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Deciphering the iron isotope message of the human body

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Abstract

Mass-dependent variations in isotopic composition are known since decades for the light elements such as hydrogen, carbon or oxygen. Multicollector-inductively coupled plasma mass spectrometry (MC-ICP-MS) and double-spike thermal ionization mass spectrometry (TIMS) permit us now to resolve small variations in isotopic composition even for the heavier elements such as iron. Recent studies on the iron isotopic composition of human blood and dietary iron sources have shown that lighter iron isotopes are enriched along the food chain and that each individual bears a certain iron isotopic signature in blood. To make use of this finding in biomedical research, underlying mechanisms of isotope fractionation by the human body need to be understood. In this paper available iron isotope data for biological samples are discussed within the context of isotope fractionation concepts and fundamental aspects of human iron metabolism. This includes evaluation of new data for body tissues which show that blood and muscle tissue have a similar iron isotopic composition while heavier iron isotopes are concentrated in the liver. This new observation is in agreement with our earlier hypothesis of a preferential absorption of lighter iron isotopes by the human body. Possible mechanisms for inducing an iron isotope effect at the cellular and molecular level during iron uptake are presented and the potential of iron isotope effects in human blood as a long-term measure of dietary iron absorption is discussed.

Keywords: Multicollector-ICP-MS; TIMS; Iron; Isotope fractionation; Iron metabolism

1. Isotope effects in basic and applied research

Stable isotope abundances of most elements are highly constant in nature but they are not invariant. Element transfer between physical and chemical compartments can alter the isotopic composition of an element slightly provided that the transfer process is incomplete and sensitive to the isotope's masses. This phenomenon, known as mass-dependent isotope fractionation, has been known since decades for the lighter elements such as hydrogen, carbon, oxygen, and nitrogen. These elements are now used widely in a number of scientific disciplines such as the Earth and environmental sciences, plant science and food science [1]. In their pioneering studies, De Niro and Epstein measured changes in carbon and nitrogen isotopic composition along the food chain [2,3]. They found that there is only minor fractionation of carbon isotopes by animals, and that the carbon isotopic composition of body tissues closely reflects that of their diet. $^{13}C/^{12}C$ in animal tissue typically increases by ca. 1‰ in the heavier isotopes compared with the animal's diet. Nitrogen isotopes show a similar phenomenon, with an increase in the $^{15}N/^{14}N$ isotope ratio by about 2–3‰ per trophic level (see Fig. 1) [4].

Until very recently it appeared impossible to resolve isotope effects in nature for the heavier elements. Isotope fractionation principles dictate that isotope effects become smaller with increasing atomic weight as fractionation processes are governed by the relative and not the absolute mass differences of the isotopes of an element. Although it has been hypothesized for decades that isotope effects must exist in nature for the heavier elements too, they were well beyond detection limits. The situation has changed in recent years with the development of new mass spectrometric techniques, namely multicollector inductively coupled plasma mass spec-

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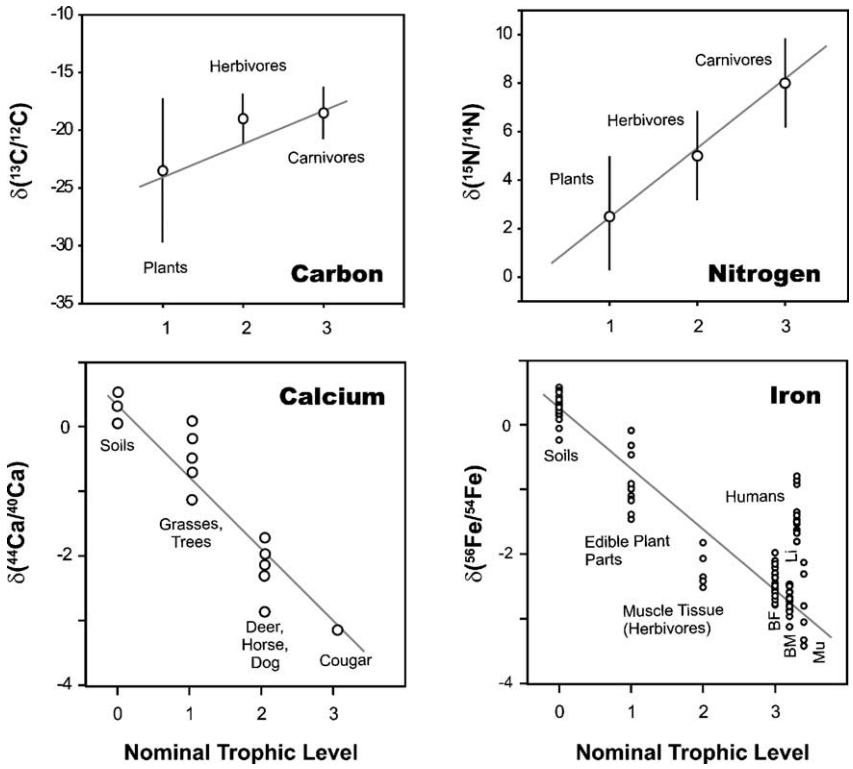

Fig. 1. Isotope fractionation along the food chain. Carbon and nitrogen isotopes were measured in bone collagen [4], calcium isotopes in soils, plants and animals ([6,7], reprinted with permission by D. DePaolo). Iron isotopes were measured in human blood, liver, muscle tissue and dietary iron sources by Walczyk and von Blanckenburg [14], with some added blood and tissue samples from this study and new data on soils and vegetables (von Blanckenburg, unpublished data); BF: blood female; BM: blood male; Li: liver tissue; Mu: muscle tissue. Isotope ratios are plotted as δ-values (for definition, see Section 2.1), i.e., as relative deviations in parts per thousand relative to a reference material. Carbon and nitrogen are fractionated towards an enrichment in their heavier isotopes as they proceed through the food chain, probably due to preferred respiration of light C and N from the lung [2,3]. Iron and calcium are fractionated towards an enrichment in their lighter isotopes along the food chain, pointing to a preferred uptake of lighter isotopes for essential minerals and trace elements.

trometry (MC-ICP-MS) and double spike thermal ionization mass spectrometry (TIMS). These techniques now permit stable isotope ratio measurements at precisions of better than 0.1‰ for the heavier elements which is sufficient to resolve even small differences in isotopic composition between samples [5]. Interestingly, this development has taken place entirely within the Earth Science community, pioneered mainly by geochemists. Resulting from these research activities, the $^{44}Ca/^{40}Ca$ isotope ratio was found to decrease by about 1‰ with each trophic level (Fig. 1, adapted from [6,7]). This was explained by the balance between calcium incorporated into bones and that incorporated into soft tissue [7].

Similar to calcium, iron exhibits relatively large relative mass differences between its four stable isotopes which occur in nature at abundances of 5.8% (^{54}Fe), 91.8% (^{56}Fe), 2.1% (^{57}Fe) and 0.3% (^{58}Fe). Earlier studies indicated that iron isotope effects may exist in the geosphere and in the biosphere [8,9] but only as recently as 1999 Beard and Johnson were able to determine small iron isotope effects in natural samples [10]. A considerable database has been produced since then showing significant variations of up to 3‰ in the $^{56}Fe/^{54}Fe$ ratio in Earth and environmental samples [11]. A debate soon emerged as to whether iron isotope effects could be used as tracers of microbial activity in the environment and even in extraterrestrial samples [11,12]. However, abiotic processes were found to produce isotope fractionations of similar magnitude which clearly jeopardizes their usefulness for this purpose. Iron reduction in aqueous solution was found to increase the $^{56}Fe/^{54}Fe$ ratio by up to 3‰ in model experiments [13]. In nature, these variations are mostly produced in aqueous low-temperature environments at the Earth's surface, which highlights the potential of iron isotope effects for studying the iron redox cycle, and the evolution of atmospheric oxygen in the Earth's history [13]. In contrast, iron isotope fractionation at high temperatures appears to be limited to variations of lower than 1‰ in rocks of the deeper Earth [13].

While geochemists mainly concentrated to date on iron isotope effects induced by bacterial activity in the environment [12], much stronger iron isotope effects were found recently in higher organisms. Using MC-ICP-MS, Walczyk and von Blanckenburg discovered that all iron in the biosphere, including the human body, is depleted in the heavier iron isotopes [14]. The $^{56}Fe/^{54}Fe$ isotope ratio appears to decrease by ca. 1‰ per trophic level (see Fig. 1). In addition, human individuals bear a distinct iron isotopic signature in blood with females having on average more of the heavier iron isotopes in blood than males. A pilot survey in human tissue

and foods of plant and animal origin has indicated that the iron isotope effect in blood is primarily determined by preferential absorption of lighter iron isotopes in intestine and that the iron isotopic signature in blood reflects differences in iron absorption. Our finding of a distinct iron isotopic signature in human blood was verified independently by Ohno et al. [15]. They confirmed our earlier hypothesis that the iron isotopic composition should be stable over months and even years due to the slow turnover of body iron.

Iron isotope effects could potentially become a new isotopic tool to study iron metabolism in vivo. It is estimated that 30% of the world population are affected by anemia from which some 600–700 million suffer from iron deficiency anemia [16]. This makes it the most common micronutrient deficiency on a global scale. Consequences of iron deficiency and iron deficiency anemia are multiple and include reduced work performance, reduced resistance to infection, a higher mortality of mother and child around birth and reduced intellectual and psycho-motoric development during childhood [17]. Stable iron isotopes (^{57}Fe and ^{58}Fe) are an important tool for the evaluation of food-based strategies for combating iron deficiency and iron deficiency anemia [18]. Contrary to radioisotopes, they can be used safely during infancy, childhood and pregnancy. Available stable isotope methods, however, resemble closely earlier radioisotope techniques in which iron isotopes are used basically as tracers for studying iron absorption in the intestine and its utilization for physiological function. Iron isotope effects may add a new dimension to this field of research which we will illustrate by summarizing first the basic principles of isotope fractionation and technical aspects of measuring iron isotope effects by MC-ICP-MS; further we will evaluate available data on iron isotope effects in the human body in relation to human iron metabolism and present new data on iron isotope distribution within the body.

2. Principles of isotope fractionation

2.1. Terminology

Because variations between stable isotope ratios are usually small, they are commonly measured relative to a reference material. In relative measurements, systematic bias in isotopic analysis is minimized as it, ideally, cancels out. Data are commonly presented in the delta-notation in parts per thousand (per mil, ‰).

$$\delta^{56}\mathrm{Fe}_{\mathrm{IRMM14}} = \left(\frac{^{56}\mathrm{Fe}/^{54}\mathrm{Fe}_{\mathrm{Sample}}}{^{56}\mathrm{Fe}/^{54}\mathrm{Fe}_{\mathrm{IRMM14}}} - 1 \right) \times 1000 \qquad (1)$$

with ^{56}Fe/^{54}Fe$_{\mathrm{Sample}}$ being the iron isotope ratio in the sample and ^{56}Fe/^{54}Fe$_{\mathrm{IRMM14}}$ the isotope ratio in the reference material. For iron, data are commonly reported relative to IRMM-014, a reference material of certified iron isotopic composition [19].

