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PAPER

Isotopic evidence of unaccounted for Fe and Cu erythropoietic pathways†

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Despite its potential importance for understanding perturbations in the Fe–Cu homeostatic pathways, the natural isotopic variability of these metals in the human body remains unexplored. We measured the Fe, Cu, and Zn isotope compositions of total blood, serum, and red blood cells of ~50 young blood donors by multiple-collector ICP-MS after separation and purification by anion exchange chromatography. Zinc shows much less overall isotopic variability than Fe and Cu, which indicates that isotope fractionation depends more on redox conditions than on ligand coordination. On average, Fe in erythrocytes is isotopically light with respect to serum, whereas Cu is heavy. Iron and Cu isotope compositions clearly separate erythrocytes of men and women. Fe and Cu from B-type men erythrocytes are visibly more fractionated than all the other blood types. Isotope compositions provide an original method for evaluating metal mass balance and homeostasis. Natural isotope variability shows that the current models of Fe and Cu erythropoiesis violate mass balance requirements. It unveils unsuspected major pathways for Fe, with erythropoietic production of isotopically heavy ferritin and hemosiderin, and for Cu, with isotopically light Cu being largely channeled into blood and lymphatic circulation rather than into superoxide dismutase-laden erythrocytes. Iron isotopes provide an intrinsic measuring rod of the erythropoietic yield, while Cu isotopes seem to gauge the relative activity of erythropoiesis and lymphatics.

Introduction

Although still at the exploratory stage, the study of natural isotopic variability of metallic elements may help identify some critical aspects of the homeostatic regulation of metals. Fractionation of stable isotopes is a quantum mechanical effect, rooted in the Heisenberg uncertainty principle, which requires that the lowest level of vibrational energy of the compound is non-vanishing. The existence of zero-point energy favors the concentration of heavy isotopes in the tighter bonds. The development of multi-collector inductively-coupled plasma source mass spectrometry (MC-ICP-MS) has made it possible to assess the isotopic variability of metals, such as Cu, Zn,[1,2] and Fe.[3] Isotope fractionation in excess of the permil level has been observed for Fe isotopes within the human body[4] and for Zn isotopes among the organs and the body fluids of sheep.[5] Biological fractionation of metal isotopes often exceeds the range of isotope fractionation in inorganic natural processes. The metal-rich organs, notably the liver, are depleted in the light isotopes of Fe and Zn isotopes with respect to blood and skeletal muscle. In the iron overload disease known as hemochromatosis, the $^{56}Fe/^{54}Fe$ isotope ratio of blood correlates with iron accumulation.[6] Although it is still to be understood whether such a strong fractionation of metal isotopes results from kinetic effects or from different coordinations in biological media, *e.g.*, ferritin *versus* hemoglobin for Fe, there is strong incentive to map isotopic effects in organs and in blood. The present investigation reports the Fe, Cu, and Zn isotope compositions of total blood, serum, and red blood cells from ~50 young anonymous blood donors and attempts to relate the isotopic variability to the available gender and blood-group information.

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Experimental

Reagents

Macroporous anion-exchange resin AGMP-1 100–200 mesh were purchased from Biorad Laboratories. Concentrated technical HCl and HNO_3 provided by Merck 64271 Darmstadt, Germany, were redistilled at low temperature in Picotrace fluoropolymer stills. H_2O_2 30% Suprapur was purchased from Merck. Demineralized water is produced in a Millipore Synergy system.

Procedures

The samples were provided as congealed splits by the Etablissement Français du Sang in Lyon. All experiments

Table 1 Summary of analytical conditions

Instrument	Agilent 7500 cx	Nu500 HR	Nu1700
RF power	1500 W	1350 W	1350 W
External flow	15 L min^{-1}	14 L min^{-1}	14 L min^{-1}
Nebuliser gas flow rate	1.1 L min^{-1}	1 L min^{-1}	1 L min^{-1}
Integration time per isotope	0.1 s	10 s per cycle	10 s per cycle
Total integration time	120 s	2 × 20 cycles	2 × 20 cycles
Collision/reaction gas	He	N/A	N/A
Flow rate	6 mL min^{-1}	N/A	N/A
Nebuliser	GE AR351FM04	GE AR351FM01	GE AR351FM02
DSN neb pressure	N/A	30 psi	31 psi
Sample cone	Ni	Ni	Ni
Skimmer cone	Ni	Ni	Ni

