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ORIGINAL ARTICLE

Mitochondrial DNA mutation analysis from exome sequencing—A more holistic approach in diagnostics of suspected mitochondrial disease

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Abstract

Diagnostics for suspected mitochondrial disease (MD) can be challenging and necessitate invasive procedures like muscle biopsy. This is due to the extremely broad genetic and phenotypic spectrum, disease genes on both nuclear and mitochondrial DNA (mtDNA), and the tissue specificity of mtDNA variants. Exome sequencing (ES) has revolutionized the diagnostics for MD. However, the nuclear and mtDNA are investigated with separate tests, increasing costs and duration of diagnostics. The full potential of ES is often not exploited as the additional analysis of "off-target reads" deriving from the mtDNA can be used to analyze both genomes. We performed mtDNA analysis by ES of 2111 cases in a clinical setting. We further assessed the recall rate and precision as well as the estimation of heteroplasmy by ES data by comparison with targeted mtDNA next generation sequencing in 49 cases. ES identified known pathogenic mtDNA point mutations in 38 individuals, increasing the diagnostic yield by nearly 2%. Analysis of mtDNA variants by ES had a high recall rate (96.2 ± 5.6%) and an excellent precision (99.5 ± 2.2%) when compared to the gold standard of targeted mtDNA next generation sequencing. ES estimated heteroplasmy levels with an average difference of 6.6 ± 3.8%, sufficient for clinical decision making. Taken together, the mtDNA analysis from ES is of sufficient quality for clinical diagnostics. We therefore propose ES, investigating both nuclear and mtDNA, as first line test in individuals with suspected MD. One should be aware, that a negative result does not exclude MD and necessitates further test (in additional tissues).

KEYWORDS

bioinformatics, diagnostics, mitochondrial disorders, muscle biopsy, nuclear DNA

Abbreviations: ES, exome sequencing; LHON, Leber hereditary optic neuropathy; mtDNA, mitochondrial DNA; mtDNA-NGS, mitochondrial DNA massive parallel sequencing; MELAS, myopathy, encephalopathy, lactic acidosis and stroke-like episodes; nuMT, nuclear mtDNA segments.

1 | INTRODUCTION

Mitochondria are key organelles in energy metabolism, free radical generation and apoptosis. Genes encoding mitochondrial proteins reside in both the nuclear DNA and in the mitochondrial DNA (mtDNA). The mtDNA is a maternally inherited 16 569 bp circular genome encoding two rRNAs, 22 tRNAs, and 13 polypeptides. Variants in more than 300 nuclear genes as well as mtDNA copy number alterations (mtDNA depletion, single or multiple mtDNA deletion(s)) and numerous mtDNA point mutations are known to cause human diseases. For a summary of mitochondrial disease genes see Reference [1].

Achieving a molecular diagnosis in patients with a suspected mitochondrial disorder is often a time consuming process including invasive procedures (eg, muscle biopsy). This is because of the aspects as outlined in Table 1.

Exome sequencing (ES) is targeted sequencing of the coding regions of the genome, which are specifically enriched. However, a large proportion of the sequenced DNA fragments derive from outside the targeted regions ("off target reads"); these are, for example, mtDNA, intronic and regulatory regions.[2]

The diagnostic yield of ES for individuals suspected of having nuclear DNA related mitochondrial disorders is 39% to 53%, indicating that still many individuals undergoing ES remain without a genetic diagnosis.[3-5]

In present-day practice long-range PCR with consecutive gel electrophoresis, Sanger sequencing of selected point mutations or targeted massive parallel sequencing of the mtDNA (mtDNA-NGS) from leucocyte derived DNA are often performed as the initial genetic test, especially in adult individuals suspected of having a mitochondrial disorder.[6] These methods allow the analysis of point mutations and mtDNA deletion(s). Due to the tissue specificity of mitochondrial DNA variants, it can be necessary to investigate additional tissues (bladder epithelia cells from urine, muscle).

However, the potential of ES is underrealized as the analysis of the mtDNA by ES is possible due to the relatively high abundance of mtDNA copies with respect to the nuclear genome leading to a significant coverage by "off-target reads" without the requirement of specific mtDNA enrichment.[7]

Noteworthy, using off-target reads and not specifically targeting the mtDNA is the reason why sequenced reads do not represent nuclear mtDNA segments NUMTs. Enrichment of the mtDNA using probes would result in increased sequencing of NUMTs as these are represented frequently on the nDNA.

