# POLR3-RELATED LEUKODYSTROPHY: FROM EXPLORING NOVEL GENETIC CAUSES AND INVESTIGATING CLINICAL FEATURES TO EXPANDING THE SPECTRUM OF DISEASE

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# ABSTRACT

Leukodystrophies encompass a spectrum of inherited neurological disorders associated with central nervous system white matter abnormalities, impacting either the development or maintenance of the myelin sheath. Within this disease group, hypomyelinating leukodystrophies are characterized by a substantial lack of myelin deposition during development and are typically diagnosed using brain magnetic resonance imaging (MRI) patterns, along with molecular genetic testing.

Advances in genetic sequencing technologies have facilitated the discovery of an abundance of novel genes associated with hypomyelinating leukodystrophies over recent years. Although this has led to the genetic diagnosis of many patients with rare hypomyelinating disorders, there remain a proportion of patients whose causal genes remain unidentified. Using next generation sequencing, we sought to investigate the genetic etiology of a cohort of patients presenting with hypomyelination on MRI, but without an identified genetic cause. Genetic variants for each patient were custom filtered and evaluated for pathogenicity using the American College of Medical Genetics guidelines. Genetic diagnoses were identified in 41% (7/17) of patients. In one patient, pathogenic variants in the gene *POLR3K* were identified, including a large deletion and a missense variant, leading to the third report worldwide of an individual harbouring pathogenic variants in this gene and a hypomyelinating phenotype.

One of the most common types of hypomyelinating leukodystrophies is RNA polymerase III-related hypomyelinating leukodystrophy (POLR3-HLD). As an autosomal recessive disorder, POLR3-HLD is caused by biallelic pathogenic variants in specific genes encoding for subunits of the transcription enzyme RNA polymerase III, including *POLR3A*, *POLR3B*, *POLR1C*, and *POLR3K*. POLR3-HLD is also known as 4H leukodystrophy due to the commonly associated combination of neurological and non-neurological features, including hypomyelination, hypodontia, and hypogonadotropic hypogonadism. As endocrine and growth abnormalities are often seen in this patient population, we sought to systematically investigate and characterize these features in a large cohort of patients with genetically-confirmed POLR3-HLD. We performed an international cross-sectional study on 150 patients to evaluate endocrine and growth measures, as well as neurological and non-neurological features. Pubertal abnormalities and short stature were found to be the most common endocrine features, and thyroid abnormalities were reported in a

portion of patients. Next, we aimed to expand the clinical and molecular spectrum of POLR3-HLD by investigating a cohort of patients with an extremely severe phenotype compared to the typical disease form. Clinical, MRI, and genetic features for all six patients were reviewed. Each had an early disease onset in the first few months of life, presenting with developmental delay, failure to thrive, and severe dysphagia. On MRI, an atypical pattern of progressive basal ganglia and thalamic abnormalities was identified. Genetically, each patient harboured similar pathogenic variants in *POLR3A*, including a variant leading to a premature stop codon on one allele, and *in trans*, a splicing variant which was investigated using *in vitro* studies to identify aberrant splicing transcripts. To further explore this novel phenotype, pathology samples from three deceased children of different ages were examined, revealing a progressive disease with involvement of the dorsal striatum, globus pallidus, and thalamus. Overall, the identification of genotype-phenotype correlations and study of disease progression provide insight into the complex pathophysiology underlying POLR3-HLD. As a whole, these studies expand understanding of the genetic basis, clinical presentation, and disease pathophysiology of hypomyelinating disorders, thus laying the knowledge foundation necessary for the development of future therapeutic approaches.

# RÉSUMÉ

Les leucodystrophies (LD) sont des maladies génétiques de la substance blanche cérébrale. Au sein de ce groupe de maladies, les LD hypomyélinisantes se caractérisent par un manque substantiel de dépôt de myéline au cours du développement et sont diagnostiquées à l'aide des résultats d'imagerie par résonance magnétique (IRM), ainsi que de tests génétiques moléculaires.

Les progrès des technologies de séquençage ont facilité la découverte de plusieurs nouveaux gènes associés aux LD hypomyélinisantes au cours des dernières années. Bien que cela ait conduit au diagnostic génétique de nombreux patients, il reste une proportion de patients dont les gènes responsables restent inconnus. En utilisant le séquençage de nouvelle génération, nous avons cherché à étudier l'étiologie génétique d'une cohorte de patients présentant une hypomyélinisation à l'IRM sans cause génétique identifiée. Les variants génétiques de chaque patient ont été filtrés et évalués pour leur pathogénicité sur la base des directives de l'*American College of Medical Genetics*. Des diagnostics génétiques ont été identifiés chez 41% (7/17) des patients. Chez un patient, des variants pathogènes du gène *POLR3K* ont été identifiés, incluant une grande délétion et un variant faux-sens, conduisant au troisième recensement mondial d'un individu porteur de variants pathogènes dans ce gène et démontrant un phénotype hypomyélinisant.

L'un des types les plus courants de LD hypomyélinisantes est la LD hypomyélinisante liée à l'ARN polymérase III (POLR3-HLD). En tant que trouble autosomique récessif, la POLR3-HLD est causée par des variants pathogènes biallélіques dans des gènes codant pour des sous-unités de l'enzyme de transcription ARN polymérase III, notamment *POLR3A*, *POLR3B*, *POLR1C* et *POLR3K*. La POLR3-HLD est également connue sous le nom de LD 4H en raison ses manifestations neurologiques et non neurologiques, notamment l'hypomyélinisation, l'hypodontie et l'hypogonadisme hypogonadotrope. Comme des anomalies endocriniennes et de croissance sont souvent observées dans cette population de patients, nous avons cherché à caractériser systématiquement ces manifestations. Nous avons réalisé une étude transversale internationale sur 150 patients avec un diagnostic prouvé génétiquement en évaluant les mesures endocriniennes, de croissance, les caractéristiques neurologiques et non neurologiques. Les anomalies pubertaires et la petite taille se sont révélées être les plus fréquentes et des anomalies thyroïdiennes ont été signalées chez certains patients. Puis, nous avons cherché à élargir le spectre clinique et

moléculaire de la POLR3-HLD en étudiant une cohorte de patients présentant un phénotype extrêmement sévère. Les caractéristiques cliniques, à l'IRM et génétiques des six patients ont été examinées. Chaque patient a eu une apparition de la maladie au cours des premiers mois de vie, présentant un retard de développement sévère, un retard de croissance et une dysphagie. À l'IRM, un schéma atypique d'anomalies progressives des ganglions de la base et du thalamus a été identifié. Génétiquement, chaque patient était porteur de variants pathogènes similaires dans *POLR3A*, incluant un variant causant un codon stop prématuré sur un allèle, et *en trans*, un variant d'épissage qui a été étudié *in vitro* pour identifier les transcrits anormalement épissés. Pour explorer davantage ce nouveau phénotype, la pathologie de la maladie a été examinée chez trois enfants d'âges différents, révélant une maladie progressive avec atteinte du striatum dorsal, du globus pallidus et du thalamus. Dans l'ensemble, les corrélations génotype-phénotype et l'étude de la progression de la maladie donnent un aperçu de la physiopathologie complexe sous-jacente à la POLR3-HLD. Ces études élargissent notre compréhension de la base génétique, présentation clinique et physiopathologie des LD hypomyélinisantes, jetant ainsi les bases de connaissances nécessaires au développement de futures approches thérapeutiques.

# ACKNOWLEDGEMENTS

Over the past six years, I have been extremely fortunate to have received a wealth of guidance, support, and inspiration from a great number of individuals. I truly appreciate everyone who has been a part of this journey and would like to express my sincere gratitude to all who have supported me from the beginning of my degree to the completion of this thesis.

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wonderfully memorable. To Alex, for giving me the first opportunity to be a “mentor”; watching you grow as a scientist has been so rewarding and I can’t wait to see everything you will achieve. And above this, I am so thankful for your friendship inside and outside of the lab, for always being there to share in-depth talks over oreos and coffee. To Julia, Mack, and Xiaoru for helping me navigate new protocols and for being enthusiastic to bounce back ideas and discuss hypotheses, and to Chia-Lun and Neeti, for your hard work in carrying on this research. To the past lab members who taught me many techniques, Kether, Valerio, and Lama, thank you for your patience and for extending your vast amounts of knowledge. To Simon, for generously translating my thesis abstract and for always being willing to help out in any way possible. To Laurence and Félixe, I am so lucky to have been able to work with you on these projects, thank you for your diligence and expertise. A big thank you to all of the past and present members on our clinical research team, it was a pleasure to work with all of you and I am grateful for your support. Finally, to my past lab mates and close friends, Travis, Alexa, and Aaron, thanks for making my grad student experience unforgettable, I know we will remain friends for many years to come.

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I am thankful to have had the opportunity to be involved in several rewarding extracurricular groups outside of research, including the Graduate Students’ Association for Neuroscience, the McGill Summer Academy for Neuroscience, the McGill Rare Disease Interest Group (RareDIG), and the McGill Journal of Medicine - thank you to all of the incredible individuals who I’ve had the opportunity to work with.

And finally, to my friends and family near and far, I couldn't have reached the end of this degree without your constant support. To my parents, Diana and David, for being the most encouraging, loving, and supportive parents I could have ever asked for. You have shaped me into the individual I am today, and I am so thankful for your never-ending encouragement to follow my dreams. Thank you for being there through the ups and downs, and for inspiring me to put forth my best effort in everything I do. To my brothers, Michael and Christopher, my grandparents, Marlene, Zdenka, and Stefan, as well as my aunts, uncles, and cousins, you are always there for me when I need it, and I am thankful for all of your love and support. To my lifelong friends, Katie, Taylor, and Marissa, for cheering me on from the sidelines and always being in my corner. And to Dylan, we started this journey in Montréal, and even from afar you have been there every step of this degree, from listening to my endless presentations to making sure I never miss an Oxford comma, and being an "extended brain" when I need to think out a new idea or plan. Your friendship is invaluable, thank you for always being there and for making me feel like my potential has no limits. To all my family and friends, I am eternally grateful for all of your love and support, you mean so much to me.

I dedicate this thesis to all of the resilient patients and families who have been impacted by POLR3-related leukodystrophy, whose courageousness inspires researchers to keep searching for answers.

# TABLE OF CONTENTS

# PREFACE AND CONTRIBUTIONS

## I. Thesis Preface

This doctoral thesis has been written in the manuscript-based formatting style according to the guidelines presented by the McGill University Department of Graduate and Postdoctoral Studies. The described work was completed in the MyeliNeuroGene Laboratory at the Research Institute of the McGill University Health Centre under the supervision of Dr. Geneviève Bernard. This thesis centers around the study of hypomyelinating leukodystrophies, from the investigation of causal genes to the classification of clinical features associated with one of the most common hypomyelinating disorders, POLR3-related leukodystrophy, followed by the presentation of a severe form of the disease, which was studied on a genetic, clinical, radiological, and neuropathological level.

This thesis is composed of the following six chapters:

**Chapter 1** provides an introduction to the work, including the objectives and rationale of this thesis, as well as a review of the background literature underlying the main concepts. This chapter includes original text, as well as excerpts from a narrative review on POLR3-related leukodystrophy (published in *Frontiers of Cellular Neuroscience*), and a textbook chapter written on hypomyelinating disorders (submitted for publication in the *Handbook of Clinical Neurology: White Matter Disorders*).

**Chapter 2** focuses on the identification of causal genes underlying hypomyelinating disorders, and is presented in three sections, including two manuscripts in preparation for submission, and one letter to the editor published in the journal *Child Neurology Open*.

**Chapter 3** describes a large cohort study of the endocrine and growth abnormalities associated with POLR3-related leukodystrophy and has been published in the *Journal of Clinical Endocrinology and Metabolism.*

**Chapter 4** presents an expanded phenotypic spectrum of POLR3-related leukodystrophy, describing a novel severe phenotype, and includes two manuscripts. The first is an original research article published in the journal *Neurology Genetics*, and the second is a correspondence article published in the *American Journal of Medical Genetics Part A.*

**Chapter 5** involves further investigation into the neuropathology and pathophysiological processes underlying the severe POLR3-related leukodystrophy phenotype and is presented as a manuscript in preparation to be submitted following the completion of minor remaining experiments.

**Chapter 6** relates each chapter through a united discussion and explores future directions, with an except from a narrative review article on potential therapies for POLR3-related leukodystrophy, published in the journal *Frontiers of Cellular Neuroscience*.

Finally, **Chapter 7** concludes the thesis through a general summary of this work.

## II. Manuscripts Contained in this Thesis

Contained in this thesis are the following first or co-first author manuscripts written by the candidate, which have either been published in academic journals or textbooks, or will be submitted for publication as indicated. Signed agreements have been obtained from co-first authors for inclusion of respective manuscripts in this thesis. In the citations below, co-first authorship is indicated by an asterisk (*), and co-senior authorship by an arrowhead (^).

**Chapter 1:**

Excerpts from the following two works have been included in the Literature Review as indicated.

**Perrier, S.**, Gauquelin, L., & Bernard G. **Myelin Disorders - Hypomyelination (Chapter 13).** *Handbook of Clinical Neurology (White Matter Disorders)*. Editors: Lynch DS & Houlden H. Elsevier; In press, 2022. [Book Chapter]. *Permission for use granted by Elsevier.*

**Perrier, S.***, Michell-Robinson, M. A.*, & Bernard, G. (2021). **POLR3-Related Leukodystrophy: Exploring Potential Therapeutic Approaches.** *Frontiers in Cellular Neuroscience*. 14, 631802. doi:10.3389/fncel.2020.631802. PMID: 33633543. [Narrative Review Article]. *CC-BY 4.0 Permission.*

**Chapter 2:**

**Part A:**

**Perrier, S.**, Guerrero, K., Tran, L.T., Michell-Robinson, M.A., Legault, G., Brais, B., Sylvain, M., Dorman, J., Demos, M., Köhler, W., Pastinen, T., Thiffault, I.^, & Bernard, G.^ **Challenges in the genetic diagnosis of rare white matter diseases: Lessons learned from a series of cases solved by next generation sequencing.** [*Original research article to be submitted for publication*].

**Part B:**

**Perrier, S.,** Maegawa, G.H.B., Misiaszek, A.D., Tran, L.T., Müller, C.W., Pastinen, T., Thiffault, I.^, & Bernard, G.^. **Novel pathogenic variants in *POLR3K* cause POLR3-related leukodystrophy.** [*Original research article to be submitted for publication*].

**Part C:**

**Perrier, S.**, Matovic, S., & Bernard, G. (2020). **Classifying Hypomyelination: A Critical (White) Matter.** *Child Neurology Open*. 7, 2329048X20983761. doi: 10.1177/2329048X20983761. PMID: 33490304. [Letter to the Editor]. *CC BY-NC 4.0 Permission.*

**Chapter 3:**

Pelletier, F.*, **Perrier, S.***, Cayami, F.K.*, Mirchi, A., Saikali, S., Tran, L.T., Ulrick, N., Guerrero, K., Rampakakis, E., van Spaendonk, R.M.L., Naidu, S., Pohl, D., Gibson, W.T., Demos, M., Goizet, C., Tejera-Martin, I., Potic, A., Fogel, B.L., Brais, B., Sylvain, M., Sébire, G., Lourenço, C.M., Bonkowsky, J.L., Catsman-Berrevoets, C., Pinto, P.S., Tirupathi, S., Strømme, P., de Grauw, T., Gieruszczak-Bialek, D., Krägeloh-Mann, I., Mierzewska, H., Philippi, H., Rankin, J., Atik, T., Banwell, B., Benko, W.S., Blaschek, A., Bley, A., Boltshauser, E., Bratkovic, D., Brozova, K., Cimas, I., Clough, C., Corenblum, B., Dinopoulos, A., Dolan, G., Faletra, F., Fernandez, R., Fletcher, J., Garcia Garcia, M.E., Gasparini, P., Gburek-Augustat, J., Gonzalez Moron, D., Hamati, A., Harting, I., Hertzberg, C., Hill, A., Hobson, G.M., Innes, A.M., Kauffman, M., Kirwin, S.M., Kluger, G., Kolditz, P., Kotzaeridou, U., La Piana, R., Liston, E., McClintock, W., McEntagart, M., McKenzie, F., Melançon, S., Misbahuddin, A., Suri, M., Monton, F.I., Moutton, S., Murphy, R.P.J., Nickel, M., Onay, H., Orcesi, S., Özkınay, F., Patzer, S., Pedro, H., Pekic, S., Pineda Marfa, M., Pizzino, A., Plecko, B., Poll-The, B.T., Popovic, V., Rating, D., Rioux, M.F., Rodriguez Espinosa, N., Ronan, A., Ostergaard, J.R., Rossignol, E., Sanchez-Carpintero, R., Schossig, A., Senbil, N., Sønderberg Roos, L.K., Stevens, C.A., Synofzik, M., Sztriha, L., Tibussek, D., Timmann, D., Tonduti, D., van de Warrenburg, B.P., Vázquez-López, M., Venkateswaran, S., Wasling, P., Wassmer, E., Webster, R.I., Wiegand, G., Yoon, G., Rotteveel, J., Schiffmann, R., van der Knaap, M.S., Vanderver, A., Martos-Moreno, G.Á., Polychronakos, C.^, Wolf, N.I.^, & Bernard, G.^ (2021). **Endocrine and Growth Abnormalities in 4H Leukodystrophy Caused by Variants in *POLR3A, POLR3B*, and *POLR1C*.** *The Journal of Clinical Endocrinology and Metabolism*. 106(2), e660–e674. doi: 10.1210/clinem/dgaa700. PMID: 33005949. [Original Research Article]. *CC-BY 4.0 Permission.*

**Chapter 4:**

**Part A:**

**Perrier, S.***, Gauquelin, L.*, Fallet-Bianco, C., Dishop, M.K., Michell-Robinson, M.A., Tran, L.T., Guerrero, K., Darbelli, L., Srour, M., Petrecca, K., Renaud, D.L., Saito, M., Cohen, S., Leiz, S., Alhaddad, B., Haack, T.B., Tejera-Martin, I., Monton, F.I., Rodriguez-Espinosa, N., Pohl, D., Nageswaran, S., Grefe, A., Glamuzina, E., & Bernard, G. (2020). **Expanding the phenotypic and molecular spectrum of RNA polymerase III-related leukodystrophy.** *Neurology Genetics*. 6(3), e425. doi:10.1212/NXG.0000000000000425. PMID: 32582862. [Original Research Article]. *CC BY-NC-ND Permission.*

**Part B:**

**Perrier, S.**, Gauquelin, L., Wambach, J.A., & Bernard, G. (2022). **Distinguishing severe phenotypes associated with pathogenic variants in *POLR3A***. *American Journal of Medical Genetics Part A*. 188(2), 708-712. doi: 10.1002/ajmg.a.62553. PMID: 34773388. [Correspondence]. *Permission for use granted by John Wiley and Sons.*

**Chapter 5:**

**Perrier, S.**, Peña, L.D.M., Saenz Ayala, S., Berklite, L., Leino, D., Bernieh, A., Gauquelin, L., Cohen, S., Deisch, J., Zuppan, C.W., Chen, Z., Fallet-Bianco, C., & Bernard, G. **Delineating the progressive neuropathology of the severe striatal form of POLR3-related leukodystrophy.** **[***Original research article to be submitted for publication*].

**Chapter 6:**

Excerpts from the following narrative review have been included in the discussion as indicated.

**Perrier, S.***, Michell-Robinson, M. A.*, & Bernard, G. (2021). **POLR3-Related Leukodystrophy: Exploring Potential Therapeutic Approaches.** *Frontiers in Cellular Neuroscience*. 14, 631802. doi:10.3389/fncel.2020.631802. PMID: 33633543. [Narrative Review Article]. *CC-BY 4.0 Permission.*

## III. Other Publications by the Candidate

The following are publications to which the candidate contributed as an author, but are not included as chapters in this thesis.

**Perrier, S.*,** Gauquelin, L.*, Tétreault, M., Tran, L.T., Webb, N., Srour, M., Mitchell, J.J., Brunel-Guitton, C., Majewski, J., Long, V., Keller, S., Gambello, M.J., Simons, C., Care4Rare Canada Consortium, Vanderver, A., & Bernard, G. (2018) **Recessive mutations in *NDUFA2* cause mitochondrial leukoencephalopathy**. *Clinical Genetics*. 93(2), 396-400. doi:10.1111/cge.13126. PMID: 28857146. [Case Report].

Michell-Robinson, M.A., **Perrier, S.**, Lucia, C., Tran, L.T., Thiffault, I., Köhler, W., & Bernard, G. (2022). **Oculodentodigital Dysplasia: A Cause of Hypomyelinating Leukodystrophy in Adults.** *Neurology*. 98(16), 675–677. doi:10.1212/WNL.0000000000200228. PMID: 35190466. [NeuroImage].

## IV. Author Contributions

**The research contained in this thesis was carried out primarily by Stefanie Perrier, under the supervision of Dr. Geneviève Bernard.** For each chapter described within, **Stefanie Perrier** had a leading role in performing experimental design, data collection, results interpretation, figure and table generation, and manuscript writing. **Dr. Geneviève Bernard** supervised the work, participated in conception of the studies and experimental design, as well as manuscript editing and review.

Additional author contributions for each chapter are described below:

**In Chapter 1,** Laurence Gauquelin contributed to writing and editing the book chapter, and Mackenzie A. Michell-Robinson contributed to writing and editing the narrative review.

**In Chapter 2, Part A:** Kether Guerrero coordinated receival of genetic datasets and contributed to interpretation of results. Mackenzie A. Michell-Robinson contributed to review of clinical data and interpretation of results. Geneviève Legault, Bernard Brais, Michel Sylvain, James Dorman, Michelle Demos, and Wolfgang Köhler referred patients to the study, provided clinical notes and medical opinion, and assisted in results interpretation. **In Parts A and B**, Luan T. Tran recruited patients to the study and coordinated receival of patient medical records. Isabelle Thiffault performed next generation sequencing, data generation, and contributed to interpretation of results. Tomi Pastinen also contributed to performing next generation sequencing and generation of results. **In Part B**, Gustavo H. B. Maegawa referred patients to the study, provided clinical notes and medical opinion, and assisted in results interpretation. Agata D. Misiaszek and Christoph W. Müller provided expertise, contributed to variant analysis through protein modelling, and generated the corresponding figure. **In Part C,** Sara Matovic contributed to drafting and editing the manuscript.

**In Chapter 3,** Félixe Pelletier and Ferdy K. Cayami performed data collection and results interpretation, contributed to figure and table generation, and assisted with manuscript preparation. Amytice Mirchi performed chart review and contributed to generating the raw dataset. Emmanouil Rampakakis assisted with statistical analysis. Stephan Saikali analyzed pathology specimens, performed immunohistochemistry, and prepared the pathology figure images. Luan T. Tran and Kether Guerrero coordinated receival of medical charts and genetic information from participants. Gabriel Á. Martos-Moreno, Constantin Polychronakos, and Nicole I. Wolf provided additional expertise in results interpretation and presentation as well as clinical significance. The remaining 106 authors referred patients to this study and provided clinical notes and medical opinion on their respective patients.

**In Chapter 4, Part A,** Laurence Gauquelin reviewed the medical records and contributed to the MRI interpretation, table and figure generation and writing of the manuscript. Mackenzie A. Michell-Robinson and Lama Darbelli performed the immunoblots and results interpretation for the respective figure. Kether Guerrero assisted in molecular and genetic results interpretation. Luan T. Tran recruited patients to the study and coordinated receival of patient medical records and imaging. Catherine Fallet-Bianco performed pathology specimen analysis, including immunohistochemistry, analysis and interpretation of results, with preparation of pathology images in the respective figure. Megan K. Dishop performed pathology specimen collection and analysis, and results interpretation. Myriam Srour and Kevin Petrecca contributed control brain tissue used in the study. The remaining 13 co-authors referred patients to this study and provided clinical notes and medical opinion on their respective patients. **In Part B,** Laurence Gauquelin and Jennifer A. Wambach contributed to result interpretation and reviewing the manuscript.

**In Chapter 5,** Catherine Fallet-Bianco performed pathology specimen analysis, including immunohistochemistry, analysis and interpretation of results, and generated the pathology images within the respective figures. Zesheng Chen assisted in pathology specimen analysis and interpretation of results. Loren D.M. Peña, Sofia Saenz Ayala, and Seth Cohen referred patients to this study and provided clinical notes and medical opinion on their respective patients. Laurence Gauquelin contributed to review of clinical data and interpretation of results. Lara Berklite, Daniel Leino, Anas Bernieh, Jeremy Deisch, and Craig W. Zuppan performed pathology specimen collection during autopsy and contributed to analysis and interpretation of results.

## V. Contribution to Original Knowledge

This thesis describes several original studies which contribute to the understanding of hypomyelinating leukodystrophies, including POLR3-related leukodystrophy, on various levels, involving molecular, genetic, clinical, and pathological analyses. Specifically, this work contributes to original academic knowledge as follows:

**1)** Chapter 2 describes the identification of pathogenic variants in genes associated with hypomyelinating disorders in patients with previously genetically undiagnosed disease, along with the experience and lessons learned on the path to confirming genetic diagnoses.

**2)** Chapter 2 also describes the third patient worldwide with novel pathogenic variants in *POLR3K,* including a novel missense variant and a large deletion, associated with a hypomyelinating phenotype and cardinal POLR3-related leukodystrophy features.

**3)** Chapter 3 describes the first large-scale cohort study investigating the endocrinological and growth abnormalities in patients with POLR3-related leukodystrophy. This study also investigates the pituitary gland pathology of one patient, which contributes to understanding of the disease pathophysiology.

**4)** Chapter 4 expands the phenotypic spectrum of POLR3-related leukodystrophy through the description of a cohort of patients with a more severe phenotype than that previously reported, along with molecular studies and the first pathological investigation of a patient with this phenotype. This expands knowledge of disease presentations and provides insight into POLR3-related disease mechanisms.

**5)** Chapter 5 describes the pathology of two additional patients with severe POLR3-related leukodystrophy, demonstrating novel insight into the progressive neuronal course of disease.

# CONTENT LISTS

## I. List of Abbreviations

| | |
|---|---|
| 4H | Hypomyelination, hypodontia and hypogonadotropic hypogonadism |
| aa | Amino acids |
| AAV | Adeno-associated virus |
| ACTH | Adrenocorticotropic hormone |
| AD | Autosomal dominant |
| AR | Autosomal recessive |
| bp | Base pairs |
| cDNA | Complementary deoxyribonucleic acid |
| CHX | Cycloheximide |
| CI | Confidence interval |
| CNP | Cyclic nucleotide phosphodiesterase |
| CNS | Central nervous system |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CSF | Cerebrospinal fluid |
| DMEM | Dulbecco's modified Eagle's medium |
| DNA | Deoxyribonucleic acid |
| DTI | Diffusion tensor imaging |
| ER | Endoplasmic reticulum |
| ES | Exome sequencing |
| FBS | Fetal bovine serum |
| FSH | Follicle-stimulating hormone |
| GC | Guanine-Cytosine |
| gDNA | Genomic deoxyribonucleic acid |
| GFAP | Glial fibrillary acidic protein |
| GH | Growth hormone |
| GI | Gastrointestinal |
| GnRH | Gonadotropin-releasing hormone |
| GPC | Glial progenitor cell |
| GS | Genome sequencing |

| | |
|---|---|
| H-ABC | Hypomyelination with atrophy of the basal ganglia and cerebellum |
| H&E | Hematoxylin and eosin |
| HEMS | Hypomyelination of early myelinating structures |
| HGH | Human growth hormone |
| HLD | Hypomyelinating leukodystrophy |
| IBA1 | Ionized calcium-binding adapter molecule 1 |
| IGF-1 | Insulin-like growth factor 1 |
| iPSC | Induced pluripotent stem cells |
| KABC-II | Kauffman Assessment Battery for Children |
| KB | Klüver-Barrera stain |
| LD | Leukodystrophy |
| LH | Luteinizing hormone |
| LHRH | Luteinizing hormone releasing hormone |
| MAF | Minor allele frequency |
| MAG | Myelin associated glycoprotein |
| MBP | Myelin basic protein |
| MOG | Myelin oligodendrocyte glycoprotein |
| MRI | Magnetic resonance imaging |
| mRNA | Messenger ribonucleic acid |
| MRS | Magnetic resonance spectroscopy |
| MSC | Mesenchymal stem cells |
| NAA | N-acetyl aspartate |
| nc-RNA | Non-coding ribonucleic acid |
| NGS | Next generation sequencing |
| NMD | Nonsense mediated decay |
| NSC | Neural stem cells |
| ODDD | Oculodentodigital dysplasia |
| OPC | Oligodendrocyte progenitor cell |
| PCR | Polymerase chain reaction |
| PLIC | Posterior limb of the internal capsule |
| PLP | Proteolipid protein |
| PMD | Pelizaeus-Merzbacher disease |
| PMLD | Pelizaeus-Merzbacher-like disease |

| | |
|---|---|
| PNS | Peripheral nervous system |
| PSI | Processing speed index |
| POLR3 | RNA polymerase III |
| POLR3-HLD | RNA polymerase III-related hypomyelinating leukodystrophy |
| PVDF | Polyvinylidene difluoride |
| RIPA | Radioimmunoprecipitation assay |
| RNA | Ribonucleic acid |
| RPC10 | RNA polymerase III subunit C10 (POLR3K) |
| RT-PCR | Reverse transcription PCR |
| SD | Standard deviation |
| sgRNA | Single guide ribonucleic acid |
| SDS-PAGE | Sulfate-polyacrylamide gel electrophoresis |
| SPG2 | Spastic paraplegia type 2 |
| T4 | Thyroxine |
| tRNA | Transfer ribonucleic acid |
| TSH | Thyroid stimulating hormone |
| WISC-IV | Wechsler Intelligence Scale for Children – Fourth Edition |
| WMI | Working memory index |
| WRS | Wiedemann-Rautenstrauch syndrome |
| WT | Wildtype |

## II. List of Figures

## III. List of Tables

# CHAPTER 1.

## Introduction and Literature Review

The following thesis introduction and literature review includes excerpts from the following publications by the author cited below, including a narrative review published in *Frontiers of Cellular Neuroscience* on POLR3-related leukodystrophy and potential therapeutic approaches, as well as a book chapter submitted for publication in the *Handbook of Clinical Neurology (White Matter Disorders)* focusing on hypomyelinating leukodystrophies. Excerpts from each publication are marked by a footnote, with formatting adapted for this thesis. Additional sections of text were written within the literature review to expand on relevant topics for this thesis.

**POLR3-Related Leukodystrophy: Exploring Potential Therapeutic Approaches**

Narrative Review in *Frontiers in Cellular Neuroscience (Issue: Myelin Repair: At the Crossing-Lines of Myelin Biology and Gene Therapy)* 14, 631802. doi: 10.3389/fncel.2020.631802. Reproduced under CC-BY 4.0 permission.

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**Inherited White Matter Disorders: Hypomyelination (Myelin Disorders)**

Chapter 13 in the *Handbook of Clinical Neurology (White Matter Disorders; Editors: David S. Lynch and Henry Houlden)*, Submitted for publication, 2022; Permission for use granted by Elsevier.

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# I. Thesis Introduction: Rationale and Objectives

The primary constituent of the white matter in the brain is the myelin sheath, an essential insulator which allows for rapid transmission of nerve impulses, and ultimately, proper physiological function of the central nervous system (CNS). Over the past several decades, mutations in specific genes with various functions have been identified as associated with inherited disorders involving myelin development and maintenance. These genetically determined white matter disorders are termed leukodystrophies, the etymology of which stems from Greek roots, with leuko, dys, and trophy meaning white, lack of, and growth, respectively. The coupling of the evolution of magnetic resonance imaging (MRI) pattern recognition with advancements in genetic sequencing technology has facilitated the discovery of numerous novel genetic causes of leukodystrophies. To this end, genetic causes for leukodystrophies span a wide range, from variants in genes encoding for structural proteins exclusively found in myelin to metabolic enzymes, and to transcription and translation-associated proteins, amongst many others. In most cases, leukodystrophies are clinically progressive, resulting in premature death due to neurodegeneration, as treatment is limited to supportive care.

Hypomyelinating leukodystrophies are a subtype of leukodystrophy characterized by the abnormal formation of myelin during development, as opposed to myelin deterioration. RNA polymerase III-related hypomyelinating leukodystrophy (POLR3-HLD) falls in this category as one of the most common hypomyelinating disorders. The genetic cause of POLR3-HLD was first resolved in a cluster of French-Canadian families, together with international patients, in which biallelic pathogenic variants in the *POLR3A* and *POLR3B* genes were identified (Bernard et al., 2011; Tetreault et al., 2011). Later, the genotypic spectrum expanded to include *POLR1C* (Thiffault et al., 2015), and more recently, *POLR3K* (Dorboz et al., 2018). In 2014, the first large

cohort study of patients with POLR3-HLD defined the clinical and MRI features of 105 patients with mutations in *POLR3A* or *POLR3B*, as well as the brain pathology of one patient (Wolf, Vanderver, et al., 2014). Since then, individuals with POLR3-HLD have been identified worldwide.

The overall objective of this thesis is to provide insight into hypomyelinating disorders, with a focus on POLR3-HLD, using various approaches. In this thesis, investigations span from identifying the initial genetic cause of disease, to the classification of specific clinical features, to in depth studies to investigate pathophysiology, including both molecular and tissue level studies.

The body of this thesis begins with a focus on the genetics of hypomyelinating leukodystrophies. In the clinical setting, after a specific pattern of white matter abnormalities is identified on MRI, the next step in a patient's diagnostic workup is to determine the genetic cause of disease and diagnose the subtype of leukodystrophy. Although next generation sequencing (NGS) technologies have advanced in recent years and numerous novel disease-causing genes have been identified, there still remain patients without a molecular diagnosis. This could partly be due to limitations in sequencing methods and analysis techniques. On a research basis, gene discovery often relies on harnessing knowledge of these limitations to form specialized approaches for the analysis of genetic variants. To this end, the first study in this thesis involves investigating the genetic basis of a cohort of patients with hypomyelination or delayed myelination on MRI, but without a genetic diagnosis. Using data re-analysis as well as re-sequencing methods, diagnoses were identified in a proportion of patients. Numerous lessons were learned in regard to the challenges faced when investigating genetic diagnoses, which are described in the first study. Notably, in one patient, novel variants were uncovered in *POLR3K*, a recently identified gene associated with POLR3-HLD, of which only two patients were reported in the past.

The next chapter of this thesis begins to focus on POLR3-HLD, with investigation of two common clinical findings: endocrinological and growth abnormalities. Through a cross-sectional study of a large cohort of patients, this study aimed to systematically characterize these features, while also exploring genotype-phenotype correlations. Moreover, the phenotypic characterization of specific features in a large cohort of individuals is especially important to provide clinicians with knowledge of expected disease progression. This study also emphasises the importance of involving pediatric endocrinologists in the multidisciplinary care team for patients with POLR3-HLD.

Previous research into the disease progression of POLR3-HLD has shown that most patients with a typical disease presentation have similar features, involving onset in childhood with progressive neurological involvement, and a brain MRI pattern involving diffuse hypomyelination with preservation of specific structures. In Chapter 4 of this thesis, a novel severe disease presentation was investigated, involving a very early disease onset in the first few months of life, and an atypical MRI pattern with striatal involvement and only mild myelin abnormalities which were further studied via neuropathological investigations. A strong genotype-phenotype association was noted, involving a specific splicing variant on one allele, and a variant causing a truncated protein product on the other. This study aimed to study the impact of the splicing variant on a molecular level to explore disease pathophysiology. Likewise, in the following chapter, this severe POLR3-HLD phenotype was further studied via additional neuropathological investigations of two patients who passed at different ages, allowing the disease progression to be explored longitudinally.

Furthermore, these studies highlight the complexities in studying genetic neurological diseases to form a complete picture of understanding from genetic and molecular implications to

clinical and MRI features, and finally in tissue pathology and mechanisms of disease pathophysiology. The global objective of this thesis is to advance research into hypomyelinating disorders, including POLR3-HLD, by providing insight on disease progression and pathophysiology to ultimately shed light on potential avenues for therapeutic interventions.

# II. Literature Review

## 1.1 Myelin Development in the CNS

### 1.1.2 The Myelin Sheath: Insulation of Neurons in the CNS

In the human CNS, rapid propagation of electrical signals between neurons is essential in order to facilitate the coordinated information transfer required for proper physiological function. This enhanced signal transmission is achieved via an insulating membranous sheath, termed the myelin sheath, which wraps around the individual axons of neurons. The myelin sheath is formed by layers of compacted membrane, which are extensions of the processes of specialized glial cells termed oligodendrocytes. These myelinated axons and their associated oligodendrocytes are the main constituent of the white matter of the brain.

### 1.1.3 Myelination in Development [1]

"Myelination is a dynamic process, involving many signalling cues, proteins, and enzymes, that begins *in utero*. The formation of myelin begins in the CNS with the development and migration of oligodendrocyte progenitor cells (OPCs), which extend their processes to contact neuronal axons and begin ensheathment (Michalski & Kothary, 2015)" (Figure 1.1; reproduced with permission, Chang et al, 2016). "Upon initial axon-glial contact, key myelin membrane components are synthesized and transported to begin extension of the processes around the axon (Emery, 2010; Mitew et al., 2014). As the processes wrap the axons and begin to compact into several thin layers, the OPCs develop into mature oligodendrocytes, thereby forming the myelin sheath (Baron & Hoekstra, 2010). Compacted myelin allows for rapid propagation of action potentials between neurons, while also providing structural protection to axons. Additionally, complex networks of microtubules in the myelin membrane support the high metabolic demand of

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

the axon by facilitating transport of proteins, metabolites, and other molecules (Lee et al., 2012; Roth et al., 2006). Typically, myelin deposition begins during the 4th month of gestation *in utero*, and myelination of most major tracts is essentially complete by age 2 years (Dietrich et al., 1988; van der Knaap & Valk, 2005). Myelination continues on a smaller scale into the first and second decades of life, with an increase of approximately 12% in total white matter volume to age 22 (Giedd et al., 1999). Additional changes in white matter volume into adulthood are both regionally and temporally associated with cognitive development and synaptic plasticity, and are also likely associated with axonal factors including pruning, branching, and packing (Sampaio-Baptista & Johansen-Berg, 2017)."

### 1.1.4 Myelin Composition, Structure, and Function

The myelin sheath is composed of concentric layers of plasma membrane containing both lipids and specialized proteins. On electron microscopy, the layers are classified as dense layers, in which the intracellular cytoplasmic surfaces are in contact (i.e., myelin dense line), and light layers, in which the extracellular surfaces are in contact (i.e., intraperiod line) (Gray, 1959; Robertson, 1955) [Figure 1.2, reproduced with permission (Potter et al., 2011)]. Near the end of each myelin segment, there are also small paranodal junctions or loops formed by expanded cytoplasmic pockets (Kosaras & Kirschner, 1990; Peters, 1960) (Figure 1.2C). Lipids comprise approximately 70-80% of the total mass of myelin (by dry weight), with proteins comprising the remaining 20-30% (O'Brien & Sampson, 1965). The main types of lipids found in myelin include cholesterol, phospholipids (e.g., ethanolamine-containing plasmalogens, lecithin, sphingomyelin), and glycosphingolipids (e.g., galactosylceramide and sulfatide) (Chavko et al., 1993; O'Brien, 1965). The ratios of these lipids in myelin vary compared to other biological membranes, with

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[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

cholesterol, phospholipids, and glycolipids comprising 40%, 40%, and 20% of lipids, respectively (O'Brien, 1965). Within the myelin sheath, lipids function to enable proteins and other molecules to pack closely in an organized manner. This allows myelin the ability to retain a high degree of stability over a long period of time, which is necessary for the long-term function and health of the CNS as oligodendrocyte populations have little turnover (Yeung et al., 2014).

CNS myelin contains a number of unique proteins responsible for different structural, adhesion, signalling, and metabolic functions. The three most abundant proteins are proteolipid protein (PLP), myelin basic protein (MBP), and cyclic nucleotide phosphodiesterase (CNP) constituting approximately 38%, 30%, and 5%, of total proteins, respectively (Jahn et al., 2020). Both PLP and MBP are structural proteins involved in the compaction of myelin and distributed in the mature compact myelin sheath. PLP is a transmembrane protein which interacts extracellularly to facilitate membrane adhesion, while MBP functions intracellularly in the cytoplasmic membrane layers (Duncan et al., 1987; Popko et al., 1987) (Figure 1.2D). CNP has enzymatic activity in hydrolyzing substrates, however its biological role is less understood, and it is also thought to perform a structural role in the noncompact paranodal loops of the myelin sheath (Edgar et al., 2009; Snaidero et al., 2017). Other notable but less abundant proteins include myelin oligodendrocyte glycoprotein (MOG) and myelin associated glycoprotein (MAG). MOG is localized to the outermost layer of compact myelin expressed in late stages of myelinogenesis, and is thought to act as a differentiation marker for mature oligodendrocytes or in maintenance of the structural integrity of myelin (Johns & Bernard, 1999; Scolding et al., 1989; von Büdingen et al., 2015). Contrarily, MAG is localized to periaxonal regions in the myelin membrane adjacent to the axon and thought to have roles in cell to cell signalling and adhesion between the neuron and oligodendrocyte (Dashiell et al., 2002; Myllykoski et al., 2018; Sternberger et al., 1979).

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

Furthermore, the entire myelin proteome is complex, including over three hundred low-abundance proteins involved in the development and maintenance of the myelin sheath (Jahn et al., 2020).

The myelin sheath functions to increase the speed of action potential transmission by acting as an electrical insulator to the axons of neurons. To overcome the process of slow sweeping depolarization and repolarization down the axonal membrane, myelin provides an alternative route for signal transmission by confining the movement of action potentials to periodically interspaced segments of unmyelinated axons termed Nodes of Ranvier (Mitew et al., 2014). Nodes of Ranvier are enriched with clusters of voltage-gated sodium channels, which promote the transmission of action potentials in a process called saltatory conduction, in which excitation of the axonal membrane jumps from node to node during signal propagation with action potentials becoming renewed at each node (Huxley & Stämpeli, 1949; Waxman, 1980). Moreover, myelin facilitates the rapid conduction velocity of impulses by providing an express route for transmission, and ultimately decreasing transverse capacitance while increasing transverse resistance across the axon (Hartline & Colman, 2007; Rasminsky & Sears, 1972). Through modulation of signal transmission speed, myelin is an essential factor for the specific synchrony of communication within neural circuits, which is required for precise motor skills, cognitive function, and proper sensory information integration (Pajevic et al., 2014).

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.

[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

## 1.2 Leukodystrophies: Inherited White Matter Disorders

### 1.2.1 Leukodystrophies Overview [1]

"Leukodystrophies are a class of heterogeneous inherited neurological diseases characterized by the predominant impairment of the central nervous system (CNS) white matter, with specific involvement of glial cells (van der Knaap & Bugiani, 2017; Vanderver et al., 2015). Affected patients typically present in childhood or adolescent years with psychomotor regression and/or neuropsychiatric manifestations. Magnetic resonance imaging (MRI) patterns, followed by genetic investigations, are used to confirm diagnoses (Parikh et al., 2015). Most leukodystrophies run a progressive disease course, with slow to rapid deterioration after onset, ultimately leading to early death. Collectively, leukodystrophies affect approximately one in 7500 individuals, however, there are many different subtypes with varying individual incidence rates (Adang et al., 2017; Bonkowsky et al., 2010; Parikh et al., 2015). Next-generation sequencing has proven to be a valuable first-line diagnostic tool for determining the genetic basis of disease, and has facilitated the discovery of a variety of causal genes encoding proteins with diverse biological functions (Boycott et al., 2014; Srivastava et al., 2014; Vanderver et al., 2016). Although some leukodystrophies have successful restorative treatments if started early following diagnosis [i.e., pre- or early symptomatic stages (Krivit, 2004; Krivit et al., 1999; van den Broek et al., 2018)], most treatments address specific clinical features, providing supportive care (Adang et al., 2017)."

### 1.2.2 Hypomyelinating Leukodystrophies [1]

"Hypomyelinating leukodystrophies (HLDs) are a defined subcategory of leukodystrophies, characterized by defects in initial myelin production and formation during development (Costello et al., 2009; Pouwels et al., 2014). HLDs are diagnosed using MRI patterns,

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

notably involving hyperintensity of the white matter compared to gray matter on T2 weighted imaging, and variable signal (i.e., hyperintensity, hypointensity, or isointensity) of white matter on T1 weighted imaging compared to gray matter structures (Barkovich & Deon, 2016; Schiffmann & van der Knaap, 2009; Steenweg et al., 2010). Hypomyelination can be diagnosed in a single MRI in children older than 2 years of age, but not in younger children. Indeed, in children below 2 years, the diagnosis of hypomyelination (vs. myelination delay) requires that myelination does not progress between two MRIs taken 6 months apart, with the second performed after 2 years of age (Pouwels et al., 2014; Schiffmann & van der Knaap, 2009; Steenweg et al., 2010). As myelination of most key brain areas is virtually complete by 2 years of age, a lack of progression in myelin development seen at this age will likely result in permanent hypomyelination (Steenweg et al., 2010)."

"Classically, HLDs were primarily known to be caused by pathogenic variants in genes encoding for proteins directly associated with the development, structure, or integrity of the myelin sheath. For example, the prototypical HLD Pelizaeus-Merzbacher disease results from pathogenic variants in *PLP1*, a gene encoding a structural myelin protein (Garbern, 2007b). However, a recently growing class of white matter disorders encompasses those caused by pathogenic variants in proteins that play key roles in transcription and translation. For example, pathogenic variants in several genes encoding for aminoacyl tRNA synthetases (e.g., *DARS1, RARS1, EPRS1*) are known to cause HLDs (Mendes et al., 2018; Ognjenovic & Simonovic, 2017; Park et al., 2008; Taft et al., 2013; Wolf, Salomons, et al., 2014). Within the category of white matter disorders caused by defects in transcription/translation-related genes is POLR3-related hypomyelinating leukodystrophy (POLR3-HLD), which is now considered one of the most common HLDs (Schmidt et al., 2020). POLR3-HLD is caused by biallelic pathogenic variants in genes encoding

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

subunits of the transcription complex RNA polymerase III (POLR3), namely *POLR3A, POLR3B, POLR1C,* and *POLR3K* (Bernard et al., 2011; Daoud et al., 2013; Dorboz et al., 2018; Tetreault et al., 2011; Thiffault et al., 2015)."

#### 1.2.2.1 Pelizaeus Merzbacher Disease and PLP1-related Disorders [2]

"Pelizaeus-Merzbacher disease (PMD; OMIM: 312080) is the prototypic early onset hypomyelinating leukodystrophy. It was first described in 1885 and 1910 by Pelizaeus and Merzbacher as an X-linked disorder affecting the cerebral white matter (Merzbacher, 1910; Pelizaeus, 1885). The molecular basis of the disease was elucidated when the *PLP1* gene was identified as causal approximately a century after the initial description (Hudson et al., 1989; Trofatter et al., 1989; Willard & Riordan, 1985)."

