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, 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. 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. 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.

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. 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. 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. 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. 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.
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. The disease was initially described as a juvenile onset disease with slowly progressive ataxia, spasticity and dorsal column dysfunction. Patients have recessive mutations in the DARS2 gene, encoding the mitochondrial aspartyl-tRNA synthetase (mtAspRS). 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. 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 aminoacylation, 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.\textsuperscript{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.\textsuperscript{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 these mutations are ‘leaky’,\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{38,41} Two different MLC phenotypes can be distinguished: a classic, deteriorating phenotype and a remitting phenotype.\textsuperscript{42} Classic MLC is caused by recessive mutations in the \textit{MLC1} gene in the majority of cases: this variant is called MLC1.\textsuperscript{43} Few patients with classic MLC have recessive \textit{GLIALCAM} mutations, this variant is called MLC2A.\textsuperscript{43} Patients with dominant \textit{GLIALCAM} mutations have remitting MLC: this variant is called MLC2B.\textsuperscript{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 \textbf{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{48,49} These observations suggest 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.\textsuperscript{47} These clinical observations prompted further study of the \textbf{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.\textsuperscript{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 \textit{MLC1} or \textit{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.\textsuperscript{51} GlialCAM is a chaperone of MLC1 which ensures its localization in the membrane of astrocytic endfeet.\textsuperscript{52} Cell swelling studies have demonstrated that MLC1 is involved in astrocyte volume regulation and disturbed brain ion and water homeostasis.\textsuperscript{40,53} To study the basis of seizures in MLC, Dubey \textit{et al.} studied the effect of astrocyte dysfunction on neuronal networks in MLC mouse models.\textsuperscript{47} MLC mice display progressive intramyelinic vacuolization, making them excellent models to study the pathophysiology of MLC.\textsuperscript{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\textsuperscript{+}) from the extracellular space. K\textsuperscript{+} release occurs during repetitive action potential firing and removal of excess K\textsuperscript{+} is needed to prevent action-potential induced intramyelinic edema.\textsuperscript{43} Clearance is also necessary to prevent hyperexcitability caused by accumulation of extracellular K\textsuperscript{+}. This theory was substantiated by the finding that upon synaptic stimulation, there was a significant higher rise in extracellular K\textsuperscript{+} in MLC mice as compared to controls.\textsuperscript{47} Furthermore, in MLC mice an increased excitability of neuronal networks was observed.\textsuperscript{47} The work puts forward that astrocyte dysfunction in MLC leads to disturbed K\textsuperscript{+} 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\textsuperscript{+} 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 \textit{MLC1} or \textit{GLIALCAM} lead to loss of MLC1 function and result in a similar clinical disease.\textsuperscript{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.\textsuperscript{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. 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. 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. 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.\(^6^0,6^1\) The combining of \(\alpha\)-tubulins with \(\beta\)-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.\(^6^2,6^3\) An essential feature is their dynamic instability, i.e. the ability to rapidly de- and repolymerize, allowing a fast response to the environment.\(^6^4\) The *TUBB4A* gene encodes the brain-specific tubulin \(\beta4A\), which is highly expressed in cerebellum, putamen and cerebral white matter.\(^6^2\) 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.\(^6^5,6^6\) 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.\(^6^7,6^8\) 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.\(^6^9-7^1\) 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),\(^7^2,7^3\) 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. 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. Conversely, mutations associated with isolated hypomyelination result in normal neuronal morphology, but altered oligodendrocyte morphology, myelin gene expression, and microtubule dynamics. 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. The finding that the DYT4 mutation had no impact on microtubule dynamics suggests that this mutation might act through a distinct mechanism. 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. 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. 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.

**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.\textsuperscript{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.\textsuperscript{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 \textit{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 \textit{UFM1} variant with the disease in all families. Haplotype analysis indicated that the shared \textit{UFM1} deletion originates from a common ancestor. We further validated the pathogenicity of the \textit{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 \textit{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 \textit{cellular pathology-based classification} of the disease, as well as increased understanding of the role of the \textit{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. 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. 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. Studies on natural disease course in VWM are scarce and rather small. 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. 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.
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.\(^9\) Mutations leading to absence of the protein, such as deletions and frame shift mutations, are always seen in combination with a missense mutation.\(^8\) 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. An underlying mechanism involving the cellular stress response was suspected. The identification of mutations in the \textit{EIF2B1-5} genes as disease cause confirmed this theory. 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. Important breakthroughs concern the finding that astrocytes are central in the pathomechanisms and that the integrated stress response (ISR) is disturbed. 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. 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

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

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%

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. 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.\textsuperscript{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).\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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. 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.

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.

**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).
**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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 successful if patients are presymptomatic or oligosymptomatic.\textsuperscript{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
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. 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. 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. 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 expanding 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. 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.

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. 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.

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. 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.

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.\textsuperscript{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.\textsuperscript{101} The first clinical trial of gene therapy in the field of leukodystrophies has recently shown encouraging therapeutic benefits.\textsuperscript{138} Recent studies are focused on gene therapy targeting specific cell types such as oligodendrocytes.\textsuperscript{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. \textbf{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.\textsuperscript{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.\textsuperscript{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 \textbf{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.\textsuperscript{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
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Chapter 8


