Expanding the phenotypic and molecular spectrum of RNA polymerase III–related leukodystrophy

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Abstract

Objective
To expand the phenotypic spectrum of severity of POLR3-related leukodystrophy and identify genotype-phenotype correlations through study of patients with extremely severe phenotypes.

Methods
We performed an international cross-sectional study on patients with genetically proven POLR3-related leukodystrophy and atypical phenotypes to identify 6 children, 3 males and 3 females, with an extremely severe phenotype compared with that typically reported. Clinical, radiologic, and molecular features were evaluated for all patients, and functional and neuropathologic studies were performed on 1 patient.

Results
Each patient presented between 1 and 3 months of age with failure to thrive, severe dysphagia, and developmental delay. Four of the 6 children died before age 3 years. MRI of all patients revealed a novel pattern with atypical characteristics, including progressive basal ganglia and thalami abnormalities. Neuropathologic studies revealed patchy areas of decreased myelin in the cerebral hemispheres, cerebellum, brainstem, and spinal cord, with astrocytic gliosis in the white matter and microglial activation. Cellular vacuolization was observed in the thalamus and basal ganglia, and neuronal loss was evident in the putamen and caudate. Genotypic similarities were also present between all 6 patients, with one allele containing a POLR3A variant causing a premature stop codon and the other containing a specific intronic splicing variant (c.1771-7C>G), which produces 2 aberrant transcripts along with some wild-type transcript.

Conclusions
We describe genotype-phenotype correlations at the extreme end of severity of the POLR3-related leukodystrophy spectrum and shed light on the complex disease pathophysiology.

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RNA polymerase III-related hypomyelinating leukodystrophy (POLR3-HLD; MIM: 607694, 614381, 616494), or 4H leukodystrophy, is one of the most common hypomyelinating leukodystrophies, typically associated with the cardinal clinical features of hypogonadotropic hypogonadism and hypodontia.1–3 POLR3-HLD commonly presents in childhood, with motor delay or regression, prominent cerebellar features, mild pyramidal signs, and variable cognitive involvement.1 Typical brain MRI pattern includes diffuse hypomyelination with relative preservation (T2 hypointensity) of the anterolateral nucleus of the thalamus, globus pallidus, dentate nucleus, optic radiations, and pyramidal tracts in the posterior limb of the internal capsule, along with cerebellar atrophy and thinning of the corpus callosum.4–6

POLR3-HLD is caused by biallelic pathogenic variants in POLR3A, POLR3B, POLR1C, or POLR3K, which encode subunits of RNA polymerase III (POLR3), an enzyme responsible for transcription of several noncoding RNAs (nc-RNAs), including transfer RNAs (tRNAs), 5S ribosomal RNA, U6 small nuclear RNA, 7S RNAs, and other small nucleolar RNAs.7–15 The precise mechanism underlying the pathogenesis of hypomyelination remains to be fully elucidated; 2 main mechanistic hypotheses include (1) defects in transcription capability of POLR3 causing disruptions in tRNA levels, thereby altering global translation during myelination, which require large production of essential myelin proteins, or (2) impairments in specific POLR3-transcribed nc-RNAs required for myelin development.7,10,16

Here, we expand the phenotypic spectrum of POLR3-HLD through description of clinical, radiologic, and molecular features of six patients with an extremely severe phenotype and present functional and neuropathologic investigations on one patient.

Methods

Patients and study design
An international cross-sectional study was performed between 2016 and 2019, including a retrospective chart review of 6 patients (P1-6) from 5 families with atypical phenotypes identified from a repository of genetically proven POLR3-HLD patients.

Standard protocol approvals, registrations, and patient consents
This research was approved by the Montreal Children’s Hospital and McGill University Health Center Research Ethics Boards (11-105-PED; 2019-4972). Informed consent was obtained from all patients or legal guardians.

Neuroradiology
Brain MRI review was performed on latest available scans by L.G. and G.B. based on previously published criteria for hypomyelination and POLR3-HLD imaging characteristics.1,5,6,17–19 The earliest studies were also analyzed when available. Only one study was available for P5 and P6.

Neuropathology
Neuropathologic investigations were performed on post-mortem brain tissue from P2; details are provided in supplemental methods (links.lww.com/NXG/A257).

Genetic analysis
Variants in POLR3A were identified by exome sequencing using genomic DNA extracted from blood samples, according to standard protocols. Variants were validated by Sanger sequencing and analyzed for familial segregation when DNA was available.

Cell culture and cycloheximide treatment
To evaluate the presence of nonsense mediated decay (NMD), fibroblasts derived from P2 were subjected to treatment with cycloheximide. Experimental details are described in supplemental methods (links.lww.com/NXG/A257).