"Over the years, novel phenotypes associated with *PLP1* variants were delineated, with documented genotype-phenotype correlations (Cailloux et al., 2000; Grossi et al., 2011; Regis et al., 2008). It is now established that *PLP1*-related disorders encompass a broad spectrum of disease severity ranging from the neonatal, connatal form of PMD at the most severe end of the continuum, to spastic paraplegia type 2 (SPG2, complicated and uncomplicated; OMIM: 312920) at the milder end. One decade ago, a novel brain MRI pattern characterized by hypomyelination of early myelinating structures (HEMS) was described, with family history suggesting X-linked inheritance (Steenweg et al., 2012; Tonduti et al., 2013). Altered *PLP1* splicing and point mutations in PLP1-specific regions were identified as the molecular basis of the disorder (Kevelam et al., 2015)."

"*PLP1*-related disorders are caused by a range of different pathogenic variants in the gene *PLP1*, which encodes for proteolipid protein 1, a transmembrane lipid protein mainly localised to

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

the myelin sheath of the CNS (Gencic et al., 1989; Hudson et al., 1989). PLP1 is one of the most abundant proteins of the CNS myelin sheath, and along with its isoform DM20, constitutes approximately 50% of the total CNS myelin protein content (Diehl et al., 1986; Eng et al., 1968; Lees & Bizzozero, 1992). PLP1 is minimally expressed in Schwann cells of the peripheral nervous system (PNS), composing less than 1% of myelin proteins (Garbern et al., 1997; Kamholz et al., 1992). Located on the long arm of chromosome X at the position Xq22, the *PLP1* gene is 17 kb in length, containing 7 exonic regions which encode 276 amino acids (Diehl et al., 1986). The DM20 isoform originates from an alternative splicing event which excludes 35 amino acids from exon 3 (positions 117-151; often referred to as exon 3B), and in contrast to PLP1, DM20 is expressed prior to myelination, i.e., in early stages of embryonic development (Ikenaka et al., 1992; Nave et al., 1987; Schindler et al., 1990). PLP1 is the main isoform expressed during and following CNS myelination (LeVine et al., 1990). Upon myelin sheath development and oligodendrocyte maturation, PLP1 quantity increases to become one of the most abundant myelin protein constituents (Yang & Skoff, 1997). PLP1 is thought to function as a structural protein in mature myelin, acting to stabilize the compact myelin membrane (Baumann & Pham-Dinh, 2001; Boison et al., 1995; Griffiths, Klugmann, Thomson, et al., 1998; Yool et al., 2002). In addition, it is thought to be involved in maintenance of mature myelin (Klugmann et al., 1997). However, the roles of PLP1 and DM20 are not fully understood in glial progenitors and other neural progenitor cell types during development and pre-myelination stages. Studies suggest possible additional protein functions include extracellular signalling, progenitor cell migration regulation, cytoplasmic protein-lipid interactions, as well as neuroprotection and axonal support (Griffiths, Klugmann, Anderson, et al., 1998; Gudz et al., 2002; Harlow et al., 2014; Laukka et al., 2016; Werner et al., 2007; Yin et al., 2006)."

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.

[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

"Within the myelin membrane, PLP1 is incorporated as a transmembrane protein, with four alpha helix domains spanning the lipid bilayer and amino- and carboxyl- termini located in the cytoplasm along with a single intracellular loop (Popot et al., 1991; Weimbs & Stoffel, 1992). Two extracellular loops are located on the membrane surface, where the largest interact between layers of external membrane surfaces in compacted myelin (Gow et al., 1997). Prior to reaching the surface of the myelin membrane, PLP1 is synthesized in the endoplasmic reticulum (ER), processed through the Golgi apparatus to become associated with lipid rafts, and transported in vesicles to the cell surface for incorporation into the membrane (Krämer et al., 2001; Nussbaum & Roussel, 1983; Simons et al., 2002; Simons et al., 2000; Winterstein et al., 2008). It is thought that disruption of the intrinsic balance between PLP1 protein expression, proper folding, and/or trafficking to the membrane could have a critical impact on myelin sheath formation, either due to destabilization of the compacted membrane or by impacting the viability of the oligodendrocyte itself (Garbern, 2007a; Inoue, 2005; Woodward, 2008)."

#### 1.2.2.1.2 Pelizaeus-Merzbacher-Like Disease [2]

"Pelizaeus-Merzbacher-like disease (PMLD) was originally described as a hypomyelinating leukodystrophy with clinical and neuroradiologic features similar to classic X-linked PMD but not caused by variants in *PLP1* (Abrams, 2019; Hobson & Garbern, 2012; Schiffmann & Boespflug-Tanguy, 2001). In 2004, a subset of male and female patients with the PMLD phenotype were found to harbor variants in *GJC2* (previously *GJA12*), and the entity was described as PMLD or Leukodystrophy, Hypomyelinating 2 (HLD2, OMIM: 608804) (Bugiani et al., 2006; Uhlenberg et al., 2004)."

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.

[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

#### 1.2.2.1.3 Imaging Characteristics of PMD, HEMS, and PMLD [2]

"PMD is the prototypic hypomyelinating disorder, with classic neuroradiologic features of diffuse hypomyelination (Figure 1.3B). Brain MRI shows insufficient myelin deposition, characterized by mild T2/FLAIR hyperintensity and corresponding variable T1 signal intensity (hyperintense, isointense or mildly hypointense) of the white matter, compared to grey matter structures (Schiffmann & van der Knaap, 2009; Steenweg et al., 2010; van der Knaap & Bugiani, 2017). The T1 signal in PMD tends to be hypointense, with a strikingly homogeneous hypersignal on T2-weighted sequences (Steenweg et al., 2010). Cerebral atrophy develops over time (Sarret et al., 2016; van der Knaap & Bugiani, 2017). The degree of hypomyelination and white matter volume loss (especially in the corpus callosum) may correlate with disease severity and progression (Laukka et al., 2013; Plecko et al., 2003; Sarret et al., 2016; van der Knaap & Valk, 1989). Milder changes are seen in SPG2. Connatal PMD is associated with more pronounced T2 hyperintensity and supratentorial atrophy (Wang et al., 1995). Various nonspecific patterns on magnetic resonance spectroscopy (MRS) have been reported in patients with PMD, sometimes reflecting hypomyelination followed by demyelination (Nezu et al., 1998; Pizzini et al., 2003; Plecko et al., 2003; Spalice et al., 2000; Takanashi et al., 2002; Takanashi et al., 1997)."

"In most hypomyelinating disorders, including PMD, the anatomic structures that normally myelinate at term birth and early after contain relatively more myelin than those that myelinate later (Steenweg et al., 2010). Patients with HEMS exhibit a characteristic brain MRI pattern (Figure 1.3C), with hypomyelination of early myelinating structures, including the brainstem (caudal pons and medulla), hilus of the dentate nucleus and peri-dentate area, optic radiations, posterior limb of the internal capsule (PLIC), frontoparietal periventricular white matter, and tracts connected with the pericentral cortex (Kevelam et al., 2015; Steenweg et al., 2012; Tonduti et al.,

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

2013). The PLIC shows a distinctive pattern of alternating hyperintense-hypointense-hyperintense T2 signal. In addition, the anterolateral thalamus shows mild T2 hypointensity compared with the rest of the thalamus, which shows slight T2 hyperintensity (Steenweg et al. 2012, Tonduti et al. 2013, Kevelam et al. 2015). Nonspecific spectroscopy findings are reported in two patients (Tonduti et al., 2013)."

"Brain MRI in PMLD shows diffuse hypomyelination, with a pattern very similar to *PLP1*-related disorders (Figure 1.3D) (Hobson & Garbern, 2012). There is a distinctive aspect of the pons, with prominent T2 hyperintensity reported in several patients (Steenweg et al., 2010; Wolf, Cundall, et al., 2007). Otherwise, marked areas of T2 hypersignal are seen in the periventricular, deep, and subcortical white matter. With disease progression, white matter volume loss occurs with thinning of the corpus callosum. Cerebral and cerebellar atrophy are seen later in the disease course. SPG44 may be associated with milder changes on brain MRI (Abrams, 2019)."

#### 1.2.2.2 POLR3-Related Hypomyelinating Leukodystrophy [2]

"POLR3-related or 4H leukodystrophy (OMIM: 607694), an autosomal recessive disorder, is one of the most common hypomyelinating leukodystrophies (Soderholm et al., 2020). Its name was coined following identification of the first two causal genes [i.e., *POLR3A* and *POLR3B* (Bernard et al., 2011; Daoud et al., 2013; Saitsu et al., 2011; Tetreault et al., 2011)] as disease-causing for five entities: Leukodystrophy with Oligodontia (Atrouni et al., 2003), Ataxia Delayed Dentition and Hypomyelination (Wolf, Harting, et al., 2007), 4H syndrome (Timmons et al., 2006), Hypomyelination with Cerebellar Atrophy and Hypoplasia of the Corpus Callosum (Sasaki et al., 2009), and Tremor-Ataxia with Central Hypomyelination (Bernard et al., 2010; Osterman et al., 2012; Tetreault et al., 2012). Since the discovery of the first genes, two others have been identified:

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

*POLR1C* (Thiffault et al., 2015) and *POLR3K* (Dorboz et al., 2018). Additionally, the disease spectrum has expanded significantly, with hypomyelination not being obligate (La Piana et al., 2016), suggesting that this group of disorders should be referred to as POLR3-related disorders."

#### 1.2.2.2.1 Cardinal Clinical Features of POLR3-HLD [2]

"POLR3-related hypomyelinating leukodystrophy (POLR3-HLD) is also known as 4H leukodystrophy, with the four H's corresponding to the three cardinal features of the disease: hypomyelination, hypodontia and hypogonadotropic hypogonadism (Bernard & Vanderver, 2017; Gauquelin et al., 2019; Wolf, Vanderver, et al., 2014). Disease presentation typically occurs in early childhood with motor delay or regression. However, later disease onset is also possible, often with cognitive manifestations, typically learning or intellectual disability. The disease manifestations can be divided between neurologic and non-neurologic features." The main clinical features associated with POLR3-HLD are outlined in Table 1.1 and Figure 1.4.

"Neurologic features are centered around prominent cerebellar involvement. Indeed, patients have marked and progressive cerebellar features, including gait ataxia, dysarthria, and dysmetria. Smooth pursuits are abnormal, and gaze-evoked nystagmus can be seen. Ocular saccades are also abnormal, and vertical gaze may be limited (Wolf, Vanderver, et al., 2014). Most patients will also exhibit some degree of pyramidal signs, including spasticity, typically involving more lower than upper extremities. Of note, pyramidal signs are not as prominent as cerebellar signs, and are not as severe as in PMD or PMLD. Extrapyramidal signs are also seen in the majority of patients, most commonly dystonia (Al Yazidi et al., 2019; Osterman et al., 2012), but are typically not significant enough to require pharmacologic therapy. Dysphagia is common, especially with disease progression. Cognitive involvement is also common and includes learning

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

disability, cognitive plateauing, and cognitive regression. In most patients, cognitive impairment is not as prominent as motor manifestations. Seizures are occasionally seen. With disease progression, ambulation is mostly affected by ataxia. Wheelchair dependency occurs around adolescence, although about half of patients with the typical phenotype remain ambulatory in adulthood (Wolf, Vanderver, et al., 2014). Death usually occurs in the second or third decade (Wolf, Vanderver, et al., 2014)."

"The most common non-neurologic symptom is myopia, which typically progresses over several years and becomes severe. Various dental abnormalities are also seen, including hypodontia, oligodontia, delayed teeth eruption, and natal tooth/teeth (Bernard & Vanderver, 2017; Gauquelin et al., 2019; Wolf, Vanderver, et al., 2014; Wolff et al., 2010). The most common endocrinologic manifestation is hypogonadotropic hypogonadism, presenting as delayed, arrested, or absent puberty (Wolf, Vanderver, et al., 2014)", with additional features described in Chapter 3 below, including short stature (Pelletier et al., 2021).

"A few patients have been incidentally diagnosed with osteosclerosis (Wolf, Vanderver, et al., 2014). Patients with biallelic pathogenic variants in *POLR1C* can also exhibit craniofacial abnormalities reminiscent of Treacher Collins syndrome, which is not surprising considering that biallelic pathogenic variants in *POLR1C* also cause Treacher Collins syndrome, a congenital disorder of craniofacial development (Gauquelin et al., 2019)."

#### 1.2.2.2.2 POLR3-HLD Phenotypic Spectrum of Disease [2]

"Since the identification of the causal genes, the POLR3-HLD disease spectrum has widened considerably. On the most severe end is an early-onset form of disease with prominent striatal involvement" (Harting et al., 2020; Perrier et al., 2020; Wu et al., 2019), described in

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

Chapters 4 and 5 below. “At the mildest end of the spectrum are adult patients who have been identified incidentally when a brain MRI performed for an unrelated indication revealed hypomyelination and a compatible MRI pattern (DeGasperis et al., 2020; Perrier et al., 2020). Genotype-phenotype correlations are present at both ends of this spectrum”, also described below. “Other milder forms include those without hypomyelination (La Piana et al., 2016) or only mild white matter abnormalities, such as the spastic paraparesis and spastic ataxia phenotypes (Di Donato et al., 2021; Gauquelin et al., 2018; Minnerop et al., 2019; Minnerop et al., 2017; Rydning et al., 2019), the mild striatal form of disease (Azmanov et al., 2016; Harting et al., 2020; Hiraide, Kubota, et al., 2020), and patients with isolated hypogonadotropic hypogonadism (Richards et al., 2017). Additionally, patients with unique bone phenotypes have been reported, including those with endosteal sclerosis and cerebellar hypoplasia caused by *POLR3B* variants, as well as those with endosteal hyperostosis and oligodontia caused by *POLR3GL* variants (Ghoumid et al., 2017; Terhal et al., 2020). Specific biallelic pathogenic variants in *POLR3A, POLR3B,* and *POLR3GL* are also associated with Wiedemann-Rautenstrauch syndrome (OMIM: 264090), a neonatal progeroid disorder characterized by intrauterine growth retardation, a progeroid appearance, distinctive facial features (i.e., triangular face, low set ears, prominent forehead with visible scalp veins and sparse scalp hair), and lipodystrophy with localized fat accumulation (Beauregard-Lacroix et al., 2020; Jay et al., 2016; Lessel et al., 2018; Paolacci et al., 2018; Temel et al., 2020; Wambach et al., 2018; Wu et al., 2021). Finally, *de novo* variants in *POLR3B* have been associated with a disorder characterized by ataxia, spasticity, demyelinating neuropathy, with or without epilepsy (Djordjevic et al., 2021).”

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.

[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

#### 1.2.2.2.3 Imaging Characteristics of POLR3-HLD [2]

"In the typical form of POLR3-HLD, brain MRI reveals hypomyelination, with diffuse hyperintensity of the white matter on T2-weighted images, together with hyper- iso- or slightly hypointense signal of the white matter on T1-weighted images, compared to grey matter structures (Figure 1.3E) (Schiffmann & van der Knaap, 2009; Steenweg et al., 2010). The typical MRI pattern is characterized by T2 hypointensity of specific white matter structures where myelination is relatively preserved, including the dentate nucleus, optic radiations, anterolateral nucleus of the thalamus, globus pallidus, and in some patients, the corticospinal tracts at the level of the PLIC (Steenweg et al., 2010; Wolf, Vanderver, et al., 2014). On 3.0T MRI, punctiform T2 hypointensities can be seen in the white matter, representing areas of better myelin deposition, referred to as myelin islets. Moreover, there is relative hypointensity of the medical lemniscus in the pons, a sign referred to as the "closed eye sign" (Cayami et al., 2018)."

"Different MRI patterns are associated with other disease forms. In the severe striatal form, there is cerebral atrophy, and insufficient myelin deposition without frank hypomyelination (Figure 1.3G)", as described in Chapter 4 below. "In patients with the very mild form of the disease, MRI findings are similar to the typical pattern, but milder, including greater myelin deposition (DeGasperis et al., 2020; Di Donato et al., 2021; Gauquelin et al., 2018; La Piana et al., 2016; Minnerop et al., 2019; Minnerop et al., 2017; Rydning et al., 2019; Wolf, Vanderver, et al., 2014). Patients with the mild striatal form of disease may have completely normal myelination, but with signal abnormalities and atrophy in the striatum and thalamus (Figure 1.3F). Signal abnormalities are also reported in the red nucleus and along the intracranial course of the third cranial nerve (Azmanov et al., 2016; Harting et al., 2020). There appears to be a spectrum of disease severity between the severe and mild striatal forms, with some overlap. The MRI patterns

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

of other phenotypic forms of disease are variable, and include normal MRI findings, specific involvement of the corticospinal tracts, isolated cerebellar atrophy, and non-specific white matter abnormalities with or without cerebellar atrophy (La Piana et al., 2016)."

#### 1.2.2.2.4 Genetics of POLR3-HLD [2]

"POLR3-HLD is an autosomal recessive disorder caused by biallelic pathogenic variants in *POLR3A, POLR3B, POLR1C,* and *POLR3K* (Bernard et al., 2011; Daoud et al., 2013; Dorboz et al., 2018; Tetreault et al., 2011; Thiffault et al., 2015). Types of causal variants range from point mutations, such as missense, nonsense, synonymous, intronic, and splicing variants, as well as large exonic deletions, or small indels (Al Yazidi et al., 2019; Bernard et al., 2011; Daoud et al., 2013; Gauquelin et al., 2019; Gutierrez et al., 2015; Harting et al., 2020; Hiraide, Nakashima, et al., 2020; Jurkiewicz et al., 2017; La Piana et al., 2016; Perrier et al., 2020; Potic et al., 2012; Richards et al., 2017; Takanashi et al., 2014; Terao et al., 2012; Tetreault et al., 2011; Thiffault et al., 2015; Wolf, Vanderver, et al., 2014). Complete loss of POLR3 function is incompatible with life, which is exemplified by the embryonic lethality of Polr3a-null mice as well as the lack of reported patients with biallelic amorphic variants (Choquet et al., 2017). Moreover, pathogenic variants associated with POLR3-HLD are hypomorphic, resulting in a partial loss of function."

"The clinical course of patients with POLR3-HLD follows a relative pattern of severity depending on the causal gene. *POLR1C* variants usually result in the most severe course, followed by *POLR3A* variants, and *POLR3B* variants respectively (Gauquelin et al., 2019; Wolf, Vanderver, et al., 2014). As only two reported patients have *POLR3K* variants, additional investigations are needed before assessing comparative severity (Dorboz et al., 2018). Over 90% of reported patients with POLR3-HLD harbor pathogenic variants in *POLR3A* and *POLR3B*, with the remaining <10%

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harbouring variants in *POLR1C* or *POLR3K* (Bernard et al., 2011; Daoud et al., 2013; Tetreault et al., 2011; Wolf, Vanderver, et al., 2014)."

"While POLR3-HLD is the most common disease presentation, there are several other phenotypes associated with variants in POLR3 subunits, ranging from extremely severe to very mild. Very mild phenotypes, in which hypomyelination was discovered during unrelated investigations, have been associated with the common pathogenic *POLR3B* variant inherited in a homozygous state [c.1568T>A (p.Val523G)] (DeGasperis et al., 2020; Perrier et al., 2020). As well, some patients with *POLR3B* variants only manifest abnormal endocrine features (Richards et al., 2017). Spastic ataxia and spastic paraparesis have also been described in patients with specific *POLR3A* variants (Gauquelin et al., 2018; La Piana et al., 2016; Minnerop et al., 2017; Rydning et al., 2019)."

"Also on the severe end of the spectrum is neonatal progeria (Wiedemann-Rautenstrauch syndrome; WRS), most commonly caused by combinations of variants in *POLR3A*, in which most patients harbour a loss of function variant with a splicing variant (Jay et al., 2016; Lessel et al., 2018; Paolacci et al., 2018; Temel et al., 2020; Wambach et al., 2018). Variants in *POLR3B* and *POLR3GL* have only recently been reported in few cases of WRS (Beauregard-Lacroix et al., 2020; Wu et al., 2021). Variants in *POLR1C* are associated with both POLR3-HLD and autosomal recessive Treacher Collins syndrome, however some intermediate phenotypes exist (Gauquelin et al., 2019). *POLR1C* variants were initially thought to be disease specific, however some were found to be shared in both disease entities (Gauquelin et al., 2019). Finally, in contrast to the autosomal recessive inheritance pattern of POLR3-HLD, a unique cohort of patients was reported with *de novo* variants in *POLR3B*, where functional studies revealed that variants impair POLR3B interactions with other individual subunits (Djordjevic et al., 2021)."

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

#### 1.2.2.2.5 POLR3-HLD Pathophysiology [2]

"Biallelic pathogenic variants in genes encoding specific subunits of the transcription enzyme RNA polymerase III (POLR3) are known to cause POLR3-HLD, including *POLR3A, POLR3B*, *POLR1C*, and *POLR3K* (Bernard et al., 2011; Daoud et al., 2013; Dorboz et al., 2018; Tetreault et al., 2011; Thiffault et al., 2015). POLR3 is a 17 subunit complex which functions to transcribe a range of small non-coding RNAs, including transfer RNAs (tRNAs), 5S ribosomal RNA, 7SL RNA, 7SK RNA, vault RNAs, some microRNAs, and small nucleolar RNAs such as U6 snRNA (Dieci et al., 2013; Dieci et al., 2007; Lesniewska & Boguta, 2017; White, 2011; Wu et al., 2012). While these RNAs do not code for proteins, they are essential for cellular processes including protein synthesis, RNA processing and splicing, and other gene expression mechanisms (Dumay-Odelot et al., 2010). Within the POLR3 complex, POLR3A and POLR3B are the two largest subunits, forming the catalytic site, while POLR1C is a shared subunit of both RNA polymerase I and III complexes."

"The direct link between the specific hypomyelination phenotype in POLR3-HLD and hypofunction of a ubiquitously expressed transcription complex is not fully understood, however, there are two main hypotheses. The first involves hypofunction of POLR3 causing decreased transcriptional activity and disruption in tRNA production, resulting in a general impact on gene expression and global protein synthesis during critical periods of development, including myelination. As myelination is metabolically expensive and requires synthesis of large amounts of myelin-specific proteins in a short developmental window, oligodendrocytes may be more susceptible to a reduced translational capacity (Anitei & Pfeiffer, 2006; Elbaz & Popko, 2019; Pfeiffer et al., 1993; Torrent et al., 2018). Likewise, pathogenic variants in several tRNA aminoacyl synthetases are associated with hypomyelination (e.g., *DARS1, RARS1, EPRS1*), further

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[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

supporting a link between translational capacity and white matter pathology (Mendes et al., 2018; Taft et al., 2013; Wolf, Salomons, et al., 2014). The other hypothesis involves hypofunction of POLR3 causing a lack in production of POLR3-specific transcripts important for transcription, translation, or RNA processing, which could impact expression of specific proteins that are essential for the development and function of oligodendrocytes or neurons, and thus myelination (Choquet, Forget, et al., 2019; Tetreault et al., 2011; Thiffault et al., 2015). In considering these hypotheses, it is possible a combination of both contribute to disease pathogenesis."

"The pathophysiologic mechanisms underlying POLR3-HLD have yet to be fully resolved on a cellular level, however, various molecular defects resulting from POLR3 subunit mutations have been uncovered (Lata et al., 2021). Studies involving variant mapping have shown that pathogenic variants may cause mechanistic disruption to POLR3 function on different degrees, including impaired assembly of the POLR3 complex, alteration to the catalytic cleft structure, impaired binding of the POLR3 complex to DNA, and disrupted subunit-subunit interactions (Bernard et al., 2011; Girbig et al., 2021; Ramsay et al., 2020; Tetreault et al., 2011; Thiffault et al., 2015). *POLR1C* mutations also result in protein mislocalization, causing defects in nuclear import of POLR3 and altered complex assembly, consequently impacting POLR3 target gene binding (Thiffault et al., 2015). In fibroblasts and brain tissue from patients with POLR3-HLD and pathogenic variants in *POLR3A*, decreased POLR3A protein expression was notable (Bernard et al., 2011). Transcriptional defects have also been demonstrated through *in vitro* functional studies of *POLR3A* variants in humans and yeast (Choquet, Forget, et al., 2019; Moir et al., 2021)."

"Attempts at generating a representative animal model of POLR3-HLD have proven challenging. Polr3a-null mice are embryonic lethal, and mice with a full-body knock-in of a common disease-causing *POLR3A* point mutation [c.2015G>A (p.Gly672Glu)] do not display

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neurologic abnormalities (Choquet et al., 2017). Contrarily, mice with a full-body knock-in of the *Polr3b* c.308G>A (p.Arg103His) variant are embryonic lethal (Choquet, Pinard, et al., 2019). In another approach, mice harbouring both the latter *Polr3b* variant in heterozygous form and the *Polr3a* variant in homozygous form also do not exhibit neurologic abnormalities (Choquet, Pinard, et al., 2019). Recently, a conditional mouse model has been developed which harbours a homozygous double allele knock-in of two variants (p.Trp671Arg and p.Gly672Glu; initially studied in *S. cerevisiae*) under an Olig2-Cre driver for targeted expression in the oligodendrocyte lineage (Merheb et al., 2021; Moir et al., 2021). Mice displayed myelination defects, as well as impaired growth and neurobehavioural phenotypes (Merheb et al., 2021). Albeit lacking the motor deficits seen in the POLR3-HLD patient population, this mouse provides an initial valuable means to study the impact of impaired POLR3 on myelination. Moreover, as pathogenic variants in POLR3 subunits are also associated with syndromes that involve a spectrum of neurologic and non-neurologic features (i.e., spastic paraplegia, Wiedemann-Rautenstrauch syndrome, Treacher Collins syndrome, amongst others), the pathophysiology surrounding POLR3 disorders is complex and will require deeper cellular and molecular studies before mechanisms of pathogenesis are fully understood."

#### 1.2.2.2.6 POLR3-HLD Pathology [1]

"Neuropathological investigations of the typical phenotype of POLR3-HLD suggest a complex pathologic process, however, the most prominent feature remains insufficient myelin deposition. The two published cases of typical POLR3-HLD pathology revealed a marked loss of oligodendrocytes, with severity varying in different brain regions (Vanderver et al., 2013; Wolf, Vanderver, et al., 2014). Moderate axonal loss was evident, thought to be secondary to white matter

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[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

abnormalities due to its apparent proportionality to lack of myelin. Despite the uniform hypomyelinating pattern seen on MRI, it has been hypothesized that POLR3-HLD is a complex heterogenous leukodystrophy with prominent neuroaxonal and glial involvement (Vanderver et al., 2013)."

## 1.3 Diagnosis of Rare Diseases in the Era of Next Generation Sequencing

### 1.3.1 Next Generation Sequencing Methods

Next generation sequencing (NGS) refers to the sequencing technology developed following the traditional "first-generation" Sanger sequencing methods. Moreover, past standard practice to identify causal variants involved using Sanger sequencing to analyze phenotypically compatible genes followed by manual screening of chromatograms (Sanger et al., 1977). Since then, NGS has revolutionized standard practices for uncovering a genetic etiology for many rare inherited disorders, and has been key in resolving the genetic origin of numerous leukoencephalopathies, including hypomyelinating leukodystrophies (Boycott et al., 2014; Helman et al., 2020; Kaur et al., 2021; Kevelam et al., 2016; Srivastava et al., 2014; Vanderver et al., 2016; Yan et al., 2021).

Also known as "second-generation" short-read sequencing, NGS methods involve massive parallel sequencing of short clonally-amplified DNA (Slatko et al., 2018). There are several different subtypes of NGS, however, all involve a similar workflow, which includes library preparation, target enrichment, sequencing, base calling, alignment to the reference genome, and annotation of genetic variants (Morey et al., 2013; Reuter et al., 2015). The two predominant types of NGS used in diagnostics of rare inherited diseases are genome sequencing (GS) and exome sequencing (ES) each of which vary based on the target enrichment step prior to sequencing

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

(Stranneheim & Wedell, 2016). As its name suggests, ES involves sequencing of the majority of protein-coding regions, or exons (and the surrounding exon-intron boundaries), which account for approximately 1-2% of the genome (Cheng et al., 2005; Ross et al., 2020). This method provides a higher depth of sequencing at a lower cost compared to GS, and despite the exonic regions only accounting for a small portion of the genome, has proven to be highly effective in diagnostics given that the vast majority of pathogenic variants are concentrated in protein-coding regions or in exon-intron boundaries (Dixon-Salazar et al., 2012; Tetreault et al., 2015). In phenotypically similar but genetically heterogeneous neurological diseases, using a broad sequencing approach like ES is especially useful when more targeted clinical diagnostic methods, such as multi-gene panel screening, are unsuccessful (Neveling et al., 2013; Xue et al., 2015).

As both GS and ES techniques generate a large amount of sequencing data, standardized variant interpretation criteria must be used when evaluating pathogenicity to identify the causal gene. Detailed guidelines for classifying variant pathogenicity have been well-described by the *American College of Medical Genetics and Genomics*, including a five-tier classification system (Richards et al., 2015). This includes description of variants based on supporting evidence and criteria as either pathogenic, likely pathogenic, of uncertain significance, likely benign, or benign. Several criteria are used to interpret variants, including assessment of healthy control population data for reported frequency of variants (i.e., under 1% minor allele frequency for rare diseases) and reported variant types in population databases (i.e., absence of homozygous individuals for recessive disorders or heterozygous individuals for dominant disorders). Additionally, computational data can be used to predict pathogenicity of variants, including *in silico* prediction tools and conservation scores. The functional impact of variants should also be considered, including the type of variant (e.g., frameshift or nonsense variant associated with loss of function),

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

and whether full or partial loss of gene function is an established mechanism of pathogenicity based on scientific evidence. Reports of unrelated individuals with the same disease phenotype also provide evidence for variant pathogenicity and genotype-phenotype associations. Finally, segregation analysis can be used to confirm parental inheritance patterns and carrier status of affected or unaffected family members. Furthermore, these criteria provide a standardized approach to evaluating variant pathogenicity when identifying and prioritizing candidate genes based on specific lines of evidence.

### 1.3.2 Limitations of Exome Sequencing

Although ES offers a cost-effective and efficient method to identify disease-causing variants, it can be associated with inherent limitations when investigating the cause of rare monogenic diseases (Burdick et al., 2020). First, ES is not reliable in the detection of CNVs or structural rearrangements, including large deletions or duplications, as read depth does not directly correlate with true copy number (Teo et al., 2012). Next, ES is less effective in detecting variants in areas with high guanine-cytosine (GC) content (e.g., exon 1), as well as repetitive regions (e.g., trinucleotide repeats) (Meienberg et al., 2016; Ross et al., 2013). Finally, by design, it is not possible for ES to detect all potential pathogenic variants, such as deep intronic variants, intragenic variants, or methylation defects. Coverage of exons may also be variable, leading to undetected variants, and reads may not properly align to the reference genome, leading to false positive variant detection (Meienberg et al., 2015).

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

### 1.3.3 NGS in Diagnostics of White Matter Disorders

Leukodystrophies can offer a diagnostic challenge as they are largely genetically heterogeneous and often phenotypically similar with an overlapping clinical picture and MRI presentation. Studies on the use of ES and GS for determining the genetic origin of cohorts of patients with white matter disorders present a range of diagnostic yields (Cohen et al., 2020; Kevelam et al., 2016; Vanderver et al., 2016; Yan et al., 2021). Moreover, despite increasing rates of genetic diagnoses, there remain limitations to using NGS, which include inherent sequencing techniques, analysis methods, and even knowledge of novel gene functions when evaluating variants. Current research aims to overcome limitations associated with sequencing and analysis techniques to uncover disease causing variants that may not be detected following conventional NGS sequencing.

The studies described herein focus on investigating hypomyelinating leukodystrophies on a range of levels, from genetic investigations to clinical phenotyping, and finally in the study of genotype-phenotype correlations and disease pathology of specific disease subtypes. Furthermore, these studies aim to shed light on this class of rare inherited neurological disorders, providing the foundation for development of future pre-clinical trials before the development of therapeutic treatments.

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.

[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

**Figures and Tables**

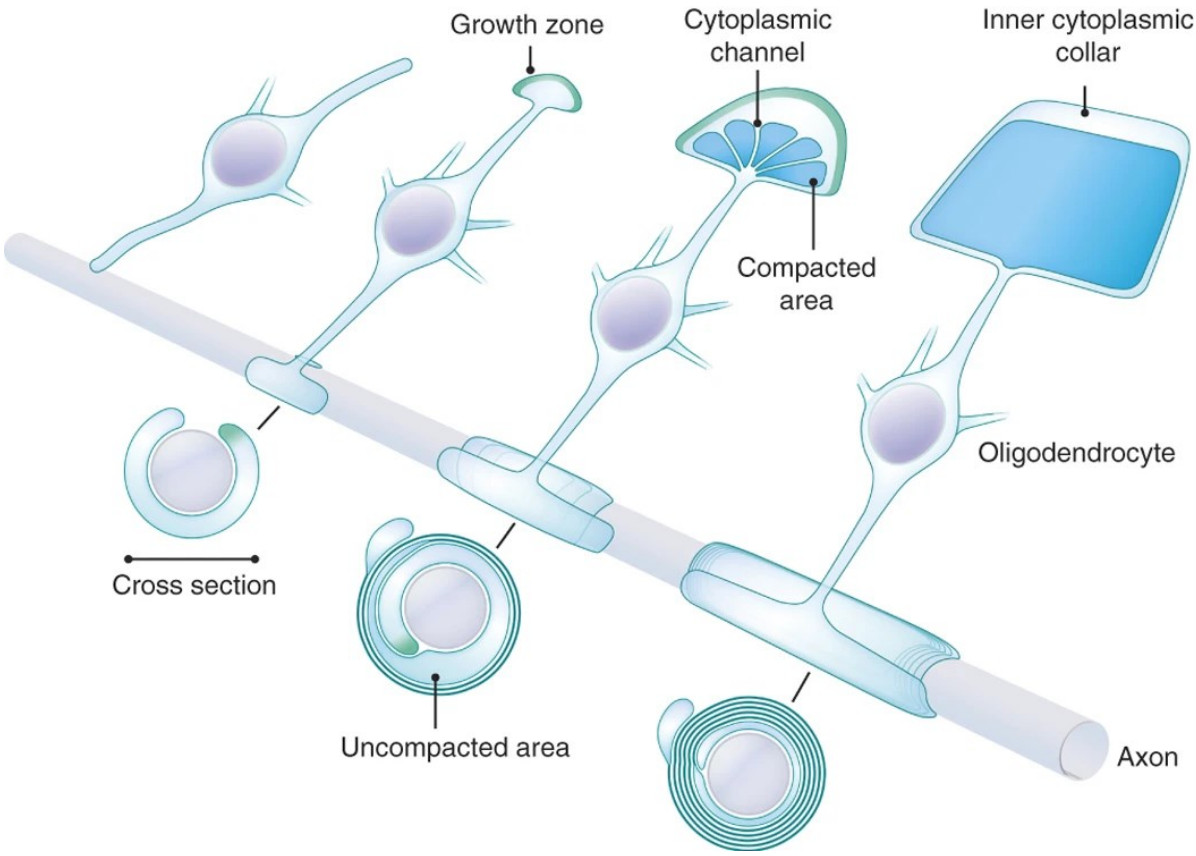


**Figure 1.1.** Formation of the myelin sheath by oligodendrocytes. OPCs extend processes to contact the axon, and ensheathment begins as the membrane wraps around the axon to form layers. Following growth of myelin radially and longitudinally, intracellular compaction occurs to form the compacted myelin sheath. Figure reprinted with permission from Springer Nature (Chang et al., 2016); Copyright 2016.

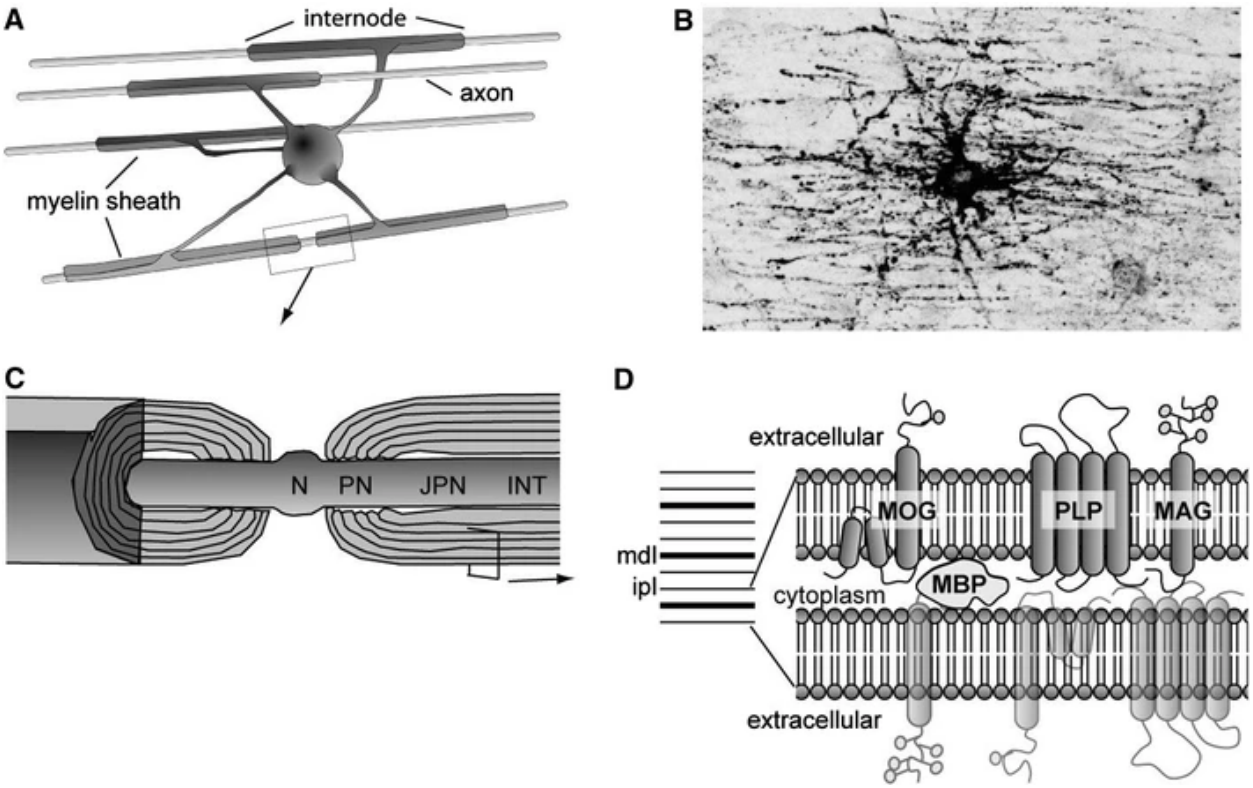


**Figure 1.2. Composition and architecture of the myelin sheath. (A, B)** CNS myelin is formed by oligodendrocytes wrapping layers of membrane around axons. A single oligodendrocyte can wrap multiple axons simultaneously. **(C)** Nodes of Ranvier (N) are located between myelinated segments on the axon and facilitate saltatory conduction. The paranodal (PN) region is located adjacent to the node, followed by the juxtaparanode (JPN) and internodal region (INT). **(D)** The myelin membrane is composed of both lipids and proteins, each of which are arranged within the outer and inner myelin leaflets, denoted by major dense lines (mdl) and intraperiod lines (ipl). Notable myelin proteins include PLP (proteolipid protein), MBP (myelin basic protein), MOG (myelin oligodendrocyte glycoprotein), and MAG (myelin-associated glycoprotein). Figure reprinted with permission from Springer (Potter et al., 2011); Copyright 2011.

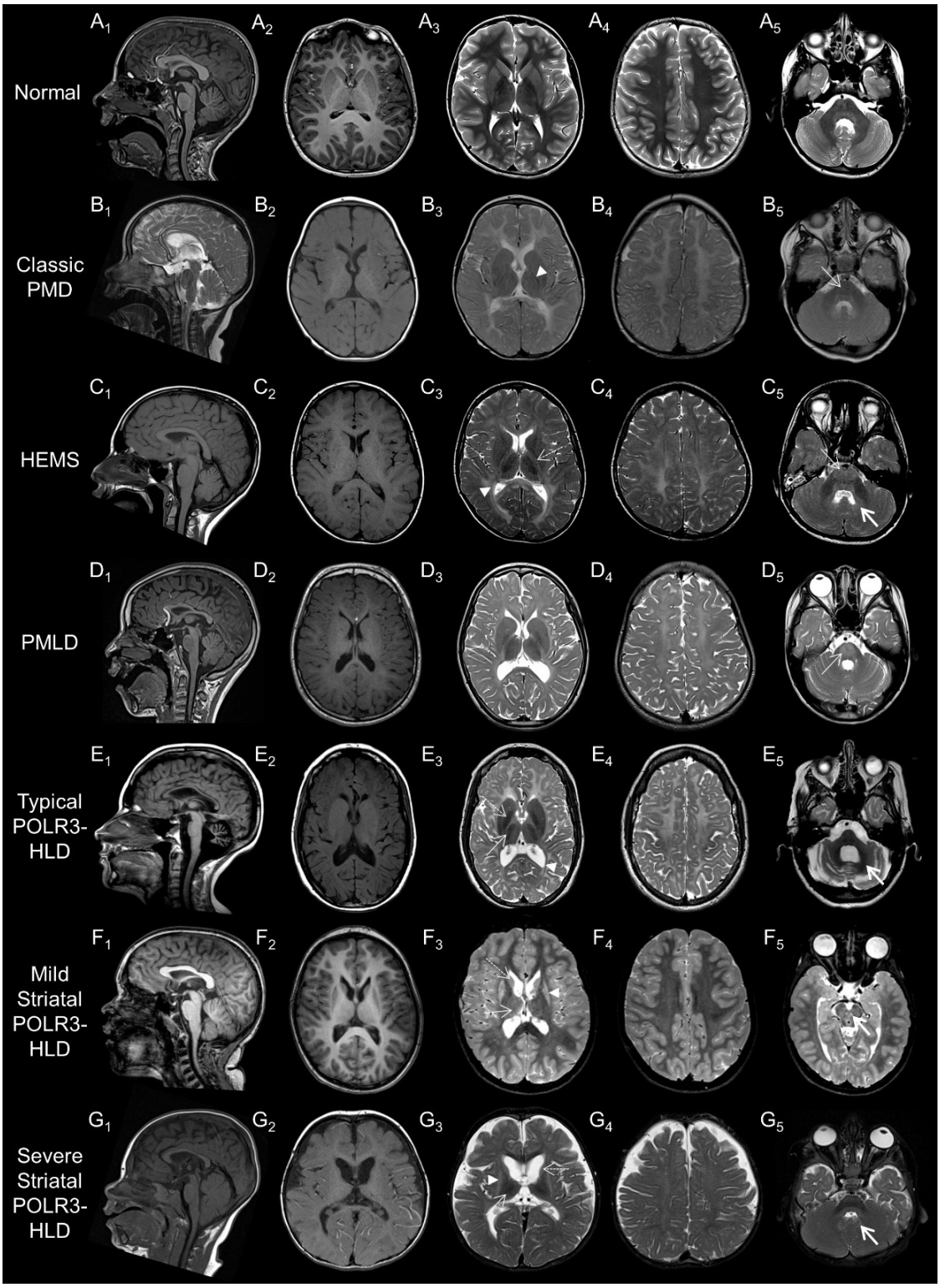


**Figure 1.3. MRI features associated with common hypomyelinating leukodystrophies.**[2] "Columns, from left to right: (A-$G_1$) midline Sagittal T1 (except $B_1$, sagittal T2), (A-$G_2$) axial T1 at the level of the basal ganglia, (A-$G_3$) axial T2 at the level of the basal ganglia, (A-$G_4$) axial T2 at the level of centrum semi ovale and (A-$G_5$) axial T2 at the level of the pons (except F5, at the

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.

[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

level of the midbrain). Brain MRI of a healthy 9-year-old child ($A_{1-5}$), showing normal midline structures ($A_1$) and normal myelination ($A_{2-5}$). Brain MRI of a 3-year-9-month-old male with classic Pelizaeus-Merzbacher Disease ($B_{1-5}$), showing diffuse supratentorial hypomyelination, i.e., isointense signal of the white matter on T1 ($B_2$), together with diffuse hyperintense signal of the white matter on $T_2$, compared to grey matter structures. There is relative preservation of the myelination, i.e., T2 hypointensity of the posterior limb of the internal capsule (PLIC) (arrowhead) and pons (thin arrow). Brain MRI of a 9-year-9-month-old male with Hypomyelination of Early Myelinating Structures ($C_{1-5}$). Hypomyelination is also seen, with mild hyperintensity of the white matter on T1, together with hyperintensity of the affected white matter structures on T2, compared to grey matter structures. Hypomyelination of immediate periventricular fibers of the optic radiations (arrowhead) as well as the pons (thin arrow) and dentate nucleus (thick arrow) is seen. A typical pattern of T2 hyper- hypo- and hyper- intensity is seen in the PLIC (dotted arrow). In panel $C_4$, there is hypomyelination of the corticospinal tracts, while the more frontal subcortical white matter is better myelinated. Brain MRI of a 9-year-3-month-old patient with Pelizaeus-Merzbacher-Like Disease ($D_{1-5}$) showing diffuse hypomyelination with relative preservation of the corticospinal tracts in the PLIC and the splenium of the corpus callosum ($D_3$). There is typical involvement of the pons (thin arrow). Brain MRI of a 23-year-old patient with typical POLR3-related leukodystrophy ($E_{1-5}$), showing a thin corpus callosum and cerebellar atrophy ($E_1$), diffuse hypomyelination ($E_{2-4}$) with relative preservation, i.e., T2 hypointensity of the optic radiations (arrowhead), anterolateral nucleus of the thalamus (thin arrow), globus pallidus (dotted arrow) and dentate nucleus (thick arrow). Brain MRI of an 8-year-6-month-old male with the mild striatal form of POLR3-related disorders, showing normal midline structures ($F_1$) and myelination ($F_{2-4}$), with specific involvement of the putamen (arrowhead), caudate nucleus (dotted arrow) and thalamus (thin arrow), best appreciated on T2 weighted images. Involvement of the red nucleus is also seen in the midbrain (thick arrow). Brain MRI of a 15-month-old patient with the severe striatal form of POLR3-related disorders showing cerebral atrophy and insufficient myelin deposition without frank hypomyelination ($G_{2-4}$). As seen in the mild striatal form of the disease, there is atrophy and T2 hyperintensity of the putamen (arrowhead), caudate (dotted arrow), and thalamus (thin arrow). In the posterior fossa, hyperintensity of the red nucleus can be seen (not shown), as well as involvement of the posterior pons and hilus of the dentate nucleus (thick arrow).” Figure reprinted with permission from Elsevier (Perrier et al., submitted 2022).[2]

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.

