High diagnostic yield of direct Sanger sequencing in the diagnosis of neuronal ceroid lipofuscinoses

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Abstract

Background: Neuronal ceroid lipofuscinoses are neurodegenerative disorders. To investigate the diagnostic yield of direct Sanger sequencing of the CLN genes, we reviewed Molecular Genetics Laboratory Database for molecular genetic test results of the CLN genes from a single clinical molecular diagnostic laboratory.

Methods: We reviewed electronic patient charts. We used consent forms and Research Electronic Data Capture questionnaires for the patients from outside of our Institution. We reclassified all variants in the CLN genes.

Results: Six hundred and ninety three individuals underwent the direct Sanger sequencing of the CLN genes for the diagnosis of neuronal ceroid lipofuscinoses. There were 343 symptomatic patients and 350 family members. Ninety-one symptomatic patients had molecular genetic diagnosis of neuronal ceroid lipofuscinoses including CLN1 (PPT1) (n = 10), CLN2 (TPP1) (n = 33), CLN3 (n = 17), CLN5 (n = 7), CLN6 (n = 10), CLN7 (MFSD8) (n = 10), and CLN8 (n = 4) diseases. The diagnostic yield of direct Sanger sequencing of CLN genes was 27% in symptomatic patients. We report detailed clinical and investigation results of 33 NCL patients. Juvenile onset CLN1 (PPT1) and adult onset CLN6 diseases were non-classical phenotypes.

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**Conclusion:** In our study, the diagnostic yield of direct Sanger sequencing was close to diagnostic yield of whole exome sequencing. Developmental regression, cognitive decline, visual impairment and cerebral and/or cerebellar atrophy in brain MRI are significant clinical and neuroimaging denominators to include NCL in the differential diagnosis.

**KEYWORDS**
CLN genes, developmental regression, epilepsy, neuronal ceroid lipofuscinoses, visual loss

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1 | **INTRODUCTION**

Neuronal ceroid lipofuscinoses (NCL) are neurodegenerative, lysosomal storage disorders, characterized by a history of developmental regression in motor and cognitive functions, seizures, visual problems, and early death. The estimated incidence of NCL ranges from 1.3 to 7 per 100 000 live births. NCL are the most common neurometabolic neurodegenerative disease with an estimated prevalence of 1.5 to 9 per million population. The most prevalent NCL are CLN3 disease (MIM#204200) and CLN2 (TPP1) disease (MIM#204500). The onset of first symptoms varies within the same genetic subtype for the vast majority of NCL. In three of the subtypes, enzyme activity can be measured in a blood dot spot including cathepsin D (EC 3.4.23.5), encoded by CTSD (MIM#116840), palmitoyl-protein thioesterase (EC 3.1.2.22), encoded by PPT1 (MIM#600722) and tripeptidylpeptidase 1 (EC 3.4.14.9), encoded by TPP1 (MIM#607998). The electron microscopic examination of lymphocytes, skin cells or cells from conjunctival biopsy has been performed to identify lysosomal inclusions, called lipopigments and described as granular osmiophilic, curvilinear and fingerprint profiles. Pathogenic variants in 13 different genes are known to cause NCL including CLN1 (PPT1), CLN2 (TPP1), CLN3 (MIM#607042), CLN5 (MIM#256731), CLN6 (MIM#601780), MFSD8 (CLN7) (MIM#611124), CLN8 (MIM#607837), CTSD (CLN10), KCTD7 (MIM#611725), ATP13A2 (MIM#610513), CTSF (MIM#603539), GRN (MIM#138945), and DNAJC5 (MIM#611203). Treatment is symptomatic for the majority of NCL. Recently, intracerebroventricular infusions of cerliponase alfa for the treatment of CLN2 (TPP1) disease were approved.

In this study, we investigated the diagnostic yield of direct Sanger sequencing of the CLN genes for the molecular genetic diagnosis of NCL in our Molecular Genetics Laboratory. This laboratory is one of the reference laboratories providing direct Sanger sequencing for eight CLN genes for patients with suspected NCL.

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SYNOPSIS

The diagnostic yield of direct Sanger sequencing of the CLN genes was 27% in symptomatic patients and developmental regression, visual impairment and cerebral and/or cerebellar atrophy in brain MRI are significant clinical and neuroimaging denominators of neuronal ceroid lipofuscinoses.

2 | **MATERIALS AND METHODS**

Institutional Research Ethics Board (Approval#1000059020) approved this study. We included all individuals, who underwent Sanger sequencing of the CLN genes in the Molecular Genetics Laboratory. We entered clinical features, family history and investigation results of all individuals into an Excel Database who had medical records at our institution. If the patients with confirmed molecular genetic diagnosis of NCL were from other centers, we informed physicians for this study, who requested genetic investigations for NCL diagnosis in their patients. We provided a study introduction letter and a release of information consent form to inform patients and families for this study. Patients and families contacted us and signed two consents: (a) consent to enroll to the study; (b) consent for release of information for their physicians. After consents are signed, we sent invitation for Research Electronic Data Capture (REDCap) questionnaire to the physicians, where they entered their patients’ clinical information, family history, and investigation results.

