Characterization of The Developmental Trajectory of Patients with RNA Polymerase III -Related (4H) Leukodystrophy

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Abstract

Leukodystrophies are a heterogeneous group of rare, inherited diseases affecting the cerebral white matter [1]. Altogether, they have a population incidence of one per 4733 live births [2]. The vast majority of leukodystrophies are neurodegenerative diseases, resulting in increased morbidity and ultimately mortality in the months to years following onset [3]. Leukodystrophies can be categorized into two groups based on their MRI characteristics: either hypomyelinating (HLD) or non-hypomyelinating [1]. The former refers to insufficient myelin deposition during development while the latter corresponds to abnormal myelin homeostasis [1]. This project focuses on one of the most common HLDs, known as RNA polymerase III-related hypomyelinating leukodystrophy (POLR3-HLD) [2]. POLR3-HLD is caused by biallelic pathogenic variants in genes encoding four distinct subunits of the RNA polymerase III complex: *POLR3A, POLR3B, POLR1C,* and *POLR3K* [4-8]. POLR3-HLD typically affects previously healthy children, has a disease onset in early childhood and has no cure [3]. Therefore, there is a tremendous need to find a treatment for this devastating disease. It is also referred to as 4H leukodystrophy which describes its cardinal features: hypomyelination, hypodontia, and hypogonadotropic hypogonadism [3]. Among these symptoms, developmental delay and regression are observed as well [9]. However, there is a lack of information about the developmental trajectory of these patients.

The first section of this thesis focuses on the adaptation of the Longitudinal Online Research Information System (LORIS) MyeliNeuroGene Rare Disease Database to accurately capture detailed information from medical records. Optimizing this database was critical for complete and comprehensive data collection.

The subsequent section of this thesis focuses on the characterization of the developmental trajectory and developmental regression of patients with POLR3-HLD. We conducted a cross-sectional and retrospective study to examine the developmental progression of 96 patients with genetically confirmed POLR3-

HLD. Specifically, we looked at the acquisition and loss of psychomotor milestones and how the onset of the disease affects the development of these patients. As no disease-specific therapies exist for this patient population, our study focused on the natural developmental trajectory of these patients, only with supportive and preventive care. We show that patients with POLR3-HLD achieve early milestones at a normal age and maintain these milestones over time. In particular, we found that patients with *POLR3A* mutations were more likely to achieve milestones at a normal age and exhibit an earlier loss of developmental milestones, whereas patients with *POLR3B* or *POLR1C* mutations show delayed acquisition of milestones and have a slower developmental regression. Additionally, we noted that patients with an early onset of disease, i.e., zero to nine months, exhibit delayed acquisition of milestones, whereas those who develop symptoms after two years exhibit normal early development. These findings suggest patients carrying *POLR3A* mutations would be the best candidates for potential clinical trials due to their more rapid disease progression.

In summary, in this study, we delineated the developmental trajectory of patients with POLR3-HLD for the first time and therefore contributed to the description of its natural history, an important first step in understanding the disease clinically. This will allow for better counseling to patients and their families on the expected developmental course and disease progression. This work will also contribute to helping design clinical trials by selecting the best candidate patients.

Résumé

Les leucodystrophies sont un groupe hétérogène de maladies héréditaires rares affectant la substance blanche cérébrale [1]. Ensemble, ils ont une incidence dans la population d'une pour 4733 naissances vivantes [2]. La grande majorité des leucodystrophies sont des maladies neurodégénératives, entraînant une augmentation de la morbidité et finalement de la mortalité des mois à des années

après leur apparition [3]. Les leucodystrophies peuvent être classées en deux groupes sur la base de leurs caractéristiques IRM et de la pathologie de la maladie comme hypomyélinisantes ou non hypomyélinisantes [1]. Les leucodystrophies hypomyélinisantes ont un dépôt de myéline insuffisant ou insuffisant au cours du développement, tandis que ceux qui ne sont pas hypomyélinisantes, ont une homéostasie anormale de la myéline [1]. Ce projet se concentre sur l'un des leucodystrophie hypomyélinisante les plus courants [2], connu sous le nom de leucodystrophie hypomyélinisante liée à l'ARN polymérase III (POLR3-HLD). POLR3-HLD est causé par des variants pathogènes bialléliques dans des gènes codant pour quatre sous-unités distinctes du complexe ARN polymérase III : POLR3A, POLR3B, POLR1C et POLR3K [4-8]. POLR3-HLD affecte des enfants auparavant en bonne santé, a une apparition de la maladie dans la petite enfance et n'a pas de remède [3]. Par conséquent, il y a un énorme besoin de trouver un traitement pour cette maladie dévastatrice. Elle est également appelée leucodystrophie 4H qui décrit ses caractéristiques cardinales: hypomyélinisation, hypodontie et hypogonadisme hypogonadotrope [3]. Parmi ces symptômes, un retard de développement et une régression sont également observés [9]. Cependant, il y a un manque d'informations sur la trajectoire de développement de ces patients.

La première partie de cette thèse porte sur l'adaptation de la base de données sur les maladies rares MyeliNeuroGene du système d'information longitudinale en ligne sur la recherche (LORIS) pour capturer avec précision des informations détaillées à partir des dossiers médicaux. L'optimisation de cette base de données était essentielle pour une collecte de données complète et exhaustive.

La section suivante de cette thèse se concentre sur la caractérisation de la trajectoire de développement et de la régression développementale des patients atteints de POLR3-HLD. Nous avons mené une étude transversale et rétrospective pour examiner la progression du développement de 96 patients atteints de POLR3-HLD génétiquement confirmé. Plus précisément, nous avons examiné l'acquisition et la perte d'étapes psychomoteurs et comment l'apparition de la maladie affecte le développement de ces patients. Comme aucune thérapie spécifique à la maladie n'existe pour cette population de patients, notre étude s'est concentrée sur la

trajectoire de développement naturelle de ces patients, uniquement avec des soins de soutien et préventifs. Nous montrons que les patients atteints de POLR3-HLD atteignent des étapes précoces à un âge typique et maintiennent celles-ci au fil du temps. En particulier, nous avons constaté que les patients porteurs de mutations *POLR3A* étaient plus susceptibles d'atteindre des étapes à un âge normale et de présenter une perte plus précoce d'étape de développement, tandis que les patients porteurs de mutations *POLR3B* ou *POLR1C* présentent une acquisition retardée des étapes et une régression développementale plus lente. De plus, nous avons noté que les patients avec un début précoce de la maladie, c'est-à-dire de zero à neuf mois, présentent une acquisition tardive des étapes, alors que ceux qui développent des symptômes après deux ans présentent un développement précoce typique. Ces résultats suggèrent que les patients porteurs de mutations *POLR3A* seraient les meilleurs candidats pour des essais cliniques potentiels en raison de leur progression plus rapide de la maladie.

En résumé, dans cette étude, nous avons délimité pour la première fois la trajectoire développementale des patients atteints de POLR3-HLD et avons donc contribué à la description de son histoire naturelle, une première étape importante dans la compréhension clinique de la maladie. Cela permettra de mieux conseiller les patients et leurs familles sur l'évolution attendue du développement et la progression de la maladie. Ce travail contribuera également à aider à concevoir des essais cliniques en sélectionnant les meilleurs patients candidats.

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Contribution of Authors

Chapter 1: Introduction

Helia Toutounchi wrote this section in collaboration with Dr. Geneviève Bernard.

Chapter 2: Hypothesis and Rational

Helia Toutounchi wrote this section in collaboration with Dr. Genevieve Bernard.

Chapter 3: Methods

Helia Toutounchi designed experiments with guidance from Dr. Geneviève Bernard and in collaboration with Aline Laurendeau.

Chapter 4: **Results**

Data collection and data entry was done by Helia Toutounchi in collaboration with Aline Laurendeau and Dr. Genevieve Bernard, data analysis was done with the guidance of Emmanouil Rampakakis.

Chapter 5: Discussion and Conclusion

Helia Toutounchi wrote this section in collaboration with Dr. Genevieve Bernard.

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4H	Hypomyelination, Hypodontia, Hypogonadotropic Hypogonadism
5S rRNA	5S ribosomal RNA
7SK	7SK small nuclear RNA
ABCD1	ATP-binding Cassette, Subfamily D, Member 1 gene
AD	Autosomal dominant
ADAR	Adenosine deaminase, RNA-specific gene
ADDH	Ataxia, Delayed Dentition, and Hypomyelination
ADL	Activities of Daily Living
AGS	Aicardi-Goutières syndrome
ALD	Adrenoleukodystrophy
AIMP1	Aminoacyl-tRNA Synthetase Complex-Interacting Multifunctional Protein 1 gene
AR	Autosomal recessive
ARSA	Arylsulfatase A gene
BC200	Brain cytoplasmic 200 RNA 1
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CARASIL	Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CI	Confidence Interval
CFCS	Communication Function Classification System
CMTX	X-Linked Charcot Marie Tooth Disease
CNS	Central nervous system
CT	Computed Tomography
DARS1	Aspartyl-tRNA Synthetase 1 gene
DARS2	Aspartyl-tRNA Synthetase 2 gene
df	Degrees of Freedom
DNA	Deoxyribonucleic acid
DDTS-II	Denver Developmental Screening Test II
EDACS	Eating and Drinking Ability Classification System
EARS2	Glutamyl-tRNA synthetase 2 mitochondrial gene
EIF2B1-5	Eukaryotic Translation Initiation Factor 2B, Subunit one to five genes

EMG	Electromyography
EPRS1	Glutamyl-Prolyl-tRNA synthetase 1
ERCC6	Excision Repair Cross-Complementing, Group 6 gene
ERCC8	Excision Repair Cross-Complementing, Group 8 gene
FAM126A	Family with Sequence Similarity 126, Member A gene
FEES	Fiberoptic Endoscopic Evaluation of Swallowing
FSH	Follicle Stimulating Hormone
GALC	Galactosylceramidase
GCDH	Glutaryl-CoA Dehydrogenase
GFAP	Glial Fibrillary Acidic Protein gene
GJB1	Gap Junction Protein, Beta-1 gene
GH	Growth Hormone
GLA	Galactosidase, Alpha gene
GLB1	Galactosidase, Beta-1 gene
gLE	Genetically determined leukoencephalopathy
GLIA	Global Leukodystrophy Initiative
GM1	GM1 Gangliosidosis
GM2	GM2 Gangliosidosis
GMFCS	Gross Motor Function Classification System
GM2A	GM2 Ganglioside Activator
H-ABC	Hypomyelination with Atrophy of the Basal Ganglia and Cerebellum
HR	Hazard Ratio
HBSL	Hypomyelination with Brainstem and Spinal cord involvement and Leg Spasticity
HCC	Hypomyelination with Congenital Cataracts
HEMS	Hypomyelination of Early Myelinated Structures
HEPACAM	Hepatic and glial cell adhesion molecule gene
HLD	Hypomyelinating leukodystrophy
HSPD1	Heat-Shock 60-KD Protein 1 gene
IFIH1	Interferon induced with helicase C domain 1 gene
LBSL	Leukoencephalopathy with Brainstem and Spinal Cord Involvement and Lactate Elevation
LD	Leukodystrophy
LORIS	Longitudinal Online Research Information System

LO	Leukodystrophy with Oligodontia
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone Releasing Hormone
LTBL	Leukoencephalopathy with thalamus and brainstem involvement and high lactate
MACS	Manual Ability Classification System
MitChap60	Heat-shock 60-Kilo Dalton Protein 1 (*HSPD60*-related) gene
MLC	Megalencephalic Leukoencephalopathy with Subcortical Cysts
MLC1	Modulator of volume-regulated anion channel (VRAC) Current 1 gene
MLD	Metachromatic Leukodystrophy
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
miRNA	Micro RNA
ncRNA	Non-coding ribonucleic acid
NCS	Nerve Conducting Study
OR	Odds Ratio
PLP1	Proteolipid protein 1 gene
PMD	Pelizaeus-Merzbacher Disease
PMLD	Pelizaeus-Merzbacher-like disease
PNS	Peripheral nervous system
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
POLR1C	Ribonucleic Acid Polymerase I Subunit C gene
POLR3	Ribonucleic Acid Polymerase III
POLR3A	Ribonucleic Acid Polymerase III Subunit A gene
POLR3B	Ribonucleic Acid Polymerase III Subunit B gene
POLR3GL	Ribonucleic Acid Polymerase III, Subunit GLike gene
POLR3-HLD	Ribonucleic Acid Polymerase III-related Hypomyelinating leukodystrophy
POLR3K	Ribonucleic Acid Polymerase III Subunit K gene
PSAP	Prosaposin gene
RARS1	Arginyl-tRNA Synthetase 1 gene
RARS1-related	Arginyl-tRNA Synthetase 1 related disorders
RNA	Ribonucleic acid
RNASEH2A	Ribonuclease H2 subunit A gene

RNASEH2B	Ribonuclease H2 subunit B gene
RNASEH2C	Ribonuclease H2 subunit C gene
rRNA	Ribosomal RNA
SAMHD1	SAM and HD domain containing deoxynucleoside trisphosphate triphosphohydrolase 1 gene
SLC16A2	Solute Carrier Family 16 Member 2 gene
SLC17A5	Solute Carrier Family 17 Member 5 gene
SQL	Structured Query Language
TACH	Tremor-Ataxia with Central Hypomyelination
TCS	Treacher Collin's Syndrome
TREX1	Three prime repair exonuclease 1 gene
tRNA	Transfer ribonucleic acid
TUBB4A	Tubulin, Beta-4A gene
UFM1	Ubiquitin-Fold Modifier 1 gene
VFSS	Videofluoroscopic Swallow Study
VWM	Vanishing white matter
WRS	Wiedemann-Rautenstrauch syndrome
XL	X-linked

1 Introduction

Leukodystrophies are a group of rare diseases affecting the cerebral white matter, that are typically neurodegenerative and ultimately result in mortality months to years following onset [3]. Leukodystrophies can be categorized into two groups, hypomyelinating (HLD) or nonhypomyelinating [1]. One of the most common HLDs, RNA polymerase III-related hypomyelinating leukodystrophy (POLR3-HLD), is also referred to as 4H leukodystrophy to describe its cardinal features: hypomyelination, hypodontia, and hypogonadotropic hypogonadism [3]. It is caused by biallelic pathogenic variants in the genes *POLR3A, POLR3B, POLR1C,* and *POLR3K,* which encode subunits of the RNA polymerase III complex [4-8]. This thesis will focus on this disease which affects previously healthy children and leads to a variety of neurological and non-neurological symptoms, including developmental delay and regression [3, 9]. However, there is a lack of information about the developmental trajectory of these patients.

This project aims to characterize the developmental trajectory and developmental regression of patients with POLR3-HLD. This project will aid in understanding the disease course and ultimately, along with future natural history data, aid clinical trial design.

2 Literature Review

2.1 White Matter

Throughout evolution, the human brain's white matter has expanded more than grey matter to encompass half of the brain [10]. Historically, the importance of white matter in neural function has been neglected in research but it is now known to be an essential component of neural networks [11]. White matter consists of axons and their myelin sheath, glial cells, and blood vessels [12]. Myelin, formed by the oligodendrocytes (in the central nervous system (CNS), is a specialized plasma membrane that wraps around axons to serve as an electrical insulator for axons,

increasing propagation of nerve impulses through saltatory conduction, a form of propagation that allows impulses to jump from one node de Ranvier (areas of unsheathed axons) to another. Impulses are re-generated at the nodes of Ranvier and since the myelin sheath has a high resistance and low capacitance, current flows down the axon to the next node, rather than through the axon itself [13, 14]. Myelin also provides trophic support and protection for axons [13, 14]. It is therefore essential for effective connectivity in the CNS and the conduction of brain signals necessary for the complexity of human behavior. The multilayered myelin surrounding axons in the CNS is also able to regulate the maturation, survival, and regeneration of axons [11]. Trophic support and signaling molecules provided by oligodendrocytes allow axons to reach their maximal diameter and support axonal development and integrity [11]. Brain myelination starts *in utero* during the 3rd trimester of gestation and has a specific spatiotemporal pattern, starting at the brainstem and progressing caudal to rostral, central to peripheral, and posterior to anterior [15, 16]. Although myelination is almost complete by two years of age, it continues to develop until adulthood [15, 16]. The CNS glial cells include microglia, astrocytes, and oligodendrocytes. Each plays a pivotal role in the normal functioning of the white matter. Microglia are the immune cells residing in the brain [11]. Astrocytes are involved in numerous processes including establishing the bloodbrain barrier, ion and osmotic homeostasis, and favouring neuronal homeostasis through neurotransmitter uptake and synaptic activity [11]. Oligodendrocytes are the main drivers in CNS myelination as they are the cell type responsible for myelin sheath production [11]. Oligodendrocyte development is a highly regulated process including oligodendroglial cell (oligodendrocyte progenitor cells and oligodendrocytes) migration, proliferation, differentiation, and maturation [17]. Only mature oligodendrocytes are capable of producing myelin and ensheathing nearby nude axons, highlighting the importance of proper oligodendrocyte development for healthy brain functioning. In fact, the maturation of oligodendrocytes strengthens or inhibits connections by providing up to 100 myelin segments along and across axons [17].

2.2 Disorders of the white matter

On account of the importance of myelin in the nervous system, it is undeniable that alterations to myelin can have great consequences for health and disease. Indeed, that is the case for leukoencephalopathies, which are disorders predominantly affecting the CNS white matter [12]. Leukoencephalopathies may be acquired or inherited. In 2015, Vanderver et al., published a consensus statement on the classification of inherited white matter disorders, classifying disorders into genetic leukoencephalopathies (gLEs) and leukodystrophies. Specifically, leukodystrophies were defined as a group of heritable CNS white matter disorders with or without the involvement of the peripheral nervous system (PNS) and typically have a characteristic glial-centric neuropathological profile [1].

Genetically determined leukoencephalopathies (gLEs) (Table 1.1) encompass the remaining inherited disorders of the white matter that do not meet the strict criteria of leukodystrophies [1]. For example, CNS diseases that are primarily neuronal (e.g., *AIMP1* or *AIMP2*-related disorders)[1, 18, 19], are not considered leukodystrophies but rather as gLEs. The same applies to inborn errors of metabolism (e.g., GM1 or GM2 gangliosidoses) associated with white matter abnormalities [1, 20-22], as well as vascular diseases (e.g., Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CARASIL), etc.) [1, 23, 24].

Another leukodystrophy classification based on pathomechanisms of white matter abnormalities was recently proposed by van der Knaap & Bugiani (2017)[12]. This classification accounts for the disease's possible effects on multiple cell types, such as astrocytes, neurons, microglia, and blood vessels. In the first group myelin disorders, oligodendrocytes and myelin are the primary cell types affected and these disorders include hypomyelinating, demyelinating, and myelin vacuolization leukodystrophies. Astrocytopathies include disorders that are caused by defects in astrocyte-specific gene products or dysfunctions in astrocytes. In comparison, white matter disorders that occur secondary to neuronal or axonal defects and lead to abnormal axo-glial interactions and subsequent white matter degeneration belong to

the leuko-axonopathies group. Finally, microgliopathies are caused by defects in microglia-specific gene products and leukovasculopathies are caused by vascular pathologies [12].

In addition to cellular pathology, leukodystrophies can likewise be classified into hypomyelinating (HLD) (Table 1.2) and non-hypomyelinating leukodystrophies (Table 1.3) [25, 26]. HLDs are a result of a lack of myelin deposition during development, whereas nonhypomyelinating leukodystrophies result from altered myelin homeostasis [1]. These two groups of leukodystrophies can be distinguished by their brain magnetic resonance imagining (MRI) characteristics. HLDs display mildly hyperintense white matter signal on T2-weighted images relative to grey matter, and hyperintense, isointense, or mildly hypointense white matter signal relative to grey matter structures on T1-weighted images [25, 26]. Conversely, non-HLDs display prominent hyperintense white matter signals on T2-weighted images and prominent hypointense white matter signals on T1-weighted images, relative to grey matter structures [25, 26].

Disease Name	**Inheritance Pattern**	**Mutated Gene (s)**
AIMP1-related	AR	*AIMP1* [27]
AIMP2-related	AR	*AIMP2* [19]
Allan-Herndon-Dudley Syndrome	XL	*SLC16A2 [28]*
CADASIL	AD	*NOTCH3 [29]*
CARASIL	AR	*HTRA1 [23]*
Fabry Disease	XL	*GLA [30]*
Glutaric Aciduria Type I	AR	*GCDH [31]*
GM1 and GM2- Gangliosidosis, Infantile onset	AR	*GLB1 [20, 22], GM2A [21]*
HSP60-chaperonopathy (MitChap60)	AR	*HSPD1 [32, 33]*
X-Charcot Marie Tooth Disease (CMTX)	XL	*GJB1 [34]*

Table 2.1: Non-exhaustive List of Genetically Determined Leukoencephalopathies. This includes examples of gLEs, their inheritance pattern and causative genes.

Disease Name	Inheritance	Gene(s)
Cockayne Syndrome	AR	*ERCC6 [35]* *ERCC8 [36]*
EPRS1-related leukodystrophy	AR	*EPRS1 [37]*
Fucosidosis	AR	*FUCA1[38]*
Hypomyelination with atrophy of basal ganglia and cerebellum (HABC)	AD AR	*TUBB4A [39]* *UFM1 [40]*
Hypomyelination with congenital cataracts (HCC)	AR	*FAM126A [41]*
Hypomyelination of early myelinating structures (HEMS)	XL	*PLP1[42]*
Hypomyelinating leukodystrophy with brainstem and spinal cord involvement and leg spasticity (HBSL)	AR	*DARS1[43]*
Pelizaeus-Merzbacher disease (PMD)	XL	*PLP1[44, 45]*
Pelizaeus-Merzbacher-like ase dise (PMLD)	AR	*GJC2 [46]*
POLR3-related(4H)leukodystrophy	AR	*POLR3A [4]* *POLR3B [7]* *POLR1C [47]* *POLR3K [5],*
RARS1-related hypomyelinating leukodystrophy	AR	*RARS1 [48]*
Sialic acid storage disease	AR	*SLC17A5 [49]*

Table 2.2: Non-exhaustive List of Hypomyelinating Leukodystrophies. This includes examples of HLDs, their inheritance pattern and causative genes.

Disease Name	Inheritance	Gene(s)
Adrenoleukodystrophy (ALD)	XL	*ABCD1 [50, 51]*
Aicardi-Goutières syndrome (AGS)	AR AD, *de novo* AR AR AR AR AR AR, *de novo*	*ADAR [52]* *IFIH1 [53]* *RNASEH2A [54]* *RNASEH2B [54]* *RNASEH2C [54]* *RNU7-1 [55]* *SAMHD1 [56]* *TREX1 [57]*
Alexander's disease	AD, de *novo*	*GFAP [58]*
Canavan's Disease	AR	*ASPA [59]*
Krabbe's disease	AR	*GALC [60]*
Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL)	AR	*DARS2 [61]*
Leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL)	AR	*EASR2 [62]*
Megalencephalic leukoencephalopathy with subcortical cysts (MLC)	AR *De novo* AR/AD AR	*AQP4 [63]* *GPRC5B [63]* *HEPACAM [64]* *MLC1 [65]*
Metachromatic Leukodystrophy (MLD)	AR	*ARSA [66]* *PSAP [67]*
Vanishing White Matter(VWM)	AR	*EIF2B1 [68]* *EIF2B2 [69]* *EIF2B3 [68]* *EIF2B4 [68]* *EIF2B5 [69]*

Table 2.3: Non-exhaustive List of Non-Hypomyelinating Leukodystrophies. This includes examples of nonHLDs, their inheritance pattern and causative genes.