Western blot
ImmunobLOTS were performed using brain tissue protein extracts of P2 and an age/sex-matched control. Detailed protocols are outlined in supplemental methods (links.lww.com/NXG/A257).

Data availability
Data supporting this study’s findings are available on reasonable request. Raw data from participants (i.e., raw genetic data and MRI data sets) are not made publicly available to protect patient privacy.

Results

Clinical characteristics
Patients 1–6 (P1-6) presented during infancy, between ages 1 and 3 months, with prominent feeding difficulties and failure to thrive. They exhibited severe developmental delay and motor regression before age 1 year. None achieved independent walking. Clinical characteristics are summarized in table 1 and table e-1 (links.lww.com/NXG/A257).

Of the 6 patients, 3 (3/6, 50%) had laryngomalacia and 2 underwent supraglottoplasty. All had dysphagia and required
enteral tube feeding, with 5 (5/6, 83%) requiring a gastrostomy or gastrojejunostomy tube placement between ages 5 and 15 months. Four patients (4/6, 67%) developed severe respiratory insufficiency, and 3 required supplemental oxygen and/or noninvasive respiratory support between ages 5 and 15 months, with 1 later having a tracheostomy at age 13 months. In addition, 2 patients (2/6, 33%) had suspected paroxysmal episodes of dysautonomia, with excessive sweating and retching.

Non-neurologic features typical of POLR3-HLD included delayed dentition (3/6, 50%) and ophthalmologic abnormalities, including hyperopia and cortical visual impairment (4/6, 67%). All patients were too young for hypogonadotropic hypogonadism to be appreciated.

Neurologic examination revealed acquired microcephaly in 4 patients (4/6, 67%). Five (5/6, 83%) had a combination of axial hypotonia and upper motor neuron signs (spasticity and/or hyperreflexia) in the limbs. Generalized dystonia and/or chorea was seen in all patients. Restricted upgaze and abnormal saccades were occasionally noted. Two patients exhibited hypomimia.

Progressive decline and respiratory complications led to the death of P1, P2, and P3 before age 2 years and P4 at age 3 years. P5 and P6 are alive and currently aged 5 and 3 years, respectively.

**Radiologic characteristics**

Brain MRI characteristics of P1-6 are summarized in table 2 and figure 1, which compares a typical POLR3-HLD MRI to P3. All 10 studies available for the 6 patients showed evidence of insufficient myelin deposition, but criteria for diffuse hypomyelination were not met (figure 1, E–K). Overall, there was more myelin than usually seen in POLR3-HLD and additional distinctive MRI characteristics. T2 hyperintensity of the hilus of the dentate nucleus, associated with T2 hypointensity (preserved myelination) of the dentate nucleus itself and peridentate region, was seen in all studies (figure 1F). In 9/10 studies (90%), the posterior brainstem exhibited similar features, with T2 hyperintensity (decreased myelin content) of the posterior medulla, posterior-inferior pons, and posterior aspect of the middle cerebellar peduncles, in a pattern suggestive of axonal degeneration (figure 1, F, I, and J). The latest imaging studies of 2 patients (2/6, 33%), obtained at ages 10 and 11 months, also revealed T2 hyperintensity of the red nucleus (figure 1K). In addition, 8/10 studies (80%) revealed abnormal signal of the lentiform nuclei, which appeared hyperintense on T2 sequences compared with gray matter and iso intense to unmyelinated white matter. The same 8 studies also showed atrophy of the thalami (figure 1G). The 2 scans without these findings were the 2 earliest studies (P2, age 2 months; P3, age 3 months); however, follow-up MRIs showed that these changes developed over time. Basal ganglia atrophy was seen only in the 5 latest scans obtained between ages 10 and 15 months. Cerebellar atrophy was not seen in any studies; however, mild to severe supratentorial atrophy was present in all cases (figure 1, G–H). No signs of pituitary involvement were noted.

**Neuropathology**

Preserved brain tissue of P2, who died at age 13 months from respiratory complications, was subjected to neuropathologic study (figure 2). The brain weighed 777 g, below expected brain weight and comparable to typical weight at age 8 months. Macroscopic examination revealed normal symmetry with well-formed cerebral hemispheres and cerebellum (figure 2A). On gross examination, white matter was slightly reduced, but demonstrated normal appearance without gray discoloration or cavitation. The lateral ventricles and cerebellum had normal size (figure 2B), and corpus callosum thickness was normal for age.

Histologic analysis of the neocortex and hippocampus revealed some ischemic neurons because of the final hypoxic-ischemic injury preceding death. No mineralization of cortical neurons or evidence of inflammatory infiltrate, necrosis, or microglial nodules was present.

