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What's up doc?—High-precision isotopic analysis of essential metals in biofluids for medical diagnosis

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Introduction

The introduction of mass spectrometers permitting measurement of isotope ratios with higher precision has changed our view on the isotopic composition of elements.[1] Of course, scientists were aware that when one or more of the isotopes of an element is/are radiogenic—i.e. formed by decay of a naturally occurring and long-lived radionuclide—its isotopic composition shows natural variation. This is, for example, the case for Sr (^{87}Sr is the daughter nuclide formed upon β^--decay of ^{87}Rb) and for Pb (^{206}Pb, ^{207}Pb and ^{208}Pb are the stable end products of the decay chains of ^{238}U, ^{235}U and ^{232}Th, respectively). Isotopic analysis of these elements is carried out in the context of, for example, geochronological dating and provenance determination, i.e. determination of the geographical origin of, for example, the raw materials used in the manufacturing of a historical object, agricultural products of plant or animal origin or environmental pollution.[2] For the light elements, it was also realised that the isotopic composition is variable due to isotope fractionation effects accompanying physical processes and (bio)chemical reactions. The lighter of two isotopes reacts slightly faster (kinetic effect), the heavier one shows a slight preference for the stronger bonding environment (thermodynamic or equilibrium effect). Isotopic analysis of H, C, N, O and S accomplished via gas source isotope ratio mass spectrometry (IRMS) has proven its utility over a wide application range. However,

more recently, it has been demonstrated that isotope fractionation actually causes *all* elements with two or more isotopes to show natural variation in their isotopic composition and that an element's isotopic analysis can be exploited in a wider variety of applications.

The introduction of multi-collector inductively coupled plasma-mass spectrometry (MC-ICP-MS) in the 1990s played a pivotal role in this context.[3] Before, thermal ionisation mass spectrometry (TIMS) was the gold standard for isotopic analysis of the "heavier" elements, but the low sample throughput of this technique and the limitation to elements with an ionisation energy $\leq 7.5\,eV$ (at least when M$^+$ formation is considered) prevented analysis of larger sample collections and excluded some elements from being analysed. The ICP is characterised by a much higher ionisation efficiency and is—in contrast to the ion source with TIMS—operated at atmospheric pressure, thus enabling straightforward sample introduction, typically by means of solution nebulisation. The simultaneous monitoring of the ion beams of interest via an array of Faraday cups corrects to a large extent for the "noisy" character of the ICP source, thus providing an isotope ratio precision down to 0.002% RSD under optimum conditions. This precision is sufficient to study the natural variation in the isotopic composition of metallic and metalloid elements due to isotope fractionation effects. While for some applications, TIMS might still be superior, most

of the isotope ratio papers published today report on results obtained using MC-ICP-MS.

The isotopic composition of metallic and metalloid elements typically varies within very narrow ranges only. Most often, the isotope ratio within the sample investigated ($^{n1/n2}R_{sample}$) is expressed as a relative difference to that of an isotopic reference material ($^{n1/n2}R_{standard}$) in permil:

$$\delta^{n1/n2}R = \frac{^{n1/n2}R_{sample} - {}^{n1/n2}R_{standard}}{^{n1/n2}R_{standard}} \times 1000 \ (‰)$$

To a large extent, applications based on isotopic analysis via MC-ICP-MS are to be found in geo- and cosmochemistry. However, a small number of research groups worldwide are currently investigating the possibility of using high-precision isotopic analysis of metals in a biomedical context. The final goal is to develop methods for medical diagnosis on the basis of isotopic analysis of mineral elements in biofluids, for diseases that can otherwise only be established at a later stage or via a more invasive method (e.g., a biopsy) and/or for prognosis purposes.

Isotopic analysis of biofluids

The start of this research field was a publication by Walczyk and von Blanckenburg in *Science* in 2002, in which they reported that Fe in whole blood is isotopically lighter than in the diet and that the male cohort of the population studied showed a lighter isotopic composition than did

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the female cohort.[4] Independently from one another, researchers at both Ghent University (Belgium) and the Ecole Normale Supérieure de Lyon (France) demonstrated this gender-based difference to result from the response of the organism to the Fe loss accompanying menstruation.[5,6] While in the latter paper, only post-menopausal women were considered,[6] the former[5] also included data for young women, who were not menstruating as a result of the use of a hormone-releasing intra-uterine anti-conception device, thus excluding age as the governing factor. In contrast, for the same reason, whole blood Cu is isotopically heavier in men than in women.

