Defective iron homeostasis and hematological abnormalities in Niemann-Pick disease type C1 [version 2; peer review: 2 approved]

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Abstract

Background: Niemann-Pick disease type C1 (NPC1) is a neurodegenerative lysosomal storage disorder characterized by the accumulation of multiple lipids in the late endosome/lysosomal system and reduced acidic store calcium. The lysosomal system regulates key aspects of iron homeostasis, which prompted us to investigate whether there are hematological abnormalities and iron metabolism defects in NPC1.

Methods: Iron-related hematological parameters, systemic and tissue metal ion and relevant hormonal and proteins levels, expression of specific pro-inflammatory mediators and erythrophagocytosis were evaluated in an authentic mouse model and in a large cohort of NPC patients.

Results: Significant changes in mean corpuscular volume and corpuscular hemoglobin were detected in Npc1−/− mice from an early age. Hematocrit, red cell distribution width and hemoglobin changes were observed in late-stage disease animals. Systemic iron deficiency, increased circulating hepcidin, decreased ferritin and abnormal pro-inflammatory cytokine levels were also found. Furthermore, there is evidence of defective erythrophagocytosis in Npc1−/− mice and in an in vitro NPC1 cellular model. Comparable hematological changes,
including low normal serum iron and transferrin saturation and low cerebrospinal fluid ferritin were confirmed in NPC1 patients.

**Conclusions:** These data suggest loss of iron homeostasis and hematological abnormalities in NPC1 may contribute to the pathophysiology of this disease.

**Keywords**
Niemann-Pick disease type C, iron, haematology, lysosome, lysosomal storage diseases
Iron is an essential element required as a cofactor for many metalloproteins including hemoglobin (HGB), myoglobin and cytochromes. Systemic iron homeostasis is achieved by controlling the level of circulating iron via its deposition and release from hepatic stores so as to prevent detrimental iron deficiency or excess. It is also influenced by the demand for erythropoiesis and conditions of inflammation and infection. Disruption of systemic iron homeostasis impairs erythropoiesis and systemic oxygen utilization; therefore, mammals have evolved complex absorption, recycling, distribution and storage mechanisms to regulate systemic iron metabolism.

The lysosome degrades and recycles macromolecules, including iron regulators, transporters and storage proteins. Cellular iron release from transferrin (Tf) endocytosis, as well as that derived from ferritinophagy, the autophagic process through which intracellular iron is increased via lysosomal degradation of ferritin and mitophagy, the selective degradation of damaged mitochondria takes place in the endosome/lysosome system. Iron is subsequently released from the lysosome and distributes it to other subcellular organelles, e.g., mitochondria. Erythrophagocytosis recycles iron from heme derived from senescent red cells that are ingested by tissue macrophages and delivered into the phago-lysosomal pathway.

Niemann-Pick disease type C1 (NPC1) is a lysosomal storage disorder, caused by mutations in either NPC1 (95% of cases) or NPC2 and occurs at a frequency of approximately 1:120,000 live births. The exact biological functions and molecular interactions of the NPC1 and NPC2 proteins remain elusive, however, the disease is characterized by reduced calcium ion content of the lysosome (acidic store calcium) and accumulation of un-esterified cholesterol and sphingolipids in the late endosomal/lysosomal system that result in part from failure in lysosome: endosomal/lysosomal system (ER) contact site formation. NPC1 typically presents as a progressive neurodegenerative disease of infancy/childhood, but adulthood onset forms have been described.

In the current study, we investigated hematological changes and iron metabolism in an authentic murine model of NPC1 (Npc1<sup>−/−</sup>) and in NPC1 patients. We found low serum iron, HGB and mean corpuscular HGB (MCH) in Npc1<sup>−/−</sup> mice and low iron and Tf saturation levels in NPC1 patients. Furthermore, the decreased systemic iron in Npc1<sup>−/−</sup> mice correlates with systemic inflammation, significantly increased circulating hepcidin levels and impaired phagocytic clearance of erythrocytes. Comparable hematological changes and cerebrospinal fluid (CSF) ferritin deficiency were detected in NPC1 patients. These studies suggest that loss of systemic iron homeostasis induces hematological changes in NPC1 via multiple mechanisms and that NPC1 patients may be at risk of iron deficiency.

**Methods**

**Reagents**
Reagents were from Sigma-Aldrich unless otherwise specified.

**Animals**
Niemann-Pick disease type C1 mice (BALB/cNctr-Npc1<sup>−/−</sup>; Npc1<sup>−/−</sup>) were housed at the University of Oxford. Food and water were available ad lib. Iron content in the diet was 200 mg/kg (Teklad Global 16% protein rodent diet, Harlan Laboratories). Animal studies were authorized by the UK Home Office (Animal Scientific Procedures Act, 1986). Npc1<sup>−/−</sup> mice have a lifespan of 10–12 weeks (average 10.5 weeks) with neurological symptoms presenting from seven weeks of age. Mice were sampled at multiple ages; early pre-symptomatic (three-weeks-old), pre-symptomatic (five-weeks-old), early-symptomatic (seven-weeks-old), late-symptomatic (nine-weeks-old) stage and late end stage (eleven-weeks-old). Male animals were used in all studies, except where indicated.

All experiments involving animals were conducted under the authority of project licence number PPL P8088558D, approved...
by the University of Oxford Animal Welfare and Ethical Review Body and granted by the United Kingdom Home Office (Animal Scientific Procedures Act, 1986). Animals were housed in the Biomedical Research Services facilities, University of Oxford. All licensed procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. All efforts were made to ameliorate any suffering of animals including adapting water and food provision during the symptomatic phase of the Npc1 deficient mice used in this study. The study design for each investigation involving the use of animals or animal-derived tissues adhered to criteria designated by the ARRIVE Essential Checklist. Mice were generated from in-house breeding colonies, genotyped, sex and age matched and allocated randomly for investigation. Where possible, mice of the two genotypes were co-housed.

Mouse hematological analysis
Mice were sacrificed at the indicated ages using approved Schedule 1 methods including overdose of anaesthetic. Blood (volumes < 2ml) was collected by cardiac puncture. Multiple hematological parameters were determined using a Pentra ES 60 system blood analyzer (HORIBA-ABX). Blood samples were collected from mice and analyzed immediately. Blood smears were stained with Wright-Giemsa reagent, examined on a Zeiss Axiosplan 2 microscope and images captured using Axiovision 2.0 software.

Mouse serum preparation and iron tissue determinations
Blood samples were collected and allowed to clot, spun at 3,000 rpm for 15 minutes and serum stored at -20°C. Sera were diluted 200-fold with 1% nitric acid at 70°C overnight. Mouse tissues were flash frozen and digested in nitric acid (69%). Samples were diluted 50-fold in water (VWR, 83877.290). Total elemental iron was measured by inductively coupled plasma mass spectrometry (ICP-MS) as previously described34. In brief, tissues were heat-digested completely in nitric acid and metal content quantified on a Thermo Finnigan Element 2 Sector-Field ICP-MS. Rhodium (1ng/g) was spiked into each sample as an internal standard. Iron concentrations were normalized to starting tissue weight.

Hepcidin measurements
Blood samples were collected, serum prepared and hepcidin levels were determined using the Hepcidin Murine-Compete ELISA kit according to the manufacturer’s instructions (Intrinsic Life Sciences).

