ADULT GENETIC LEUKOENCEPHALOPATHIES: IDENTIFYING NEW ENTITIES USING ADVANCED MRI TECHNIQUES, NEXT GENERATION SEQUENCING AND CLINICAL PROFILING

Roberta La Piana

Integrated Program in Neuroscience

McGill University, Montreal, Canada

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ABSTRACT

Genetic leukoencephalopathies are considered rare in the adult population. However, several lines of evidence suggest that there is a larger than expected number of adult patients with genetic leukoencephalopathies who are currently not diagnosed.

The study of leukoencephalopathies - either genetically-proven or unsolved - relies greatly on MRI pattern-recognition, which allowed the characterization of the majority of genetic white matter disorders. The advent of next generation sequencing (NGS) revolutionized the field, by disclosing new phenotypes associated to known genetic white matter conditions and identifying novel genes responsible for unsolved leukoencephalopathies.

The goals of my study were

1. the application of advanced neuroimaging tools to define and characterize white matter abnormalities of known and novel genetic leukoencephalopathies
2. the clinical and demographic characterization of subjects with adult genetic leukoencephalopathies
3. the identification of genes responsible for new forms of hereditary white matter disorders, using NGS techniques.

We applied an integrated approach which combined clinical phenotyping with MRI pattern-recognition and NGS data.

This work led to a) the description of a cohort of 68 adult subjects with leukoencephalopathy of probable genetic origin, 59 of which were included in our study, b) the identification of the causal mutations (16 in total, 11 novel) in genes known to be associated with leukoencephalopathies in 14/59 subjects (23.7%), and c) the broadening of clinical and imaging phenotypes of known disorders: POLR3-related disorders, vanishing white matter disease, Krabbe disease, MTFMT-related disorders. We demonstrated that MRI family studies can be

crucial in adult leukoencephalopathies to define the modality of transmission of unclear white matter disorders within families.

In conclusion, we documented that adult genetic leukoencephalopathies are an emerging problem in clinical neurosciences. MRI family studies and the recognition of disease-specific MRI features are critical to guide the diagnostic process. Despite the access to NGS techniques, more than 70% of our subjects remain without a diagnosis. The international sharing of MRI and NGS on adult leukoencephalopathies will allow the identification of subjects with same phenotypes or mutated genes and ultimately lead to the description of new genetic entities.

RÉSUMÉ

Les leucoencéphalopathies génétiques sont considérées rares dans la population adulte. Cependant, plusieurs sources de données suggèrent qu'il existe un nombre plus élevé qu'attendu de patients adultes atteints par une leucoencéphalopathie qui n'ont pas encore de diagnostic.

L'étude des leucoencéphalopathies – autant confirmées génétiquement que non résolues – dépend grandement de l'identification de caractéristiques diagnostiques obtenues par IRM, qui permettent la caractérisation de la majorité des maladies génétiques de la substance blanche. L'avènement du séquençage de dernière génération (NGS) a révolutionné ce domaine, en permettant la découverte de nouveaux phénotypes associés à des maladies génétiques connues de la substance blanche et l'identification de nouveaux gènes responsables pour des formes non résolues.

Les objectifs de mon étude ont été

1. l'application de techniques de neuroimagerie avancées dans le but de définir et caractériser les anomalies de la substance blanche de leucoencéphalopathies connues et nouvelles ;
2. la caractérisation clinique et démographique des sujets adultes atteints de leucoencéphalopathies génétiques ;
3. l'identification de gènes responsables de nouvelles formes de maladies héréditaires de la substance blanche, en utilisant des techniques NGS.

Nous avons appliqué une approche intégrée combinant phénotypage clinique et identification de critères obtenus par IRM et données générées par NGS.

Cette étude a permis l'obtention de données concernant a) la description d'une cohorte de 68 sujets adultes atteints d'une leucoencéphalopathie d'origine supposée génétique, dont 59 ont été

inclus dans notre étude, b) l'identification de mutations causales (16 au total, dont 11 nouvelles) dans des gènes connus pour être associés à des leucoencéphalopathies dans 14/59 sujets (23.7%), c) l'expansion des phénotypes cliniques et radiologiques de leucoencéphalopathies connues: les désordres associés à POLR3, maladie VWM (*Vanishing White Matter disease*), maladie de Krabbe, les désordres associés à MTFMT. Nous avons démontré que les études familiales par IRM sont un outil crucial dans le domaine des leucoencéphalopathies adultes pour la définition de la modalité de transmission – quand cette dernière était jusqu'à lors inconnue.

En conclusion, nous avons documenté que les leucoencéphalopathies génétiques chez les patients adultes sont un problème émergeant dans les neurosciences cliniques. Les études familiales par IRM et l'identification de caractéristiques IRM spécifiques de chaque maladie sont déterminantes pour guider le processus diagnostique. Malgré l'accès aux techniques NGS, une grande majorité (plus de 70%) de nos sujets demeure sans diagnostic. Le partage de données IRM et NGS de leucoencéphalopathies adultes dans des réseaux internationaux permettra l'identification de sujets avec les mêmes phénotypes ou gènes mutés, et portera finalement à la description de nouvelles entités génétiques.

LIST OF ABBREVIATIONS

1.5T: 1.5 Tesla
3T: 3 Tesla
7T: 7 Tesla
4H : hypomyelination, hypogonadotropic hypogonadism and hypodontia
ADC: apparent diffusion coefficient
ADLD: autosomal-dominant leukodystrophy with autonomic disease
BN-PAGE: blue native polyacrylamide gel electrophoresis
CADASIL: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CADD: combined annotation dependent depletion
CARASIL: cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy
cDNA: complementary DNA
CNS: central nervous system
COL4A1: collagen type IV alpha 1 chain
COX1: mitochondrial cytochrome c oxidase subunit 1
COX II: cytochrome c oxidase subunit 2
CSF1R: colony stimulating factor 1 receptor
DNA: desossiribonucleic acid
DWI: diffusion weighted imaging
eIF2B: eukaryotic initiation factor 2B
EIF2B1: eukaryotic translation initiation factor 2B subunit alpha
EIF2B2: eukaryotic translation initiation factor 2B subunit beta
EIF2B3: eukaryotic translation initiation factor 2B subunit gamma
EIF2B4: eukaryotic translation initiation factor 2B subunit delta
EIF2B5: eukaryotic translation initiation factor 2B subunit epsilon
EVS: exome variant server
ExAC: exome aggregation consortium
FA: fractional anisotropy
FLAIR: fluid attenuated inversion recovery
FOXC1: forkhead box C1
GFM1: elongation factor G1
GJC1: gap junction protein gamma 1
GM1: GM1-gangliosidosis
H-ABC: hypomyelinating leukodystrophy with atrophy of basal ganglia and cerebellum
HRR: hardy rand and rittler test
HSP: hereditary spastic paraparesis
LAMA2: laminin subunit alpha 2
LBSL: leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation

LHON: Leber's hereditary optic neuropathy
LMNB1: lamin B1
MAF: minor allele frequency
MELAS: mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
MERRF: myoclonic epilepsy with ragged red fibers
MLC: megalencephalic leukoencephalopathy with subcortical cysts
MLC1: megalencephalic leukoencephalopathy with subcortical cysts 1
MRI: magnetic resonance imaging
MS: multiple sclerosis
MT: MutationTaster
mtDNA: mitochondrial DNA
MTFMT: mitochondrial methionyl-tRNA formyltransferase
MTR: magnetization transfer ratio
ND1: NADH dehydrogenase 1
ND4: NADH dehydrogenase 4
ND5: NADH dehydrogenase 5
NGS: next generation sequencing
NOTCH3: notch receptor 3
OXPHOS: oxidative phosphorylation
PD: proton density
PMD: Pelizaeus-Merzbacher disease
PMDL: Pelizaeus-Merzbacher-like disease
Pol III: RNA Polymerase III
POLR1C: DNA-directed RNA polymerase I subunit C
POLR3: DNA-directed RNA polymerase III
POLR3A: DNA-directed RNA polymerase III subunit A
POLR3B: DNA-directed RNA polymerase III subunit B
PP2: PolyPhen-2
PPMS: primary progressive multiple sclerosis
RNA: ribossinucleic acid
SIFT: sorting intolerant from tolerant
SPG35: hereditary spastic paraplegia 35
TACH: tremor-ataxia with central hypomyelination
TOP: terminal oligopyrimidine tract
TUFM: Tu translation elongation factor, mitochondrial
VUS: variant of unknown significance
VWMD: vanishing white matter disease
WES: whole exome sequencing
WGS: whole genome sequencing

LIST OF FIGURES

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I would also like to thank Dr. Geneviève Bernard, with whom I share the interest on genetic leukoencephalopathies and the background in pediatric neurology. Working with her on POLR3-related disorders and reviewing so many MRI studies together was a great learning occasion to consolidate my skills in the MRI pattern-recognition approach.

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This thesis is dedicated to my children, Yongbo and Ester, for keeping me down to earth every single day (and night), and motivating me to work hard to leave them and the next generations a better world.

PREFACE

This thesis is written according to the guidelines of the McGill University Graduate and Postdoctoral Studies Office. It is presented in the manuscript-based format for a Doctoral thesis. The studies described herein were performed under the co-supervision of Dr. Bernard Brais and Dr. Donatella Tampieri, and in close collaboration with Dr. Geneviève Bernard and Dr. Gilbert B. Pike.

The subject of this thesis is adult genetic white matter diseases and, specifically, the contribution of an integrated multidisciplinary approach to characterize them and identify new entities.

This thesis is composed of eight Chapters, subdivided in two Sections. **Chapter 1** is a general introduction to the topic of genetic leukoencephalopathies in the adult population. **Section I** describes the contribution of the MRI pattern-recognition approach to the study of POLR3-related disorders. This section includes Chapters 2 and 3: **Chapter 2** is a manuscript published in the *Journal of Child Neurology* describing the classic MRI pattern associated to POLR3-related disorders. **Chapter 3** was published in *Neurology* and demonstrated the broadening MRI phenotype of POLR3-related disorders by documenting that hypomyelination is not an obligate feature. **Section II** summarizes the most relevant clinical, radiological and genetic results from our study on a cohort of 68 adult subjects with suspected genetic leukoencephalopathies. The section is composed of Chapters 4, 5 and 6. **Chapter 4** describes the expanding clinical phenotype associated with vanishing white matter disease and was accepted for publication in *The Journal of Neuropsychiatry and Clinical Neurosciences*. **Chapter 5** recollects the clinical and radiological data and functional characterization of a unique presentation of MTFMT-related disorders, diagnosed by whole exome sequencing. It was published in *Neurogenetics*. **Chapter 6** is a manuscript published in the *Journal of Clinical Neuroscience* demonstrating the usefulness

of familial MRI studies in the diagnostic process of genetic leukoencephalopathies with unclear origin; this approach has proven successful in a family with Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL). **Chapter 7** includes the general discussion and the future directions of the project. **Chapter 8** provides the conclusions of this thesis.

CONTRIBUTIONS OF AUTHORS

Chapter 2

The study was performed with the following collaborators: *Davide Tonduti, Heather Gordish Dressman, Johanna L. Schmidt, Jonathan Murnick, Bernard Brais, Geneviève Bernard, Adeline Vanderver*. DT, GB, AV and I analysed and interpreted the clinical and radiological data. DT and I wrote the paper. I prepared the figures and tables. GB and AV conceived and designed the study and, along with BB, critically revised the manuscript for important intellectual content. JLS and AV acquired the data. HGD performed the statistical analysis and JM analyzed the neuroradiological data. HDG and JM critically revised the manuscript.

Chapter 3

The study was performed with the following collaborators: *Ferdy K. Cayami, Luan T. Tran, Kether Guerrero, Rosalina van Spaendonk, Katrin Õunap, Sander Pajusalu, Tobias Haack, Evangeline Wassmer, Dagmar Timmann, Hanna Mierzewska, Bwee T. Poll-Thé, Chirag Patel, Helen Cox, Tahir Atik, Huseyin Onay, Ferda Ozknay, Adeline Vanderver, Marjo van der Knaap, Nicole I. Wolf, Geneviève Bernard*. FKC and I collected, analyzed, and interpreted patient data, and wrote the manuscript. I prepared the figures and tables. LTT, KO, SP, EW, DT, BTPT, CP, HC, TA, HO, FO, AV, MSVK collected, analyzed, and interpreted patient data and reviewed the article. KG, RVS, TH were responsible for molecular genetic analysis and reviewed the article. NIW and GB designed and supervised the study, collected data, and reviewed the article.

Chapter 4

The study was performed with the following collaborators: *Andrea Accogli, Bernard Brais, Donatella Tampieri*. AA wrote the draft of the manuscript. BB and DT collected the clinical and radiological data and revised the article for important intellectual content. I conceived and

supervised the study, interpreted the radiological, clinical and genetic data, prepared the figure, and revised the article for intellectual content.

Chapter 5

The study was performed with the following collaborators: *Woranontee Weraarpachai, Luis H. Ospina, Martine Tetreault, Jacek Majewski, Care4Rare Canada Consortium, G. Bruce Pike, Jean-Claude Decarie, Donatella Tampieri, Bernard Brais, Eric A. Shoubridge*. I collected, analyzed, and interpreted the radiological and clinical data, and wrote the manuscript. I prepared the radiological figure. WW performed the functional analyses and prepared the relative figure. LHO collected and interpreted the ophthalmological data and reviewed the manuscript. MT and JM were responsible for the whole exome sequencing data analysis and reviewed the manuscript. Care4Rare Canada Consortium was responsible for the generation of the whole exome sequencing data. GBP reviewed the MRI data analysis and interpretation. JCD collected and analyzed the radiological data and reviewed the manuscript. DT interpreted the radiological data and reviewed the manuscript. BB collected and interpreted the clinical data and reviewed the manuscript. EAS designed and supervised the study, reviewed the manuscript for important intellectual content.

Chapter 6

The study was performed with the following collaborators: *Ilana R. Leppert, G. Bruce Pike, Sylvain Lanthier, Bernard Brais, Donatella Tampieri*. I conceived the study, collected and analyzed the clinical and radiological data, and wrote the manuscript. I prepared the figures. IRL and GBP participated to the MRI study design, contributed to the MRI data analysis and interpretation, revised the manuscript. SL and BB acquired and contributed to the interpretation

of the clinical and genetic data, and revised the manuscript. DT supervised the study, contributed to the analysis of the radiological data, and revised the manuscript.

ORIGINAL CONTRIBUTION TO KNOWLEDGE

The work included in this thesis contributes significantly to the study of genetic leukoencephalopathies in the adult population, an emerging field of neurosciences. Specifically, the original contribution of this thesis focuses on the application of the MRI pattern-recognition approach to known disorders, and the integration of clinical, radiological and genetic datasets to investigate unsolved leukoencephalopathies of suspected genetic origin.

Chapter 2 provides the first systematic review of the neuroradiological findings in a cohort of patients with genetically proven POLR3-related disorders. Furthermore, it demonstrates that the POLR3-associated MRI pattern is characteristic and can distinguish these disorders from other hypomyelinating leukoencephalopathies.

Chapter 3 reports two new MRI patterns associated with POLR3-related disorders, the first characterized by isolated cerebellar atrophy and the second by the selective corticospinal tracts involvement. In addition, a major advancement provided by this paper was that diffuse hypomyelination is not an obligate feature of POLR3-related disorders.

Chapter 4 expands the clinical phenotype associated to vanishing white matter disease, one of the most common genetic leukoencephalopathies, by adding long-standing psychiatric features to the more classic clinical manifestations of the disease in the adult population.

Chapter 5 broadens the clinical and radiological phenotypes associated with *MTFMT* mutations by describing a milder presentation dominated by the selective involvement of the visual pathway. The functional characterization of the novel mutation identified in our subject demonstrated that the biochemical defect is similar in all reported affected individuals, despite dramatically different phenotypes.

Chapter 6 demonstrates the usefulness of familial MRI studies to clarify the modality of transmission of unsolved white matter disorders. In addition, the manuscript reported in this chapter documents the high intrafamilial clinical and MRI variability associated with Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL).

CHAPTER 1

1.1 Defining genetic leukoencephalopathies

Genetic leukoencephalopathies is a broad nosographic term which includes all inherited disorders characterized by white matter involvement. Historically, they comprise the leukodystrophies, genetic disorders in which the myelin is primarily affected, and secondary white matter disorders, in which the white matter is involved as the end result of pathogenic processes not directed to the myelin (e.g. vascular leukoencephalopathies, inherited disorders of metabolism, mitochondrial disorders, etc.)[1-4]. The distinction between leukodystrophies and other genetic leukoencephalopathies is based on the known causal genetic defect and, as proposed recently, the pathological changes and the underlying pathogenetic mechanisms [5]. To date, a considerable number of genetic leukoencephalopathies still remains unclassified, despite major developments in genetics and imaging techniques. Consequently, the more general term of genetic leukoencephalopathies is commonly used to refer to white matter disorders of inherited origin.

Two major technical advancements have revolutionized the study of genetic leukoencephalopathies. The first was Magnetic Resonance Imaging (MRI), starting in the 1980s, which demonstrated a high sensitivity for white matter abnormalities, thus allowing the classification of white matter disorders according to their different patterns of involvement. The second, since 2005, was the advent of Next Generation Sequencing (NGS), which had a major impact in identifying the cause of unsolved forms and also contributed to expand significantly the phenotypic spectrum of known leukoencephalopathies.

Nowadays, the work of researchers interested in genetic white matter disorders relies heavily on MRI and NGS and the common perception is that, in the near future, the majority of genetic

leukoencephalopathies will be solved. This is especially true for pediatric forms, which are the object of the larger part of the research in the field to date. In fact, genetic leukoencephalopathies are the most common cause of white matter disorders in childhood[6, 7]. Conversely, they are rarely included in the differential diagnosis of adult patients with white matter abnormalities[8-11], even though several lines of evidence that I will review in the next section suggest that it should not be the case.

1.2 CNS white matter disorders in adults

While inflammatory-demyelinating and vascular causes are commonly considered in the differential diagnosis of CNS white matter abnormalities in adult subjects, genetic leukoencephalopathies are rarely taken into account unless there is a clear family history, a symmetrical and more diffuse distribution of the white matter changes or a slowly progressive deterioration. Even though no single adult genetic leukoencephalopathy has an estimated prevalence higher than 1 in 20,000, some authors state that the cumulative incidence of all genetic white matter disorders is not dissimilar than that of multiple sclerosis (MS)[12, 13]. Definite epidemiological data on the frequency of adult genetic leukoencephalopathies are lacking; however, probably in relation with the widespread use of MRI, adult forms are increasingly recognized.

Two main groups of genetic white matter disorders can be identified in adults[1, 8]. The first includes the adult presentations of classic genetic leukoencephalopathies that usually manifest earlier in life. X-linked adrenoleukodystrophy, especially in its adrenomyeloneuropathy form, Alexander disease, Krabbe disease, metachromatic leukodystrophy, and vanishing white matter disease are the most common and known entities[14]. Among the hypomyelinating forms, POLR3-

related disorders, Hypomyelinating leukodystrophy with Atrophy of Basal ganglia and Cerebellum (H-ABC), Pelizaeus-Merzbacher disease, and Pelizaeus-Merzbacher-like disease are those most often diagnosed in adult patients[10]. Pediatric and adult forms differ significantly in terms of onset, evolution and findings, with the general rule that the earlier the onset, the more severe and rapid the clinical course[15]. In adult patients, the clinical onset may be insidious and the symptoms non-specific: oftentimes, slowly-progressive cognitive deterioration or psychiatric disturbances dominate at the disease's onset. Pediatric and adult forms of the same leukoencephalopathy may also differ substantially in their MRI white matter involvement, and sometimes present almost opposite patterns. As an example, in its pediatric form, Alexander disease typically shows a frontal predominance of white matter involvement, while the adult form presents with characteristic posterior fossa abnormalities[6, 14, 16, 17]. Krabbe disease is another disorder with distinct MRI phenotypes in children and adults: a predominant periventricular involvement is typically seen in early-onset forms, while adults present with an antero-posterior gradient and specific corticospinal tract involvement[14, 18, 19]. All these clinical and imaging differences may contribute to delayed diagnosis in adult subjects.

The second group of adult genetic leukoencephalopathies includes forms manifesting exclusively at adult age[1, 8]. Cerebral Autosomal Dominant Arteriopathy with Sub-cortical Infarcts and Leukoencephalopathy (CADASIL), Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL), hereditary diffuse leukodystrophy with axonal spheroids, autosomal-dominant leukodystrophy with autonomic disease (ADLD) and cerebrotendineous xanthomatosis belong to this group of disorders[1].

Interestingly, many adult leukoencephalopathies – especially in the group of disorders presenting exclusively at adult age - are characterized by multifocal white matter involvement that can be

easily misinterpreted as acquired and therefore mimic multiple sclerosis or cerebral small vessel diseases of ischemic or arteriosclerotic origin[8, 11].

As opposed to what is observed in pediatrics, few studies have tried to help neurologists by designing diagnostic algorithms for the known forms of adult inherited leukoencephalopathies[12, 13, 20]. These are based mainly on the distinct clinical and, to a lesser extent, radiological features, that can differentiate among the entities. Despite the usefulness of algorithms, the diagnosis of adults with genetic leukoencephalopathies remains often elusive. Approximately 20-30% of all patients with presumed genetic leukoencephalopathy remain without a clear diagnosis, a percentage that increases to more than 50% in adult cases[2, 7, 9, 11, 21]. These patients and their families are faced with what is referred to as "the diagnostic odyssey" in the rare diseases field, namely an extended period of time, often of decades, during which a final diagnosis is searched using all available investigations. The identification of the causal gene is the first step for clinicians and researchers to ensure genetic counselling, understand the underlying pathophysiology, and foresee therapeutic strategies.

The diagnosis of adult leukoencephalopathies is complicated by extra factors. First, the adult population is more heterogeneous and there is a radiological and clinical overlapping with acquired disorders such as severe ischemic small vessel disease or "atypical MS"[11]. Primary progressive MS (PPMS) poses a particularly difficult differential diagnosis since, by definition, it has a steadily progressive disease course and is treatment-resistant. These two features are also characteristic of genetic leukoencephalopathies, hence the possibility that subjects classified as PPMS might have yet to be recognized as having genetic conditions[22, 23]. The genetic disorders that are more easily misdiagnosed as MS are genetic vasculopathies (CADASIL, CARASIL,

COL4A1-related disorders), hereditary diffuse leukodystrophy with axonal spheroids, vanishing white matter disease, ADLD, adrenomyeloneuropathy and mitochondrial disorders[11, 24-29].

A second factor that can complicate the achievement of a definite diagnosis is that familial DNA is not often available in adult patients to verify the segregation of the disease status. Finally, many genetic neurological conditions such as hereditary ataxias and hereditary spastic parapareses (HSPs) are often associated with white matter abnormalities that are not investigated further[30]. A study showed that 11% of adult subjects with HSP have an underlying, unrecognized genetic leukoencephalopathy[31]. Adrenomyeloneuropathy and Krabbe disease are classic examples of genetic leukoencephalopathies that present as spastic paraparesis in adults[31, 32]. In addition to them, in the recent years, several reports described entities that present clinically as HSPs and have either MRI or genetic features compatible with known leukoencephalopathies. This is the case of leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL), POLR3-related disorders, and HSP type 35[33-36]. The discussion is ongoing as to whether some of these disorders should be classified as HSPs with white matter involvement or genetic leukoencephalopathies presenting clinically with spastic paraparesis[37, 38].

As a result, adult patients experience a further delay in the diagnosis or are erroneously considered as having an acquired disorder with important implications on their health and their families. Together all these factors delaying diagnosis underline the need for implementing clinical research units that combine cutting-edge imagery and genetics to accelerate the investigation of adults affected by genetic leukoencephalopathies.

1.3 MRI pattern-recognition in children and adults

The study of white matter disorders is strongly linked to the development of Magnetic Resonance Imaging. Since its advent, in the 1980s, this technique has shown a high sensitivity for white matter changes. The interpretation of brain MRI became central to the growing classification of genetic white matter disorders[39]. The MRI pattern-recognition approach is based on the evidence that each leukoencephalopathy presents a distinctive pattern of white matter involvement which is consistent among affected subjects and different from other leukoencephalopathies[6, 39, 40]. Following this approach, diagnostic MRI algorithms were designed and applied successfully by clinicians and researchers for decades, especially in the pediatric population[6, 41]. In adults, the presence of key MRI features can orient towards a specific diagnosis, as summarized in different reviews[10-13, 21]. Only a single pioneer retrospective study utilized the MRI pattern-recognition approach in a cohort of undiagnosed adult patients with the goal of predicting the final diagnosis[9]. This was the first comprehensive, nation-wide study on adult inherited leukoencephalopathies and it included 154 subjects. As opposed to specific patterns used in the approach to pediatric leukoencephalopathies, Ayrignac et al. decided to classify patients according to three main categories of white matter involvement: vascular, cavitary and other. The MRI-oriented diagnostic screening allowed a diagnosis in 64% of cases, with the highest rates in the vascular and cavitary groups (75 and 76% respectively).

Except for the work by Ayrignac et al. [9], the MRI pattern-recognition approach has not been applied to adults for several reasons. First, the radiological variability that exists in some leukoencephalopathies, especially between pediatric and adult forms (e.g. Alexander and Krabbe diseases), complicates the application of an MRI pattern-recognition approach to adult subjects[16-19]. Second, adult patients present a higher rate of acquired co-morbidities, such as hypertension or metabolic syndromes, whose associated white matter abnormalities may impact on the

recognition of an underlying genetic leukoencephalopathy pattern. Last, due to the insidious onset and slow progression, adults with genetic leukoencephalopathy might seek medical attention at a late stage of the disease course, when the white matter abnormalities have become confluent and therefore have lost any specific pattern[6, 14].

Conventional MRI as acquired in the clinical setting has limitations in thoroughly assessing the neuroradiological features of white matter diseases. In fact, it cannot always discriminate between different underlying pathological processes. For example, an abnormal signal in the white matter – hypointense in T1-weighted and hyperintense in T2-weighted sequences - can be variably due to demyelination, hypomyelination, inflammation, edema or gliosis[6]. Higher-field (3T and 7T) MRI provides extra-details to the characterization of white matter abnormalities in inherited disorders. As an example, 3T MRI in POLR3-related disorders disclosed the presence of additional features which were less apparent in 1.5T images, namely myelin islets within hypomyelinated areas, corpus callosum cyst-like lesions and spinal cord hypomyelination[42].

