Hereditary hemochromatosis is reflected in the iron isotope composition of blood

(Iron isotope composition in hereditary hemochromatosis)

1 Pierre-Alexandre Krayenbuehl, 2 Thomas Walczyk, 3 Ronny Schoenberg, 3 Friedhelm von Blanckenburg, 1 Georg Schulthess

1 Department of Internal Medicine, University Hospital of Zurich, Zurich, Switzerland
2 Laboratory of Human Nutrition, Institute of Food Science and Nutrition, Swiss Federal Institute of Technology (ETH) Zurich, Rüschlikon, Switzerland
3 Institute of Mineralogy, University of Hannover, Hannover, Germany

This work was supported by the Lixmar foundation, Switzerland, and through funding of the mass spectrometry laboratory at the University of Hannover, Germany, by the Volkswagen Stiftung and DFG grant BL562-1

Authors for correspondence :

Georg Schulthess MD (for submission process)
Department of Internal Medicine, Medical Policlinic, University Hospital of Zurich, CH-8091 Zurich, Switzerland (georg.schulthess@usz.ch)

Thomas Walczyk PhD (for correspondence after publication)
Laboratory of Human Nutrition, Institute of Food Science and Nutrition, Swiss Federal Institute of Technology (ETH) Zurich, Schmelzbergstrasse 7, CH-8092 Zurich, Switzerland (thomas.walczyk@ilw.agrl.ethz.ch)

Word counts:  Text = 3643; References, Tables, and Legends = 1198; Abstract = 191

Key words: HFE C282Y mutation – iron – intestinal absorption – isotope – multicollector inductively coupled plasma mass spectrometry (MC-ICP-MS)

Heading: Clinical Observations, Interventions, and Therapeutic Trails
Abstract

It has recently been shown that the iron isotopic composition of blood differs between individuals and genders which is supposed to reflect individual differences in iron metabolism. We hypothesized that patients suffering from hereditary hemochromatosis would demonstrate alterations in the iron isotopic composition of blood due to persistent upregulation of intestinal iron absorption. Blood of 30 patients with homozygous C282Y hemochromatosis was analyzed for iron isotopic composition by a newly developed technique using multicollector inductively coupled plasma mass spectrometry (MC-ICP-MS). Blood of hemochromatosis patients is characterized by a higher $^{56}$Fe/$^{54}$Fe isotope ratio than blood of normal individuals, which are either members of an age-matched control group (n=10, p=0.0003) or young adults (n=36, p<0.0001). In patients with hereditary hemochromatosis, the $^{56}$Fe/$^{54}$Fe isotope ratio of blood significantly correlates with total body iron accumulation, severity of clinical disease, and the need for regular phlebotomies to prevent iron re-accumulation. We conclude that blood of patients with hereditary hemochromatosis contains more of the heavier iron isotopes than blood of normal individuals. Primary determinant of the iron isotopic composition of blood appears to be isotope-sensitive iron absorption in the intestine and the efficiency of this process.
Abbreviations:

MC-ICP-MS, multicollector inductively coupled plasma mass spectrometry

DMT-1, divalent metal transporter-1 (DCT1, Nramp2)
**Introduction**

Hemochromatosis is a frequent genetic disorder in populations of European ancestry that is characterised by progressive iron overload of tissues due to an ineffective control of intestinal iron absorption. Following identification of the HFE gene,¹ several studies showed that about 90% of the patients with clinical features of hemochromatosis are homozygous for a C282Y mutation of the HFE gene.² Estimates of gene frequency range from 5-10% with a homozygote frequency of 0.2-0.5% in Caucasian populations.³⁻⁵ Typical clinical manifestations are hepatic fibrosis, arthropathies, diabetes mellitus, cardiopathy, and hypogonadism. Epidemiological studies indicate, however, a low clinical penetrance of the homozygous HFE C282Y mutation.⁶⁻⁷

