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REVIEW

High-precision isotopic analysis of essential mineral elements: capabilities as a diagnostic/prognostic tool

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Abstract

All elements with two or more isotopes show natural variation in their isotopic composition as a result of the isotope fractionation that accompanies (bio)chemical reactions and (bio)physical processes. Multicollector inductively coupled plasma-mass spectrometry (MC-ICP-MS) offers the precision required to reveal and quantify the differences in isotope ratios thus caused, although they are often of sub-permil magnitude only. Using MC-ICP-MS, it has been shown that (a) in different body compartments, essential mineral elements may display different isotopic compositions, and (b) disease conditions may alter the isotopic composition of an essential mineral element in a biofluid and/or tissue. As a result, high-precision isotopic analysis of these elements is a powerful way to unravel the actual role these essential mineral elements play in specific biochemical processes. Moreover, isotope ratio shifts also show promise as a diagnostic or prognostic tool. Despite the intensive sample pretreatment preceding MC-ICP-MS isotopic analysis and the high purchase and running costs of the instrumentation, this approach may be valuable, especially for diseases that can otherwise only be established at a later stage and/or via a more invasive approach. This review paper describes the basics of "biomedical isotopic analysis" and uses selected cases from the literature to sketch the state-of-art and illustrate in which context isotope ratio markers could be exploited in a clinical context.

KEYWORDS

diagnosis, homeostasis, isotope ratio, metals, multicollector ICP-mass spectrometry (MC-ICP-MS), prognosis

1 | ISOTOPES: A HISTORICAL BACKGROUND

Isotopes of a given element show the same number of protons in their nuclei and the same number of electrons in their electron cloud. They only differ from one another in the number of neutrons in their nuclei. As a result, they have the same atomic number Z, but different mass numbers A. Studies on naturally occurring radioactive elements revealed the existence of isotopes—the term introduced by

Todd and Soddy itself stems from the old Greek language and indicates that isotopes of an element can be found on the "same place" in the periodic system of the elements. By using a mass spectrograph, in 1913 Thomson experimentally separated the isotopes of Ne (^{20}Ne and ^{22}Ne), although he insisted that the "second line" in the mass spectrum had to be attributed to another gas than Ne. It was Aston who realized that the two lines indicated the existence of two "forms" of Ne. In the next 20 years, Aston in the United Kingdom and Dempster in the United States continued using mass spectrometry to experimentally demonstrate the existence of isotopes for a larger suite of elements.[1] By now, we know that only some 20 elements are naturally mononuclidic, that is, they have one naturally occurring nuclide only.[2]

Despite the fact that the concept of isotopes was already introduced more than a century ago, many chemistry courses still barely touch upon the subject, at best. The reason for this is that chemical reactions involve the valence (outer) electrons only and thus, the isotopes of an element should show exactly the same chemical behavior. For the lightest elements (H, C, N, O, and S), it was realized that as a result of the large *relative* difference in mass, the isotopes may behave to some extent differently in physicochemical processes. This slightly different behavior exhibited by the isotopes of an element leads to isotope fractionation and thus, to natural variation in the isotopic composition of these elements that can be revealed and quantified using gas source isotope ratio mass spectrometry.[3] Natural variation in the isotopic composition was also recognized for elements with one or more radiogenic nuclides, such as Sr or Pb.[4] Such a radiogenic nuclide is additionally produced as a function of time as a result of the radioactive decay of naturally occurring long-lived radionuclides (in the case of Sr, this is ^{87}Rb; in the case of Pb, ^{232}Th, ^{235}U, and ^{238}U). However, even at a university level, it is often stated that for all *other* elements (ie, all elements except the lightest ones and those with a radiogenic nuclide), the isotopes show an identical behavior. A first aim of this review is to refute this idea, which is still quite widespread.

Some form of mass spectrometry (MS) was already used by Thomson and later on by Aston and Dempster to reveal the isotopic composition of elements and as MS is one of the only approaches that provides nuclide-specific information, it is still the most commonly used tool for such investigation. However, the level of accuracy and precision attainable has been improved massively. Especially as a result of the introduction of multicollector ICP-mass spectrometry (MC-ICP-MS) in the 1990s,[5] which has facilitated getting hold of information on the isotopic composition of the elements at high precision, it is now generally accepted that all elements with two or more isotopes show natural variation in their isotopic composition as a result of isotope fractionation during the physicochemical processes they are involved in. Basic rule of thumb in this context is that the lighter of any two isotopes reacts slightly faster (lower activation energy), whereas the heavier of any two isotopes shows a slight preference for strong and hard chemical bonds at chemical equilibrium.

2 | INSTRUMENTATION FOR ISOTOPIC ANALYSIS OF ESSENTIAL MINERAL ELEMENTS

In the following, the isotopic analysis of essential mineral elements (metals) will be focused on and for those elements, thermal ionization mass spectrometry (TIMS) has been the golden standard for several decades, despite some inherent limitations. Next to a very demanding sample pretreatment (target element isolation), especially the modest ionization efficiency (only elements with an ionization potential up to 7-7.5 V are efficiently converted into M^+ ions) and the low sample throughput (partly to be attributed to the ionization in vacuum *via* resistive heating) have always hampered a (more) widespread use of TIMS.

This situation changed with the introduction of MC-ICP-MS in 1992.[5] In MC-ICP-MS, an inductively coupled plasma or ICP (known from ICP-optical emission spectrometry ICP-OES and from ICP-MS) is used as ion source. This powerful ionization source not only shows a vastly higher ionization power, but it is also generated at atmospheric pressure, such that samples (solutions) can be introduced *via* nebulization, as common in many instrumental techniques. Unfortunately, sample preparation is still rather demanding as the need for chromatographic isolation remained, as will be discussed later on.

Both TIMS and MC-ICP-MS have been and still are widely used in geo- and cosmochemistry for various purposes, for example, for geochronological dating (determination of the age of rocks or minerals) or for revealing the conditions (eg, temperature) that were prevailing during geological processes. Also, environmental studies have profited profoundly from isotopic analysis using TIMS (eg, on lead pollution) and MC-ICP-MS (eg, on lead and mercury pollution).

Based on the insight that—self-evidently—there should be no fundamental reason whatsoever why biochemical processes in the (human) body would not be accompanied by isotope fractionation, some researchers started investigating the isotopic composition of essential mineral elements in body fluids and tissues in search of natural variation. To the best of the authors' knowledge, TIMS was very rarely deployed in this context only, but the higher sample throughput of MC-ICP-MS "seduced" researchers to explore this atypical application field.