High-field strength MRI coupled with advanced techniques can be used to increase the understanding of the pathophysiology of white matter abnormalities. In fact, quantitative parameters - such as apparent diffusion coefficient (ADC), fractional anisotropy (FA), MR spectroscopy and magnetization transfer ratio (MTR) - provide important information on the microstructure and chemical profile of the affected white matter, sometimes at a very early stage of disease[43]. MTR is a particularly interesting technique, as it has been demonstrated to be a reliable index of myelin content, with decreased MTR indicating white matter damage and myelin loss[44]. For this aspect, MTR has found a solid application in research on MS[44-48], while it has had a limited use in genetic leukoencephalopathies to date. This might be due to the fact that

signal changes in genetic leukoencephalopathies are often diffuse, therefore difficult to assess, as sometimes no normal white matter is present in affected subjects to allow for quantitative comparison with damaged tissue. Nonetheless, a few studies have demonstrated that quantitative parameters, among which MTR, are successful in discriminating between different types of leukoencephalopathies (i.e. demyelinating versus hypomyelinating)[49].

The neuroradiological characterization of white matter abnormalities, and the MRI pattern-recognition approach in particular, have a critical role in the definition of unknown genetic white matter disorders[6, 34, 50-53]. Researchers working in this field have used the concept that novel genetic leukoencephalopathies could be defined by their specific MRI pattern of involvement. In the early 2000s, the identification of mutations in *EIF2B1-5* and *MLC1* in vanishing white matter disease and megalencephalic leukoencephalopathy with cysts (MLC) respectively was the proof of principle that novel MRI-defined leukoencephalopathies can be used to guide genetic analysis[54-57]. Since then, causal genes were identified for MRI-defined conditions using different genetic techniques as described in the next section.

1.4 The impact of NGS on unsolved white matter disorders

The contribution of genetics to the diagnosis of new forms of leukoencephalopathies has increased significantly in the last three decades. This was made possible by the identification of large or consanguineous families with multiple affected members. The French Canadian and indigenous populations of Quebec are particularly suited to this type of approach due to regional founder effects[28, 58, 59]. First Nation Cree leukoencephalopathy and Cree encephalitis are Quebec-specific subtypes of genetic leukoencephalopathies – vanishing white matter disease and Aicardi-Goutières syndrome respectively[60, 61] – discovered through linkage analysis. The same

technique was successfully used to identify the causal chromosome 5q23 duplication and the implication of *LMNB1* in ADLD in a large French Canadian family[28]. Bernard et al. combined clinical and MRI phenotypes with a geographical clustering approach to map and identify *POLR3A* as the first gene responsible for Tremor-Ataxia with Central Hypomyelination, now referred to as POLR3-related disorders or 4H syndrome[62-65].

The advent of NGS, in 2005, has revolutionized the field of undiagnosed genetic leukoencephalopathies. An application of NGS that quickly became clinically available was the targeted sequencing and analysis of candidate genes for a definite group of disorders, e.g. all genes associated with known genetic leukoencephalopathies. In contrast to the time-consuming classic Sanger sequencing of single genes, NGS gene panels allowed the fast identification of variants in a growing number of genes at once. Moreover, the overall cost of gene panels has significantly dropped over the years, making it more affordable than the consecutive sequencing of several candidate genes[66].

Whole exome sequencing (WES) involves the sequencing of the protein-coding DNA sequences, where 85% of causal mutations for Mendelian diseases have been located to date[67]. Approximately 20,000-25,000 variants are generated by WES, and about half of these are nonsynonymous, therefore having a potential deleterious impact on the encoded protein[68, 69]. The large majority of the nonsynonymous variants are common (i.e. present in >5% of the general population), thus unlikely to cause a rare disorder. However, despite filtering them out, one can remain with more than 1000 variants, which need to be analysed according to several features - such as the *in silico* pathogenicity prediction, segregation with disease status in the family, and conservation across species – in order to establish their potential association with the studied disease in a family. Therefore, despite its great potential for the diagnosis of unsolved disorders,

the interpretation of data, in particular in single cases, represents a major challenge in WES application.

The most significant contribution of WES in the field of inherited white matter disorders has been the identification of the causal genes of several unsolved entities and ultra-rare diseases[2, 70-74]. Hereditary diffuse leukodystrophy with axonal spheroids, a relatively common adult genetic leukoencephalopathy, was one of the first white matters disorders whose causal gene, *CSF1R*, was identified through WES[70]. Using WES, POLR3-related disorders were associated to a third causal gene, *POLR1C*[75].

Besides contributing to solve several undetermined leukoencephalopathies, NGS gene panels and WES have had a major role in broadening the mutational, clinical and radiological spectrum of known genetic white matter disorders[35, 76-80].

In pediatric leukoencephalopathies, thanks to the contribution of NGS, the rate of diagnosis increased from less than 40% in the 2000s, to 50% in 2010, to approximately 80% in 2016[2, 81]. In a cohort of 100 adult subjects with unsolved leukoencephalopathies, WES allowed the diagnosis in 26 of them[21]. Depending on how stringent the inclusion criteria are and the used technique – either single gene testing, NGS gene panels or WES-, the rate of diagnosis in studies on adult subjects with genetic leukoencephalopathies ranges between 13.3% to 64%[9, 21, 82]. As a result, undiagnosed leukoencephalopathies in adults have become an emerging field in neurosciences.

1.5 Rationale and hypotheses

The study of leukoencephalopathies - either genetically-proven or unsolved - relies greatly on MRI pattern-recognition, which allowed the characterization of the majority of genetic white matter disorders. The advent of NGS revolutionized the field, by disclosing new phenotypes associated to known genetic white matter conditions and identifying novel genes responsible for unsolved leukoencephalopathies.

Based on these two observations, my first hypothesis (developed in Section I) was that:

-MRI pattern recognition can be applied to the newly described POLR3-related disorders to distinguish them from other leukoencephalopathies, while an expanding spectrum of MRI and clinical phenotypes of POLR3-related disorders are revealed as new mutation-proven subjects are identified by NGS.

The high rate of undiagnosed leukoencephalopathies in adults, representing an emerging research field in neurosciences, and the challenging discrimination between inherited and acquired white matter disorders led me to the second, main hypothesis (developed in Section II):

-A multimodal approach combining clinical phenotyping, MRI pattern-recognition approach and next generation sequencing data will enable the identification of adult inherited leukoencephalopathies currently not yet diagnosed and their genetic basis.

1.6 Research goals

The goals of my study were:

1. the application of advanced neuroimaging tools to define and characterize white matter abnormalities of known and novel genetic leukoencephalopathies;

2. the clinical and demographic characterization of subjects with adult genetic leukoencephalopathies;
3. the identification of genes responsible for new forms of hereditary white matter disorders, using next generation sequencing techniques.

SECTION I

MRI pattern-recognition in POLR3-related disorders: from classic to novel imaging phenotypes

PREFACE

The approach of MRI pattern recognition is a well-recognized methodology to investigate inherited white matter disorders[6, 41]. By applying this method to a cohort of subjects with genetically proven POLR3-related disorders, we demonstrated that POLR3-disorders present with specific MRI features that can distinguish them from other hypomyelinating diseases (Chapter 2). In addition, we showed that the same approach is crucial to document atypical neuroradiological findings, not initially associated with POLR3-related disorders (Chapter 3). To this regard, we demonstrated how the combination of NGS data with MRI analysis can bring to the description of new phenotypes, since three subjects here reported were diagnosed solely based on NGS data, despite MRI findings not suggestive of a specific leukoencephalopathy.

CHAPTER 2

Brain MRI pattern recognition in Pol III-related leukodystrophies[83].

Roberta La Piana[1], Davide Tonduti[2], Heather Gordish Dressman[3], Johanna L Schmidt[3], Jonathan Murnick[4], Bernard Brais[5], Geneviève Bernard[6], Adeline Vanderver[3].

[1]Department of Neuroradiology, Montreal Neurological Institute and Hospital, McGill University, Montreal, Quebec, Canada

[2]Child Neuropsychiatry Unit, Department of Brain and Behavioral Sciences, University of Pavia, Italy

[3]Department of Neurology, Children's National Medical Center, Center for Genetic Medicine Research (CGMR), Washington DC, USA

[4] Department of Radiology, Division of Neuroradiology, Children's National Medical Center, Washington DC, USA

[5]Departments of Neurology and Neurosurgery and Human Genetics, Montreal Neurological Institute and Hospital, McGill University, Montreal, Quebec, Canada

[6]Division of Pediatric Neurology, Montreal Children's Hospital, Montreal, Quebec, Canada

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Abstract

Pol III-related leukodystrophies are caused by mutations in *POLR3A* and *POLR3B* genes and all share peculiar imaging and clinical features.

The objectives of this study are: 1) to define the neuroradiologic pattern in a cohort of *POLR3A* and *POLR3B* subjects; 2) to compare the neuroradiological pattern of Pol III-related leukodystrophies with other hypomyelinating disorders. The MRI exams of 13 patients with *POLR3A* and *POLR3B* mutations and of 14 patients with other hypomyelinating disorders were analyzed. All the subjects with Pol III-related leukodystrophies presented hypomyelination associated with T2 hypointensity of the thalami and/or the pallida. Twelve subjects (92%) presented T2 hypointensity of the optic radiations. Cerebellar atrophy was observed in most patients (92%). The combination of the analyzed criteria identified patients with Pol III-related leukodystrophies with a sensitivity of 84.6% and a specificity of 92.9%.

Introduction

Hypomyelinating leukodystrophies represent the majority of leukoencephalopathies of undetermined origin in infancy[1]. MRI pattern recognition is now an essential tool in categorizing them and has enabled the definition of new entities, in particular among hypomyelinating disorders[2, 3]. Pol III-related leukodystrophies constitute a recently genetically defined group of hypomyelinating leukodystrophies which include different previously described conditions with often specific MRI white matter involvement[4-6]. Leukodystrophy with oligodontia was the first entity of this group of diseases to be described in 2003 in a Syrian family[7]. In 2005, Wolf et al4 described four patients with a similar disorder characterized by hypomyelination, ataxia and abnormal dentition[4,5]. Following these descriptions, a growing number of patients with hypogonadotropic hypogonadism in association with hypomyelination and abnormal dentition

were identified by different groups and referred to as 4H syndrome[8,9-11]. The original family with leukodystrophy with oligodontia and Quebec families with an overlapping phenotype were found to be allelic, with mapping to 10q22 region[12] for leukodystrophy with oligodontia and 10q22.3-10q23.31 for tremor ataxia with central hypomyelination respectively[13]. In 2011, mutations in two genes (*POLR3A*[14,15] and *POLR3B*[15,16]) encoding the two largest subunits of RNA polymerase III (Pol III) were identified in subjects with leukodystrophy with oligodontia, 4H and tremor ataxia with central hypomyelination. At this time, a total of five different overlapping conditions, including 4H syndrome, leukodystrophy with oligodontia,[7] Ataxia Delayed Dentition and Hypomyelination,[4, 5] Tremor-Ataxia with Central Hypomyelination,[13] and Hypomyelination with Cerebellar Atrophy and Hypoplasia of the Corpus callosum [15, 17], have been found to be caused by recessive mutations in *POLR3A*[13-15] or *POLR3B*[15,16]. These disorders likely represent a continuum of clinical manifestations. No detailed phenotype-genotype correlation has been completed to date. These disorders are now globally referred to as Pol III-related leukodystrophies.

The 2010 manuscript by Steenweg et al.[3] described a distinctive neuroradiological pattern in subjects with 4H syndrome prior to the identification of mutation. This pattern includes: supratentorial diffuse hypomyelination variably associated with T2 hypointensity of the optic radiations, the pyramidal tracts at the level of the posterior limb of the internal capsule, the anterolateral nuclei of the thalami and the pallida. The presence of cerebellar atrophy, mostly vermian, and a relative T2 hypointensity of the dentate nuclei compared to the surrounding cerebellar white matter completes the picture.

Our study aims at further describing the neuroradiological findings of Pol III-related hypomyelinating leukodystrophies as well as validating the above MRI characteristics in a sample of mutation-proven patients.

Methods

Participants and data collection

Brain MR images of 13 patients (five females, eight males; mean age at MRI 17.8 years, range 4-32 years) with a clinical diagnosis of 4H or tremor ataxia with central hypomyelination were collected. The diagnosis of Pol III-related leukodystrophy was confirmed by Sanger sequencing in all patients, revealing pathogenic recessive mutations in either the *POLR3A* (ten subjects) or *POLR3B* (three subjects).

Fourteen control subjects (two females, twelve males; mean age at MRI 6.2 years, range 4 months-32 years) with other identified hypomyelinating disorders were selected among our database. The clinical features and the age at MRI of Pol III subjects and controls are summarized in Supplemental Table 1. Given the rarity of these diseases, matching controls for age and sex were sought, but the mean age of the Pol III mutation positive subjects was greater, partly because of the relatively milder phenotype of Pol III-related leukodystrophies compared with the other hypomyelinating leukodystrophies, and partly because of the greater likelihood that they were identified after the expected age of puberty. For both reasons, Pol III mutation subjects are more likely to have MRIs at an older age. Due to the potential biases that could arise from the age difference between the two groups, the presence of the analyzed criteria was evaluated in relation to the age of the subject.18 The control group included six subjects with Pelizaeus-Merzbacher disease, two with Pelizaeus-Merzbacher-like disease caused by *GJC2*

mutations, two with Salla disease, two with hypomyelination with atrophy of the basal ganglia and cerebellum and two with infantile GM1 gangliosidosis. Except for hypomyelination with atrophy of the basal ganglia and cerebellum, these were the same disorders studied in the original algorithm for hypomyelinating leukodystrophies by Steenweg et al.[3]

As the MR images were performed in different centres and sent to our team for diagnostic purpose, variability in the technique and acquisition of images was observed. However, sagittal T1-weighted images and axial T2- and axial FLAIR-weighted images were available for all the subjects, allowing the evaluation of the selected criteria. For ten patients, axial T1 and/or sagittal T2 were also available.

Table S2.1: Clinical features of subjects with Pol III-related leukodystrophies (a) and of control subjects (b).

S2.1a

Subject	Sex	Age at MRI (years)	Clinical Diagnosis	Mutated Gene
1	F	30	4H	*POLR3A*
2	F	20	4H	*POLR3A*
3	F	32	4H	*POLR3A*
4	F	4	4H	*POLR3B*
5	M	26	4H	*POLR3A*
6	M	25	4H	*POLR3A*
7	M	8	4H	*POLR3A*
8	M	22	TACH	*POLR3A*
9	M	11	TACH	*POLR3A*
10	M	7	4H	*POLR3B*
11	F	20	4H	*POLR3A*
12	M	17	4H	*POLR3B*
13	M	10	TACH	*POLR3A*

Legend: 4H: hypomyelination, hypogonadotropic hypogonadism and hypodontia; TACH: tremor-ataxia with central hypomyelination.

S2.1b

Subject	Sex	Age at MRI (years)	Diagnosis
1	M	0.83	GM1
2	M	0.75	GM1
3	F	2	HABC
4	M	9	HABC
5	M	0.33	PMD
6	M	14	PMD
7	M	1.5	PMD
8	M	2	PMD
9	M	2	PMD
10	M	11	PMD
11	M	32	PMDL
12	M	1	PMDL
13	F	9	Salla
14	M	1.5	Salla

Legend: GM1: GM1-gangliosidosis; HABC: hypomyelinating leukodystrophy with Atrophy of Basal ganglia and Cerebellum; PMD: Pelizaeus-Merzbacher Disease; PMDL: Pelizaeus-Merzbacher-like Disease.

Data analysis

The neuroradiologic findings were analyzed according to the following criteria: presence of diffuse hypomyelination, cerebellar atrophy, and T2 hypointensity of the anterolateral thalami, pallida nuclei, pyramidal tracts, posterior periventricular white matter (in the region of the optic radiations) and dentate nuclei.

All images were reviewed by our team collegially (A.V., G.B., D.T. and R.L.). Team disagreements were resolved by consensus. In addition, images were reviewed on two different occasions by an experienced neuroradiologist (J.M.), without specific experience in leukodystrophies who was blind to the clinical and molecular data. The two reviews of the images were separated in time by six weeks, in order to avoid memory of individual subjects.

Statistical Analysis

Logistic regression analysis was used to describe how the proposed criteria by Steenweg et al (2010)[3] compare in Pol III-related leukodystrophies versus other hypomyelinating leukodystrophies. A model including all examined criteria was initially used to evaluate the association between all the criteria and the diagnosis of Pol III-related leukodystrophies as well as the sensitivity and specificity of these criteria. The fit of the model was tested using the Homer-Lemeshow goodness of fit test. Moreover, criteria were analyzed individually and in pairs of similar findings: T2 hypointensity of the pyramidal tracts and optic radiations individually and together; T2 hypointensity of the pallida nuclei and anterolateral nuclei of the thalami individually and together; cerebellar atrophy; and T2 hypointensity of the dentate nuclei. These analyses were performed using the team evaluation data. All statistical analyses were performed using Stata V11 (College Station, TX).

Inter-operator agreement (namely, agreement between team and radiologist's reading 1 and reading 2) and intra-operator agreement (namely, the agreement between radiologist's reading 1 and 2) were evaluated using Kappa statistics. According to Landis[19], we considered Kappa statistics ≥ 0.81 as almost perfect agreement, 0.61 to 0.80 as substantial, 0.41-0.60 as moderate, 0.21-0.40 as fair and 0-0.20 as slight agreement.

Results

The data presented below corresponds to the review by the evaluating team as a group, unless otherwise specified (see Inter-operator and Intra-operator agreement paragraph).

Neuroradiological findings in Pol III-related leukodystrophies

The presence of diffuse hypomyelination was observed in all 13 mutation-proven Pol III-related leukodystrophy patients (100%) (Figure 2.1). It was associated with T2-weighted hypointensity in the regions of the pallida and/or thalami in all subjects (13/13; 100%) (Figure 2.2ab). In ten patients (10/13; 77%) the low T2 signal was seen both in the anterolateral portion of the thalami and in the pallida nuclei, while in two patients (2/13; 15%) it was limited to the anterolateral portion of the thalami and in one (1/13; 8%) to the pallida nuclei.

A relative low T2 signal of the dentate nuclei in comparison to the surrounding white matter was observed in 9 patients (9/13; 69%) (Figure 2.2c). Twelve subjects (12/13; 92%) presented T2 hypointense signal in the posterior periventricular white matter; the optic radiations were hypointense in T2-weighted images in all twelve patients while in one subject the T2 hypointensity extended to the adjacent tapetum (Figure 2.2b). The pyramidal tracts at the level of the posterior limb of the internal capsule presented a T2 hypointense signal in five subjects (5/13; 38%). Cerebellar atrophy was observed in 12 patients (12/13; 92%), involving both the

cerebellar hemispheres and the vermis (Figure 2.3). Three patients (23%) presented all MRI features analyzed.

Figure 2.1: Hypomyelination in Pol III-related leukodystrophies. Sagittal T1- (a) and axial T2-weighted images (b,c) demonstrate the presence of diffuse and homogeneous hypomyelination in two patients: 17 year-old male subject with *POLR3B* mutation shown in Figures a, b; 10 year-old male subject with *POLR3A* mutation shown in Figure c. Hypomyelination is defined here as isointense or hyperintense T1 signal and hyperintense T2 signal.

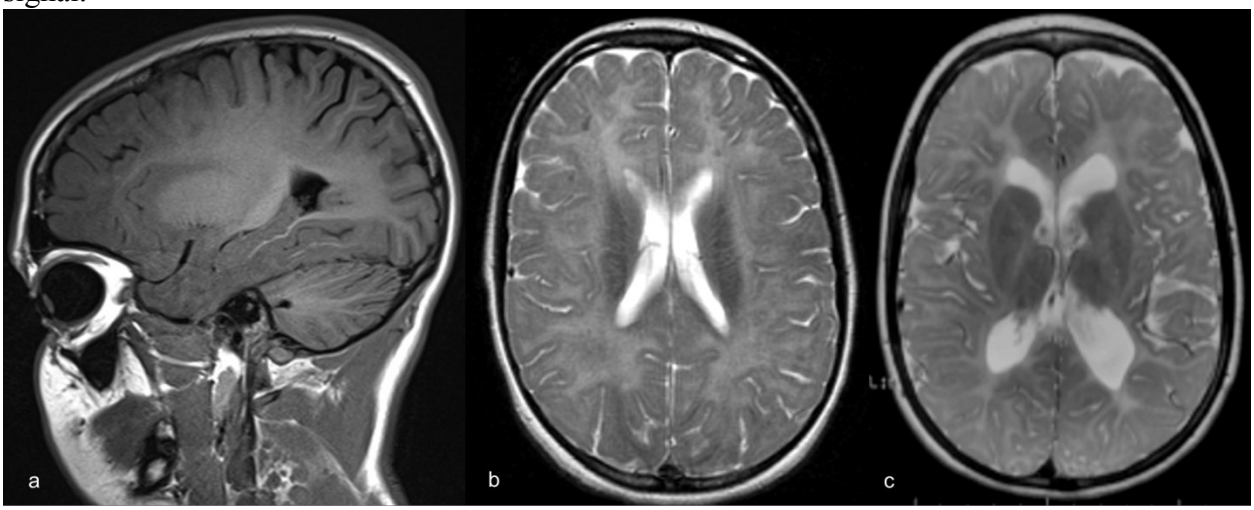


Figure 2.2: Features associated with diffuse hypomyelination in Pol III-related leukodystrophies. 22 year-old male subject with *POLR3A* mutation in figure a; 17 year-old male subject with *POLR3B* mutation in figure b; 11 year-old male subject with *POL3A* mutation in figure c. Axial T2-weighted images showing the presence of hypointense signal at the level of the antero-lateral nuclei of the thalami and of the pallida bilaterally (a, b), of the optic radiations (a, b) and corticospinal tracts (b) and of the dentate nuclei (c).

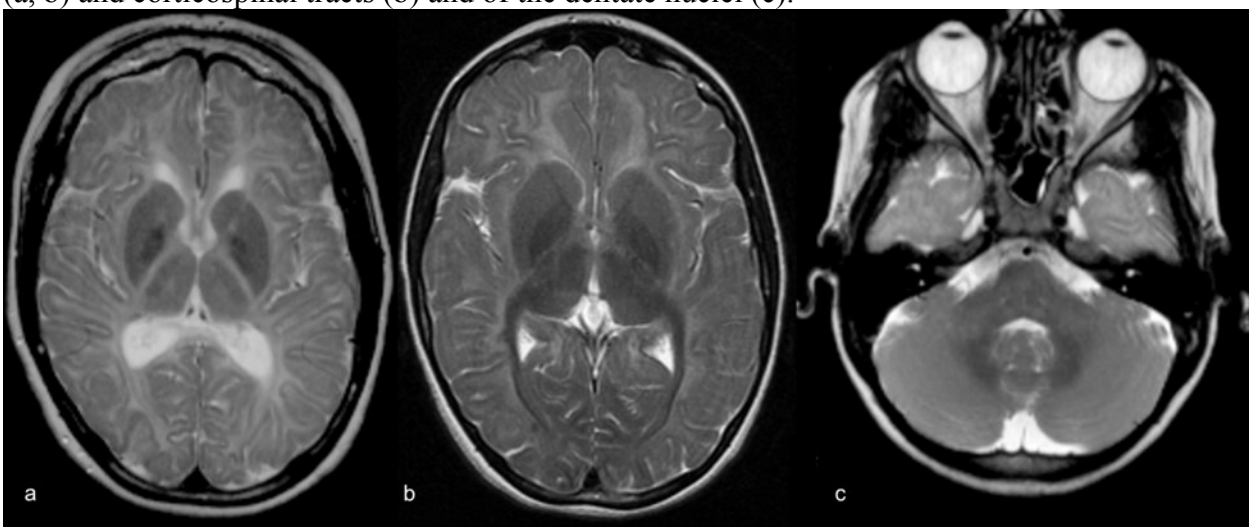
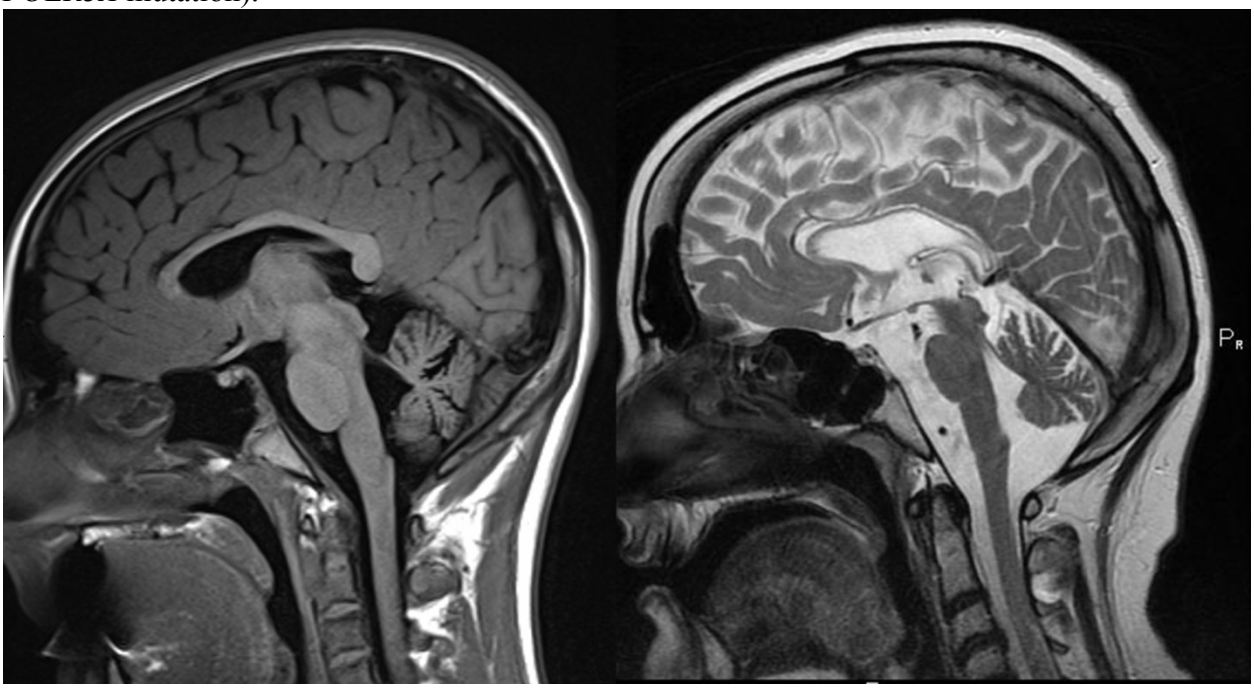


Figure 2.3: Cerebellar atrophy in Pol III-related leukodystrophy. Midline sagittal T1- (left) and T2-(right) weighted images in two patients showing variable degrees of vermian atrophy (left, 17 year-old male subject with *POLR3B* mutation; right, 8 year-old male subject with *POLR3A* mutation).



