Menopause Effect on Blood Fe and Cu Isotope Compositions

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KEY WORDS menopause; sex isotope difference; non-traditional stable isotopes; red blood cells; iron isotopes; copper isotopes

ABSTRACT Iron (δ^{56}Fe) and copper (δ^{65}Cu) stable isotope compositions in blood of adult human include a sex effect, which still awaits a biological explanation. Here, we investigate the effect of menopause by measuring blood δ^{56}Fe and δ^{65}Cu values of aging men and women. The results show that, while the Fe and Cu isotope compositions of blood of men are steady throughout their lifetime, postmenopausal women exhibit blood δ^{65}Cu values similar to men, and δ^{56}Fe values intermediate between men and premenopausal women. The residence time of Cu and Fe in the body likely explains why the blood δ^{65}Cu values, but not the δ^{56}Fe values, of postmenopausal women resemble that of men. We suggest that the Cu and Fe isotopic fractionation between blood and liver resides in the redox reaction occurring during hepatic solicitation of Fe stores. This reaction affects the Cu speciation, which explains why blood Cu isotope composition is impacted by the cessation of menstruations. Considering that Fe and Cu sex differences are recorded in bones, we believe this work has important implications for their use as a proxy of sex or age at menopause in past populations. Am J Phys Anthropol 153:280–285, 2014.

The stable isotopes of elements behave slightly differently in biochemical processes. Isotopic fractionation attests to differences in bond energies and hence is influenced by ligand configuration, redox conditions, and kinetics. Walczyk and von Blanckenburg (2002) demonstrated that the iron (Fe) in women's blood is isotopically heavier relative to that of men, that is to say enriched in Fe heavy isotopes, a result confirmed by further studies (Krayenbuehl et al., 2005; Albarède et al., 2011). Such an isotopic sex effect has also been described for copper (Cu) isotopes, but in this case, Cu in women's blood is isotopically lighter relative to that of men (Albarède et al., 2011). We further demonstrated that the blood sex isotopic differences were recorded in bones, which could bring additional information for sex assessment for incomplete past human remains (Jaouen et al., 2012).

These sex isotopic differences have been first attributed to differential intestinal absorption between men and women (Walczyk and von Blanckenburg, 2002, 2005). Women are supposed to have higher Fe intestinal absorption because of higher Fe needs due to menstrual losses. However, Hotz et al. (2012) challenged this hypothesis by observing that bloodletting led to a quick enrichment of Fe heavy isotopes in blood. Recently, Van Heghe et al. (2013) demonstrated a correlation between Fe status indicators and blood isotope composition, which would indicate that Fe sex isotope difference could be due to higher hepatic mobilization of stores by women. The reason is that liver, the main Fe storage reservoir, is enriched in ^{56}Fe (δ^{56}Fe $\sim -1.5‰$; Fig. 1) relative to blood Fe, the circulating reservoir (δ^{56}Fe $\sim -2.7‰$; Fig. 1) (Walczyk and von Blanckenburg, 2005). Concerning Cu, no hypothesis has been proposed to explain the observed sex isotopic differences at this time.

Whatever the mechanism at the origin of the Fe and Cu sex isotopic differences, we speculate to observe isotopic changes after menopause due to the cessation of blood losses. The Fe and Cu isotope compositions of women's blood would be expected to shift after menopause toward men's values.

So far, isotopic studies have been conducted on young adults only, so the effect of menopause is still undocumented. Using multicollector inductively coupled plasma mass spectrometry (MC-ICP-MS), we measured the Fe and Cu isotopic ratios of red blood cells (RBC) from aging people (>55 year olds) and the Fe and Cu isotopic ratios of liver biopsies for one woman and seven men. We compared our results to those obtained on young individuals obtained by Albarède et al. (2011) using identical experimental conditions. We also discussed the molecular and redox processes, which could account for the isotope variability occurring over the course of women's life.

MATERIAL

The donation center "Etablissement Français du Sang" provided blood samples for 19 postmenopausal women aged from 55 to 66 and for 15 men aged from 60 to 65. The samples were fully documented for sex, age, and age at menopause. Our samples were provided by the same institute than for the study of Albarède et al. (2011), for which the age of the donors ranged from 18 to 35 years old. RBC samples of Albarede et al. (2011)

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Received 3 September 2013; accepted 5 November 2013

DOI: 10.1002/ajpa.22430
Published online 22 November 2013 in Wiley Online Library (wileyonlinelibrary.com).

