Metallomics

PAPER

View Article Online
View Journal

Hepatic metabolic response to restricted copper intake in a Niemann–Pick C murine model†

Cite this: DOI: 10.1039/c4mt00056k

Graciela Argüello,[ab] Pablo Martinez,[c] Juan Peña,[a] Oscar Chen,[d] Frances Platt,[d] Silvana Zanlungo[bc] and Mauricio González*[ab]

Niemann–Pick C disease (NPC) is a vesicular trafficking disorder primarily caused by mutations in the *Npc1* gene and characterized by liver dysfunction and neuropathology. Altered hepatic copper metabolism has recently been reported in NPC disease. Therefore, we aimed to analyze the effects of a copper deficient diet and copper chelation using D-penicillamine on copper homeostasis in the liver of *Npc1*$^{-/-}$ mice of different ages. We examined liver metal ion content by AAS, and copper and iron metabolism gene expression in the liver using qPCR in *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. We found higher copper and lower iron content in the liver of *Npc1*$^{-/-}$ mice of different ages, compared to controls; these changes in copper and iron content were correlated with increased *ceruloplasmin*, *metallothionein 1*, and *transferrin receptor* gene expression and decreased gene expression of *Commd1*, *ferritin-light chain* and *ferroportin* in the liver of *Npc1*$^{-/-}$ mice of different ages. *Npc1*$^{-/-}$ mice responded to a copper-deficient diet with a decrease in copper content in the liver, bile and heart. These results correlated with a reduction in the hepatic expression of *ceruloplasmin* and *metallothionein 1* during the first week of treatment. D-penicillamine revealed hepatic adaptive response and an improvement in hepatic function in *Npc1*$^{-/-}$ mice without any effect on neurological functions. Our results confirm that the NPC1 protein is required for copper and iron homeostasis. To our knowledge, this is the first report documenting the hepatic adaptive response to low-copper intake in a *Npc1*$^{-/-}$ mouse model.

Received 25th February 2014,
Accepted 21st May 2014

DOI: 10.1039/c4mt00056k

www.rsc.org/metallomics

Introduction

Niemann–Pick type C (NPC) disease is a rare lysosomal disorder caused by the genetic loss of NPC1 or NPC2 function, characterized by liver dysfunction and progressive neurodegeneration.[1,2] Approximately 95% percent of NPC patients have mutations in the *Npc1* gene, and 5% have mutations in the *Npc2* gene.[3] At the cellular level, mutations in the *Npc1* gene result in multiple vesicular trafficking defects and accumulation of lysosomal material, mainly cholesterol, that are potentially deleterious to health.[4,5] Thus, a deficiency in this protein alters intracellular lipid homeostasis, membrane properties and intracellular trafficking of organelles.[6] Although neurodegeneration is a major feature of NPC, many patients present neonatally with acute liver disease that can be fatal, but if they survive the disease spontaneously resolves.[1,7]

Iron and copper are essential dietary components required to meet the demands from cell growth, differentiation, and optimal homeostasis.[8,9] However, both are redox active metals and through the Fenton reaction, both cuprous and ferrous ions transform the weak oxidant hydrogen peroxide into hydroxyl radicals, one of the most reactive species in nature.[10,11] Iron and copper depend, at least in part, on vesicular trafficking for their cellular uptake[12] and efflux,[13] respectively. In hepatocytes, the principal uptake pathway of iron bound to transferrin (TF) is through endocytosis mediated by the transferrin receptor (TFR).[12] Once the iron–TF/TFR complex is internalized *via* endocytosis, it is delivered to the early/sorting endosome through vesicular trafficking.[14] Interestingly, inefficient recycling of TFR has been reported in NPC cells.[15] After release from TF, iron is transported across endosomal membranes into the transit pool within the cytosol, where it regulates ferritin (*Ft*) translation.[16] Previous studies in liver tissues from NPC patients described FTH and FTL scarcity and suggested that injuries in NPC1 block the intracellular utilization of not only cholesterol but also iron, impairing the synthesis of cytosolic FT.[17] Also, recently Hung *et al.* reported altered iron and copper homeostasis in a murine model and in NPC patients[18] and

[a] *INTA, Laboratorio de Bioinformática y Expresión Génica, Universidad de Chile, El Libano 5524, Macul, Santiago, Chile. E-mail: mgonzale@inta.uchile.cl; Fax: +56 2 2214030; Tel: +56 2 9781440*
[b] *FONDAP-Center of Genome Regulation (CGR), Santiago, Chile*
[c] *Departamento de Gastroenterología, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago, Chile*
[d] *Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, UK*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4mt00056k

another study described serum free copper with low ceruloplasmin (CP) in a NPC patient.[19] Therefore, abnormal iron and copper metabolism may be components of the pathogenic cascade in NPC disease. At the cellular level, once copper is in the cytoplasm of hepatocytes, it can be transferred to ATP7B through copper chaperones such as ATOX1.[20,21] The primary role of ATP7B is to transport copper from the cytosol into the secretory compartment of the cell, where copper can be incorporated into newly synthesized cuproproteins or distributed through vesicles to the lysosomal pathway, or to the plasma membrane for copper export, while the ATP7B protein itself is recycled *via* the trans-Golgi network (TGN).[13,22,23] When cellular copper increases, ATP7B undergoes intracellular redistribution and promotes excretion of excess copper into bile.[13,24,25] Recently, reports indicated that copper excretion may be impaired in NPC hepatic cells due to the disruption of the late endosome to TGN transport[26] and that liver copper content increases in *in vivo* models of NPC disease.[27]

Here, we investigated the role of NPC1 in iron and copper metabolism by analyzing copper/iron liver storage, copper excretion and copper/iron metabolism-related gene expression. We compared *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice at 4, 7, and 8 weeks of age; this age range of mice represents an early to intermediate time period before the premature death of *Npc1*$^{-/-}$ mice on the BALB/c background that typically occurs at the age of 10–12 weeks.[28,29] We found higher copper and lower iron content in the liver of *Npc1*$^{-/-}$ mice with respect to controls (*Npc1*$^{+/+}$) at all ages; copper and iron content correlated with changes in copper and iron gene expression pattern in the liver of four- and seven-week-old *Npc1*$^{-/-}$ mice. Considering that excess liver copper has recently been reported in a NPC mouse model and associated with hepatic damage,[27,30] we analyzed the effects of a copper-deficient diet and the effects of the copper chelator D-penicillamine (DPA) on copper homeostasis in *Npc1*$^{-/-}$ mice at different ages. We examined the copper content of the liver, bile, plasma and heart by AAS, and copper metabolism gene expression in the liver by qPCR. *Npc1*$^{-/-}$ mice responded to a copper-deficient diet with a decrease in copper content in the liver, bile and heart. These results were correlated with a reduction in the hepatic expression of *Cp* and *metallothionein 1* (*Mt1*) in the first week of treatment. DPA reduced the transaminase levels in eight-week-old *Npc1*$^{-/-}$ mice. Our results suggest that NPC1 function is relevant for copper and iron homeostasis. To our knowledge, this is the first report documenting the hepatic adaptive response to low copper intake in a NPC mouse model.

Materials and methods

Animals

Niemann–Pick type C1 mice (BALB/c Nctr-Npc1^{m1N}/J, *Npc1*$^{-/-}$ mice) carrying a mutation in the *Npc1* gene[31] were from an established colony. Genotypes were identified using PCR-based screening, as described previously.[32] Immediately after weaning, Balb/c male mice were fed *ad libitum* for 4 weeks with a copper adequate (Cu-A) or copper-deficient (Cu-D) AIN-76A rodent diet (Research Diets, Inc., New Brunswick, NJ, catalogue D18106 and D18104, respectively) containing 6 mg and 0.3 mg Cu kg^{-1}, respectively. All mice had free access to double-distilled water.[33] Mice of 4, 5, 7 or 8 weeks of age were fasted for 2 hours and then anesthetized by intraperitoneal injection of ketamine (80–100 mg kg^{-1}) and xylazine (5–10 mg kg^{-1}) for tissue sampling. Animal studies performed in Departamento de Gastroenterología, Facultad de Medicina, Pontificia Universidad Católica de Chile were conducted using protocols defined by the Public Health Service Policy on Human Care and Use of Laboratory Animals in the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals[34] and were approved by the review board for animal studies at our institution (Comité de Ética y Bienestar Animal, CEBAMedUC; Approval ID#005-2011). Animal studies performed in the Department of Pharmacology, University of Oxford, were conducted using protocols approved by the UK Home Office for the conduct of regulated procedures under license (Animal Scientific Procedures Act, 1986).

Quantification of iron and copper

Liver and heart metal content was quantified as previously described.[35] Briefly, iron and copper were measured using a graphite furnace AAS (Perkin Elmer, SIMMA 6100). Iron and copper contents in plasma and bile were measured using a graphite furnace AAS (Perkin Elmer, SIMMA 6100) without pretreatment with nitric acid.

Echocardiography

Transthoracic M-mode of the left ventricle was obtained *via* echocardiogram equipped with an 8 MHz transducer (ATL 5000 ultrasound machine), using a method previously described.[36] The cavity sizes of left ventricular end-diastolic and end-systolic internal dimensions (LVIDd and LVIDs) and left ventricular anterior (LVWT) and posterior wall or interventricular septum (IVS) thickness were measured. The heart rate was monitored and the left ventricular fractional shortening and the left ventricular ejection fraction were calculated using a modification of the American Society of Echocardiography method.[37] All echocardiography examinations were performed and interpreted by a single operator blinded to the experiment.

