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Identification of a haptoglobin-hemoglobin complex in human blood plasma

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ABSTRACT

Blood plasma metalloproteins that contain copper (Cu), iron (Fe), zinc (Zn) and/or other metals/metalloids are potential disease biomarkers because the bloodstream is in permanent contact with organs. Their quantification and/or the presence of additional metal-entities or the absence of certain metalloproteins in blood plasma (e.g. in Wilson's disease) may provide insight into the dyshomeostasis of the corresponding metal (s) to gain insight into disease processes. The first step in investigating if the determination of plasma metalloproteins is useful for the diagnosis of diseases is their definitive qualitative identification. To this end, we have added individual highly pure Cu, Fe or Zn-containing metalloproteins to plasma (healthy volunteer) and analyzed this mixture by size-exclusion chromatography (SEC) coupled to an inductively coupled plasma atomic spectrometer (ICP-AES), simultaneously monitoring the emission lines of Cu, Fe and Zn. The results clearly identified ceruloplasmin (Cp), holo-transferrin (hTf), and α_2-macroglobulin (α_2M), which verifies our previous assignments. Interestingly, another major Fe-peak in plasma was identified as a haptoglobin (Hp)-hemoglobin (Hb) complex. This Hp-Hb complex is formed after Hb, which is released during the hemolysis of erythrocytes, binds to the plasma protein Hp. The Hp-Hb complex formation is known to be one of the strongest interactions in biochemistry ($K_d \approx 1$ pmol/L) and is critical because it prevents kidney toxicity of free Hb. Hence, the simultaneous determination of Cp, hTf, α_2M and the Hp-Hb complex in plasma in < 25 min has the potential to provide new insight into disease processes associated with the bioinorganic chemistry of Cu, Fe and Zn.

1. Introduction

About 1/3 of all mammalian proteins have a metal cofactor [1] and these metalloproteins serve a variety of vital biochemical roles in the context of maintaining the health and wellbeing of mammals [2]. All mammals have a minimum daily requirement for Cu, Fe and Zn [3] and adequate amounts must therefore be absorbed from the gastrointestinal tract into the systemic blood circulation for their subsequent transport to organs where metalloprotein assembly takes place [4]. The transport of Cu, Fe and Zn from the bloodstream to organs and their homeostasis therein is orchestrated by intricate biomolecular mechanisms, many of which involve metal-specific plasma transport proteins, such as Tf [5] and Cp [6]. Considering that blood plasma also transports metal-containing metabolites from organs for excretion (e.g. the kidneys), the determination of plasma metalloentities may also provide insight into metal/metalloid dyshomeostasis and its association to disease processes. Wilson's disease, for example, is associated with the absence of the plasma metalloprotein Cp owing to a genetic defect that disrupts the insertion of Cu into this protein in the liver [7].

In an attempt to explore the inherent potential of plasma metalloproteins for diagnostic purposes [8–10], we have previously reported on the systematic development of a metallomics method to simultaneously detect ~12 Cu, Fe and Zn metalloproteins in rabbit plasma in < 25 min [11]. This metallomics method can directly analyze blood plasma/serum based on size-exclusion chromatography (SEC) using PBS-buffer followed by the detection of the separated metalloentities with an inductively coupled plasma atomic emission spectrometer (ICP-AES). The choice of the mobile phase has been demonstrated to be critical to prevent artefacts resulting from the disruption of metal-protein bonds during the analysis [12]. When this metallomics method was previously applied to analyze fresh rabbit blood plasma consecutively every 30 min, three Cu-metalloproteins disintegrated over a 2 h period (i.e. they were only detectable 30 min after blood collection), whereas one Cu-metalloprotein and all Fe and Zn-metalloproteins remained stable and produced reproducible results [11]. With regard to the number of binding sites of the aforementioned metals on their respective plasma proteins and the concentration of the metalloproteins of interest in human plasma/serum, the reader is referred to previous publications [11,13]. An overview of the concentrations of plasma proteins and metalloproteins in health and specific disease states is available by the Foundation for Blood Research [8].

Of the four detected Cu-metalloproteins (fresh rabbit plasma), the

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peak that eluted first was assigned to blood coagulation factor V (Cf^V) [14] based on the analysis of column fractions using an enzyme immunoassay. The rapid disappearance of Cf^V in plasma was consistent with the established lability of the Cu-protein bond in Cf^V [15]. The second Cu-peak was assigned to transcuprein based on the previously reported order of elution of Cu-metalloproteins in rat plasma using a similar LC-approach [16]. The Cu-peak that eluted thereafter was identified as Cp based on the analysis of column fractions using an oxidation assay [11], while the fourth Cu-peak was assigned to serum albumin-bound Cu based on its co-elution with the most intense sulfur-peak of this highly abundant plasma protein. A single Cu-peak eluted in the inclusion volume, which corresponded to low molecular weight (LMW) Cu that had been previously observed by others [17].

With regard to Zn, ~five Zn-metalloproteins were detected in rabbit plasma, some of which were not baseline separated [11]. Using an enzyme immunoassay, the first Zn-peak was qualitatively identified as α_2-macroglobulin (α_2M), whereas the last Zn-peak was assigned to serum albumin-bound Zn, based on its co-elution with Cu bound to the same protein. Because of their poor separation, the remaining Zn-metalloproteins could not be qualitatively identified.

Two Fe-peaks were detected in rabbit plasma with the first one being much less intense than the second. By employing enzyme immunoassays of column fractions, the first and second Fe-peak were assigned to ferritin (Ft) and holo-transferrin (hTf), respectively [11]. Thus, the majority of individual metal peaks in rabbit plasma were qualitatively assigned either based on the analysis of collected column fractions by enzyme immunoassays (CfV, Cp, Ft, hTf, α_2M), by analogy to previous studies (transcuprein), by co-elution with HSA (Cu-HSA, Zn-HSA) or by their retention time (LMW-Cu).

With the goal of applying the developed SEC-ICP-AES method for the analysis of human plasma [10], we previously reported a similar number of Cu, Fe and Zn-metal peaks therein, but considerable differences of the relative intensity of the peaks corresponding to Cf^V, Cp and hTf were observed [13]. These findings are not entirely unexpected given that mammalian species are known to differ in terms of their bioinorganic chemistry and correspondingly their metalloprotein plasma concentrations. While the retention times of Cp, Cu-HSA, LMW-Cu, hTf, α_2M and Zn-HSA were similar in rabbit and human blood plasma [13], the retention time of what appeared to be Ft was ~100 s shorter in human plasma, which was difficult to explain at the time.