TABLE 1. Average isotopic compositions and concentrations in red blood cells and liver

	δ^{56}Fe (‰)		δ^{65}Cu (‰)	
Aging men (OM)	−2.65 ± 0.54	*15*	0.68±0.49	*14*
Young men (YM)[a]	−2.72 ± 0.36	*21*	0.67±0.36	*21*
Postmenopausal women (OW)	−2.59 ± 0.42	*19*	0.71 ± 0.54	*14*
Premenopausal women (YW)[a]	−2.49 ± 0.39	*28*	0.43 ± 0.48	*28*
Liver	−1.33 ± 1.34	*9*	−0.26 ± 0.22	*9*
	[Fe] (μg/g)		[Cu] (μg/g)	
Aging men (OM)	3,061 ± 1,554	*15*	1.74 ± 0.80	*15*
Young men (YM)	3,112 ± 928	*20*	1.76 ± 0.66	*20*
Postmenopausal women (OW)	3,226 ± 2,136	*18*	1.75 ± 0.80	*19*
Premenopausal women (YW)	3,193 ± 1,336	*20*	2.15 ± 1.22	*20*
Liver	491 ± 1,286	*8*	43 ± 64	*8*

Fe and Cu isotope compositions are in delta units. Fe and Cu concentrations are in μg/g (dry weight). Standard deviation: 2σ. The number of samples is given in italic. Typical analytical uncertainties are 0.05 ‰. Concentration data are from this study, as well as isotopic data for postmenopausal women and aging men.
[a] Data from Albarède et al (2011).

TABLE 2. Statistical results using Student and Kruskal-Wallis tests performed for Fe and Cu isotope compositions and concentrations presented in Table 1 between pairs of age and sex groups

	δ^{56}Fe		δ^{65}Cu	
	Student		Kruskal–Wallis	
	t value	*p*-value	χ^2	*p*-value
OM-OW	−0.86	0.40	0.27	0.40
OM-YM	1.62	0.13	0.06	0.81
OM-YW	−1.95	0.07	8.7	3.10^{-3}**
OW-YW	1.61	0.12	11.49	6.10^{-4}**
OW-YM	−2.2	0.04*	1.14	0.29
	[Fe]		[Cu]	
	Kruskal–Wallis		Kruskal–Wallis	
	χ^2	*p*-value	χ^2	*p*-value
OM-OW	0.29	0.59	0.41	0.52
OM-YM	0.40	0.52	0.12	0.73
OM-YW	0.40	0.53	4.07	0.04*
OW-YW	9.10^{-4}	0.98	3.81	0.05
OW-YM	0.25	0.62	3.10^{-3}	0.96
YW-YM	0.16	0.68	4.4	0.04*

T tests were performed when values follow a normal distribution. For two populations ($k - 1$) and a level of significance of 5% (±0.05), χ^2 equals 3.84. Significant results are illustrated by * when $p < 0.05$ and by ** when $p < 0.005$.
OM, old men; OW, postmenopausal women; YM, young men; YW, premenopausal women.

were reused for dry weight concentration assessments that had not been done in the previous study. Considering the values we obtained for in-house standards and the fact that we used the same equipment and methods than Albarède et al. (2011), we consider that the differences between the two studies will not be due to experimental bias, but will result in differential metabolism between age groups. Liver samples are biopsies coming from healthy areas of livers diagnosed with primary cancer. The biopsies were conducted in the Hepatobiliary Center (U785 INSERM) at the Paul Brousse hospital (Villejuif, France). All blood and liver donors gave written consent to participate in the study. All experiments were performed in compliance with the relevant laws of the Etablissement Français du Sang and the Ecole Normale Supérieure de Lyon.