This method was shown to be feasible when compared to Sanger sequencing in 46 subjects.[8] Recently, a retrospective analysis of 1059 cases referred for exome sequencing revealed three additional diagnoses when mtDNA analysis was added to the diagnostic algorithm.[9]

Here, we evaluate if mtDNA analysis as an additional filter step in the clinical ES evaluation increases the overall diagnostic lead. Furthermore, we evaluate if the quality of mtDNA analysis by ES is comparable to that of targeted mtDNA-NGS with respect to recall rate, precision and heteroplasmy levels. Hence, this study aims to establish ES as a single, more holistic diagnostic first line approach evaluating both the nuclear DNA and the mtDNA in individuals suspected of a having mitochondrial disorder.

TABLE 1 Why is the diagnosis of mitochondrial disease so challenging?

1. Mitochondrial disorders can present at any age, can affect any organ and can cause any symptom ("any age, any organ, any symptom").
2. Mutations in the same gene (eg, *POLG)* can cause a variety of phenotypes (eg, Alpers-Hüttenlocher syndrome, Chronic progressive external opthalmoplegia (CPEO)) even with interfamilial variation ("one gene—many phenotypes")
3. The same phenotype (eg, Leigh syndrome) can be caused by mutations in different genes (eg, m.8993T>G (*MT-ATP6*), *SURF1, NDUFS1* and numerous more nuclear genes) ("one phenotype—many genes)
4. Many disorders mimick mitochondrial disease and vice versa ("mitochondrial mimickry")
5. The proteom of the mitochondrion is under bigenic control, disease genes are known both in the nuclear and in the mitochondrial DNA ("nuclear DNA – mitochondrial DNA").
6. MItochondrial disease can follow any mode of inheritence ("de novo," autosomal-dominant, autosomal-recessive, X-linked, maternal).
7. Mitochondrial DNA mutations show tissue specificity (eg, higher heteroplasmy of a causative mtDNA variant in muscle than in blood in mitochondrial myopathy)

2 | MATERIALS AND METHODS

2.1 | Informed consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients or their legal guardians for being included in the study. The study was approved by the local ethics committee of the Technical University Munich (#5360/12 S).

2.2 | Cohort and samples

2111 cases (761 adults, 1350 children, median age of all patients 10 years, 1171 male, 940 female), referred as

proband-only or child-parents-trios for ES in a clinical setting, were included in this study. Our laboratory has a strong focus on neurological disorders, especially mitochondrial disorders which is reflected by the indications for referral for ES ($n = 463$ suspicion of mitochondrial disorder). A detailed composition of the reasons for referral of all cases included in the present study can be found in Figure S1.

Of note, samples of—mostly adult—individuals suspected of having mtDNA based mitochondrial syndromes such as Leber hereditary optic neuropathy (LHON) or myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) are regularly referred to our laboratory for targeted mtDNA testing, but not for ES.

For 20 of 2111 samples and 29 additional samples both data from ES and targeted mtDNA-NGS were available.

2.3 | ES and analysis of the mtDNA from ES

DNA was extracted from peripheral blood leucocytes, fibroblasts or cryopreserved brain tissue using standard protocols. Exome capture was performed with Agilent SureSelect Human All Exon kits V5/V6. Sequencing was performed on HiSeq 2500/4000 machines (Illumina) with paired end reads, with an insert size of 205 ± 30 bp and an exome wide coverage of 151 ± 28×, with 77.7 ± 1.5% on target reads. The average mtDNA coverage for the examined samples was 58 ± 38× (Figure S2). Of note, coverage was not uniform across the mtDNA (Figure 1).

Alignment was performed with Burrows-Wheel Aligner (BWA) 0.7.5a, using the mem algorithm, to a custom reference, based on the hg19 reference sequence, where the mtDNA sequence has been replaced with the revised Cambridge Reference Sequence (rCRS). The choice of the rCRS is intended to facilitate the evaluation of the variants through online available diagnostic resources like Mitomap. Variant calling was carried out with GATK 3.8.

A custom annotation script has been used to annotate mtDNA variants. Gene names and positions were derived from Gencode v20. To avoid nuclear mtDNA segments (NUMT)s contamination, read pairs from NUMT regions where one of the pairs aligns to targeted regions in the nuclear genome were removed (https://www.mitomap.org/foswiki/bin/view/MITOMAP/PseudogeneList).

