Metallomics

CRITICAL REVIEW

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Medical applications of Cu, Zn, and S isotope effects

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This review examines recent applications of stable copper, zinc and sulfur isotopes to medical cases and notably cancer. The distribution of the natural stable isotopes of a particular element among coexisting molecular species varies as a function of the bond strength, the ionic charge, and the coordination, and it also changes with kinetics. *Ab initio* calculations show that compounds in which a metal binds to oxygen- (sulfate, phosphate, lactate) and nitrogen-bearing moieties (histidine) favor heavy isotopes, whereas bonds with sulfur (cysteine, methionine) favor light isotopes. Oxidized cations (*e.g.*, Cu(II)) and low coordination numbers are expected to favor heavy isotopes relative to their reduced counterparts (Cu(I)) and high coordination numbers. Here we discuss the first observations of Cu, Zn, and S isotopic variations, three elements closely related along multiple biological pathways, with emphasis on serum samples of healthy volunteers and of cancer patients. It was found that heavy isotopes of Zn and to an even greater extent Cu are enriched in erythrocytes relative to serum, while the difference is small for sulfur. Isotopic variations related to age and sex are relatively small. The $^{65}Cu/^{63}Cu$ ratio in the serum of patients with colon, breast, and liver cancer is conspicuously low relative to healthy subjects. The characteristic time over which Cu isotopes may change with disease progression (a few weeks) is consistent with both the turnover time of the element and albumin half-life. A parallel effect on sulfur isotopes is detected in a few un-medicated patients. Copper in liver tumor tissue is isotopically heavy. In contrast, Zn in breast cancer tumors is isotopically lighter than in healthy breast tissue. $^{66}Zn/^{64}Zn$ is very similar in the serum of cancer patients and in controls. Possible reasons for Cu isotope variations may be related to the cytosolic storage of Cu lactate (Warburg effect), release of intracellular copper from cysteine clusters (metallothionein), or the hepatocellular and biosynthetic dysfunction of the liver. We suggest that Cu isotope metallomics will help evaluate the homeostasis of this element during patient treatment, notably by chelates and blockers of Cu trafficking, and understand the many biochemical pathways in which this element is essential.

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Introduction

For most people not involved in the fields of geochemistry and physics, whenever the word 'isotope' is heard or read, it calls attention to radioactive nuclides used for dating, such as carbon 14, and for medical applications such as cobalt 60. It is also suggestive of nutrition studies in which enriched stable isotopes are added to the diet of volunteers to monitor the transit of a particular element.[1] Stable-isotope probing (SIP) is a related technique used in microbial ecology.[2] All these techniques are invasive in the sense that they interfere with the normal metabolism, even if it is usually to a trivial extent. Natural fractionation of the stable isotopes involving major elements such as C, H, O, N, and S has found only rare medical applications.[3,4] Metals such as alkaline-earth Ca and Mg and transition elements such as Cu and Zn, however, are more promising because of their much smaller number of functional roles in biology and also because their turnover rate in the body is relatively short. Copper plays a major role in oxidizing iron and controlling electron fluxes, while Zn is a cofactor of hundreds of important enzymes.[5] Iron is involved in a large number of biological functions and, because of the very large stores contained in red blood cells, muscles and the liver, its overall turnover time is of several years.[6] The purpose of the present essay is to review some appealing applications of Cu,

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Zn, and S isotopes in medicine, notably their relevance to medical diagnostics and treatment follow-up.

Isotope variability is known as the isotope effect, a term describing the mass-dependent variations of natural isotope abundance of a particular element. The isotope effect is a consequence of the Heisenberg uncertainty principle on the distribution of energy levels of molecular vibrations. Quantum mechanics rules state that the velocity and the position of a particle cannot be simultaneously known with an infinite precision. Bonds never come to rest and their lowermost energy

Francis Albarede

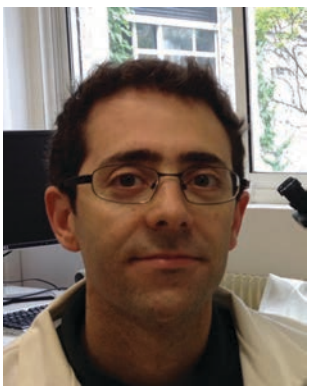
Francis Albarede, PhD, is an Emeritus Professor at the Ecole Normale Supérieure de Lyon, France. His primary field is isotope geochemistry with applications to Earth and Planetary Sciences. He was with Philippe Telouk one of the first to explore the potential of MC-ICP-MS in natural sciences, and over the last decade focused in particular on the theory and applications of metal isotope fractionation in biology.

Philippe Télouk

Philippe Telouk, PhD, is a CNRS research engineer. He built the INSU-ENS Lyon ICP-MS facilities in Lyon and led some transformative developments for the analysis of metal isotopes. Philippe Telouk initiated the technology transfer from geochemistry to biology and medicine in 2006 and his research on the applications of high precision measurements of metal isotopes as cancer biomarkers (breast, colorectal, liver…) led to fruitful collaborations with French medical cancer research centers (Centre Léon Bérard, Institut Gustave Roussy).

Vincent Balter

Vincent Balter, PhD, received his MD in geology from Paris VI University and his PhD in anthropology from Bordeaux I University. Since 2002, he has been a CNRS researcher, first at the Lyon I University, and subsequently at the Ecole Normale Supérieure de Lyon. His main current scientific interest focuses on the use of stable isotopes to highlight metal homeostasis under normal and pathological conditions.



Victor P. Bondanese

Victor P Bondanese, PhD, is a molecular biologist. After graduating in Biotechnology from the Polytechnic University of Marche in Italy, he obtained a PhD in Biomedical Sciences from the University of Southampton UK. Recently, working in the Lyon group, he has applied his interests in cell signalling and regulation of gene expression to understand the processes that regulate copper isotopic composition in human cells.

Emmanuelle Albalat

Emmanuelle Albalat, PhD, is a scientific and technical member of staff at the Ecole Normale Supérieure de Lyon. Her PhD was dedicated to the fractionation of the stable isotope of rare-earth elements in planetary material and to neutron capture by the lunar surface. Her current interest focuses on sulfur isotopes in particular on biological materials.

