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Apolipoprotein E Genotype and Neurological Disease Onset in Niemann–Pick Disease, Type C1

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Niemann–Pick disease, type C1 (NPC1) is a lipid storage disorder that results in progressive neurological impairment. The NPC1 phenotype is extremely variable and at the individual level is likely influenced by other genetic traits. In addition to residual function of NPC1 protein, we hypothesize that modifier genes, as frequently observed with other autosomal recessive diseases, influence the NPC phenotype. The NPC1 phenotype includes progressive dementia, and the NPC pathology has some overlap with the pathology of Alzheimer disease (AD). Thus, we examined apolipoprotein E (ApoE) and microtubule-associated protein tau (MAPT) polymorphisms in a cohort of 15 NPC1 patients with well characterized longitudinal disease progression. Although we did not find any correlations between disease severity and tau polymorphisms, we found significant associations between ApoE polymorphisms and phenotypic severity. Specifically, *ApoE4* and *ApoE2* alleles were associated, respectively, with increased and decreased disease severity in this cohort of NPC1 patients. These data support the hypothesis that ApoE may play a role in modulating NPC1 neuropathology.

Key words: neurodegenerative disease; lysosomal storage disease; apolipoprotein E; tau

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INTRODUCTION

Niemann–Pick disease, type C (NPC) is a lysosomal storage disorder with a wide spectrum of clinical symptoms that are a consequence of dysfunction of either the NPC1 or NPC2 protein. Understanding the factors that contribute to this phenotypic variation will provide insight into pathological mechanisms and potential therapeutic interventions. NPC is caused by mutations of either *NPC1* or *NPC2*. To date, 244 *NPC1* and 18 *NPC2* mutant alleles have been reported (http://npc.fzk.de/). The majority of NPC cases, and the cases in this report, are due to mutation of *NPC1*. The NPC1 phenotype is variable in both age of neurological onset and symptoms [Yanjanin et al., 2010]. Although there currently is no good assay to quantify NPC1 function, it is likely that residual NPC1 function accounts for a large degree of this phenotypic variability. However, residual NPC1 function does not explain phenotypic variability within family and between individuals with the same genotype [Lossos et al., 1997]. Modifier genes frequently contribute to the phenotype in autosomal recessive disorders. For example, monoamine oxidase type B (*MOAB*) is a modifier gene in phenylketonuria [Ghozlan et al., 2004], and polymorphisms of both mannose-binding lectin2 (*MBL2*) and transforming growth factor beta 1 (*TGFβ1*) influence pulmonary outcome in cystic fibrosis [Accurso and Sontag, 2008]. This study was conducted to investigate the impact of potential modifier genes on NPC severity.

Although NPC1 is a distinctly different disease than Alzheimer disease (AD), common pathological processes may contribute to both disorders. These include aberrant cholesterol metabolism [Mann et al., 2004] and increased amyloid-β (Aβ) [Yamaguchi et al., 2001]. AD is pathologically characterized by amyloid plaques and neurofibrillary tangles [Tiraboschi et al., 2004]. Neurofibrillary tangles are a common feature of NPC1, and their immunohistochemical and ultrastructural appearances are identical to those of neurofibrillary tangles in AD [Suzuki et al., 1995]. A major

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component of amyloid plaques in AD is Aβ protein generated due to aberrant processing of amyloid precursor protein (APP). Consistent with overlapping pathological processes, abnormal APP processing has been observed in cultured NPC1 neurons [Jin et al., 2004], and levels of Aβ protein are significantly elevated in cerebral spinal fluid from NPC1 patients [Mattsson et al., 2011]. Although the characteristic amyloid plaques that are a cardinal finding in AD are not observed in NPC, Saito et al. [2004] reported the presence of diffuse plaques in 3/9 NPC patients. Of particular note, the ApoE genotype for these three patients was ApoE4/ApoE4.