An especially interesting application could be the assessment of one's Fe status via isotopic analysis of the whole blood or serum Fe. Conventional diagnosis of Fe-related health problems currently relies on the measurement of the serum Fe concentration, the transferrin saturation (transferrin is the chaperon protein binding Fe for its transport via blood) and ferritin (ferritin is the form under which Fe is stored in the liver). However, inflammatory conditions, for instance, will influence these parameters, such that the Fe status cannot always be adequately assessed in this way, leaving a large number of patients at risk for Fe depletion or Fe overload. Researchers from the National University of Singapore and ETH Zürich (Switzerland) reported that the natural isotopic composition of whole blood Fe is an indicator of the efficiency of dietary Fe absorption,[7] while researchers from Ghent University have shown that, for a reference population (assumed healthy), the isotopic composition of whole blood Fe correlates well with the serum ferritin level, typically seen as a measure for the amount of Fe stored in the liver.[8] Moreover, it was also shown that patient groups suffering from haemochromatosis, a hereditary disease characterised by excessive Fe uptake, or from anaemia of chronic disease (ACD), a form of anaemia seen in chronic inflammation, display deviating whole blood Fe isotopic compositions, biased heavy[8,9] and light,[8] respectively. Further research is required to investigate whether Fe isotopic analysis can be deployed instead of or as a
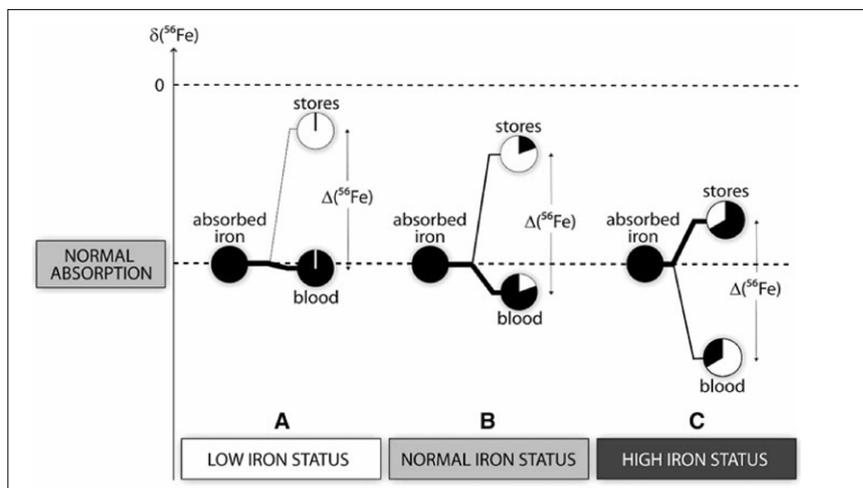

Figure 1. Link between an individual's iron status and the isotopic composition of his/her whole blood Fe (expressed as $\delta^{56/54}$Fe). The isotopic composition of the absorbed Fe is assumed identical in the three cases depicted. The lower the Fe status, the larger the fraction of the absorbed Fe used for erythropoiesis (i.e. the production of red bloods cells or erythrocytes). The higher the Fe status, the larger the fraction of the absorbed Fe stored in the liver under the form of ferritin. Due to the isotope fractionation accompanying storage (liver Fe is isotopically lighter than blood Fe), the blood Fe isotopic composition is governed by the fraction of the absorbed Fe that is stored and thus, by the individual's Fe status. Taken from Reference 10 with kind permission from Springer Science and Business Media. Other papers[7,8] rather suggest that the extent of isotope fractionation accompanying intestinal Fe uptake is affected by the degree of Fe absorption. When the uptake is upregulated (low Fe status or hemochromatosis) there is less fractionation and the isotopic composition of the absorbed Fe is closer to that of the diet. The more the uptake is down regulated, the lighter the isotopic composition of the absorbed Fe. Up/down regulating of the Fe absorption is regulated by the protein hepcidin, synthesised in the liver.

complementary tool to the parameters currently used for Fe status assessment (Figure 1).