3 | BIOMEDICAL APPLICATION OF HIGH-PRECISION ISOTOPIC ANALYSIS

Before addressing this application field and discussing its capabilities and limitations, it should be stressed that this review focuses on *natural* variation in the isotopic compositions, resulting from isotope fractionation only. The use of stable isotopes in tracer experiments for studying mineral (trace) element metabolism in humans,[6] although very interesting as well, is considered beyond the scope. This is a mature field and due to substantial differences in the precision required, both the analytical approaches and instrumentation used in this context differ substantially from those in the much younger domain of biomedical application of high-precision isotopic analysis considered here. Natural variations in isotope ratios are typically *much* smaller than the changes induced artificially by making use of enriched stable isotopes in tracer experiments and are typically of the order of magnitude of one permil (‰) or smaller, only. Isotope ratio results in this context are often expressed as a delta value, that is, as a relative difference of the isotope ratio in the sample versus that in a standard, which is preferably an internationally accepted isotopic reference material (RM). The way in which such a delta value is calculated is illustrated for the $^{56}Fe/^{54}Fe$ isotope ratio below:

$$\delta^{56}Fe = \left(\frac{\left(^{56}Fe/^{54}Fe\right)_{sample}}{\left(^{56}Fe/^{54}Fe\right)_{RM}} - 1 \right) \times 1000 \quad (in‰)$$

For the elements discussed in this review paper, the international isotopic RMs used are Mg DSM3 (Dead Sea Magnesium Ltd, Israel), Ca NIST SRM 915a (National Institute of Standards and Technology, NIST, MD, USA), Fe IRMM-014 (Institute for Reference Materials and Measurements, IRMM, Belgium), Cu NIST SRM 976, and Zn IRMM-3702.[7]

Further, this review paper does also not aim at giving a comprehensive overview of *all* papers published on the topic of biomedical application of high-precision isotopic analysis. The authors have chosen to highlight applications that could be considered breakthrough papers or that might be closer (than others) to potential use in clinical practice. As this journal is focused on biodiagnostics, preference was given to papers dealing with diagnosis/prognosis rather than to papers aiming at a profound understanding of the mechanisms governing the isotopic composition of essential mineral elements in various body compartments, although such insights (would) also contribute to a more widespread use of isotopic diagnosis/prognosis. Although there are also other approaches to discuss this field (eg, chronological or based on target element), the approach followed here is based on the type of disorder targeted.

4 | IRON STATUS AND DISORDERS AFFECTING IRON HOMEOSTASIS

In their seminal 2002 Science paper, Walczyk and von Blanckenburg[8] demonstrated differences in the isotopic composition of Fe between the body compartments blood, muscle, liver, and hair, thus confirming isotope fractionation during distribution of Fe in the human body. They also found a 0.3‰ difference in the $^{56}Fe/^{54}Fe$ isotope ratio in whole blood between the male and female cohort. Later on, Jaouen and Balter[9] and Van Heghe et al[10] demonstrated independently that this difference is due to the Fe loss accompanying menstruation and the reaction of the body in an effort to compensate for these losses (vide infra). This gender-based difference disappears in postmenopausal women and was also not found in the blood of young women who did not menstruate as a result of the use of an intrauterine anticonception device releasing the hormone levonorgestrel[10].

Walczyk and von Blanckenburg also pointed out that because the Fe isotopic composition in all human body compartments they had analyzed was lighter than that in food from both animal and plant origin, Fe absorption in the intestine must favor the uptake of the lighter Fe isotopes.[8] Using a Göttingen minipig as a model, Hotz et al[11] showed later on that the mucosal tissue of the digestive system was indeed enriched in the light Fe isotopes when compared to the isotopic composition of the diet, thus suggesting that isotope fractionation occurred at the level of the enterocytes. Flórez et al[12] proved this type of fractionation to occur using Caco-2 cells as an in vitro intestinal enterocyte model.

A first disorder that was focused on in this context was hereditary hemochromatosis (HH). As there is no active excretion mechanism for Fe (some Fe is lost through hair and nails though), in healthy conditions, homeostasis is achieved by upregulating or downregulating intestinal uptake of Fe via the hormone hepcidin produced in the liver. In HH, however, the iron absorption is not being downregulated in the presence of full body iron stores, thus leading to an excessive intestinal uptake of iron. As isotope fractionation was expected to occur at the stage of intestinal uptake and this process is affected in HH, researchers expected this disorder to be reflected in the whole blood Fe isotopic composition. The higher the uptake of Fe, the lower the degree of isotope fractionation (if the uptake efficiency would be 100%, then no isotope fractionation could occur), as a result of which the continuously high uptake results in a heavier whole

blood Fe isotopic composition for HH patients (ie, closer to that of the diet). This expectation was confirmed by the experimental data obtained in a few studies.[13–15]

Hotz et al[16] and Van Heghe et al[17] showed that the Fe isotopic composition of whole blood is a good measure for an individual's iron status. In case of an upregulated intestinal Fe absorption, the degree of fractionation is lower (the absorbed Fe then shows an isotopic composition closer to that of the diet), whereas in case the intestinal Fe absorption is downregulated, fractionation occurs to a larger extent and the absorbed Fe is manifestly isotopically lighter (enriched in the lighter isotopes) than that in the diet. As a result, a light whole blood Fe isotopic composition reflects a high iron status and a low uptake efficiency, whereas a heavy whole blood Fe isotopic composition reflects a low iron status and an enhanced uptake.