METHODS

Metal Separation

Blood samples were collected with tubes containing a separation gel, which isolates clot from serum. The clot, mainly composed of RBC, was first freeze-dried and then homogenized. RBCs powders and liver samples were dissolved by a mixture of HNO_3 15 N and H_2O_2. Dissolved samples were then evaporated, re-dissolved in 1 mL of 7 N HCl + 0.001% H_2O_2 and evaporated again in order to remove any traces of remaining nitric acid. Finally, samples were taken up in 1 mL of 7 N HCl + 0.001% H_2O_2, a 50 μL aliquot taken for elemental concentration measurements, and the remaining solution processed for isotope analysis according to the technique of Maréchal et al. (1999). Metals were then purified using the same protocol for Fe and Cu as modified by Moynier et al. (2006) for Zn, which is less time-consuming.

Isotopic Measurements

Copper isotope compositions were determined on a Nu-HR MC-ICP-MS using wet plasma, while Fe was run on a large-radius Nu-1700 operated at a resolution of 4500 as dry plasma. The samples were introduced by free aspiration in 0.05 N sub-boiled distilled HNO_3. For Cu, instrumental mass fractionation was corrected using Zn-doping and standard sample bracketing following the recommendations provided in Albarède et al. (2004). The standard error associated to isotopic ratios is 0.05 ‰. Samples were randomized and duplicated. Analytical conditions are fully described in Albarède et al. (2011) and in Jaouen et al. (2013). The data quality assessment of the results of the present work is included in Appendix TS1. Iron and copper concentrations were measured on an Agilent 7500 CX quadripole ICP-MS. Liver concentrations were converted from wet weight to dry weight according the permutation factor of Wimmer et al. (1985)

Statistical Methods

Shapiro–Wilk's tests were used to test whether data are normally distributed. Bilateral Student's *t* tests for

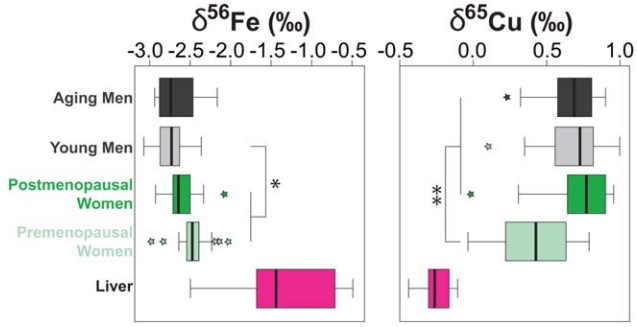

Fig. 1. Fe and Cu isotope compositions of blood and liver discussed in the text. The delta annotations are given by: $\delta^{x}Fe = [(^{x}Fe/^{54}Fe)_{sample}/(^{x}Fe/^{54}Fe)_{standard} - 1] \times 10^{3}$ with $x = 56$ or 57). The "IRMM14" solution stands for the Fe isotope standard. $\delta^{65}Cu = [(^{65}Cu/^{63}Cu)_{sample}/(^{65}Cu/^{63}Cu)_{standard} - 1] \times 10^{3}$. The "NIST-SRM 976" solution was used as Cu isotope standard. The box represents the 25th–75th percentiles (with the median as a bold vertical line) and the whiskers show the 10th–90th percentiles. Young individual data are from Albarède et al. (2011) and liver Fe data are from this study and from Walczyk and von Blanckenburg (2002). Significant differences between sex and age groups are indicated by *($p < 0.05$) and **($p < 0.005$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

each isotopic ratio were conducted between men and women. Concerning statistical tests involving data per age group, Kruskal–Wallis tests were performed for sets of data that do not follow a normal distribution.

RESULTS

All results of Fe and Cu concentration and isotopic ratios are given in Appendix TS2, TS3, and TS4

Concentrations

When all age groups are considered together, Fe concentration in RBC ranges from 1.8 to 5.6 mg/g with a mean value of 3.2 ± 1.8 mg/g in women and from 1.8 to 4.6 mg/g with a mean value of 3.1 ± 1.2 mg/g in men (Table 1). There is no significant difference between premenopausal and postmenopausal women and between men and women (Table 2). The Cu concentration in RBC ranges from 0.95 to 3.49 μg/g with a mean value of 1.95 ± 1.11 μg/g in women and from 1.08 to 2.44 μg/g with a mean value of 1.74 ± 0.40 μg/g in men (Table 1). As previously reported, young women exhibit the highest Cu concentrations in RBC (Lahey et al., 1953), whereas postmenopausal women and men have similar Cu contents. In liver, Fe and Cu concentrations range from 83 to 2,007 μg/g and from 12 to 109 μg/g, respectively (Table 1, Appendix TS4). These numbers are typical for Fe and Cu hepatic range of concentrations (Smallwood et al., 1968; Emond et al., 1999 Pierre et al., 2005).

