Valorisation
Mitochondrial diseases are the most common genetic metabolic diseases, affecting approximately 1 in 5,000 individuals [1]. Mitochondrial diseases are a clinically and genetically heterogeneous group of disorders, which generally manifest in tissues or organs with a high-energy requirement [2]. Due to this complexity and the lack of proper genotype-phenotype correlations, at the start of the work performed in this thesis, a genetic diagnosis was obtained in only 25% of patients by sequential analysis of the mtDNA and single candidate genes. The application of next-generation sequencing as a research tool to first sequence gene panels and later all genes (the exome) has increased this figure considerably, which has facilitated a rapid introduction in diagnostics and clinical care [3]. The valorisation paragraph will deal with the introduction of NGS in routine genetic diagnostics.

Next-generation sequencing: from research to diagnostics
A genetic diagnosis is important for counseling patient and relatives, for preventing the transmission, for providing a more accurate prognosis and in some cases for offering therapeutic interventions. During the past years, NGS technology has developed as the preferred tool for identifying the genetic defects in patients with clinically and genetically heterogeneous diseases (Chapter 2). NGS has contributed in identifying the genetic cause in either the mtDNA (20%) or nDNA (WES; 49%), leading to an overall diagnostic yield of 69% [4]. Given the impressive increase not only in the percentage of patients, in which a genetic diagnosis is established (from 25% to 69%), but also in the speed (from years to months) and in costs (expensive Sanger Sequencing), a rapid introduction of NGS in routine genetic diagnosis of mitochondrial disease was warranted and has been established in the last couple of years. NGS costs rapidly declined and the capacity of platforms as the Illumina NovaSeq 6000 increased tremendously. For example 200 exomes can be sequenced in a single sequencing run. Crucial in this respect was also the development of novel improved bioinformatics tools to predict variant pathogenicity and the availability of superior databases with information on exomes (Exac) and genetic variants (ClinVar). The adagium will be NGS first and guided by the genes and variants found, specific clinical and laboratory investigations can be carried out to unambiguously link the genetic defect(s) to the clinical manifestations. The laboratory findings may also be important, in case
functional validation is required. Our results show a few key points for proper introduction into the clinic:

1. **NGS of both the mtDNA and the whole exome**
   It is essential to investigate both the mtDNA and the exome to identify the genetic cause in mitochondrial disease [4-6]. Although the mtDNA can be analysed in the exome DNA, the coverage is insufficient to detect the necessary low level mutation loads in blood DNA, which might indicate a higher mutation load and follow-up in affected tissues. NGS of the mtDNA allows detection of point mutations and rearrangements alike with immediate characterization of the breakpoints. Only the mtDNA copy number has to be established by a separate test.

2. **Novel mitochondrial disease genes**
   Novel genes are still being identified as a possible cause of mitochondrial disease. These are either genes, in which no genetic defect has been found so far, or genes, in which defects have been found in apparently non-mitochondrial disease. This implies that gene panel-based approaches fall short, as the mitochondrial panel is still not complete. Complete analysis of all genes in the exome (open exome) [4] is preferable to start with to identify all relevant genetic defects in the shortest timeframe and at the lowest cost.

3. **Overlap between mitochondrial and other neuromuscular disorders**
   Our results demonstrate that many neuromuscular diseases exist, in which a mitochondrial defect can be the cause in the minority of cases. In case of gene panels, this would imply sequential analysis of multiple, and probably partly overlapping gene panels. Again, it is preferable to start with an open exome [4], as this would allow an unbiased identification of the underlying genetic causes.

4. **De novo mutations**
   As we detected de novo mutations in the clinical exome, it would be preferable to include immediately the parents for whole exome sequencing [7] and analyze the data, using an autosomal recessive and de novo dominant disease model.

5. **Multigenic disease**
   Especially in clinically heterogeneous disease and when consanguinity is involved, the option of having multiple genes causing the disease in the
patient, should be investigated. This can be done by investigating all gene defects, which could be potentially contribute to the clinical phenotype. One should not address all symptoms to the first gene identified. As this has been done in the past, it is clear that genotype-phenotype correlations from literature [8], when based on few patients and single gene analysis, should be considered with care.

