Defective iron homeostasis and hematological abnormalities in Niemann-Pick disease type C1 [version 1; peer review: awaiting peer review]

Oscar C W Chen¹*, Stephan Siebel²*, Alexandria Colaco¹*, Elena-Raluca Nicoli³¹*, Nick Platt¹*, Dawn Shepherd¹, Stephanie Newman¹, Andrew E Armitage³, Nicole Y Farhat², George Seligmann¹, Claire Smith¹, David A Smith¹, Alaa Abdul-Sada⁴, Mylvaganam Jeyakumar¹, Hal Drakesmith³, Forbes D Porter², Frances M Platt¹

¹Department of Pharmacology, University of Oxford, Mansfield Road, Oxford, Oxfordshire, OX1 3QT, UK
²Division of Translational Medicine, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, 20892, USA
³MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, Oxfordshire, OX3 9DS, UK
⁴Chemistry Department, School of Life Sciences, University of Sussex, Brighton, Sussex, BN1 9QJ, UK

* Equal contributors

First published: 20 Oct 2022, 7:267
https://doi.org/10.12688/wellcomeopenres.17261.1
Latest published: 20 Oct 2022, 7:267
https://doi.org/10.12688/wellcomeopenres.17261.1

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,
Corresponding author: Frances M Platt (frances.platt@pharm.ox.ac.uk)

Author roles: Chen OCW: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; Siebel S: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology; Colaco A: Data Curation, Formal Analysis, Investigation, Methodology, Writing – Review & Editing; Nicoli ER: Data Curation, Formal Analysis, Investigation, Methodology, Supervision, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; Shepherd D: Data Curation, Formal Analysis, Investigation, Methodology, Supervision; Newman S: Data Curation, Formal Analysis, Methodology, Writing – Review & Editing; Armitage AE: Formal Analysis, Investigation, Methodology, Writing – Review & Editing; Farhat NY: Data Curation, Formal Analysis, Investigation, Methodology, Project Administration; Seligmann G: Data Curation, Formal Analysis, Investigation, Methodology; Smith C: Data Curation, Formal Analysis, Investigation, Methodology; Platt N: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Supervision, Validation, Writing – Review & Editing; Drakesmith H: Conceptualization, Data Curation, Formal Analysis, Methodology, Supervision, Writing – Review & Editing; Porter FD: Conceptualization, Data Curation, Formal Analysis, Methodology, Supervision, Writing – Review & Editing; Platt FM: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Writing – Review & Editing

Competing interests: F.M.P. is a consultant to and co-founder of Intrabio. Other authors declare no competing interests.

Grant information: This work was supported by Wellcome [202834, https://doi.org/10.35802/202834; an Investigator Award in Science award to F.M.P.]. F.M.P. received grants from CLIMB (Children Living with Inherited Metabolic Diseases, United Kingdom), Niemann-Pick Research Foundation (NPRF, United Kingdom), Action Medical Research (AMR, United Kingdom) and the John Fell Foundation (University of Oxford). O.C-W.C. was supported by The Clarendon Fund (Clarendon Foundation, University of Oxford) and the Jason Hu scholarship (Balliol College, University of Oxford). E-R. N. was funded by Action Medical Research, UK. D. S. and N.P. were funded by Wellcome. F.D.P. received support from the Ara Parseghian Medical Research Foundation (N.Y.F), Therapeutics for Rare and Neglected Diseases program, Bench to Bedside award from the Office of Rare Diseases, and the intramural research program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development. F.M.P. is a Royal Society Wolfson Research Merit Award holder. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Chen OCW, Siebel S, Colaco A et al. Defective iron homeostasis and hematological abnormalities in Niemann-Pick disease type C1 [version 1; peer review: awaiting peer review] Wellcome Open Research 2022, 7:267 https://doi.org/10.12688/wellcomeopenres.17261.1

First published: 20 Oct 2022, 7:267 https://doi.org/10.12688/wellcomeopenres.17261.1

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
Abbreviations
Niemann-Pick disease type C1 (NPC1), hematocrit (HCT), mean corpuscular volume (MCV), hemoglobin (HGB), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), transferrin (TF), transferrin receptor (Tfrc), soluble-form of transferrin receptor (sTfrc), soluble transferrin receptor (sTfR), light chain ferritin (L-ferritin), ferroportin (Fpn), interleukin-1 beta (IL-1β), interleukin-1 alpha (IL-1α), tumor necrosis factor alpha (TNFα), hypoxanthine-guanine phosphoribosyltransferase (HPRT) cerebrospinal fluid (CSF), resistance-nodulation-cell division (RND), annual severity increment score (ASIS), endoplasmic reticulum (ER)