White matter demonstrated patchy areas of rarefaction with mild myelin pallor. Oligodendrocytes showed normal morphology and density in all studied areas, including pale areas, and features of demyelination were absent. White matter also exhibited diffuse astrocytic gliosis, both chronic (fibrillary) and subacute (protoplasmic), with activation of microglia but without macrophagic changes associated with phagocytic activity (figure 2, C–F). Changes in white matter appeared more severe in the parietal lobes (figure 2, C–D). No Rosenthal fibers or axonal spheroids were seen. Immunohistochemistry did not reveal any axonal lesions. The corpus callosum and corticospinal tracts demonstrated normal myelination.

Cellular vacuolization was seen in the thalamus and basal ganglia. Atrophy of the putamen was evident with enlarged Virchow-Robin spaces and severe neuronal loss, associated with both chronic and subacute diffuse gliosis, along with rare calcifications and considerable activation of microglia (figure 2, G–I). Discrete neuronal loss was evident in the caudate. Within the pallidum, numerous pale nuclei of Alzheimer type II glia were present due to the terminal anoxia, and no appreciable neuronal loss was evident. The adenohypophysis did not demonstrate pathologic abnormalities.

Hemisections of the brainstem demonstrated mild to moderate pyknosis in the pons and olivary nuclei of the medulla, consistent with acute ischemic changes. Patchy areas of reduced myelin were seen in the brainstem. The cerebellum demonstrated severe lesions of poorly myelinated white matter, with diffuse and mainly chronic (fibrillary) gliosis, but without notable morphological changes in oligodendrocytes (figure 2, J–L). The cerebellar cortex and dentate nucleus appeared normal, and Bielschowsky staining did not reveal...
Table 1 Clinical, MRI, molecular, and pathologic features associated with the typical and severe POLR3-related leukodystrophy phenotypes

| Feature                      | Typical phenotype                                                                 | Severe phenotype                                                                 |
|------------------------------|-----------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| **Clinical characteristics** |                                                                                   |                                                                                |
| Age at onset                 | 3–4 y                                                                             | 1–3 mo                                                                          |
| Age of death                 | Adulthood                                                                        | 1–3 y (2/6 patients still alive)                                                 |
| Symptoms at onset            | Developmental delay and motor regression                                          | Failure to thrive and developmental delay                                         |
| Developmental delay          | Mild to moderate                                                                  | Severe                                                                          |
| Dysphagia                    | Late                                                                              | Early and severe                                                                |
| Respiratory insufficiency    | End of disease course                                                            | Early and severe                                                                |
| Severe myopia                | Very common                                                                       | Too young                                                                       |
| Dental abnormalities         | Common                                                                            | Delayed dentition seen in 3/6                                                    |
| Hypogonadotropic hypogonadism| Common                                                                            | Too young                                                                       |
| **Brain MRI**                | Hypomyelination with preservation of specific structures, thinning of the corpus callosum, and cerebellar atrophy | Very atypical: more myelin than typical phenotype, supratentorial atrophy, and additional features including progressive abnormalities of the basal ganglia and thalami |
| **Genetics**                 | POLR3A, POLR3B, POLR1C, or POLR3K biallelic pathogenic variants >200 variants     | POLR3A (NM_007055.3)                                                          |
|                             | Compound heterozygous                                                            | Compound heterozygous                                                           |
|                             | Allele 1:                                                                          | Allele 2:                                                                       |
|                             | P1: c.2119C>T, p.Q707*                                                           | P1: c.1771-7C>G                                                                |
|                             | P2: c.1681C>T, p.R561*                                                           | P2: c.2119-7C>G                                                                |
|                             | P3: c.1051C>T, p.R351*                                                           | P3: c.1051C>T, p.R351*                                                         |
|                             | P4: c.1051C>T, p.R351*                                                           | P4: c.1051C>T, p.R351*                                                         |
|                             | P5: c.601delA, p.I201Lfs*18                                                       | P5: c.601delA, p.I201Lfs*18                                                     |
|                             | P6: c.3583delG, p.D1195fs*47                                                      | P6: c.3583delG, p.D1195fs*47                                                    |
|                             | Allele 2:                                                                          | Allele 2:                                                                       |
|                             | P1-6: c.1771-7C>G                                                                | P1-6: c.1771-7C>G                                                              |
| **Pathology**                | Prominent and diffuse decreased myelin, secondary axonal loss, and relative preservation of myelin in perivascular regions 26 | Patchy areas of decreased myelin, neuronal loss in the putamen and caudate, and vacuolization in the thalamus and basal ganglia |
| ID | Patient 1 | 6 | − | − | +/mild | − | + | + | + | + | − | + | − | +/mild |
| ID | Patient 2 | 15 | − | − | +/mild | − | + | + | + | − | + | − | + | +/mild |
| ID | Patient 3 | 7 | − | − | + | − | + | + | + | + | − | + | − | +/mild |
| ID | Patient 4 | 10 | − | − | +/mild | − | + | + | + | − | + | + | + | +/mild |
| ID | Patient 5 | 8 | − | − | +/mild | − | + | + | + | − | + | − | + | +/mild |
| ID | Patient 6 | 11 | − | − | +/mild | − | + | + | + | − | + | + | + | +/mild |
| ID | Patient 1 | 14 | − | − | + | − | + | + | + | − | + | − | + | +/mild |
| ID | Patient 2 | 10 | − | − | + | − | + | + | + | − | + | + | − | + | +/mild |
clear evidence of decreased axons. Moderate pyknosis was seen in Purkinje cells; however, there was no appreciable loss of neurons. In the spinal cord, patchy areas of reduced myelin were noted.