The isotopic composition of an element is changed if the element is transported from a source compartment to a target compartment provided that element transfer is both incomplete and mass-sensitive. Compartments can be either physical compartments, e.g., in diffusion or evaporation processes, or chemical compartments, i.e., different element species in chemical reactions. The resulting fractionation factor α can be calculated directly from the isotope ratios of the source compartment and the target compartment of the transfer process.

$$\alpha = \left(\frac{^{56}\mathrm{Fe}}{^{54}\mathrm{Fe}} \right)_{\mathrm{Target}} / \left(\frac{^{56}\mathrm{Fe}}{^{54}\mathrm{Fe}} \right)_{\mathrm{Source}} \qquad (2)$$

In keeping with the per mil notation, the isotope fractionation can also be expressed as a difference Δ between the target compartment and the source compartment; Δ is usually expressed in ‰.

$$\Delta = \delta_{\mathrm{Target}} - \delta_{\mathrm{Source}} \qquad (3)$$

and

$$\Delta = (\alpha - 1) \times 1000 \qquad (4)$$

2.2. Mass-dependent fractionation processes

Principles of mass-dependent isotope fractionation processes have been discussed extensively in the literature. Therefore, only a brief summary is given here. The interested reader is referred to available review articles and text books [20–23]. The isotope ratios of an element or molecule undergoing isotope fractionation are shifted during chemical reactions or during transport processes in a mass-dependent fashion. For a given element, the larger the relative mass difference between two isotopes is, the larger is the induced isotopic shift. *Equlilibrium fractionation* processes describe those isotopic shifts that occur in chemical or physico-chemical reactions that are in a state of equilibrium. Usually they take place if an element is converted from one species into another for which bond strengths are different. The theory is explained in detail by Chacko et al. [24] and Schauble [25]. Briefly, equilibrium isotope fractionations are caused by the sensitivities of molecular and condensed-phase vibrational frequencies to isotopic substitution. These result in minor shifts in the zero-point energy between equilibrated substances upon isotopic substitution. The qualitative predictions governing equilibrium fractionation have been summarized by Schauble [25] and are as follows:

(1) The magnitude of the isotope effect decreases with increasing temperature. Within the context of this study, this effect is negligible as fractionation processes discussed here take place at room or body temperature.
(2) Isotope effects are more pronounced, the larger the reduced mass $m' = (m_1 - m_2)/(m_1 \times m_2)$ of the involved

elemental or molecular species is with and m_1 *and* m_2 being the masses of the different isotopic species. Thus, isotope effects are larger for light elements which often show large relative mass differences between isotopes when compared to heavier elements or elements bound to biomolecules.

(3) Heavier isotopes are concentrated in those chemical compartments, i.e., elemental species, in which the element forms the strongest bonds.

Kinetic fractionation occurs in two distinct situations. Either transport is uni-directional, thereby preventing equilibration of atoms/molecules; or a chemical reaction takes place for which time is insufficient to reach equilibrium. This means that the rate of isotope exchange is slower than the reaction or transfer rate. Typical examples are evaporation of a gas with instant removal of the vapour, rapid crystallisation of a solid from a liquid, or diffusion of element species through a membrane. Other kinetic fractionations may result if isotopic exchange is limited by the activation energy of the exchange process.

2.3. Mass balance concepts

An essential precondition for stable isotope variations to be found in nature is that the source component and the target component of a transfer process separate from each other, such that they can be isolated and measured separately. Examples are separate sampling of the gas phase and the liquid phase for an evaporation process, separation of distinct element species involved in a chemical reaction or sampling of different physiological compartments in organisms, e.g., different tissues or excreta. Another important prerequisite for making a potential isotope effect visible is incomplete element transfer from one compartment to the other. Assume, for example, that a source component is completely converted into a target component. The isotopic composition of the target would be the same as the composition of the source, regardless of the actual fractionation factor α between target and source (see Eq. (2)). If, in contrast, only an infinitesimally small fraction of source is converted into a target, then the difference between target and source would be exactly of the size of the fractionation factor.

In reality, isotope fractionation usually takes place between these two endpoints. Consider the case, for example, of a considerable fraction of source component being transferred into the target compartment, and that this transfer is associated with a constant isotope shift Δ. In this case, the isotopic composition of the target is shifted opposite to that of the source as isotopes cannot get lost. The magnitude of this shift depends on the fraction of source component that is transferred to the target compartment. The relevant formalisms are called "Rayleigh equations" and are explained at length in the literature [5,26], see Fig. 2. In this example, an element is transferred from a source compartment

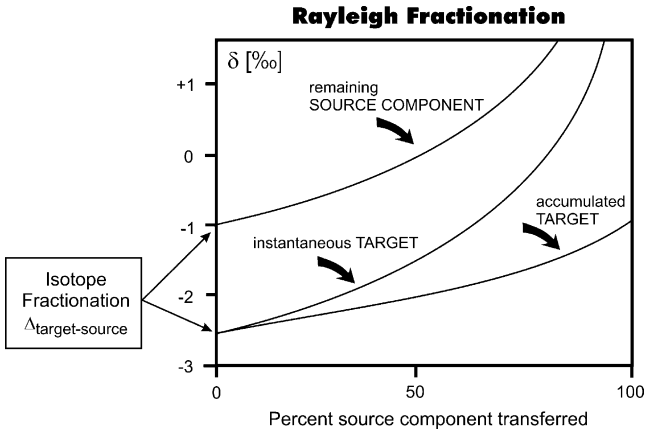

Fig. 2. Mass-balance calculations showing the fractional transfer of a source component into a target compartment, while undergoing a constant isotope fractionation $\Delta_{\text{Target–Source}}$ (Rayleigh fractionation). For underlying equations the reader is referred to the literature [23,26]. The instantaneous target component corresponds to each infinitesimally small increment of target component produced, while the cumulative target corresponds to the integral of all instantaneous products. Respective isotope ratios are expressed as δ-values (for definition see Section 2.1). The isotopic composition of the source changes with transfer efficiency as it becomes continuously depleted in the lighter isotopes with every incremental mass transfer.

with an initial isotopic composition of −1‰ and the isotopic shift Δ is −1.5‰ for this process, i.e., the isotope ratio of each infinitesimally small fraction of transferred element is always lower by 1.5‰ than that of the source compartment. Because the source compartment is constantly loosing light isotopes, it becomes more enriched in the heavier isotopes in proportion to the amount of the component transferred. If transferred fractions are collected quantitatively, the accumulated isotopic composition of transferred element in the target compartment is the integral over all instantaneously transferred element fractions. If the transfer process is complete, the amount of element accumulating in the target compartment has the original isotopic composition of the source compartment.

2.4. Element transfer processes under steady-state conditions

In natural systems, physical or physiological compartments are often in *steady state* regarding the mass fluxes of the respective element. We explain this concept using the example of iron turnover in the human body. Steady state means that the body is in iron balance. Physiological losses are fully compensated by iron influx into the body from the diet. The rate of iron influx (mass per unit time) into the body F_{in} is the same as the rate of iron efflux F_{out}, such that the iron inventory of the human body I remains constant with time. In this case

$$\frac{\mathrm{d}I}{\mathrm{d}t} = F_{\text{in}} - F_{\text{out}} = 0 \tag{5}$$

which allows us to calculate the residence time τ of iron in the human body:

$$\tau = \frac{I}{F_{\text{in}}} = \frac{I}{F_{\text{out}}} \quad (6)$$

For example, let us assume an average body iron content of 2.5 g for adult females and 4.0 g for adult males. To remain in iron balance, 1.0 mg iron has to be absorbed from the diet by males while about 1.3 mg has to be absorbed by females to replace additional iron losses through menstruation. For this model, the resulting mean iron residence time in the human body is 5 years for females and 11 years for males.

We can also define an *isotopic steady state*, which is not necessarily the same as the steady state by mass flux. At isotopic steady state, the isotopic composition of an element entering a reservoir is invariant with time, as are any fractionation factors between reservoirs. Therefore, the isotopic composition of the efflux from a reservoir is also invariant with time, and the isotopic composition of the sum of all influxes $\delta_{\text{total influx}}$ equals that of the sum of all effluxes $\delta_{\text{total efflux}}$. This translates into

$$\delta_{\text{total influx}} = \delta_{\text{total efflux}} \quad (7)$$

If a compartment's isotopic composition is governed by more than one efflux and the system is in steady state, the isotopic composition δ_{A} of the compartment A is defined as:

$$\delta_{\text{A}} = \frac{F_{\text{Out1}} \times (\delta_{\text{out1}} - \Delta_{\text{out1-A}}) + F_{\text{Out2}} \times (\delta_{\text{out2}} - \Delta_{\text{out2-A}})}{F_{\text{Out1}} + F_{\text{Out2}}} \quad (8)$$

with F_{Out1} and F_{Out2} being the mass fluxes out of the compartments, δ_{out1} and δ_{out2} the isotopic composition of the effluxes and $\Delta_{\text{out1-A}}$ and $\Delta_{\text{out2-A}}$ the corresponding fractionation factors for the effluxes.

Based on these considerations, fundamental steady-state models for describing element transfer processes can be discussed regarding their potential to alter the isotopic composition of the compartments itself or the element efflux from the compartment (Fig. 3A–F). Models are applicable both to chemical transfer processes, i.e., chemical reactions, as well as physiological transfer processes, e.g., between physiological compartments such as blood and liver.

(A) A single compartment model in which element uptake is complete, and isotopes are not fractionated during intake or loss (see Fig. 3A). In this case, the isotope ratios of influx (δ_{in}), efflux (δ_{out}), and the compartment itself (δ_{A}) are the same.

(B) A single compartment model in which the element is taken up without any fractionation (no fractionation occurs during uptake and/or uptake is complete), but fractionation occurs during element loss from the compartment (see Fig. 3B). In this model, the isotopic composition of the compartment differs from that of the influx (δ_{in}) and the efflux (δ_{out}) which are equal. This might appear counterintuitive. If, however, heavy isotopes are lost preferentially from the compartment (a positive $\Delta_{\text{out-A}}$), a light permanent residue has to exist in the compartment under steady-state conditions for reasons of mass balance. Similar phenomena are known from stable isotopes in the oceans, where a dissolved boron or a molybdenum reservoir exists in seawater that is different from both influx and efflux [27,28].