Philippe Oger

Phil Oger, PhD, is a CNRS senior researcher in Lyon. The research in his group is focused on the adaptation of archaea to environmental constraints, including high pressure, temperature and heavy metals, to understand the functioning of hydrothermal ecosystems today and at the origins of life, as well as to develop innovative industrial applications for metal and pollutant removal from the environment.

state is referred to as the zero-point energy. This energy depends on the mass M of the bonding atoms, a character that is at the origin of isotopic variability of elements between different parts of a system such as different biological compartments.

Otto Warburg and Adolf Krebs found in 1928[7] that serum copper levels increased in various chronic diseases and several types of cancers, resulting in systemic and oncogenic[8] copper accumulation. Anomalously high Cu levels or Cu/Zn ratios were indeed observed in the serum of breast cancer,[9,10] and serum ceruloplasmin (Cp) was found to be significantly elevated in advanced stages of solid malignant tumors.[11] In itself, such observations justify that copper isotopic variability should be investigated in cancer patients. Two Cu–Zn proteins, superoxide dismutase and metallothionein, are involved in the control of hypoxia and reactive oxygen species and therefore play a role in cancer development. Sulfur present in cysteine and methionine easily bonds with both Cu and Zn, while albumin, the major sulfur carrier in serum is a critical predictor of cancer survival[12] which justifies the importance of exploring the extent of $^{34}S/^{32}S$ variations in biological samples.[13]

In contrast to organic biomarkers, isotope compositions can be analyzed in biological samples years after the samples have been taken. Metal isotopes are immune to oxidation, as they are unreactive to any chemical or biological reactions taking place in the original sample container, even when exposed to the atmosphere. Here, we will review the variations in the abundance of stable isotopes of Cu and Zn, two metals tightly related in cellular and physiological activities, naturally present in the body of humans and other organisms. The very first Cu and Zn isotope data on blood[14–25] show promising relationships of isotope Cu and/or Zn compositions with age, sex, and pathologies. Iron isotopes will be left out as they have been mostly applied to the iron-related disease of genetic hemochromatosis.[26–29] Although isotopic data on organs will also be discussed, we will focus on serum for the reason of feasibility: it is a chemically stable liquid medium, much more available in contrast to biopsies and resections, even for healthy subjects, and which is commonly accessible from bio-banks. In order to assess the role of sulfur-rich amino acids and proteins and in particular the well-established connection between zinc and sulfur biochemistry through redox control,[30] we will also review some recent observations on the sulfur isotope composition of biological samples.[13,22] A review emphasizing the analytical techniques used for the analysis of metal isotopes was recently published by Costas-Rodríguez *et al.*[31]

Toshiyuki Fujii

Toshiyuki Fujii, PhD, is currently a Professor at the Graduate School of Engineering, Osaka University. He studied the mechanisms of isotope fractionation in chemical exchange reactions and identified the nuclear field shift effect as a factor of isotope fractionation for various elements. His ab initio calculations provided estimates of the conventional mass effect via molecular vibrations and of the nuclear field shift effect.

The isotope effect

Isotope fractionation is a general term referring to the variability in the isotopic abundance 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 $M^{-\frac{1}{2}}$, while bond energy E varies with vibrational frequency ν according to $E = (n + \frac{1}{2})\, h\nu$, where h is the Planck constant and n is a non-negative integer characterizing the energy 'level'. Favoring heavier isotopes in the lowermost energy levels is therefore 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.[32–36] 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 and when the overall binding energy at the site of the metal is shared among fewer partners. Bonds involving high oxidation states (Fe^{3+}, Cu^{2+}) and sites with small coordination numbers therefore prefer heavy to 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, a short for 'depleted' or 'enriched', respectively, in heavy isotopes, the effects always remain in the range of a few parts in one thousand that only modern mass spectrometry has been able to resolve.

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: kinetic effects have been advocated as a cause of biologically mediated isotope fractionation,[37] but they require either non-steady state conditions (the system grows) or the existence of competing reaction pathways (Fig. 1). What comes around goes around: the proportion of isotopes present within a system (cell, organ, body fluid) must vary if they are imported and exported at different rates. After a time exceeding the mean turnover in the system, the input and the output must be balanced. When a pathway involves multiple outputs for a single input, the abundance of the different isotopes of a specific element may not be identical in each branch: this is the nature of isotope fractionation.

Isotopic abundance is measured using mass spectrometers. Except for hydrogen, the isotope effect is normally very small, with variations of isotope abundance 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). Precision provided by inexpensive quadrupole mass spectrometers is insufficient with respect to

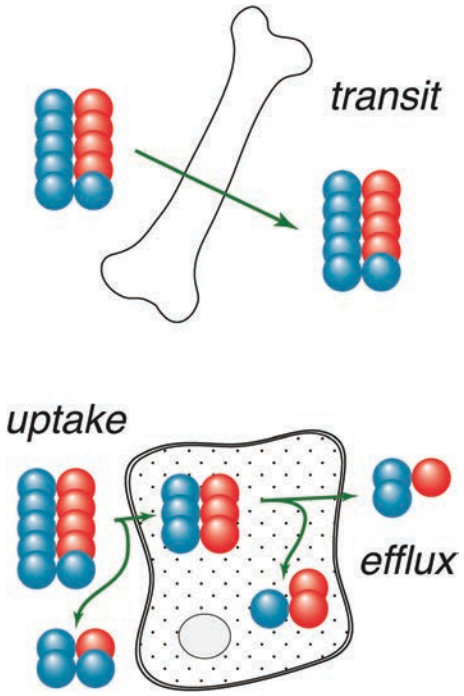


Fig. 1 Isotopes of the same element bound to a specific metalloprotein, *e.g.*, ^{63}Cu and ^{65}Cu, are depicted as blue and red spheres. Top: For transit at steady-state (no growth), the proportion of each isotope is preserved. The cell or organ is reduced to a single input and a single output. Chaperones are not expected to affect isotope compositions. Bottom: When the metal distributes itself between two coexisting channels, such as the extracellular medium and cytosol, different pathways allocate a different isotope abundance to each channel. Oxidation, biosynthesis, input, storage, and output are expected to result in isotope fractionation.