Based on the pathological overlap of NPC1 and AD we explored the hypothesis that the NPC1 phenotype could be modulated by polymorphisms of apolipoprotein E (*APOE*) or microtubule-associated protein tau (*MAPT*). *APOE* encodes a lipoprotein that is highly expressed in the brain and plays a major role in central nervous system cholesterol homeostasis [Han, 2004]. ApoE is polymorphic with three major isoforms (ApoE2, ApoE3, and ApoE4). These isoforms differ from each other only by a Cys to Arg amino acid substitution at positions 112 or 158 [Zuo et al., 2006]. ApoE3 accounts for approximately 64% of alleles [Eisenberg et al., 2010], and is considered the "neutral" ApoE genotype. ApoE4 is associated with increased risk of AD [Bignall, 1993] and impaired cognitive function [Asada et al., 1996]. In contrast, ApoE2 may be protective against AD in some populations [Tyrrell et al., 1998]. *MAPT* encodes a protein, tau, that promotes and stabilizes microtubules [Weingarten et al., 1975]. The MAPT haplotype H1 is a risk factor for AD [Shaw-Smith et al., 2006]. In this study, we correlated *ApoE* and *MAPT* genotypes with neurological disease onset and found that *ApoE4* and *ApoE2* alleles were associated, respectively, with early and later neurological disease onset.

MATERIALS AND METHODS

Study Subjects

This study was approved by the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Institutional Review Board. DNA or cell lines were available for 15 of the 19 patients for whom longitudinal disease progression had previously been characterized by chart review [Yanjanin et al., 2010]. DNA of H12 was only available for *APOE* but not *MAPT* genotyping. To determine the "age of neurological onset", the progression curve derived from the total score was extrapolated to the x-axis (age) by linear regression. Based on the calculated age of neurological onset, patients were ordered from earliest to latest onset. A similar analysis was also performed using the subset of the total score derived from the memory and cognition subscores. Two of the patients had no change in their subscores over multiple visits. H10, with an E3/E4 genotype, had a subscore of 3 stable from 10 to 18 years old; and H12, with an E3/E3 genotype, had a subscore of 7 stable from 26 to 32 years old. In order to include these patients in the analysis, we determined the mean (0.44 points/year) progression slope for these two domains based on data from the other 13 patients. We then used this value combined with the latest age for which data were available to rank these patients. We felt that this conservative estimate was more appropriate than exclusion of these data.

APOE and *MAPT* Genotyping

APOE genotype was determined by PCR Restriction Fragment Length Polymorphism (PCR-RFLP) assay using a previously published protocol with minor modifications [Hixson and Vernier, 1990]. Briefly, PCR was used to amplify a 218 bp fragment of genomic DNA which included *APOE* codons 112 and 158. The PCR was carried out with the outer primer sequences (APOE-F: 5′-CGGCTGTCCAAGGAGCTGC-3′ and APOE-R: 5′ GGCCT-GGTACACTGCCAGG-3′), amplifying a 218 bp DNA product. The PCR was performed in a 50 µl reaction mixture containing 100 ng gDNA, 50 pmol/µl primer, 10× RAB PCR buffer with $MgCl^{2+}$ (Roche Applied Science, Indianapolis, IN), 100 mM dNTPs, 5U/L Taq polymerase, and 5 M Betaine. One cycle of denaturation at 94°C for 4 min, was followed by 35 cycles at 94°C for 45 sec, 64°C for 45 sec, and 72°C for 45 sec. The reaction was concluded with a 10 min extension step at 72°C. An aliquot of the PCR-amplified product was digested with restriction enzyme HhaI overnight at 37°C. For genotype analysis, the small fragment-sized cleavage products of HhaI (48, 72, 81, and 91 bp) were electrophoresed through 20% polyacrylamide gels which were stained with ethidium bromide and visualized with ultraviolet illumination.

MAPT genotype was determined by PCR amplification based on a previously reported method [Baker et al., 1999]. The PCR reaction utilized the forward primer sequence 5′-GGAAGACGT-TCTCACTGATCTG-3′ and reverse primer 5′-AGGAGTCTGG-CTTCAGTCTCTC-3′ to detect a 238 bp deletion in the H2 haplotype, yielding products of 484 or 245 bp for the H1 or H2 haplotype, respectively. The reaction was carried out in a 20 µl reaction mixture containing 50 ng DNA and final concentrations of 500 nM of each primer, 1X PCR buffer containing 1.5 mM $MgCl_2$, 250 µM dNTPs, and 2 U Taq polymerase (Roche). Denaturation at 95°C for 4 min was followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec; then an additional cycle of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec; and a final extension at 72°C for 8 min. PCR products were electrophoresed through 2% agarose gels and visualized by ethidium bromide staining and ultraviolet illumination.