Introduction
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 and mitophagy, takes place in the endosome/lysosome system which releases and distributes it to other subcellular organelles, e.g., mitochondria. Erythrophagocytosis recycles iron from heme derived from senescent red cells that are ingested 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: endoplasmic reticulum (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−/−) and in NPC1 patients. We found low serum iron, HGB and mean corpuscular HGB (MCH) in Npc1−/− mice and low iron and Tf saturation levels in NPC1 patients. Furthermore, the decreased systemic iron in Npc1−/− 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-Npc1m1NIJ; Npc1−/−) 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−/− 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 Axioplan 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 described22. 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 centrefugation 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 RNeasy 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 5µg/ml) and FITC-conjugated anti-mouse CD71 antibodies (BioLegend, 113805, used at 10µg/ml) and labelled using LIVE/DEAD Fixable Dead Cell Stain Kit (Life Technologies). Stained cells were analyzed on a FACSort flow cytometer (Becton Dickinson) for forward and side scatter and CD71 with TER-119 fluorescence using Diva software (Becton Dickinson). Mean fluorescence intensity (MFI) values were recorded and used to quantify the percentage of Tfr2+ TER-119+ TER-119/CD71+ cells in each sample.

**Table 1. Details of primers used for Q-PCR analysis.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Product size</th>
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<tr>
<td>mTfrc set2F</td>
<td>TCCGCTCTGAGACTACCT</td>
<td>140bp</td>
</tr>
<tr>
<td>mTfrc set2R</td>
<td>ACAATAGGGCAGACAGGATG</td>
<td></td>
</tr>
<tr>
<td>mFerroportin F</td>
<td>TGGCAGAGCTTTGCTGCTA</td>
<td>161bp</td>
</tr>
<tr>
<td>mFerroportin R</td>
<td>TGGAGTTCTGCACACCATTGAT</td>
<td></td>
</tr>
<tr>
<td>mTNFalpha F</td>
<td>CCCTCACACTCATCTTCT</td>
<td>61bp</td>
</tr>
<tr>
<td>mTNFalpha R</td>
<td>GCTAGACGTGGCTACAG</td>
<td></td>
</tr>
<tr>
<td>mIL-1alpha F</td>
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<td>107bp</td>
</tr>
<tr>
<td>mIL-1alpha R</td>
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<tr>
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<td>CAAAATTTGCTTCTCGTGT</td>
<td>101bp</td>
</tr>
<tr>
<td>mIPRT R</td>
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<td>112bp</td>
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<tr>
<td>mACTB F</td>
<td>GCCGTTATCCCTCCTCATCG</td>
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<tr>
<td>mACTB R</td>
<td>CAGTGTGTAACATGGCCATGT</td>
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<td>NPCwt F</td>
<td>TCTACGGTGATCAACACACA</td>
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</tr>
<tr>
<td>NPCwt R</td>
<td>AACACCCGTCTCCTCAATGT</td>
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</tr>
</tbody>
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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 TFC 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. CSF samples were obtained by lumbar puncture within the L4/L5 interspace. 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.

**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 (Dotmatics). Unpaired two-tailed Student’s t test or ANOVA were used to determine significance. *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 1Ai). 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.
Figure 1. Altered erythrocytic indices and erythrocyte morphology in Npc1−/− 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−/− mice. B. Histograms representing reduced hematocrit (i), increased red cell distribution width (ii) and decreased hemoglobin (iii) in eleven-week Npc1−/− 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+/+ and Npc1−/− mice. Arrowheads indicate examples of erythrocytes with altered morphology. Scale bar represents 10 μm. Images of blood smears were taken on Zeiss Axioplan 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<sup>−/−</sup> mice. Serum iron levels are significantly lower in nine-week-old Npc1<sup>−/−</sup> mice than in age-matched control animals (i) whereas serum sTfrc are enhanced. Iron data are mean ± SEM, *<sup>p</sup> < 0.05, **<sup>p</sup> < 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, **<sup>p</sup> < 0.01 **<sup>p</sup> < 0.0001 n=4 per group, unpaired t test with Welch’s correction. (iii). B. Hepatic iron is significantly reduced in seven-week-old Npc1<sup>−/−</sup> mice (filled circles) but not different from controls (filled triangles) at three and eleven weeks of age. Data are mean ± SEM, ****<sup>p</sup> < 0.0001 n=5–8 per group, 2-way ANOVA (i). C. Reduced ferritin content of liver and spleen in nine-week-old Npc1<sup>−/−</sup> mice. Western blot of liver and spleen lysates (i) quantification of specific bands (ii) Data are mean ± SEM, **<sup>p</sup> < 0.01 ****<sup>p</sup> < 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<sup>−/−</sup> mice (filled columns) as compared to Npc1<sup>+/+</sup> mice (open columns). Data are mean ± SEM, *<sup>p</sup> < 0.05 ***<sup>p</sup> < 0.001 n=8 per group. Data are representative of three independent experiments.
Hepatic expression of Tfrc mRNA was significantly higher \((p < 0.05; n=8)\) (Figure 2Di) as was \(fpm\) 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\(^a\). 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\(^1\) we measured hepatic expression of specific pro-inflammatory cytokines. Transcription of TNF\(\alpha\) was significantly greater at five and eight-weeks-of-age \((p < 0.01\; n=8)\). (Figure 3Bi), IL-1\(\beta\) was not changed (Figure 3Bii), but IL-1\(\alpha\) 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\(^3\) and we therefore examined the erythrocytic 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

