Evaluation of the accuracy of exchangeable copper and relative exchangeable copper (REC) in a mouse model of Wilson’s disease.

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EVALUATION OF THE ACCURACY OF EXCHANGEABLE COPPER AND RELATIVE EXCHANGEABLE COPPER (REC) IN A MOUSE MODEL OF WILSON’S DISEASE.

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ABSTRACT

Wilson's disease (WD) is caused by mutations in the ATP7B gene responsible for a toxic copper overload mainly in the liver and the central nervous system. Phenotypic heterogeneity may challenge the diagnostic confirmation. Exchangeable copper (CuEXC) has recently been proposed as a new marker of WD, and its ratio to the total serum copper (Cus), Relative Exchangeable Copper (REC=CuEXC/Cus), as a diagnostic marker. This study aimed to investigate whether this could be confirmed in Atp7b⁻/⁻ mice, an engineered WD animal model. Atp7b⁻/⁻ (n=137) and wild type (WT; n=101) mice were investigated under the same conditions at 6-8, 20, 39, or 50 weeks of age. Twenty-four Atp7b⁻/⁻ mice received D-penicillamine treatment from 39 to 50 weeks of age. Serum and liver [histology and intrahepatic copper (IHCu)] data were evaluated. In the WT group, all serum and liver data were normal. Atp7b⁻/⁻ livers developed a chronic injury from isolated moderate inflammation (6-8 weeks: 16/33=48%) to inflammatory fibrosis with cirrhosis (50 weeks: 25/25=100% and 16/25=64% respectively). Cus and CuEXC increased until week 39, whereas IHCu and REC were stable with increasing age and much higher than in WT mice (mean±SD: 669±269 vs. 13±3 μg/g dry liver and 39±12 vs. 11±3%, respectively). A threshold value of 20% for REC provided a diagnostic sensitivity and specificity of 100%, regardless of sex, age, or the use of D-penicillamine. Eleven weeks of 100 mg/kg D-penicillamine reduced liver fibrosis (p=0.001), IHCu (p=0.026) and CuEXC (p=0.175). In conclusion, this study confirms REC as a WD diagnostic marker in a mouse model of chronic liver disease caused by copper overload. Further studies are needed to assess the usefulness of CuEXC to monitor the evolution of WD, particularly during treatment.
List of abbreviations:

WD: Wilson’s disease; IHCu: Intrahepatic copper; CuEXC: exchangeable copper; REC: relative exchangeable copper; Cus: total serum copper; LEC: Long-Evans Cinnamon; WT: wild type; ALT: serum alanine aminotransferase; AST: serum aspartate aminotransferase; LOD: lower limit of detection; SD: standard deviation; QC: quality control.

Keywords: Exchangeable copper, REC, Atp7b−/− mice, diagnostic test, cirrhosis

Introduction

Wilson’s disease (WD) is an autosomal recessive inherited disorder of copper metabolism due to loss-of-function mutations in the gene encoding the ATP7B protein, a Cu-transporting ATPase [1, 2]. In humans, mutations in ATP7B lead to decreased biliary copper excretion and reduced incorporation into ceruloplasmin. Consequently, copper accumulates in hepatocytes and the brain, resulting in severe hepatic and/or neurological impairment [3]. The diagnosis of WD is challenging since delay or failure to diagnose can lead to chronic copper accumulation and irreversible damage [4-6]. Currently WD diagnosis is based on a combination of clinical (hepatic and neurological signs, Kayser-Fleischer ring), biological (low ceruloplasmin, low total serum copper, high urinary copper excretion), and radiological findings (brain MRI) [5]. Clinical and biological phenotype may be heterogeneous in WD patients. Intrahepatic copper (IHCu) measurement can help diagnose WD (≥ 250 µg/g dry weight), but liver biopsy is an invasive approach. In addition, increased IHCu (≥ 250 µg/g dry weight) can also be found in
other cholestatic diseases [5, 7]. Molecular genetic tests can confirm the diagnosis, but they are not available in all countries [8]. Thus, a rapid and reliable diagnostic marker is needed.

