Whole Exome Sequencing Is the Preferred Strategy to Identify the Genetic Defect in Patients With a Probable or Possible Mitochondrial Cause

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Mitochondrial disorders, characterized by clinical symptoms and/or OXPHOS deficiencies, are caused by pathogenic variants in mitochondrial genes. However, pathogenic variants in some of these genes can lead to clinical manifestations which overlap with other neuromuscular diseases, which can be caused by pathogenic variants in non-mitochondrial genes as well. Mitochondrial pathogenic variants can be found in the mitochondrial DNA (mtDNA) or in any of the 1,500 nuclear genes with a mitochondrial function. We have performed a two-step next-generation sequencing approach in a cohort of 117 patients, mostly children, in whom a mitochondrial disease-cause could likely or possibly explain the phenotype. A total of 86 patients had a mitochondrial disorder, according to established clinical and biochemical criteria. The other 31 patients had neuromuscular symptoms, where in a minority a mitochondrial genetic cause is present, but a non-mitochondrial genetic cause is more likely. All patients were screened for pathogenic variants in the mtDNA and, if excluded, analyzed by whole exome sequencing (WES). Variants were filtered for being pathogenic and compatible with an autosomal or X-linked recessive mode of inheritance in families with multiple affected siblings and/or consanguineous parents. Non-consanguineous families with a single patient were additionally screened for autosomal and X-linked dominant mutations in a predefined gene-set. We identified causative pathogenic variants in the mtDNA in 20% of the patient-cohort, and in nuclear genes in 49%, implying an overall yield of 68%. We identified pathogenic variants in mitochondrial and non-mitochondrial genes in both groups with, obviously, a higher number of mitochondrial genes affected in...
mitochondrial disease patients. Furthermore, we show that 31% of the disease-causing genes in the mitochondrial patient group were not included in the MitoCarta database, and therefore would have been missed with MitoCarta based gene-panels. We conclude that WES is preferable to panel-based approaches for both groups of patients, as the mitochondrial gene-list is not complete and mitochondrial symptoms can be secondary. Also, clinically and genetically heterogeneous disorders would require sequential use of multiple different gene panels. We conclude that WES is a comprehensive and unbiased approach to establish a genetic diagnosis in these patients, able to resolve multi-genic disease-causes.

Keywords: mitochondrial disease, next-generation sequencing, mtDNA sequencing, whole-exome sequencing, diagnostic yield

INTRODUCTION

Mitochondrial disorders are clinically highly heterogeneous, with a broad variety of neurological and muscular symptoms involved and having significant clinical overlap with other neuromuscular disorders. Although, mitochondrial disorders are characterized by deficiencies in the oxidative phosphorylation (OXPHOS) and ATP production, biochemical deficiencies are not always detected in the lab. Besides, OXPHOS deficiencies can be a secondary phenomenon in neuromuscular or multi-system disorders with a non-mitochondrial cause (Pyle et al., 2015; Niyazov et al., 2016). Mitochondrial disorders are also genetically heterogeneous, as different gene defects can result in a similar phenotype and both the nuclear and mitochondrial genomes are involved (Rotig and Munnich, 2003; McFarland et al., 2010). These features complicate the establishment of a genetic diagnosis in mitochondrial patients (Koenig, 2008).

For pathogenic variants in the multi-copy mtDNA, which contains 37 genes and is exclusively maternally inherited, the mutation load of the so-called heteroplastic pathogenic variants also affects the clinical presentation (Thorburn and Dahl, 2001; Hellebrekers et al., 2012). The estimated number of nuclear genes involved in mitochondrial function is around 1500 (Prokisch and Ahting, 2007; Calvo and Mootha, 2010), of which only >250 genes have been shown to be involved in mitochondrial disease (Alston et al., 2017). In mitochondrial disorders (patients in group 1), OXPHOS deficiencies are often due to genetic defects in the OXPHOS complexes (subunits and assembly factors), or, more indirectly, in processes such as mitochondrial protein translation and degradation, mtDNA maintenance, fusion and fission, substrate transport, or phospholipid metabolism. Still, mitochondrial dysfunction can also be a more secondary defect in genetic syndromes or neuromuscular disease (Hui et al., 2006). Also, in patients with neuromuscular symptoms that are not specific for a mitochondrial disease (patients in group 2), pathological variants in mitochondrial genes have been reported in addition to other non-mitochondrial genetic causes.

Genetic diagnosis of mtDNA-disorders requires screening of all 37 mtDNA genes and determining the heteroplasmy levels of the variants, being either point mutations or large rearrangements. In addition, mtDNA copy-number is being tested to identify mtDNA deletions. mtDNA analysis in diagnostic setting commonly started with screening for a few relatively common point mutations using mutation-specific restriction enzymes or qPCR based methods (Fan et al., 2006; Wang et al., 2011). In case common pathogenic variants were not detected, the entire mitochondrial genome was analyzed by Sanger sequencing or chip-based methods (van Eijssden et al., 2006; Finsterer et al., 2009). However, these methods are non-quantitative, requiring a second molecular test to determine the mutation load. Besides, chip-based methods have difficulties in detecting small indels (Tang and Huang, 2010; Xie et al., 2011; McCormick et al., 2013). For the large mtDNA deletions, it was time-consuming to determine the exact breakpoints, which could be important for prognosis (He et al., 2002; Wong et al., 2003). The application of next-generation sequencing has greatly increased the possibilities for detecting, characterizing and quantifying point mutations, and rearrangements across the complete mitochondrial genome with one single technology (Li et al., 2010; Huang, 2011; Cui et al., 2013). Although, accurate determination of the heteroplasmy levels of large mtDNA deletions still requires quantitative PCR analysis. The first tissue tested is blood, but this is extended to muscle or urine in case the mtDNA could have been missed in blood (Koenig, 2008). If no pathogenic variants in the mtDNA were present, then moving into the analysis of nuclear genes traditionally relied on (stepwise) Sanger sequencing of nuclear candidate genes which were selected based on clinical and biochemical features or linkage/homozygosity mapping. Again next-generation sequencing methods have made this approach obsolete, as whole exome sequencing (WES), enabled the detection of the majority of the genetic variations in the coding part of the genome (Wortmann et al., 2015). An unbiased and complete genetic analysis is important, especially in heterogeneous mitochondrial disease, where genotype-phenotype relations can be indistinct and novel genes involved.

We have performed a complete next-generation sequencing strategy, analysing the mtDNA and exome in a cohort of 86 patients with mitochondrial disease (group 1) and 31 neuromuscular patients in whom a pathogenic variant in a mitochondrial gene could possibly be involved (group 2). The
latter group includes heterogeneous neuromuscular patients with
disease symptoms that are not specific for mitochondrial disease,
but in which a mitochondrial genetic cause has been identified in
a minority of cases. The results were obtained over a 4-year
period from 2012 to 2016. Patients were tested for pathogenic
variants in the mtDNA by next-generation sequencing and, if
negative, further analyzed by WES. WES-variants were filtered
according to the presumed genetic model of disease inheritance,
allele frequencies, conservation, and the predicted effect of the
variant. With this approach, we identified in 68% of our patients
a causative pathogenic variant in new and known disease genes,
which were either inherited or de novo.