the natural range of isotopic variations. For decades, gas source (electron bombardment) mass-spectrometers have been used to obtain precise isotopic abundances of H, C, N, O, and S, commonly from molecular compounds such as CO_2 or SO_2. Mass fractionation in the mass spectrometer itself (mass bias) would be dealt with by swiftly alternating the standard material with the samples with calibrated inlet valves. Isotopic variations are reported on a relative scale, typically the delta scale, for instance for ^{65}Cu:

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

A gas source would in most cases be inefficient for metallic elements: short of an efficient technique to correct the data for the analytical bias introduced by mass spectrometry, the variations of metal isotope abundance have until lately remained largely unexplored. 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 only found limited applications.[38] In the mid 90 s, Multiple Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICP-MS) quickly emerged as a game changer as the technique based on very efficient ionization, high transmission, combined with sample-standard bracketing would allow unprecedented precision (typically 0.01–0.05 parts per 1000) on metal samples as small as a few tens of nanograms of metal. The major difficulty of this technique is that the metals to be analyzed represent only traces in organic material loaded with major elements such as Na, Cl, P, Mg, and Ca. Isotopic analyses of unprocessed samples by MC-ICP-MS are notoriously made more inaccurate by matrix effects. Trace metals have first to be rigorously purified by ion-exchange chromatography yet with a yield very close to 100 percent. More details on analytical procedures and limitations may be found in Costas-Rodríguez *et al.*[31]

Why take the trouble of measuring metal isotopic abundance, an excruciating and occasionally daunting task, instead of relying on the concentrations of the same metal in various parts of the body? The answer is that changes in copper or zinc concentrations are in general 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 one another along all biochemical pathways, the isotopes of a given element behave similarly enough that variations in their relative abundance remain predictable. Decades ago, experimental determination of isotope fractionation of an element between coexisting compounds were the method of choice, but the results are in general perceived as much less reliable than those obtained by the so-called *ab initio* or first-principles theories and in most cases represent a formidable analytical challenge. 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,[39] a computational quantum mechanical modeling providing the ground-state electronic structure of many-body systems. This method is used to obtain ratios of reduced partition functions of different molecules differing by the substitution of one isotope (isotopologues). It may be used to calculate both equilibrium and kinetic fractionation of isotopes. Each atom is considered as being made of a nucleus and of orbiting electrons. Typically, each calculation is divided into two steps, one in which atoms are confined in a box and let 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 is calculation intensive and requires special software and consistent databases.[34,40]

Large proteins are still beyond the reach of DFT, but efforts to predict isotope fractionation of elements such as Fe, Cu, Zn, Ni, and Ca have recently been made by a small number of groups.[40–45] 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 become available for Cu and Zn. As shown in Tables 1 and 2, the data are tabulated as ratios of reduced partition functions β (usually as 1000 ln β) and

Table 1 Partition function ratios β for $^{66}Zn/^{64}Zn$ and $^{65}Cu/^{63}Cu$ in molecular species relevant to medical studies (T = 310 K). Isotopic fractionation α between two coexisting species 1 and 2 can be computed as $\alpha^{1\text{-}2} = \ln\beta_1 - \ln\beta_2$

Species	$\ln\beta_{Zn}$		Species	$\ln\beta_{Cu}$	
$ZnHPO_4(H_2O)_5$	3.309	1	Cu(I)L	1.725	5
$ZnH_3(PO_4)_2(H_2O)_4^-$	3.967	1	$Cu(I)(H_2O)_2^+$	2.667	4
$Zn_2H_2(PO_4)_2(H_2O)_4$	4.072	1	Cu(II)H(L-ascorbate)$(H_2O)_4^+$	3.087	4
Four-fold			Cu(II)H(D-ascorbate)$(H_2O)_4^+$	3.139	4
$Zn(Cys)(H_2O)_3^{2+}$	3.072	2	$Cu(II)H_3(PO_4)_2(H_2O)_3^-$	4.176	2
$Zn(Glu)(H_2O)_2^{2+}$	3.524	2	$Cu(II)H_2PO_4(H_2O)_4^+$	4.355	2
$Zn(H_2O)_4^{2+}$	3.577	2	$Cu(II)H_4(PO_4)_2(H_2O)_3$	4.382	2
$Zn(His)(H_2O)_3^{2+}$	3.647	2	$Cu(II)Ox(H_2O)_2$	4.931	4
$Zn(Met)(H_2O)_3^{2+}$	3.66	2	$CuH_2(PO_4)_2(H_2O)_2^{2-}$	5.024	2
$Zn(His)(H_2O)_2^{2+}$	3.673	2			
$Zn(Thr)(H_2O)_3^{2+}$	3.767	2	$Cu(II)(Cys)(H_2O)_4^{2+}$	3.124	2
			$Cu(II)(Met)(H_2O)_4^{2+}$	3.650	2
Six-fold			Cu(II)(GS)H0	3.892	2
$Zn(Cys)(H_2O)_5^{2+}$	2.504	2	$Cu(II)(Thr)(H_2O)_4^{2+}$	4.110	2
$Zn(Met)(H_2O)_5^{2+}$	2.734	2	$Cu(II)(Glu)(H_2O)_3^{2+}$	4.117	2
$Zn(His)(H_2O)_4^{2+}$	2.777	2	$Cu(II)(His)(H_2O)_3^{2+}$	4.148	2
$Zn(His)(H_2O)_5^{2+}$	2.921	2	$Cu(II)(His)(H_2O)_4^{2+}$	4.168	2
$Zn(H_2O)_6^{2+}$	3.026	2	$Cu(II)(H_2O)_5^{2+}$	4.220	2
$Zn(Glu)(H_2O)_4^{2+}$	3.053	2	$Cu(II)L\text{-}Lact(H_2O)_3^+$	4.359	2
$Zn(Thr)(H_2O)_5^{2+}$	3.075	2	$Cu(II)L\text{-}LactH_{-1}(H_2O)_2$	4.969	5
			$Cu(II)L\text{-}Lact_2$	5.616	5
			Cu(II)L-Lact D-Lact	5.627	6
Anhydrous					
$[Zn\text{-}Cys\text{-}H_{-1}]^+$	1.108	3			
$[Zn\text{-}Cys]^{2+}$	1.211	3			
$[Zn\text{-}Glu\text{-}H_{-1}]^+$	1.517	3			
$[Zn\text{-}His]^{2+}$	3.336	3			
$[Zn\text{-}His\text{-}H_{-1}]^+$	3.465	3			

1 – ref. 42; 2 – ref. 40; 3 – ref. 44; 4 – ref. 107; 5 – T. Fujii (this work); 6 – ref. 23.

the order and amplitude of isotopic enrichment between two compounds 1 and 2 at equilibrium can be estimated as $\ln\beta_1 - \ln\beta_2$. For example, the predicted $^{65}Cu/^{63}Cu$ ratio 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.

