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The blood copper isotopic composition is a prognostic indicator of the hepatic injury in Wilson disease†

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Wilson disease (WD) is an autosomal recessive disorder of copper (Cu) metabolism. The gene responsible for WD, *ATP7B*, is involved in the cellular transport of Cu, and mutations in the *ATP7B* gene induce accumulation of Cu in the liver and ultimately in the brain. In a pilot study, the natural variations of copper stable isotope ratios (⁶⁵Cu/⁶³Cu) in the serum of WD patients have been shown to differ from that of healthy controls. In the present study, we challenged these first results by measuring the ⁶⁵Cu/⁶³Cu ratios in the blood of treated (*n* = 25), naïve patients (*n* = 11) and age matched healthy controls (*n* = 75). The results show that naïve patients and healthy controls exhibit undistinguishable ⁶⁵Cu/⁶³Cu ratios, implying that the Cu isotopic ratio cannot serve as a reliable diagnostic biomarker. The type of treatment (D-penicillamine vs. triethylenetetramine) does not affect the ⁶⁵Cu/⁶³Cu ratios in WD patients, which remain constant regardless of the type and duration of the treatment. In addition, the ⁶⁵Cu/⁶³Cu ratios do not vary in naïve patients after the onset of the treatment. However, the ⁶⁵Cu/⁶³Cu ratios decrease with the degree of liver fibrosis and the gradient of the phenotypic presentation, *i.e.* presymptomatic, hepatic and neurologic. To get insights into the mechanisms at work, we study the effects of the progress of the WD on the organism by measuring the Cu concentrations and the ⁶⁵Cu/⁶³Cu ratios in the liver, feces and plasma of 12 and 45 week old *Atp7b*^{-/-} mice. The evolution of the ⁶⁵Cu/⁶³Cu ratios is marked by a decrease in all tissues. The results show that ⁶³Cu accumulates in the liver preferentially to ⁶⁵Cu due to the preferential cellular entry of ⁶³Cu and the impairment of the ⁶³Cu exit by ceruloplasmin. The hepatic accumulation of monovalent ⁶³Cu⁺ is likely to fuel the production of free radicals, which is potentially an explanation of the pathogenicity of WD. Altogether, the results suggest that the blood ⁶⁵Cu/⁶³Cu ratio recapitulates WD progression and is a potential prognostic biomarker of WD.

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Significance to metallomics

Previous studies have revealed variations in the copper stable isotope ratios (⁶⁵Cu/⁶³Cu) in the serum or blood of patients with hepatic diseases, including the Wilson disease characterized by a loss of function of the *ATP7B* gene. Here we present for the first time the results of blood ⁶⁵Cu/⁶³Cu ratio for treated and naïve WD patients that are compared to age-matched healthy controls. We propose a mechanism of Cu isotope fractionation associated to Wilson disease based on the Cu concentrations and the ⁶⁵Cu/⁶³Cu ratios in the liver, feces and plasma of 12 and 45 week old *Atp7b*^{-/-} mice. We finally suggest that the blood ⁶⁵Cu/⁶³Cu ratio recapitulates the disease progression and hence could be a prognostic biomarker of Wilson disease.

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Introduction

The WD is an autosomal recessive genetic disease, which was first described by Samuel Alexander Kinnier Wilson in 1912.¹ It is a rare disease (from 1/30 000 to 1/100 000 births) due to mutations in the *ATP7B* gene.² This gene encodes a transmembrane protein ATPase (ATP7B) and is found on chromosome 13. The ATP7B transporter is primarily found in the liver, and in smaller amounts in the kidneys and brain. The liver plays a key role in the homeostasis of Cu and constitutes its main storage organ. After the digestion of food in the stomach and duodenum, Cu is mainly absorbed in the small intestine.³ Cu assimilation occurs through the crossing of the apical membrane of enterocytes by a high affinity transporter hCTR1.⁴ Then, Cu binds to two different proteins, albumin and α 2-macroglobulin, in the extracellular medium and is finally deposited in the liver³ by the same hCTR1 transporter. In liver cells, the function of ATP7B is to transfer Cu first, across the trans-Golgi membrane for delivering to apo-ceruloplasmin^{5,6} and second, across the cellular membrane for excretion into the biliary canal out of the body.⁷ In WD, the loss of ATP7B function induces a failure to properly incorporate Cu into ceruloplasmin (Cp),⁸ resulting in a deficiency of Cp in the serum (less than 200 mg L⁻¹) and an elevation of the non-Cp serum Cu concentration of more than 100 mg L⁻¹.⁹ The loss of ATP7B function disrupts the Cu cellular processing resulting in Cu accumulation in the liver first, and then by an overflow effect in the brain, kidneys, and cornea, for which the Kayser-Fleischer ring is a specific clinical sign.

There is no curative treatment for WD and medication must be taken for life. The aim of existing treatments is to stabilize, and hopefully reduce, the body's Cu load using chelators. The first treatment, which appeared in 1951, was the British anti-Lewisite (BAL or 2,3-dimercatopropanol).¹⁰ Due to the many side effects of BAL, a new chelator, D-penicillamine, was introduced first by John Walshe in 1956.¹¹ While the use of BAL required painful injections, D-penicillamine is orally administered. D-Penicillamine is structurally similar to the cysteine amino-acid but with a free sulphhydryl group and has a high affinity for Cu. Some patients are however D-penicillamine-intolerant and develop a nephrotic syndrome, so Walshe introduced in 1969 triethylene tetramine dihydrochloride as a new orally active chelating agent.¹² Triethylene tetramine dihydrochloride has a polyamine structure and Cu is chelated within the four nitrogen groups. Noteworthy is the use of zinc in the treatment of WD.¹³ Recently, Brewer proposed tetrathiomolybdate (TTM) to chelate Cu.¹⁴ The Food and Drug Administration authorized TTM as WD treatment in USA but TTM is not authorized in Europe yet.

There is a general agreement that the diagnosis of WD should be based on a combination of several genetic, biological, clinical and radiological features.^{15,16} However, the Relative exchangeable Cu (REC), *i.e.* the ratio between exchangeable serum Cu (CuEXC) and total serum Cu, has been found to have a perfect sensitivity and specificity in diagnosing WD for REC > 18.5%.^{15,17} Recently, Aramendía *et al.*¹⁸ suggested that the natural variations of the stable isotope ⁶⁵Cu/⁶³Cu ratio in blood could be used for the

diagnosis of WD. It was found in this study that the ⁶⁵Cu/⁶³Cu ratio of WD patients is significantly lower than that of healthy controls, with no overlap.¹⁸ The two Cu stable isotopes, ⁶³Cu and ⁶⁵Cu, have different behaviors in biological processes due to mass difference. Mass difference between isotopes of an element induces variations in kinetic reactions and bond energies, called the isotope effect or isotope fractionation, which indicates small variations in the relative abundances of the isotopes. Isotope fractionation must however obey mass conservation, *i.e.* the total abundance of all the isotopes must be balanced in the course of a chemical reaction. For about ten years now, the use of Cu stable isotope fractionation has driven specific interest and developments in the field of biomedicine. The Cu stable isotopes largely fractionate in organs and body fluids, with a range of isotope compositions of a few permil (‰) units.^{19,20} The blood Cu isotope composition is different between human males and females²¹ due to menstrual losses.^{22,23} Copper isotope compositions are highly sensitive to disease conditions and have been suggested as a new biomarker for cancer,^{24–26} liver diseases^{20,27} and neurodegenerative disorders.^{28–31} In their pilot study Aramendía *et al.* show interest in measuring Cu isotopic composition in WD, but the study concerns five WD patients only, who were all treated.