RNA extraction

Total RNA was extracted from homogenized liver with TRI Reagent (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quality and quantity were assessed prior to and after DNase digestion by denaturing gel electrophoresis and photometric analysis (A260/280 ratio), respectively.

cDNA synthesis and quantitative real-time PCR (qPCR)

Total RNA (2 μg) was used as a template for reverse transcription to synthesize single-stranded cDNA. Quantitative real-time PCR (qPCR) was performed as previously described.[27] Gene-specific primer sets detailed in Table S1 (ESI†) were designed using Primer3Plus to amplify DNA products between 70 and 200 bp. The following standard thermal profile was used: 10 min at 95 °C,

40 cycles of 10 s at 95 °C and 15 s at 60 °C, with a final 10 s stage at 72 °C. Data were analyzed using LightCycler Software (v.3, Roche). Efficiency was determined for each sample and each gene by LinRegPCR v.7.5 using data obtained from the exponential phase of each amplification plot.[38] The products were resolved by 2% agarose gel electrophoresis to confirm the presence of DNA fragments of the expected sizes. Transcript levels of genes were normalized to the *Ppia* gene,[27] which was validated in our experimental conditions. qPCR was performed in samples from at least five mice.

D-Penicillamine treatment

Npc1$^{-/-}$ mice were treated with the copper chelating reagent DPA (100 mg per kg per day, Sigma),[39] supplemented as a dry admixture in powdered RM1 mouse chow (SDS, UK) or dissolved in drinking water (as indicated in the legends of Fig. 4–6). These treatments were administered from 3 or 4 weeks of age to evaluate neurological and hepatic effects, respectively.

Locomotor activity: AmLogger and open field rearing

Spontaneous activity of each mouse was recorded weekly using an automated activity monitor (AmLogger, Linton Instrument, UK) as described previously.[40]

Gait analysis

The CatWalk™ system (Noldus Information Technology, the Netherlands) was used for monitoring changes in movement patterns as indicative of any motor complications and to assess any gait improvement in response to treatment.[41] *Npc1*$^{-/-}$ mice were subjected to CatWalk™ analysis at 9 weeks of age, where a minimum of three ''runs'' were collected per animal. The data collected were subsequently examined using CatWalk™ XT 10.0 software to produce 177 inter-paw and intra-paw comparison parameters.

Serum aminotransferases

Serum was collected from mice treated with DPA in drinking water and then alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using the ALAT or the ASAT kit respectively (Kovalent, Rio de Janeiro, Brazil) following the manufacturer's instructions.

Statistical analysis

GraphPad Prism v5 software was used for the statistical analysis. Mann–Whitney *U* test and unpaired *t* test, as indicated in the figure legends, were performed. Data are presented as mean ± SEM as indicated. Statistical significance was defined as $p < 0.05$.

Results

Altered copper and iron homeostasis in the *Npc1*$^{-/-}$ mouse model

First, we used the AAS method to determine the total contents of copper and iron in the liver and plasma, and bile copper levels, as shown in Fig. 1A–E, from 4-, 7-, and 8-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. Fig. 1A shows a higher copper content and Fig. 1B shows a lower iron content in the liver of *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$ mice at 4, 7, and 8 weeks of age ($p < 0.05$). In addition, we determined the copper and iron contents in plasma in 4-, 7-, and 8-week-old mice as a representation of the availability of these two metals at the whole-body level, as shown in Fig. 1C and D. The same liver pattern was observed in plasma: an increase in copper and a decrease in iron were detected in 4-, 7-, and 8-week-old *Npc1*$^{-/-}$ mice compared to

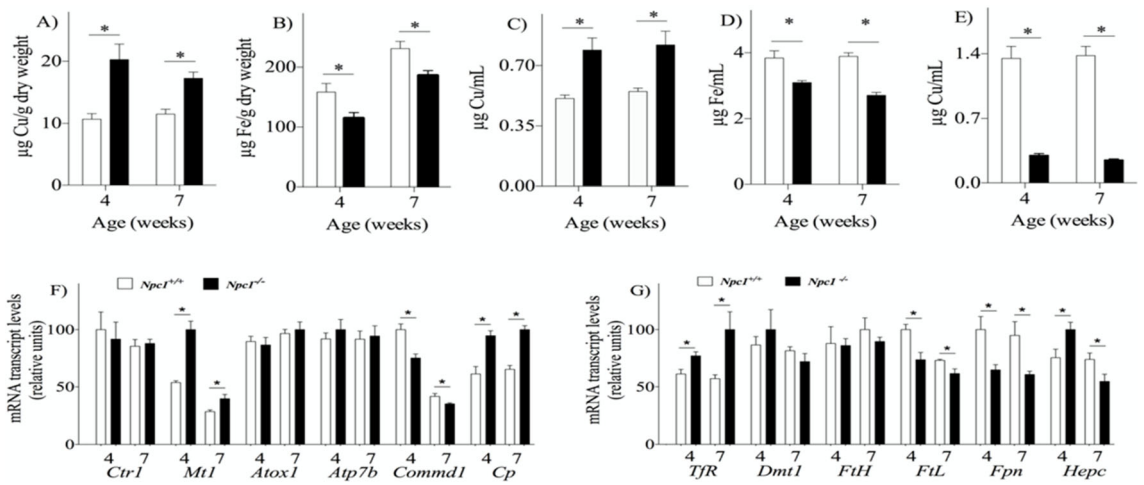

Fig. 1 Altered copper and iron homeostasis in the *Npc1*$^{-/-}$ mouse model. Levels of copper in the liver (A), iron in the liver (B), copper in plasma (C), iron in plasma (D), and copper in bile (E) of four-, seven- and eight-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice determined by AAS. Transcript levels of six copper metabolism genes (F) and six iron metabolism genes in the liver (G) of four- and seven-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice were determined by qPCR. Each gene transcript level was normalized toward *Ppia* in the corresponding samples. The results show the mean of relative abundance of the transcript value in relation to the maximum value (100%) of one gene. Data represent mean ± SEM from five biological replicates. Statistical analysis: Mann–Whitney *U* test, *$p < 0.05$.

Npc1$^{+/+}$ mice. Plasma copper was 55, 49 and 155% higher in 4-, 7-, and 8-week-old *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$ mice. Plasma iron was 19, 30 and 31% lower in 4-, 7- and 8-week-old *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$ mice. These inverse correlations between intracellular copper and iron content in hepatic tissue and plasma agree with recent studies by Hung *et al.*, also in the *Npc1*$^{-/-}$ mouse model.[18] Considering that biliary secretion is the most important excretory mechanism to eliminate hepatic copper overload,[42] we also quantified the copper content in bile, as shown in Fig. 1E. A marked decrease in copper content in the bile of *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$ mice was observed, suggesting that accumulation of copper was due to a deficiency in hepatic copper excretion in the mutant mice. Taken together, these results indicate that copper and iron homeostasis is altered by the absence of NCP1 irrespective of age (4-, 7- or 8-week-old mice) and whether pre- or post-weaning. These results suggest that copper and iron metabolism alterations in *Npc1*$^{-/-}$ mice have an early onset, during intrauterine growth or post-natal development prior to weaning.

Alterations in hepatic copper and iron metabolism-related gene expression in *Npc1*$^{-/-}$ mice

Cellular adaptation to high or low levels of metals depends on metabolic regulation mechanisms, which control uptake, intracellular handling, storage and efflux, usually by the functions of specific proteins, many of which are transcriptionally regulated.[43] To better understand the molecular mechanisms responsible for copper and iron alterations in the liver of *Npc1*$^{-/-}$ mice, we quantified the mRNA abundance of copper metabolism genes, as shown in Fig. 1F and Table S2 (ESI†), and iron metabolism-related genes, as shown in Fig. 1G and Table S3 (ESI†), in the liver of 4- and 7-week-old *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice using qPCR. We choose 4- and 7-week-old to analyze the copper and iron gene expression pattern, because until 7-week-old, *Npc1*$^{-/-}$ mice are in a steady state with respect to food intake and weight gain but not later, as described by Xie *et al.*[44]

As shown in Fig. 1F, we observed the same copper metabolism-related gene expression pattern in 4- and 7-week-old mice. The expression of the copper storage gene *Mt1* and the ferroxidase enzyme *Cp* increased, whereas the expression of the intracellular copper handling gene *Commd1* decreased significantly in the liver of *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$ mice ($p < 0.05$). We observe in Fig. 1D that *Ctr1*, *Atox1*, and *Atp7b* gene expression show no differences between genotypes. Regarding iron metabolism-related genes, we found a significant increase in the expression of the cellular iron uptake gene *TfR* and of the master regulator of iron metabolism gene *Hepc*, and a significant decrease in the expression of the iron storage and efflux genes *FtL* and *Fpn*, respectively, in the liver of 4-week-old *Npc1*$^{-/-}$ mice compared with *Npc1*$^{+/+}$ mice ($p < 0.05$). We observe in Fig. 1G that *Dmt1* and *FtH* gene expression show no differences between genotypes. At 7 weeks, Fig. 1G shows a significant decrease of *Hepc* mRNA abundance in *Npc1*$^{-/-}$ compared to *Npc1*$^{+/+}$ mice ($p < 0.05$).