(C) A single compartment model in which element uptake is incomplete and subject to an isotope effect (see Fig. 3C). The isotope composition of the compartment δ_{A} would be greater or equal than the fractionation factor $\Delta_{\text{in-A}}$ for the influx, while the complementary isotopic composition δ_{out1} is found in the fraction that has not entered the compartment. The magnitude of the fractionation effect depends on the fraction entering the compartment. The isotopic composition of the compartment would correspond to that of the accumulated target in Fig. 2, while the isotopic composition of the fraction that has not entered the compartment is described by the curve for the remaining source component. Since no isotopic fractionation occurs during loss, the isotopic composition of the efflux from the compartment δ_{out2} equals that of the compartment δ_{A}.

(D) A single compartment in which element uptake is incomplete and both uptake and loss are subject to isotopic fractionation (see Fig. 3D). The result is similar to setting C, but the isotopic shift of the compartment relative to that of the influx is greater or equal than the difference of the fractionation factors $\Delta_{\text{in-A}}$ and $\Delta_{\text{out-A}}$ between influx and efflux. The isotopic composition of the efflux δ_{out2} is the sum of the isotopic composition of the compartment δ_{A} plus the fractionation factor $\Delta_{\text{out2-A}}$ between compartment and efflux.

(E) A two compartment model with a single efflux from compartment A in which the influx from compartment A into B equals the efflux from compartment B to A (see Fig. 3E). Apart from a reversible isotope effect for influx and efflux from compartment B, no isotope fractionation occurs. Because isotope fractionation produces no net removal of isotopes from compartment A, the composition of compartment A (δ_{A}) equals that of the influx δ_{in} and the efflux δ_{out}. The isotopic composition δ_{B} of the other compartment, however, is the sum of the isotopic composition of compartment A (δ_{A}) plus the fractionation factor $\Delta_{\text{B-A}}$ for the flux between B and A.

(F) A two compartment model with a single influx and two effluxes (see Fig. 3F). This setting is identical to setting E, except that losses occur both from compartment A and B and the flux from compartment A to B is larger than vice versa. Only losses from compartment B are affected by isotope fractionation. The isotopic composition δ_{A} of compartment A differs from the isotopic composition δ_{B} of the other compartment because of isotopic fractionation during loss from compartment B. The isotope ratios of the compartments are defined by the relative fluxes following Eq. (8). The isotopic composition δ_{out1}

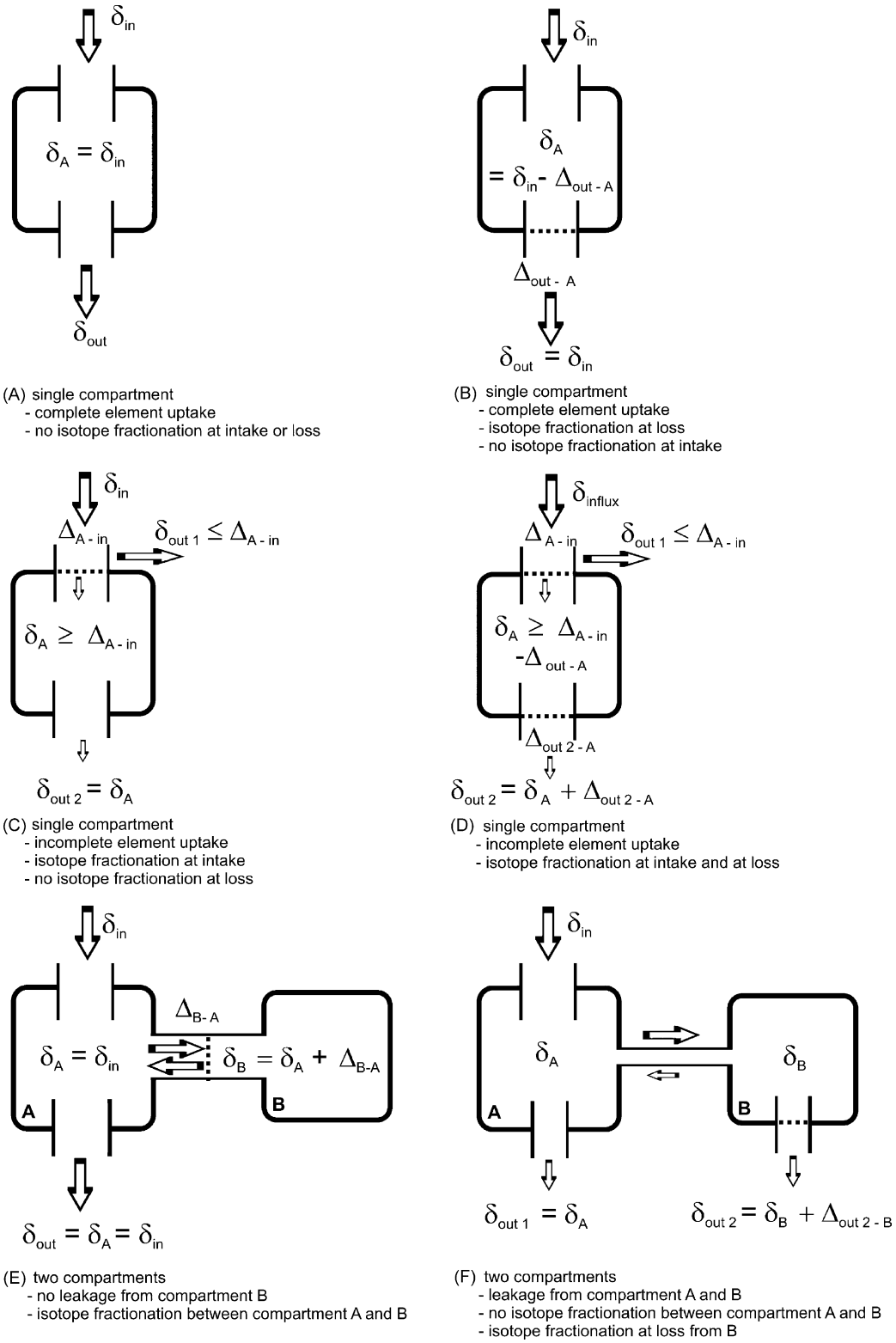

Fig. 3. Conceptual steady-state models for the distribution and isotope fractionation of elements between single and multiple compartments. Transfers are discussed regarding their potential to alter the isotopic composition δ_A or δ_B of the compartments itself, relative to an influx δ_{in} or the element efflux δ_{out} from the compartment as caused by a fractionation factor Δ_{out-A}. The size of the arrows corresponds to the size of the respective mass fluxes. Models are both applicable to mass transfer processes between chemical compartments, i.e., element species, and physiological compartments, e.g., blood, liver or muscle. The isotopic compositions of the compartments in models A, B, and E can be assessed in straightforward manner from the fractionation factors. Those in models C and D have to be assessed based on Rayleigh-type mass-balance considerations because not all of the source component is taken up by the target compartment (see Section 2.3 and Fig. 2). Isotopic compositions in model F have to be calculated from mixing equations (see Section 2.4, Eq. (8)). In organisms, an unlimited number of combinations between these basic compartments models exist.

of the efflux from compartment A equals the isotopic composition δ_A of the first compartment as efflux from compartment A is not affected by isotope fractionation. Due to the isotope effect during efflux from compartment B, the isotopic composition of the efflux equals the sum of the isotopic composition δ_B of compartment B and the fractionation factor Δ_{out2-B} (see model E).

2.5. Non-steady-state conditions

At isotopic steady-state and under steady-state conditions for mass flux, the isotopic composition and the inventory of any of the compartments in the above models is constant with time. Changes in the inventory of a compartment can be induced only if the sum of all effluxes from the system do not equal the sum of all influxes, i.e., under non-steady-state conditions for mass flux.

A transient change in the isotopic composition of a compartment can be induced in two different ways. First, a change in a fractionation factor(s) always alters the isotopic composition of the compartment, even under steady-state conditions for mass flux, but not its inventory. Second, a change in mass flux(es) per se alters the isotopic composition of the compartment if respective flux(es) are associated with an isotope effect. In either case, the inventory or the isotopic composition of the compartment changes continuously until a new isotopic steady state for the altered fractionation factor or mass flux(es) are reached. The time required to settle into a new steady state takes a few residence times τ (see Eq. (6)) and depends on the experimental capabilities to resolve a transient change. Once steady-state conditions for mass fluxes are re-established, the isotopic composition of the compartment(s) remain constant again, however, at values that may differ significantly from the original steady-state condition.

2.6. Iron isotope fractionation factors

The determination of fractionation factors for iron, as for all other metal isotopes, is still in its infancy. Data is patchy and mostly not applicable to natural systems in a straightforward manner. The current status has recently been reviewed by Anbar [11], and Johnson et al. [12]. For this study it can be summarized that oxidation of $Fe(II)_{aq}$ to $Fe(III)_{aq}$ under equilibrium conditions results in a product that is enriched in ^{56}Fe relative to ^{54}Fe by 3‰ in the ferric product, while the rapid formation of Fe(III) – bearing solids and Fe(II) – sorption leads to a depletion of ^{56}Fe relative to ^{54}Fe of 1–2‰ in the solid product [29–31]. Reduction of ferric iron leads to the opposite fractionation, with light iron isotopes being enriched in the reduced fraction. Furthermore, kinetic isotope fractionations can be expected from the analogy to biogenic carbon uptake [2]. It can be inferred that transfer of iron through the cell wall of an organism or the intestinal mucosa would also lead to a kinetic isotope fractionation favoring isotopically lighter iron in the product.

3. Iron isotope analysis: guidelines and critical remarks

Although efforts have been made to measure natural iron isotope compositions by thermal ionization mass spectrometry (TIMS), the measurements were usually not of sufficient precision to resolve the small isotope shifts found in nature [32,33]. Today, multicollector ICP-MS appears to be the method of choice for high precision iron isotope ratio measurements. The advantages are high ionization efficiency, rapid sample throughput, compensation of signal flicker, and various options to correct for instrumental mass discrimination. These techniques have been described and reviewed at length in the literature [34], and only a brief summary of the principle obstacles encountered is given here. A detailed investigation of iron isotope analysis of various inorganic and organic matrices, including the methods used for this study, is given in this issue [35].