From 2004 to 2008, we applied direct Sanger sequencing to six CLN genes (CLN1 (PPT1), CLN2 (TPP1), CLN3, CLN5, CLN6, CLN8). From 2008 to present we applied direct Sanger sequencing to two additional CLN genes (CLN7 and CLN10). All patients underwent direct Sanger sequencing of all seven CLN genes, if the laboratory received a request for NCL genetic test using patients’ genomic DNA sample. The primers were designed to cover exons and the first 50 bp of introns. The common 1.02 kb deletion in CLN3 was tested using quantitative PCR. If there was no amplification for
exons, quantitative PCR was applied. Synonymous variants were reported if they were previously reported as pathogenic or were predicted to disrupt splicing using Splice Site Finder (SSF), Maximum Entropy Scan (MaxEntScan) (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), Neural Network Splice (NNSPLICE) (Berkeley Drosophila Genome Project, http://www.fruitfly.org/seq_tools/splice.html), GeneSplicer (https://ccb.jhu.edu/software/genesplicer/). Only the first five intronic base pair variants were reported, unless there were previously reported pathogenic intronic variants, which were predicted to disrupt splicing in silico analysis tools or predicted to disrupt translation initiation.

We classified variants using Alamut variant interpretation software version 2.7-2 (http://www.interactive-biosoftware.com) and The American College of Medical Genetics and Genomics (ACMG) variant classification guidelines. Mismatches were identified using Sorting Intolerant From Tolerant (SIFT), Mutation Taster, and Polymorphism Phenotyping V2 (PolyPhen-2) and conservation in species in silico analysis prediction tools. Intronic or synonymous variants were analyzed using the SSF, MaxEntScan, NNSPLICE and GeneSplicer. We classified a variant as predicted to disrupt splicing if ≥3 of the splicing prediction programs predicted a >10% decrease in the possibility of correct splicing (minus correspondence to decrease). We also searched all variants identified in NCL patients in Genome Aggregation Database (gnomAD) (http://gnomad.broadinstitute.org/about) for their allele frequency in the general population. Homozygous or compound heterozygous variants of unknown significance in a symptomatic patient were interpreted: (A) molecular genetic diagnosis of NCL: (a) if we had clinical, neuroimaging and/or histopathology results of patients in our institution; (b) if the variant was reported previously in NCL patients; (B) no molecular genetic diagnosis of NCL: (a) if we did not have clinical, neuroimaging and/or histopathology results of patients with a novel variant.

We utilized the University College London (UCL) NCL Resource Patient Database (http://www.ucl.ac.uk/ncl/) to compare phenotypes, distribution of patients for each subgroup of NCL, as well as publicly available variants in NCL genes.

Fisher’s exact test was used for comparisons between patients with molecular genetic diagnosis of NCL and patients with no molecular genetic diagnosis of NCL. All analyses were performed using R statistical software. A P value < .05 was considered statistically significant.

3 | RESULTS

There were 693 individuals underwent direct Sanger sequencing of the CLN genes including 343 symptomatic, seven fetuses for prenatal diagnosis, and 343 parents, siblings, spouses, or relatives for carrier test or confirmation of the clinical diagnosis between April 2004 and April 2018. We confirmed molecular genetic diagnosis of NCL in 91 patients from 77 families in 343 symptomatic patients (27% of all symptomatic patients and 22% of all families with symptomatic children).

The number of patients for each gene associated with NCL is depicted in Figure S1. The most common NCL was CLN2 (TPP1) disease and the second most common NCL was the CLN3 disease. We had clinical information for 33 out of 91 patients with molecular genetic diagnosis of NCL from 27 families including 27 patients at our institution and 6 patients outside of our Institution within Canada. We did not have any clinical information for 58 out of 91 patients with confirmed molecular genetic diagnosis of NCL (35 from Canada and 23 from outside of Canada) for the following reasons: (a) physicians were not able to reach parents due to outdated contact information in their system or they were no longer taking care of the patients; (b) physicians did not want to contact families whose children had passed away so as not to cause further grief; (c) physicians did not respond to our three e-mail requests; and (d) families were not interested in the study.

All 33 patients presented with a history of developmental delay or developmental regression leading to NCL molecular genetic test request. There were seven patients with CLN1 (PPT1), five patients with CLN2 (TPP1), seven patients with CLN3 (patients 13 and 15 reported previously [9]), one patient with CLN5, four patients with CLN6, six patients with CLN7 (MFS8D) and three patients with CLN8 disease. Their clinical features and investigation results are summarized in Table 1. Eleven patients passed away and their survival period is depicted in Figure S2.