2.3 RNA Polymerase III-Related Leukodystrophy

POLR3-related Leukodystrophy is one of the most common HLDs. This disorder was first observed in a large consanguineous Syrian family with an autosomal recessive neurodegenerative disorder including myelin abnormalities, cerebellar atrophy, progressive ataxia, and pyramidal syndrome, as well as oligodontia [70]. Subsequently, in 2005, Wolf et al. (2005) described four children with early onset progressive ataxia, short stature, hypodontia, hypomyelination, and cerebellar atrophy [71]. A year later, Timmons et al., (2006), reported a series of patients with a

similar presentation. However, upon reaching adolescence, they showed signs of hypogonadotropic hypogonadism as well [72]. In the following years, two other disorders were reported: Hypomyelination with cerebellar atrophy and hypoplasia of the corpus callosum (Sasaki et al., 2009) and Tremor-Ataxia with Central Hypomyelination (TACH) [73]. It is only when the first two causal genes were identified in 2011, that these five diseases were recognized as a single entity and recognized as POLR3-related leukodystrophy (POLR3-HLD) or 4H leukodystrophy (hypomyelination, hypodontia, and hypogonadotropic hypogonadism) [4, 6, 7].

2.3.1 Genetics

POLR3-HLD is caused by biallelic pathogenic variants in *POLR3A, POLR3B, POLR1C,* and *POLR3K* [4-8]. Mutations in *POLR3A* and *POLR3B* are the most common [3, 9, 74-77] followed by *POLR1C* [47]. Only two patients with mutations in *POLR3K* have been reported so far [5]. Affected individuals harbor a wide variety of mutations, including missense, nonsense, splice site, small and large deletions [4, 7, 9, 74, 75, 77, 78]. No patient carries two nonsense variants, likely underlying the essential role of the RNA polymerase in cellular homeostasis [4].

2.3.2 Pathophysiology

Pathogenic variants in genes encoding for subunits of RNA polymerase III cause POLR3HLD. RNA polymerases are essential, highly conserved, multi-subunit complexes responsible for the transcription of DNA to RNA [79]. There are three RNA polymerases, each responsible for the transcription of a specific set of RNAs. RNA polymerase I is responsible for the transcription of ribosomal RNAs (rRNA), whereas RNA polymerase II is responsible for the transcription of all protein-coding genes [79]. RNA polymerase III has 17 subunits and is responsible for the transcription of small noncoding RNAs (ncRNA). These ncRNAs include transfer RNAs (tRNA), *5S* ribosomal RNA, *U6* small nuclear RNA, and *7SK* RNAs [80-83]. The two largest subunits, POLR3A and POLR3B constitute the active site of the enzymatic complex [80, 81]. The pathophysiology of POLR3-related disorders is still poorly understood. However, it is predicted that this disorder is caused by

hypofunctional Pol III which could result from insufficient proteinlevel production of a given subunit [4], abnormal interaction of subunits leading to complex misassembly or disrupted complex stability, and/or abnormal interaction of the complex with DNA [4, 7, 8, 81, 84]. A common hypothesis states that mutations in the subunits of the polymerase lead to hypofunction, which in turn lowers the transcription activity of the complex [8, 85]. Related to this, there are two predominant hypotheses in the literature, which may not be mutually exclusive. The first is that this decreased transcriptional activity results in insufficient production of tRNAs, thereby hindering protein synthesis. Indeed, this idea is strengthened by the rationale that insufficient protein synthesis during myelination, a critical time in development that requires the production of a large amount of proteins, could help explain the hypomyelination phenotype seen in patients and that oligodendrocytes could be especially vulnerable to this deficit in protein synthesis given the large amount of proteins needed to deposit myelin on axons [85, 86]. Meanwhile, another hypothesis states that Pol III hypofunction leads to reduced levels of specific non-coding RNAs (ncRNA) that are transcribed by Pol III, such as *BC200* [8, 85, 87].

2.3.3 Magnetic Resonance Imaging Pattern

POLR3-HLD has a distinctive MRI pattern (Figure 1). First, diffuse hypomyelination is present, i.e., a hyperintense signal of white matter compared to grey matter in T2-weighted images, and hyper-, iso- or slightly hypo-intense white matter signal of the white matter on T1-weighted images [26]. Nevertheless, relative preservation of myelination (hypointensity on T2-weighted images) in specific structures, i.e. the dentate nucleus, optic radiations, anterolateral nucleus of the thalamus, globus pallidus, and in some patients, the pyramidal tracts in the posterior limb of the internal capsule, is seen [9, 26, 74, 88, 89]. Additional features including thinning of the corpus callosum, more commonly in older patients, and cerebellar atrophy can also be seen [9, 26, 74, 88, 89]. POLR3-HLD atypical MRI patterns have also been described. Patients with milder presentations have an MRI showing hypomyelination with more residual myelin than the typical presentation [9, 77, 90-95]. In comparison, patients with a very severe clinical presentation (severe striatal form) and a specific combination of mutations have an MRI showing insufficient

myelin deposition, together with basal ganglia abnormalities [96-98]. Indeed, diffuse hypomyelination is not obligate, and focal white matter abnormalities can also be seen, as well as isolated cerebellar atrophy [77], or isolated basal ganglia involvement with completely normal myelination (mild striatal form) [96, 97, 99].

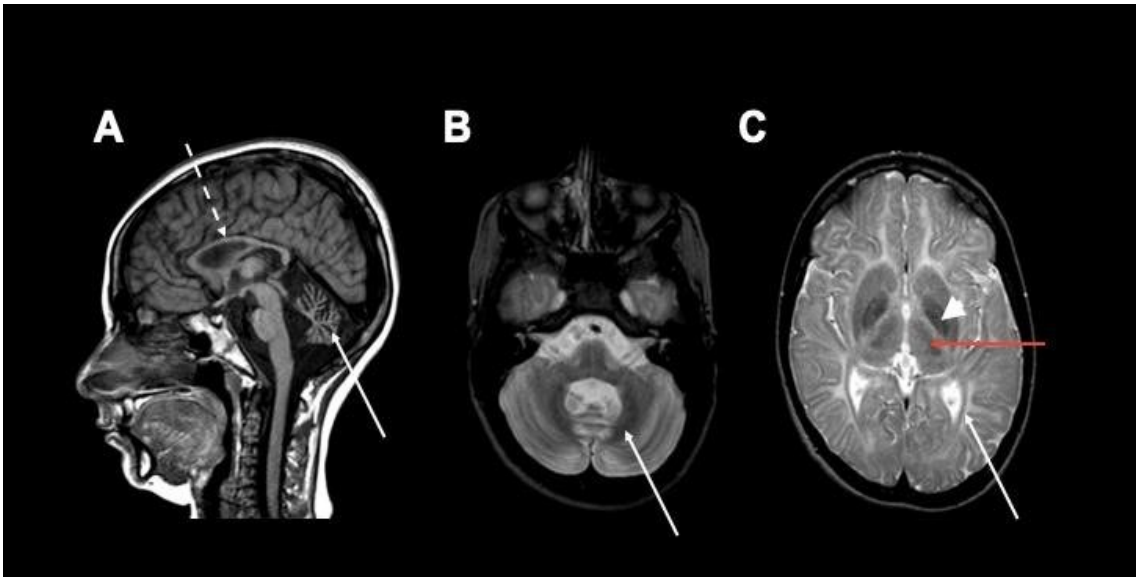

Figure 2.1 Typical brain MRI pattern of POLR3-related leukodystrophy. Sagittal T1 image at the level of the midline (A), axial T2 images at the level of the cerebellum (B) and basal ganglia (C). A. Cerebellar atrophy (white arrow), as well as thinning of the corpus callosum (dashed arrow), are seen. B-C. diffuse hypomyelination, i.e., a hyperintense signal of the white matter in the cerebrum and cerebellum is appreciated, with relative preservation, i.e., hypointensity, of the dentate nucleus (B, white arrow), optic radiations (C, white arrow), globus pallidus (C, arrowhead) and anterolateral nucleus of the thalamus (C, red arrow). This patient does not have preservation of the corticospinal tracts at the level of the posterior limb of the internal capsule, which is another feature that can be seen in this disorder (C).

2.3.4 Neurological Features

Patients with POLR3-HLD typically present as toddlers with motor delay or regression [9]. They experience a progressive disease course and develop variable cognitive difficulties and eating and swallowing difficulties later in the course of the disease [9]. POLR3-HLD neurological symptoms can be classified into cerebellar, pyramidal, and extrapyramidal features [3, 9].

Cerebellar features are observed frequently in patients and are typically the most debilitating manifestations [3, 9]. Patients experience gait ataxia, dysmetria, and dysarthria [3, 9]. On examination, abnormal extraocular movements, most specifically

saccadic pursuits (involving predominantly vertical eye movements early in the disease course), hypo or hyper metric saccades, and in some patients, gaze-evoked nystagmus, are seen [3, 9]. Gait ataxia typically starts in early childhood and leads to progressive disability, and eventually to wheelchair dependency [3, 9]. Other prominent cerebellar features seen in patients are intention tremor and dysmetria affecting all four extremities [9, 73]. Patients also experience dysdiadochokinesis and progressively acquire speech difficulties from a motor speech disorder, i.e. dysarthria, likewise cerebellar in origin [3].

Further, patients with POLR3-HLD typically exhibit pyramidal signs, which, in most cases, affect predominantly the lower extremities, are relatively mild, and do not require treatment. The pyramidal signs seen are predominantly spasticity, which, when combined with ataxia, may increase the difficulty of walking [3, 73, 100]. In fact, ambulation is compromised in this patient population due to the neurological dysfunctions mentioned above. Although some patients remain ambulatory in adulthood, others become wheelchair dependent as early as before the end of the first decade [9]. Whereas a minority of patients never achieved independent walking, those who do achieve unsupported walking before the age of two [9].

The most common extrapyramidal feature seen is dystonia, a movement disorder characterized by simultaneous contractions of agonist and antagonist muscles, leading to slow abnormal movements, abnormal postures, or both [101]. Dystonia in 4H leukodystrophy was first described in 2012 by Osterman et al., [102], however, it is only in 2019 that Al Yazidi et al. systematically investigated the implication and effects of dystonia in the POLR3-HLD population [103]. Most patients in the cohort of 20 patients exhibited multifocal dystonia affecting upper and lower limbs bilaterally and symmetrically at varying degrees. Only patients with moderate to severe dystonia exhibited neck, laryngeal, or trunk dystonia [103]. Most patients did not require treatment for their dystonia. Only one patient had severe dystonia with a paroxysmal component, which required treatment with trihexyphenidyl, tetrabenazine, and oxcarbazepine [103]. Al Yazidi et al., (2019) hypothesize that the cause of dystonia seen in patients with POLR3-HLD is a result of

a network disorder involving abnormalities in the basal ganglia connections caused by diffuse hypomyelination and neuronal loss [103].

As previously mentioned, developmental delay and regression are seen in children with 4H leukodystrophy, and typically present between one and two years of age [9]. Cognitive abnormalities and regression are also seen within this patient population; however, they vary greatly [9]. The decline of cognition typically occurs later in the disease progression. In a subgroup of patients with later onset of disease, the presentation may be cognitive, rather than motor, with academic difficulties [3].

Later in the disease course, POLR3-HLD patients progressively have increased difficulty with eating related to dysphagia, and sialorrhea also develops [3, 9]. These symptoms put patients at risk of malnutrition and aspiration. Eventually, patients may require using gastrostomy for managing nutrition [3].

2.3.5 Non-Neurological Features

POLR3-HLD also presents with non-neurological signs. Those can be divided between dental, endocrine, ocular, and bone manifestations. Dental anomalies are varied and include delayed tooth eruption, agenesis, malformed permanent maxillary central incisors, and natal teeth [104]. Specifically, delayed eruption occurs for both primary and permanent dentition, but agenesis affects permanent mandibular second premolars [104]. The incisor region, especially the maxillary central incisors is more commonly affected and may completely fail to erupt. Furthermore, the patient's erupted teeth may be abnormally shaped or placed as well [3, 104].

Endocrine abnormalities are common amongst patients with POLR3-HLD and most commonly include pubertal abnormalities and short stature. Indeed, one of the cardinal features of 4H leukodystrophy is hypogonadotropic hypogonadism, a term used to describe the absence of early pubertal changes, delayed or arrested puberty [3, 105]. Abnormal puberty is characterized by abnormal follicle-stimulating hormone (FSH), Luteinizing hormones (LH), prolactin, and testosterone levels [106]. The abnormally low levels of FSH and LH are hypothesized to result from dysfunction in the pituitary gland [105]. This further explains the lack of response to luteinizing hormone releasing hormone (LHRH) stimulating tests [72, 105] Thyroid abnormalities were also reported in a proportion of patients [105]. Patients may also

have growth abnormalities manifesting as short stature, due in some but not all patients to growth hormone (GH) deficiency [105]. Some patients also display ocular abnormalities, namely myopia and optic atrophy. Myopia is not only the most common ocular abnormality but also the most common non-neurological feature of the disease [3, 9]. It is typically pronounced and progressive, leading to it eventually becoming severe. Optic atrophy can be seen in older patients although, is not as common as myopia [3, 9]. Bone abnormalities are also seen in this patient population, including endosteal sclerosis and endosteal hyperostosis [88, 107]. As an example, patients with a unique combination of *POLR3B* variants have endosteal sclerosis with cerebellar hypoplasia [88, 107]. Similarly, patients with *POLR3GL* mutations lead to endosteal hyperostosis with oligodontia [88, 107].

2.3.6 Genotype-Phenotype Correlations

The spectrum of phenotypic severity in POLR3-HLD has motivated researchers to investigate genotype-phenotype correlations. In fact, POLR3-HLD can be considered to have a spectrum of phenotypes and phenotypic severity, at times with strong genotypical correlation [9]. In this section, we will discuss what is known about the genotype-phenotype correlations, according to the mutated gene.

2.3.6.1 POLR3A

In general, it is thought that patients with biallelic pathogenic variants in *POLR3A* have a later disease onset, a more severe disease course, and a more rapid disease progression, compared to patients with mutations in *POLR3B* [9]. This can be seen through the earlier loss of supported walking, younger survival age [9], more severe dystonia [103], and higher incidence of abnormal puberty [105]. Moreover, patients with a premature stop codon on one allele and the c.1771-7C>G splicing variant on the other allele present with an extremely severe clinical phenotype called the severe striatal form of the disease [96, 98, 108]. These patients present in early infancy with severe developmental delay and regression, failure to thrive, severe dysphagia, prominent movement disorders, and respiratory insufficiency ultimately leading to an early childhood death [96, 98, 108]. On MRI, patients with the severe striatal form of the disease have more myelin than typical 4H patients, along with supratentorial atrophy and progressive abnormalities of the basal ganglia and thalami

[108]. Moreover, when the c.1771-7C>G is inherited in a homozygous state, other in a combined heterozygous state with another splice site variant (c.1771-6C>G), and if the 17716C<G variant is inherited in a homozygous state, this leads to the mild striatal form of the disease, where patients have isolated basal ganglia involvement, without hypomyelination. When one of these two splice site variants is inherited with a point mutation on the other allele, the phenotype is variable and is most likely determined by the functional impact of the point mutation. These patients may present with severe or mild striatal forms, or with an intermediate phenotype [17, 96, 98, 99, 108]. Specific combinations of *POLR3A* variants have also been found to cause Wiedemann-Rautenstrauch syndrome (WRS), which presents with pseudohydrocephalus, neonatal progeroid appearance, diminished subcutaneous fat, and neonatal teeth [109-113].

In addition, the deep intronic mutation c.1909+22G>A in *POLR3A*, when inherited in a compound heterozygous state with another *POLR3A* variant, causes a spastic ataxia phenotype, with adolescent disease onset, tremor, and involvement of the central sensory tracts [93]. On MRI, these patients present with bilateral hyperintensities along the superior cerebellar peduncles, from the dentate nucleus and extending to the midbrain. Secondary myelin degradation is also observed in these patients, as opposed to hypomyelination which was first seen in POLR-HLD patients with *POLR3A* mutations[93]. Cerebellar abnormalities develop during the disease in this patient group, as well as cervical cord atrophy and hypoplasia of the corpus callosum. The “leaky” nature of the splice site change causes only a partial loss of the wild-type allele, which modulates the phenotype towards this milder spastic ataxia phenotype, instead of the classic hypomyelination phenotype [93].

2.3.6.2 POLR3B

Patients with biallelic pathogenic variants in *POLR3B* have an earlier disease onset, though a milder disease course with loss of supportive walking at a later age [9]. Also, individuals homozygous for the c.1568T>A (p.Val523Glu) pathogenic variants, may exhibit a milder form of the disease [9, 90, 108, 114]. The MRI features differ as well in these patients, with a greater degree of myelin preservation than is seen in typical POLR3-HLD [9, 90, 108, 114]. *POLR3B* variants have also been

associated with cerebellar hypoplasia with endosteal sclerosis [88]. These patients have short stature, delayed bone age, and increased bone density, affecting the pelvis, vertebrae, and long bones [88]. Further, certain patients with biallelic pathogenic variants in *POLR3B* present with isolated endocrine abnormalities [115]. Additionally, biallelic pathogenic variants in *POLR3B* have been reported as associated with WRS [116].

Finally, specific *de novo* pathogenic variants in *POLR3B* have recently been found to cause a different phenotype characterized by ataxia, spasticity, and demyelinating neuropathy, without hypomyelination [117].

2.3.6.3 POLR1C

Biallelic pathogenic variants in *POLR1C* are a less frequent cause of POLR3-HLD. With only approximately 36 patients reported in the literature, it is more difficult to draw strong conclusions about disease severity and genotype-phenotype correlations. The largest study looking at patients with *POLR1C*-related leukodystrophy to date included 23 patients [78]. Indeed, this study identified patients with *POLR1C* mutations have an earlier onset and more severe disease course, compared to patients with *POLR3A* or *POLR3B* mutations [78]. In this cohort, one patient presented in the neonatal period with a severe phenotype consisting of microcephaly, abnormal craniofacial development with mandibular hypoplasia, cardiac arrhythmias, respiratory distress syndrome, and adrenal insufficiency, and ultimately died at seven days [78]. One typical nonneurological feature foreseen in patients with *POLR1C* mutations is craniofacial features reminiscent of Treacher Collins Syndrome (TCS) [118, 119]. In fact, biallelic pathogenic variants in *POLR1C* are known to cause TCS [118, 119]. POLR1C is a subunit shared by both Pol III and Pol I [118, 119]. Thiffault et al. (2015) showed that leukodystrophy-associated *POLR1C* mutations alter the assembly, nuclear import, and interaction of the Pol III complex with target genes while one TCS-associated *POLR1C* mutation impacted the localization of Pol I in the nucleolus with no apparent effect on Pol III [8]. On MRI, most patients with leukodystrophy-causing *POLR1C* variants had a typical MRI pattern, though the characteristic relative preservation of myelination in the dentate

nucleus was not seen as consistently, and thinning of the corpus callosum was more frequent [78].

2.3.6.4 POLR3K

Information on POLR3-HLD caused by biallelic pathogenic variants in *POLR3K* is limited as it has only been reported in two patients [5]. However, these two patients demonstrated severe clinical manifestations, with an early age of onset, severe motor findings, and earlier age of death. Indeed, patients did not achieve independent walking, and had severe spasticity and dystonia, as well as cognitive impairment and regression [5]. These two patients also had severe upper and lower digestive dysfunctions, such as vomiting with hypoglycemia and ketosis, and dysphagia, that lead to cachexia and early gastrostomy placement [5].

2.3.6.5 POLR3GL

Biallelic pathogenic variants in *POLR3GL* have also been shown to cause disease but have never been shown to cause neurological involvement or hypomyelination. Rather, it had been shown to cause endosteal hyperostosis and dysmorphisms [107], as well as WRS [120].

2.3.7 Diagnosis

POLR3-HLD is primarily diagnosed through clinical findings, MRI pattern recognition, and molecular genetic testing [25, 26]. With the recent advances in genetic testing (i.e., multigene panels, exome sequencing, genome sequencing) the presence of biallelic pathogenic variants in *POLR3A, POLR3B, POLR1C,* and *POLR3K* can be easily identified, allowing for rapid genetic confirmation of a diagnosis [3]. Recognition of the typical 4H MRI features and clinical features described above can help guide targeted genetic testing [3].

2.3.8 Assessments of disease severity

Disease severity can be assessed using different tools. For example, time to an adverse outcome such as dependency on tube feeding, can be used to assess disease severity. To assess the severity of specific clinical features, tools, and scales can also be used, including standardized clinical assessments for spasticity (e.g., modified Ashworth scale) [114], ataxia (e.g., the scale for the assessment and rating of ataxia) [121, 122] and dystonia (e.g., global dystonia scale, FahnMarsden dystonia scale)

[123, 124]. These tools allow for the assessment of abnormalities with quantifiable metrics that can be tracked longitudinally [125]. There are also swallowing assessments to assess feeding safety with different food consistencies such as videofluoroscopic swallow study (VFSS) and fiberoptic endoscopic study evaluation of swallowing (FEES) [125]. Furthermore, functional scales that can be applied reliably when reviewing medical records [126] are used to further assess disease progression. These functional scales include Gross Motor Function Classification System (assessing gross motor function), Manual Ability Classification System (assessing fine motor function), Communication Function Classification System (assessing communication), Eating and Drinking Ability Classification System (assessing eating and swallowing) [127-131]. These scales were validated for cerebral palsy and used a common language to describe function [126]. They use a five-point ordinal grading system to assess functional capacity [126]. The GMFCS assesses gross motor ability, that is, self-initiated movement and the use of assistive devices for mobility [129]. The MACS assesses a child's typical use of hands and upper limbs [127]. The CFCS assesses both how information is communicated and received by the child, using any type of language, with both familiar and unfamiliar communication partners [130]. The EDACS assesses eating and drinking ability. More specifically, it assesses the safety and efficiency of eating and drinking [131]. It also considers the level of assistance needed, through a three-point ordinal scale, ranging from completely dependent, to requiring some assistance, to completely independent. In addition, the GMFC-MLD, a tool developed for metachromatic leukodystrophy patients, inspired by the GMFCS, is another reliable standardized assessment tool with seven levels (normal to complete loss of gross motor function) used to describe the progression of gross motor function [132]. Its seven instead of five levels, when compared to the GMFCS, may make it more suitable for a more granular assessment of gross motor abilities.

2.3.9 Standardized Outcome Measures

Clinical outcome measures can be used to evaluate POLR3-HLD patients' symptoms and functional abilities, in a standardized and objective way [133]. These assessments can be used to evaluate the development, intelligence, cognition, and

motor abilities of children. Among motor assessments, the Gross Motor Function Measure (GMFM-66 or GMFM-88) can be used to measure a patient's gross motor abilities and monitor a child's gross motor development over time [134]. The Bruininks-Oseretsky Test of Motor Proficiency Second Edition (BOT-2) can be used to measure fine and gross motor development in four motor areas, i.e., fine motor coordination, fine motor and body control, and strength and agility [135]. In addition, the Peabody Developmental Motor Scales (PDMS) can be used to measure motor abilities through six subsets, reflexes, stationary, locomotion, object manipulation, grasping, and visual-motor integration [136]. Meanwhile, the Leiter International Performance Scale-3 (Leiter-3) and the Weschler Intelligence Scale, can be used as a standardized measure of intelligence [137, 138]. Furthermore, developmental assessments, such as the Vineland Adaptive Behaviour Scales (VABS), Bayley Scales of Infant Development (BSID), and Kaufman Assessment Battery for Children (KABC), measure development, intelligence, cognition and behavior [139-141].