Neuroradiological findings in other hypomyelinating leukodystrophies (non Pol III-related)

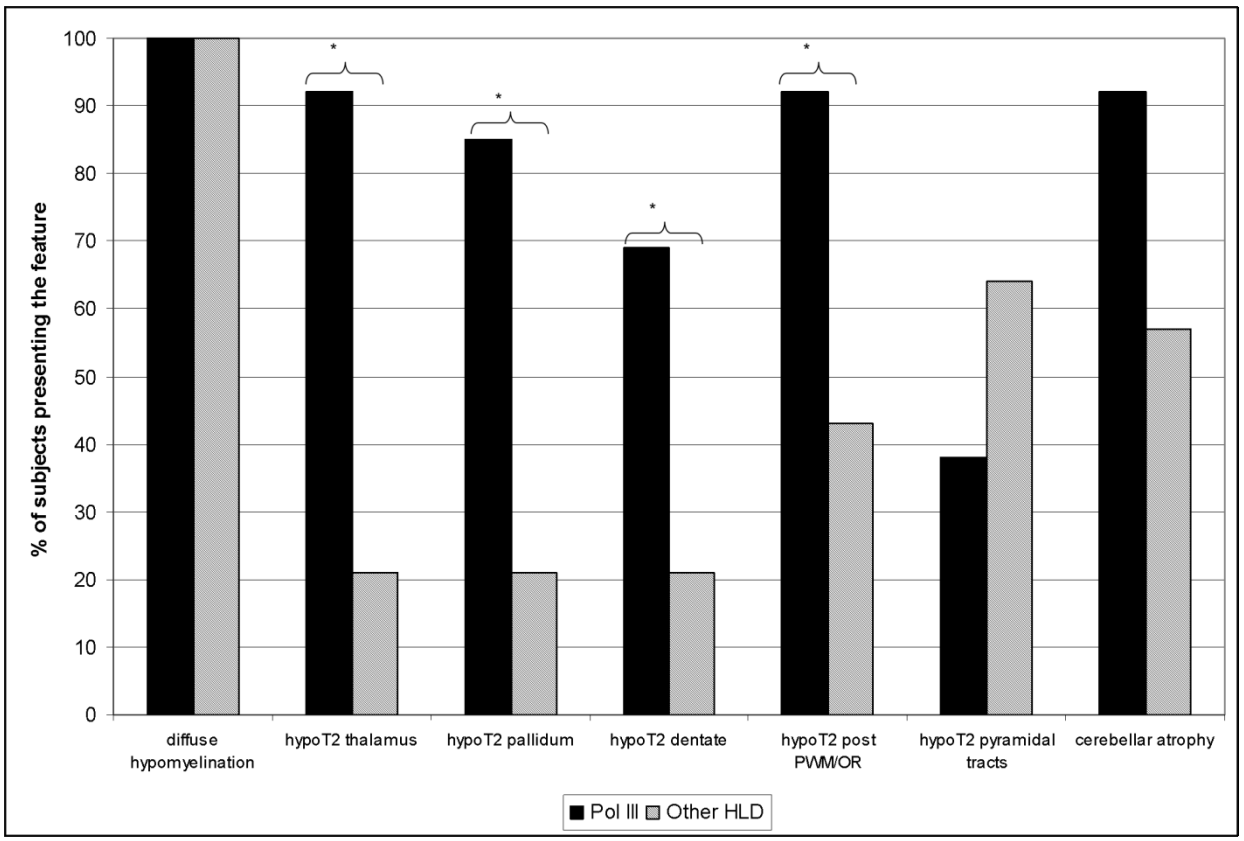
As for subjects with non-Pol III-related leukodystrophies, diffuse hypomyelination was observed in the totality of the group (14/14; 100%). T2 hypointensity in the region of the pallida and/or thalami was found in four subjects (4/14; 29%). In two patients (2/14; 14%) the low T2 signal involved both the thalami and the pallida, while in one patient (1/14; 7%) it was limited to the thalami and in another one (1/14; 7%) to the pallida. T2 hypointense signal was also observed in the pyramidal tracts at the level of the posterior limb of the internal capsule in nine patients (9/14; 64%), in the dentate nuclei in three (3/14; 21%), and in the posterior periventricular white matter in six patients (6/14; 43%). Cerebellar atrophy was present in eight subjects (8/14; 57%), including as expected the two subjects with hypomyelination with atrophy of the basal ganglia and cerebellum.

No subject from our control group presented all typical MRI features for Pol III-related leukodystrophies.

MRI pattern recognition

The direct comparison of MRI findings between patients with Pol III-related hypomyelinating leukodystrophies and the control group formed by other hypomyelinating disorders is illustrated in Figure 2.4. All the features, with the exception of cerebellar atrophy and pyramidal tract hypointense signal, were statistically significantly more likely to be seen in the Pol III-related patients than in controls with T2-weighted hypointensity in the regions of the pallida ($p=0.003$) and thalami ($p=0.002$) showing the highest significance. Pyramidal tract T2-hypointensity was more often seen in controls than in Pol III patients, though without reaching statistical significance.

Figure 2.4: Graphic representation of the different MRI features for the group of Pol III-related leukodystrophies versus the group of hypomyelinating leukodystrophies of other etiologies. The asterix (*) indicates a statically significant difference (p<0.05, Fischer's exact test) in the presence of the feature between Pol III-related leukodystrophy patients and controls. *Legend:* HLD: hypomyelinating leukodystrophies; hypoT2: hypointensity of the white matter on T2-weigthed MRI; Pol III: Pol III-related leukodystrophies; PWM: periventricular white matter; OR: optic radiations.



The combination of all criteria (1. T2-hypointensity of the globi pallidi, 2. T2-hypointensity of the anterolateral thalami, 3. T2-hypointensity of the pyramidal tracts and/or the periventricular white matter, 4. T2-hypointensity of the dentate nuclei, and 5. cerebellar atrophy) was associated by the logistic regression model to Pol III-related leukodystrophy in 88.9% of patients with a sensitivity of 84.6% and specificity of 92.9% (goodness of fit p=0.77) (Table 2.1).

The presence of each criterion, in conjunction with all the others, was more likely to be found in Pol-III related leukodystrophy (odds ratios reported in Table 2.1). However, for all the criteria

the 95% of confidence interval was very wide, and no statistical significance was reached for any individual criteria.

When logistic regression included the presence of either T2-hypointensity of the anterolateral thalami and/or pallida nuclei, all Pol III patients were correctly classified. If there was T2-hypointensity of neither the lateral thalamus nor globus pallidus, patients could therefore be reliably categorized as unlikely to have Pol III-related leukodystrophies (Table 2.2; $p<0.001$).

Table 2.1: Logistic Regression Results: Model Developed Using Team Gold Standard Assessments.

Criterion	OR	*P* value	95% CI
Hypo T2 thalamus	10.56	.144	0.44-249.86
Hypo T2 pallida	18.08	.090	0.64-511.35
Hypo T2 pyramidal tracts and/or posterior periventricular WM	11.04	.420	0.03-3801.72
Hypo T2 dentate	17.02	.095	0.61-473.78
Cerebellar atrophy	37.28	.106	0.47-2984.65

Abbreviations: CI, confidence interval; Hypo T2, hypointense signal in T2-weighted images; OR, odds ratio; WM, white matter.

Table 2.2: Presence of T2 Hypointensity of the Anterolateral Nuclei of the Thalami and/or Globi Pallidi in Pol III-Subjects Versus Controls According to the Team's Review.

Status	No	Yes	Total
Control subject	10	4	14
Pol III subject	0	13	13
Total	10	17	27

Inter-operator and Intra-operator agreements

We analyzed the agreement between the expert team review and the two neuroradiologist's reviews blind to clinical data. The agreement between the two radiologist reviews (intra-observer agreement) and between each of the radiologist reviews and the team consensus (inter-observer agreement) resulted in concordance in the majority of cases (Supplemental data Tables S2.2 and S2.3). The agreement was particularly high for the findings of T2-hypointensity of pyramidal tracts and dentate and for cerebellar atrophy. For intra-observer agreement (Table 2.1), the two different evaluations performed by the same neuroradiologist, T2 hypointensity of pyramidal tracts (κ value=0.85), T2 hypointensity of dentate nuclei (κ value=0.84), and cerebellar atrophy (κ value=0.92) resulted in perfect agreement.

Similarly, for inter-observer agreement (Supplemental data Table S2.3) each of the evaluations from the blinded neuroradiologist was compared to the consensus of the team. Although without reaching values of perfect agreement, kappa statistics were high for the same features: T2 hypointensity of pyramidal tracts (κ statistic=0.62 and 0.77); T2 hypointensity of dentate nuclei (κ statistic=0.70 and 0.70); and cerebellar atrophy (κ statistic=0.60 and 0.54).

Table S2.2: Rater agreement between the two radiologist readings (Reading 2 performed six weeks after Reading 1).

Measurement		Reading 2		% agreement	k	*P*-value
	Reading 1	No	Yes			
Hypo T2 thalamus	No	7	5	76.9%	0.52	0.002
	Yes	1	13			
Hypo T2 pyramidal tracts	No	11	2	92.3%	0.85	<0.001
	Yes	0	13			
Hypo T2 pallida	No	9	3	84.6%	0.69	<0.001
	Yes	1	13			
Hypo T2 dentate	No	9	2	92.3%	0.84	<0.001
	Yes	0	15			
Hypo T2 posterior periventricular WM	No	11	4	76.9%	0.54	0.003
	Yes	2	9			
Cerebellar atrophy	No	12	0	96.2%	0.92	<0.001
	Yes	1	13			

Table S2.3: Rater agreement between the radiologist and the team consensus.

a. Rater agreement between team and outside radiologist Reading #1:

Measurement		Reading 1		% agreement	k	*P*-value
	Team	No	Yes			
Hypo T2 thalamus	No	8	4	69.2%	0.38	0.0260
	Yes	4	10			
Hypo T2 pyramidal tracts	No	10	2	80.8%	0.62	<0.001
	Yes	3	11			
Hypo T2 pallida	No	9	4	73.1%	0.46	0.009
	Yes	3	10			
Hypo T2 dentate	No	11	4	84.6%	0.70	<0.001
	Yes	0	11			
Hypo T2 posterior periventricular WM	No	7	1	65.4%	0.35	0.020
	Yes	8	10			
Cerebellar atrophy	No	7	0	80.8%	0.60	<0.001
	Yes	5	14			

b. Rater agreement between team and outside radiologist Reading #2:

Measurement		Reading 2		% agreement	k	*P*-value
	Team	No	Yes			
Hypo T2 thalamus	No	5	7	61.5%	0.21	0.132
	Yes	3	11			
Hypo T2 pyramidal tracts	No	10	2	88.5%	0.77	<0.001
	Yes	1	13			
Hypo T2 pallida	No	9	3	73.1%	0.46	0.009
	Yes	4	10			
Hypo T2 dentate	No	11	0	84.6%	0.70	<0.001
	Yes	4	11			
Hypo T2 posterior periventricular WM	No	6	2	65.4%	0.31	0.045
	Yes	7	11			
Cerebellar atrophy	No	7	0	76.9%	0.54	0.001
	Yes	6	13			

Discussion

This is the first systematic analysis of the neuroradiological findings in a cohort of *POLR3A* and *POLR3B* mutation-proven patients. There is only one other recent qualitative study looking at the presence of cerebellar atrophy and hypomyelination in a cohort of six Japanese patients with Pol III-related leukodystrophies[20]. Our study supports the reliability of all the MRI features proposed by Steenweg et al (2010) applicable for Pol III-related leukodystrophies[3].

The association of all the criteria (i.e. T2-hypointensity of the pallida, anterolateral nuclei of the thalami, dentate nuclei and pyramidal tracts or periventricular white matter and cerebellar atrophy) was present in the large majority of Pol III-related leukodystrophies in this small sample set. No patients with non Pol III- related leukodystrophy met all of these criteria. In particular, the presence of T2-hypointensity at the level of the anterolateral portion of the thalami and/or the pallida was found to be highly useful to identify patients with Pol III-related leukodystrophies. In other words, in this cohort of mutations-proven subjects with Pol III-related compared to a cohort of other hypomyelinating leukodystrophies, the absence of T2-hypointensity of the pallida and of the anterolateral thalami makes the diagnosis of Pol III-related leukodystrophy less likely. Although in this data set no patient with Pol III related leukodystrophy had neither T2 hypointensity of the thalami nor of the globus pallidus, we cannot exclude, in view of the relatively small sample size, that this would be the case in all Pol III-related leukodystrophy patients. We suggest however, that in the presence of diffuse hypomyelination and T2 hypointensity of the anterolateral nuclei of the thalami and/or the globi pallidi the sequencing of the *POLR3A* and *POLR3B* genes should be requested. As expected based on the existing literature[5,10,13], cerebellar atrophy was found in 92% of patients with Pol III-related leukodystrophy. Consequently, the absence of cerebellar atrophy does not rule out the diagnosis

of Pol III-related leukodystrophy. The same feature was seen only in half of the subjects with other hypomyelinating leukodystrophies, among which were two subjects with hypomyelination with atrophy of the basal ganglia and cerebellum syndrome. Consequently, we suggest that in the presence of cerebellar atrophy and diffuse hypomyelination that the sequencing of the *POLR3A* and *POLR3B* genes should be considered, especially if there is no atrophy of the putamen.

The neuroradiological pattern of Pol III-related leukodystrophies is quite characteristic. However, other hereditary diseases can be included in the differential diagnosis of Pol III-related leukodystrophies based on the co-occurrence of specific features. Among hypomyelinating disorders, cerebellar atrophy can be found in hypomyelination with atrophy of the basal ganglia and cerebellum, Salla disease[3, 21, 22] and Cockayne syndrome[21, 23]. Hypomyelination can be found in association with T2-hypointensity of the globi pallidi in fucosidosis[24] and of the thalami in oculo-dento-dygital dysplasia[25].

The first limitation of this study, as is often the case in studies on rare diseases, was that the sample size was relatively small. We have used this small sample set of 27 subjects to evaluate five criteria as suggestive of the diagnosis, possibly leading to over-fitting of the model. Moreover, the mean age of two groups (Pol III versus controls) was markedly different due to the rarity of these disorders and to the milder phenotype of Pol III-related leukodystrophies. This could lead to biases in the evaluation of some MRI criteria that vary with age (i.e. T2-hypointensity of the globi pallidi). To limit this potential bias, we evaluated the presence of each criterion in relation to the age of the patient. The second limitation was that the core team of investigators was not blind to the genetic diagnosis. This was due to the fact that the study was retrospectively done and the selected patients were positive for mutation analysis of *POLR3A* and *POLR3B* genes. We are aware that this bias could lead to an overestimation of positive

findings in the case group[26]. For this reason, an experienced neuroradiologist reviewed and interpreted the images without knowledge of the final diagnosis. The neuroradiologist evaluation demonstrated good consistency with the team's review. It should be noted that although T2 hypointensity of the thalamus and globus pallidus were the best indicators of Pol-III related leukodystrophy, their intra- and inter-observer agreement where slightly less significant than that of the other criteria. The high concordance between the two reviews performed by the same neuroradiologist can be a measure of the reproducibility of our results; moreover, it suggests that the proposed criteria, when present, are evident. It is reassuring to note that the characteristic features were reliably identified by a blinded pediatric neuroradiologist with no prior experience with Pol-III related disorders. Thus, these proposed MRI criteria are likely to be useful in a clinical setting, in particular for the identification of less constant features such as the T2 hypointensity of pyramidal tracts, T2 hypointensity of dentate nuclei, and cerebellar atrophy. Finally, we are aware that our sample size is not adequate to develop and test a prediction model; nonetheless, our model shows that each of these variables might be informative for diagnosis.

In conclusion, our study confirmed that the MRI pattern recognition of patients with mutation-proven Pol III-related leukodystrophies is characterized by diffuse hypomyelination associated with cerebellar atrophy and T2-hypointensity in the pallida, anterolateral nuclei of the thalami, dentate, optic radiations and pyramidal tracts. These criteria may be helpful in the clinical setting to distinguish Pol III-related leukodystrophies among hypomyelinating disorders and, thus, to orient the clinical and molecular diagnostic work up.

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Author Contribution

Roberta La Piana and Davide Tonduti are first authors who contributed equally to this work. Adeline Vanderver and Genevieve Bernard are mentors who contributed equally to this work.

Roberta La Piana: analysis and interpretation of data; drafting the paper. Davide Tonduti: analysis and interpretation of data; drafting the paper. Heather Gordish Dressman: statistical analysis and critical revision of the manuscript. Johanna L Schmidt: acquisition of data. Jonathan Murnick: analysis of neuroradiological data; critical revision of the manuscript. Bernard Brais: critical revision of the manuscript for important intellectual content. Geneviève Bernard: conception and design; analysis and interpretation of data; critical revision of the manuscript for important intellectual content. Adeline Vanderver: conception and design; acquisition, analysis and interpretation of data; critical revision of the manuscript for important intellectual content.

Declaration of Conflicting Interests

The Authors have no conflicts of interests to declare.

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Ethical Approval

The study received internal ethical approval from the Children's National Medical Center Institutional Review Board (Ethic approval number Pro00000057) and from the Montreal Children's Hospital Research Ethics Board (Ethic approval number 11-105-PED)

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CHAPTER 3

Diffuse hypomyelination is not obligate for POLR3-related disorders[84].

Roberta La Piana[1,2,3]*, Ferdy K. Cayami[4,5]*, Luan T. Tran[3], Kether Guerrero[3], Resie M.L. van Spaendonk[5], Katrin Õunap[6,7], Sander Pajusalu[6], Holger Prokisch[8], Evangeline Wassmer[9], Dagmar Timmann[10], Hanna Mierzewska[11], Bwee T. Poll-Thé[12], Chirag Patel[13] , Helen Cox[13], Tahir Atik[14], Huseyin Onay[15], Ferda Ozkınay[16], Adeline Vanderver[17], Marjo S. van der Knaap[4,18], Nicole I. Wolf[4,18]^, Genevieve Bernard[3,19]^.

[1]Laboratory of Neurogenetics of Motion, Montreal Neurological Institute and Hospital, McGill University, Montreal

[2]Department of Neuroradiology, Montreal Neurological Institute and Hospital, McGill University, Montreal

[3]Department of Neurology and Neurosurgery, McGill University

[4]Department of Child Neurology, VU University Medical Center, Amsterdam, The Netherlands

[5]Department of Clinical Genetics, VU University Medical Center, Amsterdam, The Netherlands

[6]Department of Genetics, United Laboratories, Tartu University Hospital, Tartu, Estonia

[7]Department of Pediatrics, University of Tartu, Tartu, Estonia

[8]Institute of Human Genetics, Technical University Munich, Munich, Germany

[9]Department of Neurology, Birmingham Children's Hospital, Birmingham, United Kingdom

[10]Department of Neurology, University Clinic Essen, University of Duisburg-Essen, Essen, Germany

[11]Department of Child and Adolescent Neurology, Institute of Mother and Child, Warsaw, Poland

[12]Department of Child Neurology, AMC Academic Medical Center, Amsterdam, The Netherlands

[13]West Midlands Regional Clinical Genetics Unit, Birmingham Womens Hospital, Edgbaston, Birmingham, U.K.

[14]Department of Pediatrics, Faculty of Medicine, Ege University, Izmir/Turkey

[15]Department of Medical Genetics, Faculty of Medicine, Ege University, Izmir/Turkey

[16]Faculty of Medicine, Ege University, Izmir/Turkey

[17]Department of Neurology, Children's National Health System, Washington DC, USA

[18]Neuroscience Campus Amsterdam, Amsterdam, The Netherlands

[19]Department of Genetics, Montreal Children's Hospital, McGill University Health Center, Montreal

*Contributed equally to this work

^Dual last authors

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Abstract

Objective: To report atypical MRI patterns associated with *POLR3A* and *POLR3B* mutations.

Methods: This was a multicenter retrospective study to collect neuroradiologic, clinical, and molecular data of patients with mutations in *POLR3A* and *POLR3B* without the classic MRI phenotype, i.e., diffuse hypomyelination associated with relative T2 hypointensity of the ventrolateral thalamus, globus pallidus, optic radiation, corticospinal tract at the level of the internal capsule, and dentate nucleus, cerebellar atrophy, and thinning of the corpus callosum.

Results: Eight patients were identified: 6 carried mutations in *POLR3A* and 2 in *POLR3B*. We identified 2 novel MRI patterns: 4 participants presented a selective involvement of the corticospinal tracts, specifically at the level of the posterior limbs of the internal capsules; 4 patients presented moderate to severe cerebellar atrophy. Incomplete hypomyelination was observed in 5 participants.

Conclusion: Diffuse hypomyelination is not an obligatory feature of POLR3-related disorders. Two distinct patterns, selective involvement of the corticospinal tracts and cerebellar atrophy, are added to the MRI presentation of POLR3-related disorders.

Introduction

POLR3-related leukodystrophy is a rare autosomal recessive disease characterized by hypomyelination often accompanied by hypodontia and/or dental abnormalities and hypogonadotropic hypogonadism[1-5]. In its classical form, the association of these features is referred to as 4H syndrome[1, 2]. Mutations in the *POLR3A* and *POLR3B* genes, which encode for the two largest subunits of the RNA polymerase III (POLR3) complex, as well as in *POLR1C* also encoding a POLR3 subunit, are responsible for this disease[6-11]. With the identification of the causative genes, patients with suggestive clinical picture or MRI findings can undergo genetic testing, confirming the diagnosis[12]. The MRI pattern of POLR3-related leukodystrophy is suggestive and characterized by diffuse hypomyelination associated with relative T2 hypointensity of the ventrolateral thalamus, globus pallidus, optic radiation, corticospinal tract at the level of the internal capsule and dentate nucleus, cerebellar atrophy and thinning of the corpus callosum[12-14]. Recognition of this pattern was proven effective in detecting patients with 4H leukodystrophy caused by *POLR3A-B* or *POLR1C* mutations and is therefore used to orient the diagnostic process[12-14]. Patients without this pattern showing non-specific hypomyelination are unlikely to carry mutations in *POLR3A* or *POLR3B*[15].

Methods

We performed a multi-institutional cross-sectional observational study of the clinical, radiological and molecular data of patients that fulfilled the following inclusion criteria: presence of recessive *POLR3A* or *POLR3B* mutations and absence of typical POLR3-related MRI features[13, 14].

We identified 8 patients from 7 nonconsanguineous families, all of Caucasian ethnicity, fulfilling these criteria. Five participants underwent *POLR3A* and *POLR3B* sequencing because they presented suggestive clinical features (hypodontia or dental abnormalities, short stature, and myopia, either associated or not). In 3 participants from 2 families, *POLR3A* and *POLR3B* mutations were identified by whole-exome sequencing (WES). For all families for which DNA was available, segregation was verified. Analysis for the potential pathogenicity of novel mutations was performed, including in silico analysis. For the novel splicing mutations, we sequenced cDNA when RNA was available.

MRI images were reviewed collegially by our team. At least axial T2-weighted and sagittal T1- or T2-weighted images were available

Standard protocol approvals, registrations, and patient consents

The institutional review boards of each participating institution approved the use of clinical data for the study.

Results

Mutation findings

Six subjects carried mutations in *POLR3A* and 2 in *POLR3B* (Table 3.1). Among the 13 mutations identified, 8 were novel and they were not reported in public databases (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (http://evs.gs.washington.edu/EVS/) [June 2015]; Exome Aggregation Consortium (ExAC), Cambridge, MA (http://exac.broadinstitute.org) [June 2015]). Five mutations were intronic and affected a splice site or induced a new donor site. All new mutations were rare and predicted to be pathogenic by in silico tools[16-18] (Table S3.1) besides for the c.-35C.G change, which had

been reported in homozygous state in 2 out of over 60,000 participants (ExAC [http://exac.broadinstitute.org] [July 2015]) in patients 5 and 6. Segregation analysis in this family revealed that each of the parents carried one variant, and the healthy brother the paternal variant. WES failed to uncover other possible causal variants. Table 3.1 reports detailed information about the genetic status and mutations found in our cohort.

Table 3.1: *POLR3A* and *POLR3B* mutations identified in our cohort.

Table 1 ***POLR3A*** **and** ***POLR3B*** **mutations identified in our cohort**

	Family	Gene	Status	gDNA	Chromosomal location	Protein	Region	Previously reported	MAF	Method	Clinical features	MRI features
1	I	*POLR3A*	C Het	c.1048+1G>A	chr10:79781617C>T	—	Intron 7	No	NA	Gene testing	A, HD, SSt	PLIC
				c.1289+3A>C	chr10:79778917T>G	—	Intron 9	No	NA			
2	II	*POLR3A*	Hom	c.2710 G>A	chr10:79753032C>T	G904R	Exon 20	No	8.24E-06	Gene testing	A, HD, DD, Sp	PLIC
3	III	*POLR3A*	C Het	c.1771-6C>G	chr10:79769439C>G	—	Intron 13	rs115020338	3.30E-05	Gene testing	A, HD, DD, Sp	PLIC
				c.3205C>T	chr10:79744965G>A	R1069W	Exon 24	No	—			
4	IV	*POLR3B*	C Het	c.2084-6A>G	chr12:106848274A>G	—	Intron 19	No	0.0001769	Gene testing	A, M, DD	CA
				c.2302C>T	chr12:106850924C>T	R768C	Exon 22	rs371453512	2.48E-05			
5	V[a]	*POLR3A*	C Het	c.2381A>C	chr10:79760831T>G	Q794P	Exon 18	No	0.0001237	WES	A, M, DD, Sp	CA
				c.-35C>G	chr10:79789200 G>C	—	Exon 1 (5′UTR)	rs201700756	0.00128			
6	V[a]	*POLR3A*	C Het	c.2381A>C	chr10:79760831T>G	Q794P	Exon 18	No	0.0001237	WES	A, M, DD, Sp	CA
				c.-35C > G	chr10:79789200 G>C	—	Exon 1 (5′UTR)	rs201700756	0.00128			
7	VI	*POLR3B*	C Het	c.1244T>C	chr12:106821117T>C	M415T	Exon 13	rs199504211	0.0006182	WES	A, M, DD, SSt	CA
				c.2774C>T	chr12:106889893C>T	P925L	Exon 24	No	8.24E-06			
8	VII	*POLR3A*	C Het	c.1909 + 22G>A	chr10:79769273C>T	—	Intron 14	rs191875469	0.001326	Gene testing	A, HD	PLIC
				c.2549A>G	chr10:79759806T>C	H850R	Exon 19	No	NA			

Abbreviations: A = ataxia or other cerebellar signs; C Het = compound heterozygous; CA = cerebellar atrophy; DD = developmental delay; HD = hypodontia or dental abnormalities; Hom = homozygous; M = myopia; MAF = minor allelefrequency; NA = not available; PLIC = abnormal signal of the posterior limb of the internal capsule; Sp = spasticity; SSt = short stature; WES = whole-exome sequencing.
[a] Siblings.