Conjunctival biopsy or skin biopsy showed lipopigments suggestive of NCL in 15 patients. Granular osmiophilic profile (CLN1 (PPT1) (n = 2), CLN3 (n = 1) and CLN6 (n = 3) diseases) and mixed curvilinear and fingerprint profiles (CLN2 (TPP1) (n = 1), CLN3 (n = 2), CLN7 (MFS8D) (n = 1), and CLN8 (n = 2) diseases) were the most common lipopigments. Three patients had normal histopathology.

Twenty-two patients had brain MRI and two patients had brain CT. The most common brain MRI feature was diffuse cerebral and/or cerebellar atrophy in 21 patients. Brain MRI of a patient with CLN1 (PPT1) disease and progression of MRI features are depicted in Figure 1.

In silico analysis and ACMG variant classification of all variants are listed in Table S1. There were 52 different variants in seven genes in 91 patients including 11 novel and 41 known variants: CLN1 (PPT1) variants n = 810-12,18,19; CLN2 (TPP1) variants n = 1112-14,20-23; CLN3 variants n = 119,12,15,24,25; CLN5 variants n = 512,21,26; CLN6 variants n = 712,21,27,28; CLN7 (MFS8D) variants n = 812,16,29;
| Diagnosis/patient study ID number/sex/age/consanguinity | Presenting symptom (age of onset) | Clinical features | Histopathology (CB or SB)/ neuroimaging (age) | Genetic test results (reference) |
|--------------------------------------------------------|-----------------------------------|------------------|-----------------------------------------------|----------------------------------|
| 1/CLN1(PPT1)/23/F/16 yrs/NA                            | Seizure (7 yrs)                  | GDD, regression, FTT, seizures (MS, GTCS, focal), aggressive behavior, hallucinations, stereotypic movements | NP/cerebral atrophy, small thalami, increased T2 signal in PVWM in MRI (7 yrs) | HMZ known c.674T>C (p.Phe225Ser) in CLN1 (PPT1) |
| 2/CLN1(PPT1)/29/M/7 yrs/no                             | Developmental regression (1 yr)  | GDD, cortical blindness, seizures, ataxia, athetosis, ataxia | Normal (CB)/cerebral atrophy in MRI (2 yrs) | Compound HTZ known c.29T>A (p. Leu10*)/known c.451C>T (p. Arg151*) in CLN1 (PPT1) |
| 3/CLN1(PPT1)/51/M/18 yrs/NA                            | Vision problems (<6 yrs)         | Cognitive decline, retinitis pigmentosa, hallucinations, | GROP (CB)/cerebral, cerebellar atrophy, thin CC, increased signal intensity in caudate nuclei, putamina, and centrum semiovale in MRI | HMZ known c.223A>C (p.Thr75Pro) in CLN1 (PPT1) |
| 4/CLN1(PPT1)/62/F/18 yrs/no                            | Vision problems (8 yrs)          | Cognitive regression, seizures (AbS), retinal dystrophy | NP/cerebral atrophy in MRI (13 yrs) | Compound HTZ known c.451C>T (p. Arg151*)/known c.223A>C (p. Thr75Pro) in CLN1 (PPT1) |
| 5/CLN1(PPT1)/76/M/15 yrs/no                            | Seizures (4 yrs)                 | GDD, seizures (MS, AbS, GTCS, focal), optic atrophy, cataracts, bull's eye maculopathy, hypertonia | NP/cerebral, cerebellar atrophy, decreased T2 signal in thalamus and globus pallidi T2 signal in MRI (7 yrs) | Compound HTZ known c.541G>T (p. Val1181Leu)/novel c.289_290del (p.Gln97Glyfs*4) in CLN1 (PPT1) |
| 6/CLN1(PPT1)/112/M/2 yrs 10 mo/no                       | GDD, regression (1 yr)           | GDD, seizures (AbS, GTCS, MS), spasticity, microcephaly | NP/cerebral atrophy, small thalami decreased T2 signal in MRI (2 yrs) | Compound HTZ known c.451C>T (p. Arg151*)/known c.490C>T (p. Arg164*) in CLN1 (PPT1) |
| 7/CLN1(PPT1)/168/F/18 yrs/yes                          | Vision problems (7 yrs)          | Developmental regression, aggressive behavior, tremor, RP | GROP (SB)/NP | HMZ known c.223A>C (p.Thr75Pro) in CLN1 (PPT1) |
| 8/CLN2(TPP1)/27/M/18 yrs/no                            | Seizures (3.5 yrs)               | GDD, seizures, ataxia | NP/diffuse cerebral and cerebellar atrophy, increased FLAIR signal in MRI (9 yrs) | Compound HTZ known c.509-1G>C/known c.622C>T (p.Arg208*) in CLN2 (TPP1) |
| 9/CLN2(TPP1)/86/M/14 yrs/NA                            | Positive family history of NCL (<3 yrs) blindness | GDD, seizures (MS, GTCS, drop attacks) blindness | Normal (CB)/cerebral atrophy in MRI (11 yrs) | Compound HTZ known c.509-1G> C/known c.1064T>C (p.Leu355Pro) in CLN2 (TPP1) |
| 10/CLN2(TPP1)/117/M/12 yrs/no                          | Speech delay (3 yrs)             | GDD, seizures (AS, MS, TS, AbS), ataxia, behavioral problems | CP & FP (CB)/cerebral atrophy, increased signal in central WM, small thalami in MRI | Compound HTZ known c.509-1G>C/known c.622C>T (p.Arg208*) in CLN2 (TPP1) |
| 11/CLN2(TPP1)/170/M/9 yrs/no                           | Attention deficit (3.5 yrs)      | GDD, seizures (MS, GTCS, TS, focal), developmental regression, ASD, behavioral problems, dystonia, ataxia, chorea-athetosis | CP (SB)/arachnoid cyst in MRI (5 yrs) | Compound HTZ known c.509-1G>C/known c.229G>C (p.Gly77Arg) in CLN2 (TPP1) |