To unequivocally confirm the previous assignment of Cu, Fe and Zn-metal peaks in rabbit/human plasma to distinct metalloproteins, we acquired highly pure human Ft (MW 450 kDa), hTf (MW 79.7 kDa), Cp (MW 132 kDa) and α_2M (MW 725 kDa) standards and analyzed human plasma and human plasma that was spiked with each of these metalloprotein standards. The results represent an important step towards the full characterization of the human plasma metalloproteome, which is important to better understand bioinorganic processes that unfold in the bloodstream [18,19]. The authors are in the process of analyzing patient serum samples for metalloproteins and will publish these results in a separate paper.

2. Experimental

2.1. Chemicals and solutions

Phosphate buffered saline (PBS) powder, Krebs-Ringer bicarbonate buffer powder, sodium hydroxide (> 97%) ferritin (human liver, 1 mg, > 99% by SDS-PAGE) and Tris-buffer (Trizma base, ≥99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA), while mixed type haptoglobin from human plasma, predominantly phenotype 2-1 (1 mg, > 95% by SDS-PAGE), haptoglobin phenotype 1-1 (1 mg, > 95% by SDS-PAGE, MW 86,000), haptoglobin phenotype 2-2 (1 mg, ≥95% by SDS-PAGE, MW 170,000-900,000), holo-transferrin (human plasma, 100 mg, > 95% by SDS-PAGE), ceruloplasmin (human plasma, > 95% by SDS-PAGE) and alpha-2-macroglobulin (human plasma, 1 mg, ≥95% by SDS-PAGE) were obtained from Athens Research and Technology (Athens, GA, USA). HCl (34–37%) was purchased from SCP Science (Baie D'Urfe, Quebec, Canada). All pH measurements were done with a VWR Symphony SB20 pH meter (Thermo Electron Corporation, Beverly, MA, USA) and all aqueous solutions were prepared using DI water from a Simplicity water purification system (18.3 MΩ●cm, Millipore, Billerica, MA, USA) after filtration through 0.45-μm nylon-filter membranes (Mandel Scientific, Guelph, ON, Canada). A mixture of protein standards containing thyroglobulin (670 kDa), ɣ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). After reconstitution of the proteins in DI water following the manufacturer's protocol, the solution was injected onto the SEC system to size-calibrate the Superdex™ 200 Increase column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

2.2. Solutions and mobile phases

PBS-buffered saline (10 mM phosphate, 2.7 mM KCL and 137 mM NaCl) was prepared by dissolving PBS powder in the appropriate volume of DI water (1 packet per 1.0 L of DI water). Krebs-Ringer buffer (147.5 mM) was prepared by dissolving a Krebs-Ringer buffer package an appropriate volume of DI water followed by the addition of 1.26 g of sodium bicarbonate to 1 L. Tris-buffer (25 mM) was prepared by dissolving the appropriate amount of Trizma base in DI water. All mobile phases were adjusted to pH 7.4 by the addition of either HCl or NaOH (4.0 M) and filtered through 0.45-μm nylon-filter membranes (Mandel Scientific, Guelph, ON, Canada) before use. A 0.5 M NaOH solution for cleaning the Superdex™ 200 Increase column was prepared by dissolving NaOH pellets in an appropriate volume of DI water and filtered through a 0.45-μm nylon-filter membranes.

2.3. Blood plasma preparation

The collection of human blood from a healthy male volunteer (12 h fasting) was approved by the Calgary Conjoint Health Ethics Board (approval number E-21198). Blood was collected into trace metal grade heparinized vacutainer tubes (Becton Dickinson, Franklin Lake, New Jersey) and centrifuged at 3000 ×*g* for 20 min (4 °C) to separate blood into plasma and erythrocytes. The supernatant plasma was pooled into a 150 mL Teflon container, gently mixed and aliquots of plasma (1.0 mL or 2.0 mL) were stored in a liquid nitrogen dewar. All experiments reported in this study were conducted with plasma from this stock.

2.4. Erythrocyte lysate preparation

Following the centrifugation of whole blood (3000 ×*g*, 20 min, 4 °C) and the removal of plasma and the buffy coat, the pellet was washed twice with an equal volume of Krebs-Ringer buffer (9.5 g/L) and centrifuged (3000 ×*g*, 10 min, 4 °C). The erythrocytes were lysed with an equal volume of deionized (DI) water (22 °C) followed by centrifugation at 20,000 ×*g* for 60 min to remove cell debris. The clear lysate was pooled into a 150 mL Teflon container, gently mixed and aliquots of the lysate (1.0 mL or 2.0 mL) were flash frozen and stored in a liquid nitrogen dewar.

2.5. Sample preparation of human plasma

Human plasma aliquots (1.0 mL) were thawed at room temperature for 45 min, gently mixed and 500 μL were directly injected onto the SEC column (triplicate analysis). To identify the metal peaks in plasma, individual human metalloprotein standards were added to plasma. Spiking involved the addition of a Cp solution (prepared by dissolving 1.0 mg of Cp in 100 μL of DI water and adding this solution to 600 μL of plasma), α_2M solution (prepared by dissolving 1.0 mg of α_2M in 100 μL DI water and adding this solution to 900 μL plasma), Ft solution (prepared by dissolving 1.0 mg of Ft in 100 μL DI water and adding this solution to 700 μL plasma) and hTf (prepared by dissolving 100 mg of

