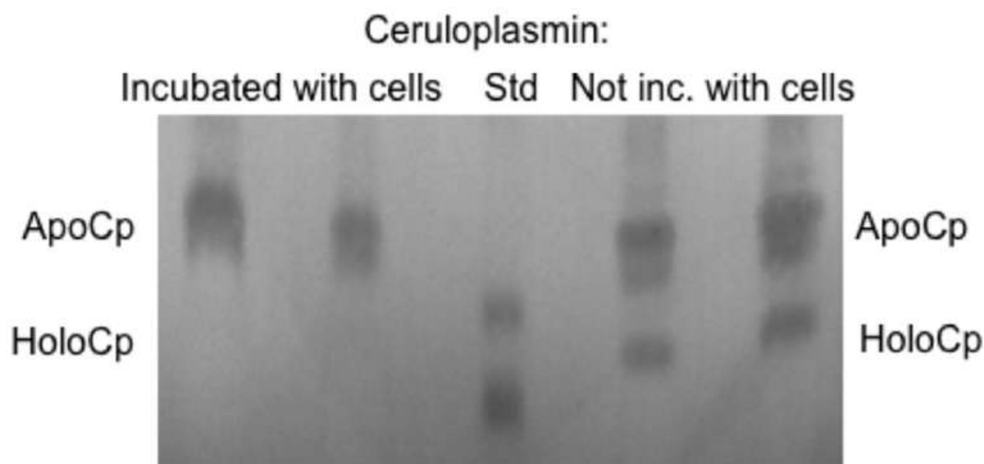


Fig. 4B

254x190mm (72 x 72 DPI)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

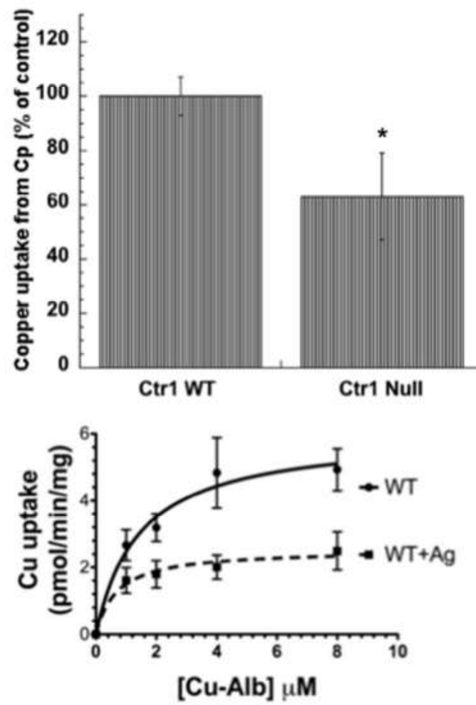


Fig. 4C

254x190mm (72 x 72 DPI)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

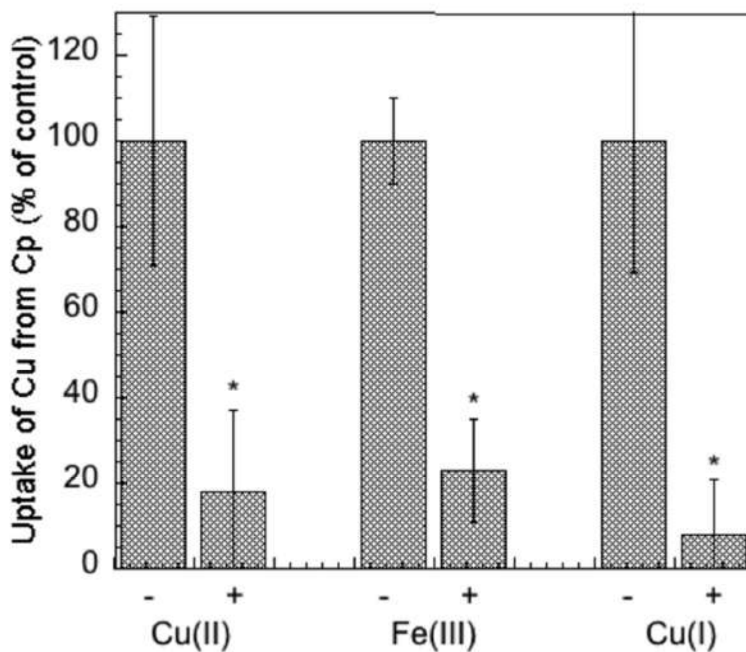


Fig. 4D

254x190mm (72 x 72 DPI)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

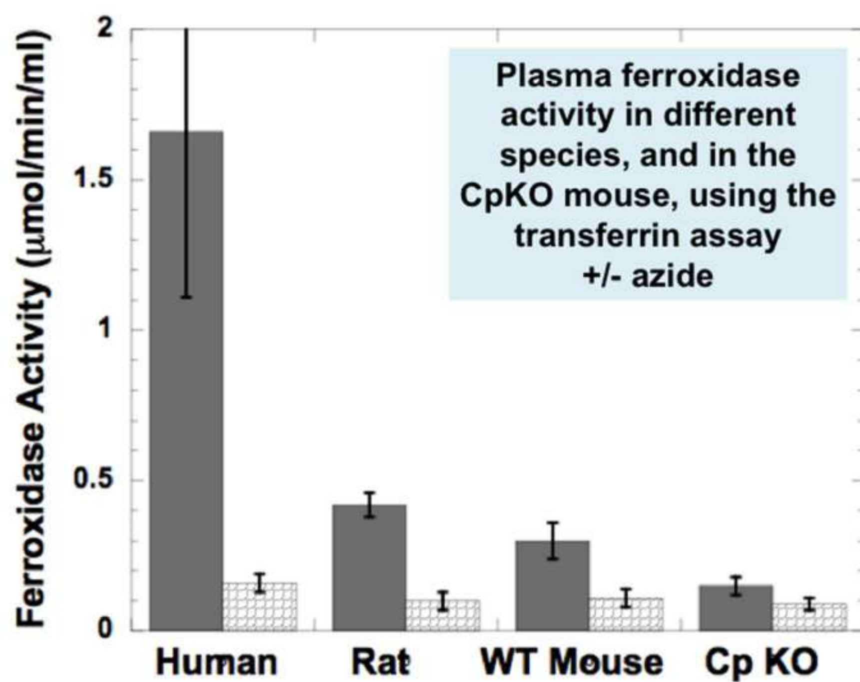


Fig. 5

254x190mm (72 x 72 DPI)

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.

Table 1 Copper binding components of the blood plasma and other fluids

Component	Relative abundance and function(s)
Ceruloplasmin*	Main copper binding glycoprotein in blood plasma, accounting for 40-70% of total plasma copper; also present in milk and intestinal, cerebrospinal, testicular and amniotic fluids; multifunctional protein: acute phase-reactant, antioxidant defense, oxidative inactivation of NO and biogenic amines; ferroxidase, inhibited by azide, involved in cellular iron efflux; delivers copper directly to cell membrane transporters with formation of apoceruloplasmin. GPI-linked form is tethered to some cell surfaces.
Albumin	The most abundant protein in blood plasma, also present in other body fluids in lower concentrations; in most mammals it has a high affinity copper binding site at the N-terminus, involving a histidine (Kd 10^{-12}-10^{-17} M); probably accounts for 10-15% of plasma copper. Major constituent of the exchangeable copper pool of blood plasma; probably can deliver copper to most cells, but preferentially delivers to those in liver and kidney. Transports many other nutrients and metabolites, from fatty acids and bile salts (like bilirubin), to tryptophan and other metal ions.
Transcuprein/ macroglobulin	Alpha-2-macroglobulin (α2M) in humans; alpha-1-inhibitor 3 macroglobulin in rodents. Tetrameric α2M binds two Cu atoms with higher affinity than albumin. Major constituent of the plasma exchangeable copper pool, accounting for 5-15% of plasma copper; probably can deliver copper to most cells but preferentially delivers to those in liver and kidney. Also carries Zn^{2+} and hepcidin (regulator of the iron efflux transporter, ferroportin).