In the present study, we focused on naïve (untreated) WD patients ($n = 11$), whose blood was collected at diagnosis, and WD patients treated with different chelators, *i.e.* D-penicillamine or “Trolovol” ($n = 19$) and triethylene tetramine dihydrochloride or “Trientine” ($n = 6$). The results obtained on the population of WD patients were compared to those of an age-matched healthy population, with new ⁶⁵Cu/⁶³Cu values of young (from 1 to 13 years old, $n = 28$) completed by ⁶⁵Cu/⁶³Cu values of adults (>18 years old, $n = 47$) from the study of Albarède *et al.*²¹ In addition, the ⁶⁵Cu/⁶³Cu values in plasma, liver and feces in 11–12 week old and 45 week old *Ath7b*^{-/-} mice have been measured.

Materials and methods

Reagents

Ultrapure water (resistivity 18.2 M Ω cm⁻¹) was produced in a Millipore Synergy system (France). Concentrated technical grade HCl and HNO₃ provided by Carlo Erba (France) were distilled at low temperature in Savillex PFA equipment. H₂O₂ 30% Suprapur was purchased from Merck (Germany). Macroporous anion-exchange resin AG MP-1, 100–200 mesh, was purchased from Biorad Laboratories.

Samples

All the young controls of this study as well as the adult controls from Albarède *et al.*²¹ were prepared and analyzed in the same conditions in the Laboratoire de Géologie de Lyon, Ecole Normale Supérieure de Lyon, France. Total blood samples from supposedly healthy controls ($n = 47$) between 18 and 35 years old are from the Etablissement du Sang.²¹ Total blood samples from supposedly healthy controls between 1 and 13 years old

($n = 28$) are from the Department of Biochemistry and Molecular Biology, Hôpital Edouard Herriot, Hospices Civils de Lyon, France. The samples were collected originally for suspicion of lead exposition. Total blood samples from WD patients treated with chelators ($n = 25$) and naïve patients ($n = 11$) between 3 and 52 years old are from French National Reference Center for Wilson's Disease, Hôpital Femme Mère Enfant, Hospices Civils de Lyon, France. The samples were collected in Vacutainer tubes. Samples were stored at 4 °C until analysis.

WD patients. The diagnosis of WD was based on clinical, biochemical, histologic and genetics findings. All patients had two disease-causing *ATP7B* mutations. Phenotypic classification was based on the Leipzig score.¹⁶ This scoring system includes a combination of clinical and biochemical tests such as the absence or presence of the Kayser-Fleischer ring, neurologic symptoms, Coombs-negative hemolytic anemia, serum ceruloplasmin concentration, hepatic Cu concentration, urinary Cu concentration and presence of mutations. Patients were considered to have severe fibrosis (F3–F4) when they have clinical or morphological features of cirrhosis (ascites, hepatic dysmorphism with signs of portal hypertension), presence of bridging fibrosis or cirrhosis on histology or fibroscan >10 kPa on two different measures in the absence of severe cytolysis (transaminases < 5 × ULN). The study was conducted in the French National Wilson disease Center of Lyon, in the Pediatric Hepatology Department of the Children's Hospital and the Hepatology Department of Edouard Herriot Hospital, qualified as evaluation of daily practice and was approved by our local ethic committee (hôpital Femme-Mère-Enfant, Hospices civils de Lyon). Informed consent was obtained from all participants.

***Atp7b*^{-/-} mice.** The *Atp7b*^{-/-} mouse model used in this study was developed by Buiakova *et al.*³² and kindly provided by Prof. Svetlana Lutsenko and Dr Dominik Huster. *Atp7b*^{+/-} heterozygous mice, maintained on a mixed 129S6/SvEv × C57BL/6J genetic background, were bred in our animal care facility and used to generate *Atp7b*^{-/-} mice. Mouse breeding, housing and experiments were performed according to the protocols approved by the ethics committees (C2EA – 12 Comité d'éthique ComEth Grenoble and C2EA – 44 CETEA – CEA DSV IdF), the veterinary authorities, and the French Ministry for Research. The mice were kept on a 12 h/12 h light/dark cycle and fed with maintenance dry food #3469 (Kliba Nafag CH), containing 14 mg kg⁻¹ of Cu. Mice at 11–12 weeks ($n = 12$) and at 45 weeks ($n = 12$) were transferred to metabolism cages for 24 hours fasting and feces collection. Then, the mice were exposed to carbon dioxide, before blood collection in sodium heparin-treated tubes (Becton Dickinson) by cardiac puncture and liver dissection. Within one hour of collection, blood samples were centrifuged at 2000g and 4 °C for 15 min to collect plasma supernatants. Liver, plasma and feces specimens were stored at –20 °C until analysis.

Blood sample preparation

An aliquot of about 500 µL of total blood was digested in Savillex PFA beakers using 2 mL of HNO₃ (14 M) and 0.2 mL

of H₂O₂ (30%) at 100 °C overnight. Dissolved samples were then evaporated to dryness at 100 °C and redissolved in 1 mL of HNO₃ (0.5 M). A 100 µL aliquot was taken for the measurement of Cu concentration, and the remaining solution was evaporated and redissolved with 1 mL of HCl (7 M) + H₂O₂ (0.001%) for chromatographic separation according to the technique of Maréchal *et al.*³³ Briefly, Cu was separated on quartz columns containing 1.6 mL of macroporous anion-exchange resin AG MP1. Samples were loaded onto the columns and rinsed with 10 mL of HCl (7 M) + H₂O₂ (0.001%). Copper was eluted using 20 mL of HCl (7 M) + H₂O₂ (0.001%). Copper was further purified using the same protocol.

Mouse sample preparation

Liver and feces samples were digested in Savillex PFA beakers using 5 mL of HNO₃ (14 M) at 100 °C for 7 days and regularly degassed. After cooling, 0.5 mL of H₂O₂ (30%) was added and the beakers were replaced at 100 °C for 4 days. Dissolved samples were then evaporated to dryness and a second mineralization was performed using the same protocol to ensure complete dissolution. Plasma samples were digested in Savillex PFA beakers using 1 mL of HNO₃ (14 M) and 0.2 mL of H₂O₂ (30%) at 100 °C overnight. Dissolved samples were then evaporated to dryness. Liver and feces samples were redissolved with 3 mL of HNO₃ (0.5 M) and plasma samples with 1 mL of HNO₃ (0.5 M). A 30 µL aliquot was taken for the measurement of Cu concentration, and the remaining solution was evaporated. Liver and feces samples were redissolved with 2 mL of HCl (7 M) + H₂O₂ (0.001%) and the quantity needed to get about 600 ng of Cu was taken for chromatographic separation as previously described. Plasma samples were redissolved with 1 mL of HCl (7 M) + H₂O₂ (0.001%) for chromatographic separation.

