Leukodystrophies

Contributions of clinical phenotyping

Eline Hamilton

Leukodystrophies

Contributions of clinical phenotyping

ELINE HAMILTON

Leukodystrophies: Contributions of clinical phenotyping

©2019, E.M.C. Hamilton, Amsterdam, The Netherlands

The research in this thesis was funded by Stiching Optimix. The printing of this thesis was kindly supported by Chipsoft and Stichting Researchfonds Kindergeneeskunde, VU University medical center, Amsterdam, The Netherlands.

Printed by: Ridderprint

Cover Design: Snejana Granatkina

ISBN: 978-94-6375-193-3

An online version of this thesis is available at www.publicatie-online.nl/publicaties/e-hamilton

All rights reserved. Any unauthorized reprint or use of this material is prohibited. No part of this thesis may be reproduced, stored or transmitted in any form or by any means, without written permission of the author or, when applicable, of the publishers of the publications.

VRIJE UNIVERSITEIT

Leukodystrophies:
Contributions of clinical phenotyping

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor
aan de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. V. Subramaniam,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Geneeskunde
op dinsdag 16 april 2019 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Eline Marie Cécile Hamilton

geboren te Nieuwegein

promotoren: prof.dr. M.S. van der Knaap

prof.dr. B.M.J. Uitdehaag

“Life is a kind of music, a symphonic interplay

between genes, cells, organs,

body, and environment”

Denis Noble, The Music of Life

CONTENTS

List of abbreviations		8
Chapter 1	General introduction and outline of this thesis	9
Part I	**Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL)**	
Chapter 2	Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation: clinical and genetic characterization and target for therapy	27
Chapter 2.2	Reply: *DARS2* gene clinical spectrum: new ideas regarding an underdiagnosed leukoencephalopathy	53
Part II	**Megalencephalic leukoencephalopathy with subcortical cysts (MLC)**	
Chapter 3	Megalencephalic leukoencephalopathy with subcortical cysts: characterization of disease variants	59
Part III	**Hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC)**	
Chapter 4	Hypomyelination with atrophy of the basal ganglia and cerebellum: further delineation of the phenotype and genotype-phenotype correlation	79
Chapter 4.2	Reply: *TUBB4A* novel mutation reinforces the genotype-phenotype correlation of hypomyelination with atrophy of the basal ganglia and cerebellum	109
Chapter 4.3	Reply: A novel *TUBB4A* mutation suggests that genotype-phenotype correlation of H-ABC syndrome needs to be revisited	115
Chapter 5	*UFM1* founder mutation in the Roma population causes recessive variant of H-ABC	119
Part IV	**Vanishing White Matter (VWM)**	
Chapter 6	The natural history of Vanishing White Matter (1): disease course	143
Chapter 7	The natural history of Vanishing White Matter (2): quality of life	167

Part V **Conclusions**

Chapter 8 Summary and general discussion 193

Part VI **Appendices**

Chapter 9 Nederlandse samenvatting 233

Overview of publications 243

Dankwoord 245

About the author 249

LIST OF ABBREVIATIONS

AD	Autosomal dominant
AR	Autosomal recessive
ARS	Aminoacyl-tRNA synthetase
CFCS	Communication Function Classification System
CNS	Central nervous system
DYT4	Dystonia type 4
EYFP	Enhanced yellow fluorescent protein
FLAIR	Fluid-attenuated inversion recovery
GMFCS	Gross Motor Function Classification System
GNDS	Guy's Neurological Disability Scale
H-ABC	Hypomyelination with atrophy of the basal ganglia and cerebellum
HRQL	Health-related quality of life
HSC	Hematopoietic stem cell
HUI	Health Utilities Index
LBSL	Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation
LOD	Logarithm of the odds
MACS	Manual Ability Classification System
MLC	Megalencephalic Leukoencephalopathy with Subcortical Cysts
MLPA	Multiplex ligation-dependent probe amplification
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Multiple Sclerosis
mtAspRS	Mitochondrial aspartyl-tRNA synthetase
NGS	Next generation sequencing
SNP	Single nucleotide polymorphism
UBLs	Ubiquitin-like proteins
VWM	Vanishing White Matter
WES	Whole exome sequencing
WM	White matter

Chapter 1

General introduction and outline of this thesis

INTRODUCTION

It was 2012 when a 2-year-old boy was suddenly faced with a life changing disease. Up to that moment, he had been a healthy, active child with a normal development. Now, he suffered from a bacterial infection with high fever and had problems with coordination and gait. He rapidly deteriorated, lost the ability to walk and was admitted to the intensive care unit with reduced consciousness. An MRI scan was made, revealing profound, symmetric signal abnormalities of the white matter of the brain. The physicians suspected a leukodystrophy, a group of brain diseases with often extremely poor prognosis. The parents were informed that their son's life expectancy was estimated to be less than a year, a devastating prospect. After recovering from the infection, the boy surprisingly regained motor ability. He learned to crawl again, and after few months he was able to walk like before his illness. Only some subtle coordination problems persisted. The exact diagnosis remained unknown. At the age of 4 years, he had a small accident while playing in the playground and fell on his head. He lost the ability to walk and developed a tremor of his hands. A new brain scan was made, again revealing white matter abnormalities. The cause was unclear. In the meantime, the parents were expecting a second child; they were told there was a significant risk that this child would also have the leukodystrophy. In the absence of a diagnosis, it was not possible to perform prenatal testing. At the age of 2 years, the second child exhibited symptoms that were reminiscent of the same disorder. Finally, after pursuing multiple second opinions, it became clear that the children had Vanishing White Matter (VWM), a hereditary white matter disorder. Genetic testing confirmed the diagnosis. For the parents, it came as a relief to receive a diagnosis and see the pieces come together, although many questions remained. Even though there was no cure for the disease, the diagnosis allowed the family to focus on adequate medical and preventive care and quality of life for their children, and to perform prenatal testing in a subsequent pregnancy.

"White matter disorders" are all disorders that exclusively or preferentially affect the white matter of the central nervous system. Leukodystrophies are the genetic white matter disorders. There are many different leukodystrophies, with equally many different underlying genetic defects. All are rare. There are, however, so many leukodystrophies, that as a group they are not so rare. They are often highly debilitating. Unfortunately, there is only a limited understanding of the mechanisms underlying leukodystrophies and there is a lack of knowledge of disease characteristics and prognosis. As there is no cure for the majority of diseases, they have an immense impact on the lives of patients and families. During the last three decades, the basic concepts, classification, diagnostic approach and medical management of these disorders have progressed, but there is still a lot to unravel. The study of leukodystrophies is challenging, also owing to the limited number of affected patients known for each disease.

The research reported in this thesis was performed at the Center for Childhood White Matter Disorders in Amsterdam. The purpose of the center is to optimize diagnostics, information and medical care for patients with white matter disorders worldwide and to

obtain new insights into the disease mechanisms for development of treatment. Clinical phenotyping is an important part of this. We here describe the clinical and genetic characteristics of four different leukodystrophies.

WHITE MATTER AND LEUKODYSTROPHIES

The nervous system is the body's electrical information highway and is essential for behavior and ability to think, talk and move. The central nervous system (CNS), comprising the brain and spinal cord, consists of two types of tissue: grey and white matter. The grey matter contains the nerve cell bodies (neurons) that fire to carry messages. The white matter contains the nerve fibers (axons) that are responsible for the communication between nerve cells and the transport of information to the rest of the body. It derives its white color from myelin: a lipid-rich multilamellar membrane coating, which insulates the axons, accelerates the propagation of impulses, and provides trophic support to axons (Figure 1). The production of the myelin sheath (myelination) predominantly takes place in the first 2 years of life by an interplay of oligodendrocytes, astrocytes and axons. Oligodendrocytes and astrocytes are collectively called "macroglia" or "glia". Glia were initially believed to be passive cells that physically supported neurons, hence the name glia meaning "glue". Nowadays it is known that glia play an active role in brain development as well as many central homeostatic processes.[1] Oligodendrocytes' main function is the formation of myelin. Astrocytes are highly versatile cells that serve a wide range of adaptive functions in the CNS, including preservation of the blood brain barrier, signal processing, regulation of neurogenesis and maintenance of extracellular ion balance.

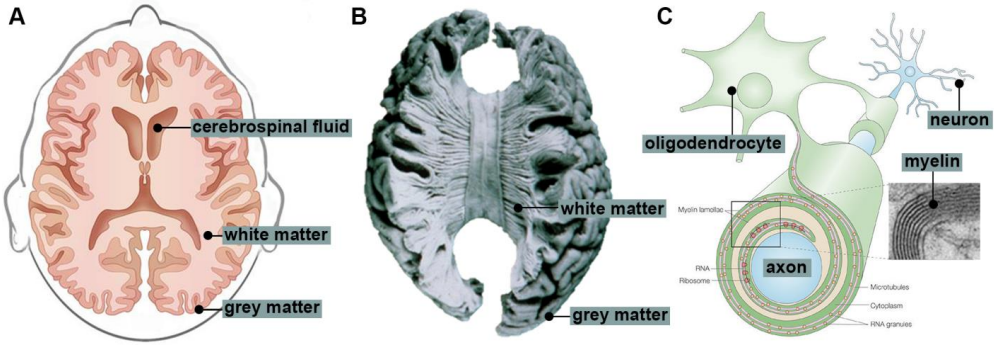


Figure 1 | Myelin and white matter. (**A**) Transverse section of the brain illustrating the surface of the brain consists of a cortex of grey matter, overlying the central white matter. (**B**) Gross dissection of the human brain, disclosing the white matter fibers. (**C**) Mechanism of axon ensheathment by oligodendrocytes, including an electron microscopic picture of the myelin sheath. Adapted with permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience,[2] copyright 2005.

White matter disorders or leukoencephalopathies ("leuko" means white) comprise a broad group of diseases, both acquired and genetic, that exclusively or predominantly affect the white matter of the brain. In the adult population, Multiple Sclerosis (MS) is the most common white matter disorder; the disease is acquired and mostly presents in early-adulthood. Along with aging, there is an increasing incidence of vasculopathy-related white matter changes. In children, white matter diseases are most often genetic. Genetically determined white matter disorders are referred to as leukodystrophies. A prerequisite for the term leukodystrophy is primary involvement of the CNS white matter, irrespective of the structural white matter component involved, the molecular process affected and the disease course.[3,4] If this criterion cannot be met, but white matter abnormalities are present as a phenomenon secondary to neuronal dysfunction or degeneration, the term genetic leukoencephalopathy is applied.[3]

The mechanisms underlying leukodystrophies are diverse, including a primary defect in oligodendrocytes or myelin [permanent deficit in myelin deposition (hypomyelination); loss of previously deposited myelin (demyelination) or myelin vacuolization]; astrocytopathies; leuko-axonopathies; microgliopathies; and leukovasculopathies.[4] Although each disorder is rare, over 30 distinct clinical genetic entities have been defined[3] and with improving gene discovery, the number of identifiable leukodystrophies increases rapidly.[5,6] Collectively, the incidence of inherited white matter disorders is relatively high, with an estimated frequency of one in ~7,600 live births.[7]

CLINICAL MANIFESTATIONS

Leukodystrophies generally manifest with nonspecific clinical signs and symptoms that most often do not allow distinction between different disorders. This causes major difficulties to the diagnosis of patients by physicians. Involvement of the white matter tracts almost universally leads to motor problems, with hypotonia, hypertonia or delayed acquisition of motor milestones in early childhood and regression of motor skills at older ages. Clues that point to the direction of white matter diseases are spasticity, hyperreflexia and ataxia.[8] Cortical grey matter abnormalities on the other hand, are more likely to present with severe seizures and cognitive decline in early stages, whereas diseases primarily affecting deep grey matter structures typically lead to extrapyramidal movement abnormalities like chorea or dystonia.[8] These are not strictly separated features and there often is overlap between these groups. Leukodystrophy phenotypes vary widely between and also within the different disease entities; a single leukodystrophy can be associated with a wide spectrum of phenotypes. Onset is often in childhood or adolescence, but ranges from antenatal life up to late-adulthood. Disease course is mostly progressive, with loss of motor skills. In advanced disease stages, motor dysfunction may significantly impair vital functions including swallowing, chewing, coughing and breathing, leading to premature death. A retrospective surveillance among over 100 children with various leukodystrophies in a period of 9 years revealed an overall mortality of 34% with an average age at death of 8 years.[7] Some leukodystrophy patients, however, show a static or even improving clinical course and some live a

relatively normal life.[4] Additional neurological features, such as changes in head circumference, skin abnormalities, eye abnormalities or extrapyramidal movement abnormalities can support the differentiation between various disorders.[9] Few white matter diseases present with highly specific clinical features that greatly facilitate diagnosis, such as hypodontia in 4H leukostrophy[10] or tendinous xanthomas in Cerebrotendinous Xanthomatosis.[11] The diagnostic work-up may further be aided by focused laboratory and neurophysiological testing.[5]

CLASSIFICATION OF WHITE MATTER DISORDERS

For a long period of time, neuropathology was the only technique to study brain diseases. The first research on white matter disorders dates back to 1838, when Robert Carswell published an illustration of a "peculiar disease state" characterized by greyish areas of atrophy of irregular shape in the brainstem and spinal cord.[12] In the eighteen sixties, Jean-Martin Charcot was impressed by a pattern of tremor and paralysis observed in young adults, who at autopsy were noted to have multiple grey patches scattered throughout the spinal cord, brainstem and brain. He named this pattern "sclérose en plaque dissiminée";[13] the disease is now known as MS. At the end of the 19th century another entity was identified with cases of diffuse lack of myelin in the white matter of the brain, referred to as "diffuse sclerosis".[14] The neuroscientists Pelizaeus and Merzbacher were the first to describe the neuropathology of a white matter disorder with a genetic cause, now referred to as the leukodystrophy Pelizaeus-Merzbacher disease.[15,16] In the 20th century, the first investigators started to recognize separate entities of white matter disorders and pathological descriptions became more refined with the help of histochemistry. Over time, various different classifications have been proposed on the basis of clinical and histological features, biochemical abnormalities or metabolic defects.[17-19]

Because patients with a white matter disorder present non-specific clinical features, establishing a diagnosis is a major challenge. With the introduction of magnetic resonance imaging (MRI) in the nineteen-eighties, the recognition and classification of white matter disorders has taken a tremendous step forward; various novel disease entities were identified and defined.[20,21] Nowadays, imaging of the brain by MRI is the most important first test in the case of a suspected white matter disorder, as it confirms the presence or absence of white matter abnormalities and can also reveal disease-specific features that can lead to diagnosis.[20-22] The concept of MRI pattern recognition is based on the selective vulnerability of different brain structures in different white matter disorders, reflecting the complex functional topography of the brain.[20-22] The core MRI feature in the diagnosis of a leukodystrophy is the presence of hyperintensity of the white matter on T2-weighted images. The first major discriminator is to distinguish between hypomyelinating disorders and other types of white matter disorders.[22] Hypomyelination is characterized by a mild hyperintensity of the white matter on T2-weighted images in combination with a hyperintense (normal), isointense or mildly hypointense white matter signal relative to the cortex on T1-weighted images; other

white matter disorders are characterized by prominent hyperintensity on T2-weighted images and prominent hypointensity on T1-weighted images.[22] The next discriminator is the aspect of the white matter abnormalities (confluent or isolated and multifocal) and the preferential involvement of different brain structures.[9] Additional MRI features, such as swelling or atrophy, calcifications, the presence of cysts or white matter rarefaction (visualized best by use of the fluid-attenuated inversion recovery (FLAIR) sequence) and/or contrast enhancement assist in the further differentiation between different white matter disorders. Some diseases have biochemical signatures that can be identified by proton MR spectroscopy (MRS).

For patients and families the establishment of a specific diagnosis and phenotypic designation are of major significance. Having a diagnosis often brings relief, particularly to those families who have been seeking a diagnosis for a long time. It opens doors to medical facilities, assists in prognostication, guides disease management and helps families to make decisions on medical procedures. Although leukodystrophies are now frequently recognized on MRI, establishing a specific diagnosis remains a difficult task.

GENETICS

When a white matter disorder is suspected on the basis of MRI features, certain disease characteristics argue for a genetic rather than acquired disease: the presence of confluent and bilaterally symmetric white matter abnormalities as compared to multifocal isolated lesions with an asymmetrical distribution on MRI,[22] familial occurrence, the absence of features suggestive of acquired conditions (e.g. inflammatory or vascular abnormalities) and the presence of clinical, radiological or biochemical findings that point to a specific genetic disorder.

Leukodystrophies are caused by mutations in single genes and follow a Mendelian inheritance pattern. The traits can be autosomal recessive with homozygous or compound heterozygous gene variants, X-linked recessive, autosomal dominant with paternal or maternal inheritance, autosomal dominant *de novo,* or mitochondrial with maternal inheritance.

Rare monogenic disorders have provided unique opportunities to identify genes mutated in humans and address biological questions on the underlying pathogenesis. Throughout the years, the landscape of Genetics has undergone incredible advancements. The history of the identification of causal gene mutations started in the eighties, with the “candidate gene approach”: testing whether mutations in a known gene coding for a protein with a specific biological role are involved in the genetic predisposition to a human disease. This approach was also successful for the field of white matter disorders; focusing on myelin associated genes resulted in the identification of several mutated genes, such as the X-linked myelin gene proteolipid protein 1 (*PLP1*) for Pelizaeus-Merzbacher disease,[23] and the *GALC* gene, encoding the enzyme galactocerebrosidase, which is involved in the metabolism of a major myelin lipid, for Krabbe disease.[24,25] Next, genetic linkage studies were initiated to detect additional genes mutated in leukodystrophies. This approach requires genotyping of relatively large

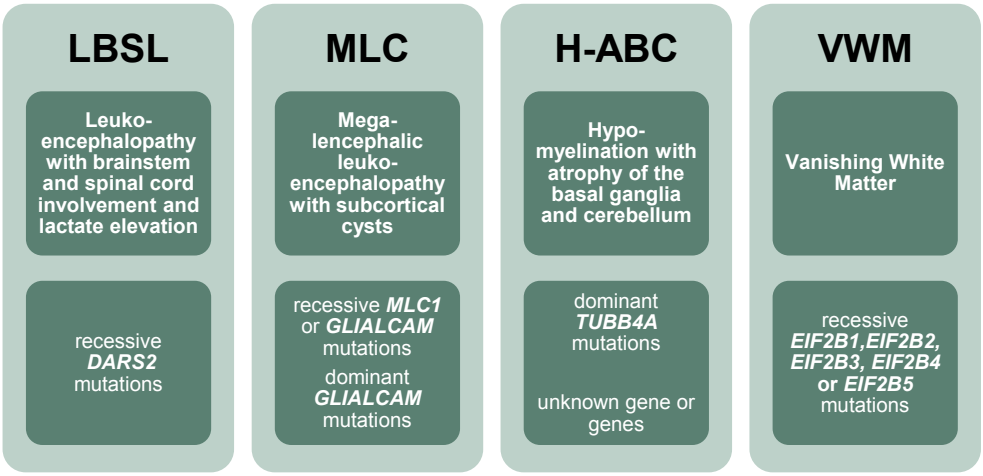
numbers of samples from patients and unaffected family members to identify the chromosomal location of the mutated gene and then narrow down the critical region.[26] The genes located in the candidate region are screened using the Sanger sequencing technique.[27] Linkage studies resulted in the identification of numerous genes such as *TREX1*, encoding 3-prime repair exonuclease 1, mutated in Aicardi-Goutières syndrome,[28] *DARS2*, encoding a tRNA synthetase, in Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation (LBSL),[29] and *MLC1*, encoding a membrane protein, in Megalencephalic leukoencephalopathy with subcortical cysts (MLC).[30] Linkage studies are extremely laborious and have often been unsuccessful due to small kindred sizes, genetic heterogeneity and *de novo* inheritance patterns. Alternative strategies, such as the application of quantitative proteomic analysis to identify *GLIALCAM* as the second causal gene for MLC,[31] have resulted in a few additional successes, but still many leukodystrophy patients remained without a molecular diagnosis.

The recent introduction of high-throughput, genome-wide technologies that are able to rapidly sequence the base pairs of the entire genome or exome (consisting of all coding regions of the genome) led to revolutionary changes in the field of genetics. As of 2010, multiple research groups have demonstrated the power of Next-Generation-Sequencing (NGS) through the identification of pathogenic mutations in previously intractable genetic disorders. Whole exome sequencing (WES) is presently the most successful NGS technique; it involves a query in the entire coding sequence of the human genome by capturing and sequencing the exons of all annotated protein-coding genes.[32] Its application led to an exponential increase in the number of molecularly determined, ultra-rare leukodystrophies. In a study in a cohort of 71 unsolved patients suspected of a leukodystrophy, the application of WES resulted in the detection of diagnostic pathogenic variants in 35%.[33] A retrospective study among three cohorts of unclassified leukodystrophy patients demonstrated that with the introduction of WES, the number of classified cases has risen from 50% in 2010 to 80% in 2016.[34] Interestingly, the yield did not only involve new genes, but also led to the expansion of the clinical spectrum of known disorders as well as the recognition of variants in genes that had thus far only been associated with entirely different disease phenotypes, not specifically involving the white matter or even the CNS.[34]

The advances in genetic testing have been a great step forward, as a definitive, genetically confirmed diagnosis is crucial for genetic and clinical counseling and enables functional studies that enhance the understanding of the molecular basis of a disorder.

OVERVIEW OF DISEASES DISCUSSED IN THIS THESIS

The work described in this thesis is focused on four relatively "new" leukodystrophies: LBSL, MLC, H-ABC and VWM.



AR, autosomal recessive; AD, autosomal dominant

Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL)

LBSL was identified in 2003 in 8 patients, on the basis of a distinct MRI pattern consisting of inhomogeneous cerebral white matter abnormalities and selective involvement of brainstem and spinal cord tracts (Table 1).[35,36] Patients generally have a normal early development and display motor deterioration in childhood or adolescence, with signs of ataxia and spasticity, involving the legs more than the arms. At neurological examination, patients typically have decreased position and vibration sense.[35] Most patients have elevated lactate in the abnormal white matter on MRS (Figure 2), suggesting a defect in mitochondrial energy production. In 2007, genetic linkage analysis revealed that patients have recessive mutations in *DARS2*, a nuclear gene encoding the mitochondrial aspartyl-tRNA synthetase (mtAspRS), an enzyme involved in the mitochondrial protein synthesis.[29] This discovery enabled further study of the clinical disease spectrum and the disease mechanism at a molecular and cellular level. Amino acylation assays revealed that different *DARS2* mutations have different effects on the expression, enzyme activity, localization and dimerization of mtAspRS.[37] Most LBSL patients have one mutation in intron 2 of *DARS2. A* splicing reporter construct revealed that such mutations have different effects on the splicing of exon 3 in different cell types.[38]

Table 1 | MRI criteria for the diagnosis LBSL[36]

	Signal abnormities of:
Major criteria	Cerebral white matter (relative sparing of subcortical white matter)
	Dorsal columns and lateral corticospinal tracts of the spinal cord
	Pyramids at the level of the medulla oblongata or decussation of the medial lemniscus or both
Minor criteria	Splenium of the corpus callosum
	Posterior limb of the internal capsule
	Superior and inferior cerebellar peduncles
	Intraparenchymal part of the trigeminal nerve
	Mesencephalic trigeminal tracts
	Anterior spinocerebellar tracts in medulla oblongata
	Cerebellar white matter

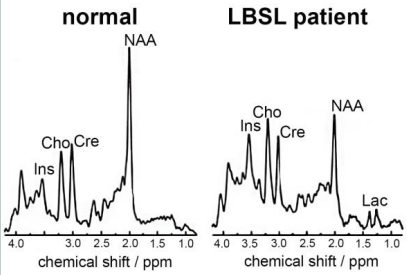


Figure 2 | Lactate elevation in MRS. In the LBSL patient a lactate peak is visible at 1.3 ppm in the abnormal white matter.

Megalencephalic leukoencephalopathy with subcortical cysts (MLC)

MLC is an infantile onset disease characterized by macrocephaly and delayed onset of neurological deterioration. The disease was recognized in the nineties independently in India and in the Netherlands.[39,40] A few years after disease onset, patients develop cerebellar ataxia and spasticity; they may become wheelchair dependent after several years. Epilepsy is a common feature.[41] No systematic study of lifespan has been performed, but mortality is generally low.[41] MRI shows diffusely abnormal and swollen cerebral white matter and subcortical cysts (Table 2).[22,41]

Table 2 | MRI criteria for the diagnosis MLC[22,41]

Diffuse cerebral white matter signal abnormality
Swelling of the abnormal white matter
Subcortical cysts, defined as areas with same signal intensity as CSF on all pulse sequences including FLAIR, or rarefaction (signal intensity close to CSF but not the same) in the anterior temporal region and possibly also in the frontal and parietal regions
Central structures including corpus callosum and internal capsule are relatively spared
Cortical and central grey matter structures are not affected
Brainstem and cerebellar white matter show no or mild signal abnormality and are not swollen

Brain biopsies collected from MLC patients revealed intramyelinic vacuoles within the white matter and the end-feet of astrocytes (Figure 3).[42,43] In 2000, genetic linkage studies performed by a Turkish-French consortium revealed a locus for MLC on the long arm of chromosome 22.[44] A year later, the information of multiple families present at the VU University medical center in Amsterdam was used to identify the mutated gene, *KIAA0027,* which was renamed into *MLC1*.[30] The gene's function was unknown at that time, and experiments were initiated to elucidate its features. In 2010 it was described that some MLC patients without an *MLC1* mutation showed major MRI improvement and lacked motor decline. MRI abnormalities initially resembled those of patients with classic

MLC, but from the second year of life, the white matter abnormalities decreased and largely or complete normalized; the cysts decreased in size and sometimes disappeared.[45] It was found that in addition to *MLC1, GLIALCAM* was a second gene mutated in this disease: patients with recessive mutations have the classic phenotype and patients with dominant mutations have the improving phenotype.[31]

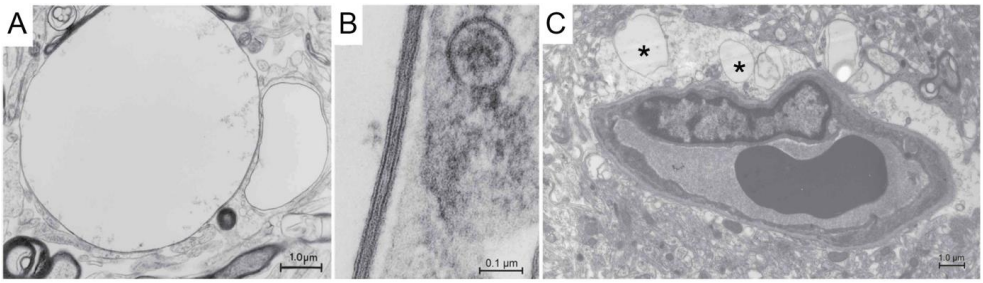


Figure 3 | Patient brain tissue with vacuoles. (**A**) Electron microscopy images of a brain biopsy sample from an MLC patient show vacuoles in a myelin sheath. (**B**) Magnification shows that the vacuoles are lined by a membrane with the typical periodicity of myelin. (**C**) Vacuoles are also present in astrocytic endfeet (asterisks). Adapted from van der Knaap et al.[42] with permission from Springer Nature, copyright 1996 and from Duarri *et al*.[43] with permission from Elsevier, copyright 2011.

During the past years, the understanding of the disease mechanism of MLC has taken major steps forward. It is now known that MLC1 is an astrocyte specific membrane protein involved in brain ion and water homeostasis[46,47] and that GlialCAM is a chaperone of MLC1 that ensures its localization in the membrane of astrocytic endfeet.[31,48] Mutations in these genes result in a defect in brain volume regulation by astrocytes and with that, white matter edema. Research on MLC has increased the knowledge of not only the disease, but also brain volume regulation in general, which is an elementary aspect of multiple neurological diseases.

Hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC)

In 2002, H-ABC was recognized as a new disease entity in a group of patients with variably delayed early development, extrapyramidal movement abnormalities, ataxia and spasticity.[49] They exhibited uniform and highly characteristic brain MRI findings consisting of diffuse hypomyelination and atrophy of the basal ganglia (neostriatum) and the cerebellum (Table 3).[49] The disappearance of the basal ganglia without evidence of remaining scar tissue on MRI was indicative of atrophy by means of apoptosis rather than necrosis. In keeping with the MRI features, histopathology studies showed a lack of myelin in the cerebral hemispheres with evidence of both hypomyelination as well as low-grade myelin loss and a subtotal degeneration of the putamen with loss of neuronal cells.[50] The findings suggested that the pathophysiological mechanism in H-ABC relies on two processes: a disturbance of normal development and degeneration.
As parents of H-ABC patients were not related and no siblings were affected, a dominant

de novo inheritance pattern was suspected, restraining the application of linkage studies to identify the underlying genetic defect. The arrival of NGS facilitated new search strategies: in 2013, 11 patients were selected on the basis of very strict clinical and MRI criteria. WES analysis revealed they all harbored the same heterozygous *de novo* mutation in the *TUBB4A* gene, encoding tubulin β-4A.[51] In the same period, also Dystonia type 4 (alias "whispering dysphonia") was found to be associated with dominant *TUBB4A* mutations.[52,53] Patients presented at adolescent or adult age and MRI of the brain revealed no abnormalities. It was puzzling that such divergent syndromes were caused by defects in the same gene.

Table 3 | MRI characteristics of H-ABC[49]

No abnormalities of the cerebral cortex
Diffuse cerebral hypomyelination; over time further myelin loss and white matter atrophy
Major criterion: small or absent putamen, without evidence of lesions in this region
Minor criterion: reduced size of the head of the caudate nucleus
No abnormalities of thalamus and globus pallidus
Atrophy of the cerebellum, especially at the vermis, characterized by shrunken folia and large cerebellar fissures

Vanishing White Matter (VWM)

In 1993, 1994 and 1996 a disease was described in three groups of children, including some affected siblings.[54-56] Patients exhibited similar clinical features[54-56] and shared an MRI pattern of diffuse, homogeneous white matter abnormalities with a signal almost identical to the signal of the ventricles (Figure 4B, Table 4).[56] MRS revealed profound decrease or absence of brain metabolites in the abnormal white matter.[55,56] Autopsy findings substantiated disappearance of the cerebral white matter and the disease was named Vanishing White Matter (Figure 4D).[56,57]

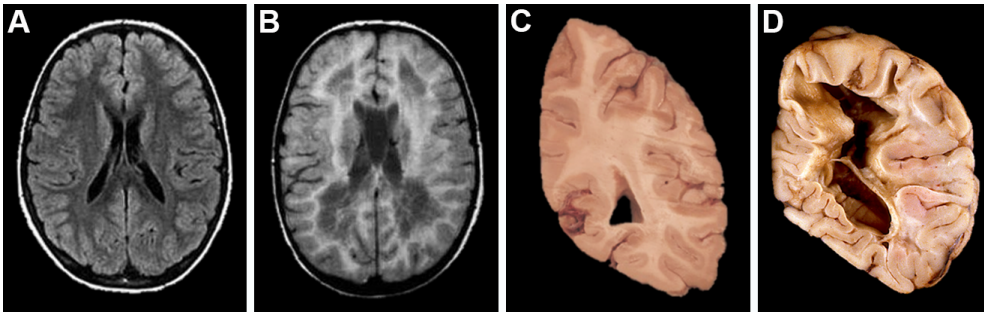


Figure 4 | MRI and pathology in VWM. (**A**) Axial FLAIR image of a control shows a hypointense signal of the cerebral white matter relative to the cortex; (**B**) in the VWM patient the subcortical white matter has an abnormal hyperintense signal and the deep white matter has a low signal intensity, similar to CSF, indicative of cystic degeneration. (**C**) Macroscopic coronal section of a cerebral hemisphere in a control and (**D**) in a VWM patient with end stage disease, showing that virtually all white matter is absent.

The clinical phenotype of VWM consists of chronic progressive neurological deterioration with ataxia and spasticity, as well as unusual episodes of rapid and major decline provoked by stressors such as fever and minor head trauma.[56] The disease was initially described as a fatal disorder affecting young children,[55,56,58] but over time it became clear that there is wide variability in age of onset and severity.[57,59] Although still rare, VWM turned out to be one of the more prevalent leukodystrophies.[7,20] Considering the familial occurrence, autosomal recessive inheritance was suspected. A genetic linkage study was established, focusing on Dutch patients.[60,61] Most of the families involved originated from a rural region in the east of the Netherlands where the population tented not to migrate or intermarry with people from other regions. A founder effect was suspected and a common ancestor was identified, which enabled identification of linkage with a locus on chromosome 3 and helped narrowing down of the candidate region, resulting in the detection of the first gene mutated in VWM: *EIF2B5*.[60-62] Another Dutch founder effect in the south of The Netherlands led to the detection of a second gene: *EIF2B2*.[62] Subsequently, it was shown that VWM can be caused by mutations in any of the genes (*EIF2B1-5*) encoding the five subunits of the pentameric complex eukaryotic translation initiation factor eIF2B (eIF2Bα, β, γ, δ, and ε).[61,63] The factor eIF2B is an enzyme that is present in all cells and is essential for the initiation of translation of mRNAs into proteins and for the regulation of the rate of protein synthesis under different conditions, especially stress.[64,65]

Table 4 | MRI criteria for the diagnosis of VWM[56]

Major criteria	Either diffuse or extensive signal abnormalities of the cerebral white matter; the immediately subcortical white matter may be spared
	Part or all of the abnormal white matter has a signal intensity close to or the same as CSF on proton density or FLAIR images, suggestive of white matter rarefaction or cystic destruction
	If proton density and FLAIR images suggest that all cerebral white matter has disappeared, there is a fluid-filled distance between ependymal lining and cortex, but not a total collapse of the white matter
	The disappearance of the cerebral white matter occurs in a diffuse “melting away” pattern
	The temporal lobes are relatively spared, in the extent of the abnormal signal, degree of cystic destruction, or both
	No contrast enhancement
Minor criteria	Within abnormal white matter there is a pattern of radiating stripes on sagittal and coronal T1-weighted or FLAIR images; on axial images dots and stripes are seen within the abnormal white matter as cross-sections of the stripes
	Lesions within the central tegmental tracts in the pontine tegmentum
	Involvement of the inner rim of the corpus callosum, whereas the outer rim is spared

PATIENT CARE

With the growing number of defined disorders and the advances in imaging technology and genetic testing, the majority of leukodystrophy cases now receive a molecular diagnosis. A diagnostic test alone is, however, meaningless if not placed into a clinical context. In order to adequately counsel patients and families, a profound knowledge of the natural disease course of a disorder is warranted, including the variability in clinical severity. As leukodystrophies share many of the clinical signs and symptoms, a common

approach to symptomatic and preventive treatment is feasible. A helpful guideline for standard care was recently developed by a leukodystrophy expert group, comprising treatment goals for the most common symptoms such as motor impairment, seizures and spasticity, an overview of common symptomatic medication and strategies for potentially preventable medical complications.[66] Nonetheless, specific leukodystrophies also come with disease-specific symptoms and disease-specific risk factors: stringent phenotyping and systematic study of prognostic factors are therefore elementary for the improvement of medical care. Such studies are scarce in the field of leukodystrophies due to the rarity of the disorders. Especially in newly defined disorders, the knowledge of the clinical variability and natural disease course is very incomplete, hampering counseling and adequate patient care.

Owing to recent technological developments such as gene therapy and stem cell therapy, there is hope for therapeutic options for a growing number of leukodystrophies.[67] Also for the planning and evaluation of treatment trials, an in-depth insight in the phenotypic spectrum of a disorder is essential. Although the results of the first human trials are promising,[68-70] for most leukodystrophies a curative therapy is still years away. Until then, clinicians should aim for the most optimal medical care and quality of life decisions in patients suffering from leukodystrophy today.

AIMS AND OUTLINE OF THIS THESIS

The general theme of this thesis concerns the clinical and genetic aspects of four recently identified leukodystrophies. The project had three aims of equal importance. The first aim was to better delineate the phenotypic and genotypic spectrum of leukodystrophies in order to improve patient and genetic counseling. The second aim was to provide natural history data, which is fundamental for the planning and evaluation of future therapeutic trials. The third aim was to enhance the understanding of the underlying disease mechanisms, a crucial step for the development of treatment strategies.

In chapter 2, we describe the clinical characteristics and *DARS2* mutations of a cohort of 78 LBSL patients and explore the application of agents that may modulate the genetic defect. In chapter 3 we address the clinical and radiological characteristics of different variants of MLC among 242 patients and the identification of differentiating features. In chapter 4 we give an overview of phenotypic characteristics and *TUBB4A* mutations in 42 H-ABC patients and establish a genotype-phenotype correlation. A small group of H-ABC patients remained without a molecular diagnosis. In chapter 5 we report on the identification of the second gene mutated in H-ABC in 16 patients with a shared Roma ethnic background. In chapter 6 we present the results of a 12.5 year natural history study on disease course in 296 genetically proven VWM patients. Next, in chapter 7 we provide insight into dimensions of disability, health-related quality of life and age of onset related differences in disease course in VWM. The main findings and implications of these studies are summarized and discussed in chapter 8.

REFERENCES

1. Domingues HS, Portugal CC, Socodato R, Relvas JB. Oligodendrocyte, Astrocyte, and Microglia Crosstalk in Myelin Development, Damage, and Repair. Front Cell Dev Biol 2016;4:71.
2. Sherman DL, Brophy PJ. Mechanisms of axon ensheathment and myelin growth. Nat Rev Neurosci 2005;6:683-690.
3. Vanderver A, Prust M, Tonduti D, et al. Case definition and classification of leukodystrophies and leukoencephalopathies. Mol Genet Metab 2015;114:494-500.
4. van der Knaap MS, Bugiani M. Leukodystrophies: a proposed classification system based on pathological changes and pathogenetic mechanisms. Acta Neuropathol 2017.
5. Kohlschutter A, Eichler F. Childhood leukodystrophies: a clinical perspective. Expert Rev Neurother 2011;11:1485-1496.
6. Kevelam SH, Steenweg ME, Srivastava S, et al. Update on Leukodystrophies: A Historical Perspective and Adapted Definition. Neuropediatrics 2016.
7. Bonkowsky JL, Nelson C, Kingston JL, Filloux FM, Mundorff MB, Srivastava R. The burden of inherited leukodystrophies in children. Neurology 2010;75:718-725.
8. Barkovich AJ. An approach to MRI of metabolic disorders in children. J Neuroradiol 2007;34:75-88.
9. Parikh S, Bernard G, Leventer RJ, et al. A clinical approach to the diagnosis of patients with leukodystrophies and genetic leukoencephelopathies. Mol Genet Metab 2015;114:501-515.
10. Wolf NI, Harting I, Boltshauser E, et al. Leukoencephalopathy with ataxia, hypodontia, and hypomyelination. Neurology 2005;64:1461-1464.
11. Cali JJ, Hsieh CL, Francke U, Russell DW. Mutations in the bile acid biosynthetic enzyme sterol 27-hydroxylase underlie cerebrotendinous xanthomatosis. J Biol Chem 1991;266:7779-7783.
12. Carswell R. Pathological anatomy: illustrations on elementary forms of disease. London: Longman, 1938.
13. Charcot J. Histologie de la sclérose en plaques. Gaz Hôp Civils Milit (Paris) 1868;41:554-558.
14. Heubner O. Uber diffuse Hirnsklerose. Charite-Ann 1897;22:298-310.
15. Pelizaeus F. Ueber eine eigenthilmliche Form spastischer Lahmung mit Cerebralerscheinungen auf hereditarer Grundlage (Multiple Sklerose). Arch Psychiat Nervenkr, 1885;16:698-710.
16. Merzbacher L. Eine eigenartige familiärhereditäre Erkrankungsform (Aplasia axialis extra-corticalis congenita). Z Gesamte Neurol Psychiat 1910;3:1-138.
17. Poser CM. Leukodystrophy and the concept of dysmyelination. Arch Neurol 1961;4:323-332.
18. Poser C. The dysmyelinating diseases. In: Baker A, Joynt R, eds. Clinical neurology vol 3, chap 34. Philadelphia: Harper and Row, 1987.
19. Blackwood W, Cumings JN. A histological and chemical study of three cases of diffuse cerebral sclerosis. J Neurol Neurosurg Psychiatry 1954;17:33-49.
20. van der Knaap MS, Breiter SN, Naidu S, Hart AA, Valk J. Defining and categorizing leukoencephalopathies of unknown origin: MR imaging approach. Radiology 1999;213:121-133.
21. van der Knaap MS, Valk J, de Neeling N, Nauta JJ. Pattern recognition in magnetic resonance imaging of white matter disorders in children and young adults. Neuroradiology 1991;33:478-493.
22. Schiffmann R, van der Knaap MS. Invited article: an MRI-based approach to the diagnosis of white matter disorders. Neurology 2009;72:750-759.
23. Koeppen AH, Ronca NA, Greenfield EA, Hans MB. Defective biosynthesis of proteolipid protein in Pelizaeus-Merzbacher disease. Ann Neurol 1987;21:159-170.
24. Ben-Yoseph Y, Hungerford M, Nadler HL. The nature of mutation in Krabbe disease. Am J Hum Genet 1978;30:644-652.
25. Rafi MA, Luzi P, Chen YQ, Wenger DA. A large deletion together with a point mutation in the GALC gene is a common mutant allele in patients with infantile Krabbe disease. Hum Mol Genet 1995;4:1285-1289.
26. Pulst SM. Genetic linkage analysis. Arch Neurol 1999;56:667-672.
27. Sanger F, Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol 1975;94:441-448.

28. Crow YJ, Hayward BE, Parmar R, et al. Mutations in the gene encoding the 3'-5' DNA exonuclease TREX1 cause Aicardi-Goutieres syndrome at the AGS1 locus. Nat Genet 2006;38:917-920.
29. Scheper GC, van der Klok T, van Andel RJ, et al. Mitochondrial aspartyl-tRNA synthetase deficiency causes leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation. Nat Genet 2007;39:534-539.
30. Leegwater PA, Yuan BQ, van der Steen J, et al. Mutations of MLC1 (KIAA0027), encoding a putative membrane protein, cause megalencephalic leukoencephalopathy with subcortical cysts. Am J Hum Genet 2001;68:831-838.
31. Lopez-Hernandez T, Ridder MC, Montolio M, et al. Mutant GlialCAM causes megalencephalic leukoencephalopathy with subcortical cysts, benign familial macrocephaly, and macrocephaly with retardation and autism. Am J Hum Genet 2011;88:422-432.
32. Ng SB, Turner EH, Robertson PD, et al. Targeted capture and massively parallel sequencing of 12 human exomes. Nature 2009;461:272-276.
33. Vanderver A, Simons C, Helman G, et al. Whole exome sequencing in patients with white matter abnormalities. Ann Neurol 2016;79:1031-1037.
34. Kevelam SH, Steenweg ME, Srivastava S, et al. Update on Leukodystrophies: A Historical Perspective and Adapted Definition. Neuropediatrics 2016;47:349-354.
35. van der Knaap MS, van der Voorn P, Barkhof F, et al. A new leukoencephalopathy with brainstem and spinal cord involvement and high lactate. Ann Neurol 2003;53:252-258.
36. Steenweg ME, van Berge L, van Berkel CG, et al. Early-onset LBSL: how severe does it get? Neuropediatrics 2012;43:332-338.
37. van Berge L, Kevenaar J, Polder E, et al. Pathogenic mutations causing LBSL affect mitochondrial aspartyl-tRNA synthetase in diverse ways. Biochem J 2013;450:345-350.
38. van Berge L, Dooves S, van Berkel CG, Polder E, van der Knaap MS, Scheper GC. Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation is associated with cell-type-dependent splicing of mtAspRS mRNA. Biochem J 2012;441:955-962.
39. van der Knaap MS, Barth PG, Stroink H, et al. Leukoencephalopathy with swelling and a discrepantly mild clinical course in eight children. Ann Neurol 1995;37:324-334.
40. Singhal BS, Gursahani RD, Udani VP, Biniwale AA. Megalencephalic leukodystrophy in an Asian Indian ethnic group. Pediatr Neurol 1996;14:291-296.
41. van der Knaap MS, Boor I, Estevez R. Megalencephalic leukoencephalopathy with subcortical cysts: chronic white matter oedema due to a defect in brain ion and water homoeostasis. Lancet Neurol 2012;11:973-985.
42. van der Knaap MS, Barth PG, Vrensen GF, Valk J. Histopathology of an infantile-onset spongiform leukoencephalopathy with a discrepantly mild clinical course. Acta Neuropathol 1996;92:206-212.
43. Duarri A, Lopez de Heredia M, Capdevila-Nortes X, et al. Knockdown of MLC1 in primary astrocytes causes cell vacuolation: a MLC disease cell model. Neurobiol Dis 2011;43:228-238.
44. Topcu M, Gartioux C, Ribierre F, et al. Vacuoliting megalencephalic leukoencephalopathy with subcortical cysts, mapped to chromosome 22qtel. Am J Hum Genet 2000;66:733-739.
45. van der Knaap MS, Lai V, Kohler W, et al. Megalencephalic leukoencephalopathy with cysts without MLC1 defect. Ann Neurol 2010;67:834-837.
46. Ridder MC, Boor I, Lodder JC, et al. Megalencephalic leucoencephalopathy with cysts: defect in chloride currents and cell volume regulation. Brain 2011;134:3342-3354.
47. Dubey M, Bugiani M, Ridder MC, et al. Mice with megalencephalic leukoencephalopathy with cysts: a developmental angle. Ann Neurol 2015;77:114-131.
48. Capdevila-Nortes X, Lopez-Hernandez T, Apaja PM, et al. Insights into MLC pathogenesis: GlialCAM is an MLC1 chaperone required for proper activation of volume-regulated anion currents. Hum Mol Genet 2013;22:4405-4416.
49. van der Knaap MS, Naidu S, Pouwels PJ, et al. New syndrome characterized by hypomyelination with atrophy of the basal ganglia and cerebellum. AJNR Am J Neuroradiol 2002;23:1466-1474.
50. van der Knaap MS, Linnankivi T, Paetau A, et al. Hypomyelination with atrophy of the basal ganglia and cerebellum: follow-up and pathology. Neurology 2007;69:166-171.
51. Simons C, Wolf NI, McNeil N, et al. A de novo mutation in the beta-tubulin gene TUBB4A results in the leukoencephalopathy hypomyelination with atrophy of the basal ganglia and cerebellum. Am J Hum Genet 2013;92:767-773.

52. Hersheson J, Mencacci NE, Davis M, et al. Mutations in the autoregulatory domain of beta-tubulin 4a cause hereditary dystonia. Ann Neurol 2013;73:546-553.
53. Lohmann K, Wilcox RA, Winkler S, et al. Whispering dysphonia (DYT4 dystonia) is caused by a mutation in the TUBB4 gene. Ann Neurol 2013;73:537-545.
54. Schiffmann R, Moller JR, Trapp BD, et al. Childhood ataxia with diffuse central nervous system hypomyelination. Ann Neurol 1994;35:331-340.
55. Hanefeld F, Holzbach U, Kruse B, Wilichowski E, Christen HJ, Frahm J. Diffuse white matter disease in three children: an encephalopathy with unique features on magnetic resonance imaging and proton magnetic resonance spectroscopy. Neuropediatrics 1993;24:244-248.
56. van der Knaap MS, Barth PG, Gabreels FJ, et al. A new leukoencephalopathy with vanishing white matter. Neurology 1997;48:845-855.
57. van der Knaap MS, Kamphorst W, Barth PG, Kraaijeveld CL, Gut E, Valk J. Phenotypic variation in leukoencephalopathy with vanishing white matter. Neurology 1998;51:540-547.
58. Schiffmann R, Fogli A, van der Knaap MS, Boespflug-Tanguy O. Childhood Ataxia with Central Nervous System Hypomyelination/Vanishing White Matter. In: Pagon RA, Adam MP, Ardinger HH, et al., eds. GeneReviews(R). Seattle (WA)1993.
59. van der Knaap MS, van Berkel CG, Herms J, et al. eIF2B-related disorders: antenatal onset and involvement of multiple organs. Am J Hum Genet 2003;73:1199-1207.
60. Leegwater PA, Konst AA, Kuyt B, et al. The gene for leukoencephalopathy with vanishing white matter is located on chromosome 3q27. Am J Hum Genet 1999;65:728-734.
61. Leegwater PA, Pronk JC, van der Knaap MS. Leukoencephalopathy with vanishing white matter: from magnetic resonance imaging pattern to five genes. J Child Neurol 2003;18:639-645.
62. Leegwater PA, Vermeulen G, Konst AA, et al. Subunits of the translation initiation factor eIF2B are mutant in leukoencephalopathy with vanishing white matter. Nat Genet 2001;29:383-388.
63. van der Knaap MS, Leegwater PA, Konst AA, et al. Mutations in each of the five subunits of translation initiation factor eIF2B can cause leukoencephalopathy with vanishing white matter. Ann Neurol 2002;51:264-270.
64. Dever TE. Gene-specific regulation by general translation factors. Cell 2002;108:545-556.
65. Pavitt GD. eIF2B, a mediator of general and gene-specific translational control. Biochem Soc Trans 2005;33:1487-1492.
66. Van Haren K, Bonkowsky JL, Bernard G, et al. Consensus statement on preventive and symptomatic care of leukodystrophy patients. Mol Genet Metab 2015;114:516-526.
67. Helman G, Van Haren K, Bonkowsky JL, et al. Disease specific therapies in leukodystrophies and leukoencephalopathies. Mol Genet Metab 2015;114:527-536.
68. Eichler F, Duncan C, Musolino PL, et al. Hematopoietic Stem-Cell Gene Therapy for Cerebral Adrenoleukodystrophy. N Engl J Med 2017;377:1630-1638.
69. Leone P, Shera D, McPhee SW, et al. Long-term follow-up after gene therapy for canavan disease. Sci Transl Med 2012;4:165ra163.
70. Gupta N, Henry RG, Strober J, et al. Neural stem cell engraftment and myelination in the human brain. Sci Transl Med 2012;4:155ra137.

Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation

Chapter 2

Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation: clinical and genetic characterization and target for therapy

Laura van Berge,* Eline M.C. Hamilton,* Tarja Linnankivi, Graziella Uziel, Marjan E. Steenweg, Pirjo Isohanni, Nicole I. Wolf, Ingeborg Krägeloh-Mann, Nils J. Brautaset, P. Ian Andrews, Brigit A. de Jong, Malak al Ghamdi, Wessel N. van Wieringen, LBSL Research Group, Bakhos A. Tannous, Esther Hulleman, Thomas Würdinger, Carola G.M. van Berkel, Emiel Polder, Truus E.M. Abbink, Eduard A. Struys, Gert C. Scheper, Marjo S. van der Knaap

*These authors contributed equally to this work

Brain. 2014;137:1019-1029

ABSTRACT

Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation is a disorder caused by recessive mutations in the gene *DARS2*, which encodes mitochondrial aspartyl-tRNA synthetase. Recent observations indicate that the phenotypic range of the disease is much wider than initially thought. Currently, no treatment is available. The aims of our study were (i) to explore a possible genotype–phenotype correlation; and (ii) to identify potential therapeutic agents that modulate the splice site mutations in intron 2 of *DARS2*, present in almost all patients. A cross-sectional observational study was performed in 78 patients with two *DARS2* mutations in the Amsterdam and Helsinki databases up to December 2012. Clinical information was collected via questionnaires. An inventory was made of the *DARS2* mutations in these patients and those previously published. An assay was developed to assess mitochondrial aspartyl-tRNA synthetase enzyme activity in cells. Using a fluorescence reporter system we screened for drugs that modulate *DARS2* splicing. Clinical information of 66 patients was obtained. The clinical severity varied from infantile onset, rapidly fatal disease to adult onset, slow and mild disease. The most common phenotype was characterized by childhood onset and slow neurological deterioration. Full wheelchair dependency was rare and usually began in adulthood. In total, 60 different *DARS2* mutations were identified, 13 of which have not been reported before. Except for 4 of 42 cases published by others, all patients were compound heterozygous. Ninety-four per cent of the patients had a splice site mutation in intron 2. The groups of patients sharing the same two mutations were too small for formal assessment of genotype-phenotype correlation. However, some combinations of mutations were consistently associated with a mild phenotype. The mitochondrial aspartyl-tRNA synthetase activity was strongly reduced in patient cells. Among the compounds screened, cantharidin was identified as the most potent modulator of *DARS2* splicing. In conclusion, the phenotypic spectrum of leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation is wide, but most often the disease has a relatively slow and mild course. The available evidence suggests that the genotype influences the phenotype, but because of the high number of private mutations, larger numbers of patients are necessary to confirm this. The activity of mitochondrial aspartyl-tRNA synthetase is significantly reduced in patient cells. A compound screen established a ‘proof of principle’ that the splice site mutation can be influenced. This finding is promising for future therapeutic strategies.

INTRODUCTION

Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL, MIM 611105) is a rare autosomal recessive disease that was initially described as a relatively mild disorder, characterized by juvenile onset of slowly progressive ataxia, spasticity and dorsal column dysfunction.[1-5] LBSL is associated with a highly distinctive MRI pattern, consisting of signal abnormalities in the periventricular cerebral white matter and specific brainstem and spinal cord tracts.[1,6] In most patients, proton magnetic resonance spectroscopy of the abnormal white matter reveals increased lactate.[1,7,8] The definitive diagnosis of LBSL is established by the demonstration of mutations in the gene *DARS2* (MIM 610956). No treatment is available for LBSL. *DARS2* encodes mitochondrial aspartyl-tRNA synthetase, the enzyme that attaches the amino acid aspartate to the correct mitochondrial transfer RNA. Aspartyl-tRNA is necessary in the translation of mitochondrial messenger RNA into protein. It is striking that almost all patients are compound heterozygous for two *DARS2* mutations and that one of the mutations is almost invariably a splice site mutation in intron 2, upstream of exon 3.[6] As a consequence of such a mutation, exon 3 is not included in the messenger RNA, leading to a frameshift, premature stop and absence of functional protein. These splice site mutations are, however, 'leaky'. This means that for part of the mutated messenger RNAs, exon 3 is included, from which normal full-length protein is formed.[9] Recent observations indicate that the phenotypic spectrum in LBSL is much wider than originally assumed. Adult-onset oligosymptomatic cases were described,[7,8,10,11] as well as patients with infantile onset, rapid neurological deterioration and early demise.[12-14] The explanation for this wide clinical variation is unclear. The first aim of the present study was to explore a possible genotype-phenotype correlation in LBSL. We made an inventory of the clinical characteristics and *DARS2* mutations in a cohort of 78 LBSL patients and evaluated a possible genotype-phenotype relationship. The second aim of the study was to investigate the potential application of agents modulating the common intron 2 splice site mutations, with the aim to increase the amount of normal enzyme produced. A compound screen was performed to identify modulators of the splicing event.

MATERIALS AND METHODS

The study was performed with approval of the Institutional Review Board of the 'VU University Medical Center', Amsterdam. Written informed consent for research on patients' cells was obtained from all patients or guardians of patients participating in the study, in agreement with the Declaration of Helsinki.

Phenotypic inventory

A cross-sectional observational study was performed including all 68 patients with two *DARS2* mutations present in the Amsterdam LBSL patient database up to

December 2012. The database contains patients referred to the VUMC Center for Childhood White Matter Disorders in Amsterdam for genetic testing. Additionally, 10 Finnish patients, investigated in the Helsinki University Central Hospital, were included. Clinical questionnaires were completed primarily by the patient's physician (77% of the patients). If this source was not available, the information was derived from medical records, supplemented by information provided by the family. To avoid differences in rating of the clinical phenotype, robust outcome measures were chosen: age of onset, age at achieving unsupported walking, age at loss of walking without support, full wheelchair dependency, Gross Motor Function Classification System (GMFCS, Expanded and Revised version) and Manual Ability Classification System (MACS) scores, cognitive ability and age at death. The GMFCS and the MACS are international standards for the classification of locomotion and hand function in patients with cerebral palsy (Supplementary Table 1).[15,16] For cognitive ability we used a 3-point score: normal level of function, learning disabilities and severe cognitive impairment, as assessed by developmental milestones, school performance and occupation. Magnetic resonance images of all patients were available for evaluation. The studies were evaluated according to a standard protocol.[17]

Genotypic inventory

Mutation analysis of *DARS2* was performed as described previously.[6] Several new primers were used, which are listed in Supplementary Table 2. The presence of the c.228-20T>C polymorphism on the same allele as another intron 2 mutation was determined by sequence analysis of the equivalent PCR fragment obtained from the parental DNA. If both parents carried the c.228-20T>C polymorphism, the patient's PCR fragment was cloned into a pGEM-T vector (Promega), which was then transformed into E. coli and individual clones were sequenced. Genotype data of all 68 patients of the Amsterdam database up to December 2012 and the 10 patients of the Helsinki database were documented. Additionally, the mutations of 42 published patients with two *DARS2* mutations were reviewed.[10,11,13,18-21]

Statistical analysis

Descriptive statistics were used to review the phenotypes and genotypes. To test whether there is an association between age of onset and disease progression in the first 10 years after disease onset, we performed a non-parametric resampling procedure with 100 000 permutations to study the differences between the empirical cumulative distribution functions over the ordinal severity scale of consecutive age-of-onset groups. Cox regression analysis was performed to study the relation between the continuous covariate 'age of onset' and disease duration at the time of loss of ambulation. The recursive partitioning technique of Hothorn *et al.* (2006)[22] was used to analyze whether categories of patients with different ages of onset could be formed. Kaplan-Meier curves were constructed to visualize the probabilities for the loss of the ability to walk without support and full wheelchair dependency relative to the duration of the disease, with a distinction for age of onset. For patients in whom

the event of loss of walking without support or full wheelchair dependency had not yet occurred at the last clinical evaluation, the event was indicated as censored. The log rank test was used to compare subgroups. Student's t-test was used to assess the difference in the levels of *DARS2* intron 2 inclusion. Analyses were performed using the statistical software program R and SPSS for Windows version 20.

Cell culture

Control and patient-derived lymphoblasts were obtained and cultured as described previously.[23] HEK293T cells were grown in Opti-MEM® (Invitrogen) and 10% fetal bovine serum. Cells were cultured at 37°C and 5% CO2.

Enzyme assay

Mitochondria were isolated from lymphoblasts using the Mitochondrial Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol. Isolated mitochondria were resuspended in reaction buffer [50mM HEPES - KOH pH 7.6, 25mM KCl, 12mM MgCl2 with protease inhibitors (Roche)]. Mitochondrial extracts were obtained after incubation in 0.5% NP40 for 10 min and centrifugation at 13 000 rpm for 10 min. Mitochondrial extracts were run on a SDS-PAGE gel with 0.5% trichloroethanol and protein loading was analyzed with the Gel Doc™ EZ system (Bio-Rad). Aminoacylation assays were carried out in 50mM HEPES -KOH pH 7.6, 25mM KCl, 12mM MgCl2, 2.5mM ATP, 0.2 mg/ml bovine serum albumin, 1mM spermine, 32 mM stable-isotope labelled aspartate, 40 mM total E. coli tRNA (Roche) and mitochondrial extract at 37°C. NaAc (0.1mM) pH 5.2 was added to the samples at indicated time points to stop the reaction. Transfer RNAs were isolated immediately, using acidic phenol/CHCl3/ IAA and ethanol precipitation. Aspartate was released from the transfer RNA by incubation in 62.5mM borate buffer at pH 10.0 for 1 h at 42°C. Stable-isotope labelled aspartate was measured by liquid chromatography–tandem mass spectrometry.

Compound screen

For the compound screen, a splicing reporter construct was used, containing the most common mutation in patients with LBSL (c.228-21_-20delTTinsC), transfected in HEK293T cells together with a pmCherry-N1 plasmid (Clontech) as control. Splicing reporter constructs contained exon 2, intron 2, exon 3, intron 3 and part of exon 4 of *DARS2* fused to enhanced yellow fluorescent protein (EYFP), as previously described.[9] This reporter construct leads to expression of EYFP when exon 3 is skipped. Compounds from the Spectrum Collection (Microsource), a library of 2000 FDA approved and natural compounds, were screened for their effect on expression of the splicing reporter construct. HEK293T cells were transfected with polyethylenimine (Polysciences) at 50% confluency. Cells were co-transfected with a pmCherry plasmid to control for the number of cells, transfection efficiency and possible other effects of the compounds on, for example, protein stability and cell viability. At an earlier stage, we had found that sodium orthovanadate increased

correct splicing of the third exon of *DARS2*, and this compound was therefore used as a positive control. The stock solution of each compound was 100 mM dissolved in 1% dimethyl sulphoxide. Compounds were added 24 h after transfections at a final concentration of 1 mM. After incubation for 24 h, cells were fixed in 4% paraformaldehyde and EYFP and mCherry expression was measured on a Cellomics ArrayScan VTI HCS Reader. The screen was done in duplicate. The 2000 screened compounds were ranked based on the EYFP/mCherry ratio with the rank product method.[24] The top five hits from this analysis were used for further validation on transfected HEK293 cells in different concentrations. The compounds that were used for validation were obtained from Sigma. These selected compounds were also used in combination with two additional constructs, one with intron 2 mutation c.228-20_-11delinsCCCCCCCCCG and the other with c.228-20_-15delinsCCCCCA, to demonstrate that the effects were not restricted to one single mutation, c.228-21_-20delTTinsC.

RNA isolation and quantitative polymerase chain reaction

Transfected cells were harvested 48 h after transfection and 24 h after addition of the compounds. Total RNA was extracted using TRIzol® (Invitrogen). First-strand complementary DNA synthesis was carried out with SuperScript® III RT (Invitrogen). PCR was done on complementary DNA with Platinum® Taq according to the manufacturer's protocol (Invitrogen). Quantitative PCR was performed using SYBR® Green (Roche) on a LightCycler 480 (Roche). The primers are described in Supplementary Table 2.

RESULTS

Phenotypic spectrum

The databases contained 78 patients with two *DARS2* mutations. Patients for whom no clinical information could be collected (n = 11) were excluded from the study. One patient was excluded because of co-morbidity (serious hypoplasia of the right cerebellar hemisphere).[25] For some patients, the available clinical information was incomplete; these patients were only excluded from the analysis for the subject of the missing information. Sixty-six patients with LBSL (36 female and 30 male patients, 58 families, see Supplementary Table 3) were available for the clinical inventory. The average age of the patients at the latest clinical evaluation was 24 years [standard deviation (SD) 14 years, range 1–59 years].

Age of onset

The mean age at which patients first showed neurological signs, was 8 years (SD 8 years, median 5 years, range 0.4-40 years). In 53% of patients, the disease began before the age of 6 and in 88% before the age of 18 years (Figure 1). Onset in late teens was rare. Eight patients had adult onset, seven of whom were female. The most common neurological sign was cerebellar ataxia, especially gait ataxia. One

patient (Patient LBSL236) has thus far been asymptomatic. He had been referred for a neurological examination at the age of 3 months because of abnormal muscle tone. Cranial ultrasound examination revealed hyperechogenecity of both cerebral hemispheres and MRI showed abnormalities typical of LBSL, after which the diagnosis was confirmed by DNA analysis. He is presently a normal boy of 2 years.

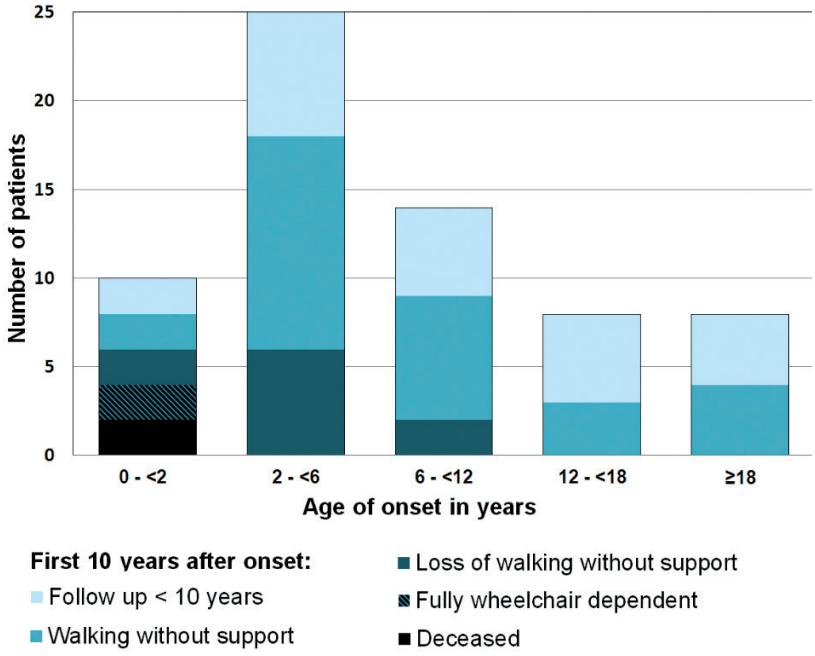


Figure 1 | Age of onset and disease progression in the first 10 years. Plot of the number of patients with LBSL per age category of disease onset, with a subclassification for disease progression in the first 10 years after disease onset. Patients not being followed for 10 years are represented in light blue, unless they were already categorized in the most severe groups (deceased or fully wheelchair dependent).

Early motor development

The majority of patients achieved unsupported walking at a normal age. Nine of 63 patients showed a delayed development and three patients never achieved unsupported walking.

Loss of ambulation

Only eight patients required an aid for walking before the age of 12 years and only four became fully wheelchair dependent before 12 years. Patients in their teens were more likely to have lost walking without support, but not to be fully wheelchair dependent. In patients of 18 years and older, 50% required support for walking and 13% were fully wheelchair dependent.

GMFCS and MACS

The majority of patients was able to walk without (GMFCS I-II) or with (GMFCS III) support. Most patients had no (MACS I) or limited (MACS II) problems with handling objects. Manual ability was generally less severely affected than ambulation. It was exceptional for patients to score on the highest level V of both systems, corresponding to complete wheelchair dependency and a severely limited manual ability. This was only the case in a small number of patients with disease onset before 2 years and rapid deterioration. Two middle-aged patients are currently also severely handicapped (GFMCS IV or V and MACS IV).

Cognitive ability

Most patients had a normal cognitive ability, whereas 20% (12/62) required special education. Serious intellectual impairment was observed in only two patients.

Mortality and survival

In this cohort of 66 patients, 15 individuals had reached the age of at least 35 years. Only two patients had died. Both had infantile disease onset, rapid disease progression and death before the age of 2 years.

Magnetic resonance imaging characteristics

Except for three patients, all patients in the databases fulfilled all major and one or more minor MRI criteria for LBSL.[14] Two siblings (Patients LBSL160/161) and Patient LBSL223 lacked the major criterion of signal abnormalities throughout the pyramidal tracts; there was no involvement of the pyramids at the medulla oblongata and the lateral corticospinal tracts of the spinal cord. Patient LBSL223 lacked abnormalities in cerebellum and brainstem at the age of 2 years (Supplementary Figure 1). Patient LBSL229 showed additional signal abnormalities in the anterior funiculus over the entire length of the spinal cord. In general, in late onset mildly affected patients, the cerebral white matter abnormalities were less profound than in severely affected patients (Supplementary Figure 2).

Correlation between age of onset and disease severity

Forty-two patients were available for the comparison between age of onset and disease severity after 10 years. We divided the patients in age of onset categories that are in common use in medicine: infantile (0 to 52 years), early juvenile (2 to 56 years), late juvenile (6 to 512 years), teenage (12 to 518 years), and adolescent and adult (18 years and older). When comparing these patient categories, earlier onset was related to a more severe neurological deterioration in the first 10 years after disease onset (p=0.00011, Figure 1). When studying the relationship between the continuous covariate age of onset and the time at loss of ambulation, only the time that patients became fully wheelchair dependent correlated with the age of onset (cox regression analysis, p=0.014). Based on the time at full wheelchair dependency, evidence was found for the categorization into two groups of patients (age of onset

0–1.5 years and age of onset 41.5 years; p-value=0.022). Patients with an infantile onset lost the ability to walk without support (p=0.0001) soonest after disease onset and became fully wheelchair dependent sooner (p<0.0001, Figure 2). All the adult onset patients were still ambulant, without or with support, at the last clinical evaluation.

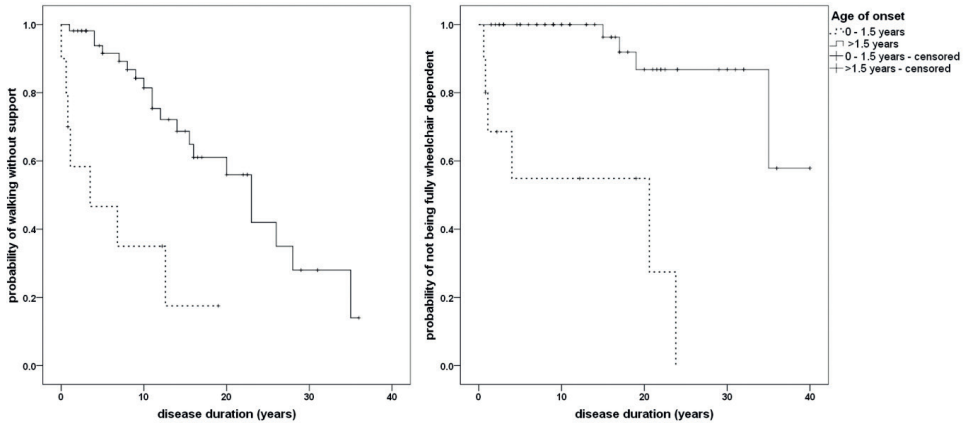


Figure 2 | Loss of ambulation in relation to age of onset. Kaplan-Meier plots showing the probability of walking without support (left) and not being fully wheelchair dependent (right) in relation to disease duration for patients with an onset before or after the age of 1.5 years, respectively.

Genotypic variation

Since the discovery of *DARS2* mutations in 38 patients with LBSL in 2007,[6] we have confirmed the diagnosis of LBSL by DNA analysis in 40 additional patients (39 families). Of the mutations found, 13 have not previously been published. In addition, 42 patients with two pathogenic mutations have been published by others.[8,10-13,18,19,21] An overview of all 60 known mutations is shown in Figure 3 and the mutations of our patients are listed in Supplementary Table 3. The mutations are spread over the entire *DARS2* gene (Figure 3). Ninety-four per cent (113/120) of the patients had an intron 2 mutation in the polypyrimidine tract just upstream of exon 3. Thirteen different mutations were found in this region with c.228-21_-20delTTinsC being the most common (88/120). In all Amsterdam database patients, these intron 2 mutations co-segregated with a single nucleotide polymorphism (c.228-20T>C) on the same allele, which resulted in a modified description of some earlier published mutations (Supplementary Table 3). All patients in our database were compound heterozygous. Four patients (two families) have been described with homozygous mutations.[10,12] In Patient LBSL44 three mutations were found that were predicted to be pathogenic. For this patient, no parental DNA was available to investigate the allelic distribution.

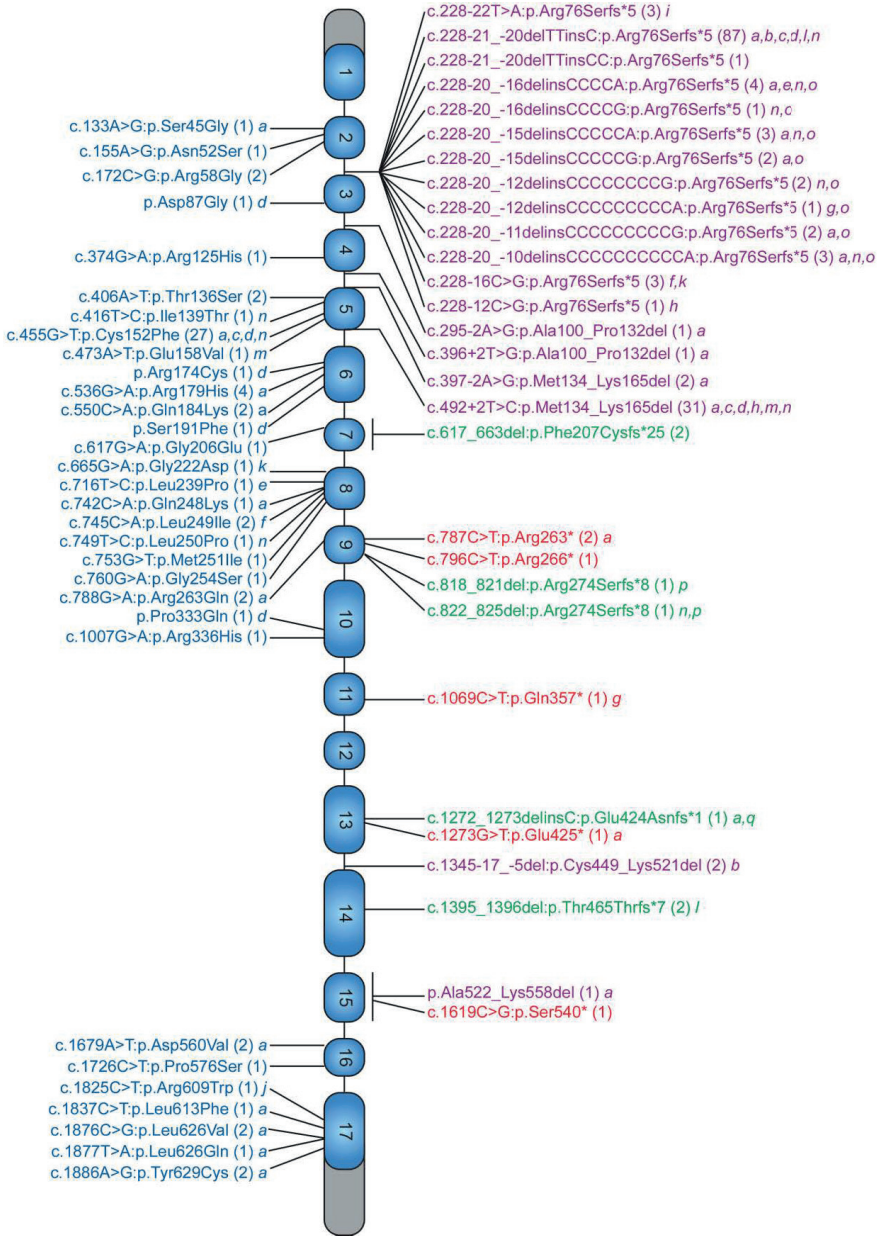


Figure 3 | *DARS2* mutations. Schematic representation of the *DARS2* gene and the distribution and frequencies (in brackets) of patients in our databases and all published mutations. The exons are displayed in proportion, the introns are shortened. Splice site mutations are indicated in purple, missense mutations in blue, deletions in green, and nonsense mutations in red. **a**: Scheper *et al.*, 2007;[6] **b**: Uluc *et al.,* 2008;[5] **c**: Isohanni *et al.*, 2010;[25] **d**: Mikhaylova *et al.*, 2009;[19] **e**: *Lin et al.*, 2010;[26] **f**: Labauge *et al.*, 2011;[20] **g**: Sharma *et al.*, 2011;[27] **h**: Galluzzi *et al.,* 2011;[13] **i**: Miyake *et al.*, 2011;[12] **j**: Synofzik *et al.*, 2011;[10] **k**: Huang *et al.,* 2012;[21] **l**: Tzoulis *et al.,* 2012;[18] **m**: Moore *et al.,* 2012;[11] **n**: Steenweg *et al.*, 2012;[14] **o**: mutation was previously published with a different annotation; **p**: p.Arg274Serfs*9 in current HGVS nomenclature; **q**: p.Glu424Asnfs*2 in current HGVS nomenclature.

Genotype-phenotype relation

Of 66 phenotyped patients with LBSL, 25 patients had a private mutation and 31 patients had a unique combination of mutations. The fact that only 22 patients had a combination of *DARS2* mutations shared by other unrelated patients hampered the study of a genotype–phenotype correlation. In the 45 patients who had the common c.228-21_-20delTTinsC mutation and different mutations on the second allele, the clinical severity ranged from childhood onset disease with loss of ambulation before adulthood to very slowly progressive adult onset disease. There was no evident relationship between the location or type of the second mutation and disease severity. Interestingly, the four patients with the most severe phenotype did not have the common mutation, but had another intron 2 mutation. Two groups could be formed with multiple patients sharing the same combination of mutations: c.228-21_-20delTTinsC together with c.455G>T (n = 9) or with c.492+2T>C (n = 11). All patients in these groups had a benign phenotype characterized by an onset in childhood up to young adulthood followed by mildly progressive neurological deterioration. There was no significant difference in clinical outcome between these groups or between these groups and the rest of the patients. Two additional patients with the same genotype (Patients LBSL103 and LBSL166, c.228-21_-20delTTinsC, c.397-2A>G) had a mild disease with an adult onset. Only four patients did not have an intron 2 mutation affecting the splicing of exon 3. This number was too small to conclude whether their phenotypes are different. It was, however, striking that two of these patients (Patients LBSL160/161) lacked some major MRI criteria, as described above. Another patient who lacked some of the classical MRI characteristics (Patient LBSL223) had a unique intron 2 mutation and a rather severe disease course. In seven of eight affected sibling pairs, the first symptoms occurred within the same phase of life for both patients, with a maximum difference of 3 years; in Patient HEL3 the symptoms occurred 13 years later than in his brother (Patient HEL2). Among five affected sibling pairs, mild differences in clinical severity of motor dysfunction or cognitive problems were observed.