This also explains the difference between males and premenopausal females[8–10] addressed earlier, as the menstrual blood loss of the latter leads to an enhanced absorption to replenish the Fe stores and thus, a lower degree of fractionation during intestinal absorption, resulting in a heavier serum Fe isotopic composition. The situation becomes a bit more complicated when taking into account the release of hepatocyte-stored Fe from the liver into the bloodstream (in the case of low Fe status) or the other way around (in the case of high Fe status) on top of the isotope fractionation during intestinal Fe absorption, but these transfers influence the isotopic composition of blood in the same way as storage Fe in the liver is isotopically heavier than blood Fe.[11]

Ohno et al[18] demonstrated that red blood cells (RBCs) do not show rapid variation in Fe isotopic composition as a function of time. The total amount of Fe in the human body is about 4.0 g for males and 2.5 g for females. About 60% of the body Fe is present in the blood, >99.8% of which in the RBCs (in hemoglobin). By taking into account these numbers, an average daily loss of Fe of about 1 mg and an average RBC lifetime of 120 days, the observation of Ohno et al can be easily understood.[13,19] In contrast, serum is a more dynamic compartment, even showing some diurnal variation,[20] but for this matrix, contamination from the RBCs has to be avoided at all cost as >99% of the whole blood Fe is present in the RBCs. Anoshkina et al[21] demonstrated that also the serum Fe isotopic composition reflects an individual's Fe status. Moreover, this parameter was shown to remain reliable in chronic kidney disease (CKD), a condition in which the parameters traditionally used to assess one's iron status (such as such as serum ferritin, transferrin saturation, and soluble transferrin receptor[22]) can no longer be trusted. As a result, a distinction could be made between Fe-deficiency anemia and erythropoietin-related anemia in CKD patients (Figure 1).

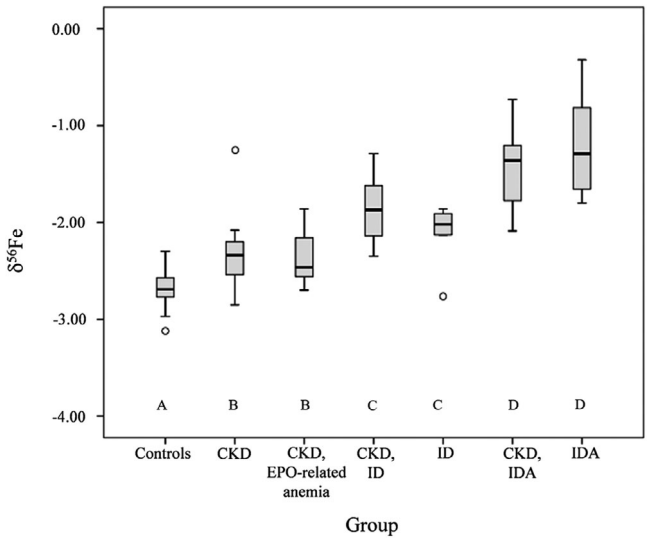

FIGURE 1 Although iron deficiency (ID, group C) and, to an even larger extent, iron deficiency anemia (IDA, group D) lead to a heavier serum Fe isotopic composition (higher δ^{56}Fe value) in both otherwise healthy individuals and chronic kidney disease (CKD) patients, erythropoietin (EPO)-related anemia has no effect on the serum Fe isotopic composition (see group B). The boxplots compile the median, quartiles, and extreme values. The open circles are outliers. Reproduced with permission from the Royal Society of Chemistry[21]

5 | BONE FORMATION AND DESORPTION: BONE DISEASES

Skulan et al[23] used TIMS for isotopic analysis of Ca (via monitoring of the ^{44}Ca/^{40}Ca isotope ratio) in urine from participants to a study concerning the effects of space flight, as mimicked by extended bed rest (17 weeks), on the human skeleton. The results obtained indicated that the urine Ca isotopic composition reflects the bone mineral balance, as determined by the relative rates of bone formation and resorption. The Ca isotopic composition in urine shifts to a heavier Ca isotopic composition (slightly enriched in the heavier isotopes) during bone formation and to lighter values (slightly enriched in the lighter isotopes) during bone resorption. Moreover, such changes are revealed more rapidly and thus with higher sensitivity by natural variation in the urine Ca isotope ratio than by bone mineral densitometry measurements with dual-energy X-ray absorptiometry. Although TIMS has the advantage that the major Ca isotope ^{40}Ca is accessible, while this is not the case with MC-ICP-MS due to isobaric overlap of the ^{40}Ca-signal with that of ^{40}Ar (coming from the plasma gas), for the benefits elaborated before, this research group also developed a MC-ICP-MS protocol suited for this purpose, by focusing on the ^{43}Ca/^{42}Ca and ^{44}Ca/^{42}Ca isotope ratios instead.[24] Morgan et al[25] have subsequently used

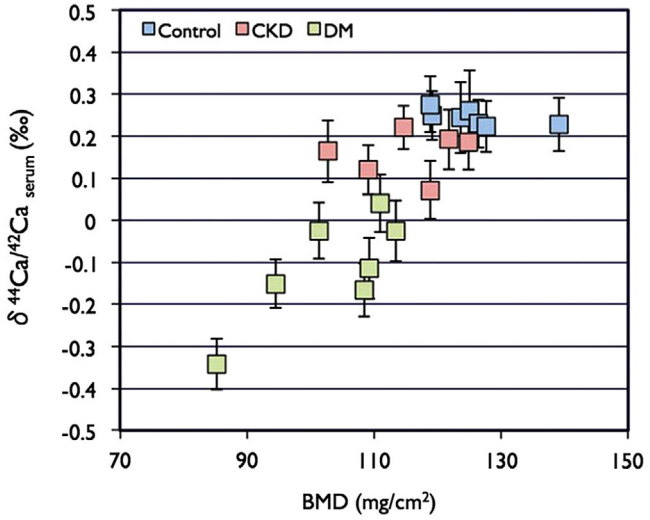

FIGURE 2 Serum Ca isotopic composition ($^{44}Ca/^{42}Ca$ isotope ratio, expressed as $\delta^{44}Ca$) versus bone mineral density for healthy rats (Control), rats with chronic kidney disease (CKD), and rats with diabetes mellitus (DM). Reproduced with permission from the Royal Society of Chemistry[28]

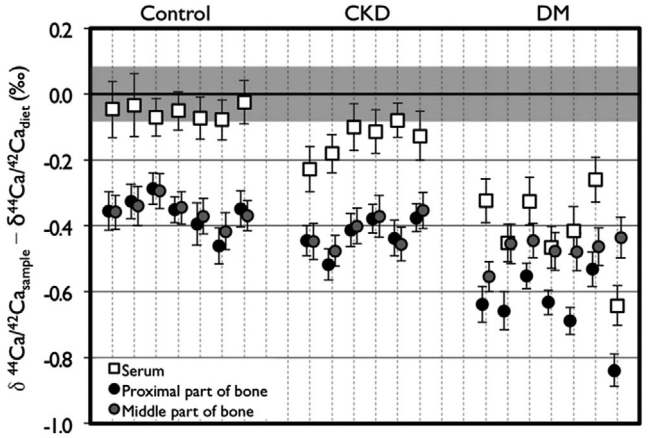

FIGURE 3 Difference between the diet $^{44}Ca/^{42}Ca$ isotope ratio (set at 0.0, see gray bar) and the $^{44}Ca/^{42}Ca$ isotope ratio in serum, the proximal part of tibia diaphysis, and the middle part of tibia diaphysis in healthy rats, rats with chronic kidney disease (CKD), and rats with diabetes mellitus (DM). Reproduced with permission from the Royal Society of Chemistry[28]