Instrumentation and measurement protocols

The Cu concentrations were determined by quadrupole inductively coupled plasma mass spectrometry (ICP-MS) on an Agilent 7500 CX. Samples were diluted in 0.5 M sub-boiled distilled HNO₃ and indium at 2 µg L⁻¹ was used as an internal standard. The Cu isotope compositions were measured by multiple-collector inductively coupled plasma mass spectrometry (MC-ICP-MS) on a Nu 500 HR instrument. Instrumental mass discrimination was corrected using Zn-doping (Zn JMC-Lyon) and standard sample bracketing following the recommendations provided by Albarède *et al.*³⁴ Generally, Cu and Zn concentrations were adjusted to 200 µg L⁻¹ in 2 mL of 0.05 M sub-boiled distilled HNO₃. Copper fractions were otherwise dissolved in the minimal volume needed for an isotopic analysis (~700 µL) and Zn concentrations were adjusted to that of Cu. Wet plasma was used to avoid instrumental bias that occurs in the membrane of the desolvating systems. Copper isotope composition is reported with the standard delta notation relative to the Cu isotopic standard NIST SRM 976, according to the formula

$$\delta^{65}\text{Cu} = \left\{ \left[\frac{({}^{65}\text{Cu}/{}^{63}\text{Cu})_{\text{sample}}}{({}^{65}\text{Cu}/{}^{63}\text{Cu})_{\text{standard}}} \right] - 1 \right\} \times 1000.$$

Statistical analysis

To compare two groups, statistical significances were calculated using the non-parametric Mann-Whitney/Wilcoxon test with the R software. The level of significance established of the *p*-values was 0.05.

Results

The results are summarized in Table 1 for humans. The blood Cu concentration in WD patients ($384 \pm 134 \mu\text{g L}^{-1}$, $n = 35$) is significantly lower (p -value $< 10^{-4}$) than in healthy controls ($997 \pm 302 \mu\text{g L}^{-1}$, $n = 75$, Fig. 1A). The blood Cu concentration in treated WD patients ($388 \pm 116 \mu\text{g L}^{-1}$, $n = 25$) and in untreated WD patients ($373 \pm 178 \mu\text{g L}^{-1}$, $n = 10$) is not significantly different (p -value = 0.6, Fig. 1A). The blood Cu concentration in WD patients treated with Trientine ($374 \pm 44 \mu\text{g L}^{-1}$, $n = 6$) is similar to that in WD patients treated with Trolovol ($392 \pm 132 \mu\text{g L}^{-1}$, $n = 19$, p -value = 0.88, Table 1). The blood $\delta^{65}\text{Cu}$ value is similar in control and untreated patients (p -value = 0.32, Fig. 1B) but in treated WD patients ($-0.97 \pm 0.70\%$, $n = 25$) it is significantly lower compared to that in untreated WD patients ($-0.04 \pm 0.85\%$, $n = 11$) (p -value = 0.0025) and healthy controls ($-0.03 \pm 0.27\%$, $n = 75$, p -value $< 10^{-4}$, Fig. 1B). To ensure that any treatment effect is not at the origin of this difference, we compared the $\delta^{65}\text{Cu}$ values at different times and for some patients on the day of the

diagnosis and after treatment. The blood Cu concentration ($n = 7$) and the $\delta^{65}\text{Cu}$ values ($n = 8$) have been measured at two time points (Table 1). For two WD patients (PALA and DIMA), the blood Cu concentration decreases over time (Fig. 2A), while other patients are stable (Fig. 2A). The blood Cu isotope composition remains constant in all patients over time (Fig. 2B). For four of them (AZWA, MOKA, PAPH, and JEAN), the average value was considered for statistical analysis because the clinical situation was unchanged between the two time points. For three of them (CREM, DIMA and PEEV), the blood samples were taken at diagnosis and after more than three years of treatment, so that the two measurements were considered as independent. Finally, for PALA, the treatment was changed between the two time points, so the two measurements were also considered as independent.

We next examine the influence of sex and age on the blood $\delta^{65}\text{Cu}$ values which are known to be influenced by menstruation.^{22,23,35} Women patients aged more than 15 years old, who are supposed to be menstruated, have a lower $\delta^{65}\text{Cu}$ value ($-1.18 \pm 0.81\%$, $n = 12$) than the rest of WD patients ($-0.44 \pm 0.78\%$, $n = 24$, p -value = 0.0137). In controls, the difference of the $\delta^{65}\text{Cu}$ value between menstruated women ($0.00 \pm 0.20\%$, $n = 25$) and men ($0.16 \pm 0.16\%$, $n = 22$) aged more than 15 years old is also significant (p -value = 0.0072, Fig. 3).

Bearing in mind that anthropological factors affect the blood $\delta^{65}\text{Cu}$ value, we now explore the pathological effects of

Table 1 Anthropological (age and sex), chemical (Cu concentration, Cu isotope composition and REC) and clinical (MELD, fibrosis and phenotype) data of the WD patients and healthy controls

	WD patients				Controls
	Treated	Untreated	All WD		
Cu ($\mu\text{g L}^{-1}$)	388 ± 116 ($n = 25$)	373 ± 178 ($n = 10$)	384 ± 134 ($n = 35$)		997 ± 302 ($n = 75$)
	Trientine	Trolovol			
	374 ± 44 ($n = 6$)	392 ± 132 ($n = 19$)			
$\delta^{65}\text{Cu}$ (‰)	-0.97 ± 0.70 ($n = 25$)	-0.04 ± 0.85 ($n = 11$)	-0.69 ± 0.86 ($n = 36$)		-0.03 ± 0.27 ($n = 75$)
REC (%)	53.0 ± 57.8 ($n = 25$)	40.7 ± 22.4 ($n = 11$)	49.2 ± 49.7 ($n = 36$)		
MELD	7.6 ± 0.9 ($n = 21$)	7.5 ± 3.1 ($n = 10$)	7.6 ± 1.9 ($n = 31$)		
Age	20.4 ± 11.9 ($n = 25$)	13.6 ± 8.2 ($n = 11$)	18.3 ± 11.3 ($n = 36$)		16.7 ± 8.5 ($n = 75$)
$\delta^{65}\text{Cu}$ (‰) F ≥ 15 years			-1.18 ± 0.81 ($n = 12$)		0.00 ± 0.20 ($n = 25$)
$\delta^{65}\text{Cu}$ (‰) all M; F < 15 years			-0.44 ± 0.78 ($n = 24$)		
$\delta^{65}\text{Cu}$ (‰) M ≥ 15 years					0.16 ± 0.16 ($n = 22$)
$\delta^{65}\text{Cu}$ (‰) M and F < 15 years			-0.15 ± 0.80 ($n = 14$)		-0.21 ± 0.27 ($n = 28$)
$\delta^{65}\text{Cu}$ (‰) M and F ≥ 15 years			-1.03 ± 0.71 ($n = 22$)		0.08 ± 0.20 ($n = 47$)
Level of fibrosis					
$\delta^{65}\text{Cu}$ (‰) F0–F2			-0.14 ± 0.84 ($n = 15$)		
$\delta^{65}\text{Cu}$ (‰) F3–F4			-1.09 ± 0.62 ($n = 21$)		
Phenotypic form					
$\delta^{65}\text{Cu}$ (‰) pre-symptomatic			0.32 ± 0.40 ($n = 2$)		
$\delta^{65}\text{Cu}$ (‰) hepatic			-0.44 ± 0.73 ($n = 24$)		
$\delta^{65}\text{Cu}$ (‰) neurologic			-1.48 ± 0.62 ($n = 10$)		
Level of fibrosis and phenotypic form					
$\delta^{65}\text{Cu}$ (‰) pre-symptomatic F0–F2			0.32 ± 0.40 ($n = 2$)		
$\delta^{65}\text{Cu}$ (‰) hepatic F0–F2			-0.09 ± 0.81 ($n = 12$)		
$\delta^{65}\text{Cu}$ (‰) hepatic F3–F4			-0.80 ± 0.43 ($n = 12$)		
$\delta^{65}\text{Cu}$ (‰) neurologic F0–F2			-1.60 ($n = 1$)		
$\delta^{65}\text{Cu}$ (‰) neurologic F3–F4			-1.47 ± 0.65 ($n = 9$)		