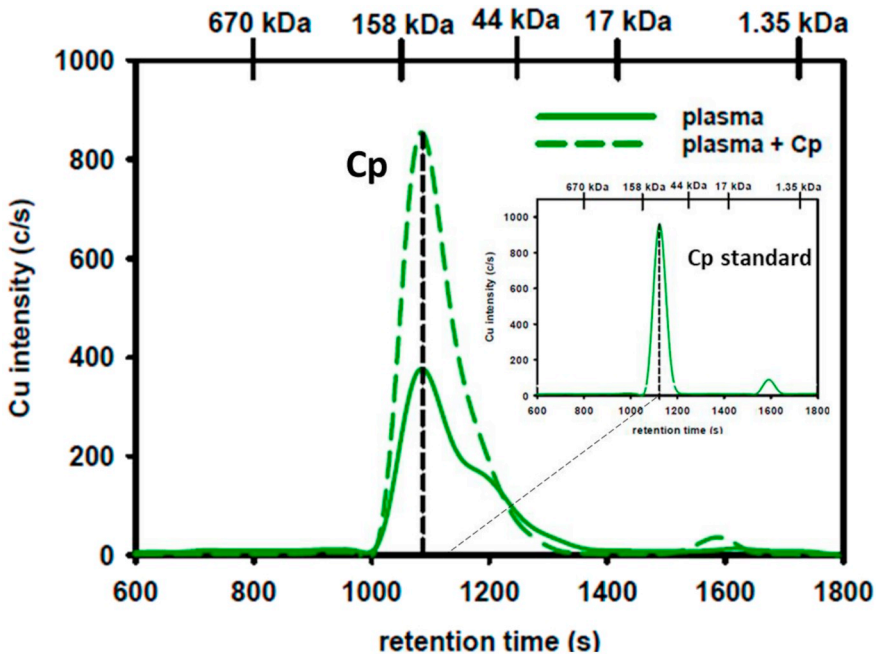

Fig. 1. Representative SEC-ICP-AES derived Cu-specific chromatograms obtained after the analysis of human plasma (green solid line) and human plasma spiked with a Cp-standard (green dashed line). Column: Superdex 200 Increase 10/300 GL (30 × 1.0 cm I.D., 13 µm particle size), temperature: 22 °C, mobile phase: 150 mM PBS buffer (pH 7.4), flow rate: 0.75 mL/min, injection volume: 500 µL. ICP-AES detection at 324.750 nm (Cu). Spiking involved the addition of a solution containing 1.0 mg of Cp in 100 µL DI water to 600 µL plasma. After mixing for 2 min, the Cp-spiked plasma was injected. Retention times of molecular weight markers are depicted on top of the figure, while the retention time of Cp is depicted as vertical dashed lines. *Inset*: Results obtained after the injection of a solution containing 1.0 mg of Cp in 100 µL DI water, which was mixed with 900 µL of PBS-buffer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

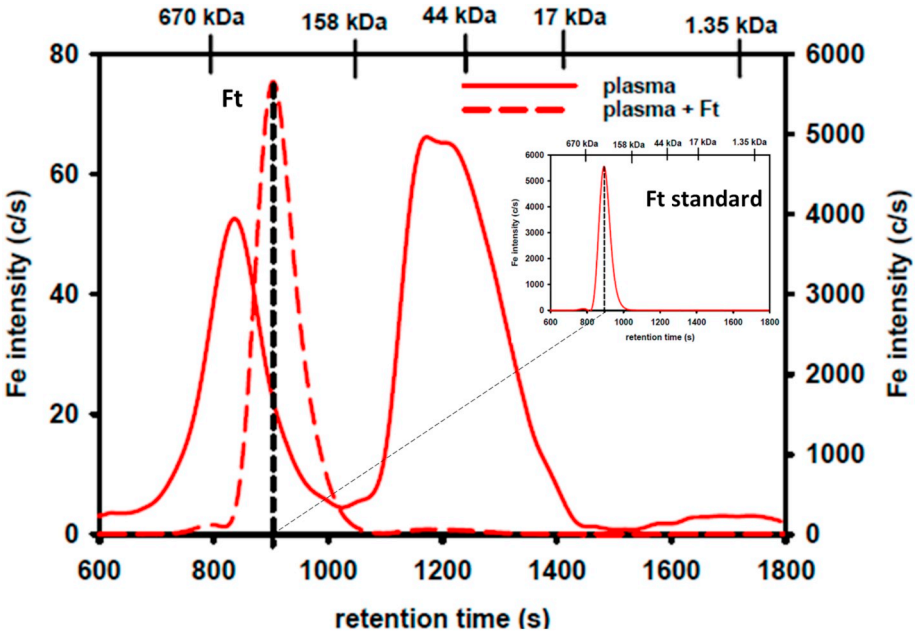

Fig. 2. Representative SEC-ICP-AES derived Fe-specific chromatograms obtained after the analysis of human plasma (red solid line which corresponds to the y-scale on the left) and human plasma spiked with a Ft-standard (red dashed line which corresponds to the y-scale on the right). Column: Superdex 200 Increase 10/300 GL (30 × 1.0 cm I.D., 13 µm particle size), temperature: 22 °C, mobile phase: 150 mM PBS (pH 7.4), flow rate: 0.75 mL/min, injection volume: 500 µL. ICP-AES detection at 259.940 nm (Fe). Spiking involved the addition of a solution containing 1.0 mg of Ft in 100 µL DI water to 700 µL plasma. After mixing for 2 min, the Ft-spiked plasma was injected. Retention times of molecular weight markers are depicted on top of the figure, while the retention time of Ft is depicted as vertical dashed lines. *Inset*: Results obtained after the injection of a solution containing 1.0 mg of Ft in 100 µL DI water, which was mixed with 700 µL of PBS-buffer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

h-Tf in 935 µL DI water and adding 100 µL of this solution to 700 µL of plasma). After mixing, each of the spiked human plasma sample was injected on the SEC-ICP-AES system.

An aqueous haptoglobin (Hp)–hemoglobin (Hb) complex was prepared by mixing 4 µL of RBC lysate with 200 µL of a solution prepared by reconstituting 1.0 mg of Hp in 300 µL DI water. To this solution, 800 µL of DI water was added and the obtained solution was injected after mixing for 2 min. Thereafter, human plasma (850 µL) was spiked with 150 µL of the aforementioned Hp-Hb complex solution and this solution was injected after mixing for 2 min (duplicate analysis).

2.6. Electrospray ionization-mass spectrometry (ESI-MS) analysis of column fractions

An aqueous solution of the Hp-Hb complex was prepared as described under Sample Preparation Of Human Plasma and the obtained solution was injected on the SEC column using 25 mM Tris buffer as the mobile phase (flow-rate of 0.75 mL/min). A fractions containing the Hp-Hb complex (from 16 min 30 s to 17 min 55 s; 0.94 mL) was collected and analyzed on an Agilent 1200 Series HPLC coupled to an Agilent 6520 Accurate-MS. The mobile phase for the HPLC separation was 70% acetonitrile (ACN) with 0.1% formic acid and 30% water with 0.1% formic acid (flow rate of 0.2 mL/min). The source temperature of the MS was 250 °C and we used a drying gas flow rate of 11 L/min and a nebulizer pressure of 25 psi. All attempts to qualitatively identify the Hp-Hb complex in fraction 1 using maximum entropy deconvolution to elucidate the stoichiometry of the Hp-Hb complex were unsuccessful. We also tried to desalt the protein solution by running the sample through a Zorbax Eclipse Plus C_{18} column, using gradient elution from 0.1% formic acid in water (solvent A) to 0.1% formic acid in ACN (solvent B), but no proteins were observed. This fraction, however, had a faint red color, which implies that Hb is present.