Western blotting
Tissues or serum were collected, and lysates prepared by homogenization in cell lysis buffer (Cell Signaling Technology) containing protease inhibitors (Complete EDTA-free protease inhibitor cocktail, Merck) on ice, followed by centrifugation to remove insoluble protein. Protein concentration of supernatants was determined by BCA assay (Sigma). Appropriate volumes of lysates were mixed with SDS Blue loading buffer (BioLabs) and heated to 95°C for 5 min and then rapidly cooled on ice. Samples were loaded onto NuPAGE™ Bis-Tris Gels (ThermoFisher) and run in NuPAGE™ MOPS SDS running buffer (ThermoFisher). Novex Sharp Pre-stained protein standard (ThermoFisher) was used to indicate the extent of protein migration and specific protein mass. Gels were transferred onto Immuno-Blot PVDF membrane (Bio-Rad) using BIORAD transblot turbo transfer system (Bio-Rad). Membranes were blocked with 5% skimmed milk in PBS with 0.1% Tween 20 (Sigma) for 1h at room temperature and then incubated with primary antibody (rabbit polyclonal anti-mouse light chain ferritin (L-ferritin) antibody was from Abcam (ab69090), used at 1: 1000; rabbit anti-mouse soluble transferrin receptor (sTfrc) polyclonal antibody was from Fisherscientific (17278842), used at 1: 500, diluted in PBS containing 2.5% skimmed milk with 0.1% Tween 20 and sodium azide overnight at 4°C. Membranes were washed with PBS containing 0.1% Tween 20 three times for 20 min each and then incubated with HRP-conjugated donkey anti-rabbit polyclonal antibody from Jackson Immunochemicals (711-035-152), diluted to 1:5000 in PBS containing 2.5% skimmed milk and 0.1% Tween 20 for 1h at room temperature. Membranes were washed as before and then developed with SuperSignal West Femto substrate (ThermoFisher) or Pierce ECL western blotting substrate (ThermoFisher). Membranes were re-probed with anti-b-actin antisera (Sigma) to evaluate equivalent protein loading. Images were obtained using a ChemiDoc XRS system (Bio-Rad) and processed and analyzed with ImageLab 5.1 software (Bio-Rad) with BioRad Universal Hood.

Q-PCR
Mouse tissues were snap frozen in liquid nitrogen. RNA was isolated using RNaseasy kits (Qiagen) according to manufacturer’s protocol and quantified using Nanodrop spectrophotometer (Thermo Scientific). cDNA generated with iScript cDNA synthesis reagents (Bio-Rad) and duplicate qPCR reactions set up with 5ng template cDNA/RNA, PowerUP SYBR Green Master Mix (Thermo Fisher Scientific) and specific primers (Table 1). Reactions were run on CFX96 Real-Time PCR Detection System (Bio-Rad) with amplification of HPRT as internal housekeeping control. Cycling conditions were: UDG activation, 50°C for 2 min; Dual-Lock DNA polymerase, 95°C for 2 min and 40 cycles of denaturation at 95°C for 15 sec and anneal/extend at 60°C for 30 sec. Gene expression levels were calculated from Ct values using comparative Ct methodology and plotted as relative to the HPRT control.

Histology and immunohistochemistry
Mice were sacrificed by Schedule 1 protocol, perfused with 4% paraformaldehyde, tissues removed and embedded and 4 µm paraffin sections stained with hematoxylin/eosin and Masson trichrome images were collected on a Zeiss Axioskop 2 microscope using Axiovision 2 software.

Flow cytometry
Single-cell spleen suspensions from nine-week-old mice were prepared by physical disruption and passage through single cell strainers (Fisher) and stained with PE-conjugated anti-mouse TER-119 (BD Bioscience, 553673; used at 5mg/ml) and
Table 1. Details of primers used for Q-PCR analysis.

| Name           | Sequence                  | Product size |
|----------------|---------------------------|--------------|
| mTfrc set2F    | TCCGCTCGTGGAAGACTACTTT   | 140bp        |
| mTfrc set2R    | ACATAGGCGGACAGGAGTGG     |              |
| mFerroportin F | TGGAGTTCGTGCACACCATGAT   | 161bp        |
| mFerroportin R | TGGGACACGCTGCACACCATGAT  |              |
| mTNFalpha F    | CCCTCACTCAGTATCCTT       | 61bp         |
| mTNFalpha R    | GCTACGACGTCGGCTCAG       |              |
| mIL-1alpha F   | CGCTTGAGTCGGCAAAAGAAA    | 107bp        |
| mIL-1alpha R   | AGATGGTCAATGGGAACTGT     |              |
| mIL1beta F     | AAGAGAAACAAGACACGAAAA    | 213bp        |
| mIL1beta R     | TGGGAAACCTCGAGCAGACTAATT |           |
| mHPRT F        | CAAACTTTGCTTTCCTTGTT     | 101bp        |
| mHPRT R        | TCTGCGCTGTATCCCAACCTTC   |              |
| mACTB F        | GGCCTGATTTCCTCCATCG      | 154bp        |
| mACTB R        | CCAGTTGGAACATTGCAACTGT   |              |
| NPC1wt F       | TCTACGCTGTACCAACACACACA  | 112bp        |
| NPC1wt R       | AACACCCTGGCTCAGAAATG     |              |

FITC-conjugated anti-mouse CD71 antibodies (BioLegend, 113805, used at 10μg/ml) and labelled using LIVE/DEAD Cell Viability Assay Kit (ThermoFisher L34955). Live cell data were acquired on a FACSCanto II Flow Cytometer (BD Biosciences) and analysed using FlowJo 10.2 software, LLC.

In vitro Ox-RBC phagocytosis assay
RAW 264.7 MΦ were obtained from ATCC and maintained in RPMI 1640 (Sigma) containing 10% (v/v) foetal bovine serum, 1% penicillin-streptomycin and 1% L-glutamine. Cells were passaged at regular intervals to maintain viability > 90%. Cells were treated with vehicle (DMSO) or 2 μg/ml U18666A (Merck) for 24 h prior to erythrophagocytosis assay. Sheep red blood cells (TCS Biochemicals) were labelled with CellTracker Green CMFDA Dye (Invitrogen), incubated with 0.2 mM CuSO₄ and 5 mM ascorbic acid for 1 h, overlaid onto MΦ plated onto glass coverslips and co-incubated at 37°C. At the times indicated, non-ingested erythrocytes were removed by washing, phagocytes fixed and examined by confocal microscopy (Leica TPC SP8) running Leica Application Suite software (LAS X) and images analyzed using ImageJ (NIH, USA). Ingestion by a minimum of 300 MΦ was determined for each condition.

Patient sample collection
NPC1 patients were enrolled in a longitudinal observational study at the National Institutes of Health, Bethesda, USA approved by the NICHD Institutional Review Board (06-CH-0186). Written informed consent, and assent was obtained as appropriate. Diagnosis was established by biochemical testing/mutation analysis. Phenotypic severity was determined using the annualized severity increment score developed by Yanjanin et al., that measures symptoms in nine major and eight minor clinical areas, which are primarily neurological. Scores ranged from one to 35 (max severity on this scale is 50). Serum samples were excluded from this study if they were collected from patients with a history of splenectomy (n=2) or thalassemia (n=1). Blood was analyzed immediately at the NIH Clinical Center Department of Laboratory Medicine (DLM). Iron, transferrin and percent saturation were measured using the Dimension Vista® System 1500 at the NIH DLM. Data from only one sampling time point was used for each individual participant. CSF samples were obtained by lumbar puncture within the L4/L5 interspace. CSF was stored at -80°C prior to assay by Medical Neurogenetics, LLC. STROBE reporting guidelines were adhered to in the observational studies.

Patient soluble-transferrin receptor, C-reactive protein and ferritin analysis
Patient blood samples were collected and processed to obtain serum. sTfR was measured using Quantikine ELISA Kit (R&D Systems) according to the manufacturer’s protocol. Plasma C-reactive protein (CRP), MULTIGENT CRP Vario Kit, with high sensitivity calibrators and ferritin (Architect Ferritin Assay) were analyzed using the Abbott Architect 2000R automated analyzer (Abbott Laboratories) at Birmingham Heartlands hospital (Birmingham, UK).

Enzyme linked immunosorbent assay (ELISA) for human TNF-α
Serum TNF-α levels of 22 patients and 14 control individuals of comparable age and gender distribution were measured by ELISA (Thermo Scientific) according to the manufacturer’s instructions.

