Copper isotope ratios in serum do not track cancerous tumor evolution, but organ failure

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Abstract

Relative to healthy controls, lighter copper isotopic compositions have been observed in the serum of breast cancer and end-stage liver disease patients, raising the possibility that Cu isotope ratios could be used as a tracer for disease progression. Here, we assess the potential of natural Cu isotopic variations (expressed as δ^{65}Cu) as diagnostic tools for cancer progression and/or liver failure by performing a first-order analysis of Cu isotopic cycling in the human body. Using a box model, we simulate the kinetics of Cu mass transfer throughout significant reservoirs in the body, allowing isotopic fractionation to occur during Cu uptake/release from these reservoirs. With this model, we determine under which conditions the serum δ^{65}Cu values would reflect perturbation related to cancer growth and/or liver failure at a level resolvable with modern mass spectrometry. We find that tumor growth alone is unable to explain the light isotopic signature observed in serum. Instead, we find that metabolic changes to the liver function resulting in a ∼1‰ isotope fractionation during Cu uptake from the blood into the liver can readily explain the long-term serum isotopic shift of ∼0.2‰ observed in cancer patients. A similar fractionation (∼1.3‰) during Cu uptake into the liver also readily explains the −1.2‰ shift observed in the serum of cirrhosis patients with ascites, suggesting a potentially common driver of isotopic fractionation in both cases. Using this model, we then test hypotheses put forward by previous studies and begin to probe the mechanisms behind the measured isotopic compositions.

Keywords: copper, isotopes, box model, cancer, liver cirrhosis, diagnostics

Graphical abstract
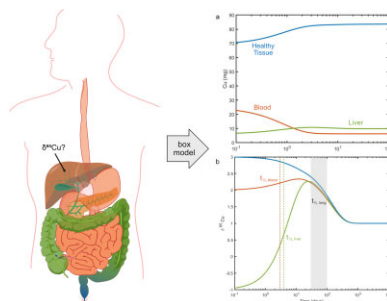
Copper (Cu) isotopic compositions have been observed to be lighter in the serum of individuals with certain cancers and liver disease relative to those of healthy controls. We utilize box modeling of the human body to quantitatively assess the conditions under which serum copper isotopic ratios would reflect disease-related perturbations. Using several test cases, we provide recommendations on the potential for Cu isotope ratios to act as a diagnostic tool.

Introduction

Stable isotopes are formidable tracers of physico-chemical processes involving mass transfer at all scales, capable of unraveling the details of galactic chemical evolution, planetary differentiation, human migrations, or mineral crystallization.[1] In the late 1990s, the advent of the Multi-Collector Inductively Coupled Plasma Mass Spectrometer (MC-ICP-MS) finally made possible high-precision isotopic characterization of metabolically critical transition metals (e.g. Fe, Cu, and Zn) in blood, serum, and other medically relevant samples.[2] Pioneering studies in this still-emerging field, named *isotope metallomics*,[3,4] have since documented significant isotopic variability in both healthy patients and patients with diseases affecting organs in which transition metals play a fundamental metabolic role (e.g. breast cancer/liver disease/Alzheimer's for Cu,[5–14] hereditary hemochromatosis for Fe,[15,16] breast cancer/Alzheimer's for Zn,[13,17,18] osteoporosis for Ca).[19–26]

Copper, in particular, has been the focus of sustained attention. Indeed, the average copper isotope composition (^{65}Cu/^{63}Cu ratio, hereafter expressed in permil notation as δ^{65}Cu) was found to be
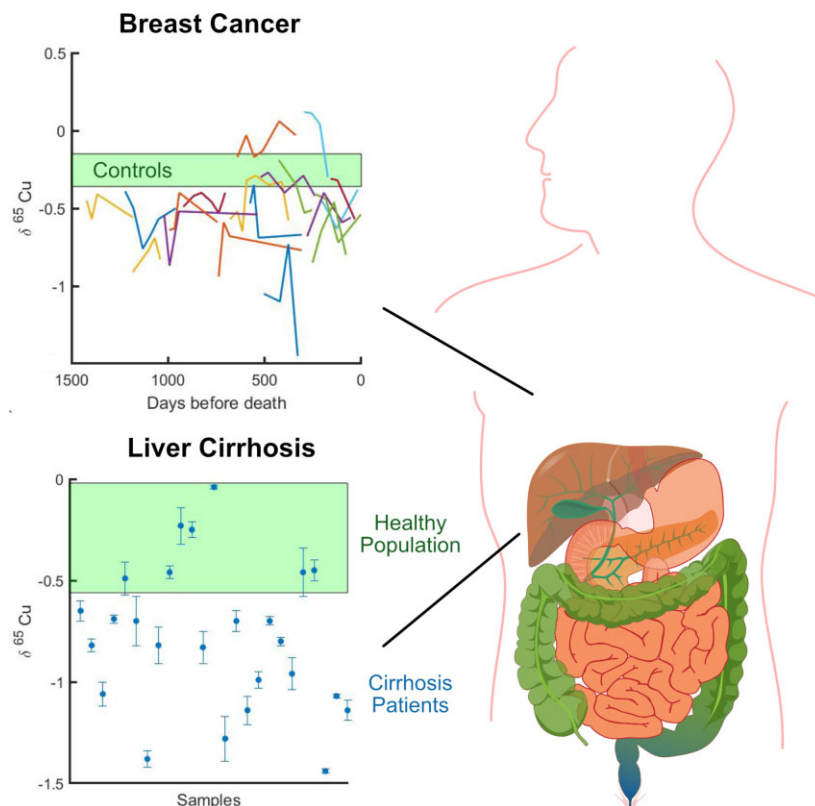
Fig. 1 Serum copper isotopic compositions in the body. Existing mapping studies have shown consistently lower Cu isotopic compositions in the serum of breast cancer (top) and liver cirrhosis (bottom) patients relative to healthy controls. Breast cancer serum Cu isotopic values are replotted from Télouk et al.[5] Liver cirrhosis serum Cu isotopic values are from Costas-Rodríguez et al.[7]

significantly lower in the serum of breast cancer patients relative to healthy controls.[5] In hepatocellular carcinoma (HCC) patients, red blood cells have been shown to exhibit lower $\delta^{65}Cu$ values relative to those of healthy patients, while cancer tissue displays $\delta^{65}Cu$ values ∼0.5‰ higher than surrounding healthy tissue.[6] Furthermore, end-stage liver disease patients have serum Cu isotopic compositions that span a larger range of values compared to healthy controls, and are on average lower by ∼0.6‰.[7] These initial results led to optimism that such signatures could be introduced as a novel method of diagnosing or characterizing their respective diseases (Fig. 1).[5–7] In recent years, additional studies have revealed a more complex picture, where both the mechanisms behind these isotopic variations and their sensitivity as potential biomarkers have been the subject of debate.[8–12,14] For instance, Bondanese et al.[8] have proposed that hypoxic tumor environments can explain the observed Cu isotopic fractionation in HCC, bringing to light the potential broader impacts of oxidative stress in fractionation. Building on these results, Telouk et al.[14] have proposed that the bimodal nature of Cu isotopic shifts in the serum of liver disease patients reflects a switch in resistance of hepatic tissues to oxidative stress, in turn leading to development of HCC. These studies warrant exploration of the relative magnitudes of impact that various factors can have on a patient's serum Cu isotope composition.

In light of these findings, this study seeks to build a quantitative framework to assess the potential for Cu isotope ratios to act as a diagnostic tool for disease onset and/or evolution and to probe the mechanisms behind the serum Cu isotope variations observed in humans. To determine the feasibility of using Cu isotope ratios as a biomarker, we must first ask the question, '*what makes an ideal*

medical diagnostic tool?' It should satisfy the following general criteria to act as a pragmatic, competitive option:

(1) Samples may be collected with a simple procedure.
(2) Sample processing and measurement methods should be consistent and efficient.
(3) Signatures must be resolvable by the instrument of choice, and
(4) Signatures should appear and respond rapidly to the onset or evolution of the disease.

Point (1) is easily addressed, as much of the existing Cu isotope data primarily comes from serum samples, which are collected through routine wellness check procedures. With regard to Point (2), chemical processing methods for high-yield Cu separation and measurement from samples are well documented in the literature and may be applied.[2,27–37] A current limitation, however, is that existing methods tend to require experienced users and are most commonly applied to small sample batches. Because many of the methods were designed for applications in research, they are currently not scalable to the needs of the medical field. As a result, they must be streamlined to focus on higher throughput for efficiency before this point is fulfilled. Points (3) and (4) are closely linked and are the focus of this study; given the difference in median serum Cu isotopic composition of healthy individuals relative to those with cancer or liver cirrhosis, the key is to determine how early the signature surpasses the instrument resolution threshold, and whether this shift occurs early enough in the disease progression to be a useful diagnostic tool. At this writing, the typical analytical uncertainty of $\delta^{65}Cu$ using state-of-the-art methods is approximately ±0.05‰, so the smallest measurable

deviation from a control is ∼0.1‰. We will primarily focus on exploring the possibility of signature detection given the restrictions of this current framework. In later sections, we also consider possible changes in these conclusions with future technological improvement.

A challenge to any quantitative assessment of the potential for Cu isotope ratios to act as tracers of disease is the limited knowledge of the mechanisms driving Cu isotope fractionation in the human body. Performing a first-order analysis of the system, however, circumvents the need to account for all the individual molecular mechanisms while still providing quantitative results for interpretation. This method is well established and has been used successfully by others in the metallomics field to better understand trace metal isotopic systems, such as zinc in the human body.[38] Here, we thus quantitatively model the human body to explore some of the unanswered questions regarding trends seen in previous Cu isotope studies, such as:

(i) When can Cu isotopic fractionation due to tumor growth alone in an otherwise healthy body create measurable serum isotopic differences?

(ii) When does complete liver failure (e.g. from HCC) cause measurable Cu isotopic differences in serum?

(iii) How may the isotopic fractionation of tissue affected by metabolic changes linked to the progression of cancer or liver disease (e.g. oxidative stress) be reflected in serum Cu composition?

