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Assessing the iron delivery efficacy of transferrin in clinical samples by native electrospray ionization mass spectrometry.

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Serum transferrin is a key player in iron homeostasis, and its ability to deliver iron to cells via the endosomal pathway critically depends on the presence of carbonate that binds this protein synergistically with ferric ion. Oxalate is another ubiquitous anionic species that can act as a synergistic anion, and in fact its interaction with transferrin is notably stronger compared to carbonate, preventing the protein from releasing the metal in the endosomal environment. While this raises concerns that high oxalate levels in plasma may interfere with iron delivery to tissues, concentration of free oxalate in blood appears to be a poor predictor of impeded availability of iron, as previous studies showed that it cannot displace carbonate from ferro-transferrin on a physiologically relevant time scale under the conditions mimicking plasma. In this work we present a new method that allows different forms of ferro-transferrin (carbonate- vs oxalate-bound) to be distinguished from each other by removing this protein from plasma without altering the composition of the protein/metal/synergistic anion complexes, and determining their accurate masses using native electrospray ionization mass spectrometry (ESI MS). The new method has been validated using a mixture of recombinant proteins, followed by its application to the analysis of clinical samples of human plasma, demonstrating that native ESI MS can be used in clinical analysis.

Introduction

Iron is an essential element that is required for nearly all living organisms. There are 3.5 grams of iron present in a healthy adult body with over 2 grams found in hemoglobin of erythrocytes.\(^1\) Despite the attention paid in the field of nutraceuticals to iron dietary supplements, the majority of iron circulating in plasma is actually recycled from reticuloendothelial macrophages through the degradation of erythrocytes as well as other cells.\(^2\) Although it is one of the most abundant elements in the Earth’s crust, iron bioavailability is limited due to the extremely low solubility of the ferric ion (Fe\(^{3+}\), the predominant form under aerobic conditions), forcing all living organisms to devise various strategies to solubilize this element. In vertebrates, this problem is solved using proteins that bind iron tightly while in circulation and release it in cells via receptor-mediated endocytosis.\(^3,4\) Human serum transferrin (Tf), a protein intimately involved in iron homeostasis, transports iron to cells that need this metal and express the Tf receptor on their surface; meanwhile, Tf sequesters iron from pathogens that also require this element for growth and proliferation.

Tf is an 80 kDa bilobal glycoprotein, with each lobe capable of binding Fe\(^{3+}\) strongly (\(K_d \sim 10^{22}\)) but reversibly at physiological pH (7.4).\(^5\) Following its association with the Tf receptor at the cell surface, Tf is internalized and releases iron under the mildly acidic conditions of the endosome, before being recycled back to the cell surface and released to circulation for another cycle of iron acquisition and delivery.\(^6\) An intriguing feature of Tf (shared across the entire family of Tf-related proteins, including lactoferrin and ovotransferrin) is the presence of a synergistic anion (typically carbonate, CO\(_3^{2-}\)) in the Fe\(^{3+}/Tf\) complex, which is required to complete the metal’s coordination sphere\(^7\) (see Supplementary Material for more detail). Oxalate (C\(_2\)O\(_4^{2-}\)) is another ubiquitous anionic species capable of acting as a synergistic anion.\(^8\) While its concentration in serum (10-30 µM) is significantly lower compared to the total pool of carbonate (20-30 mM), it has significantly higher Tf affinity.\(^9\)

The documented ability of oxalate to prevent iron release from Tf under endosomal conditions\(^10\) has led to concerns that it may interfere with iron trafficking by inhibiting its release from Tf inside the endosome and, therefore, deprive cells of this essential nutrient even though there is no iron deficiency in the diet or circulation. Under these circumstances the clinical symptoms of anaemia would not correlate with the results of laboratory testing, which commonly relies on total iron and protein-bound iron as the biomarkers. While anaemia is involved in the etiology of a range of pathologies,\(^11\) iron deficiency is particularly devastating for the function of the central nervous system, adversely affecting inter alia sleep, attention, and cognitive development.\(^12-15\) In the past decade
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several studies have reported an increased prevalence of iron deficiency in autistic children, suggesting the involvement of anaemia in the etiology of autism spectrum disorders, although more recent studies failed to confirm this correlation.

