Chapter 1

General introduction
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The main subject of this thesis is the inherited childhood white matter disorder ‘Leukoencephalopathy with brain stem and spinal cord involvement and lactate elevation’, or LBSL. Inherited childhood white matter disorders form a large group of neurological diseases with many different genetic causes. Most of these disorders are very rare; sometimes only a few affected children are known worldwide.

White matter disorders

The central nervous system is composed of grey and white matter. Grey matter refers to the greyish tissue of the brain and spinal cord that contains nerve cell bodies, dendrites, and bare (unmyelinated) axons. The white matter consists largely of the myelinated nerve fibers that are bundled into tracts. The myelin sheaths are formed by oligodendrocytes and increase the conduction speed and fidelity of action potentials along the axon.

White matter disorders or leukoencephalopathies are disorders that exclusively or predominantly affect the white matter of the brain. The word ‘leukoencephalopathy’ refers to all forms of white matter abnormality and can be both inherited and acquired. The term ‘leukodystrophy’ was coined specifically for progressive, inherited demyelinating disorders, but is nowadays used for all genetic white matter disorders, progressive or not, demyelinating or not. Acquired leukoencephalopathies can be caused by inflammatory conditions, infections, nutritional disorders, toxic substances, and disturbances of blood flow. One of the most common and best-known examples is multiple sclerosis, an inflammatory disorder of the central nervous system. Hereditary white matter disorders often manifest during childhood and are often progressive, although there is a large variety in age of onset, from neonatal to adulthood, and not all inherited white matter disorders are progressive. White matter disorders most often lead to motor dysfunction, but can also lead to a variety of different signs caused by sensory and cognitive dysfunction.

Magnetic resonance imaging in white matter disorders

In the beginning of the nineties, approximately 60% of the children with a leukoencephalopathy remained without a specific diagnosis, despite extensive investigations. Since then magnetic resonance imaging (MRI) has been shown to be very useful to distinguish between disorders. Different white matter disorders selectively affect different combinations of brain structures. MRI pattern recognition is now used for diagnosis of leukoencephalopathies and to identify and define novel disease entities.

LBSL

LBSL is one of the childhood white matter disorders in which characteristic MRI patterns were essential for defining the disease.
**MRI and MRS of LBSL**

The pattern of abnormalities that are seen on brain MRIs of LBSL patients is different from the patterns observed in all other known leukoencephalopathies, both the classic leukoencephalopathies and those recently defined, making the pattern diagnostic (Figure 1) ³. 

There are extensive signal changes within the periventricular and deep cerebral white matter, relatively or completely sparing the temporal lobes and the U-fibers. The abnormal white matter has an inhomogeneous, spotty aspect in most patients, but is diffuse and homogeneous in others ⁴. Abnormalities are found in the posterior corpus callosum and sometimes also in the anterior part, the posterior limb of the internal capsule and the cerebellar white matter. The pyramidal tracts extending downward through the posterior limb of the internal capsule and the brain stem into the lateral corticospinal tracts of the spinal cord are affected over their entire length. The sensory tracts, including the dorsal columns in the spinal cord, the medial lemniscus up to the level of the thalamus, and the corona radiata are also affected over their entire length. Cerebellar connections are selectively involved. Within the brain stem, the superior and inferior cerebellar peduncles are involved early, whereas the middle cerebellar peduncles can become affected in a later stage. There is a consistent involvement of the intraparenchymal trajectories of the trigeminal nerve and the mesencephalic trigeminal tracts ³.

![Figure 1 Characteristic MRI pattern of LBSL. (A) Inhomogeneous signal abnormalities in the periventricular and deep cerebral white matter. (B) Involvement of the posterior limb of the internal capsule (pl) and the splenium of the corpus callosum (s). (C) At the level of the pons, abnormalities are seen in the pyramidal tracts (pt), medial lemniscus (ml), mesencephalic trigeminal tracts (mtt), intraparenchymal parts of the trigeminal nerves (tn) and the superior cerebellar peduncles (scp). (D) At the level of the medulla the pyramids (p), anterior spinocerebellar tracts (ast), inferior cerebellar peduncles (icp) and decussation of the medial lemniscus (dml) are affected. (E) Tracts in the spinal cord are affected over the entire length (arrowheads). At the level of cervical (F) and thoracic (G) spinal cord abnormalities can be seen in the lateral corticospinal tracts (lct) and the dorsal columns (dc). (H) An increased lactate (lac) level is detected in the abnormal white matter by MRS. Taken from ⁶.](image-url)
Lactate elevations in the abnormal white matter can be found in most patients by magnetic resonance spectroscopy (MRS) \(^5\). Often a decrease in N-acetylaspartate and increase in myoinositol are found in the white matter of patients and white matter choline can be mildly increased \(^3\).