CSF ferritin analysis
CSF was collected from patients as part of their clinical evaluation. CSF ferritin was measured using a human ferritin ELISA kit (Abnova) according to the manufacturer’s instructions.

Statistical analysis
Data are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 9 (Dotmatrics). Unpaired two-tailed Student’s t test or ANOVA were used to determine significance. p < 0.05 were considered significant. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Results
Altered erythrocyte parameters and erythrocyte morphology in Npc1-/- mice
To investigate the impact of lysosomal dysfunction on erythropoiesis, we measured multiple erythrocyte-related parameters in Npc1-/- mice at different stages of disease progression (five weeks, pre-symptomatic; seven weeks, early symptomatic; nine weeks, late symptomatic and eleven weeks, end
stage). Mean corpuscular volume was significantly decreased in Npc1−/− mice at seven-weeks-of-age and remained lower at all later time points (p < 0.0001; n=10–20) (Figure 1Ai). Corpuscular hemoglobin (MCH) was significantly lower from five-weeks-of-age and was decreased at all subsequent ages (p < 0.0001; n=10–18) (Figure 1Aii). Hematocrit (HCT) was significantly reduced at eleven-weeks-of-age (p < 0.0001; n=10) (Figure 1Bi); red cell distribution width (RDW) was increased (p < 0.0001; n=9–10) (Figure 1Bii) and HGB reduced (p < 0.0001; n=10) (Figure 1Biii) but were not significantly different at earlier ages. Mean corpuscular hemoglobin concentration (MCHC) and red blood cell number were broadly unchanged at all time points (Extended Figure 1). Wright-Giemsa staining revealed that blood from nine-week Npc1−/− mice contained erythrocytes which were microcytic and irregularly shaped (Figure 1C).

Decreased serum iron and increased sTfR in Npc1−/− mice

Compared with age-matched control littersmates, nine-week-old Npc1−/− mice had significantly lower serum iron (p < 0.05; n=5) (Figure 2Ai). Serum sTfR was significantly increased (approximately 12-fold) in nine-week-old Npc1−/− mice (Figures 2Aii and iii).

Reduced hepatic L-ferritin, increased hepatic transferrin receptor (Tfrc) and increased duodenal ferroportin (fpn) expression

To better understand tissue iron homeostasis, we quantified hepatic iron, L-ferritin and Tfrc and duodenal fpn expression. Although hepatic iron was significantly reduced in seven-week-old Npc1−/− mice (p < 0.01; n=3), there was no significant difference between genotypes in older mice (Figure 2Bi). Brain iron was not significantly changed (Extended Figure 2). L-ferritin content of liver and spleen was significantly reduced in nine-week-old Npc1−/− mice (Figures 2Ci and ii). Hepatic expression of Tfrc mRNA was significantly higher (p < 0.05; n=8) (Figure 2Di) as was fpn in the duodenum (p < 0.05; n=8) (Figure 2Dii). There was no significant difference in the iron content of brains taken from Npc1−/− and Npc1−/− mice at all time points examined (Extended Figure 2).

Significantly increased systemic hepcidin and hepatic pro-inflammatory cytokines in Npc1−/− mice

In light of decreased serum iron, we measured systemic hepcidin, which has a central role in systemic iron regulation. Hepcidin levels were increased significantly in male and female Npc1−/− mice at seven-weeks (p < 0.01 and p < 0.005 respectively, n=5) and at nine-weeks-of-age (p < 0.0001 and p < 0.05 n=5) (Figure 3A).

As hepcidin is induced by inflammatory mediators we measured hepatic expression of specific pro-inflammatory cytokines. Transcription of TNFα was significantly greater at five and eight weeks-of-age (p < 0.01 n=8), (Figure 3Bi), IL-1β was not changed (Figure 3Bii), but IL-1α was significantly elevated in pre-symptomatic and symptomatic Npc1−/− mice (p < 0.005 and p < 0.01, n=8), (Figure 3Biii).

Splenomegaly and hepatomegaly altered erythropoiesis and disrupted splenic organization in Npc1−/− mice

Anaemia affects erythropoiesis and we therefore examined the erythropoietic compartment in mutant mice. Nine-week-old Npc1−/− mice exhibited splenomegaly (p < 0.005; n=5) (Figures 4Ai and ii). Livers from nine-week-old Npc1−/− mice were paler in appearance and had significantly greater wet weight mass (p < 0.01, n=8) (Figures 4Ai and iii). We analyzed the frequency and maturation of splenic erythroid lineage cells by flow cytometry (Figure 4Bi). At nine-weeks-of-age, there was a significant increase in Ter119+ erythroblasts in Npc1−/− mice (p < 0.01, n=8) consistent with enhanced erythropoiesis (Figure 4Bi). There were significantly more orthochromatic erythroblasts (Ter119+ CD71−; Figure 4Bi, Region IV) indicating a higher frequency of nucleated red cells in Npc1−/− mice (p < 0.01 n=7–9) (Figures 4Bi and iii). However, maturation of splenic pro-erythroblasts into chromatophilic erythroblasts was unaltered (Figure 4Bi, gates I–III). Furthermore, histochemical staining revealed disorganized splenic architecture. Lymphoid follicles appeared relatively normal albeit with evidence of vacuolated cells. In contrast, red pulp regions were disorganized and contained large numbers of vacuolated cells (Figure 4Ci).

Impaired erythrophagocytosis in Npc1−/− mice and a cellular model of NPC1 disease

Aged erythrocytes are cleared by reticuloendothelial macrophages and defective phagocytic clearance has the potential to impact upon iron recycling. Foamy macrophages were apparent in nine-week-old Npc1−/− liver (Figure 4Gi, panels e and f) but were absent from controls (Figure 4Gii, panels b and c). Non-ingested erythrocytes were frequently observed in close proximity to foamy macrophages in Npc1−/− mice (Figure 4Gii, panel f). In light of these data suggesting defective clearance of senescent RBCs in vivo, we used an in vitro phagocytosis assay to investigate phagocytosis of red cells. RAW 264.7 murine macrophages treated with U18666A, which inhibits NPC119 ingested significantly fewer oxidized sRBC (Ox-sRBC) than vehicle-treated macrophages (10 min, p < 0.05; 20 min, p < 0.005; 30 min, p < 0.0001; 40 min, p < 0.005, n=200) (Figures 4Di and ii), in line with what was observed in Npc1−/− liver.

Multiple hematological parameters tend towards the lower end of the normal range in NPC1 patients