Table S3.1. *Legend:* * indicates parents were available for testing to confirm compound heterozygosity. PP2, PolyPhen-2; MT, MutationTaster; D, probably damaging/ disease causing/ deleterious; P, possibly damaging; PM, polymorphism; SS, splice site changes; NA, new acceptor site; ND, new donor site. Mutation c.2084-6A>G in *POLR3B* (family IV) has been found in several unrelated patients.

Family	Gene	DNA change	Protein change	PP2	SIFT	MT	cDNA
*I**	*POLR3A*	c.1048+1G>A c.1289+3A>C	- -	unknown unknown	n/a n/a	D (ND) PM (ND)	Confirmation by cDNA sequencing
II	*POLR3A*	c.2710G>A	p.G904R	P	D	D	-
*III**	*POLR3A*	c.1771-6C>G c.3205C>T	- p.R1069W	unknown D	n/a D	D (NA) D	- -
IV	*POLR3B*	c.2084-6A>G c.2302C>T	- p.R768C	D D	D D	D (NA) D	- -
*V**	*POLR3A*	c.2381A>C c.-35C>G	p.Q794P -	D unknown	D n/a	D PM (SS)	- -
*VI**	*POLR3B*	c.1244T>C c.2774C>T	p.M415T p.P925L	B D	D D	D D	- -
VII	*POLR3A*	c.1909+22G>A c.2549A>G	- p.H850R	unknown D	n/a n/a	PM (SS, NA) D	- -

Clinical findings

The age of onset ranged from 6 weeks to 10 years (mean age of onset 52.3 months). The symptoms at disease onset were gait ataxia, dysarthria and tremor in 3 participants (cases 1, 4 and 8). Two participants (cases 2 and 3) presented with spasticity and diplegic gait. The patient with the earliest disease onset, at 1.5 months, presented with failure to thrive (case 7). Clinical examination revealed in all the subjects the presence of ataxia of variable severity; cerebellar tremor was documented in 4 participants (cases 1, 2, 3, and 4); pyramidal signs and spasticity were documented in 4 participants (cases 2, 3, 5, and 6). One patient had severe dystonic tremor (case 3) (Table 3.1).

Extraneurological features were found in 6 patients. Specifically, 4 participants had hypodontia, delayed dentition or other dental abnormalities (cases 1, 2, 3, and 8) suggestive of POLR3-related leukodystrophies. Two participants presented short stature (cases 1 and 7) and 4 had myopia (cases 4, 5, 6, and 7), both frequent findings in patients with mutations in *POLR3A* or *POLR3B*.

MRI findings

Mean age at the last MRI was 15 years (range 4-35 years). We identified 2 distinct MRI patterns (Figures 3.1, 3.2, S3.1, and S3.2). Four participants, all with *POLR3A* mutations, presented a selective involvement of the corticospinal tracts, which was particularly evident at the level of the posterior limb of the internal capsule as T2-hyperintense signal (cases 1, 2, 3, and 8) (Figure 3.1 and S3.1). In the other 4 patients (2 with *POLR3A* and 2 with *POLR3B* mutations), moderate to severe cerebellar atrophy was variably associated with nonspecific T2-hyperintense white matter abnormalities, as specified below, or thinning of the corpus callosum (Figure 3.2 and S3.2) (cases 4, 5, 6, and 7). We documented focal, partially confluent, T2-hyperintense white matter abnormalities located in the deep frontal and parietal white matter, suggesting partial hypomyelination, in 5 participants (cases 2, 3, 4, 5, and 6), while the remaining 3 presented adequate myelination for age.

Figure 3.1: Involvement of the corticospinal tracts. Axial (A, C, D) and coronal (B) T2-weighted images in patients 1 (A, B), 1 (C), and 8 (D) with *POLR3A* mutations show the presence of bilateral and symmetric T2-hyperintense signal at the level of the posterior limb of the internal capsules. Incomplete hypomyelination is seen in (B).

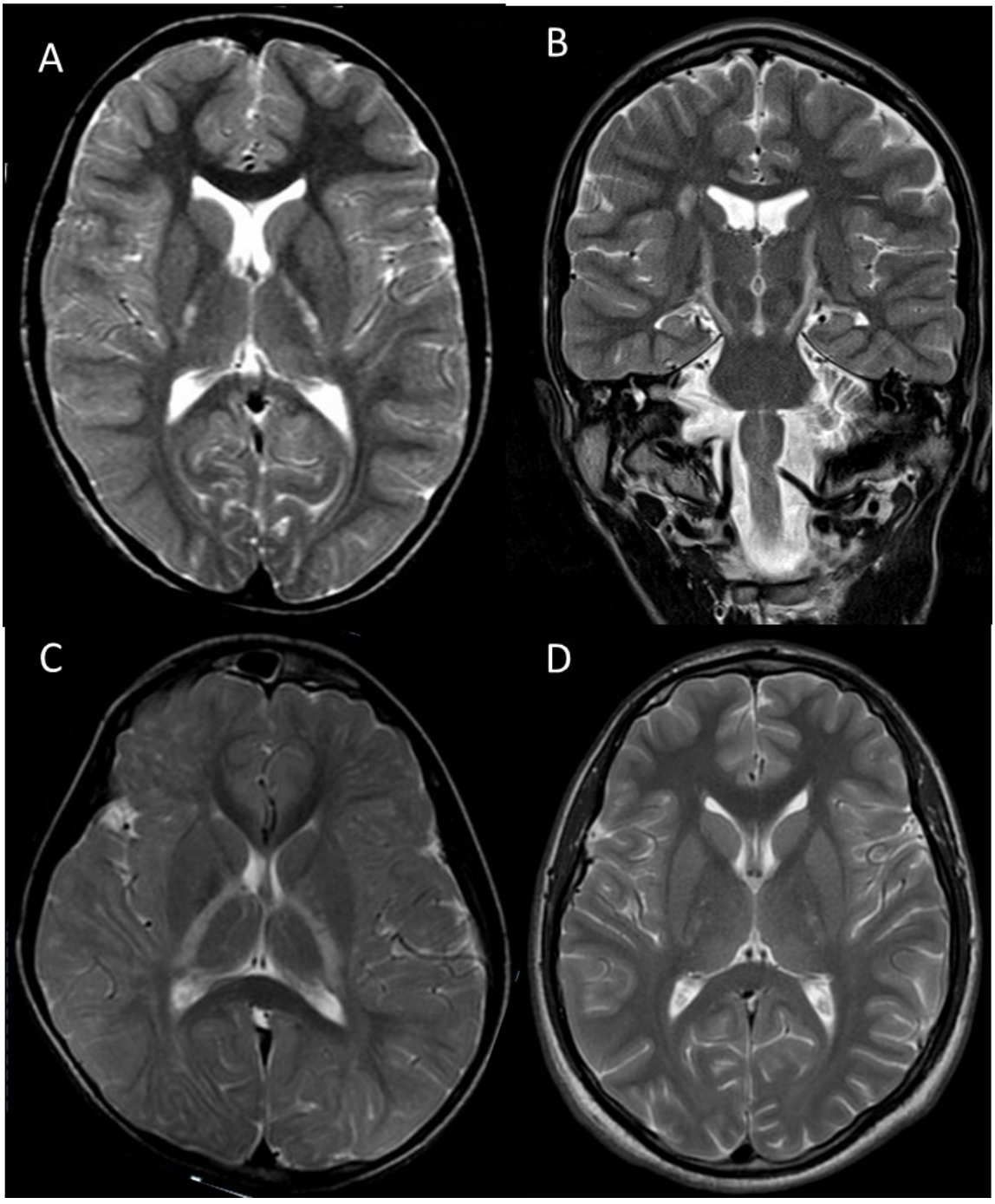


Figure 3.2: Cerebellar atrophy. (A, B) Sagittal T1-weighted images from participant 4 with *POLR3B* (A) and participant 5 with *POLR3A* (B) mutations show the presence of severe (A) and moderate cerebellar atrophy. (C, D) Axial T2-weighted and T2–fluid-attenuated inversion recovery images of the patients presented respectively in (A, B) show the presence of partial hypomyelination.

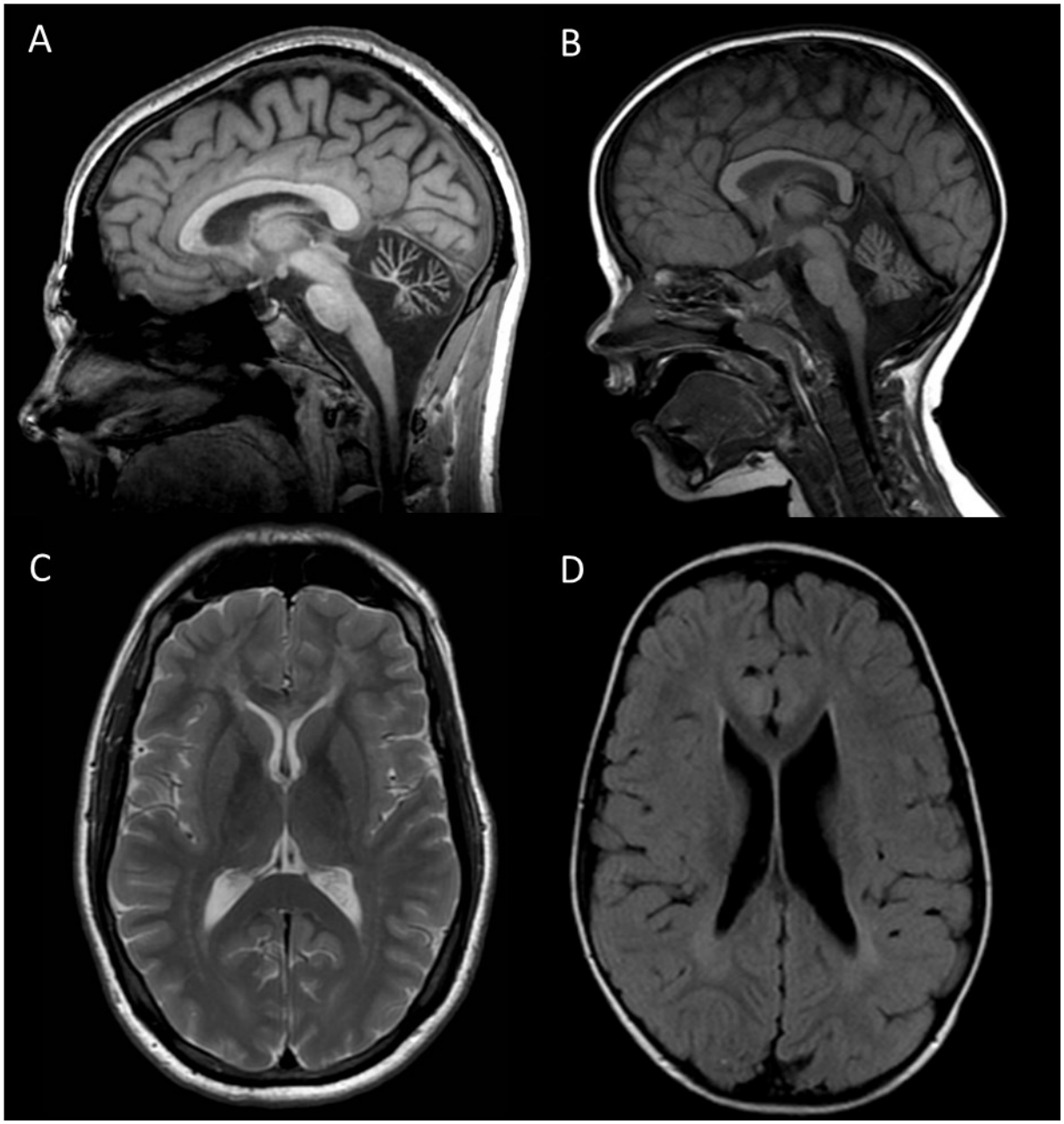


Figure S3.1: MRIs (sagittal T1-weighted (A, D, G) and T2-weighted (K) and axial T2-weighted (B, C, E, F, H, I, L, M) images) of patients 1, 2, 3 and 8 with pattern 1 (B, E, H, L; involvement of the posterior limb of the internal capsule). The anterior li limb is spared (B, E, H, L). Patient 3 shows focal, partially confluent T2-hyperintense signal of the central and periventricular white matter (H, I), sparing the U-fibers and subcortical fibres. This feature is not present in the other three patients (B, C, E, F, L, M). There is no obvious cerebellar atrophy (A, D, G).

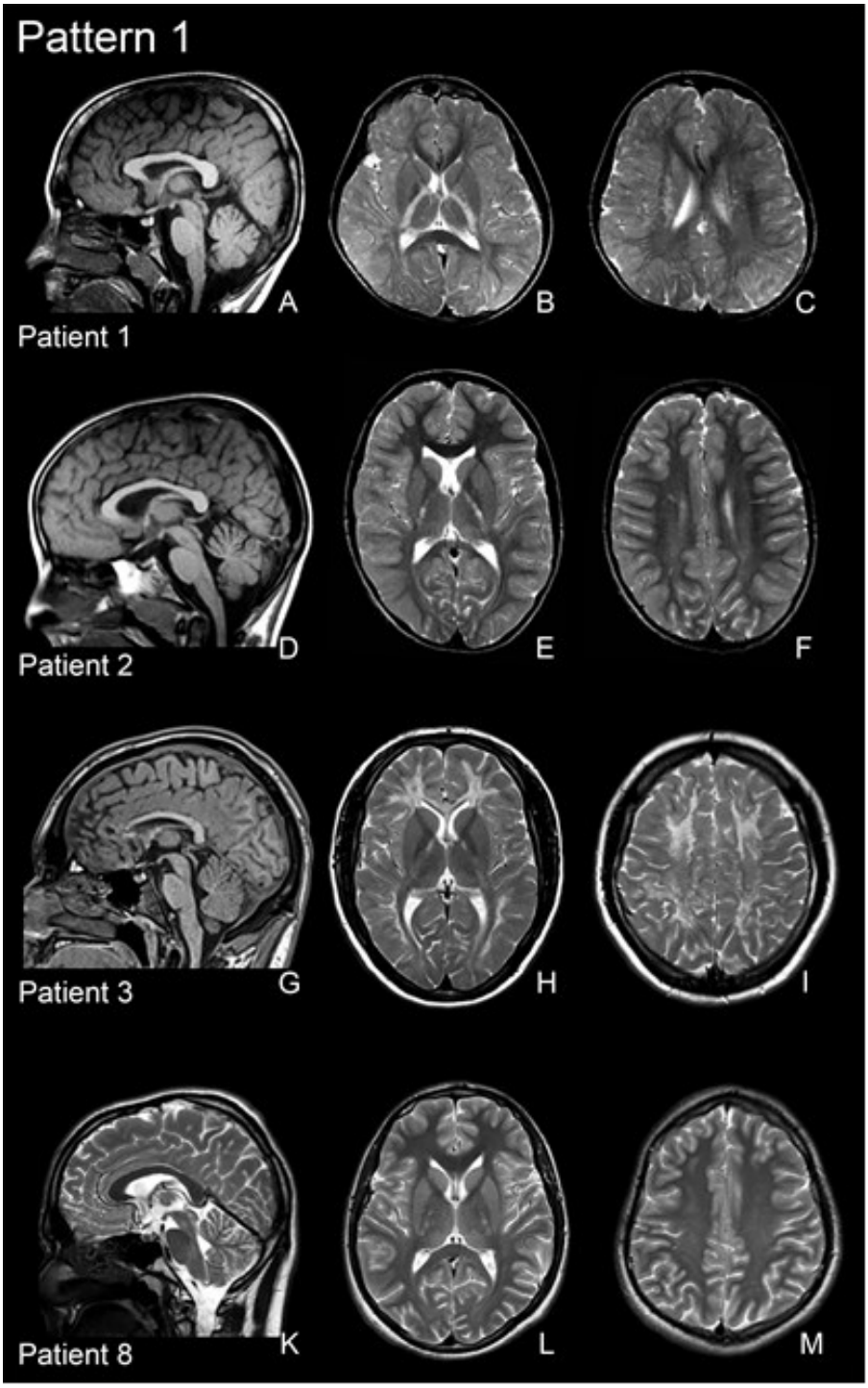
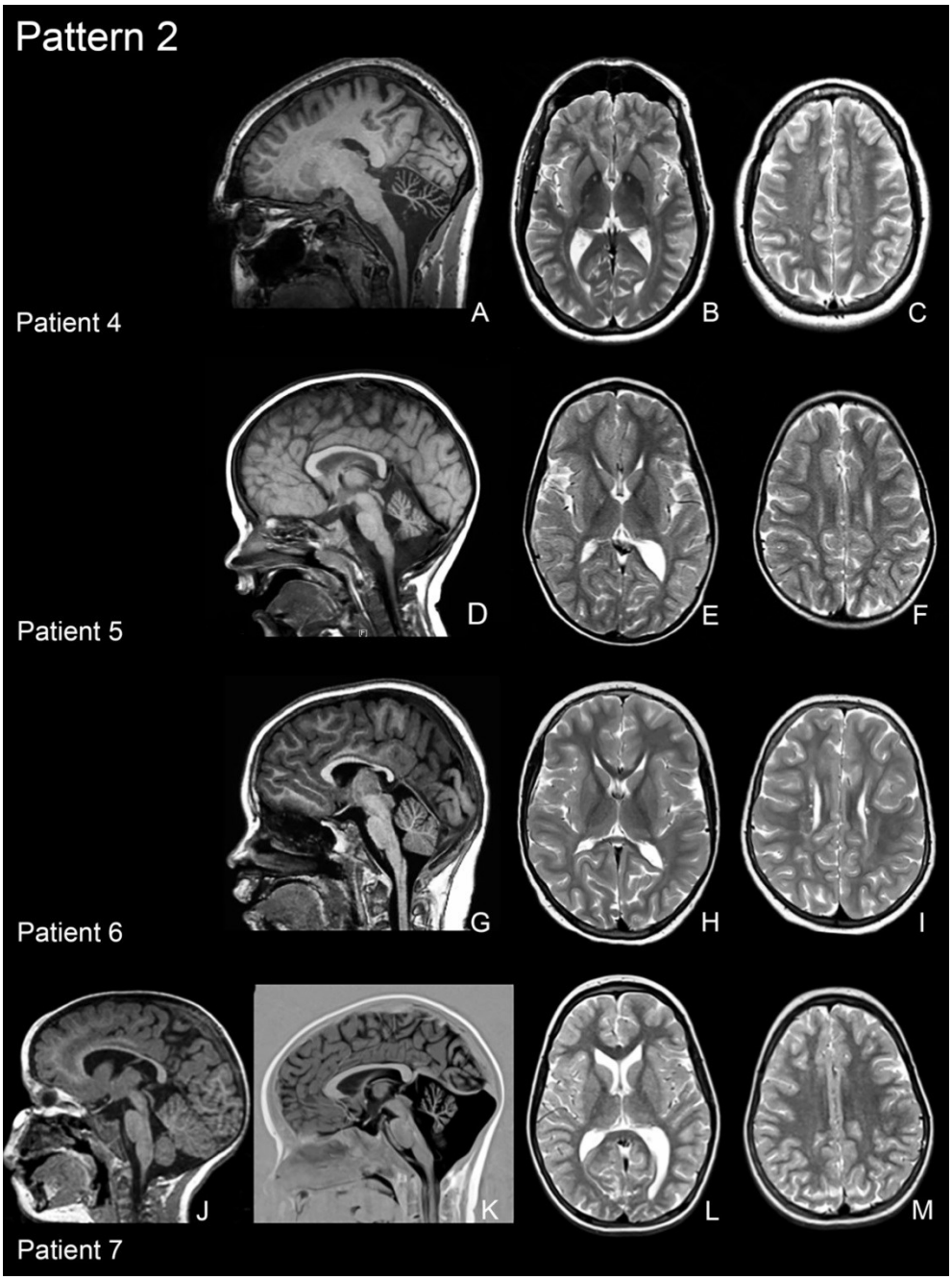


Figure S3.2: MRIs (sagittal T1-weighted (A, D, G, J, K) and T2-weighted (K) and axial T2-weighted (B, C, E, F, H, I, L, M) images) of patients 4 to 7 with pattern 2 (cerebellar atrophy). Cerebellar atrophy is severe in patients 4 and 7 (A, K), in patient 7 not being present at first imaging at age 2 years (J), but clearly visible at age 5 years (K). Patient 5 shows mild cerebellar atrophy at age 13 years, his younger sib not yet at age 6 years. Patient 4 shows focal, partially confluent T2-hyperintense signal of the central and periventricular white matter mainly of the frontal white matter (B, C), sparing the U-fibers and subcortical fibres. This feature is only very mild in patient 6 (H, I) and absent in the other two (E, F, L, M).



Discussion

Our work broadens the MRI spectrum of POLR3-related leukodystrophy by describing 2 new MRI patterns in this disease that has been known as a hypomyelinating disorder. Our results indicate that hypomyelination is not an obligatory feature. We also documented the presence of 6 *POLR3A* and 2 *POLR3B* mutations not reported before in public databases. Interestingly, 5 of the 13 mutations in our cohort were noncoding, 4 predicted to affect splicing. Pathogenicity of the variant in the 5' untranslated region in patients 5 and 6 could not be unambiguously resolved as it had been reported in homozygous state in 2 participants in a large database. However, this variant is situated in a terminal oligopyrimidine tract (TOP). The change of a pyrimidine (C) for a purine (G) shortens the TOP, and it has been shown that deletions or substitutions in this region result in unregulated translation[19, 20]. Its effect might be mild, and homozygous carriers indeed might be unaffected, but in combination with a pathogenic mutation this variant could lead to disease. Segregation analysis and the absence of other possible causal variants in WES in this family indeed support a causal role for this variant, as does the identification of other families with isolated cerebellar atrophy.

A specific involvement of the corticospinal tracts, particularly evident at the level of the internal capsule as T2-hyperintense signal, was the most striking finding in a subgroup of patients. Interestingly, in typical cases, more commonly with *POLR3B* mutations[12], the corticospinal tracts are usually one of the better myelinated structures.

The second pattern is the presence of cerebellar atrophy in the absence of diffuse hypomyelination. Cerebellar atrophy was previously known to be associated with *POLR3A* or *POLR3B* mutations in more than 80% of the participants, always in combination with diffuse hypomyelination[12-14, 21].

Focal, partly confluent T2-hyperintense white matter changes were present in some participants of both groups and located in the deep frontal and parietal white matter. The signal intensity of the abnormal areas corresponds to the one seen in hypomyelination, focal hypomyelination therefore being the most likely interpretation. These changes are obviously reminiscent of the classical MRI of 4H leukodystrophy, but sufficiently different to make a straightforward diagnosis challenging. Our results confirm white matter involvement when *POLR3A* or *POLR3B* is mutated; however, different pathogenic processes could be responsible for the variable and expanded MRI phenotypes of brain abnormalities associated with *POLR3A* or *POLR3B* mutations. Further insight into the role of POLR3 in myelin formation and maintenance as well as in axonal integrity is needed to explain the heterogeneity of the radiologic patterns.

The diagnosis of POLR3-related leukodystrophy relies both on MRI findings and clinical signs. In our cohort, the presence of typical extraneurological features oriented the clinicians towards the testing of *POLR3A* and *POLR3B* genes in 5 participants. Therefore, our study confirms the importance of the classical clinical criteria – particularly hypodontia and hypogonadotropic hypogonadism - in the diagnostic process of POLR3-related disorders, especially when cardinal MRI features are lacking. Whole exome sequencing allowed the discovery of *POLR3A* and *POLR3B* mutations in the remaining cases, thus highlighting the role of next generation sequencing in expanding the phenotypes of already known disorders.

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SECTION II

A new cohort of subjects with adult genetic white matter disorders

PREFACE

Introduction

Between 2013 and 2018, we recruited subjects with suspected inherited white matter disorders among the patients seen in different specialised clinics and in the Department of Neuroradiology of the Montreal Neurological Institute. I included subjects with either one or several of the following criteria:

-slowly progressive clinical onset;

-brain MRI findings suggestive for a genetic disorder defined as a) diffuse and symmetric involvement, or b) multifocal without evidence or history of risk factors for acquired disorders;

-positive family history for white matter disease.

Subjects were recruited from the following three main sources: the Multiple Sclerosis clinics, the Neurogenetics clinics, and the Department of Neuroradiology. A crucial role in the identification process of potential subjects was played by the White Matter Rounds. Since their launch in 2013, I am the coordinator and host of these special monthly rounds that are recognized multidisciplinary academic sessions attended by neurologists, neuroradiologists and basic scientists interested in white matter diseases. These are held at the Montreal Neurological Institute to discuss cases that have unusual white matter disorder or do not fit the classic MS criteria and could merit genetic investigations.

I identified a total of 68 subjects (38 females, 30 males; mean age at first contact 47.5 years, s.d. 17.1) with white matter disease of suspected genetic origin. For 23 of them, MS was initially suspected and therefore they were assessed in the MS clinics. The other 45 subjects have been

considered as having an inherited condition since the beginning of their investigations, based on family, clinical and/or MRI findings.

Nine of the 68 subjects were excluded because they either already carried a final genetic diagnosis (3) or they refused to undergo further investigations (3) or were lost to follow up (3). Hence, the final sample of participants with hypothesized genetic white matter disorders included 59 subjects.

Methods

For all participants, we systematically gathered data about the clinical and family history, previous MR images acquired in the clinical setting, and we performed a standardized physical examination.

MRI Study

We designed a 3T MRI study tailored to be sensitive to white matter abnormalities. The protocol includes standard anatomical scans (2D PDw/T2w (2mm isotropic); 3D T2 FLAIR (1mm isotropic)), sequences with increased specificity to myelin (3D MP2RAGE T1w (1mm isotropic); quantitative magnetization transfer (MT) imaging (3D SPGR PDw MTon/MToff) (1mm isotropic)), as well as multi-shell DWI (2mm isotropic), and MR Spectroscopy. MRI studies were performed at the McGill McConnell Brain Imaging Center.

All MRI data either acquired previously in the context of clinical investigations or generated for our research study were analysed systematically according to the qualitative analysis described in Table II.1.

The MRI analysis was formalized to answer two questions: a) do the MRI abnormalities constitute a specific pattern? and b) which information can be deducted about the underlying

pathophysiology (e.g. inflammation, demyelination, hypomyelination and/or neurodegeneration)?

An original feature of our study is that the 3T MRI exam was proposed to affected subjects and relevant family members. The fact that adult genetic leukoencephalopathies develop and progress slowly over time and might be clinically mild, suggests that the same genetic condition can be present in other family members although apparently silent clinically.

Table II.1: The systematic approach used for the qualitative analysis of the MRI images.

