Chapter 1

General introduction
LEUKODYSTROPHIES

Leukoencephalopathies can be defined as all conditions in which the white matter of the central nervous system (CNS) is predominantly or exclusively affected, assuming primary white matter involvement.\(^1\) This is opposed to white matter abnormalities secondary to Wallerian degeneration in neuronal degeneration and to white matter abnormalities in the context of a disease non-selectively affecting gray and white matter. Causes of leukoencephalopathies are numerous and include acquired conditions, such as inflammatory, autoimmune, vascular, neurotoxic, or infectious disorders, and genetic conditions.\(^2\) Genetic leukoencephalopathies are referred to as leukodystrophies. Leukodystrophies are most prevalent in children, and although each distinct leukodystrophy is rare to ultra-rare, our estimation is that the overall incidence is higher than generally appreciated. Bonkowsky \textit{et al} reported a live birth incidence of 1:7,663 (i.e. 13 leukodystrophy patients per 100,000 live births).\(^3\)

Leukodystrophies are clinically heterogeneous and can present with a wide variety of neurological signs and symptoms, ranging from a mild developmental delay or exclusively motor deficits to severe combined cognitive and motor impairment.\(^2\) Although epilepsy is seen in almost half of the cases,\(^3\) it rarely dominates the clinical picture, whereas it is more frequent and characteristic for neuronal disorders. Onset can be as early as \textit{in utero} affecting fetal brain development or later in infancy, childhood, adolescence, adulthood or even senescence.\(^2\) Initially leukodystrophies were thought to be invariably progressive.\(^4\) In more recent years it has become clear that this is not true for all disorders, as illustrated by patients with leukoencephalopathy and thalamus and brainstem involvement and high lactate (LTBL), who typically have an early infantile presentation and impressive initial magnetic resonance imaging (MRI) abnormalities with subsequent clinical amelioration and dramatic improvement of MRI abnormalities over time.\(^5\) The morbidity as well as the mortality over life time is high. Bonkowsky \textit{et al}., reported a mortality of 34% with a median age at death of 8.2 years in a cohort of 122 children with a leukodystrophy in a 9 year period,\(^3\) and another study reported a mortality of 22% in a cohort of 78 children with a genetic white matter disorder in a 5 year period, which was strikingly higher than in patients with an acquired leukoencephalopathy, of whom none died.\(^6\)

The road to reach a definitive diagnosis is often (very) long. It comprises numerous specialist visits, tests and sometimes invasive diagnostic procedures, which are a burden for patients and families and also very expensive. And even after this long diagnostic odyssey, a diagnosis cannot be established in many cases. In 2010 it was
found that despite the use of brain MRI and extensive biochemical and metabolic investigations, no diagnosis could be established in more than 50% of the patients with a leukodystrophy.\textsuperscript{3} Importantly, a genetic diagnosis can only be realized when the gene associated with that disease is known, whereas this information lacks for several defined leukodystrophies and for all the unclassified leukodystrophies.

**MRI PATTERN RECOGNITION AND LEUKODYSTROPHIES**

In the past two centuries different approaches and techniques have been used to identify and define ‘new’ leukoencephalopathies and subsequently diagnose them based on state-of-art techniques of that time. Neuropathology was the first technique available to study brain diseases. In the 19\textsuperscript{th} century, neuropathological observations in post-mortem brain tissue led to the distinction between a disorder characterized by multiple white matter lesions (‘multiple sclerosis’) and a disorder characterized by diffuse abnormality of the white matter (‘diffuse sclerosis’).\textsuperscript{7,8} In the beginning of the 20\textsuperscript{th} century Neubürger noted that the term ‘diffuse sclerosis’ was applied to very different disease entities.\textsuperscript{9} Further differentiation between these disease entities in the next decades was possible due to advances in the development of novel histochemical methods and biochemical assays of body fluids, later followed by metabolic studies on fibroblasts or muscle, specific enzyme function tests and electron microscopy.\textsuperscript{10} In the 1980s, the advent of the MRI had a major impact on the studies of leukoencephalopathies, because white matter abnormalities could be visualized in detail in vivo for the first time.\textsuperscript{11} The subsequent development of MRI pattern recognition in the early 1990s\textsuperscript{12} was a big step forward in the diagnostic workup of leukoencephalopathies and MRI became the most important tool in the diagnostic process of these disorders.\textsuperscript{12,13}