Copper in the body

Copper is an essential trace element and the third most abundant transition metal in the human body,[39] where it exists in two main oxidation states (Cu^+ and Cu^{2+}). It is versatile for performing biochemical redox reactions, acting as a useful cofactor in many enzymes (e.g. ceruloplasmin, superoxide dismutase, and lysyl oxidase), though it simultaneously poses a risk for toxicity if improperly regulated by the body. As such, Cu is almost always bound to proteins or peptides, 'free' or unbound copper is uncommon.[40]

The total mass of Cu in the average healthy adult body is ∼100 mg. Distribution of this mass between its main reservoirs (Table 1) have been well established through nutritional studies (see review in Ref. 41). Copper enters the body through food consumption, with the recommended Cu intake for a healthy adult being ∼1 mg/day (i.e. ∼100 days residence time).[42] Absorption of the nutrient occurs through the small intestine, where it binds to albumin and macroglobulins in the blood to be carried to the liver. The liver acts as the primary storage and regulatory compartment. Hepatic cells synthesize ceruloplasmin (Cp, the primary Cu protein carrier in the blood), and copper is bound to Cp or other proteins while circulating the body for delivery to various organs. Excess Cu is filtered out by the kidneys or released from the liver into the gallbladder, after which Cu exits the body through urine or fecal matter.

Though elemental Cu is already well-established as an essential trace metal for humans, much less is known about its stable isotopes and their behaviors in the body. A few select studies have approached Cu fractionation on a molecular level with a combination of experiments and first-principles calculations to quantify the fractionation of individual Cu-interacting proteins.[43–45] Such investigations are necessary steps toward achieving a mechanistic understanding of the isotopic effects, but the rate of development of these understandings is relatively slow for several rea-

Table 1. Cu distribution in organs of the human body

Organ	Mass of Cu used in Computation (mg)[*]
Liver	10
Blood	6
Bones	40
Muscle	23
Brain	8
Other	13

[*]Cu mass distribution values are taken from Modern Nutrition in Health and Disease, 11th ed.[41]

sons. There are over 50 proteins in the human Cu proteome that may contribute to net fractionation effects and would need to be characterized;[46] first principles calculations are time-intensive and computationally expensive, requiring a relatively specialized skillset, while in vitro experiments involving individual protein expression, purification, and analyses are often difficult and time-consuming, depending on the stability and other chemical characteristics of the molecules in question.

Considering these uncertainties, this study takes a macroscopic approach to investigate Cu in cancer: Whole-body Cu behavior is quantified using well-established values from nutritional literature, while assumptions about molecular-level relationships between Cu and cancer are non-essential and thus limited.

Methods

We aim to model and observe three main scenarios (see next Section for details). In the first scenario, a tumor begins to grow in an otherwise healthy body, participating in the uptake and release of Cu to aid its proliferation. Because tumor behavior differs depending on cancer type, location, and stage, our system has been defined as simulating early-stage breast cancer growth, forming a single tumor mass in the body. This specific system was selected for several reasons. First, modeling cancer allows us to make direct comparisons between our results and those of the existing studies.[5,6,8,10,14] Second, breast cancer in its early stages does not directly or immediately affect the function of important Cu reservoirs, in contrast, for instance, to hepatic cancer affecting the liver, which is the major Cu regulator of the body. This allows us to differentiate easily between the effects of the tumor vs. those of another reservoir when we built the model. Third, drawing conclusions on whether isotopic signatures can be diagnostic tools depends on the behavior of early-stage cancer growth and whether it translates into a measurable signal; modeling metastatic behavior would be less useful for this purpose. In the second scenario, liver-only fractionation is considered. This type of fractionation may represent several physical cases that could afflict this major reservoir including various stages of liver disease, from non-alcoholic fatty liver disease (NAFLD) to liver cirrhosis or even HCC. Finally, the last scenario broadly simulates altered isotopic fractionation of originally healthy tissue, due to potential effects from metabolic responses to disease (e.g. oxidative stress), to explore the sensitivity of serum Cu isotopic composition as a possible prognostic marker.

To examine the role of major elemental reservoirs in the body and the fluxes between them, we built a multi-box system. The components of interest are as follows: (1) a fixed copper influx from daily food intake according to recommended health standards, (2) the blood, which acts as a conduit through which Cu is
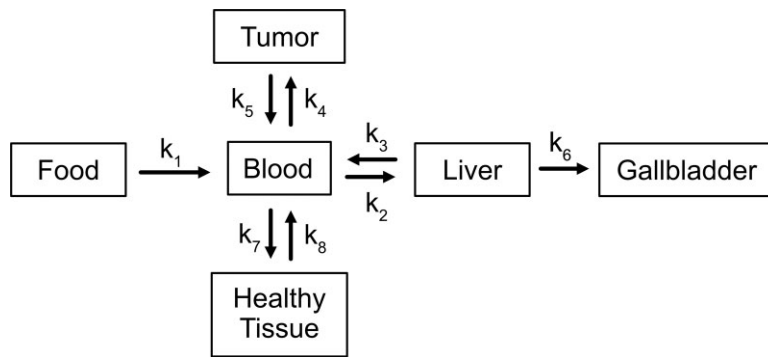
Fig. 2 Box model of major Cu reservoirs in the human body. The six groups presented in the diagram represent the major reservoirs of interest participating in copper storage and transport, with the arrows representing the allowable Cu flows between them. First-order kinetic constants for fluxes in and out of each reservoir i, k_i, are scaled based on compartmental models from existing nutritional studies.[47,48]

able to be transported throughout the body to other reservoirs, (3) healthy tissue, such as bone and muscle, which contain a large proportion of the body's copper but do not directly interact with the cancerous tumor, (4) the tumor itself, (5) the liver, which acts as the storage and regulatory compartment for Cu, and (6) the gallbladder, which we effectively treat as a waste accumulation reservoir for any Cu outflux from the body. Our treatment of the gallbladder is due to the expectation that almost all Cu released to the gallbladder from the liver is excreted via biliary incorporation into feces.[41,47] As such, the 'gallbladder' compartment is not considered in the analysis of diagnostic potential, as it is not expected to influence any of the other reservoirs. All Cu flows between reservoirs were treated as first-order reactions, the precedent for which is established by existing nutritional studies.[47,48] With the exception of unidirectional nutrient flow from the food to the blood and the liver to the gallbladder, all other flows were allowed forward and back exchange to mimic realistic behavior of the circulatory system (Fig. 2).

Two systems of ordinary differential equations (ODEs) were established to represent the elemental and isotopic mass balance of Cu in the body, using the general forms shown in Equations 1 and 2, respectively, modified from Albarède (1996).[49]

$$\frac{dN_i}{dt} = -\sum_{j\neq i} k_{i\to j}\, N_i + \sum_{j\neq i} k_{j\to i}\, N_j, \tag{1}$$

$$\frac{dR_i}{dt} = R_i \sum_{j\neq i} k_{i\to j}\left(1 - D_{i\to j}\right) + \sum_{j\neq i} k_{j\to i}\left(D_{j\to i}R_j - R_i\right)\frac{N_j}{N_i}, \tag{2}$$

In these equations, i and j are distinct reservoirs/organs of the body, N_i is the total mass of Cu in reservoir i, $k_{i\to j}$ is the first order kinetic coefficient of mass transport from reservoir i to j. In Equation 2, R_i is the ^{65}Cu/^{63}Cu ratio in reservoir i and $D_{i\to j}$ is the fractionation factor of Cu transfer from reservoir i to j (i.e. the composition of the material extracted toward reservoir j divided by the composition of parent reservoir i). Specifically, the explicit ODEs for mass change in the blood, liver, tumor, and healthy tissue, respectively are given in Equations 3–6 below:

$$\frac{dN_{blood}}{dt} = k_1 + k_3 N_{liver} - k_2 N_{blood} + k_5 N_{tumor} - k_4 N_{blood}$$
$$+ k_8 N_{healthy} - k_7 N_{blood}, \tag{3}$$

$$\frac{dN_{liver}}{dt} = k_2 N_{blood} - k_3 N_{liver} - k_6 N_{liver}, \tag{4}$$

$$\frac{dN_{tumor}}{dt} = k_4 N_{blood} - k_5 N_{tumor}, \tag{5}$$

$$\frac{dN_{healthy}}{dt} = k_7 N_{blood} - k_8 N_{healthy} \tag{6}$$

Similarly, the ODEs describing the change in isotopic composition of these reservoirs are given in Equations 7–10. In all cases, the kinetic constants are numbered according to Fig. 2. The fractionation factors are numbered to represent the reservoirs in the same order as the kinetic constants (Table 2).

$$\frac{dR_{blood}}{dt} = R_{blood}\left(k_2\left(1 - D_2\right) + k_4\left(1 - D_4\right) + k_7\left(1 - D_7\right)\right)$$
$$+ \frac{k_1\left(D_1 R_{food} - R_{blood}\right)}{N_{blood}} + k_3\left(D_3 R_{liver} - R_{blood}\right)\frac{N_{liver}}{N_{blood}}$$
$$+ k_5\left(D_5 R_{tumor} - R_{blood}\right)\frac{N_{tumor}}{N_{blood}}$$
$$+ k_8\left(D_8 R_{healthy} - R_{blood}\right)\frac{N_{healthy}}{N_{blood}}, \tag{7}$$

$$\frac{dR_{liver}}{dt} = R_{liver}\left(k_3\left(1 - D_3\right) + k_6\left(1 - D_6\right)\right) + k_2\left(D_2 R_{blood} - R_{liver}\right)\frac{N_{blood}}{N_{liver}}, \tag{8}$$

$$\frac{dR_{tumor}}{dt} = R_{tumor}\left(k_5\left(1 - D_5\right)\right) + k_4\left(D_4 R_{blood} - R_{tumor}\right)\frac{N_{blood}}{N_{tumor}}, \tag{9}$$

$$\frac{dR_{healthy}}{dt} = R_{healthy}\left(k_8\left(1 - D_8\right)\right) + k_7\left(D_7 R_{blood} - R_{healthy}\right)\frac{N_{blood}}{N_{healthy}} \tag{10}$$