MATERIAL AND METHODS

Patients
One hundred and seventeen patients, from consanguineous and
non-consanguineous families, in this study were under treatment
at Erasmus MC, Maastricht UMC+, Leiden UMC, UMC Utrecht,
AMC Amsterdam (The Netherlands). The patients had a clinical
phenotype that could be caused by a mitochondrial defect.
The age of disease onset in the cohort varied from birth to
approximately 50 years old. As 77% of our patients was below 18
years at the age of diagnosis we used the mitochondrial disease
criteria (MDC) for children (Morava et al., 2006). The MDC
include clinical signs and symptoms (max 4 points), metabolic
abnormalities and neuroimaging (max 4 points) and histologic
anomalies (max 4 points). Score 1: mitochondrial disorder
unlikely; score 2 to 4: possible mitochondrial disorder; score 5
to 7: probable mitochondrial disorder; score 8 to 12: definite
mitochondrial disorder. In group I patients were included, in
whom the diagnosis of a mitochondrial disease was probable
or definite (MDC > 5). Patients not meeting these MD-criteria
(MDC < 5, group 2) had neuromuscular symptoms that are not
specific for mitochondrial disease, but in which a mitochondrial
genetic cause has been identified in a minority of cases. All
parents were unaffected. Subjects gave written informed consent
for WES analysis in accordance with the Declaration of Helsinki.
Research was prospectively reviewed and approved by the local
ethical committee of the Maastricht University Medical Centre.

mtDNA Analysis
Sequencing of the mtDNA was performed using the Illumina
MiSeq platform. Enrichment of the entire mtDNA was
performed by a single long-range PCR using Phusion Hot Start
DNA polymerase II kit (Thermoscientific) and 100 ng of total
genomic DNA, according to the manufacturer’s instructions.
Library preparation was performed by the Illumina Nextera
XT kit according to the manufacturer’s instructions. Twelve
indexed DNA libraries were equimolarly pooled and sequenced in
a single lane of 1 MiSeq flow-cell with a 2 × 300 bp paired-end chemistry. Data demultiplexing was performed with Illumina CASAVA software (v.1.8.4.) and reads were
aligned against the revised Cambridge mitochondrial reference
sequence (but without the gap at position 3107) by BWA
software (v.0.5.9.) (Li and Durbin, 2009). For both variant
and small indel identification, Python 2.6.6., Python package
pySam 0.7.8. and SAMTools 0.1.19 software were used (Li
et al., 2009). Large deletions were identified by alignment
with the Smith and Waterman algorithm and the EMBOSS
water program (v.6.5.7.). Annotation and filtering of mtDNA
variants and indels were performed using in-house build Perl
tools and a MySQL annotation database. Calculation of the
heteroplasmic level at any nucleotide position was performed
by the read depth of the mutant vs. reference nucleotide.
We have validated the detection and quantification accuracy
of our NGS strategy by analysing DNA samples with varying
heteroplasmic levels of different substitution variants, small indels
and large deletions, as previously determined by MitoChip, RFLP
and PCR/Southern blotting (Supplementary Data Tables S1–S5;
SRA submission SUB4444333). The entire mtDNA, excluding
the highly polymorphic D-loop, was analyzed using a 2%
heteroplasmic cut-off for known pathogenic point mutations
and a 5% cut-off for the remaining positions and small indels.
If no mtDNA mutations were detected in blood of the MD-
patient (group 1) and a mtDNA disease was highly likely,
this was confirmed on muscle or urine DNA. To test for
mtDNA depletions, qPCR quantification was performed on
DNA extracted from available muscle biopsies, using SensiMix
SYBR (Bioline), where mtDNA copy-number (based on the
mitochondrial ND1 gene) was normalized to the nDNA copy-
number (based on the nuclear B2M gene).

Homozgyosity Mapping and WES
Homozgyosity mapping and CNV analysis was performed by HumanMapping 250K array (Affymetrix, Santa Clara,
California) and Genotyping console 4.0 (Affymetrix).
Homozgyosity or hemizyosity regions were defined by the
“Homozgyosity” mapper (Seelow et al., 2009), with a cutoff
of 5 MB. Exons were captured with SureSelect version 5 exome
enrichment kit (Agilent Technologies, Santa Clara, California),
including untranslated regions. Sequencing was performed on
a HiSeq2000 platform (Illumina, San Diego, California), using
a 2 × 100 bp paired-end setting. Bcl2fastq 1.8.4 (Illumina)
allowed Basecalling and demultiplexing, and Burrows-Wheeler
Aligner 0.5.9 (Broad Institute, Cambridge, Massachusetts) was
used for read alignment against human reference genome hg19.
Duplicate reads were removed by Picard software suite 1.77
(Broad Institute, Cambridge, Massachusetts) and variant calling
was performed with GATK 2.1-8 (Broad Institute).

Exome data of consanguineous families or families consisting of
> 1 patient was filtered for recessive homozygous, compound
heterozygous, and X-linked (XLR) pathogenic variants. Variants
with allele frequencie s < 1% (dbSNP151 Exome Aggregation
Consortium) were evaluated, covering missense variants,
indels, nonsense mutations, and splice variants. Non-annotated
variants were maintained, unless allele frequencies exceeded
5% prevalence in our in-house patient database. Pathogenicity
of nonsynonymous missense variants was estimated by
Polymorphism Phenotyping-2 (PolyPhen-2; Harvard, Boston,
Massachusetts), Sorting Intolerant From Tolerant (J. Craig
Venter Institute, Rockville, Maryland), Protein Variation
Effect Analyzer (PROVEAN; J. Craig Venter Institute), and
MutationTaster (NeuroCure Cluster of Excellence/Berlin)
Institute of Health, Berlin, Germany). Nonsense, frameshift, and splice-site variations were maintained. WES data of single patients from non-consanguineous families were additionally filtered for heterozygous variants in known (OMIM (Online Mendelian Inheritance in Man) disease genes with known dominant and/or de novo pathogenic variants. All identified pathogenic variants were checked for their inclusion in the MitoCarta build 2.0 database for mitochondrial localized proteins.

All variants that are listed in this manuscript were assigned as “most probably disease causing” (class 4) or “disease causing” (class 5). “Disease causing” (class 5), based on previous publications reporting patients with a similar phenotype, possibly with functional prove, or, in case of unreported variants, classified as “most probably disease causing” (class 4), according to the diagnostic standards and guidelines of the American College of Medical Genetics and Genomics (ACMG) (Richards et al., 2015). The ACMG criteria take into account, among others: allele frequencies in control databases (missense variants), amino-acid conservation, Grantham score, functional domains, and splice predictions and nucleotide conservation in case of intronic variants. Pathogenic variants were only included if these segregated correctly with the disease in the family.

RESULTS

Diagnostic Yield

A cohort of 117 patients from unaffected parents, was subject to a two-step next-generation sequencing approach (Figure 1). The age of disease onset in the cohort varied from birth to approximately 50 years old, 77% (90/117) were patients <18 years of age. We solved 20% of the cohort with a disease-causing mtDNA defect, involving 23 MD-patients from group 1 and none from group 2. Subsequent WES analysis solved an additional 49% (57 patients) of the cohort, consisting of 39 patients of group 1 and 18 patients from group 2. With this strategy, we achieved an overall diagnostic yield of 68%, with comparable yield in the group of inherited disease cases (69%) compared to the single patients from non-consanguineous families (68%).

mtDNA Next-Generation Sequencing

mtDNA sequencing resulted in the identification of a disease-causing pathogenic variants in 20% (23/117) of the patient cohort (Figure 1), solving 5% (2/42) of the inherited disease cases (consanguineous, >1 patient) and 28% (21/75) of the single patients from non-consanguineous families. As shown in Table 1, all cases with a mtDNA defect were MD-patients (group 1) and all 23 identified mtDNA defects involved known mtDNA mutations in typical mtDNA disorders, like LHON (Leber’s hereditary optic neuropathy), MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes)/MIDD (maternally inherited diabetes mellitus and deafness), Leigh syndrome and CPEO (chronic progressive external ophthalmoplegia)/Kearns-Sayre syndrome. 39% (9/23) of the mtDNA mutations were, mostly homoplasmic (8 out of 9), LHON mutations (m.11778G>A and m.14484T>G), clinically manifesting with optic atrophy. Only one LHON patient carried 80% heteroplasmy. The m.3243A>G mutation covered 30% (7/23) of the mtDNA defects, and explained both MELAS (2 patients) and MIDD (5 patients) phenotypes. Another mutation was identified in the ND5 gene (m.13513G>A), as a cause of Leigh syndrome. 26% (6/23) of the mtDNA defects were large single deletions, of which the 4,977 bp deletion (breakpoints 8482:13460) was detected in 4 patients. These patients had CPEO, sometimes in combination with additional symptoms of Kearns-Sayre syndrome (pigmentary retinopathy, cardiac conduction abnormalities). Patients with multiple mtDNA deletions or mtDNA depletion (not included in Table 1) were further analyzed by WES, as being suggestive of an underlying nuclear DNA defect in the mtDNA maintenance or replication genes.