Effect of a copper-deficient diet on hepatic copper metabolism in the *Npc1*$^{-/-}$ mouse model

Since excess copper in the tissue can create favorable conditions for redox stress and oxidative tissue damage, we treated 4-week-old *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice with a copper-deficient diet for one or four weeks to help reduce liver and plasma copper levels. Fig. 2A shows a significant decrease of copper content in the liver of *Npc1*$^{-/-}$ mice at 5 and 8 weeks of age compared to 4-week-old *Npc1*$^{-/-}$ mice ($p < 0.05$). At 8 weeks the copper content of *Npc1*$^{-/-}$ mice reached values close to those of *Npc1*$^{+/+}$ mice, including *Npc1*$^{+/+}$ mice treated for one week with the copper-deficient diet (8.0 ± 0.3 and 7.8 ± 0.7 μg Cu g^{-1} dry weight, respectively). Also, Fig. 2A shows a significant decrease of copper content in the liver of *Npc1*$^{-/-}$ mice at 5 weeks of age that were fed a copper-deficient diet compared to *Npc1*$^{-/-}$ mice fed a control diet ($p < 0.05$). Additionally, Fig. 2A shows a significant decrease of copper content in the liver of *Npc1*$^{-/-}$ mice at 8 weeks of age that were fed a copper-deficient diet compared to *Npc1*$^{-/-}$ mice fed a control diet ($p < 0.05$). The liver copper content decreased during the first week of treatment in both *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice, but the decrease was significantly greater in *Npc1*$^{-/-}$ mice. As shown in Fig. 2B, no differences were observed in the *Npc1*$^{-/-}$ mice plasmatic copper levels after the first week of treatment. However, Fig. 2B shows a significant decrease of copper content in the plasma of 8-week-old *Npc1*$^{-/-}$ mice compared to *Npc1*$^{-/-}$ mice fed with a control diet ($p < 0.05$). *Npc1*$^{+/+}$ mice showed no differences in the plasma copper level between treatments at any age, as shown in Fig. 2B. We also quantified copper in bile. Fig. 2C shows a small but significant decrease in biliary copper content in 5- and 8-week-old *Npc1*$^{-/-}$ mice fed a copper-deficient diet compared to *Npc1*$^{-/-}$ mice fed a control diet ($p < 0.05$). Fig. 2C also shows a decrease in the biliary copper content of 5- and 8-week-old *Npc1*$^{+/+}$ mice fed a copper-deficient diet compared to *Npc1*$^{+/+}$ mice fed a control diet ($p < 0.05$). Finally, we analyzed the effect of a copper-restricted diet on the body weight of *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice and on the life span of *Npc1*$^{-/-}$ mice. The copper-restricted diet did not alter the body weight of *Npc1*$^{+/+}$ mice or *Npc1*$^{-/-}$ mice (Fig. S1A). Fig. S1B (ESI†) shows that *Npc1*$^{-/-}$ mice fed with a CuA diet lived an average of 9.8 weeks and when they were fed with a low-copper diet, they lived an average of 10.4 weeks, as has been previously described by Alvarez *et al.*[45] and Maue *et al.*[29]

To understand the molecular mechanisms responsible for hepatic adaptive response in the liver of *Npc1*$^{-/-}$ mice fed a copper-deficient diet for one week, we measured the expression of genes related with the uptake, intracellular handling, storage and efflux of copper in the liver of 5-week-old *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice fed control and copper-deficient diets using qPCR. As shown in Fig. 2D and Table S4 (ESI†), the *Npc1*$^{-/-}$ mice fed a copper-deficient diet showed decreased expression of the ferroxidase enzyme gene *Cp* and the copper storage gene *Mt1* compared to *Npc1*$^{-/-}$ mice fed a control diet ($p < 0.05$). We observe in Fig. 2D that the *Ctr1*, *Atox1*, *Atp7b* and *Commd1* gene expression show no differences between treatments. The *Npc1*$^{+/+}$ mice fed copper-deficient diets for one week showed no

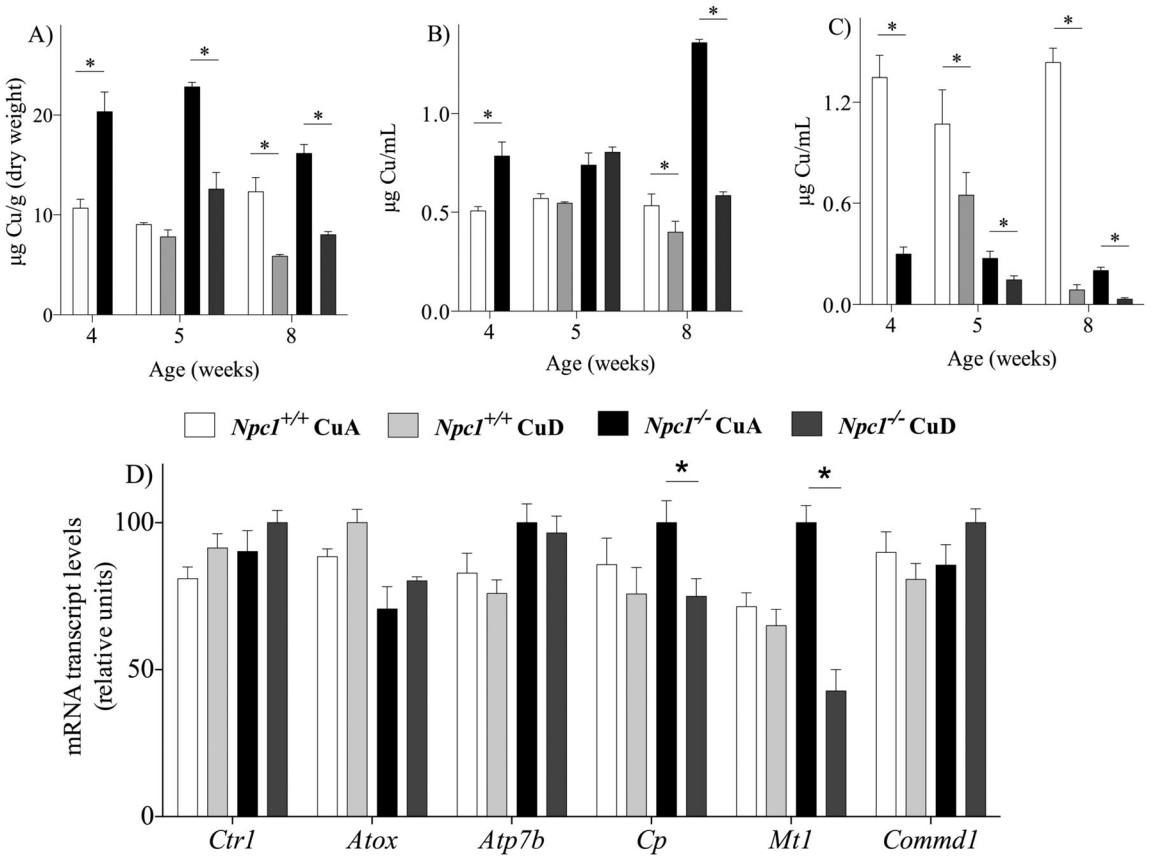

Fig. 2 Effect of a copper-deficient diet on hepatic copper metabolism in the *Npc1*$^{-/-}$ mouse model. Copper levels in the liver (A), plasma (B), and bile (C) of *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice at four, five and eight weeks of age were determined by AAS. Transcript levels of six copper metabolism genes (D) in five-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice were determined by qPCR. For each gene transcript level was normalized toward *Ppia* in the corresponding samples. The results show the mean of relative abundance of the transcript value in relation to the maximum value (100%) of one gene. Data represent mean ± SEM from five biological replicates. Statistical analysis: Mann–Whitney *U* test, *$p < 0.05$.

copper-related gene expression differences between treatments, as shown in Fig. 2D. These results indicate that hepatic adaptive response to low intake of copper in the *Npc1*$^{-/-}$ mouse model was correlated with the changes of copper-related gene expression patterns in the liver.

Effect of a copper-deficient diet on heart copper content, cardiac morphology and cardiac function in the *Npc1*$^{-/-}$ mouse model

To study the effect of a copper-deficient diet on extra hepatic copper metabolism in 5- and 8-week-old *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice we determined copper content in heart tissue. We found a significant decrease of copper content in the heart of *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice in the first week of intake of a copper-deficient diet compared with controls, *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice fed a control diet ($p < 0.05$). In Fig. 3A we observe that at 8 weeks of age *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice show no differences between treatments. Previous studies showed that mice fed a copper-deficient diet for 4 weeks changed their cardiac phenotypes, increasing the thickness of the left ventricular and interventricular walls.[46] We also analyzed the cardiac phenotype and function by echocardiography mode-M. We found a significant decrease in the thickness of the left ventricular and interventricular walls at 3 weeks in *Npc1*$^{-/-}$ compared with *Npc1*$^{+/+}$ mice fed control diets ($p < 0.05$), as shown in Fig. 3B–D. In Fig. 3C we observe that the left ventricular internal dimension shows no differences between genotypes. Analysis of the heart rate, the left ventricular fractional shortening and the ejection fraction indicated that cardiac function was not affected, as shown in Table S5 (ESI†). We also found a significant decrease in the thickness of the left ventricular and interventricular walls in 4-week-old *Npc1*$^{-/-}$ mice fed a copper-deficient diet for one week compared with 4-week-old *Npc1*$^{-/-}$ mice fed a copper-adequate diet for one week ($p < 0.05$), as shown in Fig. 3E–G. In Fig. 3F we observe that the left ventricular internal dimension shows no differences between treatments. At 5 weeks *Npc1*$^{+/+}$ mice showed no differences between treatments, as shown in Fig. 3E–G. Analysis of the heart rate, the left ventricular fractional shortening and the ejection fraction indicated that cardiac function was not affected between genotypes, as shown in Table S2 (ESI†). Together these results imply that *Npc1*$^{-/-}$ mice responded to a decrease in the dietary supply of copper by modulating both their cardiac copper content and cardiac phenotype changes.

