RESEARCH NOTE

Differential response of the liver to bile acid treatment in a mouse model of Niemann-Pick disease type C [version 1; peer review: 2 approved, 1 not approved]

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Abstract

Niemann-Pick disease type C (NPC) disease is a neurodegenerative lysosomal storage disease caused by mutations in the NPC1 or NPC2 genes. Liver disease is also a common feature of NPC that can present as cholestatic jaundice in the neonatal period. Liver enzymes can remain elevated above the normal range in some patients as they age. We recently reported suppression of the P450 detoxification system in a mouse model of NPC disease and in post-mortem liver from NPC patients. As bile acids regulate the P450 system, we tested bile acid treatment using ursodeoxycholic acid (UDCA; 3α, 7β-dihydroxy-5β-cholanic acid), a hydrophilic bile acid, which is used to treat several cholestatic disorders. In this study, we compared UDCA treatment with the bile acid cholic acid (CA), and found unexpected hepatotoxicity in response to CA in Npc1 mice, but not to UDCA, suggesting that only UDCA should be used as an adjunctive therapy in NPC patients.

Keywords

Niemann Pick disease type C, NPC1, lysosomal storage disease, p450, bile acids, ursodeoxycholic acid, cholic acid, liver
Introduction
Niemann-Pick disease type C (NPC) is a rare progressive neurodegenerative lysosomal disorder, which affects 1:100,000 live births\(^1\). In addition to the central nervous system, the liver also plays a role in this disease, either presenting in infancy as cholestatic jaundice or if these patients survive as elevated levels of liver enzymes suggestive of chronic liver dysfunction\(^1\).

We recently discovered that the hepatic cytochrome P450 system is suppressed in the Npc1 null mouse model (Npc1\(^{-/-}\)), in the feline model of NPC disease and in three NPC human liver samples (post-mortem)\(^1\). In NPC, the efflux of unesterified cholesterol from late endosomes/lysosomes to the endoplasmic reticulum (ER) is inhibited, leading to a shift in the spectrum of bile acids synthesized\(^2\), which suppresses gene expression of the P450 system\(^1\). Treatment of Npc1\(^{-/-}\) mice with ursodeoxycholic acid (UDCA; 3\(\alpha\), 7\(\beta\)-dihydroxy-5\(\beta\)-cholanic acid) rescued gene expression and P450 system enzyme activity\(^1\). In this study, we compared the effects of UDCA (soluble bile acid) and cholic acid (CA; hydrophobic bile acid) in the Npc1\(^{-/-}\) mouse and discovered hepatotoxic adverse effects on the liver in response to CA, but not to UDCA, suggesting UDCA is the bile acid of choice for clinical treatment of NPC patients.

Methods
Npc1 mouse model
Npc1\(^{-/-}\) and Npc1\(^{+/+}\) mice (n = 9-17 per group) were fed either normal chow (RM1 maintenance diet; SDS, London, UK) or normal chow supplemented with UDCA (0.5%, w/w, Sigma-Aldrich) or CA (0.5%, w/w, Sigma-Aldrich) mixed with powdered diet. Treatment started at weaning (3 weeks of age) and mice were sacrificed at 6 weeks of age by intraperitoneal injection with an overdose of phenobarbital.

Bile acid supplementation
Npc1\(^{-/-}\) and Npc1\(^{+/+}\) mice (n = 9-17 per group) were fed either normal chow (RM1 maintenance diet; SDS, London, UK) or normal chow supplemented with UDCA (0.5%, w/w, Sigma-Aldrich) or CA (0.5%, w/w, Sigma-Aldrich) mixed with powdered diet. Treatment started at weaning (3 weeks of age) and mice were sacrificed at 6 weeks of age by intraperitoneal injection with an overdose of phenobarbital.