3.1. Isobaric interferences

Molecular isobaric interferences formed in the plasma ion source, including ArO^+, ArN^+, FeH^+ ions, can potentially interfere with all iron isotope masses in the mass spectrum. Three strategies are in operation to eliminate these interferences: (a) resolving molecular interferences ion-optically by operating the instrument at high mass resolution [36]; (b) optimizing the intensity ratio of iron ions and interfering ion species by suppressing ArO^+ and ArH^+ ions with a desolvating nebuliser and by maximizing iron ion currents [14,37]; (c) collision cell technologies for suppressing argon interferences effectively [38]. Non-resolvable isobaric interferences may exist (e.g., $^{54}Cr/^{54}Fe$). These have to be minimized by removing the interfering elements during chemical purification of sample iron. Any residual interference of elemental ions is monitored and corrected for using a non-interfered isotope (e.g., ^{53}Cr). It is important that the isotope ratios of the element interfering with iron is subjected to a correction for instrumental mass bias. The presence of interferences is readily detectable when measured δ values for the $^{57}Fe/^{54}Fe$ isotope ratio are plotted against corresponding δ values for the $^{56}Fe/^{54}Fe$ isotope ratio, where all samples have to plot on a line described by the corresponding fractionation law [35].

3.2. Non-spectral matrix effects

Extraneous elements, organic matter, differences in the concentration of the solvents used to dilute samples or differences in the concentration of the element measured would all introduce a “cryptic” mass bias. It has to be pointed out that such an effect is normally not detectable. Samples would still plot on the predicted line when δ-values for two isotope ratios are plotted against each other. Even repeated analysis of samples would not necessarily disclose this effect since a matrix-related mass bias might also reproduce

[35]. Therefore, all possible precautions have to be taken to ensure that samples are presented in the same aqueous matrix and data are corrected for matrix effects as described below.

3.3. Instrumental mass bias

Isotope ratio measurements by ICP-MS are subject to an instrumental mass bias. Plasma source mass-spectrometry discriminates against light isotopes. In the plasma, these are moved to the exterior of the ion optical axis. Thus, heavy isotopes are extracted preferentially into the ion optics. This bias that amounts to a few percent in an isotope ratio is superimposed on the few per mil or less of natural mass fractionation that has to be detected. The instrumental mass bias is also not necessarily constant and may drift with time. Therefore, rigorous correction methods are required. Three different approaches are employed by MC-ICP-MS practitioners.

(a) *Standard-sample-standard "bracketing"* [37]. Mass discrimination is controlled by measuring a reference standard solution prior and subsequently to each sample which serves as the reference for calculating the δ^{56}Fe value. The ^{56}Fe/^{54}Fe isotope ratio of the reference standard at the time of the iron isotope ratio measurement of the sample is calculated by interpolation between the preceding and the subsequent standard solution. For a proper application of the bracketing technique it is essential that the mass bias is the same for sample and standard. Therefore, iron concentrations have to be matched carefully and samples have to be presented in the same matrix after removal of concomitant elements. This requires control of sample purity by AAS, optical ICP, or quadrupole ICP-MS, for example, prior to isotopic analysis [35,38]. Further, the instrument's drift in mass bias has to be small, and must follow a smooth trend. The disadvantage of the bracketing method is that a mass bias introduced by "cryptic" matrix effects would remain unnoticed and that instrument conditions have to be extremely stable.

(b) *"Doping" by an external mass discrimination monitor* [39]. Here, copper or nickel is added to the iron and isotope ratios of the doped element are measured in parallel to the iron isotope ratios. Iron isotope ratios are corrected using, for example, a reference ^{65}Cu/^{63}Cu ratio of doped copper. Usually an exponential mass discrimination law is used [35], although other empirical correlations are also in use. The advantages are that a possible matrix-induced, "cryptic" mass bias is readily detected, and that irregular changes in the instrumental mass bias during the measurement sequence are corrected for. Disadvantages are (i) samples should not contain even minute amounts of natural copper, which is often difficult to achieve with standard anion exchange techniques. Presence of natural copper of a different isotopic composition than that of the dope, which is possible by fractionation of sample copper in the ion exchange column [40], would confound measurements; (ii) the duration of an individual measurement is longer, because usually the analysis of copper involves switching of the magnetic field for alternating measurements of iron and copper isotopes, whereas measuring iron only can be done in static mode; (iii) the mass bias per mass unit is usually not the same for iron and copper, the difference also varies with instrument tuning. Therefore, copper-corrected iron isotope ratio measurements still need to be adjusted using the bracketing approach [35].

(c) *Double spike techniques* [33,41]. These techniques can be applied to elements with at least four isotopes. In principle, the sample is spiked with two enriched iron isotopes (e.g., ^{57}Fe, ^{58}Fe) of which the isotope ratio is exactly known. In a three isotope ratio space (^{56}Fe/^{54}Fe, ^{57}Fe/^{54}Fe, ^{58}Fe/^{54}Fe) the measured samples' composition is positioned on a triangular surface that is constrained by the (known) composition of the spike, the (known) composition of a natural reference material (e.g., the standard used as the reference for calculating delta values), and a curve that describes the natural mass fractionation process. This surface is intersected by a mixing line connecting the pure, unfractionated spike with the true sample's composition. The measured composition, however, deviates from this intersection by an amount determined by the instrumental mass bias. The intersection can be calculated by iteratively back-correcting the measured isotope composition using a known machine-specific fractionation law such that the point of intersection with that surface can be calculated. Finally, the natural samples' composition is found by extrapolating the found intersection line back onto the curve describing the natural fractionation process. The advantages of the double spike technique are (a) simultaneous measurement of precise concentrations by isotope dilution; and (b) a full correction of any mass bias generated within the entire procedure (including chemical separation of the element, mass spectrometric bias and its variability). This potentially increases the accuracy of the results and the reliability of the method. However, the precision might suffer as compared to the bracketing method because all measurement errors propagate into the result (e.g., measurement of three isotope ratios, spike composition, errors in the assumed fractionation laws). The most important drawback of the double spike technique is that all isotopes have to be completely free of isobaric interferences since an interference on one isotope would automatically introduce a bias on all isotope ratios. The possibility to detect the presence of interferences in a δ(^{56}Fe/^{54}Fe) versus δ(^{57}Fe/^{54}Fe) diagram does not exist when a double spike is used.

In summary, all three correction procedures have their advantages and disadvantages. They depend on the elements and sample types measured, the chemical pre-concentration,

the instrument used, and the skills of the operator. For iron isotopes, it appears that the standard-sample-standard bracketing method is the method of choice if (a) the chemical separation ensures 100% recovery; (b) chemically pure iron solutions are presented that are also free of any organic matrix; (c) the mass spectrometer can be tuned to stable and reproducible operating conditions; and (d) molecular interferences are not present. Using this approach, Schoenberg and von Blanckenburg obtained a long-term reproducibility of iron from synthetic standards and a variety of natural matrices at the 95% confidence interval of 0.049‰, 0.071‰, and 0.28‰ for $\delta(^{56}Fe/^{54}Fe)$, $\delta(^{57}Fe/^{54}Fe)$, and $\delta(^{58}Fe/^{54}Fe)$, respectively [35].

4. Iron in the human body

The aim of this paper is the identification of possible mechanisms on a physiological level that may determine the iron isotopic composition of blood. Iron metabolism is complex and involves multiple transfer processes between physiological compartments such as liver, muscle, plasma or red blood cells and chemical compartments, i.e., the various iron species involved in iron transfer. For further discussions, fundamental aspects of human iron metabolism are briefly described in the following. For more detailed overviews summarizing the current state of knowledge the reader is referred to the literature [42–45].

The human body contains ca. 4 g iron, which is distributed between different physiological compartments and corresponding iron species. Average compartment sizes are illustrated in Fig. 4. Most iron (ca. 60%) is bound in hemoglobin in red blood cells (erythrocytes) for oxygen transport from the lungs to other body compartments as well as delivery of CO_2 from the tissues back to the lungs. In muscle tissue, oxygen is transported by myoglobin in a similar fashion, which occupies ca. 10% of body iron. Both hemoglobin and myoglobin are heme-proteins, i.e., they contain the heme-subunit in which iron is bound in the center of a porphyrin ring system similar to magnesium in chlorophyll. Hemoglobin and myoglobin are tetramers of four globin polypeptide chains with each of them being bound to one heme molecule via non-covalent binding to histidine. Accordingly, one hemoglobin molecule can bind four molecules of oxygen. Iron in hemoglobin is present as Fe(II) and protected by the globin chain from oxidation to met-hemoglobin which has no oxygen binding capacity. Hemoglobin is synthesized primarily (85%) in the erythron of the bone marrow during erythropoesis (red blood cell formation), and to a minor extent in the liver. Protoporphyrin as the heme precursor is synthesized in the erythron and loaded with ferrous iron after reduction. This is accomplished before the heme molecule is incorporated into the different heme proteins by action of the enzyme ferrochelatase located at the inner mitochondrial membrane. Besides hemoglobin and myoglobin, a number of other heme-proteins (cytochromes) and iron enzymes are synthesized within the cell. Although they contribute to less than 5% to the total body iron pool, they play an essential role in cellular energy metabolism. In blood, most iron is bound in erythrocytes while a much smaller fraction of body iron (0.1%) circulates in plasma as transferrin, the major iron transport protein in the body.

Free ionized iron is toxic to the cell and has to be bound effectively to proteins to minimize oxidative stress. Excess iron is detoxified by incorporation into ferritin, the primary iron species in the liver. Ferritin is a spherical molecule composed from 24 peptide subunits which can store up to 4500 Fe atoms within its protein shell [46]. When ferritin in tissue is saturated, with about 4000 Fe atoms per ferritin molecule, ferritin is degraded by lysosomal proteases to form hemosiderin, an insoluble iron storage protein [47]. In healthy adults, about one-third of body iron is bound in ferritin, primarily in the liver and in much lower concentration in other tissues both for intracellular iron storage and iron detoxification. If iron supply of the body is not sufficient to balance iron losses, iron is mobilized from ferritin and intestinal iron absorption is up-regulated in parallel to absorb iron from the diet more efficiently [48].

Average transport rates between compartments are given in Fig. 4 based on available data in the literature [49]. Losses have to be replaced continuously through iron uptake from the diet in the intestine (Fig. 4, flux B), with most dietary iron leaving the body unabsorbed (Fig. 4, flux A). Usually, more iron enters the mucosal cell (enterocyte, see Fig. 4) than is actually delivered to the plasma for distribution within the body. This mechanism, known as the “mucosal block” is an effective mechanism of the body to modulate iron uptake and to avoid iron overload if iron supply is excessive. Through regular exfoliation and apoptosis of mucosal cells every 2–3 days, excess iron that has been stored in the mucosa and which has not been delivered to plasma is excreted in feces (Fig. 4, flux L). In addition, some iron is lost in feces through gastrointestinal secretions (Fig. 4, flux N). Once iron has been delivered from the enterocyte to the plasma (Fig. 4, step C), iron is bound to transferrin which is used for iron transport to the major iron compartments, i.e., to the bone marrow for heme synthesis and erythrocyte formation (Fig. 4, fluxes D and E); the liver for iron storage as ferritin (Fig. 4, step F); and muscle tissue for heme and myoglobin synthesis (Fig. 4, flux G). As can be seen from Fig. 4, all body iron is continuously recycled until it is finally lost from the body. Iron losses occur through blood by accidental bleedings and blood donations (Fig. 4, flux K), menstruation (Fig. 4, flux Q) and gastrointestinal bleedings due to lesions, ulcers, intestinal parasites, etc. (Fig. 4, flux M). A minor fraction is lost through urine, sweat and skin (Fig. 4, fluxes O and P). The average erythrocyte lifetime in the body is 120 days. After that, erythrocytes are broken up by macrophages in the spleen, liver and the bone marrow and iron is liberated for re-utilization (Fig. 4, flux I). The same holds for iron in myoglobin (Fig. 4, flux J) and iron in ferritin when being released from the protein shell (Fig. 4, flux F).

