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Medical Applications of Isotope Metallomics

Francis Albarède[1,2*], Philippe Télouk[1], and Vincent Balter[1]

[1]Ecole Normale Supérieure de Lyon
Université de Lyon 1 and CNRS
69007 Lyon
France
[2] Rice University
Department of Earth Science
Houston TX 77005
USA

[*] Corresponding author: Albared[redacted]s-lyon.fr

INTRODUCTION

One may wonder how a paper discussing medical applications of metal isotopes got lost in a review journal dedicated to mineralogy and geochemistry. The justifications are multiple. First, the coming of age of metal isotopic analysis in the mid '90s is largely due to the analytical creativity of the geochemical community and to corporate technical skills allowing the rise of new technologies. Second, many concepts, which can be imbedded in quantitative models testable from their predictions, are common to geochemistry, biochemistry, physiology, and nutrition: a cell, with its organelles, a body with its organ and body fluids, are systems liable to treatments similar to those used to model a lake, the ocean–atmosphere, and the mantle–crust systems. Of course, time scales and length scales differ, the complexity of biology is immense compared to that of the mineral world. Geological systems lack the hallmarks of life, genes and cell signaling. In spite of the overall complexity of the biological systems, pathways, kinetics, and chemical dynamics are better understood than their counterpart in earth sciences. Like in many fields of engineering, comparing the records of inputs and outputs is a powerful tool to identify the internal 'knobs' controlling a given system and learn how to tweak them. Third, although some of the most sophisticated techniques such as *ab initio* calculations of molecular configurations, energetics, and isotopic properties are still limited to molecules with less than a few dozens of atoms, the time is getting closer to when simulations of large molecules will become available for application to 'real' proteins with large molecular weights. The present article reviews some of the basic features of what is now known as *Metallomics* and the preliminary applications of stable isotopes to some medical cases, a discipline for which we suggest the simple term of *Isotope Metallomics*. Stable isotopes are widely used for nutrition studies: enriched stable isotopes are added to the diet of humans or animals to monitor the transit of a particular element (Umpleby and Fielding 2015) or to microbial cultures in ecology (Radajewski et al. 2000). The natural isotope fractionation of ubiquitous elements, such as C, H, O, N, and S, are only rarely used for medical purposes because are usually too unspecific relative to biological processes. In contrast, alkaline earth metals such as Ca and Mg and the transition elements Fe, Cu, and Zn are more promising because of their more specific functional roles in biology and also because their turnover rates in the body are relatively short. Copper plays a major role in oxidizing iron (oxidase) and controlling electron transfer, while hundreds of important enzymes use zinc as a cofactor. Iron is involved in a large number of biological functions and, because of the very large stores contained in red blood cells, muscle, and the

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liver, its overall turnover time is of several years (Bothwell and Finch 1962; Gropper and Smith 2012). It is an essential component of heme, a cofactor made of large heterocyclic porphyrin rings. Heme is the active component of metalloproteins known as hemoglobin and myoglobin, which are used by the body to shuttle oxygen and carbon dioxide in blood cells and muscle. Since a number of diseases act to disrupt biochemical pathways in which metalloproteins are involved, it is expected that some pathologies may induce a signal on metal isotope compositions observable in easily accessible biological samples, notably the major blood components, serum and red blood cells. The purpose of the present article is to expand the review by Albarède (2015) and Albalat et al. (2016) to some new applications of Ca, Fe, and Cu isotopes to medicine (also see Costas-Rodriguez et al. 2016). It is neither a review of isotope chemistry nor a metallomics compendium. The sections dedicated to isotope chemistry are intended to appeal to biology and medical students, while those with a more biological perspective are directed at geochemists and illustrate some potential applications of stable isotopes to the medical world.

The *isotope effect* (Lindemann 1919; Bigeleisen and Mayer 1947; Urey 1947; Galimov 1985; Criss 1999; Schauble 2004; Wolfsberg et al. 2010) refers to the small energy changes induced by the substitution of one isotope by another. This effect is a straightforward consequence of the Heisenberg uncertainty principle of quantum mechanics, which prescribes that the position and the velocity of an object cannot be both measured exactly at the same time. Even at the minimum of their potential energy, bonds never come to rest and their lowermost energy state is referred to as the zero-point energy. As the kinetic energy of a bond also depends on the mass M of bonding atoms, the zero-point energy and the successive energy levels differ for the isotopes of a same element. This is the origin of mass-dependent stable isotope fractionation in nature. Reporting the relative abundance of one pair of isotopes suffices to describe the strength of isotope fractionation: if an effect is observed with a given intensity between ^{64}Zn and ^{66}Zn (mass difference of two), the effect on ^{64}Zn and ^{68}Zn (mass difference of four) will be twice as strong. By 'intensity' we mean the deviation of isotope abundances relative to a reference value, in general a standard material typically provided by agencies such as the National Institute of Standards and Technology in the USA or the Institute for Reference Materials and Measurements in Europe. The nature and the absolute abundance of isotopes in reference materials are inconsequential to the scale of isotopic variations.

In contrast with organic biomarkers which degrade with time, isotope compositions of metals can be analyzed on biological samples years after the samples have been taken. In some less informed scientific environments, using the term isotope may lead to some confusion, as it is easily perceived as referring to radioactive nuclides, such as ^{14}C, used for labeling products, or ^{99}Tc used to visualize the inside of blood vessels and organs. It may also refer to artificially enriched stable isotopes (spikes) added to the diet of volunteers to monitor nutrition and metabolism. Contrary to C, O, H, and N, metals are not present in the atmosphere. The isotope abundances of metals in the bulk sample therefore are immune to oxidation, as they are unreactive to any chemical or biological reactions taking place in the original container, even if the sample is accidentally heated or transferred to another container. Here, we will review the variations in the abundances of stable isotopes of metals tightly related in cellular and physiological activity and *naturally* present in the body of humans and other organisms. Early metal isotope work on biological samples (Walczyk and von Blanckenburg 2002; Ohno et al. 2004; Krayenbuehl et al. 2005; Ohno et al. 2005; Stenberg et al. 2004, 2005; Albarède et al. 2011; Hotz et al. 2012; Aramendia et al. 2013; Jaouen et al. 2013; Van Heghe et al. 2014; von Blanckenburg et al. 2014; Balter et al. 2015; Costas-Rodríguez et al. 2015a,b; Larner et al. 2015; Télouk et al. 2015) showed promising relationships with age, sex, and pathologies. Although isotopic data on organs will be discussed occasionally, emphasis will be on serum for reason of feasibility: it is a chemically stable liquid medium, more readily available, commonly from bio-banks, than biopsies and resections, even for healthy subjects. Sulfur is not a metal, but is

closely related to transition metal biochemistry, and its isotopic abundances in medical materials have not been systematically explored until now (Balter et al. 2015; Albalat et al. 2016). In order to assess the role of sulfur-rich amino acids and proteins, in particular the well-established connection between zinc and sulfur biochemistry through redox control (Maret and Krężel 2007), we will therefore also review these recent observations of sulfur isotope compositions of biological samples (Balter et al. 2015; Albalat et al. 2016).

THE ISOTOPE EFFECT

Isotope fractionation is a general term referring to the variability in the isotopic abundances of a particular element among coexisting species (e.g., sulfide and sulfate for S) or reservoirs (e.g., S in serum and red blood cells) hosting this element. It can be explained in a simple way: (1) vibrational frequencies decrease approximately with $M^{-½}$, while bond energy E varies with vibrational frequency ν according to $E = (n + ½)\, h\nu$, where h is the Plank constant and n a non-negative integer characterizing the energy 'level'. Favoring heavier isotopes in the lowermost energy levels therefore is a way of reducing the total energy of the system. High temperatures work to randomize the distribution of isotopes across energy levels. At ambient temperatures, however, the total energy is minimized when heavy isotopes concentrate into the 'stiffest' bonds, those with the lowest and therefore most stable energy levels (Bigeleisen and Mayer 1947; Urey 1947; Galimov 1985; Schauble 2004; Kohen and Limbach 2005; Wolfsberg et al. 2010). For a given element, the strength of a particular bond is expected to be higher for the smaller ions with the higher charge and therefore developing the strongest field. It is also higher when the overall binding energy at the site of the metal is shared among fewer partners. The strength of a bond involving metal depends on how easily the metal loses its electrons and how eager the bonding species (O, C, N, S) are to attract the lost electrons. A crude scale of bond stiffness may be guessed from the scale of electronegativity or ionization energy (Fig. 1). Bonds involving high oxidation states (Fe^{3+}, Cu^{2+}) and sites with small coordination numbers therefore prefer heavy over light isotopes. It is worth noting at this stage that isotope variability is a very subtle phenomenon: when differences are noted between 'light' and 'heavy' zinc or copper, shorthand for 'depleted' and 'enriched', respectively, the effects are always within the range of only a few parts in one thousand, which only modern mass spectrometry can resolve.

How do we go from energy and vibrational frequencies to real-life isotope fractionation? It was Bigeleisen and Mayer's great merit in 1947 to demonstrate that the isotope effect at equilibrium (and equilibrium is always a useful reference because it is the state to which systems spontaneous relax) can be predicted by statistical quantum mechanics from the ratio β of the reduced partition function of isotopes of a molecule:

$$\beta = \prod_i \frac{u_i'}{u_i} \frac{e^{u_i/2}}{e^{u_i'/2}} \frac{1 - e^{-u_i}}{1 - e^{-u_i'}}$$

Here the product extends over all the normal vibrational modes i of the molecule, $u_i = h\nu_i/kT$, and prime/non-prime variables differentiate the two isotopes. A partition function ratio is 'reduced' when configurations equivalent under rotation are counted only once. We will see thereafter that evaluation of these reduced partition functions is at the heart of predicting isotope fractionation factors.

In addition to the effects just described for systems at thermodynamic equilibrium, the smaller activation energy of the lighter isotopes allows them to react faster (Bigeleisen and Wolfsberg 1958; Wolfsberg et al. 2010): the kinetic isotope effect (KIE) has been advocated as a cause of biologically mediated isotope fractionation (Gussone et al. 2003), but it requires either non-steady state conditions (the system grows) or the existence of competing reaction pathways.

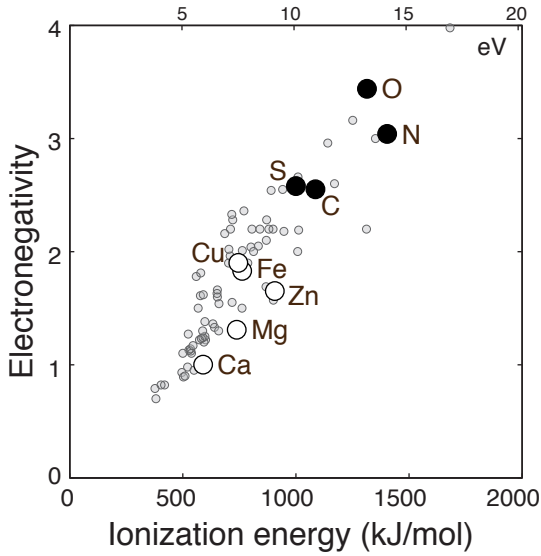

Figure 1. Strong bonds preferentially incorporate heavy isotopes. This is the case of nitrogen, as in the amino acid histidine, and oxygen, as in lactate. In contrast, sulfide bonds, as in amino acid cysteine, tend to preferentially incorporate light isotopes. The scale of bond strength can be crudely estimated using first ionization energy or element electronegativity. The electronegativity difference between bonding elements is a sensitive indicator of isotope fractionation.