Statistic Analysis

Statistical analysis was performed using Graph Pad Prism software Version 5. The Mann–Whitney Test was used for statistical comparisons of ranked data. A nominal value of $P < 0.05$ was defined as indicating a significant change. Error bars on graphs represent standard error of the mean (SEM).

RESULTS

APOE genotyping was conducted on 15 DNA samples from patients for whom longitudinal neurological progression had previously been characterized by chart review [Yanjanin et al., 2010]. This cohort of patients includes five females and 10 males, whose severity score at initial evaluation range from 1 to 27, with a mean of 9 and a median of 7. Initial evaluation age of these patients ranges from 1 to 38, with a mean of 14 and a median of 12. We previously showed that disease progression was linear, and the rate

of progression was independent of age in this group of patients. Thus, to rank patients by onset we extrapolated the progression curve to the x-axis. These values ranged from 0.5 to 35.5 years, and we refer to this value as the calculated age of neurological onset. The 15 NPC1 patients were ranked in order from earlier to later neurological disease onset; and the number of patients with childhood onset (0–12 years old), adolescent onset (13–18 years old), and adult onset (>18 years old) were 10, 3, and 2, respectively. *APOE* genotype was determined by PCR-RFLP (Fig. 1A) and *APOE* genotypes for these 15 subjects are shown in Figure 2A. Of note, *ApoE4* and *ApoE2* alleles are clustered in earlier and later onset cases, respectively. Figure 2B compares the calculated age of neurological onset for patients with and without an *ApoE2* allele and patients with and without an *ApoE4* allele. NPC1 subjects with an *ApoE4* allele had a significantly earlier onset (3.6 ± 0.8 years) than those without an *ApoE4* allele (15.5 ± 3.64 years; $P < 0.05$), and subjects with an *ApoE2* allele had a significantly later onset (19.3 ± 0.8 years) than those without an *ApoE2* allele (7.6 ± 2.7 years, $P < 0.001$).

Because memory and cognition are major issues characterizing the dementia observed in NPC1, we also ranked this cohort of NPC1 patients based on the combined score of these two subscores (Fig. 3A). In this method, NPC1 patients appear to have calculated age of neurological onset ranging from −7.5 to 33.2 years. Negative values result from extrapolation to the x-axis and only represent a tool to rank patients with regards to severity. Though not statistically significant, we observed that patients with an *ApoE4* allele and *ApoE2* allele appeared to have a trend of earlier and later onsets, respectively. Comparing the calculated age of onset values utilizing the cognitive and memory subdomains (Fig. 3B), patients with at least one *ApoE4* allele had an earlier mean age of onset (3.9 ± 1.5years) compared to patients without an *ApoE4* allele (12.2 ± 4.3 years). However, this was not significant ($P = 0.09$). In contrast, patients with an *ApoE2* allele had a later calculated age of onset compared to patients without an *ApoE2* allele (Fig. 3B) (6.5 ± 3.0 vs. 15.8 ± 1.7 years, $P = 0.07$). *MAPT* genotyping for a group of 14 NPC1 patients is shown in Figure 4. No significant correlations between disease onset and *MAPT* genotype were observed when ranking based on the total score (Fig. 4A) or the memory and cognition subscore (Fig. 4B).