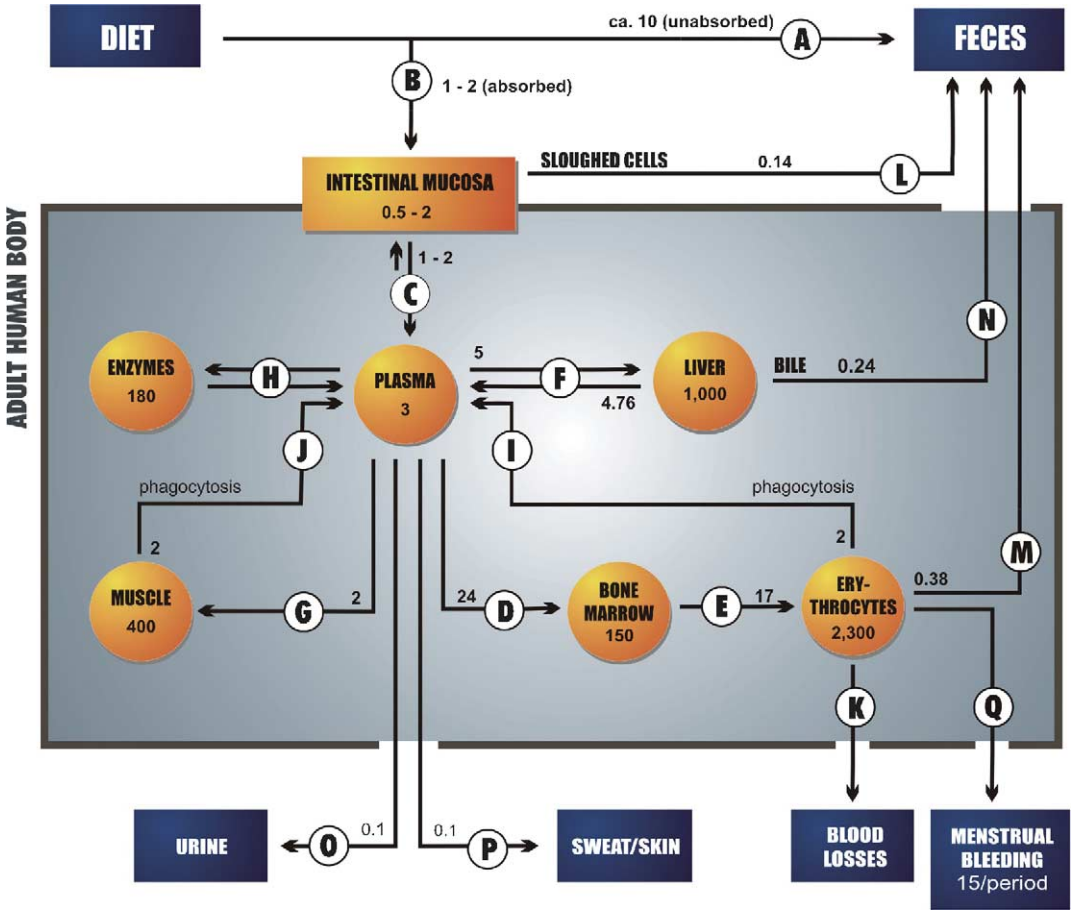

Fig. 4. Iron fluxes in the body between major iron compartments. Fluxes are given in mg iron per day and average compartment sizes in mg iron. Absorbed iron enters circulation after absorption in the upper intestine (flux B and C). Absorbed iron is distributed between bone marrow and red blood cells, liver and muscle tissue as the major iron compartments of the body. Minor amounts of iron are bound in iron-containing enzymes and metallo-proteins and in plasma to transferrin, the major iron transport protein. Basal losses occur through regular sloughing of mucosal cells every 2–3 days (flux L), gastrointestinal secretions (flux N) and blood losses in the intestine (flux M) and during menstruation (flux Q). Only minor iron amounts are lost through urine (flux O), skin exfoliation and sweat (flux P). Under balance conditions, iron turnover in adults is in the order of 1–2 mg per day at an average body iron content of ca. 4 g (after [49]).

5. Iron uptake in the intestine

Humans are omnivores and their diet contains both plant foods and foods of animal origin which contain iron of different forms. Iron in plant foods is essentially non-heme iron, while about 30% of iron in animal foods is heme-iron from hemoglobin and myoglobin. During digestion, iron is liberated from the food matrix in the stomach by action of hydrochloric acid and pepsin. Protein-bound iron is liberated by protein denaturation and pepsin digestion and solubilized by reducing it from the ferric to the ferrous state. Iron liberation from the digestive is continued by action of pancreatic proteases in the intestine. Heme is liberated during this process from myoglobin and hemoglobin in intact form while non-heme iron is liberated in ionic form. Pancreatic ductal cells secrete bicarbonate which increases the pH of the lumen and results in oxidation of Fe(II) and hydrolization of Fe(III). While availability of non-heme iron for absorption is reduced by precipitation, heme iron remains solubilized and available for absorption.

Most iron is absorbed in the duodenum and upper jejunum by enterocytes, highly specialized absorptive cells found on the intestinal villus (see Fig. 5). The vectorial passage of iron through the enterocyte entails (a) iron uptake by the enterocyte into the cytoplasma; (b) iron translocation within the enterocyte; and (c) iron release from the enterocyte into blood for transferrin binding. Over the past decade, underlying molecular mechanisms of these processes could be partly elucidated. One major finding was the identification of the transporter able to transport divalent cations through the apical membrane [50,51]. The protein has been variously termed divalent metal ion transporter (DMT1), natural resistance associated macrophage protein 2 (Nramp 2) or divalent cation transporter (DCT1). Iron uptake by DMT1 requires reduction of Fe(III) which is accomplished by action of duodenal cytochrome *b* (Dcytb), a ferrireductase (Fig. 5, steps A and B). An alternative but less well described mechanism for ferric iron absorption was proposed by Conrad et al. based on iron binding to mucin, a protein found in the intestinal lumen, and sequential iron relay to β-integrin on the apical cell surface to

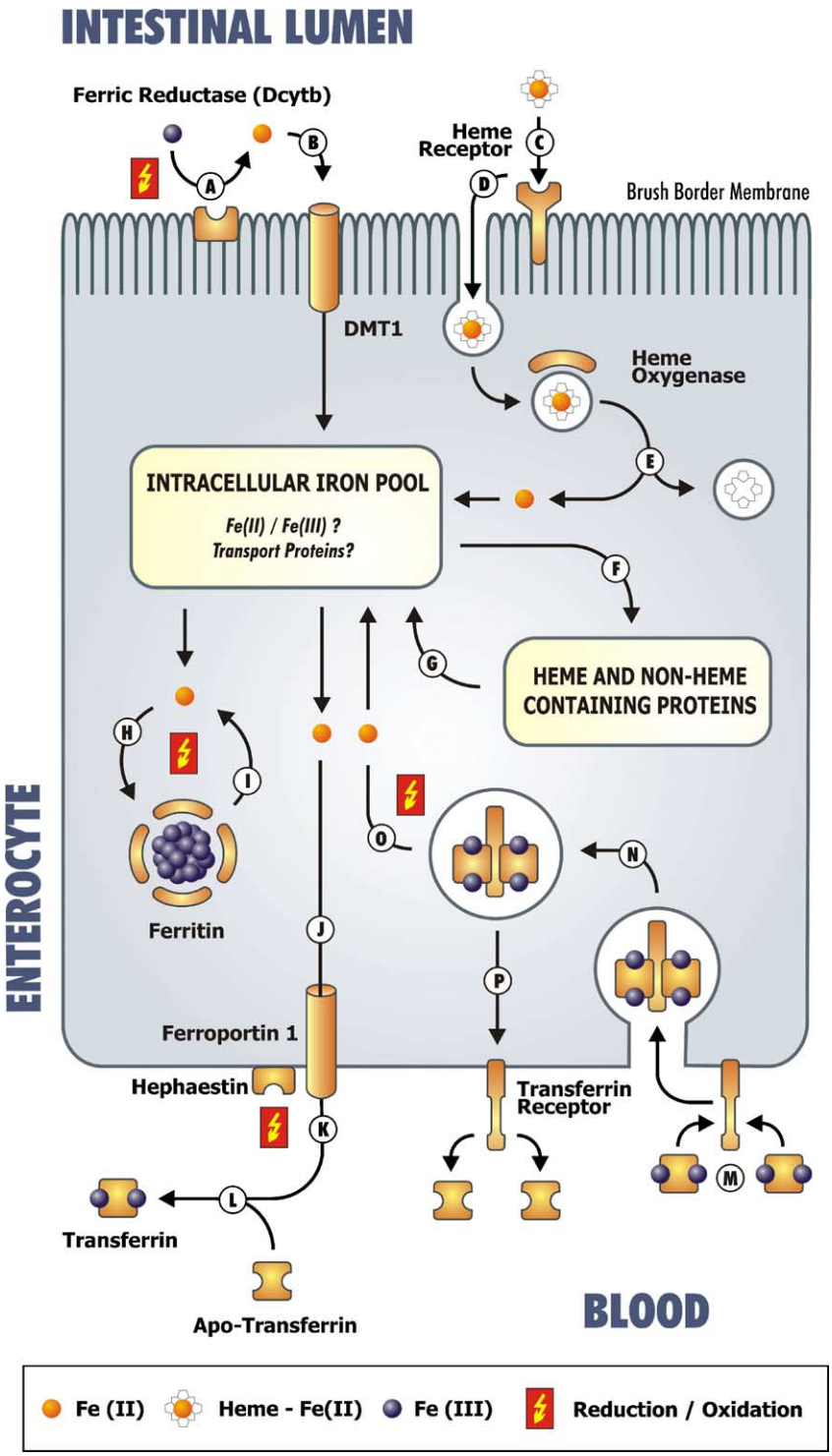

Fig. 5. Molecular mechanisms of iron absorption in the intestine. Iron is absorbed through the brush border membrane of enterocytes lining the intestinal mucosa. Non-heme iron from plant foods is mostly present as Fe(III) in the intestine. It is enzymatically reduced by duodenal cytochrome *b* (steps A and B) and taken up by the cell through divalent metal ion transporter 1 (DMT 1, step C). Heme iron from foods of animal origin is bound by an unidentified heme receptor at the cell surface (step C) and absorbed intact through endocytosis (step E). Iron is released from heme by heme oxygenase and enters with absorbed non-heme iron the common intracellular iron pool. Speciation of iron in this pool is currently unknown. Part of the absorbed iron is reversibly deposited in ferritin after oxidation (steps H and I) and incorporated in other functional metallo-proteins, mainly iron containing enzymes (steps F and G). Absorbed iron is released through ferroportin 1 at the apical side of the mucosa (step J) where it is oxidized for binding to apo-transferrin, the major iron transport protein of the body (steps K and L). Iron is taken up by the cell from plasma by binding of transferrin to specific receptor molecules (step M) and endocytosis (step N). Iron is quantitatively released into the cytoplasma (step O) while remaining apo-transferrin and receptor molecules are re-utilized (step P).