Mass spectrometry is the chief method for obtaining precise isotopic abundances.. Except for hydrogen, the isotope effect is usually very small, with variations of isotope abundances rarely exceeding one part per 1000 per unit of mass difference. Measuring such small variations requires a mass spectrometer with high transmission and a magnetic mass filter (sector). Inexpensive quadrupole mass spectrometers do not provide the precision needed for useful applications to natural abundance variations. For decades, only the isotopic abundances of elements that could be introduced into a gas source with electron bombardment, such as H, C, N, O, S, were measured, often as molecular compounds such as CO_2 or SO_2. Mass fractionation in the mass spectrometer itself (instrumental mass bias) would be dealt with by alternating standard material with the unknown samples through calibrated inlet valves (sample-standard bracketing). The actual "true" (absolute) values of isotopic ratios cannot be known with high precision, but this limitation does not really matter: only relative isotopic variations are considered, and they are typically reported on a convenient delta scale, which, for example for ^{65}Cu, is expressed as:

$$\delta^{65}\mathrm{Cu} = \frac{\left(^{65}\mathrm{Cu} / {}^{63}\mathrm{Cu}\right)_{sample}}{\left(^{65}\mathrm{Cu} / {}^{63}\mathrm{Cu}\right)_{standard}} - 1$$

It is common practice to place the heavy isotope at the numerator. Most metals are refractory. Gas sources therefore are inefficient for metallic elements, unless volatile components such as SiF_4 are used. Standardization by sample-standard bracketing could not be used on thermal ionization mass spectrometers, which is a method of choice for radiogenic isotope geochemistry. Thermal ionization is also notoriously difficult for metals such as iron. The precision of measured isotope abundances therefore was poor and their subtle variations remained largely unexplored (Shields et al. 1965). Double-spike techniques, in which the

abundance dependence of mass fractionation is used, would relieve the constraint for elements with *four* stable isotopes or more (Fe and Zn). This technique is, however, rather time consuming and has found only limited applications (Albarède et al. 2004). In the mid '90s, multiple collector inductively coupled plasma mass spectrometry (MC-ICP-MS) emerged as a game changer for the measurement of metal isotope abundances as the technique, which is based on very efficient ionization and high transmission, combined with sample-standard bracketing, allowed for unprecedented precision (typically 0.01–0.05‰) on metal samples as small as a few tens of nanograms of metal. The metal analyzed represents traces in a matrix of organic material loaded with major elements such as Na, Cl, P, Mg, and Ca. Both isobaric interferences and matrix effects seriously affect the measurement of isotopic abundances when unprocessed samples are run. The MC-ICP-MS technique therefore demands strict purification of the analyzed trace metal with a yield close to 100%. The analytical perspective provided by Costas-Rodríguez et al. (2016) is particularly useful for medical studies.

Why take the trouble to measure metal isotopic abundances, a daunting task, instead of simply relying on the concentrations of the metal in question in various parts of the body? The answer is that changes in metal concentrations in general are not amenable to quantitative predictions, whereas the direction and magnitude of the isotopic effect induced by bonding a metal with a chelate, typically an amino acid such as cysteine or histidine, can be predicted by theoretical methods. In contrast to different elements, which can never truly substitute for one another along all biochemical pathways, the isotopes of a given element behave similarly enough that variations in their relative abundances remain predictable. Decades ago, experimental determination of isotope fractionation of an element between coexisting compounds was the method of choice, but the results are now perceived as much less reliable than those obtained by the so-called ab initio or first-principles theories. In addition, the challenge of obtaining results for the very large number of relevant organic compounds is simply daunting. The most commonly used method is the Density Functional Theory or DFT (Parr 1983), a computational quantum mechanical model providing the ground-state orbital geometries and vibrational frequencies of metallic compounds. Each atom is treated as a cloud of electrons orbiting a nucleus. Typically, each calculation is divided into two steps, one step in which atoms are confined in a box and left to drift towards a stable molecular configuration and a subsequent step in which isotopes are substituted to infer the slight thermodynamic changes arising from the substitution. Obtaining results on compounds of biological interest such as the complex environment of Cu in ceruloplasmin, a blood component, and superoxide dismutase 1, an intracellular protein (Fig. 2), is calculation intensive and would require special software and consistent databases. So far, only the bonding of metals with single amino acids ligands has been explored. Even for simple cases, uncertainties on DFT results arise that are due to the difficulty of modeling a quantum mechanical effect known as the exchange and correlation interaction between electrons. Other theoretical approaches calculate the vibrational force constants of the molecular compounds. Methods specific to ^{57}Fe are based on the measure of the kinetic energy of the nucleus of interest by its Mössbauer effect, which is the resonant nuclear fluorescence of γ-rays. The nuclear kinetic energy may be measured by Mössbauer spectroscopy (Polyakov and Mineev 2000; Polyakov and Soultanov 2011), in which the relative velocity between the γ-ray emitter and absorber is determined by the Doppler shift, or by inelastic nuclear resonant X-ray scattering (INRXS) synchrotron experiments, a method giving the phonon density of states (Polyakov et al. 2007; Dauphas et al. 2012).

Large proteins are so far beyond the reach of DFT but efforts to predict fractionation of elements such as Fe, Cu, Zn, Ni, and Ca by smaller molecules have recently been made by a few groups (Seo et al. 2007; Domagal-Goldman and Kubicki 2008; Domagal-Goldman et al. 2009; Black et al. 2011; Fujii and Albarède 2012; Fujii et al. 2013, 2014; Sherman 2013). Isotope fractionation factors for ligand monomers, such as the most common amino acids (histidine, cysteine, methionine), glutathione, and carboxylic acids, such as lactate, oxalate and citrate, have

Table 1. Partition function ratios for $^{66}Zn/^{64}Zn$ and $^{65}Cu/^{63}Cu$ in molecular species relevant to medical studies on a 1000 ln β scale (reduced partition function ratios) ($T = 310$ K). Isotopic fractionation between two coexisting species 1 and 2 can be computed as $\delta^{65}Cu_2 - \delta^{65}Cu_1 \approx \ln \beta_2 - \ln \beta_1$.

Species	ln β_{Zn}	Ref	Species	ln β_{Cu}	Ref
$ZnHPO_4(H_2O)_5$	3.309	[1]	Cu(I)L-Lact	1.725	[5]
$ZnH_3(PO_4)_2(H_2O)_4^-$	3.967	[1]	$Cu(I)Cl_2^-$	2.182	[2]
$ZnH_2(PO_4)_2(H_2O)_4$	4.072	[1[	$Cu(I)HS(H_2O)$	2.529	
fourfold			$Cu(I)(H_2O)_2^+$	2.667	[4]
$Zn(Cys)(H_2O)_3^{2+}$	3.072	[2]	$Cu(I)Cl(H_2O)$	2.683	[2]
$Zn(Glu)(H_2O)_2^{2+}$	3.524	[2]			
$Zn(H_2O)_4^{2+}$	3.577	[2]	$Cu(II)H(L\text{-ascorbate})(H_2O)_4^+$	3.087	[4]
$Zn(His)(H_2O)_3^{2+}$	3.647	[2]	$Cu(II)H(D\text{-ascorbate})(H_2O)_4^+$	3.139	[4]
$Zn(Met)(H_2O)_3^{2+}$	3.66	[2]	$Cu(II)H_3(PO_4)_2(H_2O)_3^-$	4.176	[2]
$Zn(His)(H_2O)_2^{2+}$	3.673	[2]	$Cu(II)H_2PO_4(H_2O)_4^+$	4.355	[2]
$Zn(Thr)(H_2O)_3^{2+}$	3.767	[2]	$Cu(II)H_4(PO_4)_2(H_2O)_3$	4.382	[2]
			$Cu(II)O_x(H_2O)_2$	4.931	[4]
sixfold			$CuH_2(PO_4)_2(H_2O)_3^{2-}$	5.024	[2]
$Zn(Cys)(H_2O)_5^{2+}$	2.504	[2]			
$Zn(Met)(H_2O)_5^{2+}$	2.734	[2]	$Cu(II)(Cys)(H_2O)_4^{2+}$	3.124	[2]
$Zn(His)(H_2O)_4^{2+}$	2.777	[2]	$Cu(II)(Met)(H_2O)_4^{2+}$	3.650	[2]
$ZnCl(H_2O)_5^{2+}$	2.912	[2]	Cu(II)(GS)H0	3.892	[2]
$ZnSO_4(H_2O)_5^{2+}$	3.279	[2]	$Cu(II)(Thr)(H_2O)_4^{2+}$	4.110	[2]
$Zn(His)(H_2O)_5^{2+}$	2.921	[2]	$Cu(II)(Glu)(H_2O)_3^{2+}$	4.117	[2]
$Zn(H_2O)_6^{2+}$	3.026	[2]	$Cu(II)(His)(H_2O)_3^{2+}$	4.148	[2]
$Zn(Glu)(H_2O)_4^{2+}$	3.053	[2]	$Cu(II)(His)(H_2O)_4^{2+}$	4.168	[2]
$Zn(Thr)(H_2O)_5^{2+}$	3.075	[2]	$Cu(II)(H_2O)_5^{2+}$	4.220	[2]
			$Cu(II)L\text{-}Lact(H_2O)_3^+$	4.359	[2]
anhydrous			$Cu(II)L\text{-}LactH_{-1}(H_2O)_2$	4.969	[5]
$[Zn\text{-}Cys\text{-}H_{-1}]^+$	1.108	[3]	$Cu(II)L\text{-}Lact_2$	5.616	[5]
$[Zn\text{-}Cys]^{2+}$	1.211	[3]	Cu(II)L-Lact D-Lact	5.627	[6]
$[Zn\text{-}Glu\text{-}H_{-1}]^+$	1.517	[3]			
$[Zn\text{-}His]^{2+}$	3.336	[3]			
$[Zn\text{-}His\text{-}H_{-1}]^+$	3.465	[3]			

References: [1] Fujii and Albarède (2012); [2] Fujii et al. (2014); [3] Moynier et al. (2013); [4] Fujii et al. (2013); [5] T. Fujii (pers .comm.); [6] Télouk et al. (2015).

become available for Cu and Zn. As shown in Tables 1 and 2, the data are tabulated as reduced partition functions β (usually as 1000 ln β) and the order and amplitude of isotopic enrichment between two compounds 1 and 2 at equilibrium can be estimated as $\delta^{65}Cu_2 - \delta^{65}Cu_1 \approx \ln \beta_2 - \ln \beta$. For example, the predicted $\delta^{65}Cu$ value in $Cu(II)(His)(H_2O)_4^{2+}$ is $4.168 - 3.124 = 1.044$‰ higher than in $Cu(II)(Cys)(H_2O)_4^{2+}$. Table 3 shows some important stability constants for Cu and Zn chelates. Calculation of Fe and Ca fractionation factors are still largely restricted to compounds of environmental interest (Rustad et al. 2010; Fujii et al. 2014).

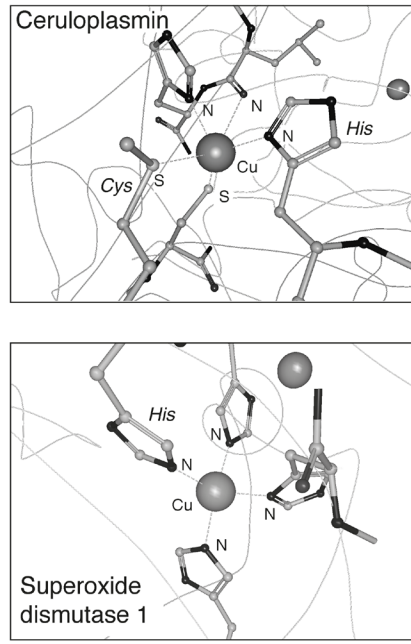

Figure 2. Example of complex coordination encountered in two metalloproteins. The ferroxidase enzyme ceruloplasmin (Cp, PDB ID: 1KCW (Zaitseva et al. 1996)) is the major copper-carrying protein in the blood, and oxidizes seric iron. The large Cu ion in the center is labeled A 1055, while the distant one is labeled A 1049. The intracellular Cu/Zn enzyme superoxide dismutase 1 (SOD1, PDB ID: 1SOS (Parge et al. 1992)) catalyzes the disproportionation of superoxide O_2^- into hydrogen peroxide H_2O_2 or di-oxygen O_2 and therefore protects the cell from free radicals. The large Cu ion in the center is labeled C 154, while the metal sphere in the background is a Zn ion labeled C 155. These two enzymes use the two oxidation states of copper to shuttle electrons. Copper is bound to N from the amino acid histidine (His, hard bond) and S from the amino acid cysteine (Cys, soft bond).