Whole Exome Sequencing

In the remaining 94 patients, prior to whole exome analysis, the DNA were subject to SNP-array analysis, using the Affymetrix GeneChip Human Mapping 250K, to detect copy number variations (CNV) and homozygosity regions. No CNVs were detected. In patients from consanguineous families, mapping of the homozygous regions provided an additional filtering criterion to select the most promising genetic variants from the exome data. When multiple affected siblings were present, the entire family, if available, was analyzed by means of SNP-arrays. This approach significantly increased the efficiency of interpreting WES results. WES identified a disease-causing gene defect in 49% (57/117) of the complete patient cohort (Figure 1). In an additional 7% of the cohort, WES analysis identified a genetic variant which could explain the patient’s phenotype, but for which definite evidence is currently lacking. Further
| Family | Patients | Pediatric/adult Symptoms | Mutation | % Mutant mtDNA (in blood) | Gene |
|--------|----------|--------------------------|----------|---------------------------|------|
| Cons., >1 patient | MD | Non-consanguineous | 2 | a | LHON | m.11778G>A | Homoplasmic | MT-ND4 |
| Non-consanguineous | 2 | a | LHON | m.11778G>A | Homoplasmic | MT-ND4 |
| Non-consanguineous, 1 patient | MD | Non-consanguineous | 1 | a | MELAS | m.3243A>G | 24% (32 y.o.) | MT-TL1 |
| Non-consanguineous | 1 | p | MELAS | m.3243A>G | 41% (18 y.o.) | MT-TL1 |
| Non-consanguineous | 1 | a | Midd, ptosis, macular degeneration | m.3243A>G | 25% (36 y.o.) | MT-TL1 |
| Non-consanguineous | 1 | a | MIDD | m.3243A>G | 26% (27 y.o.) | MT-TL1 |
| Non-consanguineous | 1 | a | MIDD | m.3243A>G | 10% (62 y.o.) | MT-TL1 |
| Non-consanguineous | 1 | a | MIDD, macular degeneration | m.3243A>G | 7% (69 y.o.) | MT-TL1 |
| Non-consanguineous | 1 | a | MIDD, cardiomyopathy | m.3243A>G | 40% (28 y.o.) | MT-TL1 |
| Non-consanguineous | 1 | p | Leigh syndrome | m.13513G>A | 72% | MT-ND5 |
| Non-consanguineous | 1 | a | LHON | m.11778G>A | Homoplasmic | MT-ND4 |
| Non-consanguineous | 1 | a | LHON | m.11778G>A | Homoplasmic | MT-ND4 |
| Non-consanguineous | 1 | a | LHON | m.11778G>A | Homoplasmic | MT-ND4 |
| Non-consanguineous | 1 | a | LHON | m.11778G>A | Homoplasmic | MT-ND4 |
| Non-consanguineous | 1 | a | LHON | m.11778G>A | Homoplasmic | MT-ND4 |
| Non-consanguineous | 1 | a | LHON | m.11778G>A | Homoplasmic | MT-ND4 |
| Non-consanguineous | 1 | a | LHON | m.11778G>A | Homoplasmic | MT-ND4 |
| Non-consanguineous | 1 | p | CPEO | de novo: single 8284 bp deletion (7462:15747) | n.d. | covers 22 mtDNA genes |
| Non-consanguineous | 1 | p | CPEO | de novo: single 6277 bp deletion (9614-15792) | n.d. | MT-ND3, MT-ND4, MT-ND5, MT-ND6, MT-CYB, tRNA's (Leu, Ser, His, Gly, Arg) |
| Non-consanguineous | 1 | p | CPEO | de novo: single 4977 bp deletion (breakpoints 8482:13460) | n.d. | MT-ATP8, MT-ATP6, MT-CO3, MT-ND3, MT-ND4, MT-ND5, MT-ND6, MT-CYB, tRNA's (Leu, Ser, His, Gly, Arg) |
| Non-consanguineous | 1 | p | CPEO | de novo: single 4977 bp deletion (breakpoints 8482:13460) | n.d. | MT-ATP8, MT-ATP6, MT-CO3, MT-ND3, MT-ND4, MT-ND5, MT-ND6, MT-CYB, tRNA's (Leu, Ser, His, Gly, Arg) |

(Continued)
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TABLE 1 | Continued

| Family               | Patients | Pediatric/adult | Symptoms          | Mutation                      | % Mutant mtDNA (in blood) | Gene                                                                                   |
|----------------------|----------|----------------|-------------------|-------------------------------|--------------------------|----------------------------------------------------------------------------------------|
| Non-consanguineous   | 1        | p              | CPEO              | de novo: single 4,977 bp deletion (breakpoints 8482:13460) | n.d.                     | MT-ATP6, MT-ATP8, MT-CO3, MT-ND3, MT-ND4, MT-ND4L, RNA's (Leu, Ser, His, Gly, Arg) |
| Non-consanguineous   | 1        | p              | Kearns-Sayre syndrome | de novo: single 4977 bp deletion (breakpoints 8482:13460) | n.d.                     | MT-ATP8, MT-ATP6, MT-CO3, MT-ND3, MT-ND4, MT-ND4L, RNA's (Leu, Ser, His, Gly, Arg) |

mtDNA point mutations and deletions were detected and quantified by mtDNA next-generation sequencing in a cohort of 117 patients. All patients with a mtDNA defect were classified as mitochondrial (group 1).

TABLE 2 | Whole-exome sequencing analysis.

| WES filtering strategy | Total % solved | Group 1 % solved | Group 2 % solved |
|------------------------|----------------|------------------|------------------|
| Consanguineous and/or >1 patient | 68% (27/40) | 65% (17/26) | 71% (10/14) |
| - Autosomal recessive (AR) |               |                  |                  |
| - X-Linked (XLR)        |               |                  |                  |
| Non-consanguineous, 1 patient | 56% (30/54) of which 15% de novo (8/54) | 59% (22/37) | 47% (8/17) |
| - Autosomal recessive (AR) |               |                  |                  |
| - X-Linked (XLR and XLD) |               |                  |                  |
| - Autosomal dominant (AD) |               |                  |                  |

94 patients were subject to WES analysis. WES-data was filtered according to the presumed pattern of disease inheritance, where consanguinity or involvement of >1 patient were indicative of a recessively inherited disorder and non-consanguineous families with a single patient were likely to cover both, recessively inherited and dominant de novo mutations.

studies should reveal which of these pathogenic variants could be classified as disease-causing.

WES-data filtering was based on the presumed genetic mode of inheritance. We applied a variant selection strategy for autosomal recessive (AR) and recessive X-linked (XLR) disorders to the inherited disease cases (consanguinity, >1 patient). As shown in Table 2, this group consisted of 40 families, where WES identified of a genetic defect in 68% (27/40) of the cases, covering homozygous, compound heterozygous and X-linked pathogenic variants (Tables 3, 4). In group 1 (MD patients), 65% (17/26) of the cases were solved, and in group 2 a disease causing gene defect was found in 71% (10/14). As expected, a broad spectrum of genes with relatively few genes with a literature reported mitochondrial function [20% (2/10)] was identified in group 2 (Table 4) compared to the proportion of such genes in group 1 [76% (13/17)] (Table 3). In 54 non-consanguineous families with a single patient, we applied a variant selection strategy for autosomal recessive (AR), X-linked (XLR and XLD) and single heterozygous pathogenic variants, expected to be dominant de novo variants. In order to limit the huge amount of heterozygous variants for dominant disease-causing mutations, variant interpretation was restricted to genes that have previously been reported in dominant disease based on the OMIM database. With this approach, we unraveled the genetic cause in 56% (30/54) of these families (Table 2), 15% of which were single dominant pathogenic variants. Follow-up investigations in the parents revealed that the pathogenic variants either occurred de novo in the patients or were present in one of the parent, who displayed subclinical symptoms upon further investigation. The diagnostic yield was 59% (22/37) in patient group 1 and 47% (8/17) in group 2, where respectively, 86% (19/22) (Table 3) and 13% (1/8) (Table 4) of the defects were located in a gene with reported mitochondrial function.