the intracellular protein mobilferrin [52]. The heme molecule is taken up intact by the enterocyte through endocytosis. Iron binds to an unknown receptor at the cell surface (Fig. 5, step C) and is internalized by endosome formation (Fig. 5, step D). After entering the cell, heme is degraded enzymatically to iron, carbon monoxide and bilirubin by heme-oxygenase (Fig. 5, step E). Both non-heme iron and iron liberated from heme enter the common intracellular iron pool for which iron speciation is poorly understood.

Absorption mechanisms and differences in the aqueous chemistry of heme-iron and non-heme iron explain why non-heme iron is absorbed less efficiently (1–10%, on average) than heme-iron (ca. 30%). Heme-iron is soluble at the neutral to alkaline pH of the upper intestine and is absorbed intact. Heme-bound iron is protected efficiently from interaction with other dietary components by its prophyrine ring system. Contrary to heme iron, absorption inhibitors such as phytic acid or polyphenolic compounds can bind to non-heme iron to form insoluble iron complexes. At the same time, other dietary components such as ascorbic acid and peptides from digested muscle tissue can enhance non-heme iron absorption by increasing Fe(III) iron solubility through reduction and chelation.

Internalized iron is partly incorporated into heme and non-heme containing iron proteins for cellular function (Fig. 5, step F) and for iron storage and detoxification (Fig. 5, step H). Iron uptake by these compartments is reversible (Fig. 5, steps G and I) and may include changes in oxidation state. Ferrous iron is oxidized to ferric iron before deposition into ferritin and vice versa (Fig. 5, steps H and I). Iron is exported from the enterocyte by ferroportin 1, also known as Ireg1 or MTP1 (see Fig. 5, step J). Transferred iron is oxidized by hephaestin (Fig. 5, step K), a copper-containing ferroxidase located at the basolateral surface of the enterocyte, before binding to apo-transferrin for plasma transport (Fig. 5, step L).

Virtually all cells in the organism take up iron from transferrin. Iron delivery to the cell occurs through binding of transferrin to transferrin receptor molecules on the cell membrane (Fig. 5, step M). Receptor-bound transferrin is taken up by the enterocyte through endocytosis (Fig. 5, step N). Iron is released from the complex by endosomal acidification and oxidized, possibly by Dcytb, before being exported into the cytoplasma by DMT1 (Fig. 5, step O) in a similar fashion as described earlier for apical iron uptake of the enterocyte (Fig. 5, steps A and B). Receptor bound apo-transferrin is finally released from the endosome by exocytosis (Fig. 5, step P).

6. Iron isotope effects in the human body

Four systematic studies on iron isotope effects in higher organisms have been conducted so far. Obtained data are illustrated in Fig. 6. The first study published by the authors in 2002 concentrated on the observation that blood of young healthy adults is depleted in the heavier iron isotopes relative to the geosphere [14]. Isotopic analysis of dietary iron sources and relevant body tissues indicated that the iron isotopic

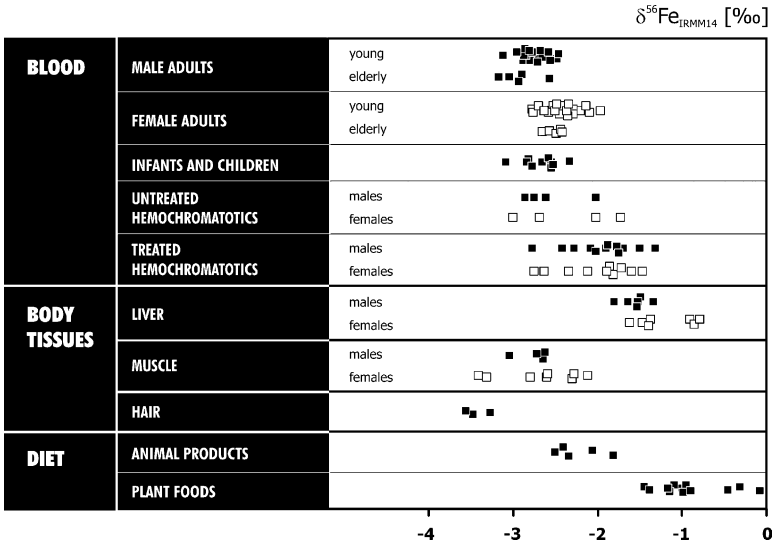

Fig. 6. Isotopic composition of iron in blood, body tissues and in the diet. Iron isotope ratios are presented on a δ-scale (for definition, see Section 2.1). More negative δ-values correspond to a lower $^{56}Fe/^{54}Fe$ isotope ratio, i.e., an enrichment in the lighter iron isotopes, compared to the reference material of non-biological origin (IRMM-014). Iron in blood of females was found to be less enriched in the lighter iron isotopes than blood of males. Differences between age groups were found to be insignificant when matched for gender [14]. Iron in the blood of treated hemochromatotic subjects was found to be less depleted in the lighter iron isotopes than blood of untreated patients and healthy controls [55]. Liver was found to be enriched in the heavier iron isotopes relative to muscle and blood ([14], this study). The isotopically lightest iron in the body was found in hair [14]. Iron in animal products (beef muscle, beef liver, pork muscle, chicken muscle, egg) contains more of the lighter iron isotopes than iron from plant foods (various cereals, salads and leafy vegetables ([14] and unpublished data from F. von Blanckenburg). For identification of statistically significant differences between sample groups, the reader is referred to the original publications [14,55] and Appendix A.

signature in human blood is primarily determined by a preferential uptake of lighter iron isotopes from the diet. Our earlier hypothesis that the iron isotope signature in blood must be stable over years has been confirmed recently by Ohno et al. who monitored the iron isotopic composition of blood in a single human subject over 1 year [15]. This finding reflects directly the slow iron-turnover of the body. In a follow-up study in collaboration with the University Hospital Zurich, we tried to test the hypothesis of a preferential absorption of lighter iron isotopes in the intestine by analyzing blood of patients suffering from hereditary hemochromatosis, a disease that is characterized by excessive iron uptake in the intestine. Following identification of the HFE gene [53], several studies showed that most patients with clinical features of hemochromatosis are homozygous for a C282Y mutation of the HFE gene [54]. Within this study, we compared the iron isotopic composition of blood obtained from patients undergoing regular phlebotomies (blood-lettings) for iron removal with the blood of untreated haemochromatotic patients as well as an age matched control group [55]. In the most recent study, which is described in Appendix A, we investigated iron isotope distribution between the major iron pools of the body, i.e., blood, liver and muscle, in 10 adult human subjects (see Fig. 7).

The major observations made so far can be summarized as follows: (a) iron in the biosphere, including plants, animals and men, has a $^{56}Fe/^{54}Fe$ ratio that is lower by two to three per mil than iron in the geosphere. Iron is progressively enriched in the lighter isotopes when being transferred from one trophic level to the next (Fig. 1). Variations in iron isotopic composition in the biosphere were found to be much stronger when compared to the geosphere; (b) each human being bears a certain iron isotopic signature in blood which is not subject to short-term fluctuations. Men and women were found to differ on average in the iron isotopic composition of their blood with women having more of the heavier iron isotopes in blood than men. Preliminary data suggests that differences in iron isotopic composition between age groups are insignificant; (c) patients suffering from iron overload due to hereditary haemochromatosis tend to have a higher proportion of heavier iron isotopes in blood relative to young and elderly healthy controls when treated by regular phlebotomies. Differences between patients who have started phlebotomy therapy only recently or had no blood-lettings at all and healthy controls did not reach statistical significance, possibly because of an insufficient subject number. The iron isotopic signature in blood was found to correlate with phenotypic expression of the disease, i.e., hepatic fibrosis by histological examination or increased serum concentrations of aminotransferases in patients without liver biopsy [55]; (d) iron isotopes are not distributed homogenously between body tissues. While the iron isotopic composition of blood and muscle tissue is very similar in the individual, iron in the liver is significantly enriched in the heavier iron isotopes (Fig. 7). Representing only a minor fraction of total body iron, the isotopically lightest iron in the biosphere was found to date in human hair.

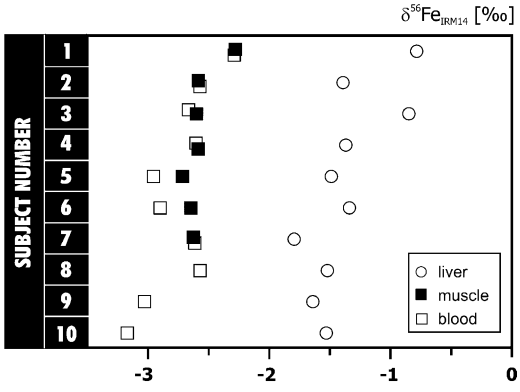

Fig. 7. Iron isotopic composition of blood, muscle and liver tissue in 10 adult subjects, representing the major iron compartments of the body (this study, see Appendix A). Data are presented on a δ-scale (see Fig. 6 for each individual subject. Iron in blood and muscle tissue was found to be of very similar iron isotopic composition while iron in the liver was found to be enriched in the heavier iron isotopes relative to muscle tissue and blood.

Mass fractionation principles dictate that a significant isotope effect can only be induced if iron transfer between compartments is incomplete. Isotope effects are stronger for the target compartment when less iron is transferred and stronger for the source compartment when more iron is transferred. At the same time, relative but not absolute mass differences between fractionating isotope species govern the magnitude of the ensuing isotope effect. Accordingly, protein bound iron is less susceptible to isotope fractionation than free solubilized iron. These basic considerations allow for a systematic analysis of the various steps of iron transfer from the intestine to the plasma and into relevant physiological compartments and their potential effect on the iron isotopic composition of blood.