Histology
Animals were euthanized at 6 weeks of age and were trans-cardiac perfused with 4% paraformaldehyde (PFA); samples of liver were fixed in 4% PFA, dehydrated and processed to wax using standard procedures. The wax blocks were cut at 4-\(\mu\)m-thick sections, mounted on SuperFrost Plus slides (Thermo Scientific, Waltham, MA, USA) and subjected to hematoxylin and eosin (H&E) staining by standard methods. Images were taken on a Nikon eclipse E200 microscope with a Leica digital camera system.

Statistical analysis
Two-way ANOVA with Tukey’s multiple comparison was used to analyse all sets of data comparing Npc1\(^{-/-}\) and Npc1\(^{+/+}\) mice. Statistical analysis was performed with GraphPad Prism version 6.

Results
We investigated the effects of UDCA and CA treatment in the Npc1\(^{-/-}\) mouse model, relative to wild type (Npc1\(^{+/+}\)) mice. Mice were fed on a diet containing either 0.5% UDCA or CA from weaning (3 weeks of age). After 3 weeks of treatment there was gross abdominal distention observed in all CA treated mice, irrespective of genotype, but the degree of distension appeared greater in the Npc1\(^{-/-}\) mice. No distension of the abdomen was observed in UDCA treated Npc1\(^{-/-}\) and Npc1\(^{+/+}\) mice. On necropsy, it was apparent that the abdominal distension in the CA treated group was due to liver enlargement (Figures 1A and B), and there was a statistically significant increase of liver weight in the Npc1\(^{-/-}\) and Npc1\(^{+/+}\) relative to UDCA and untreated mice (\(p<0.0001\)). No significant changes in liver volume were observed in the UDCA treated mice irrespective of genotype (Figures 1A and B). Npc1\(^{-/-}\) mice have a lower body weight than wild type control mice\(^1\), and so the ratio of liver/body weight was calculated (Figure 1C). The liver enlargement in response to CA as a function of body weight was greater in the Npc1\(^{-/-}\) than in the Npc1\(^{+/+}\) mice. The liver-to-body weight ratios were the same in untreated Npc1\(^{-/-}\) and Npc1\(^{+/+}\) mice and in the UDCA treated mice, irrespective of genotype. Histopathological analysis of liver (Figure 1D) showed the typical vacuolated appearance of the liver characteristic of Npc1\(^{-/-}\) mice relative to wild type mice. UDCA treatment had no impact on the histopathology of wild type and Npc1\(^{-/-}\) mice, whereas CA treatment led to hepatocyte enlargement and a foamy appearance indicative of lipid accumulation that often displaced the nucleus to the pole of the cell.

Discussion
NPC disease cells display a complex cellular pathophysiology, including impaired movement of LDL-derived cholesterol from late endosomes/lysosomes to the ER\(^1\) and the generation of non-enzymatically generated oxysterols\(^5\). As a consequence, the bile acid profile is altered in NPC disease\(^6\), and this change in bile acid composition leads to a secondary suppression of the P450 gene family, as they are bile acid regulated\(^7\). The expression of the P450 system genes could be rescued in Npc1\(^{-/-}\) mice by administering the bile acid UDCA, leading to clinical benefit\(^1\). In another study, UDCA was trialed in four clinical NPC cases and liver function improved in patients with elevated liver enzymes at baseline\(^6\).

In this study, we investigated whether CA, another bile acid used in routine clinical practice for bile acid disorders\(^1\) would also be beneficial. Therefore, we treated Npc1\(^{-/-}\) mice with UDCA or CA to investigate this further. However, three weeks into the treatment the CA mice presented with gross abdominal distension so they were culled to determine the basis for this adverse finding. It was clear on gross necropsy that the livers were significantly enlarged in CA
Figure 1. Effects of cholic acid (CA) and ursodeoxycholic acid (UDCA) on 6-week old Npc1 mouse liver. Npc1+/+ and Npc1−/− mice were treated with 0.5% UDCA or CA as admix with powdered diet from three weeks of age. The mice were euthanized at 6 weeks of age when the Npc1−/− exhibited abdominal distension due to liver enlargement. (A) Liver weights of Npc1 mice untreated, treated with 0.5% UDCA and 0.5% CA; average body weight ± SD of the mice are shown beneath each bar for each group. (B) Gross morphology of the liver. (C) Ratio of liver weight to mouse body weight. Data are presented as mean ± SEM, n = 9-17 animals per group/genotype. **** p < 0.0001 calculated using two-way Anova with Tukey’s multiple comparison test. (D) H&E histopathology of liver sections, bars represent 10μm for high magnification inset panels and 5μm the main panel.