2.3.10 Treatment Options in POLR3-HLD

POLR3-HLD currently does not have curative treatment. Patients are provided with preventive and symptomatic care aimed at prolonging life, avoiding complications, and improving quality of life [125]. Medications can likewise alleviate symptoms such as tone abnormalities (e.g., spasticity, dystonia) and sialorrhea. For example, baclofen is the most common medication used for spasticity [125, 142-144]. Similarly, there are medical interventions for dystonia, including anticholinergic medications such as trihexyphenidyl, which is one of the most efficient treatments for generalized dystonia [125, 143]. Sialorrhea can also be treated with anticholinergic agents, sublingual 1% atropine ophthalmic solution, glycopyrrolate, or botulinum toxin injections [143, 145, 146]. Other interventions are also aimed at improving the quality of life of patients and their families by easing symptoms. For example, physiatrists, physical and occupational therapists can help patients maximize their ambulatory independence, by giving them assistive devices such as orthotics, braces, gait trainers, walkers, lifts, and standers [125]. Another example is the placement of gastrostomy tubes to aid with malnutrition or dysphagia. The placement of a g-tube reduces hospitalizations, prevents respiratory complications, and allows for the safe

intake of food and medications [125]. Interventions are also available to facilitate communication between patients and their caregivers. An example of this is speech-language therapy to improve the clarity of speech or to introduce communication devices [147, 148].

3 Rationale and Hypothesis

POLR3-HLD is a debilitating disease with devastating outcomes and there is a great need to develop a treatment. Research aimed at understanding the pathophysiology of the disease and advancing our understanding of the disease clinically is imperative to progress toward therapeutic development. In particular, clinically, there is a need to track the course of the disease to identify demographic, genetic, clinical, and other variables that correlate with disease progression or specific disease outcomes. Among these other variables, developmental milestones acquisition and loss are important to understand the clinical course of the disease. This thesis investigates the developmental trajectory and outcomes of patients with POLR3-HLD. Specifically, we examined the acquisition of psychomotor milestones and the regression/loss of these milestones. We also looked at how the onset of the disease and the patients' genotype affect the developmental trajectory of these patients.

We hypothesized that the characterization of the developmental trajectory of POLR3-HLD patients, considering the effects of age of onset and genotype, can provide new knowledge about the disease progression and its impacts. To test this hypothesis, we pursued the following aims:

Aim 1: To adapt the LORIS MyeliNeuroGene Rare Disease Database for the POLR3-HLD natural history study

Aim 2: To characterize the natural developmental trajectory and regression of development in patients with POLR3-HLD

4 Material and Methods

This project was approved by the McGill University Health Center and Montreal Children's Hospital Research Ethic Boards (11-105-PED, 2019-4972). Data used for this project were extracted from the MyeliNeuroGene Biobank (2019-4972).

4.1 Adaptation of the LORIS MyeliNeuroGene Rare Disease Database for the POLR3-HLD natural history study

In collaboration with the Longitudinal Online Research and Imaging System (LORIS) team led by Dr. Alan Evans, our lab previously developed a rare disease database, the LORIS MyeliNeuroGene Rare Disease Database [149]. The LORIS platform is an open-source framework for storing and processing disease-related behavior, clinical, genetics, and imaging data. It allows handling large amounts of data longitudinally with ease and in a variety of formats. LORIS offers a variety of visualization, summarizing, and the ability to cross-link data easily and effectively. More importantly, LORIS offers an open-source codebase that could be customized to the needs of a project on the retrospective natural history of POLR3-HLD. During the years 2020-2021, we worked in collaboration with the LORIS team to optimize this database. Using structured query language (SQL), new instruments were created, and existing instruments were improved by adding, editing, or removing questions. The instruments available in our database are designed to capture the medical history of patients. For example, we can capture details of patients' medical history including their prenatal history, the achievement of their developmental milestones, their primary diagnosis, and their first symptoms of disease presentation. Instruments such as investigations (i.e., metabolic investigations, clinical investigations, MRI and CT, EMG/NCS, and swallowing evaluations), clinical evolution (i.e., age of onset of characteristic symptoms), and examination (i.e., medical visit examinations) are used to document features of the disease. The database was tested and software bugs were identified and corrected until it was fully functional.

4.2 Characterization of the natural developmental trajectory and regression of development in patients with POLR3-HLD

To study the developmental progression and regression of patients with POLR3-HLD, we used a cross-sectional and retrospective study design to gather the developmental history of 96 patients with genetically diagnosed POLR3-HLD.

We looked at the typical developmental trajectory for patients with POLR3-HLD without disease-specific therapy, i.e., only with supportive and preventive care. The inclusion criteria for this project were to have a clinical and molecular diagnosis of POLR3-related disorder. We retrospectively reviewed the medical records of patients and gathered data on the symptoms and age at disease presentation, genetic information, acquisition, and loss of developmental milestones, as well as the age of acquisition and loss.

We also created and administered a cross-sectional developmental questionnaire to collect as detailed as possible information on the acquisition and loss of patients' gross and fine motor development, speech and language development, cognitive development, social development, and activities of daily living. This questionnaire relies on responses from parents or caregivers. It allows them to indicate whether a milestone was achieved and specify the corresponding age. In order to accommodate cases where specific ages are not recalled, we included an option for parents to indicate whether the milestone was acquired at a normal age or was delayed. Additionally, the questionnaire asks to indicate the loss of milestones and the age at which they were lost. Furthermore, a comment section is provided to provide additional comments or further explanations about each milestone. The combination of both approaches allowed us to gather as complete a dataset as possible. In certain cases of patients lost to follow-up, we relied uniquely on medical records, whereas for recently recruited patients, we had both medical records and questionnaires. Questionnaire administration and medical records reviews were performed from September 2020 to April 2023. The analysis of both sources allows us to avoid recollection bias in older patients and to gather as granular and exact information as possible.

4.3 Data Entry

Data were entered in the LORIS MyeliNeuroGene Rare Disease Database. LORIS allows for easy data extraction with filters, rules, and statistical analyses. Therefore, it is ideal for managing such a complex dataset. In instances where only the age of milestone acquisition was available, we used various developmental references to determine whether the milestone was acquired at a normal age or was delayed, such as Denver Developmental Screening Test II (DDSTII) for typical development, the Nelson essentials of pediatrics, Developmental assessment of children, Pediatric Secrets and Textbook of family medicine [150-154]. This information was then incorporated into the database.

4.4 Statistical Analysis

A descriptive analysis of the variables at presentation is presented. For continuous variables, the mean, and the median, were reported. For categorical variables, we reported the total count and percentage within each subcategory. Across all developmental categories, participants were excluded from the analysis when assessing the proportion of patients who did/did not achieve specific milestones if they were too young to achieve them.

We conducted logistic regressions across genotype and age of onset for each developmental domain. Logistic regressions are ideal for our analyses since it accounts for the different sample sizes present across our different groups. Specifically, we used this statistical analysis to examine the odds of developmental milestones being achieved. We considered the earliest milestone as the reference variable and compared the odds of achieving subsequent milestones against this reference. Additionally, to examine the likelihood of different genotypes achieving milestones for each developmental domain, we added genotype as a second predictor. We alternated between the reference genotypes, *POLR3A* and *POLR1C*, in order to compare the odds across all three genes. In another model, we used four age of onset groups (0-9 months, 9-24 months, 2-4 years, and >4 years) as the second predictor. We alternated the reference between the 0-9 months and >4 years groups to assess differences in odds. Similarly, we conducted logistic regression analyses to investigate the time of milestone achievement and loss of milestones. In these cases,

we considered whether milestones were achieved at normal developmental ages and lost as the outcome. Kaplan-Meier survival plots (according to genotype and age of onset) and Cox regressions were used for capturing the age of acquisition and loss of milestones.

5 Results

5.1 Description of the Patient Cohort

A total of 96 patients, 51 males and 46 females (Figure 4.1a), with a genetically proven diagnosis of POLR3-HLD have been included in this study. Of these, 45 patients had mutations in *POLR3A (*47.42%,), 41 in *POLR3B* (42.27%,) and 10 in *POLR1C* (10.31%) (Figure 4.1b). These patients are aged from nine months to 56.25 years old, however, a patient who passed away at seven days was excluded from our analyses (table 4.1). The majority of patients had typical 4H leukodystrophy (88.54%, 85/96), while 4.16% (4/96) of patients carried the c.1771-7C>G splicing variant with a premature stop leading to the severe striatal presentation of the disease. 2.08% (2/96) presented with the mild striatal form of the disease (compound heterozygous for c.1771-7C>G and c.1771-6C>G splicing variants). Furthermore, 3.13% (3/96) of patients had the spastic ataxic phenotype (compound heterozygous for the c.1909+22G>A variant in combination with another mutation in *POLR3A*), while 2.08% (2/96) had the mild *POLR3B* phenotype (homozygous for the common *POLR3B* pathogenic variant c.1568T>A) (Figure 4.2). The age of disease onset ranged from birth to 38 years old (mean=4.26, median= 2.00). On average, patients with *POLR3A* mutations had an age of onset of 5.89 years old (median=3.00), while *POLR3B* patients had an onset of 3.53 years old (median=1.50), and patients with *POLR1C* mutations had an onset at 1.90 years old (median= 1.50) (Figure 4.3). An independent-sample median test reveals that patients with *POLR3A* mutations have a significantly (p=0.026) later disease onset than those with *POLR3B* mutations. Patients most often presented with developmental delay, gait abnormalities, and cerebellar features such as ataxia, tremors, or both (Figure 4.4). In general, patients with gait abnormalities had a later onset with a mean of onset at 5.26 years (median=3.50). In patients for whom we have data on their disease onset, those who

presented with developmental delay regardless of their genotype had an age of onset between nine to 24 months (62.50%, 5/8) (Figure 4.5b). It was most commonly observed that patients with biallelic pathogenic variants in *POLR3B* initially presented with developmental delay (100%, 8/8) or ataxia (90.9%, 10/11), while patients with mutations in *POLR3A* presented with gait abnormalities (83.33%, 5/6) or cerebellar features (52.94%, 9/17) and patients with mutations in *POLR1C* presented with tremors (57.14%, 4/7) (Figure 4.5a).

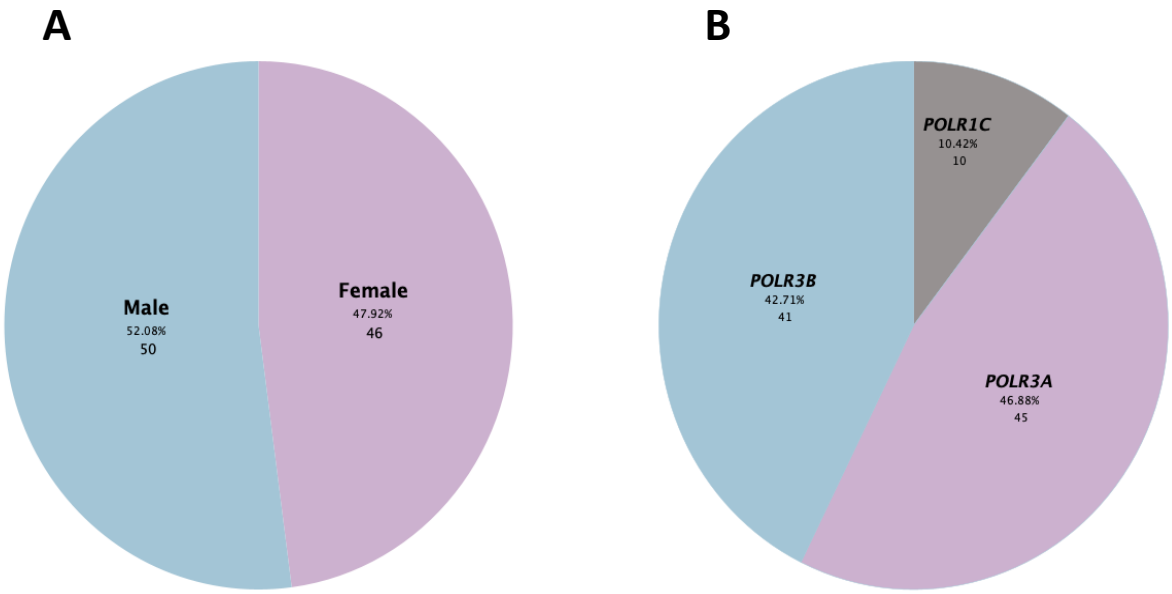

Figure 5.1. Distribution of gender (A) and genotype (B) in the patients' cohort with genetically proven POLR3-HLD. (A) Pie-chart depicting gender distribution among patients: 52.08% males (blue), and 47.92% females (purple). (B) Pie-chart depicting the distribution of genotypes in the cohort study: 46.88% *POLR3A* (purple), 42.71% *POLR3B* (blue) and 10.42% *POLR1C* (grey).

	POLR3A	***POLR3B***	***POLR1C***
Average current age (years)	18.87	14.62	7.88
Median Age (years)	16.92	14.50	6.54
Minimum Age (years)	9 Months	2.08	14 Months
Maximum (years)	56.25	45.50	15.58

Table 5.1. Summary of the age at the time of study for different genotypes in the cohort study of POLR3-HLD patients. The table presents the genotypes (*POLR3A, POLR3B,* and *POLR1C)* in columns and the average, median, minimum, and maximum age (years) in rows.

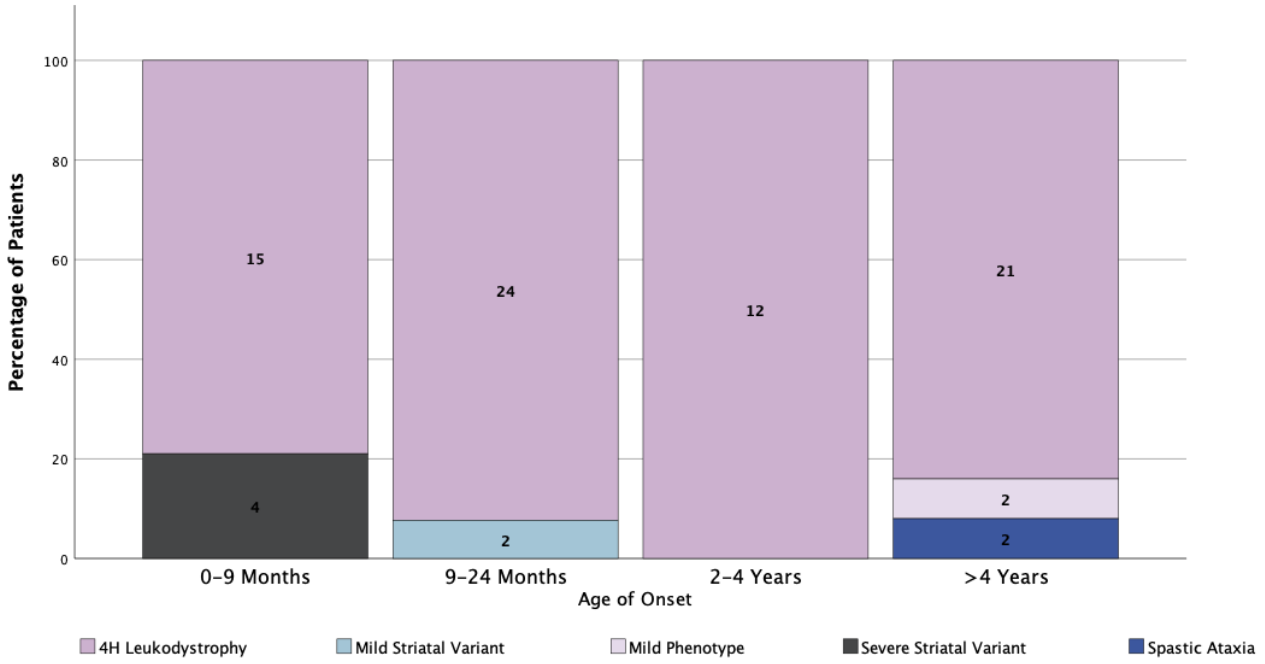

Figure 5.2. POLR3-HLD subtypes (4H leukodystrophy, mild striatal variant, mild phenotype, severe striatal variant, and spastic ataxia) by age of onset, presented as percentages of patients across four age groups: 0-9 months, 9-24 months, 2-4 years, and older than 4 years. The majority of patients had normal 4H leukodystrophy while the severe striatal variant was detected in the earliest age group (0-9 months), the mild striatal variant was only detected in two age groups (0-9 months and 9-24 months) as well as the mild phenotype (2-4 years and >4 years). Spastic ataxia was only detected in only one age of onset group (>4).

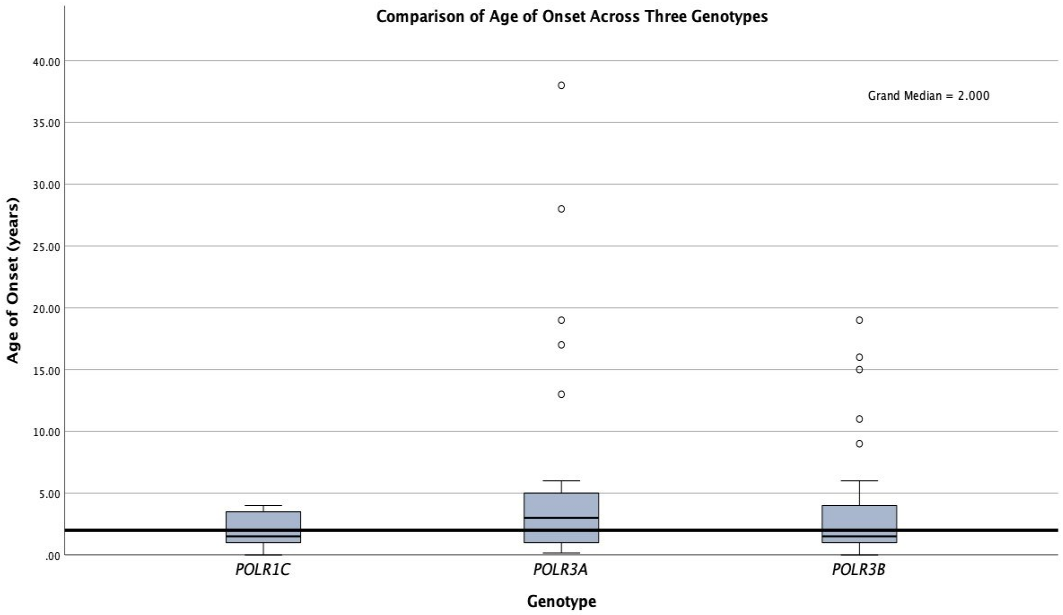

Figure 5.3. Distribution of The Age of Onset (years) by Genotype: *POLR3A*, *POLR3B,* and *POLR1C.* On average patients with *POLR3A* mutations had an age of onset of 5.89 years old (median=3.00), *POLR3B* mutations had an onset of 3.53 years old (median=1.50) and *POLR1C* mutations 1.90 years old (median= 1.50). An Independent-sample median test reveals a significantly ($p<0.026$) later disease onset for patients with *POLR3A* mutations compared to *POLR3B.*

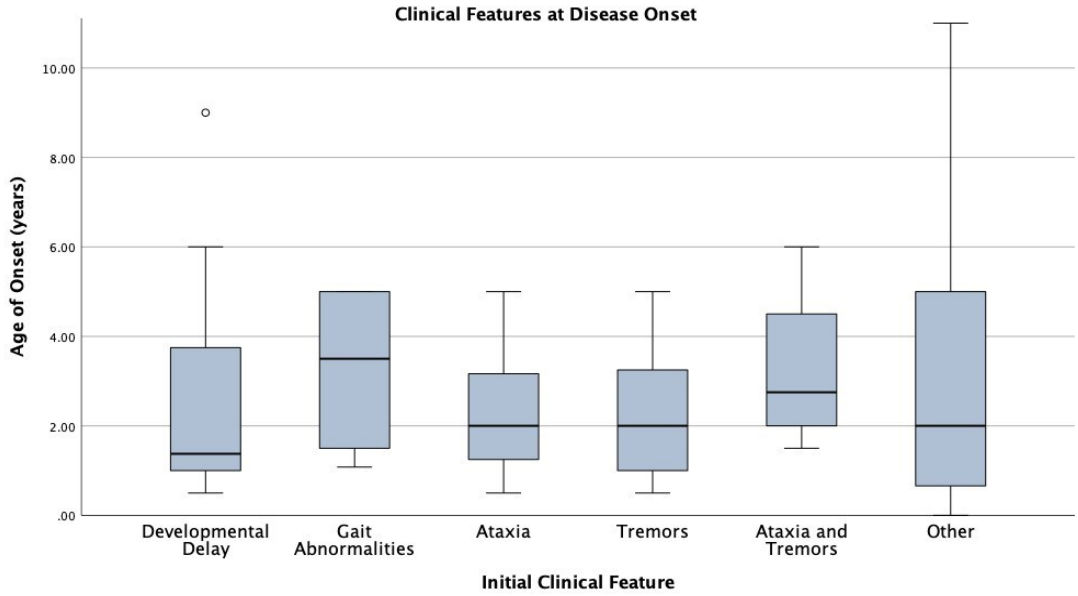

Figure 5.4. Distribution of age of onset according to clinical features at disease onset. Patients most commonly presented with developmental delay, gait abnormalities, or cerebellar features (ataxia, and/or tremors). Other symptoms include feeding difficulties, spasticity, dystonia, and behavioral difficulties. Independent-sample median test revealed no significant difference in age of onset between groups.

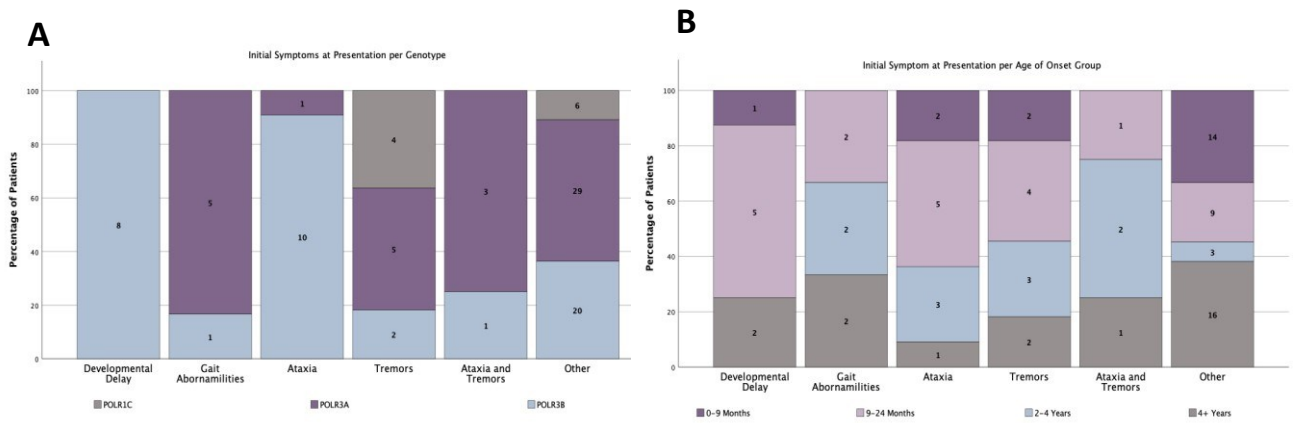

Figure 5.5. Initial symptoms at disease presentation according to genotype (A). Developmental delay is only present in the *POLR3B* genotype while tremors are only present in *POLR1C*. *POLR3A* presents with cerebellar symptoms (gait abnormalities, ataxia, tremors, and both). Initial symptoms at presentation of the disease are presented by age of onset (B). The variety of the initial symptoms is present in most of the age groups meanwhile the gait abnormalities and both ataxia and tremors together are absent in the group of 0-9 months.