Decreased mitochondrial aspartylation in patients with LBSL

Mitochondrial aspartyl-tRNA synthetase activity was measured in mitochondrial extract from lymphoblasts of four patients with LBSL (Patients LBSL8, LBSL44, LBSL160 and LBSL266) and four control subjects. The patients had different combinations of mutations and their phenotypes ranged from infantile onset disease with severe neurological deterioration (Patient LBSL266) up to adult onset, mild disease (Patient LBSL44) (Supplementary Table 3). The results show a substantial loss of activity of mitochondrial aspartyl-tRNA synthetase in all patient cells compared with control subjects (Figure 4 and Supplementary Figure 3). The level of residual enzyme activity was in the same range for all patients with overlap in values for the most severely and mildest affected patients.

Compounds altering *DARS2* exon 3 splicing

We have previously developed a yellow fluorescent protein (YFP) reporter construct, in which increased splicing efficiency of intron 2 is reflected by a decrease in the YFP signal.[9] These results were validated at both the RNA and protein level. In the present study, we used this construct with the most common splice site mutation to screen compounds for their ability to modify the splicing efficiency of intron 2. Of the 2000 compounds ranked by the rank product method, the five compounds causing the largest decrease in the EYFP/mCherry ratio in both screens were used for further studies: gentian violet, cantharidin, pyrithione zinc, celastrol and alanyl-DL-leucine (Supplementary Table 4).

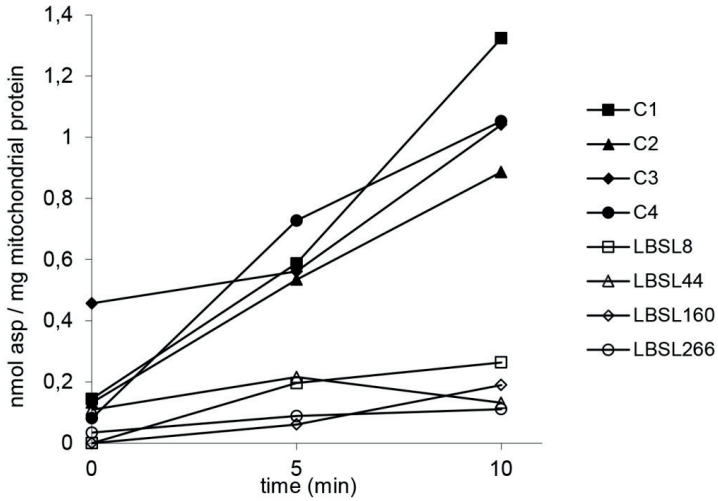


Figure 4 | Mitochondrial aspartyl-tRNA synthetase activity in lymphoblasts. The aspartylation activity in isolated mitochondria from four control and four patient lymphoblasts was measured. Activity was strongly reduced in patient lymphoblasts (Patients LBSL8, LBSL44, LBSL160 and LBSL266) as compared to control lymphoblasts (C1-C4).

Effects of all compounds except for alanyl-DL-leucine could be confirmed in separate experiments using the reporter construct. The two additional reporter constructs with different splice site mutations in the same region showed similar effects (Figure 5A). Therefore, the identified compounds affect the splicing efficiency at the intron 2/exon 3 boundary independent of the exact mutation. To directly assess the effect on messenger RNA splicing, reverse transcriptase PCR was performed to detect the messenger RNA with and without the third exon of *DARS2*. In addition to sodium orthovanadate, one of the selected compounds, cantharidin, showed a direct effect on the splicing of the third exon. Both compounds increased the amount of correctly spliced product (Figure 5B). There was a concentration-dependent effect up to 10 mM for cantharidin (Figure 5C). In experiments with a smaller number of different concentrations, we showed that the concentration- dependent effects were reproducible (Supplementary Figure 4A) and all three mutant reporter constructs respond in a similar concentration-dependent manner (Supplementary Figure 4B).

Cantharidin had a clear effect on splicing of intron 2 on the messenger RNA level (Figure 5B) and was chosen to study the effect on lymphoblasts from patients with LBSL. Two patient-derived lymphoblast cell lines and two control cell lines were treated with 10 mM cantharidin for 24 h. Quantitative reverse transcriptase PCR on RNA purified from these cells showed that cantharidin increased the correct splicing of *DARS2* exon 3 in these patient-derived cells. With the addition of cantharidin, the percentage of messenger RNA without exon 3 was decreased on average from 11.3% to 5.4% (Figure 5D).

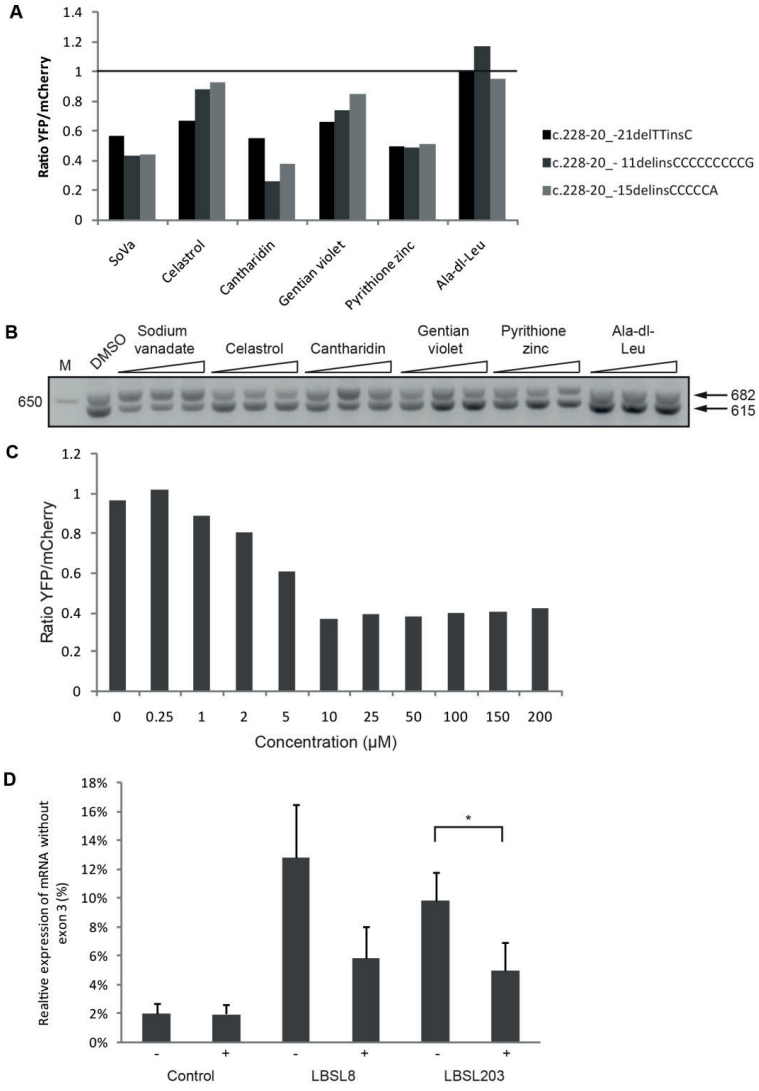


Figure 5 | Compounds affecting *DARS2* exon 3 splicing. (A) The effects of selected compounds on three different reporter constructs are shown. The effect of DMSO was set at a YFP/mCherry-ratio of 1. This ratio is decreased with all selected compounds except alanyl-DLleucine. The three different

mutations in the reporter constructs show similar results. **(B)** Reverse transcriptase PCR was performed on cells transfected with the reporter construct and treated with the indicated compound. After isolation of total RNA and complementary DNA synthesis, the inclusion or exclusion of exon 3 was visualized by PCR with primers 2Fb and GFP-R (Supplementary Table 1) before agarose gel electrophoresis. The expected sizes of the bands are 615 bp (without exon 3) and 682 bp (with exon 3) as indicated on the right. M represents a marker lane containing the Invitrogen 1Kb Plus DNA ladder and the size of the band is indicated on the left. **(C)** The effect of increasing concentrations of cantharidin on the YFP/mCherry-ratio is shown. **(D)** Relative expression of mitochondrial aspartyl-tRNA synthetase messenger RNA without exon 3 in control and patient lymphoblasts with and without treatment of 10 mM cantharidin for 24 h. The expression of the variant without exon 3 is shown as the mean percentage of total amount of mitochondrial aspartyl-tRNA synthetase messenger RNA ± standard error of the mean. The asterisk indicates $P<0.05$.

DISCUSSION

In this study, we investigated the phenotypic variation in a relatively large number of patients with LBSL. LBSL was originally described as a juvenile onset disorder with a reliable, slow progression.[1] Our study confirms that LBSL is a neurological disorder with generally slow progression and low mortality, with in some cases even later onset and slower disease course than initially described. Complete wheelchair dependency is rare, especially before the age of 18 years. Life expectancy may be normal for most patients; the oldest patient in this cohort is 59 years old. Patients with LBSL have in general a better prognosis than most other hereditary leukoencephalopathies, but the infantile onset cases are an exception. They form a distinct category characterized by a more rapid neurological deterioration and - in the most severe cases - early death. The implication of our observations is that both paediatric and adult neurologists may meet patients with LBSL and need to be aware of this disease. The patients included in the present study had been selected for analysis of the *DARS2* gene on the basis of specific MRI findings. A limitation to this approach is that, possibly, LBSL patients with an atypical MRI pattern and asymptomatic patients have been missed. So far, one index patient (Patient LBSL236) and three siblings of patients with LBSL with the same pathogenic mutations are known that are asymptomatic (Labauge et al., 2011[20] and personal observations). We suspect that until now most early-onset patients have remained undiagnosed because the severe phenotype is not widely known. Consequently, the clinical spectrum of the disease may still be wider and the distribution over ages of onset may be different to what is currently described. Almost all patients with LBSL have compound heterozygous *DARS2* mutations. Mitochondrial aspartyl-tRNA synthetase is an enzyme that is essential for life and patients never have two null mutations. These observations suggest that the range of permissive mutations resulting in mitochondrial aspartyl-tRNA synthetase activity that is high enough not to be lethal, but below a certain threshold for symptoms to appear, is narrow. The c.228-21_-20delTTinsC mutation has not been reported in the homozygous state in any LBSL patient, which is striking considering the relatively frequent occurrence of this mutation in LBSL patients, and the high carrier rate of 1:95 that has been

described in the Finnish population.[25] It is also striking that there is no patient with LBSL who is compound-heterozygous for two different intron 2 splice site mutations. The intron 2 splice site mutations are leaky and allow the production of some normal protein. It is possible that the presence of an intron 2 splice site mutation on both alleles leads to another pathology, but we suspect that this may not lead to a disease. We postulate that the remaining mitochondrial aspartyl-tRNA synthetase activity in patients' cells is largely derived from the allele with the 'leaky' intron 2 mutation, whereas the second mutation probably is a functional null allele. Patient LBSL44 had three predictively pathogenic mutations; it is most likely that the intron 2 mutation is located on one allele, and the two other mutations on the other allele, leading to one null allele. We found that a known single nucleotide polymorphism (c.228-20T>C) invariably co-segregates with mutations in the polypyrimidine tract in intron 2 in patients from the Amsterdam database. It is unknown whether this is also the case in other patients. Our finding raises the possibility that a single mutation in this intron 2 tract may only be pathogenic when occurring in combination with this single nucleotide polymorphism. Unfortunately, the heterogeneous nature of the genotypes seriously hampered the genotype-phenotype correlation study. For a proper genotype–phenotype correlation study much larger groups of patients are required. Interestingly, four severe patients do not have the common c.228-21_-20delTTinsC mutation seen in 45 milder patients. Patient LBSL263 has the same mutations as a patient described in the literature who was also severely affected (the presence of the abovementioned single nucleotide polymorphism was not mentioned). Two groups of patients with similar mutations and a mild disease course could be formed, suggesting that these genotypes are related to a benign phenotype. Striking intrafamilial differences were absent. Outside our study, only one family has been described with three affected siblings and these displayed remarkable interindividual differences.[12] Our observations support the hypothesis that there is a genotype–phenotype correlation. Intrafamilial differences are most likely explained by the influence of environmental and other genetic factors. We developed an enzyme assay to assess mitochondrial aspartyltRNA synthetase activity and for the first time confirmed its decreased activity directly in patient cells. Decreased activity of mutant enzymes has been shown previously in in vitro aminoacylation assays from overexpression studies,[6,28] but it is difficult to extrapolate these results to patient cells, where the activity is almost always determined by a combination of two different mutant alleles. We demonstrate that the tested patient cells have a significantly reduced mitochondrial aspartyl-tRNA synthetase activity. We found no clear correlation between enzyme activity and severity of the phenotype. Possibly, the assay we used was not sensitive enough to detect subtle differences. It could also be that lymphoblasts are not the most suitable cells to show the differences; they might be better detectable in other cell types, such as neuronal cells.

The high occurrence of splice site mutations makes the splicing process a promising target for therapy. We have previously demonstrated that antisense oligonucleotides can alter the splicing efficiency at the intron 2/exon 3 boundary.[9] Despite the great

progress in the field of antisense oligonucleotides in recent years, development of a therapy based on the successful delivery of antisense oligonucleotides to the CNS will probably not be possible through systemic administration.[29] Therefore, we used a compound library containing FDA-approved and natural compounds, many of which are known to be able to cross the blood–brain barrier. With this library, a screen was performed to find compounds that influence the intron 2/exon 3 splicing event. After validation of hits from this screen, cantharidin was identified as the most effective compound. It increased the inclusion of exon 3 in our reporter constructs and in lymphoblasts from patients with LBSL. Cantharidin is a protein phosphatase 1 and 2A inhibitor. It has previously been found to increase exon 7 inclusion in the *SMN2* gene, relevant for spinal muscular atrophy.[30] Reducing protein phosphatase 1 activity promotes usage of numerous alternative exons, indicating that protein phosphatase 1 activity plays a role in splice site selection.[30] Cantharidin is too toxic for use in patients,[31] but this study provides proof-of-concept that influencing the splice site mutations is possible and a highly promising therapeutic target for LBSL. Future research should be directed at less toxic variants of cantharidin[32] or other protein phosphatase 1 or 2A inhibitors.

ACKNOWLEDGEMENTS

The authors thank the patients, families and referring physicians of the LBSL Research Group for their co-operation and contribution. This study was supported by the Prinses Beatrix Fonds [grant WAR07/31] and the Optimix Foundation for Scientific Research.

The following LBSL Research Group collaborators contributed to the study: F. Alehan, Baskent University, Ankara, Turkey; R.E. Appleton, Alder Hey Children's Hospital, Liverpool, United Kingdom; E. Boltshauser, University Children's Hospital, Zürich, Switzerland; K. Brockmann, Georg August University, Göttingen, Germany; E. Calado, Hospital Dona Estefânia, Portugal; A. Carius, Universitaetsklinikum Freiburg, Freiburg, Germany; I.F.M. de Coo, Erasmus University Medical Center, Rotterdam, The Netherlands; R. van Coster, Universitary Hospital Gent, Gent, Belgium; S. El-Zind, Memorial Children's Hospital, South Bend, Indiana, U.S.A; O. Erturk, Istanbul University, Istanbul, Turkey; L. Fadeeva, Burdenko Neurosurgical Institute, Moscow, Russia; A. Feigenbaum, Hospital for Sick Children and University of Toronto, Toronto, Canada; S. Gökben, Ege University, Bornova Izmir, Turkey; M. Gorman, Boston Children's Hospital, Boston, U.S.A.; S. Gulati, All India Institute of Medical Sciences, New Delhi, India; P. Hněvsová , Faculty Thomayers Hospital, Prague, Czech Republic; K. Joost, Tartu University Hospital, Tallinn, Estonia; W. Köhler, Fachkrankenhaus Hubertusburg, Wermsdorf, Germany; A Kolk, Tartu University Hospital, Tartu, Estonia; W. Kristoferitsch, SMZ-Ost-Donauspital, Vienna, Austria; E. Lemos Silveira, Núcleo de Estudos em Psiquiatria e Genética Humana, Porto Alegre, Brazil; J. Lin, Federal University of São Paulo, Brazil; S. Lutz, University Hospital of Essen, Essen, Germany; C. Mendonça, Hospital de Faro, Faro, Portugal; C. Nuttin,

Centre Hospitalier de Luxembourg, Luxembourg; T. Opladen, University Hospital Heidelberg, Heidelberg, Germany; M. Savoiardo, Istituto Nazionale Neurologico Carlo Besta, Milan, Italy; R. Schiffmann, Baylor Research Institute, Dallas, U.S.A.; A. Seitz, University Hospital Heidelberg, Heidelberg, Germany; S. Serkov, Burdenko Neurosurgical Institute, Moscow, Russia; S. Sharma, All India Institute of Medical Sciences, New Delhi, India; S. Stockler, University of British Columbia, Vancouver, Canada; I.K. Temple, Faculty of Medicine, University of Southampton, Southampton, United Kingdom; K. Uluc, Marmara University Hospital, Istanbul, Turkey; S. Vojta, Kinderzentrum München, München, Germany; G. Wilms, University Hospital Leuven, Leuven, Belgium; B. Wong, Cincinnati Children's Hospital Medical Center, Cincinnati, U.S.A.; Z. Yapici, Istanbul University, Department of Neurology, Istanbul, Turkey.

SUPPLEMENTARY DATA

Supplementary Table 1 | Overview of the five levels of the GMFCS and MACS[33]

GMFCS: Gross Motor Function Classification System[16]

MACS: Manual Ability Classification System[15]

	GMFCS	MACS
	Motor function	**Manual ability**
LEVEL I	Walks without limitations	Handles objects easily and successfully
LEVEL II	Walks with limitations	Handles most objects but with somewhat reduced quality and/or speed of achievement
LEVEL III	Walks using a hand-held mobility Device	Handles objects with difficulty; needs help to prepare and/or modify activities
LEVEL IV	Self-Mobility with limitations; may use powered mobility	Handles a limited selection of easily managed objects in adapted situations
LEVEL V	Transported in a manual wheelchair	Does not handle objects and has severely limited ability to perform even simple actions

Supplementary Table 2 | Primers

Primers for sequencing			
Oligo name	**Exon**	**Orientation**	**Sequence**
DARS2-Ex5F	5	Forward	GCTTAAGTGATCCTCCTGTCT
DARS2-Ex5R		Reverse	TTCATGATGTGTCTACAATAAAATGC
DARS2-Ex6F	6	Forward	GTAAAACGACGGCCAGCAGTGGGCTACTTAATGATAGAAAC
DARS2-Ex6R		Reverse	CAGGAAACAGCTATGAGCAACATCTTGACCTCATGC
DARS2-Ex7F	7	Forward	TGGTAAAACGACGCCGACGAAGCCTCAGATTTGTTGTACTA
DARS2-Ex7R		Reverse	GTGTCTTGGCAGTAAATAAAAGTGGACCAAG
DARS2-Ex8F	8	Forward	GCAGGAAATTGTCTCTGTCATTG
DARS2-Ex8R		Reverse	AATCCCCTCTCACATCTACTACC
DARS2-Ex10F	10	Forward	GGCCATTAGCACAGTGTCTG
DARS2-Ex10R		Reverse	TTGCTCTAGCTCTGTAACAACG
DARS2-Ex11F	11	Forward	TCATATTGCTTAACCCATGGTAA
DARS2-Ex11R		Reverse	GCCACCACGCCTGACTAAT
DARS2-Ex13F	13	Forward	GCACAGAACTGGCACAGCTA
DARS2-Ex13R		Reverse	ATGCAGAGCAGCTCCATTTT
DARS2-Ex15F	15	Forward	CCCGTAGAACAGAAAACCAGA
DARS2-Ex15R		Reverse	GCAAACAACAACAACAACACAA
c*DARS2*-1Fa	1-3	Forward	GAGAGTGGGAACTCCTGGAA
c*DARS2*-1Ra		Reverse	TTGAACAAGCCCATCGAAAT
c*DARS2*-1Fb	1-6	Forward	TGTTGCAGAGTTCACAGAGGA
c*DARS2*-1Rb		Reverse	GGACCTCAGTCGCAGGTTAT
c*DARS2*-3Fb	10-15	Forward	TGACTTTTGCTGAGGTGCTG
c*DARS2*-3Rb		Reverse	TGCAGCTCTGCATTGTGAAT
c*DARS2*-4F	14-17	Forward	GGAGGAAAATCCCAGAGAGC
c*DARS2*-4R		Reverse	AAAATCCAAATGATGCATGAAA

Primers for qPCR and RT-PCR		
Oligo name	**Orientation**	**Sequence**
2F	Forward	ACCAACACATGTGGAGAGTTGCG
3/4R	Reverse	TGCCTTCGGTACTGAATCCATCC
2/4R	Reverse	GAGGCTGCCGACTTCGGTACTGA
2Fb	Forward	ACCATGGCATGTGGAGAGTTGCG
GFP-R	Reverse	GTTGTGGCGGATCTTGAAGT

Supplementary Table 3 | Overview of phenotype and genotype LBSL patients

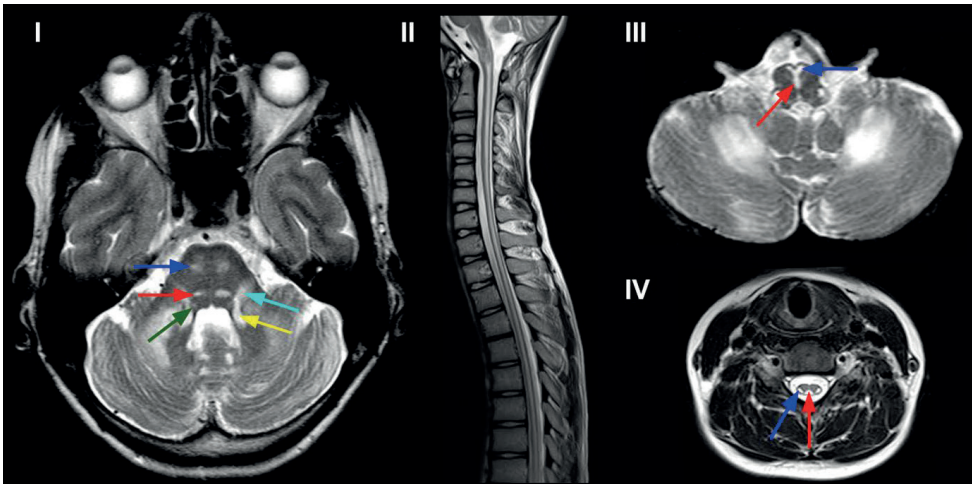
	Patient	Family	Gender	Age[a]	Mutation 1[b]	Amino acid change	Mutation 2[b]	Amino acid change	Un-supported walking	First neurological signs	Loss of un-supported walking	Full wheelchair dependency	GMFCS[c]	MACS[d]	Cognitive ability[e]	Death
1	LBSL213	1	f	18 yrs	c.155A>G	p.Asn52Ser	c.455G>T	p.Cys152Phe	18 mo	2 yrs	-	-	II	I	nl	-
2	LBSL16	2	f	27 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5		p.Ala522_Lys558del	15 mo	5 yrs	17 yrs	-	III	II	↓	-
3	LBSL222	3	m	16 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.1007G>A	p.Arg336His	14 mo	6 yrs	-	-	I	I	↓	-
4	LBSL147	4	m	19 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.1345-17_-5del	p.Cys449_Lys521del	11 mo	13 yrs	17 yrs	-	III	I	nl	-
5	LBSL72	5	f	59 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.1679A>T	p.Asp560Val	nl	27 yrs	53 yrs	-	III	II	nl	-
6	LBSL73	5	f	51 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.1679A>T	p.Asp560Val	13 mo	27 yrs	47 yrs	-	III	I	nl	-
7	LBSL2	6	m	29 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.1876C>G	p.Leu626Val	14 mo	6 yrs	-	-	I	I	nl	-
8	LBSL187	7	f	43 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.374G>A	p.Arg125His	12 mo	12 yrs	-	-	II	II	nl	-
9	LBSL166	8	f	37 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.397-2A>G	p.Met134_Lys165del	24 mo	30 yrs	31 yrs	-	II	I	nl	-
10	LBSL103	9	f	31 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.397-2A>G	p.Met134_Lys165del	11 mo	20 yrs	-	-	II	I	nl	-
11	LBSL269	10	f	11 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.416T>C	p.Ile139Thr	9 mo	6 yrs	11 yrs	-	II	I	nl	-
12	LBSL168	11	f	21 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.455G>T	p.Cys152Phe	11 mo	6 yrs	-	-	I	I	nl	-
13	LBSL80	12	m	25 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.455G>T	p.Cys152Phe	14 mo	23 yrs					nl	-
14	LBSL81	12	f	23 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.455G>T	p.Cys152Phe	15 mo	20 yrs					nl	-
15	LBSL21	13	m	24 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.455G>T	p.Cys152Phe	nl	14 yrs					nl	-
16	LBSL36	14	f	12 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.455G>T	p.Cys152Phe	11 mo	2 yrs	-	-	I	I	nl	-
17	LBSL79	15	m	20 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.455G>T	p.Cys152Phe	15 mo	11 yrs	19 yrs	-	III	II	nl	-
18	LBSL236	16	m	2 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.455G>T	p.Cys152Phe	14 mo	not yet	-	-	I	I	nl	-
19	HEL8	17	f	8 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.455G>T	p.Cys152Phe	14 mo	3 yrs	-	-	I	I	nl	-
20	HEL9	18	m	5 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.455G>T	p.Cys152Phe	38 mo	2 yrs	-	-	II	I		-
21	LBSL28	19	m	29 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.492+2T>C	p.Met134_Lys165del	14 mo	1 yrs	8 yrs	25 yrs	IV	III	nl	-
22	LBSL185	20	f	25 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.492+2T>C	p.Met134_Lys165del	14 mo	3 yrs	19 yrs	-	III	II	nl	-
23	LBSL45	21	f	24 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.492+2T>C	p.Met134_Lys165del	14 mo	3 yrs	12 yrs	-	II	II	↓	-
24	LBSL20	22	m	19 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.492+2T>C	p.Met134_Lys165del		2 yrs	16 yrs	-	III	I	nl	-
25	HEL1	23	f	39 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.492+2T>C	p.Met134_Lys165del	18 mo	10 yrs	20 yrs	-	III	II	nl	-
26	HEL2	24	m	25 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.492+2T>C	p.Met134_Lys165del	11 mo	2 yrs	24 yrs	-	II	I	nl	-
27	HEL3	24	m	22 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.492+2T>C	p.Met134_Lys165del	10 mo	15 yrs	-	-	I	I	nl	-
28	HEL4	25	f	20 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.492+2T>C	p.Met134_Lys165del	15 mo	3 yrs	-	-	II	II	nl	-
29	HEL5	26	f	14 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.492+2T>C	p.Met134_Lys165del	15 mo	9 yrs	-	-	I	I	nl	-
30	HEL6	27	m	18 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.492+2T>C	p.Met134_Lys165del	12 mo	10 yrs	-	-	II	I	↓	-
31	HEL10	28	f	59 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.492+2T>C	p.Met134_Lys165del	13 mo	9 yrs		44 yrs	IV	IV		-
32	LBSL44[f]	29	f	52 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.536G>A	p.Arg179His		22 yrs	45 yrs	-	III	I	nl	-
33	LBSL107	30	m	50 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.536G>A	p.Arg179His	nl	childhood	45 yrs	-	III	II	nl	-
34	LBSL108	30	f	46 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.536G>A	p.Arg179His	nl	childhood	-	-	II	I	nl	-
35	LBSL90	31	m	22 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.550C>A	p.Gln184Lys	13 mo	6 yrs	-	-	I	I	nl	-
36	LBSL76	31	m	20 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.550C>A	p.Gln184Lys	12 mo	7 yrs	18 yrs	-	III	III	↓	-
37	LBSL152	32	f	13 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.617_663del	p.Phe207Cysfs*25	21 mo	0.8 yrs	-	-	II	III	nl	-

	Patient	Family	Gender	Age[a]	Mutation 1[b]	Amino acid change	Mutation 2[b]	Amino acid change	Un-supported walking	First neurological signs	Loss of un-supported walking	Full wheelchair dependency	GMFCS[c]	MACS[d]	Cognitive ability[e]	Death
38	LBSL199	33	m	12 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.617G>A	p.Gly206Glu	14 mo	3 yrs	-	-	I	II	nl	-
39	LBSL91	34	m	19 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.742C>A	p.Gln248Lys	10 10 mo	2 yrs	-	-	II	I	nl	-
40	LBSL7	35	m	20 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.787C>T	p.Arg263*	24 mo	2 yrs	6 yrs	-	II	II	↓	-
41	LBSL8	35	f	24 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.787C>T	p.Arg263*	17 mo	1 yrs	14 yrs	22 yrs	IV	II	↓	-
42	LBSL15	36	f	33 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.788G>A	p.Arg263Gln	14 mo	3 yrs	8 yrs	20 yrs	IV	III	↓	-
43	LBSL 14	36	f	29 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.788G>A	p.Arg263Gln	15 mo	5 yrs	28 yrs	-	III	II	nl	-
44	LBSL 207	37	m	17 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.796C>T	p.Arg266*	13 mo	3 yrs	-	-	I	I	nl	-
45	LBSL 119	38	m	22 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs5	c.818_821del	p.Arg274Serfs*8[g]	15 mo	14 yrs	-	-	II	II	nl	-
46	LBSL247	40	m	13 yrs	c.228-21_-20delTTinsC	p.Arg76Serfs*5	c.1619C>G	p.Ser540*		4 yrs		-			nl	-
47	LBSL223	39	m	4 yrs	c.228-21_-20delinsCC	p.Arg76Serfs*5	c.1345-17_-5del	p.Cys449_Lys521del	12 mo	1.8 yrs	22 mo	-	III	II	nl	-
48	LBSL70	41	f	23 yrs	c.228-20_-16delinsCCCCA (c.228-16C>A)	p.Arg76Serfs*5	c.295-2A>G	p.Ala100_Pro132del	15 mo	14 yrs	-	-	III	I	↓	-
49	LBSL266	42	m	13 yrs	c.228-20_-16delinsCCCCA	p.Arg76Serfs*5	c.455G>T	p.Cys152Phe	never	0.9 yrs	never walked	never walked	V	IV	↓↓	-
50	LBSL191	43	m	17 yrs	c.228-20_-16delinsCCCCA	p.Arg76Serfs*5	c.716T>C	p.Leu239Pro	18 mo	2 yrs	13 yrs	-	II		nl	-
51	LBSL229	44	f	43 yrs	c.228-20_-16delinsCCCCA	p.Arg76Serfs*5	c.1876C>G	p.Leu626Val	14 mo	40 yrs	-	-	II	I	nl	-
52	LBSL275	45	f	†	c.228-20_-16delinsCCCCG	p.Arg76Serfs*5	c.822_825del	p.Arg274Serfs*8[g]	never	0.4 yrs	never walked	never walked	V	V	n/e	1.7 yrs
53	LBSL33	46	f	34 yrs	c.228-20_-15delinsCCCCCA (c.228-15C>A)	p.Arg76Serfs*5	c.133A>G	p.Ser45Gly	24 mo	6 yrs	16 yrs	25 yrs	IV	III	nl	-
54	LBSL259	47	f	8 yrs	c.228-20_-15delinsCCCCCA	p.Arg76Serfs*5	c.749T>C	p.Leu250Pro	25 mo	1 yrs	5 yrs	5.5 yrs	V	V	↓	-
55	LBSL64	48	m	15 yrs	c.228-20_-15delinsCCCCCG (c.228-15C>G)	p.Arg76Serfs*5	c.1886A>G	p.Tyr629Cys	nl	4 yrs	-	-	I	I	nl	-
56	LBSL263	49	f	†	c.228-20_-12delinsCCCCCCCCG	p.Arg76Serfs*5	c.492+2T>C	p.Met134_Lys165del	never	0.7 yrs	never walked	never walked	V	V	n/e	1.8 yrs
57	LBSL210	50	f	34 yrs	c.228-20_-12delinsCCCCCCCCG	p.Arg76Serfs*5	c.760G>A	p.Gly254Ser	22 mo	2 yrs	30 yrs	-	III	II	nl	-
58	LBSL202	51	m	19 yrs	c.228-20_-12delinsCCCCCCCCCA	p.Arg76Serfs*5	c.1069C>T	p.Gln357*	13 mo	10 yrs	-	-	II	II	nl	-
59	LBSL11	52	f	45 yrs	c.228-20_-11delinsCCCCCCCCCG (c.228-11C>G)	p.Arg76Serfs*5	c.536G>A	p.Arg179His	15 mo	5 yrs	12 yrs	20 yrs	V	IV	nl	-
60	LBSL203	53	f	20 yrs	c.228-20_-11delinsCCCCCCCCCG	p.Arg76Serfs*5	c.617_663del	p.Phe207Cysfs*25	18 mo	1 yrs	-	-	II	II	nl	-
61	LBSL216	54	m	4 yrs	c.228-20_-10delinsCCCCCCCCCCA	p.Arg76Serfs*5	c.455G>T	p.Cys152Phe	12 mo	3 yrs	-	-	I	I	nl	-
62	LBSL111	55	f	16 yrs	c.228-20_-10delinsCCCCCCCCCCA (c.228-10C>A)	p.Arg76Serfs*5	c.492+2T>C	p.Met134_Lys165del	14 mo	13 yrs	-	-	I	II	nl	-
63	LBSL208	56	f	7 yrs	c.228-20_-10delinsCCCCCCCCCCA	p.Arg76Serfs*5	c.1726C>T	p.Pro576Ser	15 mo	4 yrs	-	-	I	II	nl	-
64	LBSL161	57	m	23 yrs	c.406A>T	p.Thr136Ser	c.172C>G	p.Arg58Gly	22 mo	3 yrs	-	-	II	II	↓↓	-
65	LBSL160	57	m	15 yrs	c.406A>T	p.Thr136Ser	c.172C>G	p.Arg58Gly	24 mo	2 yrs	-	-	II	II	↓	-
66	LBSL50	58	f	36 yrs	c.1837C>T	p.Leu613Phe	c.1877T>A	p.Leu626Gln	18 mo	12 yrs	28 yrs	-	III	III	↓	-

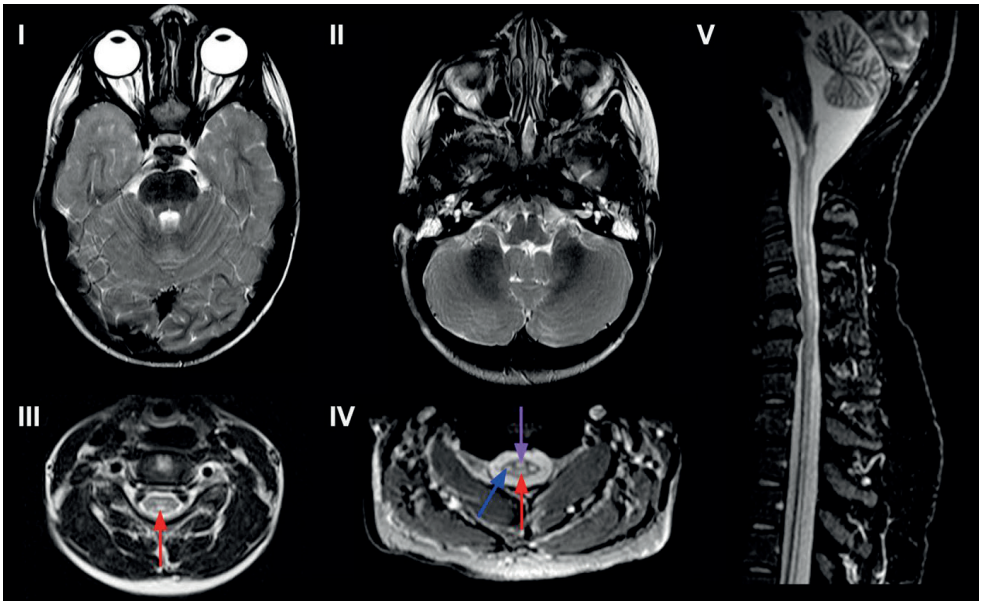
The table is organized by ascending sequence of mutation 1 and subsequently the sequence of mutation 2. Patients with similar genotypes are marked in the same color (white or green).

m, male; f, female; nl, normal; mo, months; yrs, years; n/e, not evaluable

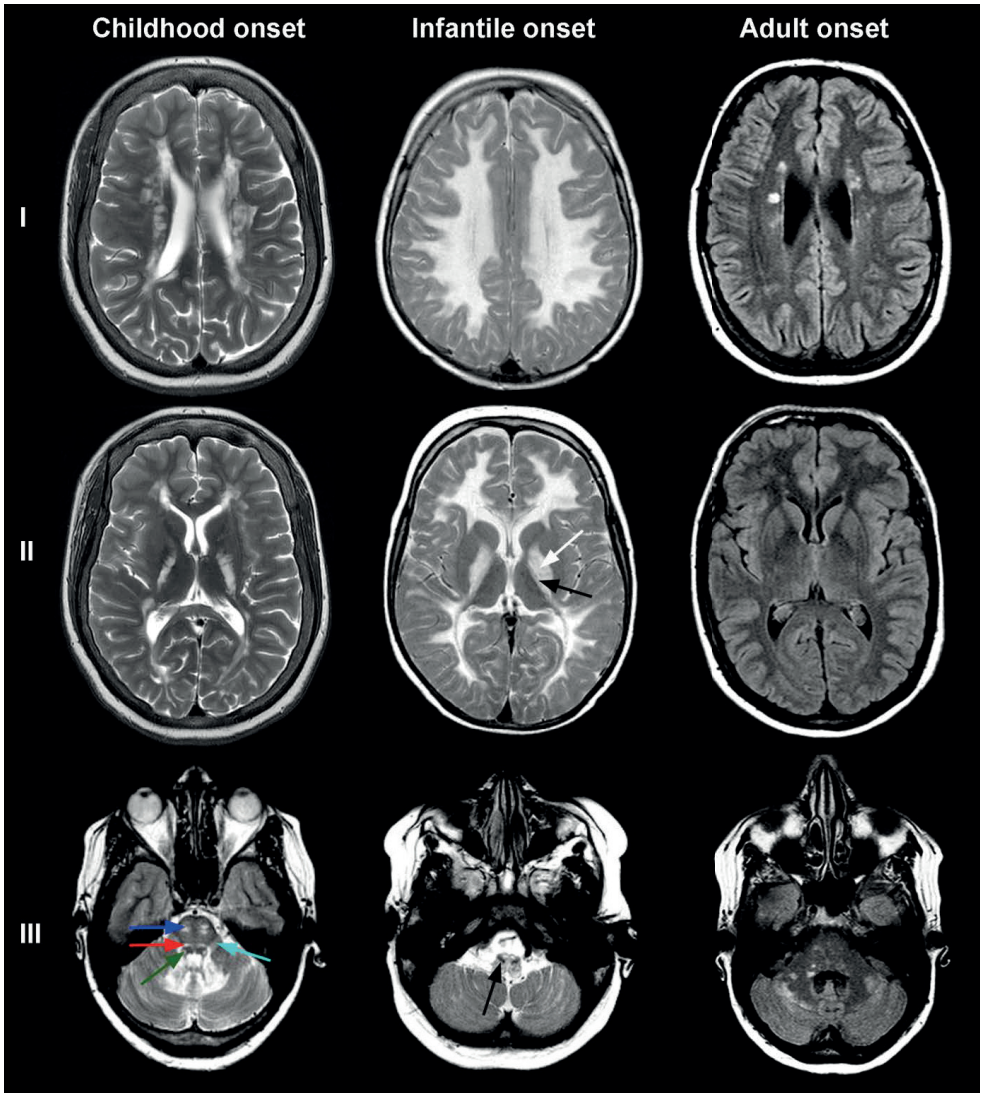
[a] Age at latest clinical evaluation; [b] Nomenclature according to http://www.hgvs.org/mutnomen/. Mutations that were published before with a different annotation are shown between brackets; [c] Gross Motor Function Classification System; [d] Manual Ability Classification System; [e] Cognitive ability: nl, normal intelligence: ↓, learning disabilities; ↓↓, severe cognitive impairment; [f] In this patient a third pathogenic mutation was found: c.1273G>T, p.Glu425*; [g] p.Arg274Serfs*9 in current HGVS nomenclature.



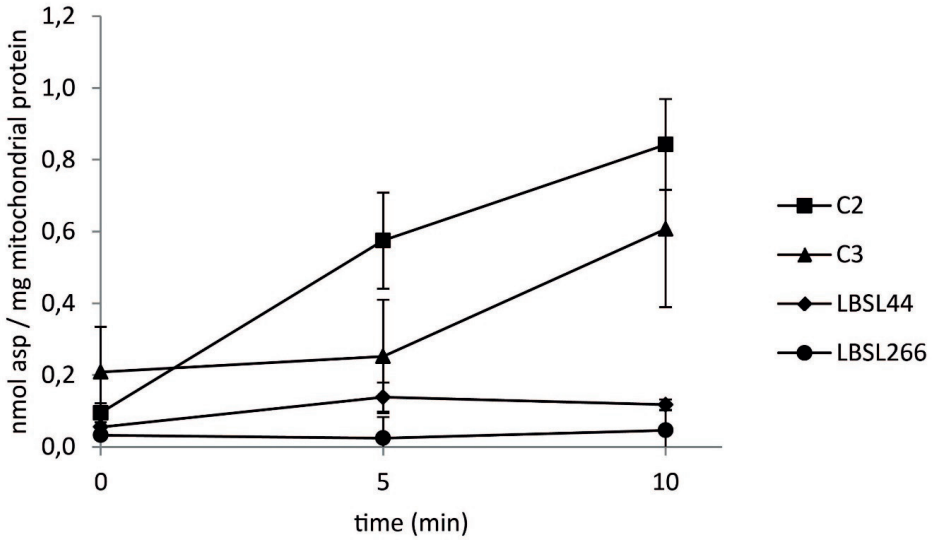
Supplementary Figure 1A | Typical MRI findings. At the level of the brainstem (**I**), an abnormal signal is seen in the medial lemniscus (red arrow), pyramidal tracts (blue arrow), intraparenchymal part of the trigeminal nerve (light blue arrow), mesencephalic trigeminal nerve tracts (green arrow) and superior cerebellar peduncles (yellow arrow). The subcortical cerebellar white matter is affected (**III**) and there are signal abnormalities throughout the spinal cord (**II**). The axial images show hyperintensity of the decussation of the medial lemniscus (red arrow) and pyramids (blue arrow) at the level of the medulla (III) and involvement of the dorsal columns (red arrow) and lateral corticospinal tracts (blue arrow) in the spinal cord (**IV**).



Supplementary Figure 1B | Atypical MRI findings. In LBSL223, there are no abnormalities in the medial lemniscus and pyramidal tracts at the level of the brainstem (**I**; major criteria). There are no cerebellar white matter abnormalities (**II**; minor criterion). The dorsal columns are affected (red arrow), but the major criterion of signal abnormalities in the lateral corticospinal tracts is missing (**III**). In LBSL229, the spinal cord is affected over the entire length (**V**), the axial image (**IV**) shows abnormalities in the dorsal columns (red arrow), lateral corticospinal tracts (blue arrow) and the anterior funiculus (purple arrow).



Supplementary Figure 2 | Variable extensiveness of MRI abnormalities. On the left, T_2-weighted images of a classical, 20 year old LBSL patient (LBSL16) are shown. The periventricular white matter (**I**), posterior limb of the internal capsule, splenium of the corpus callosum (**II**), medial lemniscus (red arrow), pyramidal tracts (blue arrow), intraparenchymal part of the trigeminal nerves in the brainstem (light blue arrow), mesencephalic trigeminal nerve tracts (green arrow) and the cerebellar white matter have an abnormal signal (**III**). In the middle, T_2-weighted images of a severe, infantile onset case (LBSL266) at the age of five years are shown. The cerebral white matter is diffusely affected (**I** and **II**) and the posterior limb of the internal capsule (black arrow) and globus pallidus (white arrow) are abnormal (**II**). In the medulla, a diffuse hyperintens signal is seen; the inferior olives (black arrow) are the only structures not affected (**III**). The FLAIR images on the right are from a 40 year old patient with mild disease (LBSL229). They show small, multifocal white matter lesions around the ventricles (**I**) and in the cerebellum (**III**). At the level of the basal ganglia the white matter is normal (**II**). The multifocal lesions in this patient are suggestive of Multiple Sclerosis, but the combination with the abnormalities in brainstem and spinal cord (supplementary figure 1B) is distinctive for the diagnosis LBSL.

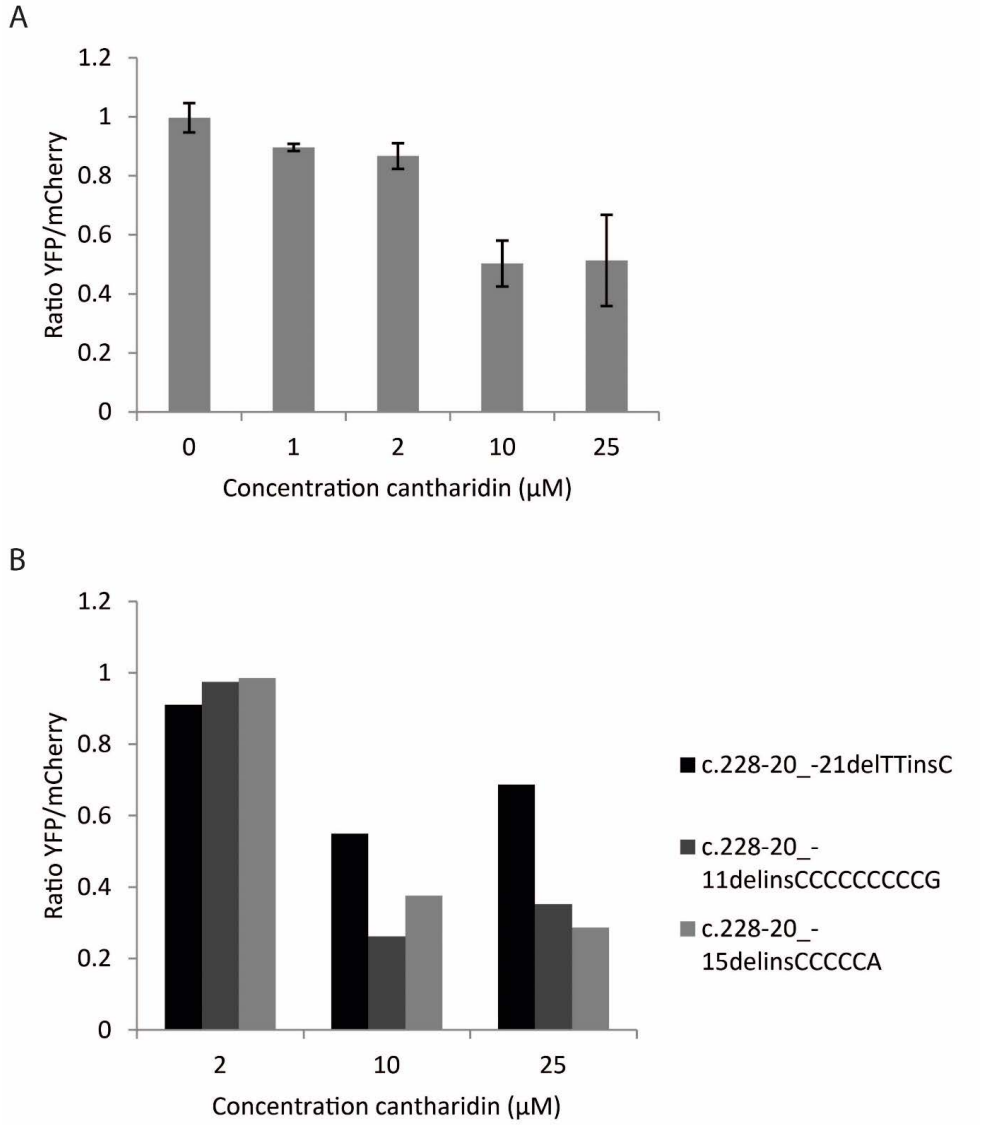


Supplementary Figure 3 | Reproducibility of mitochondrial aspartyl-tRNA synthetase activity measurements in control and patients' lymphoblasts. The aspartylation activity in isolated mitochondria from two control and two patient lymphoblast cell lines was measured as described in the methods section. The means of three separate experiments ± standard deviation are shown.

Supplementary Table 4 | Top 5 hits of compound screen

Compound	YFP/mCherry ratio[1]
Gentian violet	0,53
Cantharidin	0,59
Pyrithione zinc	0,61
Celastrol	0,63

[1]Control values observed in DMSO treated cells were set at a ratio of 1.



Supplementary Figure 4 | Cantharidin and *DARS2* exon 3 splicing. (**A**) The effect of different concentrations of cantharidin on the reporter construct containing the c.228-21_-20delTTinsC is shown. The YFP / mCherry-ratio in the presence of DMSO was set at 1. Values are means of three separate experiments ± standard deviation. (**B**) The effect of varying concentrations of cantharidin, as indicated below the figure, was tested on three different reporter constructs containing intron 2 mutations.

REFERENCES

1. van der Knaap MS, van der Voorn P, Barkhof F, et al. A New Leukoencephalopathy with Brainstem and Spinal Cord Involvement and High Lactate. Ann Neurol 2003;53:252-258.
2. Linnankivi T, Lundbom N, Autti T, et al. Five New Cases of a Recently Described Leukoencephalopathy with High Brain Lactate. Neurology 2004;63:688-692.
3. Serkov SV, Pronin IN, Bykova OV, et al. Five Patients with a Recently Described Novel Leukoencephalopathy with Brainstem and Spinal Cord Involvement and Elevated Lactate. Neuropediatrics 2004;35:1-5.
4. Tavora DG, Nakayama M, Gama RL, Alvim TC, Portugal D, Comerlato EA. Leukoencephalopathy with Brainstem and Spinal Cord Involvement and High Brain Lactate: Report of Three Brazilian Patients. Arq Neuropsiquiatr 2007;65:506-511.
5. Uluc K, Baskan O, Yildirim KA, et al. Leukoencephalopathy with Brain Stem and Spinal Cord Involvement and High Lactate: A Genetically Proven Case with Distinct Mri Findings. J Neurol Sci 2008;273:118-122.
6. Scheper GC, van der Klok T, van Andel RJ, et al. Mitochondrial Aspartyl-Trna Synthetase Deficiency Causes Leukoencephalopathy with Brain Stem and Spinal Cord Involvement and Lactate Elevation. Nat Genet 2007;39:534-539.
7. Petzold GC, Bohner G, Klingebiel R, Amberger N, van der Knaap MS, Zschenderlein R. Adult Onset Leucoencephalopathy with Brain Stem and Spinal Cord Involvement and Normal Lactate. J Neurol Neurosurg Psychiatry 2006;77:889-891.
8. Labauge P, Roullet E, Boespflug-Tanguy O, et al. Familial, Adult Onset Form of Leukoencephalopathy with Brain Stem and Spinal Cord Involvement: Inconstant High Brain Lactate and Very Slow Disease Progression. Eur Neurol 2007;58:59-61.
9. van Berge L, Dooves S, van Berkel CG, Polder E, van der Knaap MS, Scheper GC. Leukoencephalopathy with Brain Stem and Spinal Cord Involvement and Lactate Elevation Is Associated with Cell-Type-Dependent Splicing of Mtasprs Mrna. Biochem J 2012;441:955-962.
10. Synofzik M, Schicks J, Lindig T, et al. Acetazolamide-Responsive Exercise-Induced Episodic Ataxia Associated with a Novel Homozygous *DARS2* Mutation. J Med Genet 2011;48:713-715.
11. Moore SA, Kumar N, Gavrilova RH. Leukoencephalopathy with Brain Stem and Spinal Cord Involvement (and High Lactate): Raising the Bar for Diagnosis. J Neurol 2012;259:2494-2497.
12. Miyake N, Yamashita S, Kurosawa K, et al. A Novel Homozygous Mutation of *DARS2* May Cause a Severe Lbsl Variant. Clin Genet 2011;80:293-296.
13. Galluzzia P, Sacchinib M, Bartalini G, et al. Lbsl (Leukoencephalopathy with Brain Stem and Spinal Cord Involvement and High Lactate) without Sparing of the U-Fibers and Globi Pallidi: A Case Report. European Journal of Radiology Extra 2011;79.
14. Steenweg ME, van Berge L, van Berkel CG, et al. Early-Onset Lbsl: How Severe Does It Get? Neuropediatrics 2012;43:332-338.
15. Eliasson AC, Krumlinde-Sundholm L, Rosblad B, et al. The Manual Ability Classification System (Macs) for Children with Cerebral Palsy: Scale Development and Evidence of Validity and Reliability. Dev Med Child Neurol 2006;48:549-554.
16. Palisano RJ, Rosenbaum P, Bartlett D, Livingston MH. Content Validity of the Expanded and Revised Gross Motor Function Classification System. Dev Med Child Neurol 2008;50:744-750.
17. van der Knaap MS, Breiter SN, Naidu S, Hart AA, Valk J. Defining and Categorizing Leukoencephalopathies of Unknown Origin: Mr Imaging Approach. Radiology 1999;213:121-133.
18. Tzoulis C, Tran GT, Gjerde IO, et al. Leukoencephalopathy with Brainstem and Spinal Cord Involvement Caused by a Novel Mutation in the *DARS2* Gene. J Neurol 2012;259:292-296.
19. Mikhailova SV, Zakharova E, Banin AV, Demushkina AA, Petrukhin AS. [Clinical and Molecular Genetic Diagnosis of Leukoencephalopathy with Brainstem and Spinal Cord Involvement and Lactate Elevation in Children]. Zh Nevrol Psikhiatr Im S S Korsakova 2009;109:16-22.
20. Labauge P, Dorboz I, Eymard-Pierre E, Dereeper O, Boespflug-Tanguy O. Clinically Asymptomatic Adult Patient with Extensive Lbsl Mri Pattern and *DARS2* Mutations. J Neurol 2011;258:335-337.
21. Huang QH, Xiao JX, Wang JM, Jiang YW, Wu Y. [Clinical and Genetic Analysis of a Family with Leukoencephalopathy with Brain Stem and Spinal Cord Involvement and Lactate Elevation]. Zhonghua Er Ke Za Zhi 2012;50:50-55.
22. Hothorn T, Hornik K, Zeileis A. Unbiased Recursive Partitioning: A Conditional Inference Framework. J Comput Graph Stat2006: 651-674.
23. van Kollenburg B, Thomas AA, Vermeulen G, et al. Regulation of Protein Synthesis in Lymphoblasts from Vanishing White Matter Patients. Neurobiol Dis 2006;21:496-504.

24. Breitling R, Armengaud P, Amtmann A, Herzyk P. Rank Products: A Simple, yet Powerful, New Method to Detect Differentially Regulated Genes in Replicated Microarray Experiments. FEBS Lett 2004;573:83-92.
25. Isohanni P, Linnankivi T, Buzkova J, et al. *DARS2* Mutations in Mitochondrial Leucoencephalopathy and Multiple Sclerosis. J Med Genet 2010;47:66-70.
26. Lin J, Chiconelli Faria E, Da Rocha AJ, et al. Leukoencephalopathy with Brainstem and Spinal Cord Involvement and Normal Lactate: A New Mutation in the *DARS2* Gene. J Child Neurol 2010;25:1425-1428.
27. Sharma S, Sankhyan N, Kumar A, Scheper GC, van der Knaap MS, Gulati S. Leukoencephalopathy with Brain Stem and Spinal Cord Involvement and High Lactate: A Genetically Proven Case without Elevated White Matter Lactate. J Child Neurol 2011;26:773-776.
28. van Berge L, Kevenaar J, Polder E, et al. Pathogenic Mutations Causing Lbsl Affect Mitochondrial Aspartyl-Trna Synthetase in Diverse Ways. Biochem J 2013;450:345-350.
29. Southwell AL, Skotte NH, Bennett CF, Hayden MR. Antisense Oligonucleotide Therapeutics for Inherited Neurodegenerative Diseases. Trends Mol Med 2012;18:634-643.
30. Novoyatleva T, Heinrich B, Tang Y, et al. Protein Phosphatase 1 Binds to the Rna Recognition Motif of Several Splicing Factors and Regulates Alternative Pre-Mrna Processing. Hum Mol Genet 2008;17:52-70.
31. Moed L, Shwayder TA, Chang MW. Cantharidin Revisited: A Blistering Defense of an Ancient Medicine. Arch Dermatol 2001;137:1357-1360.
32. Zhang Z, Kelemen O, van Santen MA, et al. Synthesis and Characterization of Pseudocantharidins, Novel Phosphatase Modulators That Promote the Inclusion of Exon 7 into the Smn (Survival of Motoneuron) Pre-Mrna. J Biol Chem 2011;286:10126-10136.
33. Hidecker MJ, Paneth N, Rosenbaum PL, et al. Developing and Validating the Communication Function Classification System for Individuals with Cerebral Palsy. Dev Med Child Neurol 2011;53:704-710.

Chapter 2.2

Reply: *DARS2* gene clinical spectrum: new ideas regarding an underdiagnosed leukoencephalopathy

Marjo S. van der Knaap, Eline M.C. Hamilton and Laura van Berge

Brain. 2014;137:e290

Sir,

We thank Drs Bocca Vieira de Rezende Pinto and Sgobbi de Souza for their interest in our recent overview paper on a cohort of patients with leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL), caused by *DARS2* mutations.[1] Their letter[2] provides us with the opportunity to make specific points clearer.

The authors give a summary of the information published to date and pay special attention to exceptional cases, such as described by Miyake et al.[3] and Synofzik et al.[4] Additionally, they comment that magnetic resonance spectroscopy does not invariably reveal elevated lactate in patients with LBSL. They therefore suggest replacing the name LBSL by '*DARS2*-related conditions' or '*DARS2*-related spectrum disorders'.

We agree that the use of an acronym as the name for a disease may suggest that all patients fulfil all letters of the acronym at all stages of the disease. What is more, we cannot exclude the possibility that patients with a different neurological or even non-neurological phenotype may have *DARS2* mutations, which is not known because *DARS2* has not been analyzed in such patients. However, there is at present no positive evidence for the existence of an entirely different phenotype caused by *DARS2* mutations and there is, therefore, at present no information that would justify the name '*DARS2*-related conditions'. On the contrary, LBSL is a rather homogeneous disease with limited variation in symptomatology. All or virtually all known patients fulfil the 'L' for leukoencephalopathy, the 'B' for brainstem abnormalities and the 'S' for spinal cord abnormalities.[1] That not all patients had elevated lactate in magnetic resonance spectroscopy was known from the time that the name LBSL was coined.[5] The information that has become available after the first publication[5] mainly concerns the severe variants.[3,6] Strikingly, especially the unusually severe cases fulfil all letters of the acronym.

For some disorders the addition of 'spectrum' is preferred to indicate that the clinical, MRI and histopathological variation is much wider than initially indicated, and that the original name does not cover all variants. This is, for instance, the case in 'Zellweger syndrome', a name associated with a severe, infantile onset, multi-organ disease.[7] The name 'Zellweger spectrum disorders' was introduced to include all disorders caused by mutations in the same genes, and covers a much wider phenotypic range, in which numerous patients lack many of the abnormalities observed in the infantile variant.[8] The variability in phenotypes related to *DARS2* mutations, as far as currently known,[1] is in our opinion insufficient to speak of '*DARS2*-related spectrum disorders'.

Changing a name of a disease also comes with negative effects. To date, only the acronym LBSL has been used. All papers on the subject can easily be found by using this acronym. The name is informative and refers to generally shared features. Weighing the pros and cons, we conclude that there is, at present, in our opinion, insufficient reason to change the name LBSL.

We would like to indicate that cantharidin is not an 'antisense oligonucleotide', as suggested by Bocca Vieira de Rezende Pinto and Sgobbi de Souza,[2] but a

compound that influences splicing. In our paper, we describe the influence of cantharidin on the splicing defect of the *DARS2* gene in cellular assays.[1] Cantharidin is, however, too toxic for application in humans. We propose that less toxic compounds with the same effects should be searched for, such as some of the pseudocantharidins described by Zhang et al.[9] For alternative treatment options, Drs Bocca Vieira de Rezende Pinto and Sgobbi de Souza refer to the paper of Synofzik et al.,[4] in which a single patient with exercise-induced paroxysmal gait ataxia was described with the typical MRI of LBSL and a homozygous *DARS2* mutation. This patient showed an excellent dose-dependent, sustained positive response to a carbonic anhydrase inhibitor. In view of this, Bocca Vieira de Rezende Pinto and Sgobbi de Souza propose to investigate treatment of LBSL patients with acetazolamide or another carbonic anhydrase inhibitor first. Although this is an interesting option, we would like to comment that even though we know the largest cohort of LBSL patients worldwide, we have not come across another LBSL patient with exercise-induced paroxysmal ataxia. It is important to note that Synofzik et al.[4] have not proven that the exercise-induced paroxysmal ataxia is part of the LBSL phenotype. We therefore prefer an approach directed at what is known about the basic defect.

REFERENCES

1. van Berge L, Hamilton EM, Linnankivi T, et al. Leukoencephalopathy with Brainstem and Spinal Cord Involvement and Lactate Elevation: Clinical and Genetic Characterization and Target for Therapy. Brain 2014;137:1019-1029.
2. Pinto WB, de Souza PV. *DARS2* Gene Clinical Spectrum: New Ideas Regarding an Underdiagnosed Leukoencephalopathy. Brain 2014;137:e289.
3. Miyake N, Yamashita S, Kurosawa K, et al. A Novel Homozygous Mutation of *DARS2* May Cause a Severe Lbsl Variant. Clin Genet 2011;80:293-296.
4. Synofzik M, Schicks J, Lindig T, et al. Acetazolamide-Responsive Exercise-Induced Episodic Ataxia Associated with a Novel Homozygous *DARS2* Mutation. J Med Genet 2011;48:713-715.
5. van der Knaap MS, van der Voorn P, Barkhof F, et al. A New Leukoencephalopathy with Brainstem and Spinal Cord Involvement and High Lactate. Ann Neurol 2003;53:252-258.
6. Steenweg ME, van Berge L, van Berkel CG, et al. Early-Onset Lbsl: How Severe Does It Get? Neuropediatrics 2012;43:332-338.
7. Zellweger H, Maertens P, Superneau D, Wertelecki W. History of the Cerebrohepatorenal Syndrome of Zellweger and Other Peroxisomal Disorders. South Med J 1988;81:357-364.
8. Poll-The BT, Gartner J. Clinical Diagnosis, Biochemical Findings and Mri Spectrum of Peroxisomal Disorders. Biochim Biophys Acta 2012;1822:1421-1429.
9. Zhang Z, Kelemen O, van Santen MA, et al. Synthesis and Characterization of Pseudocantharidins, Novel Phosphatase Modulators That Promote the Inclusion of Exon 7 into the Smn (Survival of Motoneuron) Pre-Mrna. J Biol Chem 2011;286:10126-10136.

II

Megalencephalic leukoencephalopathy with subcortical cysts

Chapter 3

Megalencephalic leukoencephalopathy with subcortical cysts: characterization of disease variants

Eline M.C. Hamilton, MD, Pinar Tekturk, MD, Fia Cialdella, BSc, Diane F. van Rappard, MD, Nicole I. Wolf, MD, PhD, Cengiz Yalcinkaya, MD, Ümran Çetinçelik, MD, Ahmad Rajaee, MD, Ariana Kariminejad, MD, Justyna Paprocka, MD, PhD, Zuhal Yapici, MD, Vlatka Mejaški Bošnjak, MD, PhD, Marjo S. van der Knaap, MD, PhD, on behalf of the MLC Research Group

Neurology. 2018:90;e1395-e1403.

ABSTRACT

Objective: To provide an overview of clinical and MRI characteristics of the different variants of the leukodystrophy “megalencephalic leukoencephalopathy with subcortical cysts” (MLC) and identify possible differentiating features.

Methods: We performed an international multi-institutional, cross-sectional observational study of the clinical and MRI characteristics in genetically confirmed MLC patients. Clinical information was obtained by questionnaires for physicians and retrospective chart review.

Results: We included 204 patients with classic MLC, of whom 187 had recessive mutations in *MLC1* (MLC1 variant) and 17 in *GLIALCAM* (MLC2A variant) and 38 patients with remitting MLC caused by dominant *GLIALCAM* mutations (MLC2B variant). We observed a relatively wide variability in neurological disability among patients with classic MLC. No clinical differences could be identified between MLC1 and MLC2A patients. MLC2B patients invariably had a milder phenotype with preservation of motor function, while intellectual disability and autism were relatively frequent. Systematic MRI review revealed no MRI features that distinguish between MLC1 and MLC2A. Radiological improvement was observed in all MLC2B patients and also in two MLC1 patients. In MRIs obtained in the early disease stage, absence of signal abnormalities of the posterior limb of the internal capsule and cerebellar white matter and presence of only rarefied subcortical white matter instead of true subcortical cysts were suggestive of MLC2B.

Conclusions: Clinical and MRI features did not distinguish between classic MLC patients with *MLC1* or *GLIALCAM* mutations. Absence of signal abnormalities of the internal capsule and cerebellar white matter are MRI findings that point to the remitting phenotype.

INTRODUCTION

Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is an infantile-onset inherited disorder characterized by cerebral white matter edema.[1-3] Magnetic Resonance Imaging (MRI) shows diffuse signal abnormalities of the cerebral hemispheric white matter. The swelling of the abnormal cerebral white matter is most prominent in the first few years of life.[1] Subcortical cysts are typically located in the anterior temporal region and less consistently elsewhere.[4] Two different MLC phenotypes can be distinguished: a classic, deteriorating phenotype and a remitting phenotype.[5] Classic MLC is caused by recessive mutations in the *MLC1* gene (MIM 605908) in the majority of cases: this variant is called MLC1 (MIM 604004).[6] Classic MLC caused by recessive *GLIALCAM* mutations (also known as *HEPACAM*, MIM 613925) is called MLC2A.[6] Patients with remitting MLC or MLC2B (MIM 613926) have dominant *GLIALCAM* mutations.[7]

Classic MLC starts with increasing macrocephaly in the first year of life; after a few years, patients develop neurological signs, most commonly ataxia, spasticity and epilepsy.[1] Mortality is thought to be low, although no systematic study on survival has been performed. Remitting MLC initially resembles classic MLC: patients generally present with progressive macrocephaly and may have developmental delay, but neurological deterioration does not occur and the MRI abnormalities improve or normalize.

Among MLC1 patients, a relatively broad variation in disease severity has been described, also for siblings and unrelated patients with the same mutations.[8,9] So far, no clinical differences have been recognized between patients with MLC2A versus MLC1. It is unknown whether MRI features allow distinction between MLC1, MLC2A and MLC2B before improvement occurs in the latter.

MLC is a rare disorder and large studies on the disease course are scarce.[8,10,11] Systematic review of MRI characteristics has not been performed. The objective of the current study is to identify potential different and perhaps discriminating clinical and MRI features for different MLC variants, aiming at improved clinical recognition and avoidance of unnecessary genetic testing.

PATIENTS AND METHODS

Study design

We performed an international, cross-sectional observational multicenter study among all genetically proven MLC patients enrolled in the Amsterdam Database of leukoencephalopathies between January 1991 and January 2017. The database contains patients from over the world referred to VU University Medical Center for MRI review and mutational analysis. A prerequisite for genetic testing was review of clinical and MRI data in Amsterdam.[1] We performed analysis of the *MLC1* gene as previously described,[12] if necessary including analysis of *MLC1* copy DNA (cDNA)[12] and Multiplex Ligation-dependent Probe Amplification (MLPA).[13] Analysis of the

GLIALCAM gene was also performed as previously described.[7] By DNA analysis in the parents we confirmed that recessive variants were bi-allelic.

Standard protocol approvals, registrations, and patient consents

We received approval from the ethical standards committee for clinical evaluation, systematic MRI review and review of the DNA findings and obtained informed consent from the patients/guardians.

Clinical information

We analyzed clinical information available at the start of the study, supplemented by information obtained via a clinical questionnaire for physicians. We focused on measures that could be retrospectively assessed, including head circumference, motor deterioration (especially loss of ambulation), seizures, and death. As formal, standardized assessment of motor and cognitive development, autistic features, behavioral problems and current cognitive function was not feasible, these items were subjectively assessed by physicians and caretakers or derived from medical records. The patients' status at last examination was assessed by quantitative, validated and widely used 5-level classification systems for gross motor function (GMFCS), manual ability (MACS) and communication function (CFCS).[14] Scores on these scales range from I (no limitations) up to V (severe limitations; table e-1). Patients with comorbidities affecting neurological function were excluded from the study.

MRI scoring

We scored all available MRIs according to a standardized protocol.[15] Specifically, cysts were defined as areas with the same signal intensity of CSF on all pulse sequences including FLAIR, while in rarefaction the signal intensity was close but not the same.[16]

Statistical analysis

We used summary statistics to describe the clinical characteristics. Results were reported by means +/- standard deviation for continuous variables that were normally distributed, and median with 25th and 75th percentiles for non-normally distributed data and ranges. We performed time-to-event analysis of the events 'start of motor deterioration', 'start of cognitive decline', 'first seizure', 'loss of walking without support', 'loss of walking with or without support' and 'death', with age as time variable. Individuals in whom the respective event had not occurred at the last follow-up were indicated as censored for the respective analysis. Non-ambulatory patients below the age of 18 months were not included in the analysis of loss of ambulation. We estimated the median ages at which the events had occurred by plotting Kaplan-Meier curves. Group differences regarding disease variant were analyzed with the log-rank test. We performed linear regression analysis to compare GMFCS, MACS and CFCS scores in relation to age per disease variant. Statistical analysis was

performed using SPSS version 22 (Armonk, NY: IBM Corp) and GraphPad Prism version 6.07 (San Diego California USA).

RESULTS

Patients

245 MLC patients from 207 families were diagnosed with MLC by DNA analysis. Three patients were excluded because of co-morbidity (Turner syndrome, asphyxia, pituitary adenoma). In the case of limited clinical information due to loss to follow-up or nonresponse, patients were selectively included in the analyses on the basis of availability of information. Throughout the results' section we report the number of patients that were included in the different analyses in parentheses or in the respective tables.

In total 242 MLC patients, of which 187 MLC1 patients, 17 MLC2A patients and 38 MLC2B patients, were included.

Clinical characteristics

Details are presented in Table 1. Macrocephaly in the first year of life was the most common first disease sign and had been present in almost all patients in infancy. Macrocephaly persisted in more than half of patients. Secondary normocephaly was particularly common in MLC2B patients. Initial motor development was reported as mildly delayed in the majority of patients and normal in a smaller number. All patients except 12 MLC1 patients achieved unsupported walking, at a mean age of 16 months (± 8, range 10 -72). Initial cognitive development was reported as normal in just over half of patients with MLC1, MLC2A and MLC2B.

Kaplan-Meier curves on start of motor deterioration indicated that both MLC1 and MLC2A patients showed the first signs of motor deterioration at a median age of 5 years (range 6 months - 41 years); there were no significant differences between the curves (p=0.98; Figure 1A). None of the patients with MLC2B had a decline of motor function; in 34% (10/29) some clumsiness remained.

Loss of walking without support occurred in the majority of patients with MLC1 and MLC2A at ages ranging from 18 months to 43 years; Kaplan-Meier curves showed no significant differences between MLC1 and MLC2A (p=0.66; Figure 1B). Median age at loss of walking without support was estimated to be 15 years in MLC1 and 11 years in MLC2A. Patients with MLC2B all remained ambulatory.

Loss of walking with or without support (full wheelchair dependency) occurred in less than half of patients with MLC1 and MLC2A and none of the MLC2B patients. There were no significant differences between MLC1 and MLC2A patients (p=0.58; Figure 1C). Patients were most likely to become wheelchair dependent before the age of 15 years; after this age patients generally remained ambulatory, with or without support.

Behavioral problems were relatively common for all MLC variants; autism was most common among MLC2B patients. Delayed onset cognitive decline was reported in

almost half of patients with MLC1 and MLC2A, and none of the patients with MLC2B. However, stable cognitive impairment was reported in a quarter of MLC2B patients. The age at start of cognitive decline did not statistically differ between MLC1 and MLC2A (log rank p=0.10). The patients' cognitive levels at time of the inventory are presented in Table 1.

At the time of clinical phenotyping, 109 patients had had at least one seizure, of whom the majority had occasional seizures that were generally well controlled with medication (Table 1). Kaplan-Meier curve analysis indicated that approximately 75% of patients with MLC1 and MLC2A had had one or multiple seizures by the age of 20 years; the median age at the first seizure was 3 years, mode 2 years (Figure 1D). Seizures were less common among patients with MLC2B (n=4/34). There were no significant differences in onset of seizures between patients with MLC1 and MLC2A (p=0.64).

Table 1 | Clinical characteristics at latest phenotyping

	MLC1	MLC2A	MLC2B
Study characteristics			
Number of patients	187	17	38
Male / female	102 / 85	9 / 8	27 / 11
Median age at latest phenotyping [quartiles]	13 y [6 - 19 y]	15 y [7 - 23 y]	7 y [2 - 13 y]
Head circumference			
≥ 2SD below age 2 years	99% (121/122)	88% (14/16)	88% (30/34)
Currently ≥ 2SD	93% (122/131)	73% (11/15)	56% (15/27)
Walking without support			
Achieved ≤ 18 months	63% (88/138)	65% (11/17)	86% (30/35)
Achieved > 18 months	28% (38/138)	35% (6/17)	14% (5/35)
Not achieved	9 % (12/138)	0% (0/17)	0% (0/35)
Seizures			
No history of seizures	38% (50/132)	31% (5/16)	88% (30/34)
Single seizure	7 % (9/132)	19% (3/16)	3% (1/34)
Well controlled epilepsy	46% (61/132)	44% (7/16)	6% (2/34)
Poorly controlled epilepsy	9% (12/132)	6% (1/16)	3% (1/34)
History of behavioral problems			
Autistics features	9% (8/86)	0% (0/15)	25% (9/36)
Other behavioral problems	29% (31/107)	20% (3/15)	28% (9/32)
Psychiatric diagnosis	14% (15/111)	7% (1/14)	3% (1/30)
Current cognitive function			
Normal	37% (48/129)	31% (5/16)	73% (24/33)
Learning difficulties	21% (27/129)	25% (4/16)	15% (5/33)
Mild mental retardation	26% (34/129)	31% (5/16)	12% (4/33)
Severe mental retardation	16% (20/129)	13% (2/16)	0% (0/33)

y, years

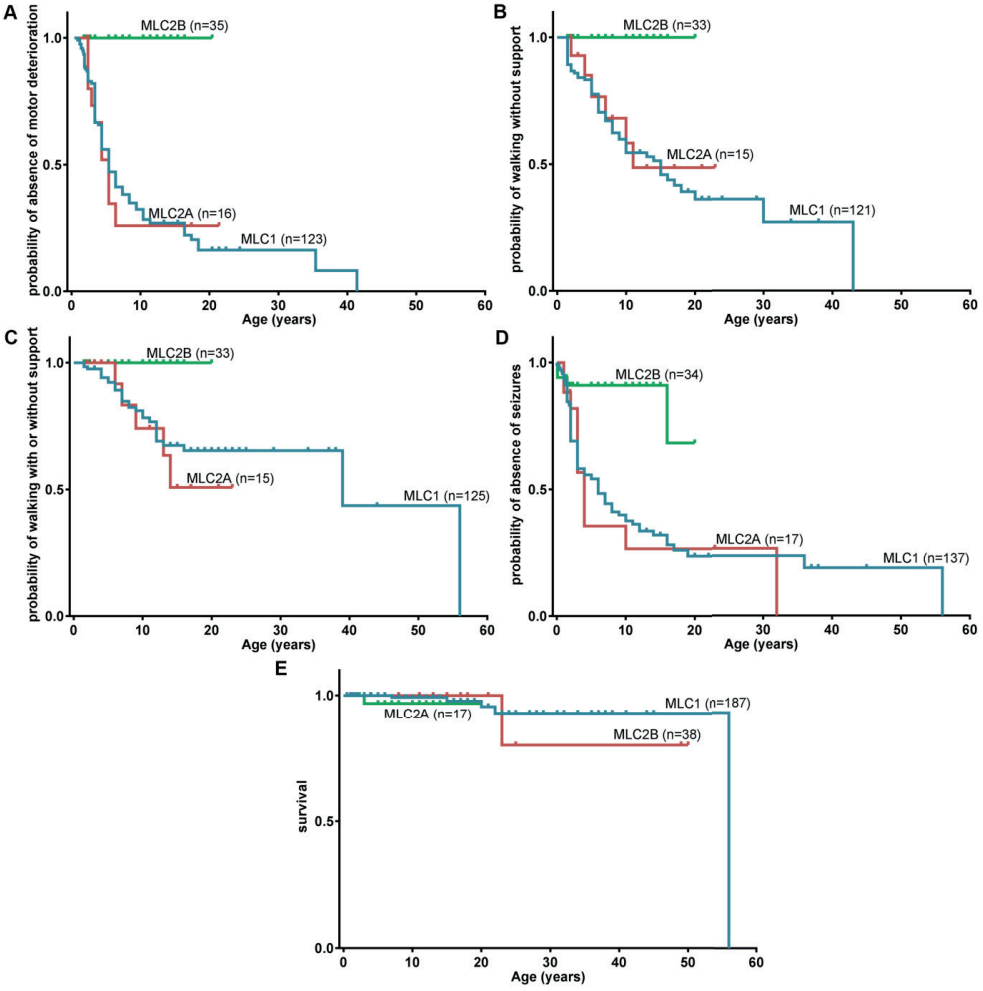


Figure 1 | Disease course. Kaplan-Meier plots on (**A**) onset of motor deterioration, (**B**) loss of walking without support, (**C**) loss of walking with or without support (full wheelchair dependency), (**D**) onset of seizures and (**E**) survival, grouped by disease variant. Censored patients (absence of motor deterioration, still walking without support, still walking with or without support, absence of seizures or still alive at last follow up) are indicated by crosses.