In a NASA-funded bed rest study, researchers at Arizona State University (US) have shown that Ca isotope ratios measured in urine indicate changes in bone mineral balance more rapidly than currently used biomarkers and especially than bone density changes as measured using X-ray densitometry (Figure 2).[11] While the aim of this study was to predict bone loss in astronauts during long travels in space, it is clear that high-precision urine Ca isotopic analysis via MC-ICP-MS can also provide invaluable information in the case of diseases affecting bone metabolism, such as osteoporosis or multiple myeloma.

Also isotopic analysis of Cu has shown promise. While for Fe, homeostasis is achieved via up/down regulating of the absorption in the intestine (there is no active mechanism of excretion), for Cu, the uptake efficiency cannot be steered. Excess Cu, however, can be removed via bile, except in individuals with obstruc-

tion of the bile ducts (cholestasis) or with Wilson's disease. In the latter case, the excess Cu is predominantly stored in the liver and the brain, causing all kinds of problems and eventually death if the patient is not treated. With a quite simple treatment, chelation of the free Cu ions, Wilson patients can have a quasi-normal life. Therefore, early diagnosis is of the utmost importance. Researchers from the University of Zaragoza (Spain) and of Ghent University have demonstrated that the isotopic composition of serum Cu is measurably lighter in Wilson patients.[12]

Researchers from the Ecole Normale Supérieure de Lyon have monitored the isotopic composition of Cu in serum from breast cancer and colorectal cancer patients under chemotherapeutical treatment as a function of time.[13] It was found that a shift in the Cu isotopic composition towards lower $\delta^{65/63}$Cu values indicated a deterioration of the condition. The fact that this shift in the isotopic composition of serum Cu preceded the change in currently used cancer biomarkers, such as CEA (carcinoembryonic antigen) and CA
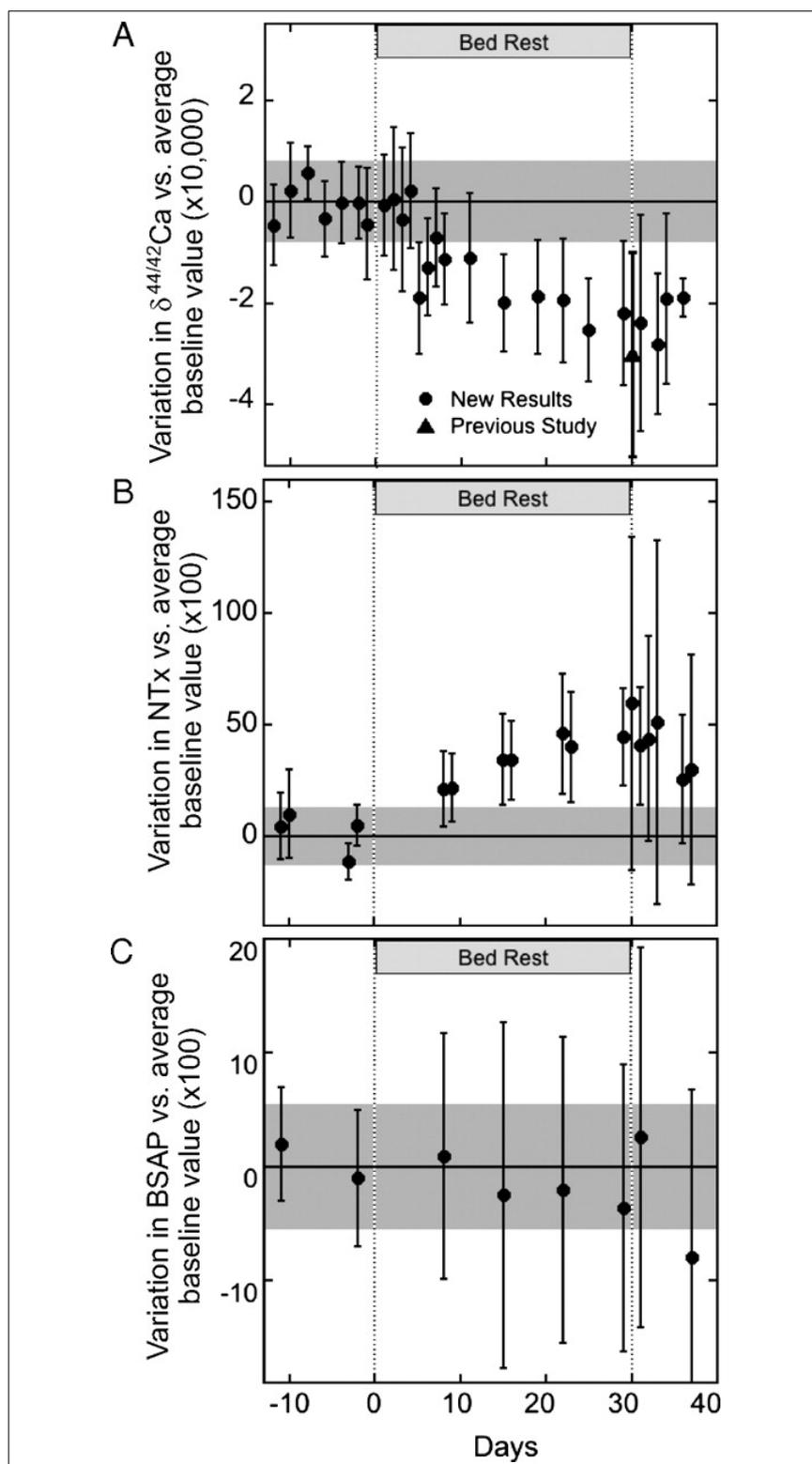
Figure 2. Variation of the urine Ca isotopic composition (expressed as $\delta^{44/42}$Ca), the N-terminal telopeptide (NTx) biomarker and the bone specific alkaline phosphatase (BSAP) biomarker during a 30-day bed rest study. Taken from Reference 11 with kind permission of PNAS. The onset of a negative bone mineral balance shifts the urine Ca isotopic composition to lighter values. While the biochemical markers reflect one process only, either bone resorption (NTx) or formation (BSAP), the urine Ca isotopic composition reflects the net effect of a change in the bone mineral balance.

