

## Oxidative stress in Niemann–Pick disease, type C

Rao Fu<sup>a,b</sup>, Nicole M. Yanjanin<sup>a</sup>, Simona Bianconi<sup>a</sup>, William J. Pavan<sup>c</sup>, Forbes D. Porter<sup>a,\*</sup>

<sup>a</sup> Program in Developmental Endocrinology and Genetics, NICHD, NIH, DHHS, Bethesda, MD 20892, USA

<sup>b</sup> Health Science Center, Peking University, Beijing, 100191, China

<sup>c</sup> Genetic Disease Research Branch, NHGRI, NIH, DHHS, Bethesda, MD 20892, USA

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### ABSTRACT

Niemann–Pick disease, type C (NPC) is a neurodegenerative lysosomal storage disorder due to impaired intracellular cholesterol and lipid transport. Increased oxidative stress has been reported in human *NPC1* mutant fibroblasts and in tissues from *Npc1* mutant mice. However, oxidative stress in NPC patients has not been established. In this study, we demonstrated increased oxidative stress in NPC patients. Evaluation of serum from 37 NPC patients, compared to control values, showed significant decreases ( $p < .01$ ) in both the fraction of reduced coenzyme Q10 (CoQ10) and trolox equivalent antioxidant capacity (TEAC). Both findings are consistent with increased oxidative stress in NPC. Supplementation with CoQ10 was not effective in correcting the decreased fraction of reduced CoQ10. Increased oxidative stress may be a contributing factor to the pathology of NPC, and demonstration of increased oxidative stress in NPC patients provides both a rationale and the biomarkers necessary to test the efficacy of antioxidant therapy in NPC.

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### Introduction

Niemann–Pick Disease, type C (NPC) is an autosomal recessive, neurodegenerative lysosomal storage disease that exhibits impaired intracellular cholesterol and lipid transport. The NPC phenotype is characterized by progressive neurological deterioration. Typical findings and symptoms include splenomegaly, ataxia, seizures, gelastic cataplexy, and dementia. The estimated incidence in Western Europeans is one in 120,000–150,000 [1]. Most cases of NPC are due to mutations of the *NPC1* gene located on chromosome 18q11 [2]. A small fraction of cases are due to mutations of the *NPC2* gene located on chromosome 14q24.3 [3]. NPC1 is a large, multipass transmembrane protein located in the late endosomal/lysosomal compartment, whereas NPC2 is a relatively small intraluminal protein [4,5]. Recent data suggest that NPC2 transfers cholesterol to NPC1, which is then hypothesized to transport cholesterol through the glycocalyx to the limiting membrane of the late endosomal/lysosomal compartment [6]. Deficiency of either NPC1 or NPC2 results in the intracellular accumulation of unesterified cholesterol and glycosphingolipids [7,8]. The impaired intracellular lipid transport then initiates a pathological cascade that includes deficient oxysterol production [9], peroxisomal dysfunction [10], abnormal sphingosine metabolism [11], neuroinflammation [12], induction of apoptosis [13], and perturbed

neurosteroid synthesis [14]. Increased oxidative stress has also been implicated as a potential pathological mechanism in NPC.

A number of studies are consistent with increased oxidative stress being a contributing factor in the pathophysiology of NPC. Compared to control fibroblasts, *NPC1*-deficient fibroblasts show a number of changes in gene expression consistent with the generation of reactive oxygen species (ROS) and reactive nitrogen species [15,16]. Treatment of primary cultured cortical neurons with U18666A, a drug that induces an NPC-like cellular phenotype, results in apoptosis secondary to increased ROS [17]. Elevated mitochondrial cholesterol levels and depletion of mitochondrial glutathione [18] have been reported in liver [19] and brain [20] tissue from *Npc1*<sup>-/-</sup> mice, thus mitochondrial dysfunction may underlie increased ROS in NPC. A study using chimeric *Npc1*<sup>-/-</sup> mice reveals elevated cholesterol oxidation products in macrophages and plasma which are associated with lower 27-hydroxycholesterol (27-HC) levels in macrophages [21].