MRI creates high-resolution anatomic images and is highly sensitive in the detection of white matter abnormalities.\textsuperscript{14} Different pulse sequences can be used that each result in different contrast between tissues. The T\textsubscript{1}- and T\textsubscript{2}-weighted sequences are best known. Unmyelinated white matter has a very long T\textsubscript{1} and T\textsubscript{2}, resulting in a lower signal than gray matter structures on T\textsubscript{1}-weighted images and a higher signal on T\textsubscript{2}-weighted images.\textsuperscript{15,16} At birth, the brain is still largely unmyelinated and the increasing deposition of myelin in the first two years of life results in shortening of first the T\textsubscript{1} and then the T\textsubscript{2}, leading to a reversal of the gray-white matter contrast on T\textsubscript{1}- and T\textsubscript{2}-weighted images.\textsuperscript{15,16} Fully myelinated white matter structures have a higher signal on T\textsubscript{1}-weighted images than gray matter structures and a lower signal on T\textsubscript{2}-weighted images than gray matter structures.\textsuperscript{15-17} Lack of myelin deposition (if permanent called ‘hypomyelination’) leads to a mildly elevated
signal on T₂-weighted images and a variable signal on T₁-weighted images, depending on the amount of myelin deposited. Abnormalities of the white matter other than lack of myelin deposition result in a much lower T₁ signal and a much higher T₂ signal than gray matter structures, allowing distinction between hypomyelination and other pathologies in vivo. Importantly, cerebrospinal fluid (CSF) has a high signal on T₂-weighted images and a low signal on T₁-weighted images, precluding differentiation between abnormal and cystic white matter. Images based on additional pulse sequences, such as fluid-attenuated inversion recovery (FLAIR) and proton density (PD) images, facilitate visualization of cystic lesions or rarefaction of tissue, because fluid has a low signal. Diffusion-weighting provides measures for the degree of freedom of movement of water molecules in brain tissue. Abnormalities on diffusion-weighted images (DWI) have to be confirmed by the apparent diffusion coefficient (ADC) values to avoid false positive results due to ‘T₂-shine through’. Restricted diffusion is seen in conditions with compression of extracellular spaces, for example due to (1) cytotoxic edema, as may occur in mitochondrial defects, (2) myelin micro-vacuolization, which may be present in neonates with maple syrup urine disease or in patients with a mitochondrial disorder, (3) high cell density as seen in the relatively spared white matter in vanishing white matter (VWM) (MIM 603896), or (4) storage of substances in lysosomal storage disorders. Increased diffusion can be observed in disorders with increased water spaces, such as megalencephalic leukoencephalopathy with subcortical cysts (MLC, MIM 604004) as the result of enlarged extracellular spaces and myelin macro-vacuolization. In some leukodystrophies, such as X-linked adrenoleukodystrophy (MIM 3001001) and Alexander disease (MIM 203450), the blood-brain barrier is grossly compromised, which can be shown by the use of contrast enhanced MR images.

MRI pattern recognition is based on the observation that individuals with the same leukoencephalopathy usually present with the same, distinct pattern of MRI abnormalities, while patients with other leukoencephalopathies have different MRI patterns. In MRI pattern recognition, major MRI discriminators are the nature of the white matter abnormalities (hypomyelination versus other pathologies), the aspect of the white matter abnormalities (e.g. confluent versus multifocal and symmetrical versus asymmetrical), the predominant location of the abnormalities (e.g. supra- or infratentorial, frontal or parietal, periventricular or subcortical) and special MRI features (e.g. cystic degeneration and/or rarefaction, contrast enhancement, diffusion restriction and microbleeds).

The starting point of the road to reach a definitive molecular diagnosis begins with the identification and classification of patients. MRI pattern recognition has not only proven to be highly successful for the recognition of known leukoencephalopathies, but also for
the identification and classification of novel disorders among the unclassified cases.\textsuperscript{12,13} In our studies we use MRI pattern recognition as a primary tool in the search for novel disorders among the unclassified leukoencephalopathies. We identify and classify the patients from an MRI database present in the Center for Childhood White Matter Disorders at the VU Medical Center in Amsterdam including over 3000 MRIs of patients with an unclassified leukoencephalopathy from all over the world.