Overview of GMFCS, MACS and CFCS scores in relation to age and disease variant showed that there was a wide variability in clinical severity among patients with MLC1 and MLC2A (Figure 2). Linear regression analysis showed no significant differences between the patients with MLC1 and MLC2A regarding GMFCS (p=0.30), MACS (p=0.18), and CFCS scores (p=0.89). Gross motor function and dexterity were generally well preserved in patients with MLC2B and communication function to a lesser degree.

Several classic MLC patients had very mild disease: 25% (17/68) of patients with MLC1 and 45% (5/11) with MLC2A who were at least 12 years at latest clinical evaluation were able to walk without support and had normal cognition or only learning problems. For ages of 12 years and above, 32% (19/59) of patients with MLC1 and 50% (5/10) with MLC2A had GMFCS, MACS and CFSC scores of I or II.

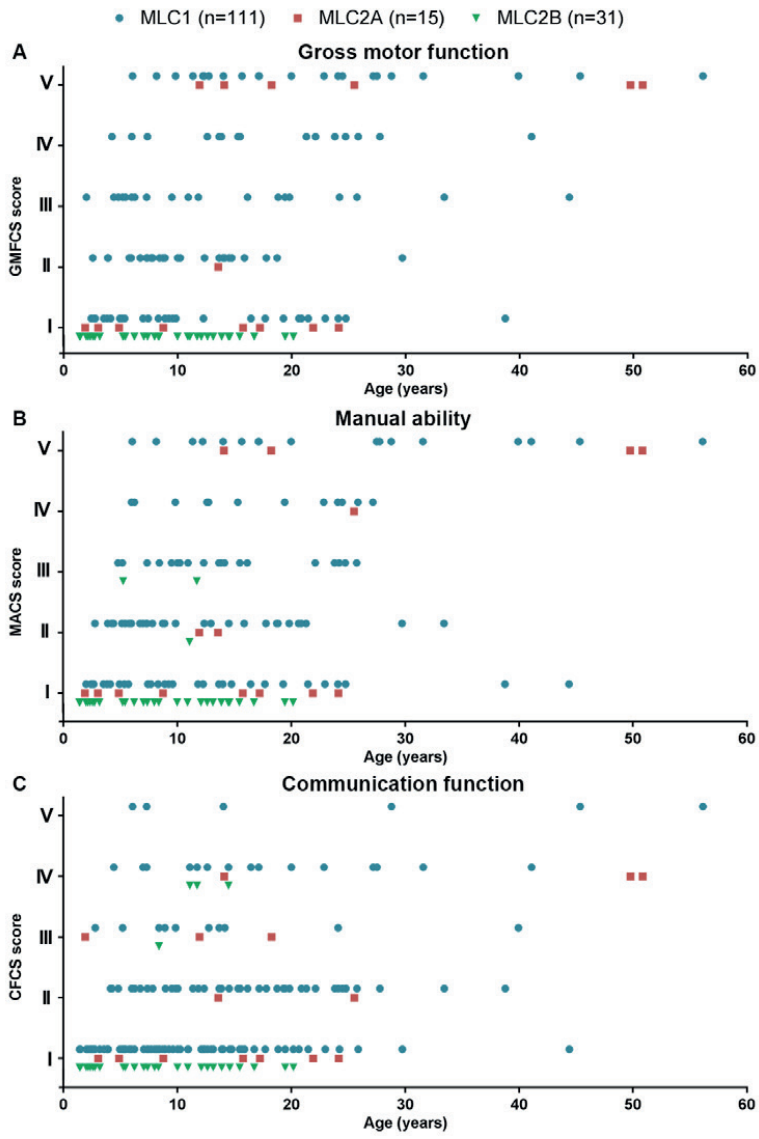


Figure 2 | Function levels. Overview of patients' scores on the Gross Motor Function Classification System (GMFCS), Manual Ability Classification System (MACS) and Communication Function Classification System (CFCS) in relation to age, grouped by disease variant. Scores range from I (no limitations) up to V (severe limitations; Supplementary Table 1).

Survival was assessed in all 242 patients; seven were deceased (Figure 1E). Five deceased patients had MLC1: four males died at age 7-22 years and one female at age 56 years. Causes of death were respiratory insufficiency after neurological deterioration following head trauma, suspected Sudden Unexpected Death in Epilepsy (SUDEP), sepsis as complication of surgical procedure, cachexia and status epilepticus. One deceased male MLC2A patient and one deceased male MLC2B patient were suspected of SUDEP at ages of 23 and 3 years, respectively.

To assess a possible genotype-phenotype correlation in patients with MLC1 and MLC2A, we compared loss of ambulation, wheelchair dependency and seizures in groups of patients with three or more patients from two or more families with the same mutations, who were at least 10 years at the clinical inventory. The number of patients with MLC2A was too small for evaluation. Six groups of informative patients with MLC1 were available for comparison; characteristics were very divergent within each group (Supplementary Table 2), arguing against a genotype-phenotype correlation.

MRI characteristics

MRIs were available for 187 patients and for 53 patients one or more follow up scans were available; we evaluated a total number of 268 MRIs. Patients had their first MRI at a median age of 2 years (range 4 months - 55 years). Follow-up scans were obtained at a median age of 5 years (range 11 months - 43 years). The median interval between the first and last MRIs was 4 years (range 3 months - 15 years).
Details of the first MRIs are presented in Supplementary Table 3. Extensive, confluent signal abnormalities of the cerebral white matter were invariably present and swelling was observed in all patients, except for one, in whom the first MRI was obtained at 30 years. All patients had sparing of the optic radiation and most patients had sparing in one or more regions of subcortical white matter (Figure 3). Several patients also had some regional white matter sparing, mostly in the occipital region (Figure 3). There were no clear differences in abnormalities between patients with MLC1 and MLC2A. They all had an abnormal signal of the posterior limb of the internal capsule, while in 54% of patients with MLC2B, in whom the MRI was obtained before the age of 2 years, the signal of the internal capsule was already normal (Figure 3). Widening of the ventricles and enlargement of the subarachnoid space was rare in MLC1 and MLC2A patients in the early disease stage and more common in MRIs performed in adolescence or adulthood. In MLC2B patients, some enlargement of the subarachnoid spaces was already observed before the age of 2 years in nearly half of patients.
Subcortical cysts were present in all MLC1 patients and almost all MLC2A patients. Forty-three percent of patients with MLC2B did not have true cysts, but only near-cystic rarefaction of subcortical white matter. Cysts or near-cystic rarefaction were almost invariably present in both anterior temporal lobes. Additional cysts were located in the frontal and parietal lobes, but never in the occipital lobe. Near-cystic rarefaction of subcortical white matter could be located in all four lobes.

The majority of MLC1 and MLC2A patients (~ 85%) had mild signal abnormalities of the cerebellar white matter, which were less pronounced than the cerebral white matter signal abnormalities (Figure 3). Patients with MLC2B never had cerebellar white matter signal abnormalities; a few patients below the age of 10 months showed some delay in myelination featured by a hyperintense rim of the subcortical cerebellar white matter (Supplementary Figure 1). The cerebellar white matter was never swollen. Almost all patients with MLC1, MLC2A and MLC2B had mild signal abnormalities in the brainstem.

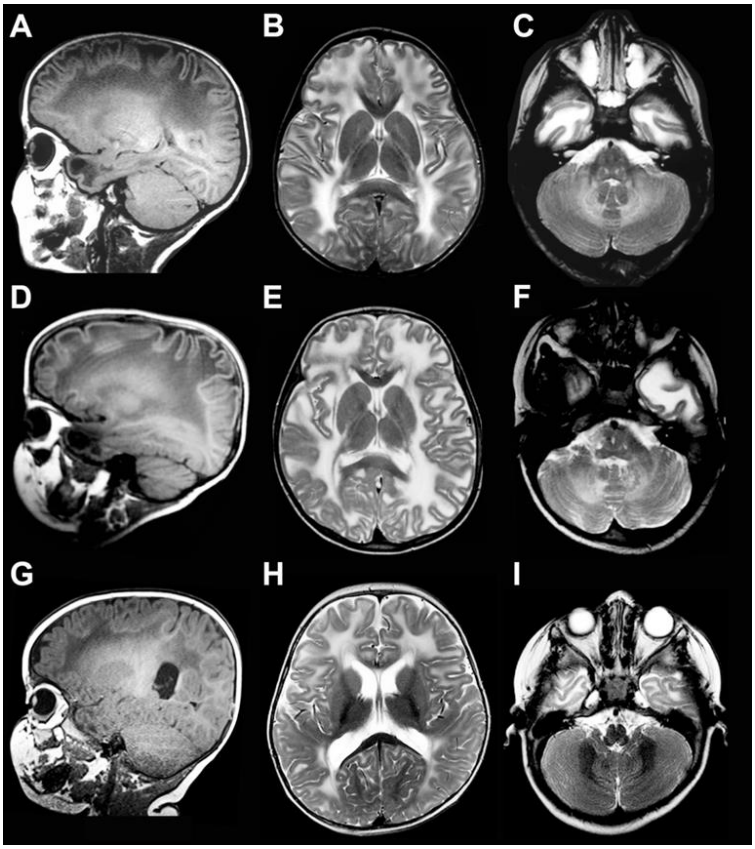


Figure 3 | MRI characteristics. MRI findings in an MLC1 patient at the age of 3 years (top), an MLC2A patient at the age of 6 years (middle) and an MLC2B patient at the age of 10 months (bottom). Sagittal T_1-weighted images show anterior-temporal and frontoparietal cysts in the MLC1 patient (A), an anterior-temporal cyst in the MLC2A patient (D) and some near-cystic rarefaction in the anterior-temporal region in the MLC2B patient (G). The T_2-weighted axial images of the hemispheres show diffuse white matter abnormalities with some swelling (B, E, H). The sagittal images show some sparing of the subcortical white matter in all patients, especially in the occipital region (A,D,G); in the MLC2B patient the white matter is relative preserved in the occipital region (G). The classic MLC patients have a double-line-shaped abnormal signal of the posterior limb of the internal capsule (B, E), while the signal is normal in the MLC2B patient (H). The T_2-weighted axial images of the cerebellum show a mildly hyperintense signal of the cerebellar white matter in the classic MLC patients (C, F) and a normal signal in the MLC2B patient (I).

Change over time

Details are presented in Table 2. Normalization of the signal and reversal of the swelling of the white matter only occurred in MLC2B patients. In MLC1 and MLC2A patients, the white matter signal abnormalities persisted or increased, with the exception of two MLC1 patients who showed improvement. The number of cysts generally remained the same or increased among patients with MLC1 and MLC2A; in MLC2B a decrease of cysts and overall quantity of cystic white matter generally occurred, although in all but one patient some cysts or near-cysts persisted.

Existing signal abnormalities of the posterior limb of the internal capsule and cerebellar white matter never improved in patients with MLC1 and MLC2A. The few patients with MLC2B, who had a rim of slightly abnormal cerebellar white matter on the initial MRI, all showed normalization over time.

Table 2 | MRI characteristics: change over time

	MLC1	MLC2A	MLC2B
Number of patients	29	5	19
Median interval [range]	4y [3m - 13y]	13y [1 - 15y]	3y [4m - 12y]
Swelling cerebral WM			
Normalization	0% (0/29)	0% (0/5)	11% (2/19)
Improvement	21% (6/29)	40% (2/5)	78% (15/19)
Unchanged	79% (23/29)	40% (2/5)	11% (2/19)
Increased	0% (0/29)	20% (1/5)	0% (0/19)
Signal abnormalities cerebral WM			
Normalization	0% (0/29)	0% (0/5)	21% (4/19)
Improvement	6% (2/29)	0% (0/5)	74% (14/19)
Unchanged	73% (21/29)	40% (2/5)	5% (1/19)
Increased	21% (6/29)	60% (3/5)	0% (0/19)
Signal abnormalities PLIC			
Not present	0% (0/26)	0% (0/5)	47% (9/19)
Normalization	0% (0/26)	0% (0/5)	26% (5/19)
Improvement	8% (2/26)	0% (0/5)	11% (2/19)
Unchanged	92% (24/26)	100% (5/5)	16% (3/19)
Enlargement ventricles and/or subarachnoid spaces			
Not present	72% (21/29)	40% (2/5)	53% (10/19)
Unchanged	4% (1/29)	0% (0/5)	0% (0/19)
Increased	24% (7/29)	60% (3/5)	47% (9/19)
Number of cysts			
Not present	0% (0/27)	0% (0/5)	37% (7/19)
Decreased	4% (1/27)	0% (0/5)	42% (8/19)
Unchanged	67% (18/27)	40% (2/5)	21% (4/19)
Increased	29% (8/27)	60% (3/5)	0% (0/19)
Signal abnormalities cerebellar WM			
Not present	11% (3/28)	40% (2/5)	89% (17/19)
Normalization	3% (1/28)	0% (0/5)	11% (2/19)
Decreased	0% (0/28)	0% (0/5)	0% (0/19)
Unchanged	86% (24/28)	60% (3/5)	0% (0/19)

m, months; y, years; WM, white matter; PLIC, posterior limb of the internal capsule

DISCUSSION

MLC is caused by mutations in *MLC1* or *GLIALCAM*. MLC1 is an astrocyte-specific membrane protein involved in brain ion and water homeostasis.[3,17] GlialCAM is a chaperone of MLC1 ensuring its localization in astrocytic endfeet.[7,18,19] So, both *MLC1* and *GLIALCAM* mutations affect MLC1 protein function. Additionally, GlialCAM is involved in transport of other proteins, such as connexin 43 and the chloride channel ClC2.[20,21] Recessive *CLCN2* mutations cause a leukoencephalopathy with childhood or adult onset, characterized by mild motor dysfunction and often retinopathy.[22] Brain MRI shows restricted diffusion in the posterior limbs of internal capsules, brain stem structures and cerebellar white matter, suggestive of myelin microvacuolization.[22] Pediatric patients have diffuse, mild cerebral white matter signal abnormalities, while these are limited in adult onset cases. By contrast, in MLC the cerebral white matter abnormalities are profound and diffusion is highly increased, consistent with myelin macrovacuolization and increased extracellular spaces.[23] Considering that GlialCAM is supposed to be a chaperone for both MLC1 and ClC2, one might expect *GLIALCAM* mutations to cause disease features of both *MLC1*- and *CLCN2*-associated diseases. The present study, however, confirms that MLC2A is indistinguishable from MLC1 and by no means shares the MRI features of the *CLCN2*- related disease. The exact roles of MLC1, GlialCAM and ClC2 in brain ion and water homeostasis and their interaction remain to be elucidated.

The knowledge obtained from our study concerning a relatively large cohort of patients can help physicians in clinical counseling of patients and families. MLC is a fairly mild and slow disorder, with low mortality compared to other leukodystrophies.[24] Nevertheless, there is a rather broad variation in clinical severity. Some patients become wheelchair dependent a few years after onset, while others remain ambulatory during adulthood. An interesting point is that slow motor deterioration often occurs from a few years after presentation onwards, but that patients who are ambulatory with or without support at the age of 15 years most likely remain ambulatory. The study confirms that epilepsy is a common feature in MLC. It is puzzling that most patients have well controlled epilepsy while only a few patients have refractory epilepsy and ~25% remains seizure free. A salient observation is that a considerable part of classic MLC patients exhibit only very mild signs during the disease course; in two MLC1 patients a considerable improvement of the MRI abnormalities occurred. The observed variability in disease severity - also among patients who share the same mutations - may depend on individual differences in compensatory volume regulatory mechanisms. The fact that remarkable improvement of cerebral swelling may occur, as mostly observed in MLC2B patients but also in a few classic MLC patients, suggests there is a window of opportunity for therapy.

The multi-institutional and observational nature of this study comes with certain limitations. In the absence of formal testing, measures like cognitive function and presence of autistic features were scored based on subjective assessment by physicians and caretakers. The application of time-to-event analysis and

standardized scales such as GMFCS resulted in a robust representation of disease progression, but missing data and inter-observer differences may have hampered the evaluation of the clinical course. There are, however, no indications that non-response was in any way systematic or that censoring in the time-to-event analyses was informative. The study limitations are at least in part compensated by the study size considering the disease is very rare. The available results give an already informative delineation of the clinical spectrum of MLC. Our ongoing data collection will help include larger numbers of MLC2A patients and extend the follow-up.

One of the aims of this study was to identify features that can help distinguish different MLC variants in early disease stages. Absence of MRI signal abnormalities of the posterior limb of the internal capsule and cerebellar white matter and presence of only rarefied subcortical white matter instead of actual cysts are features suggestive of MLC2B. This knowledge can be applied to direct genetic testing in patients suspected of the remitting phenotype. No distinguishing features have been identified to discern MLC1 and MLC2A. Considering the much higher prevalence of *MLC1* gene defects, in cases of a classic presentation of MLC, this gene should be tested first.

ACKNOWLEDEMENTS

The authors thank the patients and families for their cooperation and contribution. The following MLC Research Group collaborators contributed to the study: Hugo H. Abarca Barriga; Samer Abdelrazeq; Gül Aktan Serdaroğlu; P. Ian Andrews; Richard Appleton; Lucia Argandoña Palacios; Brenda Banwell; Florian Bauder; Gulcin Benbir; Tim Benke; Susan Blaser; Annette Bley; Cristiana Brenner; Knut Brockmann; Rafael Camino; Coriene Catsman-Berrevoets; Yanick Crow; Marguerite Dalton; María de la Luz Arenas-Sordo; Linda de Meirleir; Ana Isabel Dias; Francis J. DiMario; Maria Alice Donati; Nihal Olgac Dundar; Francois Feillet; Maria-Jose Fonseca; Emilio Franzoni; Jeremy Freeman; Katsunori Fujii; Soumya Ghosh; Scott Gold; Solange Gril; Barbara Hallinan; Agnes Herczegfalvi; Jozef Hertecant; Joannie Hui; David Hunt; Parul Jayakar; Bulent Kara; Çiğdem S. Kasapkara; Gulsen Kocaman; David M. Koeller; Wolfgang Köhler; Alfried Kohlschütter; Marja Koivusalo; Urania Kotzaeridou; Roshan Koul; Ingeborg Krägeloh-Mann; Ruzica Kravljanac; Gerhard Kurlemann; Julian Lara Herguedas; Silvia Laurentino; Richard Leventer; Bryan Lynch; Oliver Maier; Sascha Meyer; Olivera Miljanovic; José Paulo Monteiro; Ellen Moran; Teresa Moreno; Jacques Motte; Chris Moyes; Lakshmi Nagarajan; Marie-Cecile Nassogne; Slavica Ostojic; Peter Pietsch; Iliana Porfiri; Sofia Quintas; Maria Belen Ramos; Deborah Renaud; Biserka Rešić; Carolina Rivera Nieto; Jutta Rummel; Robert Rusina; Mustafa A. Salih; Sabine Scholl-Bürgi; Bitten Schönewolf-Greulich; Snehal Shah; Suvasini Sharma; Gabriella Silvestri; Komudi Siriwardena; Victoria Siu; Anne-Bine Skytte; Zeyneb Soysal; Carlos E. Speck Martins; Angela Sun; Burak Tatli; Gareth Thomas; Virpi Toivio; Leyla Tümer; Jean-Claude Turpin; Adeline Vanderver; HeleneVerhelst; Ishwar Verma; Rocio Villafuerte-de la Cruz; Roberta Vittorini; Evangeline Wassmer; Claudia Weiß; Janine Whale; Sau Wei Wong; Elif Yilmaz Gulec; Uluc Yiş.

SUPPLEMENTARY DATA

Supplementary Table 1 | Overview of the levels of the GMFCS, MACS and CFCS[14]

GMFCS: Gross Motor Function Classification System[25]

MACS: Manual Ability Classification System[26]

CFCS: Communication Function Classification System[14]

	GMFCS	MACS	CFCS
	Motor function	**Manual ability**	**Communication**
LEVEL I	Walks without limitations	Handles objects easily and successfully	Sends and receives with familiar and unfamiliar partners effectively and efficiently
LEVEL II	Walks with limitations	Handles most objects but with somewhat reduced quality and/or speed of achievement	Sends and receives with familiar and unfamiliar partners but may need extra time
LEVEL III	Walks using a hand-held mobility device	Handles objects with difficulty; needs help to prepare and/or modify activities	Sends and receives with familiar partners effectively, but not with unfamiliar partners
LEVEL IV	Self-Mobility with limitations; may use powered mobility	Handles a limited selection of easily managed objects in adapted situations	Inconsistently sends and/or receives even with familiar partners
LEVEL V	Transported in a manual wheelchair	Does not handle objects and has severely limited ability to perform even simple actions	Seldom effectively sends and receives, even with familiar partners

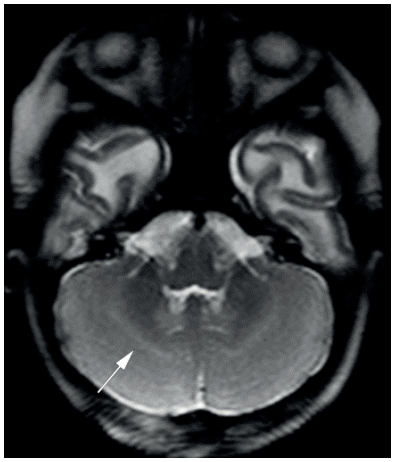
Supplementary Table 2 | Genotype-phenotype correlation

MLC1 mutations	Number of informative patients (families)	Median age at phenotyping [range]	Loss of ambulation[1]	Wheelchair dependency*	Seizures**
c.135insC, p.Cys46Leufs*34 homozygous	4 (4)	26 [14 -45] y	**(a) (b) (d)**	**(a) (b)**	**(a) (c)**
c.268_422del, p.Cys90_Ile141del homozygous	4 (3)	14 [10 - 20] y	**(a) (d)**	**(a) (c) (d)**	**(b) (c)**
c.278C>T, p.Ser93Leu homozygous	4 (4)	31 [14 - 39] y	**(a) (b) (c)**	**(a) (b) (c)**	**(c) (d)**
c.353C>G, p.Thr118Met homozygous	4 (3)	18 [12 - 24] y	**(a) (c)**	**(a)**	**(a) (c)**
c.424-3C>G, p.? homozygous	4 (2)	24 [19 - 27] y	**(b) (c)**	**(a) (b)**	**(c) (d)**
c.908_918delinsGCA, p.Val303Glyfs*96 homozygous	5 (3)	16 [12 - 20] y	**(a) (b)**	**(a)**	**(a) (b) (c)**

y, years

* **(a)**: function not lost **(b)**: Loss at age ≥ 11 years **(c)**: loss at age 6-10 years; **(d)**: loss at age 0-5 years

** **(a)**: absent; **(b)**: single seizure; **(c):** well controlled epilepsy; **(d)**: moderately controlled/refractory epilepsy



Supplementary Figure 1 | Hyperintense cerebellar rim in MLC2B. MRI findings in an MLC2B patient at the age of 5 months. The T2-weighted axial image of the cerebellum shows some delay in myelination, characterized by a mildly hyperintense rim of the subcortical cerebellar white matter, while the cerebellar white matter is otherwise adequately myelinated, as indicated by the low signal intensity.

Supplementary Table 3 | Characteristics first MRI

	MLC1		MLC2A		MLC2B	
AGE AT MRI	**<2 years**	**≥ 2 years**	**<2 years**	**≥ 2 years**	**<2 years**	**≥ 2 years**
Number of patients	50	91	5	11	28	2
CEREBRAL WM						
Signal PLIC						
normal	0% (0/49)	0% (0/89)	0% (0/5)	0% (0/11)	54% (15/28)	50% (1/2)
posterior part abnormal	2% (1/49)	7% (6/89)	20% (1/5)	9% (1/11)	14% (4/28)	50% (1/2)
double line throughout	92% (45/49)	93% (83/89)	80% (4/5)	91% (10/11)	32% (9/28)	0% (0/2)
abnormal throughout	6% (3/49)	0% (0/89)	0% (0/5)	0% (0/11)	0% (0/28)	0% (0/2)
Abnormal signal ALIC	92% (45/49)	21% (19/89)	60% (3/5)	10% (1/10)	29% (8/28)	0% (0/2)
Sparing subcortical WM						
frontal	13% (6/47)	27% (22/81)	40% (2/5)	20% (2/10)	11% (3/28)	50% (1/2)
temporal	13% (6/47)	26% (21/81)	80% (4/5)	20% (2/10)	25% (7/28)	100% (2/2)
parietal	21% (10/47)	61% (49/81)	80% (4/5)	50% (5/10)	36% (10/28)	100% (2/2)
occipital	38% (18/47)	84% (68/81)	80% (4/5)	80% (8/10)	57% (16/28)	100% (2/2)
Sparing of WM in certain brain area(s)						
frontal	0% (0/49)	6% (5/78)	0% (0/5)	0% (0/10)	0% (0/28)	50% (1/2)
temporal	0% (0/49)	3% (2/78)	0% (0/5)	0% (0/10)	4% (1/28)	50% (1/2)
parietal	0% (0/49)	6% (5/78)	0% (0/5)	0% (0/10)	14% (4/28)	100% (2/2)
occipital	4% (2/49)	12% (9/78)	20% (1/5)	10% (1/10)	11% (3/28)	100% (2/2)
Widening ventricles	6% (3/50)	25% (23/91)	0% (0/5)	27% (3/11)	7% (2/28)	50% (1/2)
Enlargement SAS	12% (6/50)	34% (31/91)	0% (0/5)	36% (4/11)	46% (13/28)	50% (1/2)
CYSTS AND NEAR-CYSTIC RAREFACTION OF THE WM						
Number of cysts						
0	0% (0/47)	0% (0/85)	40% (2/5)	10% (1/10)	43% (12/28)	50% (1/2)
1 - 2	64% (30/47)	36% (31/85)	20% (1/5)	30% (3/10)	36% (10/28)	50% (1/2)
3 - 6	34% (16/47)	52% (44/85)	20% (1/5)	40% (4/10)	21% (6/28)	0% (0/2)
> 6	2% (1/47)	12% (10/85)	20% (1/5)	20% (2/10)	0% (0/28)	0% (0/2)
Location of cysts						
frontal	30% (14/47)	54% (46/85)	40% (2/5)	60% (6/10)	21% (6/28)	0% (0/2)
temporal	98% (46/47)	99% (84/85)	60% (3/5)	90% (9/10)	57% (16/28)	50% (1/2)
parietal	17% (8/47)	37% (31/85)	20% (1/5)	20% (2/10)	7% (2/28)	0% (0/2)
occipital	0% (0/47)	0% (0/85)	0% (0/5)	0% (0/10)	0% (0/28)	0% (0/2)
Near cystic WM rarefaction	88% (42/48)	93% (79/85)	60% (3/5)	100% (11/11)	96% (27/28)	100% (2/2)
Overall quantity of cystic/near cystic WM						
none or little	6% (3/50)	93% (79/90)	40% (2/5)	18% (2/11)	32% (9/28)	50% (1/2)
moderate	88% (44/50)	93% (79/90)	40% (2/5)	64% (7/11)	64% (18/28)	50% (1/2)
large	6% (3/50)	93% (79/90)	20% (1/5)	18% (2/11)	4% (1/28)	0% (0/2)
Cavum septi pellucidi	100% (50/50)	100% (90/90)	100% (5/5)	100% (11/11)	96% (27/28)	100% (2/2)
Cavum vergae	69% (34/49)	91% (79/87)	100% (0/5)	100% (11/11)	82% (23/28)	100% (2/2)
INFRATENTORIAL FINDINGS						
Signal abn. cerebellar WM	96% (47/49)	78% (69/88)	80% (4/5)	90% (9/10)	18%* (5/28)	0% (0/2)
Signal abn. brainstem	96% (45/47)	95% (80/84)	100% (5/5)	91% (10/11)	86% (24/28)	100% (2/2)

abn., abnormalities; WM, white matter; PLIC, posterior limb of the internal capsule; ALIC, anterior limb of the internal capsule; SAS, subarachnoid space; * subtle T_2-hyperintense rim of the subcortical white matter, consistent with normal myelination stage up to ~5 months

REFERENCES

1. van der Knaap MS, Barth PG, Stroink H, et al. Leukoencephalopathy with swelling and a discrepantly mild clinical course in eight children. Ann Neurol 1995;37:324-334.
2. Singhal BS, Gursahani RD, Udani VP, Biniwale AA. Megalencephalic leukodystrophy in an Asian Indian ethnic group. Pediatr Neurol 1996;14:291-296.
3. Ridder MC, Boor I, Lodder JC, et al. Megalencephalic leucoencephalopathy with cysts: defect in chloride currents and cell volume regulation. Brain 2011;134:3342-3354.
4. van der Knaap MS, Valk J, Barth PG, Smit LM, van Engelen BG, Tortori Donati P. Leukoencephalopathy with swelling in children and adolescents: MRI patterns and differential diagnosis. Neuroradiology 1995;37:679-686.
5. van der Knaap MS, Lai V, Kohler W, et al. Megalencephalic leukoencephalopathy with cysts without MLC1 defect. Ann Neurol 2010;67:834-837.
6. van der Knaap MS, Boor I, Estevez R. Megalencephalic leukoencephalopathy with subcortical cysts: chronic white matter oedema due to a defect in brain ion and water homoeostasis. Lancet Neurol 2012;11:973-985.
7. Lopez-Hernandez T, Ridder MC, Montolio M, et al. Mutant GlialCAM causes megalencephalic leukoencephalopathy with subcortical cysts, benign familial macrocephaly, and macrocephaly with retardation and autism. Am J Hum Genet 2011;88:422-432.
8. Singhal BS, Gorospe JR, Naidu S. Megalencephalic leukoencephalopathy with subcortical cysts. J Child Neurol 2003;18:646-652.
9. Patrono C, Di Giacinto G, Eymard-Pierre E, et al. Genetic heterogeneity of megalencephalic leukoencephalopathy and subcortical cysts. Neurology 2003;61:534-537.
10. Kariminejad A, Rajaee A, Ashrafi MR, et al. Eight novel mutations in MLC1 from 18 Iranian patients with megalencephalic leukoencephalopathy with subcortical cysts. Eur J Med Genet 2015;58:71-74.
11. Cao B, Yan H, Guo M, et al. Ten Novel Mutations in Chinese Patients with Megalencephalic Leukoencephalopathy with Subcortical Cysts and a Long-Term Follow-Up Research. PLoS One 2016;11:e0157258.
12. Ilja Boor PK, de Groot K, Mejaski-Bosnjak V, et al. Megalencephalic leukoencephalopathy with subcortical cysts: an update and extended mutation analysis of MLC1. Hum Mutat 2006;27:505-512.
13. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 2002;30:e57.
14. Hidecker MJ, Paneth N, Rosenbaum PL, et al. Developing and validating the Communication Function Classification System for individuals with cerebral palsy. Dev Med Child Neurol 2011;53:704-710.
15. van der Knaap MS, Breiter SN, Naidu S, Hart AA, Valk J. Defining and categorizing leukoencephalopathies of unknown origin: MR imaging approach. Radiology 1999;213:121-133.
16. Schiffmann R, van der Knaap MS. Invited article: an MRI-based approach to the diagnosis of white matter disorders. Neurology 2009;72:750-759.
17. Dubey M, Bugiani M, Ridder MC, et al. Mice with megalencephalic leukoencephalopathy with cysts: a developmental angle. Ann Neurol 2015;77:114-131.
18. Lopez-Hernandez T, Sirisi S, Capdevila-Nortes X, et al. Molecular mechanisms of MLC1 and GLIALCAM mutations in megalencephalic leukoencephalopathy with subcortical cysts. Hum Mol Genet 2011;20:3266-3277.
19. Capdevila-Nortes X, Lopez-Hernandez T, Apaja PM, et al. Insights into MLC pathogenesis: GlialCAM is an MLC1 chaperone required for proper activation of volume-regulated anion currents. Hum Mol Genet 2013;22:4405-4416.
20. Jeworutzki E, Lopez-Hernandez T, Capdevila-Nortes X, et al. GlialCAM, a protein defective in a leukodystrophy, serves as a ClC-2 Cl(-) channel auxiliary subunit. Neuron 2012;73:951-961.
21. Wu M, Moh MC, Schwarz H. HepaCAM associates with connexin 43 and enhances its localization in cellular junctions. Sci Rep 2016;6:36218.
22. Depienne C, Bugiani M, Dupuits C, et al. Brain white matter oedema due to ClC-2 chloride channel deficiency: an observational analytical study. Lancet Neurol 2013;12:659-668.
23. van der Voorn JP, Pouwels PJ, Hart AA, et al. Childhood white matter disorders: quantitative MR imaging and spectroscopy. Radiology 2006;241:510-517.
24. Brimley CJ, Lopez J, van Haren K, et al. National variation in costs and mortality for leukodystrophy patients in US children's hospitals. Pediatr Neurol 2013;49:156-162 e151.

25. Palisano RJ, Rosenbaum P, Bartlett D, Livingston MH. Content validity of the expanded and revised Gross Motor Function Classification System. Dev Med Child Neurol 2008;50:744-750.
26. Eliasson AC, Krumlinde-Sundholm L, Rosblad B, et al. The Manual Ability Classification System (MACS) for children with cerebral palsy: scale development and evidence of validity and reliability. Dev Med Child Neurol 2006;48:549-554.

III

Hypomyelination with atrophy of the basal ganglia and cerebellum

Chapter 4

Hypomyelination with atrophy of the basal ganglia and cerebellum: further delineation of the phenotype and genotype-phenotype correlation

Eline M. Hamilton, Nicole I. Wolf and Marjo S. van der Knaap

Brain. 2015;138:e328

ABSTRACT

Hypomyelination with atrophy of the basal ganglia and cerebellum is a rare leukoencephalopathy that was identified using magnetic resonance imaging in 2002. In 2013, whole exome sequencing of 11 patients with the disease revealed that they all had the same *de novo* mutation in *TUBB4A*, which encodes tubulin β-4A. We investigated the mutation spectrum in a cohort of 42 patients and the relationship between genotype and phenotype. Patients were selected on the basis of clinical and magnetic resonance imaging abnormalities that are indicative of hypomyelination with atrophy of the basal ganglia and cerebellum. Genetic testing and a clinical inventory were performed, and sequential magnetic resonance images were evaluated using a standard protocol. The heterozygous *TUBB4A* mutation observed in the first 11 patients was the most common (25 patients). Additionally, 13 other heterozygous mutations were identified, located in different structural domains of tubulin β-4A. We confirmed that the mutations were *de novo* in all but three patients. In two of these three cases we lacked parental DNA and in one the mutation was also found in the mother, most likely due to mosaicism. Patients showed a phenotypic continuum ranging from neonatal to childhood disease onset, normal to delayed early development and slow to more rapid neurological deterioration. Neurological symptomatology consisted of extrapyramidal movement abnormalities, spasticity, ataxia, cognitive deficit and sometimes epilepsy. Three patients died and the oldest living patient was 29 years of age. The patients' magnetic resonance images showed an absent or disappearing putamen, variable cerebellar atrophy and highly variable cerebral atrophy. Apart from hypomyelination, myelin loss was evident in several cases. Three severely affected patients had similar, somewhat atypical magnetic resonance image abnormalities. The study results were strongly suggestive of a genotype–phenotype correlation. The 25 patients with the common c.745G>A mutation generally had a less rapidly progressive disease course than the 17 cases with other *TUBB4A* mutations. Overall, this work demonstrates that the distinctive magnetic resonance imaging pattern for hypomyelination with atrophy of the basal ganglia and cerebellum defines a homogeneous clinical phenotype of variable severity. Patients almost invariably have prominent extrapyramidal movement abnormalities, which are rarely seen in patients with hypomyelination of different origin. A dominant *TUBB4A* mutation is also associated with dystonia type 4, in which magnetic resonance images of the brain seem normal. It is highly likely that there is a disease continuum associated with *TUBB4A* mutations, of which hypomyelination with atrophy of the basal ganglia and cerebellum and dystonia type 4 are the extremes. This would indicate that extrapyramidal movement abnormalities constitute the core feature of the disease spectrum related to dominant *TUBB4A* mutations and that all other features are variable.

INTRODUCTION

The leukodystrophy hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC; MIM 612438) is a rare childhood disease that is clinically characterized by extrapyramidal movement abnormalities, spasticity, cerebellar ataxia and sometimes epilepsy.[1] The prominent extrapyramidal movement abnormalities are a distinguishing feature of H-ABC, because cerebellar ataxia and spasticity are the typical disease manifestations of hypomyelination. The diagnosis of H-ABC is based on MRI criteria (Figure 1 and Supplementary Figure 1).[1] The two most important MRI features are hypomyelination and an extremely small or no visible putamen, without evidence of a lesion in the region, in the presence of a normal-sized thalamus and globus pallidus. The caudate nucleus may be small. Additionally, cerebellar atrophy is present in virtually all patients. Follow-up studies have shown the progressive nature of the disease, with clinical deterioration and MRI evidence of disappearance of the putamen, further loss of myelin and cerebral atrophy in addition to the cerebellar atrophy.[2]

Since the description of H-ABC in 2002, 21 cases have been reported.[1-6] All cases were isolated and therefore the mode of inheritance was hypothesized to be dominant *de novo*, precluding the possibility of identifying the mutated gene by conventional genetic linkage analysis. Whole exome sequencing is ideal in such situations and recently led to the identification of a dominant c.745G>A *de novo* mutation in the *TUBB4A* gene (MIM 602662) in 11 patients with H-ABC.[7] *TUBB4A* encodes tubulin β-4A, which is highly expressed in the brain only. Together with α-tubulin, β-tubulin is the principal constituent of microtubules (Figure 2). Interestingly, mutations in the same gene were recently associated with dystonia type 4 (DYT4; MIM 128101), which is characterized by adolescent or adult onset 'whispering dysphonia', 'generalized dystonia' and a unique 'hobby horse' ataxic gait.[8-10] Brain MRI is reportedly normal in this disorder. The patients with dystonia all have the same missense mutation in the N-terminal autoregulatory domain of *TUBB4A*: c.4C>G.[8,9,11]

In this study, we focused only on patients with the H-ABC phenotype. Our aim was to investigate the spectrum of *TUBB4A* mutations in these patients, achieve a better delineation of the clinical spectrum including the MRI abnormalities, and evaluate a possible genotype-phenotype correlation.

PATIENTS AND METHODS

We identified patients with H-ABC in the Amsterdam Database of Leukoencephalopathies on the basis of the following MRI criteria: (i) hypomyelination, defined as a mildly elevated T_2-signal intensity of most cerebral white matter in combination with a mild T_1-hypointensity, T_1-isointensity or mild T_1-hyperintensity relative to the cortex;[12] and (ii) absent or barely visible putamen without signal abnormality in the region where the putamen should be. In addition, we

included patients with hypomyelination in whom the putamen was decreased in size, but still well visible, and who had prominent extrapyramidal signs.

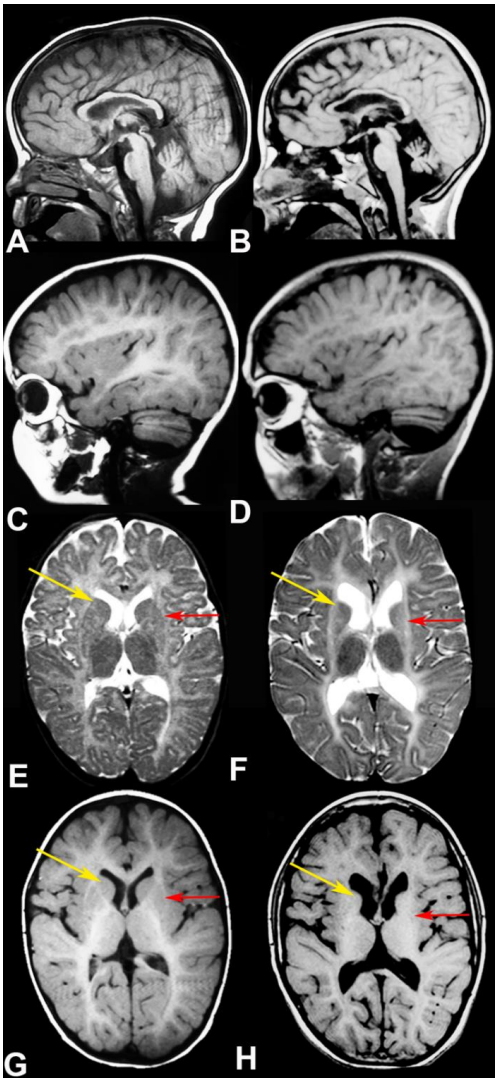


Figure 1 | MRI findings in a typical H-ABC patient. Sagittal T1-weighted (**A**–**D**) and axial T2-weighted (**E** and **F**) and T1-weighted (**G** and **H**) images in Patient HA23 with the common c.745G>A mutation at the age of 1.5 years (**A**, **C**, **E** and **G**) and 13 years (**B**, **D**, **F** and **H**).
The early MRI shows a mildly hyperintense white matter signal on T1-weighted (**C** and **G**) and T2-weighted (**E**) images, indicating a moderate lack of myelin.
In the late MRIs, the white matter T1-signal is subtly reduced, indicating loss of some myelin (**D** and **H**). The cerebellar atrophy increases over time (**A**–**D**), especially of the vermis (**A** and **B**).
At 1.5 years, a small putamen is present that has lost some of its normal grey matter signal (red arrows); the caudate nucleus is normal (yellow arrows in **E** and **G**). Note that at the age of 13 years, the putamen is no longer visible (red arrows) and that the caudate nucleus is slightly atrophic (yellow arrows in **F** and **H**). Over time, the lateral ventricles show a slight increase in size (**E**–**H**), indicating some loss of white matter volume.

We obtained DNA from patients and parents through blood or fibroblasts. Patients who fulfilled the MRI criteria but of whom no DNA was available were excluded from the study. To collect clinical information, we used a standardized data collection form for physicians, with items on disease onset, developmental milestones, growth, motor function, cognition and death. If this source was not available (5% of cases), information was provided by families, supplemented by information derived from medical records. The study received approval of the institutional review board of the VU University Medical Center and written informed consent was obtained from the parents.

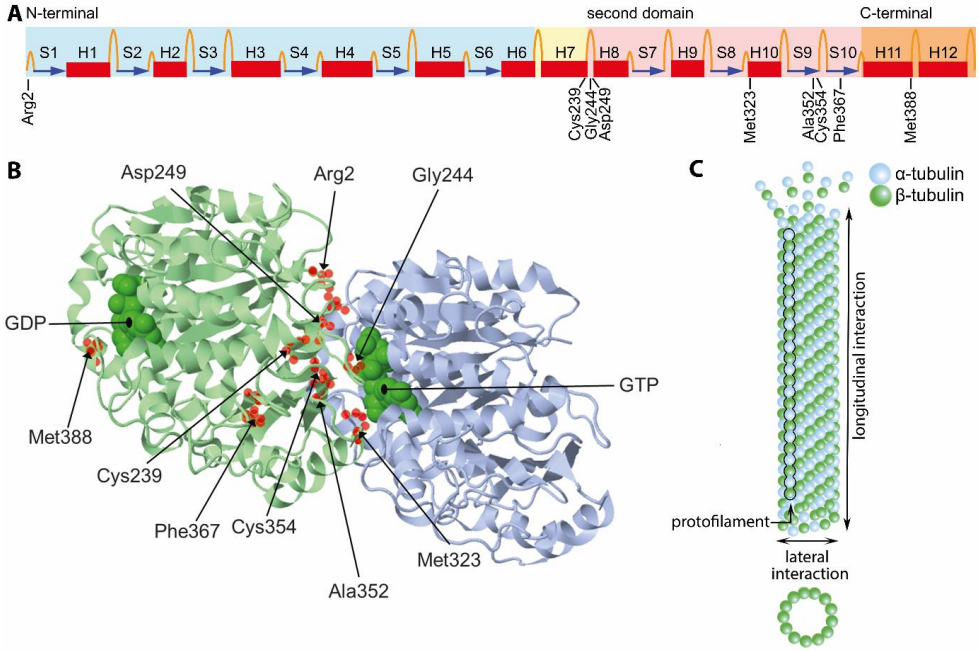


Figure 2 | Structure of tubulin. (**A**) Linear representation of the secondary structure of β-tubulin,[13] showing the positions of the amino acids mutated in the H-ABC patients described here. Colored boxes indicate the 3D structural domains: the globular N-terminal nucleotide binding domain is blue, the core helix is yellow, the second domain is pink and the C-terminal outer surface domain is orange. Helices are shown as red boxes, β-sheets as blue arrows, and loops as orange threads. Modified from Wade, with permission of Springer[14] and from Amos, with permission of Elsevier.[15] (**B**) 3D structure of the αβ-tubulin heterodimer.[13] The β-tubulin is shown in blue, the α-tubulin in green. Helices, β-sheets and loops are shown as ribbons, arrows and threads, respectively. The mutated residues are highlighted as red spheres. Most mutated residues are located at the intradimer interface. Met388 is located at the interdimer interface. (**C**) αβ-Tubulin heterodimers bind head-to-tail along their longitudinal direction into protofilaments, which then laterally interact with each other to form microtubules. Modified from Vydra and Havelka.[16]

Magnetic resonance imaging evaluation

For each patient, at least one MRI study was available. Two investigators (E.M.H. and M.S.vdK.) evaluated MRI images according to a standard protocol.[17] Because the images had been obtained at various centers, the applied pulse sequences and the quality were variable. Sagittal T_1-weighted and transverse T_2-weighted images were available for all patients. To obtain a semi-quantitative measure for the degree of myelination in relation to calendar age, 11 structures were scored for a signal relative to the cortex on T_1- and T_2-weighted images independently, as shown in Supplementary Table 1A (modified from Plecko *et al.*[18]). White matter myelination was graded with 2 points for normal to mild T_1 hyperintensity and for normal to mild T_2 hypointensity, 1 point for T_1- and for T_2-isointensity and 0 points for normal to mild T_1 hypointensity and for normal to mild T_2-hyperintensity, all relative to the cortex. Scores were shown relative to the age-related maximum score, based on the normal maturation of the brain as described by Barkovich *et al.*[19] The maximum score rose with age, depending on the progress of myelination and was 22 points for both T_1-

and T_2-weighted images in patients from the age of 18 months onwards. If not all items could be scored, the overall intensity of the cerebral hemispheric white matter was reported (Supplementary Table 1A). The putamen, caudate nucleus, globus pallidus and thalamus were scored for size (normal/small/absent) and T_2-signal intensity (normal/increased). Cerebral atrophy, defined by enlargement of the lateral and third ventricles and subarachnoid spaces, cerebellar atrophy and callosal atrophy were scored as absent, mild or severe. To reduce inter-observer variability, we only scored items on the protocol as present if they were easily evaluable and obvious; equivocal findings were scored as absent.

Mutation analysis

Genomic DNA was extracted from whole blood, lymphoblasts or fibroblasts. The four exons and intron–exon boundaries of the human *TUBB4A* gene (MIM 602662; RefSeq accession number NM_006087) of the patients were amplified by PCR and analyzed by Sanger sequencing (primer sequences in Supplementary Table 2). Pathogenicity of novel missense mutations was assessed by *in silico* analysis using SIFT (Sorting Intolerant From Tolerant) and PolyPhen.[20,21] Parental DNA was investigated for the amplicon containing the index patient's mutation.

To investigate how the mutations identified in this study might affect the microtubule structure, we mapped each affected amino acid to a predicted bovine αβ-tubulin protein secondary structure[13] and processed data from the Protein Database (1JFF) in FirstGlance (http://firstglance.jmol.org) using Jmol (http://www.jmol.org) to visualize the mutated residues in a 3D protein structure.[13]

RESULTS

In total, 42 unrelated patients (age range 2-29 years) were included in the study. In a few patients, one or two items of the clinical inventory were missing; these patients were left out of the analysis only for the subject of the missing information. Consanguinity was present in none of the families. One patient (HA127) had a half-brother from the same mother with the same phenotype and *TUBB4A* mutation. This brother was not included in the study because of lack of clinical information and MRI.

Clinical phenotype

Detailed clinical characteristics of all patients are described in Supplementary Table 1B and a summary is presented in Table 1. Patient HA103 was not included in the comparison of clinical characteristics because of comorbidity with Down syndrome.5 Median age of onset was 6 months (range birth - 3 years). The most common presenting signs were developmental delay, hypotonia, nystagmus and deterioration of motor function. Although not mentioned as a presenting sign, extrapyramidal movement abnormalities, most commonly dystonia, rigidity or both and rarely choreoathetosis, were almost invariably observed (41 of the 42 cases). Follow-up ranged between 2 and 28 years. The neurological development varied from normal early development (17%) to absence of intentional movements (10%). Walking

without support was achieved in 45% of patients (age range 10 months to 4 years), of which only 35% managed it before the age of 18 months. All patients lost the ability to walk without support between 2 and 12 years of age, except for three patients, who are presently between 5 and 7 years old. Sixty-three per cent of the patients initially acquired normal (23%) or limited (40%) speech. They started to lose their speech from a median age of 7 years (range 2-15 years). Currently only one patient has normal speech (age 7 years), whereas 11 patients have dysarthria (age range 5–22 years) and 29 have no speech (age range 2-29 years). 58% percent of patients require tube feeding. One patient received a tracheostomy at age 15 and is on continuous ventilation. Three patients are deceased (at ages of 12, 20 and 25 years).

Table 1 | Clinical data on 41 H-ABC patients

General characteristics	***TUBB4A:* c.745G>A**	**Other *TUBB4A* mutations**
Number of patients	25	16
Gender (male / female)	12 / 13	7 / 8
Median age (range)*	14 y (2 - 29 y)	10 y (3 - 25 y)
Patients with affected sibling(s)	1	0
Median age of onset (range)	1.5 y (3 mo - 3.0 y)	3 mo (birth - 6 mo)
Neurological development		
Maximum motor milestone		
walking without support	76%	0%
standing/walking with support	20%	12%
rolling over / sitting	4%	38%
touching / grasping	0%	25%
no intentional movements	0%	25%
Maximum language		
normal	38%	0%
single words/ short sentences	62%	6%
none	0%	94%
Maximum level of comprehension		
normal	32%	6%
decreased intelligence	68%	38%
social awareness only	0%	56%
Neurological symptomatology		
Spasticity	96%	94%
Ataxia	88%	31% (remainder n.e.)
Extrapyramidal movements	96%	100%
Seizures	12%	53%
Current speech		
normal	4%	0%
dysarthria	44%	0%
no speech	52%	100%
Current level of comprehension		
normal	0%	0%
decreased intelligence	100%	19%
social awareness only	0%	81%
Other characteristics		
Tube feeding (range age at start)	46% (11 - 26 y)	75% (1 - 9 y)
Height < 2SD	40%	87%
Weight < 2SD	48%	88%
Microcephaly	9%	69%
Deceased patients (age range)	4% (12 y)	13% (20 - 25 y)

*Age at time of obtaining clinical characteristics; mo, months; y, years; n.e., not evaluable

Magnetic resonance imaging characteristics

A total of 108 MRI scans were available for the 42 patients. Patients had their first MRI aged between 6 months and 23 years; 30 patients obtained one or more follow-up scans at ages between 10 months and 29 years. Results are summarized in Table 2 and Supplementary Figure 1; data about each patient are presented in Supplementary Table 1A.

Myelination: Hypomyelination was present in all patients older than 18 months (Figure 1). In younger patients, myelination was always delayed, but they were too young to establish the diagnosis of hypomyelination.[12] Only one patient (HA48) had advanced myelination on the first MRI at 5 years of age, with a mildly hyperintense T1-signal and a mildly hypointense to isointense T2-signal of the cerebral white matter relative to the cortex. Five patients had an almost complete lack of myelin on the first MRI (age range 1-3 years; Figure 3). Myelination scores in patients older than 18 months ranged from 1–22 of 22 points for T1-weighted images and 1-8 of 22 points for T2-weighted images. These T2 scores were reached on the basis of a hypointensity of the brainstem, cerebellar white matter and genu and splenium of the corpus callosum; all other structures were generally T2-hyperintense. The brainstem was the only structure that had a hypointense or isointense T2-signal relative to the cortex in all patients, followed by the cerebellar white matter (41%) and splenium of the corpus callosum (20%). In 11 of 30 patients with follow-up MRIs, progressive loss of myelin was documented (age range at latest MRI 6-29 years, Figure 1). Progression of myelination was only seen in two of the six patients who had the first MRI before the age of 18 months.

Basal ganglia and thalamus: The putamen was small in 14 and absent in 20 patients on the first MRI. A normal sized putamen was seen in only eight patients who had their first MRI at a young age (6 months to 3 years of age). On follow-up, in all patients the putamen had started to disappear or had disappeared altogether (Figures 1 and 3). The caudate nucleus was completely absent in only two patients on the first MRI and in one additional patient on follow-up (Supplementary Figure 2). The size was normal in 70% of patients on early MRI (<2 years after onset), whereas it was normal in only 10% of patients who underwent MRI later than 12 years after onset. The process of disappearance of the putamen and caudate nucleus was characterized by loss of the typical grey matter signal, resulting in a signal intensity similar to the hypomyelinated white matter on T2-weighted images, as documented for the putamen in 19 patients and for the caudate nucleus in 13 patients (Figures 1 and 3, Supplementary Figure 2). This loss of grey matter signal was followed by actual absence of the structure on follow-up, as documented for the putamen in six patients and for the caudate nucleus in one patient. The globus pallidus and thalamus were of normal size and signal in all patients at all stages of the disease.

Cerebral and cerebellar atrophy: Disappearance of the caudate nucleus led to widening of the frontal horns of the lateral ventricles. Additionally, in several patients there was moderate to severe dilatation of lateral and third ventricles (Figures 1 and 4). On the first MRI, 93% of patients had cerebellar atrophy, of which 58% was

restricted to the cerebellar vermis. On follow-up, only one patient had no cerebellar atrophy at the age of 9 years.

Table 2 | Summary MRI findings in 42 HABC patients

		MRI <2 years after onset	MRI ≥2 and ≤12 years after onset	MRI ≥12 years after onset
Number of patients		23	34	10
Age patients*		6 mo - 5 y	2 - 12 y	13 - 29 y
Myelination[1]				
Moderate lack of myelin[a]		52%	59%	30%
Severe lack of myelin[b]		35%	20.5%	50%
Almost complete lack of myelin[c]		13%	20.5%	20%
Basal Ganglia				
Putamen	normal	30.5%	3%	0%
	small	39%	38%	30%
	absent	30.5%	59%	70%
Caudate nucleus	normal	70%	47%	10%
	small	26%	44%	80%
	absent	4%	9%	10%
Atrophy				
Cerebral atrophy	absent	78%	56%	20%
	moderate	18%	38%	60%
	severe	4%	6%	20%
Atrophy corpus callosum	absent	83%	41%	0%
	moderate	17%	50%	80%
	severe	0%	9%	20%
Cerebellar atrophy	absent	9%	6%	0%
	moderate	74%	50%	10%
	severe	17%	44%	90%

*Age at moment of obtaining clinical characteristics; mo, months; y, years

[1] In patients < 12 months myelination age is scored relative to calendar age

[a] Defined as hyperintense signal of the cerebral hemispheric white matter on T1-weighted images and hyperintense signal on T2-weighted images relative to cortex

b Defined as isointense signal of the cerebral hemispheric white matter on T1-weighted images and hyperintense signal on T2-weighted images relative to cortex

[c] Defined as hypointense signal of the cerebral hemispheric white matter on T1-weighted images and hyperintense signal on T2-weighted images relative to cortex

Genotype

In 25 patients, a heterozygous c.745G>A TUBB4A mutation was identified. Thirteen other TUBB4A mutations were identified in 17 patients (Table 3), always in the heterozygous state. All affected highly conserved nucleotides and amino acids and none were present in dbSNP135 or the 1000 Genomes Project, supporting their likely pathogenicity. The mutations were all predicted to be pathogenic by in silico analysis with Polyphen, SIFT, or both. In 39 patients, the mutation was de novo. In one patient (HA163), we found the same heterozygous mutation in the asymptomatic mother's blood; we had no other sources of her DNA to prove mosaicism. For two patients, no parental DNA was available; one of them (Patient HA127) had an affected sibling.

Table 3 | Overview of *TUBB4A* mutations in H-ABC patients

Nucleotide change*	Exon	Amino acid change*	Number of patients	Protein domain & putative function
c.4C>T	1	p.Arg2Trp	2	N-terminal, globular GTPase domain -autoregulatory MREI domain
c.5G>A	1	p.Arg2Gln	1	
c.716G>T	4	p.Cys239Phe	1	Core helix - H7: connects the N-terminal and second domain; important for nucleotide binding; key part for microtubule assembly
c.730G>A	4	p.Gly244Ser	3	Second globular domain - T7-loop: Interacts with the GTP nucleotide at N-terminal side of the α-tubulin; important for longitudinal interaction between tubulins
c.731G>T	4	p.Gly244Val	1	
c.745G>A	4	p.Asp249Asn	25	
c.968T>G	4	p.Met323Arg	1	Second globular domain - H10: longitudinal interactions
c.1054G>A	4	p.Ala352Thr	2	Second globular domain - S9: Taxol binding site, important for dynamics
c.1061G>A	4	p.Cys354Tyr	1	Second globular domain - S9: Taxol binding site, important for dynamics - key part for microtubule assembly
c.1099T>A	4	p.Phe367Ile	1	Second globular domain - S10: Taxol binding site, important for dynamics
c.1099T>C	4	p.Phe367Leu	1	
c.1162A>G	4	p.Met388Val	1	C-terminal outer surface domain - H11: hypothesized to be involved in the binding of MAPs and motor proteins and in longitudinal interactions
c.1163T>C	4	p.Met388Thr	1	
c.1164G>A	4	p.Met388Ile	1	

* Nomenclature according to http://www.hgvs.org/mutnomen/
H, helix; S, β-strand; T, loops connecting helices and strands; GTP, guanosine triphosphate; MREI, methionine-arginine-glutamic acid – isoleucine; MAP, microtubule associated protein

Mutations were found in all three tubulin β-4A monomer domains that serve different functions: (i) the globular, N-terminal, nucleotide binding domain; (ii) the second globular domain; and (iii) the C-terminal outer surface domain (Figure 2A and Table 3).[11,13,15,22-24] Visualizing the mutated amino acids in a 3D map revealed that most variants are positioned at the intradimer interface (Figure 2B). Three patients, however, have a mutation at position Met388, which is located at the interdimer interface (Figure 2B and C).

Genotype-phenotype correlation

When comparing the clinical and MRI characteristics of the 25 patients with the common, previously published, c.745G>A mutation to those of the 16 patients with other *TUBB4A* mutations, the latter group was more severely affected (Table 1). The first signs occurred at an earlier age and no patient in this group achieved walking without support, in contrast to 76% of the patients with the common mutation. All patients who did not acquire any intentional movements had a mutation different from c.745G>A. Patients in this group were also more likely never to speak, have only social awareness, seizures and growth problems. Seizures occurred in half of the patients with other mutations and in 12% of patients with the common mutation. Patients with the common mutation initially all had only moderate lack of myelin on MRI (Supplementary Table 1A); some of them lost myelin over time, resulting in a

profound lack. Several patients with another mutation had profound or almost complete lack of myelin at an early stage. None of the patients with the c.745G>A mutation developed severe cerebral or callosal atrophy, whereas 24% of the patients with other mutations developed both.

Three severely affected patients (Patients HA140, HA163 and HA165) showed a similar MRI pattern (Figure 3 and Supplementary Table 1A) that was somewhat atypical for H-ABC: they initially had a normal putamen and from early on an almost complete lack of myelin. They were the only patients with a mutation located in the N-terminal domain of the β-tubulin protein structure (Table 3 and Figure 2A).

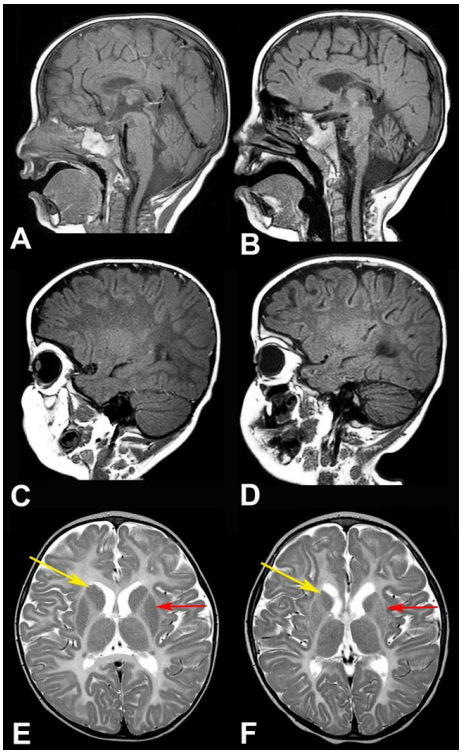


Figure 3 | Atypical MRI abnormalities.
MRI of Patient HA140, with a c.4C>T mutation at the age of 1.5 (**A**, **C** and **E**) and 4 years (**B**, **D** and **F**). From early on, there is an almost complete lack of myelin, characterized by a hypointense signal of the white matter on T1-weighted images (**C** and **D**). The white matter signal is strikingly hyperintense on T_2-weighted images, as compared to other H-ABC patients (**E** and **F**). At the age of 1.5 years, the putamen (red arrow) and caudate nucleus (yellow arrow) are normal sized (**E**). At the age of 4 years, the caudate nucleus (yellow arrow) shows slight atrophy and the putamen (red arrow) has started to lose the hypointense grey matter signal (**F**).

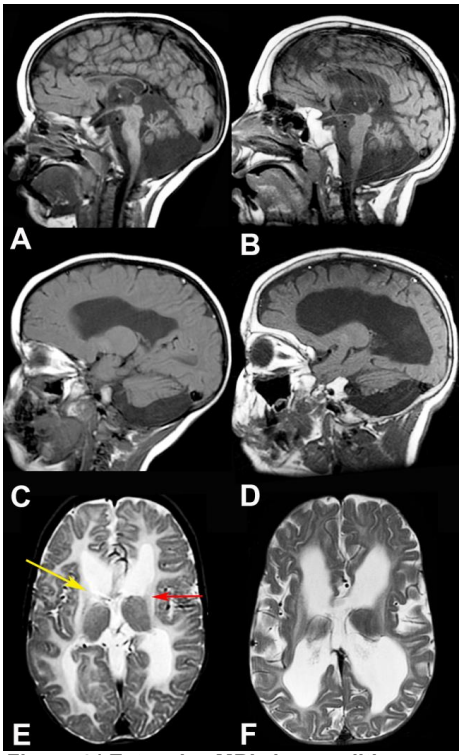


Figure 4 | Extensive MRI abnormalities.
Sagittal T1-weighted (**A**–**D**) and axial T2-weighted (**E** and **F**) images of Patient HA51 with a c.1061G>A mutation at the age of 2 (**A**, **C** and **E**) and 7 years (**B**, **D** and **F**). The white matter has an intermediate to low signal on the T1-weighted images (**C** and **D**) and a high signal on the T2-weighted images (**E** and **F**), indicating a severe, almost complete, lack of myelin. There is serious loss of cerebral white matter volume from early on (**C** and **E**), which increases over time (**D** and **F**). The cerebellar atrophy is profound, involving both the vermis (**A** and **B**) and the hemispheres (**C** and **D**). By 2 years of age, the putamen (red arrow) is absent and the head of the caudate nucleus (yellow arrow) is entirely flat (**E**).

DISCUSSION

H-ABC is defined by distinctive MRI abnormalities.[1,2] The recent elucidation of its genetic background[7] allows a more complete investigation of the H-ABC phenotypic spectrum, which is the subject of the present study.
The mutations responsible for H-ABC reside in *TUBB4A*, which encodes one of the members of the highly conserved β-tubulin protein family. β-tubulins form heterodimers with α-tubulins; these alternating α- and β-tubulin subunits form copolymers that assemble into microtubules (Figure 2C). Microtubules are an essential component of the cytoskeleton and act as a highly versatile scaffold to determine cell shape and cell shape changes, and form a backbone for cell organelle and vesicle movement.[25,26] An essential feature is their dynamic instability, i.e. the ability to rapidly de- and repolymerize, allowing a fast response to the environment.[27] Their function relies on numerous processes, such as GTP-binding and hydrolysis, heterodimer stability, binding of kinesin superfamily proteins and microtubule associated proteins and lateral and longitudinal interactions.[25] Tubulin heterodimers contain different α- and β-tubulin isotypes, which are encoded by different genes and are hypothesized to serve distinct cellular functions.[26,28]

A spectrum of neurological disorders has been associated with mutations in tubulin genes *TUBA1A*, *TUBA8*, *TUBB*, *TUBB2B* and *TUBB3*.[29-33] Many of these diseases display cortical malformations and other developmental anomalies of the brain as a result of disturbed neuronal migration. We did not observe cortical malformations in any H-ABC patients. Although most neurological disorders caused by mutations in tubulin genes are malformative, H-ABC is a degenerative disease.
Mutations observed in patients with H-ABC occur in all three predominant tubulin β-4A protein domains. They are all located near the intradimer or interdimer interface and probably affect heterodimerization or polymerization. We suspect that the common pathophysiological mechanism of the mutations is alteration of microtubule dynamics or stability. The defectuous microtubule system may hamper axonal transport, leading to axonal dysfunction and loss, as has also been shown for certain mutations in *TUBB3*.[34] Infantile onset axonal dysfunction impedes the process of myelination; ongoing axonal dysfunction is associated with myelin loss. Disrupted transport may also account for the neuronal loss in basal nuclei and cerebellar cortex.

Considering the absence of any evidence of local scar tissue on MRI, the disappearance of the putamen was previously suggested to be caused by neuronal apoptosis rather than necrosis.[1] In most patients, we saw a putamen on early MRIs, although smaller than normal, but sometimes even of normal size. On follow-up MRI, we documented a decrease in size in all cases. We also documented that the disappearance of the putamen and sometimes the caudate nucleus is preceded by a loss of grey matter signal resulting in a signal similar to the white matter, in line with the hypothesis of underlying apoptosis. A histopathology study confirmed the subtotal

degeneration of the putamen and the near-total loss of neuronal cells.[2] Why the putamen, caudate nucleus and cerebellar cortex are the most prominently affected grey matter structures in H-ABC is unclear. *TUBB4A* is expressed almost exclusively in the brain; within the brain its expression is highest in the cerebellum, followed by the putamen and white matter.[8,25] It is likely that tubulin β-4A function is most important in the latter areas.

The clinical characteristics of patients with H-ABC are rather homogeneous, but of variable severity. In our cohort of patients, a more benign phenotype was seen in patients with the common c.745G>A mutation than in the patients with other mutations. The initial motor development in the patients with the common mutation was generally normal or mildly delayed. As described by Simons *et al.,*[7] these patients generally acquired the ability to walk with or without support and lost this ability after a variable number of years. Language and cognitive function were relatively preserved, but at a later stage most patients became dysarthric and lost their speech. Patients with other mutations generally had a more severe phenotype. They showed a profound delay in motor development and never achieved walking without support; half of the patients acquired minimal or no intentional movements. Cognitive function was less well preserved and most patients never acquired any speech. A striking finding is that these patients often also showed stunted growth and microcephaly. Although patients with the common mutation showed typical MRI abnormalities of comparable severity, the severity was more variable for patients with other mutations. In particular, the degree of hypomyelination was variable in the latter patients, and in some of them, MRI evidence of myelin was almost completely lacking. The numbers of patients with other mutations are too small to further distinguish the genotype-phenotype correlation within this group, but it is interesting that the three patients with an Arg2 mutation in the N-terminal domain showed remarkably similar MRI abnormalities, with a more striking T_2-hyperintensity and T_1-hypointensity of the cerebral white matter than in the other H-ABC patients and an initially normal sized putamen. This suggests that these mutations are associated with this particular phenotype.

Intriguingly, a heterozygous change of Arg2 to glycine in *TUBB4A* has been associated with DYT4, whereas heterozygous changes to glutamine or tryptophan at the same residue result in a severe H-ABC phenotype. Arg2 is part of the methionine-arginine-glutamic acid-isoleucine domain, which has an important function in the autoregulation of β-tubulin messenger RNA stability.[11] All three changes have been shown to perturb the autoregulated instability of *TUBB4A* messenger RNA, resulting in increased β-tubulin synthesis.[11] Arg2 is positioned at the intra-dimer interface and forms a salt bridge with Asp249, one of many electrostatic interactions within and between monomers in this region.[13] Asp249 is the residue altered by the common c.745G>A mutation. One possible explanation for the strikingly different clinical outcomes is that the mutation of Arg2 to the relatively chemically inert glycine in DYT4 results in deregulation of tubulin β-4A protein levels,[35] but has limited effect on the function of the resulting tubulin β-4A protein.

Conversely, substitution of the positively charged Arg2 with either the large polar glutamine or hydrophobic tryptophan is likely to substantially alter the structure of tubulin β-4A at the intra-dimer interface in addition to perturbing *TUBB4A* messenger RNA autoregulation. Due to the methionine-arginine-glutamic acid-isoleucine domain feedback mechanism this combination of effects results in increased levels of the toxic tubulin β-4A mutant protein relative to the levels of other β-tubulins.

The designation 'dystonia type 4' (DYT4, MIM 128101) was applied to a single large pedigree with adolescent or adult onset autosomal dominant dystonia. Recently, another heterozygous variant in *TUBB4A* was found in an unrelated individual with old age onset segmental dystonia, including spasmodic dysphonia and oromandibular dystonia, and a positive family history,[9] suggesting that DYT4 may also have a broader phenotypic variation than observed in the single pedigree and may be related to more than one *TUBB4A* mutation.

Only heterozygous missense mutations have been reported in *TUBB4A*, supporting the idea that mutant *TUBB4A*-associated diseases are caused by dominant negative, toxic effects rather than loss of function of the encoded protein.[33] Within families, patients consistently have the same clinical presentation, supporting a genotype–phenotype correlation. No asymptomatic or oligosymptomatic carriers have been reported, making variable penetrance unlikely. The disease severity of H-ABC precludes offspring, which is consistent with the fact that the disease is typically caused by *de novo* mutations. The occurrence of a *TUBB4A* mutation in a patient's asymptomatic mother is best explained by maternal mosaicism, which has been described before in an H-ABC family.[7] We suspect that the patient described here with an affected sibling is another example of maternal mosaicism.

The known disease spectrum associated with *TUBB4A* mutations has now become quite broad. On the most severe end of the spectrum are neonatal onset H-ABC patients, who never acquire intentional movements and have a disturbance of normal myelin formation together with progressive myelin loss and degeneration of the putamen, caudate nucleus, cerebrum and cerebellum. On the other end of the spectrum are DYT4 patients with late adult-onset dystonia, without MRI evidence of structural brain abnormalities.[8,9] The only characteristic shared by the H-ABC and DYT4 phenotypes is the occurrence of extrapyramidal signs. It is likely that the full phenotypic spectrum associated with *TUBB4A* mutations is a continuum with the two known phenotypes as extremes, indicating that apart from extrapyramidal movement abnormalities no clinical or MRI characteristic is obligatory. The patients presented in this paper do, however, show a well-defined, rather homogeneous phenotype with highly characteristic MRI abnormalities, indicative of a distinct disease. We therefore suggest maintaining the acronym H-ABC for patients with extrapyramidal movement abnormalities and hypomyelination in combination with atrophy of the putamen and cerebellum.

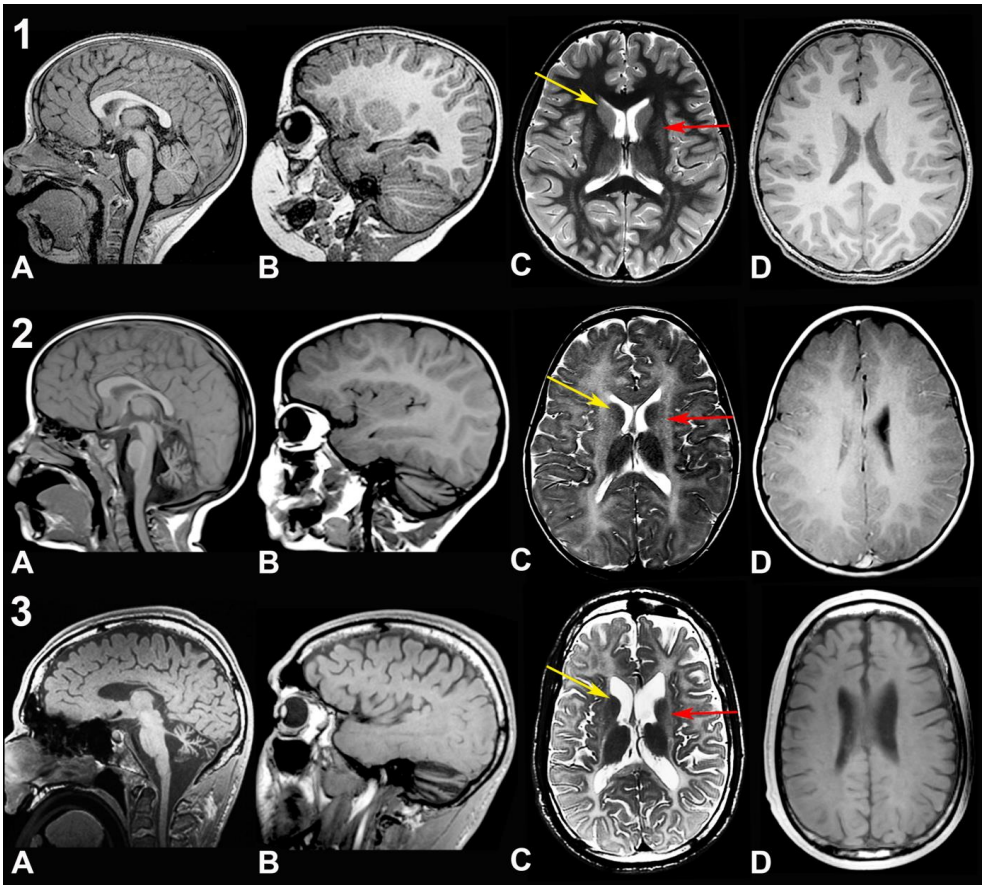
With respect to *TUBB4A* genetic analysis, extrapyramidal movement abnormalities form the only obligatory item to indicate the need for testing. Any extra finding that points to H-ABC, such as hypomyelination, putamen atrophy or cerebellar atrophy, reinforces this indication. Cerebellar atrophy is non-specific and observed in other hypomyelinating disorders, but in the context of extrapyramidal movement abnormalities, *TUBB4A* analysis is warranted. This is even truer for the combination of extrapyramidal movement abnormalities and hypomyelination if the putamen appears normal, because extrapyramidal movements abnormalities are unusual in patients with hypomyelination of different origin.

ACKNOWLEDGEMENTS

The authors express their gratitude to all patients, families and referring physicians of the H-ABC Research Group for their co-operation and contribution. The study was supported by the Optimix Foundation for Scientific Research.