One possible explanation for the lack of an obvious correlation between iron status and the occurrence/severity of autism is based on a recent observation by Konstantynowicz et al. of a three-fold greater plasma oxalate levels in autistic children compared to their symptom-free peers. Oxalate replacing Tf-bound carbonate is likely to disturb iron homeostasis by inhibiting metal release in the endosome (Figure 1). However, Mason et al. have pointed out that the serum level of oxalate could be a poor predictor of the disturbed iron homeostasis, as this anion fails to displace carbonate from transferrin on a physiologically relevant time scale in solution that has the same pH and ionic strength as blood serum. While it is possible that oxalate may easily out-compete carbonate during the iron loading of transferrin, especially under mildly acidic conditions, the molecular mechanisms of iron loading remain a subject of debate, and it remains unclear if there is correlation between serum oxalate levels and the presence of oxalate as a synergistic anion in circulating Fe₂Tf.

Clearly, a meaningful diagnostic test in this case should specifically focus on the relative amounts of oxalate and carbonate bound to Tf (rather than on the total oxalate concentration in the plasma). Together with the total amount of Tf-bound iron, this number should provide a true measure of Tf potency vis-a-vis iron delivery to cells. While the existing analytical protocols cannot accomplish this task, native electrospray ionization mass spectrometry (ESI MS) has been shown in the past to be a powerful tool capable of determining the composition of the protein/metal complexes.

However, such measurements are always carried out in vitro using solvent systems compatible with the ESI process; to the best of our knowledge, no reports have been published on applying native ESI MS to characterize metalloproteins in clinical samples. Another complication arises from the very small difference between the two synergistic anions (28 Da, which is less than 0.04% of the mass of Tf). Although modern mass spectrometry allows even smaller mass differences to be measured for polypeptide ions, these measurements are typically carried out under denaturing conditions; the gentle nature of native ESI MS typically results in formation of multiple adducts, leading to ion peak broadening and making high-resolution and high-accuracy mass measurements extremely challenging.

In this work we present a new method for the analysis of clinical blood samples that allows the composition of the endogenous ternary complexes (Fe₃CO₂⁻/C₂O₄²⁻)₂Tf to be probed using a combination of size exclusion and albumin-depletion chromatographic separations and native ESI MS detection. The new method is tested with a mixture of recombinant proteins of known composition and then applied to clinical samples. The technique is ready to be used in clinical studies, to search for a correlation between autism and iron deprivation caused by Tf-bound oxalate.

Experimental

Materials. Recombinant human Tf used in this work was a generous gift from Prof. Anne B. Mason (University of Vermont College of Medicine, Burlington, VT, USA), and the glycosylated form of human Tf was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Clinical samples of human serum from anonymous volunteers were provided by Prof. Barry Braun (University of Massachusetts-Amherst, Department of Kinesiology). Amicon UltraCel membrane microconcentrator devices (10 kDa molecular weight cut-off) were purchased from EMD Millipore (Billerica, MA, USA). Cibacron F3GA resin was purchased from Pall Corporation.