\textbf{GENETICS AND LEUKODYSTROPHIES}

In the last two decades several novel leukodystrophies have been defined using MRI pattern recognition analysis often combined with clinical and sometimes with laboratory data (e.g. VWM, MLC, hypomyelination and congenital cataract (HCC, MIM 603532), Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL) and hypomyelination, hypodontia and hypogonadotropic hypogonadism (4H syndrome, MIM 607694)).\textsuperscript{23-28} The validity of this approach has been shown by the identification of the associated genes (in 2001 and 2002 \textit{EIF2B1-5} mutations in patients with VWM\textsuperscript{29,30} and mutations in \textit{MLC1} in patients with MLC,\textsuperscript{31} in 2007 mutations in the gene \textit{FAM126A} in patients with HCC syndrome,\textsuperscript{32} in 2007 \textit{DARS2} mutations in patients with LBSL,\textsuperscript{33} and in 2011 mutations in the genes \textit{POLR3A} and \textit{POLR3B} in patients with 4H syndrome\textsuperscript{34,35}). The identification of the molecular cause of these disorders was accomplished after time-consuming, laborious and costly efforts that included linkage by positional cloning to pinpoint the chromosomal location of the candidate gene and subsequent narrowing the candidate region, followed by reconstructing which genes were located in the candidate region and sequential analysis of candidate genes in the region by Sanger sequencing.\textsuperscript{30-35} This approach required the presence of multiple, preferably large and/or consanguineous families to identify a small enough candidate region with a significant logarithm of odds (LOD) score. Although this approach was indeed successful for the disorders mentioned above, for substantial numbers of patients with an unclassified or defined, but molecularly undetermined leukodystrophy this technique was not applicable. The rarity of these disorders and the presence of only a few mostly small families hampered the success of this approach and made it impossible to successfully apply the traditional gene-discovery approaches. In addition, \textit{de novo} germ line mutations could not be identified using this technique.

The introduction of Next-Generation-Sequencing (NGS) technologies could overcome these problems and created opportunities to perform unbiased gene search approaches to identify the mutated gene in patients with extremely rare Mendelian disorders, including most leukodystrophies.
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NEXT-GENERATION SEQUENCING TECHNIQUES

A whole new genetic explorative era has emerged since the release of the first draft of the human genome sequence in 2001 and the first NGS platform release in the market in 2005. NGS is often referred to as massive parallel sequencing, which means that millions or even billions of small fragments of DNA are sequenced simultaneously, creating a massive pool of data. Whole-exome sequencing (WES) refers to massive parallel sequencing of the protein-coding part of the genome comprising 1% (30 Mb) of total human genome. Three main steps form the key elements of WES. The first step is exome enrichment (exome capture kits are offered by different companies), which is accomplished by using oligonucleotide probes to hybridize fragment of interests. The second step is high-through-put parallel sequencing which can be executed using different available platforms (e.g. Illumina HiSeq™, Life Technologies™, SOLiD™, and Roche 454 GS FLX). In our studies we work with Illumina HiSeq2000 for sequencing, which uses clonal bridge amplification and sequencing by synthesis approach. This platform creates 600 Gb of 100 bp paired-end reads in a ten-day run. The third step is bioinformatics processing of the reads including quality assessment, mapping and alignment to the human reference genome and variant calling and annotation.

The initial proof of concept of the utility of WES in disease gene discovery came in 2009 with the demonstration that the causative gene for a known Mendelian disorder, Freeman-Sheldon syndrome (MIM 193700), could be identified directly by WES. In 2010, the first discovery of the genetic cause for a rare Mendelian disorder of unknown cause, Miller syndrome (MIM 274200), was established. At the time of the start of my PhD project in September 2011, an increasing number of 30 novel genes and eight new clinical phenotypes linked to a known gene using WES were published within a time period of roughly two years. In addition, during the first month of my PhD project, the first mutations in a novel gene associated with a leukodystrophy were identified with WES (CSF1R mutations in patients with ‘Hereditary Diffuse Leukoencephalopathy with Spheroids’, MIM 221820), and soon thereafter our research group identified EARS2 mutations in patients with LTBL using WES. The exponential increase of the identification of novel genes associated with a broad variety of rare Mendelian disorders illustrates the high potential of this technique for gene finding in this field.