Table 2. Equilibrium $^{34}S/^{32}S$ enrichment in ‰ of different sulfur-bearing inorganic and organic species at 298 K (Albalat et al. 2016). The calculations include the effect of one hydrate shell on sulfate. $^{34}S/^{32}S$ fractionation α between two species may be obtained in ‰ between two coexisting species 1 and 2 and can be computed as $\delta^{34}S_2 - \delta^{34}S_1 \approx \ln\beta_2 - \ln\beta_1$.

HS^-	H_2S	Cysteine	Cystine	Glutathione	Methionine	Taurine	$SO_4^{2-}6H_2O$
4.75	11.42	16.11	17.12	15.67	20.21	71.59	73.94

Table 3. Stability constants for the successive chelates of Cu and Zn by relevant carboxylates.

Ion	Species	log β_1	log β_2	log β_3	Ref
Cu^{2+}	pyruvate	2.2	4.9		[1]
	lactate	2.52	3.9	4.28	[2]
	ascorbate	1.57			[1]
Zn^{2+}	pyruvate	1.26	1.98		[1]
	lactate	1.67	2.65	2.94	[2]
	ascorbate	1.0			[1]

References: [1] Smith and Martell (1987); [2] Portanova et al. (2003)

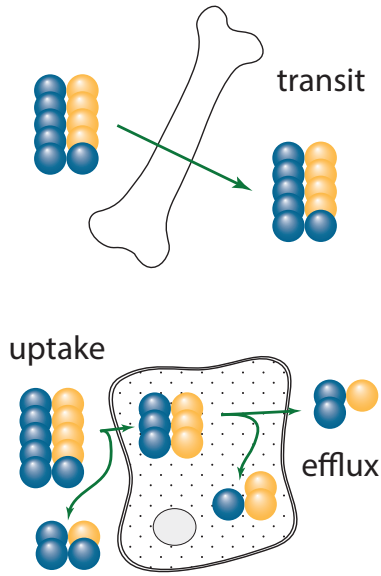

Figure 3. Isotopes of the same element bound to a specific metalloprotein, e.g., ^{63}Cu and ^{65}Cu, are depicted as spheres with two different shades of grey. *Top*: Upon transit at steady-state (no growth), the proportion of each isotope is preserved. The cell or organ is reduced to a single input and a single output. *Bottom*: When the isotopes distribute themselves between two coexisting channels, such as extracellular medium and cytosol, or the cytosol and an organelle, the different pathways allocate different isotope abundances to each channel. Oxidation, biosynthesis, input, storage, and output are expected to result in isotope fractionation.

What comes around goes around: the proportion of isotopes present within a system (cells, organs, body fluids) can only vary if they are imported and exported at different rates (Fig. 3). For a period of time exceeding the mean turnover or residence in the system, input and output must be precisely balanced. When a pathway involves multiple outputs for a single input, the abundances of the different isotopes of a specific element may not be identical in each branch: this is the nature of isotope fractionation,

The best-documented examples so far are the robust trends of Zn and Cu isotope fractionation, two elements for which fractionation by amino acids and other organic ligands have been studied. The following has been observed:

1. Isotope fractionation is less intense for Zn than for Cu
2. Cu(II) compounds are isotopically heavier than Cu(I) compounds
3. Electron donors with a strong electronegativity (N, O) and associated moieties (NH_2, SO_4, PO_4, OH, lactate and pyruvate, two carboxylic acids with a side oxygen or hydroxyl) preferentially bind to heavy isotopes relative to elements with smaller electronegativity, typically S and S-bearing amino acids (cysteine, methionine) (Fig. 1)
4. As demonstrated for zinc by the comparison between four- and sixfold coordination, preference for heavier isotopes decreases with increasing coordination numbers
5. Working out how these results relate to large proteins should attract attention in the future.

AN OVERVIEW OF Ca, Fe, Zn, Cu, AND S BIOCHEMISTRY AND HOMEOSTASIS

We will first take an introductory tour of the biochemistry of these important metals, then summarize a few important facts about sulfur-containing amino acids and proteins. A major trait shared by the ions of all these elements is that their electrostatic field is very strong, which makes their presence as bare cations harmful to many proteins. This is the reason why they are normally bound to metalloproteins. The following subsections are not intended to extensively map the pathways of metal metabolism in the human body, but to highlight some particular reactions, usually associated with transmembrane import, export, and changes in redox states, which are potential mechanisms accounting for isotope fractionation.

Calcium

Calcium is an alkaline earth metal and is always in the divalent state. It has six isotopes at nominal masses 40, 42, 43, 44, 46, and 48. The Ca content of the human body is ~ 1.4 kg and the daily intake recommended for an adult is about 1 g (Gropper and Smith 2012). Of the Ca^{2+} not stored in bones (~1 %), only ~0.1 ‰ resides in the extracellular fluid. The main intracellular Ca^{2+} stores in generic cells are the endoplasmic reticulum and the Golgi apparatus (Brini et al. 2013). The Ca content of the cytosol is particularly low but may be increased through a number of channels. The sodium-calcium exchanger (NCX), an antiporter membrane protein, and the plasma membrane Ca^{2+} ATPase (PMCA) are used to clear Ca^{2+} from the cell. Ninety-nine percent of body calcium (Ca) is in bones in the form of hydroxyapatite. It must be kept in mind that we do not inherit bone from our adolescence and that bones are renewed during our entire life. The feared bone loss with increasing age is therefore a dynamic process. Cartilage collagen is produced by chondrocytes on the outer part the new bone and is gradually replaced by bone. Chondrocytes die out and are replaced by osteoblasts, the bone building cells, which precipitate apatite. Alkaline phosphatase (ALP), an enzyme located in the membrane of osteoblasts liberates phosphate from phosphate esters, such as β-glycerophosphate (Chung et al. 1992), which allows apatite precipitation and mineralization (bone deposition). The bone demolishers, osteoclasts, are responsible for resorption, which returns Ca and phosphate to the blood stream, a process regulated by the parathyroid hormone (PTH). Deficient Ca regulation results in vascular calcification, a rather common disease of the elderly and dialysis patients (Hofmann Bowman and McNally 2012; Evrard et al. 2015). In the blood stream, Ca^{2+} exists in three forms, free ions (50 %), albumin bound (40 %), and protein bound (10 %). Free Ca is one of the most tightly regulated parameters in the body and regulates itself serum concentrations by stimulating bone resorption and Ca reabsorption by the kidney. Cysteine present in albumin, which would favor isotopically light isotopes relative to the free-ion pool, does not seem to bind significantly with Ca^{2+} (Kragh-Hansen and Vorum 1993).

Calcium, which is particularly abundant in the environment, was the first non-conventional element for which the variations of its isotopes were successfully investigated (Skulan et al. 1997). Analysis by TIMS and double-spike is still competing with sample-standard bracketing by MC-ICP-MS. Two main features have emerged: First, Ca becomes isotopically lighter as it moves through the food chain. Second, bone Ca is isotopically light (Skulan and DePaolo 1999; Reynard et al. 2010), which may come as a surprise since, as expected from the electronegativity scale, heavy Ca isotopes should favor PO_4^3 over carboxylate and carbonyl groups. Isotope fractionation inevitably reflects Ca binding with soft ligands since combination of Ca^{2+} with phosphate liberated by phosphate esters must avoid precipitation of the relatively insoluble hydroxyapatite until final delivery of its constituents to the bone.

Iron

The role of iron in human biology is particularly important because Fe(II)-bearing hemoglobin is the prime carrier of oxygen in the blood. More than 99 % of Fe in blood (Albarède et al. 2011) and 65 % in the body (Gropper and Smith 2012) is accounted for by erythrocytes. An average body of 70 kg contains ~ 3 g of Fe, and the daily requirement is 1–2 mg per day, giving a residence time of Fe in the body of ~ 5.5 years. Myoglobin is a closely related oxygen-binding protein, which allows the muscle tissue to 'hold its breath' for protracted lengths of time. Other iron stores are present in the liver, the kidney and the spleen, largely as Fe(III) ferritin, a ferrihydrite analog wrapped in a protein shell. A characteristic of iron homeostasis is that excretion is not actively regulated, with body balance being only achieved by the modulation of the intestinal input.

Iron ions exist in two oxidation states, ferrous Fe(II) and ferric Fe(III). Ferric iron binds with many inorganic and organic ligands and ferric hydroxide is highly insoluble. Iron has four major isotopes, 54, 56, 57, and 58 (nominal masses). Iron isotopic abundances are most commonly measured by MC-ICP-MS. The pioneering study of Walczyck and von Blanckenburg (2002) demonstrated the existence of large isotopic variability among organs and body fluids.

Iron homeostasis in mammalian cells has been reviewed by many authors (Fleming and Bacon 2005; Andrews 2008, 2012; Dlouhy and Outten 2013; Winter et al. 2014). After reduction of diet Fe(III) to Fe(II) by the ferric reductase duodenal cytochrome b (Dcytb), the divalent metal transporter 1 (DMT1) allows inorganic iron from the diet across the membrane into intestinal cells. Dietary iron is transported by the transmembrane copper-dependent ferroxidase hephaestin from intestinal enterocytes into the blood stream to facilitate iron loading onto the plasma iron carrier transferrin (Tf). Transferrin (Tf) binds two ferric ions and provides iron for most human cell types (Fig. 4). Intracellular iron is

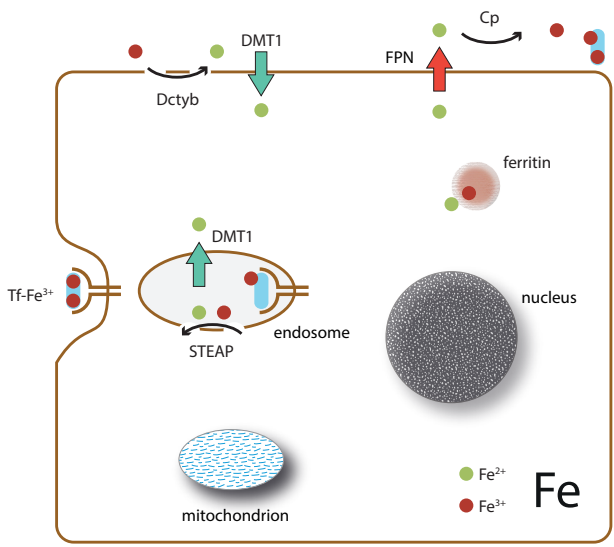

Figure 4. A sketch of iron trafficking in a generalized cell. Abbreviations: transmembrane divalent metal transporter 1 (DMT1, uptake), ferroportin (FPN, efflux), transferrin (Tf, reversible binding). Oxidase and reductase enzymes are shown as curved arrows: duodenal cytochrome B (reductase, dctyb), 6-transmembrane epithelial antigen of prostate (reductase, STEAP), ferritin (FER, oxidase and storage), ceruloplasmin (Cp, oxidase). An endosome is a membrane-bound pocket of extracellular medium created by endocytosis.

either stored in ferritin (see below) or exported by the Fe(II) iron exporter ferroportin (FPN) into the circulation where it is oxidized by ceruloplasmin for binding with transferrin (Tf), which accounts for most of iron transport in the serum. Iron-bound transferrin is imported into the cell with the cell surface Tf receptor 1, and the complex engulfed by the retracting membrane forming an endosome in a process known as endocytosis.

Further reduction of Fe(III) by the STEAP reductase and transport to the cytoplasm via DMT1 makes Fe(II) available for cell needs. Excess Fe(II) is either re-exported back to the blood stream by FPN or stored within the cell after re-oxidation as ferritin (Theil 2011; Linder 2013).

The hypoxia-inducible factor 1 (HIF-1α) links the production of red blood cells (erythropoiesis) with iron homeostasis (Haase 2013). The important observation (Krayenbuehl et al. 2005; Hotz et al. 2012) that blood Fe becomes isotopically heavier after phlebotomy (bloodletting) was interpreted as indicating that Fe is quickly retrieved from ferritin iron stores (liver, kidney) to replace lost iron, which is an important observation for the treatment of anemia. Iron is isotopically heavy in the liver, spleen, and bone marrow, and light in red blood cells (Hotz et al. 2011). A thorough inventory of Fe isotope abundances in human diet has been assembled by von Blanckenburg et al. (2013).