To ascertain whether evidence of systemic iron dysregulation and hematological abnormalities in Npc1−/− mice were relevant clinically, blood samples from NPC1 patients were analyzed. A total of 114 NPC1 patients were examined of which 54 were male and 60 were female. Fifty-two patients (45%) were taking off-label miglustat (Zavesca). Thirty-eight (33%) were taking a multi-vitamin containing iron. Exclusion of these subjects yielded results similar to when they were included. Although none of the values for MCV, HCT and MCH and HGB in NPC1 patients were statistically different from normal values many were clustered at the lower end of the normal range or were within the lower half (Figures 5A–D).
Figure 1. Altered erythrocytic indices and erythrocyte morphology in Npc1<sup>-/-</sup> mice. A. Histograms showing significantly lower mean corpuscular volume (i) and mean corpuscular hemoglobin (ii) in five-week, seven-week, nine-week and eleven-week-old Npc1<sup>-/-</sup> mice. B. Histograms representing reduced hematocrit (i), increased red cell distribution width (ii) and decreased hemoglobin (iii) in eleven-week Npc1<sup>-/-</sup> mice. Data shown are mean ± SEM, n=7–20 mice per group. * p < 0.05 ** p < 0.01 *** p < 0.001 **** p < 0.0001. 1-way ANOVA with Tukey’s. C. Wright-Giemsa stain of peripheral blood smears from nine-week-old male and female Npc1<sup>+/+</sup> and Npc1<sup>-/-</sup> mice. Arrowheads indicate examples of erythrocytes with altered morphology. Scale bar represents 10 μm. Images of blood smears were taken on Zeiss Axiosplan 2 microscope and captured using Axiovision 2.0 software. Data are representative of three independent experiments.
Figure 2. Decreased systemic iron, increased serum sTfrC, transiently reduced hepatic iron and lower L-ferritin and enhanced tfrc and fpn expression in Npc1−/− mice. Serum iron levels are significantly lower in nine-week-old Npc1−/− mice than in age-matched control animals (i) whereas serum sTfrC are enhanced. Iron data are mean ± SEM, * p < 0.05, ** p < 0.01 n=8 per group, unpaired t test with Welch's correction. Data are representative of two independent experiments. Western blot of serum protein samples, (ii) quantification of specific protein bands. Data are mean ± SEM, ** p < 0.01 n=4 per group, unpaired t test with Welch's correction. (iii). B. Hepatic iron is significantly reduced in seven-week-old Npc1−/− mice (filled circles) but not different from controls (filled triangles) at three and eleven weeks of age. Data are mean ± SEM, **** p < 0.0001 n=5–8 per group, 2-way ANOVA (i). C. Reduced ferritin content of liver and spleen in nine-week-old Npc1−/− mice. Western blot of liver and spleen lysates (i) quantification of specific bands (ii) Data are mean ± SEM, ** p < 0.01 **** p < 0.0001 n=2–4 per group, unpaired t test with Welch's correction. Data are representative of two independent experiments. Arrowheads indicate molecular mass of specific proteins. D. Increased hepatic Tfrc (i) and duodenal fpn (ii) transcripts in symptomatic Npc1−/− mice (filled columns) as compared to Npc1+/+ mice (open columns). Data are mean ± SEM, * p < 0.05 *** p < 0.001 n=8 per group. Data are representative of three independent experiments.
Serum iron, iron saturation, serum ferritin and Tf saturation are significantly different in NPC1 patients. Serum iron was significantly lower in NPC1 patients (p < 0.01, n=105), as was iron saturation (p < 0.05, n=104) and Tf saturation (p < 0.05, n=104) (Figures 6A, B and D). Serum ferritin was significantly elevated in patients (p < 0.005, n=100) (Figure 6E). None of these parameters correlated with disease severity (Extended Figure 3). Serum transferrin was not significantly different between the two populations (Figure 6C). Although serum TNFα in NPC1 patients was slightly elevated, in 6 of the 20 NPC1 patients analyzed, values were not statistically different from age-matched controls (Figure 6F). TNFα values for two patients and one control were below limit of detection. Plasma CRP was not different between the two groups (Figure 6G).

Ferritin and transferrin levels are significantly different in NPC1 patient cerebrospinal fluid (CSF). We then analyzed CSF to evaluate iron metabolism in the CNS. With the exception of a single individual, all controls had measurable levels of CSF ferritin, whereas all NPC1 patients were below the assay detection threshold (p < 0.05, n=5) (Figure 7A). Transferrin levels in patient CSF was significantly higher than in controls (p < 0.01, n=58 and n=30 respectively).

Discussion
Iron homeostasis is achieved through the coordinated activities of multiple proteins that regulate metal ion uptake, storage, efflux, and recycling, in addition to systemic hormonal regulation. Disruption of lysosome-dependent activities in
Figure 4. Hepatosplenomegaly, altered erythropoiesis, perturbed splenic architecture and evidence of impaired erythrophagocytosis in Npc1−/− mice. A. Npc1+/+ mice display increased spleen and liver mass. (i). Representative images of spleen and liver from nine-week-old Npc1+/+ and Npc1−/− mice. Histograms of spleen (ii) and (iii) liver masses from Npc1+/+ (open columns) and Npc1−/− animals (filled columns). Data are means ± SEM, ** p < 0.01 *** p < 0.001 n=5. unpaired t test with Welch’s correction. B. Perturbed erythropoiesis in Npc1−/− mice. (i) Representative FACS profiles of splenic cells from nine-week-old Npc1+/+ (left panel) and Npc1−/− mice (right panel) stained with anti-CD71 and anti-Ter119 specific antibodies. Gates indicate position of I, proerythroblasts; II, basophilic; III, polychromatic and IV orthochromatic populations. (ii) Quantification of Ter119+ cell frequencies. Mean ± SEM *** p < 0.001 n=4–8 per group. unpaired t test with Welch’s correction. (iii) frequencies of sub-populations I-IV. ** p < 0.01 **** p < 0.0001 n=7–9 per group. unpaired t test with Welch’s correction. Data are representative of three independent experiments. C. Altered splenic architecture and presence of foamy macrophages and nearby erythrocytes in livers from nine-week-old Npc1−/− mice. (i) Representative images of Masson trichrome stained spleen sections from nine-week-old Npc1+/+ (panels a-c) and Npc1−/− (panels d-f) mice. Scale bar: panels a and d, 250 μm; b and e, 100 μm; c and f, 25 μm. (ii). Hematoxylin/eosin-stained liver sections from nine-week-old Npc1+/+ (panels a–c) and Npc1−/− mice (panels d–f). Arrowheads indicate examples of macrophages with foamy appearance; arrows indicate erythrocytes in close proximity to foamy macrophages. Scale bar: panels a and d, 50 μm; b, c, e, and f, 25 μm. Images were captured with a Zeiss Axioplan 2 microscope using Axiovision 2.0 software. D. Impaired in vitro phagocytosis of oxidized sheep erythrocytes by U18666A-treated RAW 264.7 macrophages. (i) Representative confocal microscopy images of vehicle-treated (left panel) and U18666A-treated RAW 264.7 macrophages (right panel) that have been co-incubated with oxidized sheep red blood cells. Arrows indicate examples of internalized erythrocytes (green). Cyan represents actin staining. Scale bar; 20 μm. Cells were imaged on a Leica TCS SP8 confocal microscope with LAS X software (ii). Frequencies of oxidized sheep erythrocytes internalized by vehicle-treated (open columns) and U18666A-treated (filled columns) RAW 264.7 macrophages. Mean ± SEM, n=minimum of 3 x 100 cells counted for each treatment. Data is representative of three independent experiments. * p < 0.05, *** p < 0.001, **** p < 0.0001. unpaired t test with Welch’s correction.
NPC1 has the potential to affect several of these processes. We therefore explored iron metabolism in NPC1 at multiple levels in an authentic murine model and a large cohort of patients (> 100 individuals) to obtain a broad picture of clinical phenotypes and identify specific abnormalities.

Here, we report multiple hematological changes in Npc1−/− mice that include erythrocyte and hemoglobin-related parameters, systemic iron deficiency, abnormal erythropoiesis and impaired erythrophagocytosis, which confirm disrupted iron homeostasis. NPC1 patients exhibited milder erythrocytic phenotypes, including low serum iron and Tf saturation and increased serum ferritin (Table 2). This differential severity is most likely explained by the disparity between residual NPC1 activities in the two species; patients have mutations in NPC1 that encode partially functional proteins, whereas the mouse model is null for activity. However, genetic diversity may also impact upon the differential disease severity between mice and patients: Npc1−/− mice were all on an identical genetic background (Balb/c) and unidentified modifier genes may have the potential to affect patient phenotypes. The evidence of impaired iron homeostasis in NPC1 is in agreement with our previous study and independent findings reported by Bush and colleagues that suggest altered transition metal homeostasis in NPC1 mice and patients. Here, we provide important mechanistic insights and identify several pathophysiological mechanisms that may be responsible for altered systemic iron metabolism in NPC1 disease.