**Clinical phenotype**

LBSL is clinically characterized by slowly progressive signs of pyramidal, cerebellar and dorsal column dysfunction. The initial development is normal in most LBSL patients. Deterioration of motor skills usually starts in childhood or adolescence \(^3,4,7-9\) and occasionally in adulthood \(^10,11\). Most affected individuals have decreased position and vibration sense. Their legs are generally more severely affected than their arms. Patients suffer from ataxia and spasticity. Manual dexterity becomes impaired to a variable degree. Dysarthria can develop over time. Some patients develop epilepsy. Most affected children have normal intellectual capacities, but learning problems occur in some children. Cognitive decline may occur and is usually mild \(^3,4\). In several patients evidence of a peripheral neuropathy has been found \(^3,8,9,12\). The disease is slowly progressive and can result in wheelchair-dependency in the teens or twenties. However, the disease severity varies and some patients have the first signs of the disease in their twenties and still walk in their forties \(^5\). Episodes of more rapid deterioration following a minor head trauma or fever have been described in a few patients \(^4,9\). Chapter 5 of this thesis addresses the full clinical variation of the disease.

**Pathogenesis**

Although brain tissue of LBSL patients was not available until very recently, data from MR techniques provides information on the pathogenic processes that take place in the brains of these patients. Mildly elevated choline concentrations indicate enhanced membrane turnover, which may be related to mild demyelination. An increase in myoinositol in the white matter of patients indicates gliosis. MRS shows a decrease in N-acetylaspartate in the white matter, which indicates axonal damage or loss and the involvement of entire tracts suggests a primary axonal degeneration \(^3\). In almost all patients lactate elevation is found in the abnormal white matter. This suggests a defect in mitochondrial energy production. Some of the signs observed in LBSL patients, such as mild exercise intolerance and worsening of symptoms during infections, are also consistent with a mitochondrial dysfunction \(^7\). In patient-derived lymphoblasts, fibroblasts or muscle tissue, however, no abnormalities in mitochondrial activities could be detected \(^3,6\), but mitochondrial studies have not been performed in brain tissue.

A longitudinal MRI study showed that the abnormalities in the cerebral white matter in LBSL are going through different stages \(^13\). In all stages the white matter architecture appears to be relatively mildly affected. Restriction of diffusion is hypothesized to reflect the first stage of disease and to be caused by chronic myelin splitting and intramyelinic vacuole formation. This intramyelinic oedema is followed by a shift of water from the intramyelinic to the interstitial compartment resulting in a high signal on T2-weighted MR images and increased diffusivity. Subsequent loss of the interstitial water leads to intermediate signal on T2-weighted MR images, which could define the final stage of the white matter pathology. These vacuolar changes in the
white matter and myelin splitting were recently confirmed in a neuropathological report of two severe LBSL cases 14.

**Genetics**

Initially, the characteristic MRI pattern helped to diagnose LBSL in patients from multiple families. The presence of affected sibling pairs within a number of families suggested an autosomal recessive mode of inheritance. In 2007 causative mutations were found in the gene DARS2 by genome-wide linkage analysis 6. After identification of a candidate region on chromosome 1, containing 16 genes, sequencing identified mutations in DARS2 in all LBSL patients. DARS2 codes for the mitochondrial aspartyl-tRNA synthetase (mtAspRS), which functions in mitochondrial protein synthesis. Subsequent studies have further confirmed the link between DARS2 mutations and LBSL 9,12,15. The discovery of DARS2 mutations in patients with the typical LBSL pattern of MRI abnormalities has resulted in the sequence analysis of this gene in patients with MRI patterns that were suggestive of LBSL but did not fulfil all inclusion criteria 6 for the disease. The findings of this study are described in Chapter 4.

In LBSL patients, many different types of mutations in DARS2 have been found including deletions, splice-site mutations, missense and nonsense mutations. The missense mutations affect residues that are well conserved across various species. In Chapter 2 the effects of several of these missense mutations on the expression and function of the encoded protein are studied in detail. Strikingly, the presence of compound heterozygous mutations appears to be the rule in LBSL. In almost all LBSL patients, one mutation occurs in a stretch of T and C residues in intron 2, just upstream of the third exon. Controls have either three T residues preceding 11 C residues or four T residues preceding ten C residues (Figure 2). The most common mutation is an allele with only two T residues preceding a stretch of 11 C residues. In some affected individuals one of the C residues in the stretch is altered. Such changes are predicted to affect splicing of exon 3, leading to a frameshift and a premature stop, which was confirmed by cDNA analysis 6. Chapter 3 of this thesis will focus on the mutations that are found in intron 2.