(Continues)
| Table 1 (Continued) |
|----------------------|
| Diagnosis/patient study ID number/sex/age/consanguinity | Presenting symptom (age of onset) | Clinical features | Histopathology (CB or SB)/ neuroimaging (age) | Genetic test results (reference) |
| 12q/CLN2(TPP1)Y171/F/6 yrs/no | Hyperactivity (16 mo) | GDD, seizures (MS, GTCS, TS, focal), dystonia, behavioral problems, ataxia, chorea-athetosis, dyskinesia, visual impairment | NP/cerebellar atrophy in MRI (3 yrs) | Compound HTZ known c.509-1G>C \(^{12}\) known c.229G>C (p.Gly77Arg) \(^{14}\) in CLN2 (TPP1) |
| 13/CLN3/42/M/11 yrs/no | Visual problems (4 yrs) | GDD, regression, seizures (GTCS, focal, AbS, TS), behavioral problems | CP & FP (CB)/NP | HMZ known 1.02 kb deletion \(^{15}\) in CLN3 |
| 14S/CLN3/2/F/18 yrs/no | Night vision problems (10 yrs) | RP | GROP (CB)/NP | Compound HTZ known c.883G>T (p. Glu295*) \(^{9}\)/known c.917T>A (p. Leu306His) \(^{9}\) in CLN3 |
| 15S/CLN3/44/F/18 yrs/no | Visual problems (10 yrs) | RP, photophobia | NP/NP | Compound HTZ known c.883G>T (p. Glu295*) \(^{9}\)/known c.917T>A (p. Leu306His) \(^{9}\) in CLN3 |
| 16/CLN3/61/M/18 yrs/NA | Visual impairment (<9 yrs) | Cognitive dysfunction, aggressive behavior, blindness, seizures (AbS, AS, GTCS), tremor, ataxia | NP/NP | HMZ known 1.02 kb deletion \(^{15}\) in CLN3 |
| 17/CLN3/121/M/18 yrs/yes | Visual impairment (3 yrs) | GDD, regression, cortical blindness, seizures (MS, GTCS, TS, AbS) | NP/cerebral atrophy in MRI (10 yrs) | HMZ novel c.963G>A (p.Trp321*) in CLN3 |
| 18π/CLN3/46/M/18 yrs/yes | Cognitive decline (7 yrs) | GDD, optic atrophy | NP/NP | HMZ known c.1001G>A (p. Arg334His) \(^{15}\) in CLN3 |
| 19π/CLN3/144/F/18 yrs/yes | Visual impairment (6 yrs) | Seizures (GTCS, RP, cognitive dysfunction, hypokinesia) | CP & FP (CB)/NP | HMZ known c.1001G>A (p. Arg334His) \(^{15}\) in CLN3 |
| 20/CLN5/94/F/16 yrs/NA | Balance problems (3.5 yrs) | GDD, spasticity, seizures (MS, AS, AbS, focal), blindness, ataxia | NP/cerebral and cerebellar atrophy, decreased signal in thalami in MRI | HMZ novel c.545T>G (p.Met182Arg) in CLN5 |
| 21/CLN6/15/F/10 yrs/yes | Progressive motor and cognitive dysfunction (4 yrs) | GDD, regression, seizures (GTCS, MS, AbS, focal), optic atrophy | FP (CB)/cerebral, cerebellar atrophy in MRI | HMZ novel c.198+1G>A in CLN6 |
| 22γ/CLN6/178/M/44 yrs/NA | Lower limb pain (31 yrs) | Depression, dementia, seizures (MS), spasticity, rigidity, ataxia | GROP (SB)/cortical atrophy in CT | HMZ known c.445C>T (p. Arg149Cys) \(^{2}\) in CLN6 |
| 23γ/CLN6/202/F/55 yrs/NA | Lower limb pain (25 yrs) | Dementia, spasticity, rigidity | GROP (SB)/cortical atrophy in CT | HMZ known c.445C>T (p. Arg149Cys) \(^{2}\) in CLN6 |
| 24γ/CLN6/224/F/40 yrs/NA | Epilepsy (20 yrs) | Seizures (MS), dementia, ataxia, spasticity, rigidity | GROP (SB)/cortical atrophy, increased T2 signal in PVWM, decreased signal in thalami in MRI | HMZ known c.445C>T (p. Arg149Cys) \(^{2}\) in CLN6 gene |
| 25/CLN7(MFS/DF8)/68/F/14 yrs/NA | Balance and speech problems (4 yrs) | GDD, seizures (MS, GTCS, AS, focal), spastic quadriplegia | NP/NP | HMZ known c.754+2T>A \(^{16}\) in CLN7 (MFS/DF8) |