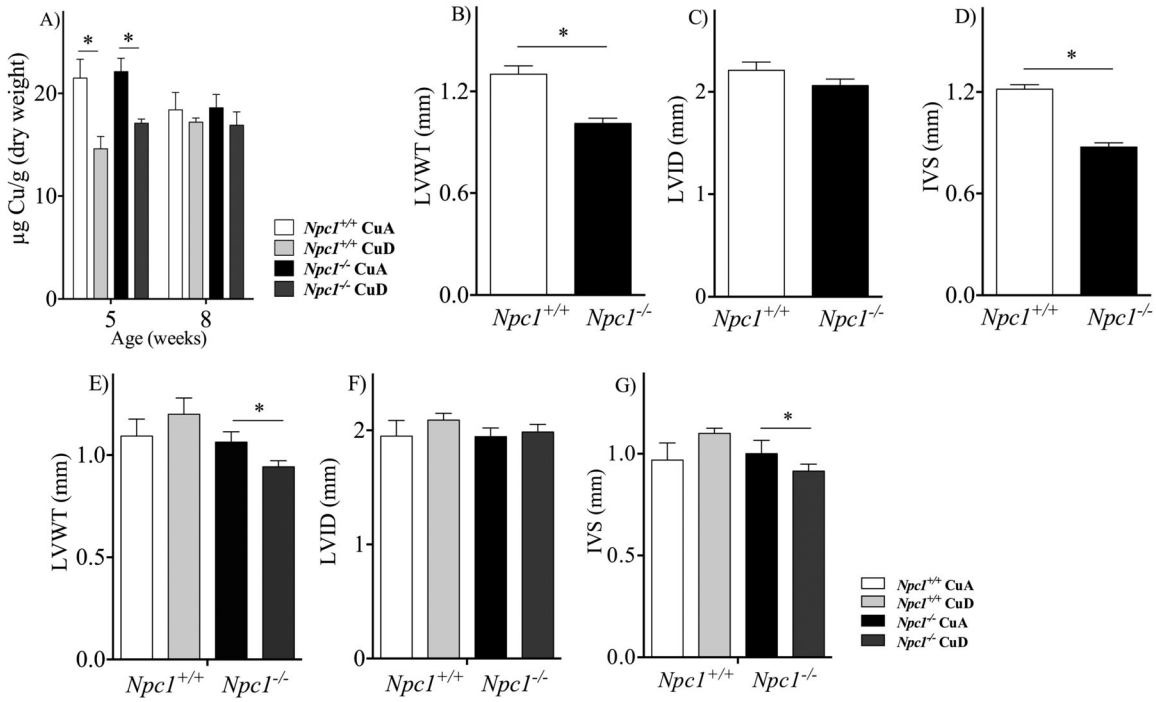

Fig. 3 Effect of a copper-deficient diet on heart copper content and on cardiac morphology in the $Npc1^{-/-}$ mouse model. Heart (A) copper levels of five- and eight-week-old $Npc1^{+/+}$ and $Npc1^{-/-}$ mice fed with an adequate (CuA) or deficient-copper diet (CuD) were determined by AAS. Quantitation of the left ventricular wall thickness (B and E), left ventricular internal dimension (C and F) and interventricular septum diastolic dimension (D and G) of $Npc1^{+/+}$ and $Npc1^{-/-}$ mice at seven (upper panel) and five weeks of age (bottom panel) by echocardiogram M-mode. Data represent mean ± SEM. Statistical analysis: Mann–Whitney *U* test, $*p < 0.05$, $n = 8$.

Effect of D-penicillamine copper chelator on copper levels in the liver, bile and plasma, and liver function in the $Npc1^{-/-}$ mouse model

In order to study the effect of a therapeutic intervention in NPC patients we treated $Npc1^{-/-}$ mice with DPA, an effective copper chelator used for treatment of Wilson's disease.[47,48] DPA was administered in drinking water (100 mg kg^{-1} weight of mice per day) from 4 until 8 weeks of age.[39] The results showed a significant decrease of copper content in the bile of $Npc1^{-/-}$ mice treated with DPA compared to $Npc1^{-/-}$ mice that received drinking water ($p < 0.05$). In Fig. 4B we observe that the bile of $Npc1^{+/+}$ mice shows no copper content differences between DPA or drinking water treatments. Liver and plasma copper content showed no differences between DPA and drinking water treatments between genotypes, as shown in Fig. 4A–C. In Fig. 5A and B we observe a significant decrease of AST and ALT plasma activity in DPA treated $Npc1^{-/-}$ mice compared to $Npc1^{-/-}$ mice that received drinking water ($p < 0.05$). As illustrated in Fig. 5A and B, the AST and ALT plasma activity showed no differences in $Npc1^{+/+}$ mice treated with DPA or drinking water. These results indicate that although DPA does not change total copper content in the liver and plasma it is capable of improving liver function in $Npc1^{-/-}$ mice.

Effect of D-penicillamine copper chelator on neurological functions of a $Npc1^{-/-}$ mouse model

Next, to evaluate the potential therapeutic effects of DPA, another group of mice were treated from 3 to 9 weeks of age

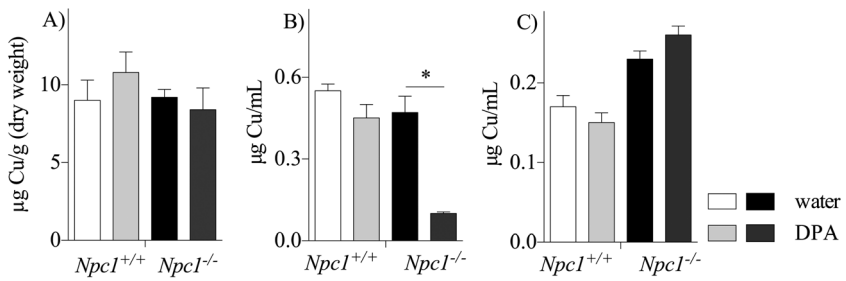

Fig. 4 Effect of D-penicillamine copper chelator on copper levels in the liver, bile and plasma of a $Npc1^{-/-}$ mouse model. Copper levels in the liver (A), bile (B), and plasma (C) of $Npc1^{+/+}$ and $Npc1^{-/-}$ mice, treated for four weeks with DPA at 100 mg per kg weight of mice per day dissolved in drinking water. Copper levels were determined by AAS. Data represent mean ± SEM. Statistical analysis: Mann–Whitney *U* test, $*p < 0.05$, $n = 5$.

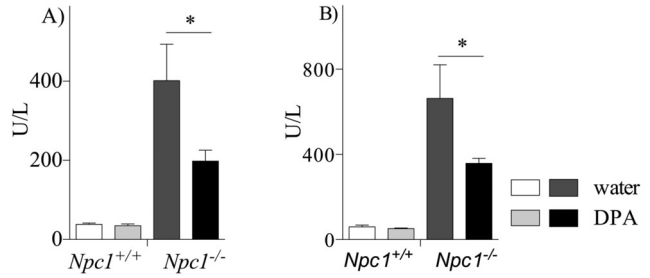

Fig. 5 Effect of D-penicillamine copper chelator on the transaminase levels in the plasma of a $Npc1^{-/-}$ mouse model. Plasma transaminase, ALT (A) and AST (B), activity was determined by the standard photometric method, using Merck Microlab 100. Data represent mean ± SEM. Statistical analysis: Mann–Whitney *U* test, $*p < 0.05$, $n = 5$.

with DPA (100 mg per kg per day) supplemented as a dry admixture and neurological functions of $Npc1^{-/-}$ were evaluated. The locomotor activity of DPA treated $Npc1^{-/-}$ mice was monitored using AmLogger.[40] There was no significant improvement in locomotor activity, including FR count, activity (S), mobile (S), rearing (S), activity and rearing counts, in DPA-treated $Npc1^{-/-}$ mice when compared with normal powder diet treated $Npc1^{-/-}$ mice, as shown in Fig. S2A–F (ESI†). The effects of DPA on gait were also measured in 9-week-old $Npc1^{-/-}$ mice.[41] As illustrated in Fig. 6A–F, no significant improvements in gait were found in DPA treated 9-week-old $Npc1^{-/-}$ mice. Therefore, these results suggested that DPA has limited therapeutic effect on neurological functions in treated $Npc1^{-/-}$ mice.