Some robust trends appear for Zn and Cu, two elements for which fractionation by amino acids and other organic ligands has been best studied:

[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, and 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).

Table 3 Stability constants for the successive chelates of Cu and Zn by relevant carboxylates. Note the potentially confusing use of β for both partition function ratios and cumulative association constants

	Species	$\log\beta_1$	$\log\beta_2$	$\log\beta_3$	
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

1 – ref. 108; 2 – ref. 109.

[4] As demonstrated for zinc by the comparison between four- and sixfold coordination, preference for heavier isotopes decreases with increasing coordination numbers.

Understanding how these results relate to large proteins should certainly attract attention in the future.

An overview of zinc, copper, and sulfur biochemistry and homeostasis

We will first take an introductory tour of the biochemistry of the two important metals Zn and Cu and then summarize a few important facts about sulfur-containing amino acids and proteins. The homeostasis of each element is depicted for a 'generalized' cell in the three panels of Fig. 2.

Zinc

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.[46] It is found in the nucleus and the cytosol of cells in all organs. About 90 percent of Zn in blood is accounted for by erythrocytes.[18] Excess cytosolic Zn is bound to metallothionein, a short sulfur-rich protein, and then transported to the 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 regulates the glutathione metabolism and metallothionein expression.[47] Zinc affects the signaling pathways and the activity of transcription factors with zinc finger domains.

Zinc homeostasis and its importance in various pathologies have been multiply reviewed.[30,48–51] Malnutrition induces cell mediated immune defects and promotes infections.[52] Zinc acts on the immune system by potentiating cytokines,[53] a mechanism that may also be controlling chronic inflammation, such as for rheumatoid arthritis.[54] Zinc in seminal fluid has been suggested as a biomarker of prostate cancer.[55] Serum albumin is the main carrier of Zn in serum. For a 'generalized' cell, the

Table 2 Equilibrium $^{34}S/^{32}S$ enrichment in ‰ of different sulfur-bearing inorganic and organic species at 298 K.[13] The calculations include the effect of one hydrate shell on SO_4^{2-}. $^{34}S/^{32}S$ fractionation α between two species may be obtained in ‰ and that between two coexisting species 1 and 2 can be computed as $\alpha^{1\text{-}2} = \ln\beta_1 - \ln\beta_2$

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

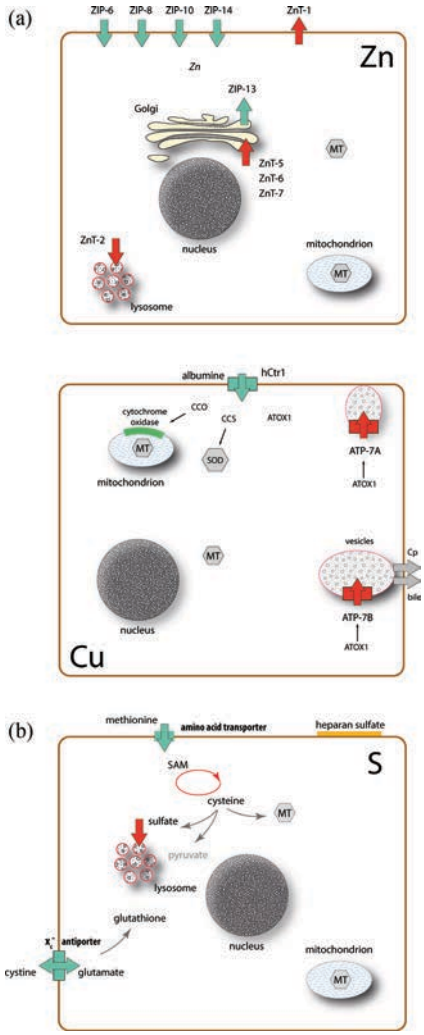


Fig. 2 A sketch of Zn, Cu, and S trafficking in a generalized cell. Abbreviations: Zn panel: Zn transporter family (ZnTx), Zn transporter ZIP family (ZIPx), metallothionein (MT), Cu, Zn-superoxide dismutase 1 (SOD1). Zn panel: human copper transporter (hCtr1), cytochrome *c* oxidase copper chaperone (CCO), copper chaperone for superoxide dismutase (CCS), antioxidant protein 1 chaperone to the copper ATPases ATP7A and ATP7B (ATOX1).

transmembrane importers consist of 14 isoforms of the ZIP family (ZIP1 to ZIP14). Different ZIP transporters are expressed specifically on different cell types.[51] DMT1 has a lower affinity for Zn.[56,57] No specific chaperone has been identified for the transfer from cytoplasm to organelles, although to some extent metallothionein may be considered one. Zn efflux from the cell and Zn storage in organelles is controlled by the ZnT protein family, which consists of 10 isoforms (ZnT1 to ZnT10). The transmembrane 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.

Copper

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.[46] About 35 percent blood copper is accounted for by erythrocytes.[18] Copper is a micronutrient and a catalytic and structural cofactor of many important enzymes involved in tumor development.[58–63] Serum ceruloplasmin is a ferroxidase enzyme synthesized 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 the cells is hCtr1 (human copper transporter),[64–66] which binds to albumin[67] and binds both Cu(I) and Cu(II).[68] Hypoxia-induced DMT1 (divalent metal transporter, notably ferrous iron) has also been invoked in copper transport into mice intestinal cells,[69,70] 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.[62,71] ATP7A and ATP7B differ in their pattern of tissue expression and cellular localization.