In a seminal study El Balkhi et al. proposed exchangeable copper (CuEXC) and relative exchangeable copper (REC) as a new biological test for WD diagnosis [9]. CuEXC corresponds to the labile fraction of serum copper that is not complexed to ceruloplasmin. It is thought to reflect blood and tissue copper and to increase in case of overload. REC is the ratio of the labile fraction to the total serum copper (Cus), REC = CuEXC/Cus. In a patient with copper overload, REC >18% has a specificity and sensitivity of 100% for the diagnosis of WD [9-11]. Recently, REC and CuEXC have been evaluated in Long-Evans Cinnamon (LEC) rats, another animal model of WD [12]. REC appeared as a reliable WD diagnostic marker and high CuEXC levels correlated with acute liver failure. Thus, the authors have suggested that CuEXC could be used as a biomarker of disease progression. However, these data were obtained in only one animal model that mostly presents acute hepatitis [13, 14] and the capacity to follow disease progression under copper chelating treatment has not been investigated.

The aim of the present study was therefore to evaluate the usefulness and reliability of REC for diagnosis and CuEXC as a marker of disease course and treatment in a mouse model of WD. We used Atp7b−/− mice from a genetically engineered rodent strain that was generated in 1999 [15-17]. As seen in human WD, these mice have hepatic intracellular copper accumulation, low serum ceruloplasmin activity, increased copper excretion in urine and chronic histological liver features of WD [18, 19].

**Material and methods**
Mice, animal care, and experimental procedure

All mice were housed under the same conditions at the animal facilities of the French atomic energy commission (Commissariat à l’énergie atomique, CEA) in Grenoble (France) that has accreditation from the French authorities under the reference C38-18510001. Animal breeding, housing, and experiments were performed in strict accordance with the French animal testing regulation. The Atp7b<sup>−/−</sup> mice have been described earlier [15-17]; they were kindly given to us by Prof. Svetlana Lutsenko from John Hopkins University. Wild type (WT) specific-pathogen-free C57Bl/6 mice (Charles River Laboratories, Saint Germain Nuelles, France) were used as controls. The mice were kept on a 12h/12h light/dark cycle and fed with maintenance dry food #3469 (Kliba Nafag CH), containing 14 mg/kg copper, 60 mg/kg zinc and 250 mg/kg iron. Food and water were provided ad libitum.

Altogether, 137 Atp7b<sup>−/−</sup> (group Atp7b<sup>−/−</sup>) and 101 C57Bl/6 (group WT) mice were included in the study and separated into 4 groups according to age (6-8, 20, 39, and 50 weeks), chosen to illustrate different stages of WD [18]. To provide insights into the effect of a Cu-chelator, 24 Atp7b<sup>−/−</sup> mice received D-penicillamine, from 39 to 50 weeks of age. D-penicillamine was administered in the morning through a gastric tube, 5 days per week at a dose of 100 mg/kg body weight; a protocol that was approved by the French Research Ministry under the reference #2015062510212698_v1(APAFIS#926).

Animals were euthanized by CO<sub>2</sub> inhalation at the indicated ages. Blood was immediately collected by cardiac exsanguination puncture either in sodium heparin tubes for enzyme measurements or in trace-element dedicated tubes (Becton Dickinson Vacutainer, #369032, Le Pont-de-Claix, France) for copper measurements. The serum was collected after coagulation and a 10-min centrifugation at 3000 rpm; it was kept at room temperature for further enzyme analysis. For copper evaluations, in each subgroup, total blood from different mice of the same sex were pooled up to ~4 mL, the volume required to perform all the copper
measurements. The serum was then prepared and frozen at -80°C for 24-48h until sent for analysis in the Trace Element Laboratory of the Edouard Herriot Hospital, Lyon. Livers were removed rapidly after exsanguination and separated into 2 parts: the left lobe was fixed in formalin for histological analyses and the other lobes immediately frozen in liquid nitrogen and kept at -80°C.

**Analysis of aminotransferase activity and bilirubin**

Serum total bilirubin, serum alanine (ALT), and aspartate (AST) aminotransferase activities were determined by automated measurement (Vista 1500, Siemens Health Care Diagnostics Inc., Newark, Delaware, US) performed routinely at the biochemistry laboratory of the Edouard Herriot Hospital. As these hepatic functions are considered not to vary over time in WT mice [20], serum ALT, AST, and bilirubin were assessed only at 20 weeks of age, whereas they were evaluated for each Atp7b−/− subgroup.