**Genetic findings**

Each patient harbored a specific combination of compound heterozygous variants, including a variant causing a premature stop codon on one allele (P1: c.2119C>T/p.Q707*, P2: c.1681C>T/p.R561*, P3&4: c.1051C>T/p.R351*, P5: c.601delA/p.I201Lfs*18, P6: c.3583delG/p.D1195Ifs*47) and a specific intronic splicing variant on the other (P1-6: c.1771-7C>G). We hypothesized that this splicing variant was leaky as complete absence of POLR3A is incompatible with life. PCR amplification using complementary DNA from fibroblasts of P2 revealed 2 additional bands compared with controls (figure 3, figure e-1, links.lww.com/NXG/A257). Sequencing of bands revealed the presence of 2 aberrant transcripts resulting from abnormal splicing, including one lacking exon 14 causing a frameshift and premature stop codon (p.P591Mfs*9) and the other lacking exons 13–14 causing loss of amino acids 548–637 (p.G548_Y637del). In addition, sequencing of the band corresponding to complementary DNA of wild-type length revealed the presence of both the nonsense transcript (c.1681C>T/p.R561*) and wild-type transcript, confirming the splice site variant is leaky (figure 3B). Thus, 4 transcripts were detected, with sequences corresponding to (1) wild-type, (2) the nonsense variant, and those resulting from aberrant splicing events including (3) lack of exon 14, and (4) lack of both exons 13–14 (figure 3C).

As transcripts containing nonsense variants are typically targeted for NMD, we hypothesized that the c.1681C>T/p.R561* variant transcript was subjected to degradation. We

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**Figure 1 MRI characteristics**

Sagittal T1-weighted (A, E) and axial T2-weighted (B–D, F–K) images. (A–D) Typical POLR3-HLD; MRI obtained at age 6 years. Hypomyelination with relative preservation (T2 hypointensity) of the dentate nucleus (red arrow; B), anterolateral nucleus of the thalamus (double-lined arrow; C), optic radiations (arrowhead; C), globus pallidus, and corticospinal tracts in the posterior limb of the internal capsule (not shown). Thinning of the corpus callosum and cerebellar atrophy are also seen. (E–K) Severe phenotype; MRI of patient 3 obtained at age 10 months. Mild insufficient myelin deposition, not meeting the criteria for diffuse hypomyelination. Loss of myelin (T2 hyperintensity) in the posterior brainstem (red arrows; F, I, J), red nucleus (red dashed arrow; K), and hilus of the dentate nucleus (double-lined arrow; F). Abnormal signal of the lentiform nucleus (arrowhead; G). Supratentorial atrophy (G–H) and diffuse atrophy of the basal ganglia and thalami (G) are also seen.
evaluated the presence of NMD in P2 fibroblasts compared with a control using cycloheximide, a compound that inhibits transcriptional elongation and consequently NMD. Following cycloheximide treatment, an increase in band 1 (corresponding to the wild-type transcript and nonsense variant transcript) was observed by semiquantitative PCR (figure 3B), indicating that the nonsense transcript is subjected to NMD under normal conditions.