Qualitative parameters	
Symmetry	**yes / no**
Pattern of involvement	isolated / multifocal / diffuse
Presumptive nature of pathological process	**demyelination / hypomyelination / cystic degeneration / vascular**
Associated features	cortical involvement / basal ganglia involvement / vascular anomalies / calcification / (micro)hemorrhage / heavy metals deposition
Regional analysis	
Location	**supratentorial / infratentorial / both**
Involved fibers	U-fibers / associative fibers / capsules / long tracts / corpus callosum
Lobar involvement	**frontal / parietal / temporal / occipital**

Genetic Analysis

Blood samples were collected from affected subjects and informative relatives for DNA extraction according to standard methods. NGS gene panels and WES were used to identify

causal genes, based on the hypothesis that shared MRI and clinical patterns are caused by mutations in the same gene or genes of the same pathway[6].

The analysis was oriented to search for rare, pathogenic variants. First, we searched for variants in genes known to cause genetic leukoencephalopathies (Table II.2). We then applied the filtering strategy illustrated in Table II.3. Considering the mode of inheritance, we looked for coding homozygous or heterozygous variants with a minor allele frequency (MAF) of less than 5% in 1000 genomes, Exome Variant Server (EVS) and ExAC. We evaluated the pathogenicity of the variants by bioinformatics prediction programs (PolyPhen-2, MutationTaster, CADD and SIFT)[85-88]. In addition, we prioritized variants present at a highly conserved position. Segregation analysis in the families was performed by Sanger sequencing.

Table II.2: List of genetic leukoencephalopathies with associated causal gene (not exhaustive).

Disease	Modality of transmission	Gene
Demyelinating leukoencephalopathies		
Adult-onset autosomal dominant leukodystrophy (ADLD)	AD	*LMNB1*
Adrenoleukodystrophy	XL	*ABCD1*
Alexander Disease	AD de novo	*GFAP*
Aicardi-Goutières Syndrome (AGS)	AR or AD	*TREX1*
	AR	*RNASEH2B*
	AR	*RNASEH2C*
	AR	*RNASEH2A*
	AR	*SAMHD1*
	AR	*ADAR1*
	AD	*IFIH1*
RNASET2 -deficient leukoencephalopathy	AR	*RNASET2*
Adult polyglucosan body disease	AR	*GBE1*
Canavan disease	AR	*ASPA*
Cerebrotendinous xanthomatosis	AR	*CYP27A1*
Cystical leukoencephalopathy without megalencephaly	AR	*RNASET2*
Krabbe (Globoid cell leukodystrophy)	AR	*GALC*
Krabbe due to saposin A deficiency	AR	*PSAP*
Hereditary diffuse leukoencephalopathy with spheroids (HDLS)	AD	*CSFR1*
L-2-hydroxyglutaric aciduria	AR	*L2HGDH*
Leukoencephalopathy with brain stem and spinal cord involvement and high lactate (LBSL)	AR	*DARS2*
Leukoencephalopathy with thalamus and brainstem involvement and high lactate (LTBL)	AR	*EARS2*
Megalencephalic Leukoencephalopathy with subcortical Cysts (MLC) type 1	AR	*MLC1*
MLC2A	AR	*HEPACAM*
Metachromatic leukodystrophy	AR	*ARSA*
Metachromatic-like (normal ARSA enzymatic activity) due to saposin B deficiency	AR	*PSAP*
Austin variant of Metachromatic leukodystrophy caused by multiple sulfatase	AR	*SUMF1*
PLOLS	AR	*TREM TYRB*
Vanishing white matter disease	AR	*EIF2B1*
	AR	*EIF2B2*
	AR	*EIF2B3*
	AR	*EIF2B4*
	AR	*EIF2B5*
Complex I deficiency - leukoencephalopathy	AD	*NUBPL*
	AR	*NDUFS1*
	AR	*SURF1*
	AR	*COX10*
	AR	*COX6B1*
	AR	*NDUFV1*
Complex II deficiency - leukoencephalopathy	AR	*SDHA*
	AR	*SDHB*

Table II.2 (continues)

Hypomyelinating leukoencephalopathies		
Pelizaeus-Merzbacher	XL	*PLP1*
Pelizaeus-Merzbacher-like	AR	*GJC2 (GJA12)*
Hypomyelination and congenital cataract (HCC)	AR	*FAM126A*
Hypomyelination with atrophy of the basal ganglia (HABC)	AD de novo	*TUBB4A*
Pol III-related leukodystrophies (4H)	AR	*POLR3A*
	AR	*POLR3B*
	AR	*POLR1C*
18q deletion	Sporadic	*18q*
Sialic acid storage disorders (including Salla disease)	AR	*SLC17A5*
Oculodentodigital dysplasia (ODDD)	AD	*GJA1*
Fucosidosis	AR	*FUCA1*
Hypomyelination with brainstem and spinal cord involvement	AR	*DARS*
Hypomyelinating leukodystrophy-15	AR	*EPRS*
Cockayne Syndrome	AR	*ERCC6*
	AR	*ERCC8*
Peripheral neuropathy, central hypomyelination, Waardenburg, Hirschsprung syndrome (PCWH)	AD	*SOX10*
Vascular leukoencephalopathies		
CADASIL	AD	*NOTCH3*
CARASIL	AR	*HTRA1*
CARASAL	AD	*CTSA*
COL4A1 -related disease	AD	*COL4A1*
COL4A2 -related disease	AD	*COL4A2*
Vascular leukoencephalopathy by Herve et al. 2012	AD	*UK*
Fabry disease	XL	*GLA*
RVCL	AD	*TREX1*

Table II.3: Filtering strategy used to identify potential pathogenic variants.

Minor allel frequency EVS, 1000genomes, ExAC	<5%
Variant features	Non-synonymous Splice-site indel
Pathogenicity (in-silico) CADD score PolyPhen2 SIFT Mutation Taster	score >10 possibly or probably pathogenic deleterious disease causing
Conservation phyloP / phastCons (UCSC)	conserved

Results

Demographics

Of the 59 subjects (34 females, 25 males; mean age at first contact 45.5, s.d. 14.9), 43 (72.9%) were of French Canadian descent; 2 (3.4%) were of mixed French Canadian/other origin; 14 (23.7%) were of other ethnicities (3 Italian, 2 African, 2 Caribbean, 2 Middle Eastern, 1 Portuguese, 1 Berber, 1 Polish, 1 Greek and 1 South American).

The majority of the subjects (42; 71.1%) were from the Montreal area, while the remaining 17 (28.9%) from other regions of Quebec.

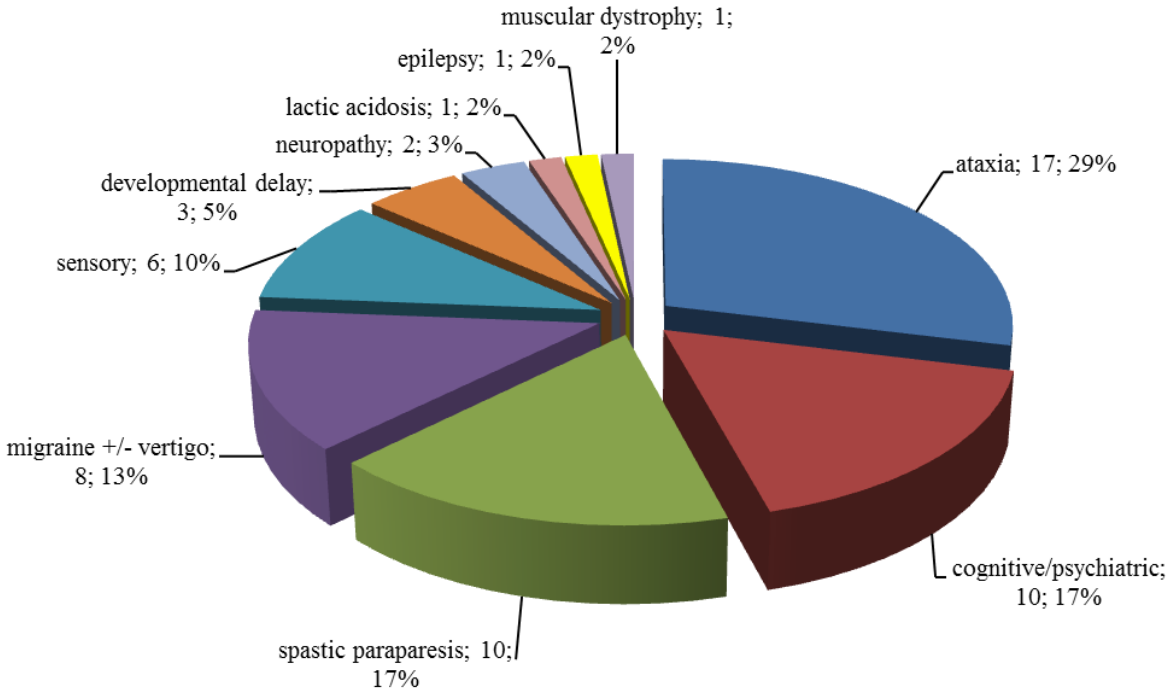
Clinical findings

The onset of symptoms was in adulthood for the majority of subjects (40/59; 67.8%). A juvenile onset was reported in 7 subjects (11.9%), while nonspecific symptoms were first noticed during childhood in the remaining 12 subjects (20.3%).

The majority of participants (37; 62.7%) presented a slowly progressive course of disease; clinical exacerbations in the context of an otherwise slow progression were noted in 9 subjects (15.3%). An acute onset with relatively rapid deterioration was reported in 13 subjects (22%).

Figure II.1 illustrates the main clinical features of the entire sample.

Figure II.1: The features dominating the clinical picture presented by subjects with adult leukoencephalopathy (total 59).



MRI patterns of white matter involvement

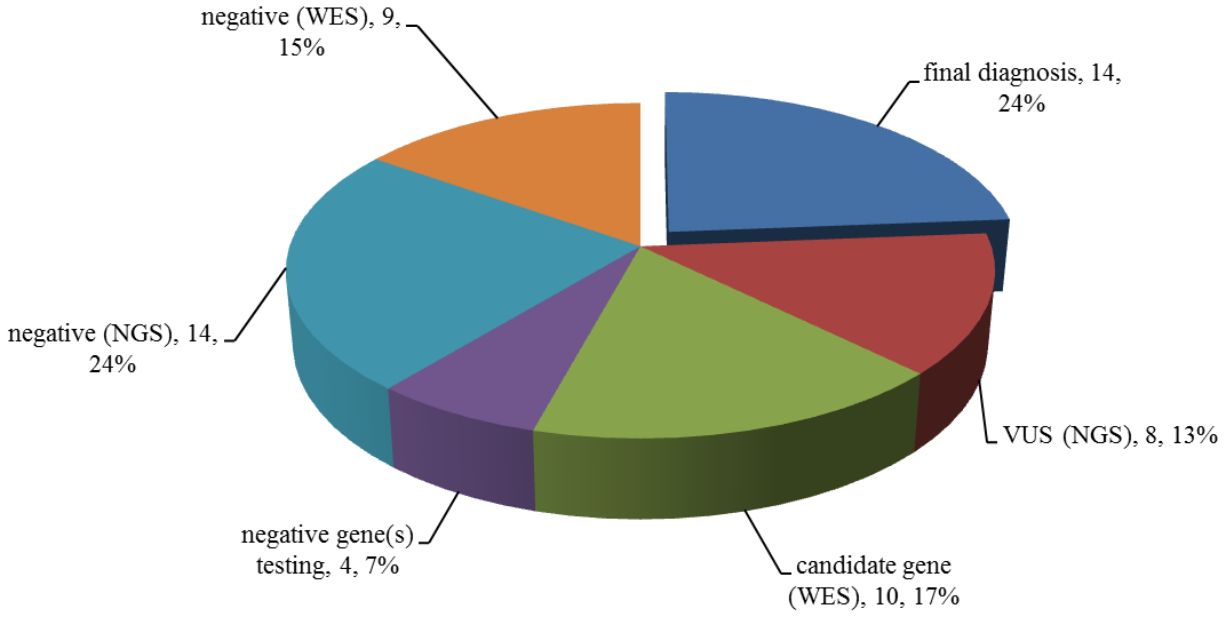
After systematic qualitative review of the MR images (Table II.1), I classified the patterns of white matter involvement into three main categories, according to Ayrignac et al.[9] Eleven subjects (18.6%) presented MR features consistent with a leukoencephalopathy of vascular origin; three subjects (5.1%) had cavitary white matter abnormalities; the majority of subjects (45/59; 76.3%) were classified as "other", which included all white matter changes that were neither vascular in origin or cavitary. In the latter, very heterogeneous group, we could identify different patterns of white matter involvement, according to the classic algorithm described by

Schiffmann and van der Knaap[6]. However, no clusters of subjects with shared MRI and clinical findings could be detected.

Next generation sequencing data

Next generation sequencing data were generated for 52 subjects: 28 underwent NGS gene panels for the known genetic white matter disorders, in some cases associated with gene panels for hereditary ataxias and spastic paraparesis. Twenty-four subjects underwent WES. In the remaining seven subjects, we performed only single gene testing, due to highly suggestive MRI and/or clinical features. The results of the genetic analyses are reported in Figure II.2.

Figure II.2: The results of the genetic analyses performed in our sample (total 59). *Legend*: WES, whole exome sequencing; NGS, next generation sequencing gene panel; VUS, variant of unknown significance.



Final diagnosis

We identified causal variants in 14/59 subjects (23.7%); the yield of diagnosis of targeted NGS panel/WES was 21.1% (11/52). All variants (16 in total) were located in genes already known to be associated with inherited white matter disorders. Eleven of the 16 variants were never reported before in public databases. A final diagnosis was reached in 36.4% of the 11 subjects (4/11) with a pattern of leukoencephalopathy of probable vascular origin, 100% of the subjects (3/3) with cavitary leukoencephalopathy, and 15.5% of subjects (7/45) with other patterns of involvement.

Table II.4 summarizes the details about the rate of final diagnosis and mutated gene in each of the three main patterns of leukoencephalopathy.

Table II.4: rate of final diagnosis and mutated gene in each of the three main patterns of leukoencephalopathy.

	vascular (tot 11)	**cavitary (tot 3)**	**other (tot 45)**
final diagnosis n (%)	4 (36.4)	3 (100)	7 (15.5)
mutated gene (n)	*NOTCH3* (2) *HTRA1* (1) *COL4A1* (1)	*MTFMT* (1) *EIF2B3* (1) *TUFM* (1)	*GALC* (1) *LAMA2* (1) *FA2H* (1) *FOXC1* (1) *PEX16* (1) *ITPR1* (1) *GJB1* (1)

Each chapter of this section is representative of the most relevant results of my research on this new cohort of subjects with genetic leukoencephalopathy. Specifically:

- Chapter 4 describes the expanding clinical phenotype of a known and relatively common leukoencephalopathy.
- Chapter 5 documents the functional characterization and expanding clinical and MRI phenotype associated to a rare mitochondrial disorder disclosed by WES.
- Chapter 6 demonstrates the usefulness of 3T MRI family studies in unsolved adult leukoencephalopathies.

PREFACE TO CHAPTER 4

The broadening of clinical phenotypes is a phenomenon that has been observed more frequently since the advent of NGS. In our cohort, the combined use of NGS – either with NGS gene panels or WES – and MRI interpretation allowed to reach the final diagnosis in subjects with clinical features not yet associated with the known disease.

As an example, in the paper by Shao et al., we reported a family in which members across two generations presented with predominant cerebellar ataxia. WES documented the presence of pathogenic variants in the *GALC* gene, therefore suggesting the diagnosis of Krabbe disease which was confirmed by compatible MRI findings[78].

In this Chapter, I describe how the recognition of disease-specific MRI findings directed the genetic testing and allowed the broadening of the phenotype associated with vanishing white matter disease to include long-standing psychiatric presentation.

CHAPTER 4

Long-standing psychiatric features as the only clinical presentation in Vanishing White Matter disease[89]

Andrea Accogli[1,2,3], Bernard Brais[4,5], Donatella Tampieri[6], Roberta La Piana[4,6].

[1]Department of Medical Genetics, McGill University Health Centre, Montreal, QC, Canada

[2]DINOGMI - Università di Genova, Italy

[3]IRCCS Ospedale Policlinico San Martino, Genova

[4]Laboratory of Neurogenetics of Motion, Montreal Neurological Institute, McGill University, Montreal, QC H3A 2B4, Canada

[5]Department of Human Genetics, McGill University, Montreal, QC H3A 1B1, Canada

[6]Department of Neuroradiology, Montreal Neurological Hospital and Institute, McGill University, Montreal, Quebec, Canada

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Vanishing white matter disease (VWMD; MIM#603896) is an autosomal recessive leukoencephalopathy caused by mutations in any of the 5 genes (*EIF2B1, EIF2B2, EIF2B3, EIF2B4* and *EIF2B5*) encoding the subunits of eukaryotic translation initiation factor 2B (eIF2B) that is essential for protein synthesis[1].

VWMD clinically ranges from antenatal or early infantile onset with poor outcome[2] to adult onset with slow progression, where the age of onset is a strong predictor for disease course[3]. Affected individuals typically have normal early development, followed by neurological deterioration triggered by stress-provoked episodes of rapid decline. Adolescent- and adult-onset patients are more likely to exhibit cognitive problems at the time or shortly after the disease onset. However, psychiatric disorders have been rarely reported as the only symptoms at the time of diagnosis,[3-7] and usually are followed by neurologic manifestations within a few years[6].

In women, VWMD can present as "ovarioleukodystrophy", a condition in which leukodystrophy is associated with primary or secondary ovarian failure[2,3]. While this could help reach a diagnosis in females, the diagnosis in adult males may be challenging, given the possible atypical onset and insidious clinical course.

VWMD is a recognizable disorder on brain MRI, usually characterized by diffuse and symmetric white matter T2-hyperintensities and progressive white matter rarefaction. The latter is a hallmark of the disease both in pediatric and adult-onset cases[7].

EIF2B5 is by far the most commonly mutated gene among adults with VWMD, with the recurrent c.338G>A(p.Arg113His) mutation accounting for the majority of adult cases[6], while c.260C>T(p.Ala87Val) is a founder *EIF2B3* VWMD mutation in the French Canadian population of Quebec[8].

Here, we report an adult male who presented with only psychiatric symptoms for more than 20 years, before the occurrence of the typical neurological signs and deterioration of VWMD.

CASE REPORT

The patient came to our attention at the age of 41 years for the suspicious of a genetic leukoencephalopathy. He was the second child to French Canadian consanguineous (first cousins) parents, originally from Matapedia, a small region in Quebec. Family history was remarkable for psychiatric illness on the maternal side: two of his mother's brothers died of suicide in the context of drug addiction and the grand-mother had schizophrenia. There were no neurological disorders segregating in the family. In terms of development, he had normal psychomotor and language acquisitions. During late childhood and adolescence, he started to present severe behavioral problems, which retrospectively fit the criteria for disruptive behavior disorder: he used to lose temper rather quickly, he was often involved in several violent fights with his peers, he was defiant towards authority both at home and school. In addition, he sexually abused a family member. The patient experienced the first episode of depression at the age of 16 when he attempted suicide by driving into the family house, causing considerable damage. This led to his removal from the family home and his placement in a residence. During this period, he did not receive a clear psychiatric diagnosis. The behavior disorder led to a significant impairment in his social interactions and did not allow him to complete his high school. He eventually worked as a truck driver until the age of 40 when he was finally diagnosed with VWMD.

Since the age of 16, he presented transient urinary incontinence that continued for many years without any other associated neurological signs. He experienced a second episode of depression at the age of 34 when he started to notice some difficulty concentrating and short-memory

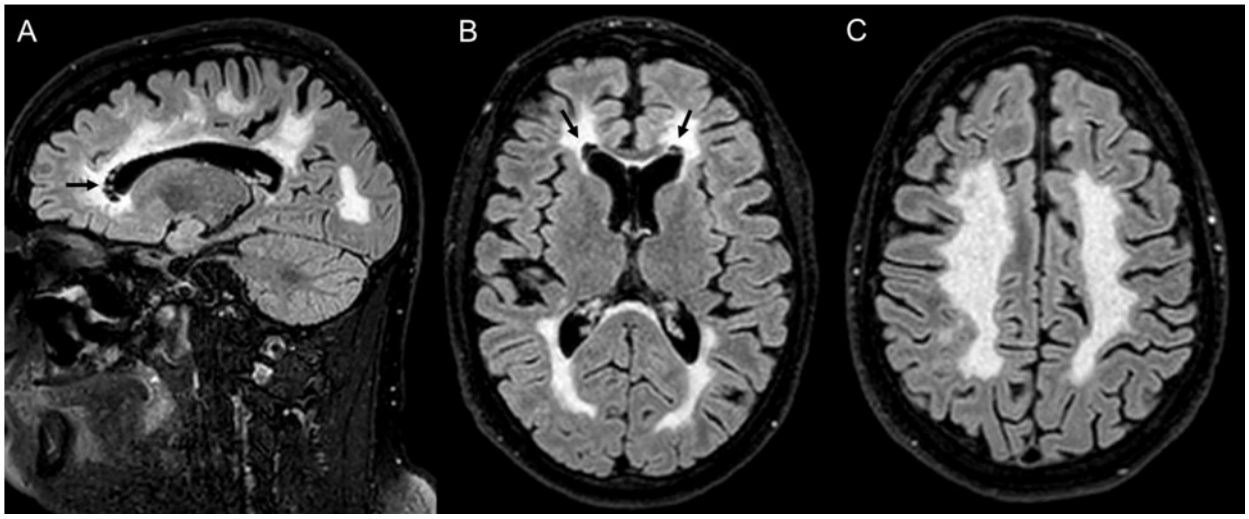
impairment. For the depressive symptoms, he was put on venlafaxine without significant benefit. Four years later he was admitted to the hospital for suicide attempt in the context of a further episode of major depression. At that time the family noticed for the first time mild gait instability while the patient complained of occasional dizziness and lack of energy.

Shortly after, at the age of 40, he was involved in a truck accident during which he lost consciousness and required a hospital admission. Since the accident, the patient has experienced cognitive decline, progressive gait deterioration with recurrent falls, increasing fine motor impairment and daily dizziness.

Neuroradiological findings

Following the car accident, a head CT scan revealed the presence of extensive, symmetric white matter disease. The brain MRI (Figure 4.1) confirmed the presence of bilateral, symmetric and diffuse white matter abnormalities. Small areas of focal white matter rarefaction were noted bilaterally at the level of the frontal horns of the lateral ventricles. The U fibers, the external rim of the corpus callosum, the internal capsule and the cerebellar white matter were relatively spared.

Figure 4.1: Brain MRI of our patient with VWMD. Sagittal (A) and axial (B,C) FLAIR T2-weighted images show the diffuse, symmetric white matter abnormalities involving the cerebral hemispheres and sparing the U fibers (C), the outer rim of the corpus callosum and the capsules (B). Areas of focal white matter rarefaction are visible around the frontal horns of the lateral ventricles (black arrows in A and B).



Physical examination

At the time of the clinical examination, the patient was still on monotherapy with venlafaxine. He was oriented and collaborative. He needed the help of his parents to recollect his personal and clinical history. He showed detachment towards the major events of his life (i.e. suicide attempts); and his mood appeared depressed. Neurological evaluation revealed mild increased tone, brisk reflexes in his lower limbs, difficulties with rapid alternating movements and tandem-gait. His gait was mildly ataxic. Given the constellation of the neuroradiological and clinical findings, VWMD was suspected.

Genetic analysis

A gene panel for leukodystrophies including the *EIF2B1-5* genes revealed the homozygous mutation c.260C>T(p.Ala87Val) in *EIF2B3* (NM_020365.3).

DISCUSSION

The long-standing psychiatric phenotype of our patient, in absence of any neurological signs apart from sphincter dysfunction, represents a unique disease course never reported before among eIF2B related disorders. Psychiatric features and cognitive decline have been described as presenting symptoms in up to 24% and 11-16% of adult onset VWMD, but other neurological signs usually occur relatively shortly after within a period of few years[2,3,6,9]. From the history, his gait and coordination were normal to the extent of working as a truck driver.

Isolated psychiatric symptoms can dominate the presentation of leukodystrophies in patients with onset in adolescence or adulthood[10-13]. For this reason, we suggest that physicians take into account the possibility of genetic leukoencephalopathies in patients with important, long-standing psychiatric symptoms especially if associated with non-specific neurological signs (e.g. sphincter dysfunction). Interestingly, our patient presented with urinary incontinence since the age of 16, whereas in VWMD it usually occurs at more advanced disease stages, when other neurological signs are already evident[3]. The predominant psychiatric presentation of our patient leads us to assume that VWMD may be undiagnosed at the early stages of disease and its frequency may be underestimated among adult patients, especially when neurological signs are absent or non-specific. Stress events, such as the car accident in our patient, dramatically influenced the course of the disease, leading to a progressive motor decline.

The identification of a leukoencephalopathy was incidental in our patient, since it was only in the context of the car accident that our patient underwent a CT scan. A test that started his neuroradiological assessment that led to the final diagnostic. The usefulness of brain MRI pattern recognition in genetic leukoencephalopathies is well known[14]. The neuroradiological phenotype of our patient perfectly overlaps with previous adult VWMD subjects, showing a diffuse and

symmetric white matter involvement with focal white matter rarefaction[15]. As reported in a recent study on adult genetic leukoencephalopathies, the presence of white matter rarefaction should promptly suggest the diagnosis of VWMD[7]. The neuroradiological findings in our patient are in line with the previous reports, confirming that anterior periventricular white matter rarefaction is a main distinctive neuroradiological feature among the adult VWMD group.

VWMD belongs to the growing group of leukodystrophies due to mutations in genes that tightly regulate mRNA translation16, resulting in irreversible white matter abnormalities through a yet poorly understood molecular mechanism. EIF2B regulates protein synthesis rates under basal and cellular stress conditions, facilitating ternary complex formation and translation initiation[1]. Failure of translation process of certain astrocytic mRNAs has been thought to be the underlying mechanism that induces degeneration of the white matter through loss of astrocyte function, failure of astrocyte-microglia crosstalk and secondary effect on both oligodendroglia and axons[17]. Several studies have recently shed light on the pathomechanism of eIF2B, showing deregulation of endoplasmic reticulum function in astrocytes of mutant mice[18] and prolonged state of translational hyperrepression in VWMD patients' cells that fail to recover from stress[19]. Although the pathomechanism underlying VWMD is becoming increasingly recognized, the reason of the broad phenotypic spectrum among eIF2B-related disorders, remain largely unknown.

Since the first report more than a decade ago, c.260C>T(p.Ala87Val) has been rarely documented[20-23]. Of note, it has been previously reported in 2 paediatric cases[8] and an adult female[15] in the Quebec population, showing variable disease course according to different age of disease onset. Our report confirm that c.260C>T(p.Ala87Val) in *EIF2B3* is a common mutation in the French Canadian population.