MC-ICP-MS for Ca isotopic analysis of urine samples and concluded that this approach enables metabolic bone disease to be diagnosed earlier and that the impact of treatments can be tracked more effectively than what was otherwise possible at the time. The same research group reported that there is a constant offset between the Ca isotope ratios in urine and serum, but that for both body fluids similar trends are seen upon bone loss, so that either can be used for diagnosis.[26] The same group also showed that variation in the isotopic composition of Ca in serum using MC-ICP-MS documented multiple myeloma disease activity[27] and it was stressed that while traditionally used techniques such as radiography document past damage only, the serum Ca isotopic composition reflects the current disease activity.

Tanaka et al[28] carried out an experiment with healthy rats, rats with CKD, and rats with diabetes mellitus (DM). This study once more confirmed that changes in bone turnover rate are reflected in the Ca isotopic composition of serum, thus "predicting" changes in bone volume. In healthy rats, serum shows a $^{44}Ca/^{42}Ca$ isotope ratio that is approximately 0.3 ‰ heavier than that in bone, indicating a preferential deposition of the lighter Ca isotopes in bone. Upon bone resorption, isotopically lighter Ca is released from bone tissue into the bloodstream, thus shifting the serum $^{44}Ca/^{42}Ca$ ratio to lower values. Compared to healthy animals, CKD rats showed a slightly lighter serum Ca isotopic composition, whereas the serum Ca isotopic composition was even lighter in the case of DM (Figure 2). Overall, the serum $^{44}Ca/^{42}Ca$ isotope ratio correlated very well with the bone mineral density of the tibia. Moreover, in DM, proximal and middle parts of the tibial diaphysis showed a different isotopic composition, providing an insight into the dynamics (onset) of bone resorption occurring as a side effect (Figure 3).

There is a continued interest in Ca isotopic analysis of body fluids, as is, for example, illustrated by a recent paper by Eisenhauer et al[29] assessing the possibility of diagnosis of osteoporosis in postmenopausal women based on Ca isotopic analysis of blood and/or urine.

6 | LIVER DISEASE

Wilson's disease is a genetic disorder, leading to excess copper in the body, mainly in the liver and in the brain. In contrast to the situation for Fe, where homeostasis is maintained via up/downregulation of the intestinal uptake, the removal of excess via the bile (and thus the feces) is key in Cu homeostasis. But, in Wilson's disease, a mutation in the ATP7B gene prevents this excretion of excess Cu via the bile. Although untreated Wilson's disease is lethal, the condition can be treated and kept under control by chelation. In a pilot study, Aramendía et al[30] showed that the serum Cu isotopic composition of Wilson's disease patients is lighter than that of a reference population. Although the number of samples investigated was very low, the observation that the isotopic composition of Cu in serum from infants already matched that of the adult reference population was considered important because in infants, the whole blood Cu concentration is typically low due to liver immaturity. As a result, Aramendía et al suggested that high-precision isotopic Cu analysis shows promise for screening for Wilson's disease at young age.

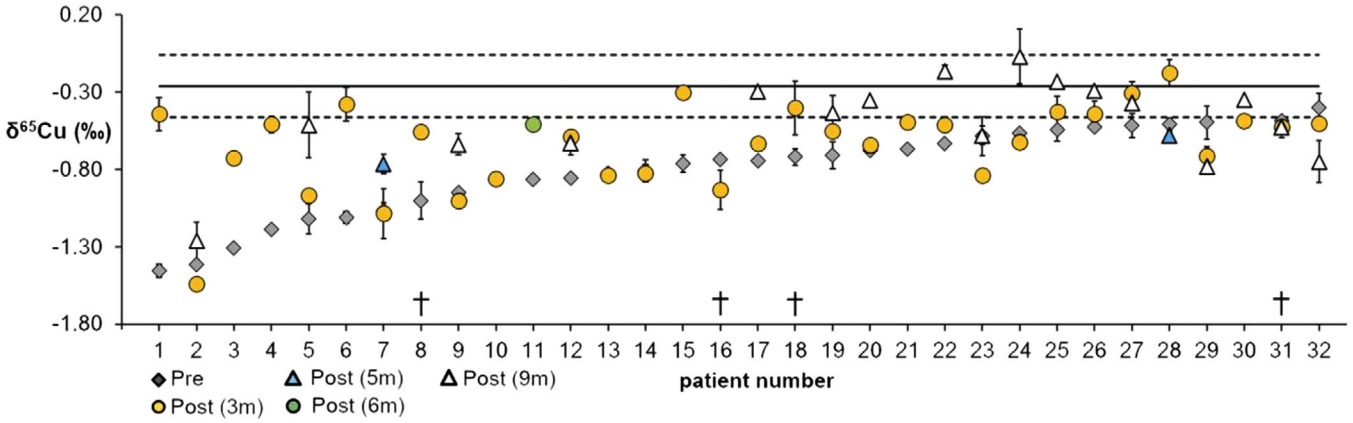

FIGURE 4 Serum Cu isotopic composition (expressed as δ^{65}Cu) for end-stage liver disease patients before (Pre) liver transplantation and at various times after liver transplantation. Crosses indicate deceased individuals. Reproduced with permission from Nature[32]