7. Deciphering the iron isotopic message of the body

Most observations made so far refer to healthy, non-anaemic, non-iron deficient adult subjects which were assumingly in steady-state conditions, i.e., normal physiological losses are fully compensated by iron uptake from the diet. For subjects being in iron balance it appears that the iron isotopic composition of blood does not show fluctuations over months and possibly years [15] and that the iron isotopic composition of blood does not differ between age groups (see Fig. 6). At an average iron isotopic composition of $\delta_{average} = -2.5‰$, blood of healthy subjects differs from the average iron isotopic composition of dietary iron. In a mixed European diet, most iron is contained in plant foods which are substantially less depleted in the lighter iron isotopes ($\delta_{average} = -0.8‰$) when compared to iron from animal products ($\delta_{average} = -2.3‰$). The difference in iron isotopic composition between dietary iron and iron in animal tissue (see Fig. 6) is even more evident when considering that relevant animals studied so far (beef, pork and chicken)

can be expected to have been raised mainly on a vegetarian diet.

Results, so far, clearly indicate that iron in human blood and muscle tissue differs from dietary iron in its isotopic composition. Accordingly, we can conclude that the iron isotopic composition of human blood is not primarily determined by the diet itself. This is confirmed by the observation that blood from vegetarians does not differ from blood of omnivores regarding isotopic composition (unpublished). Apart from dietary iron isotopic composition, other mechanisms can potentially determine the iron isotopic composition of blood: (a) heavier iron isotopes may be lost preferentially from the body which would result in an enrichment of lighter isotopes in body tissues (see Fig. 3B); (b) iron isotopic fractionation may occur within the human body by isotope-selective incorporation of iron into body compartments and relevant iron species (see Fig. 3E and F). For example, isotopically lighter iron may be incorporated into erythrocytes, while isotopically heavier iron may be deposited in other body tissues; and (c) lighter iron isotopes may be absorbed preferentially from the diet (see Fig. 3C). In our first study we argued that preferential absorption of lighter iron isotopes is the principal mechanism that determines the iron isotopic composition of blood [14]. Our recent study in hemochromatitic patients [55] and our systematic study on the iron isotopic composition of liver, muscle and blood in several individuals (this study) allow us now to discuss underlying mechanisms in more detail on a compartmental and even molecular level.

Although data are still very limited, we can exclude preferential loss of heavier iron isotopes through gastrointestinal secretions (Fig. 4, flux N), urine (Fig. 4, flux O) and through sweat skin and hair (Fig. 4, flux P) as the primary mechanism by which the body becomes enriched in the lighter iron isotopes. More than 40% of iron losses from the body occur through bleedings (see Fig. 4, fluxes K, M and Q), mostly through intestinal lesions, and menstrual blood losses. Blood losses are not subject to isotopic fractionation as iron is lost through leakage without undergoing physical or chemical processes. Losses through the other pathways, however, may differ significantly in iron isotopic composition from their source compartments. Iron delivery to these pathways is incomplete and involves physical and chemical processes which can potentially induce an isotope effect. To date, only data for human hair is available which was found to be highly enriched in the lighter iron isotopes ($\delta_{average} = -3.7‰$). Even if a significant enrichment in the heavier iron isotopes would be given for the other iron losses, the effect on iron isotopic composition of the body would be only marginal. Following fractionation concepts, lost iron can be strongly fractionated without a significant effect on the source compartment if the amount of lost iron is small compared to the iron content of the source compartment (see Fig. 2). This holds true for most iron losses apart from major bleedings. Together, they account for less than 0.5 mg iron per day which compares to a body iron content of ca. 4000 mg.

While a preferential loss of heavier iron isotopes does not seem to be a reasonable mechanism, our earlier hypothesis of a preferential absorption of lighter iron isotopes is strengthened by our findings in hemochromatotic patients. Hereditary hemochromatosis is known to affect regulatory mechanisms of iron absorption. It is well established that iron accumulates in the body in these patients by a failure in down-regulating iron absorption in presence of full body iron stores [56]. Fractionation principles predict that the iron isotopic composition of body iron should be closer to the isotopic composition of dietary iron the more effectively iron is absorbed from the intestine (see Fig. 2). This compares well with our findings in hemochromatotic patients. Remarkably, differences between hemochromatotics with no or inadequate phlebotomy therapy did not reach statistical significance relative to healthy controls ($P = 0.17$). This observation is consistent with a stronger expression of DMT1, the primary transporter of iron in the brush border membrane (see Fig. 5, step B), in treated hemochromatosis patients when compared to untreated patients [56–58]. In treated hemochromatotic patients, the body senses apparently that blood-letting results in a drop in iron stores which are lower compared to the untreated state and responds by up-regulating iron absorption to re-establish the original condition. In untreated hemochromatotic patients, iron absorption is apparently up-regulated too but not as strong as in treated patients as the body is in its predisposed, impaired balance state for iron.

Our most recent study on the iron isotope effects in the human body presented here, revealed that incorporation of iron isotopes into the major compartments for iron in the body (blood, liver and muscle tissue) is isotope selective. While blood ($\delta_{average} = -2.7‰$) and muscle ($\delta_{average} = -2.6‰$) are very similar in iron isotopic composition, liver tissue was found consistently to be enriched in the heavier iron isotopes ($\delta_{average} = -1.4‰$), see Fig. 7. Through this recent finding, our observation in hemochromatotic patients could also be explained by a transient release of isotopically heavier iron from the liver. In hemochromatotic patients, absorbed excess iron is deposited continuously in the liver as ferritin [56]. Isotopically lighter iron in blood is removed continuously from the body through blood-letting and replaced by blood in which heavier iron isotopes are incorporated that were released earlier from the liver. The absence of a distinct difference in iron isotopic composition between the blood of healthy controls and hemochromatotic patients with inadequate phlebotomy therapy or no blood-lettings at all would be in agreement with this mechanism. This clearly compromises our hypothesis of a preferential absorption of lighter iron isotopes. If the distinct iron isotopic composition of blood of treated hemochromatotic patients is not due to up-regulated iron absorption but a release of isotopically heavier iron from the liver then enrichment of lighter iron isotopes in blood of healthy subjects relative to dietary iron would result from a preferential deposition of heavier iron isotopes in the liver.

We can argue against a liver compartment driving blood iron to light compositions, however. Analysis of the molecular mechanisms of iron incorporation into erythrocytes, muscle and liver tissue reveals that iron deposition in target tissues, including liver, should not induce an isotope effect. In healthy adult subjects, the majority of freshly absorbed iron (ca. 80%) is used for hemoglobin synthesis during erythropoesis and only a minor fraction is incorporated into muscle and liver tissue (see Fig. 4). Iron is delivered to virtually all body cells through plasma transferrin by the mechanism shown in Fig. 5 (steps M, N, O and P). Because of the small relative mass differences of iron-loaded transferrin containing iron isotopes of different masses, the delivery process itself cannot result in a significant isotope effect. Release of iron from transferrin following endocytosis (Fig. 5, step O) is quantitative and, therefore, cannot result in an isotope effect either. Subsequent iron isotope fractionation processes within the cell, specifically during heme biosynthesis, do not have to be considered here as our data refer to entire cells as single compartments. Because all iron that is delivered to muscle tissue and red blood cells is finally released by phagocytosis at the end of the cell's lifetime (Fig. 4, fluxes I and J), no isotope effect can be present at the stage of iron release. Because of the absence of an isotope effect during iron uptake as well as iron release, the iron isotopic composition of both muscle tissue and blood should closely resemble that of plasma iron (see Fig. 3A). Small differences in iron isotopic composition between blood and muscle tissue in the individual, which are greater than the measurement error, can be explained by the differences in iron turnover and, thus, the recording of small changes in iron metabolism and/or residual iron in the bone marrow.

While iron delivery and release from blood and muscle tissue are closed cycles, this does not apply to liver iron. Iron is reversibly deposited as ferritin (Fig. 4, flux F) in the liver but some iron delivered to the liver cells is excreted from the body through bile (Fig. 4, flux N). While iron should not be fractionated during uptake by the liver for the same reasons as given for muscle tissue and red blood cells, it remains open if iron is fractionated during iron release from the liver into plasma or when being lost through bile. In either case, a preferential loss of lighter iron isotopes from the liver would be balanced by liver tissue being enriched in heavier iron isotopes as observed. However, under steady-state conditions this would not affect the iron isotopic composition of blood. According to fractionation theory, the iron isotopic composition of the influx δ_{in} from plasma equals that of the efflux δ_{out} from the liver (see Fig. 3B) provided that fractionation occurs during element release and not during element uptake by the liver, which we can safely assume.

Based on these considerations, preferential loss of heavier iron isotopes from the body or deposition of heavier iron isotopes in the liver cannot be the dominant mechanisms that determine the iron isotopic signature in blood. Preferential uptake of lighter iron isotopes from the diet by the body still appears to be the most probable determinant. Analysis of the molecular mechanisms of iron uptake by the enterocyte (Fig. 5, flux B) and later iron delivery to the plasma (Fig. 5, flux D) may allow identification of those steps in the absorption process which are most likely to account for an isotope effect. Dietary iron is absorbed by two distinct mechanisms. While non-heme iron released from the diet during digestion enters the enterocyte via DMT-1 (Fig. 5, steps A and B), heme iron is taken up by endocytosis (Fig. 5, steps C and D). The majority of food iron leaves the body unabsorbed which is the prerequisite for a significant isotope effect. Fractionation principles dictate that the isotope effect is stronger for the target compartment (the enterocyte) when less iron is transferred from the source compartment (the intestinal lumen). Both mechanisms, however, differ significantly in their potential for inducing an isotope effect. Relative mass differences of heme isotopomers are too small to cause an isotope effect during receptor binding at the surface of the brush-border membrane. Likewise, subsequent iron release from heme through action of heme oxygenase is of no relevance as iron release is quantitative. Uptake of non-heme iron, on the contrary, is more likely to be mass-sensitive. Non-heme iron absorption involves reduction of ferric iron (Fig. 5, step A) by Dcytb. Model experiments in aqueous solutions have shown that oxidation of ferrous iron to ferric iron results in an enrichment of the heavier iron isotopes in ferric iron [13]. Reduction of ferric iron as given at the surface of the intestinal mucosa may result in a reverse effect, i.e., enrichment of ferrous iron in the lighter iron isotopes which would subsequently enter the enterocyte by transport through DMT1 (Fig. 5, step B). Within the cell, iron is distributed between different chemical compartments, namely iron-containing proteins that are needed for cellular function (Fig. 5, steps F and G) and ferritin for deposition of excess iron that is not delivered to plasma (Fig. 5, steps H and I). Notably, this process involves oxidation of ferrous iron to ferric iron during deposition and iron reduction during release. This may lead to a preferential deposition of heavier iron isotopes into ferritin, provided this process resembles earlier observations made for iron oxidation in aqueous solution. As more iron is deposited in ferritin than released, iron in the cytoplasma would become enriched in the lighter isotopes before export through ferroportin 1 into blood (Fig. 5, step J). Oxidation of exported ferrous iron to ferric iron for transferrin binding (Fig. 5, steps K and L) are unlikely to cause an isotope effect as oxidation is quantitative. Remaining heavier iron isotopes in the mucosa are excreted by regular exfoliation of mucosal cells by which the heavier iron isotopes would leave the body through feces (Fig. 4, flux C). Because of mass balance principles, the enrichment of non-absorbed iron in the heavier isotopes is likely to be small, as ca. 90% of the dietary iron is not absorbed. Although the proposed mechanisms at molecular level are currently speculative and require iron isotopic analysis of mucosal tissue for confirmation they point clearly to specific cellular processes in the enterocyte that bear a high potential for mass-sensitive iron transport as the precondition for an isotope effect.