**Mitochondria**

LBSL is caused by a defect in a mitochondrial protein. Mitochondria are membrane-enclosed organelles within human cells. Mitochondria have four compartments: the outer membrane, the inner membrane, the intermembrane space, and the matrix, the region inside the inner membrane.
CHAPTER 1

Functions

Mitochondria perform many tasks, but the most crucial function is probably the generation of adenosine triphosphate (ATP), the energy source for many cellular processes. ATP is produced by the respiratory chain, consisting of the electron-transport chain and the oxidative-phosphorylation system. The components of the respiratory chain are located in the inner mitochondrial membrane and consist of five multimeric protein complexes (Table 1). Electrons are transported along the complexes to oxygen, which is then reduced to water. This releases energy that is used to transfer protons across the mitochondrial inner membrane from the matrix into the intermembrane space. ATP is subsequently generated by the flux of these protons back into the mitochondrial matrix through ATP synthase.

Mitochondria have many additional functions besides ATP production. For example, mitochondria are essential components in many apoptotic pathways as they can release cytochrome c which results in activation of caspases. Mitochondria are also one of the major sources of reactive oxygen species (ROS). ROS are damaging to many key components in cells, and may also influence homeostatic signalling pathways that control cell proliferation and differentiation. Additionally, mitochondria are the major source of nicotinamide adenine dinucleotide (NADH), contain parts of the pyrimidine and lipid biosynthetic pathways, regulate cellular levels of metabolites, amino acids and cofactors and have a central role in metal metabolism. Mitochondria also participate in Ca²⁺ homeostasis. The ability of mitochondria to modulate Ca²⁺ flux is crucial for controlling neurotransmitter release, neurogenesis, and neuronal plasticity. Furthermore, mitochondria provide building blocks for synthesis of certain neurotransmitters. This might make the brain more sensitive to mitochondrial defects.

Table 1 Respiratory chain complexes

<table>
<thead>
<tr>
<th>Complex</th>
<th>Name</th>
<th>Total number of subunits</th>
<th>Subunits encoded by mtDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>NADH-ubiquinone oxidoreductase</td>
<td>45</td>
<td>7</td>
</tr>
<tr>
<td>II</td>
<td>succinate dehydrogenase-ubiquinone oxidoreductase</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>ubiquinone-cytochrome c oxidoreductase</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>IV</td>
<td>cytochrome c oxidase</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>V</td>
<td>ATP synthase</td>
<td>16</td>
<td>2</td>
</tr>
</tbody>
</table>

Mitochondrial genetics

Mitochondria are the only organelles of human cells besides the nucleus that contain their own DNA, mitochondrial DNA (mtDNA), and their own machinery for synthesizing RNA and proteins. The human mtDNA is a 16,569-bp, double-stranded, circular molecule containing 37 genes. Twenty-two of these genes code for transfer RNAs (tRNAs), two code for ribosomal RNAs (rRNAs) and 13 genes are protein-encoding and code for subunits of the respiratory chain (Table 1).

Human mtDNA inheritance is exclusively maternal. There are hundreds or thousands of mitochondria per cell, and each contains approximately five mitochondrial genomes. Mutations
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in the mtDNA can be present in some but not all of these genomes. As a result, cells and tissues can contain both normal and mutant mtDNA, a situation known as heteroplasmy. The number of mtDNAs with disease-causing mutations must reach a threshold for disease symptoms to become apparent. This threshold is lower in tissues that have a high energy demand, such as brain, heart, skeletal muscle, retina, renal tubules, and endocrine glands. These tissues will therefore be especially vulnerable to the effects of pathogenic mutations in mtDNA. Due to the random redistribution of mitochondria during cell division, the proportion of mutant mtDNAs can differ between daughter cells which explains the age-related and tissue-related variability of clinical features that are frequently observed in mtDNA-related disorders.

Mitochondrial disorders

Mitochondrial dysfunction is associated with an increasingly large number of human inherited disorders, including many rare disorders, and is also becoming implicated in more common, complex diseases, such as neurodegenerative disorders. Mitochondrial disorders are a genetically heterogeneous group of different diseases. Any organ system can be affected and symptoms can start at any age. Currently, mitochondrial disorders cannot be cured, treatments are directed at relieving symptoms.

Mitochondrial disorders have an estimated prevalence of 10 to 15 cases per 100,000 persons. They can be classified into disorders due to defects in mtDNA, which are maternally inherited, and disorders due to defects in nuclear DNA, which are transmitted by mendelian inheritance.