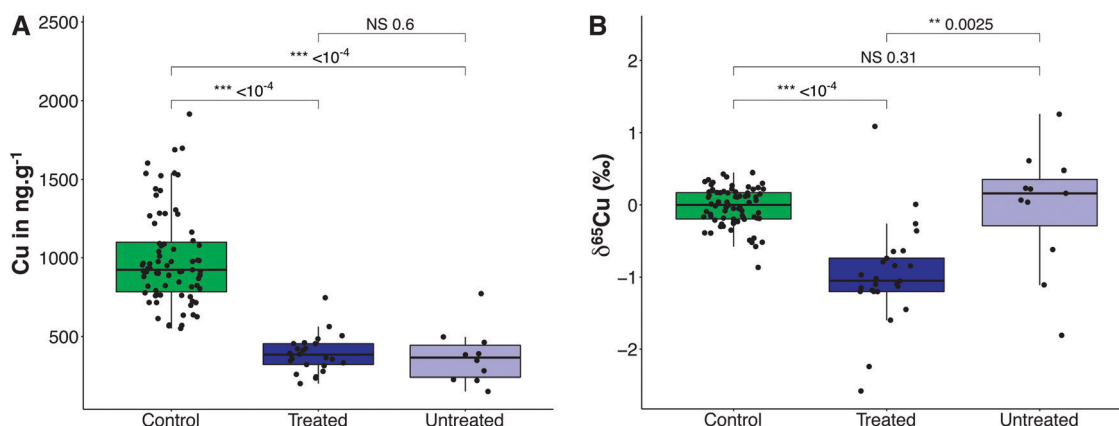


Fig. 1 (A) Distribution of the Cu concentration in the blood of healthy control, treated and untreated WD patients. (B) Distribution of the Cu isotope compositions in the blood of healthy control, treated and untreated WD patients. p -Values are non-significant (ns) $p > 0.05$; (*) $p \leq 0.05$; (***) $p \leq 0.001$.

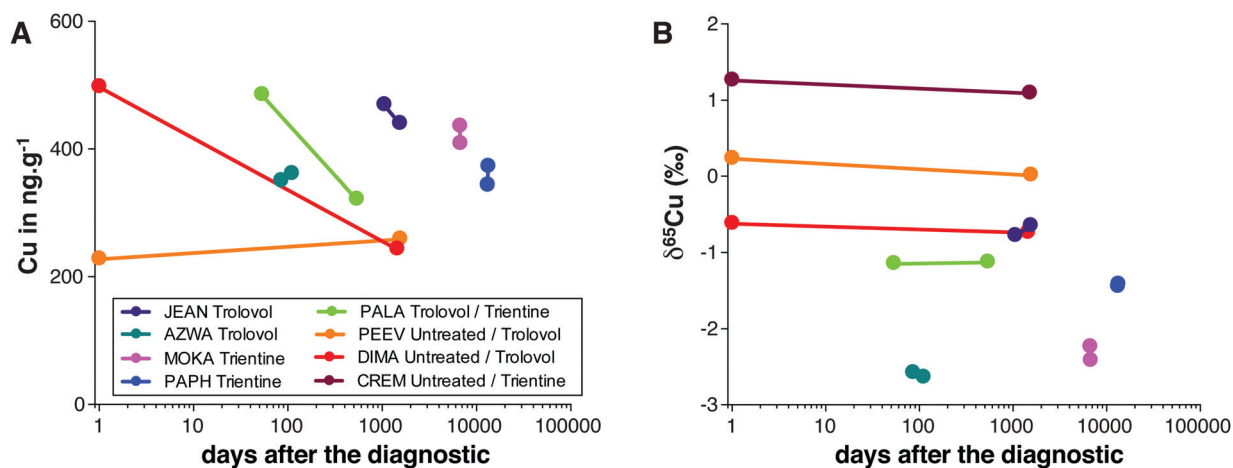


Fig. 2 (A) Evolution of the Cu concentration in blood for seven WD patients. (B) Evolution of the Cu isotope compositions in blood for eight WD patients.

the WD disease. The fibrosis stages for WD patients are summarized in Table 1. Whatever the pace of the disease, liver injury results in fibrosis, which is an excessive accumulation of extracellular matrix forming scar tissue. There are several stages of fibrosis with no fibrosis (F0), minimal fibrosis (F1), moderate fibrosis (F2), severe fibrosis (F3) and cirrhosis (F4). The comparison of the stage of fibrosis with the blood $\delta^{65}\text{Cu}$ value reveals that WD patients with severe fibrosis or cirrhosis (F3–F4) exhibit significantly lower $\delta^{65}\text{Cu}$ values ($-1.09 \pm 0.62\text{‰}$, $n = 21$) compared to WD patients with no, minimal or moderate fibrosis (F0–F2) ($-0.14 \pm 0.84\text{‰}$, $n = 15$, p -value = 0.0013, Fig. 4).

The WD usually progresses from hepatic failure to neurologic disability. Starting with the two pre-symptomatic patients at $0.32 \pm 0.40\text{‰}$, the $\delta^{65}\text{Cu}$ value decreases to $-0.44 \pm 0.73\text{‰}$ for the hepatic form ($n = 24$) and decreases again to $-1.48 \pm 0.62\text{‰}$ for the neurologic form ($n = 10$, Fig. 5). The blood $\delta^{65}\text{Cu}$ value in the pre-symptomatic form is undistinguishable from that of controls (p -value = 0.18), while the blood $\delta^{65}\text{Cu}$ value in the hepatic and neurologic forms is significantly lower than that

of controls (p -value = 0.0017 and p -value $< 10^{-4}$, respectively, Fig. 5).

Altogether, the above results show that the blood $\delta^{65}\text{Cu}$ value in WD patients decreases with the degree of the liver injury, which is reflected by the level of fibrosis and the phenotypic form. Even if the WD progress is variable among patients, there is a clear inverse correlation between the blood $\delta^{65}\text{Cu}$ value and the age of the patients (Fig. 6). The WD patients compose clusters within the relationship with little overlaps, with the hepatic F0–F2 group having the highest blood $\delta^{65}\text{Cu}$ values and the youngest age ($-0.09 \pm 0.81\text{‰}$, 12 ± 9 years old, $n = 12$), the hepatic F3–F4 group being intermediary ($-0.80 \pm 0.43\text{‰}$, 17 ± 4 years old, $n = 12$) and the neurologic F3–F4 group having the lowest blood $\delta^{65}\text{Cu}$ values and the oldest age ($-1.47 \pm 0.65\text{‰}$, 28 ± 15 years old, $n = 9$).