5.2 Gross Motor Development

5.2.1 Description of gross motor developmental milestones acquisition by the entire cohort

In patients for whom we have data on specific milestones, it was most commonly observed that they achieved the early gross motor milestones of head control (97.65%, 83/85), rollover (100%, 71/71), tripod sitting (96%, 72/75), sitting (95.65%, 88/92), crawling (92.59%, 75/81), pull-to-sit (95.89%, 70/73) and pull-to-stand (94.67%, 71/75) (Figure 4.6a). As the milestones became increasingly difficult and needed greater coordination and balance, the proportion of patients successfully achieving them decreased. Notably, 80.43% (74/92) of patients were able to achieve walking, 55.93% jumping (33/59), 53.33% hopping (24/45), 70.83% riding a tricycle (34/48), 50% riding a bicycle with (24/48), and 32% without training wheels (16/50). We conducted a logistic regression to further explore the relationship between the achievement of early and later milestones. In this analysis, we selected the earliest milestone (head control) as the reference baseline milestone, and the odds of achievement of subsequent milestones were compared to the reference. In particular, walking ($p=0.002$, OR=0.99, 95% CI=0.022-.0441), jumping ($p<0.001$, OR=0.31, 95% CI=0.007-0.136), hopping ($p<0.001$, OR=0.28, 95% CI=0.0060.126), riding a tricycle

(p<0.001, OR=0.59, 95% CI=0.013-0.271), a bicycle with (p<0.001, OR=0.24, 95% CI=0.005-0.109) and without (p<0.001, OR=0.11, 95% CI=0.002-0.052) training wheels, were less likely to be achieved than the odds of achieving head control.

5.2.2 Description of gross motor developmental milestones acquisition by genotype

In general, a logistic regression where *POLR3A* genotype is the reference gene reveals that patients with *POLR3B* mutations were significantly less likely (p<0.001, OR= 0.428, 95% CI= 0.276-0.664) to achieve gross motor milestones compared to patients with *POLR3A* mutations (Figure 4.6b). This was especially true for hopping (p=0.035, OR=0.251, 95% CI=0.070-0.908) and riding a bicycle without training wheels (p=0.036, OR=0.209, 95% CI=0.048-0.904). There was no difference in the odds of gross motor milestones achievement between patients with *POLR1C* mutations and those with *POLR3A* and *POLR3B* mutations.

5.2.3 Description of gross motor developmental milestones acquisition by Age of Onset

Patients with a disease onset before nine months old (p<0.001, OR=0.029, 95% CI= 0.0130.068) and between nine to 24 months (p=0.001, OR=0.157, 95% CI= 0.071-0.346), were less likely to achieve gross motor milestones compared to those that had an onset after four years of age (Figure 4.6c). Notably, those that have an onset before nine months are significantly less likely to achieve jumping (p=0.002, OR=0.032, 95% CI=0.004-0.268) and riding a tricycle (p=0.029, OR=0.067, 95% CI=0.006-0.755), and a bicycle with training wheels (p=0.022, OR=0.091, 95% CI=0.0120.704), whereas those that have an onset between nine to 24 months are significantly less likely to achieve hopping (p=0.047, OR=0.133, 95% CI=0.018-0.978) and riding a bicycle with training wheels (p=0.008, OR=0.052, 95% CI=0.006-0.458) compared to patients with an onset older than four years.

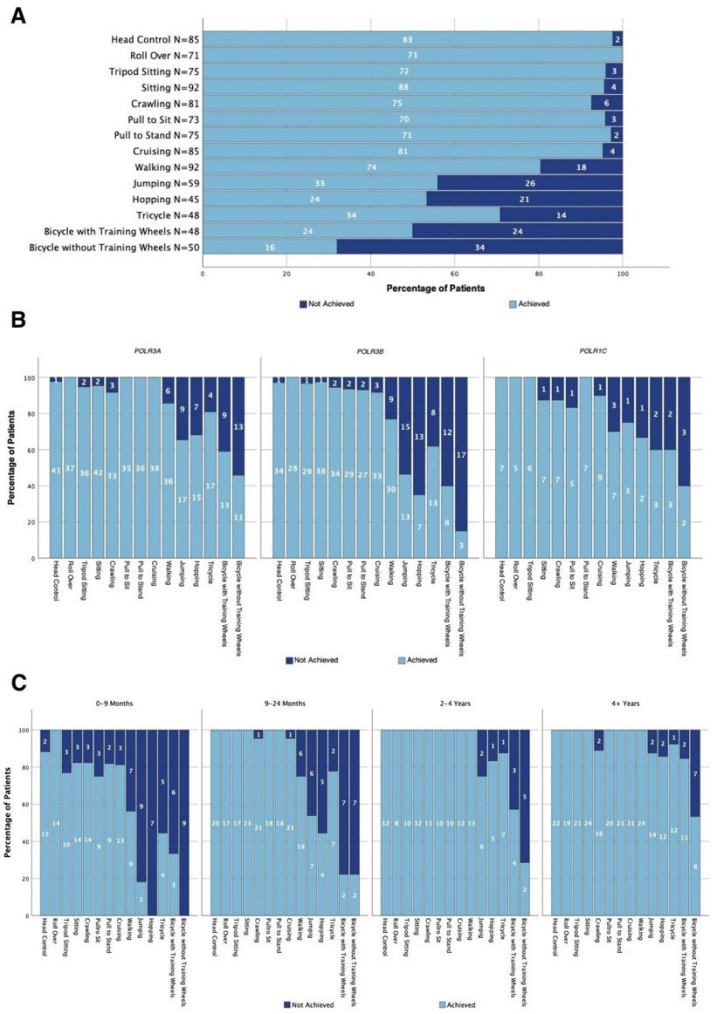

Figure 5.6. Analysis of Gross Motor Development in Patients with POLR3-related Leukodystrophy. (A) Distribution of gross motor milestone achievement (achieved vs. not achieved) for the entire cohort study, (B) presented by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (C) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) The highest achievement percentage belongs to early milestones (head control, roll-over, tripod sitting, crawling, pull-to-sit, and pull-to-stand) meanwhile later milestones (cruising, walking, jumping, hopping, tricycle, bicycle with and without training wheels) showed the lowest achievement. (B) *POLR3A* genotype is more likely to achieve gross motor milestones compared to *POLR3B* and *POLR1C*. (C) Analysis of gross motor milestone achievement by age of onset showed higher odds of achievement in the groups of 2-4 years old and >4 years old, however, the earliest group (0-9 months) was less likely to achieve gross motor milestones. Logistic regressions were performed using SPSS Statistic software.

5.2.4 Normal versus delayed development in patients who achieved gross motor developmental milestones

For a subset of patients, data on whether they had normal or delayed achievement of gross motor milestones were available. We performed similar analyses for these patients to assess whether POLR3-HLD patients achieve milestones with or without delay. Patients were most delayed in achieving pulling to stand (36.21%, 21/58) and walking (42.47%, 31/73), while for each of the other milestones, less than 35% of patients were delayed in their acquisition (Figure 4.7a). When

conducting a logistic regression analysis, we observed that rolling over (p=0.028, OR=0.397, 95% CI=0.174-0.903), sitting (p=0.027, OR=0.407, 95% CI=0.183-0.905, pulling to stand (p=0.019, OR=0.366, 95% CI=0.158-0.848), cruising (p=0.039, OR=0.415, 95% CI=0.1800.956), and walking (p=0.002, OR=0.281, 95% CI=0.127-0.625), were less likely to be achieved without delay compared to the odds of achieving head control on time.

5.2.5 Normal versus delayed development in patients who achieved gross motor developmental milestones by genotype

Furthermore, these delays in the acquisition were most common in POLR3B patients (Figure 4.7b). In fact, these patients are less likely to achieve gross motor milestones without delay (p<0.001, OR=0.520, 95% CI=0.367-0.739) than patients with POLR3A mutations. Specifically, walking (p<0.001, OR=0.145, 95% CI=0.048-0.440) was the most delayed in these patients (63.33%, 19/30).

5.2.6 Normal versus delayed development in patients who achieved gross motor developmental milestones by age of onset

Additionally, patients with an age of onset before nine months (p<0.001, OR=0.076, 95% CI=0.036-0.59) and between nine to 24 months (p<0.001, OR=0.059, 95% CI=0.030-0.116) were less likely to achieve motor milestones without delay compared to those that show initial symptoms after four years of age (Figure 4.7c). However, those with an onset between two to four years old (p<0.001, OR=21.524, 95% CI=6.991-66.265) were more likely to achieve milestones on time compared to those with an onset before nine months of age. Logistic Regression analyses showed that patients with disease onset between two to four years and over four years old had equal odds to achieve motor milestones on time. To further analyze these results, we conducted individual logistic regression models for each milestone across the four groups of ages of onset, where patients with disease onset after 4 years old were the reference variable. This analysis revealed that patients with onset before nine months old were significantly less likely to achieve sitting (p=0.039, OR=0.090, 95% CI=0.009-0.886), pull-to-stand (p=0.027, OR=0.063, 95% CI=0.005-0.724), cruising (p=0.030, OR=0.069, 95% CI=0.006-0.769), and walking (p=0.010, OR=0.100, 95% CI=0.017-0.577) without delay compared to those with onset after four years. While

those with an onset between nine to 24 months were significantly less likely to achieve sitting (p=0.006, OR=0.045, 95% CI=0.0050.403), crawling (p=0.020, OR=0.071, 95% CI=0.008-0.664), pull to stand (p=0.004, OR=0.035, 95% CI=0.003-0.345), cruising (p=0.006, OR=0.043, 95% CI=0.005-0.407), and walking (p<0.001, OR=0.062, 95% CI=0.013-0.290), at a without delay compared to those with onset after 4 years.

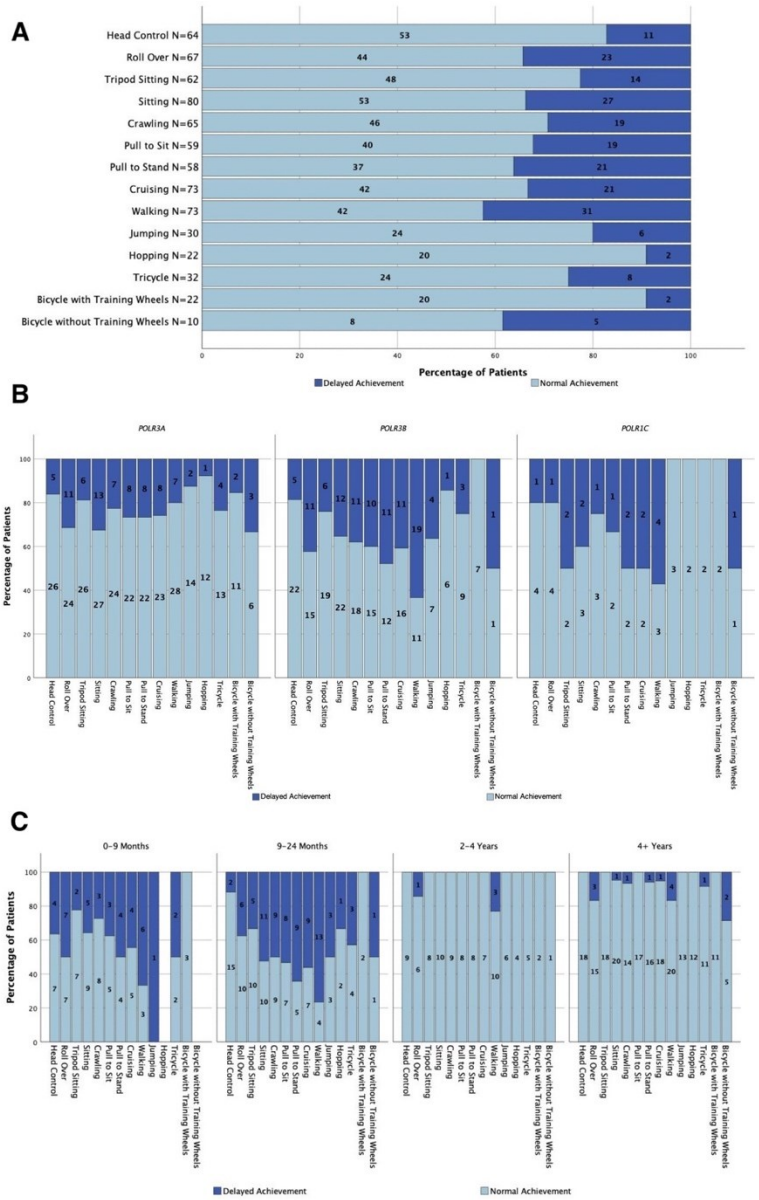

Figure 5.7. Analysis of Gross Motor Development in Patients with POLR3-related Leukodystrophy. (A) Normal vs. delayed development in patients who achieved gross motor milestones, (B) presented by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (C) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) The milestones roll-over, sitting, crawling, pull-to-sit, pull-to-stand, cruising, and walking, were more likely to be achieved with delay (B) *POLR3A* genotype is more likely to achieve gross motor milestones without delay compared to *POLR3B* and *POLR1C*. (C) Analysis of gross motor milestone achievement according to the age of

onset showed higher odds of achievement on time in the groups of 2-4 years old and >4 years old, however, the earliest group (0-9 months) was less likely to achieve gross motor milestones without delay. Logistic regressions were performed using SPSS Statistic software.

5.2.7 Description of gross motor developmental milestones loss

Similar analyses were done to investigate the gross motor developmental regression by examining the loss of milestones in patients for data were available. In particular, 75% of milestones lost were lost by patients with *POLR3A* mutations, while 17.3% were lost by *POLR3B* patients and 7.7% by *POLR1C* patients (Figure 4.8a). Logistic regression analyses suggested that *POLR3B* ($p<0.001$, OR=0.172, 95% CI=0.106-0.278) and *POLR1C* patients ($p=0.014$, OR=0.419, 95% CI=0.209-0.838), have lower odds of losing gross motor milestones than *POLR3A* patients. Patients with *POLR3B* mutations ($p=0.024$, OR=0.410, 95% CI=1.189-4.780) are also less likely to lose milestones compared to *POLR1C* patients, however the difference in sample size between these two genotypes should be taken into consideration. In contrast, it was observed that the loss of gross motor milestones across ages of onset was similar (Figure 4.8b).

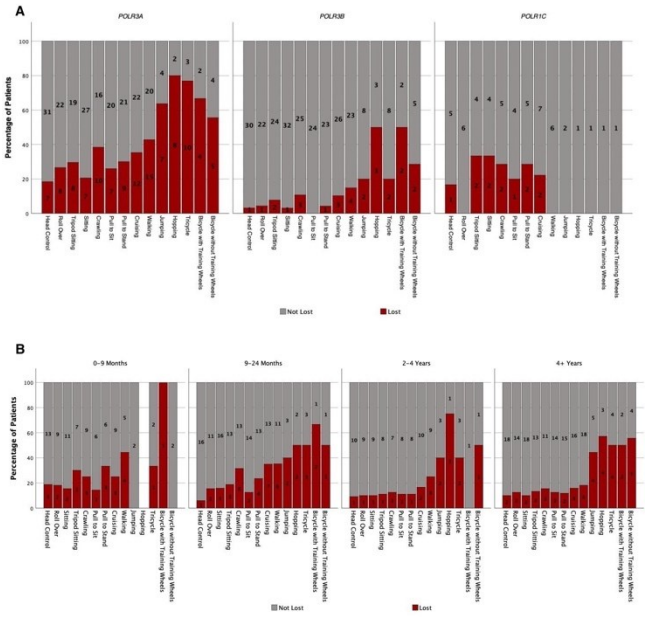

Figure 5.8. Analysis of Gross Motor Developmental Regression in Patients with POLR3-related Leukodystrophy. Distribution of gross motor milestone loss presented (A) by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (B) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) *POLR3A* genotype is more likely to lose gross motor milestones compared to *POLR3B* and *POLR1C*. (B) Analysis of gross motor milestones loss by age of onset no significant difference across groups of ages of onset. Logistic regressions were performed using SPSS Statistic software.

5.3 Fine Motor Development

5.3.1 Description of fine motor developmental milestones acquisition by the entire cohort

Patients achieved the early fine motor milestones of holding objects (93.94%, 62/66), reaching (96.83%, 61/63), putting hands together (100%, 57/57), transferring objects (93.75%, 60/64), pincer grasp (93.94%, 62/66), using a fork or spoon (93.75%, 60/64) and scribbling (96.43%, 54/56) (Figure 4.9a). As milestones required more precision and coordination, the proportion of patients successfully achieving them decreased. Notably, 84.62% (44/52) of patients were able to achieve a mature pincer grasp, 79.59% drawing a circle (39/49), 80% drawing a cross (36/45), 69.04% drawing a square (29/42), 73.17% drawing a triangle (30/41), and 75% zipping up/down zippers (36/48), 53.33% initiating zippers (24/45), 50% closing buttons (26/52), and 43.48% tying shoes (20/46). A logistic regression analysis exploring the relationship between the achievement of early and later fine motor milestones revealed that drawing a circle ($p=0.027$, OR=0.252, 95% CI=0.074-0.858), drawing a cross ($p=0.033$, OR=0.258, 95% CI=0.74-0.898), drawing a square ($p=0.002$, OR=0.144, 95% CI=0.043-0.480), drawing a triangle ($p=0.005$, OR=0.176, 95% CI=0.052-0.599), zippers (up/down) ($p=0.008$, OR=0.194, 95% CI=0.058-0.645) and zippers initiate ($p<0.001$, OR=0.74, 95% CI=0.023-0.237), buttons ($p<0.001$, OR=0.65, 95% CI=0.020-0.203), tying shoe laces ($p<0.001$, OR=0.50, 95% CI=0.015-0.159) were less likely to be achieved than the odds of achieving holding objects.

5.3.2 Description of fine motor developmental milestones acquisition by genotype

In general, patients with *POLR3A* mutations ($p<0.001$, OR= 7.516, 95% CI= 3.85714.648) and *POLR3B* mutations ($p<0.001$, OR= 5.052, 95% CI= 2.604-9.804) were significantly more likely to achieve milestones compared to patients with *POLR1C* mutations (Figure 4.9b). There was no difference in the odds of acquiring fine motor milestones between patients with *POLR3A* mutations and those with *POLR3B*.

5.3.3 Description of fine motor developmental milestones acquisition by age of onset

Furthermore, patients with disease onset before nine months ($p<0.001$, OR=0.019, 95% CI= 0.008-0.046), between nine to 24 months ($p<0.001$, OR=0.083, 95% CI= 0.037-0.188), and between two to four years ($p<0.001$, OR=0.132, 95% CI= 0.054-0.325) were less likely to achieve milestones compared to those that had an onset after four years old (Figure 4.9c). Examining individual logistic regressions for each milestone, revealed that those that have an onset between nine to 24 months ($p=0.012$, OR=0.048, 95% CI=0.004-0.515) and 2 to 4 years old ($p=0.032$, OR=0.062, 95% CI=0.005-0.785) were less likely to achieve initiating zippers than those with an onset after four years old. It was also observed that achieving the milestone zipping up/down had an important significance. Patients with an onset before nine months ($p=0.077$, OR= 0.103, 95% CI= 0.008-1.282) and between nine to 24 months ($p=0.061$, OR=0.108, 95% CI=0.010-1.113), exhibited lower odds of achieving this milestone, in contrast to those with an onset after four years. Patients with an onset of disease before nine months, between nine to 24 months and two to four years were significantly less likely to achieve closing buttons and tying shoelaces (table 4.2).

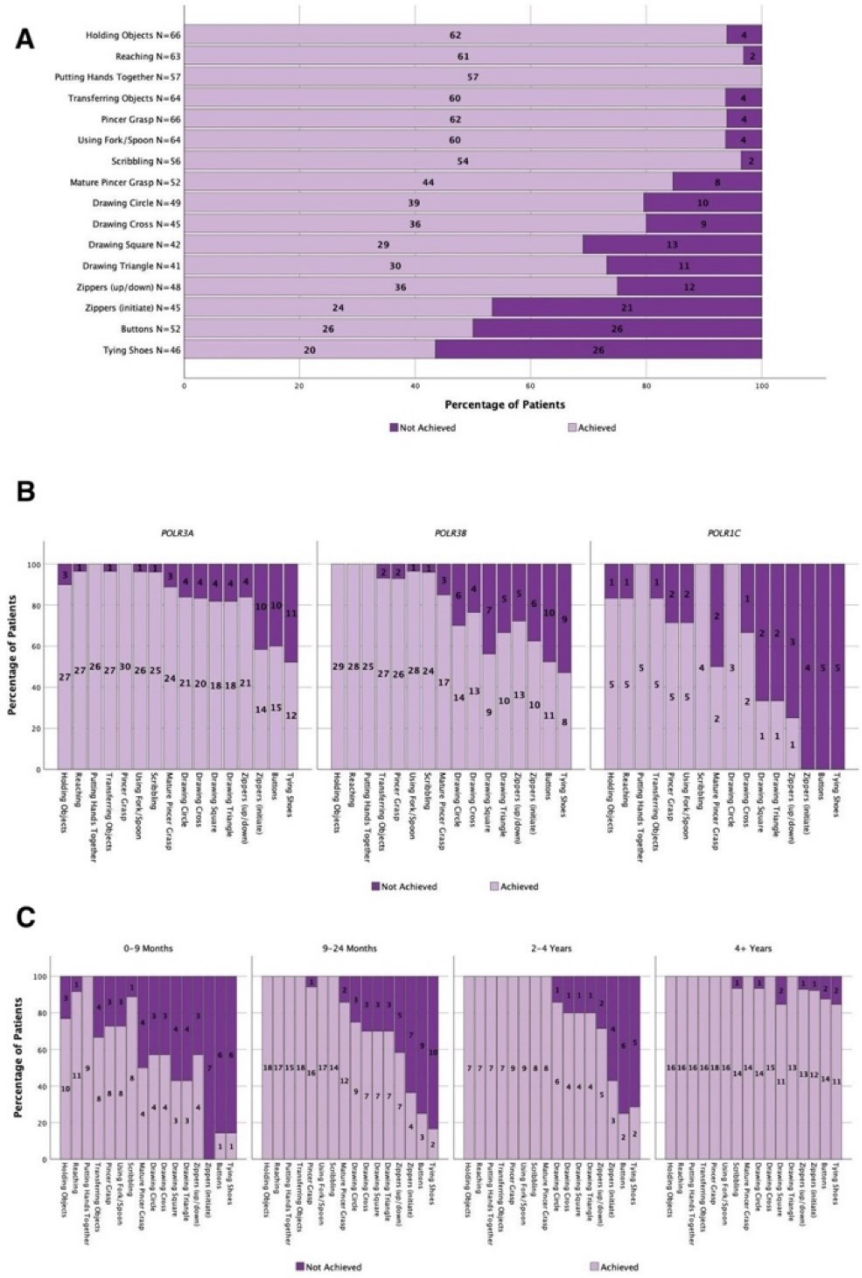

Figure 5.9. Analysis of Fine Motor Development in Patients with POLR3-related Leukodystrophy. (A) Distribution of fine motor milestone achievement (achieved vs. not achieved) for the entire cohort study, (B) presented by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (C) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) The highest achievement percentage belongs to early milestones (holding objects, reaching, putting hands together, transferring objects, pincer grasp, using fork/spoon, and scribbling) meanwhile later milestones (mature pincer grasp, drawing shapes, zippers, buttons, and tying shoelaces) showed the lowest achievement. (B) *POLR3A* genotype is more likely to achieve fine motor milestones compared to *POLR3B* and *POLR1C*. (C) Analysis of fine motor milestone achievement by age of onset showed higher odds of achievement in the groups of 2-4 years old and >4 years old, however, the earliest group (0-9 months) was less likely to achieve fine motor milestones. Logistic regressions were performed using SPSS Statistic software.

					95% C.I. for Exp (B)	
Buttons	**Age of Onset**	**df**	**Sig.**	**Exp (B)**	**Lower**	**Upper**
	4+ Years	3	0.004			
	0-9 months	1	0.005	0.024	0.002	0.315
	9-24 months	1	0.003	0.048	0.007	0.343
	2-4 years	1	0.006	0.048	0.005	0.422
Tying Shoes	4+ Years	3	0.008			
	0-9 months	1	0.008	0.030	0.002	0.407
	9-24 months	1	0.002	0.036	0.004	0.309
	2-4 years	1	0.021	0.073	0.008	0.674

Table 5.2. Logistic Regression Analysis Comparing Odds of Achieving Initiating Zippers and Tying Shoe Laces Among Different Age of Onset Groups. Patients with an onset between 0-9 months, 9-24 months, and 2-4 years are significantly less likely to achieve initiating zippers and tying shoe laces compared to the reference group of patients aged older than 4 years. The significance level is set to $p<0.05$.