Mitochondrial DNA mutations

Many mutations in the small mitochondrial genome have been identified and can cause a wide variety of clinical syndromes. Most mtDNA-related diseases share the features of lactic acidosis and massive mitochondrial proliferation in muscle, resulting in so-called ragged-red fibers. Because mitochondria are ubiquitous, every tissue in the body can be affected by mtDNA mutations, which is why mitochondrial diseases are often multisystemic. As mentioned, because of their high energy demand, the brain and muscles are often involved. A large variety of diseases is caused by point mutations or deletions or duplication in mitochondrial genes that affect specific proteins of the respiratory chain or the synthesis of mitochondrial proteins as a whole due to mutations in tRNA or rRNA genes. As a result of heteroplasmy, different tissues harbouring the same mtDNA mutation may be affected to different degrees.

Common diseases include mitochondrial encephalomyopathy, lactacidosis, and stroke-like episodes (MELAS), myoclonus epilepsy with ragged-red fibers (MERRF), Leber’s hereditary optic neuropathy (LHON), neurogenic weakness, ataxia, and retinitis pigmentosa (NARP), Kearns-Sayre syndrome (KSS), Pearson-syndrome and progressive external ophthalmoplegia (PEO). Symptoms vary a lot between mitochondrial disorders: e.g. MELAS is a multisystem disorder, particularly affecting the nervous system and muscles, whereas LHON is characterized by a bilateral, acute or subacute loss of vision in young adults.

In summary, many mutations have been identified in the small mitochondrial genome that cause
a wide variety of clinical syndromes and there seems to be no simple relation between the site of the mutation and the clinical phenotype, even with a mutation in a single gene. Mutations in one mtDNA gene can lead to different diseases. On the other hand, one disease can be caused by mutations in different mitochondrial genes. Furthermore, due to heteroplasmia, the same mutation can lead to a different severity in disease. An overview of the mutations found in the mtDNA and their associated diseases can be found in the MITOMAP database (www.mitomap.org).

**Nuclear DNA mutations**

To complicate things further, mutations in nuclear genes can also lead to mitochondrial disorders. Mammalian mitochondria contain over 1,500 proteins, which may vary in a tissue-dependent manner. Only 13 of these proteins are mtDNA-encoded, so mitochondria depend on the nucleus and other cellular compartments for the majority of their proteins. Nuclear-encoded mitochondrial proteins are actively imported into mitochondria. Mutations in several of such genes have already been identified, and it is likely that many more are yet to follow. Mutations have been identified in nuclear DNA genes encoding respiratory chain subunits or assembly factors. These can cause, for example, Leigh syndrome (LS), clinically characterized by severe, developmental psychomotor delay, cerebellar ataxia, pyramidal signs, seizures and movement disorders. Mitochondrial disorders can also be due to mutations in genes encoding components of the mtDNA maintenance machinery, including the protein synthesis machinery, of which LBSL is an example. Additionally, mutations in genes involved in coenzyme-Q synthesis, the lipid milieu, mitochondrial transport machinery and mitochondrial biogenesis all are found to cause certain forms of mitochondrial disorders.

**Mitochondrial disorders and leukoencephalopathy**

Bilateral symmetric abnormalities in basal ganglia and brain stem are regarded the most common MRI features in children with mitochondrial encephalopathies. White matter involvement has, however, recently been recognized as a common feature in patients with mitochondrial disorders that may be caused by molecular defects in either the mitochondrial genome or the nuclear genes. White matter has been described to be affected in mitochondrial disorders including MELAS, LHON, LS, KSS. Isolated deficiencies of respiratory chain complexes, often caused by nuclear defects, frequently result in LS with white matter involvement. The most common defects are in complex I. Patterns of white matter abnormalities may be characteristic depending on the molecular etiology of the mitochondrial disorder and can be used for diagnosis.

**Aminoacyl-tRNA synthetases**

As mentioned, LBSL is caused by mutations in DARS2, which encodes one of the mitochondrial aminoacyl-tRNA synthetases (ARSs). ARSs are enzymes that are ubiquitously expressed and are present in species ranging from bacteria to humans. These enzymes have an essential function in cells and defects can lead to a variety of diseases in humans.
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Function
Genetic information stored in DNA is first transcribed to messenger RNA (mRNA) and is then translated to protein to form functional products in cells. Proteins are made up of amino acids, encoded by a three nucleotide ‘codon’ in the mRNA. During translation, these codons are recognized by a complementary ‘anticodon’ of tRNAs which are charged with a specific amino acid. This ensures that the correct amino acid is attached to the growing amino acid chain. ARSs are responsible for charging the tRNAs with the correct amino acids (Figure 3). Charging of a tRNA with an amino acid is accomplished by a two-step reaction which uses an ATP molecule:

1. amino acid + ATP → aminoacyl-AMP + PPi
2. aminoacyl-AMP + tRNA → aminoacyl-tRNA + AMP

ARSs form a large and diverse family, in which each member is able to specifically attach a particular amino acid to the corresponding tRNA. The overall fidelity of protein synthesis is dependent on the accuracy of codon-anticodon recognition and aminoacyl-tRNA synthesis. ARSs make a series of contacts with both their amino acid and tRNA substrates to ensure a high specificity; ARSs display an overall error rate of about 1 in 10,000. Additionally, intrinsic proofreading activities eliminate amino acids recognized by a non-cognate ARS. The genes and proteins are named by their one-letter or three-letter amino acid designation respectively, e.g. for aspartyl-tRNA synthetase the gene is called DARS and the enzyme is abbreviated as AspRS.

Classification
Despite their conserved mechanisms of catalysis, ARSs are divided into two unrelated classes (I and II) based on mutually exclusive sequence motifs that constitute the catalytic site. Human cells contain 36 genes for ARSs, coding for both cytosolic and mitochondrial sets of ARSs. A mitochondrial set is necessary because mitochondria contain their own DNA and need the entire transcription and translation machinery to produce the encoded proteins. Mitochondrial ARSs are encoded by the nuclear DNA and upon synthesis in the cytosol the proteins are transported to the mitochondria to perform their function. In general there is a separate ARS for each of the 20 amino acids in the cytosol and mitochondria, but there are a few exceptions. The enzymes for GlyRS and LysRS function in both the cytosol and in mitochondria.
Cytosolic and mitochondrial GlyRS are generated from two translation initiation sites on the same mRNA, leading to enzymes with and without a mitochondrial targeting signal. For LysRS, the insertion or exclusion of a mitochondrial targeting signal is achieved by alternative mRNA splicing. In the cytosol GluRS and ProRS are encoded by one gene and form one protein with two activities. In mitochondria there is no GlnRS and synthesis of Gln-tRNA$^{\text{Gln}}$ proceeds via an indirect pathway. In this pathway, tRNA$^{\text{Gln}}$ is first mis-aminoacylated with glutamate by mtGluRS. A Glu-tRNA$^{\text{Gln}}$ amidotransferase, named hGatCAB, can then amidate Glu to form Gln-tRNA$^{\text{Gln}}$. The mischarged Glu-tRNA$^{\text{Gln}}$ does not compromise the fidelity of translation, as it cannot be recognized by translation elongation factors and consequently cannot participate in protein synthesis.

Mitochondrial aspartyl-tRNA synthetase

MtAspRS is a class II ARS. The gene for human mtAspRS codes for 645 amino acids. The sequence of mtAspRS contains an N-terminal mitochondrial targeting sequence which is cleaved upon translocation into mitochondria. Initially predictions suggested that this sequence was 47 amino acids long, leading to a mature enzyme of 598 amino acids. However, recent experiments have shown that a mature mtAspRS is much more stable when seven additional amino acids are added to the N-terminus, suggesting that only the first 40 amino acids are cleaved and the mature enzyme is 605 amino acids long. MtAspRS is composed of a C-terminal active site domain (amino acid 177-606) linked to an N-terminal anticodon-binding domain (amino acid 48-154) by a short hinge. The enzyme forms homodimers.

Non-canonical functions

Aminoacyl-tRNA synthetases have long been known to participate in other cellular processes in addition to protein synthesis; some examples are listed below. In the cytosol a large macromolecular complex, composed of nine different ARSs (ArgRS, AspRS, GlnRS, IleRS, LeuRS, LysRS, MetRS and the bifunctional GluRS-ProRS protein) and three ARS-interacting factors is formed. The exact role of these complexes in the cell is unclear, they might facilitate substrate channelling during protein synthesis.

Some mammalian ARSs are secreted to trigger signalling pathways. An example is human TyrRS that is secreted and processed into two fragments with either proangiogenic or immune cell stimulant activity, whereas a fragment of TrpRS has an antiangiogenic activity. Several additional functions have been described for the human LysRS. The protein is involved in the packaging of Human Immunodeficiency Virus particles, can exert transcriptional control in mast cells via its secondary catalytic product, Ap4A, and can also be secreted to stimulate immune cells. HisRS, AsnRS and SerRS can also stimulate immune cells through their interactions with cell surface chemokine receptors.

Glu-ProRS was identified as a component of the interferon-γ-activated inhibitor of translation complex. In response to interferon-γ, Glu-ProRS is phosphorylated and released from the multi-ARS complex to silence translation. Furthermore, GlnRS inhibits apoptosis through an
interaction with apoptosis signal-regulating kinase 1 27.