were performed in compliance with the relevant laws and institutional guidelines. An aliquot of about 0.5 mL of each sample was mineralized in PFA vessels at 130 °C using a mixture of HNO_3 and H_2O_2 and evaporated twice in concentrated HCl at 120 °C in a laminar flow hood. The metals analyzed here were separated on quartz columns containing 3 mL macroporous anion-exchange resin following the technique of Maréchal *et al.*[2] Samples were loaded onto the columns in 7 N HCl and rinsed in 10 mL 7 N HCl + H_2O_2 0.001%. Copper was eluted by 20 mL 7 N HCl + H_2O_2 0.001%, iron by 10 mL 2 N HCl + H_2O_2 0.001%, and zinc by 10 mL 0.5 N HNO_3. Each fraction was evaporated again, converted to nitrates and taken up in 0.05 N HNO_3 prior to analysis.

Cu and Zn isotope compositions were determined on a Nu HR multiple-collector inductively-coupled plasma mass spectrometer (MC-ICP-MS) using a wet plasma, while Fe was run on a large radius Nu 1700 MC-ICP-MS operated at a resolution of 4500 as dry plasma after desolvation in a Nu DSN. Free aspiration mode and a sample uptake rate of 100 μL min^{-1} were used throughout. Gas flow instrumental mass fractionation was controlled both by dual standard-sample bracketing and addition of an external standard (Cu to Zn samples and Zn to Cu samples). The isotope reference solutions used were NIST 976 (Cu), JMC 3-0749 L (Zn), and IRMM 014 (Fe). The precision (external reproducibility, two-sigmas) on the isotopic ratios is 0.05‰. Na, K, Mg, Ca, Fe, Cu and Zn concentrations were determined on an Agilent 7500 CX quadripole ICP-MS. The run conditions are listed in Table 1.

Results

The complete dataset is reported in Table A1 of ESI.† Average values of isotope compositions and Student-*t* are given in Table 2. The δ notation indicating the deviation in parts per 1000 of a particular isotopic ratio, here $^{56}Fe/^{54}Fe$, $^{66}Zn/^{64}Zn$,

Table 2 Average isotope compositions in delta units (permil or ‰) and 95% range (2 s) for the isotope compositions of Zn, Cu, and Fe in the serum, erythrocytes, and total blood of 49 blood donors. Typical analytical uncertainties are 0.05‰. Men–women comparison: *t* is the Student–Fisher parameter and *p* the probability that the two sets are identical

	n	av $\delta^{66}Zn$	2 s	av $\delta^{65}Cu$	2 s	av $\delta^{56}Fe$	2 s	Fe/Cu	2 s
Serum									
Women	28	0.18	0.28	−0.24	0.36	−1.35	0.93	1.06	0.96
Men	21	0.16	0.10	−0.28	0.40	−1.71	1.55	1.83	1.32
All	49	0.17	0.26	−0.26	0.40	−1.51	1.24	1.44	1.37
Outliers		3		2		0			
t men/women		0.78		1.06		2.11		−3.30	
p value		0.45		0.30		0.05		2.10^{-5}	
Erythrocytes									
Women	28	0.46	0.17	0.46	0.47	−2.49	0.39	1317	578
Men	21	0.43	0.45	0.67	0.36	−2.72	0.36	1410	396
All	49	0.44	0.33	0.56	0.5	−2.59	0.47	1361	504
Outliers		1		2		3			
t men/women		0.86		−3.87		4.44		−0.92	
p value		0.39		0		0		0.21	
Total blood									
Women	28	0.41	0.16	0.01	0.16	−2.58	0.18	409	254
Men	21	0.39	0.41	0.17	0.33	−2.72	0.4	648	180
All	49	0.4	0.37	0.09	0.32	−2.65	0.38	500	327
Outliers		3		2		3			
t men/women		1		−2.88		2.83		−5.36	
p value		0.32		0.02		0		3.10^{-9}	
Liver[a]		−1.13		−0.4		−1.63		17.43	23.08
t serum/erythrocytes									
Women		−12.76		−17.48		12.9		−27.64	
Men		−9.28		−23.24		7.91		−33.30	
All		−15.61		−24.84		14.63		−36.86	

Outliers have been eliminated using a 99% probability *t*-filter. [a] Data from Jaouen *et al.* (submitted).