Zinc

Zinc is a transition element with five isotopes, 64, 66, 67, and 68, and the very minor isotope 70. It is always in a divalent state. The Zn content of the human body ranges from 1.5 to 3 g and the daily intake recommended for an adult is about 10 mg (Gropper and Smith 2012) giving a residence (turnover) time in the body of about 225 days. This metal is found in the nucleus and the cytosol of cells in all organs. About 90 % of Zn in blood is accounted for by erythrocytes (Albarède et al. 2011). Excess cytosolic Zn is bound to metallothionein, a short sulfur-rich protein, and then transported to nucleus and organelles for storage. Zinc is a cofactor of carbonic anhydrase, which interconverts carbon dioxide and bicarbonate, and thus regulates the acid-base balance of the cytosol. Zinc is also a cofactor of superoxide dismutase, which controls reactive oxygen species. Zinc further regulates the glutathione metabolism and metallothionein expression (Cruz et al. 2015) and affects signaling pathways and the activity of transcription factors with zinc finger domains.

Zinc homeostasis and its importance in various pathologies have been regularly reviewed (Cousins et al. 2006; Maret and Krężel 2007; Lichten and Cousins 2009; Fukada et al. 2011; Maret 2013; Bonaventura et al. 2015) (Fig. 5). Adjustment of gastrointestinal absorption is the primary control of zinc homeostasis (Lowe et al. 2000). Malnutrition induces cell-mediated immune defects and promotes infections (Golden et al. 1977). Zinc acts on the immune system by potentiating cytokines (Poleganov et al. 2007), a mechanism that may also be controlling chronic inflammation, such as rheumatoid arthritis (Kawashima and Miossec 2004). Zinc in seminal fluid has been suggested as a biomarker of prostate cancer (Costello and Franklin 2008). Albumin is the main transporter of Zn in serum. For a ‘generalized’ cell, the transmembrane importers consist of 14 ‘isoforms’ of the ZIP family (ZIP1 to ZIP14). Different ZIP transporters are expressed specifically on different cell types (Bonaventura et al. 2015). DMT1 has a lower affinity for Zn (Garrick et al. 2003; Espinoza et al. 2012). No specific chaperone, a dedicated cytosolic transporter, has been identified for the transfer from cytoplasm to organelles, although to some extent metallothionein may be considered one. Zinc efflux from the cell and Zn stockade in organelles is controlled by the ZnT protein family, which consists of 10 isoforms (ZnT1 to ZnT10). The trans-membrane ZnT1 is the only isoform to be ubiquitously expressed on the cell surface, while the expression of other ZnTs depends on the type of cell and organelles where they are localized.

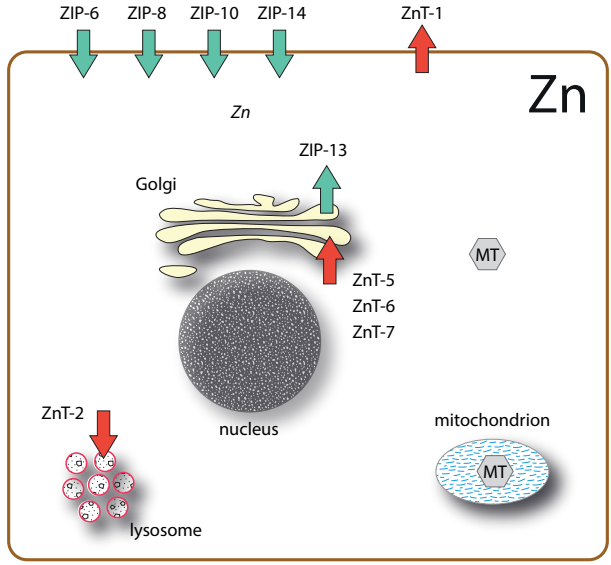

Figure 5. A sketch of zinc trafficking in a generalized cell. Abbreviations: Zn transporter family (ZnTx, uptake), Zn transporter ZIP family (ZIPx, efflux), metallothionein (MT), Cu,Zn-superoxide dismutase 1 (SOD1).

Copper

Copper has two isotopes at nominal masses 63 and 65 and its ions exist in two oxidation states, Cu(I) and Cu(II). The total copper content of the human body ranges from 50 to 150 mg and is found in all tissues and most body fluids and the daily intake recommended for an adult is about 1 mg (Gropper and Smith 2012). The residence (turnover) time in the bulk human body is therefore less than 20 days. About 35 % blood copper is accounted for by erythrocytes (Albarède et al. 2011). Copper is a micronutrient and a catalytic and structural cofactor of many important enzymes involved in tumor development (Linder and Goode 1991; Brewer 2003; Kim et al. 2008; Lutsenko 2010; Vest et al. 2013; Denoyer et al. 2015). Serum ceruloplasmin is a ferroxidase enzyme synthetized in the liver, which allows iron to be transported in the blood as harmless Fe^{3+} hydroxide. It also acts as a modulator of inflammation. A variable fraction of copper is transported by serum albumin. Cytochrome *c* oxidase is a transmembrane protein complex of the mitochondrion associated with the terminal step of electron transport and energy production. Superoxide dismutase 1 (Cu, Zn SOD1) resides mostly in the cytosol. Excess Cu may also be stored in metallothionein.

The dominant Cu importer of cells is hCtr1 (human Cu transporter) (Pope et al. 2011; Kim et al. 2013; Wee et al. 2013), which binds to albumin (Shenberger et al. 2015) and binds both Cu(I) and Cu(II) (Haas et al. 2011) (Fig. 6). Hypoxia-induced DMT1 (divalent metal transporter, notably ferrous iron) has also been invoked in copper transport into mice intestinal cells (Arredondo et al. 2003; Arredondo et al. 2014), but its relevance to other cell types is not established. Depending on the final destination, hCtr1 presents Cu^{+} to chaperones that will deliver it to specific partners: COX17 brings copper to cytochrome c oxidase (CCO) in mitochondria, CCS delivers it to SOD1, while ATOX1 is the chaperone for the copper-transporting ATPases (Cu-ATPases). The latter maintain intracellular Cu(I) levels by regulating its efflux either directly or through the secretory pathway (Lutsenko 2010; Rosenzweig and Argüello 2012). ATP7A and ATP7B differ in their patterns of tissue expression and cellular localization.

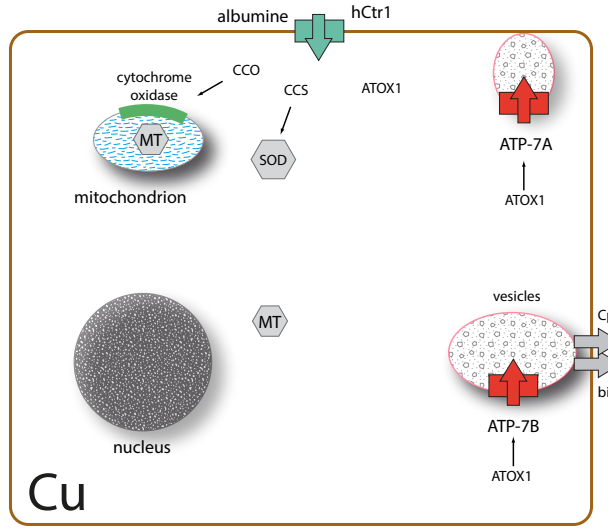

Figure 6. A sketch of copper trafficking in a generalized cell. Abbreviations: human copper transporter (hCtr1, uptake), copper ATPases ATP7A and ATP7B (efflux), cytochrome c oxidase copper chaperone (CCO), copper chaperone for superoxide dismutase (CCS), antioxidant protein 1 chaperone to ATP7A and ATP7B (ATOX1).

Sulfur

The sulfur content of the human body is about 175 g (Gropper and Smith 2012). The daily requirement of 1 g/d suggests a residence time of about 200 days which attests that S is a fairly reactive biological component. Most body sulfur is hosted by two major amino acids, cysteine a thiol ending with an -SH moiety and methionine, an *S*-methyl thioether ending with a -C–S–CH_3 moiety. Methionine is an essential amino acid, which must be obtained from the diet, and is imported by transmembrane importers, notably the Na-independent L-type amino acid transporter 1 (LAT1) (Kanai et al. 1998; Fuchs and Bode 2005; Broer and Palacin 2011). Instead, cysteine can be synthesized from methionine within the cell through the transsulfuration pathway involving methylation by *S*-adenosyl methionine (SAM). Metal binding metallothioneins are rich in cysteine, accounting for up to one third of the amino-acidic sequence (Kojima et al. 1976; Kissling and Kagi 1977).

Sulfur homeostasis is sketchily represented in Figure 7. An essential property of cysteine is the potential of two molecules to bind into cystine by forming a covalent disulfide S–S bridge, which may unlock for metal chelation in a reducing environment, such as the cytosol. Disulfide bridges are very important for the structure and stability of proteins such as serum albumin, the most abundant protein of blood serum and its main sulfur carrier. The properties of the disulfide bridge are at the basis of glutathione's function, a tripeptide essential to the control of cellular redox state by easily switching between its reduced (GSH) and oxidized (GSSG) form. Glutathione is synthetized from cystine imported from the extracellular medium in exchange of glutamate by the x_c^- 'antiporter' (Bridges et al. 2012). Intracellular cysteine is catabolized into either taurine or pyruvate, which is used for energy production, and sulfate (Stipanuk et al. 2006).

Sulfate is associated with membrane proteins known as proteoglycans, such as heparan sulfate, and is also found in heparin, an anticoagulant substance commonly used as an additive to lower the viscosity of blood samples and inhibit blood clotting.

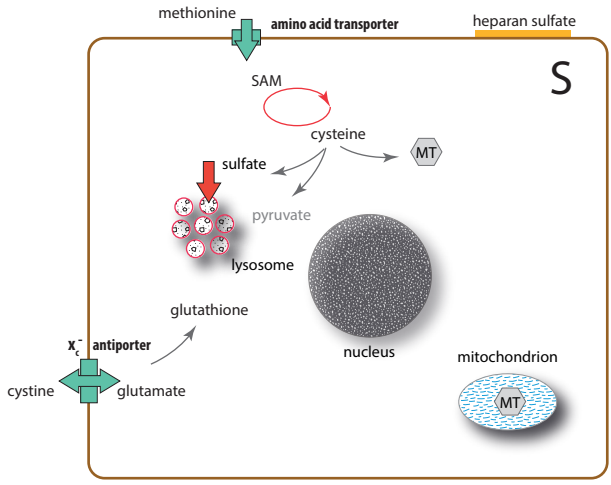

Figure 7. A sketch of sulfur trafficking in a generalized cell. Abbreviations: S-adenosyl methionine (SAM, a methylation agent involved in the intracellular biosynthesis of cysteine from methionin), metallothionein (MT, a cysteine-rich metal store). A lysosome is a membrane-bound cell organelle used to break down unused and toxic molecules and which is eventually eliminated by exocytosis. An antiporter is a two-way transmembrane exchange protein.

ISOTOPE COMPOSITIONS OF Fe–Zn–Cu–S IN THE BLOOD OF HEALTHY INDIVIDUALS

Copper and zinc contents vary in the serum of control individuals in a remarkable way. Figure 8 shows that the Cu content is high and variable among women (Milne and Johnson 1993), whereas Zn tends to be constant. In contrast, male serum tends to have a narrow range of Cu and variable Cu/Zn. The range of overlapping values is, however, relatively large. Although copper is commonly used as a biomarker to assess health status, the Zn/Cu ratio seems to have an even stronger potential (Malavolta et al. 2015; Télouk et al. 2015). Prostate cancer appears to have little effect on serum Zn levels but Cu clearly increases relative to controls. The serum of breast cancer patients seems to plot above the reference line Zn=1200 ppm, which roughly defines the average value of women controls, whereas for colon cancer patients the value plots below this line (higher Cu and/or lower Zn).

How isotope compositions of metals and sulfur vary among the organs and body fluids of a mammal was essentially unknown until a few years ago with the first studies on sheep, mice and minipigs (Balter et al. 2010, 2013; Hotz et al. 2011; Moynier et al. 2013; Tacail et al. 2014). For ethical reasons, access to human material is much more restricted. The first major observation was that, in most cases, the isotope compositions of Cu, Zn, and Fe of each organ falls, for a given species, within a narrow range of values (Fig. 9). In mice, Zn is isotopically heavy in blood and bone and light in liver and brain and independent of genetic background. Copper is specifically light in kidney. This pattern reproduces for sheep except for isotopically light Zn in blood, a feature still awaiting elucidation.