**Copper measurements in blood and liver**

Total serum copper and intrahepatic copper were measured using an inductively coupled plasma optical emission spectrometer (ICP-OES Vistapro, Agilent Technologies, Santa Clara, California, US). For intrahepatic copper measurements, liver samples were dried overnight at 40°C then 3 hours at 105°C, followed by an acid digestion (HNO₃ at 60°C overnight); results were expressed as µg of copper per gram of dry tissue. CuEXC measurement was performed by Zeeman electrothermal atomic absorption spectrometry (ETAAS, ZAA220, Agilent Technologies, Santa Clara, California, US) according to a previously reported technique [21]. The lower limit of detection (LOD) was obtained over 30 determinations of the blank (NaCl 0.9%) and calculated as threefold the standard deviation (SD). The LOD was defined as 0.003 µmol/L. The precision (coefficient of
variation, CV) was determined for the two quality control (QC) levels, Normal range and High range Trace Element Serum Control (UTAK Laboratories Inc., Valencia, California, US). For the intraday assay, 30 replicates of each QC level were processed the same day. Intraday precision ranged from 3.2 % to 5.1% for 21.7 µmol/L and 40 µmol/L of Cu in NaCl 0.9% respectively. REC was calculated by the ratio of CuEXC over total serum copper.

**Histological analysis**

Tissue sections were prepared by fixing livers in 10% buffered formalin at pH 7.4 for 3 days, transferring to 80% ethanol, and then embedding in paraffin. Each section was stained with hematoxylin eosin staining and fibrosis was investigated by Picro-Sirius red staining. Histological analysis investigated and quantified fibrosis, inflammation, activity, cellular atypia, steatosis, nodules, and biliary abnormalities. Livers that were not analyzable (e.g. for reasons of poor fixing) were excluded; livers were analyzed at a 1:2 WT to Atp7b<sup>−/−</sup> ratio.

**Statistical analysis**

Quantitative variables were expressed as means (±SD) and qualitative variables as frequencies or percentages. The main characteristics were compared between groups by using the Mann-Whitney test for continuous variables and the Chi-2 test for qualitative variables, with statistical significance set at a \( p \) value <0.05. Statistical analysis was performed with Sigma Plot 13 (Systat Software Inc.).

**Results**

**Natural history and liver histology**
A total of 101 WT and 137 Atp7b−/− mice were included (Table 1); histological analysis was performed on a total of 192 livers from 64 WT and 128 Atp7b−/− mice, including 24 Atp7b−/− treated mice. No abnormality was noted in the WT group. Histological analysis of Atp7b−/− mice showed the progressive development of chronic liver injury with significant lesions, homogeneously distributed across each age group. At 6 weeks, no obvious macroscopic abnormality in color or shape was detected. Microscopic changes were absent or mild with a moderate inflammation (16/33=48%) and no significant fibrosis (Figure 1A and B). Over time, significant injuries appeared. At 50 weeks, lesions were extensive with constant intense inflammation, cellular atypia (e.g. swollen hepatocytes, nuclear enlargement, and atypical nucleoli; Figure 1C) and an extensive perisinusoidal fibrosis (Figure 1D). Nodules were noted in 16/25=65%. The use of copper chelator treatment significantly decreased injury compared to untreated mice at the same age (fibrosis: 66% vs. 100%; p<0.001). No malignant feature, dysplastic change, or significant biliary lesions were noted in any groups.

**Hepatic functions**

Hepatic functions were assessed at 20 weeks in WT mice (mean±SD, n=5: ALT 23±10 UI/L; AST 108±45 UI/L, and bilirubin <2 µmol/L), and were in agreement with previous data from C57Bl/6 mice [20]. Serum ALT and AST regularly increased over time in the Atp7b−/− mice (n=5: from ALT 29±9 and AST 115±35 UI/L at 6-8 weeks to ALT 250±175 and AST 376±139 UI/L at 50 weeks, respectively). At 50 weeks, the mean levels of both aminotransferase activities were lowered by D-penicillamine (n=4: ALT 119±101 and AST 276±142) compared to those of untreated Atp7b−/− mice of the same age but the data scattering prevented any statistical significance. Bilirubin level increased in Atp7b−/− mice without reaching pathological values (<2 µmol/L at 6-8 weeks vs. 14.4±6.5 µmol/L at 50 weeks and 5.5±3.7 at 50 weeks + treatment).
Copper related markers