Because complete lack of POLR3A is incompatible with life, we sought to determine whether the detected residual wild-type transcript would lead to wild-type protein expression. We performed immunoblot analysis on protein extracts from frozen brain tissue of P2 and an age/sex-matched control. To ensure detection of only wild-type full-length protein, we chose a POLR3A antibody with an epitope spanning amino acid residues 607–698. In P2, this antibody cannot bind to the...
abnormal protein products as the epitope binds to residues located in the truncated POLR3A region, i.e., after the premature stop codon (p.R561*) and contained/semicontained in the deleted residues resulting from the splicing variant (p.P591Ms^9, p.G548_Y637del). Thus, this antibody only allows detection of wild-type POLR3A (figure 3C, figure e-1, links.lww.com/NXG/A257). We observed reductions in average normalized POLR3A levels both in brain gray matter (84.7% reduction, 95% CI = 69.3%–100%, d = 1.28) and white matter (54.8% reduction, 95% CI = 20.1%–89.5%, d = 1.34) of P2 compared with control (figure 3, D and E). Gray matter displayed a greater reduction in POLR3A compared with white matter (average difference 29.9%; 95% CI = 0.7%–59.0%; d = 1.77).

### Discussion

Here, we present an expanded spectrum of POLR3-HLD through description of 6 patients with a very severe phenotype and similar genotype. The dramatic clinical presentation, including prominent feeding and breathing difficulties and early death in 4 patients, is strikingly different from the typical POLR3-HLD phenotype. A large phenotypic study of POLR3-HLD revealed typical onset at age 3–4 years with mild to moderate motor delay and/or regression. Dysphagia and respiratory insufficiency were late findings. Death typically occurred in adulthood, where the youngest to die was aged 8 years.

The MRI pattern associated with this phenotype is distinct; despite very severe clinical manifestations, all patients had notably more myelin with different imaging features than typical POLR3-HLD. An evolving change in signal pattern was seen in the lentiform nuclei, with thalami atrophy, progressing to more diffuse basal ganglia atrophy. This correlated with the prominent basal ganglia and thalami pathologic abnormalities, including atrophy, calcifications, and severe neuronal loss in the putamina. Two patients also had red nuclei signal abnormalities. Recently, a similar MRI phenotype was described in patients with a c.1771-7C>G or c.1771-6C>G variant, in trans with a missense, nonsense, splice site, or synonymous variant. Clinical severity varied according to the trans POLR3A variant; patients homozygous for the splicing variant typically displayed a milder phenotype, whereas those harboring a trans loss of function variant displayed severe features with early onset. Of interest, patients homozygous or compound heterozygous for the c.1771-7C>G and/or c.1771-6C>G variants did not display white matter involvement and were described as only having the neuronal MRI features, including striatal involvement with caudate nucleus and putamen atrophy, and occasional red nuclei signal abnormalities. We hypothesize that these specific splicing variants cause a cell-specific effect (i.e., basal ganglia neurons) compared with other POLR3-HLD variants. This could explain why, when this variant is combined with a loss of function allele, patients with a severe phenotype have a specific MRI pattern (i.e., more myelin than the typical phenotype, with progressive basal ganglia involvement).

Neuropathologic examination revealed areas of reduced myelin in the brainstem. On MRI, all studies but one showed evidence of decreased myelin in specific posterior-inferior brainstem structures. Wallerian degeneration affecting specific tracts could at least partly explain these findings, although no clear axonal loss was documented on postmortem studies. The dentate nuclei appeared normal on neuropathologic analyses, consistent with the MRI pattern of preservation of the dentate nuclei and peridentate region. On MRI, reduced myelin was restricted to the hilus.

Although it is well known that hypomyelination is not obligate in POLR3-HLD, the discrepancy between the relatively mild insufficient myelin deposition and the diffuse supratentorial atrophy was highly unusual and consistent across all MRIs. Although previous studies have revealed that oligodendrocytes are primarily affected in the typical form of POLR3-HLD, our patients’ MRI and pathologic findings support the hypothesis that the severe form is primarily neuronal, with associated myelination deficits. We hypothesize that the pathophysiology associated with the severe phenotype varies substantially from typical POLR3-HLD and involves several neural cell types. As myelination is a complex process involving a multitude of signaling events between neurons and glia, it is possible that an increased disruption of POLR3 activity, or the production of aberrant transcripts, could manifest adversely in more cell types than in a milder deficit. It is known that dysregulation of transcription and translation-related genes is often associated with neurologic involvement, highlighting the importance of precise protein expression regulation during neural development. For example, defects in genes encoding aminocyl-tRNA synthetases cause a variety of phenotypes, ranging from hypomyelination to brain malformations.

Given the broad clinical spectrum of phenotypes associated with POLR3 deficiency, it is clear that pathogenic variants in POLR3 genes have distinct effects on various cellular processes. Variants in POLR3A have been associated with phenotypes ranging from spastic ataxia–related disorders to neonatal progeroid syndrome, whereas variants in POLR3B have been associated with isolated hypogonadotropic hypogonadism, without hypomyelination or hypodontia, and a distinct phenotype of cerebellar hypoplasia with endosteal sclerosis.