The following H-ABC Research Group collaborators contributed to the study:
I. Baric, University Hospital Centre Zagreb and University of Zagreb, School of Medicine, Zagreb, Croatia; R. Battini, IRCCS Stella Maris, Pisa, Italy; R. Biancheri, G. Gaslini Paediatric Institute, Genova, Italy; C.G. Bönnemann, National Institutes of Health, National Institute of Neurological Disorders and Stroke, Bethesda, USA; K. Brockmann, Georg August University, Göttingen, Germany; R. van Coster, Ghent University Hospital, Ghent, Belgium; M. Ensslen, Hauner Children‘s Hospital, University of Munich, Germany; P. Fallon, St George's Hospital, London, UK; C. de Goede, Royal Preston Hospital, Preston, United Kingdom; M. Henneke, Georg August University Göttingen, Germany; J. Kisler, Great North Children’s Hospital, Newcastle upon Tyne, UK; W. Köhler, Fachkrankenhaus Hubertusburg, Wermsdorf, Germany; L. Lagae, University Hospitals Leuven, Belgium; T. Linnankivi, Children’s Hospital, University of Helsinki and Helsinki University Central Hospital, Finland; S. Mathias, Seaside Medical Centre, East Sussex, UK; V. Mejaški Bošnjak, University of Zagreb, Croatia; J. Michaud, CHU Ste-Justine, Montréal, Canada; D. Peake, Royal Belfast Hospital for Sick Children, Belfast, UK; B. Pérez Dueñas, Hospital Sant Joan de Déu, Barcelona, Spain; V. Poisson, CHU Ste-Justine, Montréal, Canada; P. Prabhakar, Great Ormond Street Hospital, London, UK; S. Raskin, Centre for Health and Biological Sciences, Pontifícia Universidade Católica do Paraná, Curitiba PR, Brazil; I.H. Rasmussen, Stavanger University Hospital, Stavanger, Norway; D. Rating, University Children’s Hospital, Heidelberg, Germany; A. Roubertie, Hôpital Gui de Chauliac, Montpellier, France; T. Shiihara, Gunma Children’s Medical Centre, Gunma, Japan; J. Sperner, Child Neurology Service, Lübeck, Germany; S. Standridge, Cincinnati Children’s Hospital Medical Centre, Cincinnati, USA; M. Tchan, Westmead Hospital, Westmead, Australia; M.C. Waugh, The Children’s Hospital at Westmead, Westmead, Australia; R.I. Webster, The Children’s Hospital at Westmead, Westmead, Australia; S. Yoshida, Johns Hopkins School of Medicine, Baltimore, USA.

SUPPLEMENTARY DATA



Supplementary Figure 1 | MRI findings in H-ABC. Sagittal T1-weighted images (**1-3 A and B**) and axial T2-weighted (**1-3 C**) and T1-weighted (**1-3 D**) images in a 3-year-old unaffected individual (**1 A-D**), patient HA127 at the age of 3 years (**2 A-D**) and patient HA27 at the age of 29 years (**3 A-D)**. Both patients have the common c.745G>A mutation.

Note the diffusely hyperintense signal of the cerebral white matter in the patients on T2-weighted images as compared to the healthy person (**1C, 2C, 3C**). On the T1-weighted images, the cerebral white matter signal is hyperintense in the unaffected individual (**1B,1D**), mildly hyperintense in the young patient (**2B,2D**) and isointense relative to the cortex in the adult patient (**3B, 3D**). In the young patient, the corpus callosum has a normal thickness (**2A**) and a mildly hypointense signal on the T2-weighted image (**2C**), while in the adult patient, the corpus callosum is thin (**3A**) and has a high signal on the T2-weighted image (**3C**).

Note the normal anatomy of the caudate nucleus (yellow arrow) and putamen (red arrow) in the unaffected individual (**1C**). In both patients, the putamen is absent (red arrows in **2C, 3C**). The head of the caudate nucleus is a bit small in the young patient (**2C**) and is smaller in the adult patient (**3C**). The cerebellar vermis shows marked atrophy in both patients (**2A, 3A**).

Supplementary Table 1A | MRI findings

Patient number	1		2		3		4		5		6		7		8		9	
Patient ID	HA106		HA20 / LD_0440.0		HA62 / LD_0605.0		HA12		HA27		HA36		HA129		HA26		HA15	
Age at first MRI	**12 y**		**11 y**		**22 y**		**3.3 y**		**13 y**		**10 y**		**23 y**		**2.5 y**		**10 mo**	
Myelination	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2
Brainstem		↓	↑	↓	=	↓			↑	↓	↑	↓	↑	↓	↑	↓	=	=
Posterior limb internal capsule		↑	↑	↓	=	↑			↑	↑	↑	↑	↑	↑	↑	↑	=	↑
Cerebellar white matter		↑	↑	=	=	=			↑	↓	↑	↓	↑	=	↑	↓	=	=
Anterior limb internal capsule		↑	↑	↑	=	↑			↑	↑	↑	↑	=	↑	↑	↑	=	↑
Central part centrum semiovale		↑	↑	↑	=	↑			↑	↑	↑	↑	=	↑	↑	↑	=	↑
Corpus callosum splenium		↓	↑	↓	=	↑			↑	=	↑	↑	=	↑	↑	↑	=	↑
genu		↑	↑	↑	=	↑			↑	=	↑	↑	=	↑	↑	↑	=	↑
Parieto-occipital white matter deep		↑	↑	↑	=	↑			↑	↑	↑	↑	=	↑	↑	↑	=	↑
subcortical		↑	↑	↑	=	↑			↑	↑	↑	↑	=	↑	=	↑	=	↑
Frontal white matter deep		↑	↑	↑	=	↑			↑	↑	↑	↑	=	↑	↑	↑	=	↑
subcortical		↑	↑	↑	=	↑			↑	↑	↓	↑	=	↑	=	↑	=	↑
Score vs age-related max. score*	n.e.	4/22	22/22	7/22	11/22	3/22	n.e.	overall ↑	22/22	6/22	21/22	4/22	14/22	3/22	20/22	4/22	11/21	2/13
Basal ganglia																		
Putamen (size, T_2-signal)	absent		absent		absent		absent		absent		small, ↑		small, ↑		absent		absent	
Caudate nucleus (size, T_2-signal)	small, =		nl, =		small, =		absent		nl, =		nl, =		nl, =		small, =		small, ↑	
Atrophy																		
Ventricular enlargement	+		-		+		+		-		-		+		-		+	
Enlargement subarachnoid spaces	+		-		-		+		-		-		+		-		-	
Atrophy corpus callosum	+		-		+		-		+		+		+		+		+	
Cerebellar atrophy vermis	+		-		++		++		+		+		++		+		+	
hemispheres	+		-		+		+		-		-		+		-		-	
Age at latest MRI	**16 y**		**20 y**						**29 y**		**20 y**				**9 y**		**10 y**	
Myelination	T_1	T_2	T_1	T_2					T_1	T_2	T_1	T_2			T_1	T_2	T_1	T_2
Score vs age-related max. score*	n.e.	4/22	17/22	n.e.					15/22	4/22	11/22	4/22			22/22	4/22	overall ↓	1/22
Basal Ganglia																		
Putamen (size, T_2-signal)	absent		absent						absent		smaller, ↑				absent		absent	
Caudate nucleus (size, T_2-signal)	smaller, =		small, =						smaller, =		small, =				small, =		absent	
Atrophy																		
Ventricular enlargement	++		-						-		-				-		++	
Enlargement subarachnoid spaces	++		-						+		-				-		+	
Atrophy corpus callosum	++		+						+		+				+		++	
Cerebellar atrophy vermis	++		+						++		++				++		++	
hemispheres	+		-						+		+				+		+	
Change over time																		
Change in myelination	n.e.		less myelin						less myelin		less myelin				no		less myelin	
Progression atrophy putamen	absent putamen		absent putamen						absent putamen		yes				absent putamen		absent putamen	
Progression cerebral atrophy	yes		no						yes		no				no		yes	
Progression cerebellar atrophy	yes		yes						yes		yes				yes		yes	

Patient number	10		11		12		13		14		15		16		17		18	
Patient ID	HA04		HA07		HA44		HA23		HA165**		HA48		HA54		HA51		HA71	
Age at first MRI	8 y		1.8 y		12 y		1.5 y		3 y		5 y		4 y		2.3 y		7 y	
Myelination	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2
Brainstem	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓
Posterior limb internal capsule	↑	↑	↑	↑	↑	↑	↑	↑	↓	↑	↑	=	↑	↑	n.e.	↑	↑	↑
Cerebellar white matter	=	=	↑	=	↑	=	↑	↓	=	↑	=	↓	=	↓	=	↓	↑	↓
Anterior limb internal capsule	↑	↑	↑	↑	=	↑	↑	↑	↓	↑	↑	↓	↑	↑	n.e.	↑	↑	↑
Central part centrum semiovale	↑	↑	↑	↑	↑	↑	↑	↑	↓	↑	↑	=	=	↑	↓	↑	↑	↑
Corpus callosum splenium	↑	↑	↑	↑	↑	↑	↑	↓	=	=	↑	↓	↑	↑	n.e.	↑	↑	=
genu	↑	↑	↑	↑	=	↑	↑	↓	=	↑	↑	↓	↑	↑	n.e.	↑	↑	↓
Parieto-occipital white matter deep	↑	↑	↑	↑	↑	↑	↑	↑	↓	↑	↑	=	↑	↑	↓	↑	↑	↑
subcortical	↑	↑	=	↑	↑	↑	↑	↑	↓	↑	=	=	=	↑	=	↑	↑	↑
Frontal white matter deep	↑	↑	↑	↑	↑	↑	↑	↑	↓	↑	↑	=	=	↑	↓	↑	↑	↑
subcortical	=	↑	=	↑	=	↑	=	↑	↓	↑	=	=	=	↑	=	↑	↑	↑
Score vs age-related max. score*	20/22	3/22	20/22	3/22	19/22	3/22	21/22	8/22	5/22	3/22	19/22	16/22	16/22	4/22	overall ↓	4/22	22/22	7/22
Basal ganglia																		
Putamen (size, T_2-signal)	small, ↑		absent		absent		small, ↑		nl, =		small, ↑		absent		absent		small, ↑	
Caudate nucleus (size, T_2-signal)	small, =		nl, =		nl, =		nl, =		nl, =		nl, =		small, =		absent		nl, ↑	
Atrophy																		
Ventricular enlargement	-		-		-		-		+		-		+		++		-	
Enlargement subarachnoid spaces	-		-		+		-		+		-		+		-		-	
Atrophy corpus callosum	+		-		+		-		++		-		+		+		-	
Cerebellar atrophy vermis	+ +		+		+		+		++		-		+		++		+	
hemispheres	+		-		-		-		++		-		+		++		-	
Age at latest MRI	10 y		8 y				13 y		18 y		9 y		11 y		7 y			
Myelination	T_1	T_2	T_1	T_2			T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2		
Score vs age-related max. score*	18/22	3/22	20/22	3/22			18/22	4/22	5/22	2/22	22/22	5/22	11/22	2/22	overall ↓	3/22		
Basal Ganglia																		
Putamen (size, T_2-signal)	small, ↑		absent				absent		smaller, ↑		absent		absent		absent			
Caudate nucleus (size, T_2-signal)	small, =		small, =				small, =		smaller, ↑		small, ↑		small, =		absent			
Atrophy																		
Ventricular enlargement	-		-				+		++		-		+		++			
Enlargement subarachnoid spaces	-		-				-		++		-		+		+			
Atrophy corpus callosum	+		+				+		++		-		+		++			
Cerebellar atrophy vermis	+ +		++				++		++		-		++		++			
hemispheres	+		+				+		++		-		+		++			
Change over time																		
Change in myelination	less myelin		no				less myelin		no		no		less myelin		no			
Progression atrophy putamen	no		absent putamen				yes		yes		yes		absent putamen		absent putamen			
Progression cerebral atrophy	no		no				yes		yes		no		no		yes			
Progression cerebellar atrophy	no		yes				yes		no		no		yes		no			

Patient number	19		20		21		22		23		24		25		26		27	
Patient ID	HA136		HA82		HA97		HA32		HA132		HA90		HA66 / LD_0313.0		HA40		HA75	
Age at first MRI	14 y		5 y		2.9 y		8 mo		7 y		6 y		4 y		9 mo		4 y	
Myelination	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2
Brainstem	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓	=	↓	↑	↓	↑		↑	↑
Posterior limb internal capsule	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	=	↑	↑	↓	↑		↑	=
Cerebellar white matter	=	↓	n.e.	n.e.	↑	↓		↓	↑	=	=	↑	↑	↑	↑		=	↑
Anterior limb internal capsule	=	↑	↑	↑	↑	↑	↑	↑	↑	↑	=	↑	↑	↑	=		↑	↑
Central part centrum semiovale	=	↑	n.e.	↑	↑	↑	↓	↑	↑	↑	↓	↑	↑	↑	=		↑	↑
Corpus callosum splenium	↑	↑	↑	↑	↑	↓		↑	↑	↑	=	↑	↑	↓	↑		=	↑
genu	=	↑	↑	↑	↑	↓		↑	↑	=	=	↑	↑	↓	↑		=	↑
Parieto-occipital white matter deep	=	↑	↑	↑	↑	↑	↓	↑	↑	=	=	↑	↑	↑	=		↑	↑
subcortical	=	↑	=	↑	=	↑		↑	=	↑	↓	↑	↑	↑	=		=	↑
Frontal white matter deep	=	↑	↑	↑	↑	↑	↓	↑	↑	↑	=	↑	↑	↑	=		↑	↑
subcortical	=	↑	=	↑	=	↑		↑	=	↑	↓	↑	↑	↑	=		=	↑
Score vs age-related max. score*	14/22	4/22	overall ↑	overall ↑	20/22	8/22	overall ↓	4/12	20/22	5/22	8/22	2/22	22/22	8/22	17/21	n.e.	17/22	3/22
Basal ganglia																		
Putamen (size, T_2-signal)	absent		absent		small, ↑		nl, ↑		small, ↑		absent		small, ↑		small, n.e.		absent	
Caudate nucleus (size, T_2-signal)	small, ↑		nl, =		nl, =		nl, =		nl, =		small, ↑		nl, ↑		small, =		nl, =	
Atrophy																		
Ventricular enlargement	+		-		-		-		-		+		-		-		-	
Enlargement subarachnoid spaces	+		-		-		-		-		+		-		+		-	
Atrophy corpus callosum	+		-		-		-		-		+		-		-		-	
Cerebellar atrophy vermis	++		n.e.		+		+		+		+		+		++		++	
hemispheres	+		n.e.		-		-		-		+		-		+		+	
Age at latest MRI			8 y		7 y		3 y		10 y				9 y		3.3 y			
Myelination			T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2			T_1	T_2	T_1	T_2		
Score vs age-related max. score*			20/22	4/22	18/22	6/22	overall ↓	3/22	overall ↑	5/22			18/22	4/22	18/22	5/22		
Basal Ganglia																		
Putamen (size, T_2-signal)			absent		absent		small, ↑		absent				absent		absent			
Caudate nucleus (size, T_2-signal)			nl, =		nl, =		small, =		small, =				nl, ↑		smaller, =			
Atrophy																		
Ventricular enlargement			-		-		+		+				-		-			
Enlargement subarachnoid spaces			-		-		+		+				-		+			
Atrophy corpus callosum			-		-		+		-				-		+			
Cerebellar atrophy vermis			+		+		++		+				+		++			
hemispheres			-		-		+		+				-		+			
Change over time																		
Change in myelination			no		less myelin		no		no				less myelin		no			
Progression atrophy putamen			absent putamen		yes		yes		yes				yes		yes			
Progression cerebral atrophy			no		no		yes		yes				no		no			
Progression cerebellar atrophy			n.e.		no		yes		yes				no		no			

Patient number	28		29		30		31		32		33		34		35		36	
Patient ID	HA149		HA172		HA173		HA127		HA86 / LD_0345.0		HA107		HA124		HA143		HA140	
Age at first MRI	4 y		6 mo		1.0 y		4 y		1.9 y		3.8 y		3.5 y		1.2 y		1.5 y	
Myelination	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2
Brainstem	↑	↑	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓	↑	↑	↑	↓	=	=
Posterior limb internal capsule	↑	↓	↑	↓	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	=	↑	↑	↑
Cerebellar white matter	↑	↑	↑	↓	↑	=	↑	=	↑	↓	↑	↓	↑	=	=	=	↑	=
Anterior limb internal capsule	=	↓	=	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	=	↑	↑	↑
Central part centrum semiovale	↑	↑	=	↑	=	↑	↑	↑	↑	↑	↑	↑	↑	↑	=	↑	↑	↑
Corpus callosum splenium	↑	↑	↑	↓	↑	=	↑	↑	↑	↓	↑	=	↑	↑	↑	=	↑	↑
genu	↑	↑	↑	=	↑	↑	↑	↑	=	=	↑	=	↑	↑	↑	=	↑	↑
Parieto-occipital white matter deep	↑	↑	=	↑	=	↑	↑	↑	↑	↑	↑	↑	↑	↑	=	↑	↑	↑
subcortical	↑	↑	=	↑	=	↑	↑	↑	=	↑	↑	↑	↑	↑	=	↑	↑	↑
Frontal white matter deep	↑	↑	=	↑	=	↑	↑	↑	↑	↑	↑	↑	↑	↑	=	↑	↑	↑
subcortical	↑	↑	=	↑	=	↑	=	↑	=	↑	↑	↑	↑	↑	=	↑	↑	↑
Score vs age-related max. score*	21/22	4/22	16/20	9/8	17/22	4/17	21/22	3/22	19/22	7/22	22/22	6/22	22/22	3/22	14/22	5/18	1/22	2/22
Basal ganglia																		
Putamen (size, T_2-signal)	small, ↑		nl, =		nl, =		absent		small, ↑		absent		small, ↑		small, ↑		nl, =	
Caudate nucleus (size, T_2-signal)	nl, =		nl, =		nl, =		nl, =		nl, =		small, ↑		nl, ↑		small, ↑		nl, =	
Atrophy																		
Ventricular enlargement	-		-		-		-		-		-		-		+		-	
Enlargement subarachnoid spaces	-		-		-		-		-		-		-		-		-	
Atrophy corpus callosum	-		-		-		-		-		-		-		-		-	
Cerebellar atrophy vermis	+		++		+		+		+		+		+		+		+	
hemispheres	-		+		-		-		-		-		-		-		-	
Age at latest MRI	6 y		6 y		8 y				2.8 y						2.8 y		4 y	
Myelination	T_1	T_2	T_1	T_2	T_1	T_2			T_1	T_2					T_1	T_2	T_1	T_2
Score vs age-related max. score*	21/22	4/22	19/22	8/22	14/22	3/22			21/22	8/22					13/22	5/22	1/22	2/22
Basal Ganglia																		
Putamen (size, T_2-signal)	small, ↑		little smaller, ↑		little smaller, =				small, ↑						absent		small, ↑	
Caudate nucleus (size, T_2-signal)	nl, =		nl, ↑		little smaller, =				nl, =						smaller, ↑		small, =	
Atrophy																		
Ventricular enlargement	-		-		+				-						+		+	
Enlargement subarachnoid spaces	-		-		-				-						+		+	
Atrophy corpus callosum	-		-		+				-						+		-	
Cerebellar atrophy vermis	+		++		++				+						++		+	
hemispheres	-		++		++				+						+		+	
Change over time																		
Change in myelination	no		more myelin		less myelin				no						no		no	
Progression atrophy putamen	no		yes		yes				no						yes		yes	
Progression cerebral atrophy	no		no		yes				no						yes		yes	
Progression cerebellar atrophy	no		n.e.		yes				yes						yes		yes	

Patient number	37		38		39		40		41		42	
Patient ID	HA176		HA113		HA146		HA103		HA163		HA159	
Age at first MRI	**1.0 y**		**2.5 y**		**4.0 y**		**8 mo**		**1.0 y**		**10 mo**	
Myelination	**T_1**	**T_2**	**T_1**	**T_2**	**T_1**	**T_2**	**T_1**	**T_2**	**T_1**	**T_2**	**T_1**	**T_2**
Brainstem	↑	↓	=	↓	↑	↓	↑	↓	=	=	↑	↓
Posterior limb internal capsule	↑	↑	=	↑	↑	↑	↑	↑	↓	↑	↑	↑
Cerebellar white matter	↑	↓	=	↑	=	↓	=	=	↓	↑	↑	=
Anterior limb internal capsule	↑	↑	=	↑	=	↑	=	↑	↓	↑	↑	↑
Central part centrum semiovale	=	↑	=	↑	↑	↑	=	↑	↓	↑	=	↑
Corpus callosum splenium	↑	↑	=	↑	↑	↑	=	↑	↓	↑	↑	↑
genu	↑	↑	=	↑	↑	↑	=	↑	↓	↑	↑	↑
Parieto-occipital white matter deep	=	↑	=	↑	↑	↑	=	↑	↓	↑	↑	↑
subcortical	=	↑	=	↑	=	↑	=	↑	↓	↑	=	↑
Frontal white matter deep	=	↑	=	↑	=	↑	=	↑	↓	↑	↑	↑
subcortical	=	↑	=	↑	=	↑	=	↑	↓	↑	=	↑
Score vs age-related max. score*	17/22	4/17	11/22	2/22	17/22	4/22	13/21	3/12	1/22	1/18	19/21	3/13
Basal ganglia												
Putamen (size, T_2-signal)	nl, =		absent		absent		absent		nl, =		nl, =	
Caudate nucleus (size, T_2-signal)	nl, =		small, ↑		nl, =		small, ↑		nl, =		nl, =	
Atrophy												
Ventricular enlargement	-		+		-		+		-		-	
Enlargement subarachnoid spaces	-		-		-		-		-		-	
Atrophy corpus callosum	+		+		+		-		-		-	
Cerebellar atrophy vermis	++		+		+		+		+		-	
hemispheres	+		+		-		-		+		-	
Age at latest MRI	**6 y**				**4.4 y**		**12 mo**		**3.3 y**		**2.2 y**	
Myelination					**T_1**	**T_2**	**T_1**	**T_2**	**T_1**	**T_2**	**T_1**	**T_2**
Score vs age-related max. score*	15/22	4/22			17/22	3/22	13/22	3/17	1/22	2/22	22/22	8/22
Basal Ganglia												
Putamen (size, T_2-signal)	small, ↑				absent		absent		small, =		small, ↑	
Caudate nucleus (size, T_2-signal)	small, =				nl, =		small, ↑		nl, =		nl, =	
Atrophy												
Ventricular enlargement	-				-		+		+		-	
Enlargement subarachnoid spaces	-				-		-		-		-	
Atrophy corpus callosum	++				+		-		+		-	
Cerebellar atrophy vermis	++				+		+		++		+	
hemispheres	+				-		-		++		+	
Change over time												
Change in myelination	less myelin				no		no		no		more myelin	
Progression atrophy putamen	yes				absent putamen		absent putamen		yes		yes	
Progression cerebral atrophy	no				no		no		yes		no	
Progression cerebellar atrophy	no				no		no		yes		yes	

The table is organized by descending age of patients. * If not all items could be scored, the overall intensity of the cerebral hemispheric white matter is indicated.
y, years; mo, months; vs, versus; max, maximum; T_1, T_1-weighted image; T_2, T_2-weighted image; ↑, hyperintense; ↓, hypointense; =, isointense relative to the cortex; n.e., not evaluable; nl, normal; -, not present; +, present; ++, severe
** this case had been previously published in Wolf et al.[36]

Supplementary Table 1B | Clinical characteristics of 42 H-ABC patients

Patient number	1	2	3	4	5	6	7	8	9
Patient ID	HA106	HA20 / LD_0440.0	HA62 / LD_0605.0	HA12	HA27	HA36	HA129	HA26	HA15
Year of birth, age*	1980, 25 y	1980, 22 y	1982, 20 y	1983, 11 y	1983, 29 y	1984 / 29 y	1987, 23 y	1989, 23 y	1989, 23 y
Gender	m	m	f	m	m	f	f	m	f
Consanguinity parents	-	-	-	-	-	-	-	-	-
Unaffected / affected siblings	1 / 0	2 / 0	2 / 0	2 / 0	2 / 0	1 / 0	2 / 0	2 / 0	0 / 0
Mutation	c.730G>A	c.745G>A	c.745G>A	c.1164G>A	c.745G>A	c.745G>A	c.745G>A	c.745G>A	c.716G>T
Age at first signs	4 mo	3 y	9 mo	4 mo	2.5 y	9 mo	3 y	1 y	2 mo
First signs	no head control	motor deterioration	motor delay	motor delay, spasticity	motor deterioration	nystagmus	motor delay, gait problems	motor delay	no head control, decreased vision
Initial motor development	delayed	nl	mildly delayed	delayed	nl	mildly delayed	mildly delayed	mildly delayed	delayed
Unsupported walking	never	14 mo	3 y	never	16 mo	never	18 mo	never	never
Maximum motor milestone	supported walking	unsupported walking	unsupported walking	rolling over	unsupported walking	supported walking	unsupported walking	supported walking	holding objects
Loss of unsupported walking	n.a.	10 y	7 y	n.a.	12 y	n.a.	4 y	n.a.	n.a.
Full wheelchair dependency	7 y	12 y	10 y	n.a.	16 y	8 y	8 y	2.3 y	n.a.
Maximum spoken language, up to age	single words, 10 y	single words, 6 y	single words, 7 y	none	nl, 6 y	single words, 15 y	nl, 8 y	single words, 3 y	none
Maximum level of comprehension	nl	nl	decreased intelligence	social awareness only	decreased intelligence	decreased intelligence	nl	decreased intelligence	decreased intelligence
Current speech	no speech	dysarthria	dysarthria	no speech	no speech	no speech	no speech	no speech	no speech
Current level of comprehension	decreased intelligence	decreased intelligence	decreased intelligence	social awareness only	decreased intelligence	decreased intelligence	decreased intelligence	decreased intelligence	decreased intelligence
Truncal hypotonia	+	+	+	+	+	+	+	+	+
Spasticity	+	+	+	+	+	+	+	+	+
Ataxia	+	+	+	-	+	+	+	+	+
Choreoathethosis	-	+	-	+	-	-	-	+	+
Dystonia / ridigity	+	+	+	+	+	+	+	+	+
Seizures	-	-	-	-	-	-	-	-	+
Special findings	-	-	-	blind	perceptive hearing loss**	-	-	-	15 y: tracheostomy and ventilation
Tube feeding since	5 y		21 y	2.5 y	26 y	26 y	20 y	16 y	8 y
Height, weight, head circumference	<2 SD, <2 SD, <2 SD	nl, nl, nl	<2 SD, < 2 SD, < 2 SD	<2 SD, <2 SD, <2 SD	nl, < 2 SD, nl	nl, nl, nl	nl, nl, nl	<2 SD, nl, nl	<2 SD, nl, nl
Age and cause of death	25 y, resp. failure	status unknown	status unknown	20 y, peritonitis	-	-	-	-	-

Patient number	10	11	12	13	14	15	16	17	18
Patient ID	HA04	HA07	HA44	HA23	HA165***	HA48	HA54	HA51	HA71
Year of birth, age*	1989, 23 y	1991, 21 y	1992, 20 y	1993, 9 y	1994, 18 y	1994, 18 y	1995, 12 y	1996, 16 y	1996, 10 y
Gender	f	m	f	m	f	m	m	m	m
Consanguinity parents	-	-	-	-	-	-	-	-	-
Unaffected / affected siblings	1 / 0	0 / 0	1 / 0	0 / 0	5 / 0	0 / 0	2 / 0	1 / 0	unknown
Mutation	c.745G>A	c.745G>A	c.745G>A	c.745G>A	c.5G>A	c.745G>A	c.1054G>A	c.1061G>A	c.745G>A
Age at first signs	9 mo	1.75 y	1 y	6 mo	1.5 mo	3 y	4 mo	birth	1.5 y
First signs	motor delay, hypotonia	motor delay	motor delay	motor delay, hypotonia	nystagmus	dystonia, dysarthria	motor delay, hypotonia	nystagmus, hypotonia	spasticity
Initial motor development	mildly delayed	mildly delayed	mildly delayed	delayed	delayed	nl	delayed	delayed	mildly delayed
Unsupported walking	33 mo	never	17 mo	never	never	15 mo	never	never	3 y
Maximum motor milestone	unsupported walking	supported walking	unsupported walking	supported walking	no intentional movements	unsupported walking	supported standing	rolling over	unsupported walking
Loss of unsupported walking	7 y	n.a.	5 y	n.a.	n.a.	10 y	n.a.	n.a.	5 y
Full wheelchair dependency	13 y	9 y	6 y	15 y	n.a.	10 y	n.a.	n.a.	-
Maximum spoken language, up to age	nl, 6 y	nl, 5 y	short sentences, 3 y	syllables, 15 y	none	nl, 3 y	none	none	unknown
Maximum level of comprehension	decreased intelligence	decreased intelligence	decreased intelligence	decreased intelligence	decreased intelligence	nl	social awareness only	social awareness only	decreased intelligence
Current speech	no speech	dysarthria	dysarthria	no speech	no speech	no speech	no speech	no speech	no speech
Current level of comprehension	decreased intelligence	decreased intelligence	decreased intelligence	decreased intelligence	social awareness only	decreased intelligence	social awareness only	social awareness only	decreased intelligence
Truncal hypotonia	+	+	+	+	+	-	+	+	-
Spasticity	+	+	+	+	+	+	+	+	+
Ataxia	+	+	+	+	n.e.	+	-	-	+
Choreoathethosis	+	+	+	+	-	-	+	-	-
Dystonia / ridigity	+	+	+	+	+	+	+	+	+
Seizures	-	-	-	-	+	-	+	+	-
Special findings	-	-	-	-	-	-	-	blind	-
Tube feeding since	19 y	-	16 y	-	9 y	-	4 y	9 y	unknown
Height, weight, head circumference	nl, < 2SD, nl	nl, < 2 SD, nl	nl, <2 SD, <2 SD	nl, <2 SD, nl	<2 SD, <2SD, <2 SD	nl, <2 SD, nl	<2 SD, <2 SD, nl	<2 SD, <2 SD, <2 SD	<2 SD, <2 SD, nl
Age and cause of death	-	-	-	-	-	-	-	-	-

Patient number	19	20	21	22	23	24	25	26	27
Patient ID	HA136	HA82	HA97	HA32	HA132	HA90	HA66 / LD_0313.0	HA40	HA75
Year of birth, age*	1992, 15 y	1998, 14 y	1998, 14 y	2000, 12 y	2000, 10 y	2000, 11 y	2001, 11 y	2005, 10 y	2002, 11 y
Gender	m	m	m	f	f	f	m	m	m
Consanguinity parents	-	-	-	-	-	-	-	-	-
Unaffected / affected siblings	1 / 0	1 / 0	3 / 0	0 / 0	2 / 0	0 / 0	2 / 0	1 / 0	1 / 0
Mutation	c.745G>A	c.745G>A	c.745G>A	c.730G>A	c.745G>A	c.968T>G	c.745G>A	c.1054G>	c.745G>A
Age at first signs	4 mo	1.5 y	6 mo	4 mo	3 y	2 mo	1.5 y	6 mo	4 mo
First signs	motor delay	motor delay	hypotonia, ataxia	hypotonia	motor deterioration	hypotonia, no head control	gait problems, dysarthria	hypotonia	motor delay, spasticity
Initial motor development	delayed	mildly delayed	mildly delayed	delayed	nl	delayed	nl	delayed	mildly delayed
Unsupported walking	never	24 mo	27 mo	never	20 mo	never	17 mo	never	never
Maximum motor milestone	crawling	unsupported walking	unsupported walking	no intentional movements	unsupported walking	no intentional movements	unsupported walking	rolling over	supported walking
Loss of unsupported walking	n.a.	6 y	10 y	n.a.	6 y	n.a.	5 y	n.a.	8 y
Full wheelchair dependency	n.a.	8 y	11 y	n.a.	7 y	n.a.	-	n.a.	n.a.
Maximum spoken language, up to age	single words, 6 y	nl, present	single words, 7 y	none	nl, 8 y	none	nl, unknown	none	short sentences, 6 y
Maximum level of comprehension	nl	decreased intelligence	nl	social awareness only	decreased intelligence	decreased intelligence	decreased intelligence	decreased intelligence	nl
Current speech	no speech	dysarthria	no speech	no speech	dysarthria	no speech	dysarthria	no speech	dysarthria
Current level of comprehension	decreased intelligence	decreased intelligence	decreased intelligence	social awareness only	decreased intelligence	social awareness only	decreased intelligence	social awareness only	decreased intelligence
Truncal hypotonia	+	+	+	+	+	+	+	+	-
Spasticity	+	+	+	+	+	+	+	+	+
Ataxia	-	+	+	n.e.	+	n.e.	+	n.e.	+
Choreoathethosis	-	-	-	-	+	-	+	-	-
Dystonia / ridigity	+	+	+	+	+	+	+	+	-
Seizures	-	-	+	+	+	-	-	-	-
Special findings	-	-	-	-	-	-	-	-	-
Tube feeding since	-	11 y	11 y	-	-	-	-	9 y	-
Height, weight, head circumference	<2 SD, <2 SD, unknown	nl, <2 SD, nl	<2 SD, <2 SD, nl	<2 SD, <2 SD, <2 SD	nl, <2 SD, nl	<2 SD, <2 SD, nl	<2 SD, nl, nl	<2 SD, <2 SD, <2 SD	<2 SD, nl, nl
Age and cause of death	-	-	-	-	12 y, cachexia, respiratory failure	-	-	-	-

Patient number	28	29	30	31	32	33	34	35	36
Patient ID	HA149	HA172	HA173	HA127	HA86 / LD_0345.0	HA107	HA124	HA143	HA140
Year of birth, age*	2004, 7 y	2004, 8 y	2005, 8 y	2005, 5 y	2005, 5 y	2006, 6 y	2007, 5 y	2007, 5 y	2007, 5 y
Gender	m	f	f	f	f	f	f	f	m
Consanguinity parents		-	-	-	-	-	-	-	-
Unaffected / affected siblings	2 / 0	2 / 0	1 / 0	3 / 1	1 / 0	1 / 0	0 / 0	0 / 0	1 / 0
Mutation	c.745G>A	c.1099T>A	c.1163T>C	c.745G>A	c.745G>A	c.745G>A	c.745G>A	c.1099T>C	c.4C>T
Age at first signs	2 y	6 mo	6 mo	2 y	1 y	2 y	2 y	2 mo	birth
First signs	motor delay, gait problems	hypotonia, ataxia	nystagmus, motor delay	extrapyramidal movements	motor delay	motor and language delay	motor and language delay	motor delay, epilepsy	nystagmus
Initial motor development	mildly delayed	delayed	delayed	mildly delayed	mildly delayed	nl	mildly delayed	delayed	delayed
Unsupported walking	22 mo	never	never	48 mo	23 mo	13 mo	18 mo	never	never
Maximum motor milestone	unsupported walking	holding objects	rolling over	unsupported walking	unsupported walking	unsupported walking	unsupported walking	no intentional movements	supported sitting
Loss of unsupported walking	-	n.a.	n.a.	4.5 y	2 y	4 y	-	n.a.	n.a.
Full wheelchair dependency	-	n.a.	n.a.	-	-	6 y	-	n.a.	n.a.
Maximum spoken language, up to age	nl, present	none	none	short sentences, 7 y	single words	single words, 3 y	short sentences, 4 y	none	none
Maximum level of comprehension	decreased intelligence	social awareness only	decreased intelligence	decreased intelligence	decreased intelligence	decreased intelligence	decreased intelligence	social awareness only	social awareness only
Current speech	nl	no speech	no speech	dysarthria	no speech	no speech	dysarthria	no speech	no speech
Current level of comprehension	decreased intelligence	social awareness only	decreased intelligence	decreased intelligence	decreased intelligence	decreased intelligence	decreased intelligence	social awareness only	social awareness only
Truncal hypotonia	-	-	+	-	+	-	-	+	+
Spasticity	-	+	+	+	+	+	+	+	+
Ataxia	-	+	+	-	+	+	+	n.e.	-
Choreoathethosis	-	-	-	-	+	-	-	-	-
Dystonia / ridigity	+	+	+	+	+	+	+	+	+
Seizures	-	-	-	-	-	+	-	+	+
Special findings	-	-	-	-	-	-	-	-	-
Tube feeding since	-	7 y	8 y	-	-	-	-	1 y	3 y
Height, weight, head circumference	nl, nl, nl	<2 SD, <2 SD, <2 SD	<2 SD, nl, <2 SD	<2 SD, nl, nl	<2 SD, nl, nl	nl, nl, nl	<2 SD, nl, nl	<2 SD, <2 SD, <2 SD	<2 SD, <2 SD, <2 SD
Age and cause of death	-	-	-	-	-	-	-	-	-

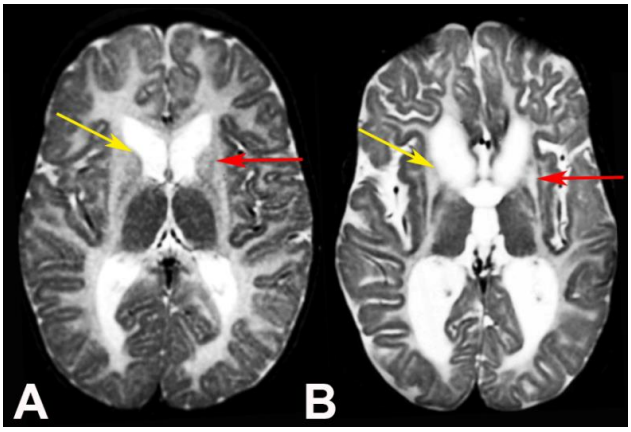
Patient number	37	38	39	40	41	42
Patient ID	HA176	HA113	HA146	HA103****	HA163	HA159
Year of birth, age*	2007, 5 y	2007, 4 y	2007, 5 y	2008, 4 y	2009, 3 y	2010, 2 y
Gender	m	m	f	m	f	f
Consanguinity parents	-	-	-	-	-	-
Unaffected / affected siblings	1 / 0	0 / 0	1 / 0	0 / 0	0 / 0	3 / 0
Mutation	c.730G>A	c.731G>T	c.745G>A	c.1162A>G	c.4C>T	c.745G>A
Age at first signs	5 mo	birth	1.25 y	5 mo	3 mo	3 mo
First signs	developmental delay	nystagmus	gait problems	nystagmus	nystagmus, hypotonia	nystagmus, mild hypotonia
Initial motor development	delayed	delayed	nl	delayed	delayed	mildly delayed
Unsupported walking	never	never	10 mo	never	never	18 mo
Maximum motor milestone	reaching for objects	touching objects	unsupported walking	no intentional movements	supported sitting	unsupported walking
Loss of unsupported walking	n.a.	n.a.	-	n.a.	n.a.	2 y
Full wheelchair dependency	n.a.	n.a.	-	n.a.	n.a.	-
Maximum spoken language, up to age	none	none	single words, 5y	none	none	short sentences, 2 y
Maximum level of comprehension	social awareness only	social awareness only	nl	social awareness only	decreased intelligence	nl
Current speech	no speech	no speech	dysarthria	no speech	no speech	no speech
Current level of comprehension	social awareness only	social awareness only	decreased intelligence	social awareness only	social awareness only	decreased intelligence
Truncal hypotonia	+	+	+	+	+	+
Spasticity	+	+	+	+	-	+
Ataxia	+	-	+	n.e.	-	+
Choreoathethosis	-	+	-	-	+	+
Dystonia / ridigity	+	+	+	+	-	+
Seizures	-	+	-	-	-	-
Special findings	-	-	-	-	-	-
Tube feeding since	3 y	2.5 y	+	3 y	-	-
Height, weight, head circumference	<2 SD, <2 SD, nl	nl, <2 SD, nl	nl, nl, nl	nl, nl, <2 SD	nl, <2SD, <2SD	nl, nl, nl
Age and cause of death	-	-	-	-	-	-

The table is organized by descending age of patients. * Age at moment of obtaining clinical characteristics; ** this patient has a deletion of the *OTOA* gene; *** this case had been previously published in Wolf et al.[36]; **** this patient has Down syndrome.
f, female; m, male; nl, normal; mo, months, y, years; -, not present; + present; n.a., not applicable; n.e., not evaluable.

Supplementary Table 2 | Primer used for *TUBB4A* sequencing

Primer name	Sequence
TUBB4A-exon1F	5'-GTCTCCGCCGCATCTTCC-3'
TUBB4A-exon1R	5'-TCCAAAGACTCCCAGGTTGC-3'
TUBB4A-exon2+3F	5'-GGCTTCCCCTCTCTCAACTC-3'
TUBB4A-exon2+3R	5'-CACCCCATCTCTGGTCTGTG-3'
TUBB4A-exon4F-a	5'-CCAGGTGGTGGAGAGACAGA-3'
TUBB4A-exon4R-a	5'-AGAAGTGCAGGCGAGGAAAG-3'
TUBB4A-exon4F-b	5'-CAACGAGGCACTCTACGACA-3'
TUBB4A-exon4R-b	5'-CTGGTCAGGGGTGCGAAG-3'
TUBB4A-exon4R-b2	5'-GTTCTTGGCATCGAACATCTGC-3'
TUBB4A-exon4F-c	5'-CTTTCCTCGCCTGCACTTCT-3'
TUBB4A-exon4R-c	5'-ACAGGTGGGAAGCGATGG-3'
TUBB4A-exon4F-3'	5'-ATGGACGAGATGGAGTTCAC-3'
TUBB4A-exon4R-3'	5'-AGGTCTGCAAAGTTAAAGGT-3'

F, forward; R, reverse



Supplementary Figure 2 | Loss of grey matter signal basal ganglia. Axial T_2-weighted images of patient HA15 at the age of 1 year (**A**) and 11 years (**B**). In **A**, note the absence of a visible putamen (red arrow). The caudate nucleus (yellow arrow) is small and has a signal similar to the abnormal white matter signal, instead of a grey matter signal. 10 years later, the caudate nucleus has entirely disappeared (yellow arrow in **B**).

REFERENCES

1. van der Knaap MS, Naidu S, Pouwels PJ, et al. New Syndrome Characterized by Hypomyelination with Atrophy of the Basal Ganglia and Cerebellum. AJNR Am J Neuroradiol 2002;23:1466-1474.
2. van der Knaap MS, Linnankivi T, Paetau A, et al. Hypomyelination with Atrophy of the Basal Ganglia and Cerebellum: Follow-up and Pathology. Neurology 2007;69:166-171.
3. Matta AP, Ribas MC. Hypomyelination with Atrophy of the Basal Ganglia and Cerebellum: Case Report. Arq Neuropsiquiatr 2007;65:161-163.
4. Mercimek-Mahmutoglu S, van der Knaap MS, Baric I, Prayer D, Stoeckler-Ipsiroglu S. Hypomyelination with Atrophy of the Basal Ganglia and Cerebellum (H-Abc). Report of a New Case. Neuropediatrics 2005;36:223-226.
5. Narumi Y, Shiihara T, Yoshihashi H, et al. Hypomyelination with Atrophy of the Basal Ganglia and Cerebellum in an Infant with Down Syndrome. Clin Dysmorphol 2011;20:166-167.
6. Wakusawa K, Haginoya K, Kitamura T, et al. Effective Treatment with Levodopa and Carbidopa for Hypomyelination with Atrophy of the Basal Ganglia and Cerebellum. Tohoku J Exp Med 2006;209:163-167.
7. Simons C, Wolf NI, McNeil N, et al. A De Novo Mutation in the Beta-Tubulin Gene Tubb4a Results in the Leukoencephalopathy Hypomyelination with Atrophy of the Basal Ganglia and Cerebellum. Am J Hum Genet 2013;92:767-773.
8. Hersheson J, Mencacci NE, Davis M, et al. Mutations in the Autoregulatory Domain of Beta-Tubulin 4a Cause Hereditary Dystonia. Ann Neurol 2013;73:546-553.
9. Lohmann K, Wilcox RA, Winkler S, et al. Whispering Dysphonia (Dyt4 Dystonia) Is Caused by a Mutation in the Tubb4 Gene. Ann Neurol 2013;73:537-545.
10. Wilcox RA, Winkler S, Lohmann K, Klein C. Whispering Dysphonia in an Australian Family (Dyt4): A Clinical and Genetic Reappraisal. Mov Disord 2011;26:2404-2408.
11. Yen TJ, Machlin PS, Cleveland DW. Autoregulated Instability of Beta-Tubulin Mrnas by Recognition of the Nascent Amino Terminus of Beta-Tubulin. Nature 1988;334:580-585.
12. Schiffmann R, van der Knaap MS. Invited Article: An Mri-Based Approach to the Diagnosis of White Matter Disorders. Neurology 2009;72:750-759.
13. Lowe J, Li H, Downing KH, Nogales E. Refined Structure of Alpha Beta-Tubulin at 3.5 a Resolution. J Mol Biol 2001;313:1045-1057.
14. Wade RH, Garcia-Saez I, Kozielski F. Structural Variations in Protein Superfamilies: Actin and Tubulin. Mol Biotechnol 2009;42:49-60.
15. Amos LA, Lowe J. How Taxol Stabilises Microtubule Structure. Chem Biol 1999;6:R65-69.
16. Vydra T, Havelka D. Post Processing of Results of Em Field Simulators. In: Katsikis VN, ed. Matlab - a Fundamental Tool for Scientific Computing and Engineering Applications. Rijeka: InTech, 2012: p.37.
17. van der Knaap MS, Breiter SN, Naidu S, Hart AA, Valk J. Defining and Categorizing Leukoencephalopathies of Unknown Origin: Mr Imaging Approach. Radiology 1999;213:121-133.
18. Plecko B, Stockler-Ipsiroglu S, Gruber S, et al. Degree of Hypomyelination and Magnetic Resonance Spectroscopy Findings in Patients with Pelizaeus Merzbacher Phenotype. Neuropediatrics 2003;34:127-136.
19. Barkovich AJ, Kjos BO, Jackson DE, Jr., Norman D. Normal Maturation of the Neonatal and Infant Brain: Mr Imaging at 1.5 T. Radiology 1988;166:173-180.
20. Kumar P, Henikoff S, Ng PC. Predicting the Effects of Coding Non-Synonymous Variants on Protein Function Using the Sift Algorithm. Nat Protoc 2009;4:1073-1081.
21. Adzhubei IA, Schmidt S, Peshkin L, et al. A Method and Server for Predicting Damaging Missense Mutations. Nat Methods 2010;7:248-249.
22. Little M, Luduena RF. Structural Differences between Brain Beta 1- and Beta 2-Tubulins: Implications for Microtubule Assembly and Colchicine Binding. EMBO J 1985;4:51-56.
23. Nogales E, Whittaker M, Milligan RA, Downing KH. High-Resolution Model of the Microtubule. Cell 1999;96:79-88.
24. Nogales E, Wolf SG, Downing KH. Structure of the Alpha Beta Tubulin Dimer by Electron Crystallography. Nature 1998;391:199-203.
25. Leandro-Garcia LJ, Leskela S, Landa I, et al. Tumoral and Tissue-Specific Expression of the Major Human Beta-Tubulin Isotypes. Cytoskeleton (Hoboken) 2010;67:214-223.
26. Tischfield MA, Engle EC. Distinct Alpha- and Beta-Tubulin Isotypes Are Required for the Positioning, Differentiation and Survival of Neurons: New Support for the 'Multi-Tubulin' Hypothesis. Biosci Rep 2010;30:319-330.

27. Mitchison T, Kirschner M. Dynamic Instability of Microtubule Growth. Nature 1984;312:237-242.
28. Lopata MA, Cleveland DW. In Vivo Microtubules Are Copolymers of Available Beta-Tubulin Isotypes: Localization of Each of Six Vertebrate Beta-Tubulin Isotypes Using Polyclonal Antibodies Elicited by Synthetic Peptide Antigens. J Cell Biol 1987;105:1707-1720.
29. Abdollahi MR, Morrison E, Sirey T, et al. Mutation of the Variant Alpha-Tubulin Tuba8 Results in Polymicrogyria with Optic Nerve Hypoplasia. Am J Hum Genet 2009;85:737-744.
30. Breuss M, Heng JI, Poirier K, et al. Mutations in the Beta-Tubulin Gene Tubb5 Cause Microcephaly with Structural Brain Abnormalities. Cell Rep 2012;2:1554-1562.
31. Jaglin XH, Poirier K, Saillour Y, et al. Mutations in the Beta-Tubulin Gene Tubb2b Result in Asymmetrical Polymicrogyria. Nat Genet 2009;41:746-752.
32. Keays DA, Tian G, Poirier K, et al. Mutations in Alpha-Tubulin Cause Abnormal Neuronal Migration in Mice and Lissencephaly in Humans. Cell 2007;128:45-57.
33. Tischfield MA, Baris HN, Wu C, et al. Human Tubb3 Mutations Perturb Microtubule Dynamics, Kinesin Interactions, and Axon Guidance. Cell 2010;140:74-87.
34. Niwa S, Takahashi H, Hirokawa N. Beta-Tubulin Mutations That Cause Severe Neuropathies Disrupt Axonal Transport. EMBO J 2013;32:1352-1364.
35. Sriram SM, Kim BY, Kwon YT. The N-End Rule Pathway: Emerging Functions and Molecular Principles of Substrate Recognition. Nat Rev Mol Cell Biol 2011;12:735-747.
36. Wolf NI, Willemsen MA, Engelke UF, et al. Severe Hypomyelination Associated with Increased Levels of N-Acetylaspartylglutamate in Csf. Neurology 2004;62:1503-1508.

Chapter 4.2

Reply: *TUBB4A* novel mutation reinforces the genotype-phenotype correlation of hypomyelination with atrophy of the basal ganglia and cerebellum

Marjo S. van der Knaap, Eline M.C. Hamilton and Laura van Berge

Brain. 2014;137:e290

Sir,

We thank Dr Carvalho and colleagues for their insightful letter,[1] which compliments our recent paper on hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC).[2] The authors present two H-ABC patients with a *TUBB4A* mutation. One mutation is novel, one has been reported before in three patients.[1,2] Carvalho *et al.* stress that their patients presented with a more severe phenotype than observed in the group of now 30 patients with the common c.745G>A, p.Asp249Asn mutation, reinforcing our previous suggestion of a genotype-phenotype correlation. In our paper, we compared the phenotypes of H-ABC patients with the common *TUBB4A* mutation and H-ABC patients with other *TUBB4A* mutations, but refrained from correlations within the group of patients with other mutations.[2] The growing number of publications now allows further analysis of the genotype–phenotype relationship. The overview of all published cases by Carvalho *et al.* in Table 2[1] provided us with the opportunity to look at the MRI and clinical characteristics in more subgroups of patients with the same mutations.

Initially, in the absence of a DNA diagnostic test, the diagnosis H-ABC was entirely based on strict MRI criteria, of which hypomyelination and disappearance of the putamen were the most important.[3] Since Simons *et al.*[4] described the association between H-ABC and *TUBB4A* mutations, DNA testing became the gold standard for the diagnosis. The disease spectrum associated with *TUBB4A* mutations has been expanding since. In our paper, we included patients with a less typical MRI, showing late and only mild putaminal atrophy.[2] The new literature on patients with hypomyelination and a *TUBB4A* mutation makes clear that some patients do not have evident atrophy of the putamen.[5-10] Several of these reports concern young children; considering that in less typical presentations of H-ABC, atrophy of the putamen may become apparent only after a few years,[2] it is possible that the putaminal atrophy will develop later in these patients. In other reports, however, MRIs made at more advanced ages (8, 10, 16, 38, 42 and 45 years) still show no atrophy of putamen,[6-8] indicating that putaminal atrophy does not occur in all patients with hypomyelination and a *TUBB4A* mutation. Additionally, it is becoming clear that the degree of hypomyelination varies. In some H-ABC patients, MRI features indicate a severe to almost complete lack of myelin in the cerebral white matter,[2,3] while recent publications describe patients with MRI features suggesting milder hypomyelination.[5,7,8,11] At the mildest end of the disease spectrum is late-onset dystonia type 4, which lacks brain MRI abnormalities altogether.[12-14]

When looking at groups consisting of more than two patients with the same *TUBB4A* mutation, it becomes evident that, despite the wide range of phenotypes associated with a *TUBB4A* mutation, particular mutations are consistently associated with a specific phenotype (Table 1). Patients with a c.4C>G, p.Arg2Gly mutation have a ‘dystonia type 4’ phenotype. Patients with a c.730G>A, p.Gly244Ser and the common c.745G>A, p.Asp249Asn mutation have a ‘classic’ H-ABC phenotype, but more

severe for the first mutation than for the second. Most patients with c.785G>A, p.Arg262His and c.1228G>A, p.Glu410Lys mutations are initially diagnosed as 'unclassified hypomyelinating leukoencephalopathy' and are subjected to extensive evaluations to establish a diagnosis. Their MRI shows hypomyelination and cerebellar atrophy, but no or only equivocal putamen abnormalities. The three patients with a c.1228G>A, p.Glu410Lys mutation show a milder lack of myelin and a milder phenotype than the other patients with a hypomyelinating leukoencephalopathy caused by a *TUBB4A* mutation. So, the genotype definitely influences the phenotype.

A remaining question concerns the diagnostic work-up. In patients fulfilling the MRI criteria for H-ABC, *TUBB4A* analysis should be the first and only test. In patients with hypomyelination and no evident putaminal atrophy on MRI, the diagnosis becomes a challenge. In our overview paper, we suggest that in cases of hypomyelination without putaminal atrophy, prominent early extrapyramidal abnormalities should prompt *TUBB4A* testing. This opinion is shared by several.[1,7] Other authors stress the absence of extrapyramidal movement abnormalities in their patients and emphasize the difficulty of establishing the correct diagnosis in such patients.[9,10] In our experience, early extrapyramidal signs are present in most patients with a *TUBB4A* mutation facilitating the diagnosis. In cases of hypomyelination without extrapyramidal signs and without evident putaminal atrophy on MRI, *TUBB4A* is only one of the genes to be considered.

It remains puzzling that mutations in the same gene can lead to a spectrum of brain abnormalities and clinical phenotypes. Apparently, different *TUBB4A* mutations have diverse effects on tubulin function. The description of genotype-phenotype associations may contribute to a better understanding of the pathophysiological consequences of *TUBB4A* mutations.

Table 1 | MRI & clinical characteristics in patients with the same heterozygous *TUBB4A* mutation

Mutation	Number of patients (references)	MRI features	Clinical characteristics
c.4C>G, p.Arg2Gly	**20** (12-14)	Normal brain MRI	- Normal psychomotor development - Onset at 13-42 y - Segmental or generalized dystonia, 'hobby horse gait', spasmodic dysphonia - Variable severity
c.730G>A, p.Gly244Ser	**4** (1,2)	- Moderate to severe hypomyelination - Atrophy of putamen - Cerebellar atrophy	- Onset at 4-5 months - Delayed motor development or hypotonia - Maximum motor milestone ranged from no intentional movements to walking with support - Progressive, severely disabling motor dysfunction - Extrapyramidal signs reported in all patients
c.745G>A, p.Asp249Asn	**30** (2,4,6,7)	- Moderate hypomyelination - Atrophy of putamen - Cerebellar atrophy	- Normal or mildly delayed early motor development - Onset at 3 mo-4.5 y - All achieved walking with or without support - Progressive, disabling motor dysfunction - Extrapyramidal signs reported in all patients but one
c.785G>A, p.Arg262His	**3** (6,7,10)	- Moderate hypomyelination - No evident atrophy of putamen (age range 7 mo-8 y) - Initially normal cerebellum, progressive cerebellar atrophy in follow up of two patients	- Onset at 2-3 months - Delayed motor development - Maximum motor milestone ranged from no head control at 1 y to unsupported sitting for a few seconds - Severe motor disability - In two patients no extrapyramidal signs reported at 1-3 y
c.1228G>A, p.Glu410Lys	**3** (5,7,11)	- Mild hypomyelination - No evident abnormalities of putamen (age range 6-38y) - Progressive cerebellar atrophy	- Normal or mildly delayed early development - Onset at 1 y - All achieved unsupported walking, but ataxic gait - Onset of slow motor deterioration at 4-20 y - Loss of walking: not yet / 12 y / 25 y for the three patients reported - Mild dystonia in all three patients

mo, months; y, years

REFERENCES

1. Carvalho D, Santos S, Martins B, Marques FP. TUBB4A novel mutation reinforces the genotype-phenotype correlation of hypomyelination with atrophy of the basal ganglia and cerebellum. Brain 2015;138:e327.
2. Hamilton EM, Polder E, Vanderver A, et al. Hypomyelination with atrophy of the basal ganglia and cerebellum: further delineation of the phenotype and genotype-phenotype correlation. Brain 2014;137:1921-1930.
3. van der Knaap MS, Naidu S, Pouwels PJ, et al. New syndrome characterized by hypomyelination with atrophy of the basal ganglia and cerebellum. AJNR Am J Neuroradiol 2002;23:1466-1474.
4. Simons C, Wolf NI, McNeil N, et al. A de novo mutation in the beta-tubulin gene TUBB4A results in the leukoencephalopathy hypomyelination with atrophy of the basal ganglia and cerebellum. Am J Hum Genet 2013;92:767-773.
5. Blumkin L, Halevy A, Ben-Ami-Raichman D, et al. Expansion of the spectrum of TUBB4A-related disorders: a new phenotype associated with a novel mutation in the TUBB4A gene. Neurogenetics 2014;15:107-113.
6. Ferreira C, Poretti A, Cohen J, Hamosh A, Naidu S. Novel TUBB4A mutations and expansion of the neuroimaging phenotype of hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC). Am J Med Genet A 2014;164A:1802-1807.
7. Miyatake S, Osaka H, Shiina M, et al. Expanding the phenotypic spectrum of TUBB4A-associated hypomyelinating leukoencephalopathies. Neurology 2014;82:2230-2237.
8. Pizzino A, Pierson TM, Guo Y, et al. TUBB4A de novo mutations cause isolated hypomyelination. Neurology 2014;83:898-902.
9. Purnell SM, Bleyl SB, Bonkowsky JL. Clinical exome sequencing identifies a novel TUBB4A mutation in a child with static hypomyelinating leukodystrophy. Pediatr Neurol 2014;50:608-611.
10. Shimojima K, Okumura A, Ikeno M, et al. A de novo TUBB4A mutation in a patient with hypomyelination mimicking Pelizaeus-Merzbacher disease. Brain Dev 2015;37:281-285.
11. Sasaki M, Takanashi J, Tada H, Sakuma H, Furushima W, Sato N. Diffuse cerebral hypomyelination with cerebellar atrophy and hypoplasia of the corpus callosum. Brain Dev 2009;31:582-587.
12. Hersheson J, Mencacci NE, Davis M, et al. Mutations in the autoregulatory domain of beta-tubulin 4a cause hereditary dystonia. Ann Neurol 2013;73:546-553.
13. Lohmann K, Wilcox RA, Winkler S, et al. Whispering dysphonia (DYT4 dystonia) is caused by a mutation in the TUBB4 gene. Ann Neurol 2013;73:537-545.
14. Wilcox RA, Winkler S, Lohmann K, Klein C. Whispering dysphonia in an Australian family (DYT4): a clinical and genetic reappraisal. Mov Disord 2011;26:2404-2408.

Chapter 4.3

Reply: *TUBB4A* novel mutation reinforces the genotype-phenotype correlation of hypomyelination with atrophy of the basal ganglia and cerebellum

Marjo S. van der Knaap, Eline M.C. Hamilton and Laura van Berge

Brain. 2015;138:e371

Sir,

We thank Dr Erro and colleagues[1] for their interest in our overview paper on the clinical and genotypic spectrum in patients with hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC).[2] In our cohort, we found a more benign phenotype in 25 patients with the common c.745G>A mutation than in 16 patients with other mutations in *TUBB4A*, suggesting a relation between genotype and phenotype. We pointed out that the disease spectrum associated with *TUBB4A* mutations has become quite broad and put forward the suggestion that the full *TUBB4A*-related disease spectrum is a continuum with severe variants of H-ABC[2] and the adult dystonia type 4 (DYT4) as extremes.[3,4] In contrast to what their title suggests, the letter of Erro *et al.*[1] endorses this concept. The authors describe a patient fulfilling the MRI criteria of H-ABC, harboring a novel *TUBB4A* mutation, with a milder presentation than so far seen in other H-ABC patients. A milder disease course has previously been described in several *TUBB4A* mutated patients with hypomyelination in the absence of basal ganglia lesions.[5,6] So far, all reports of *TUBB4A* mutated patients support the existence of a clear genotype-phenotype correlation. However, it should be noted that, as we showed for the common c.745G>A mutation, each individual mutation is also associated with certain variation in disease severity. In single cases with a novel mutation, it is not possible to be certain of the associated clinical variation. In this respect, we should wait for more reports of the c.941C>T mutation before conclusions can be drawn on the associated clinical spectrum. We agree that functional studies are needed to better understand how mutations in β-tubulin disrupt affected cells. One clue was recently provided by Pizzino *et al.*,[7] describing patients with isolated hypomyelination harboring *TUBB4A* mutations that were predicted to disrupt the lateral contacts between tubulin protofilaments, while the common c.745G>A mutation seen in H-ABC patients is predicted to interfere with longitudinal polymerization.

REFERENCES

1. Erro R, Hersheson J, Houlden H, Bhatia KP. A Novel Tubb4a Mutation Suggests That Genotype-Phenotype Correlation of H-Abc Syndrome Needs to Be Revisited. Brain 2015;138:e370.
2. Hamilton EM, Polder E, Vanderver A, et al. Hypomyelination with Atrophy of the Basal Ganglia and Cerebellum: Further Delineation of the Phenotype and Genotype-Phenotype Correlation. Brain 2014;137:1921-1930.
3. Hersheson J, Mencacci NE, Davis M, et al. Mutations in the Autoregulatory Domain of Beta-Tubulin 4a Cause Hereditary Dystonia. Ann Neurol 2013;73:546-553.
4. Lohmann K, Wilcox RA, Winkler S, et al. Whispering Dysphonia (Dyt4 Dystonia) Is Caused by a Mutation in the Tubb4 Gene. Ann Neurol 2013;73:537-545.
5. Carvalho D, Santos S, Martins B, Marques FP. Tubb4a Novel Mutation Reinforces the Genotype-Phenotype Correlation of Hypomyelination with Atrophy of the Basal Ganglia and Cerebellum. Brain 2015;138:e327.
6. Hamilton EM, Wolf NI, van der Knaap MS. Reply: Tubb4a Novel Mutation Reinforces the Genotype-Phenotype Correlation of Hypomyelination with Atrophy of the Basal Ganglia and Cerebellum. Brain 2015;138:e328.
7. Pizzino A, Pierson TM, Guo Y, et al. Tubb4a De Novo Mutations Cause Isolated Hypomyelination. Neurology 2014;83:898-902.

Chapter 5

Hypomyelination with atrophy of the basal ganglia and cerebellum: further delineation of the phenotype and genotype-phenotype correlation

Eline M. Hamilton, Nicole I. Wolf and Marjo S. van der Knaap

Brain. 2015;138:e328

ABSTRACT

Objective: To identify the gene defect in patients with hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC) who are negative for *TUBB4A* mutations.

Methods: We performed homozygosity mapping and whole exome sequencing (WES) to detect the disease-causing variant. We used a Taqman assay for population screening. We developed a luciferase reporter construct to investigate the effect of the promoter mutation on expression.

Results: Sixteen patients from 14 families from different countries fulfilling the MRI criteria for H-ABC exhibited a similar, severe clinical phenotype, including lack of development and a severe epileptic encephalopathy. The majority of patients had a known Roma ethnic background. Single nucleotide polymorphism array analysis in 5 patients identified one large overlapping homozygous region on chromosome 13. WES in 2 patients revealed a homozygous deletion in the promoter region of *UFM1*. Sanger sequencing confirmed homozygosity for this variant in all 16 patients. All patients shared a common haplotype, indicative of a founder effect. Screening of 1,000 controls from different European Roma panels demonstrated an overall carrier rate of the mutation of 3 - 25%. Transfection assays showed that the deletion significantly reduced expression in specific CNS cell lines.

Conclusions: UFM1 encodes ubiquitin-fold modifier 1 (UFM1), a member of the ubiquitin-like family involved in posttranslational modification of proteins. Its exact biological role is unclear. This study associates a *UFM1* gene defect with a disease and sheds new light on possible UFM1 functional networks.

INTRODUCTION

Hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC) (MIM 612438) is a rare leukodystrophy that was identified by MRI pattern analysis.[1] The 2 most important MRI features are hypomyelination and a very small or absent putamen. The disease is associated with dominant *de novo* mutations in the *TUBB4A* gene (MIM 602662), encoding tubulin β-4A.[2,3] In the Amsterdam Database of Leukoencephalopathies a small number of patients fulfilling the MRI criteria of H-ABC did not harbor a pathogenic *TUBB4A* mutation, suggesting that mutations in at least one other gene are involved in the disease. In this study, we aimed at identifying the causal genetic defect in this group of unsolved H-ABC cases.

METHODS

Standard protocol approvals, registrations and patient consents

We received approval from the ethical standards committee for gene identification research on patients with unclassified leukoencephalopathies at the VU University Medical Center Amsterdam. Written informed consent was obtained from the guardians of the patients participating in this study.

Patients

Sixteen patients from 14 families fulfilled the following MRI criteria for H-ABC: (i) hypomyelination, defined as a mildly elevated T_2-signal intensity of most cerebral white matter in combination with mild T_1-hypointensity, T_1-isointensity or mild T_1-hyperintensity relative to the cortex;[4] and (ii) very small or absent putamen without signal abnormality indicating lesion or scarring in the region where the putamen should be. We obtained DNA from all patients, parents and unaffected siblings and obtained clinical data by a standardized form for physicians. We performed Kaplan-Meier analysis in SPSS version 22 (SPSS Inc., Chicago, IL) to estimate the median survival. For each patient, at least one MRI was available. Two investigators evaluated MRIs by visual assessment, as previously described.[3]

Analysis of the *TUBB4A gene*

Sanger sequencing of the *TUBB4A* gene was performed in 16 patients as previously described.[3] In addition, we performed multiplex ligation-dependent probe amplification (MLPA) analysis of the *TUBB4A* gene in 9 patients according to manufacturer's instructions (MRC-Holland, Amsterdam, the Netherlands; complete description of the MLPA probe mix is available upon request).

Single nucleotide polymorphism (SNP) array analysis

We executed SNP array analysis (CytoScan HD array; Affymetrix, Santa Clara, CA) according to the manufacturer's protocol in 5 patients to identify runs of homozygosity larger than 1 Mb and overlapping regions (Nexus version 7 [BioDiscovery,

Hawthorne, CA]). SNP array-based genotypes were created using Chromosome Analysis Suite 2.1.0.16 (Affymetrix).

Whole exome sequencing (WES)

We performed WES on genomic DNA from 2 patients and analyzed data as previously described.[5] Based on family data indicating consanguinity and shared Roma (Gypsy) ethnic background for several families, variant filtering was executed under the hypothesis of a homozygous recessive inheritance model. We focused on rare variants, exonic as well as intronic, located in the identified overlapping homozygous region, filtering for a minor allele frequency of less than 1% in public databases (dbSNP, 1000 Genomes Project, Exome Variant Server, and NHLBI Exome Sequencing Project) as well as an occurrence below 1% in the heterozygous state and absence in the homozygous state in our in-house WES control database.

Segregation of the *UFM1* variant and exclusion of other mutations in the region of interest

After identification of the Chr13(GRCh38): g.38349765_38349767del *UFM1* variant by WES, hereafter called c.-273_-271delTCA (NM_001286704.1), we performed segregation analysis in all patients, parents and healthy siblings by Sanger sequencing. In 3 patients, we investigated all exons and intron-exon boundaries of the protein encoding genes present in the overlapping homozygous region that was identified by SNP array analysis by Sanger sequencing. Primers were designed using Primer 3, V.0.4.0. (Supplementary Tables 1 and 2).[6]

Microsatellite marker haplotype analysis

To analyze a possible founder effect in all patients and see if the shared haplotype identified by SNP array analysis could be further narrowed down, we genotyped microsatellite and SNP markers spanning 2.4 Mb around *UFM1* (Supplementary Table 3) in 16 patients, 24 parents and six siblings. For the microsatellite marker analysis we analyzed PCR products with an Applied Biosystems (Mulgrave, Australia) Genetic Analyzer 3730 with GS-500 Liz as a size standard. For the SNP marker analysis we performed Sanger sequencing. We used control DNA from CEPH individual 1347-02 as a reference and analyzed the data with GeneMapper v3.7.

Carrier frequency rate analysis

We tested a panel of 670 Roma controls from a range of subisolates collected for population genetic and genetic epidemiology studies[7] for the c.-273_-271delTCA *UFM1* mutation using custom-designed TaqMan SNP Genotyping Assays (Applied Biosystems, primers in Supplementary Table 4). Because one individual originating from a community in Eastern Slovakia was homozygous for the variant, we subsequently screened an additional panel of 273 samples from Roma adults from Eastern Slovakia[8] and a panel of 57 samples from inhabitants of the community in question.

Logarithm of the odds (LOD) score

Upon identification of the candidate variant, we calculated a LOD score on the basis of the following assumptions: autosomal recessive pattern of inheritance, complete penetrance and equal distribution between male and female participants. We applied 2 calculations, one based on all 12 families in which DNA of both parents was available, and one excluding the families that were used for the original candidate region and candidate variant selection to avoid possible ascertainment bias. Unaffected siblings were also included.

Construction of *UFM1* promoter reporters and transfections

We cloned the wild-type and mutant *UFM1* promoter (c.-1889 to c.-1 of NM_001286704.1) into the pNL1.1 reporter (Promega, Madison, WI) using the infusion protocol (Clontech; Mountain View, CA; oligonucleotide primers in Supplementary Table 5). The pNL1.1 plasmid encodes nanoluciferase, a sensitive reporter protein for chemiluminescence-based assays (Promega). We transfected the *UFM1* promoter reporters into HeLa (cervix carcinoma), SY-5Y (neuroblastoma), H02-F2 (oligodendrocytoma), and U373 (astroglioma) cell lines. The pNL1.1 empty vector was included as negative control. To normalize for transfection differences, we cotransfected the pGL3 plasmid (Promega) that expresses firefly luciferase driven by the SV40 promoter (Promega). All cells were grown in DMEM/F-12 with 10% fetal bovine serum at 37˚C under 5% CO_2. We performed the transfections in white, half area 96-well-plates with clear bottom. One day before transfection we seeded 3,000 cells per well. We transfected 5 ng pNL1.1-based vector and 75 ng pGL3 vector using Fugene 6 (1:3 ratio of Fugene 6:DNA; Promega). We measured firefly luciferase and nanoluciferase activity with a microplate reader (Victor2; Perkin-Elmer Life Sciences, Waltham, MA) 40 hours post-transfection according to the manufacturer's protocol. Nanoluciferase activity was normalized to firefly luciferase activity. Results were expressed as mean of 2 independent experiments performed in duplicate. Factor correction was applied to eliminate between-session variation.[9] Differences were analyzed by Student t-test.

RESULTS

Sixteen patients from 14 families were included in the study; 3 patients had similarly affected deceased siblings for whom we did not have DNA. Parental consanguinity was reported in 7 families and 2 families were related. All but 2 families were known to originate from the Roma population, which led us to suspect a common founder effect. In our search strategies, we therefore chose to focus on a rare variant with homozygous autosomal recessive inheritance.

The clinical phenotype consists of a severe encephalopathy with early death

Detailed clinical characteristics of all patients are described in Supplementary Table 6. In Table 1 we compared the present cohort with our published cohort of 41 patients with H-ABC with *TUBB4A* mutations, separately considering patients with the common *TUBB4A* mutation and patients with a different mutation, who exhibited a more severe phenotype.[3]

All current patients demonstrated severe developmental delay, typically without intentional movements and language development. Almost all patients exhibited spasticity and extrapyramidal movement abnormalities, mostly dystonia. Seizures were frequent and present in all patients 18 months and older. The epilepsy was often severe and drug-resistant, including West syndrome. Stunted growth was frequent and all patients had microcephaly. Six patients underwent tracheostomy between 6 and 17 months, 4 of whom were on intermittent or permanent ventilation. Median survival was 2 years. Nine patients died at ages between 7 months and 7 years, most often due to respiratory insufficiency. Compared to the cohort of *TUBB4A*-mutated patients, the present patients were all at the most severe end of the H-ABC spectrum.

MRI shows severe hypomyelination, putamen atrophy and distinctive caudate nucleus abnormalities

Detailed MRI findings are outlined in Supplementary Table 7, and a summary is presented in Table 2, comparing the MRI characteristics of this cohort of patients to the early MRI characteristics in *TUBB4A*-mutated patients with H-ABC.[3]

The first MRIs invariably revealed severe lack of myelin (Figure 1) suggesting hypomyelination, although a single MRI does not allow an MRI-based diagnosis of hypomyelination in infants. None of the patients showed a normal putamen and in all patients the caudate nucleus was small. In all the lateral part of the head of the caudate nucleus showed an area of abnormally high signal on T_2-weighted images, resulting in a signal intensity similar to the hypomyelinated white matter. By contrast, in patients with *TUBB4A* mutations, the caudate nucleus signal was normal (74%) or hyperintense throughout (26%).[3] Atrophy of the caudate nucleus led to widening of the anterior horns of the lateral ventricles. In addition, in 50% of patients there was a moderate dilation of lateral and third ventricles. On the first MRI, 56% of patients had mild cerebellar atrophy, restricted to the vermis.

The follow-up MRIs showed slight progress of myelination after 3 - 13 months in 3 out of 5 patients with a follow-up scan, but myelination remained severely deficient, confirming hypomyelination. In 2 patients progression of the cerebral atrophy was observed. In 4 patients who initially lacked cerebellar atrophy, follow-up MRIs revealed cerebellar atrophy after 3 - 15 months.

Table 1 | Clinical data on *TUBB4A* and *UFM1* mutated H-ABC patients

General characteristics	*TUBB4A* common mutation (c.745G>A)[a]	Other *TUBB4A* mutations[a]	*UFM1* founder mutation
Number of patients	25	16	16
Sex (male / female)	12 / 13	7 / 8	8 / 8
Median age (range)[b]	14 y (2 - 29 y)	10 y (3 - 25 y)	18 mo (3 mo - 7 y)
Patients with affected sibling(s)	1	0	7 (5 families)
Median age at first signs (range)	1.5 y (3 mo - 3.0 y)	3 mo (birth - 6 mo)	2 mo (birth - 3 mo)
Neurological development			
Maximum motor milestone			
walking without support	76%	0 %	0%
sitting to walking with support	24%	50%	0%
touching / grasping / holding	0%	25%	6%
no intentional movements	0%	25%	94%
Maximum language			
single words up to normal	100%	6%	0%
none	0%	94%	100%
Maximum level of comprehension			
normal or decreased	100%	44%	0%
social awareness only	0%	56%	100%
Neurological symptomatology			
Spasticity	96%	94%	81%
Ataxia	88%	31% (remainder n.e.)	n.e.
Extrapyramidal movements	96%	100%	75%
Seizures	12%	53%	75% (100% ≥ 18
Current speech			
normal or dysarthric speech	48%	0%	0%
no speech	52%	100%	100%
Current level of comprehension			
decreased intelligence	100%	19%	0%
social awareness only	0%	81%	100%
Other characteristics			
Tube feeding (range age at start)	46% (11 - 26 y)	75% (1 - 9 y)	81% (6 mo - 4 y)
Tracheostomy (range age at start)	0%	6% (15 y)	38% (10 - 17 mo)
Height < 2SD	40%	87%	73%
Weight < 2SD	48%	88%	62%
Microcephaly	9%	69%	100%
Deceased patients (age range)	4% (12 y)	13% (20 - 25 y)	56% (7 mo - 7 y)

[a]Data from Hamilton et al.[3]; [b]Age at time of obtaining clinical characteristics
mo, months; y, years; n.e., not evaluable because of lack of intentional movements

Genetic analysis identifies *UFM1* as the only candidate gene

Sanger sequencing was negative for *TUBB4A* mutations in 16 patients, and MLPA analysis revealed neither *TUBB4A* deletions nor duplications in the 9 patients who were investigated.

An SNP array identified a 0.8-Mb overlapping homozygous region on chromosome 13q13 (genomic coordinates 37,940,556-38,786,096 GRCh38) shared by all 5 patients in whom the SNP array was performed (Figure 2A). This region encompasses long intergenic noncoding RNAs, the *UFM1* gene and the first six exons of the *FREM2* gene (NM_207361.5). *UFM1* encodes ubiquitin fold modifier 1.

FREM2 encodes FRAS1-related extracellular matrix protein 2 and is associated with Fraser syndrome, a developmental and malformative disorder involving multiple organs.
WES analysis of 2 patients revealed a single homozygous 3 bp deletion in the promoter of *UFM1* [MIM 610553] as candidate causal mutation: c.-273_-271delTCA (NM_001286704.1, dbSNP rs747359907). No other rare variants were detected in the candidate region. The variant has been reported to dbSNP in heterozygous state by 2 independent submitters with unknown allele frequency. *UFM1* mutations have not been associated with a disease phenotype. The deleted nucleotides are moderately to highly conserved based on PhyloP conservation scores ranging from 0.32 to 2.09 and Phast conservation scores from 0.89 to 0.98.