5.3.4 Normal versus delayed development in patients who achieved fine motor developmental milestones

Considering the patients for which data on whether they had normal or delayed achievement of fine motor milestones, we observed that patients were most delayed in achieving a pincer grasp (52.94%, 18/52), a mature pincer grasp (40.74%, 11/27), using a fork or a spoon (32.07%, 17/53), and closing buttons (41.67%, 10/14), while for each of the other milestones, less than 25% of patients were delayed in their acquisition (Figure 4.10a). When conducting a logistic regression analysis, we observed that there was no significant difference in the odds of achieving milestones without delay.

5.3.5 Normal versus delayed development in patients who achieved fine motor developmental milestones by genotype

Delays in the acquisition of fine motor milestones were more likely to be observed in both *POLR3B* ($p<0.001$, OR=0.186, 95% CI=0.117-0.295) and *POLR1C* ($p<0.001$, OR=0.128, 95% CI=0.061-0.270) patients, compared to patients with

POLR3A mutations. These patients have diminished odds to achieve fine motor milestones on time compared to patients with *POLR3A* mutations (Figure 4.10b). Specifically, individual logistic regression models for each milestone across genotypes revealed that the milestones pincer grasp (p=0.035, OR=0.250, 95% CI=0.0690.906), using a fork or a spoon (p=0.021, OR=0.184, 95% CI=0.044-0.778), drawing a circle (p=0.028, OR=0.078, 95% CI=0.008-0.765), and zipping zippers (p=0.023, OR=0.067, 95% CI=0.006-0.690) had lower odds of being achieved without delay by *POLR3B* patients compared to *POLR3A* patients. Given the limited availability of data on *POLR1C* patients, making comparisons, and drawing conclusions regarding their fine motor milestone achievement in comparison to *POLR3B* patients was not possible.

5.3.6 Normal versus delayed development in patients who achieved fine motor developmental milestones by age of onset

Regarding the age of disease onset, patients with an age of onset between two to four years (p=0.002, OR=6.383, 95% CI=1.618-15.448) and over four years (p<0.001, OR=4.609, 95% CI=2.143-9.916) were significantly more likely to achieve fine motor milestones on time compared to those with disease onset before nine months (Figure 4.10c). Interestingly, those with an onset between nine to 24 months (p=0.008, OR=0.443, 95% CI=0.236-0.831) were 0.008 times less likely to achieve milestones on time compared to those with an onset before nine months of age. Logistic Regression analyses showed that patients with disease onset between two to four years and over four years old had equal odds to achieve fine motor milestones on time.

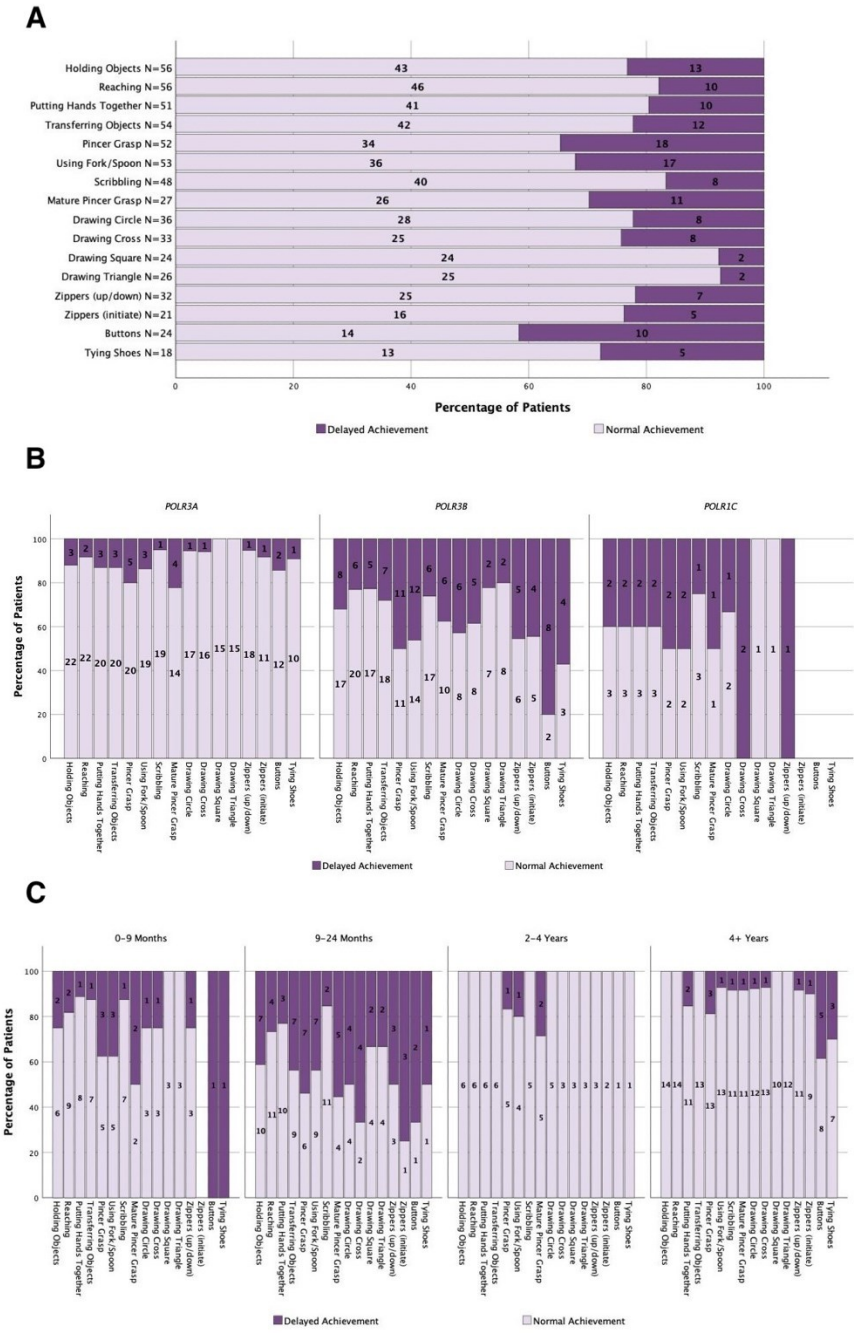

Figure 5.10. Analysis of Fine Motor Development in Patients with POLR3-related Leukodystrophy. (A) Normal vs. delayed development in patients who achieved fine motor milestones, (B) presented by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (C) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) The milestones of pincer grasp, using fork/spoon and buttons, were more likely to be achieved with delay (B) *POLR3A* genotype is more likely to achieve fine motor milestones on time compared to *POLR3B* and *POLR1C*. (C) Analysis of fine motor developmental milestones achievement by age of onset showed higher odds of achievement on time in the groups of 2-4 years old and >4 years old, however, the group 9-24 months was less likely to achieve fine motor milestones without delay. Logistic regressions were performed using SPSS Statistic software.

5.3.7 Description of fine motor developmental milestone loss

Examination of the available date on the loss of milestones showed that 83.9% of milestones lost were lost by patients with *POLR3A* mutations, while 11.6% were lost by *POLR3B* patients and 4.57% by *POLR1C* patients (Figure 4.11a). Logistic regression analyses suggest that patients with biallelic pathogenic variants in *POLR3B* have lower odds of losing milestones compared to *POLR3A* ($p<0.001$, OR=0.102, 95% CI=0.055-0.189) and *POLR1C* patients ($p=0.012$, OR=0.234, 95% CI=0.075-0.725). Investigating individual logistic regressions for each milestone across the three genotypes suggests that patients with *POLR3B* mutations are less likely to lose pincer grasp ($p=0.021$, OR=0.076, 95% CI=0.009-0.675) and using a fork or a spoon ($p=0.016$, OR=0.069, 95% CI=0.008-0.608) compared to the other two genotypes. In contrast, it was observed that there was no significant difference in the odds of loss of fine motor milestones across the age of onset groups (Figure 4.11b).

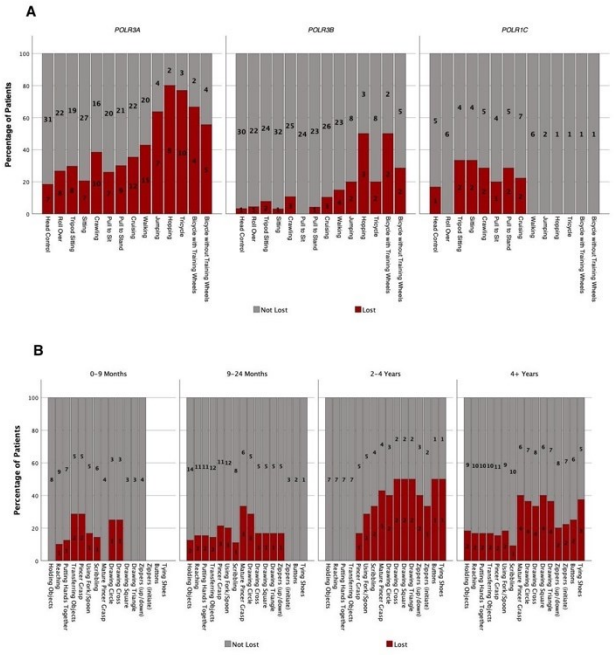

Figure 5.11. Analysis of Fine Motor Developmental Regression in Patients with POLR3-related Leukodystrophy. Distribution of fine motor milestone loss presented (A) by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (B) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) *POLR3B* genotype is less likely to lose fine motor milestones compared to *POLR3A* and *POLR1C*. (B) Analysis of fine motor milestone loss by age of onset no significant difference across groups of ages of onset. Logistic regressions were performed using SPSS Statistic software.

5.4 Speech and Language Development

5.4.1 Description of language developmental milestones acquisition by the entire cohort

Early expressive language milestones, such as cooing (96.15%, 75/78), babbling (94.87%, 74/78), and first word (95.40%, 83/87), were successfully achieved by the vast majority of patients. Proportions of achievement of expressive language decreased with later milestones such as 2-word sentences (86.90%, 73/84), 3-word sentences (86.84%, 66/76), and pronouns (81.81%, 54/66) (Figure 4.12a). However, all receptive language milestones were achieved by most patients. Specifically, 97.5% (78/80) patients achieved understanding their own name, 98.71% (77/78) achieved understanding no, 96.15% (75/78) achieved understanding 1-step commands, 94.20% (65/69) achieved understanding 2-step commands, and 91.04% (61/67) achieved understanding 3-step commands. A logistic regression analysis with cooing as the reference baseline milestone revealed that the milestones 2-word sentences (p=0.048, OR=0.265, 95% CI=0.071-0.990), and pronouns (p=0.010, OR=0.180, 95% CI=0.048-0.669) are significantly less likely to be achieved by POLR3-HLD patients, compared to the milestones cooing. However, 3-word sentences showed an important significance as well. Specifically, patients were 0.264 times (p=0.050, OR=0.264, 95% CI=0.070-1.000) less likely to achieve this milestone compared to cooing.

5.4.2 Description of language developmental milestones acquisition by genotype

When conducting logistic regressions, we observed that language milestone acquisition is similar across genotypes. However, the proportion of achievement for more difficult spoken milestones, such as 2-word sentences, 3-word sentences, and pronouns, were higher for both *POLR3A* (2-word sentences: 86.49%, 32/37; 3-word sentences: 75.76%, 28/33; pronouns: 80%, 24/30) and *POLR3B* (2-word sentences: 86.49%, 32/37; 3-word sentences: 75.76%, 28/32; pronouns: 85.18%, 23/27) patients (Figure 4.12b).

5.4.3 Description of language developmental milestones acquisition by age of onset

In contrast, patients who had a disease onset before nine months, were least likely to achieve language milestones (Figure 4.12c). Specifically, patients who exhibited initial signs of the disease between nine to 24 months ($p<0.001$, OR=18.570, 95% CI=6.167-55.916), and older than 4 years ($p<0.001$, OR=26.736, 95% CI=7.762-92.094), demonstrated higher odds of language milestone achievement compared to those who experienced disease onset before nine months old.

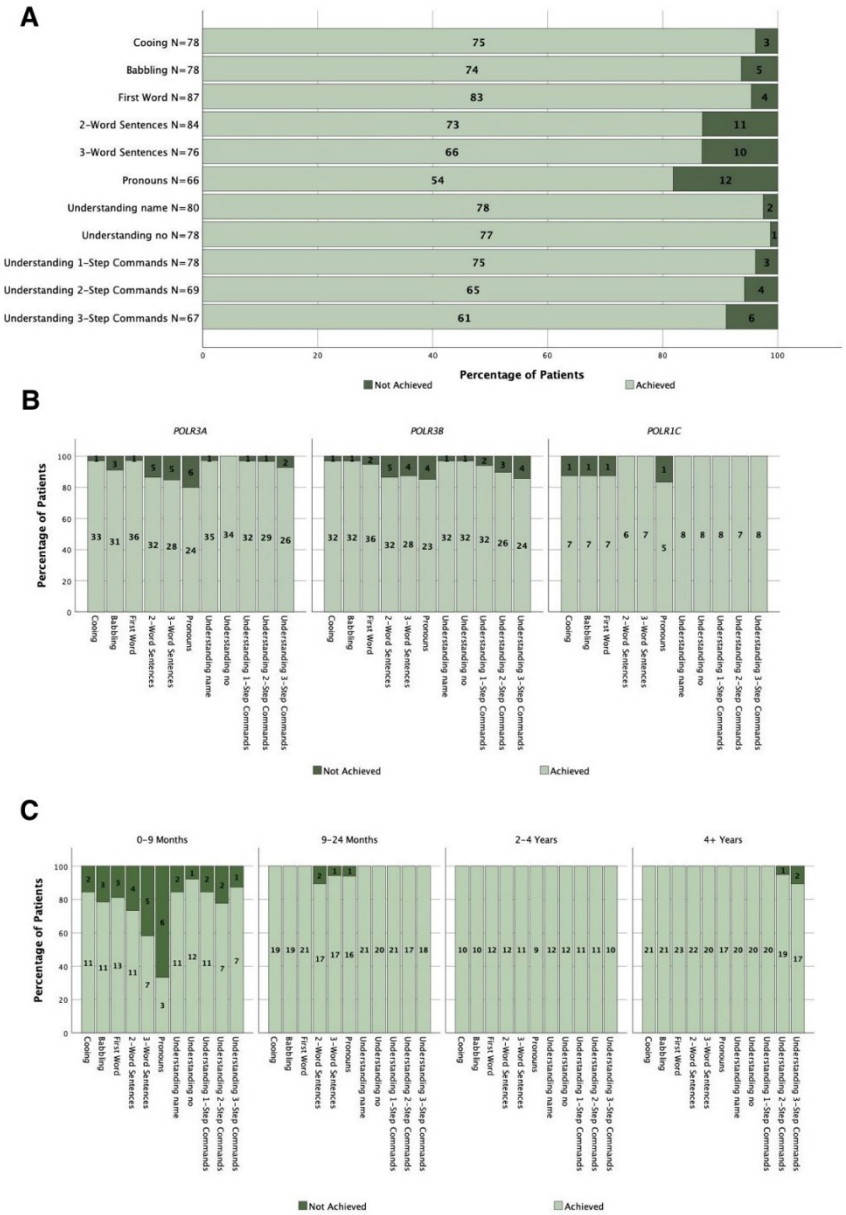

Figure 5.12. Analysis of Speech and Language Development in Patients with POLR3-related Leukodystrophy. (A) Distribution of language milestone achievement (achieved vs. not achieved) for the entire cohort study, (B) presented by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (C) by age of onset (0-9 months, 9-24 months, 2-4 years, older than 4 years). (A) The highest achievement percentage belongs to early expressive language milestones (cooing, babbling, and first word) and

receptive language milestones, meanwhile, later expressive language milestones (understanding name, no, 1-step, 2-step, and 3-step commands) showed the lowest achievement. (B) language milestones acquisition is similar across genotypes. (C) Analysis of language milestones achievement by age of onset showed patients who had a disease onset before 9 months old, were least likely to achieve language milestones meanwhile >4 years were most successful. Statistical analysis was performed using SPSS Statistic software.

5.4.4 Normal versus delayed development in patients who achieved language developmental milestones

For the subset of patients for whom data on whether they had normal or delayed achievement of language milestones were available, we observed that patients did not show statistically significant differences in odds of the time of achievement between language milestones. First word (29.03%, 18/80) and using pronouns (21.74%, 10/46) milestones showed slightly more proportions of delay in achievement, while for each of the other milestones less than 20% of patients were delayed in their acquisition (Figure 4.13a).

5.4.5 Normal versus delayed development in patients who achieved language developmental milestones by genotype

Nevertheless, patients with *POLR3B* ($p<0.001$, OR=0.446, 95% CI=0.288-0.690) mutations and *POLR1C* ($p=0.002$, OR=0.368, 95% CI=0.193-0.703) mutations were more likely to be delayed in the acquisition of milestones compared to patients with *POLR3A* mutations (Figure 4.13b). Given the limited availability of data on *POLR1C* patients, making comparisons, and drawing conclusions regarding their achievement of language milestones in comparison to *POLR3B* patients was not possible

5.4.6 Normal versus delayed development in patients who achieved language developmental milestones by age of onset

Furthermore, patients with disease onset before nine months ($p=0.005$, OR=0.322, 95% CI=0.147-0.705), between nine to 24 months ($p<0.001$, OR=0.209, 95% CI=0.107-0.406), between two to four years ($p<0.012$, OR=0.381, 95% CI=0.179-0.811) were less likely to achieve milestones without delay compared to those with an onset after four years of age (Figure 4.13c).

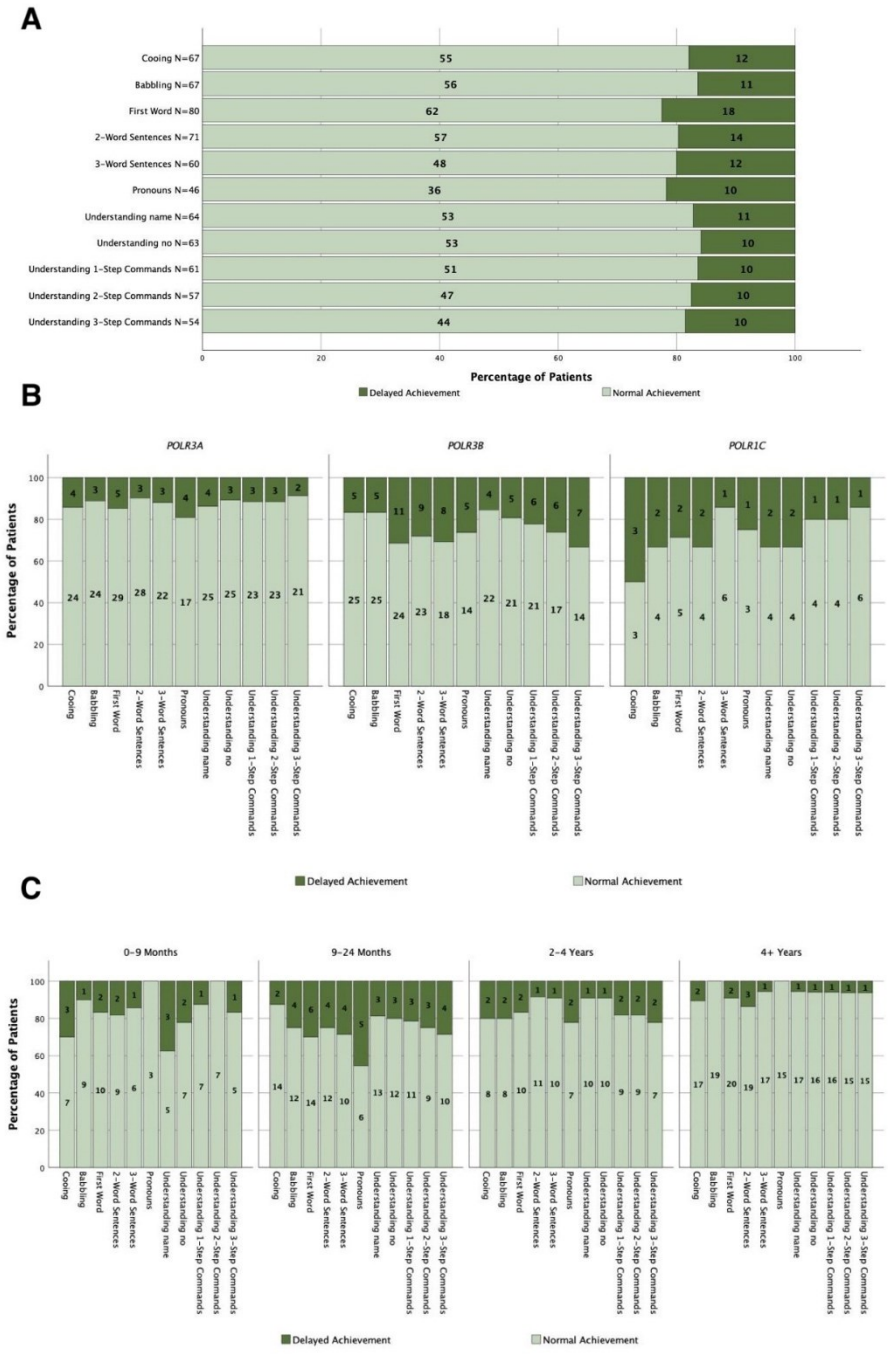

Figure 5.13. Analysis of Speech and Language Development in Patients with POLR3-related Leukodystrophy. (A) Normal vs. delayed development in patients who achieved language milestones, (B) presented by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (C) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) Patients did not show a significant difference in achieving different language milestones without delay (B) *POLR3A* genotype is more likely to achieve language milestones on time compared to *POLR3B* and *POLR1C*. (C) Analysis of language milestones achievement by age of onset showed patients with disease onset after 4 years had the most probability of achieving milestones without delay. Logistic regressions were performed using SPSS Statistic software.

5.4.7 Description of language developmental milestone loss

We further investigated language developmental regression by examining the loss of milestones in patients for whom data were available. When considering all speech and language milestones together, we noted that 80% of milestones lost were lost by patients with *POLR3A* mutations, while 10% were lost by *POLR3B* patients and 10% by *POLR1C* patients (Figure 4.14a). Logistic regression analyses suggest that *POLR3B* has lower odds of losing milestones compared to *POLR3A* ($p<0.001$, OR=0.081, 95% CI=0.33-0.196) and *POLR1C* patients ($p=0.005$, OR=0.177, 95% CI=0.053-0.587). Investigating individual milestones across the three genotypes suggested that patients with *POLR3B* mutations are less likely to lose understanding of 2-word sentences ($p=0.048$, OR=0.071, 95% CI=0.005-0.980) compared to *POLR1C* patients. Compared to *POLR3A* patients, *POLR3B* patients were less likely to lose understanding of 2-word sentences ($p=0.016$, OR=0.071, 95% CI=0.008-0.613) and 3-word sentences ($p=0.011$, OR=0.116, 95% CI=0.022-0.615). The overall speech and language developmental milestones loss was similar across different ages of disease onset, however, it was found that those who experienced a decline tended to lose predominantly expressive language milestones, whereas their receptive language skills remained relatively well maintained (Figure 4.14b).