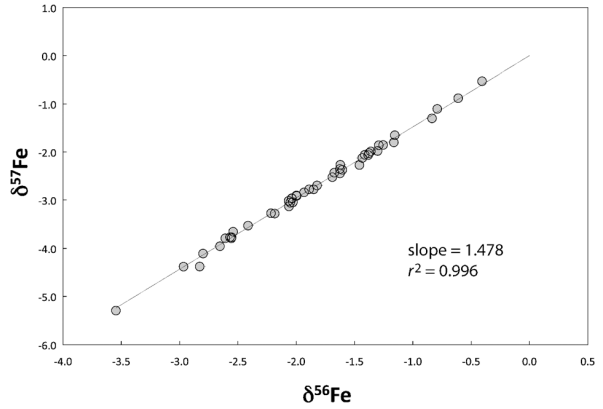

Fig. 1 Isotopic variations of $^{57}Fe/^{54}Fe$ and $^{56}Fe/^{54}Fe$ follow a mass-dependent fractionation relationship with a slope not significantly different from the theoretical value of ln(57/54)/ln(56/54) ≈1.487.

and $^{65}Cu/^{63}Cu$, with respect to a reference material, is used throughout.

The mass-dependent character of isotope fractionation, which has been repeatedly documented for Zn (*e.g.*, ref. 2), is demonstrated here for Fe in Fig. 1 by the linear relationship between $\delta^{56}Fe$ and $\delta^{57}Fe$. The slope of the straight-line is, as expected, of ~3/2, *i.e.*, within error bars equal of the theoretical value of ln(57/54)/ln(56/54) = 1.487.

The Fe, Cu, and Zn concentrations are consistent with literature values.[7] Average values are listed in Table 2 together with data averages on liver tissue discussed elsewhere (Jaouen *et al.*, submitted). Mass balance applied to Fe concentration data indicates that RBC makes up ~50 percent of the blood volume. Mass balance applied to the Mg/Fe (or Ca/Fe) and Mg/Cu ratios also shows that RBC accounts for most (99.8 ± 0.1%, 95% uncertainty) of the total Fe in blood, and 34 ± 14% of Cu. Using the Ca/Zn ratios, the mean proportion of 89 ± 6% Zn in erythrocytes is compatible with literature findings.[8] All of these values are mutually consistent, regardless of the normalizing element (Na, Ca, Fe, Mg).

Zinc

Concentrations in serum tend to be more concentrated for men than for women, whereas the Zn isotope compositions overlap for both genders (Table 2). In contrast, Zn tends to be more concentrated in the red blood cells of female subjects. The erythrocyte Zn shows no sex-related isotopic difference. The spread of $\delta^{66}Zn$ is twice as large for men. The lack of gender bias for Zn contents in total blood reflects that women have fewer erythrocytes than men. Zn is on average 0.2 permil heavier in erythrocytes with respect to serum. Erythrocyte–serum isotopic differences for Cu and Zn are not visibly correlated.

Iron

Concentrations in serum do not differ between men and women in contrast to Fe isotope compositions (p = 0.05). Iron is less concentrated in the red blood cells of men relative to women but total blood shows almost complete overlap because women's blood carries fewer erythrocytes. The $\delta^{56}Fe$ of both the serum and erythrocytes is negative, which is a feature common to all

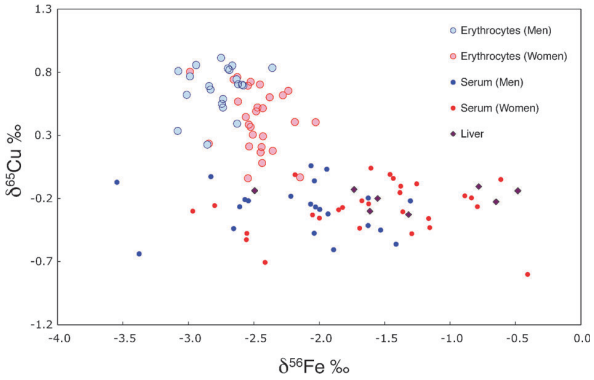

Fig. 2 Iron and Cu isotope fractionation in erythrocytes and serum of men and women (see ESI† for the data). Also shown are the liver data from Jaouen *et al.* (submitted). The gender effect reflects faster processing and smaller iron stores in women. Caveat: as a result of very different Cu and Fe apportionments φ, mass conservation upon combination of blood components, notably that of serum and erythrocytes into total blood, does not produce straight-lines but strongly curved arrays, which accounts for the overall L-shape of the data distribution. The overlap of isotope compositions between liver and serum reflects the production of ceruloplasmin in the liver.

biological material.[4] The $\delta^{56}Fe$ of erythrocytes is conspicuously lower than in serum by about 1–2 permil (Table 2 and Fig. 1). Iron of men's erythrocytes and whole blood is isotopically lighter. In women's serum, it is only marginally heavier than for men's (p = 0.05), but the spread of the data is large. The liver $\delta^{56}Fe$ data of Jaouen *et al.* (submitted) overlap with the serum values.