Stable Isotope Compositions

Reported uncertainties are two standard deviations (2σ), unless specified. The mean $\delta^{56}Fe$ value of postmenopausal women RBC is −2.59‰ ± 0.42 and falls between premenopausal women and men. Iron isotope ratios of postmenopausal women differ significantly from those of young men (Table 1). The difference between Fe isotope compositions of pre- and postmenopausal women values becomes significant only 5 years into menopause (Fig. 1). The Fe and Cu isotope compositions of old and young men are not significantly different. The mean Cu isotope composition of postmenopausal women RBC ($\delta^{65}Cu$=0.71‰±0.54) is significantly higher than that of premenopausal women ($\delta^{65}Cu$ = 0.43‰ ± 0.48). The $\delta^{56}Fe$ and $\delta^{65}Cu$ values in blood do not correlate ($R^2 = 0.008$; $p = 0.635$), nor do they correlate with Fe concentrations ($\delta^{56}Fe$ vs 1/[Fe]: $R^2 = 0.106$; $p = 0.064$). The correlation between Cu isotopic ratios and concentrations is very small but the *P* value of the coefficient of determination indicates a significant trend with 95% of confidence. ($\delta^{65}Cu$ vs 1/[Cu]: $R^2 = 0.156$; $p = 0.034$). Among postmenopausal women, we distinguish two groups (Fig. 2): Blood of women who undergone menopause since less than 5 years is characterized by high $\delta^{65}Cu$ values ($\delta^{65}Cu$ = 0.71‰ ± 0.52) close to that of men ($\delta^{65}Cu$ = 0.67‰ ± 0.42), but $\delta^{56}Fe$ values similar to that of young women ($\delta^{56}Fe$ = −2.36‰ ± 0.42). Women whose menopause occurred for more than 5 years also show high $\delta^{65}Cu$ values ($\delta^{65}Cu$ = 0.72‰ ± 0.58), but lighter $\delta^{56}Fe$ values ($\delta^{56}Fe$ = −2.67‰ ± 0.28) relative to younger women. Liver mean $\delta^{56}Fe$ value (−1.33‰ ± 1.34) (Fig. 1) is consistent with the data reported by Walczyk and von Blanckenburg (2002, 2005). The mean $\delta^{65}Cu$ value for the liver is −0.26‰ ± 0.22.

DISCUSSION

Fe and Cu Isotope Composition of RBC after Menopause

Our results suggest that the cessation of menses affects the sex isotopic differences observed between young men and women. At menopause, the Cu concentrations and isotope compositions of women's blood become indistinguishable from those of men (Figs. 1 and 2, Table 1). Compared to premenopausal women, Cu in the blood of postmenopausal women becomes significantly less concentrated and isotopically heavier (Fig. 1, Tables 1 and 2). In contrast, there is no immediate response of Fe concentration to menopause, but although newly postmenopausal women are still similar to premenopausal individuals, an isotopic shift becomes visible 5 years after the menopause (Fig. 2). A woman's body contains about 3 g of Fe and 80 mg of Cu. After menopause, the daily requirements for Fe and Cu are 1 and 1.6 mg, respectively (Hunt et al., 2009). We assume steady-state (daily requirement cover daily losses) and that the residence times of Fe and Cu in the body can be approximated by the ratio between the metal burden and daily requirement. Iron is replaced in about 8 years and Cu in only 50 days. These estimations explain why, immediately after menopause, the $\delta^{65}Cu$ in women's blood becomes similar to that of men, whereas the $\delta^{56}Fe$ difference lags for years (Fig. 2).