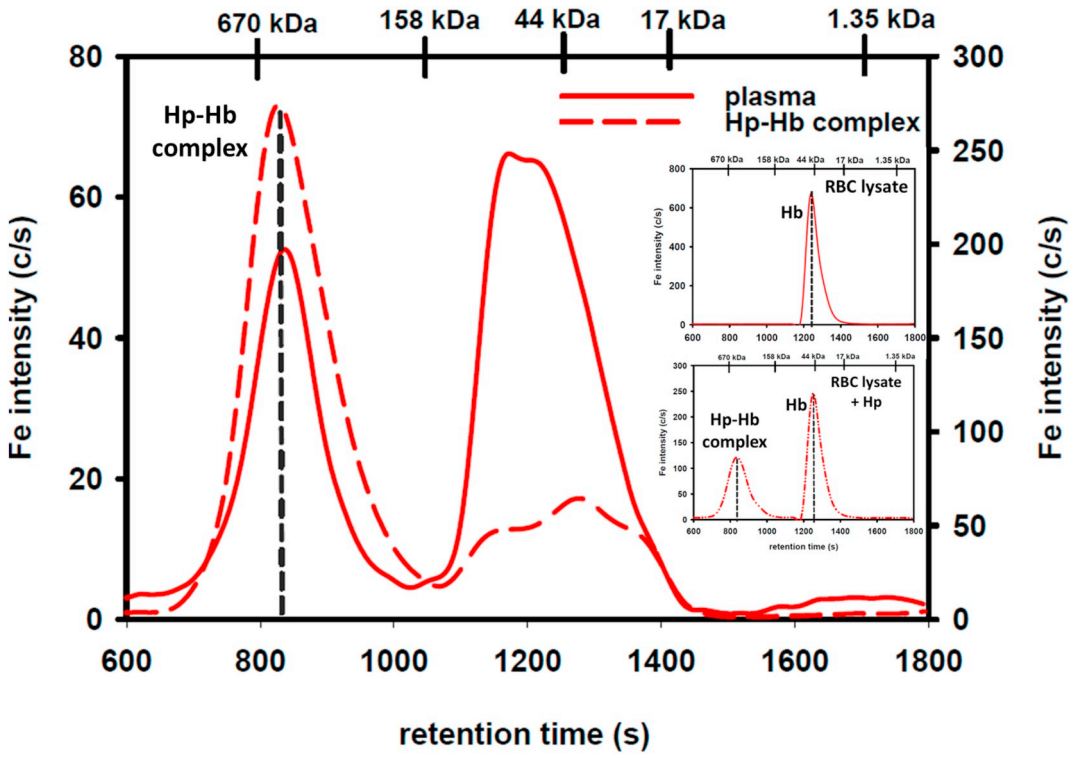

Fig. 3. Representative SEC-ICP-AES derived Fe-specific chromatograms obtained after the analysis of human plasma (red solid line which corresponds to the y-scale on the left) and human plasma spiked with Hp-Hb complex prepared with mixed type Hp 2-1 (red dashed line which corresponds to the y-scale on the right). Column: Superdex 200 Increase 10/300 GL (30 × 1.0 cm I.D., 13 μm particle size), temperature: 22 °C, mobile phase: 150 mM PBS buffer (pH 7.4), flow rate: 0.75 mL/min, injection volume: 500 μL. ICP-AES detection at 259.940 nm (Fe). Human plasma (850 μL) was spiked with 150 μL of the Hp-Hb complex solution. After mixing for 2 min, the Hp-Hb-spiked plasma was injected. Retention times of molecular weight markers are depicted on top of the figure, while the retention time of the Hp-Hb complex and Hb are shown as vertical dashed lines. *Inset* (*upper*): Results obtained after the injection of a solution prepared by adding 4 μL of erythrocyte lysate to 1000 μL of DI water. *Inset* (*lower*): Results obtained after the injection of a solution prepared by reconstituting 1.0 mg Hp (mixed type, phenotype 2-1) in 300 μL DI. To this solution, 800 μL of DI water were added and the solution was injected. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.7. Instrumentation

The SEC-ICP-AES system was comprised of a Smartline 1000 high-performance liquid chromatography (HPLC) pump (Knauer, Berlin, Germany) and a Rheodyne 9010 PEEK injection valve (Rheodyne, Rhonert Park, CA, USA), that was equipped with a 0.5 mL PEEK injection loop. A prepacked Superdex™ 200 Increase 10/300 GL Tricorn™ high performance SEC column (30.0 × 1.0 cm I.D., particle size: 13 μm, GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA) was used for all analyses (fractionation range ~ 600 and 10 kDa) in conjunction with a PBS–buffer mobile phase (pH 7.4) at a flow rate of 0.75 mL/min (column temperature: 22 °C). A size calibration standard (Bio-Rad Laboratories Hercules, CA, USA) was injected to determine the void volume and the molecular weight of the metalloproteins peaks observed. The exit tubing of the SEC column was connected to the Meinhard concentric glass tube nebulizer of the ICP-AES with 30 cm of Teflon tubing (inner diameter 0.5 mm). All elements of interest were simultaneously detected in the column effluent with the Prodigy high-dispersion, radial-view ICP-AES (Teledyne Leeman Labs, Hudson, NH, USA). Carbon (size calibration) was detected at 193.091 nm, while Cu was detected at 324.754 nm, Fe at 259.940 nm and Zn at 213.856 nm using an Ar flow of 19 L/min, a radio frequency (RF) power of 1.3 kV, a nebulizer gas pressure of 35 psi using a nebulizer gas flow rate of 1.4 L of Ar/min. A 360 s delay was implemented between injection and data acquisition. A data acquisition rate of 1 data point per 2 s was used. Raw data (Salsa software version 3.0) were imported into SigmaPlot 13.0, smoothed using the bisquare algorithm and peak areas and retention times were determined (Origin Pro 2017).