Our proposed diagnostic strategy for most mitochondrial diseases will be NGS of the mtDNA, followed by whole exome sequencing of patients and parents, followed by analysis of all genes, using an autosomal recessive or de novo dominant genetic model. In case of new genes or unclassified variants, functional follow-up studies should be offered to demonstrate pathogenicity. In case an OXPHOS deficiency is observed in patient-derived fibroblasts, this can be done straightforward by complementation assays. If this is not the case, animal studies might provide the answer, which will often go beyond the capabilities of routine diagnostic labs. An in vivo model as zebrafish could fill this gap, but standardization, validation and high-throughput processing is required to keep-up with the rapid generation of unclassified variants and novel genes by WES and WGS these days.

As the combined approach will identify at max 69% of the genetic causes, still 31% of the patients will remain without a genetic diagnosis. As these are carefully selected patients from our cohort, it is likely that that there will be technical issues, prohibiting the identification of the underlying defects. Applying WGS could solve the issue, but routine use is still limited by the financial costs and the complexity of data interpretation. Rapid and life-saving genetic diagnosis in less than 24 hours using rapid WGS (rWGS) is possible and already offered for early intervention cases within paediatric Intensive Care Units (ICUs) as discussed in Chapter 7. Introducing rWGS showed a diagnostic yield of 42 to 57% in serious ill infants in ICUs, and changes in medical management in 30 to 72%, and finally altered outcomes in 24 to 34% of the cases [9]. Sequencing the transcriptome by RNA-Seq seems at this moment a more realistic addition to WES to solve the remaining cases. RNA-Seq allowed analysis of the transcriptome at an unprecedented depth and reported the identification of causative, non-exonic regulatory variants in an additional 10% diagnostic yield of mitochondrial patients using RNA-Seq [10]. In another study of rare muscular disorders this was 35% yield, which clearly shows
the added value of this RNA-Seq approach [11]. It is beyond any doubt, that WGS and RNA-Seq will bring the paradigm shift started by WES to the next level: for a genetic diagnosis, geneticists and expert clinicians will no longer need to narrow down the differential clinical diagnosis to request sequencing of a specific gene or sets of genes, but all genetic information will be available at once from start. The results of this genetic analysis will directly guide the diagnostic patient evaluation, moving towards personalized patient care and treatment.

**Gene-based treatment options**

The identification of the gene defect in the patients has unfortunately not led to the anticipated major breakthrough in novel treatments. Only in few cases, a gene specific-treatment is possible and early start with these treatments prevents symptoms from occurring and can be lifesaving, as for example administration of thiamine to patients with fatal Leigh Syndrome due to SLC19A3 mutations [5]. In case of SLC25A32 mutations riboflavin is also the preferred treatment [12]. Our study revealed that SLC25A32 patients improved when treated with riboflavin as a vitamin B2 precursor of FAD which will compensate the lack of FAD functioning as a crucial co-factor in the mitochondrial transport chain [13]. In these cases, the genetic defect directly revealed a successful treatment option.

In other cases, clinical improvement by specific treatments at a moment when the genetic defect was not known, revealed when the genetic defect was found, which patients might benefit from a comparable treatment. We demonstrated that again riboflavin could be beneficial for patients in case of ACAD9 defects [14]. Since increasing the levels of the riboflavin restored Complex I activity by compensating a disturbed ACAD9 and FAD binding or by stimulating Complex I assembly factors. However, not all ACAD9 patients are treatable with riboflavin [15]. A recent study on ACAD9 disease-causing variants in 70 patients showed improvement of at least 20/31 (65%) ACAD9 deficient patients to riboflavin and 11/31 (35%) patients had no effect, although 20 patients were not treated and additional data about the treatment were missing for approximately 19 patients in this cohort. However, this justifies administration of riboflavin to every ACAD9 patient. Given the high frequency of ACAD9 deficiency, we also propose that early riboflavin administration for phenotypically consistent patients whilst their genetic investigations are still underway [16]. Another, successful treatment of infantile-onset ACAD9-related cardiomyopathy was achieved by a combination of sodium...
pyruvate, beta-blocker, and coenzyme Q_{10} [17]. Finally, also a high fat diet resulted in clinical improvement in a patient with a OXPHOS Complex I deficiency due to a mutation in the \textit{TMEM126B} gene [18].

Conclusively, still this is a minority, and gene and pathway based treatment strategies might fall short. More general, gene-correction/editing (CRISPR/Cas9) or cell-based strategies are more promising as a general treatment strategy, complemented by classical compound-screening strategies of the pharmaceutical industry.
References