Histopathology revealed lipid laden distended hepatocytes in the CA treated Npc1−/− mice. These data suggest CA is more hepatotoxic in mice than UDCA, consistent with the differential chemical properties of these two bile acids. The NPC1 protein, deficient in most cases of NPC disease, is a conserved member of a protein family, the RND permeases. In bacterial systems they serve to efflux multiple classes of substrates across the periplasmic space, allowing bacteria to thrive in hostile environments. CA’s hydrophobic chemistry suggest it may be a potential substrate for NPC1, so it may itself be stored in the late endocytic system in NPC1 deficient cells and adversely affect the acidic compartment of the cell leading to further lipid storage. This hypothesis merits further investigation.

When four NPC1 patients were treated with UDCA, benefit was observed based on improved liver function. Very interestingly, one patient was switched from UDCA to CA and their clinical status declined but was recovered on switching back to UDCA, and this has striking parallels with this study in mice. Taken together, these data strongly support the use of UDCA as an adjunctive therapy.
to treat residual liver disease in NPC patients, but CA is contraindicated due to acute hepatotoxicity that is most pronounced in an NPC1 deficient background.

Data availability
Raw data have been uploaded to the online data repository OSF: http://dx.doi.org/10.17605/OSF.IO/5A2F9

Competing interests
FP is a co-founder and consultant to IntraBio.

Grant information
This work was supported by the Wellcome Trust [202834] to FP; Action Medical Research to ERN; Niemann-Pick Research Foundation to DS and LM; Royal Society Wolfson Research Merit Award to FP.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements
We thank Will Evans for helpful comments on the manuscript.

References

Roberto Giugliani

National Institute on Population Medical Genetics, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

This report is based in an interesting experiment, and the conclusions are supported by the data presented. The research problem was adequately addressed, and the paper is well written. The experiment was designed after the discovery that the hepatic cytochrome P450 system is suppressed in NPC animal models and also in NPC human liver (post-mortem analyses). As previously described, the inhibition of the efflux of unesterified cholesterol from the lysosomal system to the endoplasmic reticulum changes the spectrum of bile acids synthesized, and this eventually suppresses gene expression of the P450 system. It was already demonstrated that treatment of the NPC models with ursodeoxycholic acid (UCDA) rescued P450 activity. In this study, therapy with UCDA was compared with therapy with cholic acid (CA), being hepatotoxic adverse effects observed with CA but not with UCDA, which could be further explored as a potential adjunctive therapeutic agent in NPC.

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?
Competing Interests: No competing interests were disclosed.

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

Stephen D Turley
Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, USA

Although the rationale for this study is clearly explained, the relevance of the data obtained to the management of liver disease in NPC1 deficiency is questionable for reasons relating mainly but not fully to the methodological approaches that were used. The following points warrant close consideration.