To constrain the values of the kinetic constants (Table 2), a careful examination of the sparse literature data was done. First, the dietary intake of Cu for a healthy adult was set. Though the recommended daily intake value is ∼1 mg Cu, only ∼0.8 mg of it is absorbed by the intestine and enters the circulatory system; therefore, we use $k_1 = 0.8$ mg/day.[41,42] The other kinetic constants controlling Cu flow between the blood and relevant organs/tissues were constrained by two conditions. First, Cu reservoirs must return to expected steady-state mass values after an imposed perturbation. The Cu inventory of organs/tissues in a healthy body is

**Table 2.** Rate constants and fractionation factors for box model reservoirs

Reservoir transfer, i → j	Rate constants	Steady state values[1]	Fractionation factor	Range of values considered[2]
Food → Blood	k_1	0.8	D_1	1
Blood → Liver	k_2	0.3	D_2	1−1.001
Liver → Blood	k_3	0.11	D_3	0.999−1
Blood → Tumor	k_4	0.00 002	D_4	1−1.003
Tumor → Blood	k_5	0.00 001	D_5	1
Liver → Gallbladder	k_6	0.081	D_6	1−1.001
Blood → Other Healthy Tissue	k_7	0.8	D_7	1
Other Healthy Tissue → Blood	k_8	0.06	D_8	0.997−1

[1]Steady state k values were approximated based on Cu half-life measurements in different organisms from Levenson & Janghorbani.[51]
[2]Possible fractionation factors were based on *ab initio* calculations of copper ion binding to amino acids in aqueous solution from Fujii *et al.*[67] and Cadiou *et al.*[43]

relatively well known, and this condition represents the homeostatic response of a healthy body to small fluctuations in Cu (e.g. variations in diet).[41,50] Second, the rate at which reservoirs responded to perturbations had to be consistent with the available experimental data.[47] Approximate values satisfying the conditions for kinetic constants were drawn from existing nutritional studies that utilized mice for compartmental modeling of short- and long-term Cu turnover in the body.[47,51] The values were then tuned in small increments to ensure the stability of the system, such that it would respond to deviations or perturbations from the expected mass of each reservoir to re-equilibrate it to healthy, steady-state values (e.g. if the mass of Cu is suddenly raised to 8 mg in the blood due to a Cu-rich diet, the model would reflect the body's attempt to lower it back to the healthy expected value of 6 mg). Figure 3A demonstrates the response to perturbation of Cu masses of each reservoir compared to steady state, and the ability of the system to recover over time and maintain homeostasis, while Fig. 3B demonstrates that the model's short and long turnover timescales closely match existing studies.[51,52]

The next condition to be established was tumor growth behavior. Different subtypes of breast cancer have differing growth parameters, and approximate doubling times for primary breast cancer range extremely widely—from a few months to over a year.[53,54] Furthermore, because many factors determine the spatial and temporal growth of tumors at different stages of its progression (e.g. diffusion-limitation of nutrients, allowable space in the host, successful vascularization), the growth does not follow a simple exponential curve over time, contrary to what would be expected from uncontrolled cell division alone. Due to the variable and uncertain nature of tumor growth, we decided to use a linear growth model, which is one of several commonly used models for cancer development.[55,56] This type of growth most accurately characterizes tumor growth after the initial exponential replication phase but prior to metastasis, which is a time frame of interest for diagnostic purposes. The next step was to determine the rate of Cu uptake by the tumor to fuel its growth. The average amount of time a primary breast cancer tumor has been growing prior to detection ranges from 1 to 4 years.[57] In 2010, at time of detection, 67% of breast tumors were < 2.0 cm, with ∼27% of these tumors falling in the 1–2 cm range, which is the upper bound for what is generally considered a 'smaller' tumor.[58] Assuming a density of 1 g/cm³ for the cell, which is mainly composed of water, a spherical tumor with 2 cm diameter would weigh ∼4.2 g. Measurements of Cu concentration in breast tumors range from 2 to 10 μg/g (wet mass basis).[59,60] Therefore, a relatively fast-growing tumor (2 cm diameter growth over 1 year) would need to uptake and sequester ∼0.04 mg Cu/year. This rate was taken as a first-
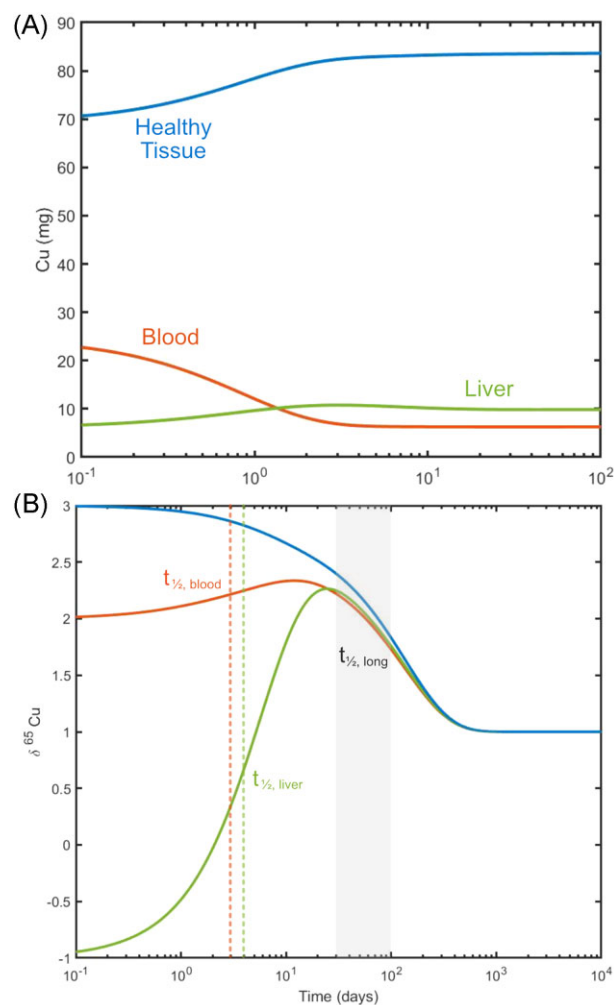
Fig. 3 Model response to mass and isotopic perturbation. (A) Cu masses of each reservoir were strongly perturbed from expected steady state values, simulating the case of an intravenous injection of Cu. The reservoirs return to expected steady state Cu masses over time to maintain homeostasis, demonstrating the robustness of the model. (B) All reservoirs were initialized to random δ^{65}Cu values and allowed to return to steady state. All reservoirs in the system converge to the isotopic composition of the food input (δ^{65}Cu = 1‰) over time. Red and green dotted lines represent the expected short half-lives ($t_{1/2}$) of plasma and liver, respectively, presented in Levenson & Janghorbani[51] and Scott & Turnlund.[52] These short half-lives represent the expected immediate behavior to a perturbation. The gray band represents the range of expected half-lives of long-term Cu turnover ($t_{1/2, long}$) in the reservoirs from Levenson & Janghorbani.[51]

Table 3. Parameters in 'Scenarios and Results' section

Literature reference	Condition	Serum δ^{65}Cu (‰)	
		Median or mean	Range
Télouk et al. (2015)	Healthy/Control ($n = 30$)	Median: −0.26	−0.80 to 0.05
	Breast cancer ($n = 86$)	Median: −0.52	−1.45 to 0.12
Van Campenhout et al. (2019)	Healthy/Control ($n = 22$)	Median: −0.13 (males), −0.26 (females)	−0.49 to 0.05 (males), −0.46 to −0.08 (females)
	NAFL ($n = 10$)	Median: −0.87	−1.01 to −0.32
Costas-Rodríguez et al. (2015)	Healthy/Control ($n = 29$)	Mean: −0.29	−0.54 to −0.06
	Liver Cirrhosis ($n = 25$)	Mean: −0.78	−1.44 to −0.04
Télouk et al. (2022)	Healthy/Control ($n = 105$)	Median: −0.11	−0.63 to 0.28
	HCC ($n = 98$)	Median: −0.51	−1.71 to 0.07
Lauwens et al. (2016)	ESLD, pre-LTx ($n = 32$)	Median: −0.74	−1.45 to −0.40
	ESLD, 3 months post-LTx ($n = 32$)	Median: −0.55	−1.54 to −0.18
	ESLD, 9 months post-LTx ($n = 17$)	Median: −0.45	−1.27 to −0.09
Costas-Rodríguez et al. (2019)	Sham-operated mice, 4 weeks post-operation	Mean: −0.67	nr
	CBDL mice, 4 weeks post-operation	Mean: −1.14	nr

All experimental studies referenced in this section reported δ^{65}Cu values using the international Cu isotopic standard NIST SRM 976. nr, not reported; NAFL, non-alcoholic fatty liver; HCC, hepatocellular carcinoma; ESLD, end stage liver disease; LTx, liver transplantation; CBDL, common bile duct ligation.

order estimate for the slope of the linear growth curve and used to calibrate k_4 ($= 0.00002$ day^{-1}). This growth rate reflects the most optimistic scenarios for detection of a small tumor, i.e. the faster the tumor grows, the quicker it sequesters Cu and may be detected by isotopic measurements. Changing this growth rate by one order of magnitude (higher or lower) also did not impact our conclusions (see next section).

For each scenario tested, the box model was initialized with steady-state values of Cu in each reservoir: 6 mg in the blood, 10 mg in the liver, and 84 mg in healthy tissue (Table 1). The fractionation factors were all set to $D_{i \to j} = 1$, indicating no fractionation between any of the reservoirs. At $t = 0$, the system was perturbed by the introduction of tumor growth, and a fractionation factor was applied to the reservoir of interest. A range of fractionation factors (Table 2) were considered for each scenario described below.