(Continues)
| Diagnosis/patient study ID number/sex/age/consanguinity | Presenting symptom (age of onset) | Clinical features | Histopathology (CB or SB)/neuroimaging (age) | Genetic test results (reference) |
|---------------------------------------------------------|----------------------------------|------------------|--------------------------------------------|-------------------------------|
| 26/CLN7(MFSD8)/78/M/10 yrs/yes | Speech delay (1.25 yrs) | GDD, regression, seizures (MS, GTCS, AS) visual impairment, microcephaly, hypotonia | CP&FP (CB)/cerebral, cerebellar atrophy, small thalami, increased T2 signal PVWM in MRI | HMZ novel c.1241_1242insGAAT (p. Ile414Metfs*109) in CLN7 (MFSD8) |
| 27/CLN7(MFSD8)/93/F/15 yrs/NA | Cognitive decline regression (5 yrs) | GDD, seizures (GTCS, focal), visual impairment, ataxia | FP (CB)/cerebral, cerebellar atrophy, increased T2 signal PVWM and thalami in MRI | HMZ known c.1394G>A (p. Arg465Gln)\(^1\) in CLN7 (MFSD8) |
| 28/CLN7(MFSD8)/100/F/12 yrs/NA | Developmental regression (2.75 yrs) | GDD, regression, seizures (GTCS, MS, AbS, focal), hypertonia, ataxia | NP/cerebellar atrophy, increased T2 signal PVWM in MRI | HMZ known c.881C>A (p. Thr294Lys)\(^1\) in CLN7 (MFSD8) |
| 29/CLN7(MFSD8)/116/F/7 yrs 7 mo/NA | Ataxia (3.5 yrs) | GDD, seizures (MS, GTCS, AbS), optic atrophy, ataxia | NP/cerebellar atrophy, increased T2 signal PVWM in MRI | HMZ known c.416G>A (p. Arg139His)\(^1\) in CLN7 (MFSD8) |
| 30/CLN7(MFSD8)/129/M/18 yrs/NA | Seizures (7 yrs) Physician diagnosed | GDD, regression, seizures (GTCS, MS, AbS, TS), ataxia, bradykinesia, tremor | Normal (CB)/cerebral atrophy, cerebellar, increased FLAIR signal PVWM in MRI | HMZ novel c.863+4A>G in CLN7 (MFSD8) |
| 31/CLN8/64/F/18 yrs/yes | Developmental regression (3 yrs 8 mo) | GDD, seizures (MS, GTCS, focal, AS), regression, severe optic atrophy | NP/NP | HMZ known c.473A>G (p. Tyr158Cys)\(^1\) in CLN8 |
| 32/CLN8/85/M/11 yrs/yes | Developmental regression (2.5 yrs) | GDD, spasticity, seizures (GTCS, MS) | CP&FP (CB)/cerebral atrophy, small thalami in MRI | HMZ known c.473A>G (p. Tyr158Cys)\(^1\) in CLN8 |
| 33/CLN8/73/F/13 yrs/no | Developmental regression (3 yrs) | GDD, seizures (GTCS, MS), visual impairment, dystonia | CP&FP (CB)/cerebellar atrophy, increased T2 signal PVWM in MRI | HMZ known c.473A>G (p. Tyr158Cys)\(^1\) in CLN8 |

\(^a\)Deceased age.
\(^b\)Siblings.