Successful WES depends firstly on the variant being detected in the captured portion of the genome and secondly on our ability to identify the variant of interest. Thorough prioritization of variants is therefore crucial to the gene finding process. After sequencing and bioinformatics processing, approximately 20,000 to 25,000 variants can be identified...
in each individual exome.\textsuperscript{38,43,46} The challenge is to pinpoint the variant of interest within this large pool of insignificant variants. Filtering strategies to identify mutations usually rely on certain assumptions. For most Mendelian disorders prioritization assumes that the mutation has a potentially large effect and is therefore unique or extremely rare (i.e. only present in patients), located within the protein coding part of the genome and is predicted to have a direct effect on the function of the protein (e.g. non-synonymous single nucleotide variants (SNVs), insertions/deletions (indels) or splice-site mutations). The initial filtering approach commonly used is the exclusion of common variants (e.g. SNVs present in public databases such as dbSNP, 1000Genomes, and the NHBI Exome Variant Server genome database), which can reduce the number of potential candidate variants substantially by 90-95\%\textsuperscript{46} After that, a total of 150-500 rare non-synonymous SNVs, indels or splice-site mutations will remain for further prioritizing.\textsuperscript{46} Leukodystrophies can present with any inheritance pattern, and information on inheritance can further reduce the number of candidate variants substantially (Figure 1).

Figure 1. Different inheritance patterns with different approaches for WES analysis.
Circles represent females; squares males. Blue solid symbols indicate affected individuals; symbols with a dot indicate unaffected carriers. In the situation of a consanguineous autosomal recessive inheritance (A), homozygosity mapping in combination with WES of sib-pairs can be helpful. With an autosomal dominant inheritance pattern (B) in a large family additional linkage analysis with WES of multiple family members is an effective approach. When an X-linked recessive disorder is suspected (C), focusing first on the X-chromosome and sequencing multiple affected male patients is useful. When a recessive disorder is suspected but no consanguinity (D) is present sib-pair analysis (with or without parents) can be performed best. In case of a strong suspicion of a de novo disorder, trio analysis (E) is the appropriate first approach.
Different approaches can be applied to achieve the most efficient reduction of candidate variants for a certain supposed inheritance pattern. De novo mutations can be identified using a trio-based approach. WES is performed in both unaffected parents and the affected child, and the data are subsequently filtered for de novo variants. Theoretically this would be extremely efficient because the average exome only contains 0-3 true de novo mutations.\(^4\) In the case of an autosomal recessively inherited disorder with reported consanguinity additional genetic techniques like a single nucleotide polymorphism (SNP) microarray can reduce the number of candidate variants by selection of an overlapping homozygous region between affected patients. In large families with an autosomal dominant disorder conventional genetic linkage analyses preceding WES could be helpful by narrowing down the candidate search area.\(^4\) Although the combination of these filtering strategies will ultimately reduce the number of candidate variants significantly, comparison of identified variants among unrelated patients with (presumably) the same disorder is often required to pinpoint the candidate gene, especially in disorders caused by a novel gene. Importantly, effective intra-group comparison depends on precise phenotyping of the patients and the absence of genetic heterogeneity.

It is important to realize that every filtering step used can discard the pathogenic variant. For example, the inheritance pattern is often not definitively known and although causal variants are expected to be novel or extremely rare, this is not always the case, as illustrated by the high carrier frequency (1:95 in the Finnish population) of the DARS2 splice mutation in intron 2.\(^4\)\(^8\)\(^4\) Furthermore, in addition to SNVs, patients can carry copy number variations (CNVs), inversions and deletions, which can be missed by WES due to limitations of this technique.\(^5\)\(^0\)\(^5\)\(^1\) Also, insufficient exome coverage, which is most often caused by the presence of extreme GC rich regions hampering good exome capture and sequencing, may result in negative results. Furthermore, it is estimated that 85% of the disease-causing mutations are located in functional and coding regions of the genome,\(^5\)\(^2\)\(^5\)\(^3\) the remaining part (non-coding causal mutations) is refractory to WES analysis and requires whole-genome sequencing (WGS).