Figure 1. Iron delivery to cells by Tf via receptor-mediated endocytosis (top) and inhibition of this process by oxalate acting as a synergistic anion instead of carbonate (bottom). Top (counter-clockwise, from upper left corner): binding of (Fe₃CO₂²⁻/C₂O₄²⁻)₂Tf to TfR at the cell surface is followed by internalization of this complex in an endosome. Activation of proton pumps (blue) leads to the endosome acidification, a process that eventually triggers iron release from Tf and its subsequent transport from the endosomal compartment to the cytosol, while the iron-free Tf is recycled back to the cell surface, where it is made available for another cycle of iron delivery. Bottom: presence of oxalate prevent iron dissociation from Tf at mildly acidic endosomal pH.
 samples were directly infused at a flow rate of 3 µL/min. A 4500 V set to a flow of 4.6 L/min and a temperature of 200 °C. Absorption at 470 nm was used to identify eluting Tf. Multiple injections were used to collect Tf-containing fractions, which were subsequently pooled. Removal of serum albumin from these pooled fractions was carried out using a home-made gravity-driven affinity column packed with Cibacron F3GA resin (BDR). A step gradient was used to allow hTf to be eluted from the column while retaining HSA. Three buffers were used for the step gradient: a no-salt buffer (pH 6.8, 150 mM ammonium acetate), a low salt buffer (pH 6.8, 0.25 M NaCl, 150 mM ammonium acetate), and a high salt buffer (pH 6.8, 2 M NaCl, 150 mM ammonium acetate). All holo-Tf eluted during the no-salt step, which was collected and buffer-exchanged to 150 mM ammonium acetate (pH 6.8) using a microconcentrator (vide supra).

Native ESI MS Analyses. All mass spectral data were acquired with a Solaris 7T Fourier transform ion cyclotron resonance (FT ICR) mass spectrometer (Bruker Daltonics, Billerica, MA, USA). All samples were directly infused at a flow rate of 3 µL/min. A 4500 V capillary voltage was used for all measurements. The dry gas was set to a flow of 4.6 L/min and a temperature of 200°C. Each measurement had a 0.52 second transient time and a 32,000-point time-domain. All spectra were acquired in a 3,500-5,000 m/z range with 400 scans were averaged for each measurement to achieve adequate signal-to-noise ratio. The ESI source parameters were adjusted to minimize collisional activation in the ESI interface region in order to preserve the integrity of the protein/metal/synergistic anion complexes. Reference mass spectra of metal- and synergistic anion-free protein for each sample were acquired by lowering the pH of the protein solution to 3.7. Data analysis was performed using Compass Data Analysis software (Bruker Daltonics). Generally, the m/z value was assigned for each Tf charge state peak observed. Mass shifts were calculated by subtracting m/z values, of the same charge state, and multiplying the difference by the charge state. Experimentally determined mass shifts were compared to expected calculated values in order to assign the synergistic anion and metal composition (an example of using this procedure is shown in Supplementary Material).

Results and Discussion

Rationale. The unequivocal proof that oxalate does disturb iron homeostasis in a specific patient can only be provided by measuring the fraction of Tf molecules in circulation in which carbonate is replaced with oxalate. Existing methods that measure plasma oxalate do not provide such information; however, the ligand composition of Tf/metal complexes can be probed by native ESI MS in vitro. In the past, we used this technique to determine the presence of oxalate as a synergistic ion in a fragment of Tf molecule (its N-lobe), but the subsequent attempts to extend this method to the full-length protein produced mixed results, as the broad shape of the mass spectral peaks typical of native ESI MS prevented us from being able to make unequivocal assignments, while collisional desorvation led to facile dissociation of the synergistic anion form the protein prior to the mass measurement. Another problem related to the use of native ESI MS for the analysis of a clinical sample is the presence of significant amounts of strong electrolytes (e.g., NaCl), which are incompatible with the ESI process and must be removed/replaced with volatile electrolytes (e.g., CH₃COONa) during the sample preparation step. The problem here lies with the possibility of altering the composition of the Tf/metal/synergistic anion complexes prior to MS analysis, which would obviously render the results of the testing meaningless.