Abbreviations: AbS, absence seizures; AS, atonic seizures; CB, conjunctival biopsy; CC, corpus callosum; CP, curvilinear profile; CT, computed tomography; d, day(s); dec, deceased; FP, fingerprint profile; GDD, global developmental delay; GROP, granular osmiophilic profiles; GTCS, generalized tonic-clonic seizures; HMZ, homozygous; HTZ, heterozygous; mo, months; MRI, magnetic resonance imaging; MS, myoclonic seizures; NCL, neuronal ceroid lipofuscinoses; NP, not performed; PV, periventricular; RP, retinitis pigmentosa; SB, skin biopsy; TS, tonic seizures; WM, white matter; y, year(s).
The number of missense and truncating variants for each gene is depicted in Figure S3. The most common variant type was truncating in CLN3, CLN5, and CLN6. According to ACMG variant classification, 25 variants were classified as pathogenic, and 24 variants were classified as likely pathogenic. Three variants were classified as variant of unknown significance including two novel (c.545T>G; p.Met182Arg in CLN5 and c.863+4A>G in CLN7) and one known (c.445C>T; p.Arg149Cys in CLN6[12]). All of those patients were listed in Table 1 for their clinical information with a molecular genetic confirmation of NCL. We did not have any clinical information for three patients with five variants (homozygous or compound heterozygous, one known pathogenic and four novel variants of unknown significance) in CLN2, CLN6, and CLN8 and did not include them into our list of patients with molecular genetic confirmation of NCL. We had clinical features and histopathology and neuroimaging results of 76 out of 343 symptomatic patients with no molecular genetic diagnosis of NCL at our institution. Distribution of clinical features and histopathology and neuroimaging results of 33 patients with molecular genetic

![FIGURE 1](image-url) Two brain MRIs and disease progression in a patient with CLN1 (PPT1) associated disease (patient 5, ID#76 in Table 1). Top row 4y2m (A-D): Sagittal T1 weighted image (A) demonstrates a normal corpus callosum, brainstem and vermis. Axial T2 weighted image (B) reveals mild widening of the interfoliate sulci (arrow) of the cerebellar hemispheres. Axial FLAIR image (C) shows dark signal intensity of the thalami (arrow) and increased signal of the periatrial white matter. Mild widening of the supratentorial sulci is noted (arrow) on T2 weighted axial image (D). Bottom row 7y8m (E-H): Marked thickening of the calvarium is shown on all images. There is volume loss of the corpus callosum and moderate atrophy of the vermis (arrow) on T1 weighted sagittal image (E). Progressive widening of the interfoliate sulci (arrow) of the cerebellar hemispheres is shown on axial T2 weighted image (F). There is persistent loss of thalamic signal and volume (arrow) on axial FLAIR image (G). On the same image, loss of globus pallidus signal is present (short arrow) and the white matter is diffusely abnormally increased in signal. Marked widening of the supratentorial sulci (arrow) and moderate ventricular enlargement is noted on axial T2 weighted image (H), reflecting both central and peripheral volume loss.
diagnosis of NCL and 76 patients with no molecular genetic diagnosis of NCL are summarized in Table 2. The history of regression, cognitive decline and visual impairment and presence of cerebral and cerebellar atrophy in brain MRI and presence of lipopigments in biopsy histopathology were significantly different in patients with molecular genetic diagnosis of NCL (Fisher’s exact test \( P < .05 \)). Hypotonia, infantile spasms, normal biopsy histopathology and white matter abnormalities in brain MRI were significantly different in patients with no molecular genetic diagnosis of NCL (Fisher’s exact test \( P < .05 \)).

Twenty-four out of 76 symptomatic patients were heterozygotes for 22 variants including five known pathogenic and three likely pathogenic (two known and one novel) variants. All those variants and their ACMG variant classification are listed in Table S3.

### 4 | DISCUSSION

We report 27% diagnostic yield of direct Sanger sequencing of the \( CLN \) genes (22% in families with more than one symptomatic child). \( CLN2 \) (\( TPP1 \)) disease was the most

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**Table 2** Distribution of clinical features, neuroimaging features, and histopathology results of patients with and without molecular genetic diagnosis of NCL are listed. Fisher's exact test was applied for the comparison of proportions for each finding between two groups and are listed