Sanger sequencing confirmed that segregation of the *UFM1* deletion was in perfect agreement with inheritance of the disease in all families.

Sanger sequencing of all exons and intron-exon boundaries in the shared haplotype in 3 patients revealed no other possible pathogenic variants.

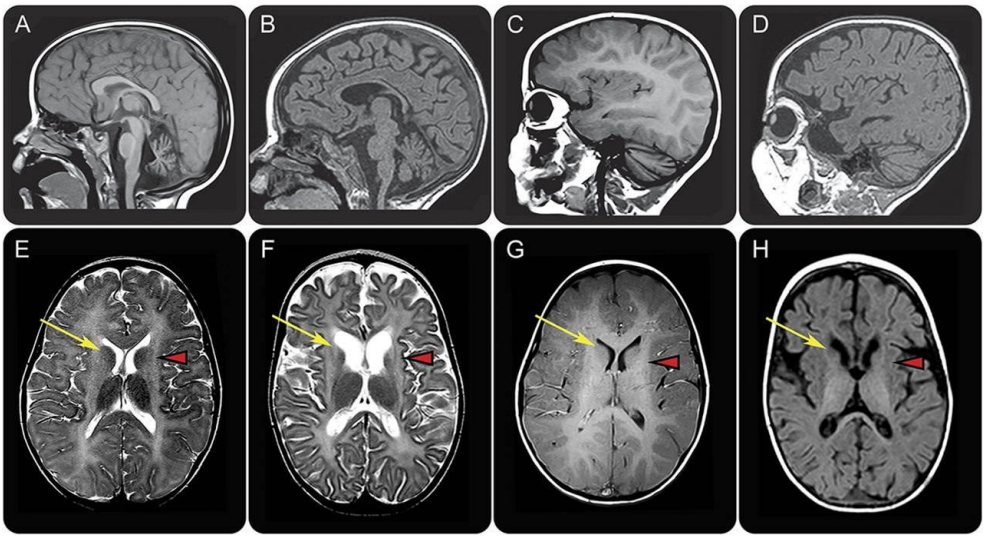


Figure 1 | MRI findings in *TUBB4A* (left) and *UFM1* (right) mutated patient with hypomyelination with atrophy of the basal ganglia and cerebellum. Sagittal T1-weighted (**A**-**D**) and axial T2-weighted (**E**, **F**) and T1-weighted (**G**, **H**) images in a patient with the common dominant c.745G>A *TUBB4A* mutation at age 3 years (**A**, **C**, **E**, **G**) and a patient with the homozygous recessive *UFM1* mutation at age 13 months (**B**, **D**, **F**, **H**). The patient with the common *TUBB4A* mutation shows a mildly hyperintense white matter signal on T1-weighted (**C**, **G**) and T2-weighted (**E**) images, indicating a moderate lack of myelin. In the *UFM1*-mutated patient, the white matter T1 signal is hypointense, indicating a profound lack of myelin (**D**, **H**). This patient also shows mild cerebral atrophy (**F**, **H**). Both patients have a mild cerebellar atrophy (**A**-**D**), most notable at the vermis (**A**, **B**). In both patients, there is no putamen visible and there is no visible lesion in this region (arrowheads in **E**-**H**). In the *TUBB4A*-mutated patient, the caudate nucleus has a normal signal (arrow in **E**). In the *UFM1*-mutated patient, the caudate nucleus is atrophic and the lateral part of the head has an abnormal hyperintense T2 signal (arrow in **F**) and hypointense T1 signal (arrow in **H**).

Table 2 | Summary early MRI findings in *TUBB4A* and *UFM1* mutated patients

		TUBB4A*-mutated patients MRI <2 years after onset [a]	***UFM1*-mutated patients***
Number of patients		23	16
Age of patients[b]		6 mo - 5 y	3 mo - 2 y
Myelination[c]			
Moderate lack of myelin[d]		52%	0%
Severe lack of myelin[e]		35%	87%
Almost complete lack of myelin[f]		13%	13%
Basal ganglia			
Putamen	normal	30.5%	0%
	atrophic	39%	13%
	not visible	30.5%	87%
Caudate nucleus	normal	70%	0%
	atrophic	26%	100%
	not visible	4%	0%
Abnormal signal of lateral aspect		0%	100%
Atrophy			
Cerebral atrophy	absent	78%	37%
	present	22%	63%
Atrophy corpus callosum	absent	83%	69%
	present	17%	31%
Cerebellar atrophy	absent	9%	19%
	present	91%	81%

mo, months; y, years

[a] Data from Hamilton et al.[3]

[b] Age at MRI (for the *UFM1* mutated patients in the case of multiple MRIs the latest MRI is ranked)

[c] In patients < 12 months myelination is scored relative to calendar age

[d] Defined as hyperintense signal of the cerebral hemispheric white matter on T_1-weighted images and hyperintense signal on T_2-weighted images relative to cortex

[e] Defined as isointense signal of the cerebral hemispheric white matter on T_1-weighted images and hyperintense signal on T_2-weighted images relative to cortex

[f] Defined as hypointense signal of the cerebral hemispheric white matter on T_1-weighted images and hyperintense signal on T_2-weighted images relative to cortex

LOD score calculation confirms linkage and haplotype analysis reveals a founder effect

LOD score calculations for the *UFM1* variant showed a maximum LOD score of 9.18 when all 12 families were included in the calculation, and a maximum LOD score of 5.32 when the families used for identification of the *UFM1* variant were excluded.

Haplotype analysis showed that all patients were homozygous for an identical haplotype containing the *UFM1* mutation, indicative of a founder effect (Supplementary Table 3). The smallest putative shared region was flanked by microsatellite markers D13S219 and D13S1288 (Figure 2B).

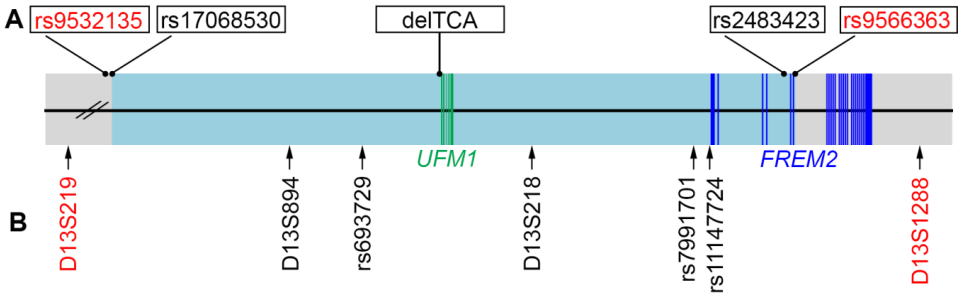


Figure 2 | Founder mutation haplotype analysis. Schematic representation of the candidate region on chromosome 13q13 containing *UFM1* and part of *FREM2*. The area of overlapping haplotype is highlighted in light blue: section **A** depicts the results of the single nucleotide polymorphism (SNP) array in 5 patients: 238 overlapping homozygous SNPs starting with rs17068530 and ending with rs248423 (shown in black), flanked by rs9532135 and rs9566363 (shown in red). Section **B** depicts the microsatellite and SNP markers analyzed in the candidate region, which confirmed the presence of a common haplotype in all patients (markers depicted in black), flanked by microsatellite markers D13S219 and D13S1288 (shown in red).

Population screening reveals high carrier rates in Roma subisolates

An important step for validation was to prove absence of homozygosity for the *UFM1* deletion among healthy Roma individuals. Screening of 670 Roma controls revealed 30 carriers with an overall carrier rate of 4.5% among different Roma communities across Europe and one individual who was homozygous for the deletion. Retrospective review of this case revealed that this sample was derived from a boy suffering with severe encephalopathy with spasticity, who had died at age 2.5 years and had erroneously been included among the controls. He had a similarly affected sibling, who had also died. No imaging had been performed, but the severe phenotype was similar to that of the patients in this study. The child lived in an endogamous community in Eastern Slovakia with a high rate of consanguinity. An additional panel of Eastern Slovak Roma samples revealed a carrier frequency of 3.3% (9 out of 273). Subsequent investigation of the carrier rate in the specific community where the homozygous individual came from revealed a carrier rate of approximately 25% (14 out of 57 individuals).

The promoter mutation reduces reporter gene expression in specific CNS cell lines

To study the effect of the deletion on promoter activity in different cell types, the candidate promoter sequence with or without the deletion was cloned into luciferase vectors and transfected into different cell lines to test its activity. The deletion significantly reduced promoter activity in SY-5Y and U373 but not in HeLa and HOG-F2 cell lines (Figure 3).

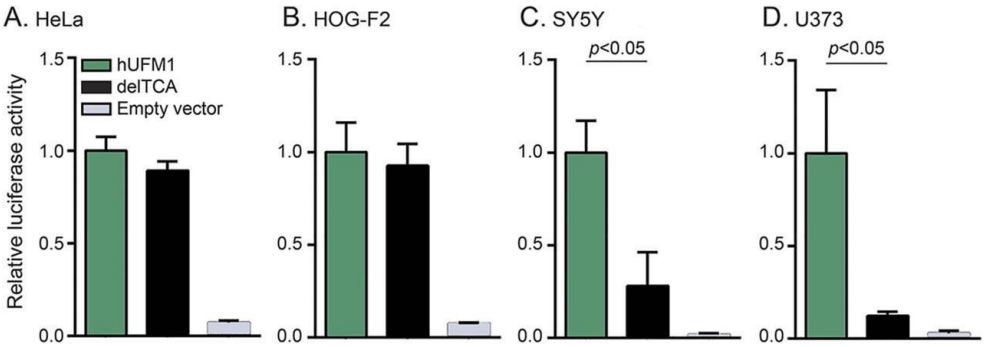


Figure 3 | c.-271_-271delTCA reduces UFM1 promoter activity in SY-5Y neuroblastoma and U373 astroglioma cell lines. (**A**-**D**) c.-273_-271delTCA in human *UFM1* (hUFM1) promoter significantly reduces reporter gene expression in SY5Y (p 5 0.001) and U373 (p 5 0.014) but not in HeLA (cervix carcinoma) or HOG-F2 (oligodendrocytoma) cell lines. Promoter activity was measured with dual luciferase reporter assays. The nanoluciferase/firefly luciferase ratio measured in cells transfected with wildtype hUFM1 was arbitrarily set at 1 on the y-axis. delTCA indicates cells transfected with human *UFM1* promoter harboring c.-273_-271delTCA. Data represent mean 6 SD values obtained from 2 independent experiments testing the luciferase activity in duplo and were analyzed by 2-tailed t test.

DISCUSSION

This study, focused on identifying the mutated gene in patients with H-ABC without *TUBB4A* mutations, revealed a homozygous 3 bp deletion in the *UFM1* promoter area, which perfectly segregates with the disease. Most patients were known to have a Roma background, and haplotype analysis indicated that the shared *UFM1* deletion originates from a common ancestor. Within the candidate region the *UFM1* promoter deletion was the only candidate. The Taqman screening assay confirmed a high carrier frequency and absence of homozygosity for the mutation among healthy Roma controls. Worldwide, our centers in Amsterdam and Washington know by far the highest number of patients with H-ABC and until now we have not identified other *UFM1* mutations in unrelated families. The genetically isolated Roma population harbors several unique autosomal recessive disorders caused by “private” founder mutations in genes, in which no other mutations have been found until now.[10, 11] In a luciferase assay we showed that the *UFM1* promoter deletion results in a significantly reduced transcription activity only in selected neural, but not in other cell lines. This finding supports the pathogenicity of the mutation and suggests a cell-specific effect.

UFM1 encodes ubiquitin-fold modifier 1 (UFM1), a ubiquitin-like (UBL) protein, which is ubiquitously expressed, including in brain.[12] UFM1 is hypothesized to post-translationally modify (“ufmylate”) proteins in a manner analogous to ubiquitination.[12,13] Ubiquitin and UBL pathways are involved in control of numerous functions, including signal transduction, transcriptional regulation and stress response.[14] Several studies indicate an association between the UFM1 pathway and

both neurodevelopment and neurodegeneration.[15, 16] Its exact biological role and working mechanism are poorly understood. Most evidence points to a role of UFM1 in endoplasmic reticulum homeostasis and protection against apoptosis.[17-19]

Genetic defects in the ubiquitin-proteasome system have been associated with several neurologic disorders, particularly cerebellar ataxias.[20, 21] Recently, various recessive mutations in *UBA5*, encoding UFM1 activating enzyme 5 (UBA5), were associated with a progressive, childhood-onset cerebellar ataxia.[19, 22, 23] The most severe variants presented with infantile onset encephalopathy with hypotonia, spasticity, movement abnormalities, refractory epilepsy, microcephaly, failure to thrive, and cerebellar atrophy,[19, 22] similar to the encephalopathy in our patients. Strikingly, biochemical and experimental findings indicated that the described *UBA5* mutations result in UFM1 system impairment.[19, 22, 23] Drosophila models with knockdown of *UBA5* and *UFM1* homologues exhibited a neurologic phenotype with reduced motor activity and shortened lifespan, with *UFM1* knockdown resulting in the most severe phenotype.[23] CNS-specific knockout of *Ufm1* in mice caused neonatal death with microcephaly and apoptosis of neurons in specific brain regions.[22]

Altogether, existing data suggest that the UFM1 system is crucial for neuronal development, function, and protection against apoptosis.[14-19, 22, 23] This concept is substantiated by the current study, showing association of *UFM1* mutations with an infantile onset, severe epileptic encephalopathy and failure of development. Intriguingly, while the *Ufm1* knockout mouse showed apoptosis in restricted brain areas, MRI also suggests apoptosis of selected neuronal cell populations in both *TUBB4A* and *UFM1* related H-ABC, substantiated by histopathology in the first.[1, 24] Muona *et al.* suggest that ufmylation may be spatiotemporally regulated and cell type-specific.[22] Specificity is reinforced by the results of our transfection studies showing that only selective neural cell lines are vulnerable for the promoter mutation.

The relationship between H-ABC caused by monoallelic *TUBB4A* mutations and H-ABC caused by biallelic *UFM1* mutations is unclear. *TUBB4A* mutations are associated with a disease spectrum ranging from early infantile, severe encephalopathy to adult onset dystonia type 4, with a strong genotype-phenotype correlation.[3] All patients with the c.-273_-271delTCA *UFM1* promoter mutation have an early infantile, severe encephalopathy with early death. Although the *UFM1*-mutated patients fulfill the MRI criteria of H-ABC, they consistently have an additional feature: signal abnormality of the lateral part of the head of the caudate nucleus suggestive of local apoptosis. This feature has not been observed in *TUBB4A*-related H-ABC and may be pathognomonic for *UFM1*-related H-ABC. Whether *UFM1*-related disease and *TUBB4A*-related disease are basically variants of one disease or unrelated phenocopies is still to be clarified. The striking similarity between the two suggests that the encoded proteins may be at least partially involved in the same processes. A possible link could be that ufmylation is involved in the regulation of microtubule dynamics, which has been shown for other UBLs.[25] Recently, recessive

ZNF335 mutations were described in a single family, in which patients displayed features compatible with early onset severe H-ABC.[26] Further confirmation and characterization are necessary, but if the finding is confirmed in other families, studies focused on possible crossroads of tubulin β-4A, ubiquitin-fold modifier 1 and zinc finger protein 335 are warranted. Considering that a few patients with H-ABC are presently still genetically unclassified despite targeted Sanger sequencing and whole exome and genome sequencing, some more genes and proteins may be added.
The current study sheds new light on possible UFM1 functional networks and directly benefits patients and families. The identification of the disease-causing variant enables better clinical and genetic counseling, the possibility of prenatal testing and the option of carrier testing in populations with a high carrier frequency.

ACKNOWLEDGEMENTS

The authors express their gratitude to all patients and families for their cooperation and contribution. Of the VU University Medical Center, Amsterdam, The Netherlands we thank Carola G. van Berkel, Emiel Polder and Nienke L. Postma, technicians, Department of Child Neurology, Marjan M. Weiss, molecular geneticist, Department of Clinical Genetics, and Robbert Zalm, technician, Department of Functional Genomics, for their excellent laboratory assistance. We thank Matthijs Verhage, head of the Department of Functional Genomics for the use of his laboratory, and Zoltán Bochdanovits, Department of Clinical Genetics, for the statistics advice. We thank Marzia Pollazzon, Clinical Genetics Unit Arcispedale S.Maria Nuova – Reggio Emilia, Italy and Francisco Menor, Radiología Infantil. Hospital Universitari i Politecnic La Fe, Valencia, Spain for their contribution to the acquisition of clinical data. We thank Chiara Aiello and Lorena Travaglini of the Unit of Neuromuscular and Neurodegenerative Disorders, Laboratory of Molecular Medicine, Bambino Gesu' Children's Hospital, Rome, Italy for their contribution to the genetic testing.
This study received financial support from the ZonMw TOP grant 91211005, the Optimix Foundation for Scientific Research and the Italian Ministry of Health.

The following Recessive H-ABC Research Group collaborators contributed to the study: Adele D'Amico, Bambino Gesù Children's Hospital; Ana Boban Raguž, Clinical Hospital Mostar; Paola la Boria, Spedali Civili; Graziella Cefalo, San Paolo Hospital, University of Milan; Argirios Dinopoulos, "Attikon" Hospital, University of Athens; Sira Domènech, Hospital Universitari Germans Trias i Pujol; Maria A. Donati, Children's Hospital A. Meyer, University of Firenze; Daniele Frattini, Arcispedale S. Maria Nuova; Serena Gasperini, Children's Hospital A. Meyer, University of Firenze; Lucio Giordano, Spedali Civili; Elena Procopio, Children's Hospital A. Meyer, University of Firenze; Anita Rauch, University of Zurich; Agusti Rodriguez-Palmero, Hospital Universitari Germans Trias i Pujol; Komudi Siriwardena, The Hospital for Sick Children; Miguel Tomás-Vila, Neuropediatría Hospital Universitari i Politecnic La Fe.

SUPPLEMENTARY DATA

Supplementary Table 1 | Primers Sanger sequencing *UFM1* gene

exon 1-forward	5'-TAAAAGCGCCATCTAGCATG-3'
exon 1-reverse	5'-CGAGCCAGGAAAGTAGATGAA-3'
exon 2-forward	5'-TGTTCTGGATTCATTCCGGCA-3'
exon 2- reverse	5'-CATTGACTCCTGGTGTTTTCA-3'
exon 3-forward	5'-CCAGATTGGCTACAGTAGTGGAA-3'
exon 3- reverse	5'-CATGGAAATGATGGGTCAAA-3'
exon 4-forward	5'-GGTATTTGTACCCCATTCTTGC-3'
exon 4- reverse	5'-TTGGGGCTTTTTGTATTTAATTTT-3'
exon 5-forward	5'-TGGCAAATCTCTTTCCTTGC-3'
exon 5-reverse	5'-ATAACCCCACCCACTCCTCT-3'
exon 6-forward	5'-AAAGCTTGGAATTGGGGTCT-3'
exon 6-reverse	5'-TGCCTGATTTCAATTTTACAACAA-3'

Supplementary Table 2 | Primers Sanger sequencing exon 1 - 6 *FREM2* gene

exon1-1-forward	5'-GGCGGTGTCTCTTGTTGTCT-3'
exon1-1-reverse	5'-CCTCCACCTCCAGTACCAGT-3'
exon1-2-forward	5'-CTGCAGCTGCGCTATGAC-3'
exon1-2-reverse	5'-CCTCCAATTCCAGTTCAAAGA-3'
exon1-3-forward	5'-CCTTCACTCAGAGGGATCTGC-3'
exon1-3-reverse	5'-GTTTCACCCTCTGCCAGTGT-3'
exon1-4-forward	5'-CCCATCACCTTAGTGCCT-3'
exon1-4-reverse	5'-GGATGCAAGTAAAGGGTAAAGG-3'
exon1-5-forward	5'-GGCTACTCGAGTGGCCCA-3'
exon1-5-reverse	5'-TCATCAGTTTCCTCATTGGTC-3'
exon1-6-forward	5'-TGTCTTAGAAAATGGGGCTACTG-3'
exon1-6-reverse	5'-TCATTGGTGGGAATGATTACA-3'
exon1-7-forward	5'-TTCTGATGGCATTAACTTTTCA-3'
exon1-7-reverse	5'-CGTCTCTGTAATAAGCCATGTCC-3'
exon1-8-forward	5'-ATGGCAACAGATTTAGATTC-3'
exon1-8-reverse	5'-CATCATCAGCTGTGTGGATGT-3'
exon1-9-forward	5'-TTCACTCAGCTGCAACTGGC-3'
exon1-9-reverse	5'-TGAAGCCACATTCTCAGGAA-3'
exon1-10-forward	5'-GACCCCAAGTGATGAAGATC-3'
exon1-10-reverse	5'-TCTCAGGAAACAAGGATAACCAA-3'
exon2-forward	5'-TGTTTCTGTTAGGAGGAAAAAGG-3'
exon2-reverse	5'-TTGTCTACAGCAAATACCAGGTT-3'
exon3-forward	5'-TGTCAGCGATTGAATGTATGTG-3'
exon3-reverse	5'-TTCCTGCCAATTTTTATACCAGA-3'
exon4-forward	5'-TTTCAGGATAAAATGACATGCAA-3'
exon4-reverse	5'-TTCCATTTTCCTGTCAACACA-3'
exon5-forward	5'-TTCCTCCTTTTCCTCCCACT-3'
exon5-reverse	5'-CGTCAAAGGTGAAATCCTGAA-3'
exon6-forward	5'-TGACCATTAAAATGCTGTGTATGA-3'
exon6-reverse	5'-TCATTGTTTTAGATTCCCATTTTT-3'

Supplementary Table 3 | Markers around the founder *UFM1* c.-273_-271delTCA mutation

Microsatellite marker / SNP	Physical position[a] (chr.13, GRCh38)	Result
D13S219	36,584,422	Variable length
D13S894	38,164,372	All patients homozygous and identical
rs693729	38,253,841	All patients homozygous and identical
D13S218	38,458,195	All patients homozygous and identical
rs7991701	38,664,953	All patients homozygous and identical
rs11147724	38,686,522	All patients homozygous and identical
D13S1288	38,949,158	Variable length

Overview of markers and results of genotyping. Physical genomic location *UFM1* gene: chr13:38,349,770 - 38,363,006.
[a] For microsatellite markers the physical position of the 5' end of the PCR product is shown

Supplementary Table 4 | Primers Taqman assay

forward	5'-GCCATCTAGCATGAGCTCTTAGG-3'
reverse	5'-CCTCCGAAGTTGTTGCTATCCAA-3'

Supplementary Table 5 | Infusion primers *UFM1* reporter construct

forward	5'-GGTAAAGCCACCATGAATGTATTTTCTGAAGCAGCA-3'
reverse	5'-AGTGTGAAGACCATGGTGGTGCCGGAATGAATC-3'

Supplementary Table 6 | Clinical characteristics of 16 patients with autosomal recessive H-ABC

Patient number (family number)	**1** (1)	**2** (2)	**3** (2)	**4** (3)	**5** (4)	**6** (5)	**7** (6)	**8** (7)
Patient ID	HA128	HA79	HA189	HA119	HA152	HA186	HA195	HA116
Roma ethnic background	yes	yes	yes	yes	yes	no	yes	yes
Year of birth, age[a]	2009, 7 y	2004, 5 y	2013, 16 mo	2010, 2 y	2011, 2 y	2012, 2 y	2013, 2 y	2009, 18 mo
Sex	m	f	m	m	m	f	f	m
Consanguinity parents	unknown[b]	+	+	+	+	+	-	+
Healthy / affected sibs	unknown[b]	0 / 1	0 /1	2 / 0	0 / 1	0 / 0	0 / 0	0 / 0
Age at first signs	3 mo	birth	3 mo	2 mo	1 mo	2 mo	3 mo	3 mo
First signs	hypotonia, motor deterioration	hypotonia, poor feeding	hypertonia, irritability	motor delay	motor delay	hypotonia, motor delay	dysphagia	motor delay
Maximum motor milestone	none	none	none	none	none	holding objects	none	none
Maximum spoken language	none	none	none	none	none	none	none	none
Maximum level of comprehension	social awareness only	social awareness only	social awareness only	social awareness only	social awareness only	social awareness only	social awareness only	social awareness only
Truncal hypotonia	+	+	+	+	+	+	+	+
Spasticity	-	+	+	+	+	+	+	+
Ataxia	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
Choreoathethosis	-	+		-	-	-	-	+
Dystonia / rigidity	+	+	+	+	-	+	+	+
Seizures, severity	+, refractory; EEG: hypsarrhythmia	+	+	+, severe epilepsy	+, refractory epilepsy	+, controlled; EEG: intermittent hypsarrhythmia	+, severe epilepsy	+, complex febrile seizures
Special findings				perceptive hearing loss		perceptive hearing loss	decreased vision	perceptive hearing loss
Tracheostomy from age, ventilation	10 mo, -	- , -	- , -	- , -	6 mo, -	-, -	13 mo, +	- , -
Tube feeding from age	10 mo	4 y	11 mo	-	6 mo	9 mo	9 mo	-
Height, weight, head circumference	nl, nl, <2 SD	<2 SD, <2 SD, <2 SD	<2 SD, <2SD, <2 SD	<2 SD, nl, <2 SD	<2 SD, <2 SD, <2 SD	nl, >2 SD, <2 SD	<2 SD, <2SD, -	<2 SD, <2 SD, <2 SD
Age and cause of death	7y, cardio-respiratory complications	5 y, respiratory insufficiency	-	2 y, cardio-respiratory arrest	-	2 y, respiratory insufficiency	2 y, cause unknown	2 y, cardio-pulmonary arrest

Patient number (family number)	**9** (8)	**10** (9)	**11** (10)	**12** (10)	**13** (11)	**14** (12)[c]	**15** (13)	**16** (14)
Patient ID	HA133	HA201	HA205	HA204	HA169	HA211	HA214	HA194
Roma ethnic background	yes	yes	yes	yes	yes	yes	no	yes
Year of birth, age[a]	2011, 18 mo	2014, 18 mo	2014, 16 mo	2015, 3 mo	2012, 12 mo	2016, 11 mo	2016, 9 mo	2014, 7 mo
Sex	m	f	f	f	m	f	m	f
Consanguinity parents	-	-	+	+	-	-	-	+
Healthy / affected sibs	0 / 0	1 / 1	0 / 1	0 / 1	7 / 1	4 / 0	0 / 0	2 / 0
Age at first signs	birth	1 week	2 mo	2 mo	birth	3 mo	3 mo	2 mo
First signs	hypotonia, poor feeding	stridor, dyspnea	Axial hypotonia	axial hypotonia, breathing problems	difficulty breathing	psychomotor regression	deafness, hypertonia	hypotonia, no eye contact
Maximum motor milestone	none	none	none	none	none	head control	none	none
Maximum spoken language	none	none	none	none	none	none	none	none
Maximum level of comprehension	social awareness only	social awareness only	social awareness only	social awareness only	social awareness only	social awareness only	n.e.	social awareness only
Truncal hypotonia	+	+	+	+	+	+	+	+
Spasticity	+	+	+	+	+	-	+	-
Ataxia	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
Choreoathethosis	-	-		-	-	-	-	
Dystonia / rigidity	-	+	+	+	-	+	+	-
Seizures, severity	+, severe epilepsy	+, severe epilepsy	-	-	-	+, refractory epilepsy	+, severe epilepsy	-
Special findings	perceptive hearing loss	tracheomalacia			blind	blind, hearing loss	perceptive hearing loss	blind and deaf, laringomalacia
Tracheostomy from age, ventilation	12 mo, +	17 mo, +	- , -	- , -	- , -	10 mo, +	-, +	-, -
Tube feeding from age	6 mo	11 mo	11 mo	-	6 mo	8 mo	8 mo	6 mo
Height, weight, head circumference	nl, nl, <2 SD	<2 SD, <2SD, <2 SD	< 2 SD, nl, < 2SD	nl, nl, -	< 2 SD, nl, < 2SD	<2 SD, <2SD, <2 SD	<2 SD, <2SD, <2 SD	-, <2SD, <2SD
Age and cause of death	-	-	-	-	1 y, respiratory insufficiency	-	9 mo, respiratory insufficiency	7 mo, respiratory insufficiency

The table is organized by descending age of patients; siblings are paired.
[a] Age at moment of obtaining clinical characteristics; [b] adopted; [c] second cousin of patients 2 and 3.
f, female; m, male; nl, normal; mo, months, y, years; -, not present; + present; n.e., not evaluable

Supplementary Table 7 | MRI findings in 16 patients with autosomal recessive H-ABC

Patient number	1		2		3		4		5		6		7		8	
Patient ID	HA128		HA79		HA189		HA119		HA152		HA186		HA195		HA116	
Age at first MRI	6 mo		11 mo		5 mo		4 mo		17 mo		5 mo		13 mo		7 mo	
Myelination	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_1	T_2	T_2	T_2	T_1	T_2	T_1	T_2
Brainstem	↑	↓	↑	↓	=	↓	=	↓	=	↓	↑	↓	=	↓	↑	↓
Posterior limb internal capsule	↑	↓	↑	↑	↑	↓	=	↑	=	=	↑	=	↑	↓	↑	↓
Cerebellar white matter	=	=	=	=	=	=	=	=	=	↓	=	=	=	=	=	=
Anterior limb internal capsule	=	↑	↑	↑	=	↑	↓	↑	=	↑	=	↑	=	↓	=	↑
Central part centrum semiovale	↓	↑	=	↑	↓	↑	↓	↑	=	↑	↓	↑	↓	↑	=	↑
Corpus callosum splenium	↑	↓	↑	↑	=	=	↓	=	=	=	↑	↓	↑	↓	↑	=
genu	=	=	↑	↑	=	=	↓	↑	=	=	=	=	↑	↓	↑	=
Parieto-occipital white matter deep	↓	↑	=	↑	↓	↑	↓	↑	=	↑	↓	↑	↓	↑	↓	↑
subcortical	↓	↑	=	↑	↓	↑	↓	↑	=	↑	↓	↑	↓	↑	↓	↑
Frontal white matter deep	↓	↑	=	↑	↓	↑	↓	↑	=	↑	↓	↑	↓	↑	↓	↑
subcortical	↓	↑	=	↑	↓	↑	↓	↑	=	↑	↓	↑	↓	↑	↓	↑
Score vs age-related max. score[a]	9/19	8/8	16/22	3/17	7/17	7/7	3/16	4/5	11/22	7/21	9/17	7/7	9/22	11/17	15/21	7/9
Basal ganglia																
Putamen (size, T_2-signal)	absent		absent		absent		small, ↑		absent		absent		absent		absent	
Size caudate nucleus	nl		small		small		small		small		small		small		small	
T_2-signal caudate nucleus	partially ↑*		partially ↑*		partially ↑*		partially ↑*		partially ↑*		partially ↑*		partially ↑*		partially ↑*	
Atrophy																
Ventricular enlargement	-		+		+		-		+		-		+		-	
Enlargement subarachnoid spaces	-		-		-		-		+		-		+		-	
Atrophy corpus callosum	-		+		+		-		-		-		+		-	
Cerebellar atrophy vermis	-		+		+		-		+		-		+		-	
hemispheres	-		-		-		-		-		-		-		-	
Age at latest MRI	12 mo		2 y				17 mo				20 mo					
Myelination	T_1	T_2	T_1	T_2			T_1	T_2			T_1	T_2				
Score vs age-related max. score[a]	14/22	9/17	16/22	5/22			14/22	4/21			8/22	8/22				
Basal ganglia																
Putamen (size, T_2-signal)	absent		absent				small, ↑				absent					
Size caudate nucleus	small		small				smaller				small					
T_2-signal caudate nucleus	partially ↑*		partially ↑*				partially ↑*				partially ↑*					
Atrophy																
Ventricular enlargement	-		+				+				-					
Enlargement subarachnoid spaces	-		+				-				-					
Atrophy corpus callosum	-		+				+				-					
Cerebellar atrophy vermis	+		+				+				+					
hemispheres	-		-				-				-					
Change over time																
Change in myelination	more myelin		no				more myelin				no					
Progression atrophy putamen	absent putamen		absent putamen				no				absent putamen					
Progression cerebral atrophy	no		yes				yes				no					
Progression cerebellar atrophy	yes		no				yes				yes					

Patient number	9		10		11		12		13		14		15		16	
Patient ID	HA133		HA201		HA205		HA204		HA169		HA211		HA214		HA194	
Age at first MRI	**5 mo**		**4 mo**		**10 mo**		**3 mo**		**7 mo**		**7 mo**		**7 mo**		**3 mo**	
Myelination	**T_1**	**T_2**	**T_1**	**T_2**	**T_1**	**T_2**	**T_1**	**T_2**	**T_1**	**T_2**	**T_1**	**T_2**	**T_1**	**T_2**	**T_1**	**T_2**
Brainstem	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓	↑	↓	=	↓	=	=
Posterior limb internal capsule	↑	=	↑	=	↑	↓	=	=	↑	↓	↑	↓	=	↓	↑	↓
Cerebellar white matter	=	=	↑	↓	=	↓	=	=	=	=	=	=	=	↑	↓	↑
Anterior limb internal capsule	↑	↑	=	↑	=	=	=	↑	↑	↑	=	↑	=	=	↓	↑
Central part centrum semiovale	↓	↑	↓	↑	↓	↑	↓	↑	=	↑	↓	↑	↓	↑	↓	↑
Corpus callosum splenium	↑	=	=	↑	↑	↓	↓	↑	↑	=	↑	↓	↑	↓	=	↑
genu	=	=	=	↑	↑	↓	↓	↑	↑	=	↑	↓	↑	↓	↓	↑
Parieto-occipital white matter deep	↓	↑	↓	↑	↓	↑	↓	↑	=	↑	↓	↑	↓	↑	↓	↑
subcortical	↓	↑	↓	↑	↓	↑	↓	↑	=	↑	↓	↑	↓	↑	↓	↑
Frontal white matter deep	↓	↑	↓	↑	↓	↑	↓	↑	=	↑	↓	↑	↓	↑	↓	↑
subcortical	↓	↑	↓	↑	↓	↑	↓	↑	=	↑	↓	↑	↓	↑	↓	↑
Score vs age-related max. score[a]	10/17	6/7	9/16	5/5	10/21	11/13	5/11	4/4	16/21	7/11	10/21	9/11	8/21	9/11	4/11	3/4
Basal ganglia																
Putamen (size, T_2-signal)	absent		absent		absent		small, ↑		absent		absent		absent		absent	
Size caudate nucleus	small		small		small		small		small		small		small		small	
T_2-signal caudate nucleus	partially ↑*		partially ↑*		partially ↑*		partially ↑*		partially ↑*		partially ↑*		partially ↑*		partially ↑*	
Atrophy																
Ventricular enlargement	+		-		+		+		-		-		-		-	
Enlargement subarachnoid spaces	+		-		+		+		+		-		+		-	
Atrophy corpus callosum	-		-		-		-		+		-		-		-	
Cerebellar atrophy vermis	+		-		+		-		+		+		+		-	
hemispheres	-		-		-		-		-		-		-		-	
Age at latest MRI															**6 mo**	
Myelination															**T_1**	**T_2**
Score vs age-related max. score[a]															6/19	6/8
Basal ganglia																
Putamen (size, T_2-signal)															absent	
Size caudate nucleus															small	
T_2-signal caudate nucleus															partially ↑*	
Atrophy																
Ventricular enlargement															-	
Enlargement subarachnoid spaces															-	
Atrophy corpus callosum															-	
Cerebellar atrophy vermis															+	
hemispheres															-	
Change over time																
Change in myelination															more myelin	
Progression atrophy putamen															absent putamen	
Progression cerebral atrophy															no	
Progression cerebellar atrophy															yes	

y, years; mo, months; vs, versus; max., maximum; [a]From Hamilton *et al.*[3], T_1, T_1-weighted image; T_2, T_2-weighted image; ↑, hyperintense relative to cortex;
↑*, hyperintense signal of the lateral aspect of the head of the caudate nucleus; ↓, hypointense relative to cortex; =, isointense relative to cortex; nl, normal; -, not present; +, present

REFERENCES

1. van der Knaap MS, Naidu S, Pouwels PJ, et al. New syndrome characterized by hypomyelination with atrophy of the basal ganglia and cerebellum. AJNR Am J Neuroradiol 2002;23:1466-1474.
2. Simons C, Wolf NI, McNeil N, et al. A de novo mutation in the beta-tubulin gene TUBB4A results in the leukoencephalopathy hypomyelination with atrophy of the basal ganglia and cerebellum. Am J Hum Genet 2013;92:767-773.
3. Hamilton EM, Polder E, Vanderver A, et al. Hypomyelination with atrophy of the basal ganglia and cerebellum: further delineation of the phenotype and genotype-phenotype correlation. Brain 2014;137:1921-1930.
4. Schiffmann R, van der Knaap MS. Invited article: an MRI-based approach to the diagnosis of white matter disorders. Neurology 2009;72:750-759.
5. Wolf NI, Salomons GS, Rodenburg RJ, et al. Mutations in RARS cause hypomyelination. Ann Neurol 2014;76:134-139.
6. Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics 2007;23:1289-1291.
7. Kalaydjieva L, Morar B, Chaix R, Tang H. A newly discovered founder population: the Roma/Gypsies. Bioessays 2005;27:1084-1094.
8. Bozikova A, Gabrikova D, Sovicova A, et al. The frequency of factor V Leiden and prothrombin G20210A mutations in Slovak and Roma (Gypsy) ethnic group of Eastern Slovakia. J Thromb Thrombolysis 2012;34:406-409.
9. Ruijter JM, Thygesen HH, Schoneveld OJ, Das AT, Berkhout B, Lamers WH. Factor correction as a tool to eliminate between-session variation in replicate experiments: application to molecular biology and retrovirology. Retrovirology 2006;3:2.
10. Varon R, Gooding R, Steglich C, et al. Partial deficiency of the C-terminal-domain phosphatase of RNA polymerase II is associated with congenital cataracts facial dysmorphism neuropathy syndrome. Nat Genet 2003;35:185-189.
11. Hantke J, Chandler D, King R, et al. A mutation in an alternative untranslated exon of hexokinase 1 associated with hereditary motor and sensory neuropathy -- Russe (HMSNR). Eur J Hum Genet 2009;17:1606-1614.
12. Komatsu M, Chiba T, Tatsumi K, et al. A novel protein-conjugating system for Ufm1, a ubiquitin-fold modifier. EMBO J 2004;23:1977-1986.
13. Tatsumi K, Sou YS, Tada N, et al. A novel type of E3 ligase for the Ufm1 conjugation system. J Biol Chem 2010;285:5417-5427.
14. Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998;67:425-479.
15. Homrich M, Wobst H, Laurini C, Sabrowski J, Schmitz B, Diestel S. Cytoplasmic domain of NCAM140 interacts with ubiquitin-fold modifier-conjugating enzyme-1 (Ufc1). Exp Cell Res 2014;324:192-199.
16. Martin DD, Ladha S, Ehrnhoefer DE, Hayden MR. Autophagy in Huntington disease and huntingtin in autophagy. Trends Neurosci 2015;38:26-35.
17. Lemaire K, Moura RF, Granvik M, et al. Ubiquitin fold modifier 1 (UFM1) and its target UFBP1 protect pancreatic beta cells from ER stress-induced apoptosis. PLoS One 2011;6:e18517.
18. Zhang Y, Zhang M, Wu J, Lei G, Li H. Transcriptional regulation of the Ufm1 conjugation system in response to disturbance of the endoplasmic reticulum homeostasis and inhibition of vesicle trafficking. PLoS One 2012;7:e48587.
19. Colin E, Daniel J, Ziegler A, et al. Biallelic Variants in UBA5 Reveal that Disruption of the UFM1 Cascade Can Result in Early-Onset Encephalopathy. Am J Hum Genet 2016;99:695-703.
20. Ciechanover A, Brundin P. The ubiquitin proteasome system in neurodegenerative diseases: sometimes the chicken, sometimes the egg. Neuron 2003;40:427-446.
21. Ronnebaum SM, Patterson C, Schisler JC. Emerging evidence of coding mutations in the ubiquitin-proteasome system associated with cerebellar ataxias. Hum Genome Var 2014;1:14018.
22. Muona M, Ishimura R, Laari A, et al. Biallelic Variants in UBA5 Link Dysfunctional UFM1 Ubiquitin-like Modifier Pathway to Severe Infantile-Onset Encephalopathy. Am J Hum Genet 2016;99:683-694.
23. Duan R, Shi Y, Yu L, et al. UBA5 Mutations Cause a New Form of Autosomal Recessive Cerebellar Ataxia. PLoS One 2016;11:e0149039.
24. van der Knaap MS, Linnankivi T, Paetau A, et al. Hypomyelination with atrophy of the basal ganglia and cerebellum: follow-up and pathology. Neurology 2007;69:166-171.

25. Lytle BL, Peterson FC, Qiu SH, et al. Solution structure of a ubiquitin-like domain from tubulin-binding cofactor B. J Biol Chem 2004;279:46787-46793.
26. Sato R, Takanashi J, Tsuyusaki Y, et al. Association Between Invisible Basal Ganglia and ZNF335 Mutations: A Case Report. Pediatrics 2016;138: e20160897.

IV

Vanishing White Matter

Chapter 6

The natural history of Vanishing White Matter (1): disease course

Eline M.C. Hamilton, Hannemieke D. W. van der Lei, Gerre Vermeulen, Jan A. M. Gerver, Charles M. Lourenço, Sakkubai Naidu, Hanna Mierzewska, Bernard M.J. Uitdehaag, Birgit I. Lissenberg-Witte, VWM Research Group and Marjo S. van der Knaap

Chapter 6 and 7 are published in Ann Neurol. 2018;84:274-288 as a joint paper

ABSTRACT

Objective: To comprehensively describe the natural history of Vanishing White Matter (VWM), aiming at improving counseling of patients/families and providing natural history data for future therapeutic trials.

Methods: We performed a longitudinal multicenter study among 296 genetically confirmed VWM patients, 134 males and 162 females. Clinical information was obtained via questionnaires for physicians and chart review.

Results: First disease signs occurred at a median age of 3 years (mode 2 years, range before birth - 54 years); 60% of patients were symptomatic before the age of 4 years. The nature of the first signs and symptoms varied for different ages of onset. Overall, motor problems were the most common presenting sign, especially in children. Adolescent and adult-onset patients were more likely to exhibit cognitive problems early after disease onset. 102 patients were deceased. Multivariable Cox regression analysis revealed a positive relation between age of onset and both preservation of ambulation and survival. Absence of stress-provoked episodes and absence of seizures predicted more favorable outcome. In patients with onset before 4 years, earlier onset was associated with more severe disability and higher mortality. For onset from 4 years on, disease course was generally milder, with a wide variation in severity. There were no significant differences for sex or for the five eIF2B gene groups. The results confirm the presence of a genotype-phenotype correlation.

Interpretation: The VWM disease spectrum consists of a continuum with extreme wide variability. Age of onset is a relatively strong predictor for disease course.

INTRODUCTION

Vanishing White Matter (VWM; OMIM 603896)[1-3] is one of the more prevalent leukodystrophies.[4] It is caused by recessive mutations in any of the genes *EIF2B1-5*.[5,6] Patients typically have a normal early development, followed by chronic progressive neurological deterioration with ataxia and spasticity and additionally stress-provoked episodes of rapid decline.[2] No curative treatment is available.[7] While VWM was initially recognized as a disorder of young children,[1,2,8] it has become apparent over time that disease onset and severity vary widely, from antenatal or early-infantile onset disease with rapid demise[9-11] to adult onset slow disease.[3,12] There is evidence for a genotype-phenotype correlation.[13-15] It has been suggested that sex influences disease course.[15,16]

Profound knowledge of the epidemiology and natural history of VWM is essential for physicians to counsel patients and families and make decisions regarding the management of the disease. In addition, studies of the natural history of diseases are valuable for enhanced understanding of the pathophysiology, evaluation of genotype-phenotype correlations and establishment of baselines for therapeutic trials. Studies on natural disease course in VWM are scarce and rather small.[14,16,17] We here report the results of a 12½-year natural history study of VWM, focusing on the occurrence of neurological signs/symptoms in relation to age and disease duration and the identification of prognostic factors. Standardized inventory of dimensions of disability will be addressed in a separate paper.

PATIENTS AND METHODS

Study design

Between January 2004 and October 2016 we performed a multicenter longitudinal observational study on all genetically proven VWM patients enrolled in the Amsterdam Database of leukoencephalopathies. The database contains all VWM patients referred for mutational analysis and 17 additional patients referred for Magnetic Resonance Imaging (MRI) analysis and genetically tested elsewhere. Written informed consent for research was obtained from the patients participating in the study or their guardians. The study was approved by the ethical standards committee of the VU University Medical Center, Amsterdam.

Clinical information

We obtained information on disease course by customized clinical questionnaires completed by the patient's physician or the patient and/or family members in consultation with the authors. If these sources were not available, we performed retrospective chart review. The questionnaires aimed at robust parameters that could easily be assessed by physicians, also when reviewing the patient file retrospectively, and by parents. The inventory involved items on demographic details, pregnancy and delivery, early motor development, early cognitive development, disease onset and presenting signs,

provoking factors, episodes of deterioration, disease course, neurological signs and symptoms, and survival. Data on medical history were obtained retrospectively when the patient entered the study. From that time onwards, data were collected prospectively. Data were checked for internal consistency and consistency with other data. Patients with another disease affecting neurological function in addition to VWM were excluded from the study.

We used age of onset to categorize the patients into the following six groups: antenatal-early infantile: <1 year **(1)**, late infantile: 1 - <2 years **(2)**, early juvenile: 2 - <4 years **(3)**, juvenile: 4 - <8 years **(4)**, late-juvenile-adolescent: 8 - <18 years **(5)** and adult-onset: ≥18 years **(6)**. Clinical disease onset was defined as the age at which the first neurological sign had been noted retrospectively. Clinical disease duration was defined as the time from disease onset onwards.
Patients were scored as having lost walking without support when they could no longer walk without equipment or help of another person; they were scored as having lost walking with or without support when fully wheelchair-dependent. In order to avoid introducing a bias by omitting patients who were more seriously affected and did not achieve walking, we scored patients who presented before 18 months and never achieved walking as having lost ambulation at disease onset and scored patients who had mildly delayed early development and never achieved walking, but presented with signs of neurological deterioration later than 18 months, as having lost ambulation at 18 months. Patients who died before 18 months or were not followed until that age, were not included in analyses concerning ambulation.
The disease course was defined as exacerbating if one or more episodes of acute major neurological deterioration occurred. Presence or absence of ovarian failure was assessed in females from 16 years at latest follow-up.

Statistical analysis

We used summary statistics to describe the clinical characteristics. Patient characteristics were reported by median and quartiles and/or ranges for non-normally distributed data. Nominal and ordinal data were analyzed by Chi-square test, Fisher's exact test or Kruskal-Wallis test to study differences between age-of-onset groups. We performed time-to-event analysis of the events 'disease onset', 'loss of walking without support', 'loss of walking with or without support', 'start of tube feeding', 'start of clear cognitive decline', 'first seizure' and 'death' with age and disease duration as two separate time variables. Individuals in whom the respective event had not occurred were censored for the item at the latest follow-up. We estimated median ages and disease durations at events by Kaplan-Meier curves. Group differences regarding age of onset, sex, exacerbating disease course and affected gene were analyzed with log-rank test.
For analyses regarding affected genes, each genotype (combination of two mutations) was represented once. If one genotype was observed in multiple patients, the median score was calculated by survival analysis. We used Cox proportional hazards models to calculate hazard ratios for predictors of survival and loss of walking with or without support with age of onset as continuous variable and sex, presence of episodes and

presence of seizures as categorical variables. Because for the variable 'seizures' the number of missing values was considerably higher than for other variables, we performed multiple imputation for this parameter, with the other variables as predictive values, including cumulative hazards for 'loss of walking with or without support' and 'survival', creating five datasets on which Cox regression analysis was performed. Statistical analysis was performed using SPSS version 22 (Armonk, NY: IBM Corp) and GraphPad Prism version 6.07 (San Diego California USA).

RESULTS

Patients

In total 305 VWM patients were eligible for the study, referred from 198 centers worldwide. Nine patients were excluded because of co-morbidity (i.e., Down syndrome, biliary atresia, galactosemia, glutaric aciduria type 1, encephalocele and cortical dysplasia, Leigh syndrome due to LRPPRC mutations, and perinatal asphyxia), leaving 296 patients (134 males and 162 females) from 261 families. Genetic analysis revealed that five patients had mutations in EIF2B1, 49 in EIF2B2, 23 in EIF2B3, 22 in EIF2B4, and 197 in EIF2B5. In the case of missing data, patients were selectively included in the analyses on the basis of available information. Throughout the results section we report the number of patients that were included in the different analyses in parentheses or in respective tables.

Clinical and demographic characteristics

The characteristics of the studied cohort are shown in Table 1. The age of the patients at the latest follow-up or death ranged from 3 months to 62 years and disease duration ranged from 1 week up to 39 years. For 63% of patients we obtained clinical questionnaires completed by physicians, in 10% questionnaires were completed by the patient and/or family members in consultation with the authors. For the remaining patients information was obtained by chart review. Patients geographically originated from Africa (n=1), Australia and New Zealand (n=11), Asia (n=26), South America (n=28) North America (n=56) and Europe (n=174). The database contains all known Dutch VWM patients (n=33); based on our numbers, the incidence in the Netherlands is estimated to be 1:80.000 live births or higher. Parental consanguinity was reported in 20% (42/210) of the families.

Table 1 | Characteristics of the VWM natural history study cohort

Number of patients	296
Number of families	261
Male / female	134 / 162
Affected gene	
EIF2B1	5
EIF2B2	49
EIF2B3	23
EIF2B4	22
EIF2B5	197
Median age at latest follow-up [quartiles] n=296	11 [5 - 25] years
Median disease duration at latest follow-up [quartiles] n=291	6 [2 - 13] years

Age of onset

The distribution of age of onset was heavily skewed to the right; on the interval 18-54 years it was rather uniform (Figure 1A). Median age of onset was 3 years (mode 2 years, range before birth - 54 years). 87 percent of the patients had an onset before 18 years and 60% before 4 years. 34 patients were symptomatic before the age of 1 year, seven of whom had an antenatal onset. When categorizing the patients in the six age of onset groups, early juvenile (2 - <4 years) onset was most common (Figure 1B). An overview of clinical characteristics classified by age of onset group is depicted in Supplementary Table 1. An outline of disease course per age of onset group is depicted in Figure 2.

Presentation

An overview of all presenting signs and symptoms is given in Table 2. Motor problems, especially gait problems, were the most common presenting sign. The nature of the first signs or symptoms varied for patients with different ages of onset (Supplementary Table 2). Antenatal onset was characterized by intrauterine growth retardation, oligohydramnios, reduced fetal movements, or contractures at birth, in variable combinations. After birth, these patients often presented signs of encephalopathy with irritability, somnolence and seizures. Several severe, mostly infantile and early-juvenile onset cases presented with similar signs of encephalopathy. Loss of acquired motor skills was common among all age of onset groups. Patients with late-juvenile, adolescent or adult-onset were more likely to present with non-motor signs, such as cognitive or psychiatric problems.

Fifty-three percent of presentations had occurred after a provoking event (Supplementary Table 1). Infection (often with fever) was the most common trigger (54%). Common types of infection were upper respiratory tract infection, flu, urinary tract infection, otitis, gastro-intestinal infection and viral rashes. Head trauma was the second most common trigger (44%); most often concerning mild trauma such as a fall while playing. Occasional triggers were vaccination and acute fright.

Seven patients were asymptomatic at study closure. Three of them had not had any disease signs: one had been genetically tested because of an affected sibling and was clinically asymptomatic at 5 years; two patients, currently 6 and 18 years, had been identified by incidental findings in brain imaging performed because of head trauma and headache. The other four patients had been identified by brain imaging for transient neurological signs: vertigo, lowered consciousness and anopia after head trauma, unprovoked dizziness and one-sided vision loss. They are currently asymptomatic at the ages of 8, 12, 12 and 43 years.

Early development

Early motor development was delayed in 25% of patients (69/276). For patients with onset <1 year (group 1), in 47% (15/32) no developmental problems had been noticed until subacute deterioration occurred at 3-8 months. Remaining patients (53%) had mild or severe development delay. The majority had reached some motor milestones ranging from head control to unsupported sitting, but 19% (6/32) never developed intentional movements.

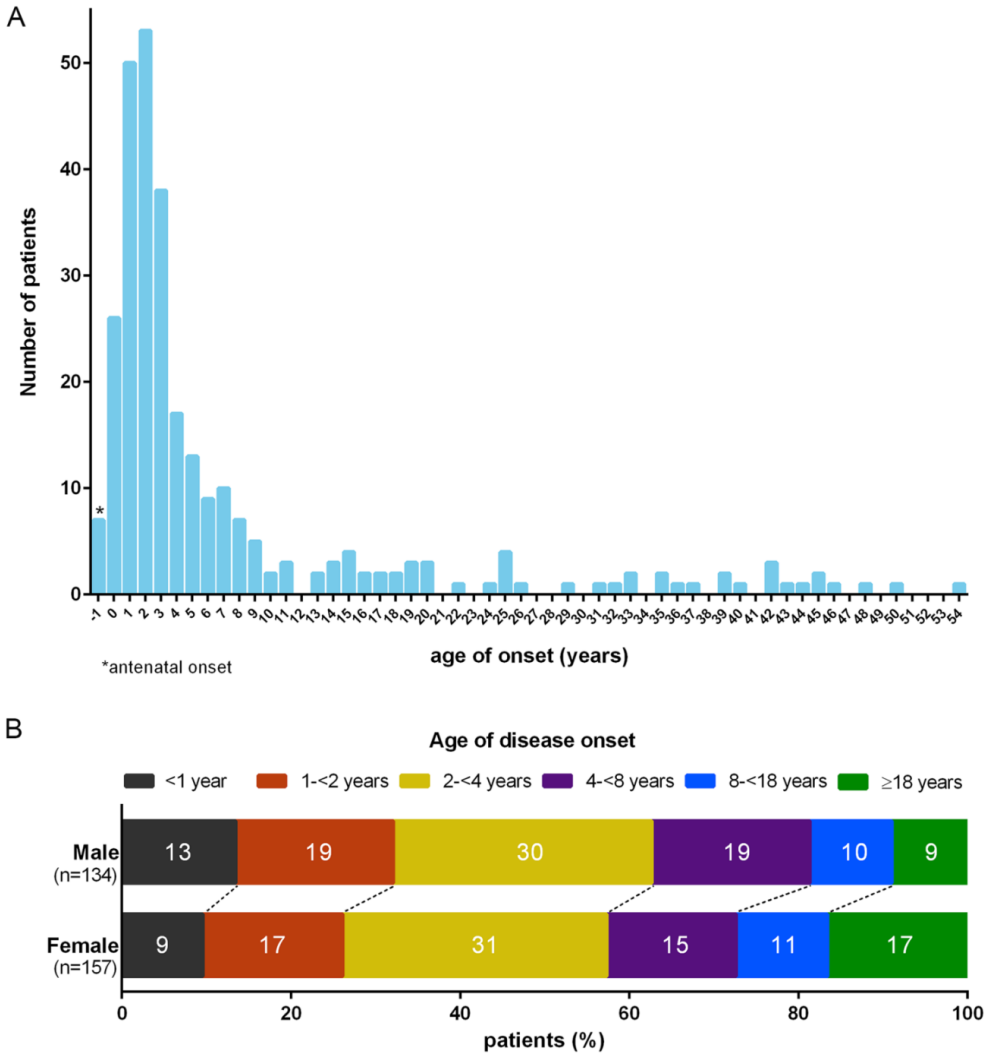


Figure 1 | Distribution of age of onset. (**A**) Histogram representing the number of patients per age of onset (years) for 291 VWM patients. (**B**) Distribution of patients per age of onset group per sex.

In most patients with onset 1 - <2 years (group 2), early motor development was reported as normal. Forty percent (n=20/50) had mild developmental delay. Among these patients, maximum motor milestones ranged from crawling (n=2), supported walking (n=6) to unsupported walking (n=11).

Ninety percent (174/194) of patients with onset ≥2 years (groups 3, 4, 5 and 6) had a normal early motor development. All four age groups contained a few patients who were reported to have had mild developmental delay (overall 10%). Early cognitive development was abnormal in 14% of patients, predominantly in patients with onset <2 years (Supplementary Table 1).

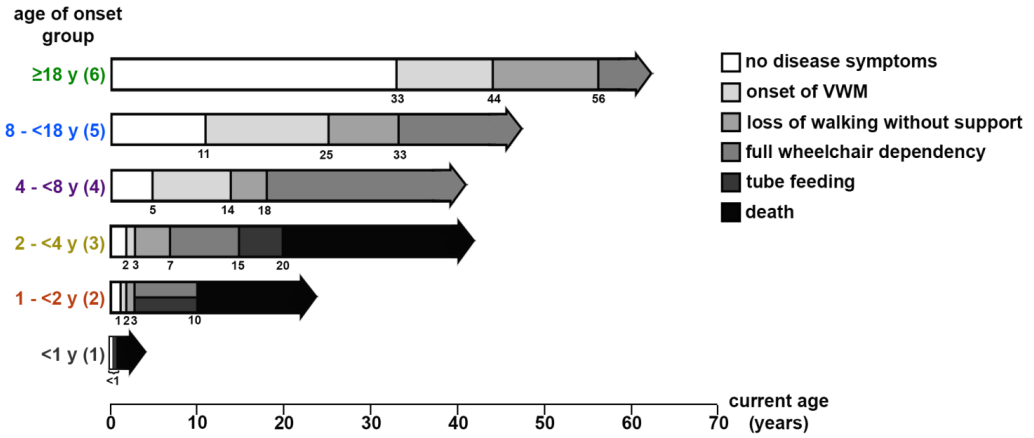


Figure 2 | Disease course per age of onset group. Age at onset of VWM and age at times of loss of walking without support, full wheelchair-dependency, start of tube feeding and death among 291 patients (numbers of patients for each item are shown in Supplementary Table 1). The horizontal arrows range from birth to the longest follow up in the respective age of onset group. The numbers below the horizontal arrows indicate the median age (years) at the respective event as estimated using Kaplan-Meier curves.

Ambulation and loss of ambulation

Unsupported walking was achieved in none of the patients with onset <1 year (group 1), in 74% of patients with onset 1 - <2 years (group 2) and in all patients with clinical onset ≥2 years (groups 3- 6). There were significant differences in age and disease duration at loss of walking without support and wheelchair-dependency when comparing the 6 age of onset groups (Supplementary Table 1, Figure 3A). Sub-analysis of groups 4, 5 and 6 (onset ≥4 years) revealed no significant difference regarding disease duration at loss of walking without support (log-rank p=0.55) and loss of walking with or without support (p=0.82).

Neurological signs

Table 3 presents an overview of the patients' current status at the last clinical inventory, with a subdivision for disease duration. Spasticity was often present; ataxia was the second most common neurological sign. Speech was affected in most patients; swallowing was affected in less than half of patients. Vision and hearing were generally well preserved. Optic atrophy had been reported in 17 patients, but was not investigated systematically. Seizures had occurred in 60% of patients; later disease onset was associated with a lower incidence and adult-onset patients were least likely to develop seizures (Supplementary Table 1). In few patients the first seizure had occurred prior to other presenting signs that led to the diagnosis VWM. The nature of seizures was variable, including non-motor seizures and focal onset seizures with and without impaired awareness. Generalized onset tonic-clonic seizures were most common. Most patients had occasional seizures, mostly well controlled with medication, less often moderately controlled. Few patients had refractory epilepsy; this was reported most often in groups 1 and 2 (onset <2 years) and also occurred in groups 3-5 (onset ≥2 - <18 years), but never in group 6 (onset ≥18 years).

Table 2 | Overview of presenting signs/symptoms in 288 patients

Presenting symptoms/signs	Frequency	Median age at presentation [range]	Provoked by trigger*
Gait problems	106	3y [14 mo - 45 y]	55 / 97
Ataxia / clumsiness	51	3 y [11 mo - 35 y]	28 / 45
Weakness / hypotonia	24	2 y [3 mo - 40 y]	17 /23
Loss of motor skills following infection	24	22 mo [3 mo -16 y]	24 / 24
Seizures	22	19 mo [2 mo - 25 y]	12 / 20
Loss of motor skills	21	2 y [2 mo - 33 y]	0 / 11
Cognitive / memory problems	20	11 y [18 mo - 54 y]	1 / 18
Loss of motor skills following head trauma	19	2 y [14 mo - 19 y]	19 / 19
Somnolence / coma following infection	16	2 y [8 mo - 14 y]	16 / 16
Developmental delay	11	18 mo [8 mo - 2y]	0 / 7
Spasticity/hypertonia	11	3 y [18 mo - 20 y]	6 / 9
Psychosis /confusion / behavioral change	10	19 y [2 - 43 y]	3 / 9
Irritability / encephalopathy	8	14 mo [8 mo - 14 yrs]	5 / 5
Antenatal signs	7	antenatally	0 / 7
Severe headache / migraine	7	13 y [3 - 33 y]	3 / 7
Speech problems / dysarthria	7	3 y [13 mo - 26 y]	3 / 6
Somnolence / coma following minor head trauma	6	6 y [23 mo - 19 y]	6 / 6
Fatigue / lethargy	6	2 y [8 mo - 33 y]	2 / 5
Dizziness / vertigo	5	8 y [2 - 45 y]	1 / 4
Depression	4	35 y [25 - 43 y]	0 / 4
Asymptomatic [incidental finding / affected sibling]	3	2 y [18 mo - 6 y]	2 / 3
Vision problems	3	31 y [4 - 33 y]	1 / 3
Amenorrhea / infertility	2	22 y [19 - 25 y]	0 / 2
Deterioration following vaccination	2	9 mo [5 - 14 mo]	2 / 2
Paresthesia	1	19 y	0 / 1

* the proportion among informative patients is presented; y, years; mo, months

Mortality

Survival analysis revealed that overall median survival was 38 years (Figure 3C, left), at median disease duration of 24 years (Figure 3C, right); 102 patients were deceased. OF deceased patients, median age at death was 6 years (range 3 months - 60 years), median disease duration at death was 3 years (range 1 week - 30 years). There was a significant difference in age and duration at death for the six age of onset groups (Figure 3D, Supplementary Table 1).

Death rate was considerably higher in groups 1-3 (onset <4 years), in which overall 47% (82/174) of patients were deceased, than for groups 4-6 (onset ≥4 years) in which 15% (18/117) had died. No significant difference regarding survival duration (log rank p=0.77) and disease duration at death (p=0.74) between groups 4-6. Deceased patients were generally in an advanced, often vegetative stage. Ninety-eight percent was wheelchair-dependent and 80% received tube feeding. Respiratory failure was the leading cause of death (61%). In a high proportion of patients, deterioration preceding death was triggered by infection (40%). Other causes of death were discontinuation of life support, coma, refractory epilepsy and cachexia.

Table 3 | Status and neurological signs in relation to disease duration

	Overall (n=214)	Disease duration 0 - <5 y (n=83)	Disease duration 5 - <10 y (n=57)	Disease duration ≥10 y (n=74)
Spasticity	82% (175/214)	75% (62/83)	79% (45/57)	92% (68/74)
Clumsiness / ataxia[1]	81% (153/189)	70% (46/66)	87% (47/54)	87% (60/69)
Hypotonia	52% (69/133)	71% (41/58)	38% (13/34)	37% (15/41)
Extrapyramidal signs	28% (29/104)	25% (9/36)	26% (7/27)	32% (13/41)
Speech[2]				
normal	32% (58/183)	43% (24/55)	26,5% (14/53)	27% (20/75)
dysarthria	44% (80/183)	33% (18/55)	47% (25/53)	49% (37/75)
no speech	24% (45/183)	24% (13/55)	26,5% (14/53)	24% (18/75)
Dysphagia	40% (70/177)	38% (23/60)	43% (20/47)	38% (27/70)
Vision				
normal	65% (117/181)	66% (38/58)	69% (38/55)	60% (41/68)
decreased	26% (48/181)	24% (14/58)	22% (12/55)	33% (22/68)
blind	9% (16/181)	10% (6/58)	9% (5/55)	7% (5/68)
Hearing				
normal	90% (158/176)	81% (45/56)	92% (47/51)	96% (66/69)
decreased	7% (13/176)	14% (8/56)	4% (2/51)	4% (3/69)
deaf	3% (5/176)	5% (3/56)	4% (2/51)	0% (0/69)
Cognition				
normal	38% (55/144)	56% (24/43)	42% (18/43)	22% (13/58)
borderline functioning/mild deficit	35% (50/144)	18% (8/43)	37% (18/43)	45% (26/58)
moderate up to profound deficit	27% (39/144)	26% (11/43)	21% (9/43)	33% (19/58)
Current activity - children				
normal school	32% (29/90)	50% (16/32)	14% (5/36)	36% (8/22)
special education	43% (39/90)	19% (6/32)	58% (21/36)	55% (12/22)
learning not possible	25% (22/90)	31% (10/32)	28% (10/36)	9% (2/22)
Current activity - adults				
normal employment	14% (7/49)	33% (1/3)	43% (3/7)	8% (3/39)
adjusted employment	12% (6/49)	0% (0/3)	14% (1/7)	13% (5/39)
day-care	45% (22/49)	67% (2/3)	29% (2/7)	46% (18/39)
bedridden	29% (14/49)	0% (0/3)	14% (1/7)	33% (13/39)

[1] nine patients were left out as coordination was not evaluable due to absence of intentional movements

[2] scored in patients from 2 years of age

y, years

Disease course and provoking factors

An exacerbating disease course was described in 82% of patients (Supplementary Table 1). The median number of episodes was 3, mode 1 (often at first presentation), range 1-25 episodes (n=162 patients). In 99% of patients the episodes involved motor problems (197/198 patients), most often gait problems, ataxia and paresis. Cognitive function was affected in 50% (71/142 patients), including loss of language, cognitive slowing, memory problems and loss of social interaction. Sixty-two percent of the patients had had one or more episodes of coma or altered consciousness (Supplementary Table 1). Coma and irritability were mostly observed in patients with early onset: 87% of patients with coma (55/63 patients) and 92% of patients with irritability (58/63) had a disease onset <8 years.

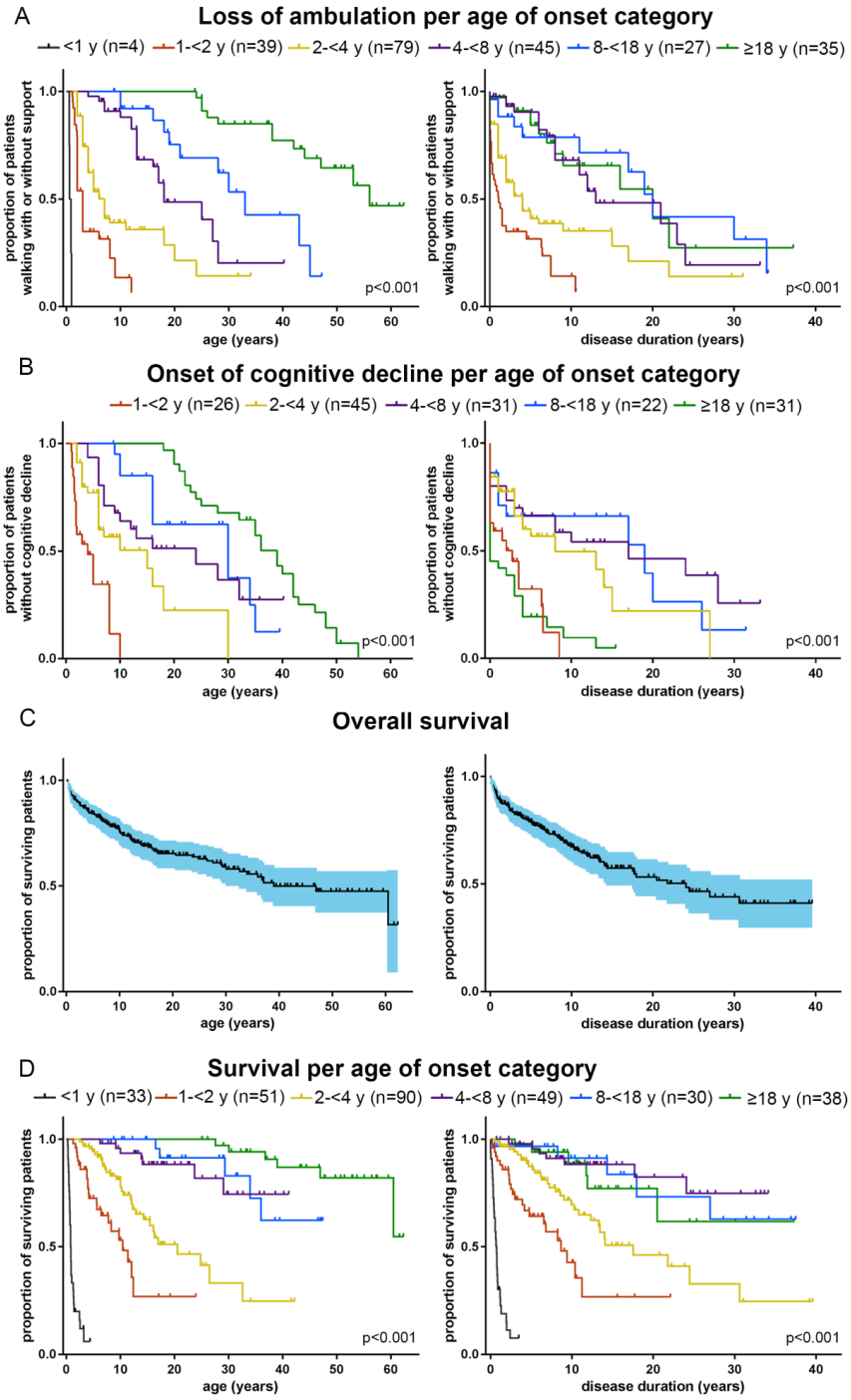


Figure 3 | Disease progression. Kaplan-Meier plots on **(A)** full wheelchair-dependency (loss of walking with or without support) in relation to age (left) and disease duration (right), per age of onset group; **(B)** onset of cognitive decline in relation to age (left) and disease duration (right), per age of onset group; **(C)** overall survival in VWM patients in relation to age (left) and disease duration (right; the 95% confidence limits are depicted by shaded areas); and **(D)** survival in relation to age (left) and disease duration (right), per age of onset group. In all plots censored patients (still ambulant, absence of cognitive decline or alive at last follow up) are indicated by crosses.

Episodes of deterioration were provoked by febrile infections in 80% (154/193) of patients with episodes, head trauma in 58% (106/182) and infection without fever in 27% (42/155). Other provoking factors mentioned less often were anesthesia in 16% (15/92), acute psychological stress or fright in 18% (27/150) and heat in 5% (8/83). In many more patients heat was mentioned as a factor adversely affecting function, but in most recovery occurred immediately after cooling down. There were incidental reports of deterioration after the use of alcohol, sleep deprivation, growth spurt, vaccination and a severe allergic reaction. Seizures were also reported as provoking factor for loss of motor function, although they may also have occurred secondary to deterioration.

Earlier disease onset was associated with higher sensitivity to febrile infections; fever caused deterioration in 86% of patients with exacerbating disease course and onset <4 years, versus 50% of patients with onset ≥18 years. Head trauma as provoking factor was reported with the highest rate in patients with onset ≥2 - <8 years (Supplementary Table 1).

After an episode of deterioration, patients rarely (10%) showed complete recovery (19/183 episodes); only few (3%; 5/183) showed almost complete recovery; the majority of episodes (51%; 93/183) were followed by partial recovery or persisting severe handicap (23%, 42/183). In 13% (24/183) death was a direct consequence of an episode of deterioration, most often concerning patients with disease onset <2 years.

Analysis of the influence of episodic deterioration on survival and preservation of ambulation by Kaplan-Meier plots indicated that an exacerbating disease course had an unfavorable effect on outcome (Figure 4A).

Sex

A VWM prevalence ratio of males to females of 1:1.21 was found. The distribution of age of onset categories per sex (Figure 1B) showed that imbalance was largest in the adult-onset group, but statistical analysis of distribution did not reveal significant differences (p=0.43). Time-to-event analysis of survival and loss of walking without and with support showed some differences between sexes, but not statistically significant (Figure 4B).

Prognostic factors

Kaplan-Meier plots indicated that exacerbating disease course had unfavorable effects on survival and preservation of ambulation (Figure 4A). Multivariable Cox regression analyses revealed that earlier age of onset, episodic deterioration and seizures had significant positive associations with death and loss of ambulation as independent prognostic indicators, while there were no sex-based differences in outcome (Table 3).

Reproductive organs

Female patients: information on ovarian function was available for 56 of 74 women above 16 years. In 86% signs of ovarian failure were reported. Eleven patients had primary and 17 had secondary amenorrhea. Four patients had amenorrhea or infertility without further

specification; 16 patients had irregular menses. Additionally, ovarian dysgenesis was found at ultrasound examination in one patient at the age 3 years and at autopsy in two patients who died at 10 months and 6 years.[2,18] Six women had offspring. There were no reports of episodic deterioration during or shortly after pregnancy.
Male patient: except for one case of infertility due to oligozoospermia, there were no reports of involvement of reproductive organs. Of the 26 males older than 16 years, four had offspring.

Other organs

Involvement of other organs was observed in seven patients with onset <1 year (group 1), which involved congenital cataract (n=4), renal hypo-dysplasia (n=2) and hepato(spleno)megaly (n=2). Furthermore, autopsy of a girl with antenatal disease onset revealed mild pancreatitis.[18] Several patients with disease onset at later ages also presented diseases of other organs, but these were never reported in >1 patient and will not be considered here.

Other features

Height was within normal ranges in 82% of patients (133/163), 18% had a height below normal (29/163, mainly patients with onset <8 years). Weight was normal in 79% (125/159), 16% of patients was underweight (26/159) and 5% overweight (8/159). Head circumference was normal in the majority of patients (132/169), 12% had microcephaly (21/169, most with onset <4 years) and 10% had macrocephaly (16/169, mainly patients with age of onset groups 1-3 in advanced disease stages). Two patients received a ventriculoperitoneal shunt because of suspected increased intracranial pressure, without clinical improvement. Headache was the most commonly reported additional problem, described in 26 patients, often referred to as migraine and reported to be severe in approximately half of the cases. Dizziness was described in six patients, including vertigo and orthostatic hypotension. Signs of peripheral neuropathy were described in three patients and scoliosis in seven patients.
History of depression had been described in 21 patients (median age at start 30 years, range 12-47 years). In 23 patients, enhanced aggression, erratic behavior or other behavioral problems had been reported, mostly in adulthood.

Genotype-phenotype correlation

Among all 296 patients, the total number of different mutation combinations was 157. No statistically significant differences were observed for the five eIF2B gene groups regarding age of onset (log rank p=0.31) and survival (log rank p=0.65). Only for the parameter “loss of walking without support” an overall significant difference was observed (log rank p=0.025). Ambulation was better preserved in the group of patients with EIF1B1 mutations, but this group was very small (only five patients) and therefore not necessarily representative. When excluding these five patients from the analysis, no overall differences remained for the groups EIF2B2-EIF2B5 (log rank p= 0.12).

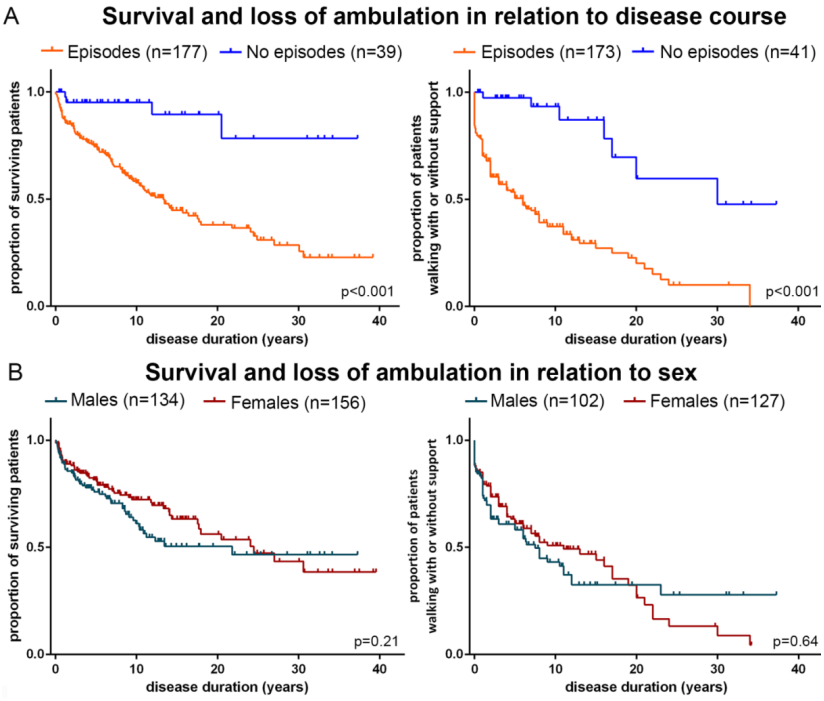


Figure 4 | Disease progression in relation to episodic deterioration and sex. (A) Survival (left) and loss of walking with or without support (right) in relation to disease duration, grouped by disease course with and without episodic deterioration. **(B)** Survival (left) and loss of walking with or without support (right) in relation to disease duration, grouped by sex. In all plots censored patients (alive or still ambulant at last follow up) are indicated by crosses.