Iron homeostasis is maintained by the body by regulating iron absorption in the proximal small intestine.⁸⁻⁹ Heme-bound iron such as myoglobin (and hemoglobin) in foods of animal origin is taken up by an endocytotic process in which the porphyrin ring of the heme molecule is broken up within the epithelial cell.¹⁰⁻¹¹ Uptake of non-heme iron, mostly from plant foods, is mediated by ferric reductase and divalent metal transporter-1 (DMT-1)¹²⁻¹⁴ or, possibly, by the integrin-mobilferrin-paraferritin pathway.¹⁵⁻¹⁶ An iron-regulated transporter 1 (IREG1) is involved in subsequent iron transfer from the epithelial cell to blood circulation.¹⁷ There is no satisfactory technique available to date to measure regulation of intestinal iron absorption. Measurements by conventional tracer techniques using stable or radioactive isotopes are jeopardized by physiological day-to-day variations of iron absorption.

All iron in nature consists of four stable isotopes which differ in their masses and natural abundances: ⁵⁴Fe (5.8%), ⁵⁶Fe (91.8%), ⁵⁷Fe (2.1%) and ⁵⁸Fe (0.3%).¹⁸ In general, the iron isotopic composition of an element can be altered by physical processes such as diffusion or evaporation if the transfer is mass-dependent or by chemical processes if breaking/formation
of chemical bonds depends on the masses of the isotopes involved. Mass balance principles dictate, however, that element transfer between compartments must be incomplete to generate an isotope effect.

Substantial isotope effects are known in nature for a number of light elements such as hydrogen, oxygen and carbon. Until recently, isotope effects were considered to be too small to be measurable for most of the heavier elements such as iron. Using multicollector inductively coupled plasma mass spectrometry (MC-ICP-MS), Walczyk and Blanckenburg demonstrated that human tissues are depleted in heavy iron isotopes compared to the diet and the geosphere. Each individual bears a distinct $^{56}$Fe/$^{54}$Fe isotope ratio in the blood which is, on average, lower in males than in females. It has been hypothesized that the iron isotopic composition is altered during intestinal absorption. Stability of the iron isotopic signature in blood over one year was recently shown in a healthy subject.

In the present study, we show that hemochromatosis patients have an altered iron isotopic composition of blood when compared to normal controls. This strengthens the hypothesis that the iron isotopic composition of blood is primarily determined by the efficiency at which iron is absorbed from the diet.
Materials and Methods

Study Design

Iron isotopic composition of blood was compared between 30 patients with homozygous C282Y hemochromatosis and 2 control groups, i.e., an age-matched control group of 10 elderly subjects, and 36 young adults included in a previous study. 19

Study Patients

The study protocol was approved by the ethical committee of the University Hospital of Zurich, Switzerland. All patients gave written informed consent to participate in the study. 30 patients with established diagnosis of hemochromatosis were enrolled showing a homozygous C282Y mutation of the HFE gene and serum ferritin concentration above the reference range at time of diagnosis. Clinical manifestations, histological findings, laboratory results, and amounts of iron removed by phlebotomy were assessed by patients’ history and from their medical records. As an age-matched control group, 10 patients without history of blood donation or disorders of iron metabolism were recruited. They were not tested for HFE genotype as they all had transferrin saturations below 45%. Four of 5 females included in this control group did not have menstrual blood loss for ≥ 10 years.

Assessment of biochemical variables and clinical manifestations

When included in this study, 22 of 30 hemochromatosis patients had completed iron depletion therapy and continued regular maintenance therapy (referred to as treated patients). In 8 patients, either no phlebotomy therapy ever (2 patients), no phlebotomies during the last 2 years (5 patients), or inappropriate phlebotomy therapy (1 patient) had been carried out (referred to as untreated patients). Iron removal by phlebotomy (500 ml blood ≈ 250 mg iron) was assessed as (a) total amount of iron removed to achieve serum ferritin within the reference range (iron depletion therapy) and (b) iron removed per year to maintain ferritin
within the reference range (maintenance therapy). Maintenance therapy was assessed in 20 patients having continued this therapy for at least 2 years.