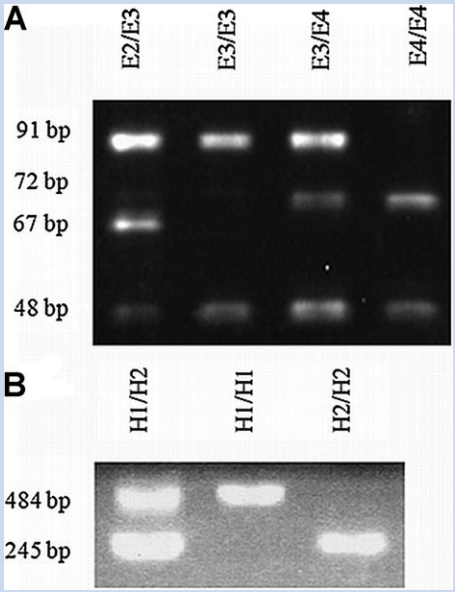
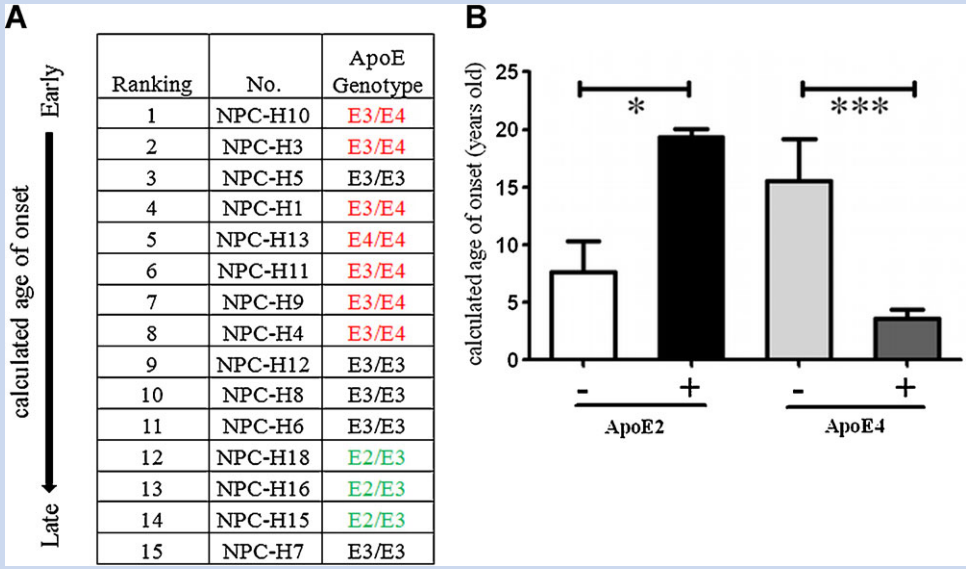


FIG. 1. Apolipoprotein E (*APOE*) and microtubule-associated protein tau (*MAPT*) genotyping. A: *ApoE* genotype was determined by PCR/Restriction Fragment Length Polymorphism (PCR-RFLP) analysis. *ApoE* alleles were identified by the DNA fragmentation pattern (ApoE2: 67 and 91 base pairs; ApoE3: 48 and 91 base pairs; ApoE4: 48 and 72 base pairs). B: MAPT genotype was determined by PCR. H1 and H2 haplotypes correspond to DNA fragments of 484 base pairs and 245 base pairs, respectively.

DISCUSSION

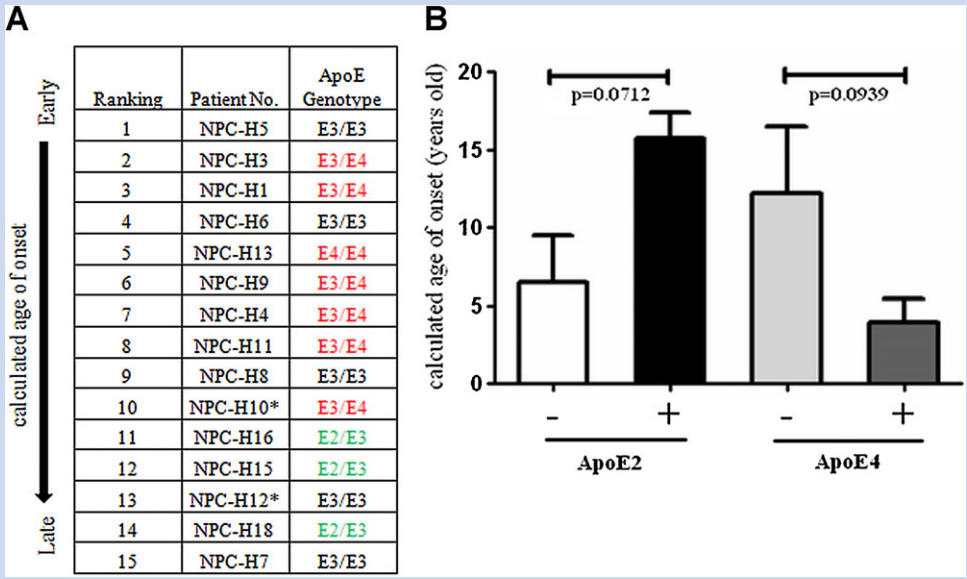
AD and NPC1 have some common pathological overlap including increased levels of both Aβ40 and Aβ42, and the presence of neurofibrillary tangles. The neurofibrillary tangles reported in NPC are immunologically and ultrastructurally identical to those observed in AD [Suzuki et al., 1995], and Mattsson et al. [2011] have recently shown that both Aβ40 and Aβ42 are abnormally increased in CSF from patients with NPC1 disease.