6

Table 3 | Multivariable Cox regression analyses of factors affecting survival and loss of walking with or without support

	Survival			Preservation of ambulation		
Factor	**n**	**Hazard ratio [95% CI]**	**p value**	**n**	**Hazard ratio [95% CI]**	**p value**
Age of onset						
continuous variable	259	1.07 [1.02-1.13]	0.009	213	1.05 [1.01-1.09]	0.008
Sex						
male	118	1.0 (reference)	0.28	95	1.0 (reference)	0.53
female	141	1.26 [0.83-1.93]		118	1.13 [0.78-1.64]	
Exacerbating course						
absent	46	1.0 (reference)	0.033	40	1.0 (reference)	0.001
present	213	0.33 [0.12 -0.91]		173	0.25 [0.11-0.56]	
History of seizures*						
absent	97	1.0 (reference)	0.016	88	1.0 (reference)	<0.001
present	162	0.42 [0.21-0.85]		125	0.27 [0.16-0.45]	

*information was imputed for 75 patients

To assess the mutation-specific genotype-phenotype correlation, we compared all available groups of at least three patients from different families with the same mutation combination. In most groups of patients with similar genotype, severity measures, such as age of onset and survival, were rather consistent, but some variability was present, especially for mutations associated with a milder phenotype (details in Supplementary Table 3). The homozygous p.Arg113His mutation in EIF2B5 was most frequent (n=36) and most often, but not invariably, associated with a mild phenotype.[33]

Intrafamilial variation

There were 31 sibling-pairs and two families with three affected siblings. The majority siblings were categorized in the same age of onset group (18/33). In all other families, siblings were categorized in two subsequent age of onset groups (15/33), without exception. Disease course and severity among siblings were relatively similar. Siblings with early onset disease (<2 years) showed homogeneous phenotypes with rapid deterioration. With childhood or adult-onset, more individual variation was observed and siblings could show divergent phenotypes with involvement of different domains.

DISCUSSION

The present study was designed to delineate the natural history of VWM. Although often referred to as an early childhood onset, rapidly fatal disorder, VWM is a heterogeneous disorder with an extremely broad phenotypic range. Age of onset is an important determinant of prognosis in VWM.[14,19] To study the effect of age of onset in more detail, we discerned six groups on the basis of observed and expected clinical differences.

Patients with antenatal-early infantile onset (<1 year) invariably have a devastating, rapidly progressive disease course. They are less likely to show any recovery after an episode of deterioration and often remain encephalopathic or comatose. Many patients suffer from refractory epilepsy. Involvement of extra-cerebral organs is seen in this group only. In all patients death occurs several months after presentation.

Patients with late-infantile onset (1 - <2 years) typically show rapid decline after presentation, with loss of supported gait followed by wheelchair-dependency after months to a few years. Death occurs after several years in the majority of patients.

Presentation in the third year of life is most common. Patients with early-juvenile onset (2 - <4 years) often present with motor deterioration after a provoking event. Disease course in this group is more variable, but generally fairly progressive. Some patients immediately lose ambulation, while in the majority ambulation is preserved in the first months to years and wheelchair-dependency follows after a median time of 7 years. Numerous patients die after one to several years, but other patients reach adulthood, although invariably severely handicapped.

Patients with juvenile onset (4 - <8 years) mostly present with motor problems, with or without a provoking event; a few patients present with learning difficulties. The variation in decline of motor function is rather wide, with some patients becoming wheelchair-

dependent after a few years or even decades, while others remain ambulant. Mortality is low.
Patients with late-juvenile to adolescent onset (8 - <18 years) often present with motor problems and some with behavioral or learning problems or migraine. Rate of loss of ambulation, cognitive decline and mortality are similar to the juvenile onset group.
Adult-onset patients (≥18 years) present with a broad palette of signs and symptoms. Gait problems are common, but various less typical presentations occur, such as cognitive, psychological or fertility problems or seizures. Rate of loss of ambulation and mortality do not significantly differ from patients with onset from 4 - <18 years, while cognitive decline soon after presentation is much more common among adult-onset patients.

Thus, within the VWM spectrum, two key disease courses can be distinguished: presentation before 4 years (60% of cases in the current cohort) is generally followed by a rapidly progressive, motor-dysfunction dominated course with a profound age of onset effect on disease course. Presentation after 4 years (40%) is associated with a more heterogeneous, less progressive course with low mortality, independent of the exact age of onset. These are not strictly separated phenotypes and much variation is seen, with some early-onset patients having an unexpectedly slow disease course and some late-onset patients experiencing rapid decline and death. The fact that the VWM spectrum is a continuum with antenatal onset, rapidly fatal disease and late-adult-onset, mild disease as extremes, suggests that perhaps even more extreme phenotypes are currently missed. It is unknown how many miscarriages and stillbirths are caused by *EIF2B1-5* mutations. On the other hand, mild adult-onset variants of VWM may be largely underdiagnosed, due to the less typical presentation and the lack of awareness among adult neurologists, and perhaps some adults never become symptomatic. For most leukodystrophies, later age of symptomatic onset is generally associated with a slower rate of clinical progression.[20] It is an interesting finding that in VWM, apart from the longer symptom-free interval, presentation in adulthood is not associated with a more favorable disease course from clinical onset onwards than presentation in later childhood or adolescence. Strikingly, the variation between affected siblings is limited for any age of onset, suggesting a predominant effect of genetic factors over environment. The presence of acute episodic deterioration is an important negative influencer of the chronic disease course in VWM for all ages of onset, but stressors cannot be simply classified as environment; their effect is related to the intrinsic vulnerability of the patient. In any case, the finding underlines the importance of preventive measures such as avoiding head trauma, antibiotics and antipyretics for all patients.[7]

Independent of the age of onset, most females who reach adulthood have ovarian failure and few have children. Also few men who reach adulthood have children; oligospermia was found in one. Whether men with VWM may have testicular dysfunction has not been investigated.

Prior studies have addressed sex-related differences among VWM patients, such as the male:female imbalance among adolescent/adult-onset patients and possible differences in disease severity.[15-17] It has been argued that females would be more susceptible to VWM[16] or have a more benign disease course than males.[15] We further investigated the effect of sex, but did not confirm consistent clinical differences between males and females. Although we also observed an overrepresentation of females among adult-onset cases, this finding was not statistically significant. No female or male predisposition for death or loss of ambulation could be objectified by Cox regression analysis. Still larger numbers of patients are required to definitively address the question of sex differences in VWM.

We aimed for robust parameters of disease progression. We should, however, bear in mind that certain parameters, like wheelchair-dependency, may partially be influenced by other factors, such as motivation. For survival, differences in clinical practice, medical resources and cultural preferences on how to deal with end stage disease impact outcome. Several items were scored based on subjective assessment by patients, caretakers and physicians. In the absence of formal testing, parameters like onset of cognitive decline, vision and hearing are rather rough indicators of the respective items. In addition, inter-observer differences, selection and information bias may hamper the evaluation of the clinical course in VWM patients. In natural history studies, assessments would optimally be prospective and take place at regular intervals from disease onset until death in all patients. As a result of the multi-center nature of the study and the wide variability in disease course, we had to deal with incomplete datasets and censored data; survival analysis is the appropriate statistical method to analyze such datasets. Nevertheless, being the largest study until now, most limitations are lessened or overcome by the large number of patients included in this study, delineating the clinical spectrum of VWM.

There are currently no curative treatment options for VWM, but the technological progress in the field of leukodystrophies is promising.[21,22] The prospect of curative therapy has intensified the need to document the natural history of VWM. Natural history derived estimates facilitate study design of clinical trials, selection of clinically relevant endpoints and determination of which patient population to target. In this view, the current work may serve as a reference point to design and evaluate future therapeutic interventions.

ACKNOWLEDGEMENTS

The authors express their gratitude to all patients, families and referring physicians for their cooperation and contribution. This study received financial support from ZonMw AGIKO grant (920-03-308) and the Optimix Foundation for Scientific Research.

The following VWM Research Group collaborators contributed to the study: N. Abbas Al-Sannaa; H. Aldhalaan; D. Alves; D.J. Amor; R. Appleton; E.A. Arslan; R. Arteaga; S. Ayta; M. Baethmann; J.F. Bale; B. Banwell; C. Barbot; N. Bass; T Ben-Omran; E. Bertini; A. Biebl; A. Bley; L. Bollen; E. Boltshauser; J. Bonkowsky; S. Bower; C. Brandt; A. Bravo Oro; C. Brenner; J. Brown; K. Brožová; E. Calado; M.M. Campos; M. Carmo Macário; L. Carr; A.K.J. Chan; M. Chopra; A. Clarke; T. Covanis; Y. Crow; B. Csányi; I.F.M. de Coo; M. Del Rossario Aldao; M. D'Hooghe; M. di Rocco; T. Dzwiniel; J El Helou; P. Fallon; A. Feigenbaum; A. Ferlini; J.M. Ferro; A. Fiumara; N.A. Fletcher; J. Fluss; J.M. Fock; L. Fontenelle; R.J. Forsyth; W. Garming; C. Garone; J. Gascon-Bayarri; M. Geldhoff; E. Glamuzina; F. Góes; A.L. Gomes; V. Gonzalez; C. Guarda; S. Gulati; S. Güler; E. Gut; P. Hansen; R. Horvath; I.S. Ivanov; S. Jagadeesh; M. Kaczorowska; P. Kankirawatana; D. Karall; A. Kariminejad; M. Käselau; T. Kerr; M.D. King; M. Kolnikova; I. Krägeloh-Mann; R. Kruschewsky; A. Lehman; Z. Liptai; J.H. Livingston; E. López-Laso; R.E. Madrid; M. Maes; K. Majdi; A. Majumdar; K. Maltby; H. Mandel; M. Matsui; M. McEntagart; M.A. McShane; V. Mejaški Bošnjak; S. Mercimek-Andrews; B.R. Miller; A. Misbahuddin; I. Moroni; R. Morton; C.F. Moura de Souza; N. Muelas; H. Mundy; P. Munzar; G. Musuwadi Subramanian; K. Naess; K. Naismith; J.P. Neau; R.W. Newton; M.J. Noetzel; B O'Brien; K. Okálová; N. Olabarrieta; J.R. Østergaard; Q.S. Padiath; A. Pato Pato; J. Pera; R. Peralta; B. Pérez Dueñas; S. Perlman; M. Philippart; M. Pineda; B. Plecko; K. Prass; J. Radić; L. Régal; J.D. Reggin; D. Renaud; C.M. Rice; E. Rossignol; J.P. Rubin; D. Salgado; F. Salvi; H. Sampaio; J. Sánchez Herrero; M. Santos; E. Santos; R. Schiffmann; M. Sessa; S. Sharma; J. Shearn; J. Shoffner; H.S. Sivri; J.S. Skranes; C.G. Spaeth; S.P. Sparagana; E. Storey; L.S. Sveberg; M. Szlago; L. Sztriha; B. Tatli; P. Tekturk; M. Tennison; S. Tirupathi; L. Toledo Bravo de Laguna; C. Torres; M.A. Tuna; V. Udani; G. Uziel; A. Valverde; R. van Coster; K. van Haren; A. Vanderver; M. Vasconcelos; J. Vilchez; H. Vogt; J.C. von Kleist-Retzow; E. Wassmer; A. Wedell; B. Weschke; C. Wilson; S.S.N. Wong; E. Wood; Z. Yapici; H. Yavuz; S. Ygberg.

SUPPLEMENTARY DATA

Supplementary Table 1 | Clinical characteristics classified by age of onset group

	Overall** *n=296*	<1 year (1) *n=33*	1 -<2 years (2) *n=51*	2 - <4 years (3) *n=90*	4 - <8 years (4) *n=49*	8 - <18 years (5) *n=30*	≥18 years (6) *n=38*	p-value
Disease onset								
Median age of clinical onset [quartiles]	3 y [22 mo-7 y] *n=291*	4 [2-7] mo *n=33*	18 [17-22] mo *n=51*	*2 [2-3] y* *n=90*	5 [4-6] y *n=49*	11 [8-15] y *n=30*	33 [23-42] y *n=38*	n.a.
Onset provoked by trigger	53% *n=138/258*	43% *n=12/28*	66% *n=29/44*	72% *n=58/81*	40% *n=18/45*	54% *n=14/26*	21% *n=7/34*	<0.001
Survival								
***Estimated median age of death**	38 y *n=102/296*	9 mo *n=28/33*	10 y *n=23/51*	20 y *n=31/90*	n.a. *n=7/49*	n.a. *n=5/30*	n.a. *n=6/38*	<0.001
***Estimated median time to death (duration)**	24 y *n=100/291*	8 mo *n=28/33*	8 y *n=23/51*	17 y *n=31/90*	n.a. *n=7/49*	n.a. *n=5/30*	n.a. *n=6/38*	<0.001
Median age of death [quartiles]	6 [1 - 13] y *n=102*	9 [6 - 14] mo *n=28*	4 [2 - 8] y n=*23*	9 [6 - 15] y *n=31*	13 [9 - 23] y *n=7*	29 [16 - 34] y *n=5*	37 [29 - 50] y *n=6*	<0.001
Median disease duration at death [quartiles]	3 y [9 mo - 9 y] *n=100*	7 [3 - 10] mo *n=28*	2 [1 - 6] y *n=23*	7 [3 - 13] y *n=31*	*6 [5 - 17] y* *n=7*	14 [4 - 22] y *n=5*	10 [4 - 14] y *n=6*	<0.001
Neurological development								
Abnormal cognitive development	*14%* *n=31/221*	63% *n=10/16*	*21%* *n=7/33*	*9%* *n=6/65*	*9%* *n=4/44*	*11%* *n=3/28*	*3%* *n=1/35*	<0.001
Achieved walking without support	94% *n=237/253*	0% *n=0/4*	74% *n=34/46*	100% *n=88/88*	100% *n=48/48*	100% *n=30/30*	100% *n=37/37*	<0.001
Ambulation* **								
***Estimated median age of loss of walking without support**	9 y *n=174/235*	8 mo *n=4/4*	2 y *n=37/42*	3 y *n=65/81*	14 y *n=31/46*	25 y *n=16/27*	44 y *n=21/35*	<0.001
***Estimated median time to loss walking without support (duration)**	3 y *n=174/235*	0 mo *n=4/4*	2 mo *n=37/42*	1 y *n=65/81*	8 y *n=31/46*	15 y *n=16/27*	7 y *n=21/35*	<0.001
***Estimated median age of full wheelchair dependency**	18 y *n=125/229*	8 mo *n=4/4*	3 y *n=31/39*	7 y *n=47/79*	18 y *n=20/45*	33 y *n=11/27*	56 y *n=12/35*	<0.001
***Estimated median time to full wheelchair dependency (duration)**	8 y *n=125/229*	0 mo *n=4/4*	1 y *n=31/39*	4 y *n=47/79*	13 y *n=20/45*	20 y *n=11/27*	20 y *n=12/35*	<0.001

Other neurological functions								
***Estimated median time to start tube feeding (duration)**	*27 y n=75/219*	5 mo *n=16/22*	3 y *n=23/38*	15 y *n=21/65*	n.a. *n=7/42*	n.a. *n=5/24*	n.a. *n=3/28*	<0.001
***Estimated median time to cognitive decline (duration)**	*4 y n=96/155*	n.e.	2 y *n=19/26*	8 y *n=22/45*	17 y *n=16/31*	19 y *n=11/22*	0 y *n=28/31*	<0.001
***Estimated median time to first seizure (duration)**	*5 y n=103/173*	6 mo *n=17/22*	2 y *n=25/36*	8 y *n=30/50*	9 y *n=13/24*	11 y *n=11/21*	n.a. *n=7/20*	<0.001
Disease course								
Exacerbating disease course	82% *n=212/259*	84% *n=21/25*	88% *n=43/49*	93% *n=80/86*	76% *n=34/45*	68% *n=17/25*	59% *n=17/29*	<0.001
Episode(s) of coma or reduced / altered consciousness	62% *n=135/219*	81% *n=17/21*	75% *n=33/44*	72% *n=47/65*	49% *n=20/41*	48% *n=10/21*	30% *n=8/27*	<0.001
Deterioration following febrile infection	64% *n=154/239*	61% *n=14/23*	77% *n=36/47*	83% *n=65/78*	58% *n=23/40*	35% *n=8/23*	29% *n=8/28*	<0.001
Deterioration following head trauma	46% *n=106/228*	0% *n=0/19*	34% *n=14/41*	67% *n=48/72*	57% *n=25/44*	52% *n=12/23*	24% *n=7/29*	<0.001

y; years, mo; months, n.a.; not applicable, n.e.; not evaluable
* Kaplan-Meier estimates
** for five patients the age of onset was unknown, therefore the numbers in the “overall” column do not always correspond to the sum of the numbers per age of onset group
*** only patients who achieved age ≥ 18 mo were included; patients who presented <18 mo and never achieved walking were scored as having lost ambulation at onset and patients who never achieved walking, but presented with signs of neurological deterioration later than 18 mo were score as having lost ambulation at 18 mo

Supplementary Table 2 | Overview of presenting signs/symptoms per age of onset group

Presenting symptoms/signs	<1 year (1) n=33	1 - <2 years (2) *n=51*	2 - <4 years (3) *n=87*	4 - <8 years (4) *n=49*	8 - <18 years (5) n=30	≥18 years (6) n=38
Antenatal signs	21%	-	-	-	-	-
Irritability / encephalopathy	3%	8%	1%	-	3%	-
Seizures	21%	12%	2%	2%	7%	11%
Somnolence / coma / lethargy / fatigue	6%	16%	14%	4%	10%	5%
Deterioration following vaccination	3%	2%	-	-	-	-
Developmental delay	3%	16%	1%	-	-	-
Weakness / hypotonia	21%	6%	5%	6%	13%	8%
Gait problems	-	35%	49%	47%	33%	32%
Ataxia / clumsiness	3%	14%	25%	33%	7%	11%
Spasticity/hypertonia	-	10%	1%	6%	-	5%
Loss of motor skills	39%	35%	24%	10%	17%	8%
Speech problems / dysarthria	-	4%	2%	2%	-	5%
Cognitive / memory problems	-	2%	-	18%	3%	24%
Psychosis /confusion / behavioral change	-	-	2%	2%	3%	16%
Depression	-	-	-	-	-	11%
Severe headache / migraine	-	-	1%	-	17%	3%
Dizziness / vertigo / paresthesia	-	-	1%	-	7%	8%
Vision problems	-	-	-	2%	-	5%
Amenorrhea / infertility	-	-	-	-	-	5%
Asymptomatic (incidental finding / affected sibling)	-	2%	1%	2%	-	-

Supplementary Table 3 | Phenotypic characteristics in patients with similar genotypes

Genotype[1]	Number of informative patients (families)	Age of onset categories[2]; median age of onset[3] [range]	Median survival [age range]	Number of deaths [age range]	Number of patients who lost walking without support [duration range]	Median time (duration) to loss of walking without support[4]
EIF2B2						
p.Gly200Val, p.Glu213Gly	9 (9)	**[2]** **[3]** 2 y [12 mo - 2 y]	11 y [4 - 34 y]	0	4 / 9 [0 - 4 y]	n.a.
p.Gly200Val, p.Pro291Ser	5 (3)	**[1]** 3 mo [ant. - 4 mo]	5 mo [3mo - 2 y]	5 [3 mo - 2 y]	no ambulation	no ambulation
p.Glu213Gly homozygous	12 (10)	**[3]** **[4]** 3 y [2 - 6 y]	22 y [6 - 38 y]	0	9 / 12 [0 - 24 y]	8 y
EIF2B3						
p.Ala87Val homozygous	3 (3)	**[3]** **[5]** **[6]** 17 y [2 - 22 y]	25 y [4 - 29 y]	0	2 / 3 [1 - 11 y]	11 y
EIF2B5						
p.Thr91Ala, homozygous	8 (6)	**[3]** **[4]** **[5]** 6 y [3 - 16 y]	37 y [16 - 46 y]	3 [16 - 29 y]	7 / 8 [0 - 19 y]	6 y
p.Thr91Ala, p.Arg113His	4 (3)	**[3]** **[5]** **[6]** 14 y [3 - 35 y]	21 y [6 - 47 y]	0	3 / 4 [1 - 7 y]	3 y
p.Thr91Ala, p.Arg339Trp	4 (3)	**[2]** **[3]** 23 mo [21 mo -2 y]	8 y [4 -14 y]	4 [4 -14 y]	4 / 4 [0 - 2 mo]	0 mo
p.Leu106Phe homozygous	6 (4)	**[2]** **[3]** 22 mo [12 mo- 3 y]	6 y [4 - 16 y]	2 [6 - 16 y]	5 / 6 [0 - 1 y]	2 mo
p.Arg113His homozygous	36 (35)	**[2]** **[3]** **[4]** **[5]** **[6]** 17 y [18 mo - 54 y]	33 y [8 - 62 y]	4 [30 - 60 y]	16 / 32 [0 - 22 y]	16 y
p.Arg113His, p.Arg299His	5 (4)	**[3]** **[4]** **[5]** 6 y [3 - 9 y]	17 y [6 - 35 y]	4 [6 - 23 y]	5 / 5 0 - 10 y]	5 y
p.Arg113His, p.Arg315His	5 (5)	**[3]** **[4]** **[5]** 4 y [3 - 11 y]	16 y 12 - 22 y]	0	4 / 5 [0 - 10 y]	7 y
p.Arg113His, p.Arg339Gln	7 (6)	**[2]** **[3]** 2 y [18 mo - 3 y]	11 y [3 - 16 y]	2 [3 - 8 y]	6 / 6 [0 - 3 y]	1 y
p.Arg113His, p.Arg339Trp	3 (3)	**[3]** 2 y [2 - 3 y]	10 y [7 - 25 y]	1 [10 y]	3 / 3 0 - 2 y]	1 y
p.Arg113His, p.Ala403Val	3 (3)	**[2]** **[3]** 3 y [18 mo - 3 y]	8 y [7 - 10 y]	1 [10 y]	3 / 3 [0 - 4 y]	0 y
p.Arg136Cys homozygous	3 (3)	**[2]** **[3]** 23 mo [18 mo - 2 y]	6 y [5 - 6y]	1 [5 y]	1 / 1 [6 mo]	6 mo
p.Arg269Gln homozygous	5 (4)	**[1]** **[2]** 18 mo [6 - 18 mo]	2 y [15 mo - 10 y]	3 [15 mo - 2 y]	4 / 4 [0 - 8 mo]	2 mo (3x no ambulation)
p.Arg315Gly homozygous	5 (4)	**[2]** **[3]** 2 y [18 mo - 3 y]	4 y [2 - 26 y]	2 [4 - 26 y]	2 / 3 [0 y]	2 mo

[1] mutations are represented according to the following transcripts: *EIF2B2*: NM_014239.3, *EIF2B3*: NM_020365.3, *EIF2B5*: NM_003907.2

[2] **(1)** onset < 1 year, **(2)** onset 1 -< 2 years, **(3)** onset 2 - < 4 years, **(4)** onset 4 - <8 years, **(5)** onset 8 - < 18 years, **(6)** onset ≥ 18 years

[3] y, years; mo, months; ant., antenatally

[4] calculated by Kaplan-Meier survival analysis

REFERENCES

1. Schiffmann R, Moller JR, Trapp BD, et al. Childhood ataxia with diffuse central nervous system hypomyelination. Ann Neurol 1994;35:331-340.
2. van der Knaap MS, Barth PG, Gabreels FJ, et al. A new leukoencephalopathy with vanishing white matter. Neurology 1997;48:845-855.
3. van der Knaap MS, Kamphorst W, Barth PG, Kraaijeveld CL, Gut E, Valk J. Phenotypic variation in leukoencephalopathy with vanishing white matter. Neurology 1998;51:540-547.
4. Bonkowsky JL, Nelson C, Kingston JL, Filloux FM, Mundorff MB, Srivastava R. The burden of inherited leukodystrophies in children. Neurology 2010;75:718-725.
5. Leegwater PA, Vermeulen G, Konst AA, et al. Subunits of the translation initiation factor eIF2B are mutant in leukoencephalopathy with vanishing white matter. Nat Genet 2001;29:383-388.
6. van der Knaap MS, Leegwater PA, Konst AA, et al. Mutations in each of the five subunits of translation initiation factor eIF2B can cause leukoencephalopathy with vanishing white matter. Ann Neurol 2002;51:264-270.
7. van der Knaap MS, Pronk JC, Scheper GC. Vanishing white matter disease. Lancet Neurol 2006;5:413-423.
8. Hanefeld F, Holzbach U, Kruse B, Wilichowski E, Christen HJ, Frahm J. Diffuse white matter disease in three children: an encephalopathy with unique features on magnetic resonance imaging and proton magnetic resonance spectroscopy. Neuropediatrics 1993;24:244-248.
9. van der Knaap MS, van Berkel CG, Herms J, et al. eIF2B-related disorders: antenatal onset and involvement of multiple organs. Am J Hum Genet 2003;73:1199-1207.
10. Fogli A, Wong K, Eymard-Pierre E, et al. Cree leukoencephalopathy and CACH/VWM disease are allelic at the EIF2B5 locus. Ann Neurol 2002;52:506-510.
11. Black DN, Harris R, Schiffmann R, Wong K. Fatal infantile leukodystrophy: a severe variant of CACH/VWM syndrome, allelic to chromosome 3q27. Neurology 2002;58:161-162.
12. Prass K, Bruck W, Schroder NW, et al. Adult-onset Leukoencephalopathy with vanishing white matter presenting with dementia. Ann Neurol 2001;50:665-668.
13. van der Knaap MS, Leegwater PA, van Berkel CG, et al. Arg113His mutation in eIF2Bepsilon as cause of leukoencephalopathy in adults. Neurology 2004;62:1598-1600.
14. Fogli A, Schiffmann R, Bertini E, et al. The effect of genotype on the natural history of eIF2B-related leukodystrophies. Neurology 2004;62:1509-1517.
15. van der Lei HD, van Berkel CG, van Wieringen WN, et al. Genotype-phenotype correlation in vanishing white matter disease. Neurology 2010;75:1555-1559.
16. Labauge P, Horzinski L, Ayrignac X, et al. Natural history of adult-onset eIF2B-related disorders: a multi-centric survey of 16 cases. Brain 2009;132:2161-2169.
17. Carra-Dalliere C, Horzinski L, Ayrignac X, et al. [Natural history of adult-onset eIF2B-related disorders: a multicentric survey of 24 cases]. Rev Neurol (Paris) 2011;167:802-811.
18. Boltshauser E, Barth PG, Troost D, Martin E, Stallmach T. "Vanishing white matter" and ovarian dysgenesis in an infant with cerebro-oculo-facio-skeletal phenotype. Neuropediatrics 2002;33:57-62.
19. Labauge P, Fogli A, Niel F, Rodriguez D, Boespflug-Tanguy O. [CACH/VWM syndrome and leucodystrophies related to EIF2B mutations]. Rev Neurol (Paris) 2007;163:793-799.
20. Van Haren K, Bonkowsky JL, Bernard G, et al. Consensus statement on preventive and symptomatic care of leukodystrophy patients. Mol Genet Metab 2015;114:516-526.
21. Helman G, Van Haren K, Escolar ML, Vanderver A. Emerging treatments for pediatric leukodystrophies. Pediatr Clin North Am 2015;62:649-666.
22. Ricca A, Rufo N, Ungari S, et al. Combined gene/cell therapies provide long-term and pervasive rescue of multiple pathological symptoms in a murine model of globoid cell leukodystrophy. Hum Mol Genet 2015;24:3372-3389.

Chapter 7

The natural history of Vanishing White Matter (2): quality of life

Eline M.C. Hamilton, Hannemieke D. W. van der Lei, Gerre Vermeulen, Jan A. M. Gerver, Charles M. Lourenço, Sakkubai Naidu, Hanna Mierzewska, Reinoud J. B. J. Gemke, Henrica C.W. de Vet, Bernard M.J. Uitdehaag, Birgit I. Lissenberg-Witte, VWM Research Group and Marjo S. van der Knaap

Chapter 6 and 7 are published in Ann Neurol. 2018;84:274-288 as a joint paper

ABSTRACT

Objective: To assess the degree of disability and health-related quality of life (HRQL) in Vanishing White Matter (VWM) patients, aiming at improving patient/family counseling and providing natural history data for future therapeutic trials.

Methods: We performed a longitudinal multicenter study among 296 patients. We obtained Health Utilities Index (HUI) assessments in patients ≥2 years and Guy's Neurological Disability Scale (GNDS) assessments in patients ≥8 years.

Results: HUI data were available for 171 patients and follow-up data in 63. GNDS scores were obtained in 114 patients and follow-up in 39. Disease duration at assessment ranged between 0.5-38 years. Median HUI Health Index was 0.21 (quartiles -0.14 - 0.69) on the HUI3 scale that ranges from -0.36 (most disabled state) to 1 (no handicap). Median GNDS sum score was 21 (quartiles 10 - 33) on the GNDS scale that ranges from 0 (normal) to 60 (most disabled). In patients with onset <4 years, earlier onset was associated with more severe disease course and higher mortality. For onset ≥4 years, rate of deterioration varied, independent of exact age of onset. Ambulation and dexterity were most affected, followed by cognition, which was affected earlier in adult-onset patients. In later stages, patients were more likely to develop problems with speech, bladder and bowel function.

Interpretation: Our results provide insight in the natural disease course of VWM and illustrate that the clinical spectrum is highly variable, ranging from limited disability to profound handicap in various domains. The variability is dependent on age of onset.

INTRODUCTION

Vanishing White Matter (VWM) is a leukodystrophy caused by mutations in any of the genes *EIF2B1-5,* which encode a complex involved in mRNA translation and its regulation under different stress conditions.[1] The classical phenotype is characterized by early childhood onset chronic neurological decline with spasticity and ataxia. In addition, many patients show striking sensitivity to stressors, such as febrile infection and minor head trauma, which may cause rapid neurological deterioration, coma and death.

The disease course and severity in VWM are extremely variable.[1,2] Because the few available studies on the natural disease course are of limited size,[2,3] we initiated the Vanishing White Matter natural history study. One part concerned a longitudinal study on the disease course (chapter 6). An important conclusion of this study was that age of onset is a strong predictor of the rate of disease progression and death in patients with disease onset before the age of 4 years, whereas for disease onset from 4 years on the disease course is highly divergent and independent of the exact age of onset.

The aim of the present part of the natural history study is to obtain insight into dimensions of disability and to estimate health-related quality of life (HRQL) in patients with VWM in order to facilitate counseling and provide natural history data for future therapeutic trials. We present the natural disease course in a large cohort of patients with VWM by means of two validated clinical scales on disability: Health Utilities Index (HUI) and Guy's Neurological Disability Scale (GNDS).

PATIENTS AND METHODS

Study design

Between January 2004 and October 2016 we performed a longitudinal observational study among all genetically proven VWM patients enrolled in the Amsterdam Database of leukoencephalopathies (chapter 6). For the present study, we followed patients from enrolment into the database and prospectively assessed HUI scores from the age of 2 years and GNDS scores from the age of 8 years between one and four time points. HUI and GNDS scores were assessed by proxies (physicians or parents) and sporadically by self-assessment, using postal or digital forms, telephone-interviews or in-person interviews. Informed consent was obtained from the patients or their guardians and the study was approved by the ethical standards committee of the VU University Medical Center, Amsterdam.

Instruments

Health Utilities Index: The HUI[4] is a well validated instrument to assess health status and HRQL. It has been used extensively as an outcome measure in population health surveys and clinical studies - including neurological disorders - in patients from the age of 1 year on.[5-8] The system consists of a set of distinct attributes (dimensions) of health status that enable valuation of function-specific

scores (single-attribute utility scores) as well as generic multi-attribute preference-based measures of quality life (HUI Health Index) at a point in time. We applied the HUI Mark 3 (HUI3) proxy version, which consists of eight attributes: Vision, Hearing, Speech, Ambulation, Dexterity, Emotion, Cognition and Pain. Each attribute has five or six levels[4] (Supplementary Table 1A) and single-attribute scores range between 0 (severe impairment in that domain) and 1 (no impairment; Supplementary Table 1B). Additionally, one attribute of the HUI Mark 2 (HUI2) system on Self-care was measured. We used well-established HUI decision tables to generate HUI2 and HUI3 single-attribute utility scores for each patient at each assessment.[9,10] We applied coding algorithms to obtain overall HUI3 Health Index scores, which provide a global HRQL estimation as product of the distinct attribute scales (Supplementary Table 1C).[10] The HUI Health Index score represents the patient's state on a scale from 0 (equivalent to death) to 1 (equivalent to perfect health). The minimum score is a negative score (-0.36) and all negative scores indicate a most disabled state that was rated 'worse than dead' in the preference survey among the general population.[10] A difference of 0.03 or greater in HUI Health Index score is considered clinically meaningful.[11,12]

In the case of missing data we applied inspection and logical deduction according to guidelines of Naeim *et al.,* if possible.[13] If more than 2 attribute scores were missing, the concerning inventory was excluded from the study (1% of the inventories). We used hot deck imputation with pattern similarity to impute the few remaining missing values up to a maximum of two domains.[13] Imputations were only calculated for the HUI3 attribute scores on Cognition in 3% of the inventories and for the scores on Emotion in 1% of the inventories.

In order to analyze the evolution of HUI Health Index scores over time, we gave each patient a baseline score, corresponding to age-matched reference scores from HUI Mark3 reference population data[14,15] for the time point 'disease onset'. As references scores are not available for individuals younger than 5 years, we also applied the reference score of 0.92 of the general population at age 5 for patients under 5. For death we used a score of -0.5, deviating from the HUI utility scale where death is defined as a value of 0. We did this, because numerous patients had negative scores during life, while being happy, as evaluated with the Emotion score, which is not in line with worse than dead. Also the score of such patients would otherwise rise from below 0 during life to 0 at death. Changing death status from zero to -0.5 alters the meaning of the negative scores, but does not influence the statistical characteristics of the scale. We gave deceased patients in whom no HUI questionnaire was obtained because of age younger than 2 years or because of nonresponse a score for two time points (baseline score at onset and end score at death).

Guys Neurological Disability Scale: The GNDS, also known as the Neurological disability Scale (NDS) or the UK Neurological disability Scale (UKNDS), is a reliable measure to assess neurological disability.[16] The system was created to assess outcome measures in Multiple Sclerosis (MS) in a generic, patient-oriented,

multidimensional way, not biased towards any particular disability.[16] As VWM and MS are comparable in terms of involvement of central nervous system white matter structures and chronic disease progression with additional episodic worsening, we decided to apply the GNDS to assess the neurological function in our cohort of VWM patients. Nowadays, the scale is also applied in other neurological disorders, such as spina bifida and neuropathy.[17,18] The system has proven to also be reliable when applied as a postal questionnaire.[16,19] The questionnaire is directed to assess the patient's disability in the previous month on the basis 12 separate subcategories: Cognitive disability, Mood disability, Visual disability, Speech and communication disability, Swallowing disability, Upper-limb disability (hereafter called 'Arm', Lower-limb disability (hereafter called 'Leg'), Bladder disability, Bowel disability, Sexual disabilities, Fatigue, and 'Other disabilities'. Each domain is scored according to a six level severity scale ranging from 0 (no disability) up to 5 (maximum possible disability; Supplementary Table 4). The grades in each sub-scale are arranged so that each step represents approximately the same level of disability in each domain. An overall score describing the patient's total disability (GNDS sum score) is reached by summing up all 12 sub-scores, with a minimum of 0 and a maximum of 60 in the case of maximum possible disability. A change of three or more points in the sum score is considered as clinically relevant.[16,20]

We followed the GNDS system instructions[16] for dealing with missing values: missing values on Mood disability were scored as the mean of the Cognitive disability and Fatigue scores (2% of the inventories); missing scores on Fatigue were scored as the mean of Cognitive and Mood disability (2% of the inventories). For Sexual disabilities, we imputed the score on the basis of the round off mean of the Leg, Bladder and Bowel disability scores in the case of a missing score and also in the case of young and/or celibate patients (89% of the inventories), in order to avoid an unrepresentative bias towards a good outcome. Because of the large number of imputations for Sexual disability, we left this item out in the results section except for the calculation of the GNDS sum score. In the case of missing values in other domains, we performed hot deck imputation in a manner similar to the processing of the HUI data, up to a number of two missing values. When more items were missing, the inventory concerned was excluded from analysis (5% of the inventories). For the domains Vision, Swallowing, Bladder, Bowel and Others we imputed the scores in 1% of the inventories.

Age of onset categories

We used age of onset information derived from the natural history study on disease course (Chapter 6) to categorize the patients into the same six groups for the current study: antenatal - early infantile: <1 year (**1**), late infantile: 1 - <2 years (**2**), early juvenile: 2 - <4 years (**3**), juvenile: 4 - <8 years (**4**), late-juvenile -adolescent: 8 - <18 years (**5**) and adult-onset: ≥18 years (**6**). The disease onset was defined as the age at which the first neurological sign had in retrospect been noted. The disease duration was defined as the time from disease onset onwards.

For certain analyses we preferred a less detailed subdivision of age of onset groups; on the basis of the results of the natural history study (Chapter 6) and the current study, we chose the following categories: early onset: <4 years; intermediate onset: 4 - <18 years and late onset: ≥18 years.

Analysis and statistics

We performed data analysis and descriptive statistics with SPSS version 22 (Armonk, NY: IBM Corp) and GraphPad Prism version 6.07 (San Diego California USA).

We compared HUI Health Index scores in relation to disease duration per age of onset group via linear mixed models, with fixed effects for age of onset group and disease duration, and their two-way interaction. Although not all were normally distributed, we analyzed the degree of disability per HUI attribute and GNDS domain per age of onset category by mean values, as this represented the center of the four up to six level data's distribution most accurately.
We created spider plots using eight selected HUI single attribute scores and nine selected GNDS domain scores. For the HUI, the attribute Pain was omitted because pain was not a prominent feature and the score of this attribute is known to be less reliable.[21] For the GNDS, Sexual ability, Fatigue and Other were omitted because these scores were not very informative in our patient population. The spider plot data separately concern mild, intermediate and severe disability as assessed by the HUI Health Index and GNDS sum scores. The values in the spider plots represent mean scores for the early (<4 years) and late (≥18 years) age of onset patient categories. By contrasting these two categories, we aimed at more clearly illustrating the effect of age of onset on the relative involvement of different domains along the disease course. The two age of onset categories were compared using the independent samples t-test; a p-value <0.05 was considered significant. In the plots on GNDS scores, the order of domains was based on the order of the items in the questionnaires. The order of the HUI domains was adapted to create analogy with the GNDS spider plots for overlapping items. N.B. for the HUI a higher score is associated with better function, while for the GNDS a higher score is associated with worse function.

We used linear regression to analyze the relation of GNDS sum scores and HUI Health Index scores as well as domain specific scores with comparable disabilities measured by both HUI and GNDS within patients at similar time points.

RESULTS

Study population

Two hundred ninety six patients were eligible for the natural history study. Five patients were excluded because of lack of information on age of onset, bringing the total to 291. An overview of obtained data is provided in Table 1. Two hundred sixty

two patients were eligible for formal HUI scoring on the basis of the age criterion of ≥2 years. We obtained at least one round of formal HUI inventory in 65% of patients (171/262) at disease durations ranging from 6 months to 38 years. In several patients more than one HUI inventory was performed, bringing the total number of HUI inventories on 258.

For GNDS scoring, 178 patients were eligible on the basis of the age criterion of ≥8 years. In 64% of patients (114/178) at least one round of GNDS inventory was obtained at disease durations ranging from 1 to 38 years. In several patients more than one GNDS inventory was performed, bringing the total number of GNDS inventories on 172.

Table 1 | Overview of obtained HUI and GNDS scores per age of onset group

Age of onset group	number of patients (total: 291)	Deceased patients only included in HUI baseline + end scores* (total: 60)	Number of formal HUI scores (258 scores in 171 patients)				Number of formal GNDS scores (172 scores in 114 patients)			
			1	2	3	4	1	2	3	4
<1 y (1)	33	27	2	-	-	-	-	-	-	-
1 - <2 y (2)	51	13	19	7	1	-	3	2	-	-
2 - <4 y (3)	90	16	35	15	4	1	20	5	1	1
4 - <8 y (4)	49	2	20	10	7	2	21	6	6	2
8 - <18 y (5)	30	1	12	5	3	1	12	5	3	1
≥18 y (6)	38	1	20	6	1	-	19	6	1	-
Median disease duration [quartiles]			6 y [3-11]	10 y [5-18]	16 y [10-30]	23 y [16-28]	9 y [6-15]	16 y [9-24]	24 y [15-31]	23 y [16-28]

y = years; *In these patients no formal HUI scoring had been performed because of age younger than 2 years or because of nonresponse: they received scores for two time points (baseline score at onset and end score at death).

HUI Health Index

The overall median HUI Health Index score derived from formal assessments across all time points was 0.21 (quartiles -0.14 - 0.69). Thirty-six percent of scores (93/258) was negative. In addition to the formal assessments, we gave all patients a standard baseline score at disease onset. In some patients the HUI score initially improved as a result of a better score at the first formal assessment as compared to the standard baseline score. We gave 60 deceased patients in whom no formal HUI scoring had been performed a standard baseline score and the end score of -0.5 for death. The disease course per patient on the basis of all HUI scores from disease onset up to last follow-up or death is depicted in Figure 1A. There was a wide variability in disease course, but later onset was generally associated with less disability and lower mortality. The HUI Health Index scores per age of onset group in relation to disease duration are depicted in Figure 1B and show a wide variability.

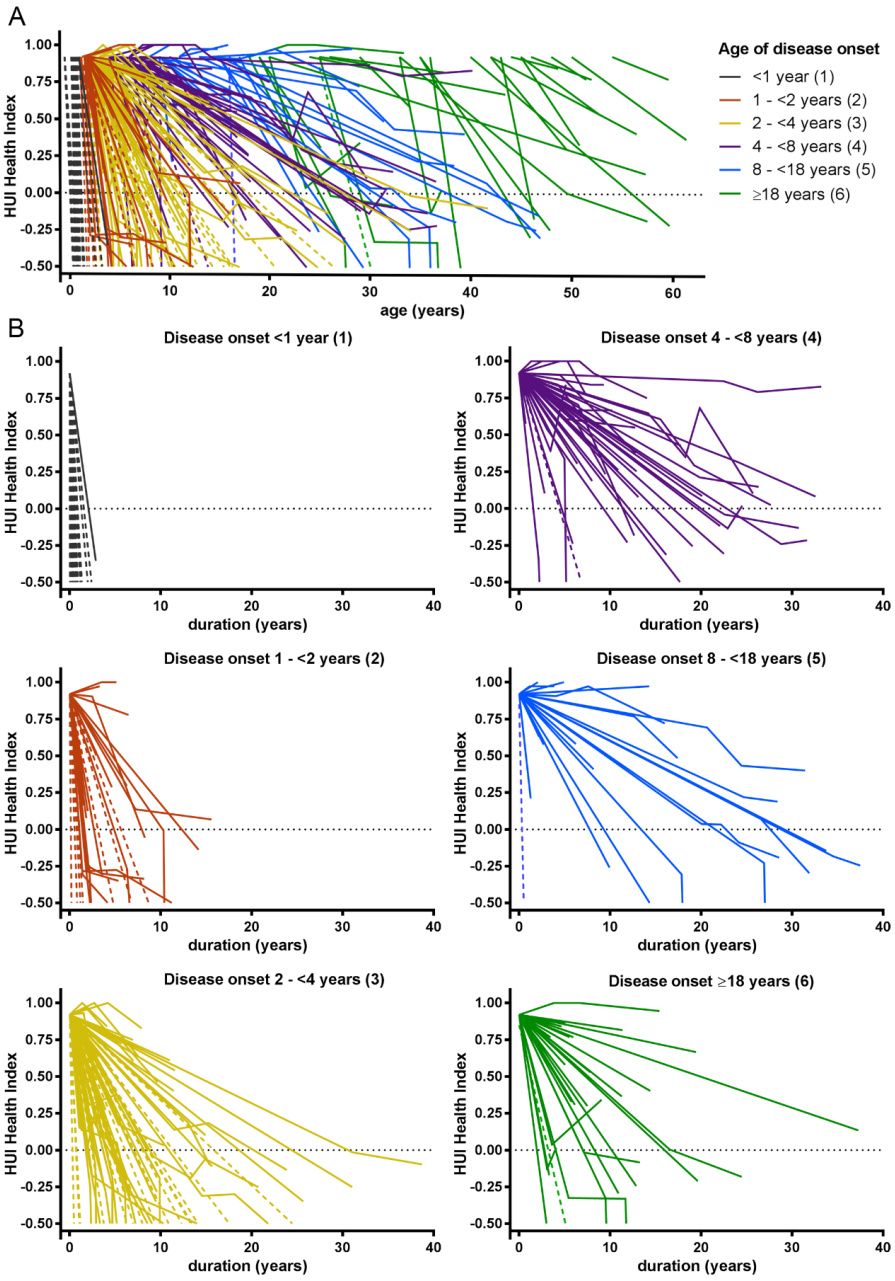


Figure 1 | Disease course in VWM patients as assessed by HUI Health Index scores. Graphs depict disease course in relation to age (**A**) and disease duration (**B**). One line represents one patient. Age of onset groups are marked by different colors. Scores range from -0.50 (dead) to 1 (perfectly healthy). Solid lines represent patients in whom one or more formal scores were obtained. Dotted lines represent patients in whom only baseline and death scores were available. In some patients the score initially improves as a result of a better score at the first formal assessment compared to the standard baseline score.

Analysis at group level by linear mixed model analysis showed that the course of HUI Health Index scores differed significantly between the age of onset groups (p <0.001; Figure 2). Disease progression was most rapid in group 1 (age of onset <1 year), followed by group 2 (age of onset 1- <2 years) and subsequently group 3 (age of onset 2 -< 4 years). Overall scores in group 6 (onset ≥18 years) were slightly lower than in groups 4 (age of onset 4-<8 years) and 5 (age of onset 8-<18 years), but there were no significant differences between the latter three groups.

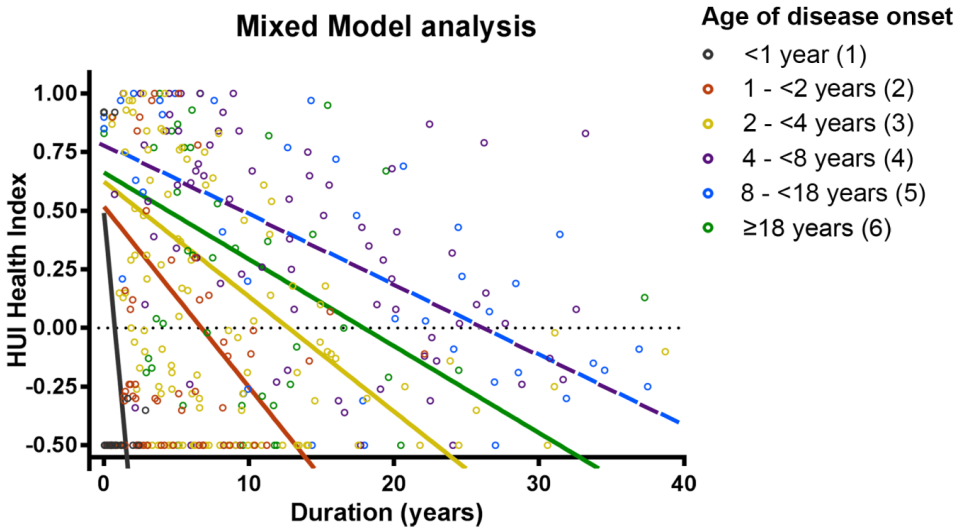


Figure 2 | Mixed models linear analysis for HUI Health Index per age of onset group. Overall there was a significant age of onset effect on disease progression (p<0.001), but there were no significant differences between groups 4 (onset 4 - <8 years), 5 (onset 8 - <18 years) and 6 (onset ≥18 years). The lines of groups 4 and 5 overlap and are therefore depicted as dashed lines.

HUI single-attribute scores

The frequency of scores per HUI attribute is displayed in Supplementary Table 3. Reduced quality of life was found in one or more of the eight attributes in all but 15 out of 258 inventories. In decreasing order of frequency, the attributes affected most severely on the basis of mean single-attribute scores were Ambulation, Self-care, Dexterity, Cognition, Speech, Vision, Emotion, Pain and Hearing.

We observed that patients with early onset disease had different clinical characteristics than patients with late onset disease: dependent on age of onset, there were differences in the distribution of disability scores in different stages of the disease. We therefore made spider plots presenting the distribution of the HUI single attribute scores (except for Pain) for the early and the late age of onset categories and separately for mild, intermediate and severe disability, as assessed by the overall HUI Health Index score (Figure 3, upper panel). In the case of mild disability, early onset was typically associated with motor signs (ambulation and dexterity

disability and loss of self-care), while late onset patients more often presented cognitive problems early in the disease course (Figure 3A). Along with disease progression, there was a profound loss of function on multiple attributes. In the intermediate disability stage, motor problems were already severe in early onset patients (Figure 3B). In the case of severe disability, profound motor problems, loss of self-care and moderate up to severe cognitive decline was invariably present in both age of onset categories (Figure 3C). Hearing and Emotion were generally spared. An overview of mean scores and confidence intervals of all HUI attributes for all three age of onset categories is presented in Supplementary Figure 1.

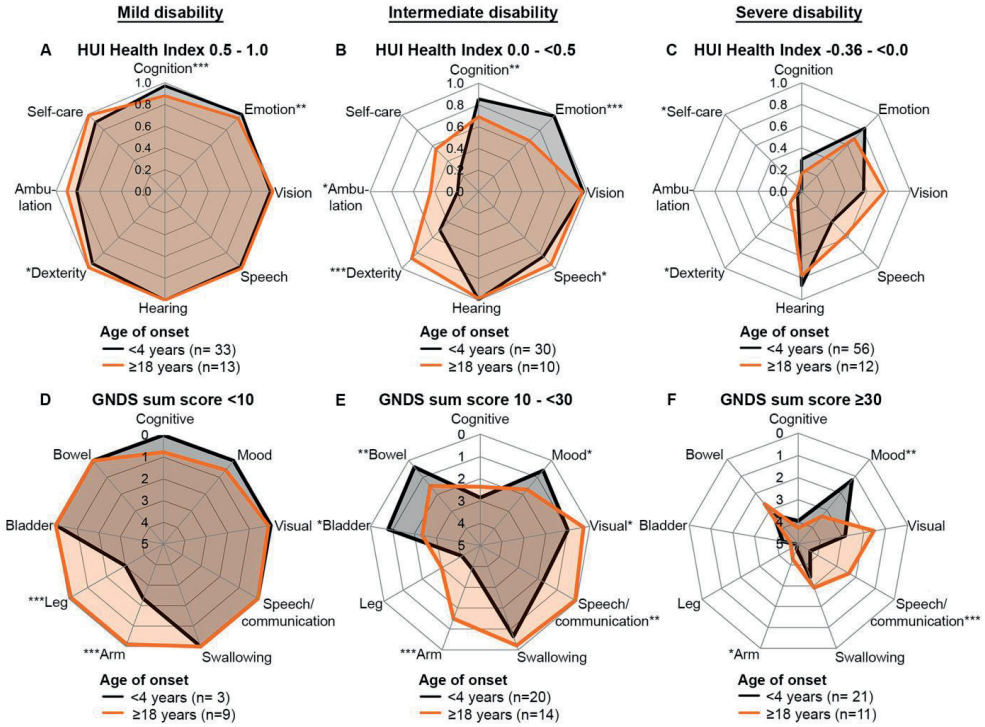


Figure 3 | Spider plots of selected HUI and GNDS scores for the early and late age of onset categories. Mean HUI single attribute scores and GNDS domain scores are shown for three different disease stages for early (<4 years, black line) and late (≥18 years, orange line) onset VWM. HUI Health Index scores of ≥0.5 - 1.0 represent mild to no disability (**A**), scores of 0 - <0.5 represent intermediate disability (**B**) and scores of -0.36 - 0 represent severe disability (**C**). HUI Attribute scores range from 0.0 (most disabled) to 1.0 (normal status). GNDS sum score <10 represents mild disability (**D**), 10 - <30 represents intermediate disability (**E**) and a score ≥30 represents severe disability (**F**). GNDS domain scores range from 0 (normal status) to 5 (total loss of function). HUI Attributes and GNDS domains in which the scores of the early and late age of onset categories are significantly different are indicated by *(p<0.05), **(p<0.01) and ***(p<0.001).
In the plots on GNDS scores, the order of domains was based on the order of the items in the questionnaires. The order of the HUI domains was adapted to create analogy with the GNDS spider plots for overlapping items. N.B. for the HUI a higher score is associated with better function, while for the GNDS a higher score is associated with worse function.

Quality of life - Impact on emotion

Considering the large number of negative formal HUI Health Index scores (n=93), representing a state considered worse than dead by the general population, we evaluated the scores on the HUI attribute Emotion (Figure 4). The majority of patients, including those with negative HUI health index scores, scored in the two most positive emotion scores ('happy and interested in life' or 'somewhat happy'). Of patients with the lowest HUI Health Index scores (<-0.2), only 28% scored in the two most severe Emotion scores ('very unhappy' or 'so unhappy that life is not worthwhile'); in patients with the second lowest HUI Health Index scores (-0.2 - <0), this was 7%. In total, only eight out of 258 Emotion scores represented a state 'so unhappy that life is not worthwhile'.

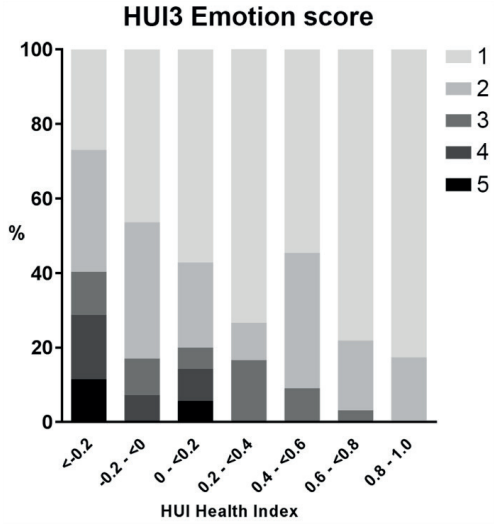


Figure 4 | Individuals' HUI Emotion scores in relation to their formal HUI Health Index scores. HUI Health Index scores range from -0.36 (most disabled state) to 1 (no handicap). 1= Happy and interested in life; 2= Somewhat happy; 3=Somewhat unhappy; 4= Very unhappy; 5= So unhappy that life is not worthwhile.

Guys Neurological Disability Scale

The overall median GNDS sum score (across all time points) was 21 (quartiles 10 - 33). The distribution of scores per GNDS domain is displayed in Supplementary Table 4. Disability was found in one or more of the 12 domains in all of the 172 inventories. In decreasing order of frequency, the leading causes of morbidity on the basis of mean scores were disabilities concerning Legs, Arms, Cognition, Bladder, Speech, Other, Fatigue, Bowel, Mood, Swallowing and Vision. The discrepancy between the HUI and GNDS scores on visual function can be explained by the difference in rating systems, where the HUI does and GNDS does not consider wearing glasses/contact lenses as abnormal (Supplementary Tables 1 and 2).

To analyze the differences in clinical characteristics in various stages of the disease between patient with early and late disease onset, we made spider plots to depict the distribution of GNDS domain scores (except for Sexual ability, Fatigue and Other) for the early and the late age of onset categories and separately for mild, intermediate and severe disability, as assessed by the GNDS sum score (Figure 3, lower panel). In correspondence with the HUI results, early onset cases typically presented motor signs in early disease stages (Arm and Leg disability), while late onset patients more often presented cognitive problems early in the disease course (Figure 3D). Along with disease progression, there was loss of bladder and bowel function. Speech/Communication was better preserved in late onset cases (Figure 3E - F). Mood was relatively spared among early onset cases. An overview of mean scores and confidence intervals of all GNDS domain scores for the three age of onset categories is presented in Supplementary Figure 2.

Other problems

In 51% of the questionnaires (87/172) other problems due to VWM were mentioned in addition to the 11 GNDS domains. The most commonly reported problems were muscle spasms/cramps, limb pain and headache, followed by dizziness and seizures (Supplementary Table 5).

Correlation HUI and GNDS scores

In order to evaluate the relation between the disability scores derived from the HUI and the GNDS, we compared the overall HRQL scores from both systems and a found significant correlation between individuals' HUI Health Index and GNDS sum score ($p<0.001$, $r^2 = 0.82$, Supplementary Figure 3). Comparison of scores on domains that were covered by both systems, obtained at the same time points, showed a significant correlation for all domains ($p<0.001$); 'HUI3 Cognition'/'GNDS Cognitive disability'($r^2 =0.57$), 'HUI3 Vision'/'GNDS Visual disability'($r^2 =0.69$), 'HUI3 Speech'/ 'GNDS Speech/communication disability', ($r^2 =0.57$), 'HUI3 Ambulation'/'GNDS Leg disability' ($r^2 =0.78$), and 'HUI3 Dexterity'/'GNDS Arm disability'($r^2 =0.77$).

DISCUSSION

The purpose of this study was to estimate HRQL in VWM patients with different ages of onset and disease courses in a standardized way and on the basis of broader-ranging domains than the robust clinical parameters applied in the other part of the natural history study (Chapter 6). Disability is an important disease consequence as it determines the patients' capabilities to perform daily activities. It is an essential parameter for health services because it defines the type and level of care needed and it determines the social and economic impact of the disease. Assessment of quality of life may contribute to better understanding between physicians, patients and caregivers. Also for proper trial design it is crucial to have information on changes on clinical scales over time.

Our results show that VWM is a heterogeneous disorder with diverse disease trajectories. Analysis of disease progression on the basis of HUI Health Index scores indicates a clear age of onset effect, although even within age of onset groups relatively broad individual variability is seen. As reported (chapter 6), disease onset <2 years is invariably associated with a rapidly progressive, fatal disease. In other age of onset groups variability is wider. No significant differences in the rate of deterioration are present between patients with juvenile onset (4 - <8 years), late-juvenile to adolescent onset (8 - <18 years) and adult onset (≥18 years). The linear mixed model gives a global indication of disease progression on group level, but no individual conclusions should be drawn from these predictions. Evaluation of patients' scores on the different HUI attributes and GNDS domains provides insight into the distribution of loss of function over time. The domains ambulation and dexterity are most severely affected. The majority of patients are dependent on carers for eating, bathing, dressing and/or toilet use. Over time, loss of bladder and bowel function occurs and in advanced disease stage speech and swallowing are affected. Compared to children, who have prominent motor problems early in the disease, adult onset patients have more prominent cognitive problems in early disease stages and they are more likely to have preserved speech function in advanced disease stages. Vision and hearing remain relatively intact in all patients. In general, pain is not a prominent feature.

The attribute utility scores of the HUI do not only represent a measure of health status, but also embody the subjective views of society on objectively defined health status features, as obtained from a random sample of 504 people from Hamilton, Ontario, Canada.[10] A remarkable finding of our study was that, despite the outcome that 1/3 of HUI Health Index scores represented a 'worse than death' health state, most patients were perceived as 'happy' or 'somewhat happy' as indicated by the scores on the HUI attribute 'Emotion'. This discrepancy suggests that the society's perspective may not correspond to the perceived quality of life of affected individuals or their care-givers. Compared to the reference point of a healthy individual, the life of a VWM patient would appear dreary. However, from the perspective of a patient, quality of life may be perceived very differently. This phenomenon is known as the 'disability paradox', the finding that people with serious and persistent disabilities report that they experience a good or excellent quality of life while to most external observers, these individuals seem to live an undesirable life.[22] Along with disease progression, patients may adjust their subjective perception of quality of life and adapt to their new functional abilities. This change of internal standards and values or 'response-shift'[23] is not represented in the HUI. The HUI detects change in symptoms and functions, but the values of health states are fixed and are not allowed to shift according to the experience of patients or carers. Importantly, in the majority of patients the responses were generated by proxies, as the patients' conditions did not allow self-report. There is evidence that both parents and health professionals tend to provide lower valuations for HRQL than affected patients themselves,[24-26] suggesting that the discrepancy between HRQL as perceived by the patients and as perceived

by society would be even greater. The here observed finding that perceived quality of life by patients and carers contrasts with HRQL as measured through public preferences has been described before in other neurological disorders, such as Duchenne muscular dystrophy and cerebral palsy.[26,27] No agreement exists on how to rate HRQL and do justice to both the objective function and the subjective judgement. Consequently, different HRQL scoring systems rate similar clinical conditions in different ways and a perfect system does not exist.

This study has some limitations. The multicenter nature of the study prohibited the application of strict intervals for the clinical scoring. Death was a common cause of missing data and together with loss to follow-up resulted in numerous missing data in all age of onset groups. We have no evidence that non-response was in any way systematic. The large number of patients included enhanced the power of the study, compensating for missing data. Another issue is that the interpretation of the data was hampered by survival effects, with longer follow-up and therefore more follow-up data on patients with milder disease course.
This study also has important strengths, including the large population size for a rare disease, the longitudinal character with a study duration of 12½ years, and the use of standardized instruments that have been extensively validated in diverse populations.[5,16,19,28,29] The latter also enables the comparison of the burden of VWM to other patient groups. The finding that evaluation of scores on domains that were covered by both the HUI and the GNDS showed a significant correlation for all domains supports the application of these questionnaires in VWM. Overall, this study provides robust insights into domains of disability in VWM and reveals age of onset related differences in disease course.

SUPPLEMENTARY DATA

Supplementary methods

Supplementary Table 1| Health Utilities Index Classification System

1A. HUI Scoring

HUI Mark 2 - SELF-CARE	
1	Eats, bathes, dresses, and uses the toilet normally for age
2	Eats, bathes, dresses, or uses the toilet independently with difficulty.
3	Requires mechanical equipment to eat, bathe, dress, or use the toilet independently.
4	Requires the help of another person to eat, bathe, dress, or use the toilet.
HUI Mark 3 - VISION	
1	Able to see well enough to read ordinary newsprint and recognize a friend on the other side of the street, without glasses or contact lenses.
2	Able to see well enough to read ordinary newsprint and recognize a friend on the other side of the street, but with glasses.
3	Able to read ordinary newsprint with or without glasses but unable to recognize a friend on the other side of the street, even with glasses.
4	Able to recognize a friend on the other side of the street with or without glasses but unable to read ordinary newsprint, even with glasses.
5	Unable to read ordinary newsprint and unable to recognize a friend on the other side of the street, even with glasses.
6	Unable to see at all.
HUI Mark 3 - HEARING	
1	Able to hear what is said in a group conversation with at least three other people, without a hearing aid.
2	Able to hear what is said in a conversation with one other person in a quiet room without a hearing aid, but requires a hearing aid to hear what is said in a group conversation with at least three other people.
3	Able to hear what is said in a conversation with one other person in a quiet room with a hearing aid, and able to hear what is said in a group conversation with at least three other people, with a hearing aid.
4	Able to hear what is said in a conversation with one other person in a quiet room, without a hearing aid, but unable to hear what is said in a group conversation with at least three other people even with a hearing aid.
5	Able to hear what is said in a conversation with one other person in a quiet room with a hearing aid, but unable to hear what is said in a group conversation with at least three other people even with a hearing aid.
6	Unable to hear at all.
HUI Mark 3 - SPEECH	
1	Able to be understood completely when speaking with strangers or friends.
2	Able to be understood partially when speaking with strangers but able to be understood completely when speaking with people who know me well.
3	Able to be understood partially when speaking with strangers or people who know me well.
4	Unable to be understood when speaking with strangers but able to be understood partially by people who know me well.
5	Unable to be understood when speaking to other people (or unable to speak at all).
HUI Mark 3 - AMBULATION	
1	Able to walk around the neighbourhood without difficulty, and without walking equipment.
2	Able to walk around the neighbourhood with difficulty; but does not require walking equipment or the help of another person.
3	Able to walk around the neighbourhood with walking equipment, but without the help of another person.
4	Able to walk only short distances with walking equipment, and requires a wheelchair to get around the neighbourhood.
5	Unable to walk alone, even with walking equipment. Able to walk short distances with the help of another person, and requires a wheelchair to get around the neighbourhood.
6	Cannot walk at all.

HUI Mark 3 - DEXTERITY	
1	Full use of two hands and ten fingers.
2	Limitations in the use of hands or fingers, but does not require special tools or help of another person.
3	Limitations in the use of hands or fingers, is independent with use of special tools (does not require the help of another person).
4	Limitations in the use of hands or fingers, requires the help of another person for some tasks (not independent even with use of special tools).
5	Limitations in use of hands or fingers, requires the help of another person for most tasks (not independent even with use of special tools).
6	Limitations in use of hands or fingers, requires the help of another person for all tasks (not independent even with use of special tools).
HUI Mark 3 - EMOTION	
1	Happy and interested in life.
2	Somewhat happy.
3	Somewhat unhappy.
4	Very unhappy.
5	So unhappy that life is not worthwhile.
HUI Mark 3 - COGNITION	
1	Able to remember most things, think clearly and solve day to day problems.
2	Able to remember most things, but have a little difficulty when trying to think and solve day to day problems.
3	Somewhat forgetful, but able to think clearly and solve day to day problems.
4	Somewhat forgetful, and have a little difficulty when trying to think or solve day to day problems.
5	Very forgetful, and have great difficulty when trying to think or solve day to day problems.
6	Unable to remember anything at all, and unable to think or solve day to day problems.
HUI Mark 3 - PAIN	
1	Free of pain and discomfort.
2	Mild to moderate pain that prevents no activities.
3	Moderate pain that prevents a few activities.
4	Moderate to severe pain that prevents some activities.
5	Severe pain that prevents most activities.

1B. HUI Single-Attribute Utility Functions

Level	Selfcare HUI 2	Vision HUI 3	Hearing HUI 3	Speech HUI 3	Ambulation HUI 3	Dexterity HUI 3	Emotion HUI 3	Cognition HUI 3	Pain HUI 3
1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
2	0.85	0.95	0.86	0.82	0.83	0.88	0.91	0.86	0.92
3	0.55	0.73	0.71	0.67	0.67	0.73	0.73	0.92	0.77
4	0.00	0.59	0.48	0.41	0.36	0.45	0.33	0.70	0.48
5	n.a.	0.38	0.32	0.00	0.16	0.20	0.00	0.32	0.00
6	n.a.	0.00	0.00	n.a.	0.00	0.00	n.a.	0.00	n.a.

From Torrence GW *et al.*[9] and Feeny D *et al.*[10]
n.a.: not applicable

1C. HUI3 Multi-Attribute Utility Function

Level X_n	Vision b_1	Hearing b_2	Speech b_3	Ambulation b_4	Dexterity b_5	Emotion b_6	Cognition b_7	Pain b_8
1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
2	0.98	0.95	0.94	0.93	0.95	0.95	0.92	0.96
3	0.89	0.89	0.89	0.86	0.88	0.85	0.95	0.90
4	0.84	0.80	0.81	0.73	0.76	0.64	0.83	0.77
5	0.75	0.74	0.68	0.65	0.65	0.46	0.60	0.55
6	0.61	0.61	n.a.	0.58	0.56	n.a.	0.42	n.a.

From Feeny D *et al.*[10]
Where x_n is the attribute level and b_n is the attribute utility score.
Formula (Dead - Perfect Health scale) $u^* = 1.371 (b1 * b2 * b3 * b4 * b5 * b6 * b7 * b8) - 0.371$
where u^* is the utility of a chronic health state on a utility scale where healthy has a utility of 1.00.
n.a.: not applicable

Supplementary Table 2 | Guy's Neurological Disability Scale

1. Cognitive disability	
0	No cognitive problems.
1	Cognitive problems not noticeable to family or friends.
2	Cognitive problems noticeable to family or friends but not requiring help from others.
3	Cognitive problems requiring help from others for normal daily affairs; patient is fully orientated in time, place and person.
4	Cognitive problems requiring help from others for normal daily affairs; patient is not fully orientated.
5	Patient is completely disorientated in time, place and person.
2. Mood disability	
0	No mood problems
1	Asymptomatic on current drug treatment.
2	Mood problems present but not affecting the patient's ability to perform any of their usual daily activities.
3	Mood problems affecting the patient's ability to perform some of their usual daily activities.
4	Mood problems preventing the patients from doing all their usual daily activities.
5	Mood problems requiring inpatient management.
X	Unknown (scored as the mean of the cognitive and fatigue disability scores rounded the nearest integer).
3. Visual disability	
0	No visual problems.
1	Visual problems (blurred vision, diplopia, scotomas) but patient is still able to read ordinary newspaper print.
2	Unable to read ordinary newspaper print.
3	Unable to read large newspaper print.
4	Unable to count fingers if they hold their hand out in front of them.
5	Unable to see hand movement if they move their hand in front of them.
4. Speech and communication disability	
0	No speech problems.
1	Speech problems which does not require the patient to repeat themselves when speaking to strangers.
2	Speech problems which require the patient to repeat themselves when speaking to strangers.
3	Speech problems which require the patient to repeat themselves when speaking to their family and close friends.
4	Speech problems making speech difficult to understand; patient is able to communicate effectively by using sign language or the help of their carers.
5	Speech problems making speech difficult to understand, patient is unable to communicate effectively by using sign language or the help of their carers.
5. Swallowing disability	
0	No swallowing problems.
1	Needs to be careful when swallowing solids or liquids but not with most meals.
2	Needs to be careful when swallowing solids or liquids with most meals; patient is able to eat food of normal consistency.
3	Needs specially prepared food of modified consistency.
4	Tendency to choke with most meals.
5	Dysphagia requiring nasogastric or gastrostomy tube.
6. Upper limb disability – referred to as "Arm"	
0	No upper limb problem.
1	Problems in one or both arms, not affecting the ability to do any of the functions listed.
2	Problems in one or both arms, affecting some but not preventing any of the functions listed.
3	Problems in one or both arms, affecting all or preventing one or two of the functions listed.
4	Problems in one or both arms preventing three or all of the functions listed.
5	Unable to use either arm for any purposeful movements.

7. Lower limb disability - referred to as "Leg"	
0	Walking is not affected.
1	Walking is affected but patient is able to walk independently.
2	Usually uses unilateral support (single stick or crutch, one arm) to walk outdoors, but walks independently indoors.
3	Usually uses bilateral support (two sticks or crutches, frame, or two arms) to walk outdoors, or unilateral support (single stick or crutch, or one arm) to walk indoors.
4	Usually uses wheelchair to travel outdoors, or bilateral support (two sticks or crutches, frame, or two arms) to walk indoors.
5	Usually uses a wheelchair indoors.
8. Bladder disability	
0	Normal bladder problems.
1	Asymptomatic on current drug treatment.
2	Urinary frequency, urgency, or hesitancy with no incontinence.
3	Occasional urinary incontinence (once or more during the last month but not every week) or intermittent catheterisation without incontinence.
4	Frequent urinary incontinence (once a week or more during the last month but not daily) or occasional urinary incontinence despite regular intermittent catheterisation.
5	Daily urinary incontinence or permanent catheter (urethral/suprapubic) or penile sheath.
9. Bowel disability	
0	No bowel problems.
1	Asymptomatic on current drug treatment or constipation not requiring any treatment.
2	Constipation requiring laxatives or suppositories or faecal urgency.
3	Constipation requiring the use of enemas.
4	Constipation requiring manual evacuation of stools or occasional faecal incontinence (once or more during the last month but not every week).
5	Weekly faecal incontinence.
10. Sexual disabilities	
0	Normal sexual functions or persons who are voluntarily celibate.
1	Reduced sexual interest.
2	Problems satisfying oneself or sexual partner.
3	Physical problems interfering but not preventing sexual function.
4	Autonomic problems interfering but not preventing sexual function.
5	Physical or autonomic problems totally preventing sexual function.
X	Unknown or not applicable (scored as the mean of the lower limb, bladder, and bowel disability scores rounded to the nearest integer).
11. Fatigue	
0	Absent.
1	Occasional fatigue (present some days).
2	Frequent fatigue (present most days).
3	Fatigue affecting the patient's ability to perform some of their usual daily activities.
4	Fatigue preventing the patient from doing all their usual daily activities.
5	Fatigue preventing the patient from doing all their physical activities.
X	Unknown (scored as the mean of the cognitive and mood disability scores rounded to the nearest integer).
12. Other disabilities	
0	Absent.
1	Asymptomatic on current drug treatment.
2	Problems, present, but are not affecting the patient's ability to perform any of their usual daily activities.
3	Problems affecting the patient's ability to perform some of their usual daily activities.
4	Problems preventing the patient from doing all their usual daily activities.
5	Problems requiring hospital admission for assessment or treatment.