For the studies described in this thesis we use WES as the main approach to identify the molecular cause of novel leukodystrophies. We consider intra-group comparison an effective additional filtering approach because this strategy will not only reduce the number of candidate variants substantially, it will also provide prove of the causality of the identified variant, when (different) variants in the same gene are found in multiple unrelated patients with the same phenotype. By using MRI pattern recognition for the classification of patients we aim at creating phenotypically homogeneous groups.
of patients that represent a reliable discovery cohort for the application WES. We will especially focus on leukodystrophies with a (suspected) mitochondrial etiology or hypomyelination.

**MITOCHONDRIAL DISORDERS**

Mitochondrial disorders constitute a large proportion of the patients presenting with an unknown leukodystrophy. They result from defective energy generation via oxidative phosphorylation (OXPHOS), which involves four electron-transferring respiratory chain complexes (complexes I-IV, CI-CIV), ATP synthase (complex V, CV) and two electron carriers coenzyme Q and cytochrome C, together comprising around 85 subunits. Additionally, numerous assembly factors are necessary to form functional complexes. In 1951, Leigh reported the first patient with a mitochondrial disorder and from about this time onwards numerous other cases were reported. Although initially deep gray matter involvement was the most frequently reported finding, white matter abnormalities are increasingly recognized as a common finding in mitochondrial disorders. Numerous cells within the brain are extremely vulnerable for mitochondrial dysfunction. Axons in the white matter have a high energy demand, required to maintain the ionic gradient and structural integrity necessary to support neurotransmission. Proper mitochondrial function is also vital for oligodendrocyte differentiation, viability and formation of myelin. Energy produced in astrocytes supports neuronal function, and several mitochondrial enzymes within astrocytes, e.g. creatine kinase, malate-aspartase, glutamate dehydrogenase and pyruvate carboxylase are required to provide high-energy substrates to neurons.

Mitochondrial disorders can be caused by defects in the mitochondrial DNA (mtDNA) or nuclear DNA (nDNA). mtDNA consists of a double-stranded circular genome of 16,569 base pairs, which is present in 2-10 copies per mitochondrion. It was the first DNA of the human genome that was fully sequenced and it can easily be analyzed because it only contains 37 genes. These genes encode 13 subunits of the OXPHOS enzyme complex, plus 22 tRNAs and two rRNAs. mtDNA is maternally inherited. In the 1990s most research concerning mitochondrial disorders was focused on mtDNA and leukodystrophies ascribed to mitochondrial syndromes were therefore associated with mutations or rearrangements in the mtDNA (e.g. mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS, MIM 540000), Leber hereditary optic neuropathy (LHON, MIM 535000) and Kearns-Sayre syndrome (MIM 530000).
1995, the first mutations in a nuclear gene (flavoprotein) involving a subunit of complex II were found in two sisters with Leigh syndrome and in 1999 recessive mutations in the gene thymidine phosphorylase were shown to be responsible for mitochondrial neurogastrointestinal encephalopathy (MNGIE, MIM 603041). The following years more and more mutations in nuclear genes were discovered, and it became evident that most mitochondrial disorders are caused by nuclear gene defects.

Of the five respiratory chain complexes present in OXPHOS, the most frequent deficiency in children is that of complex I (CI), NADH: ubiquinone oxidoreductase (EC1.6.5.3). This complex comprises seven core subunits encoded by mitochondrial DNA, 38 nuclear encoded core subunits and many (still unknown) nuclear encoded assembly factors. Besides defects of subunits and assembly factors of the respiratory chain complexes, it is increasingly shown that mitochondrial disorders can be the result of nuclear gene defects encoding mitochondrial tRNA synthetases and modification proteins, mitochondrial ribosomal proteins, proteins mediating mitochondrial mRNA translation (e.g. initiation, elongation and termination), proteins involved in mitochondrial dynamics and mitochondria quality control, defects of mtDNA maintenance, mitochondrial metabolite transporters and the import, modification and insertion of cofactors like heme, the iron-sulphur clusters and metals.