Sulfur

The sulfur content of the human body is about 175 g.[46] Most body sulfur is held in 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).[72–74] Instead, cysteine can be synthesized from methionine within the cell through the *trans*-sulfuration pathway involving methylation by *S*-adenosyl methionine (SAM). Metal binding metallothioneins are rich in cysteine, accounting for up to one third of the amino-acid sequence.[75,76]

An essential property of cysteine is the potential of two molecules to bind to cystine by forming a covalent disulfide S–S bridge, which may open 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 for the control of the cellular redox state by easily switching between its reduced (GSH) and oxidized (GSSG) forms. Glutathione is synthesized from cystine imported from the extracellular medium in exchange of glutamate by the x_c^- 'antiporter'.[77]

Cytosolic cysteine is catabolized into pyruvate, which is used for energy production, and sulfate.

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.

Isotope compositions of Zn–Cu–S in the blood of healthy individuals

Copper and zinc contents vary in the serum of control individuals in a remarkable way. Fig. 3 shows that the Cu content is high and variable among women,[78] whereas Zn tends to be constant. In contrast, the serum of men tends to have a narrow range of Cu and variable Cu/Zn concentrations. The range of overlapping values is, however, relatively large. Although copper is a commonly used biomarker to assess health status, the Zn/Cu ratio seems to have an even stronger potential.[23,79] Prostate cancer seems 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 describes 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 Cu and Zn (and Fe) vary among the organs and body fluids of a mammal was essentially unknown until the first investigations by Balter *et al.*[80,81] and Moynier *et al.*[44] of sheep and mouse. For ethical reasons, access to such a variety of human materials 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. 4). In mice, Zn is isotopically heavy in red blood cells and bone and light in the serum, liver, and brain and is not dependent on the genetic background. Copper is specifically heavy in kidneys. This pattern reproduces for sheep except for the isotopically light Zn in blood, a feature that still awaits elucidation. Buechl *et al.*[82] analyzed Zn and Cu isotopes in the brain of wild type and knockout mice and demonstrated that Zn isotopes in brain tissues are sensitive to prion-related local damage.

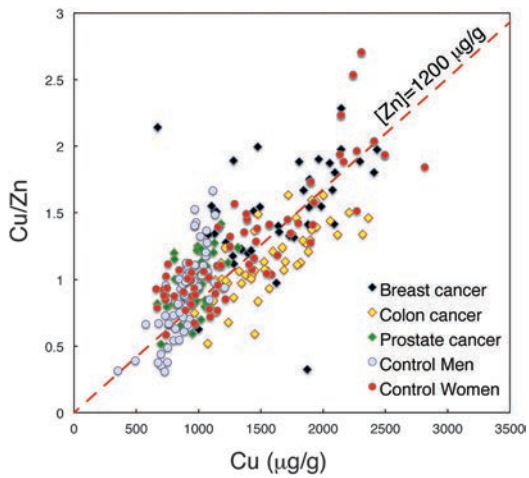


Fig. 3 Cu and Cu/Zn in the serum as indicators of cancer status. The control group[18] shows strong correlations, reflecting the tight regulation of Zn concentration in the body (note that x/y = Zn). The trends for control men and women are different, with men having, on average less Cu than women. Copper remains stable in prostate cancer patients (unpublished data, Lyon) relative to control men, but increases in colon cancer patients.[23] Zinc in the serum of breast cancer patients[23] is low and Cu is probably high relative to healthy subjects.

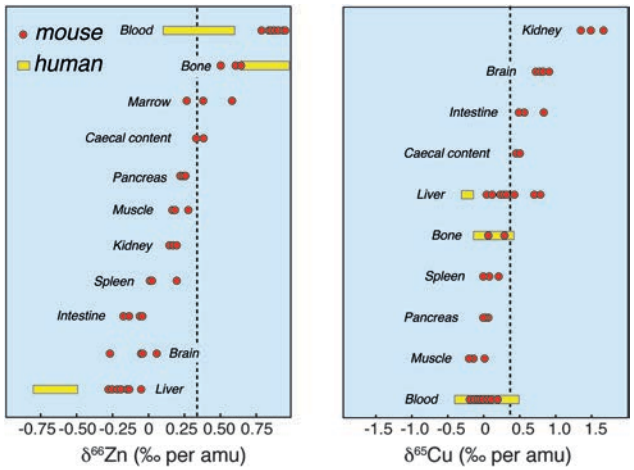


Fig. 4 Zinc and copper isotope variability among organs, bones, body fluids, and intestinal content of mice reported in delta units per mil.[80] In orange is shown the range of variations for humans.[16–18,26,28,104] Typical uncertainties are ±0.05‰ (2-sigma error).

Albarede *et al.*[18] 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, Zn and Cu are isotopically lighter in erythrocytes (red blood cells or RBC) relative to serum by ~0.3 and ~0.8‰, respectively. δ^{66}Zn values are identical in serum and RBC of both men and women as are δ^{65}Cu in serum. In contrast, Cu is isotopically heavier in men RBCs. The study found mean values of δ^{66}Zn ~ +0.17‰ and δ^{65}Cu ~ −0.26 ± 0.40‰ for serum and δ^{66}Zn ~ +0.44 ± 0.26‰

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.[110] Typical analytical uncertainties are 0.05 ‰. Men–women comparison: p is the probability that the two sets are not identical

	n	Avg. δ^{66}Zn	2s	Avg. 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	

and δ^{65}Cu ~ +0.66‰ for erythrocytes. A similar δ^{65}Cu value 0.29 ± 0.27‰ was obtained by Costas-Rodríguez *et al.*[24] on 29 serum samples. The serum–RBC difference is most significant for Cu. δ^{65}Cu is 0.2‰ heavier in the erythrocytes of men relative to women.[18,21] The erythrocyte count (hematocrit) is slightly higher for men relative to women, and the extent of δ^{65}Cu and δ^{66}Zn variation is unlikely to be large (<0.1‰), except in the case of severe anemia.