Npc1−/− mouse erythrocytes were microcytic and exhibited significantly decreased MCV and HCT, features that have been documented for iron deficiency anemia, thalassemia and
Figure 6. NPC1 patients have significantly lower serum iron, iron and transferrin saturation, but increased ferritin levels. Plots of patient A. serum iron, B. iron saturation, C. serum transferrin, D. transferrin saturation, E. serum ferritin F. Systemic TNFα and G. systemic C-reactive protein. Mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001 unpaired t test with Welch’s correction. For serum iron, n=105 for NPC1 patients, 39 for controls; iron saturation, n=104 for NPC1, n=38 for controls; serum transferrin, n=104 for NPC1, 39 for controls; transferrin saturation, n=39 for control group, 105 for NPC1 patients; serum ferritin, n=20 for control and 100 for NPC1, serum TNF-a n=11 for control and 20 for NPC1 and for C-reactive protein, n=15 for control group (mean age 12.7 ± 5.8 years), 18 for NPC1 patients (mean age 11.6 ± 7.9 years. Dashed lines indicate values at the limits of the normal range of the various parameters.\textsuperscript{30,31}. 
Figure 7. Ferritin deficiency and increased transferrin in the CSF of NPC1 patients. Plots of A. CSF ferritin and B. CSF transferrin levels in control and NPC1 patients. Mean ± SEM, * p < 0.05, ** p < 0.01. unpaired t test with Welch's correction. n=5 for ferritin determinations, n=30, n=58 for control and NPC1 patients for transferrin determinations.

Table 2. Hematological pathologies shared between Npc1−/− mice and NPC1 patients.

| Parameter                  | Npc1−/− mouse | Patient                        |
|----------------------------|---------------|--------------------------------|
| MCV                        | Decreaseda    | Lower end of normal range      |
| MCH                        | Decreaseda    | Lower end of normal range      |
| HCT                        | Decreasedb    | Lower end of normal range      |
| RDW                        | Increasedb    | Lower end of normal range      |
| HGB                        | Decreasedb    | Lower end of normal range      |
| Microcytic erythrocytes    | Yes           | Yes or absentc,d                |
| Serum iron                 | Decreased     | Decreaseda                     |
| sTfrc                      | Increased     | NR                             |
| Serum ferritin             | Increased     | Increased                      |
| Liver ferritin             | Reduced       | Reducedc,d                      |
| Hepatosplenamegaly         | Yes           | Yes or absentc,d                |

*a.* pre-symptomatic to late symptomatic mice; *b.* late symptomatic mice; *c.* 35; *d.* 36; *e.* 34; *f.* 37.

Abbreviations: MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; HCT, hematocrit; RDW, red cell distribution width; HGB, hemoglobin; sTfrc, soluble transferrin receptor; NR, not reported.

anemia of chronic diseases³⁸. However, we cannot exclude the possibility that altered cholesterol and sphingolipid composition of the plasma membrane is responsible for the structural defects in Npc1−/− erythrocytes. Four pediatric patients evaluated by Christomanou and colleagues³⁶ did not display microcytic hypochromic anemia, but the same authors report anecdotally other NPC1 patients who did show these clinical features³⁵, confirming hematological heterogeneity in this lysosomal storage disorder. Furthermore, a case report of a 29-year-old juvenile-onset patient found mild iron deficiency anemia³⁹. Although only some NPC1 patient erythrocyte phenotypes were altered, they were less acute than those of the mouse model and tended towards the lower end of the normal range, consistent with possible susceptibility to anemia. Monitoring of reticulocyte cellular indices is the basis for early diagnosis of propensity to develop anemia³⁸,⁴¹.
To characterise further the status of iron homeostasis we measured serum iron levels and expression of hemoglobin-associated molecules. Symptomatic mice and NPC1 patients had significantly reduced serum iron, but in accordance with a less acute phenotype, patient sTfrc was not significantly different from controls, but was significantly higher in Npc1<sup>−/−</sup> mice. Hung et al.<sup>34</sup> did not report changes in plasma iron in Npc1<sup>−/−</sup> mice, but analysed mice appreciably younger (three-weeks and seven-weeks of age) than animals examined here (nine-weeks of age). These authors did however confirm a significant reduction in plasma iron in patients<sup>34</sup>.

Hepatic expression of L-ferritin was significantly reduced in Npc1<sup>−/−</sup> mice, as has been described previously in patients<sup>35,36</sup>, consistent with functional iron deficiency. Analysis of the relative amount of hepatic iron in Npc1<sup>−/−</sup> mice did not reveal a consistent relationship; whilst there was a significant decrease at seven weeks, comparable to the findings of Hung et al.<sup>34</sup>, we found no significant difference at later time points. The deficiency in total liver iron in Npc1<sup>−/−</sup> mice is likely to be less pronounced because of hepatomegaly. It may be that altered distribution of iron within tissues, rather than changes in total levels, results in functional deficiency.

Low serum iron likely results from systemic iron dysregulation and systemic pro-inflammatory responses in NPC1. The peptide hormone hepcidin is a critical regulator of systemic iron via its inhibition of iron adsorption in the duodenum and release from macrophages and hepatocytes<sup>1</sup>. Hepcidin production is regulated primarily by three mechanisms: an iron-regulated pathway, an inflammatory pathway and erythropoiesis. Hepcidin synthesis is stimulated by high iron stores as well as by specific pro-inflammatory molecules, whereas increased erythropoiesis suppresses its production<sup>28</sup>. Circulating levels of hepcidin were significantly higher in symptomatic and late-symptomatic Npc1<sup>−/−</sup> animals, which is in agreement with the up-regulation of specific immune and pro-inflammatory molecules such as IL-1α that occurs during disease progression. IL-6 has been shown to induce the synthesis of hepcidin<sup>35</sup>. Whilst enhanced secretion of the cytokine by patient derived fibroblasts in culture has been documented<sup>41</sup>, to our knowledge there are no published data of systemic levels in vivo. NPC1 has a unique combination of iron-related characteristics because it shares commonalities with both iron deficiency anaemia and inflammatory induced anaemia and other LSDs such as GM2 gangliosidosis (Table 3). This would suggest that inflammation alone is unlikely to cause loss of iron homeostasis in NPC1. Inflammation may be secondary to loss of lysosomal homeostasis and induction of cellular iron deficiency, as in the case of lysosome alkalinisation<sup>44</sup>. It would be of interest to investigate whether amelioration of specific inflammatory mediators can restore iron homeostasis and normalize hepcidin levels and separately, whether neutralisation of hepcidin activity<sup>45</sup>.

### Table 3. Comparison of iron-related parameters in NPC1 disease with other disorders.

| Parameter                  | Iron deficiency anaemia<sup>a</sup> | Inflammatory induced anemia<sup>b</sup>/anemia of chronic disease<sup>c</sup> | NPC1<sup>d</sup> | GM2 Gangliosidosis<sup>i</sup> |
|----------------------------|-------------------------------------|---------------------------------------------------------------------------|-----------------|-------------------------------|
| Serum ferritin             | Reduced                             | Increased                                                                 | Increased<sup>h</sup> |                               |
| Serum iron                 | Reduced                             | Reduced                                                                  | Reduced<sup>e, h</sup> | Reduced                      |
| Transferrin                | Increased                           | Reduced or Unchanged                                                      | Unchanged       | Reduced                       |
| Tf saturation              | Reduced                             | Reduced                                                                  | Reduced         | Reduced                       |
| sTfrc                      | Increased                           | Reduced or Unchanged                                                      | Increased<sup>h</sup> | Increased                    |
| MCV                        | Reduced                             | Reduced or Unchanged                                                      | Reduced<sup>n</sup> |                              |
| Hemoglobin                 | Reduced                             | Reduced                                                                  | Reduced<sup>n</sup> |                              |
| Liver ferritin             | Reduced                             | Reduced                                                                  | Reduced<sup>n</sup> |                              |
| Microcytic erythrocytes    | Present                             | Present or absent                                                        | Present or absent<sup>l</sup> |                |
| Inflammatory cytokines     | Unchanged                           | Increased                                                                 | Atypical inflammatory profile<sup>h</sup> | Increased        |
| Serum hepcidin             | Reduced                             | Increased                                                                 | Increased<sup>h</sup> |                              |
| Erythropoiesis             | NA                                  | Reduced                                                                  | Reduced<sup>n</sup> |                              |
| MCH                        | Reduced                             | Unchanged                                                                | Reduced<sup>n</sup> | Unchanged                    |

<sup>a</sup> 46; b. 47; c. 48; d. 37; e. 34; f. 36; g. 35; h. Also in mouse, this study; i. 49.