Clinically, mitochondrial disorders present with an extreme phenotypic variability; there is no obvious correlation between the type of respiratory chain defect and the clinical presentation. Laboratory assessment of mitochondrial function in cultured fibroblasts or tissue (most often muscle biopsy) can reveal an isolated respiratory chain complex deficiency, a combined complex deficiency or even nonspecific or negative results. Elevated lactate in plasma and/or CSF is suggestive for a mitochondrial disorder, but a normal lactate does not exclude it. MRI findings suggestive of a mitochondrial disorder are involvement of both white and gray matter structures, cystic lesions in the abnormal white matter, restricted diffusion, contrast enhancement, and elevated lactate on magnetic resonance spectroscopy (MRS) of the brain. Several mitochondrial leukodystrophies are associated with a distinct MRI pattern. For example, in MNGIE, patients’ MRI shows a diffuse high T2-signal intensity of the cerebral and cerebellar white matter with sparing of the U-fibers and corpus callosum. The thalami and basal nuclei may display patchy signal abnormalities. Recessive mutations in DARS2, encoding mitochondrial aspartyl-tRNA synthase, are associated with a characteristic MRI pattern involving cerebral white matter abnormalities with signal changes in specific brainstem and spinal cord tracts and elevated lactate. Specific features seen on the MRI can also provide hints for the underlying diagnosis, like stroke-like lesions in MELAS and calcium
deposits in the globus pallidus and caudate nucleus in Kearns-Sayre syndrome.\textsuperscript{69,82} However, not all mitochondrial defects present with such a characteristic MRI pattern or clues. This is illustrated by Leigh syndrome (MIM 256000), which is a distinct phenotype defined by developmental regression and MRI signal abnormalities in the basal ganglia, thalamus and brainstem that can be caused by mutations in more than 25 genes located both in mtDNA and nDNA.\textsuperscript{83}

In 2010 it was estimated that 114 genes were known to be associated with a mitochondrial disorder.\textsuperscript{84} This is in striking contrast with the 1500 nuclear-encoded proteins that could be involved in mitochondrial functioning and if mutated could lead to disease.\textsuperscript{85} In line with this, the majority of patients with a suspected mitochondrial leukodystrophy have no identifiable genetic etiology.\textsuperscript{86} In addition to the wide phenotypic variation, genetic heterogeneity, and often poor genotype-phenotype correlation, a large proportion of the ‘mitochondrial genes’ has as yet not been associated with a disease, hampering the diagnosis in numerous patients. Until now, linkage analysis and homozygosity mapping were the main genetic techniques used in the discovery of novel mitochondrial disease genes, which have major limitations when used in small groups of patients, as discussed above.

WES would provide an unbiased approach to identify the genetic defect in rare, suspected mitochondrial leukoencephalopathies, as genes can be sequenced irrespective of their predicted role in disease pathology, or even without any evidence of mitochondrial localization. Phenotypic classification of patients groups with a suspected mitochondrial disorder is challenging due to the wide clinical and biochemical heterogeneity. We consider classification based on MRI pattern the most successful and valid approach for the identification of novel distinct mitochondrial disorders. An additional advantage of using this approach is that by identification of the causal gene defects, definition of disease entities is confirmed, which will make the diagnostic process in patients with a mitochondrial disorder more efficient and successful in the future.

**HYPOMYELINATING DISORDERS**

Patients with hypomyelination represent the largest single category among the patients with a leukoencephalopathy with unknown origin.\textsuperscript{13} At the end of the 19th century the first hypomyelinating disorder was described, Pelizaeus-Merzbacher disease (PMD, MIM 312080), which was characterized by the widespread lack of myelin staining in the white matter.\textsuperscript{87,88} On MRI, hypomyelination has a characteristic appearance. Hypomyelinated
white matter structures have a mild hyperintensity as compared to gray matter structures on T2-weighted images and, dependent on the amount of myelin deposited, a hyper-, iso, or hypointense signal relative to gray matter structures on T1-weighted images. The diagnosis hypomyelination can be made when there is a stable lack of myelin on two successive MRI scans that were performed at least six months apart with the second MRI after one year of age. At first sight hypomyelinating disorders have a rather similar appearance, both on MRI and clinically, making a definitive diagnosis in this group a challenge. Steenweg et al., showed in 2010 that using MRI-pattern recognition in patients with hypomyelination it is possible to form clusters of patients based on MRI features and that these clusters correspond to specific hypomyelinating disorders. For example, 4H syndrome presents with a specific pattern of hypomyelination characterized by T2 hypointensity of the optic radiation, pyramidal tracts at the level of the posterior limb of the internal capsule and the anterolateral part of the thalamus. Fucosidosis (MIM 230000), a lysosomal storage disorder caused by deficiency of α-L-fucosidase, is characterized by T2 hypointensity of the globus pallidus and patients with HCC syndrome have hypomyelination in combination with focal lesions of more prominent T2 hyperintensity and T1 hypointensity in the periventricular and deep cerebral white matter and a more normal appearance of the subcortical white matter on T1 weighted images. This MRI-based approach also proved to be successful in defining novel hypomyelinating disorders among the group of hypomyelinating disorders of unknown origin. One newly defined disorder was ‘Hypomyelination of Early Myelinating Structures’ (HEMS) which was identified in four male patients, suggesting an X-linked inheritance. During normal brain development tracts become myelinated at the time they become functional, resulting in a fixed spatiotemporal sequence of myelination. In patients with HEMS early myelinating structures (e.g. brainstem, hilus of the dentate nucleus, posterior limb of the internal capsule, optic tracts, and tracts to the pericentral cortex) are poorly myelinated, in contrast to structures that normally myelinate in later developmental stages. A genetic diagnosis could not be made in these patients using conventional genetic techniques (i.e. Sanger sequencing), although a gene involved in the early regulation of myelination was suspected.