In contrast to this extremely severe clinical presentation, we also identified 3 adults with a very mild phenotype and the same homozygous pathogenic POLR3B variant (c.1568T>A/p.V523E) in our patient cohort. These patients were all diagnosed incidentally in adolescence/adulthood, based on brain MRI performed for unrelated reasons, or through genetic investigation of typical POLR3-HLD affected relatives. They had minimal findings on neurologic examination, if any, and MRI revealed milder findings than usually seen in
POLR3-HLD. Two were previously described as having the mildest phenotype in a past large cohort study of POLR3-HLD, and the third, who has not been reported, is an adult woman in her late 70s for whom limited information is available. She is currently still ambulatory, and was able to reproduce, making it unlikely she had fertility concerns due to hypogonadotropic hypogonadism. She is described as having mild intellectual challenges and hearing loss from childhood of unknown etiology. She is also independent for all activities of daily living and maintained an active role in the care of her offspring. These cases highlight the extreme variability in disease severity of POLR3-HLD, which can range from very mild to exceptionally severe.

Each patient with a severe clinical presentation had a similar genotype, including a premature stop codon on one allele and
a specific splicing variant (c.1771-7C>G) on the other. In our patient, we demonstrated that this variant is leaky and causes alternative splicing events producing 2 aberrant transcripts, corresponding to results in a past study that investigated this variant in homozygous form. It is thought that this variant creates a new enhancer binding site, and competition for enhancer binding at either the native acceptor splice site (SRp40 enhancer protein) or aberrant binding site (SC35 enhancer protein) is likely the cause of incomplete inactivation of the native acceptor splice site and leaky production of the wild-type transcript.\textsuperscript{23,25} We also confirmed that the transcript containing the nonsense variant was degraded by NMD. Moreover, as POLR3 is a housekeeping gene, complete loss of its function is incompatible with life, which is further supported by the embryonic lethal Polr3a knock-out mouse.\textsuperscript{42} Thus, leaky expression of some wild-type protein is not unexpected as all patients with a severe phenotype survived until early childhood. Although we were able to detect the production of some wild-type POLR3A protein in brain tissue of P2, protein levels were significantly decreased, supporting the hypothesis that minimal production of POLR3A is insufficient for proper neurodevelopment and growth. Less POLR3A protein was detected in gray matter compared with white matter, lending further support to our hypothesis that the severe phenotype is a primarily neuronal disorder.

These findings illustrate an expanded phenotypic spectrum of POLR3-HLD through presentation of patients with biallelic pathogenic variants in POLR3A and an extremely severe phenotype. Identifying genotype-phenotype relationships advances our understanding of the disease course, providing valuable information for clinicians and allowing patients and families to have proper genetic counseling. Our functional and pathologic studies shed light on the pathogenesis of the severe form of POLR3-HLD, opening the door for the development of targeted disease interventions.

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| Name                  | Location                                      | Contribution                                                                 |
|-----------------------|-----------------------------------------------|-----------------------------------------------------------------------------|
| Stefanie Perrier, HBSc| McGill University; Research Institute of the | Designed and conceptualized the study; acquisition of data; analysis and   |
|                       | McGill University Health Centre, Montreal, QC, Canada | interpretation of data; and drafted and revised the manuscript for intellectual content |
| Laurence Gauquelin, MD, FRCPC | McGill University, Montreal, QC, Canada; The Hospital for Sick Children, University of Toronto, ON, Canada | Designed and conceptualized the study; acquisition of data; analysis and interpretation of data; and drafted and revised the manuscript for intellectual content |
Appendix (continued)

| Name                                | Location                                      | Contribution                                                                 |
|-------------------------------------|-----------------------------------------------|------------------------------------------------------------------------------|
| Catherine Fallet-Bianco, MD         | CHU Sainte-Justine, Université de Montréal, QC, Canada | Acquisition of data; analysis and interpretation of data; and drafted and revised the manuscript for intellectual content |
| Michael Renaud, MD                  |                                               |                                                                               |
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| Bader Alhaddad, MD                  | Technische Universität München, Munich, Germany | Acquisition of data and revised the manuscript for intellectual content       |
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Appendix (continued)