Copper

The present concentration data confirm earlier observations[7] that Cu is more concentrated in women's relative to men's serum. The large spread of Cu concentrations for female subjects is known to be related to estrogen intake.[9] Copper isotope compositions in serum show no significant gender bias. On average, Cu is 0.35 permil heavier in women's erythrocytes with respect to serum, and this figure increases to 0.48 permil for men. On average, Cu is isotopically 0.8 permil heavier in erythrocytes with respect to serum (Fig. 2). The liver $\delta^{65}Cu$ data of Jaouen *et al.* (submitted) (mean value −0.4‰) overlap with those of the mean serum (mean value −0.28‰).

ABO blood type

There is no observable systematics in the Fe, Cu, and Zn isotope compositions related to the ABO blood type in women except possibly for a smaller spread of group O with respect to groups A and B (Fig. 3). For men, however, a Kruskal–Wallis non-parametric test shows that B-type men erythrocytes are significantly fractionated with respect to the other blood types. This difference is shown in Fig. 4, where the statistical ('Mahalanobis') distances to the mean values in the $\delta^{65}Cu$–$\delta^{56}Fe$ space are arranged by blood types. O-type erythrocyte isotope variability for men and B-type variability for women seem to be more restricted than for other groups.

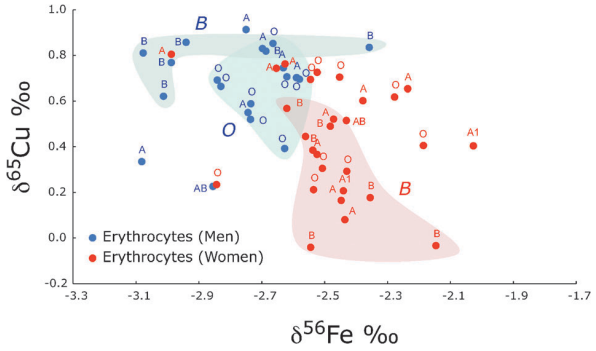

Fig. 3 Blood-type variability of Fe and Cu isotope compositions in men and women erythrocytes. The extreme composition of B-type men erythrocytes is not explained. B-type erythrocyte isotope variability of both genders is more restricted than for other groups.

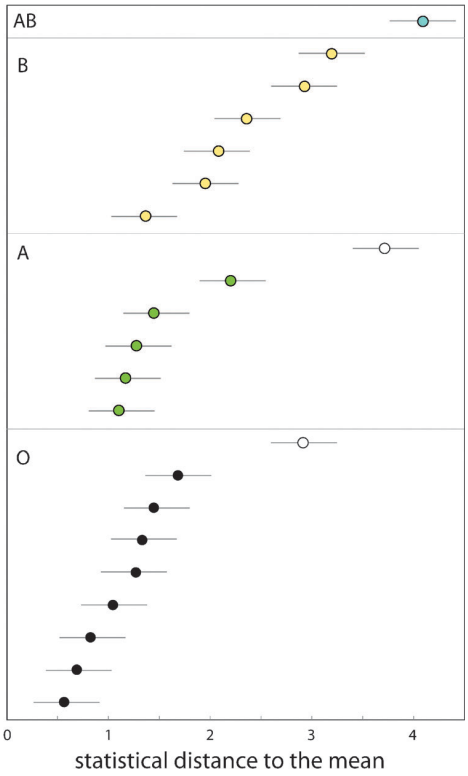

Fig. 4 Statistical ('Mahalanobis') distance in the $\delta^{65}Cu$–$\delta^{56}Fe$ space of each erythrocyte sample to the mean value (men only) arranged by the blood type. A value of one corresponds to a standard deviation along each coordinate. The B-type stands out against the other groups. The open symbols are probable outliers. The analytical uncertainties are shown as horizontal bars (0.05‰).