15.3 (cancer antigen 15-3), is especially noteworthy. The observed shift in the Cu serum isotopic composition is attributed to the so-called Warburg effect, according to which cancer cells predominantly produce energy by a high rate of glycolysis, followed by lactic acid fermentation in the cytosol, thus giving rise to lactate that can chelate Cu. As in the lactate chelates, the heavier Cu isotope is slightly enriched, serum containing Cu excreted into the blood stream is becoming isotopically lighter.

Also in the case of liver cirrhosis, the isotopic composition of serum Cu seems to have prognostic capabilities. A patient group of middle-aged female cirrhosis patients showed a markedly lighter isotopic composition of serum Cu than did a gender- and age-matched control group (Figure 3). In the context of liver disease, medical doctors would benefit from a—preferably fully objective—prognosis score, to predict the mortality risk in end-stage liver disease and thus, to prioritise liver transplants. Currently, the Model for End-stage Liver Disease score (MELD), proposed by the Mayo Clinic in 2001, is one of the most frequently used. The MELD-score can be objectively numerically calculated, but has to be corrected into the MELD-Na score to also work for patients suffering from ascites (fluid retention in the abdomen). A pretty convincing link between the isotopic composition of serum Cu and the MELD-Na score was established, indicating that a shift towards a lighter serum Cu isotopic composition seems to indicate a higher mortality risk.[14]

While also the isotopic composition of Zn has been investigated in biofluids in several of the studies referred to above, no clear trends were reported thus far. However, also this element should not yet be dismissed as researchers from the University of Oxford (UK) recently showed that its isotopic composition in breast cancer tissue differs measurably from that in the corresponding healthy tissue.[15]