Overall, we saw that 31% (13/42) of the disease-causing genes in the mitochondrial patients (group 1) were not included in the MitoCarta database for mitochondrial localized proteins at the time we identified the genetic defect (Table 3). Surprisingly, this included 3 genes with a literature reported function in mitochondrial metabolism, in which pathogenic variants are a well-known cause of mitochondrial disease (RRM2B, c19orf12, TA2). Furthermore, we identified 2 different genes (SLC19A3 (2x), SLC16A2) for which a role in mitochondrial OXPHOS is still unclear. Although a mitochondrial function cannot be excluded, it has been suggested that the OXPHOS deficiencies in the corresponding patients, might be secondary (Niyazov et al., 2016; de Beaurepaire et al., 2018). Furthermore, 5 different genes with no reported function in mitochondrial metabolism (IER31P1, IARS, CHRNE, BICD2, HPS1) have been identified. These genes require further functional testing to reveal a possible “novel” role in mitochondrial functioning or to demonstrate a secondary respiratory deficiency. In the patient with the multi-genic cause (BICD2 and HPS1), an additional, third, gene defect might have been missed. Two additional genes that were lacking in the MitoCarta database were not related to the mitochondrial symptoms of the patients, as these genes encode proteins with no mitochondrial function, but were together with a mitochondrial gene defect part of a complex multi-genic disease phenotype, in which more than a single gene defect is involved (ACY1, ANTXR2).

Genes identified in group 1 (MD-patients) were clustered according to their function (Table 5). Most genetic defects were...
| Family               | Patients | Pediatric/ adult | Symptoms (key features)                                      | Biochemistry (muscle or fibroblasts) | MDC Score | Orientation | Inheritance | Mito Carta Gene (published by our group) | Pathogenic variant                      |
|----------------------|----------|------------------|-------------------------------------------------------------|--------------------------------------|------------|-------------|------------|------------------------------------------|----------------------------------------|
| Non-consanguineous   | 3 p      | Mitochondrial myopathy, hypertrophic cardiomyopathy        | Combined OXPHOS deficiency                                   | 8         | Compound heterozygous | AR           | Yes         | AARS2 (Kamps et al., 2018) | c.1774G>A, p.(Gly85Arg); c.938G>A, p.(Arg958*) |
| Consanguineous       | 2 (partial overlap) p | Multi-system, Leigh-like, encephalopathy, 3-methylglutaconic aciduria, aminoacylase 1 deficiency, skin lesions | Normal                                                   | 8         | Homozygous, homozygous, homozygous | AR, AR, AR Yes, no, no | SERAC1, ACY1, ANTXR2 (Theunissen et al., 2017b) | c.1347_1349dup, p.(Ser450dup); c.811G>A, p.(Ala271Thr); c.1142A>G, p.(Tyr381Cys) |
| Consanguineous       | 2 p      | Encephalomyopathy, cerebellar atrophy, muscle weakness     | Combined OXPHOS deficiency                                   | 6         | Homozygous            | AR           | Yes         | SPG7                                    | c.861dup, p.(Asn288*) |
| Consanguineous       | 2 p      | Leigh-like, mitochondrial encephalopathy                   | Combined OXPHOS deficiency                                   | 8         | Compound heterozygous | AR           | Yes         | MTFMT                                   | c.1603G>A, p.(Gly54Ser); c.626C>T, p.(Arg181SerfsX3) |
| Consanguineous       | 2 p      | Leigh syndrome, cardiomyopathy                             | Combined OXPHOS deficiency                                   | 5         | Homozygous            | AR           | Yes         | QRL1 (Kamps et al., 2018) | c.850-3G>A, aberrant splicing of exon 8 |
| Consanguineous       | 1 p      | Optic atrophy, cerebellar atrophy, hypotonia, increased blood lactate | OIV deficiency                                             | 8         | Homozygous            | AR           | Yes         | SLC25A46 (Nguyen et al., 2017) | c.283+3G>T, p.(Ser237ThrfsX4) |
| Consanguineous       | 1 p      | Hearing problems, muscle hypotonia, mitochondrial encephalopathy, motor developmental delay, respiratory problems, mtDNA depletion | Gl and OIV deficiency                                       | 8         | Compound heterozygous                   | No          |                     | FRM2B                                   | c.142C>T, p.(Gln48*); c.431C>T, p.(Thr144fsX6) |
| Non-consanguineous   | 2 p      | Axonal neuropathy, mild neurodegenerative disorder         | Combined OXPHOS deficiency                                   | 5         | Compound heterozygous | AR           | Yes         | COQ7                                    | c.197T>A, p.(Ile66Asn); c.444A>G, p.(Tyr149Cys) |
| Consanguineous       | 1 miscar.| Leigh-like, dystonia, motor developmental delay           | Combined OXPHOS deficiency                                   | 5         | Homozygous            | AR           | Yes         | NDUFA5                                   | c.477A>C, p.(Leu159Pro) |
| Consanguineous       | 1 p      | Optic neuropathy, failure to thrive, 3-methylglutaconic aciduria | Combined OXPHOS deficiency                                   | 5         | Homozygous            | AR           | Yes         | TMEM126A                                 | c.163C>T, p.(Arg55*) |
| Consanguineous       | 3 p      | Microcephaly, neurodegeneration, psychomotor retardation, cerebral visual impairment, hypotonia | Combined OXPHOS deficiency                                   | 6         | Homozygous            | AR           | Yes         | PYCR2                                   | c.796C>T, p.(Arg266*) |