2.4 | Targeted mtDNA massive parallel sequencing

The entire mtDNA was amplified as a single amplicon using long-range PCR as previously published.[10] Libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) and sequenced using the MiSeq sequencing platform (Illumina) as paired-ends of 2 × 150 bp. Reads were aligned to the revised Cambridge Reference Sequence (rCRS, NC_012920.1). Variant calling was performed with the Genome Analysis Toolkit (GATK 3.8).[11]

2.5 | Data availability

Data used in this study is available at https://figshare.com/articles/mtDNA_variant_detection_by_exome_sequencing/7218266. Note, that the patients or their legal guardians did not consent the publication of next generation raw

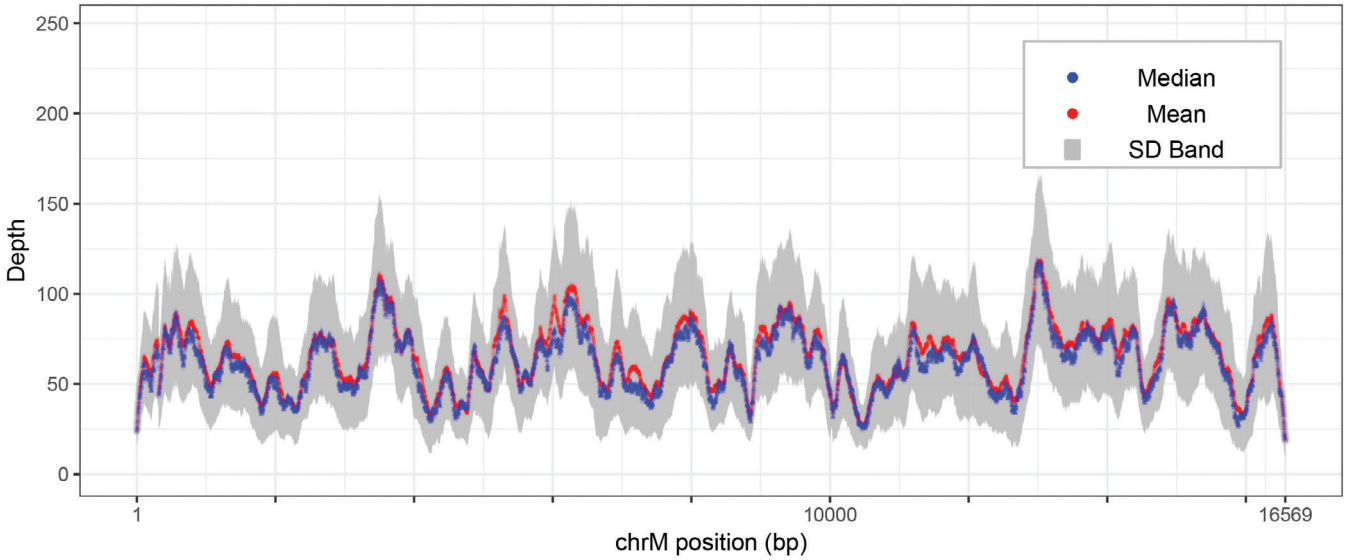

FIGURE 1 Coverage of the mtDNA as sequenced by ES across 49 samples. The figure shows the coverage of the mitochondrial DNA (mtDNA) across 49 samples. The red dotted line represents the mean coverage per position on the mtDNA whereas the blue dotted line corresponds to the median coverage. The gray band represents the SD. The figure shows nonuniformity of the coverage across the mtDNA

TABLE 2 Clinical Information, detected variants and heteroplasmy levels of patients where exome sequencing identified known pathogenic mtDNA variants