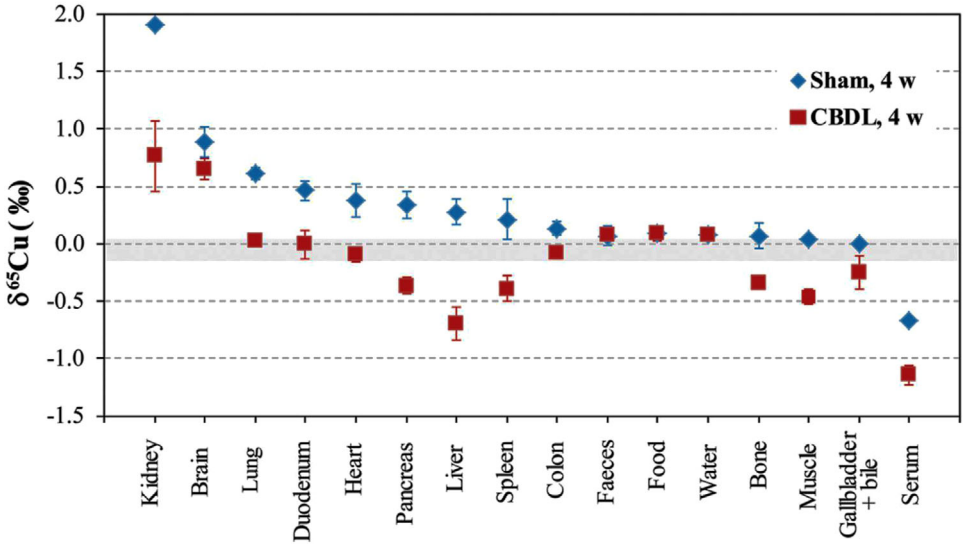

FIGURE 5 Cu isotopic composition (expressed as δ^{65}Cu) in various body compartments of mouse 4 weeks after common bile duct ligation (CBDL) or after sham operation. The gray bar represents the Cu isotopic composition of the animal chow. Reproduced with permission from the Royal Society of Chemistry[34]

Costas-Rodríguez et al[31] showed that a patient cohort suffering from liver cirrhosis of different etiologies showed a lower serum Cu isotopic composition than an age- and gender-matched control group. As the serum Cu isotopic composition correlated well with the MELD-Na score (MELD: model for end-stage liver disease), a prognostic score relied upon for assessing who should be prioritized for liver transplantation, it was concluded that the worse the condition of a patient is, the lighter his serum isotopic composition (the study was restricted to male individuals). In a follow-up study, Lauwens et al[32] first confirmed that end-stage liver disease patients systematically showed a light serum Cu isotopic composition and subsequently showed that upon successful liver transplantation the serum Cu isotopic composition is normalized in a matter of months (Figure 4). Complications after liver transplantation are reflected in a serum Cu isotopic composition that remains light or becomes even lighter than before the liver transplantation.

Both in vitro and in vivo experiments have been carried through in an attempt to identify the factors governing these shifts in the isotopic composition of Cu. Experiments with HepG2 cells, for example, pointed out that the extent of isotope fractionation upon absorption of Cu is increased under conditions of oxidative stress.[33] Systematic follow-up of the isotopic composition of Cu in various body compartments after common bile duct ligation in mice revealed a progressively lighter whole body Cu isotopic composition (Figure 5), tentatively explained by the hypothesis that excess Cu otherwise removed

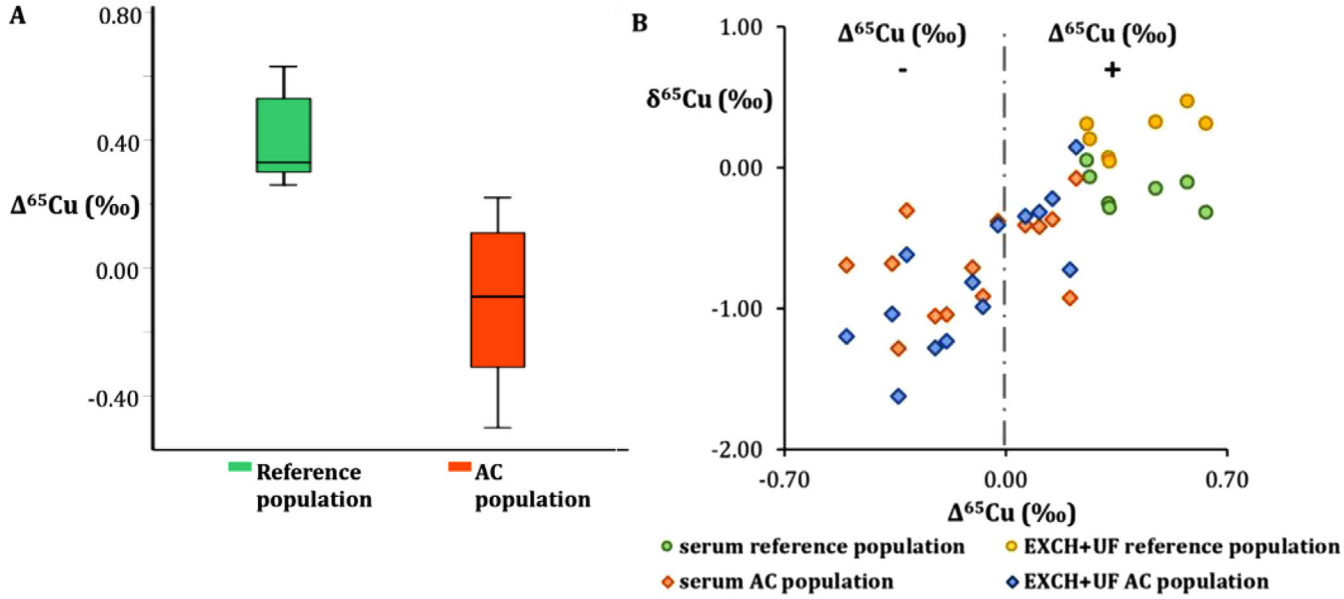

FIGURE 6 Left panel: difference between isotopic composition of Cu in the EXCH+UF fraction and that of the bulk serum Cu ($\Delta^{65}Cu = \delta^{65}Cu_{EXCH+UF} - \delta^{65}Cu_{serum}$) for alcoholic cirrhosis patients (orange) and a reference population (green). Right panel: isotopic composition of Cu in the EXCH+UF fraction and that of bulk serum versus $\Delta^{65}Cu$ as defined higher. Reproduced with permission from Elsevier[36]

via the bile and ending up in the stool is isotopically light.[34]

Nonalcoholic fatty liver disease (NAFLD) is the most widespread liver disease. NAFLD can range from simple hepatic steatosis (eg, fat accumulation and nonalcoholic fatty liver) to liver inflammation (eg, nonalcoholic steatohepatitis [NASH]), which can evolve to liver fibrosis with varying degrees of liver dysfunction. Because patients with steatohepatitis are at increased risk of fibrosis development and might even develop hepatocellular carcinoma (HCC), and no pharmacological option for any stage of NAFLD is available so far, it is of interest to detect patients early in disease progression. Van Campenhout et al[35] showed that already at the level of simple steatosis, the serum Cu isotopic composition is significantly lighter. However, the isotopic composition remains stable during further disease progression, such that it does not reflect the severity of the disease. Nevertheless, the authors point out that, as the diagnostic ability of the serum Cu isotope ratio seems to exceed that of currently used serum biomarkers, high-precision Cu isotopic analysis is a promising approach to diagnose patients with early NAFLD, such that further progression to NASH, for which no medical therapy is available so far, can be avoided.