Liver disease was assessed using as criterion either substantial fibrosis (either substantial portal fibrosis or septal/bridging fibrosis or cirrhosis) in histological examination of liver tissue or elevated serum alanine aminotransferase concentration (ALT) at time of diagnosis. Assessing total number of clinical manifestations, histological examination of liver tissue was used as primary determinant, elevated ALT was considered in patients without biopsy. All patients with liver disease were serologically tested for chronic hepatitis B and C virus infection. Habitual alcohol intake was estimated by interviewing the patients. Diabetes mellitus, cardiopathy, and hypogonadismus were taken over as established diagnosis from medical records; arthropathy of metacarpophalangeal joints was taken over as established diagnosis or assessed by clinical investigation.

Iron isotope analysis

High-precision iron isotope ratios were measured at the University of Hanover, Germany, by multicollector ICP-MS (Neptune, ThermoFinnigan, Bremen, Germany). Venous blood samples (0.5 ml) were mineralized by microwave digestion and iron was purified by anion exchange. The isotopes $^{54}\text{Fe}$, $^{56}\text{Fe}$, $^{57}\text{Fe}$, and $^{58}\text{Fe}$ were all measured simultaneously on Faraday collectors. Any residual isobaric interference of $^{54}\text{Cr}^+$ was corrected for by monitoring $^{52}\text{Cr}^+$; ArO and ArN. Interferences were resolved optically by high-mass resolution techniques. $^{\delta^{56}\text{Fe}}^{\delta^{54}\text{Fe}}$ ($\%$) was calculated as follows:

$$\delta^{56}\text{Fe}/^{54}\text{Fe}_{\text{Sample}} = \left( \frac{^{56}\text{Fe}/^{54}\text{Fe}_{\text{Sample}}}{^{56}\text{Fe}/^{54}\text{Fe}_{\text{Standard}}} - 1 \right) \times 1000.$$ 

and was measured by linear interpolation between alternating standards (IRMM-014) and samples which were matched for iron concentration. The mass spectrometric reproducibility
was assessed by repeated runs of an internal iron standard (Johnson&Matthey, Fe Puratronic wire, 99.998% purity, lot NM3688) which gave \( \delta^{56}\text{Fe}^{54}\text{Fe} = 0.422 \pm 0.044\%\text{e} \) and \( \delta^{57}\text{Fe}^{54}\text{Fe} = 0.632 \pm 0.070\%\text{e} \) (95% confidence level). Three-isotope-plots were used consistently to confirm data accuracy. The external reproducibility was assessed to be the same by multiple chemical separations of organic samples.

**Genetic analysis**

HFE gene C282Y (G845A) mutation was determined by LightCycler PCR and melting curve analyses using ToolSets with specific primers and fluorescent probes according to the manufacturers’ instructions (Roche Molecular Biochemicals, Rotkreuz, Switzerland; Genes4U AG, Zurich, Switzerland).

**Statistical Analysis**

Evaluations were performed using Statistica version 6. All comparisons of this study were done using either Student’s t-test, Chi-square test, or Spearman rank order correlation. Threshold of significance was defined as \( \alpha = 0.05 \).
Results

Iron isotopic composition of blood in hemochromatosis patients

The iron isotopic composition of blood obtained from hemochromatosis patients (n=30) was compared to that of two control groups. The first control group consisted of elderly subjects (n=10, range 44-72 years) without history of disorders of iron metabolism or blood donation who were not significantly different from the patient group regarding age (p=0.95) and gender (p=0.86). The second control group consisted of young adults (n=36, range 20-32 years) included in a previous study. Blood of patients with hereditary hemochromatosis contains more of the heavier iron isotopes than blood of normal individuals, i.e. it is characterized by a higher $^{56}\text{Fe}/^{54}\text{Fe}$ isotope ratio. Table 1 shows that the $\delta^{56}\text{Fe}/^{54}\text{Fe}$ value (relative deviation of the $^{56}\text{Fe}/^{54}\text{Fe}$ isotope ratio of blood from the isotope ratio of the iron isotopic reference material IRM-014) was -2.11±0.47‰ in hemochromatosis patients compared to -2.72±0.27‰ in the age-matched control group (p=0.0003) and -2.58±0.25‰ in normal young adults (p<0.0001).