In order to circumvent these problems, we initially worked with recombinant and commercially available protein molecules to explore the utility of thermal desorption as a tool capable of removing non-specific adducts from the Tf/metal/synergistic anion complexes in the ESI interface without altering their composition. This was followed by designing a protocol of Tf extraction from clinical samples and placing them in “ESI-friendly” solutions without altering the composition of the Tf/metal/synergistic anion complexes. The absence of any alteration of these complexes’ make-up (either due to loss/exchange of synergistic anions or due to a bias introduced by the procedure that would preferentially extract one particular form of the protein at the expense of others)
Figure 2. Zoomed views of the native ESI mass spectra of recombinant Tf reconstituted with iron using carbonate (blue trace) and oxalate (red) as synergistic anions. The black trace represents the ionic signal of the apo-form of recombinant Tf. Only peaks corresponding to ionic species at charge state +20 are shown for clarity.

Native ESI MS can make a distinction between the carbonate- and oxalate-bound forms of Fe$_2$Tf. The mass difference between carbonate and oxalate dianions is 28 Da; this number dictates the minimal level of precision that must be attained in the protein mass measurements in order for the meaningful analysis of the composition of serum transferrin to be carried out. The ability to resolve a mass difference of 28 Da would allow a distinction to be made e.g. between a mixed complex (Fe$^{3+}$CO$_3^{2-}$)$\cdot$(Fe$^{3+}$C$_2$O$_4^{3-}$)Tf and the carbonate-bound form (Fe$^{3+}$CO$_3^{2-}$)$\cdot$Tf. Although this mass difference corresponds to < 0.04% of the total protein mass, the resolving power of many modern MS instruments allows such measurements to be readily made. One complication that arises in our particular case is that such measurements must be made under the so-called native conditions, which presents two challenges. First, protein ions accumulate relatively low number of charges in native ESI MS, giving rise to the ionic signal in the high m/z range (> 3,500 for Tf$^{5+}$), where most instruments typically have sub-optimal resolution. Second, the gentle nature of native ESI MS results in production of multiple adducts, leading to broadening of ion peaks in mass spectra, which affects both the accuracy of the mass measurements and the ability to resolve closely spaced ion peaks. While mild collisional activation of ions representing protein complexes frequently enhances the ionic peak shapes (via adduct dissociation), it also leads to partial dissociation of non-covalent assemblies in the gas phase. In the case of Tf, it results in facile removal of the synergistic anion from the protein, which obviously invalidates the measurements aimed at determining the composition of Tf/ferric ion/synergistic anion complexes.

Recently we reported that such complexes exhibit surprising stability when subjected to thermal desolvation, even though the adduct ions dissociate readily, allowing high mass accuracy measurements to be made. Figure 2 shows native ESI mass spectra of recombinant human Tf reconstituted with carbonate and oxalate, where both carbonate- and oxalate-bound forms of Tf can be readily identified based on their masses. The identification becomes particularly straightforward when the ligand composition of the complex is determined based on the mass difference between the complex ion and the apo-Tf ion (228.8 Da and 287.2 Da for the peaks shown in Figure 2); the theoretical mass differences are 229.7 Da for the carbonate-bound form (calculated as a mass of 2Fe$^{3+}$ + 2CO$_3^{2-}$ + 2H$^+$) and 285.8 Da (2Fe$^{3+}$ + 2C$_2$O$_4^{3-}$ + 2H$^+$) for the oxalate-bound Tf.

Extraction of Tf from serum for the synergistic anion analysis. Although native MS with thermal ion desolvation in the ESI interface does allow the distinction to be made between the carbonate- and oxalate-bound Tf, it is important to remember that the mass spectra shown in Figure 2 were acquired using a sample prepared with volatile electrolytes (ammonium acetate). Direct analysis of a serum sample by ESI MS generates abundant, but unresolved (and, therefore, analytically meaningless) ion signal due to the presence of (i) multiple protein species and (ii) non-volatile electrolytes leading to facile cluster ion and adduct ion formation. To circumvent this problem, we ran the serum sample through a size exclusion column using an "electrospray-friendly" solvent system (150 mM ammonium acetate) whose ionic strength and pH are close to those of serum. While the protein signal spans over a...