ApoE4 is the most common genetic risk factor for AD [Bignall, 1993]. The mechanism by which *ApoE4* increases the risk for AD is not entirely understood [Verghese et al., 2011]. However, a number of pathological mechanisms, which also may influence pathological progression of NPC1 disease, have been proposed. ApoE isoforms differentially modulate amyloid β metabolism including production and clearance, and thereby may contribute to Aβ toxicity [Huang et al., 2011; Verghese et al., 2011; Ye and Zhang, 2012]. ApoE isotype may influence other pathological mechanisms that could play a role in both NPC1 and AD. ApoE4 has been reported to impair synaptic placticity [Chen et al., 2010], and ApoE isotype appears to influence the development of neurofibrillary tangles [Montine et al., 1999]. In this latter context, it is notable that Saito et al. [2004] observed increased neurofibrillary tangles in brain tissue from NPC patients who were homozygous for the *ApoE4* allele. In this study we demonstrate a correlation between *APOE* genotype and neurological disease onset in a 15-patient cohort of NPC1 patients for whom we have both longitudinal disease progression data and DNA. Our observation that an *ApoE4* allele is associated with earlier neurological disease onset is consistent with the pathological case series by Saito et al. [2004] that found that an *ApoE4* allele was associated with more severe neuropathological findings. This observation is consistent with ApoE being a modifier gene that contributes but does not determine to an earlier onset of neurological symptoms in NPC1 patients. The major determinate is likely residual NPC1 function, and one would expect that other genes also influence the pathology of NPC1 disease. A limitation of both of these studies is the small number of cases. Unfortunately this is a limitation that is inherent to the study of rare genetic diseases. However, when combined, these studies suggest that ApoE may play a role in modulating NPC neuropathology and that specifically an *ApoE4* allele may increase the severity of neurological disease in NPC1. It will also be interesting to determine if ApoE genotype



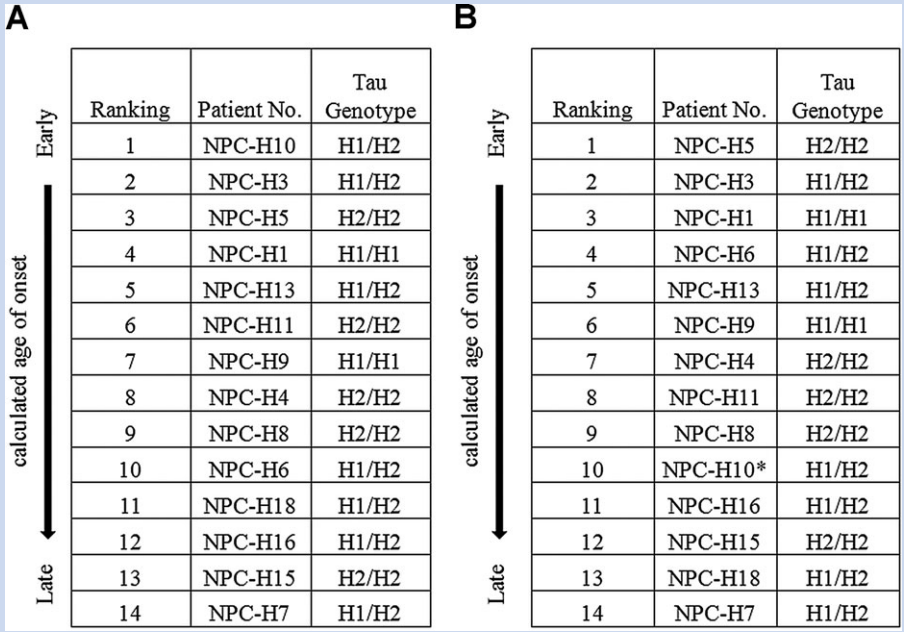
Ranking	No.	ApoE Genotype
1	NPC-H10	E3/E4
2	NPC-H3	E3/E4
3	NPC-H5	E3/E3
4	NPC-H1	E3/E4
5	NPC-H13	E4/E4
6	NPC-H11	E3/E4
7	NPC-H9	E3/E4
8	NPC-H4	E3/E4
9	NPC-H12	E3/E3
10	NPC-H8	E3/E3
11	NPC-H6	E3/E3
12	NPC-H18	E2/E3
13	NPC-H16	E2/E3
14	NPC-H15	E2/E3
15	NPC-H7	E3/E3