Among the many markers of oxidative stress, we chose to evaluate coenzyme Q10 (CoQ10) and Trolox Equivalent Antioxidant Capacity (TEAC). CoQ10 is a lipid-soluble, vitamin-like compound that cycles between a reduced (ubiquinol-10) and oxidative state (ubiquinone-10). Reduced CoQ10 functions as a coenzyme for at least three mitochondrial enzymes (complexes I, II and III) in oxidative phosphorylation [22] and as an antioxidant protecting cells from oxidative damage [12,23,24]. Multiple substances, in addition to CoQ10, contribute to total antioxidant capacity in biological samples. Trolox, or 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid, is a water-soluble derivative of tocopherol that is used as a reference substance to measure the combined antioxidant capacity in biological

**Abbreviations:** NPC, Niemann–Pick disease, type C; CoQ10, Coenzyme Q10; TEAC, Trolox Equivalent Antioxidant Capacity.

\* Corresponding author. Bld. 10, Rm. 9D42, 10 Center Dr., Bethesda, MD 20892, USA. Fax: +1 301 480 5791.

E-mail address: [fdporter@mail.nih.gov](mailto:fdporter@mail.nih.gov) (F.D. Porter).

specimens [25]. The total antioxidant capacity is then expressed as Trolox Equivalent Antioxidant Capacity (TEAC).

Although both *in vitro* and murine studies suggest that increased oxidative stress may contribute to the pathology of NPC, it has not previously been directly demonstrated in NPC patients. To test the hypothesis that increased oxidative stress is present in NPC patients, we measured both reduced and total CoQ10 levels and determined the trolox equivalents antioxidant capacity (TEAC) in serum obtained from 37 NPC patients. Consistent with our hypothesis, we found significant reduction in both the fraction of reduced CoQ10 and TEAC in serum from NPC patients compared to controls. This finding confirms the potential role of increased oxidative stress in the pathology of NPC and suggests that antioxidant therapy may be a rational adjunctive therapy for NPC.

## Materials and methods

### Patients

This clinical study was approved by the NICHD Institutional Review Board. Informed consent and, when appropriate, assent were obtained. Thirty-seven NPC patients were enrolled in a longitudinal observational study at the National Institutes of Health. Subjects were enrolled between August 2006 and November 2009. Phenotypic severity was determined utilizing the scale developed by Yanjanin et al. [26]. Age at initial NIH evaluation ranged from 1.9 to 51 years with a mean and median of 11.5 and 7.7 years, respectively. Initial severity scores ranged from 1–40 with a mean and median of 16.5 and 18, respectively. Twenty-two subjects were evaluated more than once during the period of this study. Nine patients were supplemented with CoQ10, and one patient was supplemented with other antioxidants (Chinese herb blend) at the time of at least one of the evaluations. In this cohort, 21 patients (57%) were being treated off-label with miglustat. Miglustat is an inhibitor of glycosphingolipid synthesis and has shown some efficacy in the treatment of NPC [27]. Although this study did not formally randomize treatment with miglustat, its use was primarily dictated by availability of insurance coverage. For TEAC assays, serum was also obtained from 40 healthy pediatric volunteers ranging in age from 3 to 18 years with mean and median ages of 10.7 and 11.0 years, respectively.

### Determination of CoQ10 levels and Trolox Equivalent Antioxidant Capacity

Total and reduced serum CoQ10 levels were measured by the Mayo Clinic Department of Laboratory Medicine and Pathology. Blood was collected in BD Vacutainer® Serum Collection Tubes at the National Institutes of Health Clinical Center. Independent control pediatric blood samples were not available for CoQ10 assay. Therefore, for these analyses the age appropriate Mayo Clinic Laboratory reference ranges was used. The reference ranges for reduced and total CoQ10 levels for subjects less than 18 years of age are 320–1376 mcg/L and 320–1558 mcg/L, respectively, and the normal range for the fraction of reduced CoQ10 is 93–100%. The corresponding normal ranges for subjects 18 years of age and older are 415–1480 mcg/L, 433–1532 mcg/L, and 92–98%. TEAC was measured using Trolox Equivalents Assay kits from Cayman Chemical Company. Assays were performed per manufacturer's protocol. For the TEAC assays, patient serum was stored at –80 degrees prior to assay.

### Statistical analysis

Statistical analysis was performed using Graph Pad Prism software. Values in the text are presented as mean ± standard deviation. Error bars on figures represent standard error of the mean. Student's *t*-test

was used for statistical comparisons. A nominal value of  $p < .05$  was defined as indicating a significant change.