The detection limits of Cu, Fe and Zn were determined based on calibration curves of these metals in PBS-buffer in the concentration range between 5 and 50 ppb and the measurement of a blank (n = 15). The experimentally determined detection limits (3σ) were 0.25 ppb for Cu, 0.21 ppb for Fe and 0.26 ppb for Zn, which are close to the values provided by the manufacturer for the radial view Prodigy ICP-AES. A magnesium (Mg) peak was detected in the low molecular elution range (279.553 nm), but it was not included since this element was not the focus of our investigations. Other metalloproteins that contain cobalt (Co), nickel (Ni), molybdenum (Mo), tungsten (W), selenium (Se) or manganese (Mn) that may be present in plasma could not be detected because of a combination of their low abundance and the corresponding detection limits [11].

3. Results and discussion

Metallomics-based techniques can be ideally employed to analyze biological fluids for Cu, Fe and Zn-metalloproteins [13,19–21], which in turn can provide unique insight into bioinorganic chemistry based processes in the bloodstream [18]. In this context, erythrocytes and/or blood plasma play critical roles in terms of mediating interactions between environmentally abundant toxic metals/metalloid species and essential elements [22]. Therefore, the qualitative identification of metalloproteins in blood plasma is important. In order to re-evaluate our previous metal-peak assignment in human blood plasma, aliquots of a human blood plasma stock were spiked with highly pure metalloprotein standards and analyzed for Cu, Fe and Zn-containing metalloproteins with SEC-ICP-AES. We will first describe the results that confirmed the qualitative identification of the main Cu-peak. Thereafter, we will discuss the results which allowed the identification of the two

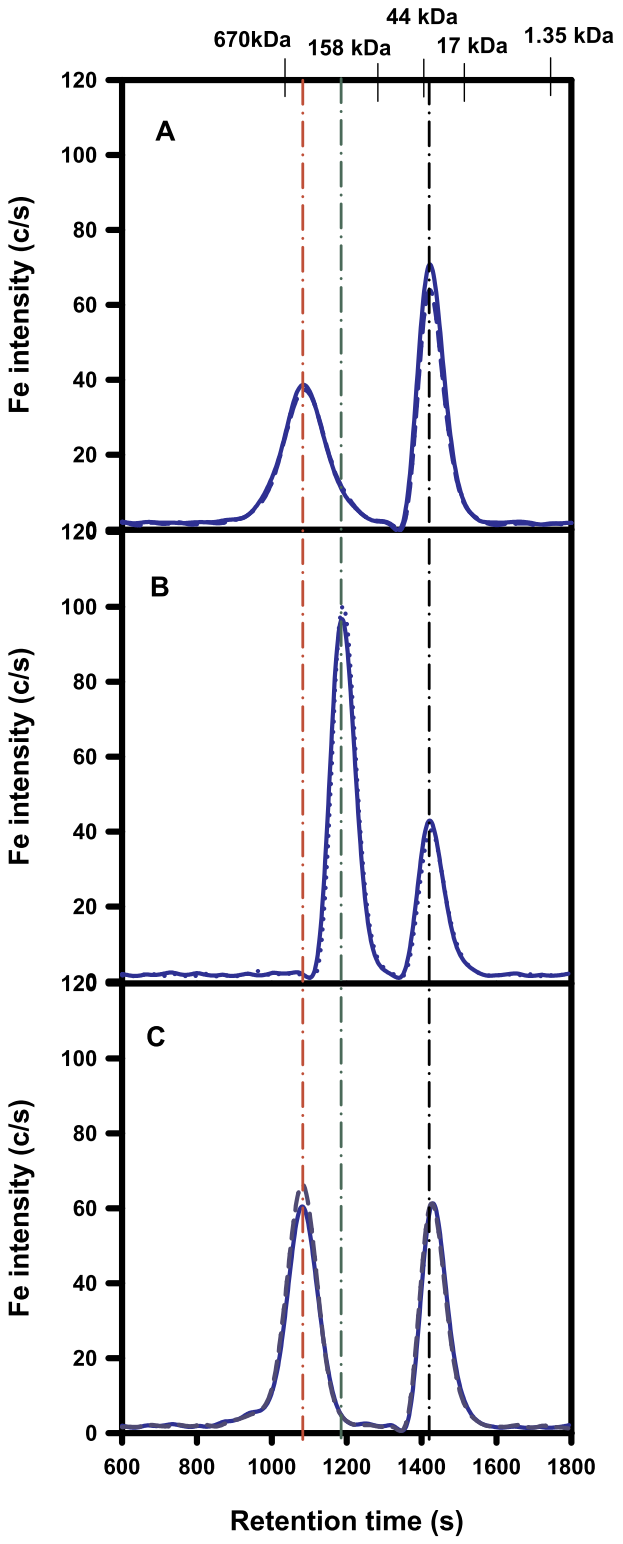

Fig. 4. Representative SEC-ICP-AES derived Fe-specific chromatograms obtained after the analysis of erythrocyte lysate spiked with Hp 2-1 (A), Hp 1-1 (B) and Hp 2-2 (C). Column: Superdex 200 Increase 10/300 GL (30 × 1.0 cm I.D., 13 μm particle size), temperature: 22 °C, mobile phase: 150 mM PBS buffer (pH 7.4), flow rate: 0.75 mL/min, injection volume: 500 μL. ICP-AES detection at 259.940 nm (Fe). Results were obtained after preparing individual Hp solutions by reconstituting 1.0 mg of each Hp phenotype with 300 μL DI. 150 μL of this solution were added to 4 μL of erythrocyte lysate, and 850 μL of DI water were added, mixed and injected.

Fe-peaks followed by the first Zn-peak that eluted.

With regard to Cu (Fig. 1, solid line), a single peak (t_r = 1086 s) was detected (intensity ~390 c/s) with a small shoulder on its long retention end corresponding to HSA-Cu complex [11]. The pure Cp-standard (Fig. 1, inset) produced an intense Cu-peak (t_r = 1124 s, intensity ~950 c/s), that eluted immediately after the 158 kDa size calibration marker, which is expected considering its MW of 132 kDa. A minor Cu-peak (t_r = 1592 s) eluted before the inclusion volume and must correspond to a Cu-containing impurity, possibly a breakdown product of Cp. Spiking plasma with the pure Cp-standard (Fig. 1, dashed line) increased the intensity of the main Cu-peak (t_r = 1086 s, intensity ~860 c/s), which unambiguously identifies this Cu-peak as Cp. In the Cp-spiked plasma we also observed a minor Cu-peak with a retention time that corresponded to the Cu-impurity that was present in the pure Cp-standard (Fig. 1, inset). We observed a ~38 s reduced retention time of Cp in the spiked plasma compared to the pure Cp-standard (see dotted line connecting the x-axes in Fig. 1). This observation is attributed to a weak association of the exogenous Cp with constituents of the plasma matrix (e.g. peptides), which results in small changes of the Cp conformation.