[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

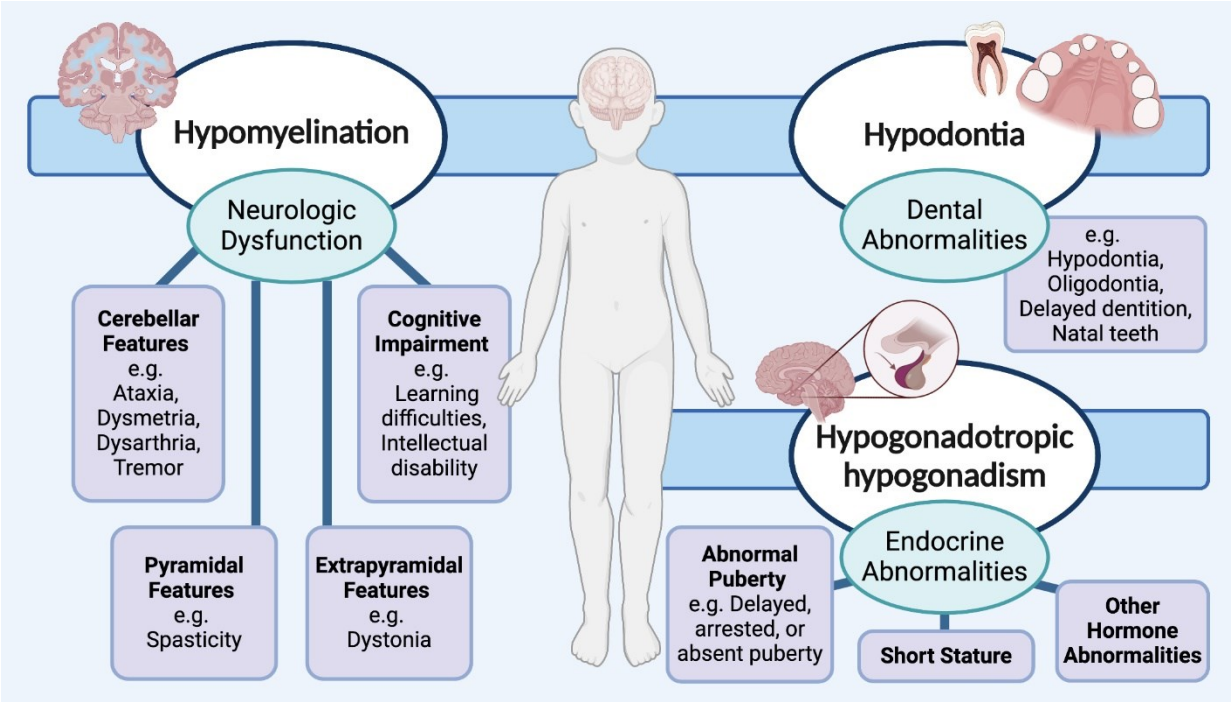


**Figure 1.4. Common clinical features seen in POLR3-related (or 4H) leukodystrophy**.[1]
Figure reprinted from Frontiers of Cellular Neuroscience (Perrier et al. 2021) under CC-BY 4.0 permission.

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.
[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

| | POLR3-related disorders<br>Most severe → Least severe | | |
|---|---|---|---|
| | **Severe Striatal** | **POLR3-HLD** | **Mild Striatal** |
| **Age of onset** | Infancy | Early childhood, rarely later | Childhood |
| **Symptoms at onset** | Severe motor delay or regression;<br>Failure to thrive | Motor delay or regression;<br>Cognitive with later onset | Motor delay; Extrapyramidal, cerebellar signs |
| **Pyramidal signs** | Spastic quadraparesis | Relatively mild c/w PLP1-related disorders | Possible but not prominent |
| **Cerebellar signs** | Possible | Prominent | Possible |
| **Additional features** | Prominent dystonia and chorea; dysphagia; laryngomalacia | Myopia; Dental abN;<br>Endocrine abN; Dystonia | Prominent extrapyramidal features, esp. dystonia |
| **Cognitive impairment*** | + | + | +/- |
| **Clinical course** | In most severe form, ambulation not achieved; Early and rapid deterioration | Prominent ataxia;<br>Wheelchair dependency around adolescence;<br>Death in second or third decade | Unknown |
| **MRI features** | Lack of myelin deposition without frank hypomyelination with striatal pattern^ and T2 hyperintensity of the hilus +/- peri- dentate nucleus and posterior brainstem | Relative preservation of dentate, optic radiations, globus pallidus, anterolateral thalamus, +/- CST in PLIC;<br>Atrophy of corpus callosum and cerebellum | Striatal pattern^ |

**Table 1.1.** Clinical and neuroimaging features of POLR3-HLD phenotypes, including the severe striatal form described in Chapter 4. Abbreviations: +: present; -: absent; abN: abnormal; CST: corticospinal tracts; c/w: compared with; esp.: especially; PLIC: posterior limb of the internal capsule, ^: T2-weighted images show hyperintensity and atrophy of the striatum (putamen, caudate nucleus) and thalamus, hyperintensity of the red nucleus. Table (partial version) reprinted with permission from Elsevier (Perrier et al., submitted 2022).[2]

[1] Text excerpt from Perrier et al. 2021. *Front Cell Neurosci.* 14: 631802.

[2] Text excerpt from Perrier et al. 2022 (submitted). *Handb Clin Neurol.* Ch. 13.

# CHAPTER 2.

# Investigation of novel genetic causes for hypomyelinating leukodystrophies

## Preface

In the clinic, hypomyelinating leukodystrophies are usually first identified based on MRI patterns, and genetic testing is then used to determine the causal gene and diagnose the specific subtype of leukodystrophy. In the following chapter, three manuscripts are presented which focus on the identification of genes associated with hypomyelinating leukodystrophies. This research began with the analysis of a cohort of 17 patients with hypomyelination or delayed myelination on MRI, but without a genetic diagnosis after clinical sequencing. Using next generation sequencing analysis, custom filtering, and variant classification, genetic diagnoses were confirmed for a subset of 7 of the described patients, leading to a solved rate of 41%. In the first manuscript, the lessons learned and challenges encountered in reaching a genetic diagnosis for 6 of these patients are presented and discussed. The next study focuses on the last patient from the analyzed cohort, for which pathogenic variants in the gene *POLR3K* were identified, representing the third patient reported worldwide with pathogenic variants in this gene and a hypomyelinating phenotype. Finally, the last manuscript discusses the importance of proper phenotyping of patients with white matter abnormalities and classification of genes associated with hypomyelination. Furthermore, these manuscripts demonstrate different aspects pertaining to the challenges associated with identifying genetic diagnoses in patients with inherited white matter disorders. For patients who remain unsolved following persistent reanalysis of data, additional methods should be considered

on a case-by-case basis, such as performing resequencing using updated technologies or using alternate advanced sequencing methods. Additionally, continued evaluation of disease progression and phenotyping of patients using clinical data and MRI patterns are imperative and should be included in genetic diagnostic protocols (Parikh et al., 2015; Stutterd et al., 2022). When investigating genetic diagnoses, it is also important to consider that although pathogenic variants in specific genes may currently be associated with specific leukodystrophy phenotypes, the spectrum of disease presentations may expand to include different mechanisms of pathogenesis. For example, this can include novel neuronal phenotypes as described in later chapters of this thesis. Moreover, with advances in sequencing technologies, development of new techniques, and increased knowledge into gene functions and disease associations, diagnostic rates will continue to increase for these rare inherited diseases.

# Chapter 2 Part A. Challenges in the genetic diagnosis of rare white matter diseases: Lessons learned from a series of cases solved by next generation sequencing

*Manuscript in preparation for submission.*

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## Abstract

Rare neurodevelopmental disorders, including inherited white matter disorders or leukodystrophies, often present a diagnostic challenge on a genetic level given the large number of causal genes associated with a range of disease subtypes. This study aims to demonstrate the challenges and lessons learned in the genetic investigations of leukodystrophies through presentation of a series of cases solved using exome or genome sequencing. Each patient had a leukodystrophy associated with hypomyelination or delayed myelination on MRI, and inconclusive clinical diagnostic genetic testing results. We performed next generation sequencing (case-based exome or genome sequencing) to further investigate the genetic cause of disease, and obtained a positive diagnosis for each case, with patients harbouring pathogenic variants in a range of genes including *TMEM106B, GJA1, AGA, POLR3A,* and *TUBB4A*. We describe the lessons learned in reaching the genetic diagnosis, including the importance of (a) utilizing proper multi-gene panels in clinical testing, (b) assessing the reliability of biochemical assays in supporting diagnoses, and (c) understanding the limitations of exome sequencing methods in regard to CNV detection, and region coverage in GC-rich areas. Furthermore, this study illustrates the importance of applying a collaborative diagnostic approach by combining detailed phenotyping data and metabolic results from the clinical environment with advanced next generation sequencing analysis techniques from the research environment to increase diagnostic yield in patients with genetically unresolved leukodystrophies.

## Introduction

Although genetic sequencing technologies have drastically evolved in recent years, identification and interpretation of rare variants associated with phenotypically similar but genetically heterogenous diseases remains a challenge. Rare inherited white matter disorders, or leukodystrophies, can be especially difficult to genetically diagnose, given the growing number of causal genes associated with different subtypes of disease (Urbik et al., 2020; Vanderver et al., 2015). Clinical presentation can be similar between patients, often involving neurological signs which can be progressive, such as ataxia, abnormal muscle tone, gait difficulties, and/or intellectual or cognitive deficits, amongst other features (Adang et al., 2017; Parikh et al., 2015). Upon MRI investigations and identification of white matter abnormalities, leukodystrophies can be categorized based on imaging characteristics as hypomyelinating leukodystrophies or as other pathologies, such as demyelinating leukodystrophies (Barkovich & Deon, 2016; Schiffmann & van der Knaap, 2009; Steenweg et al., 2010). In young children, it is important to differentiate hypomyelination from delayed myelination by repeating brain MRI as both have distinctive lists of differential diagnoses (Steenweg et al., 2010). Particular neuroimaging patterns and recognition of disease-specific MRI features can further aid in narrowing the underlying genetic cause of disease (Schiffmann & van der Knaap, 2009). On a clinical basis, molecular diagnostic procedures typically combine genetic sequencing (e.g., multi-gene panels or exome sequencing) along with metabolic investigations (e.g., enzyme deficiencies or cerebrospinal fluid (CSF) metabolite levels) to further confirm or rule out diagnoses (Parikh et al., 2015).

Since the rise of next generation sequencing use in the research environment, diagnostic rates for leukodystrophies have seen significant increases, both in report of variants within known disease-associated genes, and in the discovery of novel disease-causing genes (Helman et al., 2020;

Kaur et al., 2021; Kevelam et al., 2016; Vanderver et al., 2016; Yan et al., 2021). Despite the increase in diagnostic rates, there still remain patients who are genetically unresolved following clinical and/or research investigations, which may result from limitations within the technology itself, challenges in variant identification, or evaluation of variant pathogenicity. Along these lines, given that leukodystrophies can be associated with multi-systemic features, challenges may be faced when navigating differential genetic diagnoses, especially when presented with variants of unknown significance in multiple genes. Furthermore, when resolving the genetic basis of an unsolved leukodystrophy, it is essential to consider the patient's entire picture, including clinical presentation and disease progression, neuroimaging features, and metabolic test results.

This case series presents an overview of several lessons learned in the diagnosis of patients with genetically unresolved white matter disorders. Each patient presented with hypomyelination or delayed myelination on MRI and was referred to our study for additional genetic investigations after clinical testing remained inconclusive. We utilized next generation sequencing to determine the genetic cause of disease, noting the below challenges that were faced. The clinical and MRI features, as well as the specific genetic investigations are described below, along with a discussion of the lessons learned on the path to resolving genetic diagnoses.

## Subjects and Methods

This project was approved by the research ethics board of the McGill University Health Centre and the Montreal Children's Hospital (11-105-PED, 2019-4972), and informed consent was obtained from all participants or their parents/legal guardians. Medical records and brain MRIs were reviewed for each patient. Next generation sequencing data were either obtained from a clinical laboratory for further analysis or completed on a research basis using genomic DNA

extracted from whole blood, fibroblasts, or saliva according to standard protocols. Exome and genome sequencing were performed on a case-by-case basis as described previously (Cohen et al., 2022; Thiffault et al., 2019). Potential disease-causing variants were identified and evaluated based on the *American College of Medical Genetics Standards and Guidelines for the Interpretation of Sequence Variants* (Richards et al., 2015). All variants were validated by Sanger sequencing or confirmed by clinical genetic testing.

## Results and Discussion

In each of the six described patients, a genetic diagnosis was resolved following exome or genome sequencing completed on a research basis. We noted several challenges and lessons learned throughout these investigations, each of which are described below and summarized in Table 2.1. A summary of the clinical and MRI features are included, followed by the genetic investigations and a discussion of each lesson with insight on its application to future studies.

### Lesson I: Inclusion of phenotypically compatible genes in clinical gene-panel testing

The two patients described below demonstrate the importance of utilizing updated and broad leukodystrophy-associated targeted gene panels in the context of clinical genetic testing. Furthermore, this applies to both patients with early symptoms (Patient 1), and in adult-onset diseases (Patient 2).

#### Patient 1: Clinical/MRI Summary

Patient 1, a female, presented shortly after birth with nystagmus, episodes of rapid tremor of the hands, and tremor of the mandibula while feeding. She also had mildly reduced axial tone,

and increased tone in all four limbs. Through infancy to early childhood, she continued to have mild to moderate axial hypotonia and spastic quadriparesis, with brisk deep tendon reflexes. She also had dysmetria, bilateral sensorineural hearing loss, and dysphagia. At age 3 years, she began to demonstrate additional neurological features, including dysarthria, mild sialorrhea, dysdiadochokinesis, and a slightly instable gait. In addition, she had ophthalmic abnormalities involving strabismus and abnormal saccadic pursuits (hyper/hypometric). Despite showing an initial neurological deterioration, she later stabilized and then started improving, with developmental progress and amelioration of her dysphagia.

MRI at age 3 weeks revealed abnormal myelination, with myelin deposition being insufficient for age. At the time, due to her young age, it was not possible to determine if she had hypomyelination or myelination delay. There was also involvement of the corticospinal tracts in the c-spine, pyramids, and pons, as well as T2-hyperintensity of the cerebellar white matter. MRI at age 7 months revealed progression of the white matter signal changes, concerning both the deep and subcortical white mater and corpus callosum, and involvement of the middle cerebellar peduncles. An abnormal lactate peak was also detected in the white matter on MR spectroscopy. The optic nerves appeared normal in size, however there was an abnormal signal in the intraorbital segment, involving a slight decrease in T1-weighted imaging and increase in T2-weighted imaging, with suspicion of restricted diffusion.

**Patient 1: Genetic Investigations**

Following the initial abnormal MRI findings, genetic investigations for Patient 1 began with multi-gene panel sequencing on a clinical basis. Moreover, after screening a panel of 163 known leukodystrophy and leukoencephalopathy genes, no conclusive variants were identified.

On a research basis, exome sequencing was performed, leading to the identification of a *de novo* pathogenic variant in the gene *TMEM106B* (NM_018374.3)*:* c.754G>A (p.D252N). This variant has been reported in six unrelated individuals with a hypomyelinating leukodystrophy, appearing to be a single-nucleotide hotspot associated with this disease (Ikemoto et al., 2020; Simons et al., 2017; Yan et al., 2018). In retrospect, this gene was not included in the clinical panel, and therefore the pathogenic variant was not detected during the first genetic investigation. The length of time between the first publication of the gene as disease-causing and the completion of the clinical multi-gene panel sequencing was approximately one year.

**Patient 2: Clinical/MRI Summary**

Patient 2, whose MRI was previously published in a NeuroImages report (Michell-Robinson et al., 2022), presented in adulthood with slowly progressive gait disturbances, falls, and issues with memory, starting at age 35. As a child, he was known to have stomach malrotation and syndactyly, the latter of which was surgically corrected. Despite minor motor problems since childhood, he reported normal activities of daily life, and remained fully ambulatory. He had slight dysmetria and mild gait ataxia. He was also noted to have mild facial dysmorphisms, dental abnormalities, and severe myopia.

MRI in adulthood revealed a pattern of diffuse hypomyelination, with involvement of the posterior limb of the internal capsule, pons, and cerebellar peduncles. T2-weighted hypointensities were also noted in both the Rolandic cortex and the dentate nucleus. The corpus callosum was thin and there was mild vermian atrophy (Michell-Robinson et al., 2022).

In regard to significant family history, his mother also experienced slowly progressive neurodegeneration in adulthood, involving pyramidal signs and gait ataxia, which worsened over

several years. She experienced dysphagia, speech and cognitive difficulties, and epileptic seizures. She died in her early 80s after experiencing recurrent aspiration pneumonias.

Brain MRI of the patient's mother at age 80 showed diffuse white matter abnormalities, with involvement of the posterior limbs of the internal capsule, and the pons (corticospinal tracts and medial lemniscus), as well as significant atrophy of the posterior white matter. She also had a thin corpus callosum, cerebellar vermis atrophy, and ventriculomegaly (Michell-Robinson et al., 2022).

**Patient 2: Genetic Investigations**

Following the identification of white matter abnormalities on MRI of Patient 2, a gene panel including 122 leukodystrophy or leukoencephalopathy genes was screened on a clinical basis. The results were inconclusive, and the patient was referred to our study for further investigations. Exome sequencing was completed on DNA from the proband, revealing a heterozygous pathogenic missense variant in *GJA1* (NM_000165.5): c.413G>A; p.G138D. This variant was previously reported as pathogenic in association with autosomal dominant Oculodentodigital dysplasia (ODDD) syndrome (Fenwick et al., 2008). Parental DNA was unavailable for segregation analysis, but given the similar presentation of the mother, autosomal dominant inheritance was presumed. Notably, on the initial clinical gene panel, the *GJA1* gene (published over a decade prior) was not included, and therefore not identified on the first investigation.

**Inclusion of novel and compatible genes on clinical panels**

Gene panels which target a specific set of genes known to be associated with disease phenotypes are often used as a first-line diagnostic tool in the clinical setting. While use of gene panels may provide an effective means for identifying pathogenic variants in known genes, patients with pathogenic variants outside of those remain genetically undiagnosed, often leading to a long diagnostic odyssey. Advantages of using multi-gene panels compared to exome or genome sequencing have been studied in the past, and there remain benefits and limitations for both diagnostic techniques (de Koning et al., 2015; Lionel et al., 2018; Molina-Ramírez et al., 2022; Saudi Mendeliome, 2015; Xue et al., 2015). While multi-gene panel sequencing can be a powerful molecular diagnostic tool, without inclusion of up-to-date causal genes they remain limited in their effectiveness for investigations of rare diseases. In the case of Patient 1, the *TMEM106B* gene was published as associated with hypomyelination nearly a year prior to the initial clinical screening (Simons et al., 2017). Contrarily, Patient 2 was found to harbour a pathogenic variant in *GJA1*, which was published as associated with ODDD syndrome in the early 2000s (Paznekas et al., 2003). As neurological features are only seen in a portion of patients with ODDD, it is likely to be an often-unrecognized form of adulthood leukodystrophy. Thus, it remains possible that it was excluded from the large leukodystrophy-associated gene panel due to the leukodystrophy features in ODDD remaining overlooked, as the phenotypic bias in diagnosis may lean towards the other associated features present on clinical evaluation.

Both of the above cases had to transition from clinical to research-based studies to be resolved, thus increasing the time to diagnosis, and had the initial panel screening contained the causal genes, this time period could have been minimized. Furthermore, these cases demonstrate the importance of maintaining clinical gene panels to be both updated with newly published genes,

as well as expansive enough to include likely causative genes. In the field of medical genetics, discovery of novel disease-associated genes remains to advance at a rapid pace, with unique genetic causes of leukodystrophies continuously reported, which should involve parallel inclusion in gene panels. It is also imperative that clinicians ordering clinical gene panels are knowledgeable regarding whether the proper genes are included in the genotypic screen when there is a high index of suspicion for a phenotypically distinct condition.

**Lesson II: Reliability of biochemical assay support in confirming genetic diagnoses**

Often, variants identified on exome sequencing may be classified as being of unknown significance if they have not been previously linked to a disease, or if functional evidence is lacking. For some implicated genes, clinical biochemical assay results may be used as biomarkers to support variant pathogenicity. The following case presents a scenario in which likely pathogenic variants were identified in a causal gene; however, this finding was initially unsupported by biochemical testing results. On repeat biochemical testing, results were verified, and the genetic diagnosis was confirmed. Thus, this case demonstrates the importance of considering the validity of biochemical testing results when evaluating likelihood of variant pathogenicity, especially when contradicting evidence is provided in the case of likely pathogenic variants.

**Patient 3: Clinical/MRI Summary**

Patient 3, a female, presented to the clinic with a wobbly gait at age 4 years, and was soon after found to have diffuse abnormal myelination on MRI, either associated with a pattern of delayed myelination or hypomyelination. MRI at age 5 years was significant for diffuse hypomyelination/delayed myelination, where slight progression of myelination of the peripheral

subcortical white matter was seen on T1-weighted imaging, with the degree of myelination on T2-weighted imaging remaining stable. Upon review of her latest MRI at age 8, there was also mild thinning of the corpus callosum, mild cerebellar vermis atrophy, and a mild increase in VR spaces, with T2-hypointensity of the pulvinar.

Clinically, she had a history of global developmental delay from 6 months of age, and throughout childhood, she continued to have mild developmental delay, without focal abnormalities. She was known for ophthalmic issues, including right eye esotropia and pallor on the left optic nerve. She had mild difficulties with tandem gait. Nerve conduction studies were normal. Through to age 8, she did not experience signs of regression, and only continued to have mild developmental challenges. She was also known to have persistent mildly low platelets levels, which appeared to normalize at age 7. Initial urine oligosaccharide testing revealed a faint abnormal band, which was further investigated clinically via HPLC analysis, and reported to be unremarkable for the known lysosomal storage disorders (i.e., alpha mannosidosis, alpha fucidosis, sialidosis, galactosialidosis, GM1 gangliosidosis, GM2 gangliosidosis type Sandhoff, GSD11 Pompe disease infantile). On biochemical testing of CSF, amino acids and neurotransmitter metabolites were unremarkable.

**Patient 3: Genetic Investigations**

Exome sequencing was performed using patient genomic DNA and upon initial data analysis, two compound heterozygous nonsense variants were identified in the gene *AGA* (NM_000027.4): c.319C>T; p.R107X, and c.1018G>T; p.E340X. *AGA* encodes for the enzyme aspartylglucosaminidase, and pathogenic variants which impair its function are known to cause aspartylglucosaminuria (OMIM: 208400), an autosomal recessive neurodegenerative lysosomal

storage disease. The first *AGA* nonsense variant (p.R107X) results in a truncated protein product, lacking 240 amino acids (aa) from the 347-aa length wildtype protein. Although this specific variant has not been reported in published cases, similar truncating variants are known to be associated with AGA loss-of-function and cause disease (Isoniemi et al., 1995; Saarela et al., 2001). The second nonsense variant (p.E340X) results in a truncated protein lacking only 7-aa compared to the wildtype protein. While this variant has also not been reported previously, the nearby p.E334X variant has been studied functionally to cause a reduction in the production of an active enzyme (Saarela et al., 2001). Based on ACMG criteria (Richards et al., 2015), both variants were predicted to be likely pathogenic. Each variant was validated using Sanger sequencing, however, as the patient was adopted, parental DNA was unavailable to confirm segregation.

Regarding confirmatory biochemical testing, diagnosis of aspartylglucosaminuria is supported by screening urine oligosaccharides, as aspartylglucosamine accumulates in the urine of affected individuals (Goodspeed et al., 2021). In this patient, initial urine oligosaccharide analysis revealed an abnormal band, however, further HPLC analysis did not detect a pattern associated with diseases. This result contradicted with the predicted pathogenic variant interpretation, as both of the variants were thought to be associated with impaired function of the enzyme. Therefore, it was decided to repeat clinical urine oligosaccharide screening to verify the results, and on the second screen, an abnormal pattern associated with aspartylglucosaminuria was detected, providing support for the diagnosis. Additional tests were performed, including a sialic acid assay which showed mild elevation of total sialic acid, thought to result from abnormal accumulation of an oligosaccharide species associated with aspartylglucosaminuria. Finally, enzymatic testing demonstrated low aspartylglucosaminidase activity, further confirming the diagnosis of aspartylglucosaminuria.

**Importance of biochemical assay results in supporting genetic diagnoses**

Biochemical and functional assays are an important diagnostic component for many genetic disorders, as metabolic results often provide clues to a diagnosis, such as in lysosomal storage disorders. In the presented case, initial urine oligosaccharide screening results did not support the most likely candidate gene, and therefore difficulties were met in resolving the diagnosis. Variants were predicted to be pathogenic, however, we were unable to confirm their inheritance on trans alleles as the patient was adopted. In addition, the biochemical test provided conflicting evidence, thus causing uncertainty in either the genetic or biochemical results. The diagnosis was only confirmed after repeated biochemical screening for urine oligosaccharides, which also resulted in a prolonged diagnostic period. Therefore, this case demonstrates the importance of maintaining a degree of trust in variant interpretation guidelines when evaluating pathogenicity, and that repeat biochemical screening may be necessary should results contradict the genetic diagnosis. This case also stresses the importance of remaining cautious when presented with conflicting diagnostic evidence and approaching the reassessment of either genetic or biochemical results with a high level of clinical reasoning. Contrary to what was seen in our case, in which molecular genetic investigations allowed for the diagnosis of a metabolic disease where the biochemical genetic tests were initially normal, it is possible that genetic variants initially thought to be likely pathogenic can be reclassified as benign based on biochemical investigations. For example, in considering pathogenic variants in the *ABCD1* gene which cause adrenoleukodystrophy, the detection of true pathogenic variants is complicated by the fact that several non-functional pseudogenes exist on different chromosomes, which may result in false-positive variant detection (Wiesinger et al., 2015). In this case, it is crucial to confirm the pathogenicity of the variants with very long chain fatty acids. In conclusion, special attention

should be given to cases with conflicting genetic and biochemical evidence, and next steps for confirming or rejecting diagnoses should be considered only after critical evaluation of all provided evidence.

**Lesson III: Understanding limitations of exome sequencing in variant detection**

While the use of exome sequencing has demonstrated a high success rate in resolving the genetic cause of many rare diseases, it is well-known that this technology is associated with several limitations in its capacity to detect all disease-causing variants (Meienberg et al., 2015). The following three cases illustrate the limitations of exome sequencing in variant detection, including the lack of ability to detect CNVs (Patients 4&5) and variants in GC-rich regions (Patient 6).

**Patients 4 & 5: Clinical and MRI Information**

Patients 4 and 5 were siblings who each experienced increasing cognitive and behavioural concerns in early adulthood and were found to have hypomyelination on MRI. Patient 4, a female, had behavioural problems since childhood, with intellectual and learning disabilities. She also had severe myopia, as well as growth and pubertal abnormalities. She experienced menarche at age 14 but required hormonal treatment for irregular menstruations. In early adulthood, she was diagnosed with bipolar disorder, and further MRI investigations revealed white matter abnormalities, leading to a diagnosis of leukodystrophy. In adulthood, she experienced further neurological deterioration, exhibiting dysarthria, intention tremor, gait ataxia, as well as dystonia. She also had saccadic pursuits, with limited upgaze range. She further experienced dysphagia and required a gastrostomy. Delayed dentition was noted in childhood, and in adulthood it was reported that her teeth were becoming loose. MRI at age 30 of Patient 4 was significant for hypomyelination with a

pattern compatible with POLR3-related leukodystrophy. There was also moderate diffuse cerebral and cerebellar atrophy. Interestingly, bone abnormalities were also noted in the skull, described as diffuse thickening of the calvarium.

Patient 5, a male, had onset of behavioural issues and personality changes in his late 20s. His development was reported as normal, having graduated high school after attending special education classes. He was reported to have puberty and growth abnormalities, receiving growth hormone treatment in early adolescence. He also had severe myopia but reported no dental abnormalities through childhood. In early adulthood, he began to experience cognitive decline, with severely impaired intellectual function, while behavioral difficulties, specifically irritability, persisted. Family also noted episodes of pseudobulbar affect. He was reported to have a high-pitched voice, but no dysarthria. He had hypotonia and mild gait ataxia. MRI of the brain in adulthood also revealed hypomyelination with a pattern compatible with POLR3-related leukodystrophy. Similar to his sister, he also had thickening of the calvarium.

**Patients 4 & 5: Genetic Investigations**

Exome sequencing was first completed on both siblings, and one pathogenic missense variant was discovered in *POLR3A* (NM_007055.4): c.3013C>T, p.R1005C. This variant was previously reported in compound heterozygous form in patients with POLR3-related leukodystrophy (Bernard et al., 2011; Saitsu et al., 2011). As POLR3-related leukodystrophy is an autosomal recessive condition and only one variant was identified, data reanalysis was repeated over time to attempt to identify an alternate genetic cause, however, no strong candidates were found. Genome sequencing was then completed, identifying a maternally inherited *POLR3A* deletion of ~3 kb on 10q22.3, including exons 6-8 [(chr10:78020680-78023050)x1], thereby

resolving the genetic diagnosis as POLR3-related leukodystrophy. Notably, a similar deletion of *POLR3A* exons 6-8 (NC_000010.11: g.78020702_78023071del; c.646–687_c.1185 + 844del; p.E216_K395del) has been associated with spastic ataxia when in compound heterozygous form with the *POLR3A* c.1909+22G>A variant (Infante et al., 2020). Additionally, large deletions in the RNA polymerase III subunit gene *POLR3B*, which forms the catalytic core of the enzyme along with *POLR3A*, have also been associated with a similar phenotype of POLR3-related leukodystrophy (Gutierrez et al., 2015). The diagnosis of POLR3-related leukodystrophy aligns with the clinical features seen in both of the above patients, including the MRI pattern of diffuse hypomyelination, as well as the growth and endocrine abnormalities in both siblings, and delayed dentition in Patient 4.

**Advantages of genome sequencing in CNV detection**

Exome sequencing is known for several limitations in variant detection, inherent to its technological design. Moreover, this includes the inability to detect certain types of variants, including CNVs like the large deletion described in the above cases. This limitation is a direct result of the lack of sequencing depth uniformity in exome sequencing, as the enriched exonic regions interspaced by non-coding intronic regions are not evenly sequenced. Therefore, deletions are difficult to detect through conventional exome sequencing methods, and genome sequencing offers a more effective means to resolve the deleted region. While exome sequencing generally captures a higher sequencing depth of the targeted exonic regions, genome sequencing offers a more uniform coverage of the genome (Lelieveld et al., 2015). Indeed, due to its ability to provide more even and unbiased coverage of all exonic regions, genome sequencing delivers more accurate variant detection, especially in the recognition of large deletions (Belkadi et al., 2015). The

described cases offer a practical example of the limitations of exome sequencing in detecting CNVs and demonstrate the importance of pursuing additional investigations when a single pathogenic variant is identified in a gene associated with a phenotypically compatible autosomal recessive disease. In this case, had the *POLR3A* gene been fully investigated, either through long-range PCR, primer walking, or quantitative PCR, the genetic diagnosis may have been resolved sooner without need for repeat sequencing. However, the benefits of genome sequencing were clearly demonstrated in this case and provided an efficient means to detect the deletion. Therefore, these cases demonstrate a lesson in harnessing knowledge of the limitations associated with variant detection via exome sequencing when investigating causes of negative genetic analyses.

**Patient 6: Clinical and MRI Information**

Patient 6, a male, presented shortly after birth with nystagmus, and further experienced severe developmental delay and failure to thrive. He demonstrated neurological features including severe axial and appendicular hypotonia, moderate spasticity, and generalized dystonia. He was severely delayed in motor development, unable to gain normal head control or the ability to sit independently or walk. He also experienced sialorrhea and dysphagia, requiring a gastrostomy at age 3. He had epilepsy from early childhood, which was well controlled by age 11. He also experienced asymptomatic subluxation of both hips. In regard to ophthalmic abnormalities, he had bilateral myopia and strabismus, with bilateral optic atrophy and restriction of extraocular movements. At age 13, his condition was relatively stable, although he had slight motor deterioration, losing the ability to pick up objects with his hands.

Brain MRIs demonstrated diffuse hypomyelination with relative preservation of the cerebellum and brainstem. On follow-up MRIs, cerebral and cerebellar atrophy were noted.

**Patient 6: Genetic Investigations**

Patient 6 was first investigated using exome sequencing on a research basis, however, no strong candidates were identified despite regular data reanalysis. Five years after the initial sequencing, we opted to repeat exome sequencing using an updated platform in an attempt to identify variants which may have been previously undetected. Analysis of this data resulted in identification of a pathogenic *de novo* variant in *TUBB4A* (NM_006087.3): c.5G>A; p.R2Q, thus resolving the cause of disease. This variant has been reported in two individuals having hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC) (Hamilton et al., 2014; Miyatake et al., 2014). Indeed, although *TUBB4A* variants have mainly been associated with H-ABC, cases of isolated hypomyelination have also been described (Miyatake et al., 2014; Pizzino et al., 2014; Purnell et al., 2014; Shimojima et al., 2015), aligning with the MRI pattern of Patient 6, who did not display involvement of the basal ganglia.

Upon review of the initial exome sequencing data, the genomic region containing the *TUBB4A* variant was only covered by five sequencing reads, with only one of which carrying the variant. Therefore, due to suboptimal coverage and read depth, the variant was not detected on the first investigation. Indeed, this variant was located near the beginning of the first exon of the gene, a region generally known to contain an increased GC content (Kalari et al., 2006), and consequently associated with reduced coverage on exome sequencing.

**Coverage bias in exome sequencing and impact on variant detection**

The case of Patient 6 is a direct example of the limitations of exome sequencing in detecting variants in areas that are commonly subject to coverage bias. It is well known that exome sequencing is associated with non-uniform coverage in specific regions, including those that are

GC-rich. Uneven coverage of these regions may result from technological challenges in either library or cluster amplification, in the sequencing step itself, or through to the alignment of sequencing data (Aird et al., 2011; Chen et al., 2013; Ross et al., 2013). Updates to sequencing platforms have aimed to mitigate this through advanced correction methods, which have led to more consistent coverage in recent years. Therefore, when evaluating the best course of action for persistently unsolved cases that were sequenced several years prior, it is important to consider the benefits of re-sequencing using updated platforms. Through this case, the value of re-sequencing was certainly demonstrated as a diagnosis was easily resolved on the second round of sequencing, with the causal variant being located in an area which was not initially covered on first round sequencing, and thereby not detected. Furthermore, it is important to remain knowledgeable of the efficacy and utility of sequencing technologies used in genetic analyses, and whether updated technologies for repeat sequencing could be valuable for subsequent investigations.

## Conclusion

In this study, we illustrate several lessons learned when investigating genetic diagnoses in a subset of patients with genetically unresolved leukodystrophies following negative clinical investigations. The encountered challenges represent common lessons and pitfalls that clinicians and researchers may face when navigating pathogenic variant identification and interpretation in genetic diagnostics. The first two cases highlight the importance of utilizing appropriate gene panels in first line clinical investigations, ensuring that they contain both recently published and phenotypically compatible genes. The next case demonstrates the importance of using clinical reasoning when evaluating biochemical results that conflict with probable genetic diagnoses. Finally, the last three cases illustrate the technical limitations associated with variant detection via

exome sequencing, both of which were resolved using repeat sequencing to identify previously undetected variants. When pursuing future investigations of genetically unresolved cases, remaining knowledgeable of the challenges associated with variant identification can provide one with insight on the most beneficial and effective course of action. However, it is important to note that although there are technological limitations which may impede detection of pathogenic variants, cases may remain genetically unresolved due to other factors, such as challenges in accurate variant pathogenicity interpretation due to gaps in knowledge base, or simply because the causal gene has not yet been associated with a disease. It is imperative that unsolved cases are regularly re-evaluated as the field advances in both sequencing technologies, analysis techniques, and reports of novel genes. Furthermore, in a clinical context, resolving genetic diagnoses of rare inherited diseases is especially important for understanding of the disease prognosis, as well as tailoring supportive care, and in further advising through genetic counselling.

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## Tables

**Table 2.1.** Summary of solved cases and lessons learned on the path to genetic diagnosis.

| Patient | Pathogenic Variants | Diagnostic Challenge | Lesson Learned |
|---|---|---|---|
| 1 | ***TMEM106B*** (NM_018374.3): c.754G>A (p.D252N) | Causal gene absent on initial clinical gene panel despite publication one year prior | Importance of ensuring clinical panels include recently reported genes |
| 2 | ***GJA1*** (NM_000165.5): c.413G>A; p.G138N | Causal gene absent on initial clinical panel despite being associated with adulthood leukodystrophy (publication over a decade prior) | Importance of including phenotypically compatible genes in initial screening |
| 3 | ***AGA*** (NM_000027.4): c.319C>T; p.R107X c.1018G>T; p.E340X | Reliability of biochemical assay support in confirming genetic diagnoses | If contradicting variant interpretation and biochemical results, consider reliability and usefulness of repeat testing |
| 4&5 (sibs) | ***POLR3A*** (NM_007055.4): c.3013C>T, p.R1005C Deletion exons 6-8 (chr10:78020680-78023050del) | Limitations of exome sequencing in detecting multi-exon deletions | With identification of a single variant in a phenotypically compatible AR gene, consider secondary analysis via genome sequencing to ensure complete coverage for detection of all possible variants |
| 6 | ***TUBB4A*** (NM_006087.3): c.5G>A; p.R2Q | Limitations of exome sequencing in providing uniform coverage of all exons | If exome sequencing was completed several years prior, consider repeat exome sequencing using advanced platform for increased accuracy in variant detection |

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## Chapter 2 Part B. Novel *POLR3K* variants cause POLR3-related leukodystrophy

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## Abstract

POLR3-related leukodystrophy is a rare inherited hypomyelinating disorder caused by biallelic pathogenic variants in subunits of RNA polymerase III (Pol III). Here, we report the third patient worldwide with pathogenic variants in *POLR3K* and clinical features consistent with POLR3-related leukodystrophy. A female patient presented with mild intellectual and behavioural disturbances in childhood, with brain MRI revealing diffuse hypomyelination and a pattern consistent with POLR3-related leukodystrophy. She manifested minor motor dysfunction starting

in late adolescence and had growth delay, as well as ophthalmic abnormalities including bilateral posterior polar cataracts. Next generation sequencing revealed a paternally inherited missense variant in *POLR3K* (c.322G>T; p.D108Y), and a maternally inherited large deletion, spanning approximately 17.8kb from chr16:30,362-48,162. The missense variant is located at the C-terminus position of POLR3K, predicted to impair residue interactions and cause steric interference in enzyme conformational changes. The large deletion causes loss of the third and last exon of *POLR3K*, leading to a likely amorphic truncated protein product lacking the final 42 amino acids from the C-terminal of the total 108-amino acid length protein. This study provides further evidence for the association of pathogenic variants in *POLR3K* with POLR3-related leukodystrophy, expanding the spectrum of variants in genes encoding for Pol III subunits associated with disease.

## Introduction

RNA polymerase III-related hypomyelinating leukodystrophy (POLR3-HLD), one of the most commonly reported HLDs, is associated with biallelic pathogenic variants in genes encoding subunits of the transcription enzyme RNA polymerase III (Pol III). POLR3-HLD is typically associated with a trio of clinical findings including hypomyelination, hypodontia, and hypogonadotropic hypogonadism, and it is thereby often referred to as 4H leukodystrophy (Bernard & Vanderver, 2017). Patients with POLR3-HLD typically present in early childhood and experience progressive neurological features including motor abnormalities from cerebellar dysfunction with variable pyramidal and extrapyramidal signs, intellectual disability and cognitive impairment, as well as non-neurological features including myopia, short stature, abnormal puberty, and/or dental abnormalities (Gauquelin et al., 2019; Pelletier et al., 2021; Wolf et al.,

2014). The MRI pattern seen in patients with POLR3-HLD is specific, involving diffuse hypomyelination with preservation of specific structures, with or without cerebellar atrophy and thinning of the corpus callosum (La Piana et al., 2014).

Of the 17 Pol III subunit-encoding genes, variants in *POLR3A* and *POLR3B* were initially discovered to cause POLR3-HLD, followed by *POLR1C* and more recently, *POLR3K* (Bernard et al., 2011; Daoud et al., 2013; Dorboz et al., 2018; Saitsu et al., 2011; Tetreault et al., 2011; Thiffault et al., 2015). Indeed, the first study implicating *POLR3K* as a causal gene in 2018 described two unrelated males with hypomyelination and clinically progressive neurological features (Dorboz et al., 2018). These patients harboured the same homozygous variant in *POLR3K*, shown to decrease the expression of certain Pol III transcripts (Dorboz et al., 2018). In this report, we describe the third patient worldwide with pathogenic variants in *POLR3K* and a clinical and radiological phenotype consistent with POLR3-HLD. This study further supports the genetic association of POLR3K hypofunction in causing hypomyelination, thereby demonstrating the importance of proper Pol III complex function during myelin development.

## Methods

### Informed Consent

This research was approved by the Montreal Children's Hospital and McGill University Health Center Research Ethics Boards (11-105-PED; 2019-4972). Informed consent was obtained from all participants/parents.

### Clinical and Genetic Investigations

The available medical records and MRIs were reviewed. Clinical exome sequencing data was obtained and analyzed based on the *American College of Medical Genetics Standards and Guidelines for the Interpretation of Sequence Variants* (Richards et al., 2015), leading to the discovery of the *POLR3K* missense variant, which was confirmed by Sanger sequencing to be paternally inherited. To resolve the large deletion, genome sequencing was completed as previously described (Thiffault et al., 2019) using DNA extracted from saliva collected using the Oragene OG-500 kit, according to the manufacturer's instructions.

### Protein Modelling

Structural analysis of the human Pol III enzyme was achieved using the UCSF ChimeraX molecular visualization program (Pettersen et al., 2021). Presented structures are PDB: 7D58 for the "inside funnel" conformation (Wang et al., 2021), and PDB: 7AEA for the "outside funnel" conformation (Girbig et al., 2021). In the close-up panels, residues shown in stick representation are within 5 Å from the D108 residue. Simulation of the D108Y mutation was achieved using the swapaa command in UCSF ChimeraX.

## Results

### Clinical Presentation

#### Neurological Features

The patient, a female, was born at 36 weeks, weighing 2.045 kg. Both gross and fine motor milestones were achieved during infancy and childhood at the expected age. No dysmorphic features were noted at multiple assessments. She was raised bilingual. As a child, she experienced

learning difficulties and was diagnosed with attention deficit hyperactivity disorder (ADHD), inattentive subtype, which was managed with methylphenidate and mirtazapine. She also experienced behavioural issues and anxiety. She attended a school specializing in children with ADHD, dyslexia, and other neurodevelopmental disorders. From childhood to adolescence, she had several neuropsychological assessments due to her learning difficulties. At 8-years of age, the Wechsler Intelligence Scale for Children – Fourth Edition [WISC-IV (Wechsler, 2003)] resulted in a full-scale IQ of 87, falling in the low-average range. At 11-years of age, her WISC-IV scores showed a full-scale IQ of 73 with a processing speed index (PSI) of 80 and a working memory index (WMI) of 85. At 13-years of age, the WISC-V showed a full-scale IQ of 83 with a PSI of 98 and WMI of 107. At the age of 15-years old, the Kauffman Assessment Battery for Children (KABC-II) showed a nonverbal score of 90, which is within the average range for her age.

Neurological examination remained stable, without regression, long tract signs, or spasticity until late adolescence. Distal muscular hypotrophy was noted in the upper and lower extremities, but muscle strength was normal. Motor and sensory nerve conduction studies were normal at age 10. In adolescence, fine motor skill difficulties were reported. At age 16, she began to experience mild gross motor difficulties, reporting coordination issues while running, with occasional falls. On examination at age 17, she demonstrated mildly increased deep tendon reflexes in the upper extremities, however, she remained stable overall in balance and motor function without significant evidence of regression.

**Non-neurological features**

Regarding non-neurological features, she also experienced growth abnormalities. She had growth delay during early-mid childhood and was treated with growth hormone for a brief 8-month

period at 8 years of age, but stopped due to behavioural changes and after the leukodystrophy findings were first discovered on a standard brain MRI during GH therapy assessment. Though her short stature persisted, she maintained growth velocity, and at age 12, her IGF-1 levels were in the normal range, with increased IGFBP-3 levels. From ages 11 to 17, her height and weight z-score values fell below the general population, with an average height z-score of -1.581 (SD+/-0.205) and an average weight z-score of -1.420 (SD+/-0.297) based on yearly measurements.

She experienced normal puberty, with thelarche and pubarche at age 12 and menarche at age 13. She also had normal levels of TSH, cortisol, FSH, LH, ACTH, estradiol, and prolactin.

She had ophthalmic abnormalities, including posterior polar cataracts bilaterally (treated surgically at age 14) and photosensitivity. Visual acuity remained normal.

Additionally, she had gastrointestinal (GI) problems, including frequent abdominal pain with vomiting throughout childhood. Upper GI endoscopy was diagnostic for gastritis and cobble-stoning of the gastric mucosa, with biopsy significant for lymphoid hyperplasia in the duodenal mucosa. She was also confirmed to be lactose intolerant. In adolescence, intermittent fecal incontinence was reported, which improved over time. Constipation was also noted.

Her levels of direct bilirubin were persistently high, and the homozygous UGT1A1∗28 polymorphism of *UGT1A1* gene (c.-53_-52insTA, rs3064744) was found on clinical investigation. This gene encodes the enzyme bilirubin uridine diphosphate glucuronosyltransferase, and the UGT1A1∗28 variant denotes the presence of an additional TA repeat in the gene's promoter TATA box sequence, known to be associated with hyperbilirubinemia and Gilbert's syndrome (Bosma et al., 1995; Miners et al., 2002; Strassburg, 2008).

**MRI Features**

Brain MRI at 8 years of age revealed hypomyelination, with T2-weighted hyperintensity of the white matter, and variable white matter signal on T1-weighted images, with areas of hyperintensity, isointensity, and mild hypointensity, compared to grey matter structures (Figure 2.1). In a pattern consistent with POLR3-HLD, there was relative preservation of myelination of the optic radiations, anterolateral nucleus of the thalamus, globus pallidus, and dentate nucleus. She also had thinning of the corpus callosum and mild superior cerebellar vermis atrophy (Figure 2.1A, F, K). Besides mild improvement of myelination over time (Figure 2.1C, H, M, E, J, O) and mild cerebral atrophy on her most recent MRI at 17 years of age (Figure 2.1N), her brain MRIs appeared stable throughout childhood to late adolescence.