Non-canonical activities are also observed in the set of mitochondrial enzymes. Mitochondrial TyrRS of the mould *Neurospora crassa* works as a splicing factor through an interaction with a conserved tRNA-like structural motif in the group I intron. A similar function in splicing has been found for the yeast mitochondrial LeuRS 27. Whether mitochondrial ARSs, including mtAspRS, function outside mitochondrial protein synthesis in higher eukaryotes in unknown.

**Aminoacyl-tRNA synthetases and human disease**

*DARS2* was the first mitochondrial ARS gene shown to be involved in an inherited human disease. Now mutations in a whole range of these genes have been proven to cause a wide variety of diseases. In addition, mutations in several cytosolic ARS genes are known to cause human diseases.

**Cytosolic ARSs**

Mutations in five genes encoding cytosolic ARSs are implicated in Charcot-Marie-Tooth disease (CMT). CMT represents a genetically and clinically heterogeneous group of peripheral neuropathies, with a prevalence of 1 in 2500 individuals. The clinical features of CMT include distal muscular weakness and impaired sensation 28. CMT can be subdivided into two main types according to electrophysiological criteria. Patients with a demyelinating type (CMT1) exhibit decreased motor nerve conduction velocities and demyelination of peripheral nerve axons. Patients with the axonal type (CMT2) do not show primary demyelination but do exhibit axonal loss accompanied by decreased amplitudes of evoked nerve responses. Some forms of CMT do not fit easily into type 1 or 2 using neurophysiological criteria and the term intermediate CMT is used to refer to those patients. These types are further classified into subtypes (CMT1A, CMT2A, etc.) to characterize specific genetic causes of each of the larger categories 29.

Heterozygous missense mutations and one *de novo* deletion in *YARS*, coding for TyrRS, were detected in three unrelated families with autosomal dominant intermediate CMT type C. The missense mutations lead to a partial loss of activity and the mutant enzymes appear to have a dominant-negative effect on the functionality of the wild-type protein and distribution of TyrRS in neuronal endings 30. A heterozygous missense mutation in the *AARS* gene, coding for AlaRS, was identified in two families with patients with autosomal dominant CMT2. Functional assays strongly suggest a modified affinity of the mutant enzyme for tRNAAla and reduced aminoacylation efficiency 31. A mouse model showed that a homozygous defect in AlaRS editing is responsible for mischarging of tRNAAla with serine or glycine and leads to protein misfolding and neurodegeneration, indicating that AlaRS fidelity errors are sufficient for neurodegeneration 32. Heterozygous missense mutations in the *GARS* gene, coding for GlyRS, were found in families with CMT2D and distal spinal muscular atrophy type V (dSMA-V). dSMA-V is a neuromuscular disorder with a phenotype similar to CMT2D. Both disorders are axonal neuropathies and are associated with a more severe phenotype in the upper extremities 33. Compound heterozygous mutations in *KARS*, coding for LysRS, were found in a patient with
an intermediate CMT phenotype and an autosomal dominant missense mutation in *MARS*, coding for MetRS, was suggested to be a very rare cause of late-onset CMT2. Additionally, a missense variant in *HARS*, coding for HisRS, was identified in a patient with sporadic motor and sensory peripheral neuropathy. Interestingly, GlyRS and LysRS both function in the cytosolic and mitochondrial compartment and interference with the mitochondrial function of GlyRS and LysRS cannot be excluded as disease-causing mechanism. However, TyrRS, AlaRS, MetRS and HisRS function only in the cytosol, separate enzymes exist in the mitochondria, making a general mitochondrial defect in peripheral neuropathies less likely.

Mutations in other cytosolic ARSs are associated with disorders other than peripheral neuropathies. Interestingly, mutations in *HARS* and *KARS*, which were both associated with peripheral neuropathies, were also associated with other disorders. A different missense variant in *HARS* was associated with a variant of Usher syndrome that is characterized by progressive vision and hearing loss during early childhood. Homozygous missense mutations in *KARS* were associated with autosomal recessive nonsyndromic hearing impairment. Mutations in the *LARS* gene, coding for LeuRS, were found in a family with infantile hepatopathy. Mutations in *DARS*, coding for AspRS, cause hypomyelination with brain stem and spinal cord involvement and leg spasticity (HBSL). Interestingly, this disorder has a striking resemblance to LBSL.

**Mitochondrial ARSs**

Mutations in genes coding for mitochondrial ARSs are associated with an amazingly wide clinical spectrum.

*RARS2* – Pontocerebellar hypoplasia

Mutations in the *RARS2* gene, coding for mtArgRS, cause pontocerebellar hypoplasia type 6. After the identification in three patients, additional patients with *RARS2* mutations have been described. Splice site mutations, missense mutation and a small deletion in the *RARS2* gene were found in these patients. Patients suffer from infantile encephalopathy associated with pontocerebellar hypoplasia and multiple mitochondrial respiratory chain defects. The amount of mitochondrial tRNA is strongly reduced.