From Sharrack B *et al.*[16]
The GNDS is also known as the Neurological disability Scale (NDS) or the UK Neurological disability Scale (UKNDS)

Supplementary results

Supplementary Table 3 | Frequency distribution of HUI attribute levels

Level	HUI3 Cognition	HUI3 Emotion	HUI3 Vision	HUI3 Speech	HUI3 Hearing	HUI3 Dexterity	HUI3 Ambulation	HUI3 Pain	HUI2 Self-care
1 (normal)	77	150	136	134	238	66	46	134	71
2	46	65	72	47	4	51	31	72	45
3	10	20	3	29	2	8	18	32	3
4	46	15	10	9	4	30	30	17	139
5	31	8	17	39	0	33	30	3	n.a.
6 (most disabled)	48	n.a.	20	n.a.	10	70	103	n.a.	n.a.
Mean single-attribute score	0.65	0.89	0.85	0.76	0.95	0.53	0.39	0.90	0.43

The mode frequency is highlighted in green

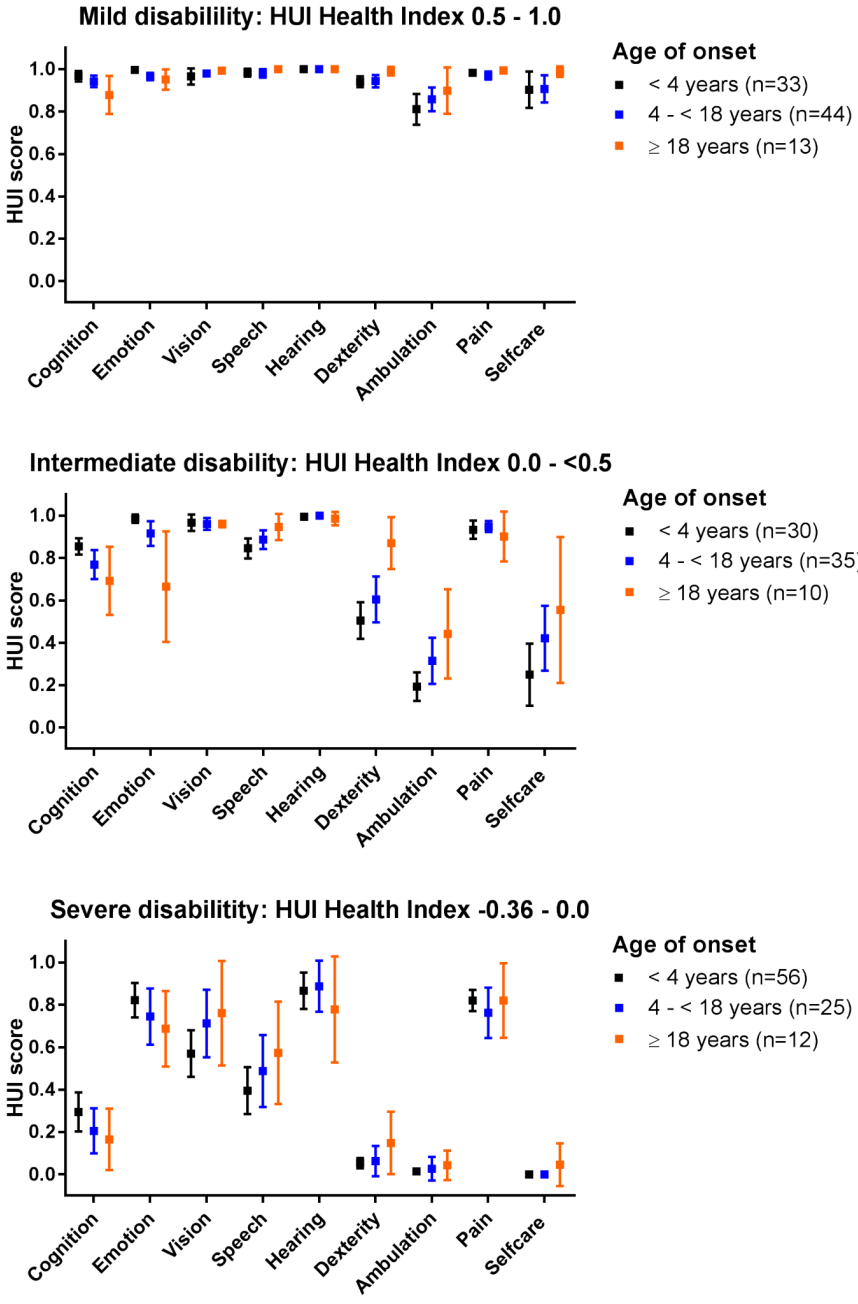
Supplementary Table 4 | Frequency distribution of GNDS score per disability domain

Level	Cognitive	Mood	Visual*	Speech / Commu-nication	Swallow-ing	Arm	Leg	Bladder	Bowel	Fatigue	Others**
0 (normal)	54	105	122	79	120	40	34	85	101	72	85
1	2	2	12	23	9	8	15	0	10	19	1
2	10	24	15	0	6	13	13	14	22	22	19
3	52	20	7	31	9	32	11	7	9	27	25
4	35	14	7	9	11	33	17	11	10	8	22
5 (total loss of function)	19	7	9	30	17	46	82	55	20	24	20
Mean domain score	2.40	1.17	0.79	1.76	1.03	2.86	3.21	2.14	1.28	1.72	1.76

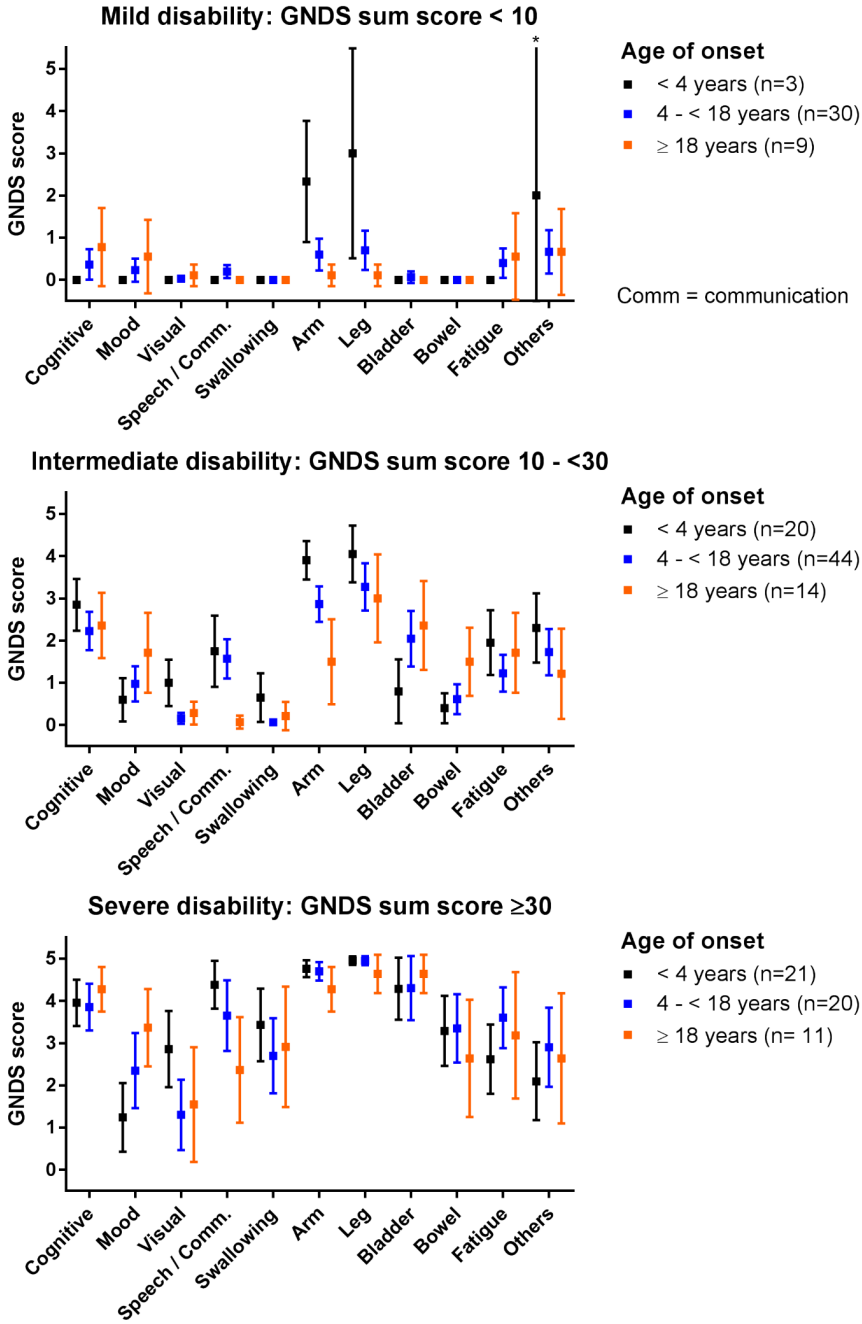
The mode frequency is highlighted in green
* The discrepancy between the HUI and GNDS scores on visual function can be explained by the difference in rating systems, where the HUI does and GNDS does not consider wearing glasses/contact lenses as abnormal (Supplementary Tables 1A and 2).
** see Supplementary Table 5

Supplementary Table 5 | Overview GNDS domain 'Other' problems

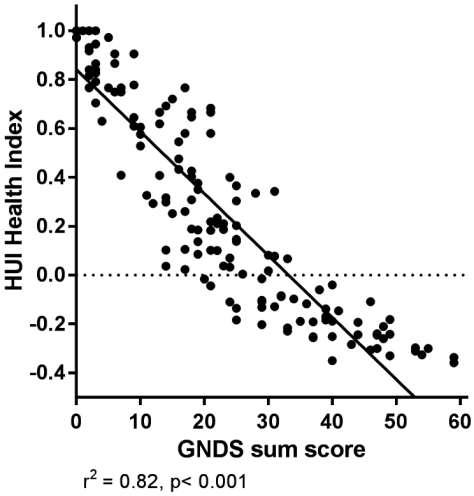
Reported problem	n=
muscle spasms / cramps	24
pain limbs	15
Headache/migraine	14
Dizziness	9
Seizures	9
Spasticity	5
Osteoporosis	3
Rigidity	3
Balance problems	1
Autonomic neuropathy	1
Scoliosis	1
Aggression	1
Gastroparesis	1
Decubitus	1



Supplementary Figure 1 | Distribution of HUI attribute levels. Mean scores per attribute level and 95% confidence intervals are shown for patients with (A) mild disability - HUI Health Index ≥0.5 - 1.0, (B) intermediate disability - HUI Health Index ≥0.0 - <0.5 and (C) severe disability - HUI Health Index -0.36 - <0.0, with a subdivision for age of onset. A single-attribute score of 1.0 represents normal status, a score of 0.0 represents a most disabled status.



Supplementary Figure 2 | Distribution of GNDS scores per domain. Mean scores per domain and 95% confidence intervals are shown for patients with (A) mild disability – GNDS sum score < 10, (B) intermediate disability - GNDS sum score 10- < 30 and (C) severe disability - GNDS sum score ≥ 30, with a subdivision for age of onset. A domain score of 0 represents normal status and a score of 5 total loss of function.
* For this function the confidence interval ranges from -3 to 7.



Supplementary Figure 3 | Correlation HUI Health Index and GNDS sum score. Linear regression analysis shows a significant correlation between individuals' HUI Health Index scores and GNDS sum scores

REFERENCES

1. van der Knaap MS, Pronk JC, Scheper GC. Vanishing white matter disease. Lancet Neurol 2006;5:413-423.
2. Labauge P, Horzinski L, Ayrignac X, et al. Natural history of adult-onset eIF2B-related disorders: a multi-centric survey of 16 cases. Brain 2009;132:2161-2169.
3. Fogli A, Schiffmann R, Bertini E, et al. The effect of genotype on the natural history of eIF2B-related leukodystrophies. Neurology 2004;62:1509-1517.
4. Horsman J, Furlong W, Feeny D, Torrance G. The Health Utilities Index (HUI): concepts, measurement properties and applications. Health Qual Life Outcomes 2003;1:54.
5. Feeny D, Leiper A, Barr RD, et al. The comprehensive assessment of health status in survivors of childhood cancer: application to high-risk acute lymphoblastic leukaemia. Br J Cancer 1993;67:1047-1052.
6. Gemke RJ, Bonsel GJ, van Vught AJ. Long-term survival and state of health after paediatric intensive care. Arch Dis Child 1995;73:196-201.
7. Fisk JD, Brown MG, Sketris IS, Metz LM, Murray TJ, Stadnyk KJ. A comparison of health utility measures for the evaluation of multiple sclerosis treatments. J Neurol Neurosurg Psychiatry 2005;76:58-63.
8. Kennes J, Rosenbaum P, Hanna SE, et al. Health status of school-aged children with cerebral palsy: information from a population-based sample. Dev Med Child Neurol 2002;44:240-247.
9. Torrance GW, Feeny DH, Furlong WJ, Barr RD, Zhang Y, Wang Q. Multiattribute utility function for a comprehensive health status classification system. Health Utilities Index Mark 2. Med Care 1996;34:702-722.
10. Feeny D, Furlong W, Torrance GW, et al. Multiattribute and single-attribute utility functions for the health utilities index mark 3 system. Med Care 2002;40:113-128.
11. Drummond M. Introducing economic and quality of life measurements into clinical studies. Ann Med 2001;33:344-349.
12. Grootendorst P, Feeny D, Furlong W. Health Utilities Index Mark 3: evidence of construct validity for stroke and arthritis in a population health survey. Med Care 2000;38:290-299.
13. Naeim A, Keeler EB, Mangione CM. Options for handling missing data in the Health Utilities Index Mark 3. Med Decis Making 2005;25:186-198.
14. Pogany L, Barr RD, Shaw A, Speechley KN, Barrera M, Maunsell E. Health status in survivors of cancer in childhood and adolescence. Qual Life Res 2006;15:143-157.
15. Fryback DG, Dunham NC, Palta M, et al. US norms for six generic health-related quality-of-life indexes from the National Health Measurement study. Med Care 2007;45:1162-1170.
16. Sharrack B, Hughes RA. The Guy's Neurological Disability Scale (GNDS): a new disability measure for multiple sclerosis. Mult Scler 1999;5:223-233.
17. Van den Berg-Vos RM, Franssen H, Wokke JH, Van den Berg LH. Multifocal motor neuropathy: long-term clinical and electrophysiological assessment of intravenous immunoglobulin maintenance treatment. Brain 2002;125:1875-1886.
18. Khan F, Amatya B, Ng L, Galea M. Rehabilitation outcomes in persons with spina bifida: A randomised controlled trial. J Rehabil Med 2015;47:734-740.
19. Rossier P, Wade DT. The Guy's Neurological Disability Scale in patients with multiple sclerosis: a clinical evaluation of its reliability and validity. Clin Rehabil 2002;16:75-95.
20. Hoogervorst EL, Kalkers NF, van Winsen LML, Uitdehaag BM, Polman CH. Differential treatment effect on measures of neurologic exam, functional impairment and patient self-report in multiple sclerosis. Mult Scler 2001;7:335-339.
21. Janse AJ, Sinnema G, Uiterwaal CS, Kimpen JL, Gemke RJ. Quality of life in chronic illness: children, parents and paediatricians have different, but stable perceptions. Acta Paediatr 2008;97:1118-1124.
22. Albrecht GL, Devlieger PJ. The disability paradox: high quality of life against all odds. Soc Sci Med 1999;48:977-988.
23. Sprangers MA, Schwartz CE. Integrating response shift into health-related quality of life research: a theoretical model. Soc Sci Med 1999;48:1507-1515.
24. Saigal S, Stoskopf BL, Feeny D, et al. Differences in preferences for neonatal outcomes among health care professionals, parents, and adolescents. JAMA 1999;281:1991-1997.
25. White-Koning M, Arnaud C, Dickinson HO, et al. Determinants of child-parent agreement in quality-of-life reports: a European study of children with cerebral palsy. Pediatrics 2007;120:e804-814.

26. Landfeldt E, Lindgren P, Bell CF, et al. Health-related quality of life in patients with Duchenne muscular dystrophy: a multinational, cross-sectional study. Dev Med Child Neurol 2016;58:508-515.
27. Petrou S, Kupek E. Estimating preference-based health utilities index mark 3 utility scores for childhood conditions in England and Scotland. Med Decis Making 2009;29:291-303.
28. Furlong WJ, Feeny DH, Torrance GW, Barr RD. The Health Utilities Index (HUI) system for assessing health-related quality of life in clinical studies. Ann Med 2001;33:375-384.
29. Hoogervorst EL, van Winsen LM, Eikelenboom MJ, Kalkers NF, Uitdehaag BM, Polman CH. Comparisons of patient self-report, neurologic examination, and functional impairment in MS. Neurology 2001;56:934-937.

V

Conclusions

Chapter 8

Summary and general discussion

White matter disorders comprise a large group of diseases with diverse pathologic mechanisms. Genetic white matter disorders, leukodystrophies, mostly affect children. They collectively have an estimated incidence of 1 in ~7500 livebirths,[1] but each individual disease is extremely rare, qualifying for the term “orphan disease”. This term implies two separate but related concepts: I) the diseases affect only small numbers of individuals, II) the diseases are largely neglected by professionals.[2] As a consequence, the field of leukodystrophies has long suffered from a deficit of medical and scientific attention and knowledge. Fortunately, over the past 3 decades major developments have been made in the classification and recognition of leukodystrophies, owing to technological developments such as advanced brain imaging techniques and advanced genetic analyses. Curative therapies are currently restricted to only a few specific diseases, but with improved understanding of the pathologic mechanisms and increasing technological possibilities, hope for therapeutic options is rising for a growing number of leukodystrophies. Such developments may, however, not be relevant for patients suffering from leukodystrophy today. Research should therefore also be focused on symptomatic and supportive patient care.

The work described in this thesis concerns clinical and genetic aspects of 4 different leukodystrophies. This chapter outlines the background of the research, summarizes and discusses the main findings and implications, and addresses future directions.

CHALLENGES IN THE FIELD OF LEUKODYSTROPHIES

Establishing a diagnosis

“Leukodystrophy” is a broad term that captures a heterogeneous patient population both in terms of clinical symptoms and age of onset. The presence of a white matter disorder is usually established by MRI, but reaching a definitive diagnosis is a challenging task. It requires detailed knowledge of clinical features and neuroimaging details. Several diseases closely resemble each other. On the other hand, phenotypes associated with one particular disease can be very divergent. Identifying the hallmark radiological features as well as key differentiating clinical phenotypes is an essential part of diagnostication.

A recent inventory by The Global Leukodystrophy Initiative (GLIA) indicated that only a minority of members of the “Society for Inherited Metabolic Disorders” and the “Child Neurology Society” felt comfortable with the neuroimaging patterns and diagnostic approaches for leukodystrophies.[3] The diagnostic work-up in leukodystrophy patients is time- and money-consuming and burdensome for patients and families. A retrospective survey among 269 patients suspected of a leukodystrophy indicated that, on average, 20 diagnostic tests were performed in each patient.[4]

An important point of note is that establishing a radiological diagnosis is only possible if the disorder has already been defined. Obtaining a molecular diagnosis is only

possible if the associated gene defect has already been identified. Fortunately, the combination of MRI, careful clinical evaluation and next generation sequencing (NGS) is extremely powerful for both expediting the diagnostic process and dramatically reducing the number of unsolved cases.

Scattered data

In phenotyping surveys, large cohorts of patients are required to reliably study disease characteristics and to relate phenotype to genetic and environmental factors. Increasing the numbers of patients in a survey increases the likeliness of identifying patterns, for instance regarding provoking or prognostic factors that may otherwise remain unnoticed. The extreme rarity of the different leukodystrophies has unfortunately hindered adequate patient enrollment in epidemiological and observational studies. Research initiatives are mostly small-scaled and mono-centered, and different investigators often apply different methods and instruments, hampering the comparison of data. Ideally, the collection of data would be multi-institutional and systematic, with consistent use of data collection instruments.

International Classification of Diseases

Over the last years, the better access to MRI has led to improvement and systematization of the diagnosis of leukodystrophies. Nevertheless, systems for reporting and tracking such diagnoses are lacking. Consequently, the leukodystrophy field is short of reliable epidemiological data on the prevalence and incidence of disease variants in national and global populations. The true incidence may be higher than currently reported.[1,5] More detailed epidemiologic studies would be helpful, also to justify financial support for research into these disorders based on their relevance to public health. An additional difficulty for epidemiologic studies concerns the lack of classification and condition-specific codes in the World Health Organization's (WHO) International Classification of Diseases (ICD). The ICD provides the international standard diagnostic classification used for epidemiology studies, health system management functions and clinical purposes. It allows monitoring of a diseases' symptoms, their incidence and prevalence, as well as for instance observing resource allocation trends.[6] There are separate ICD codes for X-linked adrenoleukodystrophy and metachromatic leukodystrophy, but more recently described leukodystrophies such as Vanishing White Matter (VWM) are not explicitly listed.[7] The WHO, in collaboration with rare diseases organization Orphanet, has now initiated an international advisory group on revision of the ICD codes. The new release of ICD-11 will more adequately code rare diseases.[8] Its recommendations should strengthen the foundation for epidemiologic and fundamental research on rare disease such as leukodystrophies, as electronic health record data can facilitate research if patients with specific diseases can be easily and reliably identified.

Clinical knowledge

The presence of an MRI diagnosis or, preferably, molecular diagnosis, is an important step for counseling of leukodystrophy patients and families. A common

misconception, also among clinicians, is that all leukodystrophies are associated with rapidly progressive loss of functions and early death. There is, however, very wide variability in disease severity and one leukodystrophy can have a wide clinical spectrum, also comprising relatively mild phenotypes. Physicians need sufficient knowledge of the disease and the associated phenotypic spectrum to place a diagnostic test into a clinical context. For the prediction of disease progression, insight in prognostic factors such as age of onset and genotype-phenotype correlations is required. For decisions on preventive and therapeutic strategies, knowledge of disease' symptoms in relation to disease stage and available interventions is necessary. For many leukodystrophies, detailed clinical information is not available, hampering the ability to provide patients and families with adequate information on signs and symptoms, rate of progression and life expectancy. Publications on rare diseases often concern the extreme and unusual cases, which introduces a bias. Systematic inventories of diseases' clinical spectra and natural disease courses are scarce. Even if clinical characteristics of a disorder have been investigated, it should be taken into account that concerned physicians often have no experience with the disease and depend on literature and guidelines for information and decision making. Centralization of patient care may therefore improve the quality and efficacy of services.[7] However, since it is also desirable to deliver supportive care close to home, optimal availability of clinical guidelines for non-expert physicians remains warranted.

Translational studies

The functionality of the brain white matter relies on an ingenious interplay between oligodendrocytes, astrocytes and axons. Accordingly, the disease mechanisms underlying leukodystrophies are very complex. Recognition of the cellular and molecular pathology behind a disease is crucial for the development of therapeutic strategies. Important insights into the pathophysiology of leukodystrophies have been achieved by neuropathological studies, for instance by their implications for the understanding of which cell type is primarily affected. Autopsy material is unfortunately scarce: for several leukodystrophies no neuropathology data are available at all. Next, in vitro studies and animal models are key to further model diseases. As the discovery of mutated genes associated with leukodystrophies allow further unraveling of pathogenesis, the growing number of discoveries of mutated genes leads to increased understanding of mechanisms of white matter injury.

Clinical trial design

Once treatment strategies become available, trial designs for rare diseases have to meet the same rigorous standards as those for trials for more prevalent diseases.[9] They must ask important scientific questions, minimize bias, be adequately powered and have prospect of achieving a scientifically acceptable answer. The power norm comes with logistic challenges with regard to recruitment of patients. Randomized controlled trials are often considered the gold standard for treatment evaluation, but

may not be feasible for leukodystrophies due to inadequate power. Randomization may also be of concern, as the different arms of a study may not be considered equivalent by the study subjects and/or their physicians.[10] An alternate design would be to use historical controls or have participants serve as their own control. These approaches may also be more efficient as they require fewer patients to be included. In any case, appropriate trial design requires a thorough knowledge of the epidemiology and natural history of a disorder, providing a reference point for the evaluation of therapeutic interventions.

OBJECTIVES

To improve the care for leukodystrophy patients worldwide in all aspects, the Center for childhood white matter disorders in Amsterdam performs multi-disciplinary research. This includes clinical phenotyping, defining of novel disorders, molecular genetic studies and the exploration of disease mechanisms. The final goal is to establish curative treatment for these patients. A crucial element for moving forward in the understanding of disease mechanisms is bringing together experts from diverse disciplines and study a disease at different levels.[11] There are different ways to approach a complex biological process. One is the “bottom-up” approach. This starts with the gene, followed by protein sequence and function and next, all additional biological levels. Balancing this with a “top-down” approach, taking the patient as the starting point, may identify additional research questions and measurable goals that are grounded in patient’s needs. All in all, studying a leukodystrophy by an integrated approach of bidirectional causation (Figure 1) can help to further unravel disease mechanisms and establish therapy designs. Observations from clinical phenotyping are often a starting point for the further unraveling of disease mechanisms, offering guidance for basic research.

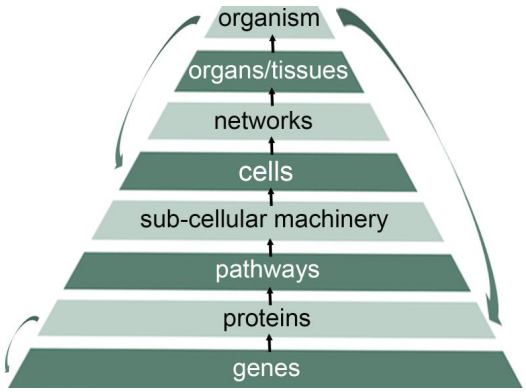


Figure 1 | System biology approach of bidirectional causation. Insights in disease mechanisms can be obtained by studying how functions arise in dynamic interactions. Loops of interacting downward and upward causation can be built between all levels of biological organization. Adapted from Noble 2006.[12]

This thesis addresses the clinical and genetic features of 4 different "new" leukodystrophies: LBSL, MLC, H-ABC and VWM. The overall objectives were threefold:

I) Better delineate the phenotypic and genotypic spectrum of leukodystrophies in order to improve patient information and genetic counseling
II) Provide natural history data for the planning and evaluation of future therapeutic trials
III) Enhance the understanding of the underlying disease mechanisms

KEY FINDINGS AND IMPLICATIONS

Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL)

LBSL is a leukodystrophy with a highly distinctive MRI pattern consisting of cerebral white matter abnormalities and selective involvement of brainstem and spinal cord tracts.[13,14] The disease was initially described as a juvenile onset disease with slowly progressive ataxia, spasticity and dorsal column dysfunction.[13,15-18] Patients have recessive mutations in the *DARS2* gene, encoding the mitochondrial aspartyl-tRNA synthetase (mtAspRS).[19] The identification of the gene defect facilitates studies on the **clinical disease spectrum** and the exploration of therapeutic targets.

In chapter 2 we describe a cross-sectional observational study among LBSL patients. Clinical data on 66 patients show that the disease severity ranges from infantile onset, rapidly fatal disease to adult onset, very slow disease that may not limit life expectancy. In most cases the disease presents in late-childhood or adolescence and follows a mild course without sudden deteriorations. The study illustrates that not all leukodystrophies are associated with rapid neurological decline and that patients may remain stable for years. Almost all LBSL patients are ambulant without or with support and they generally have preserved manual dexterity. Full wheelchair dependency is very rare and usually does not occur before patients reach adulthood. Patients presenting in their teens or in adulthood continue to be able to walk without support in at least the first 10 years after onset. Few patients present before the age of 18 months; they exhibit more profound cerebral white matter abnormalities and show severe motor problems from early on. In all variants, including severe variants, mortality is low. Of the studied cohort, 2 patients deceased. For the majority of patients, life expectancy may be normal.

We provide an overview of all 60 *DARS2* mutations that had been identified at time of the study. The large genetic heterogeneity hampers a formal genotype-phenotype study, but some mutations are consistently associated with a similar phenotype, suggesting the existence of a **genotype-phenotype correlation**. Ninety-four percent of patients had a splice site mutation in intron 2, and all but 4 patients from 2 families were compound heterozygous.[20,21] It is striking that homozygosity is very uncommon among LBSL patients. This observation suggests that the range of permissive

mutations is narrow. Mutations with a severe phenotypic effect are expected not to be compatible with life in the homozygous state. This hypothesis has been substantiated for another aminoacyl-tRNA synthetase (ARS) by an animal model.[22] Biallelic mutations with a mild phenotypic effect, on the other hand, may not give rise to a disease phenotype at all. This theory is supported by the finding that the most common *DARS2* intron 2 mutation has a high carrier rate (1 in 95 individuals) in the Finnish population.[23] Since this variant has not been reported in the homozygous state in any LBSL patient, its effect is presumable mild and homozygosity for this variant probably does not lead to disease.

Over the recent years, numerous defects in genes encoding ARSs have been associated with neurological diseases, including leukodystrophies.[24,25] ARSs are housekeeping proteins whose main recognized function is to catalyze the attachment of amino acids to their cognate tRNAs, a key step in protein synthesis.[26] For most amino acids, there are separate cytosolic and mitochondrial synthetases encoded by different genes; the latter are given the same name as the genes encoding cytosolic variants, appended with the number 2 (e.g., *DARS* encodes aspartyl-tRNA synthetase (AspRS) and *DARS2* encodes mitochondrial AspRS (mtAspRS)). Intriguingly, the highly specific brainstem and spinal cord abnormalities observed on MRI in LBSL patients (chapter 2) are also present in patients with Hypomyelination with Brain stem and Spinal cord involvement and Leg spasticity (HBSL) caused by *DARS* mutations.[27] Defining the mechanisms that underlie tissue-specific effects of mutations in various *-ARS* and *-ARS2* genes is a challenging task. An interesting feature of ARS biology is that many of these proteins have a second function, unrelated to aminocylation, concerning a wide array of activities.[28,29] Remarkably, crystal structure analysis of certain cytosolic and mitochondrial ARSs has demonstrated a similar 3D structure despite poor sequence homology of their respective genes, raising the possibility of functional overlap.[30] The **selective vulnerability** of structures observed in LBSL and HBSL suggests that perhaps the cytosolic and mitochondrial AspRSs are both involved in a currently unknown, non-canonical function. Recently, a proteomics approach revealed the coexistence of different forms of human mtAspRS; the next step is to decipher their biological roles and possible contribution to functions unrelated to the aminoacylation process.[31]

Cells have an absolute need for ATP produced by mitochondria. Consequently, the cell exerts constant surveillance and control of mitochondrial processes. It has been suggested that mitochondrial translation ensures crosstalk between cellular programs and cellular energy demands.[29] The nervous system is known to be particularly vulnerable to mitochondrial dysfunction due to its high energy demand. This may partially explain the selective involvement CNS structures in LBSL. However, considering the highly selective involvement of specific structures, additional factors must play a role in the vulnerability. Observation of phenotypic characteristics in LBSL patients, including radiological features, can provide clues for the understanding of the **cellular process** underlying the disease. All patient exhibit

involvement of multiple long-tracts. Consequently, it can be argued that LBSL might originate from a defect that primarily involves neurons and axons, rather than a defect of myelin, oligodendrocytes or astrocytes. A splicing reporter construct essay by van Berge *et al.,* indicates that the size of the effect of the intron 2 mutation differs for different cell types. For part of the mutated messenger RNAs, exon 3 is still included, resulting in normal, full-length protein. Neural cell types, particularly neuronal cells, show more pronounced exclusion of exon 3. The variability in vulnerability of different cell types could perhaps be explained by differences in presence of splicing factors in different cell types and different anatomical brain regions.[32,33] In addition, it was found that also in normal mtAspRS mRNA, inclusion of exon 3 occurs least efficiently in neuronal cells. These two effects may explain the selective vulnerability of specific axonal tracts in LBSL. The hypothesis of neuronal vulnerability for *DARS2* mutations is further substantiated by a study investigating transgenic mice in which *DARS2* was specifically depleted in forebrain-hippocampal neurons or myelin-producing cells.[34] The results indicate that loss of *DARS2* in neurons leads to strong mitochondrial dysfunction and progressive loss of cells. In contrast, myelin-producing cells seem to be resistant to cell death induced by *DARS2* depletion, arguing that LBSL might originate from a primary neuronal and axonal defect.

Insights in disease mechanisms provide guidance for therapeutic strategies. Considering that almost all LBSL patients have an intron 2 splice site mutation and that that these mutations are 'leaky',[35] increasing the correct splicing of exon 3 is a promising **target for treatment**. In chapter 2 we show that splicing efficiency can be increased by the use of certain compounds. Using a library of 2000 FDA approved compounds, cantharidin, a protein phosphatase inhibitor that dephosphorylates splicing factors, was identified as the most potent compound to increase exon 3 inclusion. Its effect has previously been reported in studies on Spinal Muscular Atrophy (SMA), shifting the proportion of SMN2 protein from a dysfunctional to a functional form.[36] Cantharidin is unfortunately a highly toxic agent, but the study does provide proof-of-concept that influencing splice site mutations is possible. This holds promise for the treatment of LBSL. Since cantharidin is also known to efficiently inhibit various tumor cell lines, several investigations have been initiated to search for viable methods to reduced its side effects and to identify analogues that inhibit protein phosphatase similar to cantharidin without high toxicity.[37] Another potential therapeutic approach would be the application of antisense oligodeoxynucleotides (ASOs), involving the application of short, chemically modified fragments of DNA that bind cognate mRNA and thereby alter splicing. The development of such therapies is still ongoing.

Megalencephalic leukoencephalopathy with subcortical cysts (MLC)

The brain is encased by a rigid bony skull. As a consequence, even small increases in tissue volume can cause significant rises in intracranial pressure and compression of brain tissue, with potentially dramatic consequences. Only in infants, in whom the

skull sutures have not closed yet, compensation through abnormal increase in head size may occur. To prevent injury caused by volume changes, the brain possesses a sophisticated system for volume regulation. The disorder MLC exemplifies the relevance of water homeostasis in the brain and the central role of astrocytes in this process. The disease starts in infancy and is characterized by white matter edema with increased brain size, accommodated by increased head size.[38-40] MRI shows diffuse signal abnormalities and swelling of the cerebral white matter as well as subcortical cysts, typically located in the anterior temporal region.[38,41] Two different MLC phenotypes can be distinguished: a classic, deteriorating phenotype and a remitting phenotype.[42] Classic MLC is caused by recessive mutations in the *MLC1* gene in the majority of cases: this variant is called MLC1.[43] Few patients with classic MLC have recessive *GLIALCAM* mutations, this variant is called MLC2A.[43] Patients with dominant *GLIALCAM* mutations have remitting MLC: this variant is called MLC2B.[44]

To systematically evaluate clinical and MRI characteristics in MLC patients, we initiated a multi-institutional observational study. In chapter 3 we report on the **clinical and radiological spectrum** in 242 MLC patients. The first aim of the study was to delineate the clinical spectrum of MLC, aimed at improved clinical counseling. We describe a fairly mild and slow clinical course in most patients with classic MLC, although the degree of disability is variable. The most common first disease sign is macrocephaly in the first year of life. Initial motor development is usually mildly delayed or normal. Almost all patients achieve unsupported walking. Slow motor deterioration generally starts several years after disease onset. The majority of patients eventually lose the ability to walk without support, at variable ages (ranging from 1 to 43 years). Patients may become fully wheelchair dependent but many remain ambulant. A new observation is that patients who are still able to walk with or without support at the age of 15 years most likely retain this ability. Mortality is low. A few patients die due to epilepsy-related causes.

MLC patients typically have early onset epilepsy. In general, epilepsy is an early and prominent sign in cortical neuronal degenerative disorders, while in white matter disorders epilepsy occurs less frequently and typically has a delayed onset, related to advanced tissue damage.[45,46] It is striking to see that in many MLC patients, epilepsy is one of the first signs of the disease, occurring before onset of motor deterioration (chapter 3). Another observation from the clinical inventory was the high susceptibility (54% of patients with seizures) to develop seizures after a mild head injury.[47] While severe traumatic brain injury is a major cause of seizures, studies on the occurrence of seizures after mild head trauma reveal no or only slightly increased risk.[48,49] These observations suggests that MLC patients may have a lowered threshold for seizures. Additionally, the occurrence of status epilepticus is relatively high among MLC patients, even though most have well controlled epilepsy, suggesting that a seizure, once begun, evolves into status epilepticus relatively easily.[47] These clinical observations prompted further study of the **mechanism of seizures** in MLC. Seizures were long thought to be primarily caused by malfunction of neurons, but in

the last decades many studies have shown that alterations in astrocyte function also play an important role in seizure pathogenesis.[50] Being equipped with important ion and water channels necessary for executing homeostatic functions, astrocytes are central cells in brain volume regulation. In MLC patients, loss of function of the MLC1 protein due to *MLC1* or *GLIALCAM* mutations leads to high water content in the brain white matter. MLC1 is a membrane protein that is highly expressed in astrocytic endfeet in the brain.[51] GlialCAM is a chaperone of MLC1 which ensures its localization in the membrane of astrocytic endfeet.[52] Cell swelling studies have demonstrated that MLC1 is involved in astrocyte volume regulation and disturbed brain ion and water homeostasis.[40,53] To study the basis of seizures in MLC, Dubey *et al.* studied the effect of astrocyte dysfunction on neuronal networks in MLC mouse models.[47] MLC mice display progressive intramyelinic vacuolization, making them excellent models to study the pathophysiology of MLC.[53] The experiments investigated the intrinsic excitability of pyramidal neurons in brain slices of MLC mice and wild type mice by using whole-cell patch-clamp recordings. Intriguingly, the excitability was unchanged in MLC mice, indicating that intrinsic hyperexcitability of principal neurons is not the cause of seizures in MLC. Building on the information that astrocyte ion and water homeostasis is disturbed in MLC, it was hypothesized that MLC1 and GlialCAM might be necessary for the clearance of potassium (K+) from the extracellular space. K+ release occurs during repetitive action potential firing and removal of excess K+ is needed to prevent action-potential induced intramyelinic edema.[43] Clearance is also necessary to prevent hyperexcitability caused by accumulation of extracellular K+. This theory was substantiated by the finding that upon synaptic stimulation, there was a significant higher rise in extracellular K+ in MLC mice as compared to controls.[47] Furthermore, in MLC mice an increased excitability of neuronal networks was observed.[47] The work puts forward that astrocyte dysfunction in MLC leads to disturbed K+ dynamics and network hyperexcitability. As a consequence of this disturbance, MLC patients have a lowered threshold for seizures; once a seizure has started, the train of action potentials enhances the K+ buffering problem and increases the risk of status epilepticus. These insights form an important step in the understanding of the pathophysiology of MLC.

Recessive mutations in either *MLC1* or *GLIALCAM* lead to loss of MLC1 function and result in a similar clinical disease.[52] [42] It is remarkable that the 2 classic forms of the disease, MLC1 and MLC2A, appear to be indistinguishable, as GlialCAM is also a chaperone for other proteins, such as connexin 43 and the chloride channel ClC2.[54,55] It could therefore be hypothesized that loss of GlialCAM would result in additional phenotypic characteristics besides the MLC phenotype. In line with this theory, another aim of the work described in chapter 3 was to identify possible features that differentiate between the different disease variants. The phenotypic inventory and the systematic MRI review indicate that the clinical course and the radiological pattern do not differentiate between MLC1 and MLC2A. One theory is that GlialCAM may not be the only chaperone for connexin 43 and ClC2; other

transporters may successfully compensate for loss of GlialCAM function.

MLC is a relatively mild disease in terms of progression rate and mortality. The fact that some patients have homozygous nonsense mutations, indicates that the MLC1 protein is not be conditional for life. All *MLC1* mutations lead to the same result, namely major reduction or absence of the plasma membrane expression of *MLC1* in astrocytic endfeet.[56] Accordingly, no clear **genotype-phenotype** correlation can be established for MLC (chapter 3). A remaining question is what then explains the observed variability in disease severity for different patients, even within families. A possible explanation is that severity may depend on individual differences in additional compensatory brain ion and water homeostasis mechanisms.

Compared to classic MLC patients, MLC2B patients have a milder phenotype with preservation of motor function, while intellectual disability and autism are relatively frequent. Radiological improvement is observed in all MLC2B patients and also in two MLC1 patients. Certain findings in MRIs obtained in the early disease stage are suggestive of MLC2B: absence of signal abnormalities of the posterior limb of the internal capsule and the cerebellar white matter and presence of only rarefied subcortical white matter instead of true subcortical cysts.

Overall, the findings of our study on the phenotypic characteristics of MLC can aid physicians with the **guidance and prognostication** of patients. Furthermore, the findings facilitate focused genetic testing in patients suspected of the remitting phenotype. The observation that the latter group initially mimics classic MLC and subsequently improves, raises the hope that if classic MLC is pharmacologically corrected at an early stage, the disease may be reversible. Influencing of astrocytic volume regulation appears to be an interesting target for therapeutic interventions for this disorder.
In the near future, additional genes involved in brain ion and water homeostasis may be discovered. By studying their effect on neuronal activity and their interaction with MLC1, GlialCAM, aquaporin-4 and other astrocyte volume regulators, we can gain more insight into the disease MLC and also learn about normal brain physiology.

Hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC)

H-ABC is characterized by hypomyelination and atrophy of neostriatum and cerebellum on both MRI and neuropathology studies.[57,58] The genetic cause of the disease was identified in 2013, by the application of a whole exome sequencing (WES) study in 11 patients selected on the basis of very strict clinical and MRI criteria.[59] All patients turned out to harbor the same heterozygous mutation in the *TUBB4A* gene, underlining the successful selection of patients on the basis of a strictly selected comparable phenotype. The discovery of the genetic cause of the disease enabled further delineation of the phenotypic characteristics. In chapter 4 we describe the clinical and genetic *TUBB4A* mutation spectrum in 42 patients fulfilling

the MRI criteria of H-ABC. Patients showed a **phenotypic continuum** with neonatal up to childhood onset, normal or delayed early development and slow to more rapid neurological deterioration. Neurological symptomatology consisted of extrapyramidal movement abnormalities, spasticity, ataxia, cognitive deficit and sometimes epilepsy. Three patients died. On MRI, the degree of hypomyelination and basal ganglia atrophy was variable. All patients had an absent or disappearing putamen. There was a variable degree of cerebellar atrophy and highly variable cerebral atrophy. Apart from hypomyelination, myelin loss was evident in several cases. Three severely affected patients had similar, somewhat atypical MRI abnormalities. The *TUBB4A* mutation observed in the first 11 patients was the most common (25 patients). Additionally, 13 other heterozygous mutations were identified. Patients with the common mutation generally had a less rapidly progressive disease course than the 17 cases with other *TUBB4A* mutations. MRI findings were very similar for patients with similar genotypes. Overall, the findings of the study were strongly suggestive of a **genotype-phenotype correlation**. Subsequent literature confirmed this correlation (see chapter 4.2 and 4.3).

An array of neurological disorders has been associated with mutations in tubulin genes.[60,61] The combining of α-tubulins with β-tubulins allows the formation of heterodimers that assemble into microtubules. Microtubules are essential components of the cytoskeleton that act as highly versatile scaffolds to determine cell shape and cell shape changes. They form a backbone for cell organelle and vesicle movement.[62,63] An essential feature is their dynamic instability, i.e. the ability to rapidly de- and repolymerize, allowing a fast response to the environment.[64] The *TUBB4A* gene encodes the brain-specific tubulin β4A, which is highly expressed in cerebellum, putamen and cerebral white matter.[62] Besides H-ABC, dominant *TUBB4A* mutations are also associated with another disorder: dystonia type 4 (DYT4). This disease typically present in adulthood, comprising spasmodic dysphonia combined with other focal or generalized dystonia, while no abnormalities are found on neuroimaging.[65,66] We speculated that there might be a **disease continuum** associated with *TUBB4A* mutations, of which H-ABC and DYT4 are the extremes. In chapter 4.2 we discuss literature describing patients with isolated hypomyelination of various degrees, without fulfilling the MRI criteria for H-ABC. In the past years, several additional cases of isolated hypomyelination caused by *TUBB4A* mutations were identified.[67,68] Some of these patients only had subtle lack of myelin and had remained without a genetic diagnosis for many years; the diagnosis was finally solved with the application of WES.[69-71] We suggested that in cases of hypomyelination without putaminal atrophy, prominent early extrapyramidal abnormalities should prompt *TUBB4A* testing. In the meantime, several patients with a *TUBB4A* mutation have been diagnosed who do not exhibit extrapyramidal signs (personal observations),[72,73] underling the large phenotypic heterogeneity of the disease spectrum related to dominant *TUBB4A* mutations. The presence of distinctive clinical and radiological phenotypes associated with different mutations in the *TUBB4A* gene suggests that mutations have diverse effects on tubulin function,

probably compromising different cell types. Recent studies have shed further light on the **cell type-specific effects** of different mutations. By using a combination of histopathological, biochemical and cellular approaches, Curiel *et al.* determined how specific *TUBB4A* mutations lead to either purely neuronal, purely oligodendrocytic or combined defects, matching their respective associated phenotypes.[74] The mutation observed in DYT4 patients, who exhibit phenotypes attributable to neuronal dysfunction, results in altered neuronal morphology, while tubulin quantity and polymerization, oligodendrocyte morphology and myelin gene expression are normal.[74] Conversely, mutations associated with isolated hypomyelination result in normal neuronal morphology, but altered oligodendrocyte morphology, myelin gene expression, and microtubule dynamics.[74] The MRI features of H-ABC, comprising hypomyelination but also cerebral atrophy and loss of the neostriatum, suggest at least in part underlying neuronal pathology. Interestingly, the *TUBB4A* mutation commonly observed in H-ABC patients has overlapping cellular defects involving both neuronal and oligodendrocyte cell types in vitro.[74] The finding that the DYT4 mutation had no impact on microtubule dynamics suggests that this mutation might act through a distinct mechanism.[74] Intriguingly, a neuropathology study on autopsy material of a patient with a severe variant with isolated hypomyelination demonstrated increased oligodendrocyte density in the white matter and accumulation of microtubules in oligodendrocytes.[75] Neuropathology findings in patients with classic H-ABC on the contrary, did not include increased oligodendrocyte numbers, but profound lack of oligodendrocytes as well as some axonal spheroids, indicative of axonal damage.[74] Altogether the findings indicate that different pathological effects of *TUBB4A* mutations probably arise from distinct molecular mechanisms (Figure 2). An important implication is that for different variants of the *TUBB4A*-disease spectrum, different **therapeutic strategies** may be required, for instance when exploring cell replacement therapy or cell targeted gene therapy.

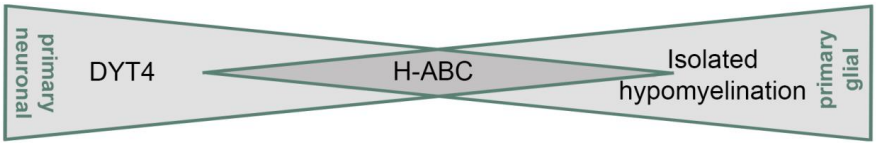


Figure 2 | Schematic representation of the disease spectrum associated with *TUBB4A* mutations. In DYT4 there is a primary neuronal dysfunction. In patients with isolated hypomyelination, oligodendrocytes appear to be primarily affected. H-ABC patients exhibit a combined phenotype: pathology and in vitro studies indicate that the cellular defect involves both neuronal and oligodendrocyte cell types. Adapted from Curiel *et al.* 2017.[74]

New disease variant: In a small group of patients fulfilling the MRI criteria of H-ABC, no pathogenic *TUBB4A* mutations could be identified. Close evaluation of the cases revealed that the majority of these patients were born to consanguineous parents and shared a Roma ethnic background. The 8-10 million European Roma/Gypsies

compose the largest European genetic isolate, consisting of geographically dispersed subisolates.[76] They represent a unique founder population. The identification of a growing number of Mendelian disorders and private mutations in this population emphasizes their unique genetic heritage.[77] Chapter 5 reports on the identification of the **second gene defect** associated with H-ABC in 16 patients. Suspecting a homozygous recessive mutation in a shared haplotype, we performed single nucleotide polymorphism (SNP) array analysis in 5 patients and found one large overlapping homozygous region on chromosome 13. Focused analysis of this region in a WES dataset of 2 patients revealed a homozygous deletion in the promoter region of the *UFM1* gene as the only candidate. It is a striking thought that the variant would have been missed if only the standard diagnostic WES pipeline at our institution would have been applied. The promoter region of a gene is not part of the standardly included region and this variant would have been excluded by the regular filtering procedure. Even if the variant would have been identified by trio analysis, its pathogenicity would have been unclear, since it is difficult often to predict the effect of variants in the promoter region. This observation stresses the value of grouping of patients on the basis of clinical, MRI and epidemiological characteristics, guiding additional investigations such as SNP arrays to identify a region of interest which can be subsequently studied in detail.

The new H-ABC variant is invariably associated with a disastrous disease course, involving lack of development, refractory epilepsy and death in early childhood. MRI shows a profound deficit of myelin and atrophy of the putamen and caudate nucleus. Systematic MRI scoring revealed an additional feature: in all patients, the lateral head of the caudate nucleus has an abnormal signal, suggestive of local apoptosis. This feature was also present in additional, recently diagnosed cases of recessive H-ABC (unpublished), suggesting it may be pathognomonic for this variant of the disorder.

As a next step, we performed Sanger sequencing to confirm segregation of the *UFM1* variant with the disease in all families. Haplotype analysis indicated that the shared *UFM1* deletion originates from a common ancestor. We further validated the pathogenicity of the *UFM1* variant by proving absence in homozygous state in 1000 healthy Roma controls.

Predictions on variants in non-coding regions of genes are generally not straightforward. In order to further substantiate the pathogenicity of the *UFM1* promoter variant, we performed transfection assays, showing that the deletion results in reduced gene expression. The effect appeared to be cell-specific: only neuroblastoma and astroglioma cell lines were affected by the mutation. The selective involvement of cells and brain structures is an interesting target for further analysis, aiming at improved **cellular pathology-based classification** of the disease, as well as increased understanding of the role of the *UFM1* gene. Neuropathology studies of brain tissue obtained in patient autopsy would be a valuable part of this process. This could, for instance, involve quantification of neuronal, oligodendroglial and astrocytic cells, and analysis of morphologic features

and molecular profiles by use of immunohistochemistry to discriminate between primary and secondary myelin involvement and to characterize the pathology in specific regions, such as the medial and lateral part of the head of the caudate nucleus.

UFM1 encodes ubiquitin-fold modifier 1 (UFM1), a member of the ubiquitin-like family. Ubiquitin is a well-established protein involved in post-translational modification. There are several additional proteins with comparable tertiary structures and supposedly comparably functions, such as UFM1 and the related process of ufmylation. The **physiological role** of ufmylation is still poorly understood. The first reports on its relevance originate from the fields of cancer, diabetes and ischemic heart disease.[78-80] Subsequent studies indicate an association between the UFM1 pathway and neurodevelopment and neurodegeneration as well as endoplasmic reticulum (ER) homeostasis and protection against apoptosis.[81-85] Colin *et al.* for instance, demonstrated that defects in the UFM1 cascade result in increased ER volume and deficient cellular response to stress induced by tunicamycin treatment.[86] The discovery that mutations in both *UFM1* (chapter 5) and *UBA5*[86-88] are associated with a severe epileptic encephalopathy led to further investigations on the role of ufmylation in brain development and function. In the meantime, additional cases have been described by Nahorski *et al,* including a *UFM1* mutation at a different location in the gene.[83] Both *UFM1* and *UBA5* mutations were shown to result in reduced, but not absent cellular ufmylation, indicating its necessity for embryonic life. This finding is consistent with the embryonic lethality of knockout models for the orthologous genes.[86,87] There are still many steps to be taken enhancing the understanding of the ufmylation cascade and its role in recessive H-ABC, before therapeutic options can be explored. Remaining questions are how to explain the striking resemblance of the MRI patterns seen in H-ABC patients with *TUBB4A* and *UFM1* mutations and explore whether the genes are involved in overlapping pathways or networks.

An important benefit from the discovery of the new disease lies in the possibility of better **family counseling**. The screening of controls from different European Roma panels demonstrates that the carrier frequency of the *UFM1* promoter variant is high (up to 25%) in certain Roma communities (chapter 5). This finding indicates that the mutation may be an important cause of early-onset leukodystrophy there. The identification of the genetic cause facilitates prenatal testing and enables carrier testing in populations with a high carrier frequency.

Vanishing White Matter (VWM)

VWM is an autosomal recessive disorder caused by mutations in any of the 5 genes encoding the subunits of translation initiation factor 2B (eIF2B), a crucial element for the initiation of protein synthesis and its regulation under conditions of cellular stress.[89-91] With a roughly estimated incidence of around 1:100,000, it is one of the more common leukodystrophies.[1,92] The actual occurrence may be underestimated due to its challenging diagnosis. Especially in regions with a high rate of

consanguinity, prevalence may be higher.[93] Our database contains all known Dutch VWM patients, enabling calculation of an estimated minimum incidence of 1:80,000 live births in the Netherlands (chapter 6). VWM is characterized by a progressive loss of cerebral white matter, causing neurological decline with ataxia and spasticity. In addition to chronic decline, rapid deterioration may occur upon exposure to various stressors.[89] Although often referred to as an early childhood onset, rapidly fatal disorder, VWM is a heterogeneous disease with an extremely broad phenotypic range. Age of onset is known to be an important determinant of prognosis.[94,95] Studies on natural disease course in VWM are scarce and rather small.[95-97] In chapter 6 we report the results of a 12½-year **natural history study** among 296 VWM patients, focusing on the occurrence of neurological signs and symptoms in relation to age and disease duration and the identification of prognostic factors. The disease spectrum is a continuum of phenotypes ranging from antenatal onset early fatal disease up to late adult-onset, relatively mild disease. Onset at the age of 2 years is most common. Presentation before the age of 4 years is associated with severe and rapid neurological decline and high mortality, while presentation after 4 years is associated with a less severe, more variable disease course independent of the exact age of onset (Figure 3). Although motor problems are the most common presenting feature, it is of importance that clinicians are aware of other possible presenting signs/symptoms. Especially in the adult onset population, patients may present with non-motor signs such as for instance cognitive decline or behavioral change. Signs related to ovarian failure may be another clue for the diagnosis VWM. There are no statistically significant differences between males and females in the studied cohort, although it is striking that the female sex is more common among the adult-onset cases. The finding that disease course was more severe for patients who had episodic deterioration and seizures stresses the importance of preventive measures and adequate treatment. Febrile infections should be avoided by vaccinations under antipyretic prophylaxis, liberal antibiotic therapy strategy, and prophylactic antibiotics, and minor head trauma should be avoided.[98] Nonetheless, it is unknown to what extent these factors influence the chronic disease course and to what extent they are manifestations of a more severe disease variant.

The analysis of groups of patients with similar mutations confirms the presence of a **genotype-phenotype correlation**. In the case of early onset, patients with similar genotypes show highly comparable phenotypes and disease progression. In patients with milder variants, more variability is observed for similar genotypes and between siblings. We hypothesize that in the latter group, there is a larger influence of other factors such as environment, e.g. exposure to factors that provoke episodic deterioration.

Because of the central role of eIF2B in mRNA translation and protein synthesis, remaining activity is conditional for life. Accordingly, VWM patients mainly have minor mutations; they never have 2 null mutations that completely abolish eIF2B activity.[90,98]

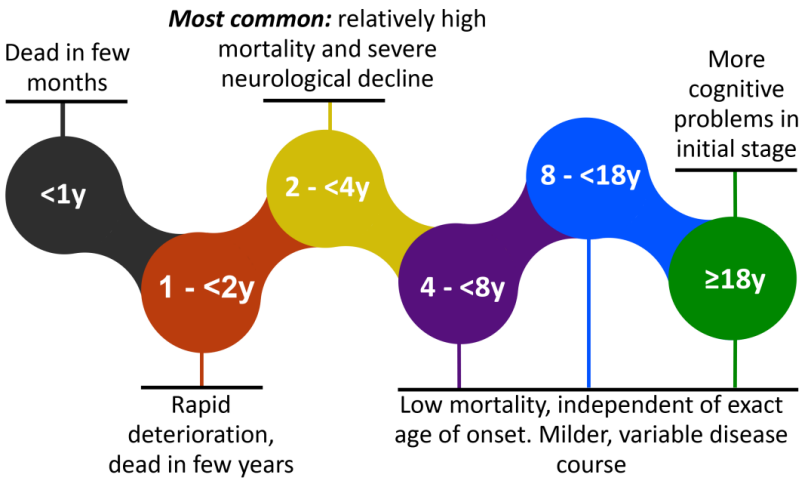


Figure 3 | Clinical course of VWM in relation to age of onset.

Missense mutations are by far the most common mutation type in VWM, comprising approximately 80% of all mutations.[99] Mutations leading to absence of the protein, such as deletions and frame shift mutations, are always seen in combination with a missense mutation.[98] We did not find significant differences in phenotypic characteristics for the five eIF2B gene groups. Exploring DNA sequence alignments becomes even more powerful when homology crystal structures are available for the sequences. It would be of interest to further study mutations of VWM patients in the context of the protein architecture of the subunits of the eIF2B complex. Mapping of mutations on a 3D structure could lead to additional insights in their putative roles in complex formation, stabilization and protein interactions. By establishing links with the clinical severity, this could lead to enhanced understanding of the molecular mechanism of the disease.

In view of the current absence of effective treatment options for VWM, the aim of medical care is to maximize quality of life from the time of diagnosis through the end of life. In that context, and also in the context of future clinical trials, it is essential to have knowledge about dimensions of disability and **health-related quality of life** (HRQL) in patients with VWM. In chapter 7 we present the natural disease course in VWM patients by means of 2 validated clinical scales on disability: Health Utilities Index (HUI) and Guy's Neurological Disability Scale (GNDS). In keeping with chapter 6, the results show that in patients with onset before the age of 4 years, earlier disease onset is associated with more severe disease course. When onset occurs from 4 years of age, the rate of deterioration is variable and independent of exact age of onset. The functions ambulation and dexterity are most severely affected, especially in young children. The next domain is cognition, which is affected sooner with disease presentation in adult-onset patients. Along with disease progression, patients are more likely to develop problems with speech and bladder and bowel function. Vision and hearing remain relatively intact. Overall, this study provides a

robust overview of domains of disability in VWM and reveals age of onset related differences in disease course.
No agreement exists on how to rate HRQL and do justice to both objective function and subjective judgement. Interestingly, we found a discrepancy between the perceived quality of life by VWM patients and/or their carers and HRQL as measured by the HUI through public preferences. In about 1/3 of scored patients, the calculated HUI Health Index score represented a 'worse than dead' health state as assessed by the general population. Nevertheless, the majority of patients received positive scores on the attribute 'Emotion'. This finding, also known as "disability paradox", might be an insightful point of note for physicians who counsels patients and families when discussing prognosis.

Clinical observations can give important clues for the establishment of studies aiming at unraveling pathogenesis. At the time, the grouping of several patients with a similar disease phenotype led to the recognition that VWM patients are vulnerable to stressors.[89] An underlying mechanism involving the cellular stress response was suspected. The identification of mutations in the *EIF2B1-5* genes as disease cause confirmed this theory.[90] Over the next years, studies applying a range of different approaches (i.e. neuropathology, proteomic and metabolomic analyses and animal models) led to gradual enhanced understanding of the disease.[100-103] Important breakthroughs concern the finding that astrocytes are central in the pathomechanisms and that the integrated stress response (ISR) is disturbed.[100-103] A consequent hypothesis is that VWM patients might benefit from treatment strategies involving interference with the ISR. In a recent study, treatment with Guanabenz, an FDA-approved centrally-acting oral antihypertensive drug that inhibits stress-induced dephosphorylation of eIF2α, was tested in a VWM mouse model.[104] There was a significant improvement of the mice' brain white matter, holding promise for the application in patients. Bearing in mind the prospects therapeutic options, the current natural history study may serve as a reference point to design and evaluate therapeutic interventions.

Table 1 | Summary of disease characteristics

	LBSL	Classic MLC	Remitting MLC	Dominant H-ABC	Recessive H-ABC	VWM
Genetic causes	recessive *DARS2* mutations	recessive *MLC1* or *GLIALCAM* mutations	dominant *GLIALCAM* mutations	dominant *TUBB4A* mutations	recessive *UFM1* mutations	recessive *EIF2B1, EIF2B2, EIF2B3, EIF2B4* or *EIF2B5* mutations
Number of phenotyped patients	66	204	38	41	16	296
Median age of onset (range)	8 y (5 mo - 40 y)	macrocephaly at 0 - 12 mo	macrocephaly at 0 - 12 mo	7 mo (birth - 3 y)	2 mo (birth - 3 mo)	3 y (before birth - 54 y)
***Median age at loss of walking without support**	30 y	15 y	n.a.	18 mo	none achieved ambulation	9 y
***Median age at full wheelchair dependency**	n.a.	n.a.	n.a.	18 mo	none achieved ambulation	18 y
Mortality	2 / 66 patients	6 / 204 patients	1 / 38 patients	3 / 41 patients	9 /16 patients	102 / 296 patients
Evidence for genotype-phenotype correlation	+/-	-	-	++	+/-	++
Likely primarily affected cell type(s)	neurons	astrocytes	astrocytes	neurons, oligo-dendrocytes, or both	to be determined	astrocytes

y, years; mo, months

* Kaplan Meier estimate; non-ambulant patients are scored has having lost ambulation at 18 months; n.a.: not applicable: probability of event <50%

8

METHODOLOGIC CONSIDERATIONS

Challenges of clinical phenotyping and natural history studies

Study population: the sample population of a study cohort should be representative of the complete disease population suffering from the respective disorder, avoiding inclusion bias.[105] A complete ascertainment is, however, impossible. One should then aim for a random sample of prevalent patients. In the studies described, we included all eligible, genetically proven patients who were referred to the Center for childhood white matter disorders for MRI opinion and/or genetic testing. We have no indications that specific subgroups have been missing out on inclusion, but we cannot rule out certain biases. Asymptomatic or oligosymptomatic cases may have been underrepresented, as well as atypical and/or strikingly mild or severe cases. An important point of note is that most patients were diagnosed on the basis of MRI

findings, applying MRI pattern recognition. This method has proven to be a very powerful tool for the grouping of patients.[106-108] The drawback is that atypical patients who do not fulfill the MRI criteria for a specific diagnosis are underdiagnosed and left out of the clinical inventory. Particularly for disorders with wide phenotypic variability, sample sizes should be large in order to provide adequate statistical power. The rarity of leukodystrophies hampers inclusion of large numbers of patients, but the reported studies do contain the largest numbers of patients described for the respective disorders so far. This was achieved by performing multicenter studies including patients from over the world. It is important to bear in mind that socio-economic influences and cultural preferences may influence the natural disease course observed in patients.

Systematic assessments and use of instruments: Ideally, clinical assessment would be prospective, performed by one single investigator and performed on regular intervals from disease onset until death. Such an approach is unfortunately not feasible, especially for rare diseases. In order to obtain large datasets, we relied on the collaboration with many referring physicians. As a consequence, we had to limit the extensiveness and frequency of assessments. We refrained from including candidates in the case of significant language barriers between researchers and referring clinicians. To promote homogeneity, we aimed for robust, easily quantified measures of clinical course, such as age at first symptoms and age at loss of ambulation. Nevertheless, certain inter-observer bias may have been introduced. To obtain a uniform description of disease course over time, researchers preferably use standardized rating scales that are validated for the disorder and for the age group of the patients. Such systems do not exist for the specific disorders studied, nor for leukodystrophies in general. For disease as rare as leukodystrophies, it does not seem feasible to create disease specific systems. In the absence of disease specific scoring systems, we aimed for the application of existing systems to would fit our patient group. For motor function, we applied the GMFCS scale: this scale is widely used to classify motor function in children with cerebral palsy (CP).[109] The described leukodystrophies are generally more heterogeneous conditions, but the conditions share the common involvement of the pyramidal tracts as underlying pathology. A recent publication by the creators of the system argues against the use of the system in disorders other than CP, as it was not validated for other disorders.[110] The fact that we observed wide variability of scores among all levels of the system, does offer some support for its application. The use of the system in children with Down syndrome for instance is disputable, since the presence of a ceiling effect is suggestive of serious content validity issues.[110] Still, it is well-founded that the creators of the GMFCS have brought about the potential need for a more generic gross motor function classification system.[110] This might also be of use for the study of leukodystrophies. For metachromatic leukodystrophy (MLD), a disease specific 7-level classification system has been developed on the basis of the conceptual ideas and structure of the GMFCS: GMFC-MLD.[111] The number of levels and the descriptions are based on data from 59 individuals with MLD. Because children with

MLD experience normal development before diagnosis, the GMFC-MLD incorporates a level 0 to indicate normal development and describe the loss of function. This would also be applicable to the majority of leukodystrophies. Since MLD is characterized by a rapid deterioration of motor function, mobility aids are less common and were therefore not included in the criteria for functional levels. Analysis of interrater reliability indicated that the GMFC-MLD is a feasible tool for standardized assessment of gross motor function in MLD, which can be used for the description of the natural course of the disease and for evaluation of therapeutic options.[111] Considering the heterogeneity of clinical course in leukodystrophies, it remains questionable if it is feasible to incorporate all possible clinical states in one general system.
To overcome the limitations of the absence of systems specifically validated for the diseases studied, we always combined the application of standardized systems with the use of customized clinical questionnaires, obtaining robust measures for disease course specific for the disorder. This also enabled us to check for internal consistency of data: in the case of inconsistencies we asked the referring clinicians to re-evaluate the data or we omitted the data from the study. The application of a more general rating system for VWM by using the Health Utilities Index (HUI), which also enables overall HRQL score calculation, facilitates comparison of the burden of disease with other diseases.[112]

With regard to MRI scoring, the heterogeneity of radiological characteristics in different leukodystrophies necessitates application of disease specific scoring systems. Consequently, comparison of features between different leukodystrophies is hampered. It could be useful if for characteristics that occur in multiple leukodystrophies, such as hypomyelination, a uniform scoring system would become available.[113]

Data analysis: One of the difficulties of phenotyping studies is handling incompleteness of data. If complete data (from disease onset to death) were available for the entire sample population, analysis would be much more straightforward (Figure 4). Considering the difficulty of obtaining clinical data in rare diseases, we commonly chose to also include patients in whom not all the requested data were obtained. Incompleteness of datasets requires specific approaches for data analysis. For survival functions, especially if patients have already reached the end point or are lost to follow up at the time of survey (Figure 4), the Kaplan-Meier technique is the most appropriate method. The obtained estimates of time-to-events are not necessarily unbiased. This is especially the case when there are large numbers of “censored” patients (patients in whom the respective event has not yet occurred at the time of survey).[114]

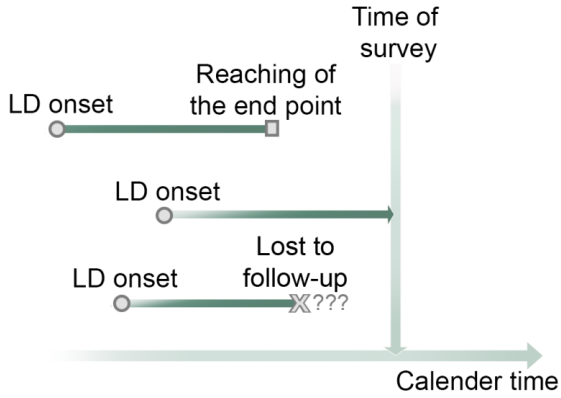


Figure 4 | Schematic representation of the distribution of patients in a cohort at the time of survey. LD = leukodystrophy. Top line: patients who have reached the endpoint before the end of the survey. Middle line: patients who have not reached the endpoint at time of study closure. Bottom line: patients who were lost to follow-up at the time of survey. Adapted from Confavreux and Compston 2006, with permission.[114]

Genetic testing

A prerequisite for patients to be included in our clinical phenotyping studies is the presence of a genetically confirmed diagnosis. If a certain leukodystrophy is suspected on the basis of characteristic MRI findings, focused Sanger sequencing can be initiated.[115] This approach results in a high diagnostic yield, due to the high sensitivity of MRI pattern recognition. When a variant is found in the sequenced gene, careful evaluation takes place on the basis of various lines of evidence such as occurrence in online variant databases or scientific literature, species conservation, *in silico* predictions and co-segregation with the disease in the family.[116] Over the years, elaboration of available information in online databases has increased the likelihood of correct interpretation of variants. If no mutations are found, additional investigations can be performed by sequencing the gene at complementary DNA (cDNA) level to detect RNA splicing defects, duplications or deletions. Multiplex ligation-dependent probe amplification (MLPA) can be performed to detect exon deletions and duplications at DNA level. The interpretation of variants in promoter and 5' or 3' non-coding regions can be substantiated in transfection studies using reporter constructs. This latter type of experiments is not part of the standard genetic investigation in most institutions and genetic diagnoses may therefore sometimes be missed.

For unsolved leukodystrophy cases, the application of WES has proven to be a successful and cost-effective method for providing a molecular diagnosis. The yield is highest when focusing on a group of patients with a similar MRI pattern.[108]The exponential rise in number of publications on the identification of genetic causes confirms the achievements of WES. Nevertheless, the technique also comes with technical and analytic limitations and little is known about the number of unsuccessful attempts. Technical challenges concern for instance incomplete sequencing

coverage of the exome or the inability to capture deep-intronic variants, variants in regulatory elements, or copy number variants.[117] Analysis of WES data involves bioinformatic tools and applications that support the several steps such as raw data quality assessment, alignment and variant prioritizing.[118] The analysis can be challenging due to genetic heterogeneity of the disease or localization of variants in areas that are difficult to interpret, for instance regions for which pseudogenes are present in the genome. Especially for the interpretation of equivocal variants, multiple patients or families with comparable phenotype and mutations in the same gene and are needed to prove pathogenicity. This underlines that, despite the tremendous improvements in bioinformatics pipeline used for WES, good clinical phenotyping remains an indispensable element of a diagnostic work-up.

THE VALUE OF A MOLECULAR DIAGNOSIS

The importance of the identification of the underlying cause in an individual with a leukodystrophy is often underestimated. First of all, an established diagnosis precludes further unnecessary testing and fruitless interventions. Quality of life often improves with diagnosis, as it brings closure and allows the patient and family to focus on optimal medical management, whether it is symptomatic or palliative care or experimental treatments. In some cases, there is limited access to supportive care or rehabilitative services when there is no diagnosis. A diagnosis may open the door to adequate services.

Next, a genetic diagnosis enables counselling of recurrence risk, family screening and prenatal testing or pre-implantation genetic diagnosis, if indicated and desired.[119] Both for established and experimental treatments for leukodystrophies, it is clear that the therapeutic window is limited to the early stages of the disease, when the brain is still relatively intact. It is therefore crucial that clinicians are aware of a disease's presenting symptoms and clinical signs to ensure early establishment of diagnoses and immediate referral if therapies are available. For instance for Metachromatic Leukodystrophy and X-linked adrenoleukodystrophy, hematopoietic stem cell transplantation (HSCT) can halt disease progression, but this is only succesful if patients are presymptomatic or oligosymptomatic.[120]

THE VALUE OF CLINICAL PHENOTYPING

Natural history studies are important pillars of epidemiologic research on rare conditions. By tracking the course of a disease over time, demographic, genetic, environmental, and various other variables can be evaluated. Knowledge on the natural course of a disease can also guide decisions for funders, health insurers, and the pharmaceutical industry. Unfortunately, large phenotyping studies tend to be unpopular, especially longitudinal studies, owing to the time taken to gather definitive results. It would be unfortunate if in the clinic, as in the literature, a shift toward genetic sequencing and analysis comes at the expense of skill and interest in clinical

phenotyping.[121,122] The items below illustrate the potential of clinical phenotyping for leukodystrophy research and clinical practice.

Grouping of patients

Diagnostics: Phenotypic information is essential at several stages of the diagnostic pathway. The challenge for genomic investigation is no longer the generation of DNA sequencing data, but its interpretation. Downstream of genetic testing, phenotyping data along with family history are necessary to filter, prioritize, and interpret potential disease causing genetic variants. The recognition of the *UFM1* gene defect (chapter 5) in H-ABC patients, for instance, would not have been possible on an individual basis.

Identification of patterns: The inventory of clinical characteristics in larger groups of patients allows for the study of similarities and differences among patients. This increases the likeliness of identifying disease patterns, which may otherwise remain unnoticed. Such patterns may involve subgroups within a uniformly classified leukodystrophy, specific risk factors and potential epidemiological differences, for instance with regards to sex or age.

Genotype-phenotype correlations: If a clear genotype-phenotype correlation has been established for a disorder, such knowledge may guide clinicians in the counseling, risk stratification and prognostication of patients. Analysis of mutation type and its molecular effects may contribute to understanding of mechanisms underlying disease phenotypes, as exemplified by the cell-specific effects observed for different *TUBB4A* mutations. As functionally pathogenic variants in vitro may not always manifest a phenotype in vivo, genotyping-phenotyping studies can provide essential knowledge on the interpretation of variants in a gene.

Clinical implications

Leukodystrophies manifest with certain overlapping signs and symptoms that allow a certain uniformity in the approach to care. But there are also disease-specific features that require special care. A genetic testing result alone cannot provide enough clarity without a comprehensive description of phenotype. Thorough knowledge of the phenotype of a disorder is essential in order for physicians to discuss with patients and families the disease and its prognosis in simple understandable terms and to help make the best medical decisions regarding the management of their disease. Insight into the prognosis is elementary for decisions on education plan, invasive treatment (e.g. gastric tube feeding) and anticipation on ergonomic adaptations (e.g. introducing a walker or a wheelchair). Literature on clinical course can guide physicians and families in making decisions on care. It should however be emphasized that clinicians should refrain from establishing a very precise prognosis for individual patients on the basis of group level data.

The availability of a detailed description of clinical features of a disorder can be of help to evaluate differential diagnostic options and adequately diagnose patients. Proper diagnosis can also have important consequences for prevention of redundant therapeutic interventions. The clinical symptomatology and MRI of LBSL, for instance, show some overlap with the most common leukoencephalopathy of young adults, multiple sclerosis (MS). Correct recognition may prevent unnecessary immunomodulating treatments in patients.

Insight in disease mechanisms and physiological processes

Clinical information and imaging patterns collected in phenotyping-genotyping studies can guide basic research aimed at better understanding of pathophysiological processes underlying leukodystrophies. For decades, the leukodystrophy field has mainly focused on myelin, its loss and the need to reconstitute it. The discovery of so many new causes for leukodystrophies and the increasing insight on cellular and molecular pathomechanisms have made clear that the myelin-centered view on leukodystrophies is no longer tenable. It has become clear that integrity and proper function of the white matter are closely related to all its structural constituents and not just to myelin. [53,123] The leukodystrophies described in this thesis comprise diverse disease mechanisms, often not primarily involving oligodendrocytes (Table 1).

Selective vulnerability: Different areas of the brain take part in different networks and have distinct functions, cellular compositions, metabolism and energy demands. The complex functional topography is reflected in the complex patterns of selective vulnerability seen in leukodystrophies. Observations from studying radiological and clinical characteristics of a disease help to gain understanding of which structures are affected. These observations can offer guidance for functional studies aimed at further understanding of the underlying disease mechanisms.

Rare diseases, common insights: An interesting aspect of research on rare diseases is that pathways or concepts may be identified that apply to a broader field. The Undiagnosed Diseases Program of the National Institutes of Health in the US has proven to successfully contribute to improvement of the understanding of more common conditions.[124] Monogenic diseases offer a unique opportunity to reveal the function of proteins in human physiology and disease. Investigations on disease mechanisms underlying leukodystrophies may function as a study model for more common, acquired diseases. The latter are generally more difficult to study due to the heterogeneity of underlying mechanisms. Acquired disorders and genetic white matter disorders may share pathomechanisms. Consequently, there may be a window for shared reparative therapies.[123,125,126] This could, for instance, be explored for disorders associated with reversible myelin vacuolization, which is observed in several genetic disorders as well as in acquired diseases caused by toxic substances and infections.

Implications for therapeutic strategies

Understanding of the underlying pathomechanism of a disorder is conditional for development of treatment strategies. The work described in this thesis illustrates that the study of a patient cohort can provide valuable insights with regard to therapeutic strategies. For instance for LBSL, the finding that ~95% of patients would benefit from a therapy that modulates splicing makes this disease an attractive candidate for therapeutic interventions. It suggests that a single strategy would suffice to treat almost all patients.

Rational, scientifically based therapy development also requires a thorough understanding of the natural history of a disease. For an intervention to receive approval, research needs to demonstrate that the intervention has a clinically meaningful effect on patients through adequate and well-controlled studies. These studies must be based on a scientific foundation that includes knowledge of the disease's natural history. It has been reported that the top reason why rare disease development programs fail at the U.S. Food and Drug Administration (FDA) is the lack of natural history information.[127] Natural history studies can shed light on the full spectrum of genotypic and phenotypic features and help identify the types of patients to study in a clinical trial, the duration of the trial, and the types of biomarkers and outcome measures to use in the trial.[128] In this view, the current work on VWM may serve as a reference point to design and evaluate future therapeutic interventions. From the data presented in chapter 6 and 7, we could for instance conclude that effect of a future therapy could be demonstrated best when targeting patients with age of onset before the age of 4 years. Patients presenting at later ages are more likely to have a less progressive disease course, which would make it less feasible to prove that a therapy is effective within a few years. Considering the high rate of loss of ambulation in patients presenting before 4 years, preservation of ambulation could be a good outcome parameter. This would exclude patients who never achieved walking, which predominantly concerns patients with disease presentation before the age of 1 year. Considering the very severe and devastating disease course observed in these patients, this might not be the most eligible group of patients to target for therapy. In addition, we conclude from our dataset that disease prevalence is very low in this subgroup of patients.

When comparing some robust parameters of the natural disease course of VWM to that of other leukodystrophies (see Table 1), it becomes clear that the different diseases would require different strategies for selection of patients likely to benefit from future therapies and clinically relevant endpoints. For instance for LBSL, a much subtler parameter than loss of walking without support would be required to establish improvement of motor function.

FUTURE PERSPECTIVES

Identification of novel genes and expansion of clinical spectra

In the coming years, the number genetically classified leukodystrophies will further increase, whether it be novel diseases or identification of genetic causes of known

leukodystrophies. With that, the mapping of all proteins and networks involved will provide further insight in biological pathways and functional networks of the brain white matter.

For monogenetic disorders, the classic “one-gene one-phenotype” paradigm is more and more negated. As a consequence of the heterogeneity of clinical phenotypes associated with different mutations in one gene, classification under one uniform header can be challenging. New discoveries may instigate a more general denomination for previously defined diseases, as could be suggested for H-ABC, after the discovery of the broad spectrum of *TUBB4A*-related disorders.

Along with expending spectra, insights in the epidemiological distribution of disorders may change. After their discovery, classic disease variants are recognized easiest, as is for instance the case for childhood onset VWM. We suspect that until now, mild variants of VWM, especially adult onset cases, have been underdiagnosed, because of the less typical presentation and the lack of awareness among adult neurologists. In general, diagnostic rates in adult onset genetic disorders tend to be lower than in children due to multiple factors. The population exhibits heterogeneous phenotypes, diagnostics are often focused on acquired diseases and parental and familial DNA is often unavailable in adult onset cases, hampering the possibility of trio-based WES or analysis of segregation of mutations. An illustrative example of a disorder with a shift in the reported epidemiological distribution is X-linked adrenoleukodystrophy, which was originally described as a rapidly progressive childhood onset disorder.[129] The initial phenotype was named ‘Childhood cerebral ALD’. Later on, the adult onset variant adrenomyeloneuropathy was recognized more and more, and is now known to be the most common form of X-ALD.[130,131]

The expected rise in the application of the whole-genome sequencing (WGS) technique rather than WES will lead to increased identification of variants in non-coding regulatory sequences, such as intronic variants resulting in altered expression or splicing. This may enhance the understanding of epigenetic mechanisms. The rapid progress in genetic diagnostic technologies will also provide new challenges in terms of care and counselling of patients and families as well as logistics regarding the storage and analysis of big data.