In conclusion, we have reported an isolated long-standing psychiatric presentation in an adult carrying an *EIF2B3* mutation, broadening the clinical spectrum associated to eIF2B-related disorders. Importantly, we underline the need of considering a thorough neurological and neuroradiological assessment in individuals presenting with psychiatric features, especially when accompanied by mild non-specific neurological symptoms, as the finding of focal white matter rarefaction on brain MRI would straight point to a VWMD diagnosis.

Recognizing this disorder at an early stage of disease is crucial in order to avoid stress-provoked episodes of rapid decline and may be critical for future therapeutic strategies.

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DISCLOSURE OF COMMERCIAL INTERESTS

The authors report that they have no competing interests.

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PREFACE TO CHAPTER 5

The identification of genotype-phenotype correlations is often a challenge in rare neurological diseases. The contribution of WES to the expansion of clinical phenotypes has opened the way to further explore the impact that different variants can have in the final phenotype presented by patients with the same disease.

In Chapter 5 we documented a milder clinical phenotype and unique MRI findings in a subject with MTFMT-related disorder diagnosed by WES. The presence of a novel mutation in such a patient prompted us to investigate further whether the variant was responsible for the observed milder and unique phenotype. Our functional studies demonstrated that the novel mutation did not affect the protein synthesis or respiratory chain complexes assembly differently than what previously reported mutations did. Therefore, besides the expansion of clinical and MRI phenotypes, our paper confirmed the complexity of genotype-phenotype correlations in MTFTM-related disorders.

CHAPTER 5

Identification and functional characterization of a novel *MTFMT* mutation associated with selective vulnerability of the visual pathway and a mild neurological phenotype[90]

Roberta La Piana[1,2], Woranontee Weraarpachai[3,4], Luis H. Ospina[5], Martine Tetreault[6], Jacek Majewski[6], Care4Rare Canada Consortium[7], G. Bruce Pike[8], Jean-Claude Decarie[9], Donatella Tampieri[2], Bernard Brais[1], Eric A. Shoubridge[3].

[1] Laboratory of Neurogenetics of Motion, Montreal Neurological Institute and Hospital, McGill University, Montreal

[2]Department of Neuroradiology, Montreal Neurological Institute and Hospital, McGill University, Montreal

[3]Department of Human Genetics, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada

[4]Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

[5]Department of Ophthalmology, Centre Hospitalier Universitaire Sainte-Justine, Université de Montréal, Montreal, Quebec, Canada

[6]Department of Human Genetics, McGill University, Genome Québec Innovation center, Montreal, Quebec, Canada

[7]Care4Rare Canada Consortium, Children's Hospital of Eastern Ontario Research Institute, University of Ottawa, Ottawa, Canada

[8]Departments of Radiology and Clinical Neurosciences, Hotchkiss Brain Institute, University of Calgary, Alberta, Canada

[9]Department of Radiology, Radiotherapy and Nuclear Medicine, Université de Montréal, Montréal, Québec, Canada

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ABSTRACT

Mitochondrial protein synthesis is initiated by formylated tRNA-methionine, which requires the activity of MTFMT, a methionyl-tRNA formyltransferase. Mutations in *MTFMT* have been associated with Leigh syndrome, early-onset mitochondrial leukoencephalopathy, microcephaly, ataxia, and cardiomyopathy. We identified compound heterozygous *MTFMT* mutations in a patient with a mild neurological phenotype and late-onset progressive visual impairment. MRI studies documented a progressive and selective involvement of the retrochiasmatic visual pathway. MTFMT was undetectable by immunoblot analysis of patient fibroblasts, resulting in specific defects in mitochondrial protein synthesis and assembly of the oxidative phosphorylation complexes. This report expands the clinical and MRI phenotypes associated with *MTFMT* mutations, illustrating the complexity of genotype-phenotype relationships in mitochondrial translation disorders.

INTRODUCTION

Mitochondria maintain an independent translation system to synthesize the 13 essential structural subunits of the oxidative phosphorylation (OXPHOS) system encoded on the mitochondrial genome (mtDNA). Defective mitochondrial translation, first documented in patients with mutations in the translation elongation factor GFM1[1], has been reported in an increasing number of neurologic conditions[2-7]. The mitochondrial protein synthesis machinery resembles that found in prokaryotes, and in both systems translation is initiated by formylmethionine. Methionyl-tRNA formyltransferase (*MTFMT*) encodes the mitochondrial MTFMT that is responsible for formylating Met-tRNA^{Met8}. Unlike the system in prokaryotes, there is only a single Met-tRNA in

mitochondria, so the ratio of fMet-tRNA^{Met} (for initiation) to Met-tRNA^{Met} (for translation elongation) must be tightly regulated to ensure efficient mitochondrial translation.

Mutations in *MTFMT* were first identified by Mito exome sequencing (nuclear-encoded mitochondrial genes plus mtDNA) in two children with Leigh syndrome and a combined OXPHOS deficiency[9]. Subsequent studies reported several new *MTFMT* mutations and expanded the clinical phenotypes to include early-onset, severe encephalopathy with white matter and basal ganglia involvement, microcephaly, ataxia, and cardiomyopathy[10-13], as well as recently, a relapsing-remitting form of demyelinating disorder[14].

Patients with *MTFMT* mutations presented either combined OXPHOS defects or isolated complex I deficiency[9-13] resulting from impaired mitochondrial translation. Strikingly, MTFMT was virtually undetectable in fibroblasts from all patients investigated, independent of the severity of the clinical phenotype[11-13]. The initial study on fibroblasts from two patients with Leigh syndrome reported a global translation defect[9]; however, two other studies[13,15] demonstrated that the protein synthesis defect is more selective, affecting most significantly three complex I subunits (ND5, ND4, ND1) and one complex IV subunit (COX II), consistent with the pattern of assembly defects in complexes I and IV observed by blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis. The defects in OXPHOS assembly appear to be disproportionately severe relative to nature and extent of the translation defects, suggesting that translation can be initiated with unformylated methionine, but that the N-terminal formyl-methionine residue is somehow important for the subsequent assembly into a functional OXPHOS complex[13].

Whole exome sequencing (WES) has revolutionized the diagnostic workup of patients with unknown rare genetic conditions, uncovering novel causal genes even when very few or single

cases from non-consanguineous families are available for analysis, and has contributed substantially to the broadening of clinical and radiological phenotypes of known diseases. The current report significantly expands the clinical and radiological phenotype associated with *MTFMT* mutations.

METHODS

Patient

We investigated the first child of healthy, non-consanguineous parents of French Canadian ancestry, with a mitochondrial disorder of undetermined origin. The Institutional Review Board of our Institution approved the research study and the subject reported here gave her consent to participate in the study.

Mutation detection and functional analysis

Variants identified by WES performed on genomic DNA from the patient were annotated and filtered as previously described[13]. Functional analyses were performed as in Hintalla et al.[13].

RESULTS

Case report

The patient was born at term after an uneventful pregnancy. The psychomotor development was always considered mildly delayed for her age. Her weight and height growth were at the third centile of the growth charts. At the age of seven years she presented with right fourth cranial nerve palsy. A brain MRI performed at that time revealed the presence of multiple areas of abnormal signal in the cerebral white matter, corpus callosum, basal ganglia, and brainstem, some of which with cystic degeneration. The involvement of the striatal nuclei raised the

possibility of a mitochondrial disease and the analysis of the respiratory chain performed on muscle biopsy tissue confirmed an assembly defect in complexes I and IV. Molecular testing for common MELAS and MERRF mutations was negative. The patient was regularly followed up by neuro-ophthalmology and the fourth cranial nerve palsy improved spontaneously over time. A follow-up MRI performed at the age of 17 showed no major changes since the previous exam (Figure 5.1A-C). At the age of 18 years, bilateral vision loss developed over a period of two months, prompting the need for help in daily life activities involving vision. The ophthalmological evaluation performed at that time revealed vision of 20/200 in each eye, almost normal color vision (8/10 HRR plates, each eye), normally reactive pupils, and normal looking optic nerves and retinas. The electroretinogram was normal and flash and pattern visual evoked potentials were decreased. Subsequent examinations showed unchanged appearance of the optic nerves for six months until mild bilateral temporal optic atrophy appeared (Figure S5.1A). At that moment vision had decreased to 20/250 OD and 20/400 OS, and color vision to 0/10 in both eyes. The follow up brain MRI showed selective bilateral involvement of the entire visual pathway, particularly severe at the level of the optic radiations (Figure 5.1D-F), and the presence of signal changes in the central portion of the brainstem. These specific findings were not present in the exam performed one year before (Figure 5.1A-C).

At the last neurological and ophthalmological examination, performed at the age of 24 years, the patient was oriented, cooperative and has mild cognitive deficit. She is autonomous for the majority of the daily life activities, requiring only minimal supervision for the visual impairment; she has a part-time employment for individuals with disabilities. The motor exam documented paratonia, hyperreflexia to the upper and lower limbs, and Babinski sign bilaterally. Her vision was 20/800 OD and 20/500 OS, and both optic nerves became severely atrophic (Supplementary

Figure S5.1B). On confrontational visual field, she also presented a left homonymous visual field deficit, in keeping with the mildly asymmetric postchiasmatic white matter changes.

Her medical history is negative for metabolic crises, diabetes, epilepsy and deafness. She never had cognitive or motor regression.

The last MRI exam was performed on a 3T Siemens TRIO machine with a specific protocol for white matter disorders (Figure 5.1G-I). The supratentorial white matter and corpus callosum abnormalities were unchanged since previous. The specific involvement of the optic pathway was again documented and appeared stable. The head of the putamina nuclei became atrophic. The MR spectroscopy did not show any lactate peak.

Figure 5.1: Brain MRI of the patient performed in 2009 (A-C), 2010 (D-F), and 2014 (G-I). A-C Axial T2-weighted images showing signal changes in the striatal nuclei, with cavitations in the putamina (B-C), involvement of the splenium of the corpus callosum (C), and multifocal white matter abnormalities in the frontal regions. D, E Axial T2- and f axial T1-weighted images documenting the symmetric involvement of the retrochiasmatic visual pathway, specifically of the optic radiations. Focal mesencephalic abnormalities are also seen (D). G, H Coronal and I axial FLAIR T2-weighted images showing the selective damage of the optic radiations (arrows) (G,H), unchanged since previous, and the progressive atrophy of the striatal nuclei (I).

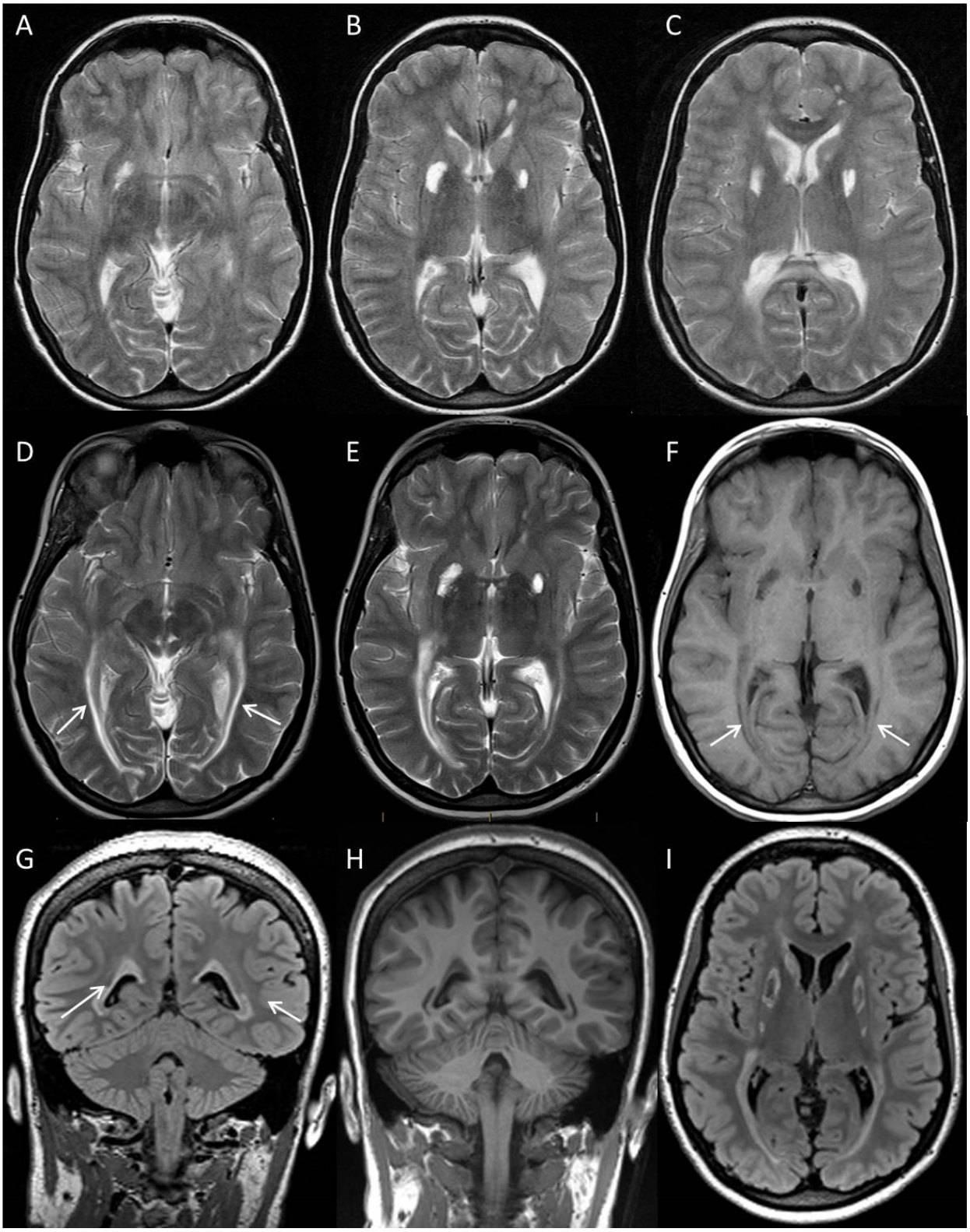
A
B
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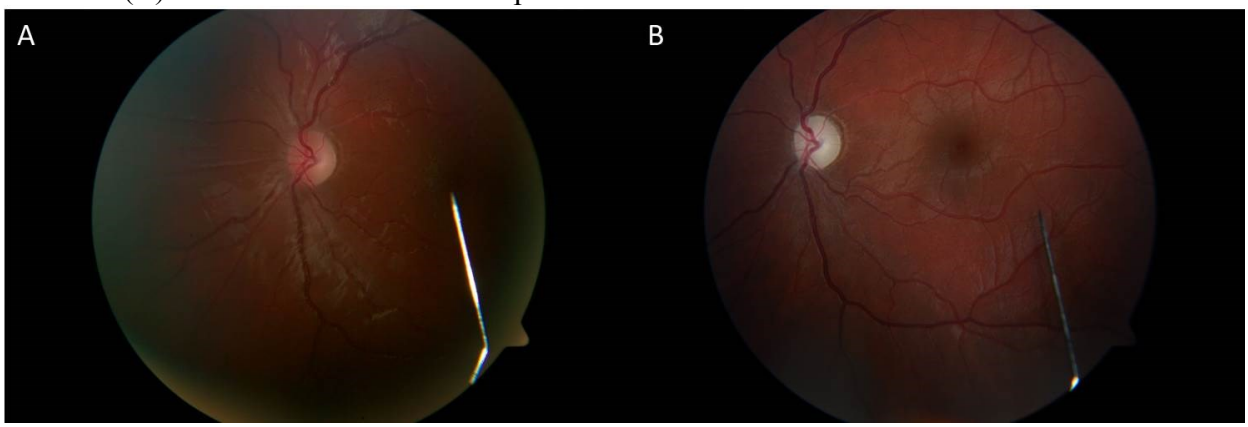
Figure S5.1: Fundus photographs showing the progressive atrophy of the left optic nerve (A) in 2010 and (B) at the last clinical follow up in 2014.



Genetic results

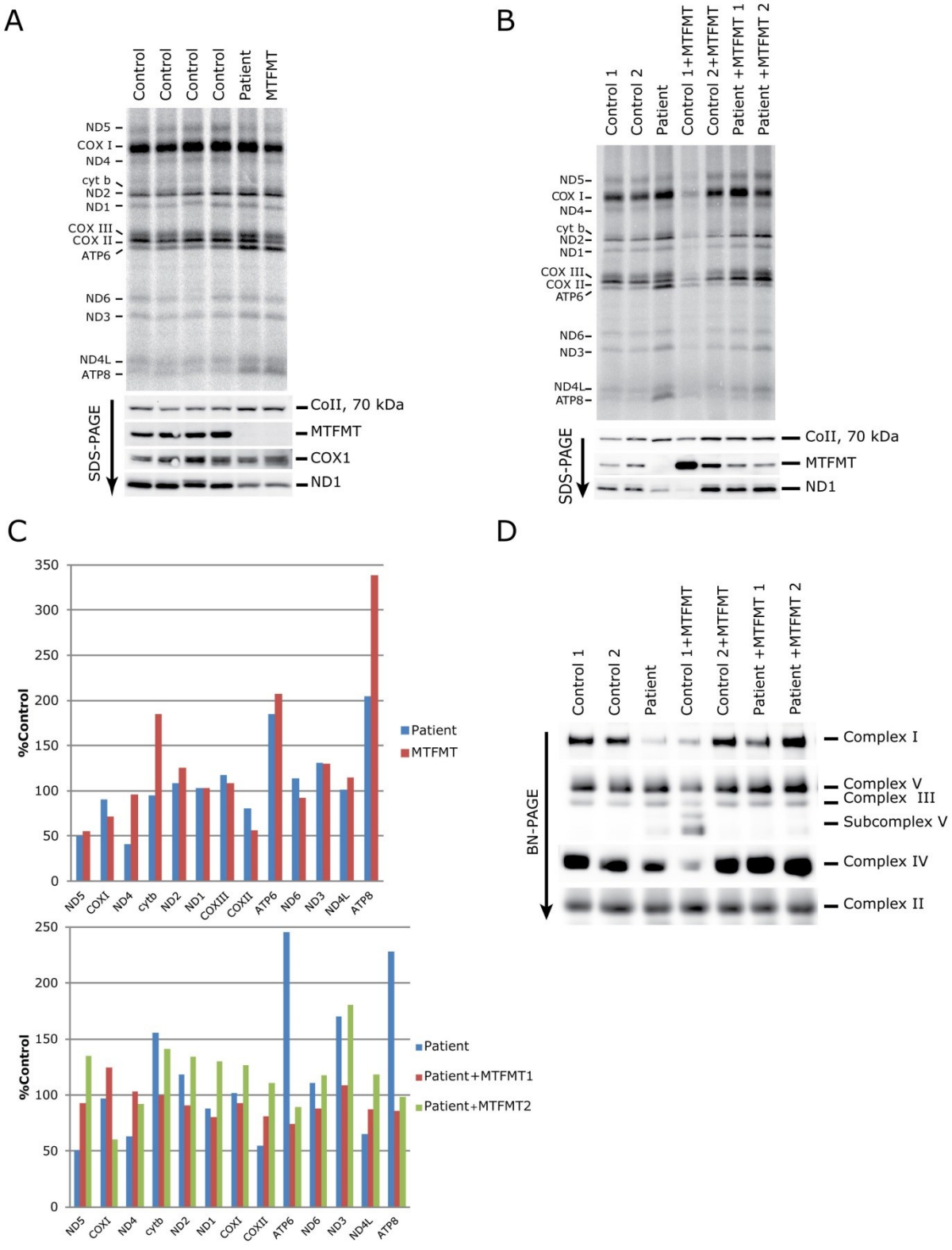
We identified two heterozygous variants in the *MTFMT* gene: the first variant (c.626C>T; p.Arg181Serfs5*) is a common, previously described, mutation[9,10,12-14], known to cause skipping of exon 4 and producing a premature stop codon. The second mutation (c.176C>T; p.Ala59Val) has never been reported before. It is predicted as damaging by SIFT[16] and probably pathogenic by PolyPhen-2[17]. Clinical analysis of the gene confirmed the heterozygous status of the mother for the variant c.626C>T.

An immunoblot analysis of whole cell extracts, from patient fibroblasts, showed that MTFMT was undetectable compared to control. This result is similar to that previously observed in an *MTFMT* patient (P1 in Figure 5.2) with the phenotypically more severe Leigh syndrome (c.626C >T; p.Arg181Serfs5* and c.994C>T; p.Arg332*) which we used as a positive control[13], and indeed, in all previously investigated patients, suggesting that the missense substitution in MTFMT destabilizes the protein. In addition, the steady-state levels of ND1 and COX1, the core structural mtDNA-encoded subunits of complexes I and IV were decreased (Figure 5.2A). A mitochondrial pulse translation assay[18] showed a decrease in the rate of synthesis of ND5 (55%), ND4 (63%), and COX II (25%). The rates of synthesis of other mtDNA-encoded polypeptides were similar or higher (especially the ATP6,8 polypeptides that are encoded on a bicistronic transcript) to those observed in controls. The global reduction in mitochondrial protein synthesis was smaller as compared to P1 (8% vs. 30%), consistent with the milder phenotype in the patient studied here (Figure 5.2A, C). An investigation of the assembly of OXPHOS enzyme complex by BN-PAGE of patient fibroblasts showed a decrease in fully assembled complexes I and IV in fibroblasts and accumulation of a small amount of a subcomplex of complex V (F1), despite the fact the both mtDNA subunits were synthesized at greater than control rates, and the level of

fully assembled complex V does not appear to be reduced (Figure 5.2D). Complex III was assembled normally and complex II was unaffected as it is encoded entirely by nuclear DNA.

To confirm the pathogenicity of the *MTFMT* mutation, we used a retroviral vector (pLSXH) to express a wild-type *MTFMT* cDNA in immortalized patient fibroblasts. Expression of *MTFMT* from the retroviral vector resulted in significantly higher steady-state levels of MTFMT than control levels in bulk culture, resulting in a dominant negative phenotype, with decreased translation of all mitochondrial polypeptides and decreased assembly of all OXPHOS complexes (Figure 5.2B,D) as we have previously observed[13]. We therefore isolated clones from the transduced patient fibroblasts to identify those expressing MTFMT at close to control levels. Analysis of two such clones showed rescue of the steady-state levels of ND1 (from 22% of control to 78 and 93% of control in clones 1 and 2, respectively); the protein synthesis defects in ND5, ND4, COXII, ATP6, and ATP8 (Figure 5.2B, C), and the OXPHOS assembly defects (Figure 5.2D). These results confirm that *MTFMT* is the gene responsible for translation and OXPHOS assembly defect in the patient.

Figure 5.2: Characterization and rescue of the biochemical defect in control and *MTFMT* patient fibroblasts. A, Pulse labeling of newly synthesized mitochondrial polypeptides in patient, a previously studied *MTFMT* patient with Leigh syndrome (P1) as a positive control (c.626C>T, c.994C>T) and control fibroblasts. The seven subunits of complex I (ND), one subunit of complex III (cyt b), three subunits of complex IV (COX), and two subunits of complex V (ATP) are indicated on the left (upper panel). Immunoblot analysis of MTFMT, COXI, and ND1 (lower panel). The complex II 70-kDa subunit was used as a loading control. B, Rescue of the translation defect in two clones expressing MTFMT near control levels and dominant negative effects associated with overexpression of *MTFMT* (control 1 plus MTFMT in lane 4) (upper panel). Immunoblot analysis of MTFMT and ND1 of the same samples used in the pulse translation experiment above (lower panel). The complex II 70-kDa subunit was used as a loading control. C, Quantification of the rate of protein synthesis in current patient compared with P1. Rates of synthesis of individual polypeptides were normalized to the steady-state level of the complex II 70-kDa subunit and are shown as the percentage of the average of four controls (top panel). Quantification of the rates of synthesis of the individual polypeptides in the two rescued clones was analyzed in C. Rates of synthesis of individual polypeptides were normalized with steady-state level of the complex II 70-kDa subunit and shown as the percentage of the average of two controls. D BN-PAGE analysis of the assembly of individual OXPHOS complexes in controls, the patient, control 1 overexpressing *MTFMT* resulting in a dominant negative phenotype (lane 4), and two rescued patient fibroblasts clones expressing wildtype *MTFMT*. Antibodies against an individual subunit of OXPHOS complexes I-V were used for immunoblotting, and complex II was used as the loading control.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Control
Control
Control
Control
Patient
MTFMT
ND5
COX I
ND4
cyt b
ND2
ND1
COX III
COX II
ATP6
ND6
ND3
ND4L
ATP8
SDS-PAGE
CoII, 70 kDa
MTFMT
COX1
ND1
B
Control 1
Control 2
Patient
Control 1+MTFMT
Control 2+MTFMT
Patient +MTFMT 1
Patient +MTFMT 2
ND5
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cyt b
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COX III
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ND5
COXI
ND4
cytb
ND2
ND1
COXI
COXII
ATP6
ND6
ND3
ND4L
ATP8
D
Control 1
Control 2
Patient
Control 1+MTFMT
Control 2+MTFMT
Patient +MTFMT 1
Patient +MTFMT 2
BN-PAGE
Complex I
Complex V
Complex III
Subcomplex V
Complex IV
Complex II

DISCUSSION

Clinical and MRI heterogeneity is not unexpected in diseases caused by dysfunction of the mitochondrial translation machinery, but it remains largely unexplained. The biochemical defects we observed in our patient are similar to those previously reported in patients with more severe phenotypes and a fatal course[11-13]. Biochemical studies of the in vitro activity of two pathogenic *MTFMT* variants showed substantially decreased activities (>36-fold compared to wild-type)[19]. This, coupled with the fact that the steady-state levels of MTFMT are below immunodetectable levels in all reported studies of patient fibroblasts, implies that the residual activity of MTFMT must be exceedingly low. It is not, however, known what level of formylation could be supported under these conditions, or whether there are tissue-specific differences in the expression or activity of the pathogenic variants. Even small differences associated with different mutations, which would be very difficult to measure accurately, could make a substantial difference in the severity of the biochemical and clinical phenotype. Neither the global translation defect nor the OXPHOS assembly defect in our patient was as severe as in the Leigh syndrome *MTFMT* patient we used as a positive control (patient 1 in [13]), consistent with the milder phenotype we observed. The initial clinical history of our patient corresponds to the spectrum of late onset Leigh disease caused by mutations in a nuclear gene[20-23]. However, the subsequent evolution, characterized by a sudden-onset, rapidly evolving visual deficit is unusual and unique.