With regard to Fe (Fig. 2, solid line), two distinct Fe-peaks with retention times of 838 s (intensity ~53 c/s) and 1174 s (intensity ~65 c/s) were detected. The pure Ft-standard (Fig. 2, inset) revealed a single Fe-peak (t_r = 894 s, intensity ~5600 c/s). After the analysis of human plasma that was spiked with pure Ft, the obtained Fe-specific chromatogram (Fig. 2, dashed line) revealed a major Fe-peak (t_r = 904 s, intensity ~5600 c/s) with an ~70 s increased retention time compared to the first Fe-peak in unspiked human plasma. Thus, our previous peak assignment for the first Fe-peak is incorrect, which is partially attributed to the fact that 1.0 mL column fractions were analyzed for Ft using enzyme immunoassays [11] and that the Ft concentration in human plasma (< 250 μg/L) [8] is too low to result in a detectable Fe-peak by ICP-AES.

To qualitatively identify the first Fe-peak, we considered the possibility that this peak may correspond to Hp [8], which tightly binds dimers of the Fe-containing protein Hb (K_d ~ 1 pmol/L) after the latter is released into the blood circulation during the intravascular hemolysis of erythrocytes [23–25]. We therefore tested the hypothesis that this Fe-peak corresponds to a Hp-Hb complex. Compared to the Fe-specific chromatogram of plasma (Fig. 3, solid line), the analysis of erythrocyte lysate (Fig. 3, upper inset) revealed a single Fe-peak (t_r = 1239 s, intensity ~700 c/s) that is identified as Hb based on its MW of 64.4 kDa and its elution just before the 44 kDa MW marker. After the addition of a pure Hp-standard to erythrocyte lysate, the analysis of the obtained solution (Fig. 3, lower inset) revealed two distinct Fe-peaks, with retention times of 864 s (intensity ~120 c/s) and 1248 s (intensity ~240 c/s), respectively. The intensity of the second Fe-peak was significantly reduced compared to the Fe-peak that was observed for untreated erythrocyte lysate and an additional Fe-peak eluted before Hb, which is identified as a Hp-Hb complex (Fig. 3, lower inset). The formation of this complex is corroborated by the fact that pure Hp (detected by its carbon emission line) eluted after this Hp-Hb complex (see electronic Supplementary information ESI S1). Previous structural studies described this latter complex as αßHb-Hp-Hp-αßHb, with a molecular weight of ~470 kDa (2Hb + 2Hp) [25], which explains its observed retention time ~100 s after the 670 kDa molecular weight marker (ESI S1). Since the retention time of the formed Hp-Hb complex (t_r = 832 s) was very close to that of the first Fe-peak (t_r = 838 s) in human plasma (Fig. 3, solid lines), we confirmed its presence therein by spiking. After the addition of a mixture obtained by adding Hp to RBC lysate (which yields the Hp-Hb complex and contains leftover Hb) to human plasma, the analysis by SEC-ICP-AES revealed an increased intensity of the first Fe-peak (t_r = 830 s, ~280 c/s; Fig. 3, dashed line). These results identify Fe-peak one in human plasma as an Hp-Hb complex. This peak assignment also provides a feasible explanation for our previous observation that the addition of *N*-acetyl-L-cysteine (NAC) to human