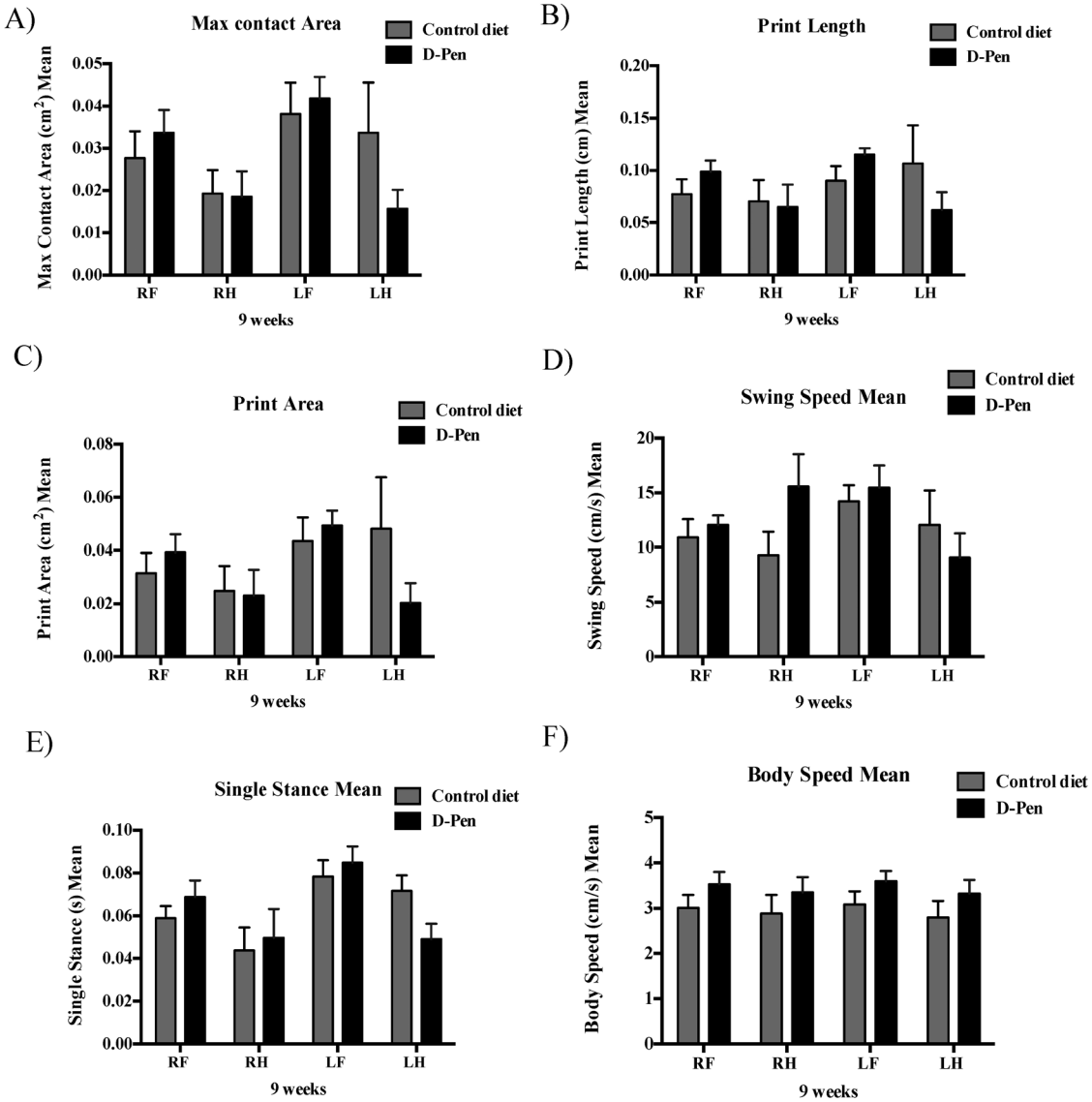

Fig. 6 D-Penicillamine treatment revealed limited therapeutic effects on gait change in treated $Npc1^{-/-}$ mice. Intra-paw and inter-paw measurements including (A) max contact area (cm^2) mean, (B) print length (cm) mean, (C) print area (cm^2) mean, (D) swing speed (cm s^{-1}) mean, (E) single stance (s) mean as well as (F) body speed (cm s^{-1}) mean were measured in control diet and D-penicillamine treated 9-week-old $Npc1^{-/-}$ mice. RF: right front limb; RH: right hind limb; LF: left front limb; LH: left hind limb. The gait changes measured using the CatWalk™ system are described in "Materials and methods". Data represent mean ± SEM, $n = 4$.

Discussion

The results of our study indicate that *Npc1*$^{-/-}$ mice, which exhibit signs of copper overload and iron depletion in the liver and plasma immediately after weaning, responded rapidly to a copper-deficient diet reducing copper content in the liver, bile and heart. These responses were correlated with changes in the copper-related gene expression profile in the liver and heart and cardiac phenotype changes, respectively. These results suggest that *Npc1*$^{-/-}$ mice can sense copper deficiency early in life. In addition, *Npc1*$^{-/-}$ mice responded to a copper chelator DPA treatment for four weeks, reducing biliary copper content and plasma transaminase, ALT and AST, levels. These results indicate that although DPA does not change total copper content in the liver and plasma, it does improve liver function in *Npc1*$^{-/-}$ mice. To our knowledge, this is the first report documenting the hepatic adaptive response to low copper intake, obtained through dietary intervention or by using a copper-chelating compound, in NPC mouse models.

Our data indicate that hepatic copper and iron metabolism alterations in *Npc1*$^{-/-}$ mice have an early onset that is maintained over time, even before weight loss and major neurological symptoms, which occur after 7 weeks of age.[3,28,44] In this context, further analyses are needed to determine whether these early changes in hepatic copper and iron metabolism are also present in models with more slowly progressing forms of NPC disease, *e.g.*, *Npc1*^{nmf164} mice.[29] Similar results were recently reported in the liver and plasma of the *Npc1*$^{-/-}$ mouse model by Hung *et al.*[18] extending our previous findings of altered hepatic copper metabolism using *Npc1*$^{-/-}$ mice.[27] Moreover, Hung *et al.*[18] reported disrupted metal homeostasis in the cerebrospinal fluid, plasma and post-mortem brain tissues of a human patient and another study described serum-free copper with low CP in a NPC patient.[19] Therefore, abnormal copper and iron metabolism may be part of the alterations involved in NPC disease.

Characterization of copper and iron metabolism in a NPC mouse model at 4 and 7 weeks of age

In order to understand the molecular mechanisms associated with alterations of hepatic copper metabolism in *Npc1*$^{-/-}$ mice, we analyzed the hepatic copper metabolism gene expression patterns in 4- and 7-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. We observed that *Mt1* and *Cp* were upregulated and *Commd1* was downregulated in *Npc1*$^{-/-}$ mice. MT1 is a metal-induced protein that has the protective function of storing excess intracellular copper to detoxify cells.[35,49] Therefore, this result suggests that the mechanisms of protection against copper overload were activated early and maintained, at least until 7 weeks of age in *Npc1*$^{-/-}$ mice. Studies have shown that CP is a copper-dependent ferroxidase necessary for iron efflux of the liver that contributes to more than 70% of total plasma copper content.[42,50,51] Therefore, an alteration in hepatic copper metabolism may affect the synthesis of holo-CP and thus also adversely affect iron metabolism. *Cp* up-regulation in the liver of *Npc1*$^{-/-}$ mice was correlated with an increase in plasma copper, which is associated with the increase of plasma CP in 7- and 8-week-old *Npc1*$^{-/-}$ mice.[52] However, it is not clear how CP activity could be increased if ATP7B function is impaired, as has been previously proposed in U18666A treated cells.[26,30,53] Our results indicated that in *Npc1*$^{-/-}$ mice the biosynthetic function of ATP7B is not altered, but rather that ATP7B-dependent copper transport to the bile is. The copper chaperone ATOX1 and COMMD1 participate with ATP7B in the biliary copper excretion process and consistently, a functional defect in these proteins leads to liver copper overload.[25,54] Although we did not observe transcriptional changes of *Atp7b* or *Atox1* in *Npc1*$^{-/-}$ mice, we found a significant decrease of *Commd1* expression in 4- and 7-week-old *Npc1*$^{-/-}$ animals. In agreement with this finding, reduced expression of *Commd1* was described at 7 and 8 weeks of age in *Npc1*$^{-/-}$ mice.[27] These results suggest that the early transcriptional alteration of *Commd1* may affect the vesicular traffic of ATP7B and copper into the bile duct. Thus, an adequate interplay among COMMD1, ATP7B and NPC1 may be necessary for an early and controlled copper efflux under physiological conditions. In fact, *in vitro* studies using a U18666A treated hepatoma cell line suggest that ATP7B function was impaired due to the disruption of the late endosome-to-TGN transport.[26]

Interestingly, the higher copper content in the liver and the lower copper content in bile observed in *Npc1*$^{-/-}$ mice have been described in other genetics models like Wilson's disease mice (*Atp7b*$^{-/-}$)[55] and in the canine models of copper toxicosis (Bedlington terrier) caused by COMMD1 mutations.[56,57] These models have hepatic copper excess in common, which is caused by disorders in biliary excretion of the metal. However, while in *Npc1*$^{-/-}$ mice copper accumulation in the liver is twice that of control mice, in Wilson's disease models and in the canine models of copper toxicosis liver copper accumulation is 10 or 100 times higher.[58] The extent of copper accumulation in the liver of *Npc1*$^{-/-}$ mice is similar to that described by Muller *et al.* in 129/SvEv mice supplemented with 6 mM copper in drinking water for one month.[59] We found a difference in the amount of copper accumulated in the liver of *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$, which suggests that biliary excretion requires the functional integrity of NPC1, ATP7B and COMMD1. Moreover, the higher copper content in the liver and plasma of *Npc1*$^{-/-}$ mice and the lower bile copper content observed in the *Npc1*$^{-/-}$ mice strongly suggest that the flux of copper from the liver into the plasma and into the bile is altered in *Npc1*$^{-/-}$ mice. The higher copper content in the plasma of *Npc1*$^{-/-}$ mice may be an example of an adaptive mechanism, which protects the liver against copper toxicity. Also, it is clear that copper transport mechanisms involved in the adaptation of increased hepatic copper storage require the activity of intracellular trafficking pathways which depend on the integrity of the NPC1 protein.

In order to understand the molecular mechanisms associated with alterations of hepatic iron metabolism in *Npc1*$^{-/-}$ mice, we analyzed the hepatic iron metabolism gene expression patterns in 4- and 7-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. Iron metabolism at the cellular level is self-regulated through iron-dependent changes in the abundance of *Ft*, which sequesters excess iron, and transferrin receptors controlling iron uptake.[60,61] When iron levels are low, *TfR* mRNA is stabilized, more receptor is synthesized,

and iron uptake increases, while *Ft* mRNA is masked and FT synthesis and iron storage are reduced.[60] During the pre-weaning period, a significant reduction in liver and plasma iron content was detected in *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$ mice, and these differences were maintained in 7-week-old animals. These results suggest that iron uptake and/or efflux processes are affected in *Npc1*$^{-/-}$ mice. The lower liver and plasma iron content in *Npc1*$^{-/-}$ mice suggests a dietary deficiency. However, under our experimental conditions, all animals were administered the same diet. In particular, the diet given during the post-weaning period contained 45 mg Fe kg^{-1}, an amount clearly over the minimum commonly used to induce dietary iron deficiency ($<$8 mg Fe kg^{-1}).[62] Thus, our results suggest that *Npc1* mutations cause iron deficiency, as has been shown in other mouse models of lysosomal disorders (murine gangliosidosis),[63] possibly affecting the uptake of the metal at the intestinal epithelium level. Iron intestinal epithelium levels should be analyzed to determine if the lower iron content in the liver of *Npc1*$^{-/-}$ mice is due to lower intestinal absorption of this metal. It is known that iron uptake in the liver occurs principally through endocytosis mediated by TF/TFRC, and these results suggest that the lower iron content in the liver of *Npc1*$^{-/-}$ mice may have been due, at least in part, to the abnormal recycling of TF/TFRC, a possibility previously proposed in CHO cell lines containing the *Npc1* mutation.[15] In addition, the amount of hepatic iron may be also affected by the expression of *Ft*. In fact, earlier studies using polyclonal and monoclonal antibodies showed low expression of H and L FT isoforms in various tissues from NPC patients.[17,64] The authors suggested that iron is sequestered in lysosomes and cannot be recycled for FT synthesis in the *Npc1*$^{-/-}$ liver, nor can cholesterol and other cargo in lysosomes.[17]