Discussion

Whether in intracellular or extracellular material, the free ions Fe^{2+}, Cu^{2+}, and Zn^{2+} are toxic to cells and therefore move around in the body as complexes within metalloproteins. Isotope fractionation of metals among their various complexes has so far been explored neither experimentally nor theoretically, and the interpretation of the isotopic variability must, therefore, resort to general principles. The energy of a bond increases with its vibration frequency and, hence, decreases with the atomic weight of the binding metal isotope. The preferential concentration of heavy isotopes into stronger over weaker bonds therefore minimizes the overall energy of the system. As a consequence, the two major potential factors of isotopic fractionation are the oxidation state and the nature of ligands. Fe(III) has a stronger electrostatic field than Fe(II) and, to a first order, oxidized compounds should be isotopically heavier than their reduced equivalent. The isotopic variability among blood components will depend on steps involving oxidoreductases, pairs of redox ions, such as Fe and Cu, and cysteine redox chemistry.[10] Likewise, ligands that are strong Lewis bases should pull their bonds more strongly than soft bases: typically, for amino acid residues, histidine with its imidazole N-bonds makes harder bonds than cysteine and methionine with their thiol and thioether S-bonds,[11] and is therefore expected to concentrate heavy isotopes. Lastly, it must be emphasized that a chemical reaction with full conversion of one single reactant into one single product cannot induce isotope fractionation. For example, little or no isotope fractionation is expected during transport by ferroportin (Fe) or Atox1 (antioxidant protein 1, Cu). In order for isotope fractionation to take place, a metal-bearing compound must dissociate, whether at equilibrium or kinetically, into two distinct metal-bearing products, as for example during partial uptake of a metal into the cell from the blood stream.

Blood-types and isotope fractionation

The Mn-loaded glycosyltransferases GTA and GTB synthesizes A and B antigens on erythrocytes.[12] The functions of the ABO blood group antigens are not known. A feature potentially relevant to erythrocytes activity is that, although GTA and GTB only differ from each other by two substitutions, one of them involves the replacement of a leucine by a methionine residue. Whether the synthesis of blood types is in some way connected to uptake of Fe by erythrocytes is not known.

Zinc

About 99% of the total zinc from the body is intracellular. Once absorbed from the gastrointestinal tract into the circulation, zinc is taken up by the liver within three hours and eventually appears in the pancreas, kidneys, and other tissues.[13] Carbonic anhydrase, in which Zn is coordinated with three histidine residues, accounts for most of this metal endowment of erythrocytes, while Zn preferentially binds loosely to albumin in the serum. In metallothionein, Zn is bound to cysteine.[14] The weak $\delta^{66}Zn$ variability in erythrocytes and serum is probably due to hard histidine *versus* soft cysteine bonding contrasts. The much weaker $\delta^{66}Zn$ contrast between erythrocytes and plasma with respect to the $\delta^{56}Fe$ and $\delta^{65}Cu$ contrasts suggests that, to a large extent, the metal oxidation state is more effective at fractionating isotopes than mere conformational differences.

Iron

Most of the bodily Fe resides as Fe(II) in hemoglobin and myoglobin, while the rest is stored largely as Fe(III) in ferritin (a ferrihydrite analog broadly distributed in tissues) and hemosiderin[15] in the cytosol of liver, spleen, and bone marrow

cells. Dietary Fe(III) iron is reduced to Fe(II) by duodenal ferric reductase (Dcytb)[16] and transported into the enterocytes by the metal transporter DMT1. Reductive uptake is a straightforward explanation for the overall negative δ^{56}Fe of all the bodily iron with respect to diet Fe.[4] Fe is released from enterocytes by ferroportin into the blood stream,[17] where it is oxidized into transferrin-bound Fe(III) by Cu-based ferroxidases hephaestin and ceruloplasmin.[18] Iron is stored in hepatocytes as ferritin-bound Fe(III) and released from the liver as serum transferrin, which is used by erythropoiesis upon Fe reduction by Cu-bearing Steap3 (six-transmembrane epithelial antigen of prostate 3) ferrireductases.[19] The present observations confirm the higher serum ferritin/hemoglobin ratio in men[20] and the large size of their iron stores.[21]

The Fe and Cu isotope differences between erythrocyte and serum must originate in erythropoiesis. Since we are dealing with blood from young adults, we assume that erythropoiesis is restricted to bone marrow.[22] We also consider that the turnover of erythrocytes is fast enough for steady-state to be achieved, or, equivalently, that there is no substantial accumulation or loss of Fe in the bone marrow. With such simple assumptions, the erythropoietic role of other homeostatic compartments, such as the bile or the spleen, can safely be disregarded. A simple examination of isotopic mass balance demands that the reduction of serum transferrin, hereafter labeled 'up', into low-δ^{56}Fe ferrous hemoglobin ($\delta^{56}Fe_{up}$ is therefore the value for serum), leaves a high-δ^{56}Fe ferric residue (the precise nature of which, ferritin or hemosiderin, being immaterial to the breakdown of the Fe inventory) and hereafter labeled 'down'. For a given individual, the isotopic mass balance equation reads

$$\delta^{56}Fe_{up} = \varphi^{Fe}_{Hb}\delta^{56}Fe_{Hb} + \varphi^{Fe}_{down}\delta^{56}Fe_{down} \quad (1)$$