The MRI findings documented in our case, in which the entire visual pathway is selectively involved with a symmetric and homogeneous pattern has not, to our knowledge, been reported before. The sensitivity of the visual pathway to mitochondrial stress is well known. Optic atrophy was previously reported in patients with *MTFMT* mutations[10,9], and is commonly associated with Leigh syndrome[24]. However, the involvement of the entire visual pathway is

novel. We cannot determine if the whole pathway was affected simultaneously; nonetheless, the delayed appearance of optic atrophy more than six months after the visual symptoms started suggests that the posterior visual pathway involvement dominated the initial clinical presentation. Furthermore, the most striking MRI finding in our case is the bilateral, symmetric and severe optic radiation damage. In Leber's hereditary optic neuropathy (LHON), another mitochondrial disorder, the microstructural involvement of the optic radiations- invisible to conventional MRI sequences- was documented with advanced imaging techniques, but it is still unclear whether it results from direct postgeniculate damage or anterograde degeneration[25,26]. In contrast to LHON, in our case the visual pathway changes were severe enough to be detected with conventional MRI sequences and they dominated the entire MRI picture. Infectious events or silent metabolic decompensation contributing to visual loss cannot be fully excluded in our case, but are unlikely given the clinical, ophthalmological and imaging presentation.

In conclusion, *MTFMT* mutations are associated with a wide variety of phenotypes that encompass very early onset and fatal disease to milder phenotypes with specific sensory involvement as illustrated here. Despite this dramatic variability, the underlying biochemical defect, virtually undetectable levels of MTFMT on immunoblot analysis, appears similar in all reported cases, suggesting that clinical expression is influenced by either very small differences in residual MTFMT activity or genetic background.

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PREFACE TO CHAPTER 6

In Chapter 6 I demonstrated the crucial role played by MRI family studies in the diagnostic process of adult genetic leukoencephalopathies. As opposed to most pediatric forms, adult genetic white matter disorders may present an insidious disease onset, a slowly progressive evolution and non-specific symptoms. Last but not least, the modality of transmission of the disorder can be difficult to ascertain, since the family history can be complicated by the presence of acquired white matter conditions and the availability of DNA of other family members can be limited. In this context, the possibility to perform brain MRI studies in informative first-degree family members acquires a critical role to clarify the mode of inheritance and, thus, drive the diagnostic process. The family described in Chapter 6 represents a good example of the usefulness of familial MRI studies in presymptomatic family members when the mode of transmission is unclear.

Furthermore, in this Chapter, we confirmed the extreme clinical and radiological intrafamilial variability associated with CADASIL. Finally, we documented a novel mutation located in exon 8 were only 3.3% of mutations are commonly found, therefore suggesting that the entire coding region of the *NOTCH3* gene should be sequenced when CADASIL is suspected.

CHAPTER 6

3T MRI study discloses high intrafamilial variability in CADASIL due to a novel *NOTCH3* mutation[91].

Roberta La Piana[1,2], Ilana R. Leppert[3], G. Bruce Pike[4], Sylvain Lanthier[5], Bernard Brais[2], Donatella Tampieri[1].

[1]Department of Neuroradiology, Montreal Neurological Institute and Hospital, McGill University, 3801 rue University, Montreal, H3A2B4, QC, Canada

[2]Laboratory of Neurogenetics of Motion, Montreal Neurological Institute and Hospital, McGill University, 3801 rue University, Montreal, H3A2B4, QC, Canada

[3]McConnell Brain Imaging Center, Montreal Neurological Institute, McGill University, 3801 rue University, Montreal, H3A2B4, QC, Canada

[4]Departments of Radiology and Clinical Neurosciences, Hotchkiss Brain Institute, University of Calgary, 3330 Hospital Dr NW, Calgary, AB, T2N 4N1, Canada

[5]Division of Neurology, Department of Medicine, Hôpital du Sacré-Cœur de Montréal, 5400 Boul Gouin O, Montréal, QC, H4J 1C5, Canada

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Abstract

In order to evaluate the usefulness of presymptomatic MRI, we performed 3T brain MRI and Sanger gene sequencing in a proband with suspected but not confirmed CADASIL and her apparently asymptomatic father. The 35-year-old proband presented with migraine with visual aura. Brain MRI showed diffuse leukoencephalopathy, suggesting CADASIL. *NOTCH3* gene sequencing (exons 3-6) was negative. Family history was unclear. The MRI study of the father documented severe, diffuse leukoencephalopathy, with involvement of the temporal poles and external capsules (not observed in the proband), and lacunar infarcts in the absence of cardiac disease or risk factors. The MRI findings were in favour of an autosomal dominant mode of transmission and reinforced the hypothesis of CADASIL. Full *NOTCH3* gene sequencing uncovered a novel exon 8 mutation (c.1337G>A; p.Cys446Tyr) outside the most commonly mutated region of *NOTCH3*.

The novel mutation leads to a typical MRI pattern but a variable overall phenotype. The study underlines the usefulness of combining full gene sequencing with familial MRI studies.

Introduction

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is a monogenic vasculopathy caused by mutations in the *NOTCH3* gene[1, 2]. Multifocal, confluent white matter changes associated with subcortical infarcts characterize the radiological picture. The involvement of the temporal poles and external capsules is highly specific.

In genetic leukoencephalopathies, MRI is extremely valuable in making the diagnosis, even in pre-symptomatic stages[1, 3]. In CADASIL, MRI changes may anticipate the clinical onset by more than ten years[1, 3].

While the MRI pattern is consistent across patients, the clinical presentation, course and severity may vary significantly, even within the same family[4, 5]. Due to this heterogeneity, the family history is sometimes difficult to interpret and the modality of genetic transmission might not be easily established[6]. Consequently, patients often experience delays in receiving an accurate diagnosis.

We report MRI and gene sequencing findings in a proband with suspected CADASIL and unclear family history to highlight the potential usefulness of brain MRI in asymptomatic family members.

Methods

This project is part of a research study aiming to identify MRI patterns to uncover mutated genes responsible for late-onset genetic leukoencephalopathies. Participants were recruited from the patients either evaluated at the Department of Neuroradiology of the Montreal Neurological Hospital or referred to the Genetic White Matter Diseases clinic.

In order to clarify the modality of transmission of the disease in the families, we recruited first-degree relatives of patients. All the participants, their relevant first-degree relatives and sex- and age-matched healthy controls underwent a 3T MRI protocol specifically designed to study white matter abnormalities: T1 MPRAGE, axial T2, 3D T2 FLAIR, and Magnetization Transfer Ratio (MTR). All images are reviewed by our team (R.L.P, D.T.). A systematic analysis was applied

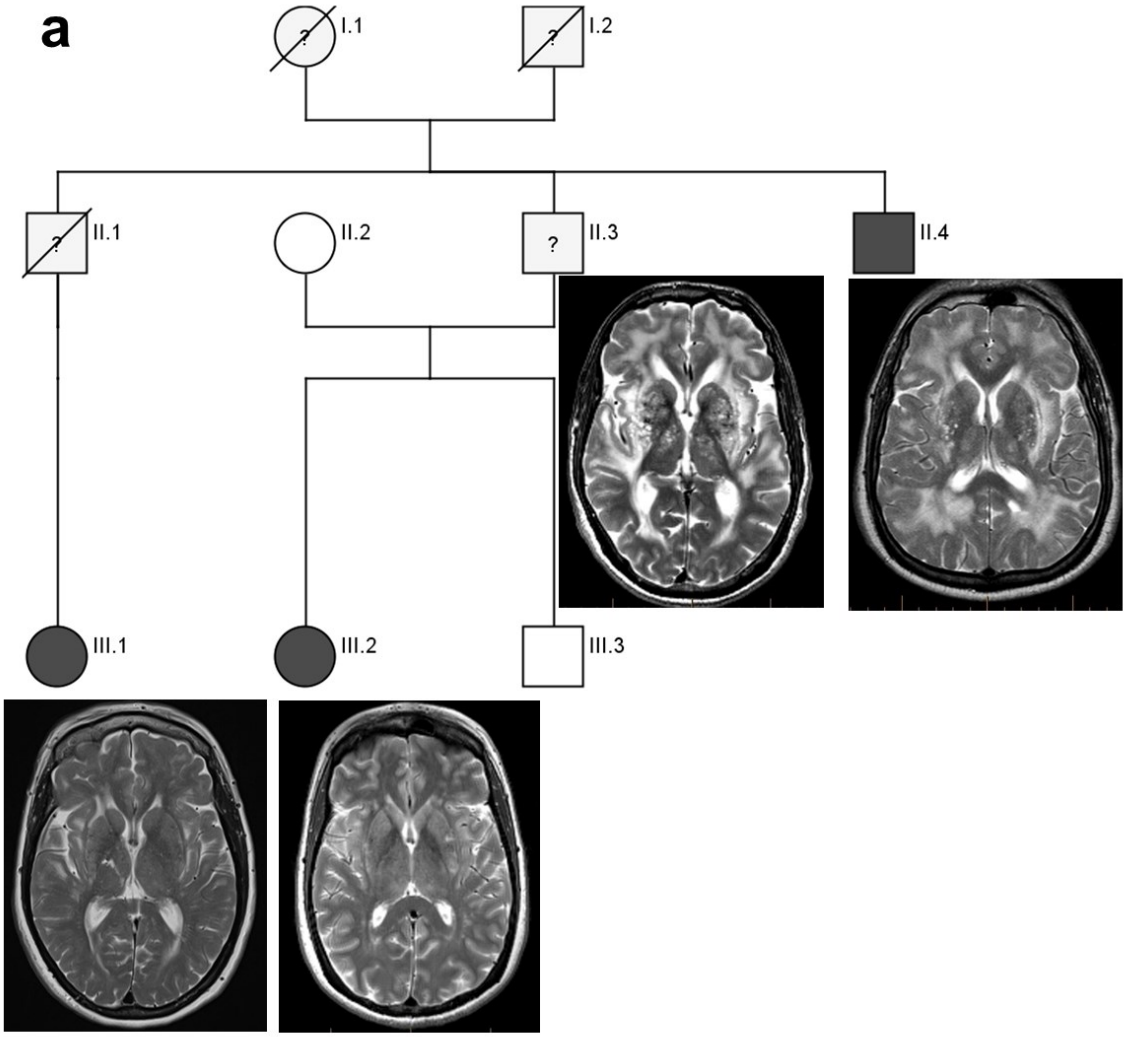
and included qualitative (e.g. regional analysis) and quantitative (MTR) parameters (Additional methods are detailed in Supplementary Data).

DNA from affected participants and first-degree relatives was extracted by standard methods for gene Sanger sequencing and, if needed, whole exome sequencing.

Results

Figure 6.1a illustrates the pedigree of the family.

Figure 6.1: Pedigree and MRI data. Family pedigree and key MRI axial T2-weighted images at the level of the basal ganglia (a). Mean whole-white matter MTR values in Subjects III.2, II.3 and five healthy controls (b). The father (II.3) of the proband showed mean MTR value significantly lower than that of the controls ($p = 0.01$) while the proband (III.2) has mean MTR value that was not significantly different from the controls ($p = 0.15$).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I.2
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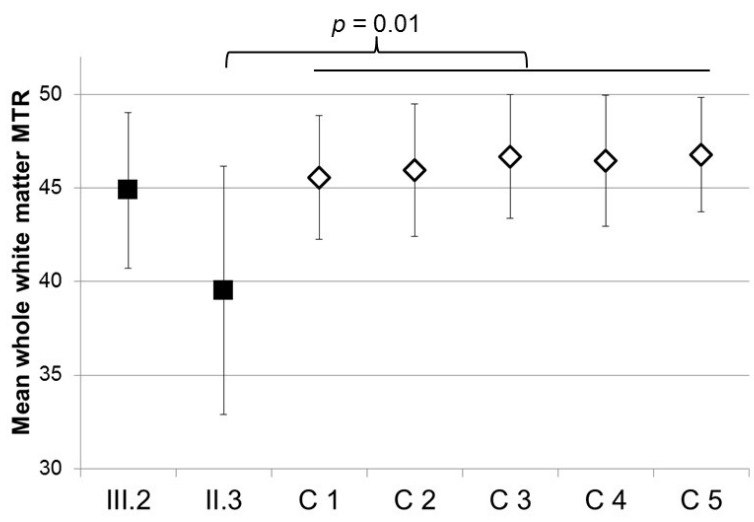

b
p = 0.01
Mean whole white matter MTR
50
45
40
35
30
III.2
II.3
C 1
C 2
C 3
C 4
C 5

Clinical findings

Subject III.2

The proband is a French Canadian 35-year-old woman referred to our clinic of Genetic White Matter Diseases. She reported severe migraines with visual aura since age of 20, which limited her daily activities. Brain MRI documented multifocal white matter abnormalities sparing the temporal poles and external capsules. The sequencing of exons 3, 4, 5 and 6 of the *NOTCH3* gene was negative. However, a skin biopsy documented characteristic granular osmophilic material supporting a diagnosis of CADASIL.

Her family history was positive for white matter disease in the following members:

Subject II.1

The older paternal uncle of the proband was affected by a white matter disorder interpreted as multiple sclerosis since the age of 40. He died at the age of 63; at that time he was bedridden, with a severe mood disorder. No neuroradiological images were available for review.

Subject II.4

The younger paternal uncle was first seen by neurologists at the age of 51 for memory loss and a transient ischemic attack. *NOTCH3* sequencing (exons 3-6) did not identify pathogenic variants. On the basis of the negative molecular test and the family history (subject II.1), multiple sclerosis was suspected. At the age of 61, he had progressive memory deficit, depression, word finding difficulties, generalized fatigue and gait imbalance with frequent falls. Brain MRI study showed multifocal confluent leukoencephalopathy with lacunar infarcts in the basal ganglia and brainstem (Figure 6.1a and Figure 6.2m-p).

Subject III.1

Subject III.1 presented at age 44 with a left-sided sensory deficit secondary to ischemic stroke. She reported 5 episodes of migraine with sensory aura since the age of 20. Brain MRI showed multifocal white matter involvement (Figure 6.1a and Figure 6.2i-l). Extensive investigation found no stroke etiology. She did not undergo *NOTCH3* analysis. She suffered no further neurological symptoms during a clinical follow-up of 3 years.

In order to clarify the disease status and the modality of transmission of the disease within the family, we asked the proband and her father to undergo our research 3T MRI study.

Subject II.3

The 69-year-old father claimed to be asymptomatic at the time of the examination. His clinical history was negative for migraine, ischemic attacks, psychiatric illness, cardiac disease and vascular risk factors. He had had two episodes of loss of consciousness in the previous two years, interpreted as vasovagal crises. His wife reported that he had mild memory difficulties that seemed to appear after his retirement two years earlier. The neurological examination was unremarkable.

3T MRI study

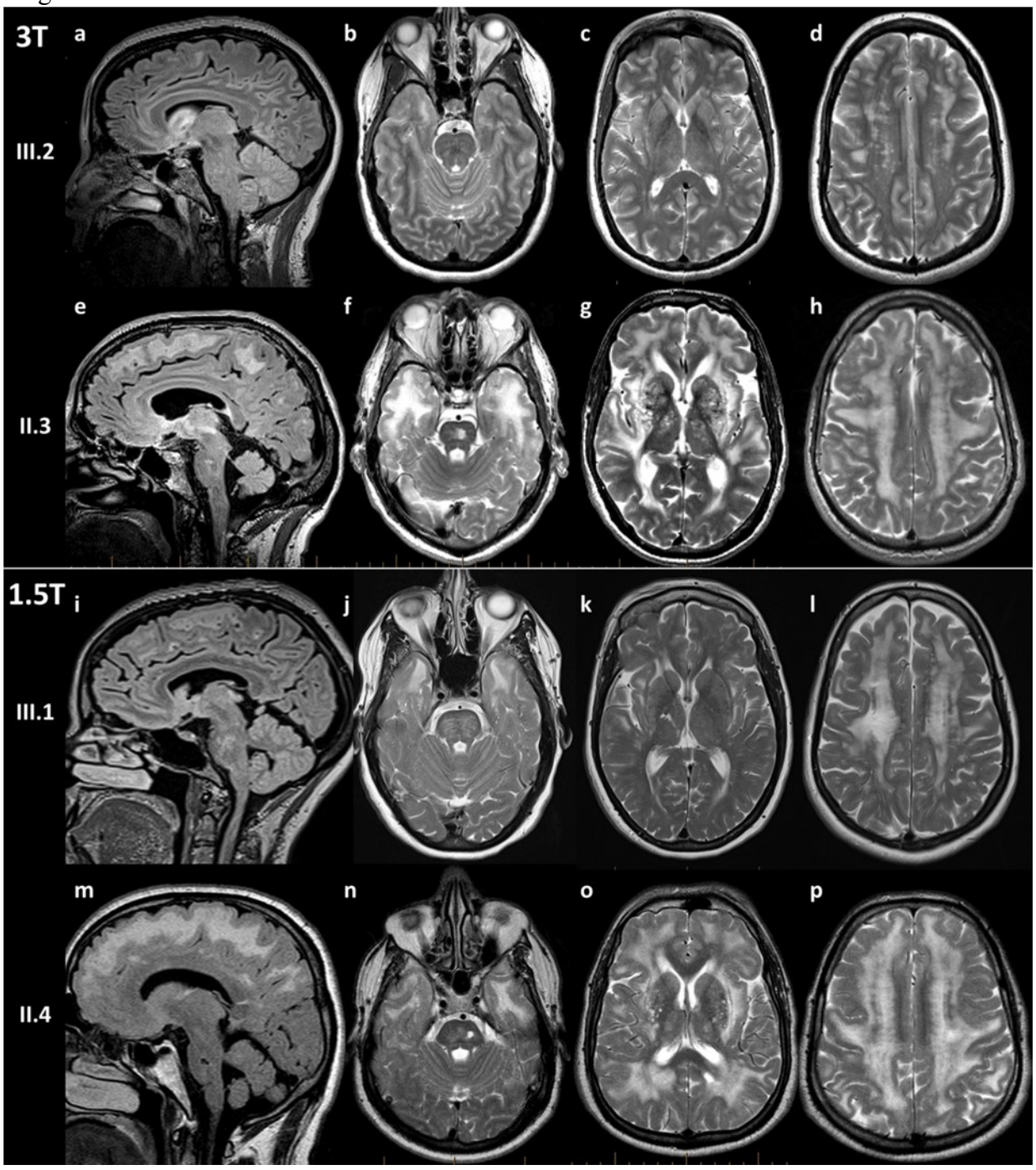
The study performed on the father (II.3) documented a severe, confluent and diffuse leukoencephalopathy (Figure 6.1a and Figure 6.2e, h), associated with basal ganglia and brainstem lacunar infarcts (Figure 6.2f, g). The proband (III.2) presented multifocal white matter abnormalities (Figure 6.1a and Figure 6.2c,d), with minimal involvement of the temporal poles (Figure 6.2b).

As expected based on the MRI pattern, the mean whole white matter MTR value was significantly different between the father and the controls ($p = 0.01$), confirming his status as

affected (Figure 6.1b). Interestingly, the mean MTR values of the affected daughter did not differ significantly from the controls ($p = 0.15$) (Figure 6.1b). For completeness, we calculated MTR values on regions of interest including areas of abnormal T2 signal on the proband, and the results did not show any statistical difference between III.2 and controls.

The 3T MRI findings and the family history of white matter disease supported the CADASIL-like autosomal dominant transmission in the family despite the initial negative exons sequencing.

Figure 6.2: Brain MRI of affected family members. Sagittal FLAIR T2-weighted midline images (a, e, i, m); axial T2-weighted images at the level of the temporal poles (b, f, j, n), basal ganglia (c, g, k, o) and centrum semiovale (d, h, l, p). The least (II.3) and the most (II.4) clinically affected subjects show the same extensive involvement of the temporal poles, external capsules, corona radiata, and centrum semiovale with severe brainstem and basal ganglia signal changes.



NOTCH3 analysis

Sequencing of all exons of *NOTCH3* uncovered a novel mutation (c.1337G>A; p.Cys446Tyr) in exon 8, where only 3.3% of mutations have been previously reported[7]. The variant is predicted to be deleterious by SIFT[8] and PolyPhen2[9]. It results in a cysteine being replaced by a Tyrosine in the Epidermal Growth Factor like repeat 11 region.

Discussion

Our study demonstrated that MRI is extremely valuable in the assessment of families with leukoencephalopathy in which the clinical data and risk factors alone are not sufficient to establish a final diagnosis. The ethical issues raised by studying asymptomatic subjects was addressed in the consent by allowing participants to request to not be informed of the results of the imaging.

We documented a high MRI and clinical intrafamilial variability, reflecting the absence of correlation between the degree of leukoencephalopathy on MRI and the severity of the clinical manifestations[10]. In fact, the same extensive leukoencephalopathy, barely without normal-appearing white matter, was observed in the least (II.3) and the most symptomatic (II.4) family members. The progression of MRI abnormalities with age is well known, so it was not unexpected to find that the two oldest subjects showed a dramatic brain involvement[11, 12]. However, the lack of correlation between the severity of symptoms, the degree of white matter involvement and the absence of vascular risk factors are highly unusual.

In the literature, other authors have reported pedigrees with atypical findings and high intrafamilial variability in terms of cerebral involvement and clinical findings[6, 13, 14]. In particular, Schubert et al. described a family in which, as in our study, the white matter

involvement was significantly more severe in the apparently not affected father than in the proband daughter. However, in the report by Schubert, the father's leukoencephalopathy could be explained by important comorbidities and, moreover, no clinical or pathology data could support the final diagnosis of CADASIL. In the family here reported, the diagnostic criteria of CADASIL – clinical, MRI, and pathological – are all fulfilled in the proband and no co-morbid conditions are identified in the father to explain the degree of leukoencephalopathy. In addition, the pattern of white matter involvement is typical for CADASIL.

Atypical multiple sclerosis is often put in the differential diagnosis with CADASIL, as the latter can sometimes mimic the first, although the two have distinct clinical and MRI features[15, 16]. Some reports even discuss whether the two entities may co-exist[17]. In our study, the lack of availability of MRI data on the paternal uncle who received the diagnosis of MS makes it impossible to conclude whether in this family MS and CADASIL co-existed or whether MS was a prolonged misdiagnosis.

The observed amino acid change supports the important role of cysteine in coupling epidermal growth factor repeats to ensure NOTCH3 function. Even though the loss of cysteine residues is common in CADASIL, the mutation is located outside the most frequently mutated region (exons 2-6)[7]. Hence, our report underlines the need to analyse the entire *NOTCH3* coding region when the clinical and MRI data are suggestive for CADASIL.

Ethics approval and consent to participate

The Institutional Review Board of our Institution approved the research study and all subjects gave consent to participate in the project. This study was conducted in accordance with the

principles of the Helsinki Declaration. Participants provided their informed consent, including consent to the publication of clinical and genetic findings.

Acknowledgements

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Supplementary Material

The systematic approach used for the qualitative analysis of the MRI images is described in the Table below:

Qualitative parameters	
Symmetry	yes / no
Pattern of involvement	isolated / multifocal / diffuse
Presumptive nature of pathological process	demyelination / hypomyelination/ cystic degeneration / vascular
Associated features	involvement of cortex / basal ganglia / vascular anomalies / calcifications / heavy metals deposition
Regional analysis	
Location	supratentorial / infratentorial/ both
Involved fibers	U-fibers / associative fibers / capsules / long tracts
Lobar involvement	frontal / parietal / temporal / occipital

For the whole-white matter MTR analysis, whole-white matter masks are obtained manually and then resampled to match the resolution of the quantitative maps. The PD/T2-weighted, T2 FLAIR, diffusion, MTon and MToff sequences are registered to the T1-weighted anatomical sequence using an affine linear registration. The mean and variance of MTR values are calculated for each individual. MTR are computed using freely-available (FSL) and in-house (MNI) software. MTR is calculated as ((MToff – MTon)/ MToff)*100.

Independent samples Student's T test is used to assess the differences between the means of the MTR values of each subject and the group of matched healthy controls. A threshold of $p = 0.05$ is selected for statistical significance.

CHAPTER 7

Discussion

In this thesis I summarized my research work on the identification and characterization of adult genetic leukoencephalopathies. The results presented were obtained through an integrated approach which combined clinical phenotyping with MRI pattern-recognition and next generation sequencing data. This work led to a) the description of an original cohort of 68 adult subjects with leukoencephalopathy of probable genetic origin, b) the identification of the causal mutations (11 novel) in genes known to be associated with leukoencephalopathies in 23.7% of the 59 subjects on which we applied our approach, and c) the broadening of clinical and imaging phenotypes of known disorders.

7.1 A new cohort of adult genetic leukoencephalopathies patients

7.1.1 An expanding French Canadian/Quebec cohort of genetic leukoencephalopathies patients

During the past five years we identified 68 subjects with suspected or confirmed genetic leukoencephalopathies referred to the Montreal Neurological Institute for investigations in the context of a white matter disease. When compared to previous studies, the size of our sample is considerable: the study by Ayrignac et al. included a total of 154 subjects which were identified throughout the entire territory of France[9]. Our study was performed at a single institution; the majority of our patients came from the Montreal region (51/68; 75%) and were of French Canadian descent (48/68; 70.6%). Therefore, due to well-recognized regional founder effects in Quebec, it is likely that the number of cases with an inherited white matter disorder in Quebec will grow in the future.