(Continued)
| Family                  | Patients | Pediatric / adult | Symptoms (key features)                                           | Biochemistry (muscle or fibroblasts) | MDC Score | Orientation | Inheritance | Mito Carta Gene (published by our group) | Pathogenic variant |
|------------------------|----------|-------------------|-------------------------------------------------------------------|--------------------------------------|------------|-------------|------------|----------------------------------------|-------------------|
| Consanguineous         | 3 p      |                   | Leigh syndrome                                                    | Normal, decreased oxygen consumption | 7          | Homozygous  | AR         | No                                     | SLC19A3 (Gerards et al., 2013) | c.20C>G, p.(Ser7*) |
| Consanguineous         | 2 p      |                   | Leigh syndrome                                                    | Normal, decreased oxygen consumption | 7          | Homozygous  | AR         | No                                     | SLC19A3 (Gerards et al., 2013) | c.20C>A, p.(Ser7*) |
| Non-consanguineous     | 2 p      |                   | Psychomotor retardation, white matter degeneration, hypo-myelination, failure to thrive | CII and CIV deficiency | 6          | X-linked    | AR         | No                                     | SLC7A2             | c.427_430+19delGCAGGTGAGTGGCCCGGACGCGC, splice donor site intron 1 deleted |
| Consanguineous         | 1 p      |                   | Leigh-like, white matter degeneration, epilepsy, dystonia, visual problems, increased blood lactate | n.d.                                  | 7          | Homozygous  | AR         | No                                     | IER3IP1            | c.128G>T, p.(Gly43Val) |
| Non-consanguineous     | 1 a      |                   | CPEO, deafness, multiple mtDNA deletions                         | N.D.                                 | 5          | Compound heterozygous | AR         | Yes                                    | POLG1              | c.752C>T, p.(Thr251Ile); c.1760C>T, p.(Pro587Leu) |
| Non-consanguineous     | 1 p      |                   | Epilepsy, multiple mtDNA deletions                               | N.D.                                 | 5          | Homozygous  | AR         | Yes                                    | POLG1              | c.1399G>A, p.(Ala467Thr) |
| Non-consanguineous     | 1 p      |                   | Leigh syndrome                                                    | CII and CIII deficiency               | 6          | Homozygous  | AR         | Yes                                    | NDUSF7             | c.364G>A, p.(Val122Met) |
| Non-consanguineous     | 1 p      |                   | Cardiomyopathy, cerebellar atrophy                               | Combined OXPHOS deficiency           | 6          | Compound heterozygous | AR         | Yes                                    | MTO1               | c.253G>A, p.(Gly85Arg); c.983G>A, p.(Arg327Gln) |
| Non-consanguineous     | 1 a      |                   | Myopathy, ptosis, spinocerebellar ataxia, ragged red fibers       | Normal                               | 6          | Compound heterozygous | AR         | Yes                                    | SPG7               | c.1529C>T, p.(Ala510Val); c.2090A>C, p.(Gln697Pro) |
| Non-consanguineous     | 1 p      |                   | Leigh syndrome                                                    | CII deficiency                       | 5          | Homozygous  | AR         | Yes                                    | NDUFA12            | c.83dup, p.(Arg29Glnfs*4) |
| Non-consanguineous     | 1 p      |                   | SNHL, psychomotor retardation, spasticity, epilepsy               | Decreased ATP production             | 5          | Compound heterozygous | AR         | Yes                                    | KARS               | c.1732_1744delGCCATTGATGCAG, p.(Gly578Serfs*20); c.683C>T, p.(Pro228Leu) |
| Non-consanguineous     | 1 p      |                   | Encephalopathy, white matter degeneration                         | CII deficiency                       | 6          | Homozygous  | AR         | Yes                                    | NDUFV2             | c.547G>A, p.(Ala183Thr) |
| Non-consanguineous     | 1 p      |                   | Leigh-like, microcephaly, mental retardation, CPEO, dystonia      | Combined OXPHOS deficiency           | 6          | Compound heterozygous | AR         | Yes                                    | MTFMT              | c.626C>T, p.(Ser209Leu); c.994C>T, p.(Arg332*) |
| Non-consanguineous     | 1 p      |                   | Leigh-like, small cerebellum, high lactate                        | n.d                                  | 6          | Compound heterozygous | AR         | Yes                                    | FBXL4 (van Rij et al., 2016) | c.292C>T, p.(Arg98*); c.1303C>T, p.(Arg435*) |

(Continued)
| Family            | Patients | Pediatric/ adult | Symptoms (key features) | Biochemistry (muscle or fibroblasts) | MDC Score | Orientation | Inheritance | Mito Carta | Gene (published by our group) | Pathogenic variant |
|-------------------|----------|------------------|-------------------------|--------------------------------------|-----------|-------------|-------------|-----------|-------------------------------|-------------------|
| Non-consanguineous | 1 p      |                  | Myopathy, muscle weakness, visual problems, mental retardation, peripheral neuropathy, mtDNA depletion | n.d. | 6 | Homozygous | AR | no | C19orf12 | c.187G＞C, p.(Ala63Pro) |
| Non-consanguineous | 1 p      |                  | Exercise intolerance, muscle weakness | CI deficiency | 5 | Homozygous | AR | yes | TMEM126B | c.635G＞T, p.(Gly212Val) |
| Non-consanguineous | 1 p      |                  | Leigh syndrome, liver failure | CI and CIV deficiency | 6 | Homozygous | AR | yes | TAMU | c.1073_1081dup, p.(Gln358_Val360dup); deletion exon2-11 |
| Non-consanguineous | 1 p      |                  | Mental retardation, ataxia, exercise intolerance, 3-methylglutaconic aciduria | CV deficiency | 7 | Homozygous | AR | yes | ATPAF2 | c.281A＞G, p.(Trp94Ser) |
| Non-consanguineous | 1 a      |                  | Leigh-like, optic atrophy, exercise intolerance | n.d. | 7 | Homozygous | AR | yes | AMPAR | c.154T＞C, p.(Ser52Phe) |
| Non-consanguineous | 1 p      |                  | Optic atrophy, muscle weakness, polyneuropathy | combined OXPHOS deficiency | 5 | Homozygous | AR | yes | C12orf65 | c.394G＞T, p.(Arg132*) |
| Non-consanguineous | 1 p      |                  | Muscle weakness, cardiomyopathy, high lactate, 3-methylglutaconic aciduria | n.d. | 7 | X-linked | AR | no | TAZ | c.646G＞A, p.(Gly216Arg) |
| Non-consanguineous | 1 p      |                  | Cerebellar atrophy, ataxia, mental retardation, white matter abnormalities, axonal peripheral sensorimotor neuropathy | n.d. | 5 | de novo | AD | yes | MFN2 | c.1058C＞T, p.(Ala353Val) |
| Non-consanguineous | 1 a      |                  | Cardiac arhythmia, CPEO, polynuropathy, multiple mtDNA deletions | Cll and CIV deficiency | 5 | unknown# | AD | yes | wrinkle (c10orf12) | c.1087T＞C, p.(Trp363Arg) |
| Non-consanguineous | 1 p      |                  | Arthrogryposis, foot deformities, muscle fat deposits, oculocutaneous albinism | CV deficiency | 6 | de novo, compound heterozygous | AD, AR | no, no | BCD2, HPS1 | c.539A＞C, p.(Asp180Ala); c.517C＞T, p.(Arg173*); c.1189delC, p.(Gln397Serfs*2) |
| Non-consanguineous | 1 p      |                  | Lung fibrosis, growth retardation, muscle weakness, failure to thrive, hepatopathy | Cll deficiency | 7 | Compound heterozygous | AR | no | IARS | c.3377dup, p.(Asn1126Lysfs*9); c.1305G＞C, p.(Thr435Cys) |
| Non-consanguineous | 1 p      |                  | PEO, mitochondrial myopathy, COX negative fibers | OXPHOS deficiency | 5 | Compound heterozygous | AR | no | CHRINE | c.1327delG, p.(Glu443Lysfs*64); c.1429delG, p.(Ala477Profs*30) |