## Results

### Reduced CoQ10 fraction was significantly decreased in NPC patients

Serum CoQ10 levels were measured in 32 NPC subjects during an evaluation when they were not supplemented with either CoQ10 or antioxidants. Initial levels for reduced and total CoQ10 are shown in Fig. 1A. For the 28 NPC patients less than 18 years of age, the mean reduced and total CoQ10 levels were  $371 \pm 20$  mcg/L and  $428 \pm 24$  mcg/L, respectively, both values being within the reference range. Only four (14%) pediatric NPC patients had a total CoQ10 level below the lower reference range. These data demonstrate that a CoQ10 deficiency is not a common finding in NPC patients. However, the fraction of reduced CoQ10 is decreased in NPC patients (Fig. 1B). The mean fraction of reduced CoQ10 was  $86.8 \pm 1.6\%$ , which is much less than the lower limit of the reference range (93%). Twenty-two patients (79%) had reduced CoQ10 fractions below 93%. Similar results were obtained for four NPC patients 18 years of age and older. Mean reduced and total CoQ10 levels were  $617 \pm 224$  mcg/L and  $674 \pm 233$  mcg/L (Fig. 1A), and the fraction of reduced CoQ10 was decreased (Fig. 1B). There was no correlation of CoQ10 levels with either disease severity or age of onset (data not shown).

### Supplementation of CoQ10 does not correct the abnormal fraction of reduced CoQ10

For patients receiving CoQ10 supplementation, we analyzed both initial and serial values. Five patients were on supplementation at their initial evaluation, and two patients were supplemented after their initial evaluation. A total of nine evaluations of patients on CoQ10 supplementation were available. As one would predict, samples obtained from CoQ10 supplemented patients had significantly higher levels of both reduced ( $p < .0001$ ) and total CoQ10 ( $p < .0001$ ) compared to unsupplemented patients (Fig. 1C). Reduced CoQ10 levels were increased 94%, and total levels were increased 128%. However, supplementation failed to correct the abnormal fraction of reduced CoQ10 (Fig. 1D). The fraction of reduced CoQ10 in supplemented patients ( $86.6 \pm 2.7\%$ ) was not significantly different ( $p = .64$ ) than that observed in unsupplemented samples ( $87.6 \pm 9.2\%$ ).

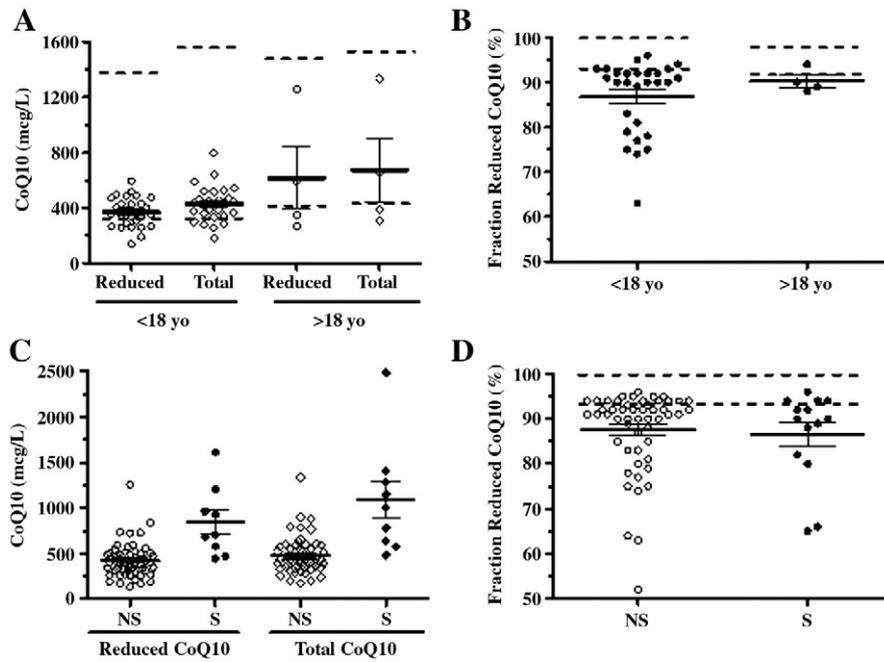
We also evaluated the effect of miglustat therapy on CoQ10 levels. Mean reduced and total CoQ10 levels were decreased in miglustat treated patients (Fig. 2A); however, miglustat therapy had no effect on the fraction of reduced CoQ10 (Fig. 2B). Consistent with this latter observation, no differences were seen in the pre- and post-miglustat treatment CoQ10 values for five patients (data not shown).