Regarding the reduction of plasma iron in *Npc1*$^{-/-}$ mice, it is known that ferroportin (Fpn) is a critical protein for the distribution of iron between tissues and its expression can be induced by iron and heme, and may be inhibited by hepcidin (Hepc) and inflammation.[65–67] Data from our study indicated an early and sustained reduction of *Fpn* in the liver of *Npc1*$^{-/-}$ mice. These results were correlated with lower iron content in the plasma and increased expression of *Hepc* in the liver of *Npc1*$^{-/-}$ mice at 4 weeks of age. The decreased expression of *Fpn* in the liver of *Npc1*$^{-/-}$ mice suggests a reduced iron efflux from the liver to the plasma in *Npc1*$^{-/-}$ mice. Previous data show that Hepc is a hormonal regulator of iron metabolism produced by the liver in mammals, which controls iron efflux to the plasma through the regulation of Fpn lysosomal degradation of the plasma membrane of hepatocytes and enterocytes.[68,69] Taken together, liver and plasmatic iron content and iron gene expression results support the hypothesis that NPC1 is necessary for adequate and efficient management of hepatocellular content of this metal. However, further investigations are required to elucidate the mechanisms involved in the NPC1-dependent changes in gene transcription of homeostatic components of iron metabolism and their pathophysiological consequences.

Effects of copper intake restriction in the Niemann–Pick C murine model

In order to reduce liver and plasma copper excess we provided 4-week-old *Npc1*$^{-/-}$ mice a copper-deficient diet for one or four weeks. The results indicate a fast (one week) hepatic and extra hepatic adaptive responsiveness to dietary copper deficiency in *Npc1*$^{-/-}$ mice, evidenced by a remarkable decrease of copper content in the liver; the same pattern was observed to a lower degree in bile and the heart. These changes were correlated with copper-related gene expression changes in the liver and cardiac phenotype alterations. The liver and heart copper content changes observed in *Npc1*$^{-/-}$ mice were similar to those described previously in wild type mice fed a copper-deficient diet in the third and fourth weeks of treatment.[70] Regarding the effect of a copper-deficient diet in the extra-hepatic organs, we observed a decrease in the left ventricular and interventricular wall thickness in *Npc1*$^{-/-}$ mice in the first week of the diet. Interestingly, FVB wild type mice fed with the same diet used in the present study manifested an increase (cardiac hypertrophy) after four weeks of diet treatment.[70] These results suggest that *Npc1*$^{-/-}$ mice have a higher sensitivity to a copper-deficient diet compared to *Npc1*$^{+/+}$ mice and FVB wild type mice. Therefore, although the *Npc1*$^{-/-}$ mice responded to the copper-deficient diet with changes in some cardiac parameters, these changes were in the opposite direction to that described in the FVB mice. This could be due to genetic background differences. Indeed, previous data have shown that the genetic background has a strong influence on NPC disease expression from the Npc1-npc nih mutation.[28] However, further study is needed to explore why *Npc1*$^{-/-}$ mice may have manifested a different response. At 8-week-old we did not find significant differences in the copper content in the heart of the wild type mice fed a copper-deficient diet or a standard diet, and between *Npc1*$^{-/-}$ mice fed with the same diets. It is possible that the lack of change shown in our results may indicate an adaptive response in the wild type; however, we do not feel this is the case because, as shown in the literature,[70] by 8 weeks the deteriorative effects of a copper-deficient diet on the heart are apparent. Furthermore, in looking at each case individually, we found that in the wild type group there were 3 outliers in the sample of 8 mice. If we remove these outliers from subsequent analysis, we find significant decreases in the copper content of the heart at 8 weeks, which is in concordance with the findings of other studies.[70,71] With respect to the *Npc1*$^{-/-}$ mice, again we feel that by 8 weeks the effects of the diet are such that we are unable to discuss an apparent adaptive response.

In order to understand the molecular mechanisms associated with alterations of hepatic copper metabolism in 4-week-old *Npc1*$^{-/-}$ mice fed a copper-deficient diet, we analyzed the hepatic copper metabolism gene expression patterns in *Npc1*$^{-/-}$ mice fed a copper-deficient diet and *Npc1*$^{-/-}$ mice fed a control diet. We observed that *Mt1* and *Cp* were downregulated and these results were correlated with a reduction of liver copper content. Eight-week-old *Npc1*$^{-/-}$ mice presented high levels of copper in the liver and liver functional damage, as evidenced by the large increases in the levels of liver disease markers such as plasmatic alanine and aspartate aminotransferases, similar to those described previously in *Npc1*$^{-/-}$ mice.[27,72,73] Evidence supports the notion that excess copper favors oxidative stress and tissue damage.[74,75] In order to decrease hepatic copper content in

Npc1$^{-/-}$ mice and to study the effect of a potential therapeutic intervention for NPC patients, we treated *Npc1*$^{-/-}$ mice with DPA, an effective and safe copper chelator that allows the removal of excessive stored amounts and prevents further accumulation of copper. DPA is currently used for the treatment of Wilson's disease.[47,48,76] Although *Npc1*$^{-/-}$ mice treated with DPA showed no total copper content differences in the liver, our results indicated a hepatic adaptive response and that *Npc1*$^{-/-}$ mice presented a decrease in copper in bile and an improvement in their hepatic function according to the significant decrease of AST and ALT levels in plasma. However, further studies are required to understand the mechanism involved in DPA improvement of liver function in *Npc1*$^{-/-}$ mice. This result suggests that if the NPC patients present hepatic damage associated to hepatic copper dyshomeostasis, then oral DPA treatment may contribute to alleviate their liver damage.

In summary our results showed that *Npc1*$^{-/-}$ mice could mount an adaptive response to a copper-deficient diet allowing a partial recovery of hepatic, biliary and plasma copper levels. Furthermore, our findings indicated an improvement in hepatic function without any effects on neurological function in *Npc1*$^{-/-}$ mice after treatment with DPA; however, further studies are required to understand the mechanism involved in DPA treatment effects and to know if DPA can be as useful to NPC patients as it is for those with Wilson's disease.

Conflicts of interest

All the authors of this work declare that they have no conflict of interest.

Acknowledgements

This work was funded by FONDECYT No. 1110427 (MG), and 1110310 (SZ), FONDAP No. 15090007, Center for Genome Regulation (MG and SZ). FMP is a Royal Society Wolfson Research Merit Award holder. CCW was supported by a Clarendon Scholarship, University of Oxford, with additional support from NPRF and CLIMB (FP). GA was supported by a CONICYT and Stekel PhD fellowship. We thank Vet's veterinary for services rendered.

References

1 M. Patterson, M. Vanier, K. Suzuki, J. Morris, E. Carsteu, *et al.*, Niemann–Pick disease type C. A lipid trafficking disorder, in *The metabolic and molecular basis of inherited disease*, ed. C. R. B. A. Scriver, W. Sly and D. Valle, Mulencer Hill, New York, 8th edn, 2001.

2 E. D. Carstea, J. A. Morris, K. G. Coleman, S. K. Loftus, D. Zhang, C. Cummings, J. Gu, M. A. Rosenfeld, W. J. Pavan, D. B. Krizman, J. Nagle, M. H. Polymeropoulos, S. L. Sturley, Y. A. Ioannou, M. E. Higgins, M. Comly, A. Cooney, A. Brown, C. R. Kaneski, E. J. Blanchette-Mackie, N. K. Dwyer, E. B. Neufeld, T. Y. Chang, L. Liscum, J. F. Strauss 3rd, K. Ohno, M. Zeigler, R. Carmi, J. Sokol, D. Markie, R. R. O'Neill, O. P. van Diggelen, M. Elleder, M. C. Patterson, R. O. Brady, M. T. Vanier, P. G. Pentchev and D. A. Tagle, Niemann–Pick C1 disease gene: homology to mediators of cholesterol homeostasis, *Science*, 1997, **277**, 228–231.

3 M. C. Patterson, E. Mengel, F. A. Wijburg, A. Muller, B. Schwierin, H. Drevon, M. T. Vanier and M. Pineda, Disease and patient characteristics in NP-C patients: findings from an international disease registry, *Orphanet. J. Rare. Dis*, 2013, **8**, 12, DOI: 10.1186/1750-1172-8-12.

4 P. Pentchev, M. Comly, H. Kruth, T. Tokoro, J. Butler, J. Sokol, M. Filling-Katz, J. Quirk, D. Marshall, S. Patel, M. Vanier and R. Brady, Group C Niemann–Pick disease: faulty regulation of low-density lipoprotein uptake and cholesterol storage in cultured fibroblasts, *FASEB J.*, 1987, **1**, 40–45.

5 M. T. Vanier, Niemann–Pick disease type C, *Orphanet. J. Rare. Dis*, 2010, **5**, 16, DOI: 10.1186/1750-1172-5-16.

6 D. C. Ko, M. D. Gordon, J. Y. Jin and M. P. Scott, Dynamic movements of organelles containing Niemann–Pick C1 protein: NPC1 involvement in late endocytic events, *Mol. Biol. Cell*, 2001, **12**, 601–614.

