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Use of Whole-Genome Sequencing for Mitochondrial Disease Diagnosis

Ryan L. Davis, PhD,* Kishore R. Kumar, MBBS, FRACP, PhD,* Clare Puttick, MStat,*
Christina Liang, MBBS, FRACP, PhD, Kate E. Ahmad, MBBS, FRACP, Fabienne Edema-Hildebrand, RN,
Jin-Sung Park, PhD, Andre E. Minoche, PhD, Velimir Gayevskiy, PhD,
Amali C. Mallawaarachchi, MBBS, FRACP, PhD, John Christodoulou, MBBS, FRACP, PhD,
Deborah Schofield, PhD, Marcel E. Dinger, PhD, Mark J. Cowley, PhD,† and Carolyn M. Sue, MBBS, FRACP, PhD†

Correspondence
Prof. Sue
carolyn.sue@sydney.edu.au

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Abstract

Background and Objectives

Mitochondrial diseases (MDs) are the commonest group of heritable metabolic disorders. Phenotypic diversity can make molecular diagnosis challenging, and causative genetic variants may reside in either mitochondrial or nuclear DNA. A single comprehensive genetic diagnostic test would be highly useful and transform the field. We applied whole-genome sequencing (WGS) to evaluate the variant detection rate and diagnostic capacity of this technology with a view to simplifying and improving the MD diagnostic pathway.

Methods

Adult patients presenting to a specialist MD clinic in Sydney, Australia, were recruited to the study if they satisfied clinical MD (Nijmegen) criteria. WGS was performed on blood DNA, followed by clinical genetic analysis for known pathogenic MD-associated variants and MD mimics.

Results

Of the 242 consecutive patients recruited, 62 participants had "definite," 108 had "probable," and 72 had "possible" MD classification by the Nijmegen criteria. Disease-causing variants were identified for 130 participants, regardless of the location of the causative genetic variants, giving an overall diagnostic rate of 53.7% (130 of 242). Identification of causative genetic variants informed precise treatment, restored reproductive confidence, and optimized clinical management of MD.

Discussion

Comprehensive bigenomic sequencing accurately detects causative genetic variants in affected MD patients, simplifying diagnosis, enabling early treatment, and informing the risk of genetic transmission.

*These authors contributed equally to this work as co-first authors.

†These authors contributed equally to this work as co-senior authors.

From the Department of Neurogenetics (R.L.D., K.R.K., C.L., K.E.A., F.E.-H., J.-S.P., C.M.S.), Kolling Institute, Faculty of Medicine and Health, University of Sydney and Royal North Shore Hospital, Northern Sydney Local Health District, St. Leonards; Kinghorn Centre for Clinical Genomics (R.L.D., K.R.K., C.P., A.E.M., V.G., A.C.M., M.E.D., M.J.C., C.M.S.), Garvan Institute of Medical Research, Darlinghurst; Department of Neurology (K.R.K., C.L., K.E.A., F.E.-H., C.M.S.), Royal North Shore Hospital, Northern Sydney Local Health District, St. Leonards; Dr. Kumar is now with Molecular Medicine Laboratory, Concord Hospital, Concord, New South Wales, Australia; Dr. Park is now with Cenyx Biotech, Jongno-gu, Seoul, South Korea; Brain and Mitochondrial Research Group (J.C.), Murdoch Children's Research Institute, Parkville, Melbourne; Department of Paediatrics (J.C.), University of Melbourne, Victoria; Prof. Schofield is now with GenIMPACT: Centre for Economic Impacts of Genomic Medicine, Macquarie University, Macquarie Park; Prof. Dinger is now with School of Biotechnology and Biomolecular Sciences, University of New South Wales, Randwick; and Prof. Cowley is now with Computational Biology Group, Children's Cancer Institute, University of New South Wales, Randwick, Australia.

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Glossary

AMACR = α-methylacyl-CoA racemase; **CNV** = copy number variation; **CPEO** = chronic progressive external ophthalmoplegia; **KSS** = Kearns-Sayre syndrome; **MD** = mitochondrial disease; **MELAS** = mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; **MIDD** = maternally inherited deafness and diabetes; **MIM** = Mendelian inheritance in man; **mtDNA** = mitochondrial DNA; **nDNA** = nuclear DNA; **SNV** = single nucleotide variant; **SV** = structural variation; **VAF** = variant allele frequency; **VUS** = variants of uncertain significance; **WGS** = whole-genome sequencing.