Summary and outlook

It should be clear that the research on isotopic analysis in a biomedical context is in a very early stage. It is known that various diseases have an influence on the uptake, metabolism and/or excre-
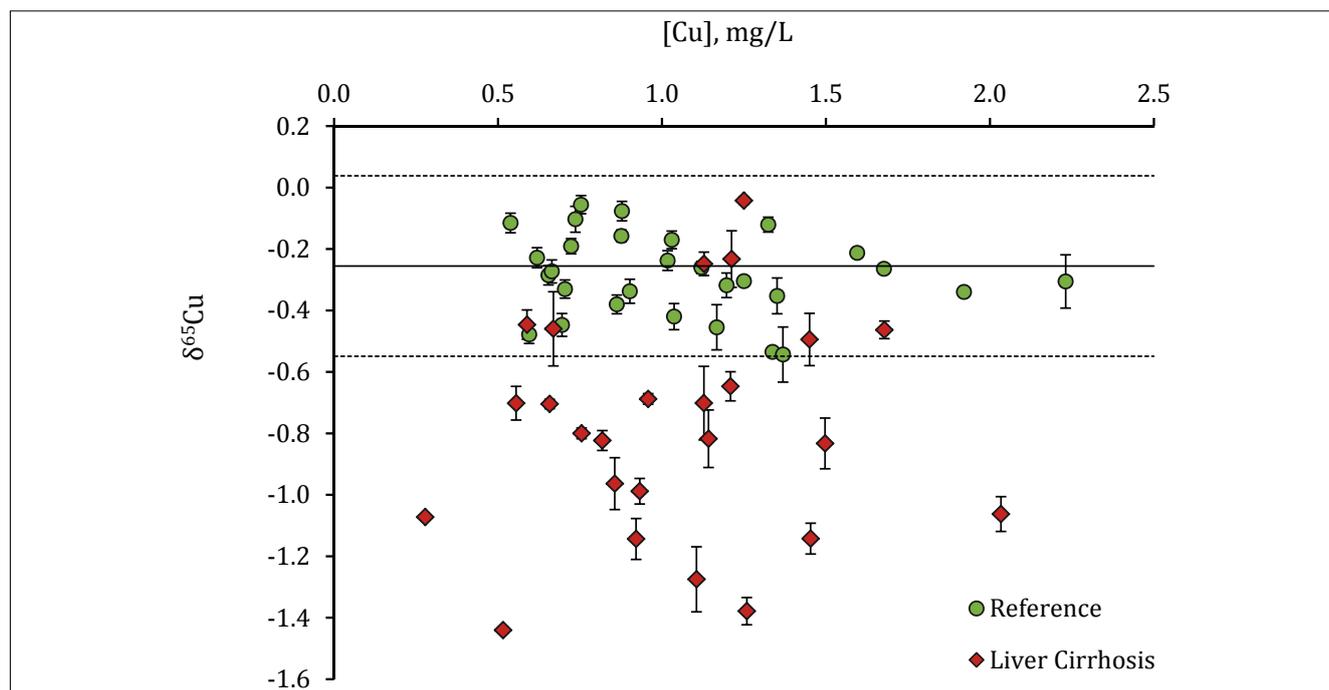
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Figure 3. Isotopic composition of serum Cu (expressed as $\delta^{65/63}$Cu) versus serum Cu concentration for a population of middle-aged female liver cirrhosis patients (red diamonds) and a gender-and age-controlled reference group (green circles). The solid line indicates the average $\delta^{65/63}$Cu value, the dotted lines indicate ±2 times the standard deviation. While the disease does not seem to affect the serum Cu concentration, it leads to a measurably lighter serum Cu isotopic composition.

tion of essential mineral elements and thus, can cause a difference in their isotopic composition in biofluids. What is currently lacking is a thorough understanding of the underlying mechanisms. Such a profound understanding will require a combination of extensive *in vivo* and *in vitro* experimental research, mass balance calculations and theoretical modelling. Some applications might seem to be close to clinical implementation, but a more complete view on the capabilities and limitations of such approaches will take many years of intense research.

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