| Name                                | Location                                      | Contribution                                                                 |
|-------------------------------------|-----------------------------------------------|------------------------------------------------------------------------------|
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| Norberto Rodriguez-Espinosa, MD     | Hospital Universitario Nuestra Señora de Candelaria, Santa Cruz de Tenerife, Canary Islands, Spain | Acquisition of data and revised the manuscript for intellectual content       |
| Daniela Pohl, MD, PhD               | Children's Hospital of Eastern Ontario, University of Ottawa, Canada | Acquisition of data and revised the manuscript for intellectual content       |
| Savithri Nageswaran, MBBS, MPH      | Wake Forest School of Medicine, Winston-Salem, NC | Acquisition of data and revised the manuscript for intellectual content       |
| Annette Grefe, MD                   | Wake Forest School of Medicine, Winston-Salem, NC | Acquisition of data and revised the manuscript for intellectual content       |
| Emma Glumuzina, MD                  | Starship Children's Hospital, Auckland, New Zealand | Acquisition of data and revised the manuscript for intellectual content       |
| Geneviève Bernard, MD, MSc FRCP     | McGill University; Research Institute of the McGill University Health Centre; Montreal Children's Hospital and McGill University Health Centre, QC, Canada | Designed and conceptualized the study; acquisition of data; analysis and interpretation of data; and revised the manuscript for intellectual content; and study supervision |

References

1. Wolf NI, Vanderver A, van Spandonk RM, et al. Clinical spectrum of 4H leukodystrophy caused by POLR3A and POLR3B mutations. Neurology 2014;83:1898–1905.
2. Timmons M, Tsokos M, Asah MA, et al. Peripheral and central hypomyelination with hypogonadotropic hypogonadism and hypodontia. Neurology 2006;67:2066–2069.
3. Bernard G, Vanderver A. POLR3-related leukodystrophy. In: Adam MP, Ardinger HH, Pagon RA, et al, editors. GeneReviews. Seattle, WA: University of Washington, Seattle; 2017.
4. Vrij-van der Bos S, Hol JA, La Piana R, et al. 4H Leukodystrophy: a brain magnetic resonance imaging scoring system. Neuropediatrics 2017;48:152–160.
5. La Piana R, Tondutti D, Gordish-Dressman H, et al. Brain magnetic resonance imaging (MRI) pattern recognition in Pol III related leukodystrophies. J Child Neurol 2014;29:214–220.
6. Steenweg ME, Vanderver A, Blaser S, et al. Magnetic resonance imaging pattern recognition in hypomyelinating disorders. Brain 2010;133:2971–2983.
7. Bernard G, Choisy E, Putron ML, et al. Mutations of POLR3A encoding a catalytic subunit of RNA polymerase Pol III cause a recessive hypomyelinating leukodystrophy. Am J Hum Genet 2011;89:415–423.
8. Saito H, Osaka H, Sasaki M, et al. Mutations in POLR3A and POLR3B encoding RNA Polymerase III subunits cause an autosomal-recessive hypomyelinating leukoencephalophathy. Ann Hum Genet 2011;89:644–651.
9. Daoud H, Teteault M, Gibson W, et al. Mutations in POLR3A and POLR3B are a major cause of hypomyelinating leukodystrophies with or without dental abnormalities and/or hypogonadotropic hypogonadism. J Med Genet 2013;50:194–197.
10. Thijsfaut I, Wolf NI, Forger D, et al. Reciprocal mutations in POLR3C cause a leukodystrophy by impairing biosynthesis of RNA polymerase III. Nat Commun 2015;6:7623.
11. Teteault M, Choquet K, Orcesi S, et al. Reciprocal mutations in POLR3B encoding the second largest subunit of Pol III, cause a rare hypomyelinating leukodystrophy. Am J Hum Genet 2011;89:652–655.
12. Gutierrez M, Thiffault I, Guerrero K, et al. Large exonic deletions in POLR3B gene cause POLR3-related leukodystrophy. Orphanet J Rare Dis 2015;10:69.

13. Dobrin I, Dumin-Odolot H, Boussaid K, et al. Mutation in POLR3K causes hypomyelinating leukodystrophy and abnormal ribosomal RNA regulation. Neurof Genet 2018;6:289.

14. Jasiak AJ, Armache KJ, Martens B, Jansen RP, Cramer P. Structural biology of RNA polymerase III: subcomplex C17/25 X-ray structure and 11 subunit enzyme model. Mol Cell 2006;23:71–81.

15. Dieci G, Fiorino G, Castelnovo M, Teschmann M, Pagano A. The expanding RNA polymerase III transcript and important regulatory RNA BC200. J Biol Chem 2019;294:7445–7459.

16. Choquet K, Forget D, Meloche E, et al. Leukodystrophy-associated POLR3A mutations down-regulate the RNA polymerase III transcript and important regulatory RNA. J Biol Chem 2020;295:12517–12528.

17. Schiffmann R, van der Knaap MS. Invited article: an MRI-based approach to the diagnosis of white matter disorders. Neurology 2009;72:750–759.