1. Although the dietary level of BA supplementation employed is in line with what is often used in animal models, the changes in bile acid pool and composition that ensue when the amount of dietary BA is set at 0.5% w/w are beyond extreme and result in shifts in cholesterol metabolism at multiple levels that would never occur in humans taking approved doses of any specific BA. At a dietary level of 0.5%, the dose used in these studies was the equivalent of around 800 mg/day/kg bw which far exceeds what most human subjects taking oral BAs consume during a 24 hour period. When BA pool size and composition change in extreme ways there are invariably marked shifts in intestinal cholesterol absorption and this in turn has a major impact on the amount of chylomicron cholesterol reaching the liver from the small intestine (and therefore in the amounts of unesterified chol –UC—becoming subsequently trapped in the late /E/L compartment when NPC1 or NPC2 are mutated). There is an excellent study by Wang et al. (2003)\(^1\) showing how a range of BAs including UDCA and CA (added to the diet at a level of 0.5%) affect intestinal cholesterol absorption in a normal mouse model (see Fig 3 in particular). While a BA pool enriched with UDCA drives down intestinal cholesterol absorption, the opposite effect occurs with CA. Another example illustrating how BA pool size and composition are major determinants of cholesterol absorption is seen in a study by Schwarz et al. (1998)\(^2\) –see solid histograms for WT mice given hyodeoxycholic acid vs cholic acid in Fig 5. Anyone who knows about the properties of CA and who also understands the biochemical consequences of NPC1 deficiency would view the effects of CA described in the current report as “totally predictable” especially given the massive dose that was fed to the mice. It would have been better to use two different doses of UDCA.

2. The bottom line in all this is that the differing impacts on liver “health” of UDCA vs CA supplementation in NPC1\(^{-/-}\) mice shown here are likely fully explainable on the basis of major differences in the amount of intestinally derived cholesterol trapped in the livers of the KOs given these two particular BAs. Although no data are presented for hepatic total chol concs (mg/g), one might predict from other studies in the NPC1 mouse model that a several fold greater level of unesterified chol (UC) would be seen in the livers of the CA supplemented mice vs those given

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1. Wang et al. (2003)
2. Schwarz et al. (1998)
UDCA. There is no question that it is the cytotoxic effects of the entrapped UC in all organs, especially the liver, that has the most damaging impact in NPC1 (or NPC2) dysfunction.

While it is recognized that this is a preliminary report, it is essential that more informed conclusions be reached about what UDCA supplementation might conceivably be doing in the human NPC1-deficient liver. To achieve this, definitely the liver UC and EC concentrations in these mice, along with their plasma/serum ALT and AST levels should be measured. Such measurements are needed if these mouse model experiments are to provide any credible guide as to how UDCA supplementation in human NPC1 patients might be acting. The benefit most likely has nothing to do with putative improvement in functionality of the P450 detoxification system.

3. As it is currently written, this report will likely attract criticism because of its lack of acknowledgement of significant published work documenting the beneficial impact of other types of treatment modalities for improving liver function in NPC1 deficiency, mostly based on in vivo experiments using primarily the NPC1Δ/Δ mouse model. In 2007 Beltroy et al. showed that the cholesterol absorption inhibitor, ezetimibe, given in the diet from the time of weaning, at doses far below those of other treatments (ie 20 or less mg/day/kg bw), reduced hepatomegaly and lowered liver cholesterol content (nearly all UC) by almost 50% (Fig 4). The benefit of ezetimibe in this disease is largely in the liver. After that, a series of definitive publications from a number of labs attesting the potency of subcutaneously administered 2HPBCD in diminishing hepatic UC content and markedly improving liver function tests (and increasing lifespan) in NPC1 mouse models helped establish this agent as one of great potential for managing multisystem disease in NPC1 deficiency.

Although this preliminary report will be of interest to many working in the field of NPCD management, it should not generate the impression that UDCA has a clear potential to become a treatment modality for liver disease in NPCD patients. Citation of the earlier papers noted above documenting the decisive benefits of ezetimibe and 2HPBCD in NPC1 mice will allow readers to gain a better perspective of what these initial data in the UDCA treated NPC1 mouse model are telling us.

References

Is the work clearly and accurately presented and does it cite the current literature? No
Is the study design appropriate and is the work technically sound?
No

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

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

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

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Control of cholesterol metabolism in the liver and other major organs in lysosomal cholesterol storage diseases resulting from mutations in the LIPA, NPC1 or NPC2 genes

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 22 Mar 2018

Fran Platt, University of Oxford, Oxford, UK

Although the rationale for this study is clearly explained, the relevance of the data obtained to the management of liver disease in NPC1 deficiency is questionable for reasons relating mainly but not fully to the methodological approaches that were used. The following points warrant close consideration.