**Abbreviations:** MCV, mean corpuscular volume; Tf saturation, transferrin saturation; sTfrc, soluble transferrin receptor; MCH, mean corpuscular hemoglobin.
has benefit. The involvement of erythroferrone, an erythroid regulator of hepcidin\(^{50}\) in NPC1 may also be relevant.

We report significant splenomegaly and hepatomegaly in \(Npc1^{-/-}\) mice. Although it has a variable age of onset, hepatosplenomegaly is a clinical feature of NPC1, particularly in early infantile forms of disease\(^{37}\), which the \(Npc1^{-/-}\) mouse model mimics most closely. Splenomegaly also occurs in other lysosomal storage diseases\(^{45}\). There was also evidence of ineffective erythropoiesis, confirmed by a significantly greater frequency of Ter119\(^{+}\) erythroblasts and accumulation of nucleated orthochromatic erythroblasts. Erythropoiesis, which occurs in the spleen, involves proliferation and differentiation of progenitors through distinct stages to yield nonnucleated reticulocytes\(^{42}\) and nucleated orthochromatic erythroblasts representing the terminal stages after which nuclei are ejected to become reticulocytes that can circulate\(^{52}\). Production of red blood cells has a major demand for iron\(^{1}\) and the disruption of the process is consistent with insufficiency. This phenotype together with evidence of disorganised splenic architecture is characteristic of iron deficiency anaemia\(^{1}\).

Because the majority of iron resides in the erythrocytic compartment and erythrocytes have a relatively short life span, efficient recycling of iron from senescent erythrocytes is critical\(^{1}\). Myeloid cells in the spleen and liver phagocytose aged erythrocytes and iron is recycled in order to meet the demand for erythropoiesis\(^{46}\). In experimental models of anaemia, senescent red cells are ingested by monocytes that accumulate in the liver\(^{37}\). We observed non-ingested RBCs in the liver \textit{in vivo} and impaired phagocytosis \textit{in vitro} consistent with defective removal of aged erythrocytes (and hence decreased recycling of iron) in \(Npc1^{-/-}\) mice. Diminished phagocytosis and the potential to impact significantly upon heme/iron recycling within the phago-lysosomal system in NPC1 is currently under investigation.

NPC1 is defined clinically as a progressive neurodegenerative disease and although we could not detect alteration in total levels of iron in the \(Ngc1^{-/-}\) mouse brain, perhaps because of the normal low levels of the metal in this organ, there was reduced CSF ferritin in patients, indicative of altered iron metabolism in the CNS. Hung \textit{et al.}\(^{35}\) reported moderate increases in iron content of \(Ngc1^{-/-}\) cerebellum and cerebrum, and it may be that our analysis of intact mouse brains obscured regional differences. A probable consequence of iron deficiency in the NPC1 brain is compromised in mitochondrial function and capacity resulting in reduced oxidative energy and further neuronal dysfunction. In light of the evidence of increased cerebellar and cerebral iron, Hung \textit{et al.}\(^{34}\) tested for benefit of iron chelation through injection of deferiprone into NPC1 mice but were unable to measure any improvement in either onset of disease symptoms or lifespan. Loss of brain iron homeostasis is implicated in the pathogenesis of common neurodegenerative disorders, including Parkinson’s and Alzheimer’s diseases\(^{55}\) and may therefore contribute to neurodegeneration in NPC1. Brain iron accumulation has been reported in other rare neurodegenerative diseases, such as pantothenate kinase-associated neurodegeneration, neuroferritinopathy, aceruloplasminemia, Kufor Rakeb syndromes and fatty acid hydroxylase associated neurodegeneration\(^{56,57}\). The finding of altered levels of iron-related molecules in patient CSF may be relevant for the identification of biomarkers to monitor therapeutic interventions. Investigation of additional CNS iron phenotypes, and distribution both regionally and at the sub-cellular level in mutant mice and patients is therefore merited.

Previously, we identified systemic iron dysregulation-induced haematological changes in murine models of GM1 and GM2 gangliosidoses\(^{46}\). We documented progressive depletion of tissue iron, including in the brain, hematological profiles indicative of iron deficiency and demonstrated that dietary iron supplementation provided functional benefit\(^{46}\). In comparison with GM1/GM2 gangliosidosis mice, \(Ngc1^{-/-}\) mice exhibited more severe hematological abnormalities, increased circulating hepcidin, abnormal hepatic pro-inflammatory cytokines profiles, erythropoiesis and erythropagocytosis defects. Hyperferritinemia, iron accumulation and elevated hepcidin are pathologies also described in Gaucher disease\(^{38}\). Disrupted iron metabolism is therefore common to multiple lysosomal storage diseases, emphasising the organelle is critical for iron homeostasis. However, the occurrence of disease-specific phenotypes suggests distinct mechanisms that will require further investigation. Furthermore, lysosomal protease activities and lysosomal acidification are crucial for the degradation of ferritin complexes and utilization of iron\(^{14,15}\). NPC1 activity might contribute to other lysosomal mechanisms that impact upon iron homeostasis, such as iron incorporation into ferritin subunits and iron export from lysosomes into the cytosol\(^{14,15}\). Iron is effluxed from the lysosome to maintain cytoplasmic concentrations\(^{45}\). It is pertinent that NPC1 protein can mediate the intracellular transport of copper\(^{2}\) and belongs to the resistance-nodulation-cell division (RND) permease superfamily that in prokaryotes function as proton symporters to the resistance-nodulation-cell division (RND) permease. Intriguingly, genetic screens to identify protein binding partners of Nrc1, the yeast orthologue of NPC1 protein, identified the iron transporter Fth1 that is responsible for the movement of intravacular iron stores\(^{42,43}\).

**Conclusions**

In conclusion, we have identified significant changes in reticuloocyte indices and alterations in iron regulatory proteins in an authentic murine model of NPC1 with some phenotypes in NPC1 patients, albeit milder. This profile includes elements characteristic of both inflammatory and non-inflammatory iron deficiencies, which to our knowledge is unique to NPC1 (Table 3). Although we were unable to detect a correlation between specific hematological parameters and clinical severity it should be noted that the latter is a scale almost entirely based upon evaluation of neurological symptoms. Importantly, loss of systemic iron homeostasis has the potential to impact upon pathogenesis. Whilst precise details of the mechanisms responsible for iron dysregulation remain to be fully elucidated, our findings have the potential to provide novel insights into the biological functions of the NPC1 protein, identify therapeutic targets and provide peripheral and CNS biomarkers.
that may be useful for analysis of disease. NPC1 patients may be at risk of systemic iron defects and monitoring of serum iron and blood counts may be important for effective clinical management and the evaluation of therapies. Although the NPC patient data shown here does not support the requirement for iron supplementation, it does highlight that clinicians, particularly those who do not regularly see individuals with NPC should be aware that iron levels may be low, but unless there is evidence of a functional problem there is not an immediate need to supplement.

Consent
Written informed consent for publication of the patients’ details and their images was obtained from the patients or guardian of the patient.