In a study of whole-blood samples on Yakut volunteers aged 18–74, Jaouen *et al.*[20] found that the ^{66}Zn/^{64}Zn ratio increases and the ^{65}Cu/^{63}Cu ratio decreases with age. Van Heghe *et al.*[21] observed that the ^{65}Cu/^{63}Cu ratio and δ^{65}Cu values in women after menopause become more similar to the values in men and concluded that the difference in the isotopic composition of Cu between whole blood from males and females is accounted for by menstruation.

Comparison of their results with Albarede *et al.*'s[18] study led Jaouen *et al.*[20] to emphasize the importance of the ethnic factor. On a small sample set, Van Hegue *et al.*[83] observed that δ^{66}Zn in whole blood is about 0.15‰ higher for vegetarians relative to omnivorous volunteers, but the outcome for Cu isotopes was less conclusive.

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.*[22] and obtained an average value $\delta^{34}S_{V\text{-}CDT}$ of 5.9 ± 1.5‰ on 11 serum samples and of 5.1 ± 1.9‰ on 20 RBC samples. On 25 serum samples of adults, Albalat *et al.*[13] obtained a very similar mean value but within a reduced interval of 6.0 ± 0.7‰, with the average $\delta^{34}S_{V\text{-}CDT}$ of women being 0.2‰ lower relative to men. On the same samples, both methods agree within one permil, with serum sulfur being a fraction of permil heavier than RBC. Albalat *et al.*[13] showed that S in children serum is only slightly heavier but more scattered (6.3 ± 1.0‰) relative to adults.

Isotope compositions of Zn, Cu, and S in cancer

Telouk *et al.*[23] measured the ^{65}Cu/^{63}Cu ratios in the serum of 20 breast and 8 colorectal cancer patients. Samples were taken at different times during the treatment, and amount to, respectively, 90 and 49 samples taken. 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, the abundance of Cu isotopes predicts mortality in the colorectal cancer group with an error probability p = 0.018 (Fig. 5). 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 δ^{65}Cu less than the threshold value of −0.35‰ (per mil) did not survive beyond a few months (Fig. 6). As a marker, a drop in δ^{65}Cu precedes molecular biomarkers such as CEA (carcinoembryonic antigen) and CA15.3 (carbohydrate antigen 15.3) by several months (Fig. 7), which is consistent

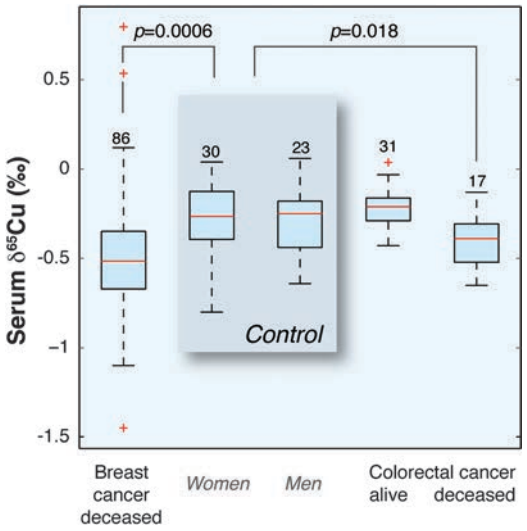


Fig. 5 Whisker plots of serum δ^{65}Cu values for healthy men and women compared to breast cancer and colorectal cancer patients.[23] Boxes represent the 75 percent middle quantiles and the whiskers 95 percent quantiles. Red lines: median; red crosses: outliers. Separation between breast cancer patients and healthy women is strong. Separation between breast cancer and colorectal cancer patients and healthy men and women seems to depend on mortality (reproduced from ref. 23 with permission from the Royal Society of Chemistry).

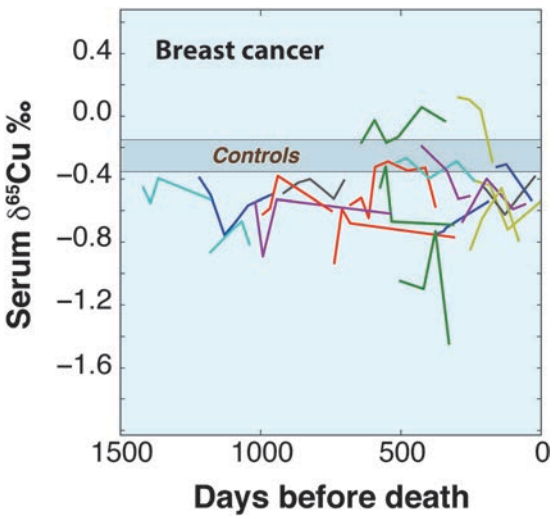


Fig. 6 Evolution of serum δ^{65}Cu for 20 breast cancer cases up to patient death.[23] Each line represents a different patient with color used for the differentiation purpose. The shaded band is the 75 percent confidence limit for the serum of control women (reproduced from ref. 23 with permission from the Royal Society of Chemistry).

with Cu turnover time in the body. The observed decrease of δ^{65}Cu in the serum of cancer patients was assigned 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 therefore strong.

So far, the number of published Zn isotope data is small, mostly because variations are limited. Larner *et al.*[25] found that the serum of five breast cancer patients and five healthy donors

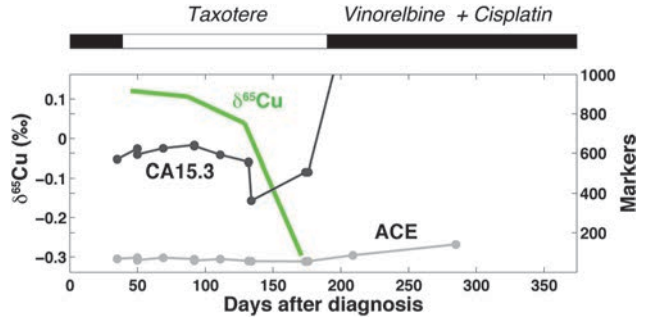


Fig. 7 Early alarm by δ^{65}Cu.[23] The plot compares the δ^{65}Cu values (left axis, green line) and the molecular biomarkers (right axis): CEA (carcinoembryonic antigen) and CA 15.3 (carbohydrate antigens). The top bar scale shows the successive therapies received by the patient. The copper isotope signal precedes the other markers by 2–3 months (reproduced from ref. 23 with permission from the Royal Society of Chemistry).