Overview of the gene defects identified by WES in 94 patients, of which 39 had a probable mitochondrial disease. Patients were grouped according to the applied WES-data filtering strategy. AR = autosomal recessive, AD = autosomal dominant, D = dominant; ‘green’ marked genes, genes with a reported mitochondrial function or localization in literature (except for ACY1, ANTXR2, which were part of a multi-genic disease); ‘purple’ marked genes, genes previously related to possibly secondary OXPHOS deficiencies; ‘red’ marked genes, no mitochondrial localization or function reported; ‘∧’, subclinical symptoms in parent revealed after follow-up investigations; ‘#’, no parental DNA available.
| Family                  | Patients | Symptoms (key features)                      | Biochemistry (muscle or fibroblasts) | MDC Score | Orientation | Inheritance | Mito Carta | Gene (published by our group) | Pathogenic variant |
|-------------------------|----------|---------------------------------------------|--------------------------------------|-----------|-------------|-------------|------------|-----------------------------|-------------------|
| Consanguineous          | 2        | SNHL, intellectual disability, cerebellar ataxia, peripheral neuropathy, mtDNA copy number increase | Normal                               | 4         | Homozygous  | AR          | Yes        | CLPP Theunissen et al., 2016 | c.21delA, p.(Ala10Profs*117) |
| Consanguineous          | 1        | Exercise intolerance, myopathy, ataxia, diabetes mellitus | Cl and CV deficiency                 | 4         | Homozygous  | AR          | Yes        | SL225A32 (Hellebrekers et al., 2017) | c.-264_31delinsCTGACAAATGCTCA, deletes start codon |
| Consanguineous          | 1        | Limb girdle muscular dystrophy               | n.d.                                 | 2         | Homozygous  | AR          | No         | CAPNS                                      | c.1906C>T, p.(Gly636*) |
| Consanguineous          | 1        | Spastic diplegia, psychomotor retardation    | Normal                               | 2         | Homozygous  | AR          | No         | AMM1                                       | c.952C>T, p.(Arg318*) |
| Consanguineous          | 1        | Encephalopathy, psychomotor retardation, epilepsy, spastic tetraplegia | Cl deficiency                        | 3         | Homozygous  | AR          | No         | ADD3                                       | c.11003>A, p.(Gly367Asp) |
| Consanguineous          | 1        | Muscle weakness, SNHL, hypotonia, peripheral neuropathy | Normal                               | 4         | Homozygous  | AR          | No         | LMOD3                                      | c.112delG, p.(Glu38Lysfs*15) |
| Non-consanguineous      | 2        | Phactomyolysis                               | Normal                               | 3         | Homozygous  | AR          | No         | LPIN1                                      | c.1162C>T, p.(Arg388*) |
| Consanguineous          | 2        | Encephalopathy, liver failure, psychomotor retardation | Normal                               | 4         | Homozygous  | AR          | No         | NBAS                                       | c.1556T>A, p.(Val519Gln) |
| Non-consanguineous      | 2        | Congenital myopathy, spasticity              | Normal                               | 4         | Compound heterozygous | AR          | No         | SCH4A                                      | c.2979C>A, p.(Gly993*); c.4949C>T, p.(Pro1650Leu) |
| Consanguineous          | 1        | NBIA, microcephaly, epilepsy, psychomotor retardation, cerebellar atrophy | n.d.                                 | 3         | Compound heterozygous | AR          | No         | RELN                                       | c.2280G>A, p.(Gln1140Asp); c.6866C>T, p.(Thr2289Ile) |
| Non-consanguineous      | 1        | Psychomotor retardation, IBD deficiency, elevated C4-acylcarnitine, PEO | n.d.                                 | 4         | Homozygous, dominant inherited\ | AR, AD      | Yes, yes | ACAD8, DNA2                                | c.289G>A, p.(Gly97Arg); c.2036, 2037insAA, p.(His679Glnfs*10) |
| Non-consanguineous      | 1        | Cerebellar atrophy, ataxia, dystonia         | Normal                               | 2         | Compound heterozygous | AR          | No         | CWF19L1                                    | c.37G>C, p.(Asp13His); c.946A>T, p.(Lys316*) |
| Non-consanguineous      | 1        | Myopathy, muscle weakness, hypotonia         | n.d.                                 | 3         | de novo     | AD          | No         | ACTA1                                      | c.169C>A, p.(Gly63Asp) |
| Non-consanguineous      | 1        | Epilepsy, hypomyelination, hypotonia, respiratory problems | Normal                               | 4         | de novo     | AD          | No         | PURA                                       | c.802G>T, p.(Gly268*) |
| Non-consanguineous      | 1        | Myopathy, epilepsy                           | Normal                               | 3         | de novo     | AD          | No         | DYNC1H1                                    | c.3364T>C, p.(Ser1122Pro) |
| Non-consanguineous      | 1        | Cerebellar ataxia, psychomotor retardation, extrapyramidal syndrome, axonal polyneuropathy | Normal                               | 5         | de novo     | AD          | No         | CTNB1                                      | c.1511G>A, p.(Trp504*) |
| Non-consanguineous      | 1        | Cerebellar ataxia, epilepsy, NBSA, retinitis pigmentosa, myopathy | Normal                               | 5         | X-linked de novo | D          | No         | WDR45                                      | c.4000C>T, p.(Arg134*) |
| Non-consanguineous      | 1        | Ataxia, spasticity, psychomotor retardation, cerebellar atrophy, encephalopathy | Normal                               | 4         | X-linked de novo (mosaic) | D          | No         | CASK                                      | c.1061T>G, p.(Leu354Arg) |

Overview of the gene defects identified by WES in 94 patients, of which 18 had a possible mitochondrial disease cause. Patients were grouped according to the applied WES-data filtering strategy. AR, autosomal recessive; AD, autosomal dominant; D, dominant; ‘green’ marked genes, genes with a reported mitochondrial function or localization in literature; ‘red’ marked genes, no mitochondrial localization or function reported.
### TABLE 5 | Mitochondrial gene functions affected in mitochondrial patients (group 1).

| Gene                  | Function                                                                 | References                                      |
|-----------------------|---------------------------------------------------------------------------|-------------------------------------------------|
| **Mitochondrial substrate metabolism** |                                                                         |                                                 |
| AMACR                | Alpha-methylacyl-CoA racemase                                             | Mubiru et al., 2004                             |
| **Mitochondrial protein metabolism; translation and degradation** |                                                                         |                                                 |
| AARS2                | Alanine-tRNA synthetase                                                   | Götz et al., 2011                               |
| MTFMT (2x)           | Mitochondrial methionyl-tRNA formyltransferase                            | Takeuchi et al., 1998                          |
| MTO1                 | tRNA modification and protein synthesis                                   | Ghezzi et al., 2012                             |
| KARS                 | lysyl-tRNA synthetase                                                     | Targoff et al., 1993                            |
| QRSL1                | glutamyl-tRNA synthetase                                                  | Nagaao et al., 2009                             |
| TRMU                 | Mitochondrial tRNA modification                                           | Yan and Guan, 2004                              |
| C12ORF65             | Release proteins from ribosomes                                           | Antonicka et al., 2010                          |
| SPG7 (2x)            | Part of the m-AAA metalloproteinase complex                               | Warnecke et al., 2007                           |
| **Mitochondrial phospholipid metabolism** |                                                                         |                                                 |
| SERAC1*              | Phosphatidylglycerol remodeling                                           | Wortmann et al., 2012                           |
| TAZ                  | Cardiolipin remodeling                                                    | Acehan et al., 2011                             |
| **ETC subunits, assembly factors, cofactors** |                                                                         |                                                 |
| NDUFA12              | Complex I subunit                                                         | Tripels et al., 2000                            |
| NDUFS7               | Complex I subunit                                                         | Visch et al., 2004                              |
| NDUFP2               | Complex I subunit                                                         | de Coo et al., 1997                            |
| COQ7                 | Coenzyme Q biosynthesis                                                   | Freyer et al., 2015                             |
| TMEM126B             | Complex I assembly factor                                                 | Andrews et al., 2013                            |
| ATPAF2               | Complex V assembly factor                                                 | Wang et al., 2001                               |
| NDUFAF5              | Complex I assembly factor                                                 | Sugiana et al., 2008                            |
| NDUFAF4              | Complex I assembly factor                                                 | Karp et al., 2004                               |
| TMEM126A             | Complex I assembly factor                                                 | Wassels et al., 2013                            |
| **mtDNA maintenance, replication and nucleotide metabolism** |                                                                         |                                                 |
| RRm2B                | Ribonucleotide reductase                                                  | Bourdon et al., 2007                            |
| FBXL4 (2x)           | Phosphorylation-dependent ubiquitination                                  | Bonnen et al., 2013                             |
| Pycr2                | Pyrroline-5-carboxylate reductase                                         | Kuo et al., 2016                                |
| Twinkle (C10ORF2)    | mtDNA helicase                                                            | Speibrink et al., 2001                          |
| POLG1 (2x)           | mtDNA polymerase                                                          | Lestienne, 1987                                 |
| **Mitochondrial fusion and fission** |                                                                         |                                                 |
| SLC25A46             | Interacts with mitochondrial fusion machinery                              | Janer et al., 2016                              |
| MFN2                 | Mitochondrial fusion                                                      | Santel and Fuller, 2001                         |
| **Mitochondrial localization** |                                                                         |                                                 |
| C19orf12             | Function unclear                                                          | Landoure et al., 2013                           |
| **No mitochondrial localization or function reported** |                                                                         |                                                 |
| SLC19A3 (2x)         | Transmembrane thiamine transporter                                        | Vernau et al., 2015                             |
| IER3IP1              | Function unclear                                                          | Yiu et al., 2004                                |
| SLC16A2              | Transporter of thyroid hormones                                          | Wurtiaka-Cabello et al., 2001                   |
| CHRNA                 | Acetylcholine receptor subunit                                            | Witzemann et al., 1996                          |
| IARS                  | Isoeucyl-tRNA synthetase                                                  | Kopatch et al., 2016                            |
| BICD2, HPS1 (multi-genic) | dynein-mediated transport, forms lysosomal complex                      | Martina et al., 2003; Neveling et al., 2013b    |

Disease causing nuclear genes, identified in patients with a mitochondrial disorder (group 1), clustered according to their function in mitochondrial metabolism. "ACY1 and ANTXR2 were excluded.