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**Figure 3.** Significantly increased systemic hepcidin and hepatic pro-inflammatory cytokines in \(Npc1^{-/-}\) mice. A. Male and female \(Npc1^{-/-}\) mice (filled circles) have higher systemic hepcidin at seven weeks of age (left panel) and nine weeks of age (right panel) than \(Npc1^{+/+}\) mice (filled triangles). Data shown mean ± SEM, * \(p < 0.05\), ** \(p < 0.01\) *** \(p < 0.001\) **** \(p < 0.0001\) \(n=5\) per group. 2-way ANOVA. B. Q-PCR data confirming (i) increased hepatic TNF\(\alpha\) transcripts, (ii) unchanged IL-1\(\beta\) and (iii) enhanced IL-1\(\alpha\) in five and eight-week-old \(Npc1^{-/-}\) mice (filled columns) relative to \(Npc1^{+/+}\) animals (open columns). Data are mean ± SEM, ** \(p < 0.01\) *** \(p < 0.001\) \(n=5\) per group, unpaired \(t\) test with Welch's correction. Data representative of two independent experiments.
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. 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.
impairment of lysosomal activities and decreased capacity of NPC1 mice to maintain iron homeostasis. We performed a series of experiments to investigate the mechanism of impaired iron homeostasis in NPC1 patients and to identify potential therapeutic targets.

**Methods**

We analyzed the hematological parameters of NPC1 patients and compared them with those of age-matched controls. We also performed in vitro experiments using NPC1 patient-derived macrophages and RAW 264.7 murine macrophages to investigate the effects of NPC1 deficiency on iron metabolism.

**Results**

We found that NPC1 patients exhibited significant decreases in serum iron, transferrin saturation, and ferritin levels compared to controls. Furthermore, we observed that the number of phagocytosed erythrocytes was significantly lower in NPC1 patient-derived macrophages compared to controls. These findings suggest that NPC1 deficiency results in impaired iron homeostasis and phagocytosis.

**Discussion**

Our results provide insights into the role of NPC1 in iron metabolism and highlight potential therapeutic targets for NPC1-related disorders. Further studies are needed to investigate the mechanisms underlying impaired iron homeostasis in NPC1 patients and to develop effective treatment strategies.
29-year-old juvenile-onset patient found mild iron deficiency anaemia\(^{36}\). 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 anaemia. Monitoring of reticulocyte cellular indices is the basis for early diagnosis of propensity to develop anaemia\(^{37,38}\).

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 \(\text{Npc1}^{-/-}\) mice. Hung et al.\(^{30}\) did not report changes in plasma iron in \(\text{Npc1}^{-/-}\) 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\(^{30}\).

Hepatic expression of L-ferritin was significantly reduced in \(\text{Npc1}^{-/-}\) mice, as has been described previously in patients\(^{32,33}\), consistent with functional iron deficiency. Analysis of the relative amount of hepatic iron in \(\text{Npc1}^{-/-}\) mice did not reveal a consistent relationship; whilst there was a significant decrease at seven weeks, comparable to the findings of Hung et al.\(^{30}\), we found no significant difference at later time points. The deficiency in total liver iron in \(\text{Npc1}^{-/-}\) mice is likely...
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 parameters34,35.
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. 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. Circulating
levels of hepcidin were significantly higher in symptomatic and late-symptomatic Npc1−/− animals, which is in agreement with the up-regulation of specific immune and pro-inflammatory molecules such as IL-1a that occurs during disease progression. NPC1 has a unique combination of iron-related characteristics because it shares commonalities with both iron deficiency anaemia and inflammatory induced anaemia (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. 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 has benefit. The involvement of erythroferrone, an erythroid regulator of hepcidin 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, which the Npc1−/− mouse model mimics most closely. Splenomegaly also occurs in other lysosomal storage diseases. 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 and nucleated orthochromatic erythroblasts representing the terminal stages after which nuclei are ejected to become reticulocytes that can circulate. Production of red blood cells has a major demand for iron 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.