Lauwens et al[36] carried out isotopic analysis of the bulk serum Cu and of its exchangeable and ultrafiltrable (EXCH+UF) versus nonexchangeable and nonultrafiltrable (NEXCH+NUF) fractions in healthy and alcoholic cirrhosis subjects. The NEXCH+NUF fraction corresponds to Cu firmly bound to ceruloplasmin, while the EXCH+UF fraction contains Cu loosely bound to proteins, such as albumin, alpha-2 macroglobulin, and other low-molecular-weight compounds, and is therefore also referred to as the labile Cu pool. The alcoholic cirrhosis patient cohort showed a higher EXCH+UF serum Cu concentration and a significantly lighter isotopic composition for both total serum Cu and the EXCH+UF fraction than did the corresponding reference population. For healthy individuals, the isotopic composition of the EXCH+UF fraction was systematically heavier (enriched in ^{65}Cu) than that of bulk serum (by about 0.4‰), whereas for AC patients shifts toward both a lighter and heavier isotopic composition of the EXCH+UF fraction were observed compared to bulk serum Cu (Figure 6). It is clear that when adding isotopic information on specific fractions of an essential mineral element, or on specific compounds containing the essential mineral element, more information is revealed, albeit at the cost of a more time-consuming and labor-intensive sample preparation.

7 | CANCER

Several authors have demonstrated an effect of "cancer" (self-evidently a term encompassing a vast array of diseases) on the isotopic composition of Cu and/or Zn.

By analysis of serum samples from a biobank, a pilot study by Télouk et al[37] showed that both breast cancer and colorectal cancer are accompanied by a lighter isotopic composition of Cu in serum. Moreover, colorectal cancer

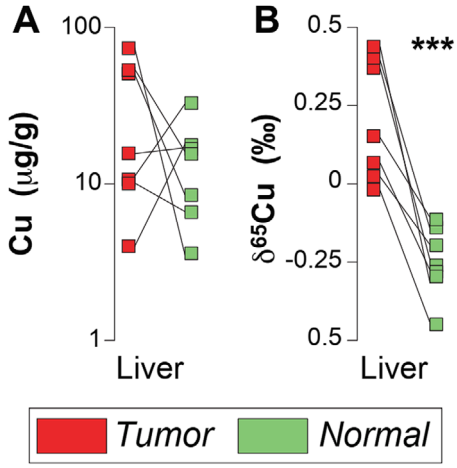

FIGURE 7 Concentration (left) and isotopic composition of Cu in tumor tissue (red) versus that in adjacent healthy (green) tissue for hepatocellular carcinoma (HCC) patients. Reproduced with permission from the National Academy of Science, USA[38]

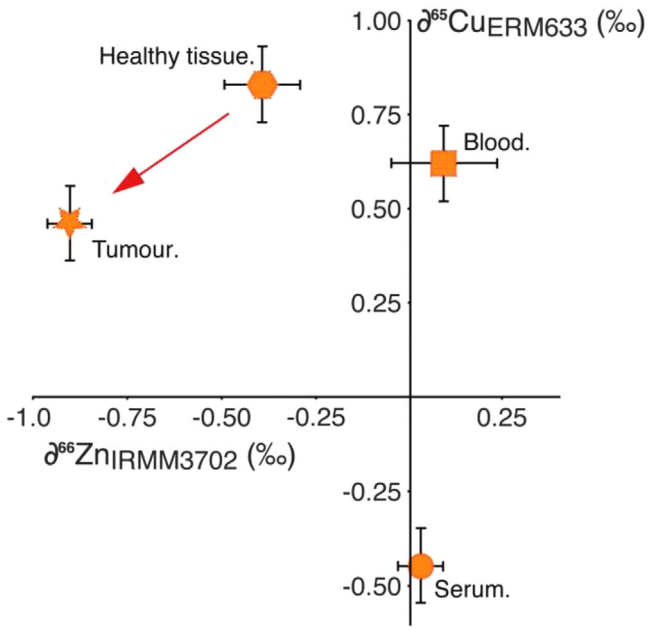

FIGURE 8 Cu and Zn isotopic compositions in whole blood, serum, tumor tissue, and adjacent healthy tissue in a breast cancer patient. Reproduced with permission from the Royal Society of Chemistry[42]

patients that deceased showed a lighter serum Cu isotopic composition than patients that survived the disease thus far. A longitudinal study observed that a significant shift in the serum Cu isotopic composition, signaling that the condition of the patient worsens, precedes a similar signal from molecular biomarkers (angiotensin converting enzyme, and cancer antigens CA 15.3 for breast cancer and CA 19.9 for colorectal cancer).

Balter et al[38] showed that HCC leads to a lighter isotopic composition of Cu (by about 0.4 ‰) in serum and RBCs. The isotopic composition of S was even affected to a larger extent with a 1.5 ‰ difference in the $^{32}S/^{34}S$ ratio in favor of a lighter isotopic composition in the case of HCC. The difference in the Cu isotopic compositions of RBC and serum between the HCC patients on the one hand and the reference population on the other was hypothetically attributed to a potential change in the redox state of Cu, thus affecting its binding to specific molecules, whereas for explaining the effect on the RBC and serum S isotopic compositions, tumor-derived sulfides were referred to. Perhaps, even more importantly, Balter et al have also analyzed biopsies of liver tumor and surrounding unaffected tissue and despite a relatively low number of samples ($N = 7$), a clear and systematic trend towards a heavier isotopic composition (enriched in ^{65}Cu) could be demonstrated for the tumor tissue versus adjacent healthy tissue (Figure 7). It is important to stress that, in contrast, the Cu concentration did not show a similar trend, as both lower and higher Cu concentrations were found in tumor tissue than in adjacent healthy tissue. Such a trend in the Cu isotopic composition was confirmed for oral squamous cell carcinoma,[39] adenocarcinoma,[20] and ovarian cancer.[40] As a heavier Cu isotopic composition in tumor tissue seems to be a robust observation, high-precision isotopic analysis might perhaps be useful in surgical tumor removal to evaluate whether all malignant tissue has been removed or to assess whether a nodule, polyp, or mass is cancerous or not.