Influence of gender, age and phlebotomy therapy

The difference in the iron isotopic composition of blood ($\delta^{56}\text{Fe}/^{54}\text{Fe}$) between treated hemochromatosis patients and the two control groups was found to be more accentuated in males than in females, see Table 1. An effect of gender on the iron isotopic composition of blood was observed in a previous study, i.e., blood of young males was found to be characterized by a significantly lower $^{56}\text{Fe}/^{54}\text{Fe}$ isotopic ratio than blood of young females. Table 1 shows a similar effect of gender for normal elderly subjects included in the present study, $\delta^{56}\text{Fe}/^{54}\text{Fe}$ was -2.92±0.22‰ in males and -2.52±0.10‰ in females (p=0.006). In contrast, no difference in the iron isotopic composition between sexes could be identified in treated hemochromatosis patients, $\delta^{56}\text{Fe}/^{54}\text{Fe}$ was -1.95±0.40‰ in males and -2.02±0.43‰ in
females (p=0.94). This might be explained by inappropriate upregulation of intestinal iron absorption in both males and females suffering from hereditary hemochromatosis. 

No significant effect of age on $^{56}\text{Fe}/^{54}\text{Fe}$ isotopic ratio of blood was detected both in hemochromatosis patients and normal individuals. This refers in particular to the subgroup of normal adult females: Table 1 shows that $\delta^{56}\text{Fe}/^{54}\text{Fe}$ did not differ significantly between young (–2.43±0.21‰) and elderly (–2.52±0.10‰) normal females (p=0.35), whereas four of five elderly females did not have menstrual blood losses for about 10 years. This points to absence or only marginal influence of menstrual blood loss on the iron isotopic composition of blood.

Differences in the iron isotopic composition ($\delta^{56}\text{Fe}/^{54}\text{Fe}$) between untreated hemochromatosis patients and control subjects did not reach statistical significance. It is worth noting, however, that the group of untreated hemochromatosis patients was small and heterogenous. Nevertheless, in untreated hemochromatosis patients, the $^{56}\text{Fe}/^{54}\text{Fe}$ isotope ratio of blood was found to correlate with biochemical and clinical manifestations of the disease (see below). Hemochromatosis appears to be generally associated with an increased $^{56}\text{Fe}/^{54}\text{Fe}$ isotope ratio of blood, with a stronger association however in patients undergoing phlebotomy therapy.

**Correlation with iron parameters**

The iron isotopic composition of blood ($\delta^{56}\text{Fe}/^{54}\text{Fe}$) significantly correlates with variables reflecting total body iron accumulation, i.e., serum ferritin concentration measured at time of diagnosis (p=0.017) and total amount of iron removed during subsequent iron depletion therapy (p=0.025), see Figure 1A-C. Linear regression analyses indicate that the association between $\delta^{56}\text{Fe}/^{54}\text{Fe}$ and the serum ferritin concentration is similar in treated and untreated hemochromatosis patients. In contrast to variables reflecting iron accumulation by the total body, no relevant association was found between hepatic iron concentration and the $^{56}\text{Fe}/^{54}\text{Fe}$
isotope ratio of blood. This might be explained either by the limited number of patients evaluated (n=8) or by previously reported absence of an association between total body iron stores and hepatic iron content.\textsuperscript{23,24}

Moreover, $\delta^{56}\text{Fe}/^{54}\text{Fe}$ was found to significantly correlate with annual amounts of iron removed by phlebotomy to maintain the serum ferritin concentration within the reference range (p=0.029), see Figure 1D. Measurements were made during maintenance therapy, i.e., in a steady state of continuous intestinal iron (hyper)absorption and regular phlebotomies to prevent iron re-accumulation.