Mitochondrial diseases (MDs) are the commonest group of inherited metabolic disorders,[1] and novel therapies in the field are now beginning to emerge.[2-5] However, targeted treatments and reproductive options rely on a precise molecular diagnosis. Limited genotypic-phenotypic correlation of MDs makes molecular confirmation challenging, and many patients remain undiagnosed despite extensive investigation over long periods of time.[6-8]

MDs are unique because they can be caused by variants in either the mitochondrial or nuclear genome[9,10] and can affect both children and adults. Although minimum prevalence studies have estimated that 1 in 4,300 live births develop MD,[11] community-based prevalence studies demonstrate that at least 1:250 people carry a pathogenic mitochondrial DNA (mtDNA) variant that puts them at risk of developing an MD.[12-14] This highlights that a large percentage of at-risk individuals carrying disease-causing variants are undiagnosed or remain asymptomatic.[7] Clinical severity of affected patients ranges from mild, oligosymptomatic disease to severe, fatal illness.[9,15] Presenting features in adults emerge at variable ages at onset, commonly including muscle weakness, fatigue, ptosis, ophthalmoplegia, hearing loss, diabetes, seizures, focal neurologic deficits, and visual loss.[1,9,10,16] Standard diagnostic criteria are based on available clinical data and include results of invasive procedures, such as muscle biopsy,[17] but a precise diagnosis requires genetic testing.[9,10]

Currently, there is no single first-line genetic test integrated into standard MD diagnostic practice. A definitive diagnosis may require sequencing of hundreds of nuclear genes and most of the mitochondrial genome[1,9,18]; a process that has been impractical to date. To complicate matters further, causative mtDNA variants in blood decline with age so may be absent or only present at low levels of heteroplasmy (the proportion of variant to wild type mtDNA genomes),[19,20] necessitating sampling of other tissues, which may be invasive. As a result, affected adult patients often endure prolonged diagnostic odysseys before achieving a genetic diagnosis,[8] delaying the benefits of informed family planning and optimal medical management.[10,15,16] Undiagnosed, oligosymptomatic adult carriers may unknowingly pass on the disease to their children[21] or receive inappropriate clinical treatment.[22]

Now, whole-genome sequencing (WGS) provides the capability to comprehensively sequence both nuclear and mitochondrial genomes simultaneously, with the potential to capture the complete spectrum of MD-causing variants in a single blood test and thereby simplify the diagnostic pathway.[15,23] Capitalizing on the ability of WGS to provide a high depth of coverage of the mitochondrial genome, we developed a bioinformatic tool[24] capable of identifying low levels of heteroplasmic mtDNA variants often found in blood. Accordingly, we determined the capacity for WGS to identify known disease-causing variants in both nuclear and mitochondrial genomes and examined the diagnostic utility of WGS as if it were applied as a front-line "genetics first"[25] blood test for a large cohort of patients with suspected MDs.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

All patients gave written consent to participate in the study, which was approved by the Northern Sydney Local Health District Human Research Ethics Committee (HREC/10/HAWKE/132). All data were deidentified.

Patient Recruitment and Samples

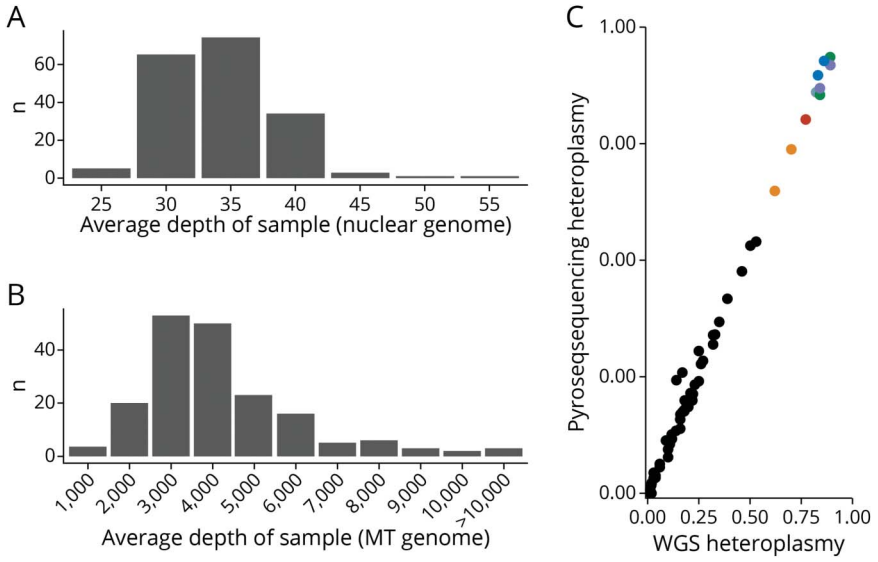
We prospectively recruited 242 consecutive patients reviewed at the Mitochondrial Disease Clinic, Royal North Shore Hospital, Sydney, Australia, between 2014 and 2020. Patients were eligible for recruitment to the study if they satisfied the possible, probable, or definite Nijmegen MD criteria.[17] Although the Nijmegen criteria were developed in children with primary MD, they were used here as the clinical manifestations included in these diagnostic criteria reflect the phenotypic variability observed in patients with MD.[9]