Scenarios and results

We present a suite of computational test scenarios that draws from several studies and their experimental observations. All experimental studies referenced in this section reported δ^{65}Cu values using the international Cu isotopic standard NIST SRM 976. Delta notation is used as follows:

$$\delta^{65}Cu_i = \left(\frac{R_i}{R_{NIST\ SRM\ 976}} - 1 \right) \times 1000, \quad (11)$$

$$\Delta^{65}Cu_i (t) = \delta^{65}Cu_i (t) - \delta^{65}Cu_i (0), \quad (12)$$

for any given reservoir i at time t. Table 3 provides a summary of pertinent observations from the studies referenced in this section.

Tumor-only fractionation: To assess whether the Cu isotope composition of the blood and organs can be affected by the tumor growth, we considered a scenario where a tumor appears in a body at steady state, and an isotopic fractionation is associated with Cu uptake/release into the tumor. As can be seen in Fig. 4, while the tumor composition directly reflects each of the applied fractionation factors, the blood, liver, and healthy tissue compositions are virtually unchanged, remaining at 0‰ for each of the cases. Sensitivity tests were performed on the tumor growth fac-
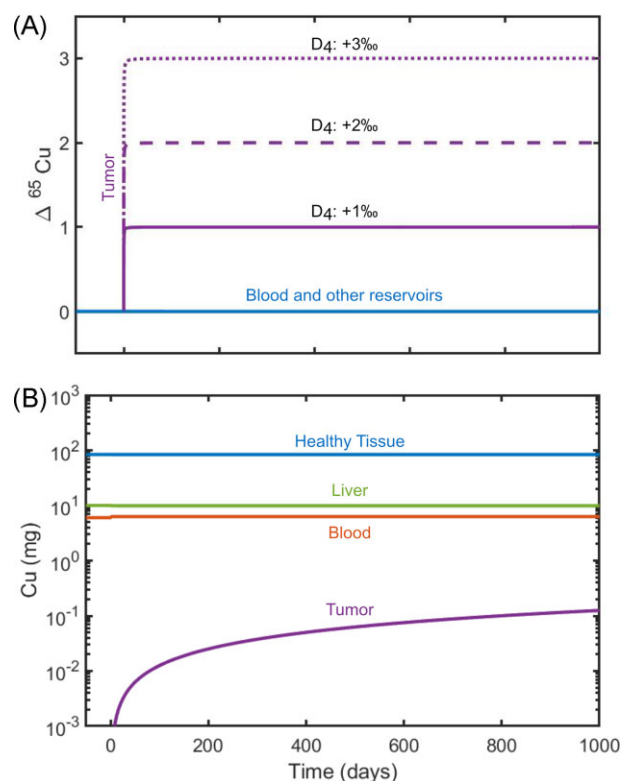
Fig. 4 Effects of tumor-only Cu isotope fractionation as a function of time. (A) The composition of each reservoir is arbitrarily set to 0 per mil at the start of the simulation. At time equal 0 days, the tumor is introduced and allowed to grow. An isotopic fractionation is associated with Cu uptake in the tumor, i.e. the fractionation factor D_4, was varied (see label on each curve), while all other reservoir interactions maintained a fractionation factor of 1. Cu isotope ratios are expressed as deviation relative to $t = 0$, just before perturbation, i.e. Δ^{65}Cu $= \delta^{65}$Cu(t)$-\delta^{65}$Cu(0). (b) The relative masses of Cu in each reservoir are plotted on a log scale. Cu mass in the tumor is orders of magnitude less than that of any other organ, resulting in its negligible contribution to the serum Cu isotopic composition, despite its ongoing exchange with the blood.

tor to gauge stability of the system under different scenarios, exploring a range of k_4 values spanning two orders of magnitude (0.00002−0.002 day^{-1}). Even at the maximum tumor growth rate tested with a 3‰ fractionation into the tumor, the drop in serum isotopic composition was < 0.05‰. This is because the absolute amount of Cu sequestered by the tumor is negligible compared to the body's Cu budget (mass of Cu in tumor is 0.012% of total body Cu, Fig. 4B). The same limitation applies in the case where the Cu isotopic fractionation is associated with Cu release from the tumor. Thus, while the tumor composition is strongly fractionated from that of the blood (and by construction, the organs as well), the $\Delta^{65}Cu$ of the blood and other reservoirs due to the tumor are also negligible (−0.0005‰). Given the expected precision of ∼0.1–0.2‰ referenced in this study, the differences in isotopic compositions of the blood due to tumor growth alone would be unresolvable.

Telouk et al.[5] proposed that serum copper of cancer patients may become isotopically lighter compared to controls due to the Warburg effect: the increased usage of anaerobic glycolysis in cancer cells that can lead to higher concentrations of L-lactate in the cytosol. This lactate can bind to free Cu^{2+}, with the chelation reaction having a strong preference for ^{65}Cu over ^{63}Cu. Based on *ab initio* calculations, the equilibrium Cu isotope composition of Cu(II)(L-lact)(D-lact) is expected to be ∼3‰ heavier than that of Cu(I)(Cys)(H$_2$O)$_4^{2+}$, which is representative of the Cu binding motif of ATP7A, one of the primary cellular transmembrane transporters of Cu. This results in isotopically heavy Cu being sequestered in the cytosol of cancer cells with lactate while ^{63}Cu is preferentially shuttled out to the bloodstream by ATP7A. While the chemistry of this hypothesis is promising, this scenario is equivalent to a Cu isotope fractionation associated solely with the tumor, as only the Cu taken up and/or released by the tumor will be isotopically fractionated. As shown in Fig. 4, such scenarios have a negligible impact on the blood Cu isotopic composition because of the overall negligible amount of Cu channeled through the tumor over time. Therefore, the Warburg effect, which applies primarily to the cancer mass, would be insufficient to explain the observed ∼ 0.4‰ $\delta^{65}Cu$ offset in the serum of cancer patients vs. healthy patients.

Liver-only fractionation: Considering the negligible effect the tumor alone has on the $\Delta^{65}Cu$ of other reservoirs, the question remains of which reservoirs *are* sufficiently large to induce a measurable change in serum $\delta^{65}Cu$. Because the liver is the primary regulator of copper and a fairly large reservoir (10% of the body's Cu budget), its dysregulation would be expected to have a noticeable effect on copper throughout the rest of the body. Some studies have investigated the effects of liver disease on serum $\delta^{65}Cu$ values at different stages, from NAFLD to HCC.[6,7,9,12,14] Van Campenhout et al.[12] found significantly (>0.5‰) lower serum $\delta^{65}Cu$ value in NAFLD patients relative to healthy controls. Costas-Rodríguez et al.[7] also observed a much lighter Cu isotope composition in the serum of patients with cirrhosis, which represents a worsening of NAFLD. More recently, Telouk et al.[14] showed that patients with further progression to HCC also demonstrated a ∼0.5‰ drop in serum $\delta^{65}Cu$ values relative to healthy controls. The consistent signature seen among these studies was used to guide the formulation of this scenario. There are two bounding cases by which Cu fractionation by the liver may result in isotopically lighter serum. The liver may preferentially uptake ^{65}Cu from the blood (Fig. 5A) or preferentially release ^{63}Cu into the blood (Fig. 5B). In stark contrast to tumor-only fractionation patterns, a fractionation of 1‰ in either

scenario can change the composition of the blood, tumor, and other healthy tissues at a readily measurable level (>0.5‰). This result demonstrates the order-of-magnitude of difference between organ-driven and tumor-driven fractionation. Intermediate cases in between these two extremes (e.g. the liver preferentially taking heavy Cu from the blood but also preferentially releasing heavy Cu back into the blood) would simply result in a dampening effect on the isotopic fractionations seen in Fig. 5. The agreement between the experimental observations and our computational results further supports ideas presented in the aforementioned works that the observed serum Cu composition is due to liver disease. Our model also aligns with observations from Lauwens et al.,[9] which demonstrated that measurable changes in liver Cu isotopic fractionation (following liver-transplant) are reflected in serum composition over the timescale of 3–9 months, consistent with the largest changes occurring within ∼200 days in the model.

After seeing this repeated trend of light serum Cu isotopic composition in liver disease patients compared to healthy controls, Costas-Rodriguez et al.[11] sought to determine more specifically the mechanism behind these results. Comparing mice populations that underwent sham operations vs. common bile duct ligation (CBDL), they observed that the overall Cu isotopic composition of the entire mouse body became ∼0.6‰ lighter 1 month post-CBDL. They hypothesized that typical excretion from the liver to the bile is isotopically light, but the CBDL-operated mice were unable to preferentially release ^{63}Cu, resulting in its accumulation in the body. Figure 6 tests this hypothesis by comparing the difference in Cu isotopic composition ($\Delta^{65}Cu$) of reservoirs from a sham-operated mouse relative to a CBDL-operated mouse. The results of the model appear to match the measurements from Ref. 43 very well, with the liver becoming 1‰ lighter and the other reservoirs nearly 0.6‰ lighter. Once again, this scenario reinforces the idea that liver-only fractionation can create resolvable compositional changes in other reservoirs.