	age at diagnosis (in years)	Indication	Lactic acidosis	Myopathy	Cardiomyopathy	Epilepsy	DD	mov.dis	MRI abnorm.	Variant (Gene)	ES heteroplasmy % blood (reads)	Targeted mtDNA-NGS heteroplasmy % blood (reads)
1	17	MD	NA	no	no	yes	yes	no	NA	m.3243A>G (MT-TL1)	85% (67/79)	83% (7625/9162)
2	4	MD	no	no	no	yes	no	yes	NA	m.3243A>G (MT-TL1)	NA*	NA
3	40	Cardiac Disease	no	no	yes	no	no	no	no	m.3243A>G (MT-TL1)	27% (10/36)	32% (634/1982)
4	17	Epilepsy	no	no	no	yes	yes	no	yes	m.3243A>G (MT-TL1)	22% (13/59)	28% (1981/7083)
5	39	Nephropathy	no	no	no	no	no	no	no	m.3243A>G (MT-TL1)	18% (6/33)	NA
6	22	MD	yes	no	no	no	no	yes	yes	m.8344A>G (MT-TK)	80% (16/20)	88% (3270/3720)
7	38	MD	yes	no	no	yes	yes	yes	yes	m.8344A>G (MT-TK)	98% (41/42)	93% (5547/5960)
8	8	Nephropathy	no	no	no	no	no	no	no	m.8344A>G (MT-TK)	86% (37/43)	NA
9	3	MD	no	yes	yes	no	no	no	no	m.8344A>G (MT-TK)	98% (54/55)	NA
10	7	MD	yes	yes	yes	no	NA	yes	yes	m.8344A>G (MT-TK)	100% (21/21)	NA
11	3	Cardiac Disease	yes	no	yes	no	NA	no	NA	m.8528T>C (MT-ATP6/8)	100% (34/34)	NA
12	7	MD	yes	NA	no	NA	yes	NA	yes	m.8993T>C (MT-ATP6)	100% (34/34)	100% (9260/9282)
13	4	MD	yes	no	no	no	yes	no	yes	m.8993T>C (MT-ATP6)	99% (89/90)	100% (7241/7258)
14	6	MD	yes	yes	no	no	yes	no	yes	m.8993T>C (MT-ATP6)	93% (99/107)	96% (11 315/11814)
15	6	MD	yes	yes	no	no	yes	no	yes	m.8993T>C (MT-ATP6)	99% (84/85)	98% (12 581/12888)
16	2	MD	no	yes	no	yes	yes	no	no	m.8993T>C (MT-ATP6)	98% (40/41)	NA
17	4	MD	yes	yes	NA	yes	NA	yes	NA	m.8993T>G (MT-ATP6)	100% (50/50)	100% (11 805/11830)
18	6	MD	yes	yes	NA	yes	NA	yes	NA	m.8993T>G (MT-ATP6)	97% (58/60)	100% (12 632/12682)
19	1	MD	yes	yes	no	no	NA	no	NA	m.8993T>G (MT-ATP6)	98% (67/68)	99% (11 682/11745)
20	0.8	Muscular Hypotonia	no	yes	no	no	yes	yes	NA	m.8993T>G (MT-ATP6)	100% (48/48)	NA
21	5	MD	yes	yes	no	no	yes	yes	yes	m.8993T>G (MT-ATP6)	95% (74/78)	NA
22	7	MD	yes	no	yes	yes	yes	no	yes	m.9176T>C (MT-ATP6)	92% (89/97)	96% (4998/5180)
23	68	Ataxia	no	no	no	no	no	yes	no	m.9176T>C (MT-ATP6)	NA**	NA
24	19	MD	yes	yes	no	no	yes	no	yes	m.9185T>C (MT-ATP6)	100% (56/56)	NA
25	3	MD	no	yes	no	no	yes	yes	NA	m.9185T>C (MT-ATP6)	100% (66/66)	NA
26	2	MD	yes	no	no	no	yes	yes	yes	m.10191T>C (MT-ND3)	97% (129/133)	91% (3347/3689)
27	4	MD	NA	NA	NA	NA	NA	NA	NA	m.10191T>C (MT-ND3)	71% (22/31)	71% (4223/5922)
28	3	MD	NA	NA	NA	NA	NA	NA	NA	m.10197G>A (MT-ND3)	97% (123/128)	94% (3664/3902)

TABLE 2 (Continued)

	age at diagnosis (in years)	Indication	Lactic acidosis	Myopathy	Cardiomyopathy	Epilepsy	DD	mov.dis	MRI abnorm.	Variant (Gene)	ES heteroplasmy % blood (reads)	Targeted mtDNA-NGS heteroplasmy % blood (reads)
29	1	MD	yes	no	no	no	yes	no	yes	m.10197G>A (MT-ND3)	89% (36/40)	91% (4096/4504)
30	8	Vision loss	no	no	no	no	no	no	no	m.11778G>A (MT-ND4)	100% (53/53)	NA
31	2	MD	yes	yes	yes	no	yes	yes	yes	m.13513>G (MT-ND5)	69% (67/97)	79% (5210/6602)
32	8	MD	no	no	no	no	yes	yes	yes	m.14459G>A (MT-ND6)	85% (73/86)	85% (3967/4665)
33	2	MD	yes	no	NA	NA	yes	NA	yes	m.14459G>A (MT-ND6)	96% (72/75)	NA
34	37	Ataxia	no	no	no	no	no	yes	no	m.14484T>C (MT-ND6)	100% (17/17)	NA
35	6	MD	no	no	no	no	yes	yes	yes	m.14484T>C (MT-ND6)	100% (74/74)	NA
36	6	MD	no	no	no	no	yes	yes	yes	m.14484T>C (MT-ND6)	100% (20/20)	NA
37	4	MD	yes	yes	no	no	yes	yes	yes	m.14484T>C (MT-ND6)	97% (69/71)	92% (4044/4396)
38	17	MD	no	no	no	no	yes	yes	no	m.5940_11960del	not applicable	48% (6588/13725)