1. Although the dietary level of BA supplementation employed is in line with what is often used in animal models, the changes in bile acid pool and composition that ensue when the amount of dietary BA is set at 0.5% w/w are beyond extreme and result in shifts in cholesterol metabolism at multiple levels that would never occur in humans taking approved doses of any specific BA. At a dietary level of 0.5%, the dose used in these studies was the equivalent of around 800 mg/day/kg bw which far exceeds what most human subjects taking oral BAs consume during a 24 hour period. When BA pool size and composition change in extreme ways there are invariably marked shifts in intestinal cholesterol absorption and this in turn has a major impact on the amount of chylomicron cholesterol reaching the liver from the small intestine (and therefore in the amounts of unesterified chol –UC—becoming subsequently trapped in the late /E/L compartment when NPC1 or NPC2 are mutated). There is an excellent study by Wang et al. (2003)1 showing how a range of BAs including UDCA and CA (added to the diet at a level of 0.5%) affect intestinal cholesterol absorption in a normal mouse model (see Fig 3 in particular). While a BA pool enriched with UDCA drives down intestinal cholesterol absorption, the opposite effect occurs with CA. Another example illustrating how BA pool size and composition are major determinants of cholesterol absorption is seen in a study by Schwarz et al. (1998)2 –see solid histograms for WT mice given hyodeoxycholic acid vs cholic acid in Fig 5. Anyone who knows about the properties of CA and who also understands the biochemical
consequences of NPC1 deficiency would view the effects of CA described in the current report as “totally predictable” especially given the massive dose that was fed to the mice. It would have been better to use two different doses of UDCA.

We thank the reviewer for their comments. We have dealt with comment 1 & 2 together, with our response given below.

1. The bottom line in all this is that the differing impacts on liver “health” of UDCA vs CA supplementation in NPC1-/- mice shown here are likely fully explainable on the basis of major differences in the amount of intestinally derived cholesterol trapped in the livers of the KOs given these two particular BAs. Although no data are presented for hepatic total chol concs (mg/g), one might predict from other studies in the NPC1 mouse model that a several fold greater level of unesterified chol (UC) would be seen in the livers of the CA supplemented mice vs those given UDCA. There is no question that it is the cytotoxic effects of the entrapped UC in all organs, especially the liver, that has the most damaging impact in NPC1 (or NPC2) dysfunction. While it is recognized that this is a preliminary report, it is essential that more informed conclusions be reached about what UDCA supplementation might conceivably be doing in the human NPC1-deficient liver. To achieve this, definitely the liver UC and EC concentrations in these mice, along with their plasma/serum ALT and AST levels should be measured. Such measurements are needed if these mouse model experiments are to provide any credible guide as to how UDCA supplementation in human NPC1 patients might be acting. The benefit most likely has nothing to do with putative improvement in functionality of the P450 detoxification system.

We thank the reviewer for their helpful comments. With regards to a more thorough investigation of liver function we refer the reviewer to a publication (to which this mouse study acts as a supplement) that investigates the effect of UDCA in a small number of NPC patients. The Evans et al clinical case report investigated the effects of UDCA on liver function in NPC patients\textsuperscript{1}. Treatment with UDCA (at between 10 and 30mg/kg/day) was associated with a marked improvement in liver function parameters (including aspartate aminotransferase (AST) and alanine transaminase (ALT)) in patients with elevated liver enzyme levels at baseline. We judged that the existence of human data demonstrating the benefit of therapeutic doses of UDCA on NPC patient liver function obviated the need for extended murine studies. The separation of murine and human studies into separate publications was an editorial requirement of Wellcome Open Research. However, the two studies should be considered together.