Figure 3. SEC chromatograms of bovine serum showing the Tf-containing fraction (highlighted in orange); native ESI mass spectrum of this fraction is shown in the inset (black trace). Serum albumin and Tf peaks are labeled with blue and red circles, respectively. The brown trace shows a mass spectrum of unfraccionated serum.
Ammonium acetate was used as a salt in the affinity column allowed holo-Tf to elute at low ionic strength while albumin
Injecting the Tf/albumin containing SEC fraction through the BDR alternative ways of albumin depletion. We accomplis hed this using
affinity column containing antibodies to the fourteen most abundant plasma proteins. Unfortunately, Tf is one of the proteins
depleted using these commercial kits, making it necessary to seek alternative ways of albumin depletion. We accomplished this using
blue dye resin (BDR), which has a high affinity to albumin. Injecting the Tf/albumin containing SEC fraction through the BDR column allowed holo-Tf to elute at low ionic strength while albumin was retained and could only be eluted under high salt conditions. Ammonium acetate was used as a salt in the affinity separation (or,

Figure 4. Zoomed views of the native ESI mass spectra of mixtures of recombinant Tf reconstituted with iron using carbonate and oxalate as synergistic anions subjected to SEC fractionation (top) and albumin depletion on a BDR column (bottom). The black traces represent the spectra acquired after the treatments, and the red-filled curves represent the mass spectra of the initial mixtures. Only peaks corresponding to ionic species at charge state +20 are shown for clarity.

Figure 5. Zoomed views of the native ESI mass spectra of the apo-form of human Tf subjected to SEC fractionation (red trace) and albumin depletion on the BDR column (blue). The black trace represents the reference mass spectrum of the apo-Tf.

The combination of SEC fractionation with albumin depletion produces a serum Tf sample suitable for the analysis of its composition vis-a-vis the synergistic anion by native ESI MS; however, it also introduces the possibility that the ratio of carbonate- vs. oxalate-bound forms of Tf is altered prior to MS analyses. This can occur through two possible mechanisms. First, it is not inconceivable that the recoveries of the two forms of Tf could be different from each other; in this case, the extraction procedure would introduce a bias. Second, both apo-Tf and mono-ferric Tf may acquire iron during the extraction and albumin depletion steps if the metal is present in the soluble form e.g. in BDR. Should this occur, a bias would be introduced favouring the carbondiated form of holo-Tf (although neither oxalate nor carbonate salts were used in preparation of solvents used for protein extraction and albumin depletion, ambient CO\textsubscript{3} is likely to contribute to formation of carbonate in solution which can be utilized as a synergistic anion by Tf upon metal binding). In order to prove that no bias is introduced prior to the MS measurements by either the SEC fractionation or the albumin depletion step, a mixture of the three forms of differ- Tf (carbonate-bound form, (Fe\textsuperscript{3+}-CO\textsubscript{3})\textsuperscript{2-};Tf; the oxalate-bound form, (Fe\textsuperscript{3+}-C\textsubscript{2}O\textsubscript{4})\textsuperscript{2-};Tf; and the mixed form (Fe\textsuperscript{3+}-CO\textsubscript{3})\textsuperscript{2-}(Fe\textsuperscript{3+}-C\textsubscript{2}O\textsubscript{4})\textsuperscript{2-};Tf) was prepared and subjected to the established Tf extraction workflow prior to native ESI MS (Figure 4). Comparison of the protein peak profiles in each case before and after the procedure provided unequivocal evidence that no detectable bias is introduced by either the SEC fractionation or the albumin depletion step. Additionally, the apo-form of Tf was tested for its ability to scavenge for iron (which could conceivably be present in either SEC or BDR columns due to contamination or carry-over) during either of the two sample preparation steps. Once again, the result of this study was negative, as neither step lead to detectable acquisition of iron by the metal-free form of the protein (Figure 5).