Isotopic Mass Balance After Menopause

The Fe cycle in the human body involves several components: hemoglobin (*Hb*) in RBC and myoglobin in muscle, both largely composed of Fe(II), and hepatocytes, which store Fe(III) as ferritin (*Ft*, Andrews, 2005). Serum Fe is essentially bound to transferrin (*Tf*) and, to a minor extent, to *Ft* as Fe(III). Extracellular Cu is hosted at 70–80% by ceruloplasmin (*Cp*) as Cu(II) (Hellman et al., 2002). In contrast, intracellular Cu is bound to several proteins, mainly to superoxide dismutase

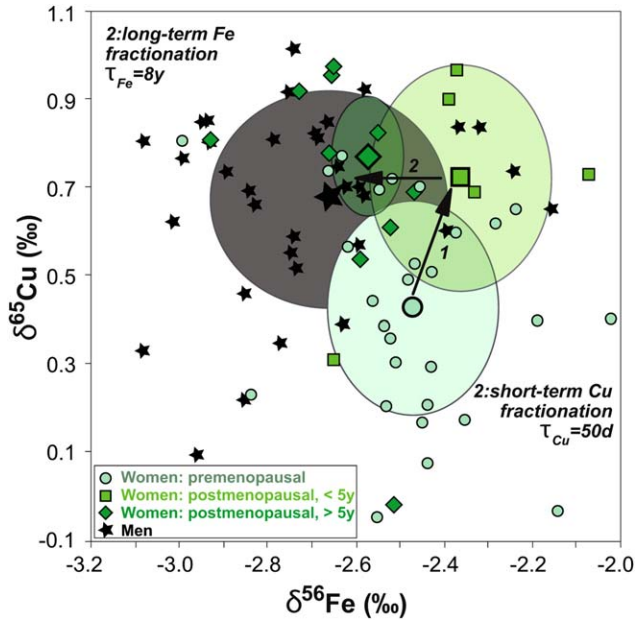

Fig. 2. Distribution of RBC δ^{56}Fe and δ^{65}Cu values for women and men. For the sake of simplicity, the young and aging men δ^{56}Fe and δ^{65}Cu values are merged. Large symbols represent average value for each group, and the corresponding shaded area one standard deviation of the mean. τ_{Fe} and τ_{Cu} stand for the residence time of Fe and Cu in the body. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

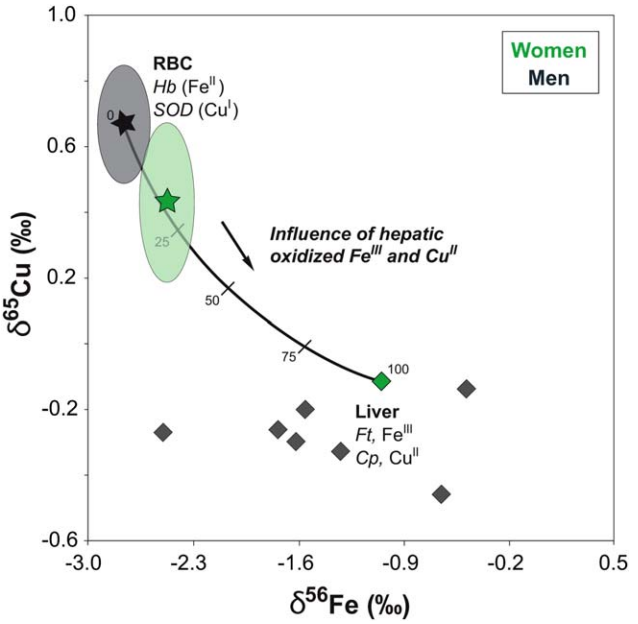

Fig. 3. Liver and RBC isotope compositions of men and women. Women draw on liver Fe and Cu more than men to compensate for menstrual blood loss. A line represents the addition of Fe and Cu from the liver and labeled with proportions calculated with data reported in Table 3. Mass balance calculations allow estimating that about 10–15% of the Fe in RBC in menstruating women has an hepatic origin. Hb, hemoglobin; SOD, superoxide dismutase; Cp, ceruloplasmin; Ft, ferritin; RBC, red blood cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(SOD) as Cu(I) (Harris et al., 1999). Liver is highly concentrated in Cu(II) because *Cp* is synthesized in this organ (Tavassoli et al., 1986).