Clotting factors V and VIII	Blood plasma proteins needed for blood coagulation, with some structural homology to ceruloplasmin, Copper may stabilize modified structures involved in coagulation activation. Factor VIII mutations are responsible for the most common form of human hemophilia.
Extracellular* superoxide dismutase (SOD3)	In blood plasma and perhaps other fluids; catalyzes same reaction as other SODs; part of mammalian antioxidant defenses. Main expression is in the lung and vascular epithelia. Knockout results in lung damage. Of current interest is its role in cancer promotion.
Amine and diamine oxidases *,**	Extracellular (and possibly intracellular) enzymes, that (like ceruloplasmin) oxidatively deaminate amines, playing a role in the inactivation or modification of bioactive amines, such as histamine, methyl histamine, tyramine, and polyamines like putrescine.
Ferroxidase II	Large, two-subunit protein containing lipid, with ferroxidase activity, first isolated by Topham and Frieden, Ferroxidase activity is not inhibited by azide (unlike that of ceruloplasmin), but may be inhibited by albumin.
Metallothionein*	Traces of this otherwise cytosolic component (present inside most cells) are in the blood plasma, probably bound to divalent metal ions, especially Cd^{2+} but also probably some Cu^{2+} and Zn^{2+}. The copper form (copper thionein) has superoxide dismutase activity.

Metallomics Accepted Manuscript

View Article Online
DOI: 10.1039/C6MT00103C

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

45 kDa protein	Identity unknown; detected by size exclusion HPLC of plasma [Cabrera].
Small Cu carriers (SCCs)	Usually found in trace amounts in normal plasma but in large amounts amounts in some conditions, including liver copper overload, and excreted in urine.

*Enzyme; reaction requires O_2 or O_2^-
**2,4,5-trihydroxyphenylalaninequinone (TOPA quinone) cofactor produced non-enzymatically by Cu [Linder 2010 review]

Published on 07 July 2016. Downloaded by University of Glasgow Library on 13/07/2016 12:52:04.
Metallomics Accepted Manuscript