(*e.g.*, ref. 23), where φ^{Fe}_{Hb} and $\varphi^{Fe}_{down} = 1 - \varphi^{Fe}_{Hb}$ represent the proportion of transferrin Fe (Tf) converted to hemoglobin (Hb) and ferric residue (Ft) (ferritin plus hemosiderin), respectively. For a given individual, the efficiency of hemoglobin conversion (iron utilization) during erythropoiesis may therefore be obtained from φ^{Fe}_{Hb} which is simply:

$$\varphi^{Fe}_{Hb} = \frac{\delta^{56}Fe_{up} - \delta^{56}Fe_{down}}{\delta^{56}Fe_{Hb} - \delta^{56}Fe_{down}} \quad (2)$$

The bone analyses by Jaouen *et al.* (submitted) suggest that $\delta^{56}Fe_{down} \approx -0.45$‰ for men and −0.12‰ for women. The values of φ^{Fe}_{Hb} plotted in the histograms of Fig. 5 correspond to a surprisingly broad range (20–100%) of transferrin–hemoglobin conversion. The magnitude of the denominator (> 2‰) is large enough that analytical uncertainties do not affect φ^{Fe}_{Hb} by more than 10 percent. The donors, having been deemed healthy from their blood counts, provide no indication that the variability of φ^{Fe}_{Hb} entails any pathological condition such as anemia. The difference of hemoglobin conversion efficiency between men and women is not significant.

Homeostatic Fe cycles (*e.g.*, ref. 24) do not, so far, include the ferric residue pathway. Such cycles violate the isotopic mass balance and need to be amended accordingly. It is only recently that ferritin has been suspected to play a significant role in Fe transport.[25] Even if physiology may have

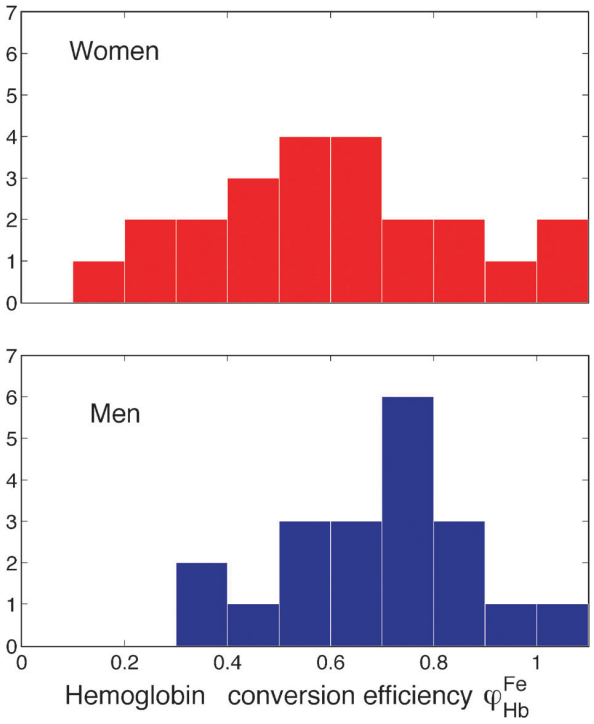

Fig. 5 Erythropoietic conversion of transferrin into hemoglobin: histograms of the yields φ^{Fe}_{Hb} calculated from the isotopic data listed in ESI† and eqn (2). Blue: men. Red: women.

underestimated the weight of the ferritin pathway, it did not escape histological observation. The erythroblastic islands of the bone marrow are composed of erythroblasts surrounding a central macrophage.[26] Whether these macrophages play a role in secreting high-δ^{56}Fe ferritin[25] or rather themselves handle the transport of ferritin to the liver and the spleen, they participate in large Fe fluxes of the same magnitude as those associated with hemoglobin (Fig. 6).

Copper in serum is hosted at 70–80% by ceruloplasmin with six Cu(II) ions,[27] a ferroxidase secreted in hepatocytes. The rest of the serum copper is bound to albumin and histidine. Ceruloplasmin is synthesized in the liver by the Cu(I)-loaded ATPase ATP7B,[28] which also eliminates excess hepatic Cu into the bile.[29] Metallothionein is also used for Cu storage in the liver. The similar δ^{65}Cu (−0.33‰) values of the liver and serum (−0.28‰) suggest a lack of Cu isotope fractionation within the liver among different exports. Serum therefore transports unfractionated liver copper, regardless of its oxidation state (Fig. 2). The isotopically lighter Cu in both the serum and the erythrocytes of women clearly reflects smaller iron stores and the much higher (×2.5) turnover rate of iron previously described in the literature.[20] Women process copper more rapidly than men and therefore produce more ceruloplasmin.