Alteration of healthy tissue metabolism/fractionation: A handful of studies investigated the mechanistic cause of serum Cu isotopic changes from liver disease and determined that oxidative stress is likely the primary contributing factor to the observed changes, with HepG2 cells under oxidative stress conditions exhibiting $\delta^{65}Cu$ values up to 2‰ heavier relative to those in normoxic environments.[8,10] If otherwise healthy cells are undergoing oxidative stress or inflammation due to any disease, including cancers or NAFLD/cirrhosis, a significant proportion of the total body mass could start to exhibit altered metabolism, which may be reflected in the serum $\Delta^{65}Cu$ value at different stages of the disease progression, and to different degrees. This scenario broadly explores the physical phenomenon of the body undergoing increasing amounts of oxidative stress for any reason (e.g. in response to cancer) by calculating what proportion of cells would need to be responding to oxidative stress to affect the blood isotopic compositions. To do so, the original healthy tissue reservoir is now divided into two: one representing healthy cells undergoing aerobic cellular respiration and the other representing formerly healthy cells metabolizing under oxidative stress conditions. Figure 7 demonstrates that greater than ∼10% mass of originally healthy tissue would have to be affected with a fractionation factor of + 3‰ in order for the blood to undergo a > 0.2‰ drop in $\Delta^{65}Cu$, which is a fairly large proportion of the body. If this is indeed the primary method by which the serum isotopic composition becomes lighter, only an extremely large or metastasized tumor could probably subject the body to such levels of oxidative
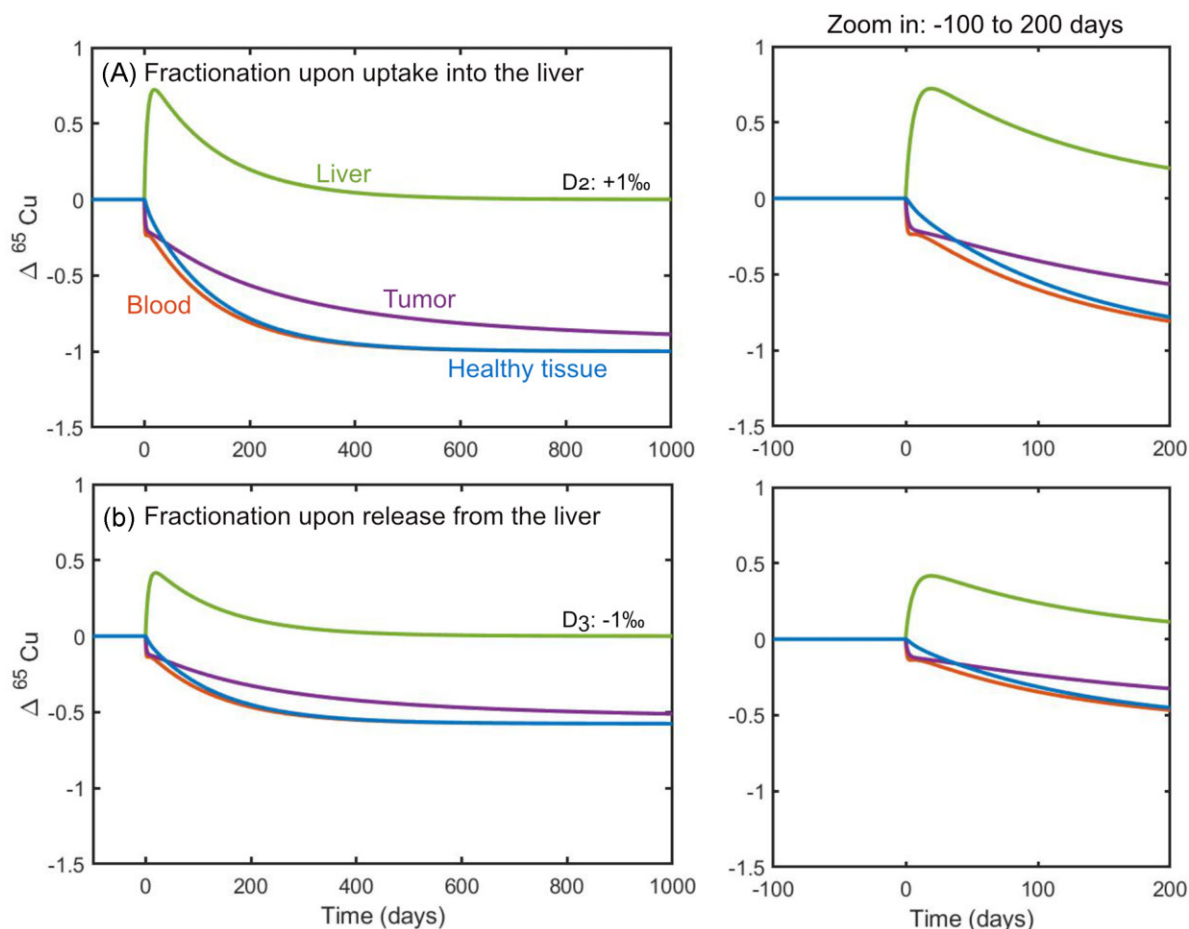
Fig. 5 Effects of liver-only Cu isotope fractionation as a function of time. The composition of each reservoir is arbitrarily set to 0 per mil at the start of the simulation. At $t = 0$ days, the tumor is introduced and allowed to grow. Cu isotope ratios are expressed as deviation relative to $t = 0$, just before perturbation, i.e. $\Delta^{65}Cu = \delta^{65}Cu(t) - \delta^{65}Cu(0)$. (A) An isotopic fractionation associated with Cu uptake from the blood to the liver, i.e. fractionation factor D_2, was set to 1.001, while all other reservoir interactions maintained a fractionation factor of 1. (B) An isotopic fractionation associated with Cu release from the liver to the blood, i.e. fractionation factor D_3, was set to 0.999, while all other reservoir interactions maintained a fractionation factor of 1.

stress to produce a detectable signature. At that point, it would very likely have already caused other serious symptoms to alert the patient to illness, and this type of isotopic analysis would not be required for detection and diagnosis. Notice that the result of this scenario is effectively an exacerbated case of the tumor-only fractionation: the blood composition recovers to its original state over time in this scenario. This contrasts with the liver-only fractionation scenario, where the blood Cu isotope composition reaches a new steady state, different from its starting composition. This is because, in the liver-only fractionation scenario, the liver composition is not only affected by forward and backward exchange with the blood, but it is also siphoned away by the gallbladder. As a result, ^{65}Cu is continuously preferentially removed from the circulatory system, and the blood cannot recover back to its original composition. In contrast, when healthy tissue is affected and undergoes altered fractionation, the long-term effects on the blood are dampened by the continual back-exchange between the affected tissue and the blood. In short, any case in which a reservoir uptakes copper with a fractionation factor but subsequently also releases said composition back into the bloodstream will create this dampening effect, ultimately resulting in the recovery of the blood back to its original (pre-perturbation) composition. Such scenarios cannot explain long terms changes on the blood Cu isotope compositions.

Discussion

Early studies raised interest in the idea that the offset in the serum Cu isotope composition of cancer patients could be used as a potentially powerful prognostic or diagnostic tool.[5–7] In agreement with more recent studies,[7,8,10–12,14] our results point to a more complex picture. We find the simple proposal that Cu isotope ratios exclusively track tumor growth/evolution is not tenable. Indeed, the model demonstrates that the growth of a tumor in an otherwise healthy body cannot create a measurable (>0.1‰) drop in blood Cu isotopic composition when any reasonable fractionation factor is imposed on the tumor (Fig. 4).

Unlike the tumor, with its small size and Cu inventory, we find that a significantly larger reservoir can alter the blood composition. Exploration of the liver-only fractionation case shows that the imposition of a fractionation factor reflecting a + 1‰ change in $\delta^{65}Cu$ uptake from the blood into the liver could successfully generate a corresponding −1‰ change in $\Delta^{65}Cu$ of the blood, which would certainly be measurable (Fig. 5). This type of alteration in liver fractionation could describe the physical scenario of liver disease, considering that liver failure is also a common effect in mid- to late-stage cancer patients. This idea is supported by several recent experimental studies, which found that liver disease of varying stages can result in a significant (0.5‰ or
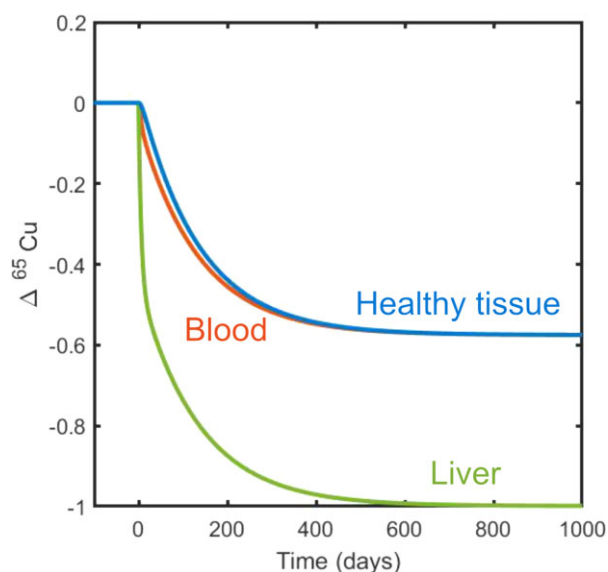
Fig. 6 Effects of bile duct ligation in mice. Cu isotopic compositions of the reservoirs of the sham-operated mouse ($\delta^{65}Cu_{sham}$) are compared to Cu isotopic compositions of the reservoirs of the common bile duct ligation (CBDL)-operated mouse ($\delta^{65}Cu_{CBDL}$), with $\Delta^{65}Cu = \delta^{65}Cu_{CBDL} - \delta^{65}Cu_{sham}$. Bile duct ligation prevents release of ^{63}Cu from the liver to the bile relative to its regular healthy process, resulting in a decrease in the $\delta^{65}Cu$ of the liver. A fractionation factor of $D_6 = 1.001$ was used to describe this physical scenario, in accordance with the observed drop in liver isotopic composition from Costas-Rodriguez et al.[11]

more) decrease in their serum isotopic compositions relative to healthy controls (Fig. 1).[7,9,12,14] Considering oxidative stress as another possible source of isotopic fractionation on a large scale, we find that >10‰ of originally healthy tissue would have to impart a Cu isotopic fractionation of ~+1‰ during Cu uptake into the tissues to create a measurable effect in the blood (Fig. 7). Further patient studies will be needed to monitor the isotopic variability induced by controls such as patient age or the degree of cancer progression, as well as clarify the specificity of these signatures.

For reasons previously discussed, our current calculations utilize a model of linear tumor growth. The integration of this model already provides clear results that can be directly compared to existing studies. However, in acknowledgment that different cancer types and subtypes exhibit different growth behaviors, other models warrant future exploration, such as exponential growth.[55] Though our calculations do not explicitly implement an exponential growth model, the tuning of the tumor growth rate, k_4, to be orders of magnitudes larger (100x) than the expected rate provides a reasonably similar comparison to exponential growth behavior. Even in this scenario, tumor growth alone still failed to create a measurable Cu isotopic composition change in the blood, indicating the low dependency of our findings to the tumor growth behavior.