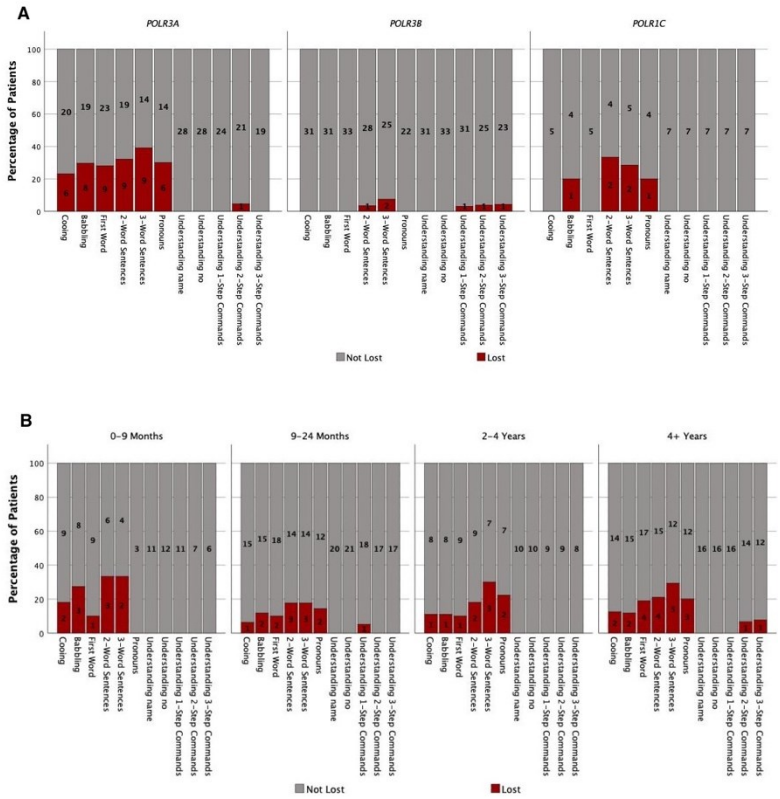

Figure 5.14. Analysis of Speech and Language Development in Patients with POLR3-related Leukodystrophy. Distribution of language milestone loss presented (A) by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (B) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) *POLR3B* genotype is less likely to lose language milestones compared to *POLR3A* and *POLR1C*. (B) Analysis of language milestones loss by age of onset showed no significant difference across groups of ages of onset. Logistic regressions were performed using SPSS Statistic software.

5.5 Social Development

5.5.1 Description of social developmental milestones acquisition by the entire cohort

All social milestones, namely, social smiling (94.59%, 70/74), laughing (95.71%, 67/70), eye contact (97.06%, 66/68), interaction with peers (96.92%, 63/65), and interactions with adults (100% 65/65), were successfully achieved amongst patients (Figure 4.15a). When conducting logistic regressions, we observed that there was no statistical difference in the odds of social milestone acquisition across genotypes and ages of onset (Figure 4.15a-c).

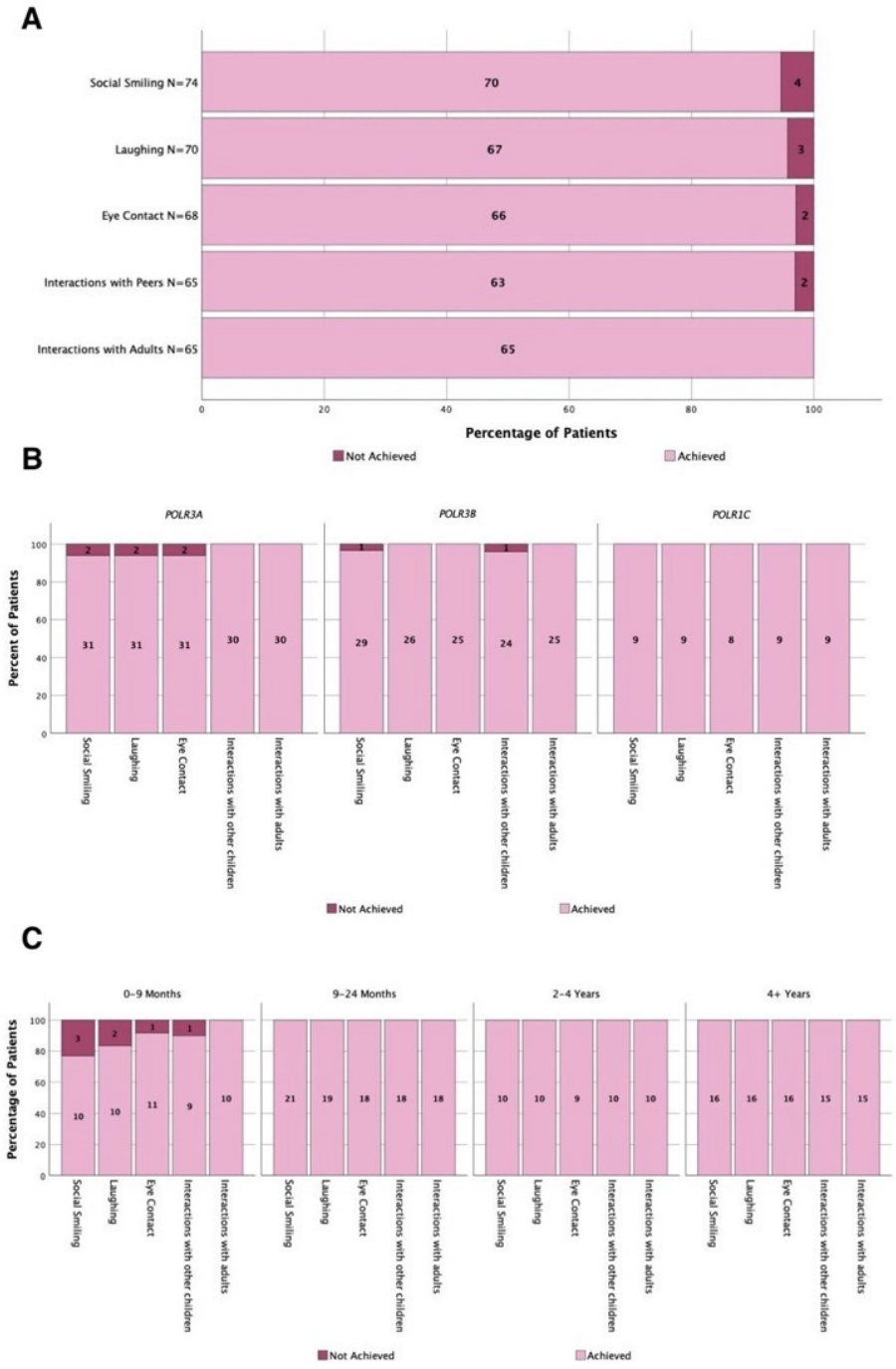

Figure 5.15. Analysis of Social Development in Patients with POLR3-related Leukodystrophy. (A) Distribution of social milestone achievement (achieved vs. not achieved) for the entire cohort study, (B) presented by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (C) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) All social milestones were successfully achieved among patients. (B) social milestone acquisition is similar across genotypes. (C) social milestone acquisition is similar across ages of onset. Statistical analysis was performed using SPSS Statistic software.

5.5.2 Normal versus delayed development in patients who achieved social developmental milestones

Patients demonstrated similar proportions of achievement between the different social milestones (Figure 4.16a). Social smiling was delayed in almost 20% of patients (18.46%, 12/65), while for each of the other milestones, less than 10% of patients were delayed in their acquisition. Notably, patients were 12.679 times (p=0.016, OR=12.679, 95% CI=1.593-100.912) more likely to achieve interactions with adults without delay compared to achieving social smiling on time.

5.5.3 Normal versus delayed development in patients who achieved social developmental milestones by genotype

Patients with all biallelic pathogenic variants in all three genes displayed similar proportions and odds of achieving of social milestones without delay (Figure 4.16b).

5.5.4 Normal versus delayed development in patients who achieved social developmental milestones by age of onset

In contrast, both patients with a disease onset between 9-24 months (p=0.049, OR=3.422, 95% CI=1.003-11.674) and after 4 years of age (p=0.013, OR=7.899, 95% CI=1.544-40.421), were statistically significantly more likely to achieve milestones without delay compared to those with an age of onset before 9 months of age (Figure 4.16c).

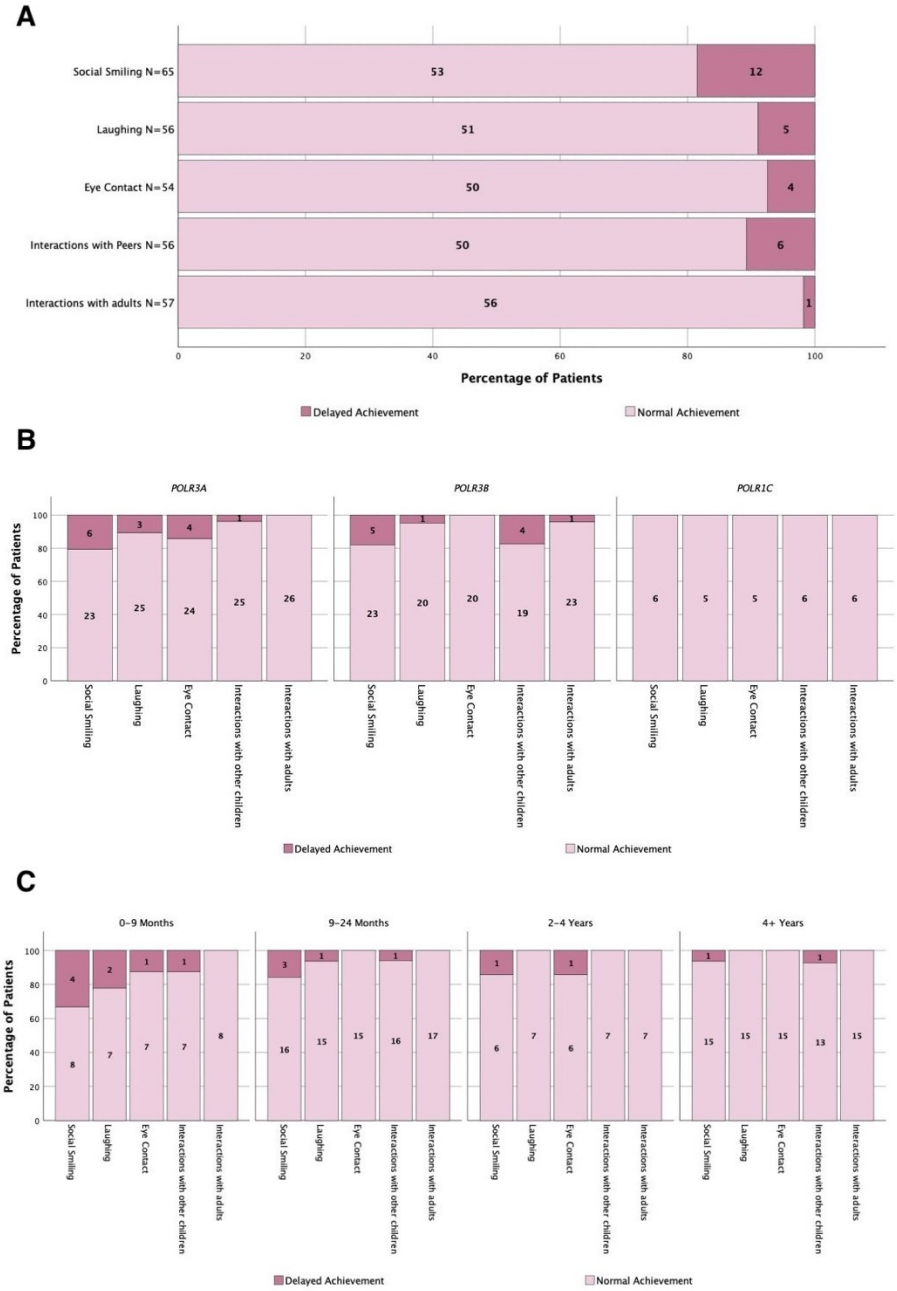

Figure 5.16. Analysis of Social Development in Patients with POLR3-related Leukodystrophy. (A) Normal vs. delayed development in patients who achieved social milestones, (B) presented genotype (*POLR3A, POLR3B,* and *POLR1C*), and (C) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) Patients showed a delay in the acquisition of social smiling (B) there was no significant difference between the three genotypes for the achievement of social developmental milestones with or without delay. (C) Analysis of social milestones achievement by age of onset showed patients with disease onset before 9 months had less probability of achieving milestones on time. Logistic regressions were performed using SPSS Statistic software.

5.5.5 Description of social developmental milestone loss

In addition, patients maintained social milestones effectively. Logistic regression analyses did not reveal any statistically significant differences in the odds of losing milestones across genotypes and ages of disease onset (Figure 4.17a-b).

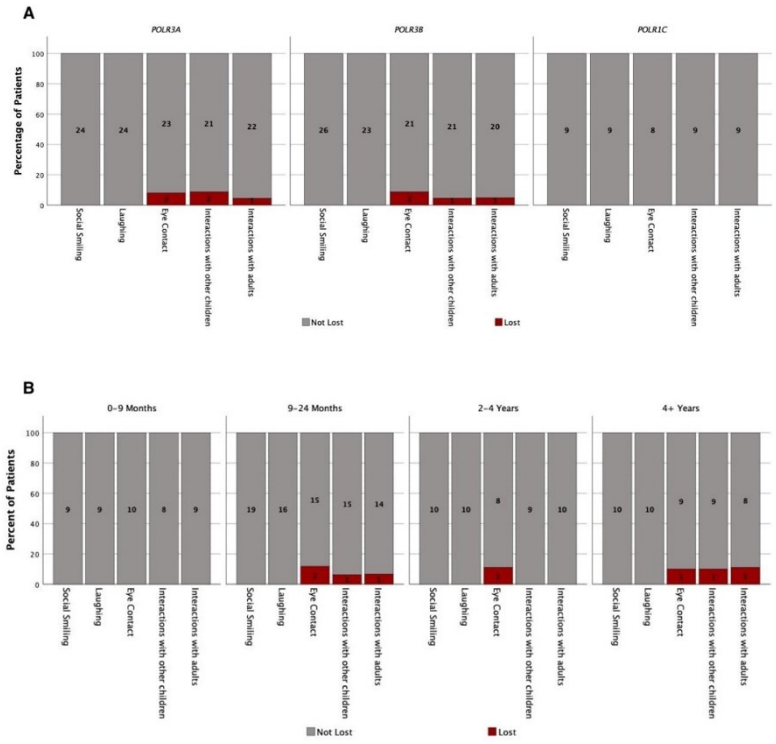

Figure 5.17. Analysis of Social Developmental Regression in Patients with POLR3-related Leukodystrophy. Distribution of social milestone loss presented (A) by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (B) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) Analysis of social milestones loss by genotype showed no significant difference across genotypes (B) Analysis of social milestones loss by age of onset showed no significant difference across groups of ages of onset. Logistic regressions were performed using SPSS Statistic software.

5.6 Cognitive Development

5.6.1 Description of cognitive developmental milestones acquisition by the entire cohort

Early cognitive milestones were successfully achieved amongst patients (Figure 4.18a). Namely, learning animal sounds (93.33%, 42/45), pointing (94.23%, 49/52) and naming (92.68%, 38/41) major body parts, pointing (89.74%, 35/39) and naming (88.89%, 40/45) complex body parts, recognizing colors (96.36%, 53/55), shapes (96%, 48/50), and letters (94.23%, 49/52). Early milestones acquired in school, such

as reading words (85.71%, 42/49), additions (85.37%, 35/41), and subtractions (82.05%, 32/39), were also successfully achieved among patients. Proportions of achieved milestones decreased with more difficult cognitive milestones such as reading short sentences (80%, 36/45) and small books (73.17%, 30/41), writing (74.42%, 32/43), multiplications (62.86%, 22/35), and division (63.64%, 21/33). A logistic regression, with the milestone animal, sounds as the reference, confirmed these results. Specifically, patients were less likely to achieve writing (p=0.041, OR=0.277, 95% CI=0.081-0.951), reading small books (p=0.033, OR=0.260, 95% CI=0.0.075-0.894), performing multiplications (p=0.004, OR=0.161, 95% CI=0.0.0470.553), and divisions (p=0.005, OR=0.167, 95% CI=0.048-0.580) compared to learning animal sounds.

5.6.2 Description of cognitive developmental milestones acquisition by genotype

Through a logistic regression analysis, we observed that patients with *POLR3B* mutations were 0.531 times less likely (p=0.010, OR=0.531, 95% CI=0.329-0.858) to achieve cognitive milestones compared to *POLR3A* patients (Figure 4.18b). Furthermore, *POLR1C* patients were found to be 0.440 times (p=0.060, OR=0.440, 95% CI=0.187-1.036) less likely to achieve cognitive milestones compared to *POLR3A* patients. However, this was only approaching statistical significance and more data is necessary to be certain of this result.

5.6.3 Description of cognitive developmental milestones acquisition by age of onset

Regarding the age of disease onset, patients who had a disease onset before nine months of age, were the least likely to achieve cognitive milestones. Specifically, patients who exhibited initial signs of the disease between nine to 24 months (p<0.001, OR=3.785, 95% CI=2.134-6.712), two to four years (p<0.001, OR=7.558, 95% CI=3.092-18.475), and older than four years old (p<0.001, OR=164.921, 95% CI=21.663-1255.574), demonstrated higher odds of cognitive milestone achievement compared to those who experienced disease onset before nine months old (Figure 4.18c).

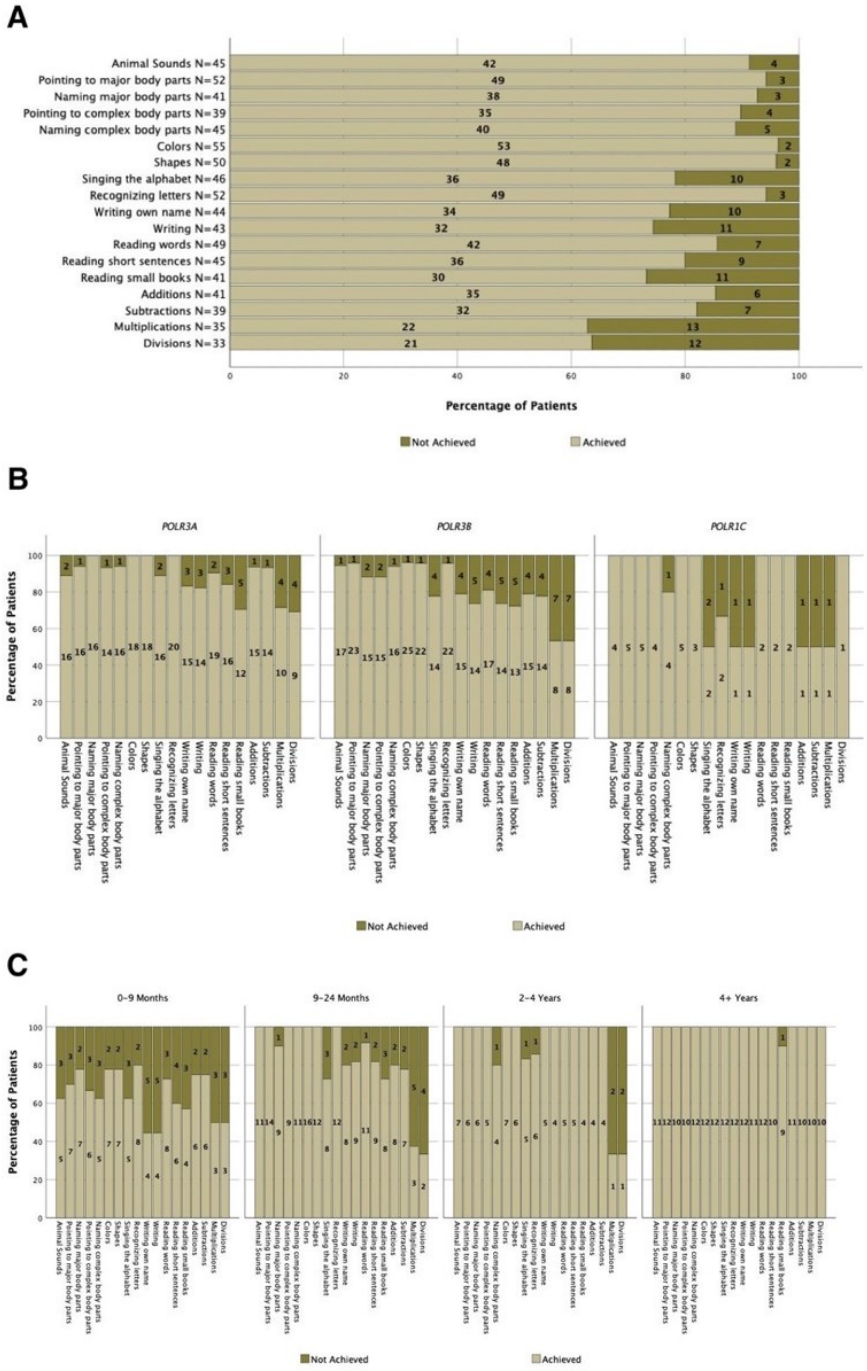

Figure 5.18. Analysis of Cognitive Development in Patients with POLR3-related Leukodystrophy. (A) Distribution of cognitive milestone achievement (achieved vs. not achieved) for the entire cohort study, (B) presented by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (C) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) Early cognitive milestones showed a higher percentage of achievement (animal sounds, pointing and naming body parts, recognizing colors, shapes, and letters) meanwhile later milestones (reading, writing, additions subtractions, multiplications, and divisions) showed the lowest percentage of achievement. (B) *POLR3A* genotype is more likely to achieve cognitive milestones compared to *POLR3B* and *POLR1C*. (C) Analysis of cognitive milestone achievement by age of onset showed higher odds of achievement in the groups of 9-24

months old and >4 years old, however, the earliest group (0-9 months) was less likely to achieve cognitive milestones. Logistic regressions were performed using SPSS Statistic software.

5.6.4 Normal versus delayed development in patients who achieved cognitive developmental milestones

Milestones acquired in school had higher proportions of delayed acquisition (Figure 4.19). Specifically, writing (23.08%, 6/26), reading words (30.30%, 10/33), short sentences (9.63%, 8/27) and small books (28%, 7/25), performing additions (25%, 7/28), subtractions (25%, 6/24), multiplications (25%, 5/20) and divisions (27.78%, 5/18) (Figure 4.19a). In contrast, the other earlier milestones (animal sounds, pointing and naming body parts, recognizing colors, shapes, and letters) showed that less than 20% of patients were delayed in their acquisition. However, when conducting individual logistic regression analyses for each cognitive milestone, we observed that the milestones reading words (p=0.022, OR=0.197, 95% CI=0.049-0.794), short sentences (p=0.030, OR=0.204, 95% CI=0.048-0.859), and small books (p=0.043, OR=0.220, 95% CI=0.051-0.956), were less likely to be achieved without delay.

5.6.5 Normal versus delayed development in patients who achieved cognitive developmental milestones by genotype

Regarding the cognitive development and genotype, we found that patients with *POLR3B* mutations were 0.236 times less likely (p<0.001, OR=0.236, 95% CI=0.136-0.410) to achieve milestones on time compared to *POLR3A* patients and 0.167 times (p=0.006, OR=0.167, 95% CI=0.046-0.520) less likely than *POLR1C* patients (Figure 20b). When conducting individual logistic regressions for each milestone, we observed an important trend where patients with *POLR3B* mutations were less likely to achieve the milestones, writing, reading words, reading short sentences, reading small books, additions, subtractions, multiplications, and divisions, on time compared to *POLR3A,* however, more data is needed to be able to see statistically significant results.

5.6.6 Normal versus delayed development in patients who achieved cognitive developmental milestones by age of onset

Regarding the age of disease onset, patients with disease onset before nine months of age ($p<0.001$, OR=0.196, 95% CI=0.084-0.458) and between nine to 24 months ($p<0.001$, OR=0.137, 95% CI=0.062-0.304) were less likely to achieve cognitive milestones without delay compared to those that had an onset after four years old (Figure 4.19c).