Albarède et al. (2011) conducted a systematic analysis of Zn, Cu, and Fe isotope compositions in human whole blood, serum, and erythrocytes (Table 4). They concluded that, on average, Fe in erythrocytes (red blood cells or RBC) is isotopically light with respect to serum, whereas Zn and Cu are isotopically heavier by ~0.3 and ~0.8‰, respectively. Male-female δ^{66}Zn and δ^{65}Cu differences were less than 0.2‰ for both serum and RBC. The study found mean values of δ^{66}Zn ~ +0.17‰ and δ^{65}Cu ~ −0.26±0.40‰ for serum

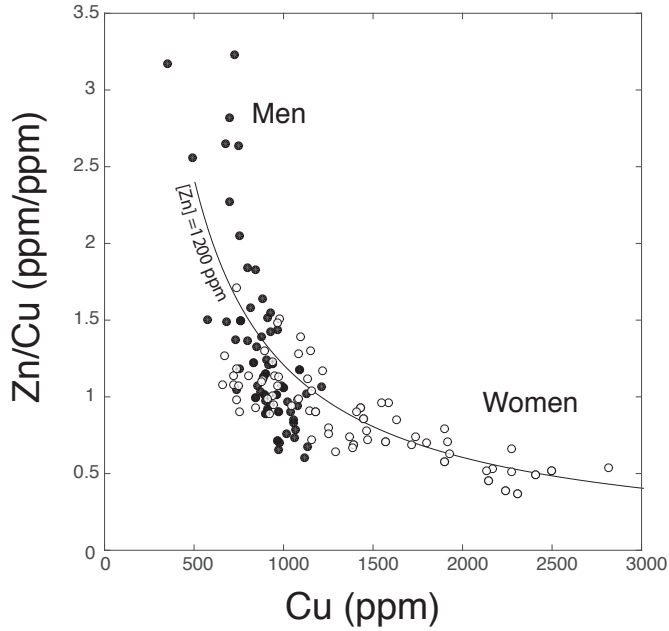

Figure 8. Plot of Zn/Cu vs Cu concentrations in the serum of healthy adults. Data from (Albarède et al. 2011). In this plot, which exacerbates the contrast between men and women, constant Zn concentrations are represented by hyperbolae.

and $\delta^{66}Zn \sim +0.44 \pm 0.26‰$ and $\delta^{65}Cu \sim +0.66‰$ for erythrocytes. A similar $\delta^{65}Cu$ value $0.29 \pm 0.27‰$ was obtained by Costas-Rodríguez et al. (2015) on 29 serum samples. The serum-RBC difference is most significant for Cu. $\delta^{65}Cu$ is $0.2‰$ heavier in men erythrocytes relative to women (Albarède et al. 2011). Total blood values depend on the erythrocyte load (hematocrit) and therefore may vary with sample preparation and should be considered less reliable than serum and RBC values. There is still no consensus on the $\delta^{56}Fe$ of human serum, in which iron concentration is very low and hemolysis (rupture of red blood cells) may obscure the results (Albarède et al. 2011; von Blanckenburg et al. 2014).

In a study of *whole-blood* samples on Yakut volunteers aged 18–74, Jaouen et al. (2013) found that the $^{66}Zn/^{64}Zn$ ratio increases and the $^{65}Cu/^{63}Cu$ ratio decreases with age. Likewise, the whole blood Fe isotope ratios of postmenopausal women or women on a hormonal anti-conception treatment leading to absence of menstruation are shifting in the direction of those characteristic of the male population (Van Heghe et al. 2013). Van Heghe et al. (2014) observed that $^{65}Cu/^{63}Cu$ ratios tend to increase in whole blood after menopause but found no age or menstruation effects on Zn isotopes. In contrast, Jaouen and Balter (Jaouen et al. 2013) showed that, while the Fe and Cu isotope compositions of blood of men are steady throughout their lifetime, postmenopausal women exhibit blood $\delta^{65}Cu$ values similar to men, and $\delta^{56}Fe$ values intermediate between men and menstruating women, an isotopic pattern easily explained by the different residence times of the metals into the body (Fe ~ 5.5 years and Cu ~ 20 days).

Comparison of their results with Albarède et al.'s (2011) study led Jaouen et al. (2013) to emphasize the importance of the ethnic factor. On a fairly small sample set, Van Heghe et al. (2012) observed that $\delta^{66}Zn$ in whole blood is about $0.15‰$ higher for vegetarian relative to omnivorous volunteers, but the outcome for Cu isotopes was less conclusive. The isotopic

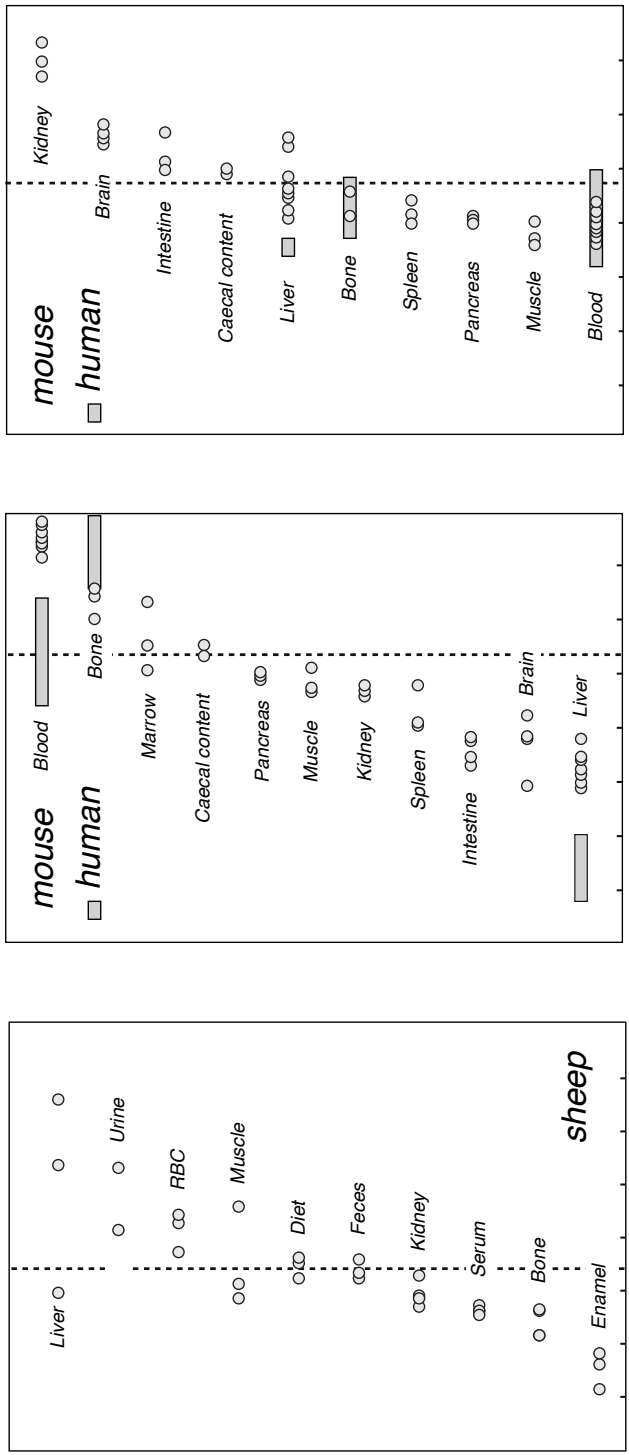

Figure 9. Zinc and copper isotope variability among organs, bones, body fluids, and intestinal content of mice (Balter et al. 2013) (left and center) and for Ca (Tacail et al. 2014) (right). All the data are reported in delta units per mil for Cu and Zn. The shaded bands show the range of variations for humans (Albarède et al. 2011; Jaouen et al. 2013). Typical uncertainties are ±0.05 ‰ (2-sigma error).

Table 4. Average isotope compositions in delta units (‰) and 95% range (2s) for the isotope compositions of Zn and Cu in the serum, erythrocytes, and total blood of 49 blood donors (Albarède 2015). Typical analytical uncertainties are 0.05 ‰. Men–women comparison: *p* is the probability that the two sets are not identical.

	n	av δ^{66}Zn	2s	av δ^{65}Cu	2s
Serum					
women	28	0.18	0.28	−0.24	0.36
men	21	0.16	0.1	−0.28	0.4
all	49	0.17	0.26	−0.26	0.4
p value (men/women)		0.45		0.3	
Erythrocytes					
women	28	0.46	0.17	0.46	0.47
men	21	0.43	0.45	0.67	0.36
all	49	0.44	0.33	0.56	0.5
p value (men/women)		0.39		0	
Total blood					
women	28	0.41	0.16	0.01	0.16
men	21	0.39	0.41	0.17	0.33
all	49	0.4	0.37	0.09	0.32
p value (men/women)		0.32		0.02	

composition of Zn was shown to be different between food products of plant and of animal origin (Costas-Rodríguez et al. 2014). Clearly, the carrier of assimilated Zn and the effect of diet deserve expanded studies on larger numbers of volunteers and with well-documented blood tests.

The first substantial set of $^{34}S/^{32}S$ values on the blood of healthy individuals were obtained by elemental analysis-isotope ratio mass spectrometry (EA-IRMS; gas source mass spectrometry) by Balter et al. (2015) and an average $\delta^{34}S_{V\text{-}CDT}$ of 5.9 ± 1.5‰ on 11 serum samples and 5.1 ± 1.9‰ on 20 RBC samples were measured. On 25 serum samples of adults, Albalat et al. (2016) obtained a very similar mean value but within a reduced interval 6.0 ± 0.7‰, with the average $\delta^{34}S_{V\text{-}CDT}$ of women being 0.2‰ lower than that of men. On the same samples, both methods agree within one permil. Albalat et al. (2016) show that S in children serum is only slightly heavier but more scattered (6.3 ± 1.0‰) than S in adult serum.

CALCIUM AND BONE LOSS

Calcium isotopic variations in urine and blood of bed rest studies (Heuser and Eisenhauer 2010; Morgan et al. 2012a,b; Channon et al. 2015) were motivated by the observation that astronauts suffer bone loss during space flight (Fig. 10). It was known that Ca in urine increases and becomes isotopically lighter with time, suggesting that this metal is liberated by osteoclasts from the bones into the blood stream (Permyakov and Kretsinger 2010). Increased loss of isotopically lighter Ca may reflect changing input, i.e., bone mineralization,

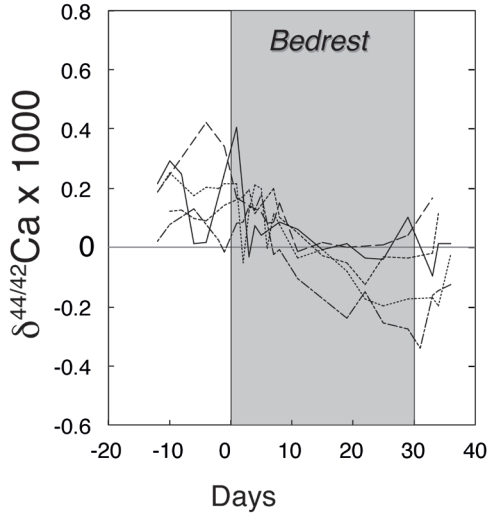

Figure 10. Isotope evolution of Ca in urine of volunteers confined to bed rest for a month. Bone Ca being isotopically light relative to diet, the study by Morgan et al. (2012a) shows that bed rest enhances bone loss.

as a response to changing proportions of free Ca to albumin-bound Ca. It may also signal an increased loss of bone Ca, which is recognized to result from enhanced expression of another calcium-binding protein, sclerostin, during bed rest (Spatz et al. 2012). Calcium isotopes show a strong potential to quantify the Ca fluxes in and out of the bones without having to resort to adding isotope tracers to the diet (Skulan and DePaolo 1999; Heuser and Eisenhauer 2010; Channon et al. 2015). This is of course relevant to the study of Ca in osteoporosis, a condition particularly common with aging women.