Data availability
Underlying data
Zenodo: Defective iron homeostasis and haematological abnormalities in Niemann-Pick disease type C1. https://doi.org/10.5281/zenodo.679243

This project contains the following underlying data:
- Data underlying Figure 1:
  - Fig 1 hom f 197.4c001 colour adjusted copy 2.tif
  - Mouse hematology data.xlsx
  - Fig 1hom m 196.5e003 copy 4.tif
  - Fig 1wt f 208.3d001 copy 4.tif
  - Fig 1wt m 196.6c001 copy 4.tif
- Data underlying Figure 2:
  - L ferritin spleen WB.
  - L ferritin liver WB
  - Serum sTfrc entire WB
  - Actin liver colour WB
  - b-Actin spleen WB
  - b-Actin liver WB
  - L ferritin & liver WB quantification.xlsx
  - Hepatic iron levels.xlsx.
  - Mouse serum iron.xlsx
  - Q-PCR ferroportin vs actin. xlsx
  - Q-PCR Tfrc rel to HPRT.xlsx
  - Serum sTfrc WB quantification.xlsx
- Data underlying Figure 3:
  - Hepcidin levels.xlsx
  - Q-PCR IL-1a vs HPRT.xlsx
  - Q-PCR IL-1b vs HPRT.xlsx
  - Q-PCR TNFa vs_HPRT.xlsx
- Data underlying Figure 4:
  - WT and NPC1 liver image
  - WT and NPC1 spleen image
  - Frequency of Splenic Ter119 Subpopulations.xlsx
  - oxRBC phagocytosis. Xlsx
  - RAW + U18666A + oxRBC image
  - RAW + vehicle + oxRBC image
  - Spleen FACS files CD71 & Ter 119 (folder)
  - Splenic Ter119 cells.xlsx
  - Npc-/- liver panel d
  - Npc-/- panel f
  - Npc-/- liver area in panel e
  - Npc-/- Liver area in panel f
  - Npc +/- Liver area in panel b
  - Npc +/- liver panel b
  - Npc +/- Liver panel c
  - Npc +/- Liver area in panel c
  - Npc +/- Liver panel a
  - Npc +/- Liver area in panel b.
  - Npc +/- Spleen panel d
  - Npc +/- Spleen panel c
  - Npc +/- Spleen panel f
  - Npc-/- Spleen area in panel d
  - Npc-/- Spleen panel e
  - Npc +/- Spleen panel b
  - Npc +/- Spleen region in panel d
  - WT and Npc-/- Spleen and liver weights. xlsx
- Data underlying Figure 5:
  - NPC Patient Haematology No names.xlsx
- Data underlying Figure 6:
  - Patient serum data.xlsx
- Data underlying Figure 7:
  - CSF Ferritin and transferrin data.xlsx

Extended data
Zenodo: Defective iron homeostasis and haematological abnormalities in Niemann-Pick disease type C1. https://doi.org/10.5281/zenodo.679243

This project contains the following extended data:
- Patient Haem vs severity score.xlsx
- Extended Figure 1. Erythrocyte number and mean corpuscular hemoglobin concentration (MCHC) are not changed in Npc1-/- mice.
- Histograms of RBC number (A) and MCHC (B) in Npc1−/− mice (open columns) and Npc1+/− mice (filled columns) at specified ages. n=5–11 mice per group

- Extended Figure 2. Brain iron levels are not significantly changed in Npc1−/− mice.

- Graph of iron content of Npc1−/− brain (filled circles) and Npc1+/− brain (filled triangles) at indicated ages. N=3 per group.

- Extended Figure 3. Serum iron levels, serum iron saturation, systemic ferritin and serum transferrin levels in NPC1 patients do not correlate with clinical severity. Plots for serum iron (n=105), serum iron saturation (n=104), serum ferritin (n=100) and serum transferrin (n=104) vs clinical severity as determined by annualized severity increment score (ASIS)26.

Reporting guidelines
Zenodo: ARRIVE checklist for ‘Defective iron homeostasis and hematological abnormalities in Niemann-Pick disease type C1’, https://doi.org/10.5281/zenodo.679243

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Version 2

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I have no further comments and approve this version for indexing.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: NPC

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 03 April 2023

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I approve the article.

Competing Interests: No competing interests were disclosed.
Reviewer Expertise: Neuroscience

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 08 November 2022

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This is an interesting idea and well written manuscript. The authors do state there is not an absolute correlation between the mice and humans and give some explanations as to why this is likely so. I think this needs further expansion including the lack of genetic variability in the mice, the early ages of the mice, and likely that NPC mice may have more liver disease than most human patients, which significantly impacts hematopoiesis.

There is a paucity of human data shown. Though the methods state there were 114 patients in the data base, it does not appear there were that many patients on the scatter plots, so this is confusing. As well, I assume this is data form one time point for the patients? This is not clear (whereas there were several time points for the mice as they aged). We don't know how these values in humans compared for those based on age, disease progression, presence or absence of overt liver disease, or presence of co-morbidities. Iron homestasis is sensitive to all these factors. Additionally, the authors note the measure hematologic parameters in the NPC1 patients were at the lower end of the normal range, a range in which we may expect to see patients with a chronic disease that results in inflammation. Additionally, many patients have nutritional concerns due to dysphagia, weight loss, need for supplementation, etc. which may impact these values. It is noted that the values do not correlate with the clinical severity of NPC. Taken together it seems that that in humans it does not appear the changes noted in iron homestasis are clinically relevant.

In the conclusion it is stated that monitoring of blood counts and iron status may be important for clinical management and the evaluation of therapies, but it does not seem the findings support this statement. Also, in chronic inflammation it is not clear iron replacement is needed or helpful. The clinical care of these patients requires extensive supportive care to ensure optimal quality of life, and (based on this data as well as general clinical experience with these patients) it does not seem that monitoring for anemia per se due to NPC1 is necessary. It is likely these patients have...
periodic blood counts for general safety monitoring (and have known thrombocytopenia). Therefore this appears to be too sweeping of a conclusion. It seems more investigation may be necessary in humans, more than can be ascertained from natural history data.

The CSF iron is interesting and decreased levels in the CSF may be expected in patients with neurodegenerative disorders. It might be interesting to determine if this could be a biomarker in the future with interventional therapies. Taken alone, its clinical relevance is not necessarily any different from an elevated Tau for example. It might be good to add some more thought on the potential utility of this finding (knowing the data is limited due to only 1 time point for the patients).

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** NPC

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Mar 2023

Fran Platt

Caroline Hastings

We thank the two reviewers for their constructive comments on our manuscript. We have sought to address each point that has been raised.

This is an interesting idea and well written manuscript. The authors do state there is not an
absolute correlation between the mice and humans and give some explanations as to why this is likely so. I think this needs further expansion including the lack of genetic variability in the mice, the early ages of the mice, and likely that NPC mice may have more liver disease than most human patients, which significantly impacts hematopoiesis. We agree that genetic background and lack of variability in Npc1−/− mice may also influence disease presentation and have included a statement to this effect. “However, genetic diversity may also impact upon the differential disease severity between mice and patients: Npc1−/− mice were all on an identical genetic background (Balb/c) and unidentified modifier genes may affect patient phenotypes”.

There is a paucity of human data shown. Though the methods state there were 114 patients in the data base, it does not appear there were that many patients on the scatter plots, so this is confusing. As well, I assume this is data form one time point for the patients? This is not clear (whereas there were several time points for the mice as they aged). We don't know how these values in humans compared for those based on age, disease progression, presence or absence of overt liver disease, or presence of co-morbidities. Iron homestasis is sensitive to all these factors. Additionally, the authors note the measure hematologic parameters in the NPC1 patients were at the lower end of the normal range, a range in which we may expect to see patients with a chronic disease that results in inflammation. Additionally, many patients have nutritional concerns due to dysphagia, weight loss, need for supplementation, etc. which may impact these values. It is noted that the values do not correlate with the clinical severity of NPC. Taken together it seems that that in humans it does not appear the changes noted in iron homestasis are clinically relevant.

We have clarified that 114 NPC patients enrolled in a natural history study are reported and numbers for each specific analysis indicated. We have confirmed that data from only one sampling time point was obtained for each individual participant. Data indicating whether patients were receiving additional dietary supplements etc. is provided within underlying data files that have been uploaded to Zenodo.