| Clinical features and investigations | Number of patients with molecular genetic diagnosis of NCL (percentage) (n = 33) | Number of patients with no molecular genetic diagnosis of NCL (percentage) (n = 76) | Fisher’s exact test \( P \) value |
|-------------------------------------|----------------------------------------|---------------------------------------------|----------------------------------|
| **Neurodevelopment and neurological features** | | | |
| GDD                                | 23 (69.6%)                             | 56 (73.7%)                                 | .8158 |
| Regression or cognitive decline     | 19 (57.5)                              | 15 (19.7%)                                 | .0017 |
| Hypotonia                          | 1 (3%)                                 | 22 (28.9%)                                 | .0017 |
| Hypertonia                         | 2 (6%)                                 | 13 (17.1%)                                 | .224 |
| **Seizures**                       | 27 (81.8%)                             | 59 (77.6%)                                 | .7993 |
| GTCS                               | 21 (63.6%)                             | 23 (30.2%)                                 | .0015 |
| Myoclonic                          | 18 (54.5%)                             | 20 (26.3%)                                 | .008 |
| Absence                            | 11 (33.3%)                             | 17 (22.3%)                                 | .2417 |
| Infantile spasms                   | 0 (0%)                                 | 12 (15.7%)                                 | .0165 |
| Partial Seizures                   | 11 (33.3%)                             | 10 (13.1%)                                 | .0188 |
| **Visual impairment**              | 22 (66.6%)                             | 13 (17.1%)                                 | <.0001 |
| **Movement disorder**              | 19 (57.5%)                             | 33 (43.4%)                                 | .2125 |
| Ataxia                             | 13 (39.3%)                             | 15 (19.7%)                                 | .0545 |
| Dystonia                           | 3 (9.1%)                               | 0 (0%)                                     | .026 |
| Tremor                             | 3 (9.1%)                               | 7 (9.2%)                                   | 1 |
| **Behavioral problems**            | 7 (21.2%)                              | 13 (17.1%)                                 | .6007 |
| Failure to thrive                  | 1 (3%)                                 | 8 (10.5%)                                  | .2722 |
| **Histopathology**                 | 18                                     | 19                                          | <.0001 |
| Normal                             | 3 (16.6%)                              | 19 (100%)                                  | |
| Granular osmiophilic inclusions    | 6                                      | 0                                           | |
| Finger print                       | 2                                      | 0                                           | |
| Curvilinear and fingerprint profiles | 6                                    | 0                                           | |
| **Brain MRI features**             | 22                                     | 67                                          | |
| Cerebral and/or cerebellar atrophy | 21 (95.4%)                             | 37 (55.2%)                                 | .0005 |
| Abnormal T2/FLAIR signal intensities | 15 (68.2%)                             | 18 (26.8%)                                 | .0008 |
| Corpus callosum abnormalities      | 1 (4.5%)                               | 17 (25.3%)                                 | .0362 |
| Delayed myelination                | 0 (0%)                                 | 12 (17.9%)                                 | .0077 |
| Hypomyelination                    | 0 (0%)                                 | 4 (5.9%)                                   | .2991 |

Abbreviations: GDD, global developmental delay; GTCS, generalized tonic-clonic seizures; MRI, magnetic resonance imaging.
common subtype (36%) and CLN3 disease was the second most common subtype (19%) of NCL in our study. Distribution of NCL in the UCL NCL Resource Patient Database (http://www.ucl.ac.uk/ncl/) is depicted in Figure S4. We report detailed clinical features of 33 NCL patients and 11 novel variants in the CLN genes. Presence of developmental regression, cognitive decline and progressive visual impairment in the history, and cerebral and cerebellar atrophy in brain MRI are significant clinical and neuroimaging denominators and should warrant physicians to investigate NCL using direct Sanger sequencing of the CLN genes.

In patients with neurodegenerative disorders, skin biopsy was applied to identify underlying causes in a few studies. The diagnostic yield of NCL based on the lipopigments was <10% in all of those studies (Williams et al., 2006). In one of those studies, 143 patients had axillary skin biopsy for the evaluation of metabolic disease. Only one patient had curvilinear profile and was morphologically diagnosed with NCL. Morphological diagnostic yield of skin biopsy was 0.7% in that study. In another study, 184 pediatric patients underwent skin biopsy including 139 patients with progressive encephalopathies and 45 patients with static encephalopathies. Morphological diagnosis of NCL was confirmed in 6.5% (12 out of 184) patients. In 2013, the experts recommended an algorithm for the diagnostic investigations of NCL in patients with suggestive symptoms including enzyme tests and biopsies to investigate for lipopigments to perform economical and adequate diagnostic investigations for the molecular confirmation of NCL. The molecular genetic tests identify variants in NCL genes in more than 90% of the patients with phenotypic features suggestive of NCL. To the best of our knowledge, there are no studies that reported the diagnostic yield of next generation sequencing or whole exome sequencing for NCL genetic diagnosis.

Morphological diagnosis of CLN1 (PPT1) (granular osmiophilic profile), and CLN2 (TPP1) (curvilinear profile) diseases have been reported in the majority of patients with the genotypic confirmation. Mixed lipopigment profiles were also reported in CLN1 (PPT1) (15%) and CLN2 (TPP1) (20%) diseases in the UCL NCL Resource Patient Database. In our study, we found mixed profiles in 33% (6 out of 18 patients with biopsy) and normal histopathology in 17% of the patients. Genotype and morphotype correlation was present in 17% of the patients (CLN1 n = 2 and CLN2 n = 1) with a confirmed molecular genetic diagnosis of NCL in our study. In centers, where molecular genetic tests are not readily available or funded through health care system, in the presence of suggestive history and brain MRI features, conjunctival or skin biopsy histopathology will likely help clinicians to guide them for direct Sanger sequencing of the CLN genes. Normal histopathology and normal NCL genetic test results are sufficient to exclude NCL.