Importantly, this NPC patient case study (Evans et al) also suggested that cholic acid is deleterious in NPC patients\textsuperscript{1}. One of the patients detailed in the case study was commenced on UDCA from 4 weeks of age due to cholestatic jaundice of uncertain aetiology. A bile acid profile at 2 months of age indicated a potential bile acid synthesis defect, and cholic acid treatment was initiated (at 4.5 months) with the aim of supressing bile acid synthesis. CA treatment was associated with elevations in aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase, bilirubin and total cholesterol levels. Upon discontinuation of CA and resumption of UDCA treatment there was quantitative improvement in multiple parameters of liver function.

We have additional data demonstrating that the beneficial effect of UDCA is not due to effects on liver cholesterol levels. There is no significant difference in the levels of liver cholesterol in the UDCA-treated Npc1\textsuperscript{1/-} mouse (relative to untreated Npc1\textsuperscript{1/-} mouse, new Figure 1D in the revised
Whilst CA treatment is associated with a statistically significant elevation in Npc1<sup>−/−</sup> liver cholesterol levels (relative to UDCA-treated Npc1<sup>−/−</sup>) the extent of this elevation is less than 1.5 fold. These data have been added to the paper, and the text of the results section expanded accordingly.

This study should also be considered in light of our previous published study which fully characterised the beneficial effect of UDCA treatment on body weight, motor function, tremor and was consistent with restoration of the P450 system in Npc1<sup>−/−</sup> mice mediating the therapeutic benefit.<sup>2</sup>


As it is currently written, this report will likely attract criticism because of its lack of acknowledgement of significant published work documenting the beneficial impact of other types of treatment modalities for improving liver function in NPC1 deficiency, mostly based on in vivo experiments using primarily the NPC1<sup>−/−</sup> mouse model. In 2007 Beltroy et al. showed that the cholesterol absorption inhibitor, ezetimibe, given in the diet from the time of weaning, at doses far below those of other treatments (ie 20 or less mg /day/kg bw), reduced hepatomegaly and lowered liver cholesterol content (nearly all UC) by almost 50% (Fig 4). The benefit of ezetimibe in this disease is largely in the liver. After that, a series of definitive publications from a number of labs attesting the potency of subcutaneously administered 2HPBCD in diminishing hepatic UC content and markedly improving liver function tests (and increasing lifespan) in NPC1 mouse models helped establish this agent as one of great potential for managing multisystem disease in NPC1 deficiency.<sup>4,5</sup>

Although this preliminary report will be of interest to many working in the field of NPCD management, it should not generate the impression that UDCA has a clear potential to become a treatment modality for liver disease in NPCD patients. Citation of the earlier papers noted above documenting the decisive benefits of ezetimibe and 2HPBCD in NPC1 mice will allow readers to gain a better perspective of what these initial data in the UDCA treated NPC1 mouse model are telling us.

We thank the reviewer for their comments. We agree that it would be beneficial to acknowledge the published work relating to other types of treatment modalities for hepatic dysfunction in NPC. We have amended the text of the paper accordingly. The intended purpose of this study was to demonstrate that there is a differential response of the Npc1<sup>−/−</sup> mouse to treatment with UDCA and CA, despite both bile acids being widely used in the treatment of cholestatic disorders. CA treatment was deleterious in both Npc1<sup>−/−</sup> mice and NPC patients and hence is contraindicated for Npc1<sup>−/−</sup>.

We would suggest, based on observed benefits to hepatic and neurological function (from this publication, the accompanying publication relating to NPC patients and previously published data...
with Npc1<sup>-/-</sup> mice), that UDCA has the potential to be a valuable addition to combination therapy for NPC<sup>1,2</sup>.


**Competing Interests:** No competing interests were disclosed.
effect of bile acid treatment in NPC in the mouse model as well as in clinics without going through
two separate publications. Nevertheless, this manuscript on its own reports interesting findings.

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.

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