In the conclusion it is stated that monitoring of blood counts and iron status may be important for clinical management and the evaluation of therapies, but it does not seem the findings support this statement. Also, in chronic inflammation it is not clear iron replacement is needed or helpful. The clinical care of these patients requires extensive supportive care to ensure optimal quality of life, and (based on this data as well as general clinical experience with these patients) it does not seem that monitoring for anemia per se due to NPC1 is necessary. It is likely these patients have periodic blood counts for general safety monitoring (and have known thrombocytopenia). Therefore this appears to be too sweeping of a conclusion. It seems more investigation may be necessary in humans, more than can be ascertained from natural history data. We are in agreement that NPC patients require extensive and broad clinical care that extends beyond management of neurological disease. In particular, informing clinicians who may not have extensive experience of caring for affected individuals of the potential for haematological/iron changes may be considered important. We have included a statement to this effect.” Although the NPC patient data shown here does not support the requirement for iron supplementation, it does highlight that clinicians, particularly those who do not regularly see individuals with NPC should be aware that iron levels may be low, but unless there is evidence of a functional problem
there is not an immediate need to supplement”.

The CSF iron is interesting and decreased levels in the CSF may be expected in patients with neurodegenerative disorders. It might be interesting to determine if this could be a biomarker in the future with interventional therapies. Taken alone, its clinical relevance it not necessarily any different from an elevated Tau for example. It might be good to add some more thought on the potential utility of this finding (knowing the data is limited due to only 1 time point for the patients).

The authors thank the reviewer for this suggestion and will consider whether it can be included in future clinical evaluation studies. We have included a statement to this effect. “The finding of altered levels of iron-related molecules in patient CSF may be relevant for the identification of biomarkers to monitor therapeutic interventions”.

**Competing Interests:** No competing interests were disclosed.

Reviewer Report 07 November 2022

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**Frank W. Pfrieger**

Centre National de la Recherche Scientifique, Institute of Cellular and Integrative Neurosciences, Universite de Strasbourg, Strasbourg, Grand Est, France

The article by Chen and colleagues follows up on previous studies reporting defects in iron homeostasis in patients and in mouse models of Niemann-Pick type C (NPC) disease. The present work adds new results from NPC1 mice and a large cohort of NPC patients and therefore corroborates the more general notion of perturbed transition metal homeostasis due to defects in NPC1. Whether these changes contribute to disease progress remains to be proven. The study is well-designed and comprehensive, experimental approaches are explained in a satisfactory manner, the data are clearly presented. The authors may consider a few points to revise their paper.

- Pg. 3, para 3. The authors explain in their Introduction the importance of lysosomal function for iron homeostasis and they mention some key processes such as ferritinophagy, mitophagy and erythrophagocytosis all requiring intact lysosomes. However, for the non-expert reader, the authors should mention the cell types that perform these tasks. A corresponding statement on pg. 10, para 2 could be moved to the Introduction.

- Figs. 1A,B; 2A,C,D; 3B; 4A,B,D: The authors should remove the black fill from columns as individual data points are barely visible on this background. They should indicate the genotype on the x axis rather than in the legend.
Pg. 6, Fig. 1A, B; legend: The panels do not show "histograms". Therefore, this term should be replaced.

Pg. 6, Fig. 1C; legend: The authors may consider to write explicitly what exactly is altered in erythrocytes from mutant mice.

Pg. 11, Fig. 5, legend: It is unclear what "+/- SEM" relates to as the graphs do not show whiskers.

Pg. 14, para 1: The authors discuss how inflammatory signals may affect hepcidin expression. Previous studies (e.g. Nemeth et al., 2004\(^2\)) showed that IL-6 rather than IL-1 or TNFa enhances the levels of the hormone. Is there evidence that IL-6 levels are changed in NPC mice or patients? The authors may consider to include this point in their discussion.

Discussion: The authors should consider to cite Hung et al. 2020\(^2\) who report the results of iron chelation.

Discussion, Table 3: It may be interesting to include in the table (and the discussion) another lysosomal disorder and to discuss whether lysosomal defects in general affect iron homeostasis.

References
1. Nemeth E, Rivera S, Gabayan V, Keller C, et al.: IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin.\*Clin Invest. 2004; 113 (9): 1271-6 PubMed Abstract | Publisher Full Text
2. Hung YH, Lotan A, Yeshurun S, Schroeder A, et al.: Iron chelation by deferiprone does not rescue the Niemann-Pick Disease Type C1 mouse model.\*Biometals. 33 (2-3): 87-95 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Fran Platt

We thank the two reviewers for their constructive comments on our manuscript. We have sought to address each point that has been raised.

Frank Pfrieger

The article by Chen and colleagues follows up on previous studies reporting defects in iron homeostasis in patients and in mouse models of Niemann-Pick type C (NPC) disease. The present work adds new results from NPC1 mice and a large cohort of NPC patients and therefore corroborates the more general notion of perturbed transition metal homeostasis due to defects in NPC1. Whether these changes contribute to disease progress remains to be proven. The study is well-designed and comprehensive, experimental approaches are explained in a satisfactory manner, the data are clearly presented. The authors may consider a few points to revise their paper.

Pg. 3, para 3. The authors explain in their Introduction the importance of lysosomal function for iron homeostasis and they mention some key processes such as ferritinophagy, mitophagy and erythrophagocytosis all requiring intact lysosomes. However, for the non-expert reader, the authors should mention the cell types that perform these tasks. A corresponding statement on pg. 10, para 2 could be moved to the Introduction. As suggested by the reviewer we have modified the text to provide more information about the processes of ferritinophagy and mitophagy to the non-expert reader and indicated relevant references.

Figs. 1A,B; 2A,C,D; 3B; 4A,B,D: The authors should remove the black fill from columns as individual data points are barely visible on this background. They should indicate the genotype on the x axis rather than in the legend.

As requested by this reviewer we have changed the black fill from columns to a paler shading and have increased the size of individual data points to enhance the clarity of the figures (for both within the main paper and extended data figures). The genotypes are indicated for each panel as the format of the individual data points.

○ Pg. 6, Fig. 1A, B; legend: The panels do not show "histograms". Therefore, this term should be replaced. We have changed the term "histograms" to "bar charts".

○ Pg. 6, Fig. 1C; legend: The authors may consider to write explicitly what exactly is
altered in erythrocytes from mutant mice. We have included the description of “microcytic and irregularly shaped”

- Pg. 11, Fig. 5, legend: It is unclear what "+/- SEM" relates to as the graphs do not show whiskers. We have changed this to “individual data points are shown, together with mean”.

- Pg. 14, para 1: The authors discuss how inflammatory signals may affect hepcidin expression. Previous studies (e.g. Nemeth et al., 2004) showed that IL-6 rather than IL-1 or TNFα enhances the levels of the hormone. Is there evidence that IL-6 levels are changed in NPC mice or patients? The authors may consider to include this point in their discussion. We have included the statement “IL-6 has been shown to induce the synthesis of hepcidin. Whilst enhanced secretion of the cytokine by patient derived fibroblasts in culture has been documented, to our knowledge there are no published data of systemic levels in vivo”.

- Discussion: The authors should consider to cite Hung et al. 2020 who report the results of iron chelation. We have added “In light of the evidence of increased cerebellar and cerebral iron Hung et al tested for benefit of iron chelation through injection of deferiprone into NPC1 mice but were unable to measure any improvement in either onset of disease symptoms or lifespan.”

- Discussion, Table 3: It may be interesting to include in the table (and the discussion) another lysosomal disorder and to discuss whether lysosomal defects in general affect iron homeostasis. The discussion includes the following statements with relation to iron homeostasis in other LSDs. “Previously, we identified systemic iron dysregulation-induced haematological changes in murine models of the LSDs GM1 and GM2 gangliosidoses. We documented progressive depletion of tissue iron, including in the brain, hematological profiles indicative of iron deficiency and demonstrated that dietary iron supplementation provided functional benefit. Disrupted iron metabolism is therefore common to multiple lysosomal storage diseases, emphasising the organelle is critical for iron homeostasis. Haematological parameters for murine GM2 gangliosidosis have been included in Table 3 for comparison.

**Competing Interests:** No competing interests were disclosed.