To get insights into the pathological mechanisms at work in the WD, we measured the Cu concentration and isotope composition in plasma, liver and feces in 11–12 week and 45 week old *Atp7b*^{-/-} mice, which are summarized in Table 2.

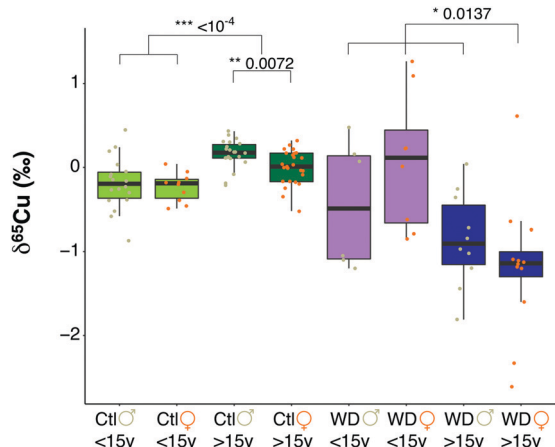


Fig. 3 Distribution of the Cu isotope compositions in the blood of healthy controls (ctl) and WD patients (WD). A threshold value of fifteen years (15y) is used to identify infants (with non-menstruated girls) from adults (with menstruated women). *p*-Values are non-significant (ns) $p > 0.05$; (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$.

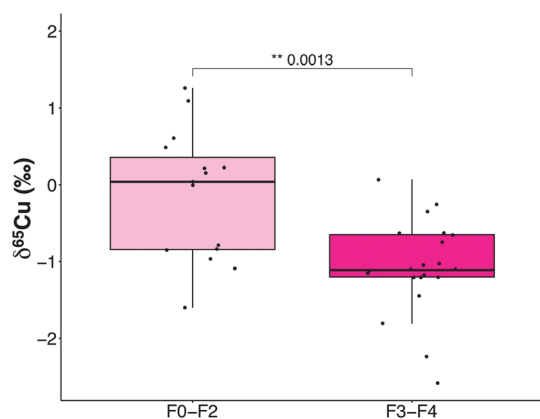


Fig. 4 Distribution of the Cu isotope compositions in the blood of WD patients with no, minimal or moderate fibrosis (F0–F2) and severe fibrosis or cirrhosis (F3–F4). *p*-Values are non-significant (ns) $p > 0.05$; (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$.

From 11–12 weeks to 45 weeks, the Cu concentration increases in the liver (p -value $< 10^{-4}$) and plasma (p -value = 0.0243), but decreases in feces (p -value = 0.0111, Fig. 7A). The Cu isotope composition was significantly lower in 45 week old mice in comparison with 11–12 week old mice (p -values $< 10^{-4}$ for plasma, liver and feces, Fig. 7B).

Discussion

Aramendía *et al.*¹⁸ previously showed that the Cu isotope composition in WD patients ($-1.70 \pm 0.66\text{‰}$, $n = 5$) is lower than in healthy controls ($-0.72 \pm 0.25\text{‰}$, $n = 18$). On comparing these results to the present ones two observations can be drawn. First, based on their results, Aramendía *et al.*¹⁸ claimed that the $\delta^{65}\text{Cu}$ value could be a potential diagnostic biomarker of WD, but we do not observe here any difference between

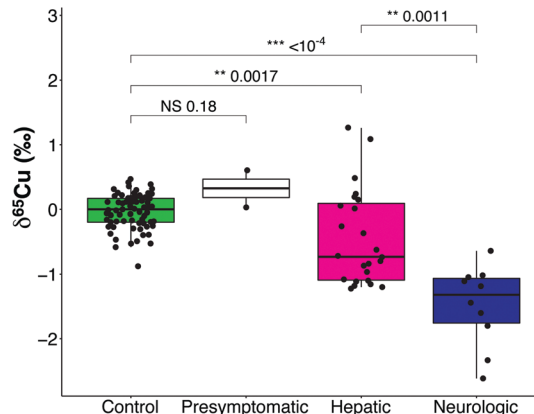


Fig. 5 Distribution of the Cu isotope compositions in the blood of healthy controls and WD patients according to the phenotypic presentation. *p*-Values are non-significant (ns) $p > 0.05$; (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$.

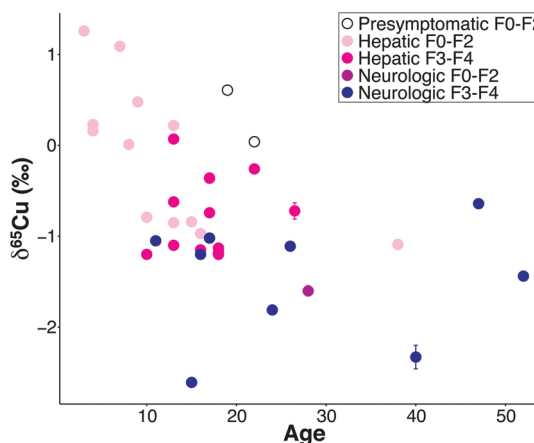


Fig. 6 Variation of the Cu isotope composition in the blood of WD patients according to the degree of fibrosis and the phenotypic presentation. *p*-Values are non-significant (ns) $p > 0.05$; (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$.

untreated WD patients ($n = 11$) and controls ($n = 75$) suggesting that the $\delta^{65}\text{Cu}$ value cannot be reliably used for WD diagnosis. The inconsistency between the two studies lies in the fact that the five WD patients in the study of Aramendía *et al.*¹⁸ were treated patients (VanHaecke, pers. comm.). Second, Aramendía *et al.*¹⁸ measured the Cu isotope composition in serum while we measured the Cu isotope composition in total blood. Copper in the serum is mostly bound to ceruloplasmin ($\sim 90\%$) and the remaining Cu is bound to albumin and alpha-2 macroglobulin. The extra compartment in whole blood is red blood cells in which Cu is bound to superoxide dismutase. Serum components and red blood cells have a different turnover, so comparing serum and whole blood $\delta^{65}\text{Cu}$ values is not appropriate at first glance. However, a comparison of serum and whole blood $\delta^{65}\text{Cu}$ values from the same sample collection tube reveals that both compartments are isotopically similar.²¹ It is obvious that working with the same type of sample is more careful, but it can happen that different types of samples can be present in the

Table 2 Cu concentration and isotope composition in 11–12 and 45 week old *Atp7b*^{-/-} mice

Age (w)	Plasma		Liver		Feces	
	Cu ($\mu\text{g L}^{-1}$)	$\delta^{65}\text{Cu}$ (‰)	Cu ($\mu\text{g L}^{-1}$)	$\delta^{65}\text{Cu}$ (‰)	Cu ($\mu\text{g L}^{-1}$)	$\delta^{65}\text{Cu}$ (‰)
11–12	0.39 ± 0.09 ($n = 10$)	-0.31 ± 0.13 ($n = 9$)	275 ± 22 ($n = 12$)	-0.53 ± 0.12 ($n = 12$)	49 ± 7 ($n = 12$)	0.33 ± 0.06 ($n = 12$)
45	0.53 ± 0.16 ($n = 12$)	-1.63 ± 0.53 ($n = 12$)	479 ± 116 ($n = 12$)	-1.86 ± 0.40 ($n = 12$)	42 ± 6 ($n = 12$)	0.06 ± 0.10 ($n = 12$)