In contrast, intracellular Cu is bound first to redox-active Cu(I–II)–Zn superoxide dismutase, an antioxidant enzyme, through histidine bonds.[30] Cu–Zn superoxide dismutase is especially abundant in liver, kidney, and red blood cells.[31]

As for Fe, the conspicuous 0.82‰ difference in δ^{65}Cu values between serum and erythrocytes leaves erythropoiesis

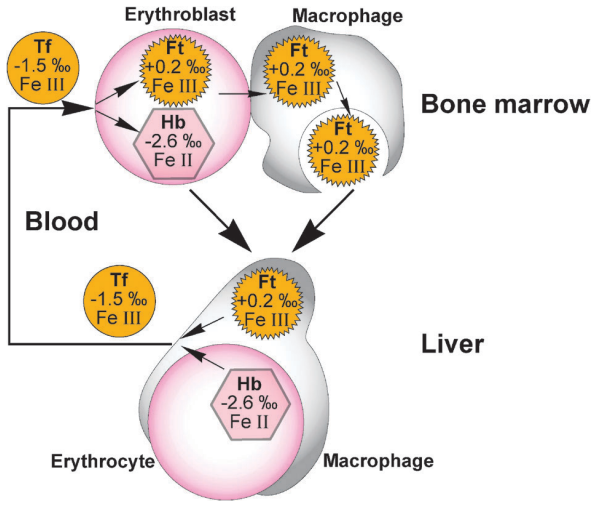

Fig. 6 Iron isotope fractionation during erythropoiesis: the missing Fe pathway. Wholesale conversion of transferrin (Fe(III), $\delta^{56}Fe = -1.2‰$) to hemoglobin (Fe(II), $\delta^{56}Fe = -2.6‰$) is not allowed isotopically. A quite substantial part of the transferrin iron must be converted into isotopically heavy ferritin (tentatively $\delta^{56}Fe = +0.2‰$) and disposed of by macrophages.

grossly imbalanced isotopically (Fig. 7). Ceruloplasmin-dominated serum Cu, which is again labelled 'up', enters bone marrow with $\delta^{65}Cu_{CP} \approx -0.26‰$, whereas CuZn-superoxide

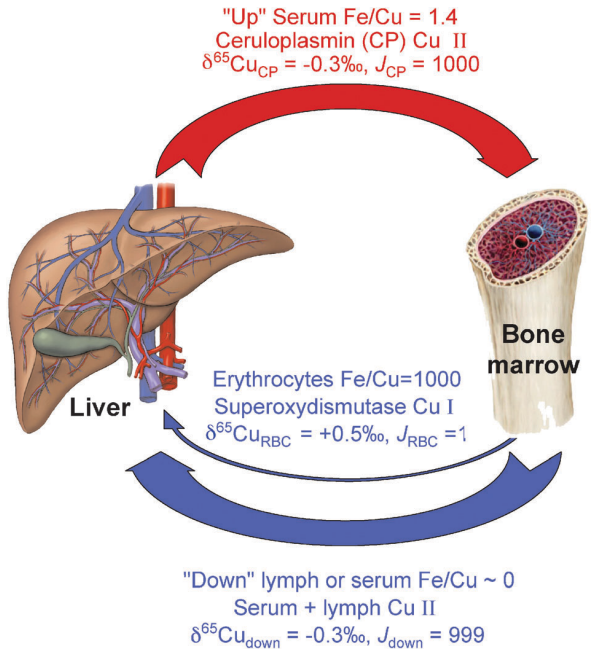

Fig. 7 Copper isotopes and Cu/Fe fractionation during erythropoiesis: the missing Cu pathway. Wholesale conversion of the plasma ceruloplasmin produced by the liver ($\delta^{56}Fe = -0.3‰$) to the CuZn superoxide dismutase present in erythrocytes ($\delta^{56}Fe = +0.5‰$) is not allowed isotopically. 99.9 percent of the copper present in serum before erythropoiesis is not used. It must be returned with a very different Cu/Fe ratio and either carried by the lymph or mixed back into post-erythropoietic serum. The variable J stands for Cu fluxes with respect to an input of ceruloplasmin of 1000.