**Correlation with clinical manifestations**

Iron isotopic composition of blood ($\delta^{56}\text{Fe}/^{54}\text{Fe}$) is significantly associated with liver disease assessed either as substantial fibrosis (substantial portal fibrosis or septal/bridging fibrosis or cirrhosis) in histological examination (p=0.0031) or, alternatively, as elevated serum alanine aminotransferase concentration (ALT) at the time of diagnosis (p=0.0009), see Table 2. Moreover, $\delta^{56}\text{Fe}/^{54}\text{Fe}$ was found to significantly correlate with ALT, as well in treated (p=0.020) as in untreated (p=0.036) hemochromatosis patients. Considering that the iron isotopic composition of blood and parameters of liver disease are associated, correlation between $\delta^{56}\text{Fe}/^{54}\text{Fe}$ and the iron content of liver tissue would be expected. However, hepatic iron concentration was available only in a limited number of patients with a similar extent of liver disease. Chronic hepatitis B and C virus infection, tested in all patients with liver disease, was never diagnosed. Estimated habitual alcohol intake was 7.4±7.6 g/d in patients with liver disease and 14.3±14.9 g/d in patients without liver disease. Hence, a confounding effect of alcohol intake on liver disease is improbable because of the higher mean alcohol intake in the group without liver disease.
A significant association was also found between the iron isotopic composition of blood (δ\(^{56}\text{Fe}/^{54}\text{Fe}\)) and arthropathy of the metacarpophalangeal joints (p=0.019), see Table 2. Less common manifestations of hemochromatosis such as diabetes mellitus, cardiopathy, or hypogonadism were also observed among the study patients, but prevalences were too low for proper statistical evaluation. Table 2 moreover shows that the \(^{56}\text{Fe}/^{54}\text{Fe}\) isotope ratio of blood significantly correlates with the number of clinical manifestations of hemochromatosis (p=0.0030). The presented data provide strong evidence that the iron isotopic composition of blood is associated with the severity of clinical disease, as well in treated as in untreated hemochromatosis patients.
Discussion

Blood of patients with hereditary hemochromatosis contains more of the heavier iron isotopes than blood of normal individuals, i.e. it is characterized by a higher $^{56}$Fe/$^{54}$Fe isotope ratio resembling more the value of dietary iron. This can be explained by iron absorption from the diet being less selective (less preferential for lighter iron isotopes) in hemochromatosis patients than in normal individuals, see below. Moreover, the $^{56}$Fe/$^{54}$Fe ratio is shown to correlate significantly with phenotypic expression of hemochromatosis, i.e., total body iron accumulation (serum ferritin concentration at time of diagnosis, phlebotomy requirements during iron depletion therapy), severity of clinical disease (prevalence of liver disease, arthropathy of metacarpophalangeal joints, total number of clinical manifestations), and the need for regular phlebotomies to prevent iron re-accumulation during maintenance therapy.

Iron of human blood contains more of the lighter iron isotopes, i.e. it is characterized by a lower $^{56}$Fe/$^{54}$Fe isotope ratio, than dietary iron. Findings can be explained by iron absorption from the diet being a mass-dependent process with preference for the lighter iron isotopes. This is true for normal subjects and – though less accentuated – hemochromatosis patients. Mass balance principles dictate that iron isotope fractionation during absorption is less accentuated the more completely iron is absorbed, i.e. the $^{56}$Fe/$^{54}$Fe isotope ratio of absorbed iron increases and approaches the value of dietary iron. Figure 2 illustrates how isotope fractionation of dietary iron may occur during the absorption process. Iron is absorbed from two major dietary pools, i.e., foods of animal origin containing primarily heme-bound iron and plant foods containing non-heme iron. Isotope effects (isotope fractionation) may be induced by preferential uptake of lighter isotopes of non-heme iron by DMT-1 and/or iron release from the epithelial cell into the circulatory system by IREG1 and/or preferential deposition of heavier iron isotopes into ferritin (which is removed from the body by regular
apoptosis and shedding of epithelial cells into the lumen of the small intestine). In contrast to non-heme iron, it is unlikely that the iron isotope composition of heme-bound iron is affected during uptake by the intestinal epithelial cell. Relative mass differences of the heme isotopomers, which are taken up intact, are too small to cause a significant isotope effect during endocytosis. Subsequent release of iron from the heme molecule by heme-oxygenase is quantitative within epithelial cells and, therefore, cannot cause an isotope effect for reasons of mass balance. Besides isotope effects during absorption, the ratio between heme-bound iron and non-heme iron absorbed from the diet may influence the iron isotope signature of blood. In an earlier survey, foods of animal origin were found to be more depleted in the heavier iron isotopes than plant foods. 19 However, results of more than 70 individuals (including unpublished data) do not indicate a significant difference in the iron isotopic composition of blood between vegetarians and omnivores.