### Mean serum TEAC was significantly decreased ( $p < .01$ ) in NPC patients

The mean TEAC value for 33 NPC patients was  $1.39 \pm 0.15$  mM, which was significantly lower than the mean value of  $2.09 \pm 0.17$  for 40 age appropriate controls (Fig. 3A). Although NPC patients supplemented with CoQ10 had a higher mean TEAC value ( $1.71 \pm 0.37$  mM) than unsupplemented patients ( $1.32 \pm 0.16$  mM) this difference was not significant ( $p = .32$ ). There was no significant correlation of TEAC values with either disease severity or age of onset (data not shown). Although the mean TEAC values were higher in miglustat-treated patients (Fig. 3B), consistent with the fraction of reduced CoQ10 data, the difference was not significant ( $p = .31$ ).

## Discussion

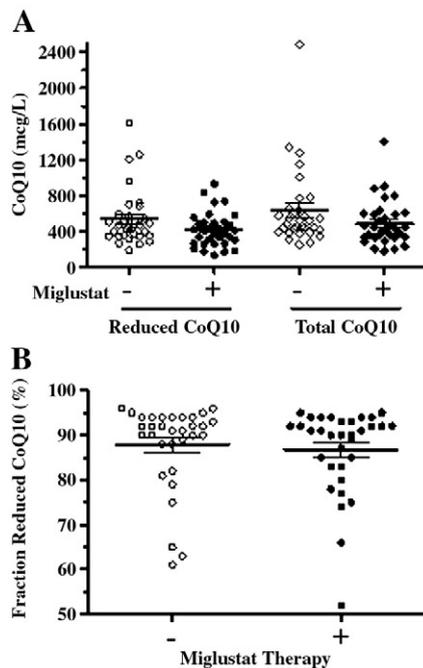
The impaired intracellular cholesterol and lipid transport that arises from abnormal NPC1 or NPC2 protein function in NPC patients



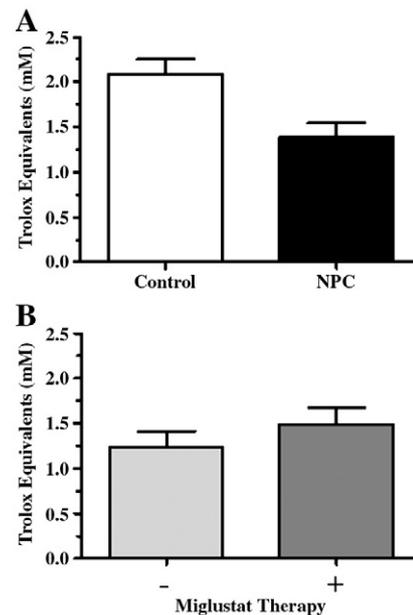
**Fig. 1.** Serum CoQ10. (A) Serum levels of reduced and total CoQ10 for 28 pediatric NPC patients (age <18 years) and 4 adult NPC patients. Patient mean values are indicated by solid lines and the reference range is indicated by dashed lines. (B) The mean fraction of reduced CoQ10 is decreased in both pediatric ( $86.8 \pm 1.6\%$ ) and adult ( $90.3 \pm 1.3\%$ ) patients. Lower reference ranges are 93% and 92% for pediatric and adult subjects, respectively. (C) CoQ10 supplementation increases serum levels of reduced and total CoQ10. Reduced CoQ10 levels were significantly ( $p < .0001$ ) increased in supplemented patients ( $845 \pm 127$  mcg/L,  $n = 9$ ) compared to unsupplemented patients ( $424 \pm 25$  mcg/L,  $n = 56$ ). Total CoQ10 levels were similarly increased ( $p < .0001$ ) in supplemented versus unsupplemented patients with mean values of  $1090 \pm 204$  and  $477 \pm 27$  mcg/L, respectively. (D) CoQ10 supplementation failed to normalize the fraction of reduced CoQ10 with mean values of 87.6% and 86.6% in unsupplemented and supplemented patients, respectively. NS: Non-supplemented; S: Supplemented.

leads to progressive neurodegeneration. While the molecular details of this disease process are not fully understood, previous *in vitro* [16–18,28] and mouse studies [19–21] suggest the presence of increased

oxidative stress in NPC. In this paper, we demonstrate evidence of increased oxidative stress in serum from NPC patients enrolled in our observational study. Specifically, NPC patients have decreased fractions of reduced CoQ10 and decreased TEAC, a measure of total antioxidant capacity.



**Fig. 2.** Effect of miglustat treatment on serum CoQ10 levels. (A) Reduced CoQ10 levels were decreased in patients receiving miglustat therapy ( $544 \pm 55$  mcg/L,  $n = 33$ ) compared to untreated patients ( $421 \pm 33$  mcg/L,  $n = 32$ ). In a similar manner, total CoQ10 levels were decreased in samples from miglustat-treated subjects ( $636 \pm 77$  mcg/L) compared to samples from untreated patients ( $491 \pm 43$  mcg/L). (B) The fraction of reduced CoQ10 did not differ significantly ( $p = .64$ ) between miglustat-treated and untreated patients.