dismutase-dominated erythrocytes move out with $\delta^{65}Cu_{RBC}$ values of $\sim +0.56‰$. A major complementary flow downstream from the bone marrow, hereafter labeled 'down', with very negative $\delta^{65}Cu$ is therefore required for closure. Using the same assumptions as for Fe, isotopic Cu mass balance reads:

$$\delta^{65}Cu_{up} = \varphi^{Cu}_{RBC}\delta^{65}Cu_{RBC} + \varphi^{Cu}_{down}\delta^{65}Cu_{down} \qquad (3)$$

while for the elemental Fe/Cu ratio:

$$\left(\frac{Fe}{Cu}\right)_{up} = \varphi^{Cu}_{RBC}\left(\frac{Fe}{Cu}\right)_{RBC} + \varphi^{Cu}_{down}\left(\frac{Fe}{Cu}\right)_{down} \qquad (4)$$

in which φ^{Cu}_{RBC} and $\varphi^{Cu}_{RBC} = 1 - \varphi^{Cu}_{down}$ represent the proportion of seric ('up') Cu converted to RBC Cu and directed to the missing pathway, respectively. Therefore

$$\varphi^{Cu}_{RBC} = \frac{\delta^{56}Cu_{up} - \delta^{65}Cu_{down}}{\delta^{65}Cu_{RBC} - \delta^{65}Cu_{down}} = \frac{(Fe/Cu)_{up} - (Fe/Cu)_{down}}{(Fe/Cu)_{RBC} - (Fe/Cu)_{down}} \qquad (5)$$

The combination of 4 and 5 shows that $\delta^{65}Cu$ and Fe/Cu, RBC, serum ('up'), whole blood, and the missing 'down' component are linearly related. The conditions $(Fe/Cu)_{RBC} > 1000$ and $(Fe/Cu)_{up} \approx 1$ require that $(Fe/Cu)_{down} < 1$ and $\varphi^{Cu}_{RBC} \ll 0.001$. Again, the uncertainty on these estimates is fairly small. Less than one part per mil of the serum Cu is therefore utilized by erythropoiesis. The huge, unaccounted for 'down' pathway, which makes up over 99.9 percent of the 'up' flow, is physiologically unexplained. Copper excess may be disposed of at other erythropoietic centers, such as the spleen, or may be entrained in the lymphatic circulation, possibly refluxed with the serum downstream from the bone marrow.[32] Copper fixation in the cytochrome oxidase of the erythroid mitochondrion membrane is clearly involved in Cu cycling.[24] A histological Cu model parallel to the erythroblastic islands model used for Fe is, however, not available and, in particular, the role of macrophages with respect to such a large Cu flux is unknown.

Potential usefulness of isotopic studies

Iron and copper isotopes carry information on erythropoiesis that cannot be superseded by more easily gathered concentration data. Collecting Fe and Cu concentrations characteristic of the different blood compartments, notably 'up' and 'down' the sites of erythrocytes production and destruction, is a formidable challenge, whereas a single anomalous isotope composition of any tissue biopsy may reveal a shift in metal processing. The similarity of $\delta^{56}Fe$ and $\delta^{65}Cu$ between liver and serum, and that of $\delta^{65}Cu$ in bone and serum (Jaouen *et al.*, submitted), determines a baseline for the 'normal' isotope compositions of these metals in bone and liver. This baseline is now in place to be used to detect anomalous conditions.

The present work clearly should be complemented by isotopic work on metal in ferritin, hemoglobin, ceruloplasmin, and membranes. Examples of potential applications abound. The Menkes and Wilson disease are controlled by perturbed Cu-ATPase activity and ceruloplasmin production[33] and would benefit from Cu isotopic analysis. The well-documented coupled imbalance of Fe and Cu in Alzheimer patients[34]

warrants further isotopic investigations to search for a distant echo of a severe erythropoietic condition. The buildup of resistance against cisplatin cancer drug may indicate that malignant cells may be able to redirect Cu-ATPases ATP7A and ATP7B[35] to modify the Cu-dependent uptake of Pt compounds through cellular membranes.[36] The apparent control of blood type may also deserve further attention since, for example, patients with blood groups A, AB, and B are 50 percent more likely to develop pancreatic cancer than those of the group O.[37]

Conclusions

The present work shows that the strong isotopic contrast of Fe and Cu between erythrocytes and serum reflects the redox conditions prevailing during erythropoiesis. The gender isotopic effect is accounted for by the different dynamics of iron stores between men and women. Full conversion of transferrin into hemoglobin is not permitted by isotopic observations and another pathway (possibly through ferritin) accounting for a substantial part of iron transit through the bone marrow is suspected, yet remains to be established. Likewise, isotopic constrains require the existence of an unknown Cu erythropoietic pathway.

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