**Molecular Genetics**

Genetic investigations began with secondary analysis of clinical exome sequencing data on a research basis, leading to the identification of a paternally inherited missense variant in *POLR3K*: c.322G>T; p.D108Y (NM_016310.5). The p.D108Y variant has not been reported in population databases, however, two variants at the same position (p.D108E and p.D108H) each have a single heterozygous individual reported, with the residue being highly conserved between species. A large maternally inherited deletion was also suspected, and genome sequencing resolved the deletion to be approximately 17.8kb, located from chr16:30,362-48,162 (GRCh38/hg38). The upstream deletion breakpoint is located in the final 3' intron of *POLR3K,* leading to deletion of the third and final exon of the gene, with a predicted loss of the final 42 amino acids from the C-terminal of the full 108 amino acid length wild-type protein, and subsequent predicted loss of function. The deleted region following the *POLR3K* gene is largely intragenic and non-translated,

only containing a portion of the long non-coding RNA gene ENSG00000260803 prior to the downstream breakpoint.

As the *POLR3K* missense variant c.322G>T (p.D108Y) is located at the final C-terminal position of *POLR3K* directly adjacent to the termination codon, we sought to investigate its impact on the protein level by modelling the variant onto the human Pol III enzyme (Girbig et al., 2021). POLR3K (also known as RPC10) is a mobile subunit of the Pol III complex, with the ability to adopt two different conformations, either located inside or outside of the polymerase funnel, dependant on the transcriptional state (Figure 2.2A,C). For the conformation in which POLR3K is inserted inside the polymerase funnel (thought to be associated with an active transcription elongation state), the C-terminal domain of the POLR3K subunit is located near the active site of the Pol III enzyme. Simulation of the D108Y variant onto the subunit in this conformation (Figure 2.2B) demonstrates that steric clashing may occur between neighbouring residues such as K96, or that stacking interactions could occur with the F94 residue. When in the outside conformation, the D108Y variant is located on the surface of the complex and does not form contacts with other residues (Figure 2.2C); however, as the subunit must undergo a large conformational change in order to insert into the funnel, it remains possible that the mutation could sterically interfere with this process, causing disruptions to RNA cleavage and/or termination.

## Discussion

This study further expands the spectrum of variants in Pol III genes associated with hypomyelination through the description of a female patient with biallelic pathogenic variants in *POLR3K*, including a missense variant (c.322G>T; p.D108Y), and a large 17.8kb deletion including the last exon of the gene. Moreover, as the large deletion results in the loss of over a

third of the total gene, the resulting product is likely amorphic. Large deletions have been reported in other instances of POLR3-HLD, including those spanning exonic regions of *POLR3B* (Gutierrez et al., 2015). Moreover, as complete loss of Pol III function is incompatible with life, the missense variant is likely hypomorphic, retaining partial function. Protein modelling of this variant showed that it is located at the end of the C-terminus, a region which is located in the active site of the enzyme. Moreover, this variant may cause disruption to the conformational changes that the POLR3K domain undergoes upon changing between active transcription states.

Of the Pol III subunits implicated in POLR3-HLD disease, pathogenic variants in *POLR3K* are rare, with only two patients previously described in the literature, both males from two unrelated consanguineous families who harboured the same homozygous variant (c.121C>T; p.R41W) (Dorboz et al., 2018). These patients demonstrated typical neurological features associated with POLR3-HLD, including cerebellar signs, dystonia, and pyramidal features, with onset in childhood and motor decline. The degree of neurological involvement at an early age was more severe compared to the patient we report, as they experienced severe spasticity before age 10. Our patient presents with a milder phenotype, with learning difficulties throughout childhood evolving to cognitive and mild motor impairments in late adolescence. These phenotypic differences in severity are consistent with the spectrum of disease seen in POLR3-HLD, for which both mild and severe presentations have been reported.

The patient's brain MRI pattern was consistent with that typically seen in POLR3-HLD, involving diffuse hypomyelination and relative preservation of myelin in specific structures, with or without thinning of the corpus callosum and mild cerebellar atrophy. Over time, the MRI pattern appeared stable, without evidence of significant progressive changes from childhood to late adolescence. In contrast, the two other published cases of POLR3-HLD caused by variants in

*POLR3K* demonstrated progressive atrophy of supratentorial and infratentorial structures, with white matter atrophy, between early to late childhood. However, given the spectrum of disease severity often seen in patients with POLR3-HLD, this is not unexpected, as rates of disease progression vary between patients with variants in the same gene (i.e., *POLR3A, POLR3B,* or *POLR1C*). Moreover, it is known that typical POLR3-HLD disease presentations are not strictly driven based on the mutated gene, but may be associated with different progression rates of both clinical and MRI features. This could be further explained by the fact that Pol III is an enzyme complex, where different pathogenic variants may impair subunit function through alternative processes with various levels of complex hypofunction associated with progressive disease phenotypes.

The patient we describe experienced non-neurological features seen in POLR3-HLD, including growth delay as well as ophthalmic abnormalities. She also experienced GI problems; a feature not usually associated with the typical POLR3-HLD phenotype caused by variants in other Pol III subunits. Interestingly, other patients with *POLR3K* variants also experienced digestive dysfunction, and in a Polr3b zebrafish model involving a genetic deletion thought to impact Polr3b-Polr3k interactions, digestive development was significantly impaired (Dorboz et al., 2018; Yee et al., 2007). This may suggest tissue-specific developmental defects are caused by distinct impairments of Pol III function involving the POLR3K subunit. However, in our patient, clinical investigations also demonstrated she harboured a variant in the *UGT1A1* gene, associated with increased bilirubin. The homozygous UGT1A1*28 allele has a relatively high prevalence in the general population, varying from 1-23% depending on ethnicity, with many individuals remaining asymptomatic (Liu et al., 2022; Marques & Ikediobi, 2010; Premawardhena et al., 2003).

In conclusion, the report of this 17-year-old female provides supporting evidence for the association of POLR3K hypofunction with impaired white matter development, further broadening the range of variants associated with POLR3-HLD and demonstrating the critical role of proper transcription machinery function during the important neurodevelopmental stage of myelination.

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## Figures

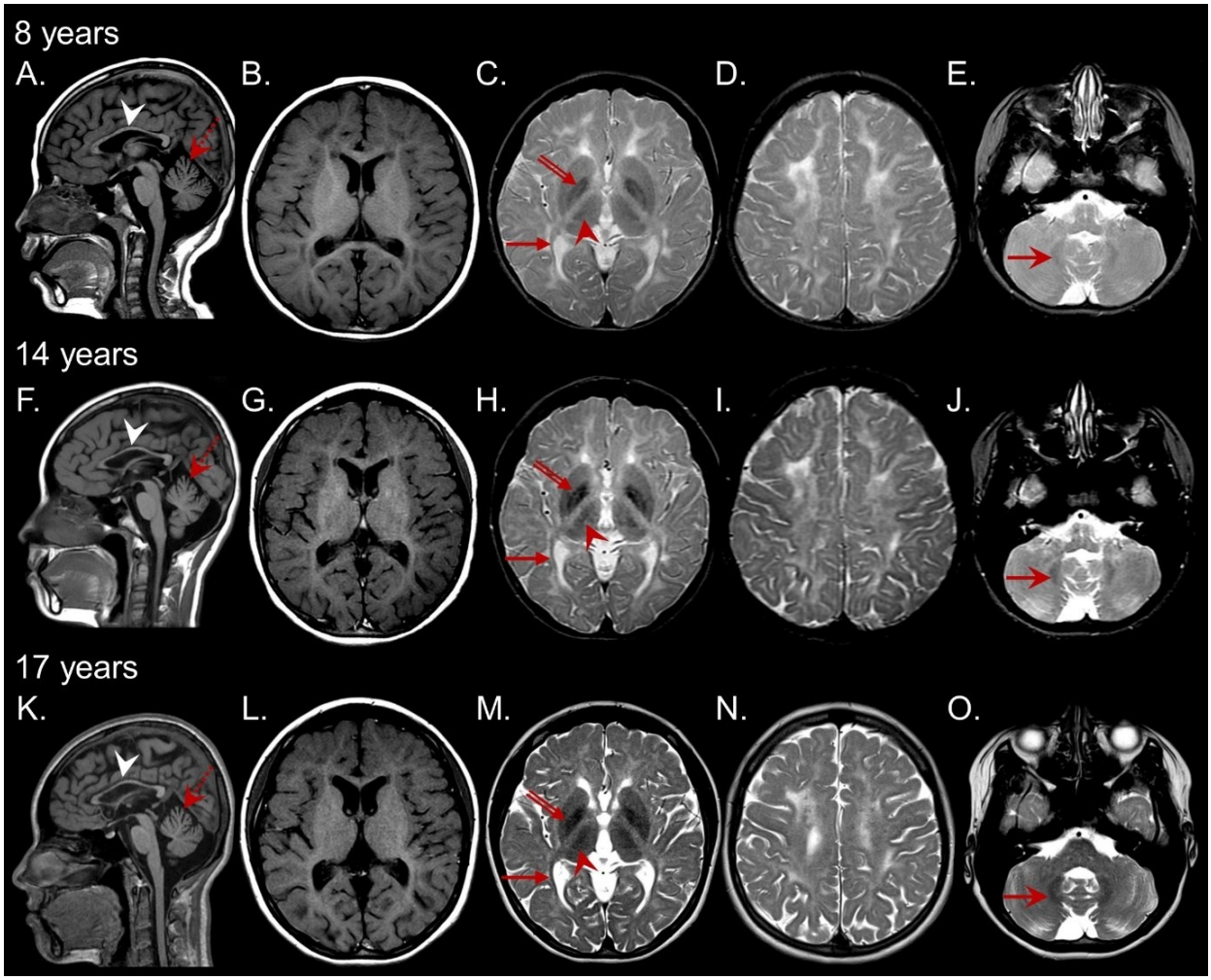


**Figure 2.1. MRI features.** Serial neuroimaging at ages 8, 14, and 17 years, including sagittal T1- (column 1: A, F, K), axial T1- (column 2; B, G, L), and axial T2- (column 3: C, H, M; column 4: D, I, N; column 5; E, J, O) weighted MRI images. Diffuse hypomyelination is evident: there is hyperintensity of the white matter on T2-weighted imaging (C, D, H, I, M, N), together with a variable signal of the white matter on T1-weighted imaging, with hyperintense/isointense/slightly hypointense areas of the white matter (B, G, L), compared to grey matter structures. Relative myelin preservation (T2-hypointensity) is seen in the optic radiations (red arrows in C, H, M), anterolateral nucleus of the thalamus (red arrowhead), globus pallidus (red double-lined arrow), and dentate nucleus (red arrow in E, J, O), with overall mildly improved myelination over time. Mild cerebral atrophy is evident by age 17. A thin corpus callosum (white arrowheads) and mild superior cerebellar vermis atrophy (red dashed arrow) are demonstrated, appearing stable over time (A, F, K).

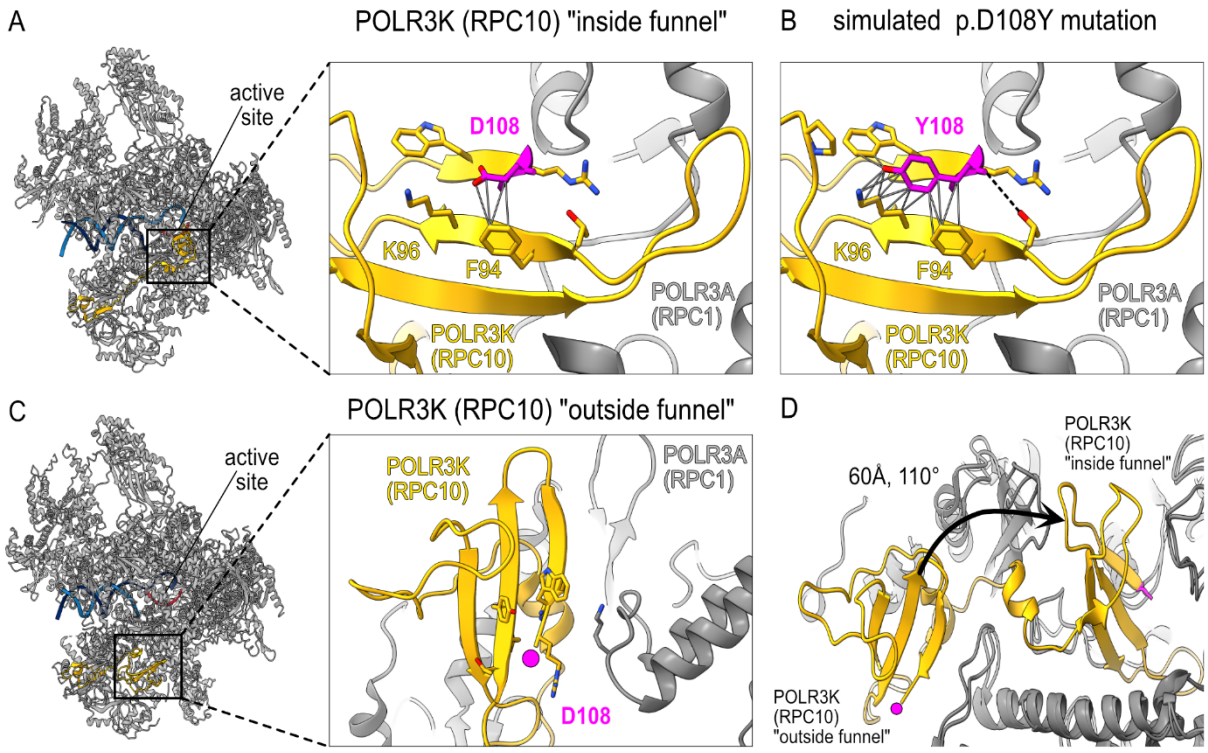


**Figure 2.2. Structural mapping of the *POLR3K* (RPC10) c.322G>T; p.D108Y variant onto the human Pol III enzyme complex (Girbig et al., 2021; Wang et al., 2021).** (A) Structure of the human Pol III complex (PDB: 7D58) with the POLR3K (RPC10) subunit (yellow) shown in the "inside funnel" conformation. (B) Simulation of the D108Y variant (magenta) demonstrating possible interactions with neighbouring residues (F94 and K96), with hydrogen bonds denoted by black dotted lines and contacts/clashes denoted by solid grey lines. (C) Human Pol III complex structure (PDB: 7AEA) with the POLR3K (RPC10) subunit (yellow) shown in the "outside funnel" conformation. Putative location of the D108 residue (not modelled in the structure) is marked with magenta dot. (D) Illustration of conformational change of the POLR3K (RPC10) subunit when inserting into the funnel and active site of Pol III, denoted with black arrow.

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## Chapter 2 Part C. Classifying Hypomyelination: A Critical (White) Matter

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## Classifying Hypomyelination: A Critical (White) Matter

*Response to: Urbik, V.M., Schmiedel, M., Soderholm, H., & Bonkowsky, J.L. (2020). Expanded Phenotypic Definition Identifies Hundreds of Potential Causative Genes for Leukodystrophies and Leukoencephalopathies. Child Neurol Open. 7: 2329048X20939003. doi:10.1177/2329048X20939003*

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**Letter to the Editor**

We read with great interest the publication by Urbik et al. titled "Expanded phenotypic definition identifies hundreds of potential causative genes for leukodystrophies and leukoencephalopathies."[1] We commend the authors for their work on this study, and for constructing such an extensive list of causative genes for genetic white matter disorders. With the utmost respect, we acknowledge the importance of this work, and truly appreciate that phenotype-specific gene lists provide guidance to both clinicians and researchers, especially when considering the diagnostic odyssey of rare inherited neurological disorders. We recognize the value of such publication and anticipate that it may inspire others to delve into the literature to create other phenotype-specific gene lists.

In their study, Urbik et al. further delineated a list of genes associated with hypomyelination based on phenotypic descriptions in currently published articles. However, after examining the literature and MRI features associated with these disorders, we noted discrepancies between the observed MRI phenotypes and proper definition of hypomyelination in a number of cases. Based on MRI patterns, hypomyelination is defined as a mild hyperintense signal on T2-weighted sequences, with variable (i.e. iso-, hyper-, or mildly hypo-intense) signal on T1-weighted sequences of white matter compared to gray matter, signifying deficiencies in myelin development, which must persist on two MRI scans at least 6 months apart if taken before age two years.[2,3] The MRI phenotypes of demyelination, dysmyelination, and delayed myelination differ significantly from this definition, and have been described extensively in the literature.[2-4] Additionally, when considering disease evolution on a clinical level, it is imperative to consider its origin, and whether it should be classified as primarily neuronal (i.e. affecting the gray matter with secondary implications on myelin development), or primarily hypomyelinating (i.e. directly

associated with a deficiency in the formation of myelin).[5,6] Herein, we aim to highlight the importance of properly identifying and classifying hypomyelination on MRI by providing selected examples of genes that should fall under different classifications, such as delayed myelination or nonspecific leukoencephalopathy.

Classic primary hypomyelination is known to be caused by pathogenic variants in a wide range of genes, many of which were appropriately identified in Urbik et al.'s "genes with hypomyelination" list. Examples span from genes encoding for proteins directly associated with myelin formation, such as the structural myelin protein PLP1 or the myelin paranodal junction cell adhesion protein CNTNAP1, to the newly emerging group of hypomyelination-associated transcription/translation-related genes, such as the amino-acyl tRNA synthetase enzymes DARS1, EPRS1, and RARS1 or the transcription enzyme RNA polymerase III subunits POLR3A, POLR3B, POLR1C, and POLR3K. These disorders have varying systemic manifestations, but a clearly identifiable hypomyelination pattern on MRI.

Progression of myelination is the key distinguishing factor between permanent hypomyelination or delayed myelination.[3,4] On a single MRI in early infancy, it can be difficult to conclude whether hypomyelination is indeed present, therefore, it is recommended to evaluate a sequential MRI after 6 months for changes in myelination.[2,3] If myelination improvement is evident, delayed myelination should be diagnosed. We note myelination delay is typical in Allan-Herndon-Dudley Syndrome, caused by pathogenic variants in *SLC16A2*, however, this gene was present on the hypomyelination-associated list by Urbik et al. Another less prominent example is *HIKESHI*, in which pathogenic variants were initially published as causing an "infantile hypomyelinating leukoencephalopathy,"[7] however, upon review of published MRI figures, delayed myelination is in fact evident. We note that incorrect classification of delayed myelination

as hypomyelination is a cause for concern in the literature, which has been highlighted in recent reviews.[8]

Additionally, although we recognize Urbik et al.'s "genes with hypomyelination" list intended to identify all genes associated with some degree of hypomyelination, we would like to stress the importance of documenting whether disorders are truly primary hypomyelinating leukodystrophies or primary neuronal diseases with associated hypomyelination or slowly progressing myelination. We do also appreciate that knowledge on disease pathology is limited in many disorders, making classifications difficult.[5] Additionally, in cases of neuronal diseases, severe atrophy can be present, making it difficult to classify the level of myelin progression, such as with the gene *PRKDC*.[9] The "genes with hypomyelination" list included several diseases that are neuronal in origin, including some associated with epileptic encephalopathies (e.g. *SLC25A12* and *SPTAN1*), or lysosomal storage disorders (e.g. *FUCA1, GLB1, NPC1, NPC2, SGSH*). Notably, some genes with a primary neuronal origin are not associated with hypomyelination, but rather nonspecific leukoencephalopathies, such as *NPC1* and *NPC2*. We also identified *TSC1,* associated with the neurocutaneous disease Tuberous Sclerosis, which we would not classify as a genetic white matter disorder. While we appreciate the depth of Urbik et al.'s hypomyelination gene list, we note one gene associated with a neuronal phenotype and hypomyelinating leukodystrophy, *AIMP1*, was mistakenly excluded.[10]

We would also like to note the presence of genes associated with treatable diseases on this list. We emphasize that screening for the genes associated with these diseases, such as folate transporter deficiency (caused by *FOLR1* variants) and phenylketonuria (caused by *PAH* variants), should be prioritized to mitigate disease progression by confirming the diagnosis and proceeding with treatment as soon as possible.

Finally, we note that some genes on this list could not be completely classified as truly associated with hypomyelination due to the lack of published MRI data. For example, several genes only had one published MRI obtained early in life, making it difficult to distinguish between hypomyelination or delayed myelination. We recommend that classifications are approached with caution if limited data are available, and to seek expert opinion when evaluating MRIs at a young age, if necessary.

To conclude, we reiterate the importance of composing phenotype-specific gene lists as demonstrated by Urbik et al. and stress the importance of proper white matter disorder characterization when considering clinical diagnoses and evaluating disease course. Moreover, incorporating genes causing myelination delay or other white matter diseases on a verified list of true hypomyelinating leukodystrophies could pose concerns during the diagnostic process (i.e. when evaluating variants for pathogenicity based on correlation to phenotype). Additionally, proper characterization of MRI features and corresponding disease classification is important in understanding the disease on a pathophysiological level. Future collaborative studies with detailed evaluation of published MRIs for each considered disorder would be extremely beneficial when considering the generation of widespread phenotype-specific gene lists. In conclusion, we thank Urbik et al. for their detailed study and emphasize the importance of proper classification of subcategories of leukodystrophies and genetically determined leukoencephalopathies.

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# CHAPTER 3

# Endocrine and growth abnormalities associated with POLR3-related leukodystrophy

## Preface

As one of the most common types of hypomyelinating leukodystrophies, the number of patients with POLR3-HLD or 4H leukodystrophy has continued to expand over the past decade since the discovery of the first causal genes, with clinicians and researchers identifying affected individuals around the globe. POLR3-HLD is known to be associated with a combination of neurological and non-neurological features, with growth and endocrine abnormalities being a common finding in this patient population and hypogonadotropic hypogonadism named as a cardinal clinical feature. Although large cohort studies defining the broad phenotypic picture have been completed in the past, endocrine and growth abnormalities had not been investigated systematically. In the following study, we reviewed these features in a large cohort of patients, analyzing their prevalence and whether they correlate with genotype. Furthermore, studying the prevalence and progression of specific features in POLR3-HLD aids in the formation of a complete clinical picture of the typical disease phenotype, further supplying clinicians with the foundation needed to provide evidence-based recommendations for supportive care.

# Chapter 3. Endocrine and growth abnormalities in 4H Leukodystrophy caused by variants in *POLR3A*, *POLR3B*, and *POLR1C*

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# Endocrine and growth abnormalities in 4H Leukodystrophy caused by variants in *POLR3A, POLR3B,* and *POLR1C*

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**Freeform/Key Words:** POLR3-related leukodystrophy, 4H leukodystrophy, hypomyelination, hypogonadotropic hypogonadism

**Abbreviations:** CI, confidence interval; CNS, central nervous system; HGH, human growth hormone; LHRH, luteinizing hormone releasing hormone; MRI, magnetic resonance imaging; POLR3, RNA polymerase III.

## Abstract

**Context:** 4H or POLR3-related leukodystrophy is an autosomal recessive disorder typically characterized by hypomyelination, hypodontia, and hypogonadotropic hypogonadism, caused by biallelic pathogenic variants in *POLR3A, POLR3B, POLR1C,* and *POLR3K*. The endocrine and growth abnormalities associated with this disorder have not been thoroughly investigated to date.

**Objective:** To systematically characterize endocrine abnormalities of patients with 4H leukodystrophy.

**Design:** An international cross-sectional study was performed on 150 patients with genetically confirmed 4H leukodystrophy between 2015 and 2016. Endocrine and growth abnormalities were evaluated, and neurological and other non-neurological features were reviewed. Potential genotype/phenotype associations were also investigated.

**Setting:** This was a multicenter retrospective study using information collected from 3 predominant centers.

**Patients:** A total of 150 patients with 4H leukodystrophy and pathogenic variants in *POLR3A, POLR3B,* or *POLR1C* were included.

**Main Outcome Measures:** Variables used to evaluate endocrine and growth abnormalities included pubertal history, hormone levels (estradiol, testosterone, stimulated LH and FSH, stimulated GH, IGF-I, prolactin, ACTH, cortisol, TSH, and T4), and height and head circumference charts.

**Results:** The most common endocrine abnormalities were delayed puberty (57/74; 77% overall, 64% in males, 89% in females) and short stature (57/93; 61%), when evaluated according to physician assessment. Abnormal thyroid function was reported in 22% (13/59) of patients.

**Conclusions:** Our results confirm pubertal abnormalities and short stature are the most common endocrine features seen in 4H leukodystrophy. However, we noted that endocrine abnormalities are typically underinvestigated in this patient population. A prospective study is required to formulate evidence-based recommendations for management of the endocrine manifestations of this disorder.

## Introduction

Leukodystrophies are a group of rare genetic diseases characterized by abnormal white matter in the central nervous system (CNS), which often result in progressive neurodegeneration and premature death (1, 2). Based on whether the white matter abnormalities seen on brain magnetic resonance imaging (MRI) are caused by insufficient initial myelin deposition or altered myelin homeostasis, leukodystrophies can be classified as hypomyelinating or non-hypomyelinating, respectively (3-5). 4H leukodystrophy, also known as RNA polymerase III (POLR3)-related leukodystrophy, is an autosomal recessive hypomyelinating leukodystrophy associated with several characteristic neurological and non-neurological clinical features, primarily including hypomyelination, hypodontia, and hypogonadotropic hypogonadism (6). Commonly presenting neurological signs include cerebellar manifestations, such as ataxia and dysmetria, as well as pyramidal, extrapyramidal, and cognitive features (5, 7). Non-neurological manifestations can include myopia and endocrine features such as growth hormone (GH) deficiency and short stature (7-12). 4H leukodystrophy is also typically associated with a unique MRI phenotype, including cerebellar atrophy, progressive thinning of the corpus callosum, and diffuse hypomyelination with relative preservation of specific structures, namely the dentate nucleus, optic radiations, anterolateral nucleus of the thalamus, globus pallidus, and corticospinal

tracts at the level of the posterior limb of the internal capsule (13, 14). 4H leukodystrophy has been found to be caused by biallelic pathogenic variants in *POLR3A, POLR3B, POLR1C,* and *POLR3K*, each of which encode subunits of the POLR3 complex (15-18).

As this class of leukodystrophies has been discovered relatively recently, secondary features that are typically associated with this phenotype have not been comprehensively described. This study presents a thorough investigation of the endocrine and growth abnormalities associated with 4H leukodystrophy through an international cross-sectional retrospective study of 150 patients with a molecular confirmation of the diagnosis. Moreover, this study provides insight on the endocrine disorders associated with this disease, and how some endocrine abnormalities may have an impact on patients' quality of life, thus highlighting the importance of considering endocrine therapeutic options and the associated impact on medical care.

## Materials and Methods

### Patients and study design

An international cross-sectional study was performed between 2015 and 2016 on a cohort of 150 patients (76 females, 74 males) with biallelic pathogenic variants in *POLR3A, POLR3B,* or *POLR1C* and hypomyelination on brain MRI. Chart review focused on endocrine data, including any available hormonal investigations and pubertal history information, as well as growth data, including height and head circumference. Other clinical features, including age at disease onset, genotype, and both neurological and non-neurological features were reviewed. This project was approved by the research ethics committee of the Montreal Children's Hospital (11-105-PED), Canada; the Children's Hospital of Philadelphia, USA; and the VU University Medical Center in Amsterdam, Netherlands. Informed written consent was obtained from all participants or their

legal guardians. Several patients have been previously published in studies describing the genetic basis of the disease or in the delineation of other clinical features.

**Pubertal status**

In females, to evaluate abnormalities in pubertal development, we primarily assessed age at menarche and considered puberty delayed if menarche had not occurred by the 16th birthday. We lacked information on breast development for most patients; however, for those that had information available, we evaluated Tanner stage of breast development at age 13 (persistence of Tanner stage 1). Of note, we considered menarche as the main criteria for evaluating abnormal puberty (i.e., if patients had delayed or absent menarche, this feature took priority over breast development stage). In males, we assessed Tanner staging for testicular growth, and considered puberty delayed if Tanner stage 1 for testicular growth persisted at age 14.

**Growth data**

Data sets of patients' height and head circumference at all available times from birth to their latest available visit were reviewed and standardized according to the appropriate sex and age references.

**Endocrine investigations**

For patients whose information was available, we retrospectively reviewed levels of estradiol, testosterone, stimulated LH and FSH, stimulated GH, IGF-I, prolactin, ACTH, cortisol, TSH, and free T4.

**Statistical analysis**

Descriptive statistics were produced for all studied parameters, including the median and minimum/maximum values for continuous variables and the count and percentage for categorical variables. For the latter, 95% confidence intervals (95% CI) were also produced using the normal approximation method.

For the growth analysis, the percentiles for each measure were determined using the World Health Organization growth charts. To compare height data accounting for age and sex, Z-scores were calculated using values and standard deviations from the World Health Organization child growth standards. Because 95% of the normal population are within 2 SDs from the mean, short stature was defined as a height value below 2 SDs for that corresponding age and sex. Similarly, for head circumference data, microcephaly was defined as a head circumference that was 2 SDs or more below the mean for that corresponding age and sex, whereas macrocephaly was defined as greater than 2 SDs above the mean.

The association between genotype and phenotype was assessed for exploratory purposes with the $\chi^2$ test. Analyses were conducted using SPSS software, version 24 (Armonk, NY: IBM Corp.).

**Pituitary gland pathology**

Pituitary gland pathology of one patient with 4H leukodystrophy and pathogenic variants in *POLR3A*, who had died of respiratory complications at age 19, was investigated via immunohistochemical staining of the adenohypophysis. Five-micrometer-thick vertico-frontal sections of the hypophysis were analyzed with immunostaining for FSH (Agilent catalog no. M3504, RRID: AB_2079146, dilution 1/400), LH (Agilent catalog no. M3502, RRID:

AB_2135325, dilution 1/400), prolactin (Cell Marque catalog no. 210A-18, RRID: AB_1516984, dilution 1/250), ACTH (Agilent catalog no. M3501, RRID: AB_2166039, dilution 1/1000), TSH (Agilent catalog no. M3503, RRID:AB_2287785, dilution 1/300), and human growth hormone (HGH; Aglient catalog no. A0570, RRID:AB_2617170, dilution 1/3000).

## Results

### Molecular genetics and clinical features

Within our cohort of 150 patients, 56 had pathogenic variants in *POLR3A*, 81 in *POLR3B,* and 13 in *POLR1C*. Seventy-six patients were females and 74 were males (Table 3.1). Both neurological and non-neurological features were noted in the patient cohort. Many patients demonstrated ataxia (94%; 49/52), dysarthria (85%; 34/40), and dystonia (84%; 26/31). Other features can be seen in Table 3.1. Non-neurological features were also evident, including myopia (87%; 90/103) and teeth abnormalities (85%; 99/117).

### Reproductive hormones and pubertal development

#### Female patients

Delayed puberty was reported in 89% (34/38; 95% CI, 80-99%) of female patients based on the clinical judgment of the treating physician. In analyzing our cohort based on the clinical information that was available for menarche and breast development, 21/25 patients (84%; 95% CI, 70-98%) were considered to have absent, delayed, or arrested puberty. Therefore, most females in our cohort presented with abnormal puberty and differing severities of early-onset hypogonadism.

Of the female patients tested for specific hormone levels, 13/19 (68%; 95% CI, 48-89%) had low levels of estradiol. Luteinizing hormone releasing hormone (LHRH) stimulation tests were performed on 13 female patients, of whom 8 (62%; 95% CI, 35-88%) had abnormally low levels of both LH and FSH. In regard to sex steroid treatment, information was available for 29 patients, of whom 20 (69%) received treatment and 9 (31%) did not. Sex steroid medications induced menstruation and/or puberty in 9/20 (45%; 95% CI, 23-67%) female patients. In one patient, treatment was clearly ineffective, and in several cases, sex steroids were not well tolerated and caused adverse reactions. Of the untreated patients, 6/9 (67%) had abnormally low sex steroid levels, and of the 2/9 (22%) patients with normal levels, one had a clinical diagnosis of delayed puberty. Only 4 of 13 patients who had menarche experienced it spontaneously with no need for sex hormone treatment, at a median age of 12 years (minimum age, 11 years; maximum age, 13 years; n = 4). In patients who were treated with sex hormones, menarche occurred at a median age of 18 years (minimum age, 16 years; maximum age, 32 years; n = 9). For those who experienced treatment-induced menarche, information on spontaneous breast development was only available for 2 patients, who reached onset of breast development at ages 12 and 19 years old. Of the patients for which menarche did not yet occur, 3/4 (75%) were treated with sex hormones, one of which was only 16 years old at the time of data collection. The remaining patient who did not experience menarche had not been treated with sex hormones (1/4; 25%). Summarized results are shown in Fig. 3.1.

**Male patients**

Overall, less information was available regarding puberty of males compared to females. Based on the physician's clinical judgment, 23/36 (64%; 95% CI, 48-80%) male patients had

delayed puberty. In analyzing our cohort based on the definition of delayed puberty (persistence of Tanner stage 1 for testicular growth at age 14), 6/7 (86%; 95% CI, 60-100%) were considered to have delayed puberty. Many patients lacked information on Tanner staging in adolescence and therefore could not be assessed for early pubertal abnormalities.

Of the male patients in our cohort tested for sex steroid levels, 11/14 (79%; 95% CI, 57-100%) had abnormally low testosterone levels. All patients with low testosterone also presented with delayed puberty (7/7, 100%). LHRH stimulation tests revealed abnormally low levels of LH in all patients that were tested (5/5, 100%). Information regarding sex steroid treatment was available for 23 male patients; approximately one-half of the patients (11/23; 48%) were treated, whereas 12/23 (52%) were not. Nearly all the males who received sex steroid medication were treated with testosterone (10/11, 91%), with the exception of one who was treated with chorionic gonadotropin (1/11, 9%). Treatment, however, was only effective in 5 patients (5/11; 45%; 95% CI, 16-75%), including 4 patients who received testosterone and the single patient who received chorionic gonadotropin treatment. Results are summarized in Fig. 3.1.

**Growth analysis**

Linear growth data were obtained for 115 patients and analyzed using Z-scores. The Z-score for all ages ranged from -4.76 to 1.70 (median, -1.48). To determine whether growth was more severely affected in participants of certain ages, median Z-scores for heights of different age groups of patients were evaluated. For each age group range, an average Z-score was calculated for each patient based on all height data if multiple records were available. Some participants are represented in multiple age groups if records were available spanning different ranges. Z-scores for ages < 5 years (n = 42), 5 to 9 years (n = 37), 10 to 14 years (n = 28), and ≥ 15 years (n = 57)

were found to be -0.56, -1.86, -1.83, and -1.16, respectively. Figure 3.2 shows boxplots of height Z-scores for all patients and for each age group. Across age groups, patients < 5 years of age had a median Z-score closest to that of the general population (0). Additionally, the < 5 years age group had a positive Z-score for the third quartile (0.31), whereas the other age groups (5-9 years, 10-14 years, and ≥ 15 years) each had negative third quartile values (-0.89, -0.57, and -0.62, respectively). Moreover, the maximum height Z-score of the < 5 years group (3.16) corresponds to the maximum value of the entire cohort, and the minimum Z-score for the < 5 years group (-3.23) is closest to 0 when comparing minimum values between all groups. These results suggest that the < 5 year age group seems to be least affected in regard to stature. In contrast, the 5 to 9 year age group is most affected as it has the lowest values for the maximum and median Z-score values of all groups. Moreover, within the 5 to 9 year age group, the median height Z-score was -1.86 and the Z-score of the first quartile was -2.76, where more than one-quarter of these patients had short stature.

Based on the clinical judgment of the treating physician, 57/93 patients (61%; 95% CI, 51-71%) were considered to have short stature. When analyzing the Z-scores of patients' heights, 53/115 patients (46%; 95% CI, 37-55%) had values lower than 2 SDs and thus by clinical definition had short stature. Of 115 patients, 67 (58%; 95% CI, 49-67%) were also reported to have a height > -1.5 SD below the mean. Additionally, 68% of these patients (78/115; 95% CI, 59-76%) had a height lower than 1 SD below the mean. Thus, even if some patients did not meet the criteria for the clinical definition of short stature, our cohort seems to be smaller than the general population.

Head circumference data were analyzed using Z-scores in patients aged 0 to 5 years (n=35). The Z-scores ranged from -2.93 to 2.44 (median, -0.04). Twenty patients (20/35, 57%; 95% CI,

41-74%) presented with a head circumference within 1 SD from the mean, whereas 31 patients (31/35, 89%; 95% CI, 78-99%) were within 2 SDs from the mean. Three patients (3/35, 9%; 95% CI, 0-18%) had values lower than 2 SDs below the mean and thus by clinical definition had microcephaly. Additionally, 14% of patients (5/35; 95% CI, 3-26%) were reported to have a head circumference lower than 1.5 SDs below the mean, and 34% (12/35; 95% CI, 19-50%) had a head circumference lower than 1 SD below the mean. One patient (1/35, 3%; 95% CI, 0-8%) met the criteria for macrocephaly, with a head circumference greater than 2 SDs above the mean.

Of our cohort of 150 patients, data regarding growth hormones were available for 12 patients, of whom 7 had a diagnosis of GH deficiency based on the clinical judgment of the treating physician (7/12, 58%; 95% CI, 30-86%). Of these 7 patients who were diagnosed with a GH deficiency, only 2 had a GH stimulation test, where one had a decreased response and the other presented with a normal response. Many patients only had a single measurement of GH; however, these results could not be analyzed as GH is secreted in a pulsatile manner, and therefore nonstimulated levels do not provide useful information for analysis. In total, 5 patients in our cohort had a GH stimulation test, in which 3 exhibited a decreased response (3/5, 60%; 95% CI, 17-100%), and the remaining 2 a normal response. Additionally, only 2 patients were treated with GH, which was ineffective in both cases. IGF-I values were only available for 27 patients, of which 19% presented with a low value (5/27, 19%; 95% CI, 4-33%). For those that had both a GH stimulation test and IGF-I levels measured, 2 patients had low levels of both tests, one had normal results for both, one had a normal GH stimulation test and low IGF-I levels, and one had a low GH stimulation test and normal IGF-I levels.

**Other endocrine abnormalities**

In patients with abnormal prolactin levels (9/22, 41%; 95% CI, 20-61%), values were found to vary in both high and low ranges. Of these patients, 4 had high levels of prolactin (4/22, 18%; 95% CI, 2-34%), wherein 3 had levels at least 50% higher than normal (3/22, 14%; 95% CI, 0-28%). In contrast, 5 patients had low prolactin levels (5/22, 23%; 95% CI, 5-40%), of whom one had levels at least 50% lower than normal (1/22, 4.5%; 95% CI, 0-13%).

Nearly all patients who were tested for cortisol levels had results within the normal range (20/21, 95%; 95% CI, 86-100%). Additionally, most patients who were tested for ACTH levels displayed normal results (8/9, 89%; 95% CI, 68-100%). The single patient with abnormal cortisol levels presented with an elevated level (1/21, 5%; 95% CI, 0-14%), although his ACTH level was not tested. The single patient who displayed high ACTH levels (1/9, 11%; 95% CI, 0-32%) had normal cortisol levels.

Thyroid function was tested in 59 patients, where 13 showed abnormal results (13/59, 22%; 95% CI, 11-33%). Approximately 10% of the patients (6/59, 95% CI, 2-18%) had abnormal TSH levels, including 2 patients with low TSH levels (2/59, 3%; 95% CI, 0-8%), and 4 patients with high TSH levels (4/59, 7%; 95% CI, 0-13%). Of the 47 patients tested for free T4 levels, 5 showed abnormal results (5/47, 11%; 95% CI, 2-19%), including 3 with high levels (3/47, 6%; 95% CI, 0-13%), and 2 with low levels (2/47, 4%; 95% CI, 0-10%).

One patient with low TSH levels, but normal T4 levels, was diagnosed with subclinical hyperthyroidism. One patient had low T4 levels and high TSH levels, which is consistent with hypothyroidism. Additionally, one patient with an unknown TSH level, and a low free T4 level was diagnosed with hypothyroidism. Only one patient was treated with thyroid hormones, but no

data were available regarding his hormonal levels. A summary of hormone levels is presented in Table 3.2.

**Relationship between genotype and endocrine abnormalities**

According to different genotypes (i.e., whether pathogenic variants were present in *POLR3A, POLR3B,* or *POLR1C*), the presence of delayed puberty and short stature in patients was analyzed as these features were most prevalent in our patient cohort. In terms of delayed puberty, significant differences were observed between genotypes ($p < 0.001$), with the highest incidence observed in patients with pathogenic variants in *POLR3A* (27/32, 84%; 95% CI, 72-97%), followed by those with variants in *POLR3B* (30/38, 79%; 95% CI, 66-92%). None of the patients with pathogenic variants in *POLR1C* (0/4; 0%) who had reached the appropriate age exhibited delayed puberty. Of the patients with pathogenic variants in *POLR3A*, 71% (22/31; 95% CI, 55-87%) had short stature, compared with 54% (32/59; 95% CI, 42-67%) with variants in *POLR3B*, and 100% (3/3) with variants in *POLR1C* ($p = 0.113$). Data on specific hormone measurements were limited, however, an analysis of levels of stimulated GH, prolactin, TSH, and free T4 between genotypes is also presented in Table 3.2.

**Pituitary gland pathology**

Pathological investigations were performed following autopsy of a 19-year-old patient who was homozygous for the *POLR3A* pathogenic variant c.2015G>A (p.G672E). Clinically, the patient did not show signs of puberty and was reported to have hypogonadotropic hypogonadism; however, results of specific hormone levels were not available. He also had short stature, falling in the 5th percentile for height at age 18 years. The patient demonstrated typical neurological

features associated with 4H leukodystrophy, including ataxia with abnormal gait, tremor, dystonia, spasticity, and dysarthria. MRI scans revealed diffuse hypomyelination with cerebellar atrophy and a thin corpus callosum, consistent with the pattern for 4H leukodystrophy. He also had epilepsy, with complex partial seizures. Dentition was abnormal, with notable hypodontia. Ocular abnormalities included myopia, mild optic nerve atrophy, and esotropia. With age, chronic progressive decline in neurological function was evident, along with decline in motor ability. He was wheelchair bound at age 8 years, eventually becoming quadriplegic with increased tone in all extremities. He had dysphagia, with frequent choking episodes, and progressively lost the ability to eat unaided, further requiring a gastrostomy. The patient had recurrent aspiration pneumonias and died at the age of 19 from complications of bilateral pneumonia. Immunohistochemical analysis of the anterior pituitary gland revealed an absence of immunostaining of anti-FSH and anti-LH antibodies (Fig. 3.3) and normal immunostaining for anti-GH, anti-TSH, anti-prolactin, and anti-ACTH antibodies. This demonstrates an absence of secretion of gonadotropic hormones (FSH and LH) by the pituitary gland.

## Discussion

Our study confirms that endocrine impairment is frequent in patients with 4H leukodystrophy and although limited data were available for the entire cohort of patients, our results reveal notable information regarding abnormalities in the pituitary-gonadotrophic axis. Delayed puberty was a common finding in our patient population. However, LHRH stimulation tests were only performed on a small percentage of patients to confirm hypogonadotropic hypogonadism. It should be noted that baseline FSH and LH levels are not useful for the diagnosis of hypogonadotropic hypogonadism, and stimulation tests should be performed for an accurate

result. There are currently no guidelines for the introduction of sex steroid treatment in patients with 4H leukodystrophy. Still, we would recommend LHRH stimulation tests to confirm the diagnosis before initiating treatment. Thus, a pediatric endocrinologist should be included in the multidisciplinary team assessing patients with 4H leukodystrophy.

Treatment of hypogonadotropic hypogonadism remains controversial in this patient population; although there are significant benefits, there are also associated risks. One significant treatment advantage is the promotion of bone health by influencing bone remodeling. Another potential benefit is the physical appearance in a period of life where being similar to his or her peers is important. Such a factor is typically not a consideration for severely neurologically impaired children. Treating hypogonadotropic hypogonadism would allow the development of secondary sexual characteristics associated with normal pubertal development, and also induce a growth spurt, which might enhance motor difficulties and behavioral problems. Currently, little information is available in the literature regarding this treatment and its effects on this patient group. It was previously suggested that the same principles of hormone replacement therapy used in patients with other forms of hypogonadotropic hypogonadism should be applied to patients with 4H leukodystrophy (10). To induce puberty, sex steroids (testosterone for boys and estrogen/progesterone for girls) are the first line of therapy for patients with hypogonadotropic hypogonadism. If fertility induction is intended, pulsatile GnRH therapy could be tried, however, may not be effective, as some individuals with hypogonadotropic hypogonadism respond poorly to short-term stimulation (19). In this case, treatment with recombinant gonadotrophins could provide an alternative option. In this cohort, sex steroid treatment did not appear to be effective in all cases, however, it could not be established by what criteria response was judged, how long treatment was pursued, and/or by what dose. Collecting additional prospective data on sex

hormone treatment for delayed puberty would allow better ascertainment of these situations and allow clinicians to provide further informed recommendations. In the meantime, we recommend that the decision to initiate sex steroid treatment is approached on an individual basis, while weighing the benefits (e.g., bone health) and disadvantages (e.g., rapid growth spurt with motor regression), together with the overall health of the patient (e.g., well vs. severely impaired) and only after measure of abnormal sex hormone levels confirms the diagnosis.

Pathological investigations of an affected patient confirmed dysfunction in the sex steroid axis as an absence of FSH and LH secretion was observed in the anterior pituitary gland. Low response to LHRH stimulation tests, observed in 72% of our patients, also supports the hypophyseal origin (20). Thus, it is likely that abnormal levels of FSH and LH are a result of pituitary gland malfunction, resulting in central hypogonadism.

Limited data were available regarding stimulated GH levels in our cohort of patients. Based on our results, decreased GH secretion could be frequent in patients with 4H leukodystrophy; however, very few regularly had levels of stimulated GH measured. Moreover, a GH stimulation test is necessary for the diagnosis of GH deficiency; random measurements of GH are not diagnostic if low and can only by useful to rule out a deficiency if high. Therefore, it is recommended that every patient with an abnormally low growth velocity should be tested with a GH stimulation test. Our data are insufficient to conclude which percentage of patients with 4H leukodystrophy would need a GH stimulation test; before we can make a general recommendation, further analysis of a larger cohort is required. As with any other child, height should be recorded and plotted at least once a year and more frequently in young children. GH treatment was reported to be ineffective in 2 patients who were treated in our cohort; however, it can be difficult to form conclusions based on these findings. GH treatment can often fail because of extraneous factors,

such as nutrition, psychological aspects, and general state of health. Additionally, limitations of testing have been reported in establishing a firm diagnosis of GH deficiency, with a high rate of false-positive diagnoses reported in the literature (21). It is also possible that late sex steroid therapy could contribute to an impaired pubertal growth spurt and mislead the conclusion of an eventual lack of efficacy of GH treatment. Our findings support the early consultation and regular follow-up with an endocrinologist, especially in patients with short stature.