Abbreviations: DD, developmental delay; ES, exome sequencing; MD, mitochondrial disease; mov.dis, movement disorder; MRI abnorm., MRI abnormalities; NA, not available; mtDNA-NGS, mitochondrial DNA targetes next generation sequencing. *Heteroplasmy in fibroblasts 18% (3/16 reads) **Heteroplasmy in brain tissue: 100% (232/232 reads).

sequencing data as this represents identifying information. Raw data was generated at the Institute of Human Genetics, Helmholtz Zentrum München and is available upon request if in line with patient's consents.

3 | RESULTS

3.1 | MtDNA analysis by ES in a clinical setting

To determine if the analysis of the mtDNA by ES results in a significant improvement of the diagnostic yield in a clinical setting, we analyzed the ES data of 2111 individuals for known mtDNA mutations that were listed as "confirmed" pathogenic in MitoMap. Variants of uncertain significance (VUS) as identified in 15/2111 were not included in the analysis. ES yielded a total of 927 diagnoses (43.9%) of which 889/2111 (42.1%) were due to variants on the nuclear DNA and 38/2111 (1.8%) due to confirmed pathogenic mtDNA variants. Table 2 gives an overview of all mtDNA variants detected, the heteroplasmy levels as determined by ES and targeted mtDNA sequencing and the indication for referral.

Altogether, 16 different known pathogenic point mutations with clinically relevant heteroplasmy levels were found in eight mtDNA genes (5× m.3243A>G in *MT-TL1*; 4× m.8344A>G; 1× m.8363A>G in *MT-TK,* 1× m.8528T>C in *MT-ATP8/6*; 10× m.8993T>G/C; 2× m.9176T>G/C; 2× m.9185T>G/C in *MT-ATP6*; 2× m.10191T>C; 2× m.10197G>A in *MT-ND3*; 1× m.13513G>A in *MT-ND5*; 3× m.14487T>C; 2× m.14459A>G in *MT-ND6*; 1× m.11778G>A in *MT-ND4;* 1× m.14484T>C in *MT-ND6*) as well as a single 6 kb deletion. If available, heteroplasmy in several tissues of the patient and the mother was evaluated and was in line with the diagnosis of the index patient (data available upon request).

The most frequent variants affected the m.8993 position within the *MT-ATP6* gene associated with Leigh syndrome; T > C and T > G in five cases each (93%-100% heteroplasmy in blood). The second most frequently found variant was the "MELAS" mutation m.3243A>G, which was found in five cases in a heteroplasmic state (18%-85% in blood). Interestingly, the LHON variant m.14484T>C was found in homoplasmic state in a female with nonspecific neurological symptoms where a history of transient visual loss was only reported after re-evaluation once the variant was identified. In one patient (individual 38) ES analysis raised the suspicion of a mtDNA 6 kb deletion due to the detection of split-reads spanning the deletion which was confirmed with long range PCR and targeted mtDNA-NGS in blood (heteroplasmy 48%) (Figure S3, m.5940_11960del).

As expected, most cases where we detected pathogenic mtDNA variants were referred for ES with the suspicion of a mitochondrial disorder (29/38, 76%). A summary of clinical findings can be found in Table 2. However, some individuals had clinical findings which were not hinting towards mitochondrial disorders at first glance: Two patients had isolated progressive kidney dysfunction, two isolated childhood-onset cardiomyopathy, one isolated muscular hypotonia, one isolated epilepsy, two had adult onset ataxia and one vision loss.

In one deceased patient (individual 23) with ataxia and lower limb weakness who had undergone extensive genetic testing during lifetime (screening for the repeat disorders spinocerebellar ataxias, linkage analysis and ES at another institute) ES was performed using frozen brain tissue, which was the only available tissue. This analysis yielded a homoplasmic m.9176T>C variant in *MT-ATP6*, which was later also confirmed in the likewise affected brother. A pedigree of the family can be found in Figure S4.