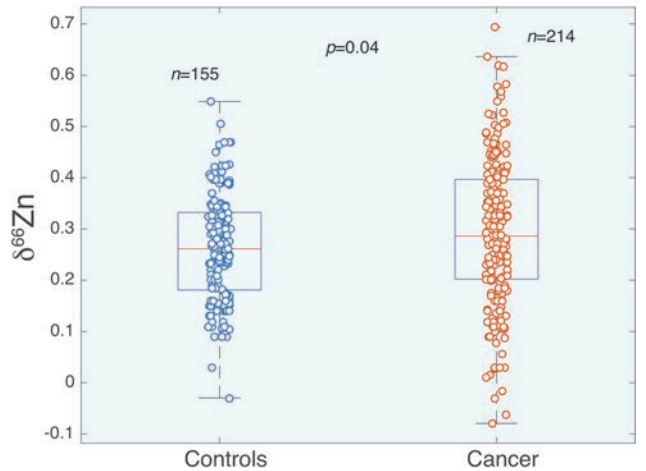


Fig. 8 Zinc isotope fractionation in the serum of cancer patients is small. This rather large δ^{66}Zn dataset consists of control serum samples including those reported by Albarede *et al.*[18] and samples from breast and colon cancer patients[23] and unpublished data on prostate cancer patients. In spite of an increased spread of δ^{66}Zn relative to controls, its overall prognostic value for cancer in general is so far limited.

cannot be differentiated. This is confirmed by a larger δ^{66}Zn data set (Fig. 8) of 155 control serum samples of adult donors, including those reported by Albarede *et al.*[18] and 214 serum samples from breast and colon cancer patients[23] and unpublished data on prostate cancer patients. The relatively large p value (p = 0.04) reflects the broader dispersion of the cancer patient data relative to controls. In contrast, Larner *et al.*[25] found that δ^{66}Zn in five tumor resections of breast cancer patients has a significantly lighter Zn isotopic composition than the serum and healthy breast tissue. 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. The Zn isotope signal is conspicuous, but the preliminary character of the study calls for confirmation on a larger dataset.

Balter *et al.*[22] found that in hepatocellular carcinoma patients, serum and erythrocyte copper and sulfur are both enriched in light isotopes relative to controls (Fig. 9, left). The magnitude of

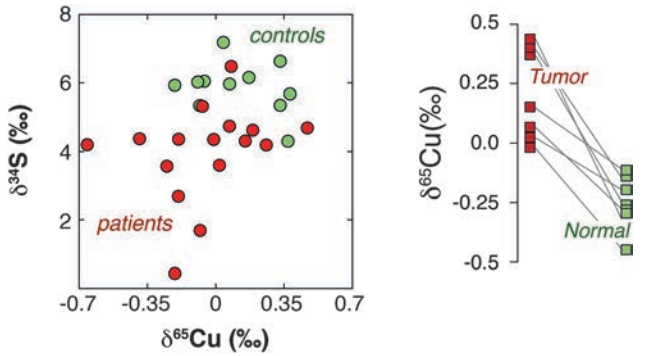


Fig. 9 Left: Isotopically light copper and sulfur in the serum of hepatocellular carcinoma patients relative to controls (reproduced from ref. 22 with permission from the Royal Society of Chemistry). Right: Isotopically heavy copper in tumor liver tissue relative to normal tissue. The opposite direction of the changes in Cu isotope abundance in serum and tumor may be explained in different ways.

the sulfur isotope effect is similar in red blood cells and the serum of hepatocellular carcinoma patients, implying that sulfur fractionation is systemic. In contrast to serum data, the δ^{65}Cu of tumor resections is notably higher relative to healthy liver tissue (Fig. 9, right). The agreement between sulfur isotope data acquired on the same samples by EA-IRMS and MC-ICP-MS by Albalat *et al.*[13] is reasonably good. Balter *et al.*[22] 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 related study by Costas-Rodríguez *et al.*[24] found lower δ^{65}Cu in the serum of patients with end-stage liver disease, with complications such as ascites, encephalopathy, and hepatocellular carcinoma (Fig. 10). These authors pointed out that δ^{65}Cu was positively correlated with the liver cirrhosis-related parameters, notably aspartate aminotransferase, INR (International Normalized Ratio for

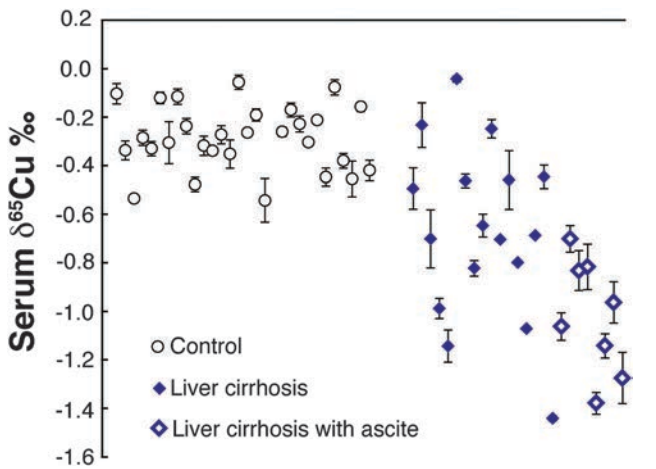


Fig. 10 δ^{65}Cu values in the serum of liver cirrhosis patients with and without accumulation of fluid in the peritoneal cavity (ascites) relative to controls.[24] Ascites is often associated with cirrhosis and metastatic cancer (reproduced from ref. 24 with permission from the Royal Society of Chemistry).

prothrombin time), bilirubin and C-reactive protein, and inversely correlated with albumin and Na. They also found a negative correlation of δ^{65}Cu with the Child-Pugh score based on albumin, bilirubin, and INR and the Mayo Clinic Model for End-stage Liver Disease score (MELD) based on creatinin, bilirubin, and INR.

Albalat *et al.*[13] analyzed sulfur isotopes in a large number of pathological samples with emphasis on serum. These serum samples deviated by a much smaller S concentration from those of healthy volunteers, which echoed the negative correlation between low serum albumin content.[84,85] The samples, however, for which the δ^{34}S deviated from the range of healthy individuals were very few and corresponded to the 'naive' (untreated) patients, in particular those analyzed by Balter *et al.*[22] Cancer and 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.