GENETIC AND INFECTIOUS DISEASES

Blood of patients with hereditary hemochromatosis contains more of the heavier iron isotopes than blood of healthy individuals (Krayenbuehl et al. 2005). This pathology is characterized by a genetically disrupted function of two critical proteins involved in control of intestinal iron absorption, hepcidin and ferroportin, leading to ineffective control of intestinal iron absorption and progressive iron overload of tissues. Iron in the blood of patients with hemochromatosis is 0.2–0.4 ‰ heavier than in healthy individuals, which suggests that the blood of hemochromatosis patients uses iron stores more heavily than the control cases. An alternative explanation (Van Heghe et al. 2013) is that, due to decreased hepcidin levels, hemochromatosis patients absorb more iron in the intestine.

Wilson's disease is a genetic disorder affecting Cu metabolism, which can lead to severe physiological and neurological symptoms. Aramendia et al. (2013) demonstrated a correlated decrease of both Cu levels and δ^{65}Cu in the serum of Wilson patients. This observation is quite remarkable and calls for an expanded data set.

It is well established that the prion protein (PrP) has an influence on cellular copper metabolism. Buechl et al. (2008) analyzed Zn and Cu isotopes in the brain of wild type and knockout mice and demonstrated that Cu and Zn isotopes in brain tissue are sensitive to prion-related local damage. They concluded that the presence of a form of PrP that does not bind Cu induces the fractionation of Cu isotopes.

ISOTOPE METALLOMICS IN CANCER

We already mentioned that calcium isotopes constitute a biomarker of bone loss. It was therefore suspected that they also have some potential to be affected by multiple myeloma (MM), a cancer of the plasma B-cells (lymphocytes produced in the bone marrow) characterized by bone destruction (Gordon et al. 2014). Radiographs do not demonstrate MM-induced abnormalities until a substantial fraction of the bone has been lost and provide no information about ongoing bone remodeling. Existing MM biomarkers do not provide an estimate of the net bone mineral balance. From the data on a limited number of patients, Gordon et al. (2014) suggested a significant relationship between serum Ca isotope abundances and myeloma activity, likely due to an MM-induced increased level of bone resorption.

Warburg and Krebs (1927) found that serum copper levels increased in various chronic diseases and several types of cancers, resulting in systemic and oncogenic (Ishida et al. 2013) copper accumulation. Anomalously high Cu levels or Cu/Zn ratios were indeed observed in the serum of breast cancer (Gupta et al. 1991; Yücel et al. 1994; Magalova et al. 1996; Piccinini et al. 1996; Koksoy et al. 1997; Wu et al. 2006; Cui et al. 2007) and cervical cancer (Cunzhi et al. 2003) patients. Zowczak et al. (2001) showed significant increase in the mean ceruloplasmin oxidase activity and total Cu concentrations in the serum of 62 patients with breast, lung, gastrointestinal, and cervical cancer relative to a control group. Serum ceruloplasmin was found to be significantly elevated in advanced stages of solid malignant tumors (Senra Varela et al. 1997). A compilation of Cu and Zn concentrations in the serum of cancer patients discussed below confirms that certain types of cancer may affect these parameters (Fig. 11). By themselves, such observations justify that copper and zinc isotopic variability should be investigated in cancer patients. The connection between Cu and Zn through proteins such as superoxide dismutase and metallothionein involved in the control of hypoxia, and therefore of

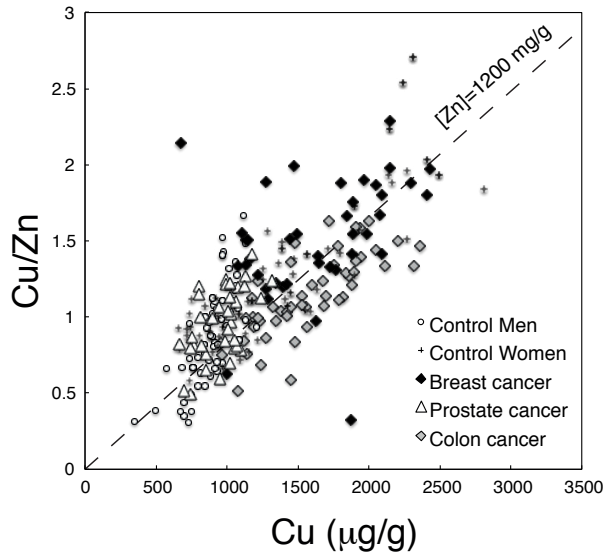

Figure 11. Copper and Cu/Zn in the serum as indicators of cancer status. The control group shows strong correlations, reflecting the tight regulation of Zn concentrations in the body (note that x/y=Zn). The trends for control men and women are different, with men having, on average, less Cu and more Zn than women.. Copper remains stable in prostate cancer patients relative to control men, but increases in colon cancer patients. Zinc in the serum of breast cancer patients is decreased and Cu probably increased relative to healthy subjects.

cancer in particular, gives grounds for interest in the amino acids that control the coordination of both metals, notably histidine, cysteine, and methionine. Hence the importance of exploring the extent of correlated Cu, Zn, and S isotopic variations.

Télouk et al. (2015) measured the $^{65}Cu/^{63}Cu$ ratios in the serum of 20 breast and 8 colorectal cancer patients (Fig. 12). Samples were taken at different times during the treatment, and amount to, respectively, 90 and 49 samples. Phenotypes and molecular biomarker were documented on most of the samples. When compared with the literature data from a control group of 50 healthy blood donors, abundances of Cu isotopes predict mortality in the colorectal cancer group with an error probability $p = 0.018$ (Fig. 13). For the breast cancer patients and the group of control women the probability falls even further to $p = 0.0006$. Most patients considered in this preliminary study and with serum $\delta^{65}Cu$ below the threshold value of −0.35‰ (per mil) did not survive beyond a few months. As a marker, a drop in $\delta^{65}Cu$ precedes molecular biomarkers such as CEA (carcinoembryonic antigen) and CA15.3 (carbohydrate antigen 15.3) by several months (Fig. 14), which is consistent with Cu turnover time in the body. The observed decrease of $\delta^{65}Cu$ in the serum of cancer patients was assigned by Télouk et al. (2015) to the extensive oxidative chelation of copper by cytosolic lactate. The potential of Cu isotope variability as a new diagnostic tool for breast and colorectal cancer seems strong.

Larner et al. (2015) analyzed zinc isotope compositions of various tissues in breast cancer patients. Resections of breast cancers were found to have a significantly lighter Zn isotopic composition than the blood, serum, and healthy breast tissue in both groups. The authors interpret the isotopically light Zn in tumors as attesting to its uptake by metallothionein in breast tissue cells, rather than in Zn-specific proteins. This reveals a possible mechanism of Zn delivery to Zn-sequestering vesicles by metallothionein, and is supported by a similar signature observed in the copper isotope abundances of one breast cancer patient. The number of samples used for this study was rather small and, hence, whether and how cancer may affect the $\delta^{66}Zn$ of serum deserves further attention.

Balter et al. (2015) found that in hepatocellular carcinoma (liver cancer) patients, serum and erythrocyte copper and sulfur are both enriched in light isotopes relative to controls (Fig. 15, bottom). The magnitude of the sulfur isotope effect is similar in red blood cells and serum of hepatocellular carcinoma patients, implying that sulfur fractionation is systemic. In contrast to serum data, the $\delta^{65}Cu$ of tumor resections is notably higher relative to healthy liver tissue (Fig. 15, top). The agreement between sulfur isotope data acquired on the same samples by EA-IRMS and MC-ICP-MS (Albalat et al. (2016) is reasonably good. Balter et al. (2015) concluded that the isotopic shift of either element is not compatible with a dietary origin, but rather reflects the massive reallocation in the body of copper immobilized within cysteine-rich metallothionein. A study by Costas-Rodrıguez et al. (2015b) also found lower $\delta^{65}Cu$ in the serum of patients with end-stage liver disease, with complications such as ascites, encephalopathy, and hepatocellular carcinoma (Fig. 16). These authors pointed out that $\delta^{65}Cu$ was positively correlated with the liver cirrhosis-related parameters, notably aspartate aminotransferase, INR (International Normalized Ratio for prothrombin time), bilirubin, and C-reactive protein, and inversely correlated with albumin and Na. They also found a negative correlation of $\delta^{65}Cu$ with Child-Pugh score based on albumin, bilirubin, and INR and the Mayo Clinic Model for End-stage Liver Disease score (MELD) based on creatinine, bilirubin, and INR.

Albalat et al. (2016) analyzed sulfur isotopes in a large number of pathological samples with emphasis on serum. The serum samples depart from those of healthy volunteers by their much smaller S concentration. This observation echoes the negative correlation between low serum albumin content and mortality (Okuda et al. 1985; Kao et al. 2015). The samples, however, for which the $\delta^{34}S$ departs from the range of healthy individuals are few and correspond to the ‘naive’ (untreated) patients, in particular those analyzed by Balter et al. (2015). Cancer and

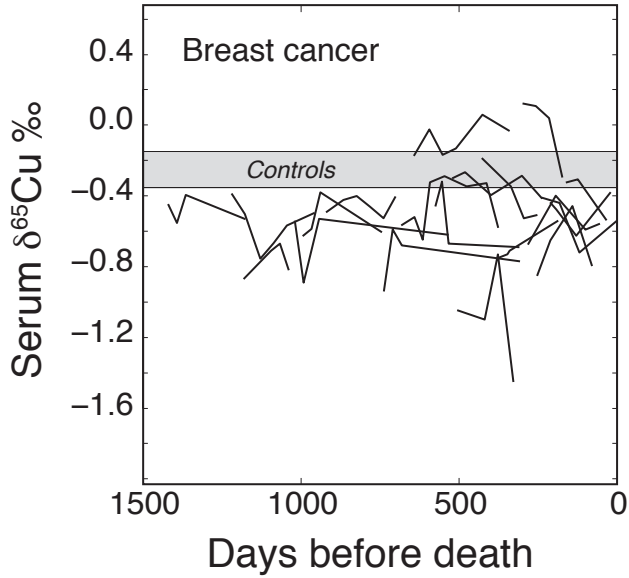

Figure 12. Evolution of serum δ^{65}Cu in 140 samples taken from 20 breast cancer cases until patient death (Télouk et al. 2015). Each line represents a different patient, with patterns used for differentiation purposes. The shaded band (controls) represents the 75 % range of δ^{65}Cu in the serum of healthy donors.

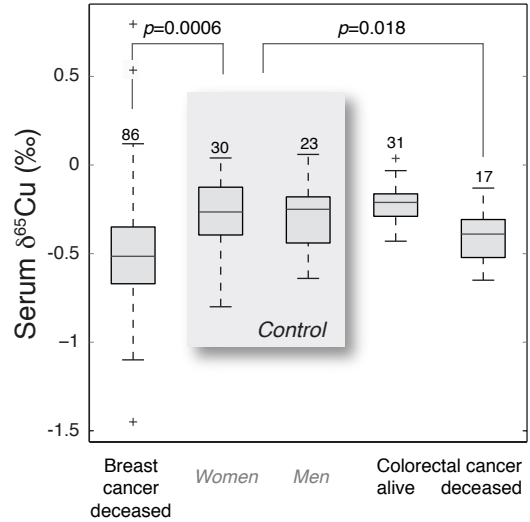

Figure 13. Whisker plots of serum δ^{65}Cu values for healthy men and women compared to breast cancer and colorectal cancer patients (Télouk et al. 2015). Boxes represent the 75 % middle quantiles and the whiskers 95 % quantiles. Parameter *p* represents the probability that two populations may be identical. Horizontal lines in the box: median; crosses: outliers. Separation between breast cancer patients and healthy women is strong. Separation between breast cancer and colorectal cancer patients and healthy men and women seems to depend on mortality.