When analyzing growth data according to age group, it was found that young patients < 5 years of age were least affected by abnormal growth. This finding is expected given that the typical age of disease onset is during the second year of life. Growth was most affected in children 5 to 9 years of age, followed by children 10 to 14 years of age. We initially hypothesized that growth would be most affected in patients ≥ 15 years of age because of the frequent occurrence of delayed puberty. Per the recommendations for treatment, patients with delayed puberty are not usually treated before age 14; it is therefore possible that our cohort of patients age 5 to 9 years are most affected by growth abnormalities because older patients may have received sex steroids to stimulate puberty and growth, thereby compensating for the deficit caused by this disease. Indeed, in our cohort of patients ≥ 15 years of age, 22 patients received treatment of sex steroids and/or growth hormone. These treatments may have stimulated their growth and puberty. Therefore, the growth data of 22/57 patients may have been affected by a treatment that helped in compensating their short stature. It is clear that, as a whole, patients with 4H leukodystrophy are smaller compared to the general population within each analyzed age group. Thus, growth and height data should be collected by treating physicians so that abnormalities can be clearly identified, thereby facilitating more rapid treatment interventions. If growth anomalies are observed, GH stimulation tests should be considered. In future studies, it would be interesting to systematically follow

growth and perform comparisons to bone age in individual patients to identify the subgroups of patients who would benefit most from GH replacement.

When analyzing head circumference data in our cohort of patients < 5 years of age, microcephaly seems to be more prevalent (9%) compared with the general population, in which there is a prevalence of 2% (22, 23). However, head circumference data were collected only for a limited number of patients. Microcephaly is typically not observed in the context of 4H leukodystrophy; only one case report describes this condition in 2 female siblings with a novel phenotype of 4H leukodystrophy with polymicrogyria and cataracts, who were also included in this study but not analyzed for head circumference because of a lack of head circumference measurements (24). As our results show an increased prevalence of small head size in the young age group < 5 years old, it would be interesting to further analyze additional data on head circumference to better characterize head size in 4H leukodystrophy.

A high percentage (41%) of patients with 4H leukodystrophy were found to have abnormal prolactin levels, where variability was seen in elevated (18%), or deficient (23%) levels. Limited information is available on the typical prolactin levels in patients with 4H leukodystrophy; 2 case reports have previously reported low prolactin levels in 2 patients, who were also included in this study (11, 12). Notably, prolactin values should be interpreted with the consideration of current treatments as some medications are known to cause hyperprolactinemia (e.g., psychoactive drugs). Because the full medication records of all patients in our cohort were not available, the interpretation of elevated results should be approached with caution. However, hypoprolactinemia does not commonly result from medications. It is known that the 2 previously published patients in our cohort with hypoprolactinemia (11, 12) were not under any treatments that could have interfered with testing, and their prolactin deficiency could not be explained by any known causes.

Therefore, this finding provides foundation for the attribution of abnormal prolactin levels to 4H leukodystrophy itself.

Most patients in our cohort presented with normal random cortisol levels (20/21; 95%), except for one whose levels were elevated. ACTH levels were also normal for most patients tested (8/9, 89%). This suggests that adrenal dysfunction is not a common feature in our cohort; however, stimulation tests would be needed to fully conclude normal adrenal function in this patient population. Of note, random cortisol levels should be interpreted with caution, as cortisol secretion follows a circadian rhythm.

The prevalence of thyroid dysfunction appears to be increased in the 4H leukodystrophy population compared with the unaffected population. However, diverse abnormal variations of TSH and T4 hormones levels were observed in our cohort, suggesting that it is not associated with a typical disease phenotype. In the general pediatric population, hypothyroidism is the most common dysfunction with a prevalence of 0.135% in young people (<22 years of age) (25). Compared with this, in our cohort, 4% of patients had hormone levels consistent with hypothyroidism.

Our results do not suggest a strong genotype-phenotype correlation between patients with biallelic pathogenic variants in the different genes associated with 4H leukodystrophy and short stature. However, a significant difference was observed between genotypes when considering delayed puberty. Patients with variants in *POLR3A* had the highest incidence of delayed puberty, followed by those with variants in *POLR3B*. Although no patients in our cohort with pathogenic variants in *POLR1C* demonstrated delayed puberty, data on only 4 patients were available and further analysis is required before definitive conclusions are formed. Additionally, because most patients in our cohort were compound heterozygous for different variants in each gene, and

variants are located across different protein domains, it is difficult to make detailed phenotype-genotype correlations between specific pathogenic variants and clinical features.

It is important to be mindful that patients with 4H leukodystrophy are at risk for endocrine abnormalities, most commonly hypogonadotropic hypogonadism and GH deficiency. Thus, the treating physicians should have a high index of suspicion when signs or clinical symptoms appear. Measurements of relevant hormone levels and stimulation tests when necessary should be made to confirm the diagnosis. Finally, the decision of whether to treat should be evaluated on an individual basis while considering the advantages and disadvantages as no definitive recommendations currently exist.

In summary, endocrine abnormalities are underinvestigated in patients with 4H leukodystrophy. A future study is required to investigate the full extent and severity of typical endocrine abnormalities. Additional data on the evolution of growth, evaluated by regular height measurements, are necessary. Because hypodontia and teeth abnormalities are commonly seen in 4H leukodystrophy, it is also important to continue to monitor dental growth and the development of permanent tooth sets. An additional interesting aspect for future assessment could involve bone age and density and their correlations with growth development and puberty. To fully determine the prevalence of delayed puberty, clear information about the stage of puberty, age of menarche and breast development, level of testicular growth, and Tanner stage of development is needed. Additional data on endocrine hormone levels would also allow a more comprehensive evaluation of the prevalence of endocrine anomalies. In this study, data to evaluate the described growth and pubertal measures were only available for a limited number of patients. This presents a limitation and raises difficulty in drawing precise conclusions. In this sense, it is also possible that puberty and growth anomalies could have been recorded more commonly in patients with abnormal clinical

findings, thus raising the frequency of these alterations in our cohort. With an expanded data set, a future objective would be to formulate evidence-based recommendations regarding the management of the endocrine manifestations of 4H leukodystrophy. In conclusion, this is the first study to systematically analyze endocrine abnormalities in a large cohort of patients affected by 4H leukodystrophy and provides the foundation for future comprehensive studies.

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## Data Availability

The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

## Figures

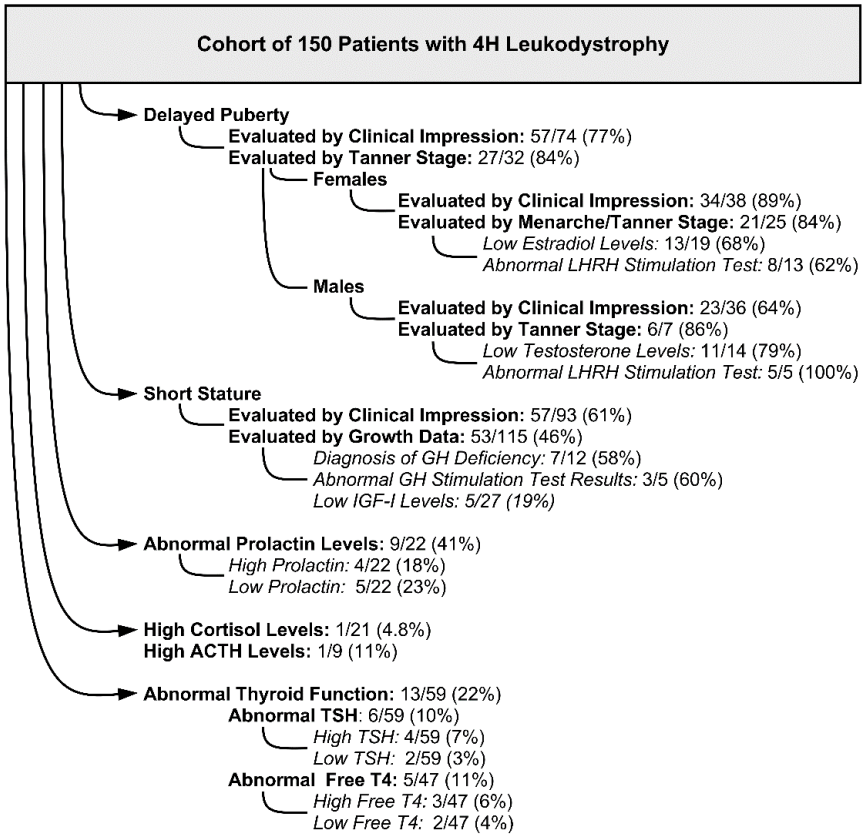


**Figure 3.1.** Summary of endocrine abnormalities in this cohort of patients with 4H leukodystrophy, according to the available clinical information on growth and endocrine features.

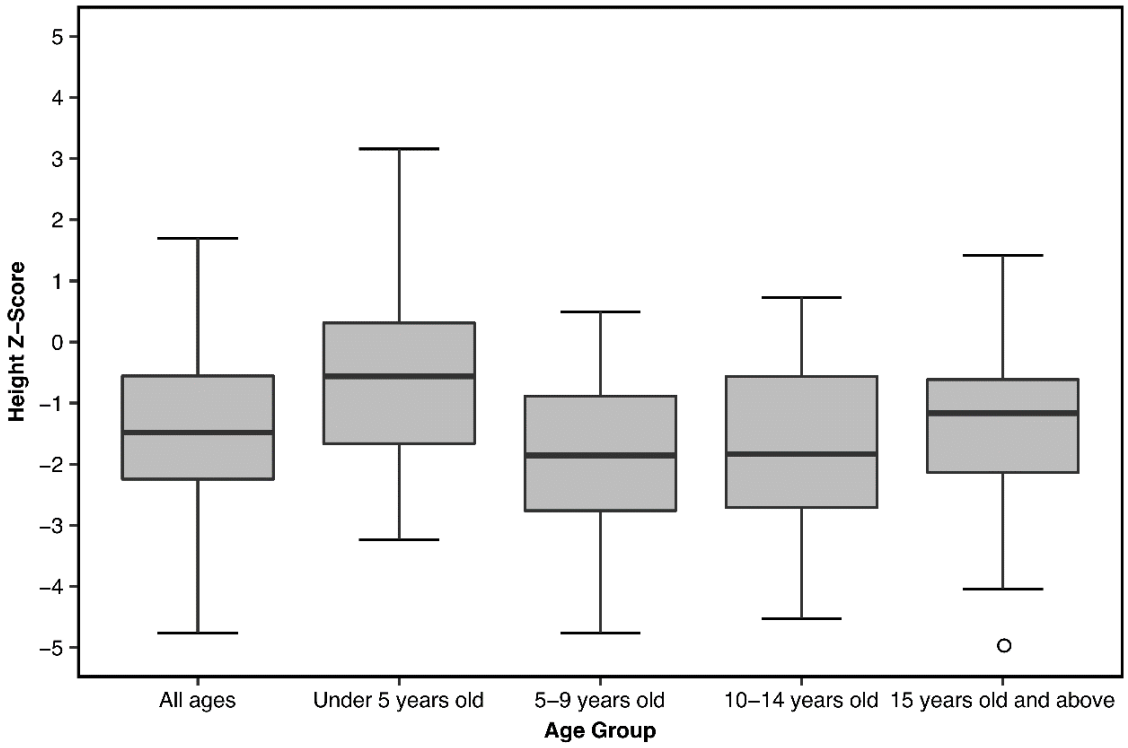


**Figure 3.2.** Box plots for Z-scores obtained for the mean height in different age categories. The height z-score for the general population (mean value) is considered to be zero (0), and the clinical definition for short stature is two standard deviations below the mean (-2).

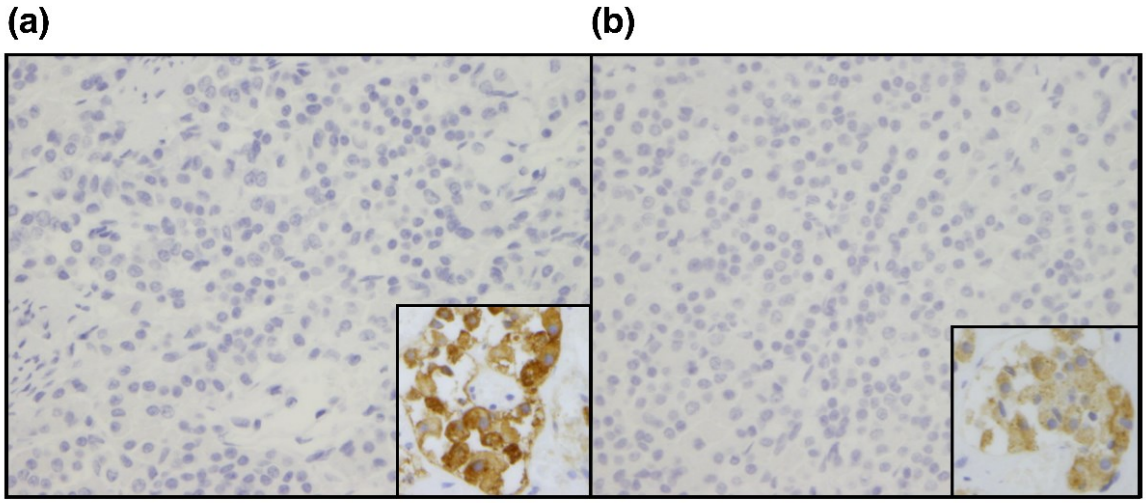


**Figure 3.3.** Immunohistochemical analysis of the anterior pituitary gland. Total lack of cytoplasmic expression of LH (A; left) and FSH (B; right) is seen relative to the external control (insert). Magnification x400.

## Tables

**Table 3.1.** Patient demographic characteristics, molecular diagnosis, clinical and endocrine features.

| Characteristic | n (N) | Percentage |
|---|---|---|
| **Gender** | | |
| Male | 74 (150) | 49.3% |
| Female | 76 (150) | 50.7% |
| **Molecular Diagnosis:** | | |
| *POLR3A* | 56 (150) | 37.3% |
| *POLR3B* | 81 (150) | 54.0% |
| *POLR1C* | 13 (150) | 8.7% |
| **Clinical Features[a]:** | | |
| ***Neurological*** | | |
| Ataxia | 49 (52) | 94.2% |
| Tremor | 48 (66) | 72.7% |
| Dystonia | 26 (31) | 83.9% |
| Dysarthria | 34 (40) | 85.0% |
| Dysphagia | 18 (37) | 48.7% |
| Sialorrhea | 12 (25) | 48.0% |
| Seizures | 17 (61) | 27.9% |
| ***Non-neurological*** | | |
| Myopia | 90 (103) | 87.4% |
| Teeth abnormality | 99 (117) | 84.6% |
| **Endocrine Features:** | | |
| Short stature | | |
| *Clinical impression* | 57 (93) | 61.3% |
| *Growth data* | 53 (115) | 46.1% |
| Delayed puberty | | |
| *Clinical impression* | 57 (74) | 77.0% |
| *Tanner stage* | 27 (32) | 84.3% |
| Abnormal thyroid function | 13 (59) | 22.0% |

n: number of identified patients per data sample. N: total number of patients in the data sample.
[a]: N values vary as clinical data were not available for all 150 patients in the cohort.

**Table 3.2.** Summary of hormonal levels according to mutated gene.

| Hormone Levels | | Genotype (No. of Patients) | | | All Patients |
|---|---|---|---|---|---|
| Hormone | Reported Levels | *POLR3A* | *POLR3B* | *POLR1C* | TOTAL |
| GH (stimulation test) | Low | 2 | 0 | 1 | 3 |
| | Normal | 2 | 0 | 0 | 2 |
| | % Abnormal | 50.0% | 0.0% | 100.0% | 60.0% |
| Prolactin | Low | 3 | 2 | 0 | 5 |
| | High | 1 | 1 | 2 | 4 |
| | Normal | 8 | 3 | 2 | 13 |
| | % Abnormal | 33.3% | 50.0% | 50.0% | 40.9% |
| TSH | Low | 2 | 0 | 0 | 2 |
| | High | 1 | 2 | 1 | 4 |
| | Normal | 22 | 24 | 7 | 53 |
| | % Abnormal | 12.0% | 7.7% | 12.5% | 10.2% |
| Free T4 | Low | 2 | 1 | 1 | 2 |
| | High | 0 | 2 | 1 | 3 |
| | Normal | 21 | 15 | 6 | 42 |
| | % Abnormal | 8.7% | 16.7% | 25.0% | 10.6% |

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# CHAPTER 4.

# Classifying the spectrum of disease severity associated with biallelic pathogenic variants in *POLR3A*

## Preface

Based on past phenotypic studies of POLR3-HLD, it was initially thought that pathogenic variants in *POLR3A* and *POLR3B* led to a relatively homogeneous presentation, with two main phenotypes. The first and most common is associated with an age of onset in early childhood involving motor delay or regression, followed by progressive neurological involvement through early adulthood. The second phenotype, initially described in a minority of patients, involves an onset later in childhood with learning difficulties, intellectual disability and/or cognitive regression with patients later developing motor features. Since then, patients have been reported with phenotypes ranging on a spectrum of severity, from mild presentations without hypomyelination seen on MRI, to severe presentations with a much earlier disease onset. As such, the following study in Part A of this chapter defines a novel severe disease presentation associated with a specific *POLR3A* genotype and features that are atypical from those usually seen in POLR3-HLD. In this study, the clinical, MRI, and molecular features are defined in a cohort of six patients, with neuropathological investigations performed on one patient who passed just after 1 year of age. Part B of this chapter follows with a discussion on this phenotypic presentation, and comparison to Wiedemann-Rautenstrauch syndrome, a distinct disorder also caused by pathogenic variants in *POLR3A*. These studies describe the severe end of the phenotypic spectrum associated with pathogenic variants in *POLR3A*, providing additional insight into the pleiotropy of disease.

# Chapter 4 Part A. Expanding the phenotypic and molecular spectrum of RNA polymerase III–related leukodystrophy

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

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## Abstract

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

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

**Results:** Each patient presented between 1 and 3 months of age with failure to thrive, severe dysphagia, and developmental delay. Four of the 6 children died before age 3 years. MRI of all patients revealed a novel pattern with atypical characteristics, including progressive basal ganglia and thalami abnormalities. Neuropathologic studies revealed patchy areas of decreased myelin in the cerebral hemispheres, cerebellum, brainstem, and spinal cord, with astrocytic gliosis in the

white matter and microglial activation. Cellular vacuolization was observed in the thalamus and basal ganglia, and neuronal loss was evident in the putamen and caudate. Genotypic similarities were also present between all 6 patients, with one allele containing a *POLR3A* variant causing a premature stop codon and the other containing a specific intronic splicing variant (c.1771-7C>G), which produces 2 aberrant transcripts along with some wild-type transcript.

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

## Introduction

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

POLR3-HLD is caused by biallelic pathogenic variants in *POLR3A, POLR3B*, *POLR1C*, or *POLR3K*, which encode subunits of RNA polymerase III (POLR3), an enzyme responsible for transcription of several noncoding RNAs (nc-RNAs), including transfer RNAs (tRNAs), 5S ribosomal RNA, U6 small nuclear RNA, 7S RNAs, and other small nucleolar RNAs.[7-15] The

precise mechanism underlying the pathogenesis of hypomyelination remains to be fully elucidated; 2 main mechanistic hypotheses include (1) defects in transcription capability of POLR3 causing disruptions in tRNA levels, thereby altering global translation during myelination, which require large production of essential myelin proteins, or (2) impairments in specific POLR3-transcribed nc-RNAs required for myelin development.[7,10,16]

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

## Methods

### Patients and study design

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

### Standard protocol approvals, registrations, and patient consents

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

### Neuroradiology

Brain MRI review was performed on latest available scans by L.G. and G.B. based on previously published criteria for hypomyelination and POLR3-HLD imaging

characteristics.[1,5,6,17-19] The earliest studies were also analyzed when available. Only one study was available for P5 and P6.

**Neuropathology**

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

**Genetic analysis**

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

**Cell culture and cycloheximide treatment**

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

**Western blot**

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

### Data availability

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

## Results

### Clinical characteristics

Patients 1–6 (P1-6) presented during infancy, between ages 1 and 3 months, with prominent feeding difficulties and failure to thrive. They exhibited severe developmental delay and motor regression before age 1 year. None achieved independent walking. Clinical characteristics are summarized in Table 4.1 and Table 4.e-1.

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

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

Neurologic examination revealed acquired microcephaly in 4 patients (4/6, 67%). Five (5/6, 83%) had a combination of axial hypotonia and upper motor neuron signs (spasticity and/or

hyperreflexia) in the limbs. Generalized dystonia and/or chorea was seen in all patients. Restricted upgaze and abnormal saccades were occasionally noted. Two patients exhibited hypomimia.

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

**Radiologic characteristics**

Brain MRI characteristics of P1-6 are summarized in Table 4.2 and Figure 4.1, which compares a typical POLR3-HLD MRI to P3. All 10 studies available for the 6 patients showed evidence of insufficient myelin deposition, but criteria for diffuse hypomyelination were not met (Figure 4.1, E–K).[6,17] Overall, there was more myelin than usually seen in POLR3-HLD and additional distinctive MRI characteristics. T2 hyperintensity of the hilus of the dentate nucleus, associated with T2 hypointensity (preserved myelination) of the dentate nucleus itself and peridentate region, was seen in all studies (Figure 4.1F). In 9/10 studies (90%), the posterior brainstem exhibited similar features, with T2 hyperintensity (decreased myelin content) of the posterior medulla, posterior-inferior pons, and posterior aspect of the middle cerebellar peduncles, in a pattern suggestive of axonal degeneration (Figure 4.1, F, I, and J). The latest imaging studies of 2 patients (2/6, 33%), obtained at ages 10 and 11 months, also revealed T2 hyperintensity of the red nucleus (Figure 4.1K). In addition, 8/10 studies (80%) revealed abnormal signal of the lentiform nuclei, which appeared hyperintense on T2 sequences compared with gray matter and isointense to unmyelinated white matter. The same 8 studies also showed atrophy of the thalami (Figure 4.1G). The 2 scans without these findings were the 2 earliest studies (P2, age 2 months; P3, age 3 months); however, follow-up MRIs showed that these changes developed over time. Basal ganglia atrophy was seen only in the 5 latest scans obtained between ages 10 and 15 months.

Cerebellar atrophy was not seen in any studies; however, mild to severe supratentorial atrophy was present in all cases (Figure 4.1, G–H). No signs of pituitary involvement were noted.

**Neuropathology**

Preserved brain tissue of P2, who died at age 13 months from respiratory complications, was subjected to neuropathologic study (Figure 4.2). The brain weighed 777 g, below expected brain weight and comparable to typical weight at age 8 months.[20] Macroscopic examination revealed normal symmetry with well-formed cerebral hemispheres and cerebellum (Figure 4.2A). On gross examination, white matter was slightly reduced, but demonstrated normal appearance without gray discoloration or cavitation. The lateral ventricles and cerebellum had normal size (Figure 4.2B), and corpus callosum thickness was normal for age.

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

White matter demonstrated patchy areas of rarefaction with mild myelin pallor. Oligodendrocytes showed normal morphology and density in all studied areas, including pale areas, and features of demyelination were absent. White matter also exhibited diffuse astrocytic gliosis, both chronic (fibrillary) and subacute (protoplasmic), with activation of microglia but without macrophagic changes associated with phagocytic activity (Figure 4.2, C–F). Changes in white matter appeared more severe in the parietal lobes (Figure 4.2, C–D). No Rosenthal fibers or axonal spheroids were seen. Immunohistochemistry did not reveal any axonal lesions. The corpus callosum and corticospinal tracts demonstrated normal myelination.

Cellular vacuolization was seen in the thalamus and basal ganglia. Atrophy of the putamen was evident with enlarged Virchow-Robin spaces and severe neuronal loss, associated with both chronic and subacute diffuse gliosis, along with rare calcifications and considerable activation of microglia (Figure 4.2, G–I). Discrete neuronal loss was evident in the caudate. Within the pallidum, numerous pale nuclei of Alzheimer type II glia were present due to the terminal anoxia, and no appreciable neuronal loss was evident. The adenohypophysis did not demonstrate pathologic abnormalities.

Hemisections of the brainstem demonstrated mild to moderate pyknosis in the pons and olivary nuclei of the medulla, consistent with acute ischemic changes. Patchy areas of reduced myelin were seen in the brainstem. The cerebellum demonstrated severe lesions of poorly myelinated white matter, with diffuse and mainly chronic (fibrillary) gliosis, but without notable morphological changes in oligodendrocytes (Figure 4.2, J–L). The cerebellar cortex and dentate nucleus appeared normal, and Bielschowsky staining did not reveal clear evidence of decreased axons. Moderate pyknosis was seen in Purkinje cells; however, there was no appreciable loss of neurons. In the spinal cord, patchy areas of reduced myelin were noted.

**Genetic findings**

Each patient harbored a specific combination of compound heterozygous variants, including a variant causing a premature stop codon on one allele (P1: c.2119C>T/p.Q707*, P2: c.1681C>T/p.R561*, P3&4: c.1051C>T/p.R351*, P5: c.601delA/p.I201Lfs*18, P6: c.3583delG/p.D1195Ifs*47) and a specific intronic splicing variant on the other (P1-6: c.1771-7C>G). We hypothesized that this splicing variant was leaky as complete absence of POLR3A is incompatible with life. PCR amplification using complementary DNA from fibroblasts of P2

revealed 2 additional bands compared with controls (Figure 4.3, Figure 4.e-1). Sequencing of bands revealed the presence of 2 aberrant transcripts resulting from abnormal splicing, including one lacking exon 14 causing a frameshift and premature stop codon (p.P591Mfs*9) and the other lacking exons 13–14 causing loss of amino acids 548–637 (p.G548_Y637del). In addition, sequencing of the band corresponding to complementary DNA of wild-type length revealed the presence of both the nonsense transcript (c.1681C>T/p.R561*) and wild-type transcript, confirming the splice site variant is leaky (Figure 4.3B). Thus, 4 transcripts were detected, with sequences corresponding to (1) wild-type, (2) the nonsense variant, and those resulting from aberrant splicing events including (3) lack of exon 14, and (4) lack of both exons 13–14 (Figure 4.3C).

As transcripts containing nonsense variants are typically targeted for NMD, we hypothesized that the c.1681C>T/p.R561* variant transcript was subjected to degradation. We evaluated the presence of NMD in P2 fibroblasts compared with a control using cycloheximide, a compound that inhibits transcriptional elongation and consequently NMD. Following cycloheximide treatment, an increase in band 1 (corresponding to the wild-type transcript and nonsense variant transcript) was observed by semiquantitative PCR (Figure 4.3B), indicating that the nonsense transcript is subjected to NMD under normal conditions.

Because complete lack of POLR3A is incompatible with life, we sought to determine whether the detected residual wild-type transcript would lead to wild-type protein expression. We performed immunoblot analysis on protein extracts from frozen brain tissue of P2 and an age/sex-matched control. To ensure detection of only wild-type full-length protein, we chose a POLR3A antibody with an epitope spanning amino acid residues 607–698. In P2, this antibody cannot bind to the abnormal protein products as the epitope binds to residues located in the truncated POLR3A

region, i.e., after the premature stop codon (p.R561*) and contained/semicontained in the deleted residues resulting from the splicing variant (p.P591Mfs*9, p.G548_Y637del). Thus, this antibody only allows detection of wild-type POLR3A (Figure 4.3C, Figure e-1). We observed reductions in average normalized POLR3A levels both in brain gray matter (84.7% reduction, 95% CI = 69.3%–100%, d = 1.28) and white matter (54.8% reduction, 95% CI = 20.1%–89.5%, d = 1.34) of P2 compared with control (Figure 4.3, D and E). Gray matter displayed a greater reduction in POLR3A compared with white matter (average difference 29.9%; 95% CI = 0.7%–59.0%; d = 1.77).

## Discussion

Here, we present an expanded spectrum of POLR3-HLD through description of 6 patients with a very severe phenotype and similar genotype. The dramatic clinical presentation, including prominent feeding and breathing difficulties and early death in 4 patients, is strikingly different from the typical POLR3-HLD phenotype. A large phenotypic study of POLR3-HLD revealed typical onset at age 3-4 years with mild to moderate motor delay and/or regression.[1] Dysphagia and respiratory insufficiency were late findings. Death typically occurred in adulthood, where the youngest to die was aged 8 years.[1]

The MRI pattern associated with this phenotype is distinct; despite very severe clinical manifestations, all patients had notably more myelin with different imaging features than typical POLR3-HLD. An evolving change in signal pattern was seen in the lentiform nuclei, with thalami atrophy, progressing to more diffuse basal ganglia atrophy. This correlated with the prominent basal ganglia and thalami pathologic abnormalities, including atrophy, calcifications, and severe neuronal loss in the putamina. Two patients also had red nuclei signal abnormalities. Recently, a

similar MRI phenotype was described in patients with a c.1771-7C>G or c.1771-6C>G variant, in trans with a missense, nonsense, splice site, or synonymous variant.[21,22] Clinical severity varied according to the trans *POLR3A* variant; patients homozygous for the splicing variant typically displayed a milder phenotype, whereas those harboring a trans loss of function variant displayed severe features with early onset.[21-24] Of interest, patients homozygous or compound heterozygous for the c.1771-7C>G and/or c.1771-6C>G variants did not display white matter involvement and were described as only having the neuronal MRI features, including striatal involvement with caudate nucleus and putamen atrophy, and occasional red nuclei signal abnormalities.[21-24] We hypothesize that these specific splicing variants cause a cell-specific effect (i.e., basal ganglia neurons) compared with other POLR3-HLD variants. This could explain why, when this variant is combined with a loss of function allele, patients with a severe phenotype have a specific MRI pattern (i.e., more myelin than the typical phenotype, with progressive basal ganglia involvement).

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

Although it is well known that hypomyelination is not obligate in POLR3-HLD,[18,25] the discrepancy between the relatively mild insufficient myelin deposition and the diffuse supratentorial atrophy was highly unusual and consistent across all MRIs. Although previous studies have revealed that oligodendrocytes are primarily affected in the typical form of POLR3-HLD,[1,26] our patients' MRI and pathologic findings support the hypothesis that the severe form is

primarily neuronal, with associated myelination deficits. We hypothesize that the pathophysiology associated with the severe phenotype varies substantially from typical POLR3-HLD and involves several neural cell types. As myelination is a complex process involving a multitude of signaling events between neurons and glia, it is possible that an increased disruption of POLR3 activity, or the production of aberrant transcripts, could manifest adversely in more cell types than in a milder deficit. It is known that dysregulation of transcription and translation-related genes is often associated with neurologic involvement, highlighting the importance of precise protein expression regulation during neural development.[27-30] For example, defects in genes encoding aminoacyl-tRNA synthetases cause a variety of phenotypes, ranging from hypomyelination to brain malformations.[31-38]

Given the broad clinical spectrum of phenotypes associated with POLR3 deficiency, it is clear that pathogenic variants in POLR3 genes have distinct effects on various cellular processes.[25,39] Variants in *POLR3A* have been associated with phenotypes ranging from spastic ataxia–related disorders to neonatal progeroid syndrome, whereas variants in *POLR3B* have been associated with isolated hypogonadotropic hypogonadism, without hypomyelination or hypodontia, and a distinct phenotype of cerebellar hypoplasia with endosteal sclerosis.[25,39-41]

In contrast to this extremely severe clinical presentation, we also identified 3 adults with a very mild phenotype and the same homozygous pathogenic *POLR3B* variant (c.1568T>A/p.V523E) in our patient cohort. These patients were all diagnosed incidentally in adolescence/adulthood, based on brain MRI performed for unrelated reasons, or through genetic investigation of typical POLR3-HLD affected relatives. They had minimal findings on neurologic examination, if any, and MRI revealed milder findings than usually seen in POLR3-HLD. Two were previously described as having the mildest phenotype in a past large cohort study of POLR3-

HLD,[1] and the third, who has not been reported, is an adult woman in her late 70s for whom limited information is available. She is currently still ambulatory, and was able to reproduce, making it unlikely she had fertility concerns due to hypogonadotropic hypogonadism. She is described as having mild intellectual challenges and hearing loss from childhood of unknown etiology. She is also independent for all activities of daily living and maintained an active role in the care of her offspring. These cases highlight the extreme variability in disease severity of POLR3-HLD, which can range from very mild to exceptionally severe.

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

growth. Less POLR3A protein was detected in gray matter compared with white matter, lending further support to our hypothesis that the severe phenotype is a primarily neuronal disorder.

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

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## Figures

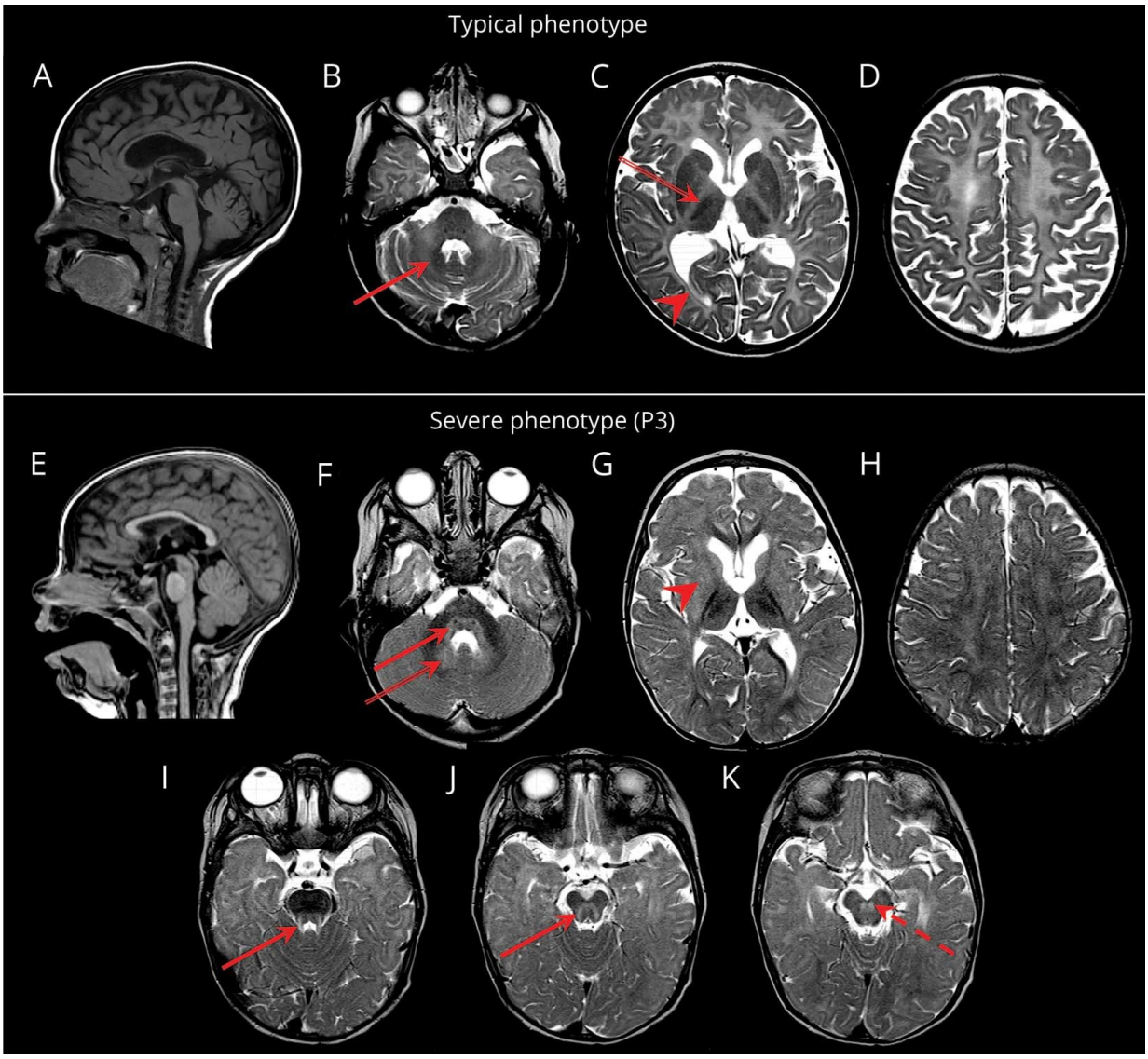


**Figure 4.1. MRI characteristics.** Sagittal T1-weighted (A, E) and axial T2-weighted (B–D, F–K) images. (A–D) Typical POLR3-HLD; MRI obtained at age 6 years. Hypomyelination with relative preservation (T2 hypointensity) of the dentate nucleus (red arrow; B), anterolateral nucleus of the thalamus (double-lined arrow; C), optic radiations (arrowhead; C), globus pallidus, and corticospinal tracts in the posterior limb of the internal capsule (not shown). Thinning of the corpus callosum and cerebellar atrophy are also seen. (E–K) Severe phenotype; MRI of patient 3 obtained at age 10 months. Mild insufficient myelin deposition, not meeting the criteria for diffuse hypomyelination. Loss of myelin (T2 hyperintensity) in the posterior brainstem (red arrows; F, I, J), red nucleus (red dashed arrow; K), and hilus of the dentate nucleus (double-lined arrow; F). Abnormal signal of the lentiform nucleus (arrowhead; G). Supratentorial atrophy (G–H) and diffuse atrophy of the basal ganglia and thalami (G) are also seen.

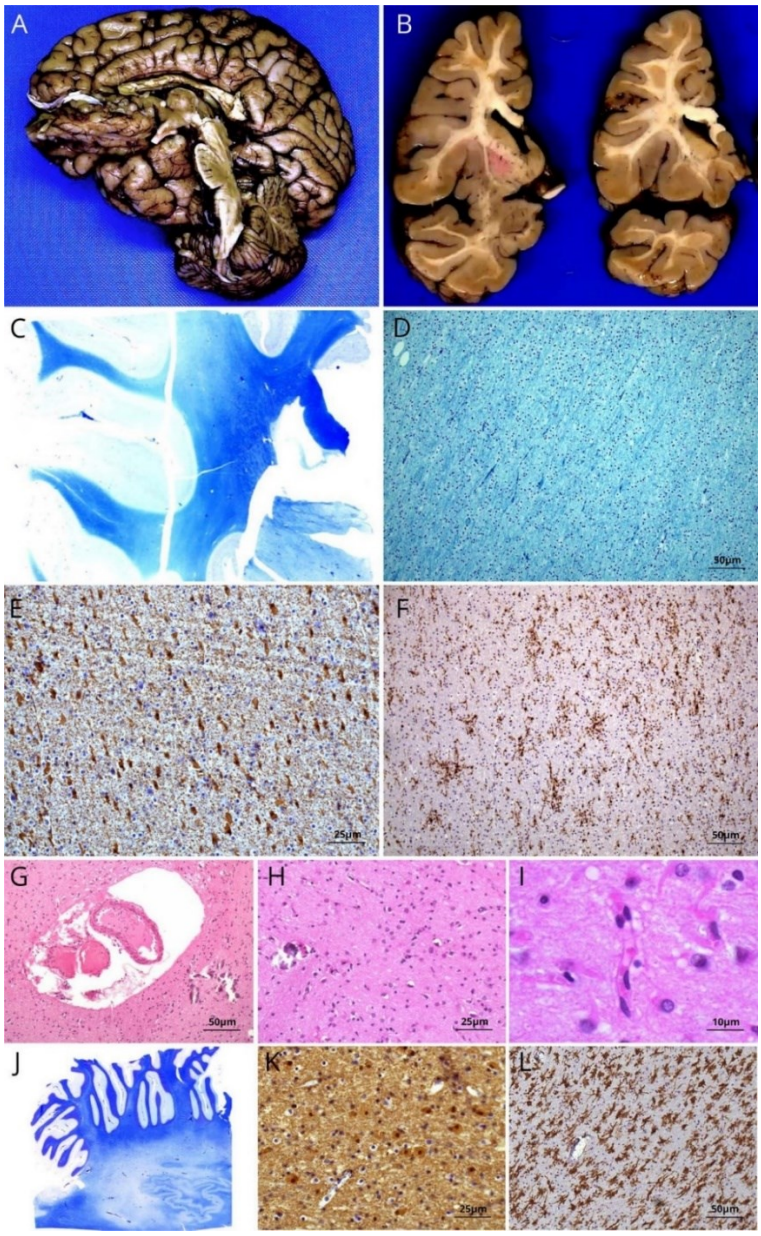


**Figure 4.2. Neuropathology of the POLR3-HLD severe phenotype (patient 2).**
(A–B) Macroscopic appearance of the (A) right cerebral hemisphere, and (B) coronal sections showing a slight decrease of the volume of the white matter without appreciable ventricular enlargement. (C) Luxol fast blue-cresyl violet (Kluver-Barrera) staining demonstrating areas of poor myelination in the parietal white matter, but a normally myelinated corpus callosum. (D) Higher magnification of poorly myelinated white matter (10×), and (E) GFAP IHC revealing astrocytic gliosis (20×). (F) IBA1 IHC revealing activated microglia of the occipital white matter (10×). (G–I) Hemalun-phloxin staining revealing abnormalities of the putamen including (G) enlarged Virchow-Robin spaces (10×), (H) neuronal loss and gliosis with few calcifications (20×), and (I) neuronal death (60×). (J) Luxol fast blue-cresyl violet stained section of the cerebellum revealing hypomyelination of the cerebellar white matter surrounding the dentate nucleus. (K) GFAP IHC demonstrating gliosis in the cerebellar white matter (20×), and (L) IBA1 IHC revealing activated microglia (10×). GFAP = glial fibrillary acidic protein; IHC = immunohistochemistry.

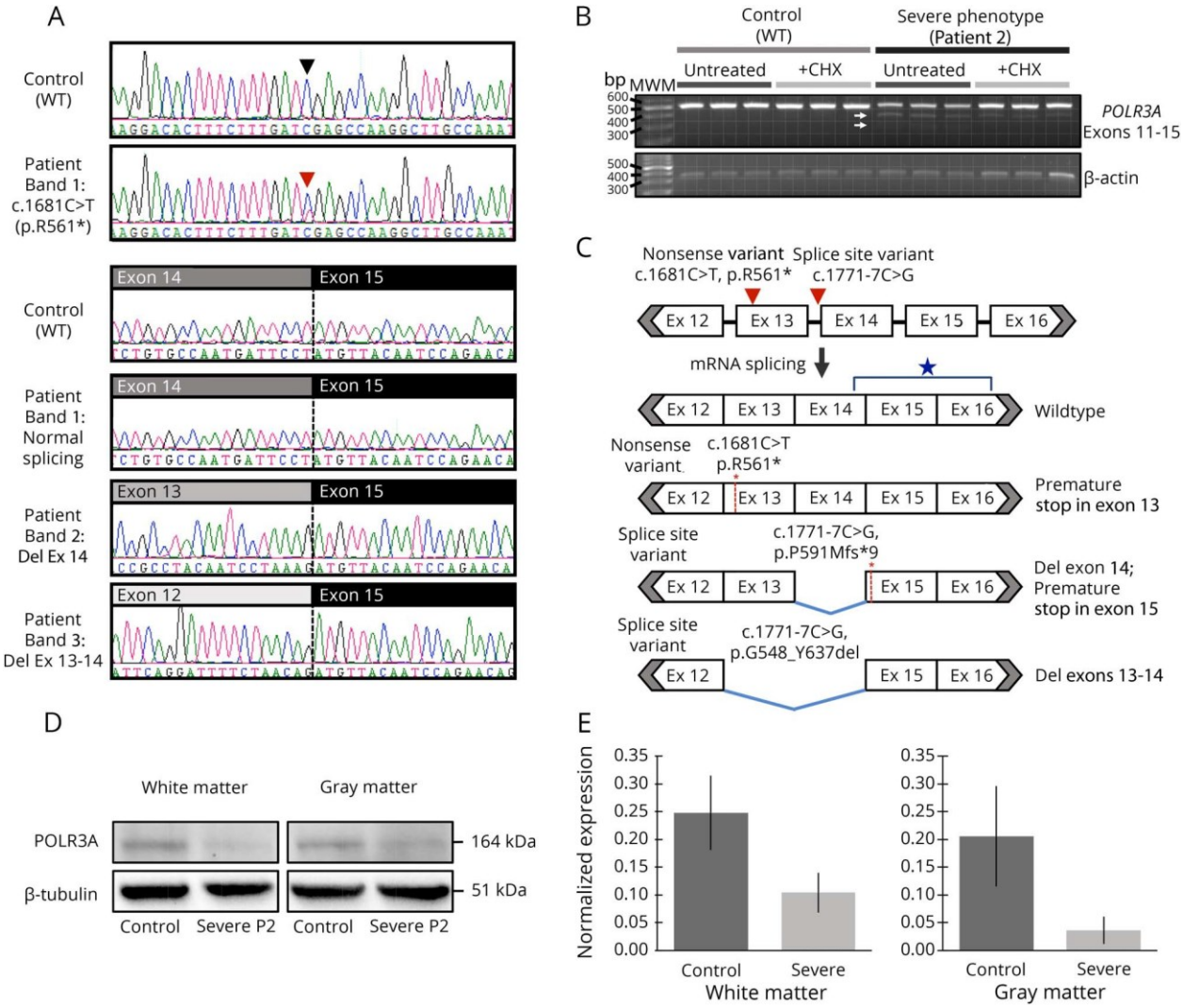


**Figure 4.3. Molecular and protein level implications of pathogenic variants in patient 2.** (A) Sanger sequencing results of RT-PCR products generated from patient 2 fibroblasts, as visualized by agarose gel electrophoresis in (B), in which 3 separate bands were excised and sequenced. In band 1, the presence of the POLR3A wild-type transcript is detected, as well as the transcript containing the paternally inherited nonsense variant (c.1681C > T; p.R561*), confirming that the splice site variant is leaky. Sequencing of the 2 additional bands confirms that the maternally inherited splice site variant (c.1771-7C>G) causes production of 2 additional transcripts, including 1 transcript with a deletion of exon 14, which produces a new open reading frame that results in a premature stop codon (p.P591Mfs*9), and the other containing a deletion of exons 13–14, which leads to the loss of amino acids 548–637 (p.G548_Y637del). (B) RT-PCR products with primers in *POLR3A* exons 11 and 15 revealing 2 additional bands in patient 2 fibroblasts compared with control fibroblasts. Cycloheximide treatment shows a stabilization of

the mRNA containing the nonsense variant (band 1), confirming that it is targeted by NMD. β-Actin is shown as a loading control. (C) Schematic summary of each transcript detected following mRNA splicing. The starred region in the wild-type transcript denotes the POLR3A antibody epitope spanning from amino acids 607–698 for the immunoblots depicted in (D). (D) Immunoblots of protein lysates from frozen brain tissue of patient 2 (age 13 months) compared with that of an age/sex-matched control (age 14 months). Samples were collected from the subcortical white matter (left) and the cortical gray matter (right). (E) Normalized expression of POLR3A in the brain of patient 2 compared to that in the control. Chemiluminescent intensity of the POLR3A signal at 164 kDa was normalized to the intensity of the β-tubulin signal at 51 kDa for each blot. Average values of normalized protein expression are derived from 4 Western blot replicates, and error bars represent standard error of the mean. Full-length POLR3A is detected in both control and patient 2 white and gray matter, with decreases seen in patient samples compared with the control. bp = base pairs; CHX = cycloheximide; Del = deletion; Ex = exon; mRNA = messenger RNA; NMD = nonsense medicated decay; P2 = patient 2; RT-PCR = reverse transcription PCR; WT = wild type.