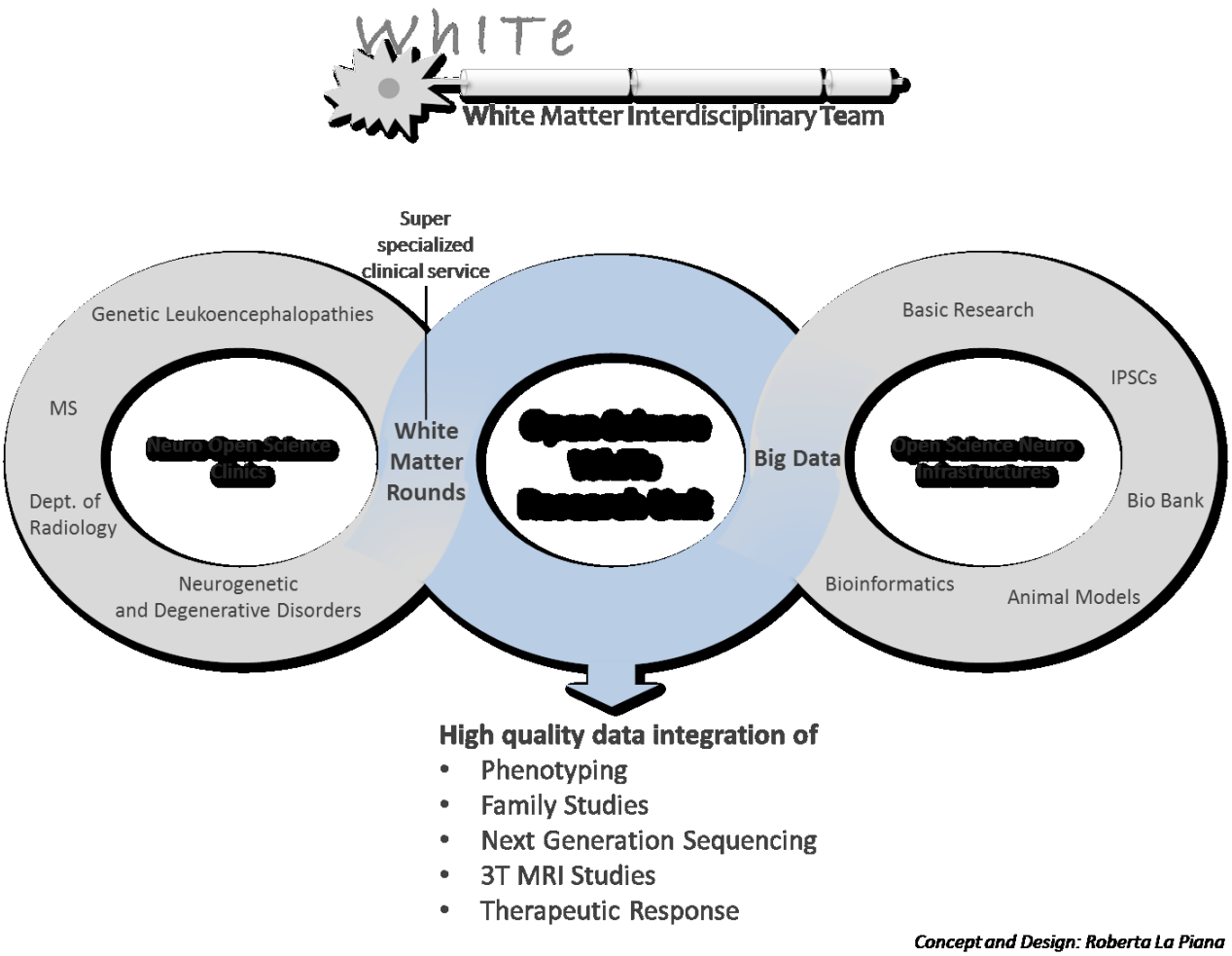
In recruiting this new expanding cohort, our research study has important clinical implications. First of all, patients and their families can benefit from genetic counselling. The second implication is especially relevant for those subjects who have been initially referred to a Multiple Sclerosis clinic for a possible atypical form of inflammatory demyelinating disorder. The identification of the correct genetic origin of their disease allows to avoid unnecessary immunomodulatory therapies associated often with significant side effects. Some have estimated that the rate of misdiagnosis of MS is approximately 10%[92]. Our results document that 33.8% (23/68) of the subjects in our cohort were initially assessed in the MS clinics.

7.1.2 The essential contribution of interdisciplinary collaborations

The collaborative work in a multidisciplinary team as the White Matter Rounds we set up at the onset of this project, amply demonstrates its value to establish the correct classification of subjects as having a suspected genetic disorder. Our work proved the crucial role of an interdisciplinary approach in the study of rare white matter disorders. As a further development of the White Matter Rounds, in the next year, we will create the White Matter Diseases Interdisciplinary Team (WhITe) at the Montreal Neurological Institute. This will consolidate our research network of MS specialists, neurogeneticists, neuroradiologists, and imagery and basic science researchers. The concept of WhITe has been designed by me and I will be its coordinator (Figure 7.1). As far as we know, the WhITe is a unique concept and we aim to make it the reference in Canada for the research in adult leukoencephalopathies of unknown etiology. The distinctive value of WhITe is the bridging role between MS clinicians, neurogenetics, imaging specialists and basic research scientists working on white matter biology. The WhITe will receive the referral of subjects with undiagnosed white matter diseases from three principal major sources: the Neurogenetics Clinics, the MS Clinics and the White Matter Diseases Rounds

at the Montreal Neurological Institute. In order to increase the identification of potential affected subjects, we will not only reinforce our already active collaboration with other centers across the province of Quebec, but also in other Provinces (CHEO, Ottawa) and outside Canada (Yale University, University of Rochester, University of Pennsylvania).

Figure 7.1: The White Matter Interdisciplinary Team. The WhITe is a unit at the core of the research on white matter disorders with a central bridging role between the clinical world and the research.



7.1.3 New clinical phenotypes associated with adult genetic leukoencephalopathies

Our study led to the description of two novel clinical phenotypes associated with known white matter disorders[78, 89]. In the paper by Accogli et al., the pathognomonic areas of white matter rarefaction around the frontal horns of the lateral ventricles were critical to suggest the diagnosis of vanishing white matter disease[89]. Therefore, we added a long-standing severe psychiatric presentation as part of the clinical phenotype of the disease. In addition, we recommended that psychiatrists consider vanishing white matter disease in the differential diagnosis of their patients when they present long-standing symptoms, particularly if refractory to treatment and accompanied by organic features. In the report by Shao et al., the integrated analysis of WES with MRI pattern-recognition allowed the diagnosis of adult-onset Krabbe disease in a family with multiple subjects affected by cerebellar ataxia and mild spastic features[78]. Our paper expanded the phenotypic spectrum of adult Krabbe disease by adding predominant cerebellar features to the classic clinical presentation usually characterized by spastic paraparesis. Hence, in both papers, we confirmed the central relevance of recognizing disease-specific MRI patterns in the diagnostic process of atypical presentations.

7.2 <u>The value of MRI pattern recognition in known and unknown leukoencephalopathies</u>

7.2.1 MRI pattern recognition in POLR3-related disorders

The causal role of *POLR3A* and *POLR3B* mutations in POLR3-related disorders was discovered in the Brais lab with the active participation of Geneviève Bernard in the early 2010s[62, 64]. Since then, Montreal has been a world-wide recognized reference for the research on this leukoencephalopathy. In this context, we were able to analyse the MRI data of a series of POLR3

patients. The first collective work performed in 2014 and reported in Chapter 2 demonstrated that some MRI features were typical of POLR3-related disorders and able to distinguish them from other hypomyelinating leukodystrophies[83]. The MRI phenotype represented by diffuse hypomyelination, cerebellar atrophy and T2 hypointensities located in specific cerebral structures (pallida nuclei, thalami, dentate nuclei) became the only recognized imaging hallmark of POLR3-related disorders. This remained valid until our paper on atypical MRI findings disclosed on POLR3 patients partly diagnosed through WES[84].

Our work on POLR3-related disorders demonstrated that the combined use of WES and MRI pattern-recognition is indispensable when studying rare white matter disorders in the NGS era. If until a decade ago, MRI pattern-recognition drove the diagnostic process and the interpretation of genetic analysis, nowadays the reverse is also true, namely the fact that novel MRI phenotypes are disclosed only after patients are diagnosed through NGS. Researchers working in this field need to be familiar with both approaches and facilitate the integrated work between specialists in imaging analysis and bioinformatics experts in NGS analysis.

Taken together, the findings of both our studies shed new light on POLR3-disorders. Indeed, patients with POLR3 mutations present commonly with a hypomyelinating disorder. However, hypomyelination may be lacking, hence the change in referring to these conditions from 4H – which includes "hypomyelination" - to the more appropriate POLR3-related disorders. Interestingly, the white matter is variably affected in these subjects: when not by hypomyelination, they present non-specific white matter abnormalities. These latter will benefit from *ad hoc* advanced MRI studies, aimed to obtain more data on the underlying pathophysiology. For instance, MTR and DWI will provide information on whether demyelination or altered water diffusion can explain the areas of abnormal signal. Cerebellar

atrophy is another finding which we confirmed to be POLR3-specific, even in subjects without white matter involvement. Consequently, POLR3-related disorders should be suspected in patients with isolated cerebellar atrophy. Future research should further explore the involvement of the cerebellum in POLR3-disorders, both in humans and animal models, as it might be critical to the underlying pathophysiology.

POLR3-related disorders were not the only leukoencephalopathies in which WES contributed to expand the neuroradiological phenotype of known conditions. In Chapter 5 we described the novel and exceptional selective involvement of the entire retrogeniculate visual pathway in a subject with *MTFMT* mutations. This pattern of white matter involvement was not only unusual for *MTFMT*-mutated patients, but also unique in the literature. The serial MRI studies mirrored the peculiar clinical evolution of the patient, which was characterized by the subacute vision loss occurred over a 2 months-period. The mild clinical and the unusual MRI phenotypes led us to explore the functional characterization of the novel *MTFMT* mutation disclosed by WES. Interestingly, we did not document any functional differences in the biochemical defects when compared to subjects with more severe phenotypes. Hence, the clinical and MRI heterogeneity associated with *MTFMT* mutations is not mutation-specific and remain unexplained.

7.2.2 The challenge of identifying new MRI entities

In the analysis of the entire sample of affected subjects, as described in the Preface of Section II, we performed a systematic analysis of the MRI data to assess whether the abnormalities constitute a specific pattern. With the exception of the group of subjects with vascular or cavitary abnormalities, we could not identify other clusters, since our population presented a high clinical and radiological heterogeneity. In the group of subjects with neither vascular nor cavitary abnormalities, we detected ten different patterns of white matter involvement, the majority of

which were multifocal. Some subjects, belonging to the same family, shared the same MRI and clinical phenotype. Our experience suggests that the landscape of MRI patterns associated with genetic leukoencephalopathies is different between pediatric and adult forms, with the latter being more heterogeneous and more multifocal. This observation is supported by previous literature, which underlined the risk for adult inherited disorders to be confused with acquired conditions[11]. Adult subjects may present vascular risk factors and changes on MRI that further complicate the interpretation of the imagery. The radiological heterogeneity, taken into account with the often non-specific clinical presentation and slowly progressive evolution, represents an extra obstacle to the correct identification of inherited conditions and their final diagnosis.

7.2.3 MRI family studies are crucial in adult leukoencephalopathies

In our research, the importance of MRI data analysis extended beyond the MRI-pattern recognition, as we reported in Chapter 6. Here we demonstrated the usefulness of MRI family studies to clarify the modality of transmission in families with adult leukoencephalopathies. The MRI exam proposed to first-degree informative relatives has been an original feature of our study. This was motivated by some specific features that distinguish adult from pediatric inherited leukoencephalopathies. In adult patients, the disease onset is often insidious and symptoms are non-specific. Therefore, we hypothesized that the same condition can be present, even though silent, in other family members, as we demonstrated in the father of the proband reported in Chapter 6. MRI features may sometimes be non-specific, at least in the initial phase of adult genetic leukoencephalopathies. In the proband described in Chapter 6, the typical characteristics of CADASIL – the involvement of temporal poles and external capsules – were not definite, even with the resolution provided by the 3T MRI machine. Contrarily and surprisingly, all diagnostic features were documented in the asymptomatic father, thus

confirming the family segregation and ultimately leading to the diagnosis. Finally, with adult patients, the recollection of family history across generations can be challenging and DNA from previous generations can be difficult, if not impossible, to obtain. Hence, the need to investigate the living family members with such an informative, yet non-invasive tool as MRI with the goal to clarify the pattern of inheritance of white matter abnormalities.

A limitation of my research is that we focused on the qualitative analysis of imaging findings and did not systematically used quantitative MR techniques, such as MTR and DWI. These advanced techniques can definitely be applied to distinguish between different pathologic processes and therefore help to clarify the origin – whether acquired or inherited – in uncertain cases. However, it is a matter of fact that quantitative techniques and other image analysis tools still have a limited application in the clinical setting. Radiologists have great expectations and hopes in the implementation of advanced techniques and artificial intelligence to medical imaging[93, 94]. In the field of white matter diseases, especially in MS, machine learning methods are among the most promising tools of innovation[95-97]. Potentially, they could be used to identify patterns of tissue involvement and, subsequently, assist the radiologist to discriminate between different conditions with a computer-aided diagnosis system. To enter this new era of neuroimaging, the collaborative work between clinicians and researchers experts in medical imaging and biostatistics will become increasingly critical. In rare genetic leukoencephalopathies, a big limitation to the application of artificial intelligence will definitely be the limited sample size, which can be partly addressed by data sharing between researchers.

7.3 NGS allowed the majority of the diagnosis despite high clinical and MRI heterogeneity

7.3.1 Advantages and limitations in the use of NGS panels and WES

In our research program, we planned to use NGS data to identify causal genes, based on the hypothesis that shared MRI and clinical patterns are caused by mutations in the same gene or genes of the same pathway[6]. Our sample size and heterogeneity did not allow the classification of subjects in clusters with shared clinical and radiological phenotypes; therefore we had to analyze NGS data on a subject/family basis. NGS findings reflected the heterogeneity of the clinical and neuroradiological data. The yield of diagnosis obtained through NGS panels/WES in our sample was 21.1%. This is comparable, and even higher[82], to what is described in the literature[21]. The Queen Square Adult Leukodystrophy Group (QSALG) is a multidisciplinary group based at the UCL Institute of Neurology, London (UK), combining experts on neuroinflammation, neurogenetics, inherited metabolic disease, cognitive neurology and neuroradiology[21]. When using WES in a cohort of unsolved leukoencephalopathies, they were able to diagnose 26% of patients. Other studies have found higher diagnostic rates. Ayrignac et al. reached a final diagnosis in 64% of their 154 cases by using biochemical and/or targeted gene testing[9]. The difference with our rate can be partly ascribed to the different inclusion criteria. While Ayrignac et al., selected subjects with exclusive symmetric and confluent white matter abnormalities, our sample included individuals with multifocal white matter involvement and clinical or family history suggestive of a genetic condition. As a consequence, our sample was more heterogeneous.

Diagnostic rates below 30% indicate that much is still to be discovered in the field of genetic white matter disorders in adults. The use of NGS gene panels and WES has definite advantages: first of all, there is no need for large families, as diagnosis can be made even on single cases or trios. WES performed on multiple subjects with homogenous phenotype has a much higher probability to uncover the mutated gene, as patients with shared clinical, radiological and genetic

findings validate each other. Undoubtedly, the greatest challenge of WES remains the interpretation of data[2]. This is facilitated when working on even small groups of subjects sharing the same features. The direct consequence of these observations is that, for rare disorders, the collaborative work among the few centers specialized in genetic white matter disorders should be fostered to be able to cluster subjects with similar phenotypes. This will facilitate the identification of novel causal genes.

The advent of WES has greatly increased the rate of diagnosis in unsolved leukoencephalopathies, especially the pediatric forms[2]. However, recent studies have documented a certain plateau in the discovery rate of novel genes[98]. It should be kept in mind that WES covers only a limited portion of the genome, despite being the region in which 85% of the causal variants have been located to date. Among the unsolved forms, some of them are caused by mutations in regulatory regions (e.g. promoters, enhancers) not covered by WES and disclosed only by the sequencing of the entire genome (whole genome sequencing, WGS). The constant price dropping of sequencing will eventually make WGS more accessible. The application of WGS will probably increase the diagnostic rate of unsolved disorders, even though the challenge of data interpretation will be even higher than what seen for WES, as the number of rare variants will significantly increase.

7.3.2 The contribution of NGS to phenotype expansion

In this thesis, I documented how NGS and WES can disclose new phenotypes associated with known genetic leukoencephalopathies. In Chapter 3 WES was partly responsible for the discovery that hypomyelination is not an obligate characteristic of POLR3-related disorders, a finding that revolutionized the concept of this group of disorders, previously defined by their hypomyelinating features. In Chapter 5, mutations in the *MTFMT* gene were associated with a

milder clinical phenotype and unique, selective involvement of the visual pathway. Therefore, in my thesis, WES contributed to the broadening of both neuroradiological and clinical spectrums of inherited conditions. These observations are in line with what is reported in the literature where it has been documented that, since the advent of NGS techniques, approximately 50% of all new genotype-phenotype correlations are due to this expanding phenotype phenomenon driven by WES[98].

Interestingly, in the years following the publication of our data on POLR3-related disorders, the literature witnessed a significant widening of the clinical spectrum associated to *POLR3A* mutations. Hypomorphic *POLR3A* mutations have been found in about 3% of patients with sporadic and recessive spastic ataxias without hypomyelination, but with dental problems[35]. Very recently, intronic *POLR3A* variants were documented in patients with Wiedemann-Rautenstrauch syndrome, an early-onset segmental progeria[99-101]. All these new reports are relevant not only for the expanding phenotype, but also because they highlight the emerging discovery of causal variants in regulatory regions of the genome[35, 84, 99-101].

Taken together, our observations and the recent reports, demonstrate that POLR3-related disorders include a much larger spectrum of conditions than initially thought in the early 2010s, before the widespread availability of NGS techniques. Moreover, they all suggest that the increasing access to WGS will lead to a further expansion of the phenotypic spectrum of many disorders.

7.3.3 The need for international consortia on white matter disorders

In my thesis, I demonstrated the crucial role of collaborative efforts to study undiagnosed white matter disorders. I already mentioned the importance of having access to different expertises (i.e. imaging analysis, bioinformatics, molecular genetics, biochemical validation, etc.). In addition,

as it is often the case in the rare diseases field, researchers need to collaborate and constantly interact to maximize the possibility to identify clusters of patients with new rare entities. In our sample, we identified VUS or variants in candidate genes in almost 30% of the subjects, all of which were sporadic cases or two affected siblings of the same family with otherwise negative family history. By working in network with other national and international research groups we will be able to share ultra-rare cases. This will facilitate the validation of candidate genes, when variants are found in subjects with homogeneous phenotypes, and will eventually lead to the discovery of new causal genes.

CHAPTER 8

Conclusions

We documented that adult genetic leukoencephalopathies are an emerging field in neurosciences. The new cohort of affected subjects identified in the past five years will likely increase in the near future, as we will continue to apply our approach and actively work to increase awareness on these disorders among neuroradiologists, MS experts, and neurogeneticists. We demonstrated the importance of an integrated approach which combines the analysis of all datasets: clinical phenotyping, neuroimaging data, and next generation sequencing data. The WhITe, a multidisciplinary team based at the Montreal Neurological Institute, has set as its unique role to connect clinicians with basic research scientists with the goal of becoming the reference for the research on genetic adult white matter diseases in Canada.

The recognition of disease-specific MRI findings is an asset in the study of genetic leukoencephalopathies. MRI features can guide the genetic analysis, as seen in the first years following the discovery of POLR3-related disorders and in clinically-atypical vanishing white matter disorders. On the other hand, the advent of NGS has enabled the identification of novel radiological phenotypes associated with known disorders. Besides the role of neuroimaging data for pattern-recognition, we demonstrated that MRI family studies can be crucial in adult leukoencephalopathies to disclose affected, apparently asymptomatic subjects and therefore clarify the modality of transmission of a disease within a family.

Despite the availability of NGS gene panels and WES, more than 70% of our sample remains without a diagnosis. The creation of international consortia of experts in adult leukoencephalopathies will allow the identification of subjects with shared phenotypes or mutated genes and ultimately lead to the description of new genetic entities.

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APPENDICES

A) Significant contributions to other projects

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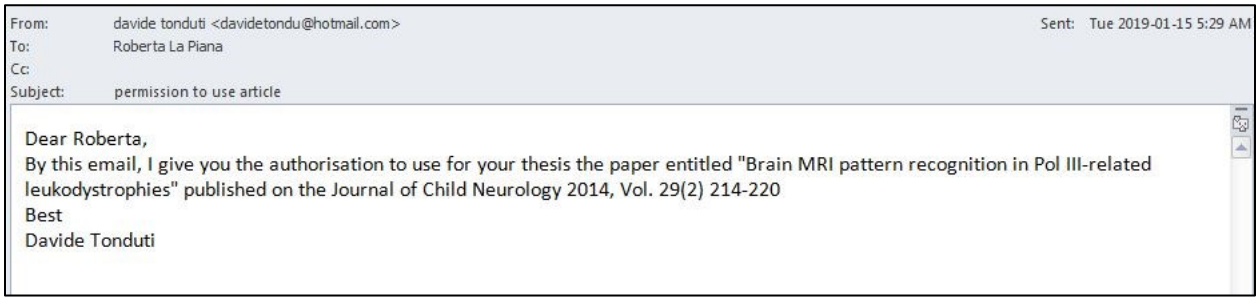

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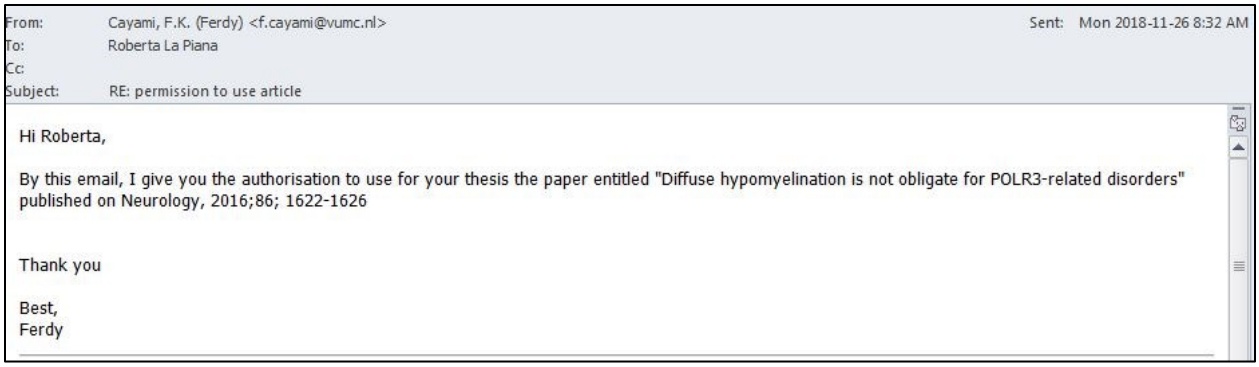

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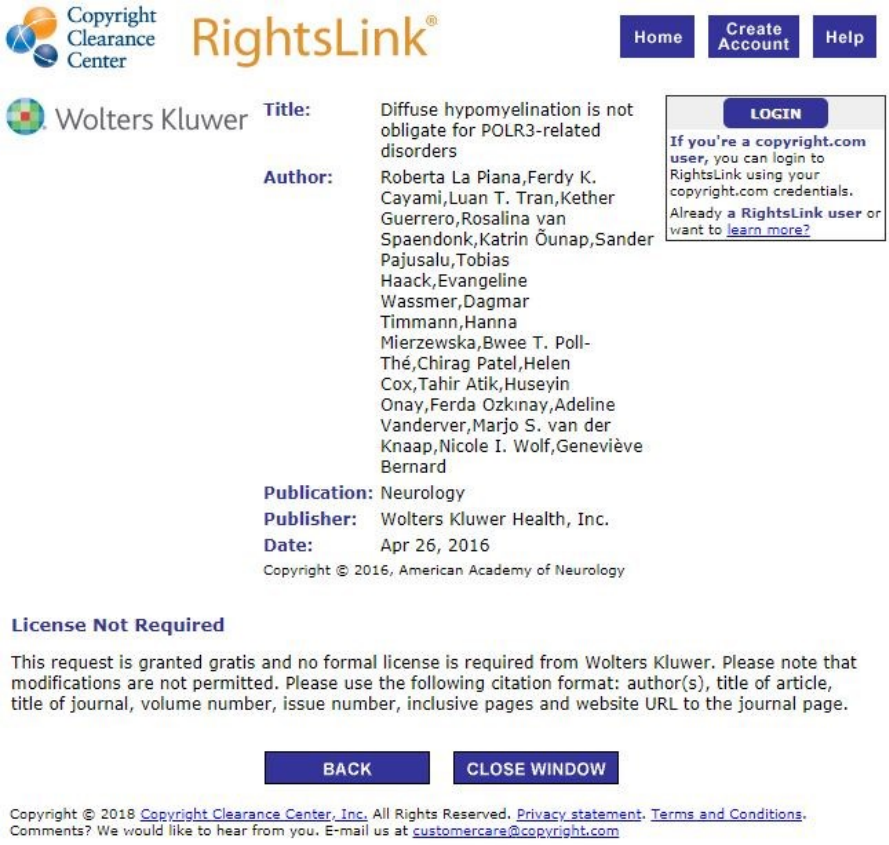

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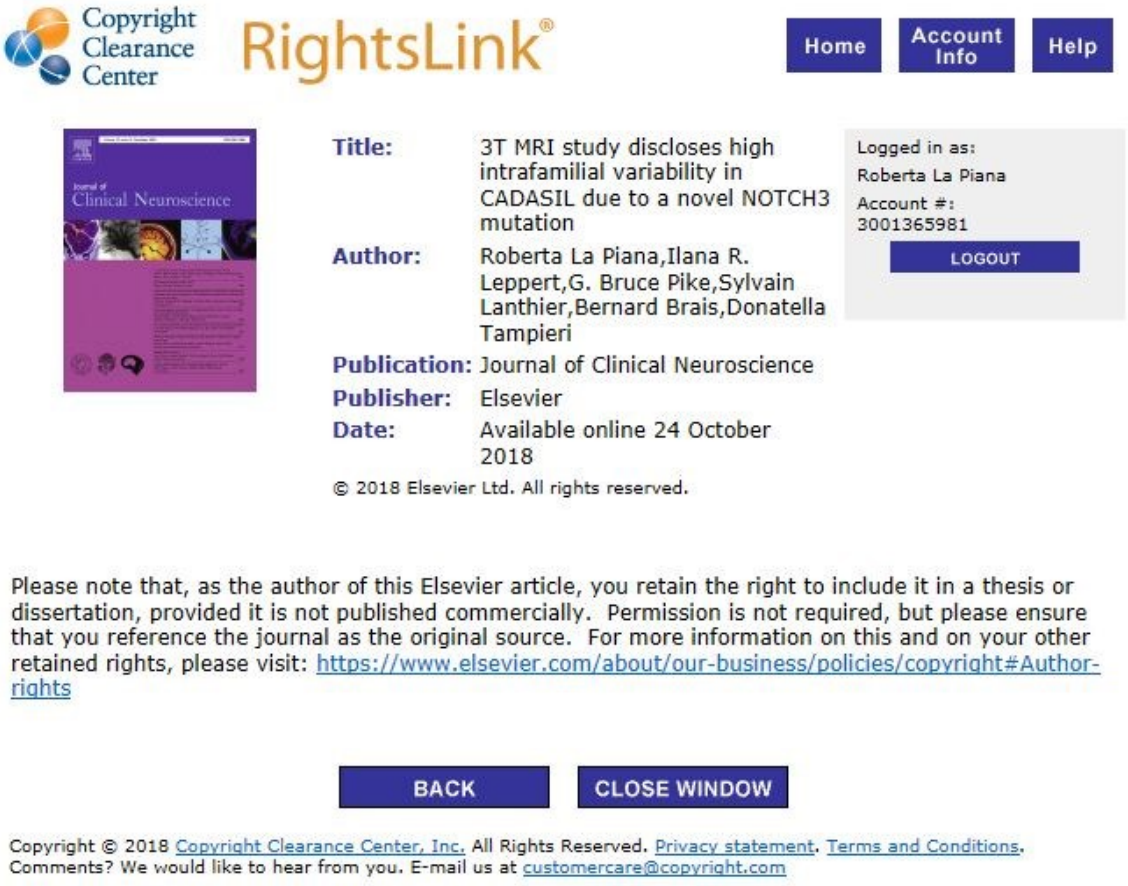

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