Chapter 6

Summary, discussion, and prospects
LBSL

LBSL is an autosomal recessive white matter disorder. Its characteristic constellation of MRI abnormalities was first described in 2003 3. Patients suffering from LBSL show signs of slowly progressive cerebellar ataxia, spasticity and decreased position and vibration sense. Mutations in the gene DARS2 were identified in LBSL patients in 2007 6. The gene DARS2 codes for the mtAspRS protein, a dimeric enzyme functioning in mitochondrial translation. Almost all patients are compound heterozygous and the large majority has a mutation in intron 2 on one allele. Identification of the causative gene has enabled studies on the underlying molecular mechanism, broadening of the clinical spectrum and a first search for therapeutic targets. The progress that has been made on these subjects is described in this thesis.

Molecular mechanisms

Missense mutations

Since the first report of mutations in DARS2 6, it has become clear that many different mutations in this gene can cause LBSL. We studied the effect of a selection of the identified missense mutations in Chapter 2 to increase our understanding of the pathological molecular mechanism. We studied the effect of these mutations on enzyme activity, protein expression, cellular localization and dimerization of mtAspRS. Mutation-mediated changes with a negative effect on any of these properties are likely to result in decreased mitochondrial aspartylation activity in patient cells. Our studies showed that mutations had diverse effects and most mutations had a substantial impact on one or more of the properties studied. This suggests that missense variants contribute only marginally to the mitochondrial aspartylation activity in the cell and that most of the residual activity must come from mtAspRS expressed from the other allele. Nearly all patients have a mutation in intron 2 on the other allele, affecting the splicing of the third exon of DARS2 and leading to a premature stop. In Chapter 3 we have studied the effect of these mutations. In patients’ lymphoblasts we could confirm that this mutation is leaky, meaning that wild-type protein is produced from this allele albeit at lower levels.

Selective vulnerability

MtAspRS has an essential function in mitochondrial translation and is expressed in all cells in the human body except erythrocytes. It is, therefore, remarkable that the abnormalities in LBSL are confined to specific structures in the nervous system. The nervous system is, among a few other tissues, known to be vulnerable to mitochondrial dysfunction due to its high energy demand. Muscle tissue is also vulnerable to mitochondrial dysfunction, but no significant mitochondrial defects could be detected in muscle tissue from LBSL patients 3. Within the nervous system some long white matter tracts are affected, which might be explained by extra sensitivity to mitochondrial defects because of the necessary long distance transport processes, but also specific smaller white matter structures are affected, negating this explanation. The selective vulnerability of the nervous system and the selective vulnerability of specific structures within the nervous system in LBSL suggest that additional factors play a role in determining the vulnerability to
**Summary, discussion, and prospects**

**DARS2 mutations.**

In Chapter 3 we studied the cell-type specific effects of intron 2 mutations and found a potential explanation for this selective vulnerability in LBSL. The magnitude of the effect of the intron 2 mutations differed depending on the cell type. The largest effect was observed in cells derived from the central nervous system; exclusion of the third exon from the mRNA was more prominent in these cell types as compared to non-neural cell types. Further comparison of different neural cell types indicated that the mutations had the largest effect in cells of neuronal origin. This is in line with the involvement of tracts in LBSL patients. Although LBSL is a white matter disorder and primary involvement of myelin, oligodendrocytes or astrocytes may be suspected, it is important to realize that tracts are determined by axons and not by oligodendrocytes or astrocytes, suggesting that the primary problem in LBSL may involve neurons or axons. The cell type specific effect of the intron 2 mutations can be explained by differences in the abundance of splicing factors between cell types. Several brain-specific splicing factors have been identified that could account for splicing differences between non-neural and neural cells. In addition, region- and cell-type-specific expression of many RNA-binding proteins has been observed in the mouse brain. It is likely that these differences in expression also exist in human brains. We do not have access to material from different brain regions of LBSL patients to study the local impact of the splicing defect and to see whether the degree of exclusion of exon 3 indeed correlates with abnormality of the area.

Only a few patients do not have an intron 2 mutation. In Chapter 3 we showed that there is also increased exon 3 skipping in neuronal cells with the wild-type sequence as compared to other cell types. This decreased splicing efficiency may make the nervous system more vulnerable to DARS2 mutations in general and could explain why an intron 2 mutation is not an absolute prerequisite for developing LBSL. We postulate that the mutations, combined with the less efficient splicing in neural cells, decrease the mtAspRS activity in the brain to a level below a threshold that is necessary for normal function, but are mild enough to spare other tissues.