Ultimately, this study sought to utilize box modeling to quantitatively assess the potential for natural Cu isotopic variations in serum to act as a diagnostic tool for breast cancer and liver cirrhosis. Through modeling, we were able to determine whether Cu isotopic compositions satisfy criteria (3) and (4) set forth for a competitive medical diagnostic tool. Criterion (3) required that the drop in Cu isotopic composition of the patient serum must be resolvable by current MC-ICP-MS capabilities, which was determined to be largely dependent on the size of the reservoir causing the fractionation. With tumor-only fractionation, the serum composition is not sufficiently modified by the growth of

the tumor due to its small size and Cu content relative to other reservoirs in the body. In contrast, isotopic fractionation associated with a larger reservoir, such as the liver or a large proportion (>10%) of healthy tissue undergoing severe oxidative stress, may cause an isotopic shift in the serum composition of the patient compared to healthy controls. Yet, only in the liver fractionation scenario does the serum composition shift permanently to a new steady-state value that is lower than initial controls. In contrast, some percentage of healthy tissue with altered metabolic behavior affects the serum temporarily under homeostatic conditions. Criteria (4) required that signatures develop relatively early in the disease, which we see to be true if the fractionating reservoir is sufficiently large. When a fractionation factor is imposed on a specific reservoir, the change in isotopic composition of other reservoirs is reflected quickly, typically on the timescale of months.[9] However, given the nature of the conditions used in the box modeling, i.e. that the body attempts to return to steady state Cu distributions if functioning properly, the results presented above may not be fully relevant to a patient who is seriously ill and unable to maintain homeostasis as expected. Although these parameters may be changed in future studies to explore other possible response timescales, we consider the estimates presented above as conservative since the inability of the body to return to steady state would only lead to shorter timescales for the expression and detection of isotopic signatures.

Concluding remarks

While much remains to be learned on the exact mechanisms driving Cu isotope fractionations during the evolution of cancer and other diseases, consideration of first-order box-model scenarios allowed us to show that the lower $\delta^{65}Cu$ observed in the serum of breast cancer patients is not a result of tumor growth itself. Instead, these signatures are much more likely due to isotopic fractionation developing during organ failure (e.g. liver failure) or in association with synergistic effects in otherwise healthy tissue, and may be used instead in pursuit of prognosis or diagnosis of liver-related diseases or cancers.[61]

The future of mass spectrometry technology will undoubtedly yield even higher resolution measurements of isotopes, decreasing the analytical uncertainty by up to several folds relative to currently achieved values. Hypothetically, it would not be unreasonable to expect resolving power to routinely reach 0.02‰ or better in the next decade. Given this possibility, re-evaluation of Points (3) and (4) regarding ideal diagnostic tools would be warranted. Point (3), pertaining to the resolvability of the signals, would be met even more easily, and the detection of changes due to any given disease would occur earlier in its progression, improving upon Point (4) as well. However, with this better precision comes the need to face the inherent difficulties associated with discerning meaningful signals from biological noise. Specifically, the precision of the measurement may exceed our understanding of the causes of variations within the system. In the case of copper, many studies have evaluated natural variations of serum $\delta^{65}Cu$ in healthy individuals and shown that they can fall within a large range (from approximately −0.6 to +0.2‰). Studies dedicated to understanding the exact drivers of isotope ratio variability for a given individual over time, as well as variability between different demographics, would have to be conducted to enable meaningful interpretations of these results with improved measurement precision.

As more kinetic and isotopic data become available, models like the one we described above may be improved and refined. In particular, one additional criterion of a diagnostic tool that should
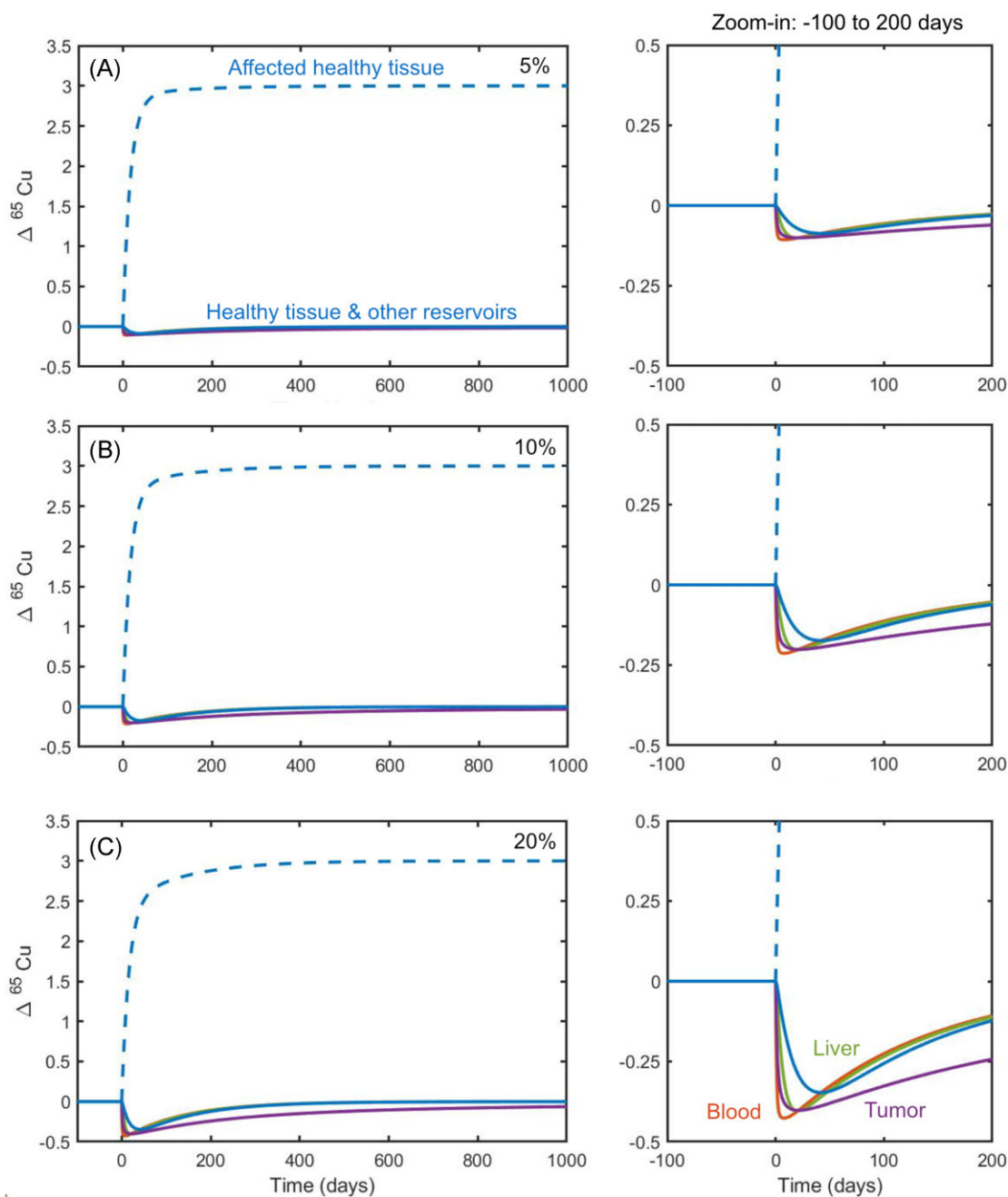
Fig. 7 Isotopic fractionation effects of Cu chelation by lactate. A designated proportion of healthy tissue is affected by oxidative stress ('affected healthy tissue') and begins to undergo anaerobic glycolysis: (A) 5%, (B) 10%, and (C) 20%. Cu isotope ratios are expressed as deviation relative to $t = 0$, just before perturbation, i.e. $\Delta^{65}Cu = \delta^{65}Cu(t) - \delta^{65}Cu(0)$. A fractionation factor of 0.997 is used to describe the expected Cu chelation effect resulting from lactate production due to glycolysis, based on *ab initio* calculations from Telouk *et al.*[5]

be explored as more clinical studies are performed is the uniqueness of the signature, i.e. whether the appearance of the signature can be directly connected to cancer, or whether other diseases (e.g. liver cirrhosis, Wilson's disease)[62] may also produce an identical result. Considering our observations that oxidative stress may generate similar signatures, it would be worthwhile to explore serum Cu isotopic compositions found in other diseases strongly linked to oxidative stress, such as cardiovascular (e.g. atherosclerosis) and neurological (e.g. Parkinson's, Alzheimer's diseases).[63] Unfortunately, due to the scarcity of serum isotopic data across the numerous other possible diseases at this writing, it is currently impossible to tell whether the drop in serum Cu isotopic compo-

sition in cancer patients is sufficiently distinctive. Finally, future considerations should include the effects of chemotherapeutic treatment on the liver. Given that cancer literature has widely documented the stress and imbalance imposed on liver metabolism in response to chemotherapy,[64–66] it would be valuable to understand whether chemotherapy may act as a confounding factor for the prognosis potential of Cu isotope ratios. Specifically, liver injury due to chemotherapy may alter serum Cu isotopic composition, adding to or potentially masking the signature from the original disease.

Most diagnostic tools work in conjunction with each other to help deduce the disease presented (e.g. masses detected on CT

scans are confirmed by immunostaining of tissue biopsies), and it seems δ^{65}Cu is no exception. To date, observations have shown that serum Cu isotope ratios may act as good non-specific markers; at a minimum, it could be used as an initial filter in routine medical examinations to signal a need for more targeted tests that can then narrow down possible disease candidates. As the field looks toward potential medical applications of Cu isotope ratios, it will certainly be worthwhile to explore their use as part of a suite of diagnostic tools, including other isotope ratios (e.g. Zn,[61] S[6]) or molecular markers.