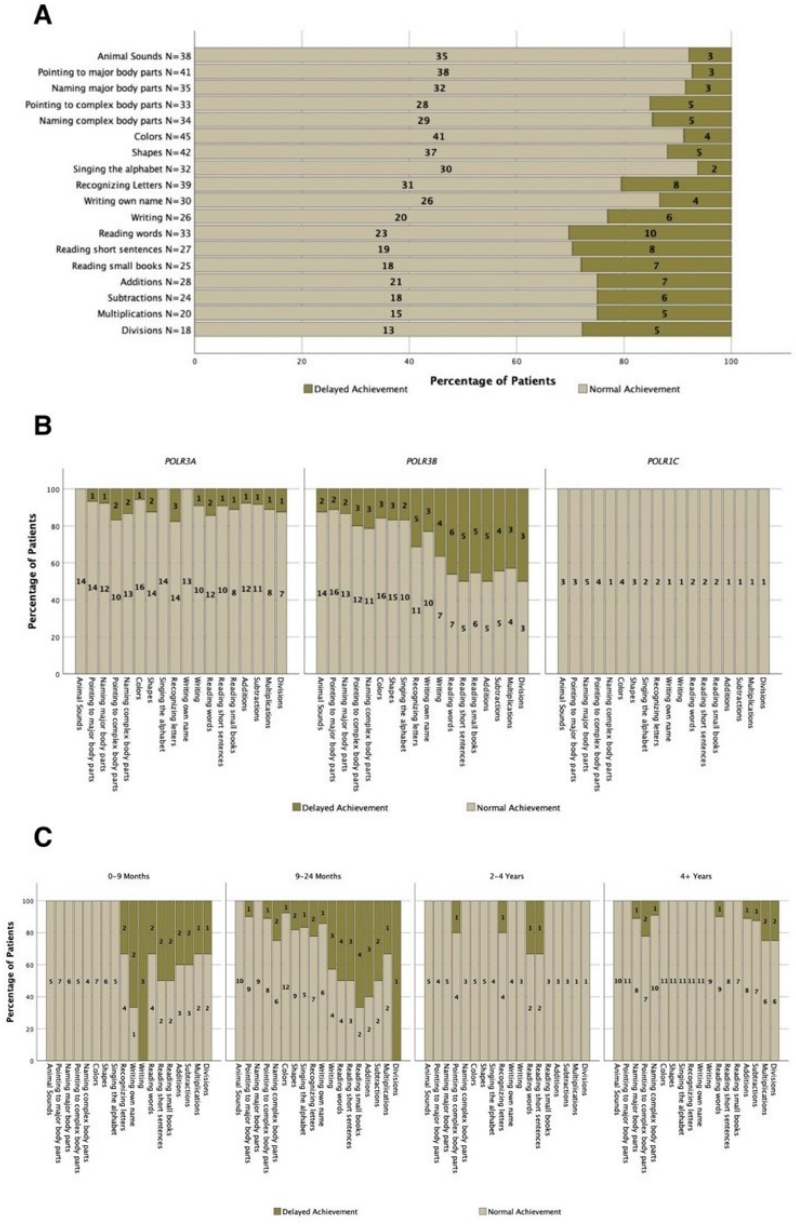

Figure 5.19. Analysis of Cognitive Development in Patients with POLR3-related Leukodystrophy. (A) Normal vs. delayed development in patients who achieved cognitive milestones, (B) presented genotype (*POLR3A, POLR3B,* and *POLR1C*), and (C) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) The

milestones of writing, reading short sentences and small books, additions, subtractions, multiplications, and divisions, were more likely to be achieved with delay (B) *POLR3A* genotype is more likely to achieve cognitive milestones on time compared to *POLR3B* and *POLR1C*. (C) Analysis of cognitive milestones achievement by age of onset showed higher odds of achievement without delay in the groups of 2-4 years old and >4 years old, however the group 0-9 months was less likely to achieve cognitive milestones on time. Logistic regressions were performed using SPSS Statistic software.

5.6.7 Description of cognitive developmental milestone loss

The loss of cognitive milestones in this cohort was prominently lost by *POLR3A* patients. In particular, 75.4% of milestones lost were lost by patients with *POLR3A* mutations, while 19.7% were lost by *POLR3B* patients and 4.9% by *POLR1C* patients (Figure 4.20a). Logistic regression analyses suggested that *POLR3B* patients have 0.171 ($p<0.001$, OR=0.171, 95% CI=0.087-0.336 lower odds of losing milestones compared to *POLR3A* patients. Given the limited availability of data on *POLR1C* patients, making comparisons, and drawing conclusions regarding their loss of milestones in comparison to *POLR3B* and *POLR3A* patients, was not possible. Regarding the age of disease onset, we found that patients with an onset of disease before 9 months old experienced the least loss of milestones (13.8%), while 25.9% of milestones lost were lost by those with an onset between nine to 24 months, 19% by those with an onset between 2 to 4 years and 41.4% by those with an onset after four years (Figure 4.20b). Logistic regression analyses suggested no statistical difference in the odds of loss of milestones between the different ages of onset.

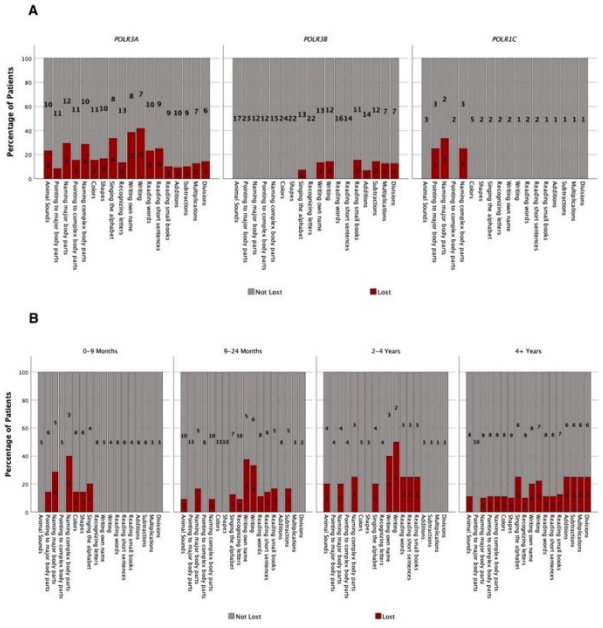

Figure 5.20. Analysis of Cognitive Developmental Regression in Patients with POLR3-related Leukodystrophy. Distribution of cognitive milestone loss presented (A) by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (B) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) *POLR3B* genotype is less likely to lose cognitive milestones compared to *POLR3A* and *POLR1C*. (B) Analysis of cognitive milestone loss by age of onset showed no significant difference across groups of ages of onset. Logistic regressions were performed using SPSS Statistic software.

5.7 Activities of Daily Living Development

5.7.1 Description of activities of daily living developmental milestones acquisition by the entire cohort

Early activities of daily living (ADL) milestones, such as finger feeding (95.16%, 59/62), and feeding with utensils (93.93%, 62/66), and milestones that required partial independence, such as undressing partly (93.85%, 61/65), dressing partly (89.23%, 58/65) and washing partly (85.71%, 42/49) were successfully achieved by most patients. Proportions of achievement of ADL milestones diminished for milestones that require independence such as, undressing completely (61.67%, 37/60), dressing completely (55.93%, 33/59), washing completely (48.89%, 22/45), cooking (31.81% 7/22) and finances (30.76%, 8/26) (Figure 4.21a). A logistic regression analysis with the earliest ADL milestone (finger feeding) confirmed that milestones requiring independence were significantly less likely to be achieved (table 4.3).

5.7.2 Description of activities of daily living developmental milestones acquisition by genotype

Activities of daily living milestones were acquired successfully across different across genotypes (Figure 4.21b). However, when comparing the odds of ADL milestone achievement across genotypes, we observed that the differences in odds of acquisition for, patients with variants in *POLR3B* (p=0.050, OR=0.615, 95% CI=0.378-0.999) and *POLR1C* (p=0.053, OR=0.479, 95% CI= 0.228-1.009) was approaching significance.

However, the proportion of achievement of ADL milestones by patients with biallelic pathogenic variants in *POLR3B* was lower than by patients with *POLR3A* mutations. Given the limited availability of data on *POLR1C* patients, making

comparisons, and drawing conclusions regarding their time of ADL milestones achievement in comparison to *POLR3B* and *POLR3A* patients, was not possible.

5.7.3 Description of activities of daily living developmental milestones acquisition by age of onset

Regarding the age of disease onset, patients who had a disease onset before nine months of age were the least likely to achieve ADL milestones (Figure 4.21c). Specifically, patients who exhibited initial signs of the disease between nine to 24 months ($p<0.001$, OR=6.865, 95% CI=3.25314.486), two to four years ($p<0.001$, OR=10.134, 95% CI=4.133-24.849), and older than four years old ($p<0.001$, OR=22.869, 95% CI=9.899-52.834), demonstrated higher odds of milestone achievement compared to those who experienced disease onset before nine months old. Specifically, individual logistic regression models for each milestone across ages of onset revealed that the milestones undressing completely ($p=0.026$, OR=7.50, 95% CI=1.276-44.085), dressing completely ($p=0.011$, OR=12.250, 95% CI=1.788-83.946), toilet training ($p=0.010$, OR=21.00, 95% CI=2.099-210.136), and washing completely ($p=0.024$, OR=10.50, 95% CI=1.360-81.053) had higher odds of being achieved by patients with an age of onset after 4 years compared to those before nine months. Patients with an onset of disease between two to four years ($p=0.045$, OR=11.00, 95% CI=1.061-114.086) were also more likely to achieve toilet training compared to those with an onset before nine months.

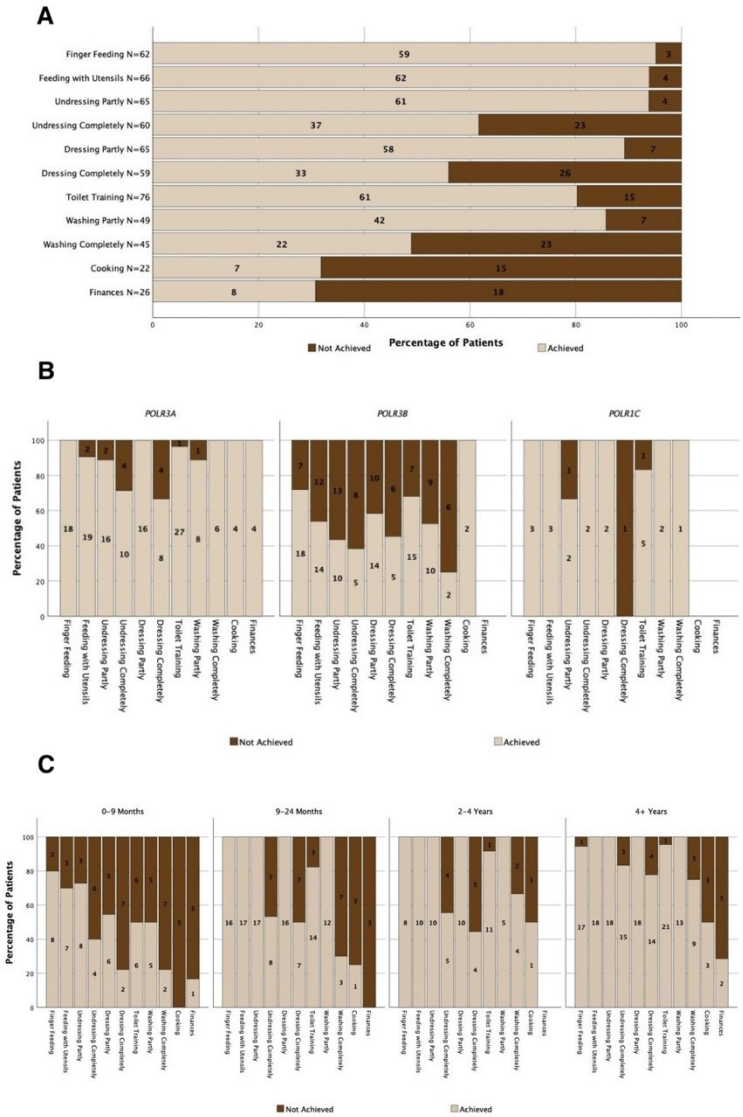

Figure 5.21. Analysis of Activities of Daily Living Development in Patients with POLR3-related Leukodystrophy. (A) Distribution of Activities of Daily Living milestone achievement (achieved vs. not achieved) for the entire cohort study, (B) presented by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (C) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) The highest achievement percentage belongs to the milestones, finger feeding, feeding with utensils, undressing partly, dressing partly, and washing partly. Meanwhile, the milestones of undressing, dressing, and washing completely, toilet training, cooking, and managing finances, showed the lowest achievement. (B) *POLR3B* genotype is less likely to achieve activities of daily living milestones compared to *POLR3A* and *POLR1C*. (C) Analysis of activities of daily living milestone achievement by age of onset showed higher odds of achievement in the groups of 2-4 years old and >4 years old, however, the earliest group (0-9 months) was less likely to achieve activities of daily living milestones. Logistic regressions were performed using SPSS Statistic software.

				95% C.I. for Exp (B)	
Age of Onset	**df**	**Sig.**	**Exp (B)**	**Lower**	**Upper**
Finger Feeding	10	<0.001			
Feeding with utensils	1	0.762	0.788	0.169	3.672
Undressing Partly	1	0.746	0.775	0.166	3.614
Undressing Completely	1	<0.001	0.082	0.023	0.292
Dressing Partly	1	0.226	0.421	0.104	1.709
Dressing Completely	1	<0.001	0.065	0.018	0.230
Toilet Training	1	0.017	0.207	0.057	0.751
Washing Partly	1	0.99	0.305	0.075	1.249
Washing Completely	1	<0.001	0.049	0.013	0.178
Finances	1	<0.001	0.024	0.005	0.103
Cooking	1	<0.001	0.017	0.004	0.079

Table 5.3. Logistic Regression Analysis Comparing Odds of Achieving Activities of Daily Living Milestones. The reference milestone is finger feeding. The milestones undressing completely, dressing completely, toilet training, washing completely, managing finances, and cookies are significantly less likely to be achieved compared to finger feeding. The significance level is set to $p<0.05$.

5.7.4 Normal versus delayed development in patients who achieved activities of daily living developmental milestones

Considering the subset of patients for whom data on whether activities of daily living were achieved with or without delay was available, we observed that patients were delayed in their acquisition of ADL milestones that required independence. Specifically, undressing completely (41.93%, 13/31), dressing completely (46.15%, 12/26), and washing completely (41.18%, 7/17) (Figure 4.22a). In contrast, we observed that less than 35% of patients were delayed in the acquisition of finger feeding, feeding with utensils, toilet training, undressing, dressing, and washing partly. Furthermore, a logistic regression, revealed that the milestones undressing partly (p=0.049, OR=0.378, 95% CI=0.144-0.996) and completely (p=0.014, OR=0.270, 95% CI=0.0950.765), dressing completely (p=0.007, OR=0.228, 95%

CI=0.077-0.671), and washing completely (p=0.041, OR=0.279, 95% CI=0.082-0.951), were less likely to be achieved without delay.

5.7.5 Normal versus delayed development in patients who achieved activities of daily living developmental milestones by genotype

Patients with *POLR3B* mutations were 0.108 times less likely (p<0.001, OR=0.108, 95% CI=0.056-0.212) to achieve ADL milestones on time, compared to *POLR3A* patients and 0.167 times (p=0.006, OR=0.167, 95% CI=0.046-0.605) less likely than *POLR1C* patients (Figure 4.22b). Specifically, individual logistic regression models for each milestone across genotypes revealed that the milestones feeding with utensils (p=0.013, OR=0.123, 95% CI=0.024-0.638), undressing partly (p=0.006, OR=0.096, 95% CI=0.018-0.519), and toilet training (p=0.023, OR=0.079, 95% CI=0.009-0.708), had lower odds of being achieved without delay by patients with *POLR3B* mutations compared to *POLR3A* mutations.

5.7.6 Normal versus delayed development in patients who achieved activities of daily living developmental milestones by age of onset

Furthermore, patients with disease onset between two to four years (p<0.001, OR=12.678, 95% CI=3.647-44.064) and after four years of age (p<0.001, OR=5.367, 95% CI=2.328-12.374), were more likely to achieve milestones on time compared to those that had an onset before nine months (Figure 4.22c). Specifically, individual logistic regression models for each milestone across ages of onset revealed that toilet training (p=0.025, OR=19.00, 95% CI=1.454-248.237) was 19 times more likely to be achieved on time by patients with disease onset after four years, compared to those with onset before nine months old.

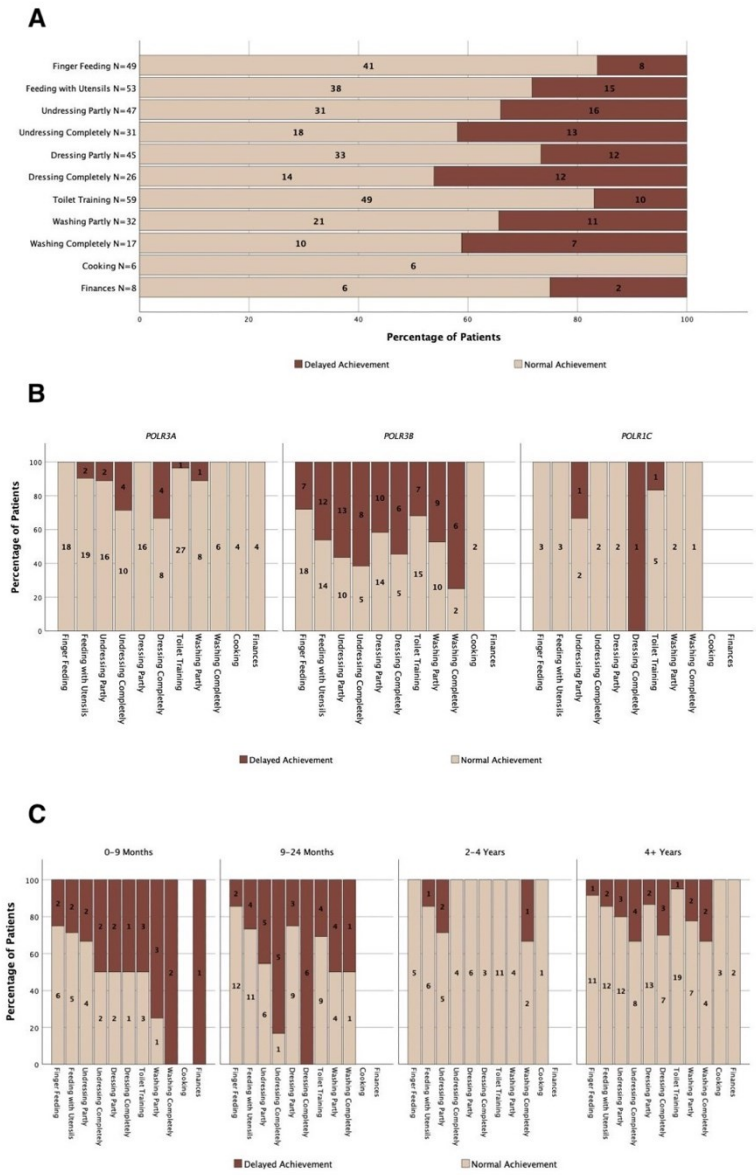

Figure 5.22. Analysis of Activities of Daily Living Development in Patients with POLR3-related Leukodystrophy. (A) Normal vs. delayed development in patients who achieved activities of daily living milestones, (B) presented genotype (*POLR3A, POLR3B,* and *POLR1C*), and (C) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) The milestones feeding with utensils, undressing partly and completely, dressing completely, and washing partly and completely, were more likely to be achieved with delay (B) *POLR3A* genotype is more likely to achieve activities of daily living milestones on time compared to *POLR3B* and *POLR1C*. (C) Analysis of achievement of activities of daily living milestones development by age of onset showed higher odds of achievement without delay in the groups of 2-4 years old and >4 years old, however the group 0-9 months was less likely to achieve activities of daily living milestones on time. Logistic regressions were performed using SPSS Statistic software.

5.7.7 Description of activities of daily living developmental milestone loss

The majority of milestones lost (85.7%) were lost by patients with *POLR3A* mutations, while 12.5% were lost by *POLR3B* patients and 1.8% by *POLR1C* patients (Figure 4.23a). Logistic regression analyses suggest that *POLR3B* patients have 0.85 ($p<0.001$, OR=0.085, 95% CI=0.370.195) and *POLR1C* have 0.055 times ($p=0.005$, OR=0.055, 95% CI=0.007-0.417) lower odds of losing milestones compared to *POLR3A* patients. Additionally, patients with an onset of disease between two to four years old experienced the least loss of milestones (4.1%) while 16.3% of milestones lost were lost by those with an onset before nine months, 34.7% by those with an onset between nine to 24 months and 44.9% by those with an onset after four years (Figure 4.23b). Logistic regression analyses suggest that patients with an onset between two to four years have 0.145 ($p=0.019$, OR=0.145, 95% CI=0.029-0.727) times lower odds of losing milestones compared to those with an onset before nine months old and 0.149 times ($p=0.012$, OR=0.149, 95% CI=0.034-0.659) lower odds compared to those with an onset after four years of age.

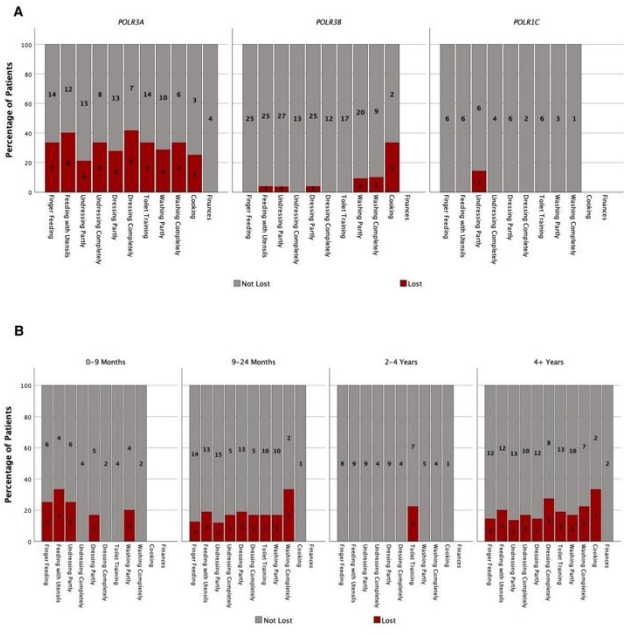

Figure 5.23. Analysis of Activities of Daily Living Developmental Regression in Patients with POLR3-related Leukodystrophy. Distribution of activities of daily living milestone loss presented (A) by genotype (*POLR3A, POLR3B,* and *POLR1C*), and (B) by age of onset (0-9 months, 9-24 months, 2-4 years, and older than 4 years). (A) *POLR3A* genotype is more likely to lose activities of daily living milestones compared to *POLR3B* and *POLR1C*. (B) Analysis of activities of daily living milestone loss by age of onset showed that patients with onset before 9 months old were more likely to lose milestones. Logistic regressions were performed using SPSS Statistic software.

5.8 Survival Analysis

5.8.1 Walking

Survival analysis using the Kaplan-Meier method was conducted to assess the time of walking achievement across the three genotypes, for the patients for which the exact age of acquisition was available: *POLR3A* (n=30), *POLR3B* (n=29), and *POLR1C* (n=6) (Figure 4.24a). The log-rank (Mantel-Cox) test was used to compare the survival curves of the three genotypes, resulting in a statistically significant difference between *POLR3A* and *POLR3B* ($p<0.001$). This suggests that *POLR3B* patients achieve walking later than *POLR3A*. However, the survival curves for *POLR3A* and *POLR1C* (p=0.417) patients as well as *POLR3B* and *POLR1C* (p=0.118) were not significantly different from each other. Performing a Cox regression further revealed that patients with variants in *POLR3B* ($p<0.001$, OR=0.384, 95% CI=0.221-0.668) are also less likely to achieve walking compared to those with variants in *POLR3A*. In addition, a survival analysis was done to assess the time of loss of walking across the three genotypes: *POLR3A* (n=35), *POLR3B* (n=30), and *POLR1C* (n=7). The log-rank (MantelCox) demonstrated that *POLR3A* patients lose walking significantly earlier than *POLR3B* patients (p=0.037) (Figure 4.24b). Furthermore, a Cox regression analysis revealed that *POLR3B* patients are 0.288 times less likely to lose walking compared to *POLR3A* (p=0.051, HR=0.288, 95% CI=0.083-1.005). However, the loss of walking for *POLR3A* and *POLR1C*

patients as well as *POLR3B* and *POLR1C* were not significantly different from each other.