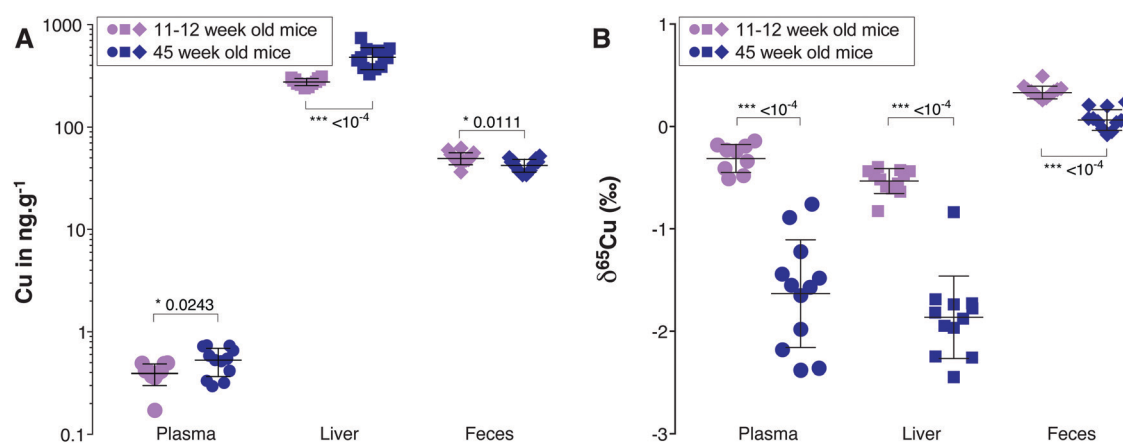


Fig. 7 (A) Distribution of the Cu concentration in the serum, liver and feces of *Atp7b*^{-/-} mice. (B) Distribution of the Cu isotope compositions in the serum, liver and feces of *Atp7b*^{-/-} mice. *p*-Values are non-significant (ns) $p > 0.05$; (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$.

same biobank. Noteworthy is the fact that hemolysis will not affect the quality of the measurement as far as Cu isotope composition is concerned.

In the present study, we found that untreated WD patients and controls have comparable $\delta^{65}\text{Cu}$ values, which are different from treated patients (Fig. 1B). In addition, WD patients, being treated or not, exhibit a $\delta^{65}\text{Cu}$ value which seems to not vary through time (Fig. 2B). Each WD patient is likely to have a Cu isotopic signature. A distribution of the $\delta^{65}\text{Cu}$ value as a function of gender and age indicates some patterns of anthropological origin (Fig. 3). While the difference between menstruated women and men has already been described and explained,^{21–23,35} an isotopic pattern yet unknown is the difference (p -value $< 10^{-4}$) of the $\delta^{65}\text{Cu}$ value between young (< 15 years old, $-0.21 \pm 0.27\text{‰}$, $n = 28$) and adult (> 15 years old, $0.08 \pm 0.20\text{‰}$, $n = 47$) controls (Fig. 3). This difference is also observed in WD patients (p -value = 0.0033). The blood $\delta^{65}\text{Cu}$ signature of WD patients thus integrates an anthropological (age and sex) and a pathological component.

The results described above show that the Cu isotope composition in total blood decreases with the severity of the liver disease and the state of liver functions, which is in accordance with a previous study²⁷ showing that the serum Cu isotope composition in female cirrhotic patients ($-0.78 \pm 0.72\text{‰}$, $n = 25$) is lower than in female healthy controls ($-0.29 \pm 0.27\text{‰}$, $n = 29$) (p -value $< 10^{-4}$). In this study,²⁷ the authors also showed that the $\delta^{65}\text{Cu}$ value decreases with the MELD score (Model of End stage Liver Disease), which confirms that the serum/blood Cu isotope composition is correlated with the severity of the liver injury. Here, the blood $\delta^{65}\text{Cu}$ value is also

inversely correlated to the MELD score ($R = -0.15$) but the correlation is not significant (p -value = 0.40).

In WD, which is a genetic disease, Cu hepatic accumulation probably begins at birth but it takes several years to observe the first hepatic symptoms. The familial genetic screening can however help to diagnose pre-symptomatic young WD patients. The hepatic injury generally happens at 2–3 years and further evolves at a variable pace, so that some WD infants can be cirrhotic at ten years and others will reach adulthood with moderate hepatic damage. The neurologic injury always follows the hepatic one, but is little symptomatic at the onset and can be ignored for a certain time. The age of the patient at diagnosis therefore generally gives first order information about the degree of the hepatic injury. In this context, the blood $\delta^{65}\text{Cu}$ value appears to be a good biomarker of WD progress and potentially of WD prognosis. Interestingly, some patients exhibit blood $\delta^{65}\text{Cu}$ values which are outside the expected range of age. This is the case for instance for AZWA who is already a F3–F4 neurologic phenotype at 15 years old only. In his/her case, the WD has been very aggressive because he/she has a very negative $\delta^{65}\text{Cu}$ value at -2.61‰ , the lowest of the dataset. Another interesting example is JEJI, for whom the WD is stable at 38 years old, with a blood $\delta^{65}\text{Cu}$ value at -1.09‰ .

To study the mechanism of the WD, we measured the Cu concentration and isotope composition in blood plasma, liver and feces in *Atp7b*^{-/-} mice at two time points, 11–12 and 45 weeks. The Cu concentration increases in the liver by 87% and plasma by 67% but slightly decreases by 15% in feces from 11–12 weeks to 45 weeks (Fig. 7A). During that duration, the Cu

isotope composition decreases in all tissues. The liver $\delta^{65}\text{Cu}$ value decreases from $-0.53 \pm 0.12\%$ ($n = 12$) to $-1.86 \pm 0.40\%$ ($n = 12$), that of plasma from $-0.31 \pm 0.13\%$ ($n = 9$) to $-1.63 \pm 0.53\%$ ($n = 12$) and that of feces from $0.33 \pm 0.06\%$ ($n = 12$) to $0.06 \pm 0.10\%$ ($n = 12$) (Fig. 7B). The difference of the $\delta^{65}\text{Cu}$ values between 45 and 11–12 weeks, hereafter annotated $\Delta^{65}\text{Cu}$, is -1.3% , -1.3% and -0.3% for liver, plasma and feces, respectively. Unfortunately, these numbers cannot be evaluated relative to the control group because we did not have access to wild type mice. Twelve week old mice can however be considered as a control group because it is at this age that copper has significantly accumulated in the liver and causes liver damage.³⁶ The evolution of the $\Delta^{65}\text{Cu}$ values of organs of *Atp7b*^{-/-} mice can be compared to those of Costas-Rodriguez *et al.*,²⁰ who studied the effects of cholestatic liver disease using a common bile duct ligation (CBDL) murine model. The comparison is interesting because, although of distinct etiologies, cholestatic liver disease and WD both result in hepatic Cu accumulation. CBDL-operated mice exhibit a dramatic increase of Cu concentration of 336% in the liver and of 170% in serum and a decrease of 16% in feces compared to SHAM-operated mice.²⁰ The bile duct ligation results in a decrease of the $\Delta^{65}\text{Cu}$ value of -1.0% , -0.6% and 0.0% for liver, serum and feces, respectively. Therefore, while the Cu accumulation in CBDL-operated mice is much more important than in *Atp7b*^{-/-} mice, the resulting decrease of the $\Delta^{65}\text{Cu}$ value is less pronounced than in *Atp7b*^{-/-} mice. This suggests that the knockdown of the *ATP7B* gene leads to a very efficient hepatic accumulation of ^{63}Cu .