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. Myeloid cells in the spleen and liver phagocytose aged erythrocytes and iron is recycled in order to meet the demand for erythropoiesis. In experimental models of anaemia, senescent red cells are ingested by monocytes that accumulate in the liver. We observed non-ingested RBCs in the liver in vivo and impaired phagocytosis 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 Npc1−/− mouse brain, perhaps because of the normal low levels of the metal ion in this organ, there was

<table>
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<th>Parameter</th>
<th>Iron deficiency anemia a</th>
<th>Inflammatory induced anemia/anaemia of chronic disease b</th>
<th>NPC1 d</th>
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<td>Increased b</td>
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<td>Reduced</td>
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<td>MCH</td>
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Abbreviations: MCV, mean corpuscular volume; TF saturation, transferrin saturation; sTfrc, soluble transferrin receptor; MCH, mean corpuscular hemoglobin.

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

a. 46; b. 47; c. 48; d. 39; e. 30; f. 32; g. 33; h. Also in mouse, this study.
reduced CSF ferritin in patients, indicative of altered iron metabolism in the CNS. Hung et al.\(^9\) reported moderate increases in iron content of Npc1\(^{-/-}\) 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. Loss of brain iron homeostasis is implicated in the pathogenesis of common neurodegenerative disorders, including Parkinson’s and Alzheimer’s diseases\(^{49}\) 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\(^{49-51}\). 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\(^{52}\). 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\(^{52}\). In comparison with GM1/GM2 gangliosidosis mice, Npc1\(^{-/-}\) mice exhibited more severe hematological abnormalities, increased circulating hepcidin, abnormal hepatic pro-inflammatory cytokines profiles, erythropoiesis and erythrophagocytosis defects. Hyperferritinemia, iron accumulation and elevated hepcidin are pathologies also described in Gaucher disease\(^5\). 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\(^{50,51}\). 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\(^{52}\). Iron is effluxed from the lysosome to maintain cytoplasmic concentrations\(^{49}\). It is pertinent that NPC1 protein can mediate the intracellular transport of copper\(^{55}\) and belongs to the resistance-nodulation-cell permease superfamily that in prokaryotes function as proton symporters in the coupled efflux of multiple substrates including metals\(^{56}\). 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 intravacuolar iron stores\(^{59,58}\).

**Conclusions**

In conclusion, we have identified significant changes in reticulocyte indices and alterations and 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.

**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.6792432](https://doi.org/10.5281/zenodo.6792432)

This project contains the following underlying data:

- **Data underlying Figure 1:**
  - Fig 1 hom f 197.4e001 colour adjusted copy 2.tif
  - Mouse hematology data.xlsx
  - Fig 1 hom m 196.5e003 copy 4.tif
  - Fig 1 wt f 208.3i001 copy 4.tif
  - Fig 1 wt m 196.6e001 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-1α vs HPRT.xlsx
  - Q-PCR IL-1β vs HPRT.xlsx
  - Q-PCR TNFα 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<sup>−/−</sup> liver panel d
  - Npc<sup>−/−</sup> panel f
  - Npc<sup>−/−</sup> liver area in panel e
  - Npc<sup>+/−</sup> Liver area in panel f
  - Npc<sup>+/−</sup> Liver area in panel b
  - Npc<sup>+/−</sup> liver panel b
  - Npc<sup>+/−</sup> Liver panel c
  - Npc<sup>+/−</sup> Liver area in panel c
  - Npc<sup>+/−</sup> Liver panel a
  - Npc<sup>+/−</sup> Liver area in panel b.
  - Npc<sup>+/+</sup> Spleen panel d
  - Npc<sup>+/+</sup> Spleen panel c
  - Npc<sup>+/+</sup> Spleen panel f
  - Npc<sup>+/+</sup> Spleen area in panel d
  - Npc<sup>−/−</sup> Spleen panel e
  - Npc<sup>−/−</sup> Spleen panel b
  - Npc<sup>−/−</sup> Spleen region in panel d
  - WT and Npc<sup>−/−</sup> Spleen and liver weights. xlsx

- Data underlying Figure 5:
  - NPC Patient Haematology No names.xls

- 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:
- Extended Figure 1. Erythrocyte number and mean corpuscular hemoglobin concentration (MCHC) are not changed in Npc<sup>−/−</sup> mice.
- Histograms of RBC number (A) and MCHC (B) in Npc<sup>−/−</sup> mice (open columns) and Npc<sup>+/−</sup> mice (filled columns) at specified ages. n=5–11 mice per group
- Extended Figure 2. Brain iron levels are not significantly changed in Npc<sup>−/−</sup> mice.
- Graph of iron content of Npc<sup>+/−</sup> brain (filled circles) and Npc<sup>−/−</sup> 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)<sup>24</sup>

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<sup>25</sup>

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

References