8. The potential of iron isotope effects in biomedical research

Although the exact biochemical pathways of iron isotope fractionation in the human body still remain to be explored, we have presented a model that appears to suggest that preferential absorption of lighter iron isotopes, rather than release of heavier iron isotopes, exerts the principle control over the iron isotopic composition of blood in healthy adults. Consequently, the human body is enriched in lighter iron isotopes, as is observed in animals in general. Similar to calcium, iron becomes increasingly enriched in the lighter isotopes with trophic level along the food chain. Given that iron in muscle tissue of animals is largely heme iron, the absence of a fractionation step suggests that intestinal absorption of non-heme iron from plant foods is responsible for the observed isotope effect. Possible mechanisms include reduction of ferric iron prior to absorption and/or kinetic effects within the enterocyte during uptake, distribution and/or release of absorbed iron. Contrary to carbon and nitrogen, iron and calcium become increasingly enriched in the lighter isotopes with trophic level (Fig. 1). In case of carbon this is supposedly due to the release of light isotopes during respiration [4]. Our observations suggest that iron, in contrast, is fractionated upon absorption.

If this is the case, then the obvious next step would be to establish whether the isotope ratio of adsorbed iron relates in a predictable and quantifiable way to the efficiency of intestinal iron absorption. It might then be possible to assess the regulation state of iron absorption in the individual by its iron isotopic signature in blood. The human body has no active means to excrete excess iron to avoid iron accumulation in body tissues. Iron balance is maintained exclusively by down-regulating iron absorption in the intestine if iron is building up in the body or by up-regulating iron absorption if more iron is lost than gained from the diet. Messaging of iron status to the enterocyte for up-regulating or down-regulating iron absorption is currently unclear and may involve concentration of ferritin in serum from sequestered erythrocytes [48] or hepcidin, a recently discovered protein that is expressed in the liver [59]. If up-regulation of intestinal iron absorption can not prevent further emptying of liver iron stores, hemoglobin concentration in blood cannot be maintained any longer and iron deficiency anemia develops with its direct consequences on physiological function. Cut-off levels have been defined for anemia by the World Health Organization (WHO). These range from 110 g/L hemoglobin in blood for pregnant women and children 6 months to 5 years of age, to 120 g/L for non-pregnant women, to 130 g/L for men in Caucasians [60]. Use of stored iron for heme synthesis is reflected in a drop in ferritin concentration in serum as a measure of iron status. In absence of infection, serum ferritin concentrations below 15 μg/L indicate iron deficiency [60]. Because of regulatory mechanisms, the efficiency of intestinal iron absorption is directly linked with iron status in the healthy individual [48].

One of the major obstacles in biomedical research are biological variations. Cut-off levels of status measures are mostly derived from population-based studies in healthy individuals. Cut-off values are obtained by measuring the respective parameter in blood in a representative population group and by substracting two standard deviations from the mean. Alternatively, functional parameters such as the absence of stainable iron in the bone marrow are assessed which indicates iron deficiency as all iron delivered to the erythron is used immediately for haemoglobin synthesis. While cut-off levels for the various available measures of iron status in blood (haemoglobin, serum ferritin, zinc protoporphyrin, serum transferrin receptor, serum iron or total iron binding capacity) allow for a reliable assessment of the prevalence of iron deficiency, iron deficiency anemia or iron overload in population groups [60], their usefulness for diagnosis in the individual is limited.

Iron isotope effects in blood have two distinct advantages over other measures of iron metabolism in the individual. First, they are independent of the element's concentration as the iron isotopic composition of blood is a property of the iron itself. Second, they are not subject to intra-individual changes within and between days as other measures of iron status in blood. Because of the slow turnover of body iron, it takes years to alter the iron isotopic composition of the body. Obviously, this disqualifies iron isotope effects in blood as a possible tool to assess acute changes in iron metabolism as is possible using conventional biochemical measures of iron status. However, they could be useful to assess long-term differences in the efficiency of iron absorption between individuals. An individuals' unique iron isotopic signature in blood would be established in the long-term by the efficiency of iron absorption while bulk fractionation factors between individuals are uniform, or by a specific bulk fractionation factor for each individual. In either case, a specific fingerprint results.

For practical reasons, it is unlikely that iron isotope effects in blood can be exploited in clinical settings and patient care in the nearby future as sample analysis is technically comparatively demanding and costly. For research purposes, however, iron isotope effects could be potentially helpful in identifying genotypes within a population that carry a genetic predisposition to absorb iron more efficiently or less efficiently which would point to a higher risk for developing iron deficiency or iron overload, respectively. Hereditary hemochromatosis is perhaps the most prominent example in this context as it leads to pathological iron accumulation due to a distinct gene defect. Phenotypic expression of other gene defects are probably less visible and, therefore, still remain hidden.

Using conventional tracer techniques, the long-term efficiency of iron absorption is practically impossible to assess in the individual as iron absorption varies strongly within and between days. This is a major obstacle for better understanding the link between iron absorption and iron status in order to identify an impaired balance state. Iron isotope effects in blood may serve here as a novel tool as they integrate over

physiological fluctuations in iron absorption. Since significant isotope effects have also been identified for calcium in higher organisms [6,7] and, more recently, for magnesium in the human body [61] it appears that MC-ICP-MS has opened a new door to biomedical research. The emerging interdisciplinary research has the potential to become a new bridge between inorganic mass spectrometry and the life sciences for improving health through isotope sciences.

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Appendix A

A.1. Study design

In this study, we present new data on the iron isotopic composition of blood, liver, and muscle obtained from the same individual. Samples for iron isotopic analysis were obtained from 10 individuals undergoing abdominal surgery at the University Hospital Zurich. All subjects gave written informed consent to participate in the study. Liver samples were obtained from removed liver tissue. Muscle samples were obtained from abdominal muscle tissue during surgery. Venous blood samples were drawn into heparinized tubes before surgery. Subject characteristics and iron status parameters assessed before surgery (University Hospital Zurich, Clinical Chemistry Department) are shown in Table A.1. The study protocol was approved by the Ethical Committee at the University Hospital Zurich.

A.2. Materials and methods

Liver and muscle samples were washed thoroughly with physiological saline (0.7% NaCl, w/w) in acid washed polyethylene tubes. Physiological saline was prepared for this study from analytical grade NaCl (p.a., Fluka, Buchs, Switzerland) and 18 MΩ water. Samples of blood (0.5 ml), liver (0.1 g) and muscle (0.2 g) were mineralized by microwave digestion (MLS 1200, MLS GmbH, Leutkirch, Germany) using a mixture of H_2O_2 (p.a., Fluka) and conc. HNO_3 (p.a., Fluka, subboiled). Digested samples were shipped to Hannover for isotopic analysis, where iron was separated and purified by anion-exchange chromatography. Isotope ratios were determined by a Thermo Finnigan Neptune Multicollector ICP-MS at the University of Hannover. The precision on δ^{56}Fe was 0.049‰, and that of ^{57}Fe was 0.071‰, respectively (2σ external reproducibility of natural samples).

A.3. Results

Iron isotope ratios of blood, muscle and liver tissue and corresponding iron status parameters for each individual are shown in Table A.1. None of the subjects were anemic. Three male subjects had serum ferritin concentrations outside the normal range (15–300 μg/L) and two subjects had elevated inflammation markers (C-reactive protein >5 mg/L). Statistically significant differences between the iron isotopic composition of blood, muscle and liver tissue were identified by paired Student's *t*-test (level of significance: 95% confidence interval, $P < 0.05$). Differences were insignificant for muscle and blood ($P = 0.13$) but were highly significant for blood and liver ($P < 0.0001$) and muscle and liver ($P < 0.001$). For statistical analysis of other data discussed in the text please refer to the corresponding, original publications [14,55].

Table A.1
Iron isotope ratios of blood, muscle and liver tissue in 10 individuals (for illustration, see Fig. 7)

Subject no.	Gender	Age	Hematology				^{56}Fe/^{54}Fe isotope ratio		
			Hemoglobin (g/L)	Serum ferritin (μg/L)	Transferrin saturation (%)	C-reactive protein (mg/L)	Blood, $\delta_{IRMM\,14}$ (‰)	Muscle, $\delta_{IRMM\,14}$ (‰)	Liver, $\delta_{IRMM\,14}$ (‰)
1	F	73	133	88	14	9	−2.290 ± 0.030	−2.280 ± 0.038	−0.784 ± 0.027
2	F	47	128	17	23	2	−2.572 ± 0.008	−2.584 ± 0.007	−1.393 ± 0.043
3	F	52	136	103	25	2	−2.668 ± 0.019	−2.601 ± 0.001	−0.850 ± 0.052
4	F	65	77	1175	9	172	−2.606 ± 0.069	−2.587 ± 0.042	−1.369 ± 0.023
5	M	72	146	637	40	2	−2.954 ± 0.037	−2.716 ± 0.016	−1.489 ± 0.068
6	M	65	126	268	42	2	−2.900 ± 0.028	−2.646 ± 0.052	−1.340 ± 0.065
7	M	66	139	407	17	23	−2.613 ± 0.034	−2.625 ± 0.005	−1.795 ± 0.024
8	M	55	143	54	27	5	−2.570 ± 0.073	–	−1.521 ± 0.023
9	M	44	132	528	21	27	−3.027 ± 0.050	–	−1.641 ± 0.042
10	M	72	145	127	41	5	−3.172 ± 0.045	–	−1.530 ± 0.016
Mean							−2.74 ± 0.27	−2.58 ± 0.15	−1.37 ± 0.32

Data are given as the mean ± 1 S.D. of two to five independent measurements of the same sample.

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