Detected in genes related to mitochondrial protein metabolism (protein translation and degradation) and OXPHOS function (ETC subunits, assembly factors, and cofactors). The first group mainly consisted of mitochondrial tRNA synthetases, transferases, and modification enzymes, where the resulting defects in mitochondrial protein synthesis (AARS2, MTFMT, MTO1, QRSL1, TRMU, C12ORF65) especially manifested with combined OXPHOS deficiencies (not measured for KARS). Among the group of OXPHOS-associated genes, defects in complex I subunits and assembly factors (NDUFA12, NDUFS7, NDUFV2, TMEM126A, TMEM126B, NDUFAF4, NDUFAF5) were most prevalent, all resulting in a significant
complex I deficiency at the biochemical level. NDUFS7 resulted in an additional complex III deficiency. Defects in NDUFS7, NDUFA12 and NDUFAF5 caused Leigh-syndrome or a Leigh-like-phenotype. The third largest group represented genes involved in mtDNA metabolism, required for mtDNA maintenance, replication and nucleotide metabolism. The RRM2B defect resulted in mtDNA depletion, whereas pathogenic variants in the RMM2B interacting protein PYCR2 did not reveal any mtDNA abnormalities. Furthermore, pathogenic variants in POLG1, *twinkle* (*c10orf2*) and DNA2 displayed multiple mtDNA deletions in muscle. Interestingly, we found the mitochondrial localized, but functionally uncharacterized, *c19orf12* gene to be associated with mtDNA depletion (mtDNA copy-number, normalized to nDNA, was 10–30% of healthy control samples). Among the mitochondrial patients (group 1), we identified pathogenic variants in 5 genes without a reported mitochondrial function or localization, but reported to result in a comparable phenotype as in our patient. 4 genes, functional in different cellular processes, including the transporter proteins SLC19A3 (thiamine) and SLC16A2 (thyroid hormones), tRNA-synthetase IARS, and acetylcholine receptor subunit CHRNE, were likely to underlie the OXPHOS deficiencies measured in these patients, although some of these genes have been related to possible secondary OXPHOS deficiency (SLC19A3 and SLC16A2). In an additional patient, the functionally uncharacterized *IER3IP1* gene was likely to explain a seemingly mitochondrial disease phenotype, yet no OXPHOS measurement was performed in this patient. As indicated in Table 6, in patient’s from group 2, 4 mitochondrial gene defects were identified, *SLC25A32, CLPP, ACAD8*, and *DNA2* (multi-genic disease cause).

**DISCUSSION**

We used a next-generation sequencing strategy in a cohort of 86 patients with a likely mitochondrial disease (group 1), as these cases met the criteria for MD, and 31 patients, who did not meet the criteria for MD, but where a mitochondrial defect could possibly cause the disease symptoms. In 68% of the patients we identified a disease causing genetic defect, of which 20% was solved by mtDNA sequencing and 49% by subsequent WES analysis. Compared to conventional Sanger sequencing methods, which only solved 11% (Neveling et al., 2013a), primarily due to limitations on the number of genes being sequenced, this is a major step forward. Selective sequencing of targeted gene panels in two studies, including 1,598 and 1,034 mitochondrial disease genes, resulted in respectively 22% (102 suspected mitochondrial patients) (Lieber et al., 2013) and 24% (42 mitochondrial patients) (Calvo et al., 2012) diagnostic yield. This is less than in our study, which might in part be explained by an incompleteness of the panels (more details below).

In a previous next-generation sequencing-based study, which included 113 pediatric patients with suspected mitochondrial disease, screening both the mtDNA and the exome, resulted in a diagnostic yield of 59% (Pronicka et al., 2016). Another study performing WES in 109 pediatric and young adult patients with suspected mitochondrial disease, established a genetic diagnosis in 39% of the patient cohort (after exclusion of mtDNA mutations) (Wortmann et al., 2015). Our pediatric patient group consisted of 90 patients, for which we could establish a genetic diagnosis in 66% (8 mtDNA and 51 nuclear gene mutations). Our higher yield could be caused by a complete analysis of both the mtDNA and the exome, with a strong selection for genetic cases, and follow-up investigations in case of an unknown gene. The relatively high number of patients from consanguineous parents could in part explain the high diagnostic yield, as a genetic cause is highly likely. As consanguineous parents have an increased risk of having children that suffer from multiple genetic diseases, WES data should always be completely analyzed and, preferably, parents should be offered preconception genetic testing. Different from previous studies on mitochondrial cohorts, we have included a more heterogeneous patient group (group 2), in which a relatively small portion is likely to be caused by a mitochondrial gene defect. In conclusion, our data shows that mtDNA sequencing followed by a complete whole exome analysis is the preferred strategy to identify the genetic basis in heterogeneous neuromuscular patients with a likely or possible mitochondrial disease cause.

As the mtDNA genome is relatively small, NGS allows cost effective and efficient testing of many patient samples within a single run, with sufficient sensitivity on blood DNA. Yet, as mtDNA mutations may disappear from blood during life, in cases where an mtDNA disorder is likely, DNA from muscle (or

| Table 6 | Mitochondrial gene functions affected in patients from group 2. |
|---------|---------------------------------------------------------------|
| Gene    | Function                                      | References   |
|---------|---------------------------------------------------------------|
| Mitochondrial substrate import |     |
| SLC25A32 | Mitochondrial folate transporter | Haitina et al., 2006 |
| Mitochondrial protein metabolism; translation and degradation |     |
| CLPP | Mitochondrial proteolytic complex | Jenkinson et al., 2013 |
| Mitochondrial substrate metabolism |     |
| ACAD8 | Isobutyryl-CoA dehydrogenase | Nguyen et al., 2002 |
| mtDNA maintenance, replication and nucleotide metabolism |     |
| DNA2 | mtDNA replication, mtDNA base-excision repair | Ronchi et al., 2013 |

Disease causing nuclear genes were classified according to their function in mitochondrial metabolism.
urine for the m.3243A>G) should be tested to prevent missing a diagnosis. Also for prognosis mutation levels in muscle are preferable. Where traditionally several complementary methods had to be used for detection and quantification of mtDNA mutations (e.g., RFLP, ARMS-qPCR, sanger sequencing, Mito-CHIP, Southern blot analysis), NGS can be used as a single method for identifying point mutations and indels, large deletion breakpoints and quantifying heteroplasmy levels of point mutations and small indels with high sensitivity and specificity (Tang and Huang, 2010). Only to accurately measure heteroplasmy levels of large mtDNA deletions and mtDNA depletions, additional qPCR based quantification is required. NGS of the mtDNA solved 20% of our patient cohort (all mitochondrial patients), mainly, but not only, consisting of patients with early disease onset (77% pediatric patients). In the pure pediatric subgroup with mitochondrial disease (group 1), mtDNA mutations accounted for 19% (8/43) of the genetic defects, which is in line with earlier reports that estimated involvement of a mtDNA defect in < 20% of the pediatric mitochondrial patients (Schaefer et al., 2008). In our adult subgroup with mitochondrial disease (group 1), mtDNA defects accounted for 75% (12/16), which is comparable to the previously estimated 70–75% (Schaefer et al., 2008; Gorman et al., 2015). As shown in Table 1, most mtDNA patients carried LHON and MIDD/MELAS causing mutations, which was also likely based on the clinical presentation. Both MIDD and MELAS symptoms were caused by the m.3243A>G mutation, in line with what has been reported before (de Wit et al., 2012). Yet, as indicated by the heteroplasmy levels (24–41% in MELAS and 7–40% in MIDD), variation in symptom manifestations among these patients could not solely be explained from the differences in heteroplasmy level in blood, complicating prognostic predictions. Using WES, we identified a disease-causing variant in an additional 49% of the patient cohort, where we applied a variant selection strategy fitting the presumed genetic mode of disease inheritance. In a group with likely inherited disease (consanguineous and/or multiple patients), filtering for recessive pathogenic variants solved 68% of the patients. A group of single patients from non-consanguineous parents was also screened for heterozygous variants in known (OMIM) AD disease genes, which resulted in an overall genetic diagnosis in 56% of the patients, of which 15% of the identified variants were de novo. To reduce the huge amount of potential dominant pathogenic variants, we have restricted our heterozygous variant selection to OMIM-reported dominant disease genes, and therefore might have missed some de novo cases. This could be overcome by WES trio-analysis. Additionally, the higher likelihood of an inherited genetic cause, the availability of homozgyosity mapping data and of exome data of affected siblings might have contributed to a higher diagnostic yield in the first group. Our strategy did not result in large differences in diagnostic yield between patients from group 1 and patients from group 2. In an additional 7% of the WES cohort we found a genetic variant (variant of unknown significance) with a clear lead to the patient’s phenotype (data not shown). For these cases, further laboratory testing or patient screening should reveal which genes can be classified as disease causing.