In the UCL NCL Resource Patient Database, CLN1 (PPT1) disease was juvenile onset (≥2 years of age) in 27% of the patients, whereas in our study, 71% of the patients had juvenile disease onset (≥4 years). Interestingly, in three out of five patients with juvenile onset CLN1 (PPT1) disease, vision problems were the presenting symptom in our study. In the UCL NCL Resource Patient Database, CLN6 disease was adult onset in 12% of the patients, whereas in our study three out of four (75%) patients with CLN6 disease had adult onset. Interestingly, in two of those patients, lower limb spasticity was the presenting symptom. The phenotypic spectrum is highly variable in CLN1 (PPT1) and CLN6 diseases and application of targeted next generation sequencing panels for seizures, intellectual disability or retinopathy, or whole exome sequencing will likely identify more patients with nonclassical NCL phenotypes. In patients with retinopathy and spastic paraplegia, targeted next generation sequencing panels should also include all CLN genes due to variable phenotype within the same genotype.

Our Research Ethics Board approved the study with the condition of two consents signed by parents or institutional Research Ethics Board approvals at each institution. The majority of clinicians did not apply to their Research Ethics Board due to single patient or small number of patients at their institution. The majority of the clinicians did not want to contact parents to provide study information, as their children had passed away due to NCL or clinical contact was lost. Due to these, we received phenotypic information in 9.4% of NCL patients (6 out of 64 patients) outside of our Institution. Additionally, we were not allowed to collect any other genetic diagnoses other than NCL in our database as per request of our Research Ethics Board. We were also requested not to report any other genetic diagnoses in patients with no molecular genetic diagnosis of NCL in the manuscript. For these reasons, we do not have any data for other genetic diagnoses in the group of 76 symptomatic patients with no molecular genetic diagnosis of NCL at our Institution. The remaining 176 symptomatic patients were referred from outside of our institution that we did not have any clinical information for those. A follow-up multicenter study would be interesting to identify the diagnostic yield of targeted next generation sequencing panels or whole exome sequencing for NCL. It is not certain if the diagnostic yield of next generation sequencing would be higher than our study results.

The most commonly reported variant in CLN1 (PPT1) was c.451C>T (p.Arg151*) in the UCL NCL Resource Mutation Database, which is a panethnic variant. This variant resulted in phenotypes ranging from infantile onset to adult onset either compound heterozygous or homozygous. We also had similar results in our small cohort of patients with CLN1 (PPT1) disease for this common variant.
In conclusion, we report 27% diagnostic yield of direct Sanger sequencing of the CLN genes in symptomatic patients (22% in families with more than one affected child). Developmental regression, cognitive decline, visual impairment and cerebral and/or cerebellar atrophy in brain MRI are significant clinical and neuroimaging denominators to include NCL in the differential diagnosis. Juvenile onset CLN1 (PPT1) and adult onset CLN6 disease phenotypes were the most common non-classical phenotypes in our study. Whole exome sequencing or targeted next generation sequencing panels for epilepsy, intellectual disability and retinopathy will likely identify more patients with nonclassical phenotypes of NCL and should be the first line investigations in patients with a history of progressive neurodegenerative encephalopathy.

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AUTHOR CONTRIBUTIONS

A.J. reviewed charts, generated database, drafted the manuscript, conducted the work, and involved in the approval of the final version. D.M. generated database from the molecular genetics laboratory, reviewed all variants for in silico analysis and classified all variants based on the ACMG variant classification guidelines, and involved in the approval of the final version. S.B. reviewed patient MRIs and provided figure and image information, and involved in the approval of the final version. S.D., J.M., A.N., C.P. provided cases from their centers and involved in the approval of the final version. L.K. generated database from the molecular genetics laboratory and involved in the approval of the final version. S.M-A involved in planning, applying, and receiving funding; conduct, drafting, and revising the manuscript; and approval of the final version.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL AND PATIENT CONSENT

Institutional Research Ethics Board (Approval#1000059020) approved this study.

Informed consent was obtained for patients outside of our institution.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5).

Proof that informed consent was obtained is available upon request.

There is no identifying information about patients included in the article.

This article does not contain any studies with animal subjects performed by the any of the authors.

DATA AVAILABILITY

The authors confirm that the part of the data supporting the findings of this study is available within the article (and/or) its supplementary material based on the Research Ethics Board approvals. The data that support the findings of this study analysis are available on request from the corresponding author. The data are not publicly available due to data containing research participants' information could compromise the privacy of research participants.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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