3.2 | Comparison of mtDNA analysis by ES and targeted mtDNA sequencing

To evaluate the method of detecting mtDNA variants by ES we examined 49 samples where targeted mtDNA-NGS was performed in addition to exome sequencing. As the mtDNA control region, where the primers for the long range PCR of the entire mtDNA are located, is the most polymorphic region of the mtDNA, we decided to exclude these regions from further analyses. This does not impair the validity of our study, as no confirmed pathogenic variants are located in the control region so far.

The recall rate or sensitivity (the percentage of the variants identified by targeted mtDNA-NGS that was also detected by ES) was 96.2% ± 5.6% when assessing 946 variants across 49 samples. The precision or positive predictive value (the percentage of variants identified by ES that was also detected by targeted mtDNA-NGS) was 99.5% ± 2.2%. With an average of 19.3 variants (pathogenic and no pathogenic) detected per sample, 0.1 variants were false positives and 0.57 false negatives implying that roughly one variant would be missed every two samples (Table 3). Of note, ES did not miss a single pathogenic variant detected by mtDNA-NGS.

3.3 | Heteroplasmy evaluation by ES

The analysis of heteroplasmy is crucial for the clinical interpretation of mtDNA variants. Therefore, we evaluated if heteroplasmy of true positive variants in the 49 samples were identical when using ES compared to targeted mtDNA-NGS. We restricted our analysis to mtDNA variants with a heteroplasmy <90%, as homoplasmic variants will reliably be identified as such by both methods consequently leading to bias. We included all variants independent from their coverage. A total number of 33 variants (average of 0.67 per sample) fulfilled these criteria. The average difference in heteroplasmy was 6.6% ± 3.8%. However, when including variants with a heteroplasmy >90% the average difference is 0.80% ± 0.84%.

TABLE 3 Comparison of quality between exome sequencing and targeted mtDNA-next generation sequencing

	Average	Standard deviation
Precision	0.995	0.022
Recall rate	0.962	0.056
True positives		
Total	944	
Per sample	19.3	10.3
False positives		
Total	5	
Per sample	0.102	0.47
False negatives		
Total	28	
Per sample	0.571	0.95

In order to evaluate whether the difference in heteroplasmy is relevant in a clinical context, we compared the data of 20 probands where we identified pathogenic mtDNA variants by ES for which targeted mtDNA-NGS was performed (Table 2). Eight mutations had heteroplasmy <90% and were eligible for analysis, the average difference in heteroplasmy was 4.1% ± 3.7%.

4 | DISCUSSION

In this study, we performed analysis of confirmed pathogenic mtDNA single nucleotide variants by ES in a cohort of 2111 individuals with various (suspected) genetic disorders, which resulted in an increase of the diagnostic yield of almost 2%. We show that analysis of mitochondrial DNA variants by exome sequencing has a high recall rate (96.2% ± 5.6%) and an excellent precision (99.5% ± 2.2%) and that ES estimates heteroplasmy levels with an average difference of 6.6% ± 3.8% when compared to the gold standard of targeted mitochondrial DNA next generation sequencing. This data highlights that the method is both feasible and robust given the challenges such as NUMTs.

The phenotypic and genetic heterogeneity of mitochondrial disorders often implicates a long diagnostic journey for affected individuals, including invasive procedures like muscle biopsy. Therefore, the increasing availability of NGS

techniques is a promising tool to speed up the diagnostic process. However, still it depends on the knowledge of the referring physician, the available techniques and knowledge of the genetic lab to combine the necessary techniques to efficiently reach a diagnosis. Hence, a more holistic approach for genetic testing of both mtDNA and nuclear DNA variants in a single first line test is highly sought after.

It has recently been shown that the analysis of copy number variants from ES data can increase the diagnostic yield by 2% without additional cost, which was a first and important step to further utilize ES data.[12] Concerning mtDNA analysis, only one study on this topic was published: Bergant et al re-evaluated a cohort of 1.059 ES cases of which 39% ($n = 413$) of cases had a neurological referral indication in the broadest sense in. They diagnosed three additional mtDNA mutations (2× m.3243A>G (60% heteroplasmy), referral indication MELAS; 1× m.14598T>C (22%heteroplasmy) referral indication LHON) hereby increasing the diagnostic yield by 0.3%.[9]

First, we aimed to evaluate the feasibility of analyzing the mtDNA by ES in a diagnostic context. We showed that we were able to increase the overall diagnostic yield in a cohort including non-neurological disease by 1.8% even in a setting where targeted mtDNA analyses were used prior to ES in clinical cases with a high suspicion of a certain pathogenic mtDNA variant (eg, LHON, MELAS).