Via the use of several human cell lines, Bondanese et al[41] demonstrated that hypoxia affects the extent of fractionation accompanying Cu uptake and leads to a heavier Cu isotopic composition in several human cell lines. Based on this observation, they hypothesized that hypoxia in the tumor environment drives the copper isotope fractionation in HCC and other solid cancers toward a local heavier isotopic composition. Toubhans et al[40] speculate that in the hypoxic tumor cell environment, the chelation of Cu by lactate preferentially binds the heavier isotope ^{65}Cu. These authors also indicate that Pt-based chemotherapy treatment further strengthens this trend as a result of overexpression of the ATP7ase gene. The isotope fractionation accompanying the transport of Cu out of the cell via the copper-transport protein ATP7A favors excretion of the light ^{63}Cu isotope.

Also, the isotopic composition of Zn in body fluids and tissues can be affected by cancer. It needs to be noted though that isotopic analysis of Zn, or more correctly the sample preparation step, is analytically more challenging due to contamination issues. For a small cohort of breast cancer patients, Larner et al[42] found a lighter Zn isotopic composition in breast tumor tissue than in healthy tissue (Figure 8). Schilling et al reported that the isotopic composition of urine Zn in pancreatic cancer is shifted to a lower

value.[43] By calculating hazard ratios and constructing time-dependent receiver operating characteristic (ROC) curves, Hastuti et al[44] demonstrated the isotopic composition of Cu and Zn in plasma to be useful for the prediction of survival among hematological malignancy patients. Patients displaying a lighter Cu isotopic composition and a heavier Zn isotopic composition in plasma showed the poorest survival. Metal concentrations did not produce similar information and also the traditionally used laboratory test values were not successful in identifying this high-risk group. Finally, time-dependent ROC curves based on the isotopic composition of plasma Cu or Zn were similar to that based on the creatinine concentration (a well-known prognostic factor in hematological malignancy).

8 | NEURODEGENERATIVE DISORDERS

A number of research groups have also started exploring the diagnostic capabilities of high-precision isotopic analysis of essential mineral elements in neurological disorders.

Büchl et al demonstrated that the expression of a single protein—in casu the prion protein PrP—can affect the isotopic composition of metals in mouse brains.[45] The misfolded isoform of this protein PrP^{Sc} is involved in fatal neurological disorders such as Scrapie and Creutzfeldt-Jacob's disease. Cellular prion protein PrP^{C} is expressed in a variety of tissues in the body, with the highest levels in the brain. PrP^{C} binds Cu with high affinity, linking it with the Cu metabolism. Büchl et al reported that mice brains expressing mutant PrP lacking the known Cu-binding domain (by genetic manipulation) had δ^{65}Cu values of on average 0.57 ‰ higher than wild-type mouse brains and suggested high-precision isotopic analysis of metals to be a promising tool for examining processes leading to brain damage and disease and for unraveling the pathways by which metals are transported through the (animal) body. Also Miller et al[46] studied Cu isotope ratio variations in the context of prion diseases. Cu isotope ratios were measured in various body compartments of mice with a targeted disruption of the prion gene of the Zurich I strain (PrP^{C} knockout mice) and the corresponding wild type. Systematic differences in the Cu isotope ratio were observed for liver tissue between the wild-type and the PrP^{C} knockout mice. Further scrutinization of the results also revealed that the difference between the serum and liver Cu isotope ratio and between the serum Cu isotope ratio and that in specific parts of the brain (hippocampus, cerebral cortex, and brainstem) was affected, indicating widespread changes in copper isotopic distribution in the transgenic mouse.

Sauzéat et al[47] analyzed cerebrospinal fluid (CSF) from patients suffering from amyotrophic lateral sclerosis (ALS), patients suffering from Alzheimer's disease (AD), and age-matched controls. Although the isotopic composition of Zn did not show significant differences between the groups, that of Cu was significantly heavier for the ALS group. The different observations for Zn and Cu are attributed to Zn being redox-inactive, whereas ionic Cu can occur in two oxidation states—Cu(I) and Cu(II). These authors concluded that Cu is implicated in ALS and refer to abnormal protein aggregation in the brain parenchyma as the possible origin of the shift in the isotopic composition of Cu. It is claimed that high-precision Cu isotopic analysis is a promising tool that can help understanding the molecular mechanisms involved in the disease.

Moynier et al[48] investigated the possible effects of AD on the isotopic composition of Zn in brain, serum, and RBCs from APPswe/PSEN1dE9 transgenic mice, which develop Alzheimer's-like disease (including Aβ deposition starting after 6 months), in comparison to wild-type controls. The motivation behind this study came from the assumption that the formation of deposits of Zn-rich amyloid-β fibrils in AD would have an effect on the homeostasis, and thus possibly also on the isotopic composition of Zn in brain tissue and body fluids. In wild-type mice, the isotopic composition of brain Zn was established to become lighter with aging, an observation hypothetically attributed to the deterioration of brain cells. Brain tissue of AD mice showed a heavier isotopic composition than that of the controls, thus signaling the involvement of Zn in the disease. Unfortunately, the isotopic composition of Zn in blood was not significantly affected upon development of AD, thus limiting diagnostic possibilities. In a later paper, Moynier et al[49] also studied the isotopic compositions of Cu in brains and serum of APPswe/PSEN1dE9 mice versus wild-type mice, because Cu is involved in the formation of Aβ fibrils. No significant differences in Cu isotopic composition could be established. Solovyev et al[50] used two murine AD models—AD transgenic male tau (Line 66, L66) and amyloid/presenilin (5xFAD) mice—and compared Fe, Cu, and Zn isotope ratios in brain tissue and serum with those in the corresponding age-matched wild-type control mice. The major observation was a significantly lighter Fe isotopic composition in the brains of L66 mice compared to controls.

Larner et al[51] characterized the isotopic composition of Cu in the Cu-containing protein superoxide dismutase (SOD) and the metallothionein (MT) fraction in post-mortem human frontal cortices. SOD was enriched in the heavier ^{65}Cu isotope relative compared to the MT fraction and to bulk brain homogenate. The binding of Cu through the nitrogen ligands of histidine residues in SOD leads

to an enrichment in the heavy ^{65}Cu isotope, whereas the binding of Cu via the cysteine-based sulfur ligands in MTs generates a preference for the light ^{63}Cu isotope. This type of protein-specific isotopic analysis provides yet another level of information that can be exploited for unraveling the role of essential mineral elements in health and disease.