**Clinical data**

LBSL was first described as a relatively mild disorder, in which patients usually develop signs of the disease in childhood or adolescence and have a slowly progressive course. The identification of mutations in the DARS2 gene allowed studies into a possible broader phenotype. Several mild cases were described in the literature and also more severe cases were identified. Chapter 4 describes a study on a group of 11 patients with a severe encephalopathy, in whom most MRI criteria for LBSL were fulfilled, but also other or additional features were observed. The MRI abnormalities were more extensive than in classical LBSL, especially in the cerebral and cerebellar white matter. Additionally, the middle cerebellar peduncles were affected and abnormalities in the globus pallidus were frequently seen. Clinical signs of the disease appeared early in life, from the antenatal period to the age of 6 years. Two of these patients died in the second year of life. In six of the 11 cases mutations in DARS2 were detected. This confirms the existence of severe LBSL forms, indicating that the clinical severity of LBSL is highly variable,
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from a severe form with early onset and early death, to a mild form with adult onset and very slow progression.

The phenotypic variation was examined in depth in Chapter 5 by a cross-sectional observational study of 78 LBSL patients. There are extreme forms at both ends of the spectrum, but in most cases the disease has an onset in late-childhood or adolescence and a relatively slow and mild course. Full wheelchair dependency is rare and most often does not occur before adulthood.

Functional data

The enzyme activities of several mutant variants of mtAspRS have been quantified previously; additional results are described in Chapter 2. Those aminoacylation assays were carried out with purified mutant mtAspRS expressed in E.coli and provided valuable insights into the effect of single mutations. The situation in LBSL patients is, however, more complex, because they are compound heterozygous. The mitochondrial aspartylation activity in the cell is determined by two different mutant alleles, and is not only affected by a direct effect of mutations on the catalytic properties, but also by effects on other aspects such as expression and dimerization (as described in chapter 2) and possibly other unknown effects. The overall effect of the two heterozygous mutations is best studied by measurement of the enzyme activity in cells of LBSL patients.

We have access to cultured lymphoblast and fibroblast cell lines derived from patients. Although there is no evidence of lymphocyte or fibroblast dysfunction in patients, these cells do carry the DARS2 mutations and could provide a relatively easily available cell model to determine the impact of the mutations. In Chapter 5 we used mitochondrial extracts from patient-derived lymphoblasts to measure aspartylation activity. We found a significantly reduced endogenous mtAspRS activity in these cells compared to control lymphoblasts. Given the apparent normal function of white blood cells in LBSL patients, the residual activity appears to be sufficient for these cells to function adequately. We hypothesize that the reduction in expression, and thereby activity, is larger in neuronal cells due to the splicing defect and that this decreased expression leads to a decrease in the enzyme activity below the threshold level needed in these cells. If correct, this hypothesis implies that the primary defect in LBSL lies in the reduced mitochondrial aspartylation activity in patients and does not suggest a contribution of another, unknown, non-canonical function of mtAspRS. It should be noted, however, that the available data does not exclude this latter possibility.

We could not find a correlation between the activity of the enzyme as measured in the lymphoblasts and the severity of the disease in the patients. Possibly such a correlation does not exist, the assay might not be sensitive enough to detect subtle differences, or the chosen cell type (lymphoblasts) does not reflect the effects in neural cells. A correlation might be found in cell types that are more relevant for the disease, such as neuronal cells.

Genotypic variation

Many different mutations have been identified in the DARS2 gene in LBSL patients. In Chapter 5
we provided an overview of all mutations that are identified in 120 cases, either published in literature or known in our database up to December 2012. 60 different mutations have been identified, including missense mutations, nonsense mutations, splice-site mutations, insertions and deletions. Mutations in intron 2 occur in 94% of the patients.

All patients are compound heterozygous, with the exception of four patients from two families for which homozygosity was described. The severity of the disease differs substantially between these patients with homozygous mutations. Three siblings with a homozygous mutation in intron 2 (c.228-22T>A) have a very severe phenotype. Two of them died at the age of 2 and 8 years. In contrast, a patient with a homozygous missense mutation in exon 17 (c.1825C>T, p.R609W) has a mild phenotype. It is surprising that homozygous mutations do not occur more often. Despite the frequent occurrence of the c.228-21_-20delTTinsC mutation in LBSL patients, with for example a carrier rate of 1:95 in the Finnish population, this mutation has thus far not been reported in a homozygous state in LBSL patients. The most likely explanation is that there is a narrow range of permissive mutations. Probably, severe mutations are not compatible with life in a homozygous state or may lead to a different, possibly multisystemic, disorder. Milder mutations, on the other hand, might not lead to clinical symptoms in a homozygous state. We suspect that individuals homozygous for c.228-21_-20delTTinsC do not have a disease.

Genotype – phenotype correlation

In Chapter 5 we investigated a possible genotype-phenotype correlation for LBSL. Due to the large number of different mutations and the compound heterozygosity, only a few small groups of patients with the same combination of mutations could be formed. A few trends could be observed, but the groups were too small to determine statistical significance.