Acknowledgement

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

References

1. H. D. Holland and K. K. Turekian *Treatise on Geochemistry*. 2nd ed. Elsevier, 2014.
2. C. N. Maréchal, P. Télouk and F. Albarède, Precise analysis of copper and zinc isotopic compositions by plasma-source mass spectrometry, *Chem. Geol.*, 1999, 156(1–4), 251–273. https://doi.org/10.1016/S0009-2541(98)00191-0.
3. F. Albarède, P. Télouk and V. Balter, Medical applications of isotope metallomics, *Non-Traditional Stable Isot.*, 2017, 82, 851–885. https://doi.org/10.1515/9783110545630-021
4. B. Mahan, R. S. Chung, D. L. Pountney, F. Moynier and S. Turner, Isotope metallomics approaches for medical research, *Cell. Mol. Life Sci.*, 2020, 77(17), 3293–3309. https://doi.org/10.1007/s00018-020-03484-0
5. P. Télouk, A. Puisieux, T. Fujii, V. Balter, V. P. Bondanese, A. P. Morel, G. Clapisson, A. Lamboux and F. Albarede, Copper isotope effect in serum of cancer patients. A pilot study, *Metallomics*, 2015, 7(2), 299–308. https://doi.org/10.1039/C4MT00269E
6. V. Balter, A. Nogueira, V. Paky, K. Jaouen and A. Lamboux, Natural variations of copper and sulfur stable isotopes in blood of hepatocellular carcinoma patients, 2015, 112, 1–4.
7. M. Costas-Rodríguez, Y. Anoshkina, S. Lauwens, H. Van Vlierberghe, J. Delanghe and F. Vanhaecke, Isotopic analysis of Cu in blood serum by multi-collector ICP-mass spectrometry: a new approach for the diagnosis and prognosis of liver cirrhosis?, *Metallomics*, 2015, 7(3), 491–498. https://doi.org/10.1039/C4MT00319E
8. V. P. Bondanese, A. Lamboux, M. Simon, J. E. Lafont, E. Albalat, S. Pichat, J. M. Vanacker, P. Telouk, V. Balter, P. Oger and F. Albarède, Hypoxia induces copper stable isotope fractionation in hepatocellular carcinoma, in a HIF-independent manner, *Metallomics*, 2016, 8(11), 1177–1184. https://doi.org/10.1039/C6MT00102E
9. S. Lauwens, M. Costas-Rodríguez, H. Van Vlierberghe and F. Vanhaecke Cu isotopic signature in blood serum of liver transplant patients: a follow-up study, *Sci Reports 2016 61*, 2016, 6, 1–9.
10. M. R. Flórez, M. Costas-Rodríguez, C. Grootaert, J. Van Camp and F. Vanhaecke, Cu isotope fractionation response to oxidative stress in a hepatic cell line studied using multi-collector ICP-mass spectrometry, *Anal. Bioanal. Chem.*, 2018, 410(9), 2385–2394. https://doi.org/10.1007/s00216-018-0909-x
11. M. Costas-Rodríguez, S. Van Campenhout, A. Hastuti, L. Devisscher, H. Van Vlierberghe and F. Vanhaecke, Body distribution of stable copper isotopes during the progression of cholestatic liver disease induced by common bile duct ligation in mice, *Metallomics*, 2019, 11(6), 1093–1103. https://doi.org/10.1039/c8mt00362a
12. S. Van Campenhout, A. Hastuti, S. Lefere, H. Van Vlierberghe, F. Vanhaecke, M. Costas-Rodríguez and L. Devisscher, Lighter serum copper isotopic composition in patients with early non-alcoholic fatty liver disease, *BMC Res. Notes*, 2020, 13(1), 1–6. https://doi.org/10.1186/s13104-020-05069-3
13. F. Moynier, B. M. Le, E. Lahoud, B. Mahan, F. Mouton-Ligier, J. Hugon and C. Paquet, Copper and zinc isotopic excursions in the human brain affected by Alzheimer's disease, *Alzheimer's Dement.: Diagn. Assess. Dis. Monit.*, 2020, 12(1), e12112. https://doi.org/10.1002/DAD2.12112.
14. P. Telouk, M. - L. Plissonnier, P. Merle, F. Zoulim, N. Fares, P. Guilloreau, R. Parent, J. Bacchetta, M. Danan, S. Carandina and F. Albarède, Copper isotope evidence of oxidative stress—induced hepatic breakdown and the transition to hepatocellular carcinoma, *Gastro Hep. Adv.*, 2022, 1(3), 480–486. https://doi.org/10.1016/j.gastha.2022.02.024
15. P. - A. Krayenbuehl, T. Walczyk, R. Schoenberg, F. Von Blanckenburg and G. Schulthess, Hereditary hemochromatosis is reflected in the iron isotope composition of blood, 2005, 105(10), 3812–3816.
16. L. Van Heghe, J. Delanghe, H. Van Vlierberghe and F. Vanhaecke, The relationship between the iron isotopic composition of human whole blood and iron status parameters, *Metallomics*, 2013, 5(11), 1503–1509. https://doi.org/10.1039/c3mt00054k
17. F. Larner, L. N. Woodley, S. Shousha, A. Moyes, E. Humphreys-Williams, S. Strekopytov, A. N. Halliday, M. Rehkämper and R. C. Coombes, Zinc isotopic compositions of breast cancer tissue, *Metallomics*, 2015, 7(1), 112–117. https://doi.org/10.1039/C4MT00260A
18. K. V. Sullivan, R. E. T. Moore, M. S. Capper, K. Schilling, K. Goddard, C. Ion, D. Layton-Matthews, M. I. Leybourne, B. Coles, K. Kreissig, O. Antsygina, R. C. Coombes, F. Larner and M. Rehkämper, Zinc stable isotope analysis reveals Zn dyshomeostasis in benign tumours, breast cancer, and adjacent histologically normal tissue, *Metallomics*, 2021, 13(6)27. https://doi.org/10.1093/mtomcs/mfab027
19. J. Skulan and D. J. DePaolo, Calcium isotope fractionation between soft and mineralized tissues as a monitor of calcium use in vertebrates, *Proc. Natl. Acad. Sci. U.S.A.*, 1999, 96(24), 13709–13713. https://doi.org/10.1073/pnas.96.24.13709
20. J. Skulan, T. Bullen, A. D. Anbar, J. E. Puzas, L. Shackelford, A. LeBlanc and S. M. Smith, Natural calcium isotopic composition of urine as a marker of bone mineral balance, *Clin. Chem.*, 2007, 53(6), 1155–1158. https://doi.org/10.1373/clinchem.2006.080143
21. A. Heuser and A. Eisenhauer, A pilot study on the use of natural calcium isotope (44Ca/40Ca) fractionation in urine as a proxy for the human body calcium balance, *Bone*, 2010, 46(4), 889–896. https://doi.org/10.1016/j.bone.2009.11.037
22. J. L. L. Morgan, J. L. Skulan, G. W. Gordon, S. J. Romaniello, S. M. Smith and A. D. Anbar, Rapidly assessing changes in bone mineral balance using natural stable calcium isotopes, *Proc. Natl. Acad. Sci. U.S.A.*, 2012, 109(25), 9989–9994. https://doi.org/10.1073/pnas.1119587109
23. M. B. Channon, G. W. Gordon, J. L. L. Morgan, J. L. Skulan, S. M. Smith and A. D. Anbar, Using natural, stable calcium isotopes of human blood to detect and monitor changes in bone

mineral balance, *Bone*, 2015, 77, 69–74. https://doi.org/10.1016/j.bone.2015.04.023

24. R. Rangarajan, S. Mondal, P. Thankachan, R. Chakrabarti and A. V. Kurpad, Assessing bone mineral changes in response to vitamin D supplementation using natural variability in stable isotopes of calcium in urine, *Sci. Rep.*, 2018, 8(1), 16751. https://doi.org/10.1038/S41598-018-34568-4

25. A. Eisenhauer, M. Müller, A. Heuser, A. Kolevica, C. C. Glüer, M. Both, C. Laue, U. V. Hehn, S. Kloth, R. Shroff and J. Schrezenmeir, Calcium isotope ratios in blood and urine: a new biomarker for the diagnosis of osteoporosis, *Bone Rep.*, 2019, 10, 100200. https://doi.org/10.1016/j.bonr.2019.100200

26. R. Shroff, M. Fewtrell, A. Heuser, A. Kolevica, A. Lalayiannis, L. McAlister, S. Silva, N. Goodman, C. P. Schmitt, L. Biassoni, A. Rahn, D. C. Fischer and A. Eisenhauer, Naturally occurring stable calcium isotope ratios in body compartments provide a novel biomarker of bone mineral balance in children and young adults, *J Bone Miner Res*, 2021, 36(1), 133–142. https://doi.org/10.1002/jbmr.4158

27. C. Maréchal and F. Albarède, Ion-exchange fractionation of copper and zinc isotopes, *Geochim. Cosmochim. Acta.*, 2002, 66(9), 1499–1509. https://doi.org/10.1016/S0016-7037(01)00815-8

28. T. F. D. Mason, D. J. Weiss, M. Horstwood, R. R. Parrish, S. S. Russell, E. Mullane and B. J. Coles, High-precision Cu and Zn isotope analysis by plasma source mass spectrometry. Part 2. Correcting for mass discrimination effects, *J. Anal. At. Spectrom.*, 2004, 19(2), 209–217. https://doi.org/10.1039/b306958c

29. T. F. D. Mason, D. J. Weiss, M. Horstwood, R. R. Parrish, S. S. Russell, E. Mullane and B. J. Coles, High-precision Cu and Zn isotope analysis by plasma source mass spectrometry, Part 1. Spectral interferences and their correction, *J. Anal. At. Spectrom.*, 2004, 19(2), 209–217. https://doi.org/10.1039/b306958c

30. C. Archer and D. Vance, Mass discrimination correction in multiple-collector plasma source mass spectrometry: an example using Cu and Zn isotopes, *J. Anal. At. Spectrom.*, 2004, 19(5), 656–665. https://doi.org/10.1039/b315853e

31. J. Bermin, D. Vance, C. Archer and P. J. Statham, The determination of the isotopic composition of Cu and Zn in seawater, *Chem. Geol.*, 2006, 226(3–4), 280–297. https://doi.org/10.1016/j.chemgeo.2005.09.025