Our recent findings support the hypothesis that the iron isotopic composition of blood is primarily determined by the efficiency at which iron is absorbed from the diet, i.e., an increased $^{56}\text{Fe}/^{54}\text{Fe}$ isotope ratio of blood reflects upregulated intestinal iron absorption:

(I) Iron homeostasis is maintained by the body by regulating intestinal iron absorption, however, control of intestinal absorption is ineffective in hereditary hemochromatosis. Inappropriately enhanced intestinal absorption of iron, more accentuated for non-heme iron than heme-bound iron25, and inappropriately upregulated intestinal expression of DMT-126 have previously been reported in patients suffering from hereditary hemochromatosis. An increased $^{56}\text{Fe}/^{54}\text{Fe}$ isotope ratio of blood as observed in hemochromatosis patients is expected to be related with the underlying mechanism of the disorder.

(II) We found a higher $^{56}\text{Fe}/^{54}\text{Fe}$ isotope ratio in treated than in untreated hemochromatosis patients. According to our hypothesis, this finding would indicate that iron absorption is
upregulated more distinctly in hemochromatosis patients undergoing phlebotomy therapy than in untreated patients. Prior studies showed that intestinal expression of DMT-1 is increased in treated but not in untreated hemochromatosis patients. Other studies have shown that phlebotomy therapy per se causes a significant increase of intestinal iron absorption in hemochromatosis patients.

After initial iron depletion therapy, hemochromatosis patients reach a steady state of continuous intestinal iron (hyper)absorption which is balanced by iron depletion (phlebotomy) therapy in regular intervals. Phlebotomy requirements for maintaining steady state conditions are supposed to be higher in patients that absorb iron more efficiently. Hence, we would expect a more pronounced increase in the $^{56}\text{Fe}/^{54}\text{Fe}$ ratio of blood in patients phlebotomized more frequently. In the present study, we have found a significant correlation between the $^{56}\text{Fe}/^{54}\text{Fe}$ isotope ratio of blood and the annual amount of iron removed by phlebotomy to maintain body iron stores in steady state.

Blood of patients with hemochromatosis contains more of the heavier iron isotopes, i.e. is characterized by a higher $^{56}\text{Fe}/^{54}\text{Fe}$ isotope ratio, than blood of normal individuals because intestinal iron absorption is upregulated and, therefore, the isotope effect (i.e. preferential transfer of lighter isotopes) is less accentuated. However, processes related to inhomogenous distribution of iron isotopes between body compartments such as blood, liver and muscle may also contribute to our findings in hemochromatosis patients. Earlier investigations have shown that liver tissue is characterized by a higher $^{56}\text{Fe}/^{54}\text{Fe}$ isotope ratio than blood in humans. If this is also the case in hemochromatosis patients, release of isotopically heavier liver iron during initial phlebotomy therapy and its subsequent utilization for erythropoiesis should result in an increase in the $^{56}\text{Fe}/^{54}\text{Fe}$ isotope ratio of blood until liver stores are emptied. However, it appears that the iron isotopic signature of blood is not primarily determined by
the release of isotopically heavier liver iron since no correlation between the iron isotope composition of blood and total duration of phlebotomy therapy (continued up to 18 years) was observed. Once inappropriate iron stores of the liver are emptied, the iron isotope composition of blood should be determined mainly by continuously absorbed dietary iron which is presumed to reflect the efficiency at which iron is absorbed from the diet. In principle, hemoglobin concentration might be associated with the iron isotopic composition of blood, too, this however has not been systematically investigated in the present study.