7 L. Liscum and J. J. Klansek, Niemann–Pick disease type C, *Curr. Opin. Lipidol.*, 1998, **9**, 131–135.

8 M. Linder and M. Hazegh-Azam, Copper biochemistry and molecular biology, *Am. J. Clin. Nutr.*, 1996, **63**, 797S–811S.

9 M. Arredondo and M. T. Nunez, Iron and copper metabolism, *Mol. Aspects Med.*, 2005, **26**, 313–327, DOI: 10.1016/j.mam.2005.07.010.

10 B. Halliwell and J. M. Gutteridge, Free radicals, lipid peroxidation, and cell damage, *Lancet*, 1984, **2**, 1095.

11 B. Halliwell and J. M. Gutteridge, Oxygen toxicity, oxygen radicals, transition metals and disease, *Biochem. J.*, 1984, **219**, 1–14.

12 T. Wileman, C. Harding and P. Stahl, Receptor-mediated endocytosis, *Biochem. J.*, 1985, **232**, 1–14.

13 M. Schaefer, R. G. Hopkins, M. L. Failla and J. D. Gitlin, Hepatocyte-specific localization and copper-dependent trafficking of the Wilson's disease protein in the liver, *Am. J. Physiol.*, 1999, **276**, G639–G646.

14 F. R. Maxfield and T. E. McGraw, Endocytic recycling, *Nat. Rev. Mol. Cell Biol.*, 2004, **5**, 121–132, DOI: 10.1038/nrm1315.

15 N. H. Pipalia, M. Hao, S. Mukherjee and F. R. Maxfield, Sterol, protein and lipid trafficking in Chinese hamster ovary cells with Niemann–Pick type C1 defect, *Traffic*, 2007, **8**, 130–141, DOI: 10.1111/j.1600-0854.2006.00513.x.

16 P. Ponka, C. Beaumont and D. R. Richardson, Function and regulation of transferrin and ferritin, *Semin. Hematol.*, 1998, **35**, 35–54.

17 H. Christomanou, M. T. Vanier, P. Santambrogio, P. Arosio, W. J. Kleijer and K. Harzer, Deficient Ferritin Immunoreactivity in Tissues from Niemann–Pick Type C Patients: Extension of Findings to Fetal Tissues, H and L Ferritin Isoforms, but also One Case of the Rare Niemann–Pick C2

Complementation Group, *Mol. Genet. Metab.*, 2000, **70**, 196–202, DOI: 10.1006/mgme.2000.3004.

18 H. Hung, N. Faux, D. Killilea, N. Yanjanin, S. Firnkes, I. Volitakis, G. Ganio, M. Walterfang, C. Hastings, F. Porter, D. Ory and A. Bush, Altered transition metal homeostasis in Niemann–Pick disease, type C1, *Metallomics*, 2014, **6**, 542–553, DOI: 10.1039/c3mt00308f.

19 B. J. Connemann, M. Gahr, M. Schmid, H. Runz and R. W. Freudenmann, Low ceruloplasmin in a patient with Niemann–Pick type C disease, *J. Clin. Neurosci.*, 2012, **19**, 620–621, DOI: 10.1016/j.jocn.2011.05.038.

20 I. H. Hung, R. L. Casareno, G. Labesse, F. S. Mathews and J. D. Gitlin, HAH1 is a copper-binding protein with distinct amino acid residues mediating copper homeostasis and antioxidant defense, *J. Biol. Chem.*, 1998, **273**, 1749–1754.

21 S. M. Hamza, M. Schaefer, L. Klomp and J. Gitlin, Interaction of the copper chaperone HAH1 with the Wilson disease protein is essential for copper homeostasis, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **9**, 13363–13368.

22 M. Y. Bartee and S. Lutsenko, Hepatic copper-transporting ATPase ATP7B: function and inactivation at the molecular and cellular level, *Biometals*, 2007, **20**, 627–637, DOI: 10.1007/s10534-006-9074-3.

23 S. Lutsenko, N. L. Barnes, M. Y. Bartee and O. Y. Dmitriev, Function and regulation of human copper-transporting ATPases, *Physiol. Rev.*, 2007, **87**, 1011–1046, DOI: 10.1152/physrev.00004.2006.

24 K. Terada, N. Aiba, X. L. Yang, M. Iida, M. Nakai, N. Miura and T. Sugiyama, Biliary excretion of copper in LEC rat after introduction of copper transporting P-type ATPase, ATP7B, *FEBS Lett.*, 1999, **448**, 53–56.

25 C. Wijmenga and L. W. Klomp, Molecular regulation of copper excretion in the liver, *Proc. Nutr. Soc.*, 2004, **63**, 31–39.

26 H. M. Yanagimoto, H. Kumemura, H. Koga, T. Kawaguchi, K. Terada, S. Hanada, E. Taniguchi, Y. Koizumi, S. Koyota, H. Ninomiya, T. Ueno, T. Sugiyama and M. Sata, Niemann–Pick C1 protein transports copper to the secretory compartment from late endosomes where ATP7B resides, *Exp. Cell Res.*, 2009, **315**, 119–126.

27 M. C. Vazquez, F. Robledo, G. Carrasco, L. Pavez, F. Olivares, M. Gonzalez, S. Zanlungo and T. del Pozo, Alteration of gene expression profile in Niemann–Pick type C mice correlates with tissue damage and oxidative stress, *PloS One*, 2011, **6**, e28777, DOI: 10.1371/journal.pone.0028777.

28 J. Parra, A. D. Klein, J. Castro, M. G. Morales, M. Mosqueira, I. Valencia, V. Cortes, A. Rigotti and S. Zanlungo, Npc1 deficiency in the C57BL/6J genetic background enhances Niemann–Pick disease type C spleen pathology, *Biochem. Biophys. Res. Commun.*, 2011, **413**, 400–406, DOI: 10.1016/j.bbrc.2011.08.096.

29 R. A. Maue, R. W. Burgess, B. Wang, C. M. Wooley, K. L. Seburn, M. T. Vanier, M. A. Rogers, C. C. Chang, T. Y. Chang, B. T. Harris, D. J. Graber, C. A. Penatti, D. M. Porter, B. S. Szwergold, L. P. Henderson, J. W. Totenhagen, T. P. Trouard, I. A. Borbon and R. P. Erickson, A novel mouse model of Niemann–Pick type C disease carrying a D1005G-Npc1 mutation comparable to commonly observed human mutations, *Hum. Mol. Genet.*, 2012, **21**, 730–750, DOI: 10.1093/hmg/ddr505.

30 M. C. Vazquez, E. Balboa, A. R. Alvarez and S. Zanlungo, Oxidative stress: a pathogenic mechanism for Niemann–Pick type C disease, *Oxid. Med. Cell. Longevity*, 2012, **2012**, 205713, DOI: 10.1155/2012/205713.

31 S. Loftus, J. Morris, E. Carstea, J. Gu, C. Cummings, A. Brown, J. Ellison, K. Ohno, M. Rosenfeld, D. Tagle, P. Pentchev and W. Pavan, Murine Model of Niemann–Pick C Disease: Mutation in a Cholesterol Homeostasis Gene, *Science*, 1997, **277**, 232–235.

32 L. Amigo, H. Mendoza, J. Castro, V. Quiñones, J. Miquel and S. Zanlungo, Relevance of Niemann–Pick type C1 protein expression in controlling plasma cholesterol and biliary lipid secretion in mice, *Hepatology*, 2002, **36**, 819–828.

33 L. M. Ruiz, E. L. Jensen, R. I. Bustos, G. Arguelloa, R. Gutierrez-Garcia, M. Gonzalez, C. Hernandez, R. Paredes, F. Simon, C. Riedel, D. Ferrick and A. A. Elorza, Adaptive responses of mitochondria to mild copper deprivation involve changes in morphology, OXPHOS remodeling and bioenergetics, *J. Cell. Physiol.*, 2014, **229**, 607–619, DOI: 10.1002/jcp.24484.

34 ILAR, *Guide for the care and uses of laboratory animals*, 1996.

35 L. Tapia, M. Gonzalez-Aguero, M. F. Cisternas, M. Suazo, V. Cambiazo, R. Uauy and M. Gonzalez, Metallothionein is crucial for safe intracellular copper storage and cell survival at normal and supra-physiological exposure levels, *Biochem. J.*, 2004, **378**, 617–624, DOI: 10.1042/bj20031174.

36 T. Masuyama, K. Yamamoto, Y. Sakata, R. Doi, N. Nishikawa, H. Kondo, K. Ono, T. Kuzuya, M. Sugawara and M. Hori, Evolving changes in Doppler mitral flow velocity pattern in rats with hypertensive hypertrophy, *J. Am. Coll. Cardiol.*, 2000, **36**, 2333–2338.

37 D. J. Sahn, A. DeMaria, J. Kisslo and A. Weyman, Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements, *Circulation*, 1978, **58**, 1072–1083.

38 C. Ramakers, J. M. Ruijter, R. H. Deprez and A. F. Moorman, Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data, *Neurosci. Lett.*, 2003, **339**, 62–66.

39 X. Zhu, J. Li, F. Liu and J. P. Uetrecht, Involvement of T helper 17 cells in D-penicillamine-induced autoimmune disease in Brown Norway rats, *Toxicol. Sci.*, 2011, **120**, 331–338, DOI: 10.1093/toxsci/kfq392.

40 E. Elliot-Smith, A. O. Speak, E. Lloyd-Evans, D. A. Smith, A. C. van der Spoel, M. Jeyakumar, T. D. Butters, R. A. Dwek, A. d'Azzo and F. M. Platt, Beneficial effects of substrate reduction therapy in a mouse model of GM1 gangliosidosis, *Mol. Genet. Metab.*, 2008, **94**, 204–211, DOI: 10.1016/j.ymgme.2008.02.005.