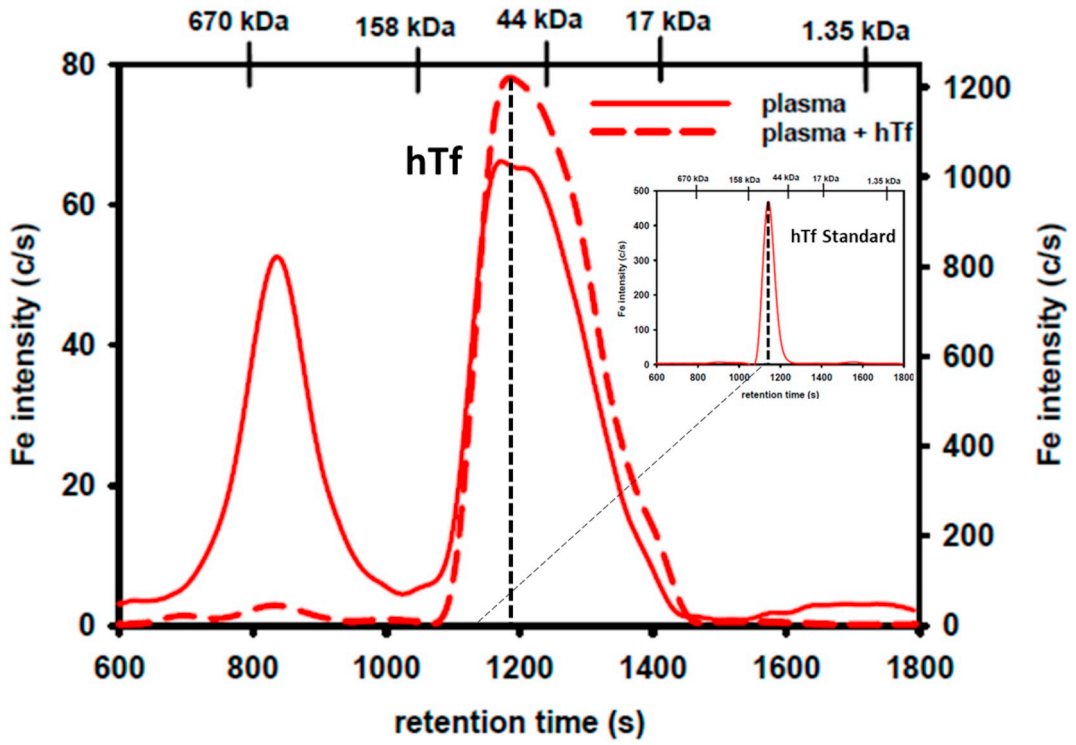

Fig. 5. Representative SEC-ICP-AES derived Fe-specific chromatogram obtained after the analysis of human plasma (red solid line which corresponds to the y-scale on the left) and human plasma spiked with a hTf-standard (red dotted line which corresponds to the y-scale on the right). Column: Superdex 200 Increase 10/300 GL (30 × 1.0 cm I.D., 13 μm particle size), temperature: 22 °C, mobile phase: 150 mM PBS (pH 7.4), flow rate: 0.75 mL/min, injection volume: 500 μL. ICP-AES detection at 259.940 nm (Fe). Spiking involved the addition of a solution containing 100 mg of hTf dissolved in 935 μL DI water. 100 μL of this hTf standard solution was added to 700 μL plasma. After mixing for 2 min, the hTf-spiked plasma was injected. Retention times of molecular weight markers are depicted on top of the figure, while the retention time of hTf is shown as vertical dashed lines. *Inset*: Results obtained after the injection of a solution containing 100 μL of the hTf standard solution in 700 μL of PBS-buffer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

plasma resulted in the detection of two baseline-separated Fe-peaks, while only one Fe-peak eluted when plasma was analyzed that had not been treated with NAC [26]. These findings may now be attributed to a NAC-mediated dissociation of the Hp-Hb complex, which contains a total of 9 disulfide bonds between the 2 αß Hb dimers of the formed αßHb-Hp-Hp-Hbßα structure. Therefore, the reduction of the most solvent accessible disulfide bond(s) in the αßHb-Hp-Hp-Hbßα complex will result in two Fe-containing fragments of different size, each of which contains two heme-groups and will result in the detection of two Fe-peaks of similar intensity after their chromatographic separation [26]. The fact that the intensity of the second Fe-peak in plasma after it was spiked with the RBC lysate plus Hp mixture was essentially unaffected compared to unspiked plasma is readily explained in terms of the binding of the excess Hb to the endogenous Hp in plasma (range 0.45–2.37 g/L [8]).

With regard to the identification of the Hp-Hb complex in human plasma (Fig. 3), it is important to point out that the phenotypes Hp 1-1 (MW 86 kDa), Hp 2-1 (MW 200 kDa) and Hp 2-2 (MW 400 kDa) are present in the mixed-type Hp-standard that we used [27], which predominantly is Hp 2-1. In order to identify the Hp phenotype that is present in the human blood plasma that was analyzed, we also added Hp 1-1 and Hp 2-2 – which have different binding affinities for Hb [28]– to erythrocyte lysate and analyzed the obtained mixtures by SEC-ICP-AES (Fig. 4). The blood plasma that was used in our experiments was from a volunteer of European descent whose phenotypic composition is expected to be 15% Hp 1-1, 50% Hp 2-1 and 35% Hp 2-2 [27]. As is evident from Fig. 4, the retention time of the Hp-Hb complex that was obtained after the addition of the mixed type Hp 2-1 to the erythrocyte lysate was ~100 s smaller than that for Hp 1-1, but rather similar to that for Hp 2-2 (Fig. 4). Based on these results the first Fe-peak in human plasma is therefore identified as mixture of the Hp 2-1 and the Hp 2-2:Hb complex [29], which based on structural studies corresponds to αßHb-Hp-Hp-Hbßα (~470 kDa) [25].

To qualitatively identify the second Fe-peak (t_r = 1174 s), which had a peak intensity of ~65 c/s (Fig. 5, solid line), we analyzed pure hTf (MW 79.7 kDa). This revealed a single Fe-peak (t_r = 1144 s) with an intensity of ~470 c/s (Fig. 5, inset). After the addition of pure hTf-standard to plasma, its subsequent analysis revealed that only the second Fe-peak (t_r = 1180 s) increased in intensity to ~1200 c/s (Fig. 5, dotted line). These results qualitatively identify the second Fe-peak in plasma as hTf. The reason as to why the retention time of pure hTf (Fig. 5, inset) was 42 s shorter than the corresponding Fe-peak in plasma is not understood at present, but may be attributed to its conformational changes in plasma vs PBS-buffer. The observation that the Fe-peak width of pure hTf (~100 s) was 150 s narrower compared to that of hTf in human plasma (~250 s) suggests that the diffusion of hTf into the pores of the SEC stationary phase is strongly affected by other plasma proteins in solution as was previously observed for a small molecular weight species on the same stationary phase [30].

The analysis of plasma for Zn revealed four Zn-peaks (Fig. 6, solid line), which is in general accord with our previous results [11]. The first Zn-peak had a retention time of 824 s (intensity ~8 c/s) and the most intense one had a retention time of 1238 s (intensity ~45 c/s). In order to qualitatively identify the first Zn-peak (t_r = 824 s), we analyzed a pure α_2M-standard, which revealed two peaks with retention times of 800 s (intensity ~8 c/s) and 1588 s (intensity ~45 c/s), respectively (Fig. 6, inset). The relative intensity of the second Zn-peak was unexpected and is attributed to a Zn-containing breakdown product of α_2M. We then spiked plasma with the pure α_2M-standard and the analysis of the obtained mixture revealed several poorly separated Zn-peaks (Fig. 6, dashed line). The first Zn-peak (t_r = 816 s, intensity ~12 c/s) had a similar retention time as unspiked plasma (Fig. 6, solid line),