*YARS2* – MLASA

Homozygous missense mutations in *YARS2*, coding for mtTyrRS, were detected in patients with myopathy, lactic acidosis, and sideroblastic anemia (MLASA). Patients presented multiple respiratory chain defects in muscle and deficient mitochondrial protein synthesis in myotubes but not in cultured skin fibroblasts. Aminoacylation activity of the mutant mitochondrial tyrosyl-tRNA synthetase showed a 2-fold decrease compared to the wild-type enzyme.

*SARS2* – HUPRA

A homozygous missense mutation in the *SARS2* gene, coding for mtSerRS, was identified in
three patients with hyperuricemia, pulmonary hypertension, renal failure and alkalosis (HUPRA syndrome) [46]. Immunohistochemical staining in muscle biopsy showed COX deficiency and enzyme assays revealed multiple oxidative phosphorylation deficiencies in these patients. The mutant enzyme specifically decreased aminoacylation of one of the two isoacceptors: tRNA$_{AGY}^{\text{Ser}}$ but not tRNA$_{UCN}^{\text{Ser}}$.

**HARS2 and LARS2 – Perrault syndrome**

Perrault syndrome is a rare genetically heterogeneous recessive disorder characterized by ovarian dysgenesis in females and sensorineural hearing loss. Missense mutations in the *HARS2* gene, coding for mtHisRS, were found in one family [47]. There were no indications for mitochondrial abnormalities. Aminoacylation activity of mutant mtHisRS was reduced and a deletion mutant was not stably expressed in mammalian mitochondria. Homozygous or compound heterozygous mutations in *LARS2*, coding for LeuRS, were identified in two other families with Perrault syndrome [48].

**AARS2 - Infantile mitochondrial cardiomyopathy**

Infantile cardiomyopathies are fatal disorders of the neonatal period or the first year of life and mitochondrial dysfunction is a common cause of this group of diseases. In a group of three patients mutations in the *AARS2* gene, coding for mtAlaRS, were found. The patients showed multiple oxidative phosphorylation deficiencies [49].

**EARS2 – LTBL**

Leukoencephalopathy with thalamus and brain stem involvement and high lactate (LTBL) is a rare disorder with a specific pattern of MRI abnormalities, including extensive symmetrical cerebral white matter abnormalities sparing the periventricular rim and symmetrical signal abnormalities of the thalami, midbrain, pons, medulla oblongata and cerebellar white matter. MRS shows increased lactate in the abnormal white matter. Two distinct groups were identified. While all patients share an infantile onset and rapidly progressive disease, patients in the ‘mild’ group partially recover and regain milestones in the following years with striking MRI improvement and declining lactate levels, whereas patients of the ‘severe’ group do not recover, show brain atrophy on MRI and a persistent lactate increase. Biochemical assays of individual mitochondrial respiratory chain complexes in cultured fibroblasts ranged from a moderate reduction of mitochondrial respiratory chain activities to hardly any detectable defect [50]. Compound heterozygous mutations in the *EARS2* gene, coding for mtGluRS, were found in all patients [50], which is similar to the situation for *DARS2* mutations in LBSL.

**MARS2 - ARSAL**

Complex rearrangements in the *MARS2* gene, coding for mtMetRS, were identified in a large cohort of French-Canadian families with autosomal recessive spastic ataxia with leukoencephalopathy (ARSAL) patients. Analysis of patient cells revealed decreased levels of mtMetRS protein and
a reduced rate of mitochondrial protein synthesis. Patient cells also exhibit reduced complex I activity, increased reactive oxygen species, and a slower cell proliferation rate \(^{51}\).

**FARS2 – Alpers syndrome**

In a Finnish family with two patients and a Saudi girl suffering from a fatal mitochondrial encephalopathy mutations were detected in the *FARS2* gene, coding for mtPheRS, by exome sequencing \(^{52,53}\). Neuropathological findings are consistent with Alpers syndrome. The missense mutations affect highly conserved amino acids and directly impair aminoacylation function and stability of mtPheRS, leading to a decrease in overall tRNA charging capacity \(^{52}\).

**Pre-mRNA splicing**

Upon the discovery of mutations in *DARS2* as cause of LBSL, it was immediately clear that many patients had mutations that affected the splicing of mtAspRS mRNA \(^6\). Most mRNAs, before they are transported to the cytoplasm to be translated, must be processed to remove its introns in a process called splicing. The most common mutation in LBSL affects splicing of exon 3.