Costas-Rodríguez et al[52] studied Cu isotope fractionation in the human neuroblastoma SH-SY5Y cell line, in a proliferating/tumor phase (undifferentiated cells), and in a differentiated state (neuron-like cells). This differentiation was induced using retinoic acid and drives the cell line toward phenotypes suitable for the research of neurological diseases (eg, AD or Parkinson's disease). Cu isotopic analysis was performed at cellular and subcellular levels and a first finding was that both undifferentiated and differentiated cells became systematically enriched in ^{63}Cu with increasing intracellular Cu content, with the mitochondria being isotopically lighter (even more enriched in ^{63}Cu). Differentiated cells showed a significant difference in Cu isotopic composition with respect to undifferentiated cells, but no difference was established for the mitochondria. Based on these observations, the authors suggested that high-precision Cu isotopic analysis can be an interesting tool for studying Cu metabolism at a (sub)-cellular level in functional neurons. Paredes et al[53] studied variations in the isotopic composition of Cu in protein fractions from lysates of differentiated SH-SY5Y cells exposed to U in vitro, as the brain is a sensitive target to this toxic element. Disparities among the protein fractions, attributed to differences in the coordination sphere and oxidation state of Cu in the proteins, open a way to obtain insight into stress-induced alteration of metabolic processes involving Cu, as well as to provide a deeper understanding of the mechanisms of disease.

It is clear that in the field of neurological disorders, the use of high-precision isotopic analysis is in a very early phase only. For further research aiming at understanding the processes involving essential metals, several areas of the brain will need to be investigated separately. For evaluating the diagnostic capabilities in this context, CSF and serum should be focused on. Especially analysis of CSF (obtained by lumbar puncture) poses analytical challenges, as a result of the limited volume of CSF typically available and the low concentration of some of the essential mineral elements in this matrix. These constraints also enhance the risk of contamination.

9 | DIABETES MELLITUS

So far, natural isotope ratio variations of Mg have received less attention, but recently, Grigoryan et al[54] showed a quite pronounced and systematic difference in the serum Mg isotopic composition between diabetes type I patients and a control group consisting of age- and gender-matched individuals. The robustness of this observation was demonstrated by analyzing serum samples from the same patients, sampled 1 year later. Although the observation at "cohort level" remained identical, individual patients sometimes showed significant differences between their year-1 and year-2 data. Further investigation is required to unravel the underlying reason for this in order to extract all information embedded in the isotopic signature.

10 | OUTLOOK

From all of the above, it is clear that isotope ratios in biofluids do hold a relevant message. It is therefore clear that high-precision isotopic analysis is a powerful and versatile tool for unraveling the role of essential minerals in biochemical processes. This approach is complementary to other high-end analytical techniques providing information on essential mineral elements, such as laser ablation ICP-MS, which is capable of quantitatively documenting the two-dimensional and sometimes even three-dimensional distribution of essential mineral elements across tissues with an optimum spatial resolution of ≤ 1 μm in a quantitative way,[55–58] X-ray absorption techniques—such as X-ray absorption near edge structure and extended X-ray absorption fine structure—that can provide in situ information on the oxidation state and chemical environment of such an essential mineral element,[56,59] and synchrotron-based X-ray fluorescence spectroscopy that provides an even better spatial resolution (some tens of nm) for bioimaging applications.[56,60]

But what about actual clinical use of high-precision isotopic analysis of essential mineral elements? Although this review paper hopefully makes clear that diagnostic/prognostic promise has been demonstrated in a number of contexts, to the best of the authors' knowledge, actual clinical applications are still very scarce if any. Below, is outlined what is actually withholding actual clinical use according to the authors.

For the most important findings, a more systematic approach involving a substantially higher number of patients is recommended, especially as the medical world is used to studies involving a much higher number of subjects than reported in these papers on isotopic analysis. It should be noted, however, that isotope ratios are more "robust" than concentrations and even tiny differences (of the order of 0.1 ‰) can be revealed and reproducibly quantified. Moreover, these larger studies should be ideally multicentered, meaning that both different hospitals, ideally

located in different geographical areas, and different labs should participate.

Efforts should also no longer be solely exploratory, aiming at revealing possible differences between the isotopic composition of one or more essential mineral elements between patients and an appropriate reference population, but should also focus on identifying and understanding the factors driving these changes in isotopic composition, as this will bring clinical confidence and move the field away from an interesting but somewhat curious novelty. Obtaining a profound insight will entail the use of in vitro cell experiments,[12,33,41,52,61] in vivo experiments with animals,[11,28,34,45,46,48-50,62, 63] and theoretical calculations[64–67] and the studies will have to be executed by interdisciplinary teams. For this unraveling of the factors governing the isotopic composition of essential mineral elements in various body compartments and in health and disease, complex processes will also have to be "dissected" and isotope fractionation needs to be studied on the level of subcellular compartments,[52] and on that of individual processes involving specific proteins or protein fractions.[36,51,53]

Another factor currently tempering clinical interest is the time-consuming and labor-intensive sample preparation and the relatively high purchase and running costs of the instrumentation. It is clear that for wider clinical acceptance, sample preparation protocols should be simplified[68,69] and/or automated.[70] Although MC-ICP-MS instruments are now versatile instruments, manufacturers could perhaps also focus on less expensive, simpler, and dedicated units for the clinical lab.

Also the specificity of the message embedded in isotope ratio shifts should be evaluated. As an example, both liver disease[30–32,35,36] and cancer[37,38,40] were reported to affect the isotopic composition of Cu in serum. Chemotherapy could, for example, also put a huge strain on the liver, which might contribute to the shift in the whole blood/serum isotope ratio observed. However, some studies explicitly report that the cancer patients did not receive any chemotherapeutic treatment at the time of sampling,[39,44] whereas the observation of a local heavier isotopic composition in tumoral tissue has been a consequent observation.[20,38–40]

Further fundamental research is thus required and is ongoing and from the current literature, it is clear that an increasing number of enthusiastic research teams are working in this nascent field. Therefore, it can be expected that if high-precision isotopic analysis can be shown capable of diagnosis of an important disorder that cannot be picked up in any other way or via a significantly more invasive approach only, it will eventually find its way into the clinic.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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