Continuous need for phenotyping

An important point is that the easier access to genetic testing and therefore to molecular diagnoses does not mean that stringent clinical phenotyping is no longer necessary. A genetic diagnosis is not sufficient to predict disease course and perform optimal clinical counseling. There are still many leukodystrophies in which systematic inventory of clinical features in large groups of patients are lacking. Especially with the prospect of therapeutic options, we stress the need for clinical phenotyping studies.

Also for the leukodystrophies described in this thesis, there is still a lot to gain from follow-up phenotyping studies. This could for instance involve longer follow-up of patients and establishment of larger datasets to answer questions on for instance gender influence or subtypes of diseases. A proposed complement would be to compare the output of questionnaires completed by different players involved (e.g. the patients themselves versus parents versus clinicians).

Analysis of radiological features is an essential element of phenotyping. With advancing MRI techniques, MRI pattern recognition becomes more sensitive. MR Imaging at 3 Tesla can results in the identification of additional features as compared to 1.5 Tesla imaging, as has been demonstrated for 4H leukodystrophy.[132] Together with advancing DNA technologies, leukodystrophies can be described in more and more detail, revealing insights in for instance which structures or cell types are involved, offering clues for basic studies and therapeutic strategies.

Phenotyping studies will always need renewing, as findings will not necessarily be applicable to patients living a few decades from now. Diseases are influenced several factors, like changing environmental circumstances and genetic effects. The latter are also impacted by evolutionary changes.[133]

Global collaborations

Collaboration is the cornerstone of progress in the world of rare diseases. The ability of the internet can greatly facilitate the formation of clinical research networks. A crucial component of success with rare-disease research is establishing strong connections with physicians and the patient community. Involvement of patient advocacy groups and online platforms results in increasing awareness and funding and facilitates the recruiting of patients and measurement of patient-reported outcomes.

An increasing amount of detailed patient-related data is being collected over time in electronic health records. This should facilitate the delineation of clinical syndromes at a low cost.[134] Advanced tools to enable systematic capture of clinical data would benefit phenotyping studies. Regardless of this, successful phenotyping studies require harmonization of instruments to describe phenotypes, improved exchange of phenotypic data and a pragmatic assessment of what data to collect under different clinical circumstances and at what point along the diagnostic pathway.

Web-based applications facilitate cross-talk between researchers working on highly specialized topics. With regard to the identification of genetic causes of rare diseases, the field would greatly benefit from enhanced data sharing. Research output is enhanced by initiatives seeking to maximize sharing of genomic data, such as GeneMatcher and the Global Alliance for Genomics and Health.[135,136]

Treatment strategies

Being monogenic disorders, leukodystrophies appear highly suitable for **gene therapy** development, aiming at counteracting or replacing a malfunctioning gene

within the cells adversely affected by the gene defect.[137] Loss-of-function mutations are amendable to the delivery of the natural functional gene, whereas gain-of-function mutations require reduction of the toxic gene product activity. As simple as the concept sounds, there are many technical challenges to overcome.[101] The first clinical trial of gene therapy in the field of leukodystrophies has recently shown encouraging therapeutic benefits.[138] Recent studies are focused on gene therapy targeting specific cell types such as oligodendrocytes.[139] Gene therapy will however not have the capability of restoring damaged tissue. Therefore, additional strategies are warranted. Autologous hematopoietic stem cell (HSC) therapy, targeting restoration of tissue macrophages, has proven to successfully cross-correct enzyme deficiencies in a subset of leukodystrophies. For most disorders other strategies are needed to halt further progression and repair existing damage. **Cell replacement therapy**, where populations of healthy glial cells (oligodendrocytes and astrocytes) or their precursors are transplanted in the CNS, is a promising treatment strategy for leukodystrophies.[140] At the Center for childhood white matter disorders, cell replacement therapy for VWM is currently tested on mouse models closely resembling the disease in humans. Issues to be addressed include which glial precursor cell population should be focused on to correct for the defect in VWM, how the brain microenvironment should be modulated for optimal improvement, how the genetic defect can be corrected and which route of administration is most effective.[141] While progress in the fields of gene and stem cell therapies is ongoing, there are still many challenges to overcome for their implementation. For the near future, a focus on **pharmacological compounds** may be the most promising approach. Understanding of the underlying mechanism of a disease and the identification of involved pathways are crucial steps in the identification of targets for therapy. Cell models or patient cell lines may be used for drug screening, followed by application in animal models representative to human diseases. Overall, interdisciplinary collaborations are essential for further advancement in the identification and development of therapeutic agents. A suggested approach is the exploration of parallels between the fields of neuroscience and oncology. The two share several important research themes, such as the influence of gene expression patterns and microenvironmental factors on pathology, and the development of targeted therapies. For leukodystrophies, this could for instance involve the identification of alternatives for cantharidin as splicing modulator for LBSL or the application of knowledge on microtubule stability from research on cytostatic drugs for H-ABC.

All in all, recent technological progress is changing the paradigm that leukodystrophies are untreatable. Multimodal approaches targeting multiple aspects of the disease are most likely to succeed in halting the disease process and repairing the multifactorial complex pathology.[142] Regardless of treatment strategy, consensus should be achieved with regard to the inclusion and exclusion criteria for patient selection, which requires comprehensive phenotyping and long term follow-up studies.

CLOSING REMARKS

Important progress is being made in diagnostic rates and understanding of pathologic mechanisms in the field of leukodystrophies. Clinical phenotyping remains essential to interpret genetic findings, perform optimal patient counseling and plan clinical trials. Multidisciplinary and international collaborations are crucial to improve quality of life for these groups of patients and eventually find a cure.

Key issues on leukodystrophies

- Leukodystrophies are a diverse group of diseases with heterogeneous causes, clinical presentation and disease progression: not all leukodystrophies are disastrous and fatal
- A molecular diagnosis is essential for clinical and genetic counseling
- Molecular understanding of disease cause and mechanisms are conditional for the development of treatment strategies
- A diagnostic test alone has little meaning if not placed into a clinical context
- Improved knowledge on natural disease course can only be achieved by international, standardized and centralized collection of data and strong connections with physicians and patient communities
- The study of genetic diseases in a multidisciplinary approach provides the best chance of crucial insights into underlying pathophysiological mechanisms
- **LBSL** has a broad phenotypic spectrum, but in the majority of cases the disease course is mild and slow. The development of a successful modulation of “leaky” splice site mutations in intron 2 is of importance for 95% of patients
- Classic **MLC** is a relatively mild leukodystrophy. Epilepsy is an early feature, often occurring before motor decline. No clinical differences have been found on the basis of affected gene (*MLC1* or *GLIALCAM*)
- The clinical course of **H-ABC** caused by *TUBB4A* mutations ranges from neonatal up to early juvenile presentation with rapidly progressive to more gradual neurological decline. There is a strong genotype-phenotype correlation
- Among unsolved **H-ABC** patients with a Roma ethnic background, the genetic cause lies in a founder effect: all patients have the same mutation in the promoter region of the *UFM1* gene and exhibit a severe, fatal epileptic encephalopathy
- **VWM**, one of the more common leukodystrophies, is associated with a highly variable disease, ranging from antenatal onset rapidly fatal disease up to adult-onset milder disease. Age of onset is an important predictor of outcome

REFERENCES

1. Bonkowsky JL, Nelson C, Kingston JL, Filloux FM, Mundorff MB, Srivastava R. The burden of inherited leukodystrophies in children. Neurology 2010;75:718-725.
2. Aronson JK. Rare diseases and orphan drugs. Br J Clin Pharmacol 2006;61:243-245.
3. Parikh S, Bernard G, Leventer RJ, et al. A clinical approach to the diagnosis of patients with leukodystrophies and genetic leukoencephelopathies. Mol Genet Metab 2015;114:501-515.
4. Richards J, Korgenski EK, Srivastava R, Bonkowsky JL. Costs of the diagnostic odyssey in children with inherited leukodystrophies. Neurology 2015;85:1167-1170.
5. Vanderver A, Prust M, Tonduti D, et al. Case definition and classification of leukodystrophies and leukoencephalopathies. Mol Genet Metab 2015;114:494-500.
6. http://www.who.int/classifications/icd/en/ [online].
7. Brimley CJ, Lopez J, van Haren K, et al. National variation in costs and mortality for leukodystrophy patients in US children's hospitals. Pediatr Neurol 2013;49:156-162 e151.
8. Ayme S, Bellet B, Rath A. Rare diseases in ICD11: making rare diseases visible in health information systems through appropriate coding. Orphanet J Rare Dis 2015;10:35.
9. Appel LJ. A primer on the design, conduct, and interpretation of clinical trials. Clin J Am Soc Nephrol 2006;1:1360-1367.
10. Griggs RC, Batshaw M, Dunkle M, et al. Clinical research for rare disease: opportunities, challenges, and solutions. Mol Genet Metab 2009;96:20-26.
11. Pennisi E. Systems biology. Tracing life's circuitry. Science 2003;302:1646-1649.
12. Noble D. The music of life. Oxford: Oxford University press, 2006.
13. van der Knaap MS, van der Voorn P, Barkhof F, et al. A new leukoencephalopathy with brainstem and spinal cord involvement and high lactate. Ann Neurol 2003;53:252-258.
14. Steenweg ME, van Berge L, van Berkel CG, et al. Early-onset LBSL: how severe does it get? Neuropediatrics 2012;43:332-338.
15. Linnankivi T, Lundbom N, Autti T, et al. Five new cases of a recently described leukoencephalopathy with high brain lactate. Neurology 2004;63:688-692.
16. Serkov SV, Pronin IN, Bykova OV, et al. Five patients with a recently described novel leukoencephalopathy with brainstem and spinal cord involvement and elevated lactate. Neuropediatrics 2004;35:1-5.
17. Tavora DG, Nakayama M, Gama RL, Alvim TC, Portugal D, Comerlato EA. Leukoencephalopathy with brainstem and spinal cord involvement and high brain lactate: report of three Brazilian patients. Arq Neuropsiquiatr 2007;65:506-511.
18. Uluc K, Baskan O, Yildirim KA, et al. Leukoencephalopathy with brain stem and spinal cord involvement and high lactate: a genetically proven case with distinct MRI findings. J Neurol Sci 2008;273:118-122.
19. Scheper GC, van der Klok T, van Andel RJ, et al. Mitochondrial aspartyl-tRNA synthetase deficiency causes leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation. Nat Genet 2007;39:534-539.
20. Miyake N, Yamashita S, Kurosawa K, et al. A novel homozygous mutation of DARS2 may cause a severe LBSL variant. Clin Genet 2011;80:293-296.
21. Synofzik M, Schicks J, Lindig T, et al. Acetazolamide-responsive exercise-induced episodic ataxia associated with a novel homozygous DARS2 mutation. J Med Genet 2011;48:713-715.
22. Seburn KL, Nangle LA, Cox GA, Schimmel P, Burgess RW. An active dominant mutation of glycyl-tRNA synthetase causes neuropathy in a Charcot-Marie-Tooth 2D mouse model. Neuron 2006;51:715-726.
23. Isohanni P, Linnankivi T, Buzkova J, et al. DARS2 mutations in mitochondrial leucoencephalopathy and multiple sclerosis. J Med Genet 2010;47:66-70.

24. Abbott JA, Francklyn CS, Robey-Bond SM. Transfer RNA and human disease. Front Genet 2014;5:158.
25. Kevelam SH, Steenweg ME, Srivastava S, et al. Update on Leukodystrophies: A Historical Perspective and Adapted Definition. Neuropediatrics 2016.
26. Scheper GC, van der Knaap MS, Proud CG. Translation matters: protein synthesis defects in inherited disease. Nat Rev Genet 2007;8:711-723.
27. Taft RJ, Vanderver A, Leventer RJ, et al. Mutations in DARS cause hypomyelination with brain stem and spinal cord involvement and leg spasticity. Am J Hum Genet 2013;92:774-780.
28. Antonellis A, Green ED. The role of aminoacyl-tRNA synthetases in genetic diseases. Annu Rev Genomics Hum Genet 2008;9:87-107.
29. Sissler M, Gonzalez-Serrano LE, Westhof E. Recent Advances in Mitochondrial Aminoacyl-tRNA Synthetases and Disease. Trends Mol Med 2017;23:693-708.
30. Kim DG, Lee JY, Kwon NH, et al. Chemical inhibition of prometastatic lysyl-tRNA synthetase-laminin receptor interaction. Nat Chem Biol 2014;10:29-34.
31. Carapito C, Kuhn L, Karim L, et al. Two proteomic methodologies for defining N-termini of mature human mitochondrial aminoacyl-tRNA synthetases. Methods 2017;113:111-119.
32. Chen M, Manley JL. Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. Nat Rev Mol Cell Biol 2009;10:741-754.
33. McKee AE, Minet E, Stern C, Riahi S, Stiles CD, Silver PA. A genome-wide in situ hybridization map of RNA-binding proteins reveals anatomically restricted expression in the developing mouse brain. BMC Dev Biol 2005;5:14.
34. Aradjanski M, Dogan SA, Lotter S, et al. DARS2 protects against neuroinflammation and apoptotic neuronal loss, but is dispensable for myelin producing cells. Hum Mol Genet 2017.
35. van Berge L, Dooves S, van Berkel CG, Polder E, van der Knaap MS, Scheper GC. Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation is associated with cell-type-dependent splicing of mtAspRS mRNA. Biochem J 2012;441:955-962.
36. Novoyatleva T, Heinrich B, Tang Y, et al. Protein phosphatase 1 binds to the RNA recognition motif of several splicing factors and regulates alternative pre-mRNA processing. Hum Mol Genet 2008;17:52-70.
37. Wang G, Dong J, Deng L. Overview of Cantharidin and its Analogues. Curr Med Chem 2018;25:2034-2044.
38. van der Knaap MS, Barth PG, Stroink H, et al. Leukoencephalopathy with swelling and a discrepantly mild clinical course in eight children. Ann Neurol 1995;37:324-334.
39. Singhal BS, Gursahani RD, Udani VP, Biniwale AA. Megalencephalic leukodystrophy in an Asian Indian ethnic group. Pediatr Neurol 1996;14:291-296.
40. Ridder MC, Boor I, Lodder JC, et al. Megalencephalic leucoencephalopathy with cysts: defect in chloride currents and cell volume regulation. Brain 2011;134:3342-3354.
41. van der Knaap MS, Valk J, Barth PG, Smit LM, van Engelen BG, Tortori Donati P. Leukoencephalopathy with swelling in children and adolescents: MRI patterns and differential diagnosis. Neuroradiology 1995;37:679-686.
42. van der Knaap MS, Lai V, Kohler W, et al. Megalencephalic leukoencephalopathy with cysts without MLC1 defect. Ann Neurol 2010;67:834-837.
43. van der Knaap MS, Boor I, Estevez R. Megalencephalic leukoencephalopathy with subcortical cysts: chronic white matter oedema due to a defect in brain ion and water homoeostasis. Lancet Neurol 2012;11:973-985.
44. Lopez-Hernandez T, Ridder MC, Montolio M, et al. Mutant GlialCAM causes megalencephalic leukoencephalopathy with subcortical cysts, benign familial macrocephaly, and macrocephaly with retardation and autism. Am J Hum Genet 2011;88:422-432.

45. Wang PJ, Hwu WL, Shen YZ. Epileptic seizures and electroencephalographic evolution in genetic leukodystrophies. J Clin Neurophysiol 2001;18:25-32.
46. Vanderver A, Tonduti D, Schiffmann R, Schmidt J, van der Knaap MS. Leukodystrophy Overview. In: Adam MP, Ardinger HH, Pagon RA, et al., eds. GeneReviews. Seattle (WA) 2014.
47. Dubey M, Brouwers E, Hamilton EMC, et al. Seizures and disturbed brain potassium dynamics in the leukodystrophy megalencephalic leukoencephalopathy with subcortical cysts. Ann Neurol 2018;83:636-649.
48. Annegers JF, Hauser WA, Coan SP, Rocca WA. A population-based study of seizures after traumatic brain injuries. N Engl J Med 1998;338:20-24.
49. Gilad R, Boaz M, Sadeh M, Eilam A, Dabby R, Lampl Y. Seizures after very mild head or spine trauma. J Neurotrauma 2013;30:469-472.
50. Coulter DA, Steinhauser C. Role of astrocytes in epilepsy. Cold Spring Harb Perspect Med 2015;5:a022434.
51. Boor I, Nagtegaal M, Kamphorst W, et al. MLC1 is associated with the dystrophin-glycoprotein complex at astrocytic endfeet. Acta Neuropathol 2007;114:403-410.
52. Lopez-Hernandez T, Sirisi S, Capdevila-Nortes X, et al. Molecular mechanisms of MLC1 and GLIALCAM mutations in megalencephalic leukoencephalopathy with subcortical cysts. Hum Mol Genet 2011;20:3266-3277.
53. Dubey M, Bugiani M, Ridder MC, et al. Mice with megalencephalic leukoencephalopathy with cysts: a developmental angle. Ann Neurol 2015;77:114-131.
54. Jeworutzki E, Lopez-Hernandez T, Capdevila-Nortes X, et al. GlialCAM, a protein defective in a leukodystrophy, serves as a ClC-2 Cl(-) channel auxiliary subunit. Neuron 2012;73:951-961.
55. Wu M, Moh MC, Schwarz H. HepaCAM associates with connexin 43 and enhances its localization in cellular junctions. Sci Rep 2016;6:36218.
56. Duarri A, Teijido O, Lopez-Hernandez T, et al. Molecular pathogenesis of megalencephalic leukoencephalopathy with subcortical cysts: mutations in MLC1 cause folding defects. Hum Mol Genet 2008;17:3728-3739.
57. van der Knaap MS, Naidu S, Pouwels PJ, et al. New syndrome characterized by hypomyelination with atrophy of the basal ganglia and cerebellum. AJNR Am J Neuroradiol 2002;23:1466-1474.
58. van der Knaap MS, Linnankivi T, Paetau A, et al. Hypomyelination with atrophy of the basal ganglia and cerebellum: follow-up and pathology. Neurology 2007;69:166-171.
59. Simons C, Wolf NI, McNeil N, et al. A de novo mutation in the beta-tubulin gene TUBB4A results in the leukoencephalopathy hypomyelination with atrophy of the basal ganglia and cerebellum. Am J Hum Genet 2013;92:767-773.
60. Bahi-Buisson N, Poirier K, Fourniol F, et al. The wide spectrum of tubulinopathies: what are the key features for the diagnosis? Brain 2014;137:1676-1700.
61. Mutch CA, Poduri A, Sahin M, Barry B, Walsh CA, Barkovich AJ. Disorders of Microtubule Function in Neurons: Imaging Correlates. AJNR Am J Neuroradiol 2016;37:528-535.
62. Leandro-Garcia LJ, Leskela S, Landa I, et al. Tumoral and tissue-specific expression of the major human beta-tubulin isotypes. Cytoskeleton (Hoboken) 2010;67:214-223.
63. Tischfield MA, Engle EC. Distinct alpha- and beta-tubulin isotypes are required for the positioning, differentiation and survival of neurons: new support for the 'multi-tubulin' hypothesis. Biosci Rep 2010;30:319-330.
64. Mitchison T, Kirschner M. Dynamic instability of microtubule growth. Nature 1984;312:237-242.
65. Hersheson J, Mencacci NE, Davis M, et al. Mutations in the autoregulatory domain of beta-tubulin 4a cause hereditary dystonia. Ann Neurol 2013;73:546-553.
66. Lohmann K, Wilcox RA, Winkler S, et al. Whispering dysphonia (DYT4 dystonia) is caused by a mutation in the TUBB4 gene. Ann Neurol 2013;73:537-545.

67. Miyatake S, Osaka H, Shiina M, et al. Expanding the phenotypic spectrum of TUBB4A-associated hypomyelinating leukoencephalopathies. Neurology 2014;82:2230-2237.
68. Pizzino A, Pierson TM, Guo Y, et al. TUBB4A de novo mutations cause isolated hypomyelination. Neurology 2014;83:898-902.
69. Lu Y, Ondo Y, Shimojima K, Osaka H, Yamamoto T. A novel TUBB4A mutation G96R identified in a patient with hypomyelinating leukodystrophy onset beyond adolescence. Hum Genome Var 2017;4:17035.
70. Nicita F, Bertini E, Travaglini L, Armando M, Aiello C. Congenital-onset spastic paraplegia in a patient with TUBB4A mutation and mild hypomyelination. J Neurol Sci 2016;368:145-146.
71. Arai-Ichinoi N, Uematsu M, Sato R, et al. Genetic heterogeneity in 26 infants with a hypomyelinating leukodystrophy. Hum Genet 2016;135:89-98.
72. Purnell SM, Bleyl SB, Bonkowsky JL. Clinical exome sequencing identifies a novel TUBB4A mutation in a child with static hypomyelinating leukodystrophy. Pediatr Neurol 2014;50:608-611.
73. Shimojima K, Okumura A, Ikeno M, et al. A de novo TUBB4A mutation in a patient with hypomyelination mimicking Pelizaeus-Merzbacher disease. Brain Dev 2015;37:281-285.
74. Curiel J, Rodriguez Bey G, Takanohashi A, et al. TUBB4A mutations result in specific neuronal and oligodendrocytic defects that closely match clinically distinct phenotypes. Hum Mol Genet 2017;26:4506-4518.
75. Duncan ID, Bugiani M, Radcliff AB, et al. A mutation in the Tubb4a gene leads to microtubule accumulation with hypomyelination and demyelination. Ann Neurol 2017;81:690-702.
76. Morar B, Gresham D, Angelicheva D, et al. Mutation history of the roma/gypsies. Am J Hum Genet 2004;75:596-609.
77. Kalaydjieva L, Morar B, Chaix R, Tang H. A newly discovered founder population: the Roma/Gypsies. Bioessays 2005;27:1084-1094.
78. Azfer A, Niu J, Rogers LM, Adamski FM, Kolattukudy PE. Activation of endoplasmic reticulum stress response during the development of ischemic heart disease. Am J Physiol Heart Circ Physiol 2006;291:H1411-1420.
79. Yoo HM, Kang SH, Kim JY, et al. Modification of ASC1 by UFM1 is crucial for ERalpha transactivation and breast cancer development. Mol Cell 2014;56:261-274.
80. Lu H, Yang Y, Allister EM, Wijesekara N, Wheeler MB. The identification of potential factors associated with the development of type 2 diabetes: a quantitative proteomics approach. Mol Cell Proteomics 2008;7:1434-1451.
81. Lemaire K, Moura RF, Granvik M, et al. Ubiquitin fold modifier 1 (UFM1) and its target UFBP1 protect pancreatic beta cells from ER stress-induced apoptosis. PLoS One 2011;6:e18517.
82. Zhang Y, Zhang M, Wu J, Lei G, Li H. Transcriptional regulation of the Ufm1 conjugation system in response to disturbance of the endoplasmic reticulum homeostasis and inhibition of vesicle trafficking. PLoS One 2012;7:e48587.
83. Nahorski MS, Maddirevula S, Ishimura R, et al. Biallelic UFM1 and UFC1 mutations expand the essential role of ufmylation in brain development. Brain 2018;141:1934-1945.
84. Homrich M, Wobst H, Laurini C, Sabrowski J, Schmitz B, Diestel S. Cytoplasmic domain of NCAM140 interacts with ubiquitin-fold modifier-conjugating enzyme-1 (Ufc1). Exp Cell Res 2014;324:192-199.
85. Martin DD, Ladha S, Ehrnhoefer DE, Hayden MR. Autophagy in Huntington disease and huntingtin in autophagy. Trends Neurosci 2015;38:26-35.
86. Colin E, Daniel J, Ziegler A, et al. Biallelic Variants in UBA5 Reveal that Disruption of the UFM1 Cascade Can Result in Early-Onset Encephalopathy. Am J Hum Genet 2016.

87. Muona M, Ishimura R, Laari A, et al. Biallelic Variants in UBA5 Link Dysfunctional UFM1 Ubiquitin-like Modifier Pathway to Severe Infantile-Onset Encephalopathy. Am J Hum Genet 2016.
88. Duan R, Shi Y, Yu L, et al. UBA5 Mutations Cause a New Form of Autosomal Recessive Cerebellar Ataxia. PLoS One 2016;11:e0149039.
89. van der Knaap MS, Barth PG, Gabreels FJ, et al. A new leukoencephalopathy with vanishing white matter. Neurology 1997;48:845-855.
90. Leegwater PA, Vermeulen G, Konst AA, et al. Subunits of the translation initiation factor eIF2B are mutant in leukoencephalopathy with vanishing white matter. Nat Genet 2001;29:383-388.
91. Schiffmann R, Moller JR, Trapp BD, et al. Childhood ataxia with diffuse central nervous system hypomyelination. Ann Neurol 1994;35:331-340.
92. Elroy-Stein O. Mitochondrial malfunction in vanishing white matter disease: a disease of the cytosolic translation machinery. Neural Regen Res 2017;12:1610-1612.
93. Fogli A, Wong K, Eymard-Pierre E, et al. Cree leukoencephalopathy and CACH/VWM disease are allelic at the EIF2B5 locus. Ann Neurol 2002;52:506-510.
94. Labauge P, Fogli A, Niel F, Rodriguez D, Boespflug-Tanguy O. [CACH/VWM syndrome and leucodystrophies related to EIF2B mutations]. Rev Neurol (Paris) 2007;163:793-799.
95. Fogli A, Schiffmann R, Bertini E, et al. The effect of genotype on the natural history of eIF2B-related leukodystrophies. Neurology 2004;62:1509-1517.
96. Carra-Dalliere C, Horzinski L, Ayrignac X, et al. [Natural history of adult-onset eIF2B-related disorders: a multicentric survey of 24 cases]. Rev Neurol (Paris) 2011;167:802-811.
97. Labauge P, Horzinski L, Ayrignac X, et al. Natural history of adult-onset eIF2B-related disorders: a multi-centric survey of 16 cases. Brain 2009;132:2161-2169.
98. van der Knaap MS, Pronk JC, Scheper GC. Vanishing white matter disease. Lancet Neurol 2006;5:413-423.
99. van der Knaap MS, Bugiani M, Boor I, Proud CG, Scheper GC. Vanishing white matter. In: Valle D, Beaudet AL, Vogelstein B, et al., eds. The Online Metabolic and Molecular Bases of Inherited Disease (OMMBID). New York: McGraw-Hill, 2010.
100. Wisse LE, Penning R, Zaal EA, et al. Proteomic and Metabolomic Analyses of Vanishing White Matter Mouse Astrocytes Reveal Deregulation of ER Functions. Front Cell Neurosci 2017;11:411.
101. Klok MD, Bugiani M, de Vries SI, et al. Axonal abnormalities in vanishing white matter. Ann Clin Transl Neurol 2018;5:429-444.
102. Dooves S, Bugiani M, Postma NL, et al. Astrocytes are central in the pathomechanisms of vanishing white matter. J Clin Invest 2016;126:1512-1524.
103. van der Voorn JP, van Kollenburg B, Bertrand G, et al. The unfolded protein response in vanishing white matter disease. J Neuropathol Exp Neurol 2005;64:770-775.
104. Dooves S, Bugiani M, Wisse LE, Abbink TEM, van der Knaap MS, Heine VM. Bergmann glia translocation: a new disease marker for vanishing white matter identifies therapeutic effects of Guanabenz treatment. Neuropathol Appl Neurobiol 2017.
105. Confavreux C, Vukusic S. The clinical course of multiple sclerosis. Handb Clin Neurol 2014;122:343-369.
106. van der Knaap MS, Valk J, de Neeling N, Nauta JJ. Pattern recognition in magnetic resonance imaging of white matter disorders in children and young adults. Neuroradiology 1991;33:478-493.
107. Schiffmann R, van der Knaap MS. Invited article: an MRI-based approach to the diagnosis of white matter disorders. Neurology 2009;72:750-759.
108. Kevelam SH, Steenweg ME, Srivastava S, et al. Update on Leukodystrophies: A Historical Perspective and Adapted Definition. Neuropediatrics 2016;47:349-354.

109. Palisano RJ, Rosenbaum P, Bartlett D, Livingston MH. Content validity of the expanded and revised Gross Motor Function Classification System. Dev Med Child Neurol 2008;50:744-750.
110. Towns M, Rosenbaum P, Palisano R, Wright FV. Should the Gross Motor Function Classification System be used for children who do not have cerebral palsy? Dev Med Child Neurol 2017.
111. Kehrer C, Blumenstock G, Raabe C, Krageloh-Mann I. Development and reliability of a classification system for gross motor function in children with metachromatic leucodystrophy. Dev Med Child Neurol 2011;53:156-160.
112. Furlong WJ, Feeny DH, Torrance GW, Barr RD. The Health Utilities Index (HUI) system for assessing health-related quality of life in clinical studies. Ann Med 2001;33:375-384.
113. Vrij-van den Bos S, Hol JA, La Piana R, et al. 4H Leukodystrophy: A Brain Magnetic Resonance Imaging Scoring System. Neuropediatrics 2017;48:152-160.
114. Confavreux C, Compston A. The natural history of multiple sclerosis. In: Compston A, ed. McAlpine's multiple Sclerosis, 4th edition ed. London: Churchill Livingstone Elsevier, 2006: 183-272.
115. Sanger F, Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol 1975;94:441-448.
116. Wallis Y, Payne S, McAnulty C, et al. Practice Guidelines for the Evaluation of Pathogenicity and the Reporting of Sequence Variants in Clinical Molecular Genetics. Association for Clinical Genetic Science and the Dutch Society of Clinical Genetic Laboratory Specialists 2013.
117. Biesecker LG, Green RC. Diagnostic clinical genome and exome sequencing. N Engl J Med 2014;371:1170.
118. Bao R, Huang L, Andrade J, et al. Review of current methods, applications, and data management for the bioinformatics analysis of whole exome sequencing. Cancer Inform 2014;13:67-82.
119. Vermeesch JR, Voet T, Devriendt K. Prenatal and pre-implantation genetic diagnosis. Nat Rev Genet 2016;17:643-656.
120. van den Broek BTA, Page K, Paviglianiti A, et al. Early and late outcomes after cord blood transplantation for pediatric patients with inherited leukodystrophies. Blood Adv 2018;2:49-60.
121. Collins C. Phenotype with a side of genotype, please: Patients, parents and priorities in rare genetic disease. Appl Transl Genom 2016;8:42-44.
122. Giovanni MA, Murray MF. Genome-first findings require precision phenotyping. Genet Med 2018.
123. Bugiani M, van der Knaap MS. Childhood white matter disorders: much more than just diseases of myelin. Acta Neuropathol 2017;134:329-330.
124. Tifft CJ, Adams DR. The National Institutes of Health undiagnosed diseases program. Curr Opin Pediatr 2014;26:626-633.
125. van der Knaap MS, Bugiani M. Leukodystrophies: a proposed classification system based on pathological changes and pathogenetic mechanisms. Acta Neuropathol 2017.
126. Back SA. White matter injury in the preterm infant: pathology and mechanisms. Acta Neuropathol 2017.
127. Workshop on Natural History Studies of Rare Diseases: meeting the Needs of Drug Development and Research [online]. Available at: https://events-support.com/events/Natural_History_Studies/page/87. Accessed May 16–17, 2012.
128. Shapiro E, Bernstein J, Adams HR, et al. Neurocognitive clinical outcome assessments for inborn errors of metabolism and other rare conditions. Mol Genet Metab 2016;118:65-69.
129. Schaumburg HH, Powers JM, Raine CS, Suzuki K, Richardson EP, Jr. Adrenoleukodystrophy. A clinical and pathological study of 17 cases. Arch Neurol 1975;32:577-591.

130. Bezman L, Moser HW. Incidence of X-linked adrenoleukodystrophy and the relative frequency of its phenotypes. Am J Med Genet 1998;76:415-419.
131. Kemp S, Pujol A, Waterham HR, et al. ABCD1 mutations and the X-linked adrenoleukodystrophy mutation database: role in diagnosis and clinical correlations. Hum Mutat 2001;18:499-515.
132. Cayami FK, Bugiani M, Pouwels PJW, Bernard G, van der Knaap MS, Wolf NI. 4H Leukodystrophy: Lessons from 3T Imaging. Neuropediatrics 2018;49:112-117.
133. Park S, Yang J-S, Kim J, et al. Evolutionary history of human disease genes reveals phenotypic connections and comorbidity among genetic diseases. Scientific Reports 2012;2:757.
134. Wei WQ, Denny JC. Extracting research-quality phenotypes from electronic health records to support precision medicine. Genome Med 2015;7:41.
135. Sobreira N, Schiettecatte F, Valle D, Hamosh A. GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. Hum Mutat 2015;36:928-930.
136. Rahimzadeh V, Dyke SO, Knoppers BM. An International Framework for Data Sharing: Moving Forward with the Global Alliance for Genomics and Health. Biopreserv Biobank 2016;14:256-259.
137. Naldini L. Gene therapy returns to centre stage. Nature 2015;526:351-360.
138. Leone P, Shera D, McPhee SW, et al. Long-term follow-up after gene therapy for canavan disease. Sci Transl Med 2012;4:165ra163.
139. Georgiou E, Sidiropoulou K, Richter J, et al. Gene therapy targeting oligodendrocytes provides therapeutic benefit in a leukodystrophy model. Brain 2017;140:599-616.
140. Osorio MJ, Goldman SA. Glial progenitor cell-based treatment of the childhood leukodystrophies. Exp Neurol 2016;283:476-488.
141. Leferink PS, Heine VM. The Healthy and Diseased Microenvironments Regulate Oligodendrocyte Properties: Implications for Regenerative Medicine. Am J Pathol 2018;188:39-52.
142. van der Knaap MS, Wolf NI, Heine VM. Leukodystrophies: Five new things. Neurol Clin Pract 2016;6:506-514.

VI

Appendices

Chapter 9

Nederlandse samenvatting

ACHTERGROND

Het is 2012 als het leven van een 2-jarige jongen plotseling drastisch verandert. Tot die tijd was hij een gezond, actief kind met een normale ontwikkeling. Nu lijdt hij aan een bacteriële infectie met koorts en heeft hij problemen met coördinatie en lopen. Hij gaat snel achteruit, verliest de vaardigheid om te lopen en wordt opgenomen op de Intensive Care afdeling met een verlaagd bewustzijn. Er wordt een MRI scan gemaakt, die uitgebreide afwijkingen van de witte stof van de hersenen toont. De artsen vrezen voor een vorm van leukodystrofie. Leukodystrofieën vormen een groep van zeldzame, erfelijke hersenziekten met vaak een slechte prognose. Niet duidelijk is om welke vorm van leukodystrofie het gaat. De ouders van de jongen krijgen te horen dat de verwachting is dat hij niet langer dan een jaar zal leven, een rampzalig vooruitzicht. Nadat hij hersteld is van de infectie, toont de jongen een verrassend herstel: hij leert opnieuw kruipen en na een paar maanden kan hij weer lopen. Er blijven enkel wat subtiele coördinatieproblemen bestaan. Het blijft onduidelijk wat de diagnose is. Als de jongen 4 jaar is, valt hij tijdens het spelen op zijn hoofd. Daarna kan hij niet meer lopen en verschijnt er een tremor aan de handen. Een nieuwe hersenscan toont opnieuw wittestofafwijkingen, maar de oorzaak is onbekend. Ondertussen verwachten de ouders een tweede kind. De artsen hebben hun verteld dat er een reële kans is dat dit kind ook leukodystrofie heeft. Het is niet mogelijk om prenatale diagnostiek te doen, aangezien er geen specifieke diagnose is. Het kind wordt gezond geboren, maar vertoont op 2-jarige leeftijd de eerste symptomen van de hersenziekte. Uiteindelijk, na vele onderzoeken en second opinions, wordt er voor beide kinderen een diagnose gesteld: zij lijden aan de leukodystrofie Vanishing White Matter (VWM). Voor de ouders is het een opluchting om te weten wat er aan de hand is en de puzzelstukjes op hun plaats te zien vallen. Hoewel er geen behandeling bestaat voor de ziekte, maakt het hebben van de diagnose het mogelijk voor de familie om zich te richten op gepaste medische en preventieve zorg en op kwaliteit van leven voor hun kinderen. Ook is het nu mogelijk om prenataal onderzoek te doen bij een volgende zwangerschap.

“Wittestofziekten” is de verzamelnaam van een groep ziekten waarbij voornamelijk of uitsluitend de witte stof van de hersenen is aangedaan. Leukodystrofieën zijn de erfelijke wittestofziekten. Er zijn vele verschillende vormen en elke ziekte is zeldzaam, maar als groep zijn ze niet zo zeldzaam. De meeste ziekten zijn zeer invaliderend. Er is helaas nog maar weinig bekend over de onderliggende mechanismen van deze ziekten en over hoe de ziekten verlopen. De impact op het leven van patiënten en families is enorm.

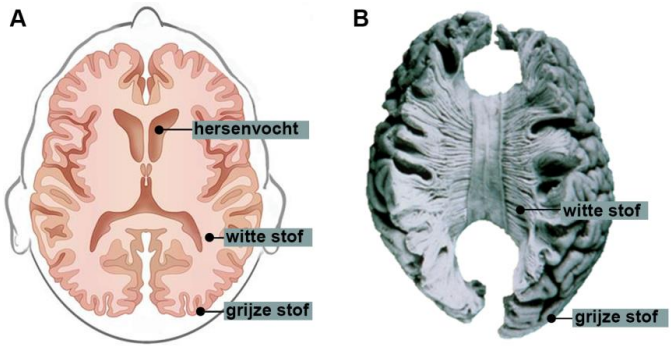
Het onderzoek dat in dit proefschrift wordt beschreven, werd verricht bij het Centrum voor kinderwittestofziekten in Amsterdam. Het doel van dit centrum is het verbeteren van de diagnostiek en patiëntenzorg voor kinderen met wittestofziekten. Het ultieme doel is om de ziekten te kunnen genezen. Het ontrafelen van de mechanismen die aan de ziekten ten grondslag liggen is een essentiële stap in deze richting. In dit

proefschrift beschrijven we de nieuwe inzichten die zijn opgedaan voor verschillende leukodystrofieën aan de hand van het bestuderen van het ziektebeloop.

De volgende twee secties bevatten achtergrondinformatie over leukodystrofieën en genetica, belangrijk voor het begrijpen van de onderzoeksresultaten.

1. Leukodystrofieën

De hersenen bestaan uit grijze en witte stof. De grijze stof bevat de zenuwcellen en is voornamelijk gelegen in de hersenschors aan de buitenkant van het brein. De witte stof bevat de uitlopers van de zenuwcellen (axonen), die verschillende delen van de hersenen onderling en met het ruggenmerg verbinden; het vormt de “bedrading” van het zenuwstelsel (Figuur 1). De witte kleur komt van de aanwezigheid van myeline, de isolatielaag van de zenuwbaan.



Figuur 1 | Witte stof. (**A**) Overzicht van een dwarsdoorsnede van de hersenen, waarop zichtbaar is dat de grijze stof zich aan de buitenkant bevindt. De witte stof is aan de binnenkant gelegen. (**B**) Foto van een geprepareerde dwarsdoorsnede van menselijke hersenen, die de wittestofbanen toont.

Bij veel neurologische ziekten wordt met name de grijze stof getroffen. Een bekend voorbeeld van een grijzestofziekte is de ziekte van Alzheimer. Een veelvoorkomende wittestofziekte is Multiple Sclerose (MS), een verworven ziekte die vooral op de volwassen leeftijd voorkomt. Wittestofziekten worden ook wel leukoencefalopathieën genoemd. Deze term stamt af van de Griekse taal: “leuko” = wit en “encefalopathie” = ziekte van de hersenen. Indien een wittestofziekte erfelijk is, wordt gesproken van leukodystrofie: “dys” = gestoord en “trofie” = groei. Bij deze groep van ziekten worden patiënten vaak al op de kinderleeftijd getroffen. Geschat wordt dat ongeveer 1 op de 7600 kinderen wordt geboren met de erfelijke aanleg die een leukodystrofie tot gevolg heeft. Motorische problemen staan doorgaans op de voorgrond en patiënten kunnen last hebben van spasticiteit en een gestoorde coördinatie. Het beloop is vaak ernstig en een aanzienlijk deel van de patiënten overlijdt ten gevolge van de ziekte. Maar er is veel variatie en er zijn ook patiënten die een redelijk normaal leven leiden.

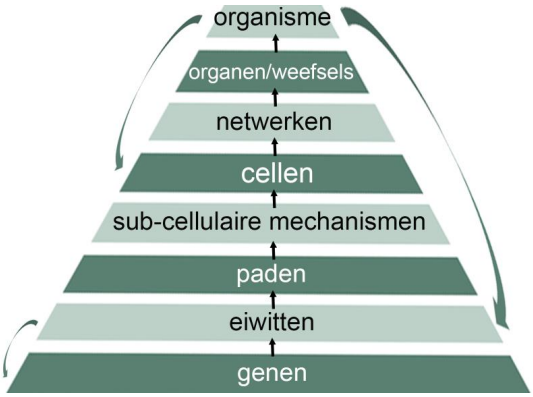
In de afgelopen 30 jaar is er veel vooruitgang geboekt op het terrein van onderzoek naar leukodystrofieën. Dat begon met de introductie van de Magnetische Resonantie

Beeldvorming (MRI) waarbij het mogelijk werd om de hersenen in detail af te beelden. Het werd duidelijk dat verschillende wittestofziekten te onderscheiden zijn op basis van gelijksoortige MRI patronen. Vervolgens werden er bij patiënten met gelijke MRI afwijkingen ook afwijkingen (mutaties) in hetzelfde gen gevonden.

2. Genetica en onderliggende ziektemechanismen

Leukodystrofieën zijn monogenetische aandoeningen: dit type van overerving wordt veroorzaakt door mutaties die optreden in één enkel gen. Een gen is een stukje erfelijke materiaal (DNA) dat informatie bevat over de vorming van een bepaald eiwit met een specifieke functie of eigenschap in het lichaam (Figuur 2). Met de revolutionaire ontwikkelingen op het gebied van genetisch onderzoek worden er nu in snel tempo nieuwe genen ontdekt die geassocieerd zijn met leukodystrofieën. Het vinden van de genetische oorzaak is een essentiële stap voor het bestuderen van onderliggende ziektemechanismen. Bovendien maakt het hebben van een genetische diagnose het door de toepassing van prenatale diagnostiek of preïmplantatie-diagnostiek mogelijk te voorkomen dat er binnen een familie opnieuw een kindje met dezelfde wittestofziekte wordt geboren.

In het Centrum voor kinderwittestofziekten worden leukodystrofieën op multidisciplinaire wijze bestudeerd. Er worden nieuw inzichten verworven op het gebied van onder andere de klinische, radiologische, biologische en neuropathologische aspecten. Het vanuit verschillende invalshoeken bestuderen van een ziekte kan door kruisbestuivingen leiden tot belangrijke nieuwe inzichten (Figuur 2). Observaties aan de hand van onderzoek naar patiëntkenmerken vormen veelal het beginpunt van het verder ontrafelen van hoe een ziekte werkt.



Figuur 2 | Overzicht van verschillende niveaus van de biologische organisatie van organismen. Genen coderen voor eiwitten, die in het lichaam een specifieke functie uitvoeren en betrokken zijn bij de vorming van netwerken, weefsel en organen. Het leven van mensen (en andere organismen) berust op een samenspel van genen, eiwitten, weefsels, organen en de omgeving. Door dit samenspel vanuit een multidisciplinaire aanpak te bestuderen worden nieuwe inzichten verkregen in functies en ziektemechanismen.

OPZET EN DOEL VAN HET PROEFSCHRIFT

Met het groeiende aantal gedefinieerde leukodystrofieën en de vooruitgang in beeldvormende technieken en genetisch onderzoek kan bij het merendeel van de patiënten met een leukodystrofie nu een specifieke diagnose gesteld worden. Een diagnostische test alleen heeft echter weinig betekenis wanneer deze niet in een klinische context geplaatst kan worden. Een belangrijk deel van de symptomen van verschillende wittestofziekten komt overeen, en daarmee ook de aanpak voor symptomatische en preventieve behandeling. Maar om patiënten en families optimaal te kunnen informeren, begeleiden en behandelen, is ziekte-specifiek inzicht in het beloop, klinische variatie en risicofactoren essentieel. Het centrale thema van dit proefschrift is het bestuderen van de klinische kenmerken (het fenotype) en de genetische kenmerken (het genotype) van een viertal relatief recent geïdentificeerde, “nieuwe” leukodystrofieën, namelijk;

- **LBSL:** leukoencefalopathie met hersenstam en ruggenmergafwijkingen en verhoogd lactaat;
- **MLC**: megalencefale leukoencefalopathie met subcorticale cysten;
- **H-ABC:** hypomyelinisatie met atrofie van de basale kernen en het cerebellum;
- **VWM** Vanishing White Matter.

Omdat genoemde ziekten nog maar kort bekend zijn, is de kennis over de ziekten zeer beperkt. Door middel van wereldwijde samenwerking met verwijzende artsen, patiënten en families hebben we ziektekenmerken kunnen beschrijven onder voor deze vier zeer zeldzame ziekten unieke aantallen patiënten.

Doelstellingen

I) Het beter definiëren van de klinische en genetische kenmerken van vier leukodystrofieën, om informatieverstrekking en patiëntenzorg door artsen te verbeteren.
II) Het verkrijgen van inzicht in het natuurlijk beloop van de ziekten om in de toekomst het evalueren van therapeutische behandelingen mogelijk te maken.
III) Het vergroten van de kennis over de onderliggende ziektemechanismen.

RESULTATEN

In deze sectie worden voor de vier leukodystrofieën de belangrijkste onderzoeksresultaten beschreven, voorafgegaan door een introductie over de ziekte.

Leukoencefalopathie met hersenstam en ruggenmergafwijkingen en verhoogd lactaat (LBSL)

LBSL is een aandoening met een heel karakteristiek MRI patroon, waarop de witte stof van de hersenen en specifieke structuren in de hersenstam en het ruggenmerg een afwijkend signaal vertonen. De ziekte wordt meestal gekenmerkt door een langzaam progressieve verstoring van de normale functie van de pyramidebanen (de motorische banen), het cerebellum (de kleine hersenen die voor coördinatie zorgen) en de achterstrengen (de banen voor het gevoel). LBSL stond initieel bekend als een relatief milde ziekte, met presentatie van verschijnselen op latere kinderleeftijd. Door de zeldzaamheid van de ziekte was er in 2012 nog maar weinig bekend over het ziektebeloop en de variatie in ernst.

Wij hebben een studie verricht onder 78 patiënten om een beter inzicht te krijgen in het spectrum van de ziekte. In hoofdstuk 2 beschrijven we dat de ziekte zeer uiteenlopend kan verlopen, van presentatie in het eerste levensjaar met snelle achteruitgang en vroeg overlijden, tot presentatie op de volwassen leeftijd met een mild ziektebeloop. Met uitzondering van kinderen bij wie de ziekte zich al op heel jonge leeftijd openbaart, gaan patiënten over het algemeen zeer langzaam achteruit. Volledige rolstoelafhankelijkheid is zeldzaam en komt doorgaans pas op volwassen leeftijd voor. Ernstige gevallen uitgezonderd, lijkt het erop dat de meeste patiënten met LBSL een normale levensverwachting hebben.

LBSL is een recessieve ziekte; patiënten erven van beide ouders een mutatie in het *DARS2* gen. *DARS2* codeert voor het eiwit “mitochondriaal aspartyl-tRNA synthetase” (mtAspRS). Dit is een enzym dat een belangrijke functie vervult in de mitochondriën, de energiecentrales van de cellen in het lichaam. We presenteren in hoofdstuk 2 een overzicht van verschillende *DARS2* mutaties die zijn gevonden bij LBSL patiënten. Een van de observaties was dat bijna alle patiënten een specifiek type mutatie hebben, een zogeheten splice-site mutatie in intron 2, die ervoor zorgt dat een deel van het mtAspRS eiwit niet gevormd wordt. Veel patiënten zouden dus baat hebben bij een therapie die het effect van deze splice-site mutatie ongedaan maakt. In hoofdstuk 2 beschrijven we hoe we via laboratoriumonderzoek een middel hebben geïdentificeerd dat aangrijpt op de splice-site mutatie en de vorming van het eiwit gunstig beïnvloedt: cantharidine. Helaas is dit middel erg giftig, waardoor het niet zomaar als behandeling kan worden ingezet. Er wordt veel onderzoek gedaan naar cantharadine omdat het ook effectief is gebleken in de behandeling van kanker. Ons onderzoek heeft aangetoond dat het principe werkt. Het zoeken naar een minder toxische variant van het middel lijkt een veelbelovende weg vooruit in de behandeling van LBSL.

Megalencefale leukoencefalopathie met subcorticale cysten (MLC)

De hersenen liggen goed beschermd in een schedel. Het gevolg van deze harde bescherming is dat er nauwelijks ruimte is voor verandering van volume van de inhoud van die schedel. Een kleine toename van het volume, door bijvoorbeeld bloeding of zwelling, leidt tot stijging van de druk en beknelling van hersenweefsel, hetgeen desastreuze gevolgen kan hebben ("inklemming"). Om dergelijke schade te beperken zijn de hersenen uitgerust met een ingenieus systeem voor volumeregulatie. Alleen in de eerste 1 à 2 levensjaren is zwelling van de hersenen minder een probleem, omdat de schedelnaden nog open zijn en versterkte groei van het hoofd toelaten. Bij de ziekte MLC is er iets mis met deze volumeregulatie, waardoor er in het eerste levensjaar zwelling van de hersenen optreedt. Toename van de hoofdomtrek (megalencefalie) is doorgaans het eerste verschijnsel van de ziekte, zonder dat er op dat moment andere symptomen zijn. De MRI van de hersenen toont dat de witte stof een abnormaal signaal heeft en gezwollen is. Ook zijn er cysten in de hersenen: holtes gevuld met vocht. Er zijn twee verschillende vormen van de ziekte: de klassieke vorm, waarbij patiënten langzaam achteruit gaan, en de verbeterende vorm, waarbij in de loop van de tijd vooruitgang optreedt.

Omdat er nooit systematisch was gekeken naar de klinische en MRI kenmerken van de ziekte in een grote groep patiënten, zijn wij een samenwerking gestart met een internationale groep artsen. Het doel was om de ziektekenmerken beter in kaart te brengen en om te kijken of er bepaalde eigenschappen zijn, die helpen bij het onderscheid maken tussen verschillende vormen van de ziekte. In hoofdstuk 3 beschrijven we de ziekte- en MRI kenmerken onder 242 MLC patiënten. Bij de meeste patiënten met klassieke MLC werd gedurende de eerste jaren na de start van de ziekte een langzame achteruitgang van de motoriek gezien. Opvallend was dat wanneer patiënten op de leeftijd van 15 jaar nog niet volledig rolstoelafhankelijk waren, zij ook hierna doorgaans het loopvermogen behielden. Er werd een laag sterftegetal gezien. De 38 patiënten met verbeterende MLC hadden een duidelijk milder ziektebeloop met behoud van motorische functies. Intellectuele beperkingen en autisme kwamen wel relatief veel voor in deze groep. We konden enkele radiologische kenmerken identificeren die in de vroege fase van de ziekte differentiëren tussen klassieke en verbeterende MLC. We vonden geen verschil tussen klassieke MLC door *MLC1* mutaties en MLC door *GLIALCAM* mutaties. De resultaten van de studie kunnen artsen van MLC patiënten helpen bij het bieden van medische zorg en verstrekken van informatie over het ziektebeloop aan patiënten en het gericht aanvragen van genetisch onderzoek naar de ziekte. Naar aanleiding van observaties op het gebied van epileptische aanvallen bij patiënten met MLC werd er een nieuwe studie opgezet waarbij een ziektemodel voor MLC werd gebruikt om aan te tonen dat de ziekte waarschijnlijk berust op een defect in de kaliumbuffering in de hersenen.

Hypomyelinisatie met atrofie van de basale kernen en het cerebellum (H-ABC)

Bij H-ABC patiënten is er een verstoring van de normale aanleg van myeline in de hersenen. Bovendien toont MRI dat specifieke centrale kernen in de hersenen afwezig zijn of in verminderde mate aanwezig. De zieke presenteert zich in de eerste levensjaren. De belangrijkste symptomen zijn spasticiteit, coördinatieproblemen en de aanwezigheid van ongewilde bewegingen. Bij de zoektocht naar de genetische oorzaak van H-ABC in 2013 werden 11 patiënten geselecteerd aan de hand van strenge MRI criteria en vergelijkbare klinische verschijnselen. Er werd ontdekt dat deze patiënten allen dezelfde dominante mutatie hadden in het *TUBB4A* gen. De identificatie van het genetisch defect maakte het mogelijk om vervolgens in een grotere groep patiënten te bestuderen wat de ziektekenmerken waren. In hoofdstuk 4 beschrijven we de ziekte en MRI kenmerken onder 42 genetisch gediagnosticeerde patiënten. Er bleken ook atypische gevallen van de ziekte te zijn en er waren sterke aanwijzingen dat de ernst en vorm van de ziekte nauw samenhangt met de specifieke lokalisatie van de mutatie in het *TUBB4A* gen. In dezelfde periode is ontdekt dat *TUBB4A* mutaties ook geassocieerd zijn met een andere neurologische ziekte, namelijk Dystonie type 4. Deze ziekte komt doorgaans pas op volwassen leeftijd aan het licht en gaat niet gepaard met MRI afwijkingen. Vervolgens bleken ook diverse gevallen van hypomyelinisatie zonder basale kernen afwijkingen te berusten op mutaties in dit gen. Deze klinische observaties in patiënten waren de basis voor vervolgstudies in het laboratorium die hebben bijgedragen aan nieuw inzichten in het onderliggende ziektemechanisme.

Bij een klein deel van de patiënten die aan de MRI kenmerken van H-ABC voldeden, kon bij DNA onderzoek geen mutatie in het *TUBB4A* gen gevonden worden. Dit deed vermoeden dat er nog een tweede gen geassocieerd is met de ziekte. Het nader bestuderen van de onopgeloste gevallen bracht het inzicht dat verschillende patiënten afkomstig waren uit families met een Roma (zigeuner) achtergrond. Ook bleken ouders van de patiënten veelal verwant te zijn. Derhalve was er een sterke verdenking op een erfelijke aandoening met homozygote overerving, waarbij patiënten van beide ouders dezelfde genetische variant overerven. Geavanceerd genetisch onderzoek toonde aan dat 16 patiënten dezelfde homozygote mutatie in het *UFM1* gen hadden (hoofdstuk 5). Alle patiënten met *UFM1* mutaties hadden een zeer ernstige ziekte met moeilijk behandelbare epilepsie en sterfte op de jonge kinderleeftijd. Screening onder verschillende Roma groeperingen toonde dat het dragerschap van deze mutatie in bepaalde delen in Europa erg hoog is. De ontdekking van de genetische oorzaak van deze vorm van H-ABC maakt het mogelijk om in risicogroepen dragerschapstesten of prenatale screenings te verrichten.

Vanishing White Matter (VWM)

Bij VWM, een van de vaakst voorkomende leukodystrofieën, is er een proces gaande waarbij de witte stof van de hersenen verdwijnt en geleidelijk wordt vervangen door vloeistof. Het gevolg is, dat de hersenen van patiënten steeds minder goed

functioneren en zij onder andere problemen krijgen met de motoriek. In eerste instantie werd VWM vooral gediagnosticeerd bij jonge kinderen, met een presentatie tussen het 2e en 6e levensjaar. Later werd duidelijk dat er ook gevallen zijn die zich op veel jongere of juist veel latere leeftijd openbaren. De ziekte berust op mutaties in 5 verschillende genen die coderen voor eiwitten die een cruciale rol spelen bij de regulatie van de vertaling van genetische informatie naar eiwit, in het bijzonder in omstandigheden waarbij cellen in stress verkeren. Een typerend kenmerk van de ziekte is dat bepaalde stressoren, zoals een infectie met koorts of een val op het hoofd, ervoor kunnen zorgen dat patiënten plotseling achteruitgaan, en zelfs in coma komen of overlijden.

Om meer inzicht te krijgen in het beloop van deze ziekte en de relatie met het genotype is er in 2004 een database gestart waarin gedetailleerde informatie over het ziektebeloop werd verzameld. In hoofdstuk 6 beschrijven we de resultaten van een 12,5 jaar durende studie naar het natuurlijke beloop van de ziekte onder 296 genetisch bevestigde patiënten. Er is sprake van een zeer uiteenlopend ziektebeloop, variërend van gevallen waarbij de eerste symptomen al voor de geboorte aan het licht komen en waarbij patiënten snel overlijden, tot presentatie op de volwassenen leeftijd met een relatief mild beloop. Een vroege beginleeftijd bleek een ongunstige voorspeller voor de uitkomst. Andere voorspellers die samenhingen met een ernstiger beloop waren de aanwezigheid van epileptische aanvallen en de aanwezigheid van episodische achteruitgang uitgelokt door stressoren. Het is dus van groot belang om maatregelen te nemen om de invloed van dergelijke stressoren tegen te gaan. De bevindingen van deze studie illustreren dat alleen het hebben van de diagnose VWM nog niet veel zegt over het te verwachten ziektebeloop. Het is voor het counselen van patiënten en families essentieel om kennis te hebben over de invloed van andere factoren, zoals de beginleeftijd.

Om meer inzicht te krijgen in de impact van de ziekte VWM op het dagelijks leven van patiënten hebben we onderzoek gedaan naar domeinen van handicap en de impact van de ziekte op de kwaliteit van leven. In hoofdstuk 7 beschrijven we het ziektebeloop aan de hand van twee gestandaardiseerde vragenlijsten over handicap, de Health Utilities Index (HUI) en Guy's Neurological Disability Scale (GNDS). De data tonen dat het merendeel van de patiënten veelal eerst problemen krijgt met het lopen en de handvaardigheid, vooral op de kinderleeftijd. Zij krijgen doorgaans pas in een later stadium problemen met het denken. Daar staat tegenover dat cognitieve problemen bij patiënten met een ziektedebuut op de volwassen leeftijd juist vaak al vroeg aanwezig zijn. In een gevorderd stadium van de ziekte doen zich ook problemen voor op het gebied van spraak en de functie van blaas en darmen.

De HUI biedt de mogelijkheid om het functioneren van patiënten af te zetten tegen het oordeel van gezonde mensen over een bepaalde gezondheidstoestand. Een opvallende bevinding was dat buitenstaanders de levenskwaliteit van patiënten met VWM doorgaans zeer laag of zelfs als ontoereikend zouden beoordelen, terwijl patiënten met de ziekte of hun of families vaak nog veel levensvreugde ervaren.

Al met al bieden de bevindingen inzicht in het ziektebeloop voor verschillende varianten van de ziekte. De in hoofdstuk 6 en 7 besproken data over het natuurlijke beloop van VWM dragen bij aan de planning van therapeutische trials.

RELEVANTIE

Dit proefschrift biedt nieuwe inzichten in het ziektebeloop van enkele leukodystrofieën. Het onderzoek draagt bij aan een betere herkenbaarheid van de ziektekenmerken en daarmee de kans op het stellen van een juiste diagnose. De opgedane kennis helpt artsen bij het beter informeren van patiënten en families met LBSL, MLC, H-ABC en VWM over het te verwachten ziektebeloop. Daarnaast dragen observaties over ziekte-eigenschappen bij aan het beter begrijpen van de onderliggende ziektemechanismen. Vooruitgangen in technologische ontwikkelingen op het gebied van therapieën, zoals gentherapie en stamceltherapie, bieden hoop voor curatieve behandeling voor een toenemend aantal leukodystrofieën. Ook voor het opzetten en evalueren van behandelingsstudies is informatie over het ziektebeloop en onderlinge variatie tussen patiënten onmisbaar. Ondanks de enorme vooruitgang op het gebied van kennis over leukodystrofieën, blijven er nog vele vragen. Gezien de complexiteit en zeldzaamheid van de ziekten is samenwerking, zowel internationaal als interdisciplinair, de sleutel voor verdere vooruitgang.

OVERVIEW OF PUBLICATIONS

Publications related to this thesis

Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation: clinical and genetic characterization and target for therapy.
van Berge L,* Hamilton EM,* Linnankivi T, Uziel G, Steenweg ME, Isohanni P, Wolf NI, Krägeloh-Mann I, Brautaset NJ, Andrews PI, de Jong BA, al Ghamdi M, van Wieringen WN, Tannous BA, Hulleman E, Würdinger T, van Berkel CG, Polder E, Abbink TE, Struys EA, Scheper GC, van der Knaap MS, LBSL Research Group. Brain. 2014;137:1019-1029.

Reply: *DARS2* gene clinical spectrum: new ideas regarding an underdiagnosed leukoencephalopathy.
van der Knaap MS, Hamilton EM, van Berge L. Brain. 2014;137:e290.

Hypomyelination with atrophy of the basal ganglia and cerebellum: further delineation of the phenotype and genotype-phenotype correlation.
Hamilton EM, Polder E, Vanderver A, Naidu S, Schiffmann R, Fisher K, Raguž AB, Blumkin L, H-ABC Research Group, van Berkel CG, Waisfisz Q, Simons C, Taft RJ, Abbink TE, Wolf NI,** van der Knaap MS.** Brain. 2014;137:1921-1930.

Reply: *TUBB4A* novel mutation reinforces the genotype-phenotype correlation of hypomyelination with atrophy of the basal ganglia and cerebellum.
Hamilton EM, Wolf NI, van der Knaap MS. Brain. 2015;138:e328.

Reply: A novel *TUBB4A* mutation suggests that genotype-phenotype correlation of H-ABC syndrome needs to be revisited.
Hamilton EM, Wolf NI, van der Knaap MS. Brain. 2015;138:e371.

***UFM1* founder mutation in the Roma population causes recessive variant of H-ABC.**
Hamilton EMC, Bertini E, Kalaydjieva L, Morar B, Dojčáková D, Liu J, Vanderver A, Curiel J, Persoon CM, Diodato D, Pinelli L, van der Meij NL, Plecko B, Blaser S, Wolf NI, Waisfisz Q, Abbink TEM, van der Knaap MS, Recessive H-ABC Research Group. Neurology. 2017;89:1821-1828.

Megalencephalic leukoencephalopathy with subcortical cysts: Characterization of disease variants.
Hamilton EMC, Tekturk P, Cialdella F, van Rappard DF, Wolf NI, Yalcinkaya C, Çetinçelik Ü, Rajaee A, Kariminejad A, Paprocka J, Yapici Z, Bošnjak VM, van der Knaap MS, MLC Research Group. Neurology. 2018;90:e1395-e1403.

Natural History of Vanishing White Matter.
Hamilton EMC, van der Lei HDW, Vermeulen G, Gerver JAM, Lourenço CM, Naidu S, Mierzewska H, Gemke R, de Vet HCW, Uitdehaag BMJ, Lissenberg-Witte BI, VWM Research Group, van der Knaap MS. Ann Neurol. 2018;84:274-288.

Other scientific publications

Novel cases of D-2-hydroxyglutaric aciduria with *IDH1* or *IDH2* mosaic mutations identified by amplicon deep sequencing.
Nota B, Hamilton EM, Sie D, Ozturk S, van Dooren SJ, Ojeda MR, Jakobs C, Christensen E, Kirk EP, Sykut-Cegielska J, Lund AM, van der Knaap MS, Salomons GS. J Med Genet. 2013;50:754-759.

Novel (ovario) leukodystrophy related to *AARS2* mutations.
Dallabona C,* Diodato D,* Kevelam SH,* Haack TB, Wong LJ, Salomons GS, Baruffini E, Melchionda L, Mariotti C, Strom TM, Meitinger T, Prokisch H, Chapman K, Colley A, Rocha H, Ounap K, Schiffmann R, Salsano E, Savoiardo M, Hamilton EM, Abbink TE, Wolf NI, Ferrero I, Lamperti C, Zeviani M, Vanderver A,** Ghezzi D,** van der Knaap MS.** Neurology. 2014;82:2063-2071.

***TUBB4A*-Related Leukodystrophy.**
Nahhas N, Conant A, Hamilton E, Curiel J, Simons C, van der Knaap M, Vanderver A. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, et al., editors. GeneReviews. Seattle (WA) 2016.

Seizures and disturbed brain potassium dynamics in the leukodystrophy megalencephalic leukoencephalopathy with subcortical cysts.
Dubey M,* Brouwers E,* Hamilton EMC, Stiedl O, Bugiani M, Koch H, Kole MHP, Boschert U, Wykes RC, Mansvelder HD, van der Knaap MS, Min R. Ann Neurol. 2018;83:636-649.

* These authors share first authorship
** These authors share senior authorship

DANKWOORD

Dit proefschrift gaat over kinderen en volwassenen met wittestofziekten. Zonder hun bijdragen zou het resultaat nooit geworden zijn wat het nu is. Daarom wil ik op de eerste plaats de patiënten en families bedanken die hebben meegewerkt aan onze studies. Het is inspirerend om te ervaren hoe persoonlijk betrokken vele families zich blijken te voelen bij het onderzoek. Ik heb bijzondere en veerkrachtige patiënten en families uit vele landen leren kennen. Wat ik geleerd heb van hun ervaringen en verhalen is veel meer dan wat er in dit proefschrift beschreven staat.

Een promotieonderzoek is een reis die je samen met anderen aflegt. Tijdens deze reis heb ik heel veel wijze, bevlogen en leuke mensen leren kennen. Alleen dat al heeft deze periode in mijn leven op een bijzondere manier verrijkt.

Graag wil ik iedereen bedanken die direct of indirect een bijdrage heeft geleverd aan de totstandkoming van dit proefschrift. Een aantal mensen wil ik in het bijzonder noemen.

Prof. dr. M.S. van der Knaap, beste Marjo, mijn eerste stappen als onderzoeker heb ik bij jou gezet tijdens mijn wetenschappelijke stage tijdens de studie Geneeskunde. Jouw aanstekelijke enthousiasme maakte dat ik al snel gegrepen was door het onderzoek. Heel veel dank voor je vertrouwen en voor de kans om uitgebreider onderzoek te kunnen doen naar wittestofziekten. Ik heb veel bewondering voor jouw enorme toewijding voor het onderzoek en de zorg voor patiënten. Tijdens het onderzoek heb ik vele uren doorgebracht in het door jou opgebouwde archief en was me er telkens weer van bewust hoe uniek deze verzameling aan data en contacten is. Dankjewel voor alle feedback die altijd snel volgde op gestuurde stukken en voor de spontane levenslessen, ik heb er veel van geleerd.

Prof. dr. B.M.J. Uitdehaag, beste Bernard, het onderzoek naar het natuurlijke beloop van leukodystrofieën staat nog in de kinderschoenen in vergelijking met het onderzoek naar MS. Veel dank voor je waardevolle suggesties over de epidemiologisch en methodologische aspecten van de studies en voor de huidige uitdagende werkomgeving.

De leden van de lees- en promotiecommissie, prof. dr. Hanne Meijers-Heijboer, prof. dr. Berry Kremer, prof. dr. Michèl Willemse, dr. Stefan Roosendaal, prof. dr. Reinoud Gemke en prof. dr. Hans van Goudoever, veel dank voor de tijd en moeite die jullie hebben gestoken in het lezen van dit proefschrift en voor het plaatsnemen in de promotiecommissie.

Ik heb het geluk gehad om in een heel multidisciplinaire onderzoeksgroep te mogen werken die bestaat uit mensen met verschillende achtergronden, persoonlijkheden, perspectieven en ideeën. Beste Truus, dank voor de inleiding in de moleculaire biologie. Het is fascinerend om te zien hoe in jouw hoofd een vraagstuk direct vertaald wordt naar een mogelijk experiment. Het was erg gezellig om de

voorbereidingen van onze gezamenlijk presentaties te combineren met een etentje. Carola, Nienke en Emiel, dank voor al jullie hulp en uitleg vanuit het lab, bij Sanger sequencing tot aan het uitvoeren van microsatellite marker analyse. Jullie hebben mij deelgenoot gemaakt van een voor mij totaal onontgonnen terrein. Nicole, jouw gedrevenheid om verder te komen met de zorg voor patiënten met wittestofziekten heeft veel indruk op mij gemaakt. Veel dank voor je onmisbare hulp bij het benaderen van artsen uit andere landen en voor alle nuttige suggesties en aanvullingen bij verschillende projecten. Marianna Bugiani, grazie voor de essentiële expertise en uitleg.

Lieve Sietske en Diane, mijn “sparring partners”, wat was het fijn om jullie bij me in de buurt te hebben op mijn werkplek! Dank voor alle gezelligheid en het delen van ups en down. Sietske, vanaf de eerste dag van mijn stage maakte jij mij wegwijs in het doen van onderzoek en in de genetica. Dank voor je positieve energie, je humor en tomeloze enthousiasme. Diane, ik herinner me nog goed het nieuws dat er een ‘hele aardige’ nieuwe collega zou komen, en niets was minder waar. Ik wil je bedanken voor de persoonlijke manier waarop je er voor me was wanneer het even tegen zat en voor alle leuke momenten die we hebben beleefd. Fijn om jou op 16 april aan mijn zijde te hebben.

Laura van Berge, dank voor de samenwerking aan LBSL. Rogier, Mohit, and Eelke, thank you for the beautiful work on MLC. Mohit, I enjoyed your enthusiastic tutor skills, whether it be patch clamping on Saturday morning, or Bollywood dancing on Saturday evening. Pinar Tekturk, thank you for contribution to the MLC MRI scoring and the rich dialogues on cultural differences in Medicine. Fia Cialdella, dank voor je bevlogen inzet tijdens je wetenschappelijke stage. Erg leuk om je weer terug te zien als semi-arts bij de Neurologie. Hannemieke, dank voor al je werk en aandacht voor de VWM database, ik heb het met veel plezier van je overgenomen. Renate en Menno, het is leuk om te zien met hoeveel enthousiasme jullie aan het vervolg van het onderzoek naar VMW zijn begonnen, heel veel succes! Collega’s van de onderzoeksgroep wittestofziekten: Vivi, Liane, Prisca, Stephanie Dooves, Ferdy, Anna, Lisanne, Gerbren, Stephanie Hoekstra, Lisa Hinz, Dwayne, Timo, Anne, Aish, Marjolein, Paulien, Lisa Gasparotto, en Claudia, dank voor alle inspirerende research meetings, creatieve kerstontbijten en leuke uitjes.

Beste Quinten Waisfisz, veel dank voor je geduldige uitleg en begeleiding bij de WES analyses.

The projects described in this thesis are the result of warm collaborations. Many colleagues from over the world were willing to include their patients in our studies. I am very grateful for all their involvement. I particularly would like to thank Adeline Vanderver for her hospitality and collaborations.

Dear Snejana Granatkina, thank you for your heartening involvement with our research and your enthusiastic help designing the cover of this thesis.

Lieve collega's van de Kindergeneeskunde op het nostalgische PK4X, we deelden hetzelfde motto: "promoveren is wél leuk!". Sandra, Katja, Bibian, Kim, Sophie, Charlotte, Stefanie, Tamara, Marc, Jolice, Suzanne, Raphaële, Marita, Gerrit, Annelies, Marleen, Willemijn, Hester, Arend, Stijn, Jonneke, Lindsay, Dana, Mirjam, Anne, Fatma, Marloes, Esmee, Lucie, Francis, Jojanneke en Britt, het was fijn om zoveel collega-onderzoekers te hebben om mee te sparren, dank voor jullie luisterende oren. Erg gezellig waren de sushi-lunches voor het vieren van successen en de legendarische Indonesische-rijsttafel-avonden van Sandra!

Tijdens mijn promotieonderzoek heb ik ook de waardevolle kans gekregen om mijn eerste ervaringen op te doen als arts-assistent. Collega's van de Neurologie, ik ben ontzettend blij dat ik nog vele jaren bij en met jullie mag werken!

Ik ben een gelukkig mens omdat ik omgeven ben met vele lieve mensen. Ik wil hen op deze plek allemaal bedanken. Een speciaal woord van dank geldt de volgende personen.

Lieve Elisa, wie had tijdens onze ontmoeting op de 1e dag van jouw carrière, 1991, in de zandbak van RKBS de Zevensprong, ooit kunnen bedenken dat we allebei zouden gaan promoveren op het gebied van de kinderneurologie. We hebben als het duo "EliHam' samen flink geoefend op diverse 'proef' proefschriften. Dank voor onze vriendschap, ik ben heel blij dat jij mijn paranimf bent.

Lieve An, Marijn, Tes, Lies, Pau, Pee, Jor, Loes, Nyn, Mar en Kim, wat ben ik blij met ons fijne clubje. Ik hoop dat we de jaarlijkse traditie om bij de haard herinneringen op te halen, bijvoorbeeld aan ons succesnummer met de gebroeders Hendriks of aan de "konijnsalade", nog lang blijven voortzetten! Jorien en Wouter, stelletje avonturiers, jullie belangstelling voor hoe het met me gaat betekent veel voor me. Dank dat ik altijd aan mag kloppen voor eerlijk advies en wijze raad. Tessa, dank voor je onuitputtelijke interesse in alles. Na een avondje met jou werd ik altijd nog enthousiaster over mijn onderzoek.

Anouk, Iris, Teska, Vera, Maud, Sofie en Laetitia, dank voor jullie vriendschap en betrokkenheid in de afgelopen jaren. Lieve Laura, tijdens de eerste jaren van mijn onderzoek maakten wij elkaars dagelijkse wel en wee van dichtbij mee, die tijd zal ik nooit vergeten. Iedereen is het er over eens: jij bent een geweldig mens!

Lieve vrienden van het USG, het Amsterdamse bos, de Bilt en omgeving en wijnclub, dank voor alle momenten die het leven zo leuk maken! Laar, Heleen, Jacq, Lies, Liek en Patty, let's keep calm & cook on!

Lieve familie, schoonfamilie en "extended" familie, dank voor alle dierbare momenten die we samen beleven.

Alexander, lieve broer, dankjewel dat je er altijd voor me bent. Ik vind het knap hoe het je lukt om steeds alle ballen in de lucht te houden en bovendien tijd te maken voor familie en vrienden. Tanja, ik ben dol op jouw “gezonde dosis creativiteit” en kijk er naar uit om te zien waar deze je nog allemaal gaat brengen. Jouw liedje “Lief leven” vind ik ontroerend mooi. Mijn lieve nichtje Jasmijn, als ik aan jou denk verschijnt er altijd een lach op mijn gezicht.

Papa en mama, jullie hebben mij altijd gestimuleerd om me te ontwikkelen en de wereld te ontdekken, daar ben ik jullie heel dankbaar voor. Het is fijn om te weten dat ik altijd kan rekenen op jullie steun en vertrouwen. Lieve pap, jij hebt mij laten zien hoe verrijkend het is om over de landsgrenzen heen te kijken. En wat was ik onder de indruk van het kerstgedicht over “myEline”. Sindsdien weet ik dat je best een beetje mee kan praten over witte stof. Lieve mama, de vele inspirerende verhalen en projecten waar jij bij betrokken was hebben bijgedragen aan mijn interesse in de zorg en in de neurologie, dank daarvoor. Jij bent voor mij en velen een rots in de branding, daar kan niemand tegenop.

Lieve Servé, bij jou thuiskomen is voor mij het grootste geluk. Ik kijk uit naar onze toekomst samen en vind het heel bijzonder dat het volgende grote project er een van ons samen is. Dankjewel dat jij er bent!

ABOUT THE AUTHOR

Eline Hamilton was born April 12 1987 and grew up in Houten, the Netherlands. In 2005 she graduated from secondary school at the Utrechts Stedelijk Gymnasium cum laude and started Medical School at the Rijksuniversiteit Groningen. In 2008 she went to London to spend six months on extracurricular courses. In 2011 she did an internship at the Pediatrics department at the Webuye district hospital in Kenya. Her interest in neuroscience and pediatric neurology led to a scientific internship at the Center for childhood white matter disorders at the VU University medical center (VUmc) in Amsterdam under supervision of prof. dr. Marjo van der Knaap. This experience sparked her fascination with MRI pattern recognition and neurogenetics. After finishing her Master's degree in Medicine in 2012 cum laude, she started a PhD project on leukodystrophies at the same center. Meanwhile, she was involved in clinical work and education at the department of Neurology at the VUmc. In 2017 she won an investigation award at the congress of the European Academy of Neurology and in 2018 she received the Jacobus Willemse award from the Dutch society for Pediatric Neurology for the research described in this thesis. In January 2018 she started her specialist registrar training in Neurology at the Amsterdam UMC/VUmc.

Leukodystrophies are a group of rare genetic disorders affecting the white matter of the brain. Disease onset may be at any age, mostly in childhood. There is only a limited understanding of the mechanisms underlying leukodystrophies and most of these disorders are not curable at this point. Clinicians are challenged by a deficit of knowledge of phenotypic characteristics and disease course. In this thesis, we delineate the clinical and genetic properties of four different leukodystrophies, aimed at improved patient counseling, enhanced understanding of underlying disease mechanisms and providing natural history data for the planning and evaluation of future therapeutic trials.