In September 2011, at the time I started my PhD project, 15 distinct inherited hypomyelinating disorders were defined, but the molecular cause was only known in nine of these disorders. The most common hypomyelinating disorder, PMD, is caused by mutations in the proteolipid protein-1 gene (PLP1), encoding proteolipid protein (PLP) and DM20. PLP and DM20 are major myelin components in the central nervous system, constituting approximately 50% and 30% of the total protein, respectively. Conformational changes of the PLP/DM20 protein result in oligodendrocyte loss
due to endoplasmic reticulum stress-induced apoptosis, while excessive PLP (gene duplications) may accumulate in the late endosomes and lead to oligodendrocyte arrested maturation, dysfunction and loss of the protein. Null mutations result in a lack of synthesis of the protein, leading to formation of compact myelin lacking PLP. Considering the role of this protein within myelin, it is no surprise that a defect in this protein disturbs myelination. It is, however, important to realize that the process of myelination is complex and depends on numerous processes, involving oligodendrocyte progenitor proliferation, migration and differentiation, and normal function and interaction of oligodendrocytes, neurons and astrocytes. This whole process is regulated by several signaling pathways, transcription and growth factors, epigenetic factors, DNA methylation and non-coding RNAs. This extensive list of potential candidate genes and proteins involved makes gene finding for both classified and unclassified hypomyelination disorders extremely difficult. Moreover, genes involved in DNA repair mechanisms; ERCC6 and ERCC8 in Cockayne syndrome (MIM 216400), and ERCC2, ERCC3, GTF2H5, and MPLKIP in patients with trichothiodystrophy with hypersensitivity to sunlight (MIM 601675 and MIM 616395) are also associated with hypomyelination, making it even more complex. In September 2011, the molecular cause of 4H syndrome, one of the most prevalent hypomyelinating disorders, was identified. Patients had recessive mutations in POLR3A or POLR3B, encoding the largest and second largest subunits of RNA Polymerase III (Pol III), RPC1 and RPC2, respectively, extending the list of potential genetic possibilities. We expected the unbiased gene search approach of WES to be of great benefit for patients with hypomyelination of unknown origin. As shown by Steenweg et al., disease classification based on MRI patterns is also a reliable method in patients with hypomyelination. One of the groups with a novel hypomyelination pattern and without molecular cause (HEMS) identified by Steenweg et al. would be an excellent cohort for the application of WES.
CONTENT OF THIS THESIS

The elucidation of the molecular bases of unclassified leukodystrophies is extremely important for patients and families because this means a DNA-confirmed diagnosis, information, prognosis and options for family planning (e.g. carrier testing, prenatal or preimplantation genetic diagnosis). Gene discovery is also the essential starting point for better insight into the white matter components and their role in disease mechanisms. Subsequently, new treatment options can be explored depending on the defect identified.

The aim of this thesis is to classify novel leukodystrophies among the unclassified ones by MRI pattern recognition analysis and to identify their underlying genetic defect by using WES.

The thesis is divided into four parts. The first three parts are classified according to the genes identified.

I Expansion of the phenotypic spectrum of a known disorder
II Novel disease entity associated with a known gene
III Novel disorder associated with a gene previously not linked to a human disorder or clear phenotype
IV Update on leukodystrophies
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