In WT mice, the mean ± SD Cus was 8.4±0.7 µmol/L at 6-8 weeks and 10.2±0.5 µmol/L at 50 weeks, IHCu concentrations was 14±4 µg/g dry liver at 6-8 weeks and 14±2 µg/g dry liver at 50 weeks, and CuEXC was 1.3±0.1 µmol/L at 6-8 weeks and 1.1±0.1 µmol/L at 50 weeks (Table 2). In Atp7b−/− mice, the mean ± SD Cus increased over 20 weeks and remained stable thereafter, IHCu was stable over time and CuEXC increased over 39 weeks and remained stable thereafter (Table 2 and Figure 2). In young mice (6-8 weeks), Cus was higher for the WT group than for the Atp7b+/− group (8.4±0.7 vs. 4.3±1.7 µmol/L, p<0.01), there was no difference at 20 weeks and Cus was significantly higher in Atp7b−/− mice after 39 weeks (Table 2). The IHCu concentration was stable over time in Atp7b−/− (Figure 2B) and always higher than in WT livers (Table 2). In young mice (6-8 weeks), CuEXC levels were similar in WT and Atp7b−/− mice and significantly higher in Atp7b−/− after 20 weeks (Table 2). Treatment of Atp7b+/− mice with D-penicillamine led to significantly reduced mean±SD Cus concentration (9.6±2 vs. 13.3±1.2 µmol/L, p<0.01) and IHCu concentration (486±263 vs. 797±402 µg/g dry liver, p=0.026) and led to a non-significant trend towards decreased CuEXC (3.2±1.1 µmol/L vs. 4.6±2 µmol/L; p=0.07) at 50 weeks of age (Table 2). In the total population, the REC was significantly higher in Atp7b−/− compared to WT mice (39±12 vs. 11±3%, p<0.01; Figure 3A). The sex of animals (Figure 3B), the use of D-penicillamin (Figure 3C) and age (Figure 3D, Table 2), did not modify the REC value in Atp7b−/− mice. A cut-off value set at 20% conferred a 100% sensitivity and specificity, irrespective of age, sex, or use of treatment.

Discussion and conclusion
Since the discovery and cloning of the human \textit{ATP7B} gene in 1993 [1, 2], our knowledge of the genetic basis of WD has increased dramatically. However, the pathogenesis has not been fully deciphered and an explanation for the phenotypic variability is still missing. Animal models of copper overload are therefore essential to understand the disease pathogenesis and improve the diagnosis and treatment of WD patients. Several rodent models have been described, for example, LEC rats and toxic milk mice are products of a spontaneous mutation in the \textit{Atp7b} gene that occurred during breeding [14, 15, 22]. However, the \textit{Atp7b} mouse, generated by Buiakova \textit{et al.} [16, 17] is the only genetically engineered rodent strain that can serve as a model for the hepatic forms of WD. The insertion of multiple stop codons in exon 2 leads to truncated mRNA that are present in the tissues although the \textit{Atp7b} protein is not produced [16].

Although copper accumulation can be detected in other organs, the highest copper concentration and the most striking phenotype is observed in the livers of \textit{Atp7b} mice [17]. \textit{Atp7b} mice are born with a copper deficiency but soon after copper accumulates in their liver and increases to peak levels between 6 and 20 weeks of age [16, 18]. In the present study, intrahepatic copper concentration was stable from 6 to 50 weeks of age and around 50-fold the concentration of that found in WT mice. This copper overload led to hepatic tissue damage but there was no direct correlation between the IHCu concentration and the severity of the histological lesions as previously reported [23]. One interpretation of these findings is that copper overload precedes tissue damage and that hepatic lesions occur not only because of copper toxicity \textit{per se} but also through several mechanisms dealing with lipid metabolism, mitochondrial injury, and hepatic nuclear receptor dysfunction [23-25]. According to the literature, no acute liver failure was observed in \textit{Atp7b} mice; rather, they developed chronic hepatitis [17]. Herein, numerous and systematic histological analyses of \textit{Atp7b} livers showed a progressive and homogenous development of chronic liver injury. At early stages of
copper accumulation (6 weeks), no macroscopic morphological changes were observed in the liver and microscopic changes were moderate. Over time, serious injuries appeared leading to severe chronic liver disease in all mice at 50 weeks. At this stage, nodules were observed in two-thirds of the animals. This nodular evolution may be related to regenerative nodes, similar to those observed in human cirrhosis, or restoration of normal liver architecture [17, 23]. Unlike previous descriptions [18], we did not observe neoplastic biliary proliferation in older mice. The Golgi membrane protein GP73 has been recently identified as a potential trigger for fibrosis and dysplasia in $Atp7b^{-/-}$ mice [26]. Further studies are needed to focus on this protein or other neoplastic processes. Some genetic drift may also have occurred since the creation of the original $Atp7b^{-/-}$ mice in 1999, leading to different phenotypes. For instance, $Atp7b^{+/+}$ mice born during the time of this study were nursed by their mother and none of them developed the neurological symptoms described earlier [16]. Despite differences with previously published data, we observed very homogenous histological lesions in each $Atp7b^{-/-}$ subgroup leading to the hypothesis of a progressive worsening of hepatic lesion over time in $Atp7b^{+/+}$ mice. Herein, we confirm that the $Atp7b^{-/-}$ mouse model is a valuable model for chronic liver disease in WD and may be used to test new diagnostic tools or treatments.