## Tables

**Table 4.1.** Clinical, MRI, molecular, and pathologic features associated with the typical and severe POLR3-related leukodystrophy phenotypes.

| Feature | Typical Phenotype | Severe Phenotype |
|---|---|---|
| **Clinical Characteristics** | | |
| Age of onset | 3-4 y | 1-3 mo |
| Age of death | Adulthood | 1-3 y (2/6 patients still alive) |
| Symptoms at onset | Developmental delay and motor regression | Failure to thrive and developmental delay |
| Developmental delay | Mild to moderate | Severe |
| Dysphagia | Late | Early and severe |
| Respiratory insufficiency | End of disease course | Early and severe |
| Severe myopia | Very common | Too young |
| Dental abnormalities | Common | Delayed dentition seen in 3/6 |
| Hypogonadotropic hypogonadism | Common | Too young |
| **Brain MRI** | Hypomyelination with preservation of specific structures, thinning of the corpus callosum, and cerebellar atrophy | Very atypical: more myelin than typical phenotype, supratentorial atrophy, and additional features including progressive abnormalities of basal ganglia and thalami |
| **Genetics** | *POLR3A, POLR3B, POLR1C,* or *POLR3K* biallelic pathogenic variants<br>>200 variants | *POLR3A* (NM_007055.3)<br>compound heterozygous<br>Allele 1:<br>P1: c.2119C>T, p.Q707*<br>P2: c.1681C>T, p.R561*<br>P3: c.1051C>T, p.R351*<br>P4: c.1051C>T, p.R351*<br>P5: c.601delA, p.I201Lfs*18<br>P6: c.3583delG, p.D1195Ifs*47<br>Allele 2:<br>P1-6: c.1771-7C>G |
| **Pathology** | Prominent and diffuse decreased myelin, secondary axonal loss, and relative preservation of myelin in perivascular regions[26] | Patchy areas of decreased myelin, neuronal loss in putamen and caudate, and vacuolization in thalamus and basal ganglia |

**Table 4.2**. MRI features of patients with the severe POLR3-related leukodystrophy phenotype.

| | | Typical POLR3-HLD Characteristics | | | | Additional Atypical Characteristics | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ID** | Age at MRI (mo) | Diffuse hypomyelination | Classic T2 hypointensity of specific structures | Thin corpus callosum | Cerebellar atrophy | More myelin than typically seen in POLR3-HLD | T2 hyperintense hilus of dentate nuclei | T2 hyperintense lentiform nuclei | T2 hyperintense posterior inferior brainstem | T2 hyperintense red nuclei | Thalami atrophy | Basal ganglia atrophy | Supratentorial atrophy |
| **Patient 1** | 6 | - | - | + / mild | - | + | + | + | + | - | + | - | + / mild |
| | 15 | - | - | + / mild | - | + | + | + | + | - | + | + | + / moderate-severe |
| **Patient 2** | 2 | - | - | + | - | + | + | - | - | - | - | - | + / mild |
| | 7 | - | - | + | - | + | + | + | + | - | + | - | + / mild-moderate |
| **Patient 3** | 3 | - | - | + / mild | - | + | + | - | + | - | - | - | + / mild |
| | 10 | - | - | + / mild | - | + | + | + | + | + | + | + | + / mild-moderate |
| **Patient 4** | 8 | - | - | + / mild | - | + | + | + | + | - | + | - | + / mild-moderate |
| | 11 | - | - | + / mild | - | + | + | + | + | + | + | + | + / mild-moderate |
| **Patient 5** | 14 | - | - | + | - | + | + | + | + | - | + | + | + / moderate-severe |
| **Patient 6** | 10 | - | - | + / thin isthmus | - | + | + | + | + | - | + | + | + / moderate |

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# Supplemental Data

## Supplemental Methods

### Genetic Analysis

Pathogenic variants in *POLR3A* were assigned by the RefSeq sequence NM_007055.3, and the ClinVar accession numbers are as follows: SCV000987272, SCV000987273, SCV000987274, SCV000987275, SCV000987276, and SCV000987277.

### Neuropathology

Tissues were analyzed initially during autopsy, followed by a second specialized neuropathological examination. During autopsy, the right cerebral hemisphere was fixed in 20% formalin for 2 weeks prior to coronal sectioning, and the left cerebral hemisphere was frozen for research. Fixed tissue was processed on a Leica ASP300 automated processor using standard protocols, and routine histologic sections were obtained at 4 μm thickness. Sections were stained with Hematoxylin and Eosin on a Leica H&E stainer, and CD68 stain was completed using the Ventana Benchmark automated immunohistochemistry stainer using Ventana anti-CD68 (KP-1) antibody. Luxol fast blue staining, Periodic acid Schiff staining, von Kossa calcium staining, and Bielschowsky staining were also completed.

For the second specialized neuropathological examination, tissue fragments were collected from the frontal, occipital and parietal white matter, basal ganglia (anterior region), cerebellum (cortex and dentate nucleus), cingular gyrus, centrum ovale, and the corpus callosum (genu, body, and splenium). Sections were embedded in paraffin, cut at 7 μm thickness, and stained with Hemalun-Phloxin and Luxol-Fast-Blue-Cresyl-Violet (Klüver-Barrera stain).

Immunohistochemistry was also completed with antibodies for GFAP (Anti-Glial Fibrillary Acidic Protein, polyclonal rabbit antibody, DAKO), IBA1 (Anti-Ionized Calcium-Binding Adapter Molecule 1, Wako (Sodobis) lot PDK 6188), and SMI32 (Anti-Neurofilament non-phosphorylated, Calbiochem).

**Cell Culture and Cycloheximide Treatment**

Primary fibroblasts derived from patient 2, as well as a control cell line, were cultured at 37°C under humidified 95% air and 5% CO2 in Dulbecco's modified Eagle's medium (DMEM, Wisent) supplemented with 10% fetal bovine serum (FBS, Wisent). Fibroblasts were plated in 6-well plates (0.5 million cells, samples in triplicate), and grown in the presence and absence of cycloheximide (CHX, 100 ng/µl) for 20 hours to inhibit translational elongation and downstream nonsense-mediated decay of variant transcripts (1, 2). RNA was isolated from each sample (QIAamp RNA Blood Mini Kit, Qiagen), and cDNA was reverse-transcribed from 500 ng of RNA (BioRad iScript Reverse Transcription Supermix) following manufacturer's protocols. PCR amplification was performed with primers in *POLR3A* (exons 11 [5'-CCACCGGACCTTCAGATTTA-3'] and 15 [5'-TGCCACTCATCAACTCACTG-3']), as well as β-actin as a loading control (exons 1 [5'-GCTCGTCGTCGACAACGGCTC-3'] and 2 [5'-CAAACATGATCTGGGTCATCTTCTC-3']), using the following PCR cycling conditions: 95°C for 10 minutes, followed by 40 cycles of [95°C for 15 seconds, 58°C for 20 seconds, and 72°C for 45 seconds], and 72°C for 10 minutes. PCR products were then separated using agarose gel electrophoresis (2.5% agarose gel; 120V, 30 minutes). Gel imaging was completed using the Gel Doc EZ System (BioRad) using ImageLab Software (BioRad, Version 6.0.1). Visible bands were

excised, products were extracted and purified (QIAquick Gel Extraction kit), and Sanger sequencing was performed to characterize the differentially spliced mRNAs.

**Western Blot**

Western blotting was completed with protein extracts from independent samples of cortical grey matter or subcortical white matter of patient 2 and an age/sex matched control. Control brain tissue was collected in the context of surgery for intractable epilepsy associated with *PIK3CA* mosaicism in a 14-month old girl with hemimegalencephaly. Protein lysates were prepared from brain tissues extracted in standard radioassay immunoprecipitation (RIPA) buffer (Thermo Scientific Pierce #89901) containing protease inhibitors (Sigma cOmplete EDTA-free protease inhibitor cocktail #4693132001) for 30 minutes, and lysates were sonicated for 2.5 minutes in ten second bursts with 10 seconds rest. Protein concentrations were determined using the Bradford assay and normalized correspondingly (3). Normalized lysates were combined in Laemmli buffer and electrophoresed using standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) techniques on 1.5 mm, 7.5% tris-glycine gels and blotted onto polyvinylidene difluoride (PVDF) membrane using a standard semi-dry protein-transfer at 25V, 2.5A for 10 minutes (Biorad Trans-Blot Turbo Transfer System). Immunoblots were incubated with a primary antibody targeted to the POLR3A amino acid residues 607-698 (Anti-POLR3A rabbit polyclonal antibody [Abcam ab247007]; 0.4 ug/mL), or with β-tubulin as a loading control (Anti-tubulin mouse polyclonal antibody [Sigma, T8328]; dilution 1:2000), with goat anti-rabbit (dilution 1:5000) or goat anti-mouse (dilution 1:10000) secondary antibodies. Blots were incubated with enhanced chemiluminescent substrate (ECL Prime, Amersham) for 5 minutes and imaged on a

Chemidoc XRS+ Imaging System (Biorad) using ImageLab Software (BioRad, Version 6.0.1). Full unedited blots are provided in Supplementary Fig. 4.e-1.

Western Blot quantification was performed using ImageLab Software (BioRad, Version 6.0.1). Chemiluminescent band intensity of four replicates from each sample was measured for POLR3A at 164 kDa, normalized to the β-tubulin signal at 51 kDa, and averaged for patient 2 and control samples. Average percent decrease was calculated for each tissue type, and average reduction of protein expression levels between grey matter and white matter were compared. Confidence intervals (95%) were calculated for each mean difference in protein expression. Effect size estimations were calculated using Cohen's d, with pooled standard deviation calculations. Statistical analysis was performed using GraphPad Prism 8 for Windows (GraphPad Software Inc. La Jolla, CA).

**Supplemental References**

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## Supplemental Figures

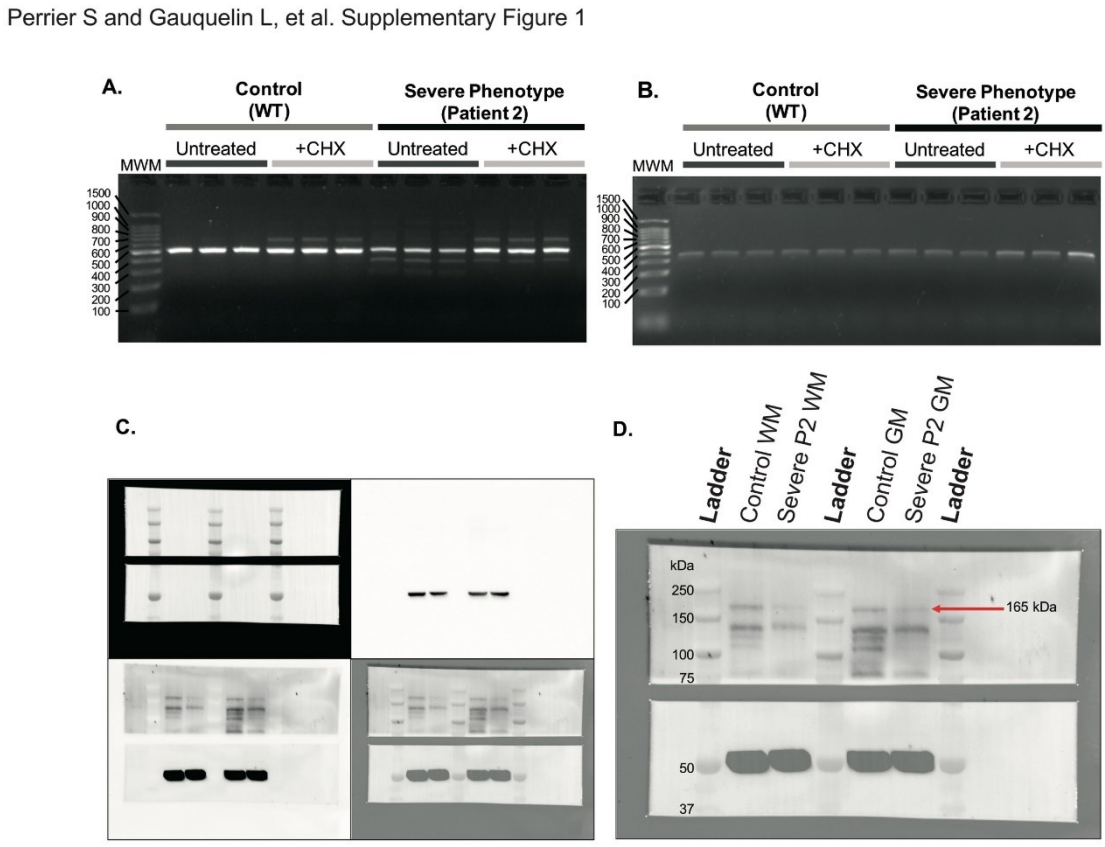


**Figure 4.e-1. Additional gel and blot images.** (A-B) Images of full agarose gels (uncropped) demonstrating RT-PCR products with primers to amplify (A) *POLR3A* exons 11-15, and (B) β-Actin. (C) Individual exposures used for assembling the composite western blot image to assess molecular weight (bottom right) including top illumination to capture the protein ladder (top-left), six second chemiluminescence exposure to capture β-tubulin for data analysis (top-right), and 400 seconds to capture POLR3A for data analysis (bottom-left). (D) Composite image showing molecular weights of protein ladder and band of interest at 164 kDa, the predicted size of POLR3A. CHX; cycloheximide. MWM; molecular weight marker. GM; grey matter. WM; white matter.

# Supplemental Tables

**Table 4.e-1.** Additional clinical characteristics and demographic information for patients included in this study.

| | Demographic characteristics | | | Clinical characteristics | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | Pheno-type | Sex | Ethnicity | Age of onset | Failure to thrive | Develop-mental delay | Age at walking without support | Cognitive impairment | Seizures | Age at motor regression | Dysphagia/ Age at G-tube | Dys-autonomia | Respiratory insufficiency | Ocular abnormalities | Dental abnormalities | Hypogonado-tropic hypogonadism | Micro-cephaly | Upper motor neuron signs | Prominent tremor | Dystonia | Age of death |
| Patient 1 | Severe | F | Caucasian | 1 mo | + | + | Not achieved | Too young | - | 10 mo | + / 5 mo | + | + L S T | Cortical visual impairment | Delayed dentition | Too young | + | Spasticity | - | + | 15 mo |
| Patient 2 | Severe | F | Asian/ Ashkenazi Jewish | 2 mo | + | + | Not achieved | Too young | - | 7 mo | + / 7 mo | - | - | - | - | Too young | + | Spasticity | - | + | 13 mo |
| Patient 3[a] | Severe | F | Maori/ Cook Island Maori/ New Zealand European | 1 mo | + | + | Not achieved | Too young | - | 2 mo | + / 7 mo | - | + L S | Hyperopia | Delayed dentition | Too young | - | Hyperreflexia | - | + C | 21 mo |
| Patient 4[a] | Severe | M | Maori/ Cook Island Maori/ New Zealand European | 3 mo | + | + | Not achieved | Too young | - | 6 mo | + / - | + | + L | Hyperopia | Delayed dentition | Too young | - | - | - | + C | 38 mo |
| Patient 5 | Severe | M | Russian | 3 mo | + | + | Not achieved | Too young | N/A | 3 mo | + / 15 mo | - | - | - | - | Too young | + | Spasticity | - | + C | N/A (Currently 5 y) |
| Patient 6 | Severe | M | Hispanic/ American | 3 mo | + | + | Not achieved | Too young | - | 12 mo | + / 12 mo | - | + | Cortical visual impairment | N/A | Too young | + | Spasticity and hyperreflexia | - | + C | N/A (Currently 3 y) |

[a] : Patients 3 and 4 are from the same family; C: Choreoathetosis; L: Laryngomalacia; N/A: Not available; mo: Months; S: Supraglottoplasty; T: Tracheostomy; y: Years.

# Chapter 4 Part B. Distinguishing severe phenotypes associated with pathogenic variants in *POLR3A*

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## Distinguishing severe phenotypes associated with pathogenic variants in *POLR3A*

*Response to: Majethia, P. & Girisha, K.M. (2020). Wiedemann-Rautenstrauch syndrome in an Indian patient with biallelic pathogenic variants in POLR3A. Am J Med Genet A. 185A: 1602-1605. doi:10.1002/ajmg.a.62115*

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## Correspondence

We read with great interest the recent publication by Majethia and Girisha, titled "Wiedemann-Rautenstrauch syndrome in an Indian patient with biallelic pathogenic variants in *POLR3A*" (Majethia & Girisha, 2021). This case report provided a detailed description of a patient with biallelic pathogenic variants in *POLR3A* (OMIM: 614258; NM_007055.4: c.1771-7C>G and c.2005C>T; p.Arg669*). We appreciate the thorough report of this patient and commend the authors for this work. After reviewing the phenotypic description of the patient, we would like to comment on the classification of POLR3-related disorders by comparing the clinical features of the reported patient to those of patients with a severe form of POLR3-related hypomyelinating leukodystrophy (POLR3-HLD).

In a recent study, we reported a cohort of six patients with a more severe phenotype compared to that typically seen in POLR3-HLD (OMIM: 607694) and a specific genotype (Perrier et al., 2020). This genotype includes the *POLR3A* splicing variant c.1771-7C>G on one allele, and on the other, a *POLR3A* variant leading to a truncated protein (i.e., nonsense variant or frameshift deletion) (Perrier et al., 2020). Likewise, the patient published by Majethia and Girisha also harbors this combination of variants (i.e., c.1771-7C>G and c.2005C>T; p.Arg669*). It was this similarity, along with the similarities in clinical course that led us to further consider the features of this patient compared to those with the severe form of POLR3-HLD.

In Table 4.3, we present a comparison between the clinical features of the patient reported by Majethia and Girisha, our cohort of patients with a severe POLR3-HLD phenotype, and five published studies of other cohorts of patients with Wiedemann-Rautenstrauch syndrome (WRS; OMIM: 264090) caused by biallelic pathogenic variants in *POLR3A* (Jay et al., 2016; Lessel et al., 2018; Paolacci et al., 2018; Temel et al., 2020; Wambach et al., 2018). Notably, the patient reported

by Majethia and Girisha was born at term with a normal weight, similar to the patients with severe POLR3-HLD, whereas in patients with WRS, intrauterine growth retardation and low birth weight are common. The clinical course of the patient was similar to those with severe POLR3-HLD, involving failure to thrive and recurrent respiratory issues. The patient also had laryngomalacia, which was reported in three out of six patients with severe POLR3-HLD. Most importantly, the patient reported by Majethia and Girisha appeared to have prominent neurological manifestations, with developmental regression, movement disorders, and upper motor neuron signs on examination. There was loss of motor skills, and walking was never achieved. Neurological features, when present, are not typically prominent in patients with WRS. Dystonia was reported in the patient described by Majethia and Girisha, a feature seen in all patients with severe POLR3-HLD, but not usually in WRS. The patient succumbed to an early death at 1.5 years of age. In patients with POLR3-related disorders, the age of premature death can range from shortly after birth to childhood or adulthood, depending the severity of the disease. Several patients with severe POLR3-HLD passed at an early age (4/6 before age 3) (Perrier et al., 2020). In the studied cohorts of patients with WRS, there was variance as to whether patients succumbed to an early death. The first patient described in the literature with WRS and biallelic pathogenic variants in *POLR3A* passed during infancy, at age 7 months (Jay et al., 2016). In three other publications of patients with WRS and published data on current age (Lessel et al., 2018; Temel et al., 2020; Wambach et al., 2018), all were living at the time of publication (11 patients; age range 11 months to 21 years). In the cohort study by Paolacci et al. (2018), the age of death was not listed for all patients, however some were previously published and noted to be deceased, including two females who passed in early to late adolescence (Paolacci et al., 2017; Rautenstrauch & Snigula, 1977), and several patients who passed away at various ages ranging from days to months after birth (specifically,

four patients within the first two weeks of life: G. Arboleda, Morales, Quintero, & Arboleda, 2011; H. Arboleda, Quintero, & Yunis, 1997; Morales et al., 2009, one patient after 1.5 months: G. Arboleda et al., 2011, and one patient after 6 months: H. Arboleda et al., 1997).

WRS typically presents in the neonatal period. Individuals are known to have characteristic facial features, including a triangular face with a prominent forehead, visible scalp veins, and sparse scalp hair (Paolacci et al., 2017). The patient reported by Majethia and Girisha did not have these facial characteristics early on, but a triangular face was only noticed around the age of 15 months, when the child had lost a significant amount of weight and has become cachectic. Contractures are also common in individuals with WRS, however the reported patient was not known for joint abnormalities. Another typical feature of WRS is lipodystrophy with localized fat deposits usually over the iliac region. The reported patient had decreased subcutaneous fat, without deposits noted in the description. Our patients with severe POLR3-HLD did not have notable facial dysmorphia or a progeroid appearance, and POLR3-HLD is not typically associated with joint abnormalities or unusual fat distribution.

Dental abnormalities appear to be common and diverse across each described phenotype. The presence of dental abnormalities between patients with different *POLR3A* genotypes and broad phenotypes is an interesting concept that exemplifies the importance of proper POLR3A protein abundance/function in the development of specific tissues.

POLR3-HLD can be associated with atypical MRI findings, without frank hypomyelination (Azmanov et al., 2016; Harting et al., 2020; Hiraide et al., 2020; La Piana et al., 2016; Perrier et al., 2020; Wu et al., 2019). The brain MRI pattern of published patients with the c.1771-7C>G variant (whether homozygous or in trans with another variant) is specific, and distinct from the typical POLR3-HLD imaging pattern (Harting et al., 2020; Perrier et al., 2020).

In the cohort of patients with a severe POLR3-HLD phenotype (and a similar genotype to the patient reported by Majethia and Girisha), all had insufficient myelin deposition not meeting the criteria for true hypomyelination, as well as additional findings including progressive abnormalities of the basal ganglia and thalami (sometimes only seen on repeat imaging) (Perrier et al., 2020).

A recent cohort study by Harting et al. (2020) also reported the combination of the c.1771-7C>G variant with an additional splicing, missense, or synonymous variant (Harting et al., 2020). Phenotypes of patients in this cohort ranged from severe (with similar features to those in our severe POLR3-HLD cohort) to a milder presentation. However, all patients had a specific MRI pattern involving striatal abnormalities, without frank hypomyelination (Harting et al., 2020). In other cohorts of patients with the adjacent c.1771-6C>G variant, whether homozygous or compound heterozygous with another variant in trans, striatal involvement was also evident on MRI (Azmanov et al., 2016; Hiraide et al., 2020; Wu et al., 2019). Thus, it would be interesting to review the MRI of the patient reported by Majethia and Girisha specifically for abnormalities of the basal ganglia and thalami, as well as for subtle evidence of insufficient myelin deposition. Moreover, it should be noted that although this MRI pattern appears to correlate to the genotype, it is possible that these abnormalities could have only been detected later, on repeat imaging, and were not evident on a single MRI obtained at 8 months.

As we mention above, genotypically, the patient described by Majethia and Girisha harbored similar *POLR3A* variants to those associated with the severe POLR3-HLD phenotype (c.1771-7C>G in addition to a nonsense variant). In this case, the nonsense variant (c.2005C>T; p.Arg669*) has also been reported in one patient with WRS (in combination with the splicing variant c.3337-11T>C) (Wambach et al., 2018). This patient was 20 years old at the time of

publication and had typical facial features associated with WRS, as well as contractures and frank generalized lipodystrophy. She had recurrent pneumonias and dysphagia. She also had cerebellar signs and intention tremors, and lost the ability to walk at 9 years of age.

These similarities are interesting and support the question of whether it is a dose-dependent amount of functional POLR3A that causes specific phenotypes, or the presence of specific variants. Indeed, the phenotypic variability associated with pathogenic variants in *POLR3A* is complex, and before distinct correlations can be formed, studies on additional patients with a similar genotype would be necessary, together with functional studies.

In conclusion, we thank Majethia and Girisha for their report of this patient and contribution to the literature on patients with biallelic pathogenic variants in *POLR3A*. While we highlight the similarities to patients with severe POLR3-HLD, as well as the differences in phenotype between this patient and those with WRS, we acknowledge that intermediate phenotypes of POLR3-related disorders may exist. Phenotypic diversity is broad in disorders associated with pathogenic variants in RNA polymerase III subunits, ranging from extremely mild to very severe. As researchers and clinicians move forward to characterize these disorders, detailed phenotyping and systematic classification of disorders are of importance for genotype-phenotype correlations to be established, knowledge of disease progression in a clinical setting, and potential therapeutic interventions.

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## Conflict of Interest Statement

Geneviève Bernard has no relevant conflict of interests. She is/was a consultant for Passage Bio Inc and Ionis. She serves on the scientific advisory board of the Pelizaeus-Merzbacher Foundation and is the Chair of the Medical Advisory Board of the United Leukodystrophy Foundation. She has received an unrestricted educational grant from Takeda (2021). In the last 2 years, Dr Bernard received research grants from the Canadian Institutes for Health Research (project grant 426534 and 201610PJT-377869), Montreal Children's Foundation, Fondation Les Amis d'Elliot, Pelizaeus-Merzbacher Disease Foundation, Foundation of Stars, Healthy Brains

Healthy Lives, Fondation le Tout pour Loo, Leuco-Action, Rare Diseases Foundation and BC Children's Foundation, Canada Summer Jobs, and McGill University Health Center Department of Medicine CAS Clinical Research Funding. She has received a Research Scholar Junior 1 award from the FRQS (2012–2016), the New Investigator Salary Award from the Canadian Institutes of Health Research (2017–2022) and the Research Scholar Senior award from the FRQS (2022–2025). Jennifer A. Wambach receives funding from the National Institutes of Health and the Children's Discovery Institute (Washington University School of Medicine/St. Louis Children's Hospital). The other authors declare that there are no conflict of interests.

## Data Availability Statement

Data sharing is not applicable to this correspondence as discussion is based on previously published articles.

## Tables

**Table 4.3.** Comparison of phenotypic and genotypic features of the patient reported by Majethia and Girisha (2021), the cohort of patients with severe POLR3-HLD (Perrier et al. 2020), and five published studies of patients with WRS (Jay et al. 2016, Lessel et al. 2018, Paolacci et al. 2018, Temel et al. 2020, Wambach et al. 2018).

| | **Majethia et al. 2021 Am J Med Genet A** | **Perrier et al. 2020 Neurol Genet** | **Jay et al. 2016 Am J Med Genet A** | **Wambach et al. 2018 Am J Hum Genet** | **Lessel et al. 2018 Hum Genet** | **Paolacci et al. 2018 J Med Genet** | **Temel et al. 2020 Eur J Hum Genet** |
|---|---|---|---|---|---|---|---|
| **Phenotype** | **? Severe POLR3-related leukodystrophy** | **Severe POLR3-related leukodystrophy** | **Wiedemann-Rautenstrauch syndrome** | **Wiedemann-Rautenstrauch syndrome** | **Wiedemann-Rautenstrauch syndrome** | **Wiedemann-Rautenstrauch syndrome** | **Wiedemann-Rautenstrauch syndrome** |
| **Pre-natal Growth** | Normal pregnancy | Unremarkable | Intrauterine growth restriction (1/1) | Intrauterine growth restriction (6/7) | Intrauterine growth restriction (3/3) | Length at birth <P3 (3/12) | Length 8 days after birth <P3 (1/1) |
| **Birth weight** | Normal weight | Normal weight (6/6) | Low birth weight (1/1) | Low birth weight (6/7) | Low birth weight (3/3) | Low birth weight (11/14) | Low birth weight (1/1) |
| **Age of death** | Death at 1.5 years | Death before age 3 years (4/6) | Death at 7 months (1/1) | Currently living at time of publication (ages 2-21 years) (7/7) | Currently living at time of publication (ages 11 months - 12 years) (3/3) | Death within first 6 months (6/15)<br>Death in early-late adolescence (2/15)<br>Age of death not reported (7/15) | Currently living at time of publication (age 6 years) (1/1) |
| **Physical appearance** | Triangular face at 15 months | No notable dysmorphia | i) Triangular face<br>ii) Alopecia<br>iii) Prominent forehead veins<br>iv) Low set malformed ears (1/1) | i) Triangular face (5/7)<br>ii) Sparse scalp hair (5/7)<br>iii) Prominent forehead veins (6/6)<br>iv) Low set ears (5/7) | i) Triangular face (3/3)<br>ii) Sparse scalp hair (2/3)<br>iii) Prominent scalp veins (3/3)<br>iv) Thin/ translucent skin (3/3) | i) Triangular face (13/14)<br>ii) Sparse scalp hair (13/13)<br>iii) Prominent scalp veins (13/13)<br>iv) Thin/ translucent skin (14/14) | i) Triangular face<br>ii) Alopecia<br>iii) Prominent scalp veins<br>iv) Low set ears (1/1) |
| **Fat distribution** | Decreased subcutaneous fat<br>Fat accumulation not reported | Unremarkable | Decreased subcutaneous fat (1/1) | Lipodystrophy (6/7)<br>Localized fat accumulation (5/7) | Lipodystrophy (3/3)<br>Fat accumulation not reported | Lipodystrophy (14/14)<br>Localized fat accumulation (6/11) | Local lipoatrophy (1/1) |
| **Dental abnormalities** | Anodontia | Delayed dentition (3/6) | Natal teeth (1/1) | Delayed dentition/ hypodontia (4/7)<br>Natal teeth (5/6) | Delayed dentition/ oligodontia (2/2)<br>Natal teeth (1/3) | Delayed dentition/ hypodontia (8/8)<br>Natal teeth (13/14) | Delayed dentition, natal teeth (1/1) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Neurological and Movement Abnormalities** | Increased muscle tone in lower limbs, ankle clonus, bilateral cortical thumbs, dystonia, walking not achieved | Axial hypotonia and upper motor neuron signs (spasticity and/or hyperreflexia) (5/6)<br>Dystonia/chorea, walking not achieved (6/6) | Abnormalities not reported (1/1) | Ability to walk/walk with assistance (6/7)<br>Tremor, cerebellar signs, inability to walk (1/7) | Ability to walk (2/2) | Tremor (2/8)<br>Hypertonia (8/13)<br>Ataxia or hypotonia (3/12) | Developmental delay (1/1) |
| **MRI features** | None reported | Basal ganglia and thalami abnormalities, insufficient myelin deposition (6/6) | MRI not performed (1/1) | MRI normal (1/7)<br>MRI not available (6/7) | Agenesis of corpus callosum (1/1)<br>MRI not available/reported (2/3) | No hypomyelination (4/4)<br>MRI not available/reported (11/15) | None reported (1/1) |
| **Genotype (Variants in *POLR3A* NM_007055.3)** | **Present report:**<br>Allele 1: c.1771-7C>G<br>Allele 2: c.2005C>T (p.Arg669*) | **6/6 Patients:**<br>Allele 1: c.1771-7C>G<br>Allele 2: Nonsense variant or frameshift deletion | **1/1 Patient:**<br>Allele 1: c.1909+18G>A<br>Allele 2: c.2617C>T (p.Arg873*) | **6/7 Patients:**<br>Allele 1: c.3337-5T>A or c.3337-11T>C<br>Allele 2: Additional splicing or nonsense variant<br>**1/7 Patients:**<br>Allele 1: c.3G>T (p.Met1?)<br>Allele 2: c.*18 C>T | **2/3 Patients:**<br>Alelle 1: c.3337-5T>A<br>Allele 2: Additional splicing or nonsense variant<br>**1/3 Patients:**<br>Allele 1: c.3G>T (p.Met1?)<br>Allele 2: Unidentified | **5/15 Patients:**<br>Allele 1: c.1909+18G>A or c.1909+22G>A & c.3337-11T>C<br>Allele 2: Additional splicing or missense or synonymous variant<br>**2/15 Patients:**<br>Allele 1: c.*18C>T<br>Allele 2: c.3G>T (p.Met1?) or c.4003G>A (p.Gly1335Arg)<br>**8/15 Patients:**<br>Allele 1: Missense or Frameshift deletion<br>Allele 2: Unidentified (ex12-15 del identified in one patient) | **1/1 Patient:**<br>Allele 1: c.3337-11T>C<br>Allele 2: c.3568C>T, (p.Gln1190*) |

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# CHAPTER 5.

# Delineating the progressive neuropathology of the severe striatal form of POLR3-related leukodystrophy

## Preface

In the previous chapter, we expanded the phenotypic spectrum of POLR3-HLD to include an atypical severe form of disease, involving prominent striatal involvement and a rapid disease progression. This severe form of disease is distinct from the typical POLR3-HLD presentation, and involves a much earlier disease onset, with severe developmental delay, failure to thrive, and a hyperkinetic movement disorder, resulting in death during childhood from respiratory insufficiency. In the typical form of disease, onset is usually in childhood, and involves progressive motor involvement, with cerebellar and pyramidal features, and is associated with death in adolescence or adulthood, dependent on the progression rate of neurodegeneration. Interestingly, in the severe form of disease, the MRI pattern also appeared distinct, including a pattern of progressive involvement of the striatum and mild insufficient myelin deposition, compared to the diffuse hypomyelination and preservation of specific structures seen in the typical form of POLR3-HLD. As this MRI pattern was distinct and involved nuclei of the basal ganglia as well as the thalamus, we hypothesized that the severe form of disease is associated with a different cellular pathophysiology. In the completed neuropathological studies on a patient at age 13 months, atrophy and severe neuronal loss were evident in the putamen, with discrete neuronal loss in the caudate. As the MRI pattern shows involvement of additional subcortical nuclei with age, it is likely that this involvement is progressive and at later ages, additional structures become affected.

In order to explore this hypothesis, in the following chapter we investigated the progression of the severe striatal form of POLR3-HLD through neuropathological investigations of two patients at ages 3 and 4. This study further explores the pathological features of the basal ganglia structures, thalamus, cerebellum, and spinal cord, providing key information into the pathophysiology of disease by characterizing progressive pathological features associated with this severe form of POLR3-HLD.

# Chapter 5. Delineating the progressive neuropathology of the severe striatal form of POLR3-related leukodystrophy

*Manuscript in preparation for submission.*

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**Keywords:** POLR3-related leukodystrophy, 4H leukodystrophy, POLR3A, neurodegeneration, striatal abnormalities, neuropathology

## Abstract

Specific combinations of biallelic pathogenic variants in *POLR3A* are known to be associated with a severe striatal form of disease, characterized by onset in early infancy, severe developmental delay, striatal abnormalities on MRI, and death in childhood. Previous neuropathological investigations of a patient deceased at 13 months of age have demonstrated involvement of the putamen and caudate nucleus, with areas of decreased myelin in the cerebellum, brainstem, and spinal cord. As this disease form is rapidly progressive, we aimed to investigate this phenotype in two additional individuals who died at ages 3 and 4 years old. Investigations revealed significant abnormalities of the striatum and thalamus. Both patients demonstrated severe atrophy of the putamen and caudate nucleus associated with neuronal loss, and atrophy of the globus pallidus, but with a milder neuronal loss. The thalamus was also atrophic, but to a lesser extent than the striatum. Patchy areas of hypomyelination were present in the hemispheric white matter, and within the brainstem and spinal cord the corticospinal tracts and ascending long

sensory tracts showed diffuse hypomyelination. In the cerebellum, hypomyelination was evident in the deep white matter, as well as the hilum of the dentate nuclei. In the second patient, the dentate nuclei also demonstrated neuronal loss. Through presentation of these cases, we demonstrate new pathological insights into the progressive involvement of specific subcortical structures, further expanding knowledge into the pathophysiology underlying this severe striatal form of disease.

## Introduction

RNA polymerase III (Pol III), the enzyme complex responsible for the transcription of many essential short non-coding RNAs, has been implicated in a spectrum of rare inherited neurological diseases resulting from pathogenic variants in specific subunit-encoding genes. Most commonly described is POLR3-related hypomyelinating leukodystrophy (POLR3-HLD), an autosomal recessive disease typically associated with the combination of hypomyelination, hypogonadotropic hypogonadism, and hypodontia (4H leukodystrophy), known to be caused by biallelic pathogenic variants in *POLR3A, POLR3B, POLR1C*, and *POLR3K* (Bernard et al., 2011; Bernard & Vanderver, 2017; Daoud et al., 2013; Dorboz et al., 2018; Saitsu et al., 2011; Tetreault et al., 2011; Thiffault et al., 2015). The typical clinical presentation of POLR3-HLD involves disease onset in childhood with motor delay and/or regression, and progressive neurological impairments, but at a much slower pace compared to the severe striatal form of disease (Gauquelin et al., 2019; Perrier et al., 2020; Wolf et al., 2014).

The severe striatal form of POLR3-HLD, caused by a specific combination of *POLR3A* variants, was described to be associated with an MRI pattern of striatal involvement and only mild insufficient myelin deposition, as opposed to the diffuse hypomyelination seen in patients with the

typical disease form (Harting et al., 2020; La Piana et al., 2014; Perrier et al., 2020; Wu et al., 2019). Patients with this severe presentation had early disease onset in the first months of life, involving severe developmental delay with regression as well as failure to thrive, and experienced progressive neurological features including a combination of dystonia and chorea. Most patients had dysphagia, with some also demonstrating laryngomalacia. The rapid progression of disease led to early death from respiratory failure in most patients.

The neuropathology of one patient with the severe striatal form of POLR3-HLD at age 13 months has been past studied, with involvement of specific structures (Perrier et al., 2020). Atrophy of the putamen was most evident, involving a severe neuronal loss and gliosis, with microglial activation. Discrete neuronal loss was seen in the caudate, however the globus pallidus and thalamus did not demonstrate significant neuronal loss. Myelin loss was not substantial as only patchy areas of reduced myelin were found in the cerebral hemispheres, brainstem, cerebellum, and spinal cord.

In this study, we present the neuropathological features of two patients with the severe form of POLR3-HLD, who died at ages 3 and 4 years. We describe the progression of specific features and provide detailed information on the neuronal and white matter abnormalities.

## Materials and Methods

### Patients' Inclusion and Ethics Approval

This research was approved by the Research Ethics Board of the McGill University Health Centre and the Montreal Children's Hospital (11-105-PED, 2019-4972), and informed consent was obtained for each patient. Available medical records and MRIs were reviewed.

### Neuropathological Investigations

Brain tissue was collected during autopsy and fixed before being transported to our centre for further neuropathological investigations. Fixation was performed in 10% formalin or Zn-formalin for a minimum of three weeks prior to transport. Tissue samples were embedded in paraffin and sections were cut at 7 µm thickness. Sections were stained with Hematoxylin-Eosin (H&E) and/or Klüver-Barrera (KB; Luxol-Fast-Blue-Cresyl-Violet). Immunohistochemistry for selected regions was completed with the following antibodies: GFAP (anti-glial fibrillary acidic protein, polyclonal rabbit, DAKO), IBA1 (anti-ionized calcium-binding adapter molecule 1, Wako, Sodobis, lot PDK 6188), SMI32 (anti-neurofilament H non-phosphorylated, mouse, Calbiochem, NoNE1023), calretinin (anti-calretinin, monoclonal mouse, DAKO, Clone DAK-Calret 1), calbindin (anti-calbindin, rabbit polyclonal, ABCAM) and MAP2 (anti-MAP2, mouse monoclonal, ABCAM). A panel of antibodies was also used to stain the pituitary gland, including anti-FSH, LH, PRL, GH, ACTH, TSH, PIT-1, T-pit, and SR1.

## Results

### Clinical, MRI, and Genetic Features

The first patient was a female who died shortly before turning 3 years of age. She passed from acute on chronic respiratory failure due to pneumonia in the left lower lobe. Clinically, her features were consistent with the severe striatal form of POLR3-HLD, with onset in early infancy. She had global developmental delay, with failure to thrive, and progressive neurological features including cerebellar features, dystonia, dyskinesia, chorea, and epilepsy. She also had laryngomalacia, and a laryngeal cleft. Due to dysphagia and chronic aspirations, she underwent a gastrostomy tube placement by age 5 months. She was hospitalized for recurrent aspiration

pneumonias and required a tracheostomy at 18 months of age. She was noted to have hemangiomas on the skin/subcutaneous tissue of the right scalp and posterior left leg.

The second patient, a male, passed at 4 years of age. He was previously described in the cohort of patients with the severe striatal form of POLR3-HLD (Patient 6 in Perrier et al. 2020). He had developmental delay and failure to thrive, with motor regression. He also had microcephaly, epilepsy, and showed pyramidal signs, along with dystonia and chorea. He was known to have dysphagia and recurrent aspirations, requiring a gastrostomy tube by age 12 months. He also had recurrent pneumonias requiring hospitalizations and succumbed to death at age 4 years.

On MRI, both patients showed progressive striatal involvement, with mild insufficient myelin deposition (Figure 5.1). Progression of myelination was noted over time in the two available studies of Patient 1 (Figure 5.1D, E). Both patients demonstrated mild to moderate cerebral atrophy (Figure 5.1B, C, E, F). Regarding subcortical nuclei abnormalities, Patient 1 demonstrated progressive T2-hyperintensities of the caudate nuclei and putamen between ages 7 and 14 months, with progressive atrophy of the putamen, caudate nuclei, and thalamus, associated with enlargement of lateral ventricles (Figure 5.1D, E). Similarly, Patient 2 demonstrated abnormal T2-weighted signal of the putamen, caudate nuclei, and thalamus at age 10 months, with atrophy of the putamen and caudate nuclei (Figure 5.1F). Both patients demonstrated preservation of the red nucleus at an early age (Figure 5.1G, I), and hyperintensities were evident on the later MRI of Patient 2 (Figure 5.1H). The brainstem of Patient 1 demonstrated progressive T2-hyperintensities of the posterior pons and hilus of the dentate nucleus, with T2-hypointensities noted in the medial lemniscus (Figure 5.1J, K). The middle cerebellar peduncles were preserved in the earlier study but became T2-hyperintense posteriorly 7 months later (Figure 5.1K). Patient 2 also demonstrated

T2-hyperintensity of the pons at age 10 months, which appeared most severe in the posterior region, along with preservation of the medial lemniscus (Figure 5.1L). T2-hyperintensities were also seen in the hilus of the dentate nucleus and the dentate nucleus itself (Figure 5.1L). In both patients, the midline structures were within normal limits (Figure 5.1M, N, O).

Genetically, both patients harboured the combination of compound heterozygous *POLR3A* (NM_007055.4) variants known to be associated with the severe striatal form of POLR3-HLD, including a specific splicing variant (c.1771-7C>G), and a variant leading to a truncated protein product *in trans* (Patient 1: c.760C>T, p.R254*; Patient 2: c.3583delG, p.D1195Ifs*47). The splicing variant has been previously reported to cause aberrant splicing and results in production of gene products lacking exon 14 (p.P591Mfs*9) as well as exons 13-14 (p.G548_Y637del) (Minnerop et al., 2017; Perrier et al., 2020).

**Neuropathological Investigations**

**Patient 1 (Female, Age 3)**

The brain of Patient 1 weighted 975 grams, significantly reduced compared to age-matched controls, with expected brain weight at 3 years being between 1263-1305g (Kayser, 1987; Molina et al., 2019). On external examination, the brain was symmetrical, with grossly normal gyration but decreased number of secondary and tertiary sulci when compared to an age-matched control, thus suggesting delayed brain development as opposed to atrophy (Figure 5.2A). The cranial nerves appeared normal. Upon midline sagittal sectioning, the corpus callosum demonstrated a normal size and thickness, with a well-developed genu, body, and splenium (Figure 5.2A). On coronal sections, the lateral ventricles appeared significantly enlarged due to severe atrophy of the striatum, mainly involving the putamen and caudate (Figure 5.2B). Enlargement of the ventricles

was most prominent in the frontal horns, with the atrium and occipital horns being less enlarged. Mild atrophy of the globus pallidus and thalamus were also appreciated. The neocortex was of normal thickness.

Microscopically, the neocortex showed a normal cytoarchitecture and differentiation, with a well-developed cortical ribbon. Rare ischemic neurons were observed. Occasional neurons appeared slightly hyper-eosinophilic, with smudgy degenerated appearing nuclei, and minimal loss of nuclear basophilia. The hippocampus showed a normal cytoarchitecture with no significant neuronal abnormalities, ischemic change, or inflammation. The amygdala appeared unremarkable, without neuronal abnormality.

The hemispheric white matter appeared relatively well-myelinated, apart from some small areas of mild hypomyelination in the parietal and occipital lobes (Figure 5.2C, D). Additionally, the white matter demonstrated non-uniform and patchy areas of pallor and rarefaction in the internal capsule and periventricular region. No evidence of demyelination was noted.

The basal ganglia demonstrated significant atrophy, varying in severity in specific nuclei. In particular, the putamen and caudate nuclei of the striatum demonstrated severe atrophy when compared to age-matched control tissue (Figure 5.2E). Further, synaptophysin staining confirmed the level of severe atrophy of the dorsal striatum, with milder involvement of the globus pallidus compared to control tissue (Figure 5.2F). Within the putamen and caudate nuclei, diffuse neuronal loss involving both small and large neurons was evident, associated with astrocytic gliosis and microglial activation (Figure 5.2G, H, J, K, L). Enlarged Virchow-Robin spaces were also identified, particularly in the putamen (Figure 5.2I). Few calretinin-positive neurons were identified in both the caudate nucleus and putamen, similarly to what was seen in the control. The globus pallidus appeared less atrophic but demonstrated gliosis with some apoptosis and mild

neuronal loss (Figure 5.2M). Calretinin-positive neurons were also preserved in the globus pallidus.

The thalamus appeared smaller compared to a control of the same age, however to a lesser extent than the level of striatal atrophy (Figure 5.2N). Mild neuronal loss was evident, with some apoptotic nuclei, particularly in the dorso-medial and ventral nuclei (Figure 5.2O, P). In other thalamic nuclei, the neuronal density was normal compared to control tissue. There were no significant changes in the geniculate bodies.

In mesencephalon of the brainstem, the corticospinal tracts demonstrated mild hypomyelination. Along the pons and medulla, the corticospinal and the ascending long sensory tracts appeared mildly smaller and paler compared with control tissue (Figure 5.3A, B, C). Within the pons, the corticospinal tracts were hypoplastic, however the pontine nuclei appeared unremarkable, with transverse fibers appearing of both normal size and myelination (Figure 5.3A). In the medulla, the olivary nuclei appeared smaller compared to control, but normal in both shape and neuronal density (Figure 5.3C). Macroscopically, the red nucleus was of normal size, and on the cellular level, the neuronal density was mildly decreased with no significant anomaly or astrogliosis (Figure 5.3E, F). The substantia nigra and cranial nerve nuclei both appeared normal.