41 A. F. Gabriel, M. A. Marcus, W. M. Honig, G. H. Walenkamp and E. A. Joosten, The CatWalk method: a detailed analysis of behavioral changes after acute inflammatory pain in the

rat, *J. Neurosci. Methods*, 2007, **163**, 9–16, DOI: 10.1016/j.jneumeth.2007.02.003.

42 M. C. Linder, L. Wooten, P. Cerveza, S. Cotton, R. Shulze and N. Lomeli, Copper transport, *Am. J. Clin. Nutr.*, 1998, **67**, 965S–971S.

43 J. C. Rutherford and A. J. Bird, Metal-Responsive Transcription Factors That Regulate Iron, Zinc, and Copper Homeostasis in Eukaryotic Cells, *Eukaryotic Cell*, 2004, **3**, 1–13, DOI: 10.1128/ec.3.1.1-13.2004.

44 C. Xie, S. D. Turley, P. G. Pentchev and J. M. Dietschy, Cholesterol balance and metabolism in mice with loss of function of Niemann–Pick C protein, *Am. J. Physiol.*, 1999, **276**, E336–E344.

45 A. R. Alvarez, A. Klein, J. Castro, G. I. Cancino, J. Amigo, M. Mosqueira, L. M. Vargas, L. F. Yevenes, F. C. Bronfman and S. Zanlungo, Imatinib therapy blocks cerebellar apoptosis and improves neurological symptoms in a mouse model of Niemann–Pick type C disease, *FASEB J.*, 2008, **22**, 3617–3627, DOI: 10.1096/fj.07-102715.

46 L. Elsherif, L. Wang, J. T. Saari and Y. J. Kang, Regression of Dietary Copper Restriction-Induced Cardiomyopathy by Copper Repletion in Mice, *J. Nutr.*, 2004, **134**, 855–860.

47 J. M. Walshe, The discovery of the therapeutic use of D-penicillamine, *J. Rheumatol., Suppl.*, 1981, **7**, 3–8.

48 R. Purchase, The treatment of Wilson's disease, a rare genetic disorder of copper metabolism, *Sci. Prog.*, 2013, **96**, 19–32.

49 A. T. Miles, G. M. Hawksworth, J. H. Beattie and V. Rodilla, Induction, Regulation, Degradation, and Biological Significance of Mammalian Metallothioneins, *Crit. Rev. Biochem. Mol. Biol.*, 2000, **35**, 35–70, DOI: 10.1080/10409230091169168.

50 N. E. Hellman and J. D. Gitlin, Ceruloplasmin metabolism and function, *Annu. Rev. Nutr.*, 2002, **22**, 439–458, DOI: 10.1146/annurev.nutr.22.012502.114457.

51 U. Merle, S. Tuma, T. Herrmann, V. Muntean, M. Volkmann, S. G. Gehrke and W. Stremmel, Evidence for a critical role of ceruloplasmin oxidase activity in iron metabolism of Wilson disease gene knockout mice, *J. Gastroenterol. Hepatol.*, 2010, **25**, 1144–1150, DOI: 10.1111/j.1440-1746.2009.06173.x.

52 M. C. Vazquez, P. Martinez, A. R. Alvarez, M. Gonzalez and S. Zanlungo, Increased copper levels in *in vitro* and *in vivo* models of Niemann–Pick C disease, *Biometals*, 2012, **25**, 777–786, DOI: 10.1007/s10534-012-9546-6.

53 C. Yanagimoto, H. Kumemura, M. Abe, H. Koga, M. Sakata, T. Kawaguchi, K. Terada, S. Hanada, E. Taniguchi, H. Ninomiya, T. Ueno, T. Sugiyama and M. Sata, Copper incorporation into ceruloplasmin is regulated by Niemann–Pick C1 protein, *Hepatol. Res.*, 2011, **41**, 484–491.

54 J. Prohaska, Role of copper transporters in copper homeostasis, *Am. J. Clin. Nutr.*, 2008, **88**, 826S–829S.

55 D. Huster, M. J. Finegold, C. T. Morgan, J. L. Burkhead, R. Nixon, S. M. Vanderwerf, C. T. Gilliam and S. Lutsenko, Consequences of copper accumulation in the livers of the Atp7b-/- (Wilson disease gene) knockout mice, *Am. J. Pathol.*, 2006, **168**, 423–434, DOI: 10.2353/ajpath.2006.050312.

56 B. van De Sluis, J. Rothuizen, P. L. Pearson, B. A. van Oost and C. Wijmenga, Identification of a new copper metabolism gene by positional cloning in a purebred dog population, *Hum. Mol. Genet.*, 2002, **11**, 165–173.

57 A. E. Klomp, B. van de Sluis, L. W. Klomp and C. Wijmenga, The ubiquitously expressed MURR1 protein is absent in canine copper toxicosis, *J. Hepatol.*, 2003, **39**, 703–709.

58 H. Fieten, P. A. Leegwater, A. L. Watson and J. Rothuizen, Canine models of copper toxicosis for understanding mammalian copper metabolism, *Mamm. Genome*, 2012, **23**, 62–75, DOI: 10.1007/s00335-011-9378-7.

59 P. Muller, H. van Bakel, B. van de Sluis, F. Holstege, C. Wijmenga and L. W. Klomp, Gene expression profiling of liver cells after copper overload *in vivo* and *in vitro* reveals new copper-regulated genes, *JBIC, J. Biol. Inorg. Chem.*, 2007, **12**, 495–507, DOI: 10.1007/s00775-006-0201-y.

60 E. C. Theil, Regulation of ferritin and transferrin receptor mRNAs, *J. Biol. Chem.*, 1990, **265**, 4771–4774.

61 R. R. Crichton, S. Wilmet, R. Legssyer and R. J. Ward, Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells, *J. Inorg. Biochem.*, 2002, **91**, 9–18.

62 K. Yokoi, M. Kimura and Y. Itokawa, Effect of dietary iron deficiency on mineral levels in tissues of rats, *Biol. Trace Elem. Res.*, 1991, **29**, 257–265.

63 M. Jeyakumar, I. Williams, D. Smith, T. M. Cox and F. M. Platt, Critical role of iron in the pathogenesis of the murine gangliosidoses, *Neurobiol. Dis.*, 2009, **34**, 406–416.

64 H. Christomanou, J. Kellermann, R. P. Linke and K. Harzer, Deficient ferritin immunoreactivity in visceral organs from four patients with Niemann–Pick disease type C, *Biochem. Mol. Med.*, 1995, **55**, 105–115.

65 D. M. Ward and J. Kaplan, Ferroportin-mediated iron transport: expression and regulation, *Biochim. Biophys. Acta*, 2012, **1823**, 1426–1433, DOI: 10.1016/j.bbamcr.2012.03.004.

66 N. Naz, I. A. Malik, N. Sheikh, S. Ahmad, S. Khan, M. Blaschke, F. Schultze and G. Ramadori, Ferroportin-1 is a 'nuclear'-negative acute-phase protein in rat liver: a comparison with other iron-transport proteins, *Lab. Invest.*, 2012, **92**, 842–856, DOI: 10.1038/labinvest.2012.52.

67 D. Chiabrando, V. Fiorito, S. Marro, L. Silengo, F. Altruda and E. Tolosano, Cell-specific regulation of Ferroportin transcription following experimentally-induced acute anemia in mice, *Blood Cells, Mol., Dis.*, 2013, **50**, 25–30, DOI: 10.1016/j.bcmd.2012.08.002.

68 T. Ganz, Systemic iron homeostasis, *Physiol. Rev.*, 2013, **93**, 1721–1741, DOI: 10.1152/physrev.00008.2013.

69 T. Ganz and E. Nemeth, Hepcidin and disorders of iron metabolism, *Annu. Rev. Med.*, 2011, **62**, 347–360, DOI: 10.1146/annurev-med-050109-142444.

70 Y. J. Kang, H. Wu and J. T. Saari, Alterations in Hypertrophic Gene Expression by Dietary Copper Restriction in Mouse Heart, *Proc. Soc. Exp. Biol. Med.*, 2000, **223**, 282–287, DOI: 10.1111/j.1525-1373.2000.22340.x.

71 D. M. Medeiros, J. Davidson and J. E. Jenkins, A unified perspective on copper deficiency and cardiomyopathy, *Proc. Soc. Exp. Biol. Med.*, 1993, **203**, 262–273.

72 E. P. Beltroy, J. A. Richardson, J. D. Horton, S. D. Turley and J. M. Dietschy, Cholesterol accumulation and liver cell death

in mice with Niemann–Pick type C disease, *Hepatology*, 2005, **42**, 886–893, DOI: 10.1002/hep.20868.

73 E. P. Beltroy, B. Liu, J. M. Dietschy and S. D. Turley, Lysosomal unesterified cholesterol content correlates with liver cell death in murine Niemann–Pick type C disease, *J. Lipid Res.*, 2007, **48**, 869–881, DOI: 10.1194/jlr.M600488-JLR200.

74 M. Valko, H. Morris and M. T. Cronin, Metals, toxicity and oxidative stress, *Curr. Med. Chem.*, 2005, **12**, 1161–1208.

75 A. Pal, Copper toxicity induced hepatocerebral and neurodegenerative diseases: An urgent need for prognostic biomarkers, *Neurotoxicology*, 2013, **40C**, 97–101, DOI: 10.1016/j.neuro.2013.12.001.

76 K. H. Weiss, F. Thurik, D. N. Gotthardt, M. Schafer, U. Teufel, F. Wiegand, U. Merle, D. Ferenci-Foerster, A. Maieron, R. Stauber, H. Zoller, H. H. Schmidt, U. Reuner, H. Hefter, J. M. Trocello, R. H. Houwen, P. Ferenci and W. Stremmel, Efficacy and safety of oral chelators in treatment of patients with Wilson disease, *Clin. Gastroenterol. Hepatol.*, 2013, **11**, 1028–1035, DOI: 10.1016/j.cgh.2013.03.012e1-2.