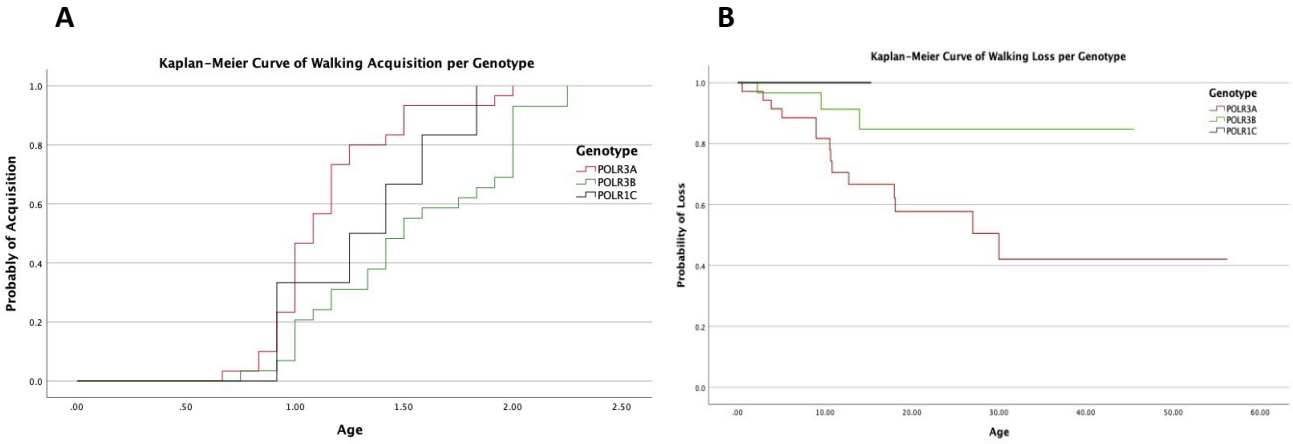

Figure 5.24. Kaplan-Meier Curve of the Age at Walking (A) Acquisition and (B) Loss according to Genotype. (A) *POLR3B* patients have delayed acquisition of walking compared to *POLR3A.* (B) *POLR3A* has an earlier loss of walking compared to *POLR3B.*

We also conducted a Kaplan-Meier analysis to assess the time of walking achievement across the four groups according to the ages of disease onset: zero to nine months (n=12), nine to 24 months (n=15), two to four years (n=11) and older than four years old (n=18) (Figure 4.25a). The log-rank (MantelCox) test revealed that patients with onset before nine months ($p<0.001$) and between nine to 24 months ($p=0.023$) are more likely to be delayed in the achievement of walking compared to those with onset after four years. Those with an onset between nine to 24 months are also delayed compared to those with an onset between two to four years ($p<0.001$). A Cox regression reveals that these two groups of early disease onset are less likely to achieve walking than those with onset after four years of age. Specifically, those with an onset before nine months are 0.383 times ($p=0.013$, HR= 0.383, 95% CI= 0.179-0.819) less likely to achieve walking and those with an onset between nine to 24 months have 0.276 times ($p<0.001$, HR=0.276, 95% CI=0.133-0.575) lower odds to achieve walking compared to those with an onset after four years.

We investigated the loss of walking across these four groups as well: zero to nine months (n=15), nine to 24 months (n=16), two to four years (n=11), and older than four years old (n=20) (Figure 4.25b). The log-rank (Mantel-Cox) test revealed no statistically significant difference in the time of loss between the four groups. A Cox

regression also resulted in no significant difference in the odds of loss of walking across these ages of onset. However, it's important to note that the odds of losing walking for those between nine to 24 months (p=0.088) approached statistical significance when compared to those with onset after four years. Specifically, the patients in this early onset group were 3.022 times (p=0.088, HR=3.022, 95% CI=0.848-10.773) more likely to lose walking than those with onset after four years of age.

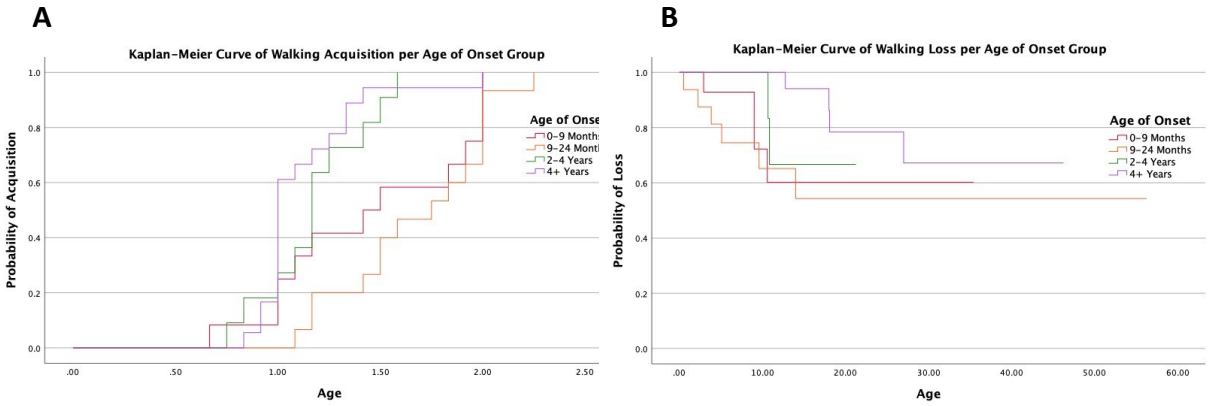

Figure 5.25. Kaplan-Meier Curve of the Age at Walking (A) Acquisition and (B) Loss according to Age of Onset. (A) age of onset group 0-9 months and 9-24 months were delayed in the acquisition of walking. (B) There was no significant difference between the ages of onset groups for the loss of walking.

5.8.2 First Word

A Kaplan-Meier analysis was done to assess the time of first-word achievement across the three genotypes: *POLR3A* (n=15), *POLR3B* (n=18), and *POLR1C* (n=4) (Figure 4.26a). The logrank (Mantel-Cox) demonstrated no statistically significant difference between the three genotypes, however after one year of age, the curve of *POLR3B* patients showed a delay in acquisition compared to the two other curves. A Cox regression analysis revealed that patients with *POLR3B* are 2.392 times (p=0.019, HR=2.392, 95% CI=1.154-4.960) more likely to achieve this milestone compared to patients with *POLR1C* mutations. Similarly, we investigated the time of achievement of first word across the four groups of ages of onset: zero to nine months (n=6), nine to 24 months (n=12), two to four years (n=6), and older than four years old (n=11) (Figure 4.26b). The log-rank (Mantel-Cox) demonstrated no statistically significant difference between the four groups, however after one year of age, the curve of patients with an onset between nine to 24 months showed some delay in acquisition compared to the two other curves. However, a Cox regression revealed that those with a disease onset between two to four years have 4.394 times

(p=0.026, HR=4.394 95% CI=1.199-16.108) higher odds to achieve this milestone compared to those with an onset before nine months.

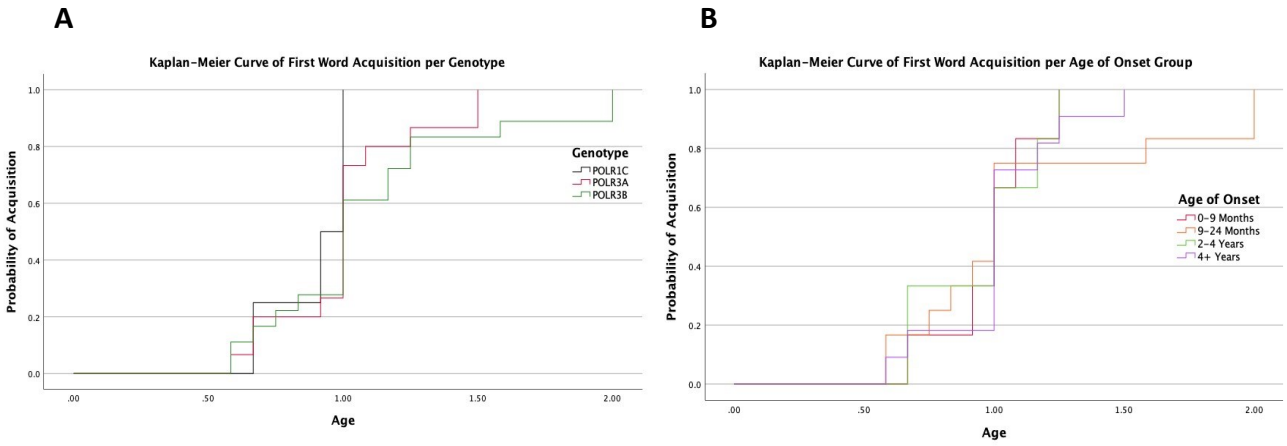

Figure 5.26. Kaplan-Meier Curve of the Age at First Word Acquisition according to (A) Genotype and (B) Age of Onset. (A) There was no significant difference between genotype groups in the acquisition of the milestone first word. (B) There was no significant difference between the age of onset groups in the acquisition of the milestone first word.

5.8.3 Continence

A Kaplan-Meier analysis was done to assess the time of continence achievement across the three genotypes: *POLR3A* (n=18), *POLR3B* (n=15), and *POLR1C* (n=6) (Figure 4.27a). The log rank (Mantel-Cox) demonstrated that *POLR3B* patients (p=0.010) achieve continence later than *POLR3A*. However, the survival curves for *POLR3A* and *POLR1C* (p=0.325) patients as well as *POLR3B* and *POLR1C* (p=0.178) were not significantly different from each other. A Cox regression analysis revealed no statistically significant difference between the three genotypes, suggesting that all three groups of patients have equal odds of achieving this milestone. Similarly, we investigated the time of achievement of continence across the four groups of ages of onset: zero to nine months (n=5), nine to 24 months (n=10), 2 to 4 years (n=7) and older than four years old (n=13) (Figure 4.27a). The log rank (Mantel-Cox) demonstrated no statistically significant difference between the four groups. A Cox regression analysis revealed no statistically significant difference between the four groups of ages of onset, suggesting that all four groups of patients have equal odds of achieving this milestone.

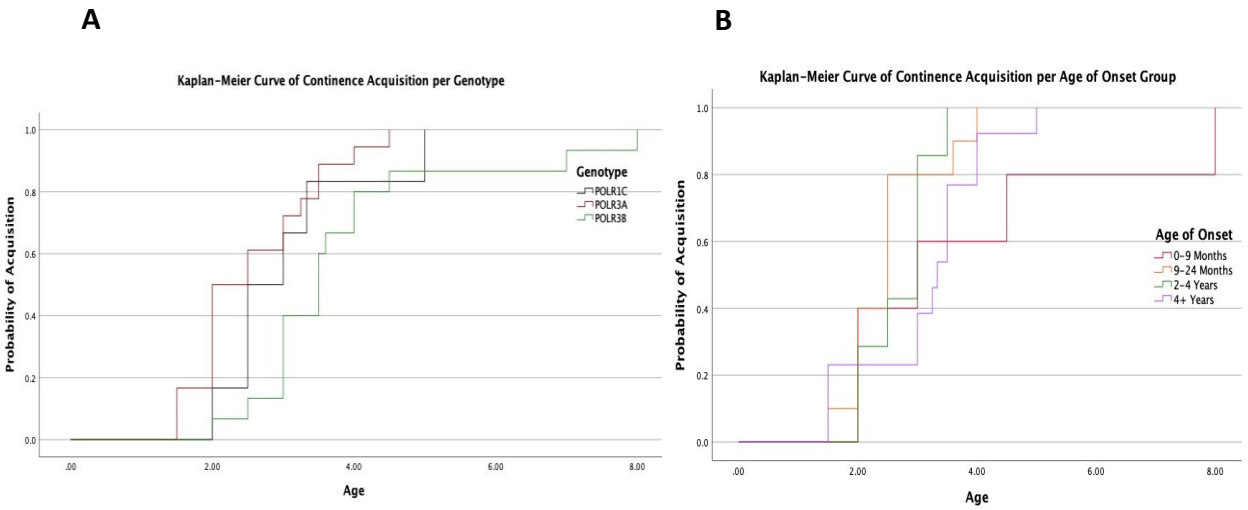

Figure 5.27. Kaplan-Meier Curve of the Age at Continence Acquisition according to (A) Genotype and (B) Age of Onset. (A) *POLR3B* patients have delayed acquisition of continence compared to *POLR3A.* (B) There was no significant difference between the age of onset groups in the acquisition of the milestone continence

6 Discussion

6.1 General Discussion

RNA polymerase III-related hypomyelinating leukodystrophy (POLR3-HLD), is one of the most common hypomyelinating leukodystrophies. This thesis aimed to investigate and characterize the developmental trajectory of patients affected by POLR3-HLD.

6.2 LORIS MyeliNeuroGene Rare Disease Database

The first section of this thesis involved adapting the LORIS MyeliNeuroGene Rare Disease Database for a retrospective natural history study (including this developmental trajectory study) of POLR3-HLD. Through collaboration with the LORIS team, we refined the database by creating new instruments and improving existing ones. These instruments enabled the capture of essential medical histories details, such as prenatal history, developmental milestones, primary diagnosis, and initial symptoms of disease presentation.

Our database also incorporated instruments for documenting investigations (metabolic investigations, clinical investigations, brain MRI and CT, EMG/NCS, and swallowing evaluation), clinical evolution (age of onset of characteristic symptoms), and examination (medical visit examinations). The database underwent rigorous testing, and any identified bugs were addressed, resulting in a fully functional and reliable data entry and management tool. Optimizing this database was essential for collecting complete and reliable data for the developmental trajectory project.

6.3 The Developmental Trajectory of Patients with POLR3-HLD

The second section focused on investigating the natural developmental trajectory and regression of patients with POLR3-HLD. Through a cross-sectional, retrospective study, we examined the acquisition and loss of six psychomotor milestones categories (gross motor, fine motor, language, social, cognitive, and activities of daily living) in 96 patients with genetically confirmed POLR3-HLD. Our results revealed that for all six neurodevelopmental domains, patients with POLR3-HLD generally achieved early milestones and when achieved they were achieved at

normal ages. These early milestones were also generally maintained over time and were less likely to be lost, compared to later milestones. This suggests that, as suggested by the literature [3, 9] the initial stages of development are relatively unaffected by the disease, and it is only later that patients present delays in milestones and developmental losses. Interestingly, significant differences were observed in the development trajectories amongst patients with biallelic pathogenic variants in *POLR3A, POLR3B,* and *POLR1C*. Furthermore, our findings suggest that the age of disease onset is a good predictor of the developmental trajectory.

6.3.1 Effects of Different Genotypes on Developmental Trajectory

Patients with *POLR3A* mutations demonstrated higher proportions of achievement for gross and fine motor development, cognitive development, and activities of daily living, compared to other genotypes. These patients also displayed higher proportions of achieved milestones at a normal developmental age for all developmental domains except social development, for which patients with all genotypes demonstrated equal proficiency in achievement. Patients with biallelic pathogenic variants in *POLR3A* also had higher odds of achieving milestones and achieving them at a normal age. This may be attributed to patients experiencing a later onset of the disease, which allows them to achieve developmental milestones within the expected timeframe before the disease onset [3, 9]. Conversely, patients with *POLR3B* mutations were less likely to achieve gross motor, cognitive, as well as activity of daily living skills compared to patients with *POLR3A* and *POLR1C* variants. They also displayed higher proportions and odds of delayed acquisition of milestones gross motor, fine motor, language, cognitive, and ADL milestones. Despite this, *POLR3A* patients exhibited an earlier regression compared to patients with *POLR3B* who experienced a slower rate of regression once these milestones were acquired. Patients with *POLR1C* mutations showed lower odds of fine motor milestone achievement compared to both patients with *POLR3A* and *POLR3B* variants. Additionally, they demonstrated delayed acquisition of language and fine motor milestones compared to patients with *POLR3A* mutations. In general, these patients had an earlier loss of fine motor milestones compared to patients with *POLR3B* mutations. However, given the limited data and small sample size (n=10) for

this genotype, it is difficult to make strong conclusions. A larger sample size would be required. These findings align with the observed differences in the presenting symptom at disease onset among these patients (Figure 4.5a). Specifically, the developmental delay was observed as an initial symptom only by individuals with *POLR3B* mutations, while those with *POLR3A* mutations displayed cerebellar signs, and those with *POLR1C* mutations presented with tremors. These initial symptoms may explain why patients with *POLR3B* mutations experience more delays in milestone acquisition and are less likely to achieve certain milestones and why those with *POLR3A* mutations exhibit a faster loss of milestones, given that they present with cerebellar symptoms which increase their difficulty in maintaining neurodevelopmental skills. In fact, patients with *POLR3B* mutations have a significantly earlier disease onset than those with *POLR3A* mutations. Therefore, it is possible that while patients with *POLR3A* mutations have a later onset which allows them to achieve milestones, the early age of disease onset of patients with *POLR3B* mutations may intervene with their achievement of milestones. Furthermore, the presence of tremors at disease onset in individuals carrying *POLR1C* mutations suggests the underlying cause of their delayed acquisition and subsequent early loss of fine motor milestones. Our results are consistent with previous research by Wolf et al. (2014), [9]. Furthermore, our results demonstrate that patients lose expressive language milestones and maintain receptive language milestones, which is consistent with our findings that motor functions are typically much more impaired than cognitive functions. Congruent with results seen in the literature [9], in our study, patients with *POLR3A* mutations had an earlier loss of expressive language milestones compared to the other two genotypic groups.

6.3.2 Effects of Different Ages of Onset on Developmental Trajectory

Furthermore, our analysis indicated that the age of disease onset also played a role in the developmental trajectory of POLR3-HLD patients. Patients with an onset of disease before nine months exhibited lower odds of achieving milestones for all six developmental domains. They were also delayed in the acquisition of gross motor and activities of daily living milestones. Patients with an age on onset between nine to 24 months exhibited delayed acquisition of fine motor milestones. In general, patients

with an age of onset after the age of two years were more likely to achieve developmental milestones at a normal age. However, the subsequent loss of milestones varied within these disease onset groups, which may be in part explained by the fact that it may be easier to lose more difficult milestones while it may be more difficult to lose early milestones, i.e., that one would need a greater deficit to lose an early milestone such as head control, compared to a later milestone such as riding a bicycle, where a milder deficit may be required. This is a hypothesis and does not exclude that additional factors are contributing. Furthermore, patients with onset before nine months of age exhibited greater difficulty in milestone acquisition, and if milestones were achieved, they experienced delays in their acquisition. Notably, a subset of these patients (21%, 4/19) are affected by the severe striatal form of the disease (Figure 4.4) [96, 98, 108]. This may explain why these specific patients experienced difficulty in attaining early milestones in all six developmental domains, which were otherwise successfully achieved by the rest of the cohort. Their increased breathing and feeding difficulties may also contribute to their difficulty in achieving expressive language milestones. However, for other patients within the early onset group, their difficulties in milestone achievement may be attributed to the severity associated with the early onset of the disease, suggested in previous literature [3, 9]. In contrast, patients with an onset between nine to 24 months, first presented with developmental delay as well as cerebellar features, this could be a reason for their delayed acquisition of fine motor skills and fast regression of gross motor milestones. Cerebellar features, such as ataxia and tremors can significantly impact ambulation, as evident from the Kaplan-Meier analysis of walking milestones [3, 9]. Predictably, patients with a later onset were more successful in achieving milestones, however, it is interesting that those with an age of onset after two years had a faster loss of cognitive milestones. This was also seen in other neurological disorders, such as cerebrotendinous xanthomatosis, where patients showed cognitive decline later in life compared to other symptoms (e.g., gait abnormalities, ataxia, CSF abnormalities) that were seen throughout life [155]. In previous POLR3-HLD research, it was also shown that those with an onset later than five years tended to present with learning difficulties [9]. Therefore, patients may reach an academic plateau with the onset of

the disease and experience cognitive regression soon after. Furthermore, patients with an onset of disease between nine to 24 months exhibit a faster gross motor decline, which is consistent with a natural history study done on Vanishing White Matter (VWM) where patients with an onset at one to less than two years typically showed a rapid decline, with wheelchair dependency after months to a few years [156].

6.3.3 Development Survival Analysis

In addition, Kaplan-Meier analyses of the time to achieve walking, first word, and continence, provided us with further evidence that *POLR3B* and early onset patients experience delay in acquiring these milestones. They also revealed that all patients acquire walking by two and a half years, first word by two years, and continence by five years, regardless of genotype and age of onset. Furthermore, our results suggest that the likelihood of achieving walking in this patient population does not differ across genotype and age of onset. However, the likelihood of achieving walking with delay does differ among these groups. Specifically, it can be expected that patients with biallelic pathogenic variants in *POLR3B* will achieve walking with delay whereas those with *POLR3A* variants will achieve walking on time. Despite this, patients with *POLR3B* mutations maintain walking longer than those with *POLR3A* variants. Conversely, it can be expected that patients with early disease onset experienced more delay in achieving walking and are also more likely to experience earlier loss of walking.

The findings of this study represent an advancement towards understanding the natural history study of POLR3-related leukodystrophy. Natural history study is imperative to perform for this disease to enable the establishment of appropriate clinical outcomes measures which are essential for future therapeutic trials. In addition, the findings of this study suggest that *POLR3A* patients and those with an early onset, are particularly suitable candidates for clinical trials. The restricted duration of these trials may be challenging for testing patients with *POLR3B* mutations, as their delayed acquisition and a slower rate of regression would necessitate a longer timeframe to observe meaningful outcomes. Similarly, conducting trials on early onset groups would be advantageous as they also exhibit a faster and

more severe regression pattern, which allows for a more efficient evaluation of potential interventions.

It is critical to recognize this project's limitations. The retrospective nature of this study makes it a challenge to acquire detailed granular data. The initial solution to this limitation was to create questionnaires for parents to answer, however, there was a challenge with parents and caregivers returning the questionnaires, but also with the level of detail when they answered it. Therefore, we had to mostly rely on retrospective data, which is limited to specific ages of acquisition and loss. Future research should focus on prospective developmental data collection with regular data collection throughout the disease course. Specifically, there should be standardized neurological assessments, and developmental assessments (e.g., GMFM-88, BOT-2, PDMS, and Leiter-3)[134-137], and questionnaires should be filled at specific time points. This will allow us to gain comprehensive and detailed data for a longitudinal study, which would be beneficial for further understanding of the developmental trajectory in POLR3-HLD patients over time.

In summary, the findings from this study have significant implications for understanding the progression of POLR3-HLD, as well as informing families about the expected disease course according to the genotype and age of onset of their child. Families will have a greater understanding of the possible difficulties their children may face by shedding light on the developmental patterns and traits found in this study. This will help families manage the complexity of their children's development and disease progression. Furthermore, our findings provide information that will be useful for designing trials to assess the efficacy of clinical interventions and potential therapeutic strategies for POLR3-HLD.

7 Concluding Remarks

In conclusion, this thesis contributes to the understanding of the natural developmental trajectory of POLR3-HLD. The findings suggest that the genotype and age of disease onset contribute to the developmental progression and regression of patients. Specifically, patients with *POLR3A* mutations acquire and lose developmental milestones earlier and faster than *POLR3B* and *POLR1C.* While patients with an early disease onset have delayed acquisition of milestones. However, further research is necessary to investigate the effects of age of onset on developmental regression. The findings from this study serve as valuable information for families, equipping them with a better understanding of the expected development and disease course of their children. Ultimately, the insights gained suggest that *POLR3A* patients are good candidates for potential clinical trials for therapies that may emerge.

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