**Mechanism**

The human genome contains over 200,000 different introns, which range in size from less than 100 to over 700,000 nucleotides \(^{54}\). Splicing is carried out by a large macromolecular complex named the spliceosome. The complexity of the spliceosome is needed to recognize the huge variety of exons and introns and to be highly accurate \(^{55}\).

The spliceosome consists of five RNA molecules, named U1, U2, U4, U5 and U6, and over 300 different proteins \(^{54}\). The RNA molecules are relatively short, less than 200 nucleotides each, and each RNA is part of a complex with at least seven protein subunits to form a small nuclear ribonucleoprotein (snRNP). These snRNPs form the core of the spliceosome \(^{55}\).

Intron removal is directed by special sequences at the intron-exon junctions called splice sites (Figure 4). At the 5’ splice site a GU dinucleotide within a larger less conserved consensus sequence marks the start of an intron. Three conserved sequence elements mark the 3’ splice site region: the branch point (usually an adenosine), followed by a polypyrimidine tract, followed by an AG at the 3’ end of the intron \(^{56}\). During the

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**Figure 4** Exons in the pre-mRNA are interrupted by introns which are removed during splicing. The 5’ splice site is marked by a GU, the branch point adenosine (A) is shown, followed by a polypyrimidine tract (YYYY) and the AG at the 3’ splice site. Splicing takes place in two steps as described in the text.
splicing reaction, recognition of the 5’ splice junction, the branch point site and the 3’ splice junction is performed largely through base-pairing between the snRNAs and the consensus RNA sequences in the pre-mRNA. After assembly of the major components on the pre-mRNA, intron excision occurs in two chemical steps (Figure 4). First, the phosphodiester bond at the 5’ splice site is cleaved by attack of the 2’ hydroxyl of the branch point adenosine. This leads to cleavage of the 5’ exon from the intron and the ligation of the intron 5’ end to the branch-point 2’-hydroxyl. In the second step the 3’ splice site is attacked by the 3’ hydroxyl of the 5’ exon resulting in exon ligation and release of the intron.

Regulation
Deep sequencing has revealed that >95% of human genes are alternatively spliced and some pre-mRNAs can have multiple positions of alternative splicing, giving rise to a family of related proteins from a single gene. Alternative splicing is recognized as the major contributor to protein diversity and often occurs in a developmental, tissue-specific or signal transduction-dependent manner.

There are different ways for alternative splicing. A complete exon can be included or excluded from the mRNA, the position of the 5’ or 3’ splice sites can be altered, increasing or decreasing the length of an exon, and finally, a complete intron can be included in the final transcript, called intron retention.

Changes in splice site choice arise from changes in the assembly of the spliceosome. There are many regulatory sequences within exons and introns that strongly affect spliceosome assembly. RNA elements that act positively to stimulate spliceosome assembly are called splicing enhancers. Other RNA sequences act as splicing silencers or repressors to block spliceosome assembly and certain splicing choices. Another way of regulation is to create an RNA secondary structure that affects splice site recognition.

Besides different RNA sequences, many regulatory proteins are involved in splice-site selection and these proteins are often combined in complex ways into multiple layers of regulation. Many of these proteins can act either positively or negatively depending on their binding context. The expression level of splicing factors varies between tissues and cell types and the expression of some splicing regulatory proteins is restricted to certain cells. Splicing can also be regulated within a given cell by external stimuli and growth conditions that can induce the expression of splicing factors, alter the phosphorylation state of certain proteins or the localization of the splicing factor within a cell. The ratio of one splicing pattern to another for a typical alternatively spliced transcript is determined by the combination of all of these factors.

Scope and outline of this thesis
In 2007, before the start of this study, the genetic defect underlying LBSL was found. This discovery made it possible to study the disease mechanism of this currently untreatable disorder. This thesis describes the research that has been done to increase our understanding of the pathophysiology of LBSL at a molecular and cellular level. Chapter 2 describes the effect of
several pathogenic missense mutations on the functioning of mtAspRS. Chapter 3 addresses the cell-specific effects of the common type of mutation that affects splicing of the intron 2-exon 3 boundary of the mtAspRS mRNA. With the knowledge of the defective gene it became possible to study whether LBSL has a broader phenotype and whether patients with a similar but in some aspects distinct MRI pattern also have a defect in the same gene. This led to the discovery of severe forms of LBSL (Chapter 4). The full phenotypic and genotypic variation in LBSL is further described in Chapter 5, which emphasizes the importance of the mutations in intron 2 as a potential therapeutic target and shows the feasibility of a compound screening approach to find possible therapeutic agents. The implications of the data presented in the experimental chapters are discussed in Chapter 6.