Considering our recent results, it appears that we now have, basically, a method at our hands to determine the efficiency of intestinal (non-heme) iron absorption. The finding that the iron isotopic composition of blood is significantly associated with phenotypic expression of hereditary hemochromatosis can be considered as an early proof of concept. The method bears potential relevance as indicator for disorders affecting regulatory mechanisms of iron metabolism. 8-9,25-28 Conventional tracer techniques are insensitive since intestinal iron absorption is subject to strong day-to-day variations. This is not the case for iron isotope effects in the blood which – if not perturbed – are stable during months or even years as recently shown. 20

Acknowledgments

We thank R.F. Hurrell, W. Vetter and F.E. Maly for supporting the study and providing infrastructure, M.H. Balsat, M. Mamberti, P. Greminger and A. Himmelmann for assistance, and all acknowledged persons for valuable discussions. We thank G. Hinz, MEDIDATA AG for help in statistical analysis.
References


Table 1. Iron isotopic composition of blood in patients with homozygous C282Y hemochromatosis and control subjects, presented as relative deviation of the $^{56}\text{Fe}/^{54}\text{Fe}$ isotope ratio of blood from the isotope ratio of the isotopic reference material IRM-014 (means ± 1 SD)

<table>
<thead>
<tr>
<th></th>
<th>Hemochromatosis patients</th>
<th>Control groups</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All patients</td>
<td>Age-matched controls</td>
<td>Young adults</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>61.8 ± 10.7 (n=30)</td>
<td>61.6 ± 9.8 (n=10)</td>
<td>23.6 ± 3.2 (n=36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>53%</td>
<td>50%</td>
<td>47%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\delta^{56}\text{Fe}/^{54}\text{Fe}$ (‰)</td>
<td>-2.11 ± 0.47</td>
<td>-2.72 ± 0.27</td>
<td>-2.58 ± 0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p=0.0003†</td>
<td></td>
<td></td>
<td>p&lt;0.0001†</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male subjects: $\delta^{56}\text{Fe}/^{54}\text{Fe}$ (‰)</td>
<td>-1.95 ± 0.40 (n=12)</td>
<td>-2.56 ± 0.37 (n=4)</td>
<td>-2.92 ± 0.22 (n=5)</td>
<td>-2.75 ± 0.17 (n=17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p=0.0001‡</td>
<td></td>
<td></td>
<td>p&lt;0.0001‡</td>
<td></td>
</tr>
<tr>
<td>Female subjects: $\delta^{56}\text{Fe}/^{54}\text{Fe}$ (‰)</td>
<td>-2.02 ± 0.43 (n=10)</td>
<td>-2.35 ± 0.59 (n=4)</td>
<td>-2.52 ± 0.10 (n=5)</td>
<td>-2.43 ± 0.21 (n=19)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p=0.025‡</td>
<td></td>
<td></td>
<td>p=0.0016‡</td>
<td></td>
</tr>
</tbody>
</table>

† when compared to all hemochromatosis patients
‡ when compared to treated hemochromatosis patients
Table 2. Association of the iron isotopic composition of blood ($\delta^{56}\text{Fe}/^{54}\text{Fe}$; means ± 1 SD) with clinical manifestations in hemochromatosis patients homozygous for the HFE G845A (C282Y) mutation.