**Fig. 3.** Serum Trolox Equivalent Antioxidant Capacity. (A) Mean serum TEAC value was significantly decreased ( $p < .01$ ) in NPC patients ( $1.39 \pm 0.15$  mM,  $n = 33$ ) in comparison to mean serum TEAC value of age appropriate controls ( $2.09 \pm 0.17$  mM,  $n = 40$ ). (B) The mean TEAC value measured for 27 samples obtained from miglustat-treated patients ( $1.50 \pm 0.18$  mM) was not significantly ( $p = .31$ ) different from the mean value measured for 31 serum samples obtained from untreated patients ( $1.23 \pm 0.18$  mM).

Altered CoQ10 ratios may directly contribute to NPC pathology. CoQ10 plays an important role in the production of chemical energy in the mitochondrial respiratory chain, and the imbalance between ubiquinone-10 (oxidized) and ubiquinol-10 (reduced) levels suggests mitochondrial dysfunction. Although a definitive pathological role has not been established, mitochondrial dysfunction is linked to oxidative stress [29] and has been observed in other neurodegenerative diseases, such as Down syndrome [30,31], Alzheimer disease [32], Parkinson disease [33], amyotrophic lateral sclerosis [34], Huntington disease [35,36], and Friedreich ataxia [37]. Mitochondrial inefficiency decreases ATP production, while promoting the production of excess reactive oxygen species, and results in further cell damage and mitochondrial inefficiency. This has been proposed to contribute to the pathology of Alzheimer disease, because brain neurons are more susceptible to the detrimental effects of excessive reactive oxygen species [38]. Reduced CoQ10 also inhibits lipid peroxidation in biological membranes, protects membrane proteins against oxidative damage [39], and inhibits peroxidation of lipids and cholesterol in low-density lipoproteins [24,40].

Based on our data, NPC patients do not have a deficiency of CoQ10. Mean levels of both total and reduced CoQ10 are normal. Thus, neither decreased dietary intake nor synthesis of CoQ10 is likely to be an issue in NPC. Our data support the hypothesis that the cycling of oxidized to reduced CoQ10 is impaired in NPC, resulting in a decreased fraction of reduced CoQ10. Our data clearly demonstrate dietary supplementation of CoQ10 is ineffective in correcting this problem. CoQ10 supplementation increases total levels, but the fraction of reduced CoQ10 remains low. In fact, although some of the highest total CoQ10 levels were observed in CoQ10-supplemented patients, these same patients had some of the lowest ratios of reduced to total CoQ10.

Increased oxidative stress would be consistent with a decreased fraction of reduced CoQ10. Measurement of serum TEAC, a proxy for total antioxidant capacity, in NPC patients showed a significant decrease compared to age appropriate control samples. Thus, both the CoQ10 and TEAC data support the hypothesis of increased oxidative stress.

Currently, there are no feasible therapeutic interventions that would correct the primary defect in NPC. Because the NPC1 protein is membrane bound, and the defect is cell autonomous [41], therapies designed to correct the primary defect in neurons (such as gene or stem cell therapy) are not likely to be practical in the near future. Chaperone therapy could address the primary defect in some patients but is not yet applicable [42]. An alternative approach would be to focus interventions on ameliorating specific downstream aspects of the pathological cascade. Ultimately, this therapeutic approach will necessitate the development of combination therapy. Although not a cure, such a therapeutic approach might slow the inevitable progression of neurological symptoms in NPC. Miglustat is an iminosugar that inhibits glycosphingolipid synthesis [43]. In addition to cholesterol, glycosphingolipids also accumulate in NPC neurons [44]. Treatment of *Npc1* mutant mice with miglustat significantly delays onset of neurological symptoms [8], and a recent clinical trial in NPC patients suggests some efficacy [45]. It is likely that the efficacy of miglustat is limited because it addresses only one aspect of the pathological cascade. Although statistical power is limited by the small number of NPC patients available for analysis, we show that miglustat therapy does not significantly improve either the fraction of reduced CoQ10 or TEAC levels in NPC patients. This suggests that oxidative stress is an independent pathological process and suggests that combination therapy with an antioxidant may be of additional benefit in NPC patients. This hypothesis is under investigation in a randomized, placebo-controlled clinical trial utilizing CoQ10 and TEAC as biomarkers for oxidative stress in NPC patients.

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