Each month, women generally lose up to 80 mL of blood (Group et al., 2004; Harvey et al., 2005). Iron loss over a menstrual cycle (~15 mg) corresponds to 40% of the monthly dietary uptake (~35 mg) (Hallberg and Rossander-Hultén, 1991; Harvey et al., 2005). Circulating Fe, which is composed by reduced and isotopically light Fe^{2+}-laden *Hb*, mainly constitutes menstrual blood losses. This overall Fe deficit must be compensated by release of storage Fe from the liver, which can only originate in oxidized, isotopically heavy Fe^{3+}. However, efficient Fe release from hepatocytes requires *Cp* because it catalyzes the oxidation of Fe(II) to Fe(III) (Tavassoli et al., 1986). Higher Fe fluxes through the body of women predict an enhanced redox *Cp* activity, which is the case: *Cp* activity is known to be more elevated in women than in men (Hunt et al., 2009). The fast Fe and Cu turnover for women reduces the differences between $\delta^{56}Fe^{3+}$ and $\delta^{56}Fe^{2+}$ on the one hand, and between $\delta^{65}Cu^{+}$ and $\delta^{65}Cu^{2+}$ on the other hand. This is illustrated by the isotopic mass balance between the men RBC end-member and the liver of either men or women (Fig. 3). The mass balance equation reads, for Fe:

$$\delta^{65}Cu_{mix} = [x \,.\, M^{Cu}.\delta^{65}Cu_{SOD} + (1-x) \,.\, M^{Cu}.\delta^{56}Fe_{Cp}] / [x \,.\, M^{Cu} + (1-x) \,.\, M^{Cu}] \quad (1)$$

where $\delta^{56}Fe_{mix}$ is the Fe isotopic composition resulting from the mixing between RBC and liver, with proportions of x and $(1 - x)$, respectively, M^{Fe}_{Hb} and M^{Ft}_{Hb}, the concentration of Fe in hemoglobin and ferritin, respectively, and $\delta^{56}Fe_{Hb}$ and $\delta^{56}Fe_{Ft}$, the expected Fe isotopic composition of hemoglobin (*Hb*) and ferritin (*Ft*), respectively (Table 3). Similarly, it reads for Cu:

$$\delta^{56}Fe_{mix} = [x \,.\, M^{Fe}.\delta^{56}Fe_{Hb} + (1-x) \,.\, M^{Fe}.\delta^{56}Fe_{Ft}] / [x \,.\, M^{Fe} + (1-x) \,.\, M^{Fe}] \quad (2)$$

where $\delta^{65}Cu_{mix}$ is the Cu isotopic composition resulting from the mixing between RBC and liver pools with proportions of x and $(1 - x)$, respectively, M^{Cu}_{SOD} and M^{Cu}_{cp}, the concentration of Cu in SOD and ceruloplasmin (*Cp*), respectively, and $^{65}Cu_{SOD}$ and $\delta^{65}Cu_{Cp}$, the Cu isotopic composition of SOD and ceruloplasmin, respectively.

Using mass balance calculations, one can estimate that about 15% of the Fe in RBC in menstruating women has an hepatic origin. The Fe and Cu isotope composition of menstruating women's RBC therefore reflects a higher proportion of hepatic high-δ^{56}Fe *Ft* and low-δ^{65}Cu *Cp* relative to men (Fig. 3). As for Fe isotopes (Albarède et al., 2011; Hotz et al., 2012; Van Heghe et al., 2013), we surmise that hepatic solicitation is the origin of the sex effect visible in the isotope compositions of blood Cu isotope compositions. However, our hypothesis does not reject higher Fe and Cu intestinal absorption by menstruating women, because otherwise, steady-state would not be maintained. Our data and model highlight the importance of the interactions between Fe and Cu pathways on the isotope compositions of these metals in blood because they exchange electrons notably through electron transfer (Collins et al., 2010). Our results suggest a true potential of stable isotope compositions of metal isotopes with multiple oxidation states