Mutations found in patients with a severe form of LBSL are also found in patients with a milder form, although not in the same heterozygous combination. As described in chapter 5, there are some indications for a possible genotype-phenotype correlation. Interestingly, most of the severe patients do not have the common intron 2 mutation (c.228-21_-20delTTinsC) that 71% of the patients have, but have another mutation in this area. Possibly, these mutations lead to a more severe disease outcome. A variation in disease severity within families is seen, although the range is quite narrow. The inclusion of more patients is needed to confirm that there is a genotype-phenotype correlation. It is likely that other genetic, epigenetic or environmental factors also influence the severity of the disease.

Therapeutic target

Understanding the underlying mechanism of disease can provide targets for therapeutic agents. The results that are discussed in Chapter 2 indicate that the missense mutations have diverse effects and it would be difficult, if not impossible, to find a general treatment for these mutations. In contrast, almost all patients have a splice-site mutation in intron 2, so most patients would benefit from a treatment that would counteract this defect. Importantly, these intron 2 mutations are leaky and allow wild-type protein to be formed from this allele. Increasing the correct splicing
of this exon thus offers great potential as treatment for LBSL. In Chapter 5 we show that this splicing efficiency can be increased by the use of certain compounds. Using a library of 2000 FDA approved and natural compounds we searched for compounds that increase exon 3 inclusion. Using this approach we identified cantharidin, which increases the efficiency of exon 3 splicing both in our cell model with splicing reporter constructs as well as in patients’ lymphoblasts. Cantharidin is a known protein phosphatase (PP)1 and PP2A inhibitor. It has been identified previously as a compound that alters the splicing of the SMN2 gene. PP1 binds to and dephosphorylates splicing factors, thereby regulating pre-mRNA splicing. Cantharidin is unfortunately too toxic to use as a therapeutic agent for LBSL. However, it provides a proof of the concept that influencing the efficiency of exon 3 inclusion is possible and a highly promising therapeutic target for LBSL.

Remaining questions and future research

Molecular mechanism

Several questions on the mechanism underlying LBSL remain. mRNA splicing most likely plays a role in determining the selective vulnerability of the nervous system, however, the exact splicing factors that are involved remain to be identified. This knowledge could also provide new targets for drugs. Furthermore, it is not completely understood how decreased mtAspRS activity leads to neuronal or axonal degeneration. Some insight into these processes has come from MRI studies, one neuropathological report and research on patient cell lines and cell models as described in this thesis. The scarcity of brain tissue from patients makes it necessary to look for other options to model the disease. Modelling the disease in mice will not be straightforward. Two mutations must be introduced, one of which would preferably be an intron 2 mutation. The corresponding region of DARS2 in mice is, however, not conserved. The use of induced pluripotent stem cells from patients’ cells might provide a better model. Upon differentiation into neuronal cells, such cells can be used to study the pathological effect of DARS2 mutations. Additionally, further studies with therapeutic agents could make use of these cells to determine the effects of compounds on neuronal cells that have the genetic background of LBSL patients.

Selective vulnerability

The cell type-specificity of the exon 3 splicing defect offers a potential explanation for the selective vulnerability of the nervous system in LBSL. In the recent years mutations in many other genes encoding mitochondrial ARSs have been found in a large variety of disorders, as explained in Chapter 1. The differences between these disorders are striking considering the similar, essential functions of these enzymes in mitochondrial translation. It is still unclear what determines the selective vulnerability for the different tissues in each of these disorders. Although splicing defects have been found in some of these disorders, like in pontocerebellar hypoplasia caused by RARS2 mutations, tissue-specific differences in splicing defects do not provide a general explanation for the selective vulnerability. Although no additional functions of the human mitochondrial ARSs have been described, a tissue-specific non-canonical function of these enzymes cannot be
excluded. This latter hypothesis is supported by the recent identification of mutations in *DARS* in HBSL \(^9\), a disorder that bears striking resemblance to LBSL. The similarity between HBSL and LBSL suggests that these disorders may share a common underlying molecular mechanism, which is not yet understood. The way in which mutations in different mitochondrial ARSs lead to such a variety of diseases should be subject of further studies.

**Therapy**

We have shown that cantharidin can alter the splicing efficiency of exon 3, but unfortunately cantharidin is not suitable for use in humans. Future research could be directed at less toxic variants of cantharidin or other PP1 or PP2A inhibitors. A study of Zhang et al. \(^95\) describes the identification of several pseudocantharidins. They identified compounds that are less toxic while still affecting splicing. It would be interesting to study the effect of these compounds on *DARS2* splicing. Additional research could focus on alternative approaches to influence exon 3 splicing. Antisense oligonucleotides are small molecules that can be designed to bind to an exact site on the pre-mRNA to alter splicing and thus could potentially offer a more specific approach than compounds. Progress in the field of therapeutic antisense oligonucleotides is ongoing, but at the moment there are still many challenges to overcome before the use of antisense oligonucleotides in the brain can be accomplished. For the near future a focus on the less toxic cantharidin derivatives and additional screening of more compounds that affect exon 3 splicing is considered the most promising way forward.