In 82% (32/39) of the likely mitochondrial patients (group 1), symptoms were caused by a gene with a reported mitochondrial function or localization. As expected, this was significantly lower in patient group 2 [17% (3/18)]. Our results showed that in patients with mitochondrial disease, most genetic defects were associated with mitochondrial protein metabolism and OXPHOS function, where a majority of the OXPHOS defects were found in complex I genes. In line with previously reported complex I deficient patients, we found that defects in NDUF7, NDUF12, and NDUFAF5 caused Leigh-syndrome or a Leigh-like-phenotype (Rahman and Thorburn, 1993). Although NDUFV2 defects have also been reported in association with Leigh syndrome (Cameron et al., 2015), our patient exclusively expressed symptoms of white matter degeneration (encephalopathy). Also, we identified TMEM126A as an underlying cause of isolated complex I deficiency, therewith supporting co-migration studies that characterized TMEM126A as an early assembly factor of complex I (Wessels et al., 2013). Patients with mtDNA abnormalities carried pathogenic variants in RRM2B, twistle (c10orf2) and DNA2, which were all functionally related to mtDNA maintenance, and presented as the third largest group. Interestingly, we also found pathogenic variants in the c19orf2 gene in a patient with an mtDNA depletion. The link between this gene and the mtDNA depletion remains to be established. In 6 mitochondrial patients (group 1), we identified a causal gene for which no direct mitochondrial function or localization has been reported. This included 6 genes involved in different cellular processes (SLC19A3, SLC16A2, IARS, CHRNE, BICD2, and HPS1). In two families with classical Leigh-syndrome we identified a defect in the thiamine transporter SLC19A3, where despite a decrease in mitochondrial oxygen consumption, complex activities seemed normal in muscle, and skin tissue (Gerards et al., 2013). Thiamine-diphosphate, the active form of thiamine, has been reported as an essential cofactor for several mitochondrial enzymes, including the pyruvate dehydrogenase complex (Ortigoya-Escobar et al., 2014). A second transmembrane transporter defect was found in the thyroid hormone (T3/T4) transporter SLC16A2, in a patient with Allan-Herndon Dudley syndrome, which resulted in a CII and CIV deficiency, where triiodothyronine (T3) has been suggested as an important regulator of mitochondrial activity (Wrutniak-Cabello et al., 2001). However, a role for SLC19A3, but also SLC16A2 as mitochondrial genes is under debate, as defects in these genes have been suggested to cause possible secondary respiratory chain deficiency (Niyazov et al., 2016; de Beaurepaire et al., 2018). In such case, patients might possibly get a false positive classification as mitochondrial (group 1) based on biochemical criteria. Still, concerning SLC19A3, either being primary or secondary, this gene should be tested in patients with Leigh syndrome, and should be in all MD panels, because it is a treatable condition, even though, having a secondary effect, it may not have been included in the MitoCarta database. Furthermore, we found a pathogenic variant in the cytoplasmic located aminoacyl-tRNA synthetase IARS to cause complex I deficiency in skin and muscle tissue of a patient with a multi-system disorder, and
a defect in the acetylcholine receptor subunit epsilon CHRNE associated with CII deficiency, COX negative muscle fibers and abnormally shaped muscle mitochondria in CPEO with myopathy. Also we measured a complex IV deficiency in a patient with a multi-genic cause, where either the identified BICD2 or HPS1 defect causes the deficiency, or a possible third defect was missed. In another patient, who was classified as mitochondrial based on the classical mitochondrial disease symptoms involved, the functionally uncharacterized IER3IP1 gene was identified. Although, no OXPHOS measurements were performed in this patient, knockdown of IER3IP1 was reported to affect mitochondrial function by decreasing the mitochondrial membrane potential and increasing cytochrome C release (Sun and Ren, 2017). The genetic defects in these patients need further functional characterization to prove or exclude a mitochondrial function, the involvement of secondary respiratory chain deficiencies, or perhaps the presence of an additional gene defect that has been missed by WES.

31% of the disease-causing genes identified in our mitochondrial patients (group 1) were not included in the MitoCarta database at the moment of identification, and could therefore be missed with panel-based sequencing approaches that rely on the MitoCarta database. In addition to the 5 genes described above, this also involved some genes with a reported function in mitochondrial metabolism, indicating that it can be difficult to keep databases or panels up to date with recent discoveries. Also, the contribution of non-mitochondrial disease genes to complex multi-genic mitochondrial disease might be missed when applying targeted sequencing methods, as illustrated by two of the mitochondrial patient cases, in which defects in more than a single gene were involved. Our data shows that WES is the most suitable approach to characterize the causative gene defect in both groups, patients with most likely a mitochondrial disease, as according to the consensus criteria for MD (group 1), and patients in who a mitochondrial gene defect could possibly be involved (group 2). With respect to group 1, we show mitochondrial gene panels are still not complete, and that a strict preselection based on the consensus criteria for MD could result in the missing of mitochondrial gene defects (SLC25A32, CLPP, ACAD8, DNA2). Also in group 2 WES will be the preferred method, because of the genetic heterogeneity, targeted approaches can easily result in inefficient and sequential use of different gene panels, and still miss the complete picture in multi-genic disease. WES is a comprehensive and unbiased approach to establish a genetic diagnosis in heterogeneous mitochondrial disease, able to resolve complex multi-genic disease manifestations. Obviously, a disadvantage of WES in diagnostics settings will be the identification of novel, non-reported gene defects that often require extensive experimental setups to validate a possible role in mitochondrial disease. Embedding within or contact with a specialized research group should therefore be preferable. Identification of the genetic defect is not only important for diagnostics, but also for therapeutic interventions. Patients with SLC25A32, SLC19A3, and TMEM126B defects showed improvement upon treatment with respectively riboflavin, biotin/thiamine and high fat-diet (Gerards et al., 2013; Hellebrekers et al., 2017; Theunissen et al., 2017a). Yet, despite the significant improvements in the genetic diagnosis of mitochondrial disorders, the development of novel therapies is lagging behind.

AUTHOR CONTRIBUTIONS

TT, MN, RK, JV, DH, and MG were responsible for analysis and interpretation of patient WES data, and validation of the variants. RG, AH, and CC were involved in the handling of patient DNA and WES preparation. BdK, AS, and RS were responsible for the bioinformatics pipeline. KS performed biochemical measures on patient material. EM, SF, YH-H, MdV, IdC, and SS was responsible for patient contact and clinical analysis and diagnosis. IdC, DH, and HS were responsible for intellectual content and study setup.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2018.00400/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.