TABLE 3. Biochemical and isotopic data of Cu and Fe body reservoirs

			$\delta^{56}Fe$ (‰)	
	Fe-ligand	M^{Fe} (mg)	Women	Men
RBC	Hb	1,800[a]	−2.47 ± 0.38	−2.76 ± 0.36
Hepatocytes	Ft	1,600[a]	−1.04	−1.41 ± 1.36[b]
			$\delta^{65}Cu$ (‰)	
	Cu-ligand	M^{Cu} (mg)	Women	Men
RBC	SOD	5[c]	0.43 ± 0.48	0.67 ± 0.36
Hepatocytes	Cp	10[c]	−0.11	−0.28 ± 0.16

[a] Andrews, 2005. Note that the Fe content of the reticulo endothelial macrophages (600 mg) has been added to that of hepatocytes (1,000 mg).
[b] The Ft isotopic values are deduced from those of bulk liver. See text for abbreviations.
[c] Hellman et al. (2002). SD: 2σ.

as a tracer of redox perturbations associated with metabolic disorders.

Anthropological Implications

We recently demonstrated that Fe and Cu isotope compositions in bones reflect patterns observed in blood (Jaouen et al., 2012), which could have consequences for the use of Fe and Cu isotope as a sex indicator for past populations. However, one must remember that bone turnover is about 10 years for human, increases with aging (Fatayerji and Eastell, 1999) and is slower in the cortical part of bones (Sealy et al., 1995). Iron and Cu isotopic changes in women's bones are expected to occur after 60 years old given an age at menopause of 50 years old. In our study on the population buried during the XVII and XVIII in Saint-Laurent de Grenoble (France), age-at-death assessment of the population gave large range of variations and we could not clearly identify whether women died over 60 years old or not (Jaouen et al., 2012). The oldest female age group, containing women dead over 45 years old did not show significant isotopic sex differences relative to the younger group. Further investigations should then focus on the record of the menopause effect in bones. Comparisons between well-off and non-industrialized populations should also be done. Past societies indeed experienced later menarche (Shorter, 1981; Eveleth, 1986), numerous pregnancies and prolonged breastfeeding periods associated to amenorrhea in comparison to the modern well-off population we analyzed in the present study. Therefore, the cumulative amount of blood lost through years could be expected to be significantly lower for past populations. Nevertheless, the use of contraceptive pill can divide by two the volume of menstrual blood loss (Nilsson and Solvell, 1967; Larsson et al., 1992), so that the amount lost during the reproductive years is not necessarily higher for well-off contracepting populations compared to poor modern populations and, by extension, to ancient ones. Future studies on modern non-contracepting societies associated to more difficult life conditions could allow us to extrapolate our findings to historical or ancient populations. Such studies are also likely to find perspectives in paleoanthropology to determine the age at menopause in ancient populations. This could be important regarding the grandmotherhood theory (Hawkes et al., 1998) or demographic reconstructions.

CONCLUSION

We show that the Fe and Cu isotope compositions of women's blood are affected by menopause. The timing of the isotopic changes depends on the element because of differential turnover between Cu and Fe in the human body. Copper isotope values of RBC immediately increase after menopause whereas Fe isotope composition starts to shift only 5 years after. We observe that Fe and Cu isotope composition in RBC and liver was related to the speciation of the element. We suggest that the redox reaction occurring during hepatic solicitation and involving a Cu-containing protein, the ceruloplasmin, explain why Cu is also isotopically affected by the cessation of the menstruations. Further work is needed to precise the link between speciation and isotope composition of Fe and Cu in the human body, which could include experimental data or *ab initio* calculations. Particular attention should also be carried on the amount of time existing between the age at menopause and its potential record in bones. Such effort will allow assessing if Fe and Cu isotopes can be used to the sex assessment of populations with high life expectancy or to determine a global age at menopause in archeological populations.

ACKNOWLEDGMENTS

The authors thank Francis Albarède for advice on the manuscript. They also thank Francis Camoin from the donation center "Etablissement Français du Sang" in Lyon, and Michelle Gigou and Christian Bréchot from the Hepatobiliary Center of the Paul Brousse hospital. Axelle Zacaï and Jean-Michel Brazier are thanked for help during samples preparation. They are grateful to Joe Skulan and an anonymous reviewer for helpful comments on the manuscript. Funding was supported by the Bullukian Foundation and the Mérieux Foundation.

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