18. La Piana R, Cayami FK, Tran LT, et al. Diagnosis of white matter disorders. Neurology 2009;72:750–759.

19. Cayami FK, Bugiani M, Pouwels PJW, Bernard G, van der Knaap MS, Wolf NI. 4H leukodystrophy: lessons from 3T imaging. Neuropediatrics 2018;49:112–117.

20. Schulz DM, Giordano DA, Schulz DH. Weights of organs of fetuses and infants. Arch Pathol 1962;74:244–250.

21. Hiraide T, Kubota K, Kono Y, et al. POLR3A variants in striatal involvement without diffuse hypomyelination. Brain Dev 2020;42:363–368.

22. Harting I, Al-Saady M, Krageloh-Mann I, et al. POLR3A variants with striatal involvement and extrapyramidal movement disorder. Neurogenetics 2020;21:121–133.

23. Armanov DN, Siru SJ, Chamov T, et al. Transcriptome-wide effects of a POLR3A gene mutation in patients with an unusual phenotype of striatal involvement. Hum Mol Genet 2016;25:4302–4314.

24. Wu S, Bai Z, Dong X, et al. POLR3-related disorders. Neurology 2016;86:1622–1626.

25. Choquet K, Yang S, Moir RD, et al. Absence of neurological abnormalities in mice homozygous for the Polr3a G672E hypomyelinating leukodystrophy mutation. Mol Cell 2006;23:71–81.

26. Taft RJ, VanderVeer A, Leventer RJ, et al. Mutations in DARS cause hypomyelination with brain stem and spinal cord involvement and leg spasticity. Am J Hum Genet 2016;86:1622–1626.

27. Mendes MI, Gutierrez Salazar M, Guerrero K, et al. Bi-allelic mutations in EPRS, encoding the glutamyl-prolyl-aminocyl-tRNA synthetase, cause a hypomyelinating leukodystrophy. Am J Hum Genet 2018;102:676–684.

28. Lee TI, Young RA. Transcriptional regulation and its misregulation in disease. Cell 2013;152:1237–1251.

29. Taft RJ, VanderVeer A, Leventer RJ, et al. Mutations in DARS cause hypomyelination with brain stem and spinal cord involvement and leg spasticity. Am J Hum Genet 2013;92:774–780.

30. Wolf NJ, Salomons GS, Redenburg RJ, et al. Mutations in RARS cause hypomyelination. Ann Neurol 2014;76:134–139.

31. Lee TI, Young RA. Transcriptional regulation and its misregulation in disease. Cell 2013;152:1237–1251.

32. Feinstein M, Markus B, Noyman I, et al. Pelizaeus-Merzbacher-like disease caused by AIMP1/p43 homozygous mutation. Am J Hum Genet 2010;87:820–828.

33. Zhang X, Ling J, Barcia G, et al. Mutations in QARS, encoding glutaminyl-tRNA synthetase, cause progressive microcephaly, cerebral-cerebellar atrophy, and intractable seizures. Am J Hum Genet 2013;94:547–558.

34. Accogli A, Russell L, Sebire G, et al. Pathogenic variants in AIMP1 cause pontocerebellar hypoplasia. Neurogenetics 2019;20:103–108.

35. Ognjenovic J, Simonovic M. Human aminocyt-tRNA synthetases in diseases of the nervous system. RNA Biol 2018;15:623–634.

36. Friedeman J, Smith DE, Issa MY, et al. Biallelic mutations in valyl-tRNA synthetase gene VARS are associated with a progressive neurodevelopmental epileptic encephalopathy. Nat Commun 2019;10:707.

37. Jay AM, Conway RL, Thi M, et al. Neonatal progeriod syndrome associated with biallelic truncating variants in POLR3A. Am J Med Genet A 2016;170:820–828.

38. Mendes MI, Gutierrez Salazar M, Guerrero K, et al. Bi-allelic mutations in POLR3A cause autosomal-recessive wiedemann-rautenstrauch syndrome. Am J Hum Genet 2010;87:108–117.

39. Abbott JA, Francklyn CS, Robey-Bond SM. Transfer RNA and human disease. Front Genet 2014;5:155.

40. Abbott JA, Francklyn CS, Robey-Bond SM. Transfer RNA and human disease. Front Genet 2014;5:155.

41. Mendes MI, Gutierrez Salazar M, Guerrero K, et al. Bi-allelic mutations in POLR3A cause autosomal-recessive wiedemann-rautenstrauch syndrome. Am J Hum Genet 2010;87:108–117.

42. Mendes MI, Gutierrez Salazar M, Guerrero K, et al. Bi-allelic mutations in POLR3A cause autosomal-recessive wiedemann-rautenstrauch syndrome. Am J Hum Genet 2010;87:108–117.