In the cerebellum, the anterior vermis demonstrated a normal macroscopic appearance, however the posterior vermis showed a slight reduction in size, especially in the declive and lower lobules. Microscopically, areas of hypomyelination were evident in the deep white matter (Figure 5.3G, H). Sections of the cerebellum showed non-uniform mild thinning of white matter tracts, with patchy areas of mild myelin rarefaction. However, in the folia, the white matter did not show significant abnormalities. Similarly, the superior and inferior cerebellar peduncles were hypomyelinated and pale (Figure 5.3I, J), though the middle cerebellar peduncle did not show

these abnormalities (Figure 5.3K). In the cerebellar cortex, the Purkinje cells and granular cells appeared normal, along with the neurons in the dentate nucleus. In the hilum of the dentate nucleus, fibers appeared hypomyelinated and pale (Figure 5.3D).

In the spinal cord, the long tracts demonstrated diffuse hypomyelination with micro-vacuolation (Figure 5.3L, N, M). No significant lesions of motor neurons were noted in the anterior horns (Figure 5.3O).

The hypophysis demonstrated normal staining for FSH, LH, PRL, GH, ACTH, TSH, and PIT-1. Staining for T-pit and SR1 appeared absent or very weak, which was likely associated with the specimen being autopsy tissue.

**Patient 2 (Male, Age 4)**

The brain weight of Patient 2 was also significantly reduced, weighing approximately 960g, compared with the expected brain weight at 4 years between 1217-1375g (Kayser, 1987; Molina et al., 2019). On gross examination, the cerebral hemispheres appeared symmetric, without foci, softening, or discolouration. The cranial nerves were unremarkable. A hypercellular exophytic lesion was noted in the third ventricle, without significant cytologic atypia or elevated proliferative indices, likely consistent with a low-grade ependymoma. This lesion was considered diminutive and an incidental finding, not thought to be related to the patient's underlying disease or contribute to the cause of death. On histology, the neocortex demonstrated a decreased neuronal density, with columnar arrangement of neurons and inconspicuous horizontal lamination. Ischemic neurons were observed in different neocortical areas, including CA1 of Ammon's horns, due to the final hypoxic-ischemic injury preceding death (Figure 5.4A, B). No significant lesions were noted in the dentate gyrus of the hippocampus.

In the hemispheric white matter, patchy areas of hypomyelination were present, without features of demyelination (Figure 5.4C, D). There were no significant changes in the morphology or density of oligodendrocytes. Chronic astrocytic gliosis was noted in the frontal and temporal white matter, and there was significant and diffuse microglial activation in all white matter areas, including those which demonstrate normal myelination. The corpus callosum (genu) appeared of normal thickness and myelination.

In examination of the basal ganglia, specific nuclei demonstrated remarkable abnormalities, similar to Patient 1. The dorsal striatum, including both the putamen and caudate nuclei, were substantially atrophic, demonstrating severe volume loss (Figure 5.4E, F). On a cellular level, the putamen and caudate nuclei showed a considerable decrease in neurons, mainly involving small neurons, with a few large neurons identified (Figure 5.4I-N). Loss of neurons was associated with a dense chronic gliosis (Figure 5.4J). Virchow-Robin spaces were particularly enlarged, thus confirming parenchymal atrophy (Figure 5.4G, H). No significant microglial reaction was demonstrated. The globus pallidus demonstrated atrophy and an obvious but less severe neuronal loss, associated with chronic gliosis, a microglial reaction, and the presence of Alzheimer type II glia (Figure 5.4O, P).

The thalamus also demonstrated a level of atrophy, albeit in smaller proportions compared to the striatum (Figure 5.4Q). Neuronal loss was present, particularly in the anterior nucleus and dorso-medial nucleus (Figure 5.4R). In other thalamic nuclei, neuronal loss appeared more focal. There was chronic astrocytic gliosis, but no significant microglial reaction (Figure 5.4S). However, in the nearby posterior limb of the internal capsule, a microglial reaction was evident (Figure 5.4T).

Abnormalities were also noted in regions of the brainstem of Patient 2, similar to Patient 1. In the mesencephalon, the corticospinal tracts demonstrated hypomyelination. Neuronal loss

associated with astrocytic gliosis was seen in the colliculi (Figure 5.5A, B). In the pons, the corticospinal tracts also demonstrated asymmetric areas of myelin pallor (Figure 5.5D, E). The cranial nerve nuclei appeared normal and the pontine transverse fibers were well-myelinated. In the medulla, hypomyelination of the inferior cerebellar peduncles, ascending sensory tracts, and the pyramids was notable (Figure 5.5C). Ischemic neurons and type II Alzheimer's glia were present in the olivary nuclei and some of the cranial nerve nuclei (Figure 5.5F, G).

In the cerebellum, areas of hypomyelination were present, particularly within the axis of the cerebellar lamellae at the hemispheres and vermis, associated with astrocytic gliosis and a diffuse microglial reaction (Figure 5.5H, I, L). The cerebellar cortex demonstrated focal neuronal loss, accompanied by Bergman glia hyperplasia and microglial reaction (Figure 5.5J, K). In the hilum of the dentate nuclei, diffuse hypomyelination was notable (Figure 5.5M). The cerebellar dentate nuclei also demonstrated neuronal loss and ischemic damage of the remaining neurons (Figure 5N, O). Hypomyelination was also seen in the middle cerebellar peduncles. The spinal cord demonstrated diffuse hypomyelination of all long tracts (Figure 5.5P, Q, R).

## Discussion

In this study, we describe the neuropathological features of two patients with the severe striatal form of POLR3-HLD. Both patients harboured the specific combination of genetic variants known to be associated with this phenotype, including the c.1771-7C>G splicing variant, and a variant *in trans* which results in a truncated protein product. While the majority of biallelic pathogenic variants in subunits of *POLR3A* are associated with a typical phenotype involving childhood onset of motor dysfunction and hypomyelination on MRI, this specific combination of

variants is well-known to cause a severe form of neuronal disease with striatal involvement (Harting et al., 2020; Perrier et al., 2020; Wu et al., 2019).

Both patients, who passed at ages 3 and 4 years, demonstrated substantial atrophy of the putamen and caudate nuclei, with severe neuronal loss. The globus pallidus was also atrophic, but to a lesser extent, with milder neuronal loss. The previously investigated patient who passed at 13 months of age also demonstrated atrophy of the putamen with severe neuronal loss, however only discrete neuronal loss was evident in the caudate (Perrier et al., 2020). Additionally, in the globus pallidus of this younger patient, no appreciable neuronal loss was present. The thalamus was normal in size and without neuronal loss in this patient, while there was mild atrophy in the older patients. This suggests a progressive involvement of specific deep nuclei, perhaps associated with basal ganglia connectivity pathways in which early neuronal loss of specific structures may lead to decreased signalling and connectivity to downstream structures through Wallerian degeneration.

The brainstem and spinal cord were also involved in both described patients, with hypomyelination of the corticospinal tracts and ascending sensory tracts. In the patient who passed at age 13 months, there were only patchy areas of reduced myelin in these structures. Additionally, in the cerebellum, the hilum of the dentate nucleus demonstrated hypomyelination in each of the cases, however only in the oldest patient at age 4 years did neuronal loss appear evident in the dentate nucleus itself. Along with the basal ganglia and thalami abnormalities, these features demonstrate a clear progression of the disease with age.

Regarding white matter abnormalities, each patient with a severe phenotype showed patchy areas of reduced myelin in the hemispheric white matter, with oligodendrocytes appearing normal in morphology. Accordingly, the MRI pattern of patients with the severe striatal phenotype is only associated with mild insufficient myelin deposition, as opposed to the diffuse hypomyelination

seen in the typical disease presentation (Harting et al., 2020; La Piana et al., 2014; Perrier et al., 2020; Steenweg et al., 2010; Wolf et al., 2014). Likewise, the neuropathological features associated with typical POLR3-HLD have demonstrated hypomyelination and reduced numbers of oligodendrocytes, with variable severity in several brain regions (Vanderver et al., 2013; Wolf et al., 2014). Moderate axonal loss was also seen in patients with a typical phenotype, thought to be secondary to the loss of myelin (Vanderver et al., 2013; Wolf et al., 2014). This contrasts with the severe presentation, which we show to be associated with significant neuronal involvement in specific structures. Thus, it is possible that each disease form is associated with unique pathophysiological mechanisms causing defects during development in different cell types, leading to varying neurological abnormalities.

Interestingly, a genotype-phenotype correlation between the splicing variants at the c.1771-7 or -6 positions and striatal abnormalities on MRI has been well-established (Azmanov et al., 2016; Harting et al., 2020; Hiraide et al., 2020; Perrier et al., 2020; Wu et al., 2019). Depending on the variant *in trans*, patients can present with a more severe or milder form of disease. The severe form occurs when an amorphic loss of function allele is inherited with the splicing variant (Harting et al., 2020; Perrier et al., 2020; Wu et al., 2019), while the milder form occurs when the splicing variant is inherited in homozygous form, or if another hypomorphic allele is inherited, such as a missense, synonymous, or a different splicing variant (Azmanov et al., 2016; Harting et al., 2020; Hiraide et al., 2020). Furthermore, it is hypothesized that this splicing variant causes a cell-specific defect, leading to the neuronal involvement seen in the striatum. While both phenotypes demonstrate similar striatal abnormalities, patients with the mild striatal form of disease who are homozygous for the c.1771-7C>G or c.1771-6C>G variants appear to have normal myelination, where slight white matter involvement is usually only seen in the severe form. In past

studies, the c.1771-7C>G and c.1771-6C>G variants were shown to cause production of aberrant splicing transcripts, as well as wild-type length transcripts, likely due to incomplete cryptic splice site activation (i.e., leaky splicing) (Azmanov et al., 2016; Perrier et al., 2020). As diffuse hypomyelination is not reported in patients with these variants, it can be hypothesized that the level of wild-type protein expression from both alleles reaches the threshold necessary for myelin development. However, in patients with a severe phenotype, it is further hypothesized that the mild insufficient myelin deposition may result from the amorphic allele *in trans*, limiting the amount of functional POLR3A that is present during development compared to those with the homozygous splicing variants. Further studies are required to investigate the impact of specific variants and the involvement of different cell types during neurological development.

Other splicing variants in *POLR3A*, including the c.1909+22G>A variant, are known to be associated with milder phenotypes, including adolescent and adult-onset spastic paraparesis and spastic ataxia (de Assis Pereira Matos et al., 2020; Di Donato et al., 2021; Gauquelin et al., 2018; Infante et al., 2020; Minnerop et al., 2017; Morales-Rosado et al., 2020; Ruggiero et al., 2020; Rydning et al., 2019). This phenotype differs from typical POLR3-HLD and does not involve diffuse hypomyelination, rather, MRI patterns show variable atrophy of the spinal cord, corpus callosum, and cerebellum, with T2-hyperintensity of the superior cerebellar peduncles (Di Donato et al., 2021; Infante et al., 2020). A recent investigation of the neuropathology in an adult with this variant, in compound heterozygous form with the *POLR3A* variant c.1051C>T; p.Arg351*, demonstrated severe degeneration of the posterior columns, spinocerebellar tracts, and anterior corticospinal tracts through the cervical and lumbar spinal cord (Sytsma et al., 2022). In this case, as both axonal and myelin loss were observed in the tracts, it was unclear whether the disease pathogenesis was primarily neuronal or related to white matter (Sytsma et al., 2022). The

neuropathological pattern appeared to resemble Friedreich's ataxia (Koeppen, 2011). The c.1909+22G>A variant has also been found to be associated with a level of wildtype length transcript expression, similar to the splicing variants discussed above. Moreover, patients with this spastic ataxia phenotype also do not display diffuse hypomyelination, further supporting the hypothesis that certain threshold levels of wild-type POLR3A protein expression are required for proper formation of myelin during brain development. Additionally, as each of these splicing variants are associated with the production of different aberrant splicing transcripts and distinct neuropathological phenotypes, it is possible that the mechanisms of disease pathogenesis may impact different cell types.

In conclusion, these cases demonstrate a progressive sequential involvement of specific structures associated with the severe striatal form of POLR3-HLD. The extrapyramidal system, involving the cerebellum, basal ganglia structures, and thalamus, are each involved. As these anatomical structures are intricately related, it can be difficult to pinpoint those in which the abnormalities may originate, or if each may be involved individually. However, in this study, and together with the previously published neuropathological case, we were able to hypothesize that the dorsal striatum is primarily involved, followed by the globus pallidus and thalamus, with the possibility of Wallerian degeneration impacting these and other structures. To further explore the disease pathophysiology, additional studies investigating the effect of this combination of variants on specific cell types are required. This study provides key information into the progression of this disease on a pathological level, and further characterizes the severe striatal phenotype associated with pathogenic variants in *POLR3A*.

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## Figures

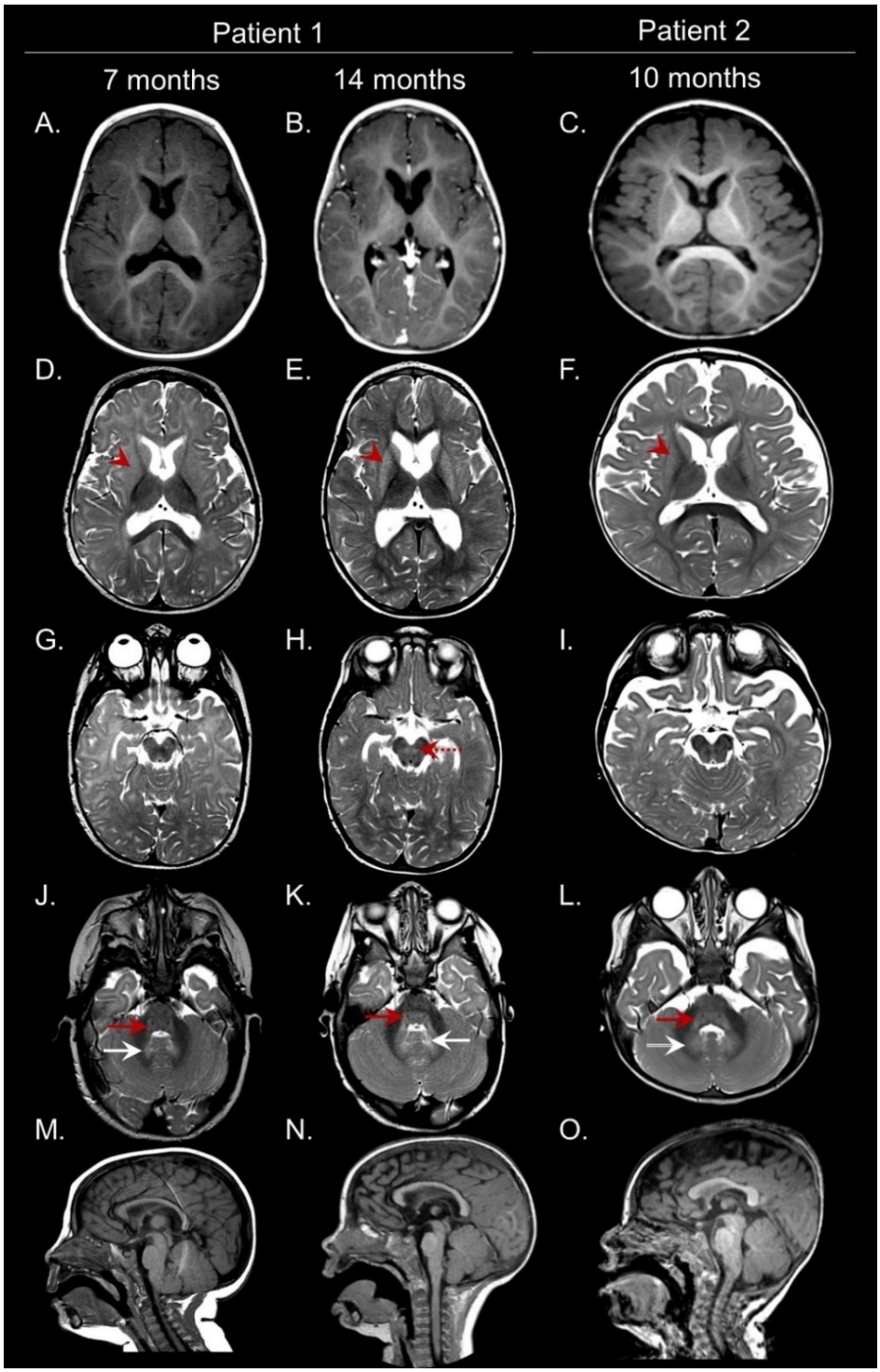


**Figure 5.1. MRI studies of Patient 1 (7 months, 14 months) and Patient 2 (10 months).** MRIs shown as axial T1 weighted images (A-C) [(A) T1 FLAIR, (B) T1 with gadolinium and (C) T1], axial T2 (D-L) and sagittal T1 weighted (M-O) images. Mild to moderate cerebral atrophy is seen

in both patients (B, C, E, F). Mild myelin deficits are evident (D-L), with progression of myelination seen in Patient 1 over time (A, B, D, E). In Patient 1 between ages 7 to 14 months, the basal ganglia demonstrated progressive T2-hyperintensities of the putamen (D, E, red arrowhead) and caudate nucleus, as well as progressive atrophy of the putamen, caudate nucleus, and thalamus with enlargement of the lateral ventricles. The brain MRI of Patient 2 at age 10 months demonstrates abnormal T2-weighted signal of the putamen (F, red arrowhead), caudate nucleus, and thalamus, with atrophy of the putamen and caudate nuclei (F). In both patients, the red nucleus appears preserved in the MRIs at an early age (G, I), and became hyperintense in Patient 1 by age 14 months (H, red dashed arrow). In the brainstem of Patient 1, the posterior pons (red arrow) and hilus of the dentate nucleus and part of the dentate nucleus (white arrow) demonstrate progressive T2-hyperintensities (J, K), with the medial lemniscus appearing T2-hypointense (J, K), and the middle cerebellar peduncles appearing preserved in the earlier study (J) but becoming T2-hyperintense posteriorly by age 14 months (K). Brain MRI of Patient 2 also demonstrated T2-hyperintensity of the pons, appearing most severe posteriorly (red arrow), with preservation of the medial lemniscus, as well as hyperintensities of the hilus of the dentate nucleus and the dentate nucleus itself (white double-lined arrow) (L). The midline structures appeared within normal limits (M, N, O).

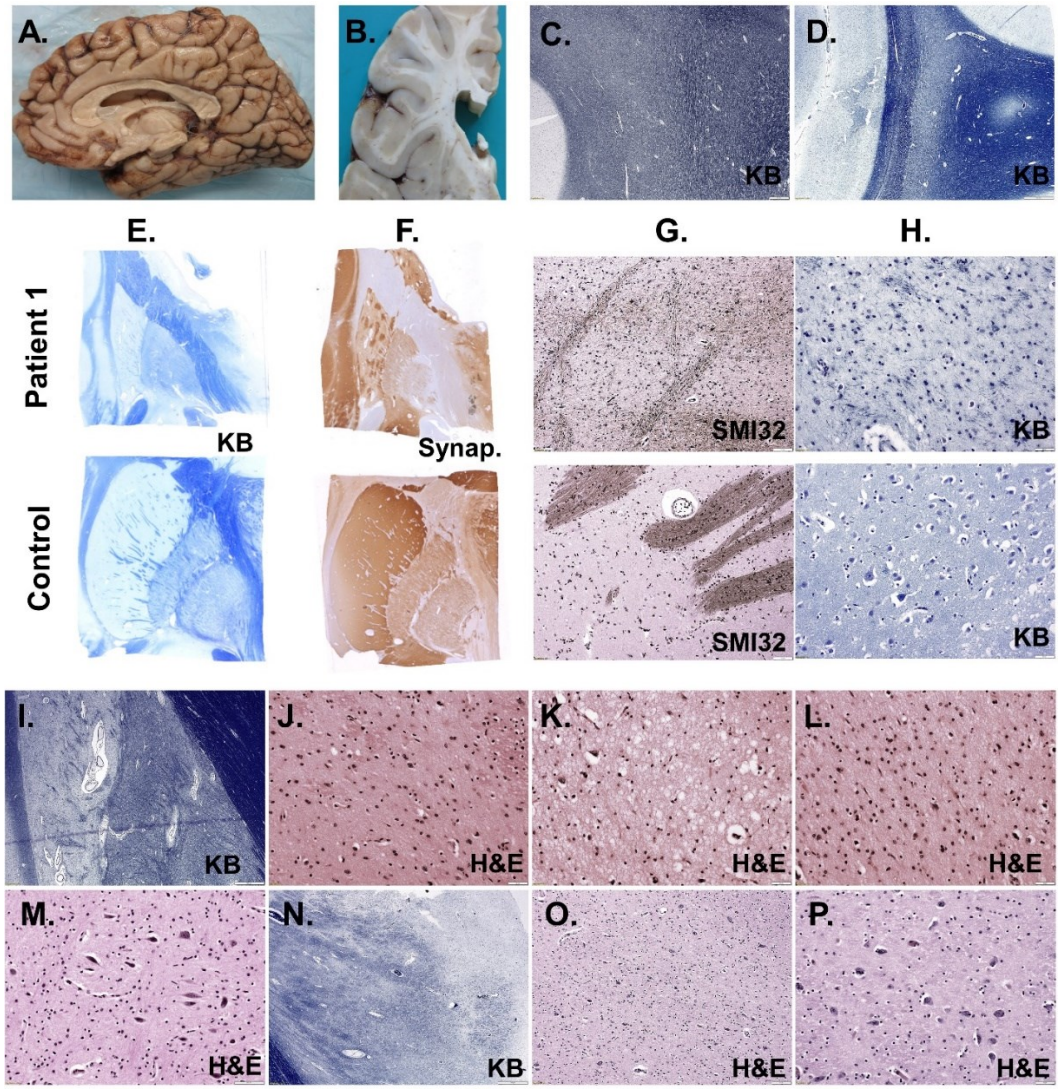


**Figure 5.2. Neuropathology of Patient 1 at age 3 years demonstrating striatal and thalamus abnormalities.** (A) Macroscopic appearance of the brain, demonstrating normal thickness of the corpus callosum. (B) Coronal section of the brain, demonstrating enlargement of the lateral ventricle and severe atrophy of the striatum. (C) White matter of the parietal lobe, with areas of pale appearance (1x, KB). (D) Small pale areas in the occipital lobe, near the calcarine fissure (1x, KB). (E,F) The lenticular nuclei including the putamen and caudate nuclei, demonstrate severe atrophy compared to control tissue visualized with Klüver-Barrera (KB) and synaptophysin staining (1x). (G) Axonal loss is evident compared to control tissue using SMI32 staining (10x). (H) Neuronal loss and gliosis are prominent compared to control tissue (20x, KB). (I) The lenticular nucleus demonstrates enlarged Virchow-Robin spaces, particularly in the putamen (1x, KB). (J) In the putamen, there is severe neuronal loss, with the appearance of numerous apoptotic nuclei (20x, H&E). (K, L) The caudate nucleus also shows severe neuronal loss, with gliosis, and spongiosis (20x, H&E). (M) The globus pallidus appears to be less involved, but demonstrates gliosis and apoptotic nuclei (20x, H&E). (N) The thalamus displays enlarged Virchow-Robin spaces, with the anterior thalamus appearing atrophied (1x, KB). (O) In the dorso-medial nucleus of the thalamus, neuronal loss is evident (10x, H&E). (P) Numerous apoptotic nuclei are visualized in the thalamus (40x, H&E).

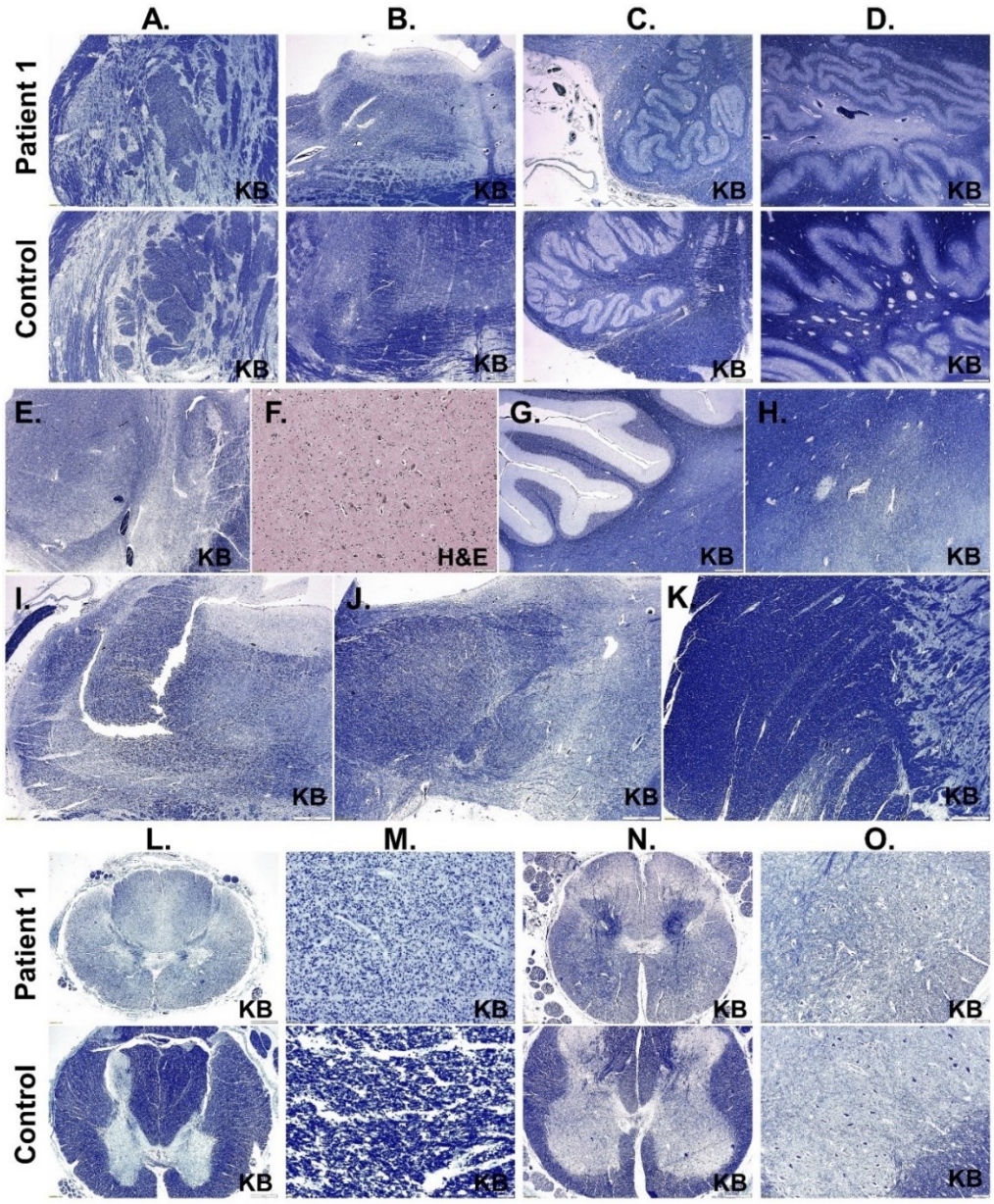


**Figure 5.3. Neuropathology of Patient 1 demonstrating abnormalities within the brainstem, cerebellum, and spinal cord.** (A) The pons demonstrates normal transverse fibers, but hypoplastic corticospinal tracts compared to control tissue (1x, KB). (B) The medial lemniscus appears pale and hypoplastic (1x, KB). (C) The medulla demonstrates pale corticospinal tracts, with a small olivary nucleus (1x, KB). (D) The hilum of the dentate nucleus appears pale compared to control tissue (1x, KB). (E,F) The red nucleus appears of normal size, with no significant neuronal loss or gliosis (1x, KB; 10x, H&E). (G, H) In the cerebellum, patchy areas of pale myelin are present in the deep white matter (1x, 2x, KB). (I) The superior cerebellar peduncle appears pale (1x, KB). (J) The inferior cerebellar peduncle also appears mildly hypomyelinated (1x, KB). (K) The middle cerebellar peduncle is well myelinated (1x, KB). (L, M, N) The spinal cord demonstrates hypomyelination and atrophy of long tracts at the cervical (L) and the lumbar (N) levels (1x, KB). (M) The corticospinal tracts appear hypomyelinated compared to control (20x, KB). (O) The anterior horn appears normal (4x, KB).

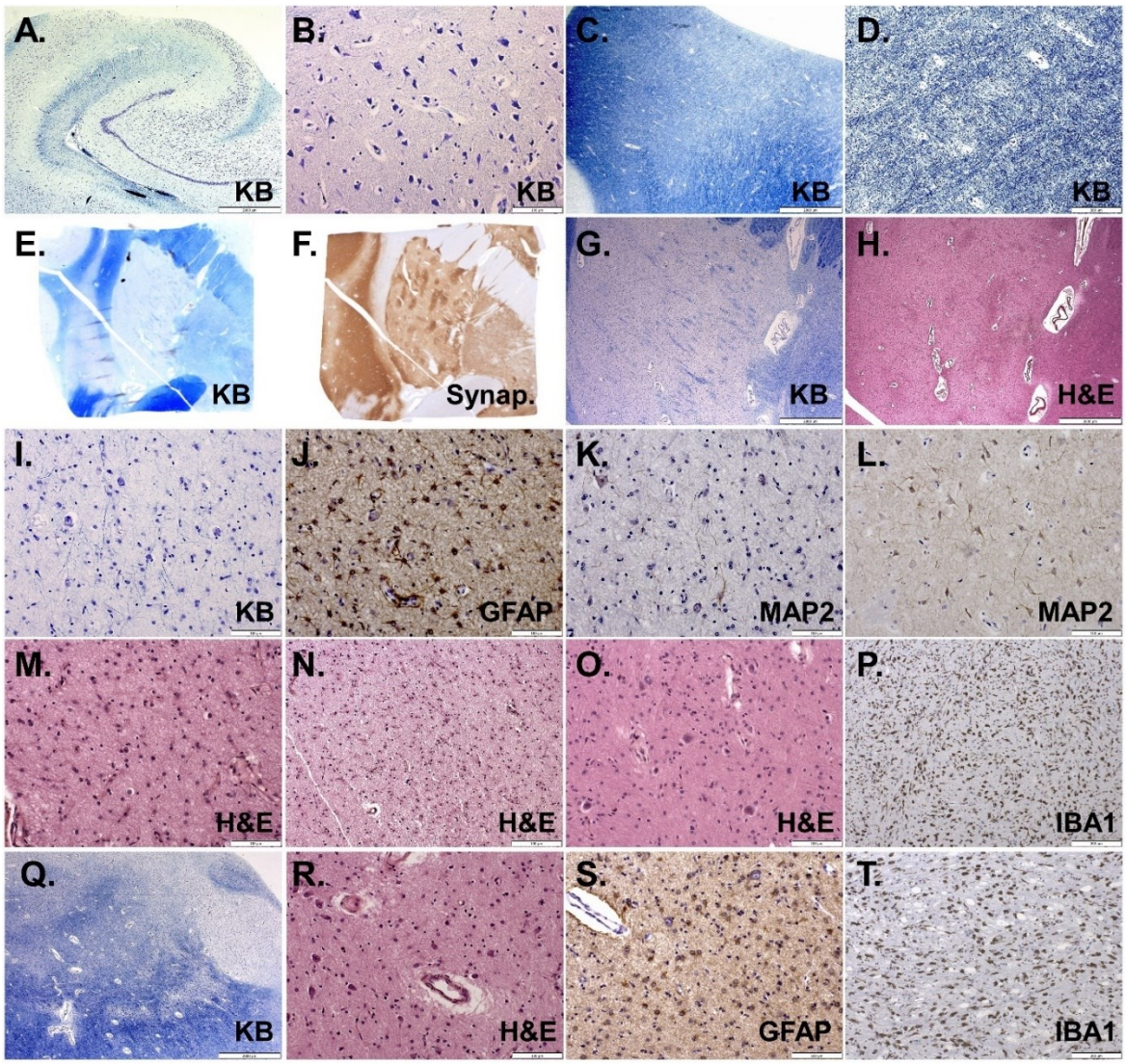


**Figure 5.4. Neuropathology of Patient 2 at age 4 years demonstrating striatal and thalamus abnormalities.** (A, B) Ammon's horn demonstrates neuronal loss and ischemic neurons in the subiculum (1x, 20x, KB). (C, D) Areas of pale myelin are evident, shown in the centrum ovale of the frontal lobe, without gliosis, but with microvacuolization (1x,10x, KB). (E, F) The striatum and pallidum appear severely atrophic (1x, KB, synaptophysin). (G, H) The atrophic putamen demonstrates enlargement of Virchow-Robin spaces (1x, KB, H&E). (I, J) Neuronal loss is evident in the putamen, with gliosis (20x, KB, GFAP). (K, L) Neuronal loss is also evident following MAP2 staining (20x). (M) In the putamen, there is severe loss of small neurons, with visualization of some large neurons (20x, H&E). (N) The caudate nucleus demonstrates neuronal loss, with loss of small neurons and a few remaining large neurons (20x, H&E). (O) The globus pallidus shows less neuronal loss, but chronic gliosis and type II Alzheimer's glia (20x, H&E). (P) A microglial reaction is evident in the white matter (20x, IBA1). (Q) The thalamus demonstrates atrophy, to a lesser extent than the striatum (1x, KB). (R, S) Neuronal loss and gliosis are evident in the anterior nucleus of the thalamus (20x, H&E). (S) Gliosis is present in the centromedial nucleus (20x, GFAP). (T) A microglial reaction is evident in the posterior limb of the internal capsule (20x, IBA1).

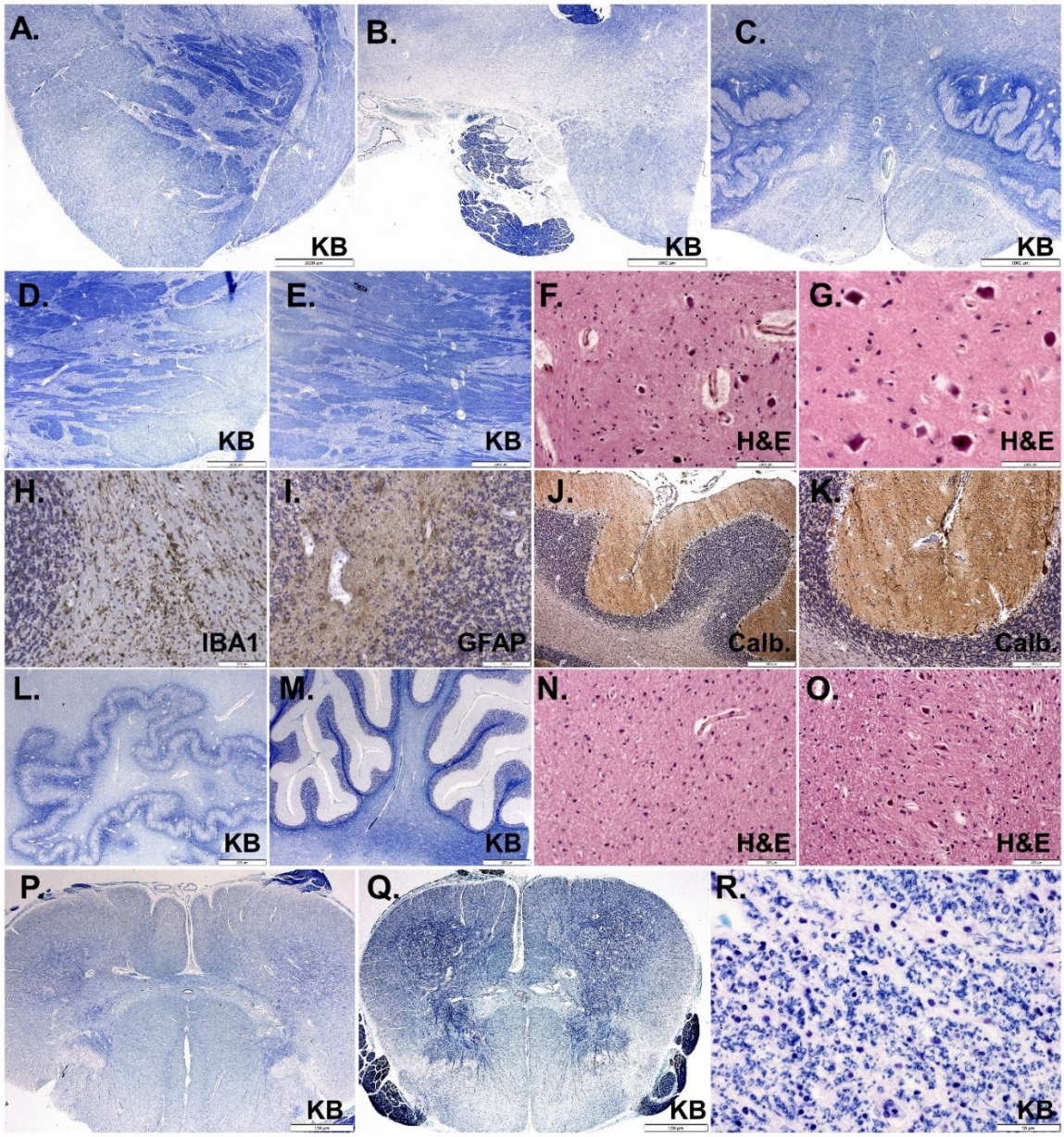


**Figure 5.5. Neuropathology of Patient 2 demonstrating abnormalities within the brainstem, cerebellum, and spinal cord.** (A, B) In the mesencephalon, large areas of myelin pallor are evident in the corticospinal tracts (1x, KB). (C) In the medulla, the pyramids appear pale (1x, KB). (D, E) Asymmetric areas of myelin pallor of the corticospinal tracts are also evident in the pons (1x, KB). (F, G) The olivary nucleus contains ischemic neurons and Alzheimer's type II glia (10x, 20x, H&E). (H) Microglial reaction is present in the cerebellar white matter (20x, IBA1). (I) Gliosis is noted in areas of hypomyelination in the cerebellum (20x, GFAP). (J, K) Loss of Purkinje cells is also evident in some areas (4x, 10x, calbindin). (L) In the white matter of the vermis, myelin pallor is present (1x, KB). (M) In the cerebellum, hypomyelination is noted in the deep white matter, in areas surrounding the dentate nucleus (1x, KB). (N) In these areas, gliosis and microglia are evident (20x, H&E). (O) Ischemic neurons and Alzheimer's type II glia are also present in the dentate nucleus (20x, H&E). (P, Q, R) Hypomyelination of the long tracts is seen in the spinal cord, shown at the cervical and lumbar levels (1x, 20x, KB).

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# CHAPTER 6.

# General Discussion and Future Directions

The following discussion includes original content as well as excerpts from the following narrative review article on POLR3-related leukodystrophy and potential therapeutic approaches for treatment, published in *Frontiers of Cellular Neuroscience*. Excerpts from this publication are marked by quotations, with formatting adapted for this thesis.

**POLR3-Related Leukodystrophy: Exploring Potential Therapeutic Approaches**

Narrative Review in *Frontiers in Cellular Neuroscience (Issue: Myelin Repair: At the Crossing-Lines of Myelin Biology and Gene Therapy)* 14, 631802. doi: 10.3389/fncel.2020.631802.



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## 6.1 General Summary

This thesis uses several approaches to investigate different aspects of hypomyelinating leukodystrophies, including underlying genetics, clinical manifestations, disease pathology, and pathophysiology. In Chapter 2, NGS was used to identify a genetic diagnosis in patients presenting with hypomyelination or delayed myelination on MRI, but without a resolved genetic cause following initial clinical sequencing. Analysis of this cohort also led to the identification of causal variants in 7/17 patients. In the first manuscript, we describe the lessons learned on the path to diagnosis, including the challenges and limitations of both clinical multi-gene panel sequencing, and exome sequencing. The second manuscript describes a patient who was initially included in this cohort, and found to have pathogenic variants in the gene *POLR3K*, a particularly rare cause of POLR3-HLD. In a correspondence, we also comment on the classification of genes associated with hypomyelinating leukodystrophies and the importance of proper characterization of white matter disorders. In Chapter 3, a large cohort of patients with POLR3-HLD was investigated in order to systematically evaluate endocrine and growth abnormalities, where it was clear that short stature and pubertal abnormalities were common findings. In Chapter 4, a severe form of POLR3-HLD was identified and further phenotypically classified and genetically studied. We performed functional studies to determine the impact of the splicing variant, which each patient shared, and investigated the effect of nonsense mediated mRNA decay. The neuropathology of one patient was also investigated, with striatal abnormalities being prominent. In an additional correspondence, this phenotype was compared to that of WRS, a severe congenital progeroid syndrome with distinct clinical features but also caused by variants in the same gene. In the following chapter, the pathology of two additional patients with the severe form of POLR3-HLD was investigated and found to have progressive striatal and thalamus abnormalities, increasing in severity with age.

This combination of work further expands knowledge into rare hypomyelinating disorders, from exploring genetic diagnoses, to investigating phenotypic features, and finally, describing a severe form of disease with insight into its pathophysiology.

## 6.2 Investigating Genetic Diagnoses Using NGS

Despite extensive clinical investigations, many rare diseases, including white matter disorders, remain undiagnosed for a period of time in part due to limitations in sequencing and analysis techniques. However, recent advances in genomics research have focused on overcoming these challenges both by developing new technologies and harnessing knowledge of sequencing limitations to improve analysis techniques and variant screening. The benefits of using NGS, including ES or GS, to diagnose rare genetic diseases have been demonstrated widely in past studies (Koboldt et al., 2013; Marinakis et al., 2021; Stavropoulos et al., 2016; Y. Yang et al., 2014).

As novel rare benign variants are often identified alongside truly pathogenic variants, challenges lie in the interpretation of sequencing data. In ES-targeted regions, over 20,000 variants are typically identified, which are further filtered and classified based on variant type and predicted functional impact when analyzing data to identify likely pathogenic variants in candidate genes. In a study of just under 50 000 participants who underwent ES, it was found that of the 783 median number of rare variants [i.e., minor allele frequency (MAF) <1%] detected in ES-targeted regions, 227 were synonymous, 379 were missense, and 20 were predicted as loss of function (Van Hout et al., 2020). Thus, a challenge is presented in identifying disease-causing variants in monogenic diseases and distinguishing them from numerous benign variants, which may be falsely associated with positive pathogenicity predictions. This demonstrates the importance of using a robust

diagnostic analysis protocol for evaluating pathogenicity of variants, including guidelines set by expert consortiums (Richards et al., 2015). Interpretation of these variants and correlation to phenotype is thereby critical in order to determine the true cause of disease. In addition, proper phenotyping and knowledge of clinical presentation is particularly important when evaluating variants for pathogenicity.

### 6.2.1 Importance of Collaborative Approaches in Genetic Diagnostics

As research progresses in the fields of both genomics and medical genetics, discovery rates for novel genes associated with disease continue to increase. This can partially be attributed to the development of global databases which allow users to submit candidate genes along with phenotypic details to match with others sharing the same information, such as GeneMatcher (Sobreira, Schiettecatte, Boehm, et al., 2015; Sobreira, Schiettecatte, Valle, et al., 2015), DECIPHER (Firth et al., 2009), and PhenomeCentral (Buske et al., 2015). Further, the Matchmaker exchange was created as a platform to merge numerous gene matching databases into a common interface to facilitate data submission and queries (Philippakis et al., 2015; Sobreira et al., 2017). The classification of variants in a novel gene as pathogenic typically requires a high level of evidence, including both functional data as well as the identification of several unrelated patients with shared phenotypes. These data sharing platforms allow for phenotypically similar individuals to be identified, thus providing foundational support to strengthen novel genes as candidates. Many studies have demonstrated the utility of these platforms, further illustrating the value of global data sharing in the resolution of genetic diagnoses (Azzariti & Hamosh, 2020; Baxter et al., 2022; Hamosh et al., 2022; Kernohan et al., 2017; McWalter et al., 2022; Osmond et al., 2022; Taylor et al., 2022; Towne et al., 2022). Furthermore, by linking phenotypic traits and

gene candidates in patient populations on a global scale using these collaborative approaches, the discovery of novel disease-causing genes can be expedited.

### 6.2.2 Investigating Genetically Unresolved Disorders

The value of periodically reanalysing data in the context of resolving genetic diagnoses has also been demonstrated; in many studies, reanalysis of NGS data using an improved bioinformatics pipeline led to increases in diagnostic yields (Al-Murshedi et al., 2019; Al-Nabhani et al., 2018; Costain et al., 2018; Ewans et al., 2018; Fung et al., 2020; Li et al., 2019; Shashi et al., 2019; Wenger et al., 2017). Reanalysis of data can be beneficial over time as new disease-causing genes are reported and as knowledge of novel protein functions develop. Bioinformatics techniques may also be adapted for reanalysis by using less-stringent filtering criteria to investigate variants which may have been overlooked on initial analyses. Over time, phenotypic information on disease spectrum may also become available and provide clues to the diagnosis and identification of gene candidates.

Sequencing additional family members can also be an effective means to increase diagnostic yield when investigating persistently unsolved cases. Moreover, with knowledge of carrier status for variants in asymptomatic individuals, strong candidate genes can be identified more easily. In addition, sequencing of parental DNA in a trio-based approach can be beneficial for the identification of *de novo* pathogenic variants (Ewans et al., 2018). The implementation of more advanced sequencing methods, such as RNA sequencing or High-Fidelity Long-Read Sequencing, can also be useful to identify variants which may not have been detected using previous methods (Hiatt et al., 2021; Marco-Puche et al., 2019).

Furthermore, in a clinical context, determining genetic diagnoses in children with rare neurological disorders allows for access to information on expected disease progression, which is useful for clinicians when determining eligibility for a clinically available therapy or clinical trials, as well as in providing supportive care. With a complete picture of other individuals and phenotypes associated with the disease, it can also inform clinicians on the specialists to be involved in the patient's multidisciplinary care team. For patients and their families, obtaining specific genetic diagnoses are especially important when making reproductive decisions, in consultation with genetic counsellors. Likewise, obtaining a genetic diagnosis enables patients and families to connect with others in disease-specific support groups, providing a level of social support through connections in the patient community, reducing the level of isolation that is often described by patients with rare disorders.

### 6.3 Investigating Clinical Manifestations of POLR3-HLD

Over the past decade, several genes encoding for subunits of the Pol III enzyme complex have been described as associated with phenotypically different conditions, with POLR3-HLD remaining the most commonly identified phenotype. Pathogenic variants in the genes *POLR3A*, *POLR3B*, and *POLR1C* are most frequently associated with POLR3-HLD, and past cohort studies have been completed to describe the prevalence of both neurological and non-neurological features (Gauquelin et al., 2019; Wolf, Vanderver, et al., 2014). Variants in *POLR3K* have only been described in three patients, including the case we report in Chapter 2. Although genes encoding for different Pol III subunits are implicated in POLR3-HLD, patients are often phenotypically similar, sharing both neurological and non-neurological features, together with a typical brain MRI pattern.