CuEXC and REC have recently been described as new biomarkers for WD diagnosis in humans [9]. In a previous report, El Balkhi et al. [21] described the ultrafiltration procedure and the instrumental determination of ultrafiltrable copper and CuEXC, and established reference values in healthy subjects. CuEXC and REC have appeared as highly sensitive and reliable tests for discriminating adults with WD from healthy subjects and also from heterozygous $ATP7B$ mutation carriers [9, 10]. The practicality of CuEXC determination may currently be a major limit of this new test. However, REC seems to represent a new specific, sensitive, and non-invasive biomarker for WD. Recently Guillaud et al. [11] confirmed these data in humans, especially in the pediatric population and in adults or children with non-WD
hepatic diseases. In this study a cut off value of 18.5% for the REC allowed to discriminate 49 WD patients from non-WD hepatic disease patients (103 adults and 49 children). Moreover, mean REC level was lower in cholestatic patients, in whom diagnosis is occasionally a problem, as increased IHCu may be observed. Further studies dealing with the measurement of the REC in other liver diseases would also be of great interest for humans and animals [27]. Recently, Schmitt et al. [12] have evaluated CuEXC and REC in LEC rats, an animal model of WD that mostly presents acute liver failure [13, 14]. The authors found that a cut off value for the REC set at 19% allowed to discriminate 24 LEC rats from 6 Long-Evans WT rats, with a sensitivity of 97.3% and a specificity of 100% [12]. In the present study, Atp7b−/− mice allowed us to include a greater number of cases but also to evaluate the evolution of copper parameters during chronic hepatitis as these do not present acute liver failure [17]. Whereas recurrent episodes of acute liver failure can be observed over the course of a LEC rat’s life, no mouse from the present study had such symptoms. Histological analysis found a progressive worsening of liver disease leading to fibrosis in all 50-week-old mice. We confirmed REC as a discriminative tool for the diagnosis of WD in mice; REC was significantly higher in Atp7b−/− mice than in WT. A cut off set at 20% gave sensitivity and specificity of REC of 100% in Atp7b−/− mice irrespective of sex, age, stage of the disease, or the use of a treatment.

Exchangeable copper (CuEXC) corresponds to the labile fraction of copper in the serum, not complexed to ceruloplasmin but complexed to albumin and other peptides [9, 21]. An increase of CuEXC is thought to reflect a blood and tissue copper overload. If CuEXC (and its derived calculated REC) appears as an important new diagnostic biomarker in WD, it seems also that it could be used in monitoring the disease course and treatment efficacy. Recently, a correlation was reported in LEC rats between CuEXC values and the severity of acute liver failure [12]; CuEXC was always lower in an animal receiving a low-copper diet. Herein, no acute liver failure was observed but CuEXC tended to increase over time, in
parallel with hepatic lesions. The sensitivity of LEC rats to dietary copper has been demonstrated [28]. The variation of CuEXC level according to the copper intake in LEC rats is consistent with the ability of CuEXC to reflect the copper overload [12]. Recently Guillaud et al. [11] showed a variability of CuEXC in human according to the observance of treatment in WD patients with significant higher CuEXC in non-compliant patients. In the present study, the use of D-Penicillamine reduced intrahepatic copper and significantly improved histological lesions. We observed a trend towards lower CuEXC in treated mice without reaching statistical difference. This result may be due to the reduced number of CuEXC values, but also to the small size of mice that does not allow measurement of CuEXC without sacrificing the animal. Moreover, we could not monitor the evolution of urinary copper concentration in treated mice to evaluate the efficacy of copper chelators for technical reasons. Additional studies are needed to assess the use of CuEXC as a monitoring marker of the disease course and of decoppering treatment efficacy in WD. Such studies should be performed on bigger mammals, if not on humans, to follow the disease course and its treatment on the same individual.

In conclusion, this study confirms REC as a discriminative tool for the diagnosis of WD in a mouse model of chronic liver disease caused by copper overload. Further studies are needed to assess the usefulness of CuEXC to monitor the evolution of WD, particularly during treatment.

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**References**


Illustrations

Table 1: *Atp7b*−/− and WT mice groups according to age and sex.