Two different processes are likely at work to explain the ^{63}Cu hepatic accumulation in *Atp7b*^{-/-} mice, and thus in WD. First, it is the preferential entry of ^{63}Cu in cells, being healthy or pathologic. Copper crosses the cellular membrane with the high affinity transporter hCTR1, which only carries reduced Cu^+ . The reduction of Cu is achieved by a Steap reductase prior to the transport by hCTR1. Because bonds involving low oxidation states (Cu^+ , Fe^{2+}) prefer light to heavy isotopes,^{37,38} the Steap activity will favor ^{63}Cu to ^{65}Cu , and thus $^{63}\text{Cu}^+$ will preferentially enter the cells. Second, it is suppression of the preferential exit of Cp-bound ^{63}Cu . Due to the effects on the isotopic distribution of Cu with different binding sites, Tennant *et al.*³⁹ predicted that the binding of Cu to apo-Cp favors ^{63}Cu . The prediction is in accordance with the observation that serum has systematically a lower $\delta^{65}\text{Cu}$ value than liver in normal conditions, *i.e.* the difference of the $\delta^{65}\text{Cu}$ values between liver and serum ($\Delta^{65}\text{Cu}_{\text{liver-ser}}$) is positive. In CBDL-operated mice, *ATP7B* is functional and can still deliver Cu to apo-Cp resulting in a $\Delta^{65}\text{Cu}_{\text{liver-ser}}$ of $+0.5\%$.²⁰ However, in *Atp7b*^{-/-} mice, Cu is no longer delivered to apo-Cp resulting in $\Delta^{65}\text{Cu}_{\text{liver-ser}}$ of -0.2% (Fig. 7B and Table 2), whatever the age of the mice.

Finally, ^{63}Cu accumulates in *Atp7b*^{-/-} mice and in WD patients because the biliary excretion is impaired by the mutation of the *ATP7B* gene,⁴⁰ but part of the accumulated Cu is still excreted out of hepatocytes by a yet unknown process. Exocytosis can be involved where Cu would be unbound,

leading to the observed increase in CuEXC in *Atp7b*^{-/-} mice⁴¹ and in WD patients.^{15,17} Interestingly, Lauwens *et al.*⁴² measured the $\delta^{65}\text{Cu}$ value of CuEXC in alcoholic liver cirrhotic patients and found comparable or lower values than in the corresponding bulk serum.

Conclusion

In this study, the Cu concentrations and isotope compositions have been measured in treated and untreated WD patients and compared to age-matched healthy controls. We show that WD patients exhibit a Cu isotopic signature that depends on anthropological parameters in addition to the degree of disease progression, which was quantified by the level of liver fibrosis and the gradient of the phenotypic presentation. To get insights into the effects of the progress of the WD, we measured the Cu concentrations and isotope compositions in the liver, feces and serum of 12 and 45 week old *Atp7b*^{-/-} mice. The evolution of the $\delta^{65}\text{Cu}$ values is marked by a decrease in all tissues. The results show that ^{63}Cu dynamically accumulates in the liver due to the preferential cellular entry of reduced $^{63}\text{Cu}^+$ and the impairment of the preferential ^{63}Cu over ^{65}Cu exit by Cp. The hepatic accumulation of monovalent $^{63}\text{Cu}^+$ and the release of ^{63}Cu in the peripheral system as CuEXC, with an unknown redox state, are likely to fuel the production of free radicals, likely supporting WD pathogenesis. The present results obtained on WD patients and *Atp7b*^{-/-} mice already suggest that the blood $^{65}\text{Cu}/^{63}\text{Cu}$ ratio recapitulates the progress of the WD and is potentially a prognostic biomarker for WD.

Conflicts of interest

There are no conflicts of interest to declare.

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References

- 1 S. A. K. Wilson, Progressive lenticular degeneration, *Br. Med. J.*, 1912, 2(2710), 1645.
- 2 P. C. Bull, G. R. Thomas, J. M. Rommens, J. R. Forbes and D. W. Cox, The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene, *Nat. Genet.*, 1993, 5(4), 327–337.
- 3 M. C. Linder and M. Hazegh-Azam, Copper biochemistry and molecular biology, *Am. J. Clin. Nutr.*, 1996, 63(5), 797S–811S.
- 4 B. Zhou and J. Gitschier, hCTR1: A human gene for copper uptake identified by complementation in yeast, *Proc. Natl. Acad. Sci. U. S. A.*, 1997, 94(14), 7481–7486.