Second, we illustrate that this method is of comparable quality to the current gold standard of targeted mtDNA-NGS by comparing almost 1000 variants across 49 samples (recall rate or sensitivity 96.2% ± 5.6%, precision or positive predictive value 99.5% ± 2.2%). There is a low rate of false negative variants implying that one variant will not be called per two samples. The main reason was low heteroplasmy levels of variants, which would only in rare cases be a problem in a diagnostic context as these variants are usually not of clinical relevance. Other variants were not identified by the bioinformatic algorithms due to a nonuniform mtDNA coverage with a low coverage of the respective position. This finding highlights that in cases with the suspicion of a mitochondrial disorder targeted mtDNA sequencing should still be considered after negative ES.

The evaluation of the clinical relevance of mtDNA variants is highly based on heteroplasmy levels in different tissues of the index patient, the mother, and other affected and unaffected family members of the maternal line. An analysis of heteroplasmy levels of variants identified by ES in comparison with targeted mtDNA sequencing resulted in an average difference of 6.6% ± 3.8% across all variants. When assessing known pathogenic mutations only, the average difference was even less (4.1% ± 3.7%). This accuracy would be sufficient for the evaluation of pathogenicity in a diagnostic context and exceeds Sanger sequencing which is often used as the standard test to assess heteroplasmy. We would like to draw attention, that we found previously unreported mtDNA variants of unknown significance in 12 individuals (0.6%). Here the evaluation of the heteroplasmy in different tissues and in different family members of the maternal line is necessary to judge pathogenicity. As this article aims on routine clinical diagnostics, mtDNA variants of unknown significance were left out of the analysis.

22% of cases (Figure S1) were referred for genetic testing because a mitochondrial disorder was suspected possibly leading to a biased increase in the diagnostic yield. On the other hand, in our laboratory, individuals with a high suspicion of a mtDNA related disorder are investigated via targeted mtDNA-NGS which might balance out this issue.

In comparison, the Bergant et al study made three additional diagnoses in 1079 individuals which equals an increase in diagnostic yield of 0.3%. This difference might also derive from differences in the coverage.

However, in some cases we were surprised that we identified disease causing mtDNA variants even though the clinical heterogeneity of mitochondrial disorders is well known.[13] Especially, two cases with isolated adult onset ataxia and two cases with adult onset kidney disease would most likely not be referred for mtDNA sequencing as the expected diagnostic yield is minimal.

As these off-target sequences are a by-product of ES data, we advise to analyze the mtDNA as part of the routine analysis of all ES samples irrespective of the indication until genome sequencing with inherent deep coverage of the mtDNA will become the state of the art method for monogenic disorders, as sequencing costs decline.

However, one should be aware of the specific limitations of ES when using the method in a clinical setting (Figure 2): Single and multiple deletions as well as depletion of the mtDNA are very difficult to detect, especially when DNA from blood is investigated. Determination of heteroplasmy as well as the sensitivity of detecting variants with low heteroplasmy is inferior to targeted mtDNA-NGS which is why it cannot be replaced by ES. The coverage of the mtDNA depends on the ES sequencing depth and is not uniform across the mtDNA (Figure 1). Therefore, mtDNA regions might be covered insufficiently for variant detection and/or evaluation of heteroplasmy levels emphasizing that ES is unable to exclude mtDNA mutations.

Independent from the sequencing method, tissue specificity that can occur in mtDNA related mitochondrial disorders can be challenging. This means that a diagnoses of a mtDNA related mitochondrial disease can be missed in blood due to a low heteroplasmy level. In these cases different patient tissues (bladder epithelial cells from urine, mucosa cells, muscle tissue or fibroblasts) can be helpful.