<table>
<thead>
<tr>
<th>Ages</th>
<th>Group <em>Atp7b</em>−/−</th>
<th>Group WT (C57Bl/6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>male</td>
<td>female</td>
</tr>
<tr>
<td>6-8 weeks</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>20 weeks</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>39 weeks</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>50 weeks*</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>50 weeks + treatment</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>All ages</td>
<td>72</td>
<td>65</td>
</tr>
</tbody>
</table>

* five mice who were to be sacrificed at 50 weeks (without treatment) died before this time point
Table 2 - Copper data from liver and serum

<table>
<thead>
<tr>
<th>Weeks</th>
<th>IHCu(^a), µg/g dry liver</th>
<th>Cus(^b), µmol/L</th>
<th>CuEXC(^b), µmol/L</th>
<th>REC(^b), %</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Atp7b(^{-/-})</td>
<td></td>
<td>WT</td>
<td>Atp7b(^{-/-})</td>
</tr>
<tr>
<td>6-8</td>
<td>14 (±4, 5)</td>
<td>617 (± 220, 16)</td>
<td>**</td>
<td>8.4 (±0.7)</td>
<td>4.3 (±1.7)</td>
</tr>
<tr>
<td>20</td>
<td>14 (±2, 5)</td>
<td>779 (±143, 9)</td>
<td>**</td>
<td>11.0 (±0.9)</td>
<td>10.1 (±1.1)</td>
</tr>
<tr>
<td>39</td>
<td>9 (±1, 5)</td>
<td>605 (±172, 16)</td>
<td>**</td>
<td>7.7 (±0.2)</td>
<td>13.5 (±4.8)</td>
</tr>
<tr>
<td>50</td>
<td>14 (±2, 5)</td>
<td>797 (±402, 9)</td>
<td>**</td>
<td>10.2 (±0.5)</td>
<td>13.3 (±1.2)</td>
</tr>
<tr>
<td>50 + treatment</td>
<td>486 (±263, 11)</td>
<td>9.6 (±2)</td>
<td>**</td>
<td>3.2 (±1.1)</td>
<td>Ns</td>
</tr>
</tbody>
</table>

\(^a\)Mean values (±standard deviation, n). \(^b\)Mean values (±standard deviation) n=5 for all serum data. * 0.01<p<0.05, ** p<0.01 for comparison WT vs Atp7b\(^{-/-}\) at each age and with vs without treatment for the 50-week-old Atp7b\(^{-/-}\) mice, ns p≥0.05

IHCu: Intrahepatic copper; Cus: total serum copper; CuEXC: exchangeable copper; REC: relative exchangeable copper.
Title and legend for figures:

**Figure 1:** Microscopic features illustrating the development of chronic liver injury over time in the *Atp7b<sup>−/−</sup>* mice.

A: no significant changes at 6 weeks (HES stain x200); B: no fibrosis at 6 weeks (Picro-Sirius red stain, x200); C: major inflammation with lobular activity and important cellular atypia at 50 weeks (HES stain x200); D: extensive perisinusoidal fibrosis of the liver at 50 weeks (Picro-Sirius red stain, x200).

**Figure 2:** Evolution of copper related markers *Atp7b<sup>−/−</sup>* mice.

Total serum copper (Cus); intra hepatic copper (IHCu); and exchangeable copper (CuEXC) are represented in light gray for *Atp7b<sup>−/−</sup>* mice at different ages and in dark gray for *Atp7b<sup>−/−</sup>* mice treated with D-Penicillamin. Each box is delimited by the 25 and 75 percentiles and shows the median value of the data. The whiskers show the min and max values of the data. Note the different scales. Non significant (ns): \( p \geq 0.05 \), ** \( p < 0.01 \). Cus increased over 20 weeks and remained stable thereafter, IHCu was stable over time and CuEXC increased over 40 weeks and remained stable thereafter. D-Penicillamin treatment reduced Cus (\( p < 0.026 \)), IHCu (\( p < 0.01 \)), and CuEXC (\( p = 0.07 \)).

**Figure 3:** Evolution of REC.

Relative exchangeable copper (REC) values are represented in % and given in terms of mean values and standard deviation. REC is significantly higher in total *Atp7b<sup>−/−</sup>* vs wild type (WT) mice (\( p < 0.01 \)). The sex of animals, the use of D-penicillamin treatment or the age of the
animal, and so the stage of the disease, did not modify the REC value in Atp7b\textsuperscript{−/−} mice (ns \( p \geq 0.05 \)).