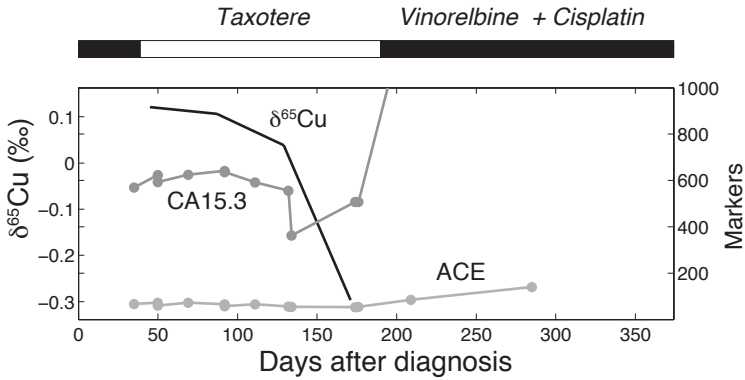

Figure 14. Early alarm by δ^{65}Cu (Télouk et al. 2015). The plot compares the δ^{65}Cu values (left axis, black line) and the molecular biomarkers (right axis): CEA (carcinoembryonic antigen, shade 1) and CA 15.3 (carbohydrate antigens, shade 2). The top bar scale shows the successive therapies received by the patient. The copper isotope signal precedes the other markers by 2–3 months.

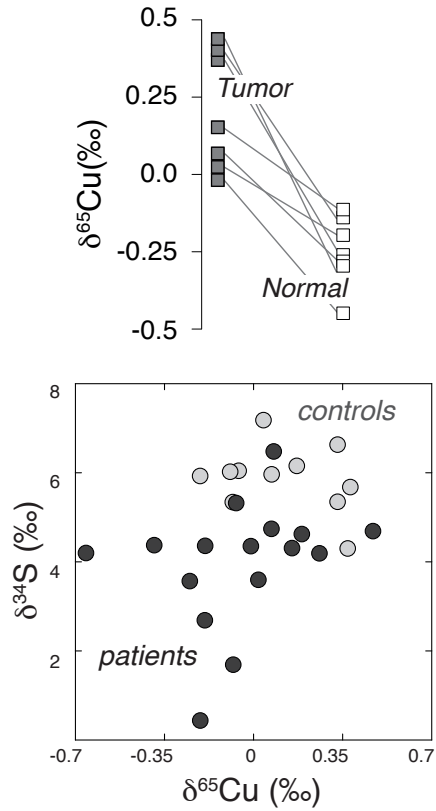

Figure 15. *Bottom*: Isotopically light copper and sulfur in the serum of hepatocellular carcinoma patients relative to controls (Balter et al. 2015). *Top*: Isotopically heavy copper in tumor liver tissue relative to normal tissue. The opposite direction of the changes in Cu isotope abundances in serum and tumor may be explained in different ways (see text).

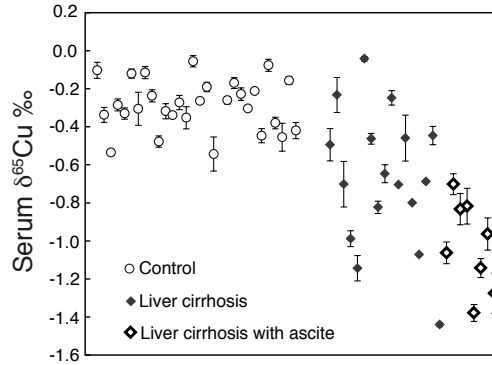

Figure 16. δ^{65}Cu values in the serum of liver cirrhosis patients with and without accumulation of fluid in the peritoneal cavity (ascites) relative to controls (Costas-Rodríguez et al. 2015b). Ascites is often associated with cirrhosis and metastatic cancer.

rheumatoid arthritis conditions increase the scatter of sulfur isotope compositions by up to a factor of two, but with little effect on the mean δ^{34}S values. It has been observed that medication brings δ^{34}S back to normal values but does not change sulfur concentrations in the serum.

Before attempting a biochemical interpretation of isotopic trends in biological samples, let us summarize the observations at hand. Most of the observations so far have been made on serum, on whole blood, occasionally on erythrocytes, and only exceptionally on organ tissues and tumors. Among all the analyzed elements, Cu and Zn isotope compositions seem to show that tumors deviate from healthy tissue (heavy Cu in liver and light Zn in breast neoplastic tissue) (Balter et al. 2015; Larner et al. 2015). In contrast to Cu, which is isotopically lighter in the serum of well over 130 cancer patients relative to a similar number of controls (colon, breast, and liver) (Balter et al. 2015; Télouk et al. 2015), our unpublished Zn isotope serum data show no difference between cancer patients and healthy donors of any age that could be used for medical purposes. (Fig. 17). Likewise, results obtained so far suggest that sulfur isotope compositions in the serum of cancer patients (colon, breast, and liver) could not in general be distinguished from the values in control patients, with the exception of some hepatocellular carcinoma patients (Balter et al. 2015; Albalat et al. 2016), but that the spread of δ^{34}S values is smaller for controls.

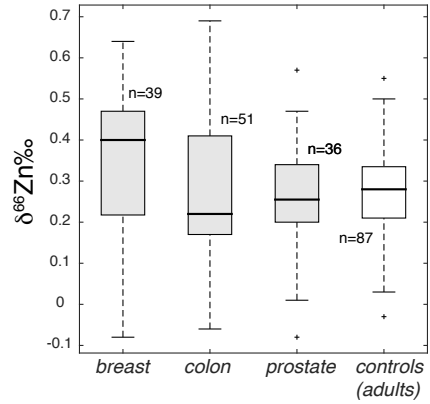

Figure 17. Whisker plot of serum δ^{66}Zn values for healthy men and women compared to breast, colorectal, and prostate cancer patients (unpublished data). The parameter n is the number of samples. Although the database is still rather small, Zn isotopes in serum show little potential as a cancer biomarker.

Both Télouk et al. (2015) and Costas-Rodrıguez et al. (2015b) suggested that low δ^{65}Cu can be used for prognosis in end-stage cancer (liver, colon, breast). Copper isotopes would complement other markers, such as the Child-Pugh score, albumin, or transaminases. The ~6 weeks turnover time (Linder and Goode 1991; Milne 1998) is close enough to the 19 days of albumin (Schaller et al. 2008) that the two parameters may have some biochemical pathways in common, one of them being that albumin is a Cu transporter. Télouk et al. (2015) pointed out that Cu isotopes seem to be reactive over time intervals of weeks to deteriorating health conditions, whereas molecular biomarkers tend to increase, whenever they do, within months.

Among possible reasons for δ^{65}Cu variations, some have been privileged:

1. Télouk et al. (2015) suggested that isotopically heavy Cu chelated by lactate, which cancer cells are known to produce massively, accumulates in the cytoplasm.
2. Balter et al. (2015) proposed that the low δ^{65}Cu value of serum is due to the release of intracellular copper from cysteine clusters in metallothionein.
3. Costas-Rodrıguez et al. (2015b) suggested that low δ^{65}Cu values reveal the hepatocellular and biosynthetic dysfunction of the liver, synergistically with inflammation and water retention.

Isotope abundances add a new 'dimension' to the overall budget of each element in cells and in the organism. None of the Cu isotope studies discussed above have attempted a mass balance evaluation including blood components, healthy tissues, and tumors, simply because the data are not available. Liver accounts for a large fraction of bodily metals such as Cu and Fe. A legitimate concern therefore is that the Cu isotope effects observed in serum and tumors cannot be directly compared until the missing data have been collected.

Three main routes by which Cu interacts with cancer cells are cellular metabolism, angiogenesis, and hypoxia. Copper is a tumor promoter and regulates oxidative phosphorylation in rapidly proliferating cancer cells inside solid tumors (Ishida et al. 2013). In normal cells, glycolysis, the first step of ATP production from glucose, is slow and its end product, pyruvate, is oxidized in mitochondria, where it fuels the much more efficient steps of the citric acid cycle and oxidative phosphorylation. Glucose degradation (glycolysis) is the primary source of ATP, which is achieved by the attachment of inorganic phosphate PO_4 to adenosine diphosphate (ADP). In normal *aerobic glycolysis* ATP is produced by a set of reactions summarized as follows:

$$\text{Glucose} + 2\ \text{ADP} + 2\ \text{NAD}^+ + 2\ \text{PO}_4 \Leftrightarrow 2\ \text{ATP} + 2\ \text{pyruvate}^- + 2\ \text{NADH} + 2\ \text{H}_2\text{O} + 4\ \text{H}^+$$

where nicotinamide adenine dinucleotide (NAD^+) is a ubiquitous electron acceptor. NADH is further re-oxidized at the surface of mitochondrion with consumption of H^+. In cancer cells, in contrast, pyruvate is used as an electron acceptor and aerobic glycolysis is replaced by *anaerobic glycolysis*:

$$\text{Glucose} + 2\ \text{ADP} + 2\ \text{PO}_4 \Leftrightarrow 2\ \text{ATP} + 2\ \text{lactate}^- + 2\ \text{H}_2\text{O} + 2\ \text{H}^+$$

a reaction known as lactic fermentation. Excess protons produced by the latter reaction are pumped out of the cell into the blood stream, which decreases its pH and greatly favors metastasis. The observation that cancer cells show enhanced glycolysis followed by lactate production in the cytosol, even in the presence of O_2, is known as the Warburg effect. Lactate levels are observed to be elevated in the serum of critically ill patients and correlate well with disease severity (Okorie and Dellinger 2011; Kinnaird and Michelakis 2015). Lactate efflux from the cell is regulated by monocarboxylate transporters (MCT) and intracellular and extracellular lactate levels are not simply related (Halestrap and Price 1999; Swietach et al. 2014). Copper is isotopically heavy in both pyruvate and lactate. However, in healthy cells pyruvate is shuttled into mitochondria for further energy processing, whereas lactate is exported from the cell by MCT and is metabolized in the liver. To a large extent, lactate is

'available' in the cell for Cu chelation (Fig. 18), whereas pyruvate is not. This is the substance of Télouk et al.'s (2015) explanation for the accumulation of copper with high δ^{65}Cu in the cell

A major role of copper in cancer is associated with hypoxia, a hallmark of both inflammation and human malignancies. In order to secure delivery of oxygen and nutrients to tumor cells, the growth of cm-sized tumors is accompanied by pervasive neovascularization (Carmeliet and Jain 2000). Several angiogenic factors, notably VEGF, tumor necrosis factor alpha (TNF-α) and interleukin (IL1), are copper activated (Nasulewicz et al. 2004). Under hypoxic conditions, ionic serum copper stabilizes the hypoxia-inducible factor (HIF-1α) (Martin et al. 2005), which is associated with tumor growth, vascularization, and metastasis (Semenza 2007; Wilson and Hay 2011).

Copper transport and uptake are still poorly understood (Lutsenko 2010), as is the mechanism of Cu reduction during uptake. It is likely that albumin is the main serum carrier presenting Cu to the cell and binds both Cu(I) and Cu(II) (Haas et al. 2011; Shenberger et al. 2015). Both Cu^{+} and Cu^{2+} are captured by the terminal amino acids of Ctr1, which introduces Cu^{+} into the cytosol. Hypoxic stimulation of the HepG2 cells (hepatocellular carcinoma) leads to a down-regulation of albumin (Wenger et al. 1995), supporting a connection between liver, copper, and albumin (Costas-Rodríguez et al. 2015b). Transmembrane Cu uptake is the primary site where isotope fractionation takes place. Fractionation restricted to storage or efflux is unlikely, as it would lead to an open-ended shift in intracellular δ^{65}Cu. Clearly, Cu isotopes may help understand the connections between tumor growth and Cu homeostasis.

ASSESSING THE POTENTIAL OF METAL ISOTOPES AS BIOMARKERS

Will metal isotopes become efficient biomarkers of disease? Efficiency here means reliability, independence relative to pre-existing biomarkers, and a potential for early diagnosis. Much work lies ahead. First, documenting the sensitivity and the specificity of metal isotopes relative to a particular pathology or a family of pathologies is a huge endeavor. Second, promoting Isotope Metallomics as a set of biomarkers will be confronted with the sample throughput capacity of the analytical groups. The medical community expects large datasets to be evaluated before metal isotopic variations can be routinely used as a diagnostic tool or as a therapeutic help. Running thousands of samples, as it is common practice in modern medical cohorts, is labor intensive and costly, and still beyond the analytical reach of existing mass-spectrometry labs.