FIG. 2. Correlation of Apolipoprotein E genotype and neurological disease onset. A: NPC1 patients were ranked based on calculated age of neurological onset and corresponding ApoE genotype is shown. ApoE genotypes including an *ApoE4* allele are in red and those including an E2 allele are in green. B: Significant differences in the calculated age of neurological onset were observed for NPC1 patients with and without an *ApoE2* allele (***$P < 0.001$) and NPC1 patients with and without an *ApoE4* allele (*$P < 0.05$). Calculated age of neurological disease onset was earlier for patients with an *ApoE4* allele and later for patients with an ApoE2 allele.



Ranking	Patient No.	ApoE Genotype
1	NPC-H5	E3/E3
2	NPC-H3	E3/E4
3	NPC-H1	E3/E4
4	NPC-H6	E3/E3
5	NPC-H13	E4/E4
6	NPC-H9	E3/E4
7	NPC-H4	E3/E4
8	NPC-H11	E3/E4
9	NPC-H8	E3/E3
10	NPC-H10*	E3/E4
11	NPC-H16	E2/E3
12	NPC-H15	E2/E3
13	NPC-H12*	E3/E3
14	NPC-H18	E2/E3
15	NPC-H7	E3/E3

FIG. 3. Correlation of Apolipoprotein E genotype and onset of cognitive and memory deficits. A: NPC1 patients were ranked based on memory and cognition scores. ApoE genotypes including an *E4* allele are in red and those including an *E2* allele are in green. *Ranking was estimated for these two subjects. B: NPC1 subjects with at least one *ApoE2* allele had a later age of onset compared to patients without an *ApoE2* allele, although this different was not significant ($P = 0.09$). Similarly, mean age of onset was later in patients with at least one *ApoE4* allele with a *P*-value of 0.07.



A

Early → Late (calculated age of onset)

Ranking	Patient No.	Tau Genotype
1	NPC-H10	H1/H2
2	NPC-H3	H1/H2
3	NPC-H5	H2/H2
4	NPC-H1	H1/H1
5	NPC-H13	H1/H2
6	NPC-H11	H2/H2
7	NPC-H9	H1/H1
8	NPC-H4	H2/H2
9	NPC-H8	H2/H2
10	NPC-H6	H1/H2
11	NPC-H18	H1/H2
12	NPC-H16	H1/H2
13	NPC-H15	H2/H2
14	NPC-H7	H1/H2

B

Early → Late (calculated age of onset)

Ranking	Patient No.	Tau Genotype
1	NPC-H5	H2/H2
2	NPC-H3	H1/H2
3	NPC-H1	H1/H1
4	NPC-H6	H1/H2
5	NPC-H13	H1/H2
6	NPC-H9	H1/H1
7	NPC-H4	H2/H2
8	NPC-H11	H2/H2
9	NPC-H8	H2/H2
10	NPC-H10*	H1/H2
11	NPC-H16	H1/H2
12	NPC-H15	H2/H2
13	NPC-H18	H1/H2
14	NPC-H7	H1/H2

FIG. 4. Microtubule associated protein tau polymorphisms and NPC1 disease onset. MAPT genotype did not significantly correlate with neurological disease onset using either the total score (A) or the memory and cognition subscores (B). *Ranking was estimated for this subject.

contributes to the severity of cholestatic liver disease that is frequently observed in the neonatal period. Although confirmation will be required after longitudinal progression has been quantified in larger cohorts of patients, identifying potential genetic modifiers in NPC1 could provide immediate insight into pathological processes that would be amendable to therapeutic intervention.

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