- 5 S. Lutsenko, N. L. Barnes, M. Y. Bartee and O. Y. Dmitriev, Function and Regulation of Human Copper-Transporting ATPases, *Physiol. Rev.*, 2007, **87**(3), 1011–1046.
- 6 P. Delangle and E. Mintz, Chelation therapy in Wilson's disease: from D-penicillamine to the design of selective bioinspired intracellular Cu(I) chelators, *Dalton Trans.*, 2012, **41**(21), 6359–6370.
- 7 A. Ala, A. P. Walker, K. Ashkan, J. S. Dooley and M. L. Schilsky, Wilson's disease, *Lancet*, 2007, **369**(9559), 397–408.
- 8 E. A. Roberts and M. L. Schilsky, Diagnosis and treatment of Wilson disease: an update, *Hepatology*, 2008, **47**(6), 2089–2111.
- 9 I. Sternlieb, The outlook for the diagnosis of Wilson's disease, *J. Hepatol.*, 1993, **17**(3), 263–264.
- 10 J. N. Cumings, The effects of B.A.L. in hepatolenticular degeneration, *Brain*, 1951, **74**(1), 10–22.
- 11 J. M. Walshe, Penicillamine, a new oral therapy for Wilson's disease, *Am. J. Med.*, 1956, **21**(4), 487–495.
- 12 J. M. Walshe, Management of penicillamine nephropathy in Wilson's disease: a new chelating agent, *Lancet*, 1969, **294**(7635), 1401–1402.
- 13 T. U. Hoogenraad, R. Koevoet and R. Korver EGWM de, Oral Zinc Sulphate as Long-Term Treatment in Wilson's Disease (Hepatolenticular Degeneration), *Eur. Neurol.*, 1979, **18**(3), 205–211.
- 14 G. J. Brewer, Zinc and tetrathiomolybdate for the treatment of Wilson's disease and the potential efficacy of anticopper therapy in a wide variety of diseases, *Metallomics*, 2009, **1**(3), 199.
- 15 O. Guillaud, A.-S. Brunet, I. Mallet, J. Dumortier, M. Pelosse and S. Heissat, *et al.*, Relative exchangeable copper: a valuable tool for the diagnosis of Wilson disease, *Liver Int.*, 2018, **38**(2), 350–357.
- 16 P. Ferenci, K. Caca, G. Loudianos, G. Mieli-Vergani, S. Tanner and I. Sternlieb, *et al.*, Diagnosis and phenotypic classification of Wilson disease1, *Liver Int.*, 2003, **23**(3), 139–142.
- 17 S. El Balkhi, J.-M. Trocello, J. Poupon, P. Chappuis, F. Massicot and N. Girardot-Tinant, *et al.*, Relative exchangeable copper: A new highly sensitive and highly specific biomarker for Wilson's disease diagnosis, *Clin. Chim. Acta*, 2011, **412**(23–24), 2254–2260.
- 18 M. Aramendía, L. Rello, M. Resano and F. Vanhaecke, Isotopic analysis of Cu in serum samples for diagnosis of Wilson's disease: a pilot study, *J. Anal. At. Spectrom.*, 2013, **28**(5), 675.
- 19 V. Balter, A. Lamboux, A. Zazzo, P. Télouk, Y. Leverrier and J. Marvel, *et al.*, Contrasting Cu, Fe, and Zn isotopic patterns in organs and body fluids of mice and sheep, with emphasis on cellular fractionation, *Metallomics*, 2013, **5**(11), 1470.
- 20 M. Costas-Rodríguez, S. V. Campenhout, A. A. M. B. Hastuti, L. Devisscher, H. V. Vlierberghe and F. Vanhaecke, Body distribution of stable copper isotopes during the progression of cholestatic liver disease induced by common bile duct ligation in mice, *Metallomics*, 2019, **11**(6), 1093–1103.
- 21 F. Albarède, P. Telouk, A. Lamboux, K. Jaouen and V. Balter, Isotopic evidence of unaccounted for Fe and Cu erythropoietic pathways, *Metallomics*, 2011, **3**(9), 926.
- 22 L. Van Heghe, O. Deltombe, J. Delanghe, H. Depypere and F. Vanhaecke, The influence of menstrual blood loss and age on the isotopic composition of Cu, Fe and Zn in human whole blood, *J. Anal. At. Spectrom.*, 2014, **29**(3), 478–482.
- 23 K. Jaouen and V. Balter, Menopause effect on blood Fe and Cu isotope compositions, *Am. J. Phys. Anthropol.*, 2014, **153**(2), 280–285.
- 24 V. Balter, A. Nogueira da Costa, V. P. Bondanese, K. Jaouen, A. Lamboux and S. Sangrajang, *et al.*, Natural variations of copper and sulfur stable isotopes in blood of hepatocellular carcinoma patients, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**(4), 982–985.
- 25 V. P. Bondanese, A. Lamboux, M. Simon, J. Lafont, E. Albalat, S. Pichat, J. M. Vanacker, P. Télouk, V. Balter, P. Oger and F. Albarède, Hypoxia induces copper stable isotope fractionation in hepatocellular carcinoma, in a HIF-independent manner, *Metallomics*, 2016, **8**, 1177–1184.
- 26 L. Lobo, M. Costas-Rodríguez, J. C. de Vicente, R. Pereiro, F. Vanhaecke and A. Sanz-Medel, Elemental and isotopic analysis of oral squamous cell carcinoma tissues using sector-field and multi-collector ICP-mass spectrometry, *Talanta*, 2017, **165**, 92–97.
- 27 M. Costas-Rodríguez, Y. Anoshkina, S. Lauwens, H. V. Vlierberghe, J. Delanghe and F. Vanhaecke, Isotopic analysis of Cu in blood serum by multi-collector ICP-mass spectrometry: a new approach for the diagnosis and prognosis of liver cirrhosis?, *Metallomics*, 2015, **7**(3), 491–498.
- 28 K. A. Miller, C. M. Keenan, G. R. Martin, F. R. Jirik, K. A. Sharkey and M. E. Wieser, The expression levels of cellular prion protein affect copper isotopic shifts in the organs of mice, *J. Anal. At. Spectrom.*, 2016, **31**(10), 2015–2022.
- 29 T. G. Enge, H. Ecroyd, D. F. Jolley, J. J. Yerbury and A. Dosseto, Longitudinal assessment of metal concentrations and copper isotope ratios in the G93A SOD1 mouse model of amyotrophic lateral sclerosis, *Metallomics*, 2017, **9**(2), 161–174.
- 30 F. Moynier, J. Creech, J. Dallas and M. L. Borgne, Serum and brain natural copper stable isotopes in a mouse model of Alzheimer's disease, *Sci. Rep.*, 2019, **9**(1), 1–7.
- 31 L. Sauzéat, E. Bernard, A. Perret-Liaudet, I. Quadrio, A. Vighetto and P. Krolak-Salmon, *et al.*, Isotopic Evidence for Disrupted Copper Metabolism in Amyotrophic Lateral Sclerosis, *iScience*, 2018, **6**, 264–271.
- 32 O. I. Buiakova, J. Xu, S. Lutsenko, S. Zeitlin, K. Das and S. Das, *et al.*, Null Mutation of the Murine ATP7B (Wilson Disease) Gene Results in Intracellular Copper Accumulation and Late-Onset Hepatic Nodular Transformation, *Hum. Mol. Genet.*, 1999, **8**(9), 1665–1671.
- 33 C. N. Maréchal, P. Télouk and F. Albarède, Precise analysis of copper and zinc isotopic compositions by plasma-source mass spectrometry, *Chem. Geol.*, 1999, **156**(1–4), 251–273.
- 34 F. Albarède, P. Telouk, J. Blichert-Toft, M. Boyet, A. Agranier and B. Nelson, Precise and accurate isotopic measurements using multiple-collector ICPMS, *Geochim. Cosmochim. Acta*, 2004, **68**(12), 2725–2744.
- 35 K. Jaouen, V. Balter, E. Herrscher, A. Lamboux, P. Telouk and F. Albarède, Fe and Cu stable isotopes in archeological

- human bones and their relationship to sex, *Am. J. Phys. Anthropol.*, 2012, **148**(3), 334–340.
- 36 D. Huster, Structural and metabolic changes in Atp7b^{-/-} mouse liver and potential for new interventions in Wilson's disease, *Ann. N. Y. Acad. Sci.*, 2014, **1315**(1), 37–44.
- 37 F. Albarède, P. Télouk and V. Balter, Medical Applications of Isotope Metallomics, *Rev. Mineral. Geochem.*, 2017, **82**(1), 851–885.
- 38 F. Albarède, P. Télouk, V. Balter, V. P. Bondanese, E. Albalat, P. Oger, P. Bonaventura, P. Miossec and T. Fujii, Medical applications of the Cu, Zn and S isotope effects, *Metallomics*, 2016, **8**, 1056–1070.
- 39 A. Tennant, A. Rauk and M. E. Wieser, Computational modelling of the redistribution of copper isotopes by proteins in the liver, *Metallomics*, 2017, **9**(12), 1809–1819.
- 40 M. Lacombe, M. Jaquinod, L. Belmudes, Y. Couté, C. Ramus and F. Combes, *et al.*, Comprehensive and comparative exploration of the Atp7b^{-/-} mouse plasma proteome, *Metallomics*, 2020, **12**(2), 249–258.
- 41 S. Heissat, A. Harel, K. Um, A.-S. Brunet, V. Hervieu and O. Guillaud, *et al.*, Evaluation of the accuracy of exchangeable copper and relative exchangeable copper (REC) in a mouse model of Wilson's disease, *J. Trace Elem. Med. Biol.*, 2018, **50**, 652–657.
- 42 S. Lauwens, M. Costas-Rodríguez, J. Delanghe, H. Van Vlierberghe and F. Vanhaecke, Quantification and isotopic analysis of bulk and of exchangeable and ultrafiltrable serum copper in healthy and alcoholic cirrhosis subjects, *Talanta*, 2018, **189**, 332–338.