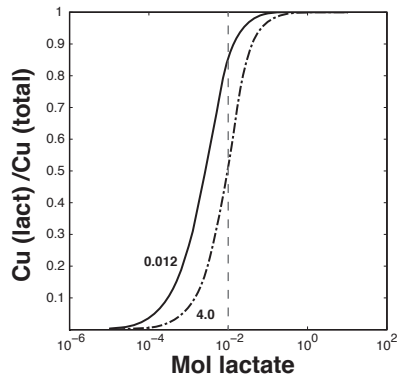

Figure 18. Extent of copper chelation by lactates in the cytosol (Télouk et al. 2015). The numbers on the curves represent the Cu^{+}/Cu^{2+} ratio for a redox potential of 0.153 V (copper ions) and for a body potential of 0.27 V (Lundblad and Macdonald 2010). The vertical dashed line corresponds to a lactate concentration of 10 mMol typical of tumor cells (Walenta et al. 2000).

Given the expectedly small sample size of the existing isotope abundance datasets, high-profile statistics will have a strong role to play. When facing a decision, geochemists are used to compare two-sigma intervals or, at best, to run t-tests on rather small populations (e.g., patients vs controls). Non-parametric tests, such as the Mann–Whitney and Kruskal–Wallis rank tests, are particularly useful to strengthen a case. When decision comes to human health, the dread of a false positive diagnose adds, however, to that of a failed detection. Receiver Operating Characteristics (ROC) curves (e.g., (Krzanowski and Hand 2009)) are particularly powerful tools of diagnostic medicine. The Y-axis of the ROC curve is the probability of a True Positive decision concluding that a patient with serum $\delta^{65}Cu \le \delta^{65}Cu_c$ died, or that a donor serum $\delta^{65}Cu$ exceeded $\delta^{65}Cu_c$ for different cutoff values $\delta^{65}Cu_c$. The Y-axis is also known as Sensitivity. The X-axis is the probability of a False Positive decision concluding that a patient with serum $\delta^{65}Cu \ge \delta^{65}Cu_c$ has died, or that a donor serum $\delta^{65}Cu$ is $\le \delta^{65}Cu_c$. The abscissa is also known as 1– Specificity. A widely used test is the area under the ROC curve (AUC), which varies between 0.5 (pure chance) and 1.0 (fully trustworthy test). The most reliable cutoff value can also be inferred by different techniques (Krzanowski and Hand 2009) from the elevation of the ROC curve above the first diagonal. In the present case of breast cancer diagnostic (Fig. 19), AUC is 0.76, while the optimum cutoff $\delta^{65}Cu_c$ value is $-0.37‰$.

Whether metal isotopes as biomarkers can compete with molecular biomarkers, such as CA 125 for ovarian cancer (Cramer et al. 2011), CA 19–9 for colorectal cancer (Hotakainen et al. 2009), and CA 15–3 for metastatic breast cancer (Fahmueller et al. 2013) seems unlikely in the foreseeable future. Metal isotopes, however, have opened a window on cellular metallomics and therefore show promise of significant return on the biochemical mechanisms associated with each disease.

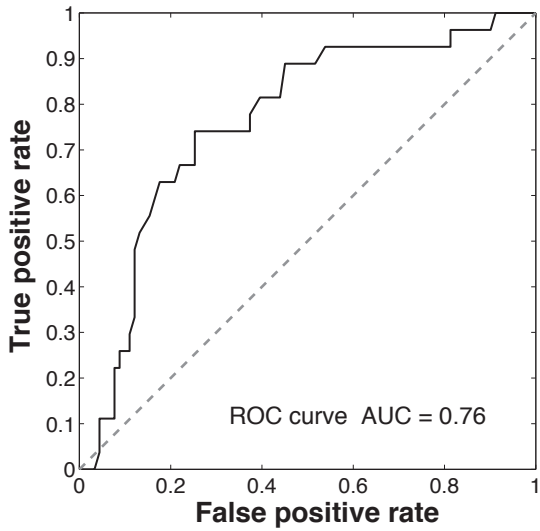

Figure 19. Receiver Operating Characteristics (ROC) curve for $\delta^{65}Cu$ in the serum of cancer patients. The ROC curve plots the probability of a True Positive decision vs the probability of False Positive decision. The optimum cutoff value for breast cancer is $\delta^{65}Cu_c = -0.37‰$. The area under the ROC curve (AUC) may vary between 0.5 (pure chance) and 1.0 (fully trustworthy test): the value of 0.76 obtained for the present data set supports the worth of $\delta^{65}Cu$ as a diagnostic tool.

COMPARTMENTALIZED MODELS OF CELLULAR HOMEOSTASIS

This field is known as Mathematical Systems Biology (e.g., ref. (Klipp et al. 2013)). Metal concentrations, as all concentrations, are prone to very large uncertainties due in particular to the elusive nature of the amount of material concentrations refer to. Elemental fractionation coefficients between coexisting media, e.g. extracellular medium and membrane, are also known with very large uncertainties. One more time, isotopic ratios help get around these problems. Models of metal homeostasis are useful as they help comparing predictions from fractionation patterns with isotope compositions observed in cells and biological fluids. An element for which modeling is particularly well advanced is zinc (Colvin et al. 2008, 2010), yet the models are still rather complex. For sake of illustration, let us consider a simpler formulation of an arbitrary compartmentalized model that captures anyway the simple features of homeostasis: uptake, efflux, storage, and catabolism. Zinc is absorbed by diffusion by transmembrane proteins from the extra-cellular fluid and stored in metallothionein, which is itself synthesized from cysteine. We leave endocytosis and exocytosis out of the model. The rate of change of the number of moles of ^{64}Zn (n), ^{66}Zn ($\tilde{n}$), cysteine (c), and Zn-loaded methionine (m) within the cytosol changes according to the following set of non-linear differential equations:

$$\frac{dn}{dt} = -k\rho\left(Dn - D^e n^e\right) - \left(Knc^i - K'm\right)$$

$$\frac{d\tilde{n}}{dt} = -\tilde{k}\rho\left(\tilde{D}\tilde{n} - \tilde{D}^e\tilde{n}^e\right) - \left(\tilde{K}\tilde{n}c^i - \tilde{K}'m\right)$$

$$\frac{dc}{dt} = -k_c\rho_c\left(D_c c - D_c^e c^e\right) - i\left(Knc^i - K'm\right) - \lambda_c c$$

$$\frac{dm}{dt} = Knc^i - K'm$$

Variables with superscript e refer to the extracellular medium, tildes (~) to the properties of ^{66}Zn compounds. For simplicity, we assume that $\tilde{n} << n$. The first term on the right-hand side of the first three equations refers to transfer through the membrane in response to a chemical potential gradient (Bonaventura et al. 2016): parameters D are membrane-cytosol and membrane-extracellular medium partition coefficients. We assume that the transmembrane protein density ρ may vary, possibly as a result of inflammation (Bonaventura et al. 2016). For simplicity again, we combined Zn uptake (ZIP) and export (ZnT) in one coefficient. The probability of cysteine catabolism per unit time is denoted l_c. K and K' are the rate constants of bonding and breakdown of Zn-bearing metallothionein. The variable i refers to the cysteine/Zn stoichiometric ratio in metallothionein.

Such a set of first-order non-linear differential equations can be solved by standard numerical packages (e.g., Matlab) or by commercial software (Klipp et al. 2013). A mock-up example is shown in Figure 20 in which the rate K' of metallothionein catabolism increases at time $t = 30$, while the methionine concentration c^e in the extracellular medium decreases. The purpose of this exercise is to show how the isotope composition of a metal in one of the compartments (including the extracellular medium) may respond to changes in biological pathways upon signaling. The actual values of the parameters chosen were not meant to reproduce natural conditions. In this respect, the cell is similar to a chemical reactor, in which the records of input and output variables can be used to document the 'state variables' within the system.

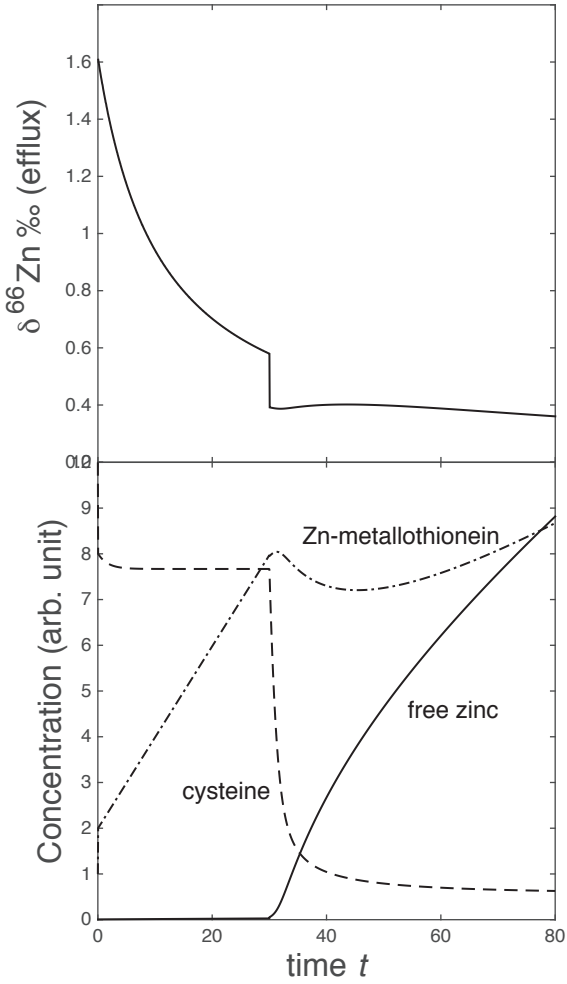

Figure 20. A mockup example of Zn transport through a cell. We assume that Zn is imported and exported through transmembrane proteins. The number of these proteins may be modulated by cell signaling. Transmembrane Zn transfer takes place by diffusion. Excess Zn is stored in Zn-metallothionein, which requires cysteine biosynthesis in the cytosol. Cysteine biosynthesis is dependent on methionine uptake. Cytosolic cysteine is broken down with a first-order rate. All the units and the parameters are arbitrary. It was assumed that at $t = 30$, the rate of metallothionein catabolism increases, while the methionine concentration in the extracellular medium decreases. *Bottom panel:* amounts of cytosolic Zn in each form. *Top panel:* δ^{66}Zn in the efflux.

PERSPECTIVES

So far, Cu has provided the strongest signal associated with a number of diseases, in particular cancer. Zinc, iron, and sulfur have not so far proved to be as informative as copper. The exploratory stage of Cu isotope variations in blood has been very fruitful. Now that this field is becoming mature, descriptive investigations need to be complemented. Data on organs are needed that only animal models can provide. Experiments should be run on cell cultures under hypoxic conditions. Protein expression, notably those controlling metal trafficking, storage, and redox, should be evaluated.

Among the upcoming challenges, several major questions need to be addressed, notably which part of the δ^{65}Cu signal is due to the cancer itself, and which part is due to other factors, such as age and, even more likely, inflammation. Our preliminary studies of athletes, which shows little or no effect exertion on δ^{65}Cu, and of patients with purely inflammatory diseases, such as rheumatoid arthritis, suggest that if there an effect of inflammation, it only manifests itself on the very long term.

Reduction of copper or ceruloplasmin levels by chelates, without causing clinical copper deficiency, was proposed for therapeutic purposes. Specific copper chelators, such as tetrathiomolybdate, D-penicillamine, and TPEN(Brewer 2001, 2005; Pan et al. 2002, 2003; Lowndes et al. 2008; Fatfat et al. 2014) have been shown to be a potent antiangiogenic and antimetastatic compound possibly through suppression of the NFκB signaling cascade. Recently, Cu-chelation therapy has been proposed as treatment of the broad spectrum of cancers containing the $BRAF^{V600E}$ mutation (Brady et al. 2014). Inhibition of copper Atox1 trafficking has also been investigated (Wang et al. 2015). The isotopic study of copper will certainly add a new dimension to the understanding of chelation pathways and copper mass balance, at the scale of both the cell and the organism, during the treatment.

The prospects of isotope variations of magnesium, which is involved in a large number of biological segments, remain unexplored. Molybdenum plays a role, notably through molybdenum hydroxylase, in a variety of hydroxylation, oxygen atom transfer, and other oxidation–reduction reactions (Hille et al. 1998; Schwarz et al. 2009) and shows promise for future isotopic work.

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