32. K. Peel, D. Weiss, J. Chapman, T. Arnold and B. Coles, A simple combined sample—standard bracketing and inter-element correction procedure for accurate mass bias correction and precise Zn and Cu isotope ratio measurements, *J. Anal. At. Spectrom.*, 2007, 23(1), 103–110. https://doi.org/10.1039/B710977F

33. D. M. Borrok, R. B. Wanty, W. I. Ridley, R. Wolf, P. J. Lamothe and M. Adams, Separation of copper, iron, and zinc from complex aqueous solutions for isotopic measurement, *Chem. Geol.*, 2007, 242(3–4), 400–414. https://doi.org/10.1016/j.chemgeo.2007.04.004

34. P. A. Sossi, G. P. Halverson, O. Nebel and S. M. Eggins, Combined separation of Cu, Fe and Zn from rock matrices and improved analytical protocols for stable isotope determination, *Geostand. Geoanalytical Res.*, 2015, 39(2), 129–149. https://doi.org/10.1111/j.1751-908X.2014.00298.x

35. S. C. Yang, L. Welter, A. Kolatkar, J. Nieva, K. R. Waitman, K. F. Huang, W. H. Liao, S. Takano, W. M. Berelson, A. J. West, P. Kuhn and S. G. John, A new anion exchange purification method for Cu stable isotopes in blood samples, *Anal. Bioanal. Chem.*, 2019, 411(3), 765–776. https://doi.org/10.1007/s00216-018-1498-4

36. K. Sullivan, D. Layton-Matthews, M. Leybourne, J. Kidder, Z. Mester and L. Yang, Copper isotopic analysis in geological and biological reference materials by MC-ICP-MS, *Geostand. Geoanal. Res.*, 2020, 44(2), 349–362. https://doi.org/10.1111/ggr.12315

37. H. Jeong, K. Ra and J. Y. Choi, Copper, Zinc and lead isotopic delta values and isotope ratios of various geological and biological reference materials, *Geostand. Geoanal. Res.*, 2021, 45(3), 551–563. https://doi.org/10.1111/ggr.12379

38. K. Jaouen, L. Pouilloux, V. Balter, M. L. Pons, J. J. Hublin and F. Albarède Dynamic homeostasis modeling of Zn isotope ratios in the human body, *Metallomics*, 2019, 11(6), 1049–1059. https://doi.org/10.1039/c8mt00286j

39. K. Jomova, M. Makova, S. Y. Alomar, S. H. Alwasel, E. Nepovimova, K. Kuca, C. J. Rhodes and M. Valko, Essential metals in health and disease, *Chem. Biol. Interact.*, 2022, 367, 110173. https://doi.org/10.1016/j.cbi.2022.110173

40. D. Ramos, D. Mar, M. Ishida, R. Vargas, M. Gaite, A. Montgomery and M. C. Linder, Mechanism of copper uptake from blood plasma ceruloplasmin by mammalian cells, *PLoS One*, 2016, 11(3), e0149516. https://doi.org/10.1371/journal.pone.0149516

41. A. C. Ross, B. Caballero, R. J. Cousins, K. L. Tucker and T. R. Ziegler, eds. *Modern Nutrition in Health and Disease*, 11th edn. Philadelphia: Lippincott Williams & Wilkins, 2012.

42. R. Russell, J. L. Beard, R. J. Cousins, J. T. Dunn, G. Ferland, K. Hambidge, S. Lynch, J. G. Penland, A. C. Ross, B. J. Stoecker and J. W. Suttie, *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*. Washington D.C.: National Academies Press, 2001.

43. J. L. Cadiou, S. Pichat, V. P. Bondanese, A. Soulard, T. Fujii, F. Albarède and P. Oger, Copper transporters are responsible for copper isotopic fractionation in eukaryotic cells, *Sci. Rep.*, 2017, 7(1), 44533. https://doi.org/10.1038/srep44533

44. A. Tennant, A. Rauk and M. E. Wieser, Computational modelling of the redistribution of copper isotopes by proteins in the liver †, *Metallomics*, 2017, 9(12), 1809. https://doi.org/10.1039/C7MT00248C

45. C. Selden, K. Schilling, A. Basu and N. Yee, Experimental investigation of amino acid binding as a mechanism for fractionating metal stable isotopes, *Annual V.M. Goldschmidt Conference.*, 2022.

46. S. Blockhuys, E. Celauro, C. Hildesjö, A. Feizi, O. Stål, J. C. Fierro-González and P. Wittung-Stafshede, Defining the human copper proteome and analysis of its expression variation in cancers, *Metallomics*, 2017, 9(2), 112–123. https://doi.org/10.1039/C6MT00202A

47. J. R. Turnlund, Human whole-body copper metabolism, *Am. J. Clin. Nutr.*, 1998, 67(5), 960S–964S. https://doi.org/10.1093/ajcn/67.5.960S

48. M. A. Dunn, M. H. Green and R. M. Leach, Kinetics of copper metabolism in rats: a compartmental model, *Am. J. Physiol. Endocrinol. Metab.*, 1991, 261(1), E115–E125. https://doi.org/10.1152/ajpendo.1991.261.1.e115

49. F. Albaréde, *Introduction to Geochemical Modeling*. Cambridge: Cambridge University Press, 1996.

50. M. C. Linder, *The Biochemistry of Copper*. New York, NY: Plenum Press, 1991.

51. C. W. Levenson and M. Janghorbani, Long-term measurement of organ copper turnover in rats by continuous feeding of a stable isotope, *Anal. Biochem.*, 1994, 221(2), 243–249. https://doi.org/10.1006/abio.1994.1408

52. K. C. Scott and J. R. Turnlund, Compartmental model of copper metabolism in adult men, *J. Nutr. Biochem.*, 1994, 5(7), 342–350. https://doi.org/10.1016/0955-2863(94)90064-7

53. L. Heuser, N. S. Spratt and H. C. Polk, Growth Rates of Primary Breast Cancers, *Cancer*, 1979, 43(5), 1888–1994. https://doi.org/10.1002/1097-0142(197905)43:5(1888::AID-CNCR2820430545)3.0.CO;2-M

54. D. Förnvik, K. Lång, I. Andersson, M. Dustler, S. Borgquist and P. Timberg Estimates of breast cancer growth rate from mammograms and its relation to tumor characteristics, *Radiat. Prot. Dosimetry*, 2016, 169(1–4), 151–157. https://doi.org/10.1093/rpd/ncv417

55. H. Murphy, H. Jaafari and H. M. Dobrovolny, Differences in predictions of ODE models of tumor growth: a cautionary example, *BMC Cancer*, 2016, 16(1), 163. https://doi.org/10.1186/s12885-016-2164-x

56. A. Brú, S. Albertos, J. L. Subiza, J. L. García-Asenjo and I. Brú, The universal dynamics of tumor growth, *Biophys. J.*, 2003, 85(5), 2948. https://doi.org/10.1016/S0006-3495(03)74715-8

57. J. S. Spratt, R. A. Greenberg, Geometry and H. L. S.,, Growth rates, and duration of cancer and carcinoma in situ of the breast before detection by screening, *Cancer Res.*, 1986, 46(2), 970–974.

58. H. G. Welch, P. C. Prorok, A. J. O'Malley and B. S. Kramer, Breast-cancer tumor size, overdiagnosis, and mammography screening effectiveness, *N. Engl. J. Med.*, 2016, 375(15), 1438–1447. https://doi.org/10.1056/NEJMoa1600249

59. H. W. Kuo, S. F. Chen, C. C. Wu, D. R. Chen and J. H. Lee, Serum and tissue trace elements in Patients with Breast Cancer in Taiwan, *Biol. Trace Elem. Res.*, 2002, 89, 1–11.

60. E. J. Margalioth, J. G. Schenker and M. Chevion, Copper and zinc levels in normal and malignant tissues, *Cancer*, 52(5), 868–872. https://doi.org/10.1002/1097-0142(19830901)52:5(868::AID-CNCR2820520521)3.0.CO;2-K

61. A. Hastuti, M. Costas-Rodríguez, A. Matsunaga, T. Ichinose, S. Hagiwara, M. Shimura and F. Vanhaecke, Cu and Zn isotope ratio variations in plasma for survival prediction in hematological malignancy cases, *Sci. Reports 2020 101*, 2020, 10, 1–12.

62. A. Lamboux, E. Couchonnal-Bedoya, O. Guillaud, C. Laurencin, L. Lion-François, A. Belmalih, E. Mintz, V. Brun, M. Bost, A. Lachaux and V. Balter, The blood copper isotopic composition is a prognostic indicator of the hepatic injury in Wilson disease †, *Metallomics*, 2020, 12(11), 1781. https://doi.org/10.1039/d0mt00167h

63. G. Pizzino, N. Irrera, M. Cucinotta, G. Pallio, F. Mannino, V. Arcoraci, F. Squadrito, D. Altavilla and A. Bitto, Oxidative stress: harms and benefits for human health, *Oxid. Med. Cell. Long.*, 2017, 2017, 1–13. https://doi.org/10.1155/2017/8416763

64. G. Ramadori and S. Cameron, Effects of systemic chemotherapy on the liver, *Ann. Hepatol.*, 2010, 9(2), 133–143. https://doi.org/10.1016/S1665-2681(19)31651-5

65. Y. Maor and S. Malnick Liver injury induced by anticancer chemotherapy and radiation therapy, *Int. J. Hepatol.*, 2013, 2013, 1–8. https://doi.org/10.1155/2013/815105

66. A. Grigorian and C. B. O'brien, Hepatotoxicity Secondary to chemotherapy, *J. Clin. Transl. Hepatol.*, 2014, 2, 95.

67. T. Fujii, F. Moynier, J. Blichert-Toft and F. Albarède, Density functional theory estimation of isotope fractionation of Fe, Ni, Cu, and Zn among species relevant to geochemical and biological environments, *Geochim. Cosmochim. Acta.*, 2014, 140, 553–576. https://doi.org/10.1016/j.gca.2014.05.051