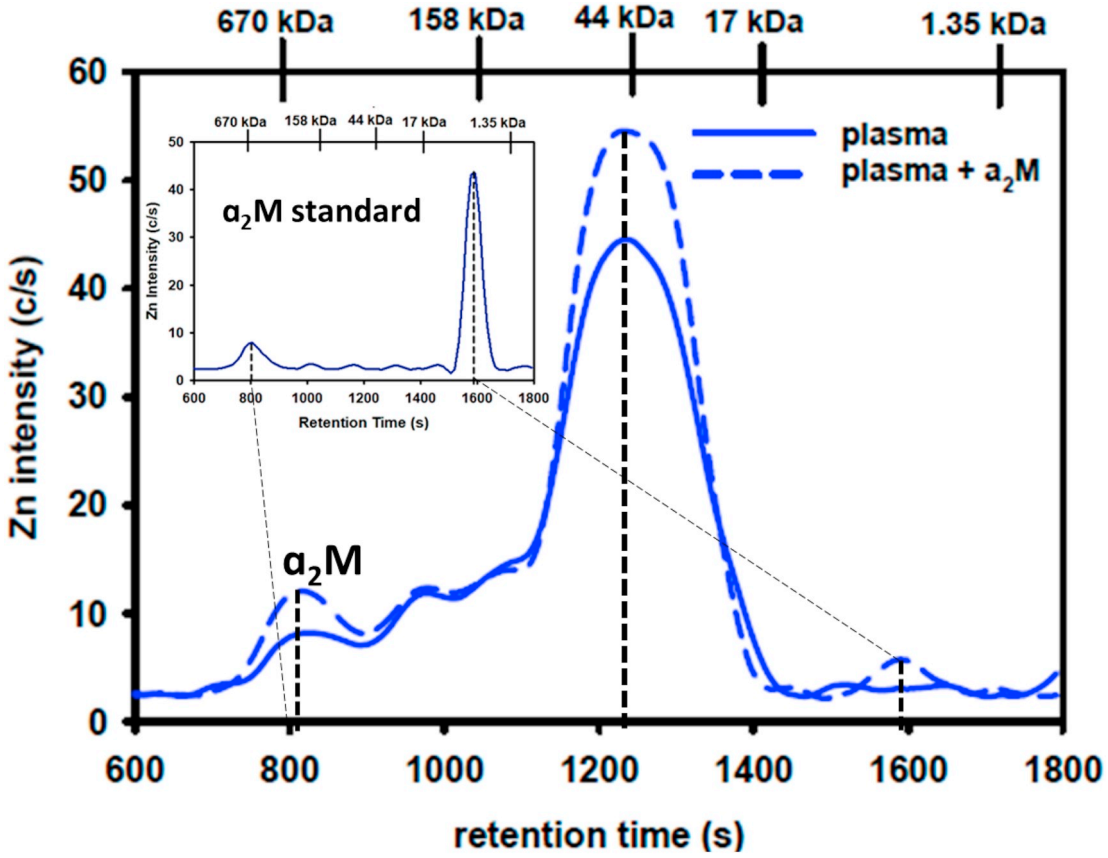

Fig. 6. Representative SEC-ICP-AES derived Fe-specific chromatogram obtained after the analysis of human plasma (blue solid line) and human plasma spiked with a α_2M-standard (blue dashed line). Column: Superdex 200 Increase 10/300 GL (30 × 1.0 cm I.D., 13 μm particle size), temperature: 22 °C, mobile phase: 150 mM PBS buffer (pH 7.4), flow rate: 0.75 mL/min, injection volume: 500 μL. ICP-AES detection at 213.856 nm (Zn). Spiking involved the addition of a solution containing 1.0 mg of α_2M in 100 μL DI water to 900 μL plasma. After mixing for 2 min, the α_2M-spiked plasma was injected. Retention times of molecular weight markers are depicted on top of the figure, while the retention time of α_2M is shown as vertical dashed lines. *Inset*: Results obtained after the injection of a solution containing 1.0 mg of α_2M in 100 μL DI water, which was added to 900 μL of PBS-buffer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

which identifies the endogenous Zn-peak as α_2M. The retention time of the most intense Zn-peak (t_r = 1234 s, intensity ~55 c/s) was in accord with the previously assigned HSA-Zn complex on a similar SEC column [11,13]. The last Zn-peak eluted before the inclusion volume (t_r = 1590 s, intensity ~5 c/s). As expected, spiking plasma with a pure α_2M-standard increased the intensity of the first Zn-peak, but Zn-bound to HSA also increased significantly (Fig. 6, dashed line). The latter result implies that the unknown Zn-containing breakdown product present in the α_2M-standard bound to HSA. Combined, these results unmistakably identify α_2M as the first Zn-peak that elutes if plasma is analyzed by SEC.

Currently, the biological complexity and large dynamic concentration range of blood plasma proteins hampers the reliable determination of bio-molecular differences between plasma from disease patients and healthy controls [9]. One possible means by which this problem can be addressed is by analyzing plasma for a sub-proteome, such as the metalloproteome [11,13]. To this end, we have previously demonstrated that ~12 metalloentities can be rapidly determined in rabbit and human plasma by SEC-ICP-AES [11,13], which is comparatively much faster (no sample preparation) and cheaper than other approaches, such as 2D-HPLC as well as those that identify proteins after 2D PAGE separation by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)-MS or LC-ESI-MS/MS [13]. Therefore, the analysis of human blood plasma by SEC-ICP-AES represents a useful approach to determine potential metalloprotein biomarkers for the early diagnosis of disease.

4. Conclusion

Only 22 plasma proteins are validated as clinical biomarkers for the diagnosis of diseases [31]. Thus, the qualitative identification of additional plasma proteins that can be easily and reproducibly measured has potential to uncover novel biomarkers to advance the early diagnosis of diseases [32,33]. To this end, metallomics methods have already been employed for the detection of Cu, Fe and Zn-metalloproteins in blood plasma [13,34,35], but their unequivocal identification is a critical prerequisite before their inherent biomarker potential can be fully explored. The qualitative identification of the metalloprotein entities in plasma from healthy controls, however, is critical before this metallomics method may be applied to determine their concentration and/or the presence of additional disease-specific biomarkers when plasma from patients is analyzed. Herein, we have definitively identified Cp, hTf, α_2M and Hp-Hb – the first Fe-metalloprotein-protein adduct – in human plasma. Since the intensity of this latter Fe-peak in plasma is linked to the hemolysis of erythrocytes and/or their hemolysis during their passage through medical devices (e.g. during blood collection) [36], the measurement of the intensity of the Hp-Hb Fe-peak in blood plasma may be linked to disease states that affect the stability of erythrocytes [37]. It has been reported that increased or decreased concentrations of the detected Cu, Fe and Zn-metalloproteins in human blood plasma are associated with clinically adverse symptoms (e.g. trauma, inflammation, chronic anemia) [8] and/or the exposure to certain environmental pollutants [13,22]. Further studies can now be

initiated to investigate which of the qualitatively identified plasma metalloentities may serve as biomarkers for the early detection of specific diseases that are known to be intricately connected to the corresponding metals [38–40]. Thus, the identification of Cp, hTf, α_2M and the Hp-Hb complex in human plasma is a starting point in terms of linking their plasma concentrations to bioinorganic processes that either unfold in the bloodstream (e.g. hemolysis) and/or in organs to diseases.

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Declaration of competing interest

The authors declare no conflict of interest.

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