Synergistic anions bound to endogenous Tf in human blood: analysis of clinical samples. Once the procedure for Tf extraction from serum followed by identification and quantitation of
synergistic anions had been validated, it was applied to the analysis of clinical samples. One significant difference between the recombinant proteins discussed above and endogenous Tf encountered in plasma is that the protein mass of the latter may differ from the mass based on the published wild-type sequence and glycosylation pattern. Tf glycosylation is known to be affected by several disorders (with alcoholism being perhaps the best known, but certainly not the only example). Glycosylation is an enzymatic post-translational modification (PTM) that generally leads to heterogeneous protein populations. Tf is rather unusual in that regard, as it exhibits surprising level of homogeneity with over 80% of all protein molecules being modified with two fully sialylated biantennary glycan chains, while other glycosylation patterns make minor contributions. Mass profiling of both commercial Tf and Tf extracted from patients’ blood confirms the paucity of minor Tf glycoforms (see Supplementary Material for more detail). However, the protein mass can also be affected by various non-enzymatic post-translational modifications as a result of stress or protein aging; the presence of Tf mutants in some patients cannot be excluded either. Therefore, confident identification of the synergistic anions bound to Tf in vivo would not be possible without the knowledge of the apo-Tf mass in each patient. To obtain this information, we carried out the analysis of each serum sample in two steps. First, following the SEC fraction collection and albumin depletion on the BDR column, the sample was analyzed by native ESI MS, yielding the total mass of the protein/metal/synergistic anion complex. After that, the sample was quickly acidified, causing the complex to dissociate, and the mass spectrum was recorded, yielding the mass of the endogenous protein in its apo-form. This allowed the total mass of the ligands (metal and synergistic anion) to be calculated as a mass difference between the two forms of the protein (Figure 6).

Five out of six anonymized blood samples revealed nearly identical MS patterns; one example is presented in Figure 6A. The extracted metal-bound Tf population consists of both mono-ferric and di-ferric species, each utilizing carbonate as a synergistic anion. The presence of the mono-ferric form of Tf is consistent with the known pattern of Tf metal loading in healthy subjects, which typically contains a distribution of apo-, mono-ferric and di-ferric protein species. Interestingly, one of the patients exhibited a very different metal loading pattern, with only di-ferric protein species present in the sample. The two distinct peaks present in the mass spectrum (Figure 6B) correspond to the oxalate-bound form ([Fe$^{3+}$-C$_2$O$_4^{2-}$]·Tf) and to the mixed form ([Fe$^{3+}$-CO$_3^{2-}$]·[Fe$^{3+}$-C$_2$O$_4^{2-}$]·Tf).

The fact that the oxalate-bound Tf species were detected in one of the clinical samples is exciting, as it clearly signals the ability of oxalate to act as a synergistic anion in vivo, and not just in vitro, as had been previously demonstrated. We note that this anomalous clinical sample did not reveal the presence of mono-ferric forms of Tf, which may be indirect evidence of the inhibition of iron release in vivo by oxalate acting as a synergistic anion. Indeed, the inability of Tf to unload iron in the endosome coupled with continuous iron uptake would result in complete saturation of the protein with the metal. Since the focus of this work was on method development, and we did not have access to the patients’ medical records, it is impossible to draw any definitive conclusions regarding the interference of Tf-bound oxalate and iron homeostasis. It is clear, however, that oxalate can act as a synergistic anion, likely interfering with the iron delivery to cells. The ability to differentiate between carbonate- and oxalate-bound Tf in clinical samples will provide clinicians with a powerful tool that can be used to establish an actual role of oxalate in symptomatic iron deprivation, as well as in the etiology of neuropathologies caused by insufficient supply of iron to the brain during its development.