<table>
<thead>
<tr>
<th>Clinical manifestations of hemochromatosis</th>
<th>$\delta^{56}\text{Fe}/^{54}\text{Fe}$ (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Histological examination</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Substantial fibrosis† (n=15)</td>
<td>-1.90 ± 0.34</td>
<td>0.0031</td>
</tr>
<tr>
<td>- No substantial fibrosis‡ in histological examination or no biopsy carried out in the presence of normal ALT (n=10)</td>
<td>-2.43 ± 0.47</td>
<td></td>
</tr>
<tr>
<td><em>Serum alanine aminotransferase concentration measured at the time of diagnosis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- ALT above the reference range (n=17)</td>
<td>-1.89 ± 0.37</td>
<td>0.0009</td>
</tr>
<tr>
<td>- ALT within the reference range (n=10)</td>
<td>-2.48 ± 0.42</td>
<td></td>
</tr>
<tr>
<td><strong>Arthropathy of metacarpophalangeal joints</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- present (n=17)</td>
<td>-1.93 ± 0.40</td>
<td>0.019</td>
</tr>
<tr>
<td>- absent (n=13)</td>
<td>-2.33 ± 0.47</td>
<td></td>
</tr>
<tr>
<td><strong>Diabetes mellitus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- present (n=3)</td>
<td>-1.88 ± 0.35</td>
<td>NS</td>
</tr>
<tr>
<td>- absent (n=27)</td>
<td>-2.13 ± 0.48</td>
<td></td>
</tr>
<tr>
<td><strong>Cardiopathy</strong> (n=1)</td>
<td></td>
<td>-1.59</td>
</tr>
<tr>
<td><strong>Hypogonadism</strong> (n=1)</td>
<td></td>
<td>-2.02</td>
</tr>
<tr>
<td><strong>Total number of clinical manifestations‡</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 2 (n=14)</td>
<td>-1.89 ± 0.37</td>
<td>0.0030</td>
</tr>
<tr>
<td>1 (n=9)</td>
<td>-2.09 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>0 (n=7)</td>
<td>-2.56 ± 0.41</td>
<td></td>
</tr>
</tbody>
</table>

† either substantial portal fibrosis or septal/bridging fibrosis or cirrhosis of liver tissue
‡ Assessing total number of clinical manifestations, liver disease was defined as substantial fibrosis in histological examination or, in patients with biopsy, elevated ALT at the time of diagnosis
NS = not significant
Legends

**Figure 1.** Correlation of the iron isotopic composition of blood ($\delta^{56}\text{Fe}/^{54}\text{Fe}$) with serum ferritin concentration at time of diagnosis in treated (A) and untreated (B) hemochromatosis patients and with the amount of iron removed by phlebotomy during iron depletion therapy (C) and maintenance therapy (D). Solid lines represent linear regression analyses; bars in panel D represent means ± 1 SD.

**Figure 2.** Transfer of heme-iron (foods of animal origin) and non-heme iron (plant foods) through the intestinal mucosa into blood. Non-heme iron is taken up by divalent metal transporter-1 (DMT-1) while heme-iron enters the epithelial cell by endocytosis. Non-heme iron as well as iron released from heme are either transported by iron regulatory protein 1 (IREG1) from the epithelial cell to the blood or deposited in ferritin. Isotope effects (isotope fractionation) may occur during transport of preferentially lighter isotopes by DMT-1 or IREG1 and/or during deposition of heavier iron isotopes into ferritin. In contrast, endocytosis of heme isotopomers (small relative mass differences) and release of iron from the heme molecule (quantitative process) are not expected to be isotope selective.
Figure 1.

(A) Serum ferritin concentration (µg/l) vs. 56Fe/54Fe (‰)

(B) Serum ferritin concentration (µg/l) vs. Iron removed during depletion therapy (g)

(C) Iron removed during depletion therapy (g) vs. 56Fe/54Fe (‰)

(D) Iron removed during maintenance therapy (g/year) vs. δ56Fe/54Fe (‰)
Figure 2.

Lumen of small intestine

Epithelial cell

Blood

Non-heme iron

DMT-1

Ferritin

isotope effect possible

Heme-bound iron

Endocytosis

no expected isotope effect

no expected isotope effect

isotope effect possible

isotope effect possible

For personal use only.
Hereditary hemochromatosis is reflected in the iron isotope composition of blood

Pierre-Alexandre Krayenbuehl, Thomas Walczyk, Ronny Schoenberg, Friedhelm von Blanckenburg and Georg Schulthess