Discussion

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. Out of the three elements, Cu and Zn seem to show a deviation of tumors from healthy tissue (heavy Cu in liver and light Zn in breast neoplastic tissue).[22,25] In contrast to Cu, which is definitely isotopically lighter in the serum of well over 130 cancer patients relative to a similar number of controls (colon, breast, liver),[22,23] Zn isotope data show little difference between cancer patients and healthy donors of any age. Likewise, it was shown 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,[13,22] but that the spread of δ^{34}S values is smaller for controls.

Both Telouk *et al.*[23] and Costas-Rodríguez *et al.*[24] suggested that low δ^{65}Cu can be used for prognosis in end-stage cancer (liver, colon, breast). Copper isotopes would definitely complement other markers, such as the Child-Pugh score, albumin or transaminases. The ~6 week turnover time (ref. 59 and 86) is close enough to the 19 days of albumin[87] that the two parameters may have some biochemical pathways in common, one of them being that albumin is a Cu transporter. Telouk *et al.*[23] 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.

Different interpretations of δ^{65}Cu variations have been suggested:

(1) Telouk *et al.*[23] appealed to the cytosolic storage of isotopically heavy Cu chelated by lactate, which cancer cells are known to produce massively.

(2) Balter *et al.*[22] suggested that the low δ^{65}Cu value of the serum could be explained by the release of intracellular copper from cysteine clusters, with MT being the most likely source.

(3) Costas-Rodríguez *et al.*[24] suggest that low δ^{65}Cu values reveal the hepatocellular and biosynthetic dysfunction of the liver, synergistically with inflammation and water retention.

Isotope abundance adds 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 that would include blood components, healthy tissues, and tumor, simply because the data are not available. Liver accounts for a large fraction of body 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 some 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.[8] 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 citric acid cycle and oxidative phosphorylation. In tissues in which anaerobic condition results from reduced access to blood flow, pyruvate is reduced to L-lactate. This is lactic acid fermentation. 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.[88,89] Lactate efflux from the cell is regulated by monocarboxylate transporters (MCT) and intracellular and extracellular lactate levels are not simply related.[90,91] Copper(II) is isotopically heavy in both pyruvate and lactate relative to Cu(I) (Table 1), but Cu(II) lactate is a particularly stable compound (Table 3). However, in healthy cells pyruvate is shuttled into mitochondria for further energy processing, whereas free 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. 11), whereas pyruvate is not. This is the substance of Telouk *et al.*'s[23] explanation for the accumulation of copper with high δ^{65}Cu in the cell.

It is clear from Table 1 that a major parameter of isotope fractionation is bidirectional conversion between Cu(I) and Cu(II), which raises the question of the redox conditions both within the cell and in the extracellular medium. 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.[92] Several angiogenic factors, notably VEGF, tumor necrosis factor alpha (TNF-α) and interleukin (IL1), are copper activated.[93] The copper-dependent Memo redox protein plays an essential role in breast-cancer metastasis.[94]

Copper transport and uptake are still poorly understood,[62] as is the mechanism of Cu reduction during uptake. Fractionation upon storage or efflux is unlikely, as it would lead to an open-

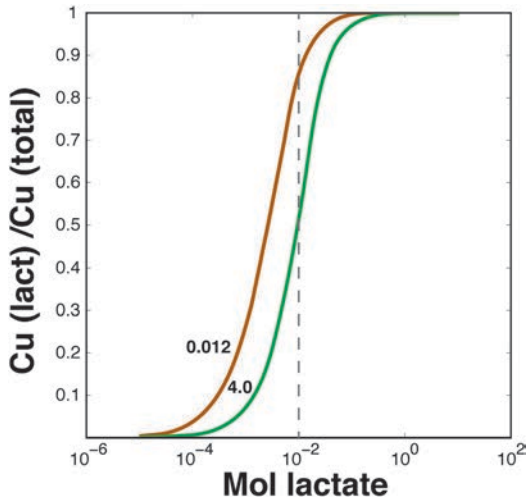


Fig. 11 Extent of copper chelation by lactates in the cytosol (reproduced from ref. 23 with permission from the Royal Society of Chemistry). The numbers on the curves represent the Cu^{+}/Cu^{2+} ratio for a redox potential of 0.153 V (copper ions) and for a body potential of 0.27 V.[105] The vertical dashed line corresponds to a lactate concentration of 10 mMol typical of tumor cells.[106]

ended shift in intracellular δ^{65}Cu. Albumin appears as the main serum carrier presenting Cu to the cell and binds both Cu(I) and Cu(II).[67,68] Although Cu^{+} and Cu^{2+} bound to albumin are likely to be isotopically very different, it must be the selective transmembrane uptake of Cu^{+} by Ctr1 which ensures Cu isotope fractionation between cells and the extracellular medium. Hypoxic stimulation of the HepG2 cells (hepatocarcinoma) leads to a down-regulation of albumin,[95] which does support a connection between liver, copper, and albumin.[24] Clearly, Cu isotopes may help understand the connections between tumor growth and Cu homeostasis.

Perspectives

So far, of all the elements discussed here, Cu has provided the strongest signal associated with a number of diseases and in particular with 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 what part of the δ^{65}Cu signal is due to cancer itself, and what is due to other factors, such as age and, even more, to inflammation. Our preliminary studies of athletes and of patients with purely inflammatory diseases, such as rheumatoid arthritis, suggest that they can document the specific effect of inflammation.

Reduction of the copper or ceruloplasmin level by chelates, without causing clinical copper deficiency, was proposed for therapeutic purposes. Specific copper chelators, such as tetrathiomolybdate, D-penicillamine and TPEN,[96–101] have been shown to be potent antiangiogenic and antimetastatic compounds possibly through suppression of the NFκB signaling cascade. Recently, Cu-chelation therapy has been proposed as a treatment for the broad spectrum of cancers containing the $BRAF^{V600E}$ mutation.[102] Inhibition of copper Atox1 trafficking has also been investigated.[103] 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.

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