So far, in the majority of cases autism has eluded attempts to discover its genetic origins, hinting at the importance of complex gene-environment interactions in the etiology of this disease. Extensive efforts to identify metabolic biomarkers of autism have also met only with limited success. Nevertheless, a relentless pursuit of autism biomarkers continues with the ultimate goal of improving both diagnosis of the disease and evaluation of the effectiveness of therapeutic interventions, and currently the most promising strategies appear to be those integrating Omics-based approaches and clinical data. Surprisingly, oxalate does not appear on the list of candidate biomarkers despite wide-spread anecdotal evidence for its involvement in autism progression, and a clinical study suggesting a correlation between the elevated levels...
of oxalate in plasma and the occurrence of autism.\textsuperscript{20} Oxalate is an endogenous anion, which is both produced internally (as a final product of metabolism of glyoxalate and glycerate), and acquired with food (especially through diets reach in leafy greens, but also from a variety of other sources ranging from chocolate to tofu). As there are no enzymes in humans that can degrade oxalate, the only channel of its elimination from circulation is through the kidney, with a typical plasma concentration in healthy adults being 10-30 µM.\textsuperscript{9} While kidney stone formation is probably the best known pathology linked to increased levels of oxalate, hyperoxaluria may also affect other organs and tissues, including the myocardium and bone marrow, through systemic oxalosis.\textsuperscript{42}

Anemia is also one of the well-documented clinical presentations of systemic oxalosis, which is linked to oxalate deposition in the bones.\textsuperscript{43} Conceivably, limited availability of iron may also be caused by oxalate interfering with iron delivery to cells (e.g., increased levels of oxalate may lead to this anion replacing carbonate from the (Fe\textsuperscript{3+} · CO\textsubscript{3}\textsuperscript{2-})\textsubscript{2} · Tf complexes in circulation; with the resulting (Fe\textsuperscript{3+} · CO\textsubscript{3}·O\textsubscript{3}·H\textsuperscript{+})\textsubscript{2} · Tf complexes unable to release iron in the mildly acidic endosomal environment, see Figure 1). Since the insufficient supply of iron to the developing brain is known to have devastating consequences,\textsuperscript{44-50} arguments have been repeatedly made that iron deprivation may also play a role in the etiology of autism. However, multiple studies of the iron status in autistic children failed to reach a consensus whether oxalate is a contributing factor to iron deprivation.

However, it is important to note that even abundant plasma oxalate may not necessarily interfere with iron delivery to the central nervous system via receptor-mediated transcytosis, as this anion fails to displace carbonate from Tf \textit{in vitro} at neutral pH on a physiologically relevant time scale.\textsuperscript{5} The ability to determine the level of Tf complexed with oxalate in clinical samples opens a host of exciting opportunities in this field by providing a powerful analytical tool to establish the role of this ubiquitous metabolite in modulating iron supply within the developing organism. Definitive proof of oxalate interference with iron delivery in autistic patients would provide an explanation for the frequent ineffectiveness of iron supplementation. Otherwise, it would bring into question the effectiveness of the aggressively marketed low-oxalate diets, at least with respect to ensuring sufficient iron supply, which appear to be common dietary interventions in autistic children.\textsuperscript{41}

**Conclusions**

Tf-bound oxalate is expected to disturb iron homeostasis by inhibiting its release during Tf receptor-mediated endocytosis, potentially leading to a range of pathological conditions triggered by iron deprivation. Although the plasma levels of oxalate can be readily determined using a variety of techniques, currently there are no methods to determine the extent of oxalate bound to Tf in circulation. We have developed an analytical procedure that uses native ESI MS to identify synergistic anions bound to Tf in clinical blood samples without introducing artefacts that alter the carbonate/oxalate ratio. Therefore, this procedure may allow direct quantitation of different forms of Tf to be carried out. Application of this new technique to the analysis of blood samples of patients with various forms of anaemia and/or hyperoxaluria will allow the role of oxalate in limiting iron bioavailability to be established. This information will be invaluable for the design of a targeted and effective treatment of various pathologies triggered by iron deprivation without relying on iron supplementation, which frequently fails. The work presented in this report had focused specifically on the composition of Tf/metal/synergistic anion complexes \textit{in vivo}. However, a similar strategy may also be used for the analysis of other clinically relevant non-covalent complexes whose composition may provide important information regarding disease diagnosis, its progression or the treatment progress.

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