Neurological features associated with POLR3-HLD predominantly involve cerebellar, pyramidal, extrapyramidal, and cognitive aspects (Al Yazidi et al., 2019; Gauquelin et al., 2019; Wolf, Vanderver, et al., 2014). As the disease progresses, neurodegeneration leads to progressive impairments in motor skills resulting in loss of independent ambulation, as well as dependency on a feeding tube as a result of dysphagia, and loss of speech due to dysarthria (Lata et al., 2021). Individuals with POLR3-HLD eventually succumb to an early death in adolescence to adulthood depending on the rate of disease progression.

Non-neurological features are not uniform across this patient population but are often seen to varying extents. Progressive myopia is a common finding, along with dental abnormalities, which can range between hypodontia, oligodontia, delayed dentition, or the presence of natal teeth (Gauquelin et al., 2019; Wolf, Vanderver, et al., 2014; Wolff et al., 2010). As described in Chapter 3, endocrine abnormalities are also common, involving delayed or absent puberty, along with growth abnormalities manifesting as short stature (Pelletier et al., 2021; Potic et al., 2012; Potic et al., 2015). Interestingly, a phenotype of isolated hypogonadotropic hypogonadism has been described in a small number of patients with pathogenic variants in *POLR3B*, who did not exhibit neurological features or dental abnormalities (Richards et al., 2017).

The range of features associated with POLR3-HLD require a robust multidisciplinary care team in the clinical setting. In addition to primary health care providers (i.e., pediatricians or family physicians), pediatric neurologists and geneticists, care teams should involve otolaryngologists, ophthalmologists, endocrinologists, rehabilitation physicians, physical and occupational therapists, speech pathologists, neuropsychologists, and dentists (Adang et al., 2017; Bernard & Vanderver, 2017). Involvement of these specialists allows for proper management of disease

features, monitoring of symptom progression, and supportive care to be provided, in order to improve quality of life.

### 6.3.1 POLR3-HLD Pathophysiology

The pathophysiology underlying POLR3-HLD is complex given that Pol III is a ubiquitously expressed housekeeping gene. Studies are ongoing to assess the association of Pol III hypofunction with the white matter pathology seen in the majority of patients. The two major hypotheses for disease pathophysiology center around reduction in Pol III activity causing a lack in production of its target nc-RNAs. The first hypothesis is that insufficient production of tRNAs leads to a reduction in global protein synthesis during myelination, a critical period in development which requires the production of large amounts of proteins and lipids over a short period (Lata et al., 2021; Thiffault et al., 2015; Watt et al., 2022). In support of this hypothesis, as early myelinating structures are spared and show relatively preserved myelination on MRI in patients with the typical disease presentation, it can be postulated that myelination arrests at a certain timepoint in neurodevelopment (i.e., after the smallest structures are myelinated) when the ability of the hypofunctional Pol III becomes insufficient to myelinate the rest of the brain (i.e., the cerebral hemispheres, which are the largest structures). This hypothesis is also supported by the report of other transcription and translation-related genes being implicated in other hypomyelinating leukodystrophies, such as amino-acyl tRNA synthetase and related genes (e.g. *AIMP1, AIMP2, DARS1, AARS1, RARS1, VARS1, EPRS1*) (Feinstein et al., 2010; Friedman et al., 2019; Mendes et al., 2018; Ognjenovic & Simonovic, 2017; Park et al., 2008; Shukla et al., 2018; Simons et al., 2015; Taft et al., 2013; Wolf, Salomons, et al., 2014). The second hypothesis involves specific nc-RNAs being required for proper development and myelination of the CNS,

such as BC200 (Choquet, Forget, et al., 2019). It also remains possible that both hypotheses may play a role in disease pathogenesis, especially given the spectrum of phenotypes associated with hypofunction of Pol III. Moreover, it is likely that different tissues and cell types may exhibit distinct responses to Pol III hypofunction and the associated decrease of nc-RNA production, leading to specific phenotypes such as the striatal form of disease. Furthermore, with the expansion of phenotypes associated with mutations in genes encoding Pol III subunits, additional insight can be provided into the underlying disease pathophysiology.

### 6.4 Unravelling a Novel Severe Phenotype of POLR3-HLD

Studying disease pathophysiology in a group of patients with highly similar phenotypes is certainly beneficial when establishing genotype-phenotype correlations. In Chapter 4, the severe form of POLR3-related leukodystrophy was described, with distinct clinical and MRI features compared to the typical form of disease. The typical form of disease associated with *POLR3A* and *POLR3B* variants has been well described in a study of 105 patients, where the majority of patients presented with early onset motor delay or regression, prominent cerebellar features, mild pyramidal features and relatively preserved cognition (Wolf, Vanderver, et al., 2014). Approximately 10% of patients had a later onset of disease with learning difficulties and development of motor impairment. In this 2014 study, it was also established that pathogenic variants in *POLR3A* generally cause a more severe phenotype compared to *POLR3B* variants (Wolf, Vanderver, et al., 2014). The MRI pattern of individuals with POLR3-HLD is also relatively consistent, involving diffuse hypomyelination with relative preservation of myelination of specific structures, and may or may not involve cerebellar atrophy or thinning of the corpus callosum (La Piana et al., 2014; Steenweg et al., 2010; Wolf, Vanderver, et al.,

2014). The patients we describe with a severe phenotype have an earlier onset in the first few months of life, a much faster disease progression, severe developmental delay and regression, failure to thrive, and a characteristic hyperkinetic movement disorder. The MRI pattern also shows prominent neuronal involvement, including the striatum and thalamus, as well as the red nucleus. However, myelination is only mildly abnormal, contrary to those with a typical presentation. This leads to the hypothesis that the severe striatal form of disease is associated with different pathophysiology on a cellular level.

### 6.4.1 Striatal Involvement in POLR3-Disorders

A strong association has been described relating splicing variants at the c.1771-7 or -6 positions in *POLR3A* with striatal abnormalities on MRI (Azmanov et al., 2016; Harting et al., 2020; Hiraide, Kubota, et al., 2020; Perrier et al., 2020; Wu et al., 2019). Further, patients are known to present with the severe form of disease when an amorphic loss of function allele is inherited *in trans* with this splicing variant (Harting et al., 2020; Perrier et al., 2020; Wu et al., 2019). However, when a hypomorphic variant is inherited on the other allele (e.g., a missense, synonymous, or splicing variant), a range of mild to intermediate phenotypes can be seen (Azmanov et al., 2016; Harting et al., 2020; Hiraide, Kubota, et al., 2020; Minnerop et al., 2017). It is hypothesized that these splicing variants are associated with a cell specific defect impacting neurons of the striatum, leading to the common phenotype seen radiologically and clinically. This is further exemplified through the neuropathological studies in Chapters 4 and 5 which demonstrate progressive involvement of the striatum and thalamus. Through these studies, it was shown that the dorsal striatum demonstrates a severe neuronal loss early in the disease, with

involvement of the globus pallidus and thalamus progressing over time. We hypothesized that this may result due to connectivity pathways in the subcortical structures and Wallerian degeneration.

Interestingly, inheritance of the c.1771-7C>G and c.1771-6C>G variants in homozygous form result in a milder phenotype involving adolescent or adult onset of spastic ataxia with extrapyramidal features (Azmanov et al., 2016; Minnerop et al., 2017). This phenotype also involves striatal abnormalities on MRI, but with normal myelination. Both of these variants have been shown to cause production of aberrant splicing transcripts, in addition to full-length wildtype transcript due to partial activation of the cryptic splice site, which results in "leaky" splicing (Azmanov et al., 2016; Minnerop et al., 2017; Perrier et al., 2020). It is possible that the production of this wildtype transcript reaches the threshold of functional Pol III required for myelination to proceed normally in patients harbouring the splicing variant in homozygous form. On the other hand, the mild insufficient myelin deposition in patients with a severe phenotype may result from the threshold for Pol III production not being fully met, as the splicing variant in present only in heterozygous form, with an amorphic loss of function variant *in trans*. Furthermore, this observation supports the hypothesis that a threshold level of Pol III function is required for proper myelination during development. Likewise, it is possible that diffuse hypomyelination occurs in patients with typical POLR3-HLD as hypomorphic variants are harboured on both alleles, without the production of wildtype protein that is associated with the "leaky" splicing variants described above. Moreover, it is hypothesized that without the presence of any wildtype normal functioning Pol III, myelin cannot properly form during development, leading to the hypomyelination seen in patients with the typical POLR3-HLD phenotype. Thus, adequate Pol III activity is likely to be a critical factor for the formation of myelin during early neurological development.

## 6.5 Future Directions: Approaching Treatment Options for POLR3-HLD

*The following sections of quoted text are reprinted from the narrative review written by the author (Perrier et al., 2021. Front Cell Neurosci. 14:631802.) under CC-BY 4.0 permission.*

"With the advent of MRI pattern recognition, and improvements in genetic technologies in the last decade, diagnostic rates for leukodystrophies, including POLR3-HLD, have risen in parallel. An important goal for POLR3-HLD research now lies in the determination of quantifiable markers of disease progression. Indeed, before therapeutic options can be considered, clinical outcome measures and surrogate markers of disease progression must be established and deemed accurately quantifiable. These markers are critical for assessing the effectiveness of treatment efficacy in future clinical trials. Advanced neuroimaging techniques, such as diffusion tensor imaging (DTI), pose an interesting route for measurement of improvements in myelination (Aung et al., 2013; Koob et al., 2016; Poretti et al., 2016; Pouwels et al., 2014; Sarret et al., 2018; van Rappard et al., 2018). The heterogeneity of POLR3-HLD presents an additional limitation for assessing the effectiveness of different therapies as difficulties could arise when comparing the progression rate of phenotypes between patients. Thus far, the clinical experience of most patients with POLR3-HLD presents a relatively similar disease course according to the gene which is mutated. Indeed, those with pathogenic variants in *POLR1C* present with the most severe disease course, followed by *POLR3A*, and then *POLR3B* (Gauquelin et al., 2019; Wolf, Vanderver, et al., 2014). The comparative severity of patients with pathogenic variants in *POLR3K* cannot yet be determined as clinical information has only been published on two patients (Dorboz et al., 2018). In recent years, it has become clear that natural history studies concerning the delineation of disease progression and identification of surrogate markers are of the utmost importance (Pouwels

et al., 2014). Hence, it is essential to complete these studies in parallel to pathophysiological investigations for clinical trials of potential therapies to progress."

"Limited knowledge of the exact pathophysiological mechanisms underlying POLR3-HLD also poses a challenge for the evaluation of the most effective treatment options. When specific mechanisms are implicated in genetic diseases, it is possible to focus on targeting alternative pathways in treatment approaches, in order to overpass the mechanism containing the defective protein (Greene & Voight, 2016). Although the cellular pathophysiological mechanisms associated with POLR3-HLD have yet to be uncovered, studies have shown that mutations in POLR3 subunits can cause disruptions on several molecular levels. For example, mutational mapping onto specific protein domains suggests association with specific mechanisms of dysfunction, including modification of the catalytic cleft structure, impaired POLR3 complex assembly, perturbed interactions between subunits, and interference within POLR3 complex binding to DNA (Bernard et al., 2011; Girbig et al., 2021; Ramsay et al., 2020; Tetreault et al., 2011). Additionally, protein localization studies have shown that disease-causing *POLR1C* variants can alter assembly and nuclear import exclusively of POLR3, resulting in a lack in binding to POLR3 target genes (Thiffault et al., 2015). Protein expression studies on patient fibroblasts and brain tissue also demonstrate a decrease in POLR3A abundance (Bernard et al., 2011). Finally, functional studies of *POLR3A* mutations associated with POLR3-HLD have demonstrated transcriptional defects when introduced in both yeast and human cells (Choquet, Forget, et al., 2019; Moir et al., 2021). Further research on molecular pathways and other POLR3 interactors will be valuable in determining whether suppression of upstream POLR3 inhibitors, such as MAF1 (Bonhoure et al., 2020; Johnson et al., 2007; Reina et al., 2006; Vorlander et al., 2020), are appropriate for future treatments. It is also possible that a small molecule screening approach could identify drugs for

the treatment of specific molecular mechanisms, such as upregulation of complex assembly cofactors or signaling molecules for nuclear import of POLR3 (Cloutier & Coulombe, 2010; Lesniewska & Boguta, 2017; Willis & Moir, 2018). However, further research is required in this avenue before a molecular target approach can be considered for the repair of myelin in POLR3-HLD. Currently, as the pathophysiological processes underlying POLR3-HLD are not well known, potential therapeutic approaches can be considered in a general manner, by focusing on the replacement of defective cells (i.e., oligodendrocytes), or directly restoring POLR3 function. Specifically, this could be accomplished by: directly transplanting stem cells containing functional protein to migrate and replicate in damaged areas, using gene therapy techniques to deliver gene products and restore the functional protein in damaged cells, or repairing genetic variants in damaged cells via delivery of gene editing constructs (Gordon-Lipkin & Fatemi, 2018; Helman et al., 2015)" (Figure 6.1).

### 6.5.1 Cell-Based Therapies: Transplantation as Treatment for Leukodystrophies

"Cellular therapies, which involve the transplantation of stem cells into an affected individual, offer an attractive approach for treating HLDs. Stem cells can self-renew and differentiate into different lineages, including OPCs, and therefore could directly repopulate lost host cells for the regeneration of myelin in leukodystrophies. Generally, the therapeutic mechanisms of cellular therapy can be two-fold, including direct replacement of lost host cells *via* migration of transplanted cells to repopulate defective tissues, and transplanted cells acting as a source of functional exogenous enzymes (De Feo et al., 2012).While delivery of stem cells for the treatment of neurological diseases has been achieved *via* both intravenous and intracerebral administration techniques, only the latter could be applicable for the treatment of POLR3-HLD.

Intravenous stem cell therapy, including bone marrow transplantation or hematopoietic stem cell transplantation, has been used in treating other monogenic neurological diseases based on the notion that monocytes could migrate through the blood-brain barrier to the CNS tissue and secrete active enzyme for cellular uptake by dysfunctional host cells, as well as differentiate into microglia and/or astrocytes that could inherently provide trophic support for diseased cells or regulate inflammation (Asheuer et al., 2004; Krivit et al., 1995; Priller et al., 2001; Sun & Kurtzberg, 2018). While this approach has been used in leukodystrophies that are associated with enzyme deficiencies [e.g., globoid cell leukodystrophy or Krabbe disease disease (Escolar et al., 2005; Laule et al., 2018; Wright et al., 2017), adrenoleukodystrophy (Mahmood et al., 2007; Matsukawa et al., 2020; Peters et al., 2004), and metachromatic leukodystrophy (Boucher et al., 2015; Groeschel et al., 2016; Martin et al., 2013; Musolino et al., 2014)], it is not applicable for treatment of the hypomyelinating phenotype associated with POLR3-HLD as neither the POLR3 enzyme complex nor its subunits are secreted extracellularly for reuptake, and myelination would be dependent on the delivery of functional OPCs or earlier lineages. Therefore, intracerebral administration of stem cells of neural lineage poses the most likely route for exploration in the treatment of POLR3-HLD."

#### 6.5.1.1 Neural Stem Cell Transplantation and Remyelination

"Neural stem cells (NSCs) are multipotent neural cells that give rise to radial glial progenitor cells, which can, in turn, give rise to neuron and glial cell populations, making them an attractive cell type for transplantation in the leukodystrophy setting (Brustle et al., 1997; Temple, 2001; Zhao & Moore, 2018). During neural development, gradients of specific signaling molecules guide the fate of NSCs and provide positional information to form different regions of the brain

(Temple, 2001; Wolpert, 1994). In the CNS, NSCs also have a temporal differentiation component, where the response to growth factors is altered over time, as the cells undergo repeated asymmetric divisions to first produce neurons, followed by glia (Okano & Temple, 2009; Qian et al., 2000). In the postnatal brain, NSC production and neurogenesis are restricted to certain brain areas but primarily occur in the subventricular zone (Gonzalez-Perez, 2012)."

"Several mouse models of dysmyelination and hypomyelination have shown that intracerebral-transplanted NSCs are effective in remyelinating the myelin-deficient brain (Duncan et al., 2011). Explored extensively is the *shiverer* mouse model, which exhibits dysmyelination and a motor phenotype due to a deletion in the *Mbp* gene, encoding for myelin basic protein, which is required for the formation of major dense lines in compact myelin (Privat et al., 1979; Roach et al., 1985). When transplanted into *shiverer* mice, NSCs can differentiate and remyelinate the brain, promoting recovery of their ataxic phenotype and prolonging survival (Low et al., 2009; Uchida et al., 2012; Yandava et al., 1999). Additionally, when transplanted into the *shiverer* spinal cord, exogenous transplanted NSCs can ensheath axons, form compact myelin, and improve nerve conduction (Buchet et al., 2011; Eftekharpour et al., 2007; Mothe & Tator, 2008). Studies on rodent models of Pelizaeus-Merzbacher disease have shown similar results; following NSC transplantation, *Plp1*-transgenic mutant mice undergo remyelination of the brain with the production of compact myelin (Gruenenfelder et al., 2020; Marteyn et al., 2016). Additionally, engraftment of NSCs into the white matter tracts of hypomyelinated mutant myelin-associated glycoprotein and nonreceptor-type tyrosine kinase Fyn (MAG/Fyn) mice produced mature oligodendrocytes and improvements in myelination (Ader et al., 2001, 2004). These studies provide evidence that mammalian NSCs can undergo functional integration into the CNS white matter, promoting remyelination and offering potential as a therapeutic approach in

hypomyelinating disorders. Besides direct remyelination, it is also thought that NSC transplantation can offer an additional advantage through a neuroprotective effect *via* the release of trophic factors, which promote tissue repair and protect endogenous cells from further damage (De Feo et al., 2012)."

"Recently, the safety of allogeneic NSC intracerebral transplantation in humans was investigated in a phase I clinical trial including four young patients with Pelizaeus-Merzbacher disease, who were monitored over the course of 5 years (Gupta et al., 2019; Gupta et al., 2012). The primary goal of this study was to assess the safety profile of the transplantation of allogeneic NSCs derived from human fetal brain tissue using intracerebral injections. Using MRI guidance, cells were delivered *via* four bilateral frontal burr holes to the deep white matter of the centrum semiovale or corona radiata, and patients underwent an immunosuppression regime. A 1-year evaluation determined that the procedure was well-tolerated without clinical or radiological adverse effects, and after 5-years, no tumor formation was evident and no other long-term adverse effects were noted. However, two patients had an immune response and developed donor-specific leukocyte antigen alloantibodies, pointing to the importance of monitoring immune response in future studies. Serial MRI and magnetic resonance spectroscopy (MRS), including DTI, were performed for evaluation of remyelination, where signal changes were observed at the injection sites and some distant regions in each patient through the second year following transplantation. In the three patients who were studied up to year 5, persistent increased signal changes were noted, however, they were described as patchy and subtle, and could not be guaranteed conclusive evidence of remyelination. Although further studies are required to optimize transplantation efficacy, this study provides support for the safety of intracerebral transplantation of progenitor cells for repopulation of myelin in HLDs."

"Should the transplanted NSCs be successful in migrating, signaling, and differentiating to form functional myelin in humans, this therapeutic approach would be optimal to treat the diffuse hypomyelination seen in POLR3-HLD. However, in considering the described rodent studies of remyelination following NSC transplantation, results should be interpreted with caution for their translation to the clinical setting as rodents have a much lower proportion of subcortical white matter in relation to cortical volume compared to humans (Hofman, 2014; Schoenemann et al., 2005). Therefore, transplantation in humans would more heavily depend on the severity of hypomyelination and the extent to which exogenous cells must migrate and reproduce. Moreover, to effectively correct CNS functioning *via* remyelination in humans, experimental studies on higher-order mammals, such as primates, would allow for a more comparable result in terms of determining the optimal dosage and regions of transplantation."

#### 6.5.1.2 Glial Progenitor Cell Transplantation: A Targeted Lineage

"Glial progenitor cells (GPCs), which are further patterned from NSCs towards a glial fate, have also been explored as a candidate for cerebral transplantation in leukodystrophies (Chanoumidou et al., 2020; Goldman, 2017; Osorio & Goldman, 2016). Similar to NSCs, GPCs can be generated from pluripotent stem cells or harvested and purified from fetal brain tissue for transplantation (Monaco et al., 2012; Nunes et al., 2003). Many studies have successfully performed intracerebral transplantation of glial cells in animal models and shown their effectiveness in remyelination remyelination (Duncan, 2005; Franklin & Ffrench-Constant, 2008; Goldman, 2011). Notably, several studies involving the transplantation of human glial lineage-specific cells into *shiverer* mice show consistent results, with evidence of robust remyelination, prolonged survival, and phenotypic rescue (Izrael et al., 2007; Mariani et al., 2019; Windrem et

al., 2004; Windrem et al., 2008; Windrem et al., 2014; Windrem et al., 2020). These results provide support for clinical exploration of this treatment, revealing that GPCs have a migratory potential and can effectively differentiate *in vivo* when transplanted into another host. Comparative efficacy between specific lineages in transplantation therapy remains to be confirmed; in one study, both NSCs and OPCs were able to remyelinate and produce compact myelin in both Pelizaeus-Merzbacher disease *Plp1*-overexpressing and *shiverer* immunodeficient mouse models, however, in the transgenic *Plp1*-overexpressing mice, NSCs more notably promoted survival and prolonged lifespan, whereas in *shiverer* mice, OPC transplantation promoted a slightly longer lifespan compared to NSCs (Marteyn et al., 2016). Nonetheless, it is important to note that the microenvironment within the CNS tissue likely had a significant impact on survival, with neuroinflammation being downregulated in NSC-grafted mice, which is an important consideration in therapy for Pelizaeus-Merzbacher disease due to the known inflammatory component of disease pathogenesis (Marteyn et al., 2016). Likewise, when OPCs were co-transplanted with mesenchymal stem cells (MSCs) into *shiverer* mice, the immune response was minimized and increased oligodendrocyte engraftment, myelination, and maturation was evident (Cristofanilli et al., 2011). Therefore, immune response could prove to be an additional important consideration when evaluating the effectiveness of stem cell therapy, and would be noteworthy to explore in POLR3-HLD pathogenesis before the development of therapeutic strategies."

#### 6.5.1.3 Induced Pluripotent Stem Cells: Patient-Derived Cell Therapy Approaches

"GPCs generated from induced pluripotent stem cells (iPSCs) have also been investigated as a prospect for cell therapy and transplantation in white matter diseases (Chanoumidou et al., 2020; Fox et al., 2014). iPSC-derived cells provide an additional advantage as they harbor the

genetic background of the individual from whom they originate, thereby adding a patient-specific approach to cell-based therapies. iPSCs can be generated *via* direct reprogramming of somatic cells using a series of pluripotency factors, reverting them into a stem-like fate with the ability to renew indefinitely or differentiate into the desired lineage (Takahashi et al., 2007; Takahashi & Yamanaka, 2006). Patient-specific cells with a renewable potential are especially appealing for the treatment of genetic disorders as they can be expanded to a large number before transplantation and downstream differentiation, and importantly they can evade the possible immunologic rejection that accompanies allogeneic stem cell transplantation. However, before iPSC-derived GPCs can be considered in a clinical setting, there are several limitations to consider and study, including the possibility of tumor formation, as well as potential safety concerns of gene editing required for correction of disease-causing mutations in patient cells, including off-target effects, immunotoxicity, and DNA damage toxicity (Neofytou et al., 2015; Uddin et al., 2020)."

"Studies on the development of iPSC-derived oligodendrocytes have progressed in the past decade, leading to increased discussion of their utility in treating neurological diseases (Chanoumidou et al., 2020). One of the first studies of human iPSC-derived OPCs aimed to investigate their myelinating potential in the lysolecithin-induced demyelinated rat optic chiasm, in which remyelination was evident following transplantation, reinforcing the potential for iPSC-derived cell transplantation (Pouya et al., 2011). Following this direction, further studies were completed transplanting human iPSC-derived cells into the *shiverer* mouse, revealing that iPSC-derived OPCs can migrate and robustly myelinate brain tissue (Ehrlich et al., 2017; Sim et al., 2011; Wang et al., 2013). iPSC-derived cell transplantation has also proven effective in other neurodegenerative disease models; transplantation studies using a mouse model for Huntington's disease recently demonstrated that iPSC-derived NSCs were capable of ameliorating their motor

phenotype and differentiating into region-specific neurons without tumor formation, thereby providing the foundation for use of iPSC-derived cells in future studies of neurological diseases (Al-Gharaibeh et al., 2017)."

"Using a direct approach to replace myelin in the brain *via* NSCs, GPCs, or OPCs is an option to consider further studying in POLR3-HLD, however, studies are needed to first determine the pathophysiological mechanisms underlying hypomyelination. The use of autologous patient-specific iPSCs is also an attractive approach due to the decreased risk of transplant rejection. In these circumstances, the concern for donor cell rejection would be limited given that the patient-derived cells are nonimmunogenic, and therefore suppression of the immune system could be avoided. Moreover, iPSCs offer an accessible and renewable source of patient-derived cells, making them an optimal option for transplantation, provided that the potential for tumor formation is deemed very low risk. Further research into the potential for genetic correction of iPSCs from POLR3-HLD patients would be required to determine whether restoration of myelin would be possible with iPSC-derived OPC transplantation."

### 6.5.2 Gene Transfer Therapy: Considerations in Leukodystrophies

"Historically, the concept of gene therapy evolved from gene transfer experiments which suggested that supplying functional transgenes to cells with corresponding dysfunctional counterparts might provide therapeutic benefit (Friedmann, 2001; Rogers, 1959, 1966, 1971; Rogers & Pfuderer, 1968; Terheggen et al., 1975; Wirth et al., 2013). Gene therapy as a field has grown beyond gene transfer therapy, to encompass techniques such as oligonucleotide and mRNA therapy (Bennett, 2019; Kowalski et al., 2019; Setten et al., 2019) as well as gene editing (discussed below). Typically, gene transfer therapy is divided into *ex vivo* and *in vivo* approaches

that make use of different viral vectors for delivery of genetic material to cells. *Ex vivo* gene therapy usually involves removing hematopoietic stem cells from the body and administering a gene therapy vector (often lentiviral) *in vitro* before re-infusing treated cells into the patient. It is methodologically similar to bone marrow transplantation therapy and has overlapping applications, with the advantage of obviating the need for long-term immunosuppression required after bone marrow transplantation. *Ex vivo* gene therapy of this type has been used successfully to treat X-linked adrenoleukodystrophy (Cartier et al., 2012; Cartier et al., 2009; Eichler et al., 2017) and metachromatic leukodystrophy (Biffi et al., 2013; Sessa et al., 2016) if administered early. *Ex vivo* gene therapy using hematopoietic stem cells is not a viable option for POLR3-HLD due to the primary defect in POLR3 activity in brain tissues, especially because the complex is not secreted and has a primarily non-metabolic function. However, *ex vivo* gene therapy may have an application using iPSCs, if treated cells are subsequently differentiated to a glial lineage and delivered into the brain. Limitations would involve similar factors to those described above (i.e., safety, route of transplantation, migration, and differentiation capacity). Primarily, *in vivo* gene transfer has been applied successfully to treat specific leukodystrophies, and may be a candidate modality for the treatment of POLR3-HLD. Here, we will focus on *in vivo* gene transfer data, which represents a majority of the literature and clinical experience with gene therapy directly targeting the brain in leukodystrophies, such as Canavan's disease and metachromatic leukodystrophy. As our knowledge of POLR3-HLD pathology continues to evolve, so too will opportunities to advance tractable strategies for developing a disease-modifying treatment."

#### 6.5.2.1 *In vivo* Approaches to Gene Therapy

"In considering *in vivo* gene therapy, several viral vectors have been proposed to achieve transgene delivery, but thus far, the most clinically successful has been adeno-associated virus (AAV). *In vivo* gene therapy for leukodystrophies began with an AAV trial for Canavan disease, an autosomal recessive leukodystrophy caused by mutations in the *ASPA* gene, encoding the enzyme aspartoacyclase which functions to degrade N-acetylaspartate (NAA) in the brain (Janson et al., 2002). AAV2-*ASPA* treatment was supported by concurrent pre-clinical rodent studies suggesting human *ASPA* gene transfer to Canavan mice and rats resulted in decreased NAA concentrations in brain tissue, along with decreased seizure frequency and histopathological improvements (Matalon et al., 2003; McPhee et al., 2005). These findings were translated into a clinical trial. Long term follow-up in a cohort of 28 patients, 13 of which were treated by intraparenchymal delivery of AAV2-*ASPA* to six sites in the brain, demonstrated a good safety profile with the most common adverse events (i.e., small subdural hemorrhage, postoperative fever) most likely associated with the neurosurgical aspect of the treatment, and no adverse events occurring after 90 days of follow-up (Leone et al., 2012). AAV2-*ASPA* was shown to decrease NAA in the brain, as measured by MRS, as well as the slow progression of brain atrophy, and was considered to have been associated with adequate safety and moderate overall clinical efficacy that warranted further clinical trials (Leone et al., 2012). This early AAV trial was instrumental in demonstrating the enhanced safety profile of AAV for *in vivo* gene therapy in leukodystrophies."

"The discovery of novel AAV serotypes in nonhuman primates and human tissues elucidated numerous aspects of AAV biology, including their differences in tissue tropism, leading to an explosion of studies exploring the use of naturally occurring AAV serotypes and recombinant AAV (Gao et al., 2002; Gao et al., 2003; Gao et al., 2004). Importantly, the AAV serotypes

identified in the course of Dr. Gao and colleagues' work especially AAV9, have been studied for their utility in transducing brain tissues. An important aspect surrounding the use of AAV9 for CNS diseases involves its enhanced ability to target the CNS, which allows for intrathecal or intravenous administration (Foust et al., 2009; Gessler et al., 2019; Mendell et al., 2017). Most notably, AAV9 was successfully used in a clinical trial for spinal muscular atrophy (Mendell et al., 2017), resulting in FDA approval of Zolgensma®, an intravenously delivered gene therapy treatment. AAV9 has also recently demonstrated effectiveness in a mouse model of Canavan disease (Gessler et al., 2017), which played a role in promoting the rAAV9-*ASPA* vector transitioning to a recent open-label clinical trial for Canavan disease (*CANaspire*, ASPA Therapeutics). Finally, an exciting recent AAV9 finding is the success of AAV9-*GALC* in treating a canine model of globoid cell leukodystrophy or Krabbe disease, improving myelination and extending lifespan more than seven times beyond the typical life expectancy for model animals (Bradbury et al., 2020). However, AAV9 is not known to efficiently mediate significant transduction of oligodendrocyte lineage cells."

"A Clade E AAV serotype identified in 2004 (Gao et al., 2004) called AAVrh.10, has been tested in the context of metachromatic leukodystrophy on a small number of patients (NCT01801709); however, the results of this trial have not been released. The initial preclinical data for this study suggested that intracerebral delivery of AAVrh.10-*ARSA* was superior to AAV5 both in terms of the overall impact on the model disease and its ability to transduce oligodendrocytes (Piguet et al., 2012; Sevin et al., 2006; Sevin et al., 2007), which led to further safety and feasibility assessments in non-human primates leading up to the clinical trial (Zerah et al., 2015). Importantly, in the preclinical assessment, Sevin and colleagues evaluated the direct impact of AAVrh.10 on oligodendrocyte transduction using a GFP-containing vector and

estimated that 9% of oligodendrocytes in the striatum were transduced directly, whereas 21% were found to contain ARSA enzyme after administration of AAVrh.10-*ARSA* (Piguet et al., 2012). These findings indicate that cross-correction of oligodendrocyte ARSA enzyme levels *via* transduction of non-oligodendrocyte targets plays a role in the observed improvement in oligodendrocyte sulfatide levels and brain pathology (Piguet et al., 2012). The AAVrh.10 trial excepted, in each of the mentioned leukodystrophies in which *in vivo* gene therapy has been tested, the putative improvement in oligodendrocyte function is thought to occur through cross-correction. Indeed, most AAV capsids are not known to efficiently transduce oligodendrocytes (Burger et al., 2004; Cearley et al., 2008; Cearley & Wolfe, 2006; San Sebastian et al., 2013). This fact has prompted studies evaluating the use of oligodendrocyte-specific promoters to drive expression in oligodendrocytes (Chen et al., 1998; Lawlor et al., 2009) as well as the pursuit of novel recombinant capsids with significant oligodendrocyte tropism as demonstrated in rodents (Powell et al., 2016), and the characterization of oligodendrocyte tropism in a novel naturally occurring AAV capsid (Hsu et al., 2020). Taken together, these study results indicate that AAV vector research continues to yield important advances toward achieving both safety and efficacy for *in vivo* gene therapy approaches to leukodystrophies. The increasing focus on understanding how AAV technology can be used to target oligodendrocyte lineage cells will be important for the development of an *in vivo* gene therapy approach to POLR3-HLD."

"Currently, the POLR3-HLD disease population is divided with the majority (≥90%) of patients having either biallelic mutations in *POLR3A* or *POLR3B* (Bernard et al., 2011; Daoud et al., 2013; Tetreault et al., 2011; Wolf, Vanderver, et al., 2014) and a minority (<10%) having mutations in *POLR1C* (Gauquelin et al., 2019; Thiffault et al., 2015) or *POLR3K* (Dorboz et al., 2018). In the future, it may be possible to treat patients by grouping according to the affected

subunit and administering a vector carrying the appropriate sequence *in vivo*. However, there are three key challenges for developing an *in vivo* gene therapy approach for POLR3-HLD that have not been addressed by prior *in vivo* leukodystrophy gene therapy studies. The first is the fact that in each of the previously mentioned diseases, cross-correction is possible and beneficial due to the nature of the defective enzymes and metabolites responsible for the disease. In POLR3-HLD, cross-correction is improbable because POLR3 subunits are unlikely to be secreted or transferred between cells and also because pathogenesis likely does not involve the accumulation of the enzymatic reactants (RNA nucleotides), as they are used by other RNA polymerases, and do not directly cause toxicity. Therefore, directly correcting the oligodendrocyte lineage is an important aspect of a putative gene therapy strategy for POLR3-HLD. Second, the pathophysiological axis of POLR3-HLD is hypomyelination, relating to a specific and yet poorly characterized deficit in the oligodendrocyte lineage that may occur well before the cells mature and myelinate the affected CNS regions. If the deficit occurs primarily in the cellular precursors of oligodendrocytes (i.e., a dividing cell population), the exponential dilution of non-integrating vector genomes such as those transduced using AAV is an important consideration. Third, attempts to produce a representative animal model of POLR3-HLD in which to perform pre-clinical testing have proven difficult (Choquet, Pinard, et al., 2019; Choquet et al., 2017), and this barrier will need to be overcome to properly test any novel therapeutic candidate. Recently, progress in generating an animal model has been made using an *Olig2*-Cre conditional double *Polr3a* mutant knock-in strategy (Merheb et al., 2021). Overcoming these challenges would elucidate the potential for POLR3-HLD gene transfer, and will also inform the future development of more advanced and/or personalized (e.g., gene editing) therapeutic strategies for POLR3-HLD."

### 6.5.3 Gene Editing Techniques: A Modern Approach

"Recently, gene-editing research has gained traction for its utilization in the development of patient-specific therapies for genetic diseases. While these techniques are not yet employed in a large-scale clinical setting, they hold promise for treating rare genetic diseases that are without curative therapies. Moreover, the design of personalized therapies is a possibility through the use of gene editing, a technique that can create alterations in precise genomic locations to correct pathogenic variants. Yet, to establish translational gene editing strategies, additional aspects must be investigated such as vehicles and delivery methods of editing systems, optimization of editing constructs, and elimination of off-target effects. Furthermore, with correct optimization, genome engineering can lead to the establishment of personalized therapies for diseases that are otherwise challenging to treat."

#### 6.5.3.1 CRISPR-Cas9 Editing System

"Since the discovery of its potential for human genome editing in 2013, clustered regularly interspaced short palindromic repeats (CRISPR)-Cas gene-editing technology has been heavily investigated for its use in studying and treating genetic diseases (Cong et al., 2013; Jinek et al., 2013). The CRISPR-Cas system harnesses the cellular machinery involved in the adaptive immune response of bacterial cells against viral particles (Horvath & Barrangou, 2010). This highly specific system can target precise genomic regions and has revolutionized modern genetic research for its capability to easily manipulate the human genome. While different systems have been engineered using a series of CRISPR/Cas components and types of Cas nucleases, the Cas9 nuclease has been most commonly used in genetic editing of mammalian cells (Makarova & Koonin, 2015). In combination with a single guide-RNA (sgRNA), Cas9 can be programmed to target and cleave

complementary DNA sequences, which can be subsequently repaired using a donor DNA template strand and the intrinsic homology-directed repair mechanism (Mali et al., 2013; Ran et al., 2013; L. Yang et al., 2014). Thus, given that the proper cell type is targeted, it is possible to use CRISPR-Cas9 technology to genetically correct mutations causing monogenic diseases, bringing to light its ability to facilitate phenotypic repair."

"CRISPR-Cas9 editing has been successfully used in many *in vitro* and *in vivo* studies to both explore disease pathophysiology through the creation of transgenic or knock-out models, and investigate treatment methods *via* targeted gene editing and correction of genetic mutations (Rodriguez-Rodriguez et al., 2019). For example, in an initial study using a murine model of hereditary tyrosinemia, injection of CRISPR-Cas9 components into the liver led to phenotypic rescue, demonstrating the potential for genetic correction *in vivo* (Yin et al., 2014). Additionally, studies using the *mdx* murine model for Duchenne muscular dystrophy have shown that delivery of CRISPR-Cas9 constructs, both at the germline level with injection into zygotes and postnatally with delivery *via* AAV9, can lead to phenotypic improvements (Long et al., 2014). *In vitro* and *in vivo* studies have also been completed using CRISPR-Cas9 to correct mutations associated with Huntington's disease, with promising results demonstrating that suppression of mutant alleles can alleviate motor phenotypes in mice (Kolli et al., 2017; Monteys et al., 2017; Shin et al., 2016; Yang et al., 2017). In the field of HLDs, a recent study has demonstrated that CRISPR-Cas9 mediated germline suppression of *Plp1* in the severe *jimpy* mouse model of Pelizaeus-Merzbacher disease leads to increased myelination and restored lifespan (Elitt et al., 2020). Most recently, the first clinical trials of CRISPR-Cas9 therapies have launched, with *ex vivo* approaches centering around cancer immunotherapy, as well as gene disruption of hematological disorders including sickle-cell anemia and β-thalassemia (Li et al., 2020; Rosenblum et al., 2020; Uddin et al., 2020).

Additionally, an *in vivo* approach has been employed in Leber congenital amaurosis, a monogenic disease associated with childhood blindness, involving the delivery of AAV5-packaged CRISPR-Cas9 constructs directly to the retina (Maeder et al., 2019). As these trials progress and with the assessment of long-term outcomes and safety, this gene-editing technology could show powerful potential for use in treating many classes of diseases. Moreover, research involving gene editing with CRISPR-Cas9 techniques in the CNS is constantly evolving; innovations and improvements to the editing system focus on optimizing editing efficiencies and reducing off-target effects, as well as exploring delivery methods *via* biological vesicles, nanoparticles, or viruses (Cota-Coronado et al., 2019; Sandoval et al., 2020)."

"While rapidly advancing, gene editing techniques would have to be studied *in vitro* and *in vivo* for their use in correcting the POLR3-HLD phenotype before they can be considered as a potential therapeutic approach. It is noteworthy that the use of CRISPR-Cas9 technology is not effective for the correction of all mutation types associated with POLR3-HLD (i.e., large exonic deletions, synonymous variants, some splice site variants), and this therapeutic approach would have to be considered on a patient-specific level. Moreover, this technique is still in the early experimental stage of study and before it can be considered in a clinical setting, its benefits and downfalls as a therapeutic tool must be explored along with the most optimal delivery methods and its potential in correcting cells of the CNS. In speculating on the use of gene editing therapy to treat the cellular pathogenesis associated with POLR3-HLD, this therapy may or may not be applicable depending on the stage of oligodendrocyte lineage that is defective. Moreover, if future studies find that early OPC proliferation or migration ability is not severely affected, and pathogenesis predominantly concerns the formation of myelin itself (due to transcriptional defects causing lack of protein availability for myelin membrane formation), the delivery of CRISPR

constructs for genetic correction of myelinating cells could show high potential for phenotypic remediation. However, there are many other potential scenarios in which different cell types or mechanisms could be affected (e.g., differentiation of NSCs to a glial fate, impairments in migration of OPCs, maturation of OPCs into oligodendrocytes, signaling between different cell types and/or other mechanisms for formation, wrapping, or compaction of the myelin membrane itself). Thus, without knowledge on the cellular pathophysiology, it is to be determined whether correcting cells after birth and the initial waves of OPC production/migration during the *in utero* period of myelin development would be applicable. Knowledge of disease pathogenesis would help predict the probability of success for delivery of gene editing constructs at certain stages of the disease progression, and whether myelination is possible."

### 6.5.4 The Future of POLR3-HLD Therapies

"Along the front of therapy development for rare inherited neurological disorders, advances in cell therapy, gene therapy, and gene editing techniques have all presented exciting results in recent years. Combination approaches have also been considered, including the use of gene transfer or editing of stem cells for transplantation to improve disease phenotypes (Meneghini et al., 2017; Ricca et al., 2015). In considering POLR3-HLD, much information remains to be uncovered regarding the pathophysiology of the disease and whether myelin restoration is possible. As pathological studies demonstrate that oligodendrocytes are primarily affected in POLR3-HLD, this review provided a cell-specific approach to the consideration of therapies. However, disease pathogenesis may involve other cell types, which could also be targeted in combination. The described therapies offer potential options for exploration, and future studies in both cellular and animal models to investigate their effectiveness and mechanisms would prove to be beneficial.

Moreover, developing disease biomarkers and tangible clinical outcome measures are of utmost importance to evaluate therapeutic efficacy and successfully translate pre-clinical findings into the clinical setting. Ongoing research on POLR3-HLD pathophysiology will surely provide a backbone for ascertaining which therapy approaches could provide the most beneficial results, and ultimately uncover the avenues for potential clinical trial development to improve patient outcomes."

**Figures**

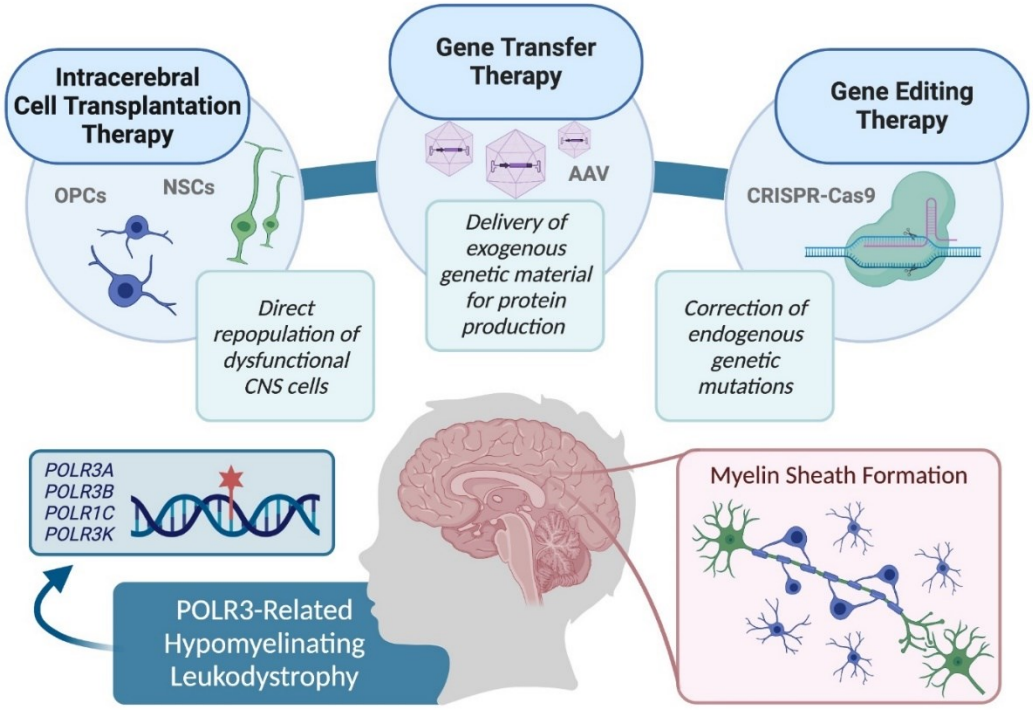


**Figure 6.1.** Summary of the therapy approaches that could be explored for use in pre-clinical studies, and eventually translated in clinical trials to treat RNA polymerase III (POLR3)-related leukodystrophy, including cell transplantation therapy, gene transfer therapy, and gene editing techniques. Figure reprinted from Frontiers of Cellular Neuroscience (Perrier et al. 2021) under CC-BY 4.0 permission.

# CHAPTER 7.

# Conclusion

As a whole, leukodystrophies are a heterogeneous group of diseases, caused by pathogenic variants in a vast array of genes. Indeed, hypomyelinating leukodystrophies were initially thought to be solely caused by variants in genes encoding for myelin-associated proteins, but in in recent years, thanks to improved molecular genetics technologies, are now known to be caused by variants in genes associated with a wide range of other functions, including the mechanisms of transcription and translation. This thesis provides insight into the genetic basis of hypomyelinating leukodystrophies and highlights the utility of performing next generation sequencing and data reanalysis on cases which remain unsolved. In addition, the described studies emphasize the importance of developing a multidisciplinary diagnostic pipeline which includes collaboration between both the clinical and research environments to obtain detailed phenotyping and metabolic information for genetic investigations. This thesis also provides insight into POLR3-HLD, by describing the prevalence of frequently seen clinical findings, including growth and endocrine abnormalities. The phenotypic variability in POLR3-HLD is substantial and further demonstrated by the cohort of patients described with a severe phenotype, involving onset that is much earlier than typically seen with unique involvement of specific basal ganglia nuclei in the brain. Notably, there is clinical value in describing novel disease presentations, such as the severe POLR3-HLD phenotype, as this informs clinicians of the progression of disease and expected outcomes when providing care to patients.

Moving forward, although there have been significant advancements in the genotypic and phenotypic characterizations of POLR3-HLD, the comprehensive molecular mechanisms

underlying disease pathophysiology have yet to be thoroughly defined. Future studies should aim to resolve the association between the impact of Pol III hypofunction on neural cell development and myelination. Ultimately, it is important to advance research into POLR3-HLD and similar hypomyelinating leukodystrophies to address the unmet medical need for therapies, and thereby improve patient outcomes. In closing, these studies provide insight into the genetic, clinical, MRI, pathophysiological and pathological features underlying inherited neurodevelopmental disorders including POLR3-HLD, thereby laying the foundation for future studies to explore potential disease mechanisms and therapeutic approaches.

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