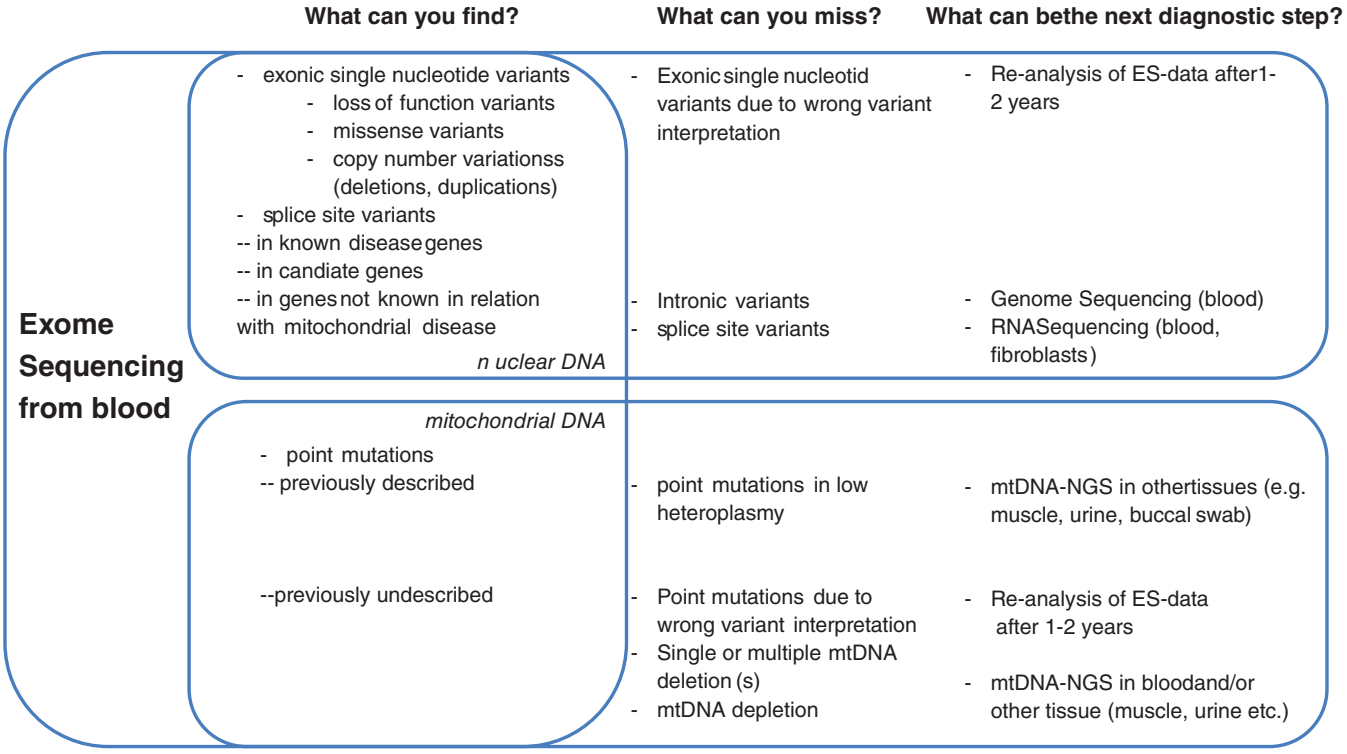

FIGURE 2 Overview of benefits and pitfalls of mitochondrial DNA diagnostics with exome sequencing mitochondrial DNA = mtDNA, exome sequencing = ES, mitochondrial DNA massive parallel sequencing = mtDNA-NGS

In summary, we show that mtDNA variants can reliably be detected by ES and that it is possible to estimate clinically meaningful heteroplasmy levels. We further show that it is feasible to perform off-target mtDNA analysis from ES data in clinical routine and that this more holistic approach significantly increases the diagnostic yield. Traditionally, the diagnostic procedure for suspected mitochondrial disorders is to first exclude mtDNA variants by direct testing followed by panel or ES testing.[14]

Taking the specific challenges for diagnosing MD as shown in Table 1, our data and recent studies claiming for an "exome first" or a combined diagnostic approach to identify mtDNA and nuclear variants in account,[1,15,16] we propose that ES from leucocyte derived DNA (blood) should be done as the initial test in all individuals with suspected mitochondrial disease. Hereby, it is important to know the limitations of this method and the necessary follow test as summarized in Figure 2. In cases with a high suspicion of a mtDNA related mitochondrial syndrome (eg, LHON) mtDNA-NGS could be considered alternatively.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

Author contributions

Study concept: M.W., B.L.-D., T.M., S.W. Bioinformatic analysis: R.B., T.S. Clinical data acquisition: U.A., J.A.M. Data analysis: M.W., R.B., B.L.-D., E.G., G.E., U.A., H.P., S.W. Drafting the manuscript: M.W., R.B., S.W. Critically reviewing the manuscript: all.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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