DNA samples from the blood of 41 participants with known pathogenic variants (30 in nuclear DNA [nDNA] and 11 in mtDNA) and the muscle tissue of a patient with Kearns-Sayre syndrome (KSS) with a 4.6 kb mtDNA deletion were used as "positive controls" for assessing the capacity of WGS to identify known variants in the 2 different genomes (eTable 1, links.lww.com/WNL/C92).

WGS and Analysis

Total genomic DNA was isolated from peripheral blood using standard methods. Sequencing libraries were prepared using robotic instrumentation and sequenced on an Illumina HiSeq X platform at the Kinghorn Centre for Clinical Genomics, Sydney, Australia. 2 × 150 bp reads yielded at least 110 Gb of raw sequencing data and a minimum 30× coverage of nDNA per lane. To determine the clinical utility of the test, initial variant analysis was performed blinded and regardless of clinical phenotype, family history, or prior known genetic results.

Figure 1 WGS Provides Deep, Uniform Coverage of Both the Mitochondrial and Nuclear Genome



The average depth of sequencing coverage across the (A) nuclear and (B) mitochondrial genomes from a cohort of adult participants with suspected mitochondrial disease (n = 242). (C) m.3243A>G variant heteroplasmy assessed by WGS and pyrosequencing for DNA extracted from blood (black) from n = 50 patients diagnosed with the m.3243A>G variant and multiple autopsy tissues (n = 10; colored points) from patients E9 and E59. Heteroplasmy was highly correlated between WGS and pyrosequencing (R^2 = 0.994). Colors in (C) are consistent with those in Figure 2 for the different tissues. MT = mitochondrial; WGS = whole-genome sequencing.

Nuclear DNA Analysis

We detected small nDNA variants using a GATK best practices pipeline[26] and interpreted them using our nuclear variant filtering analysis platform, Seave.[27,28] Raw fastq files were aligned to the hs37d5 reference genome using BWA-MEM (v0.17.10-r789), with resulting BAM file duplicate reads marked using Novosort (default settings) and read alignment improved using GATK Indel Realignment (v3.3).[26] Single nucleotide variants (SNVs) and short indels (<50 bp) were identified using GATK HaplotypeCaller, GenotypeVCFs, and VQSR (v3.3),[26] annotated with VEP (v87), converted into a GEMINI (v0.11.0) database, and imported into Seave[27] for filtration and prioritization. We detected structural variation (SV) and copy number variation (CNV) from 50 bp to whole-chromosome aneuploidy, in the nuclear and mitochondrial genomes, using ClinSV.[29] Data analysis was performed using R (v3.6.0) and RStudio (v1.2.1335), and was plotted using ggplot2.

To restrict the search space for nDNA variant analysis, we curated a panel of 249 MD genes (eTable 2, links.lww.com/WNL/C92), 400 neuromuscular disease genes,[30] and for unsolved cases, additional tailored individual searches based on clinical phenotype were made in optic atrophy, metabolic, developmental disorder, and other gene panels relevant to the phenotype. Variants were classified using the American College of Medical Genetics and Genomics 2015 guidelines,[31] with consideration of "pathogenic" or "likely pathogenic" variants. Variants of uncertain significance (VUS) bordering on, but insufficient to be classified as likely pathogenic, were classed as "VUS—favor pathogenic"[31] (eTable 3) and included in the diagnostic count because of the difficulty in obtaining supporting evidence for a Class IV variant. Pathogenic variants were confirmed using Sanger sequencing of an alternate DNA sample on an ABI3100 using the BigDye Xterminator Kit (Garvan Molecular Genetics, Garvan Institute, Sydney, Australia). Segregation studies were conducted where possible for newly identified variants.

mtDNA Analysis

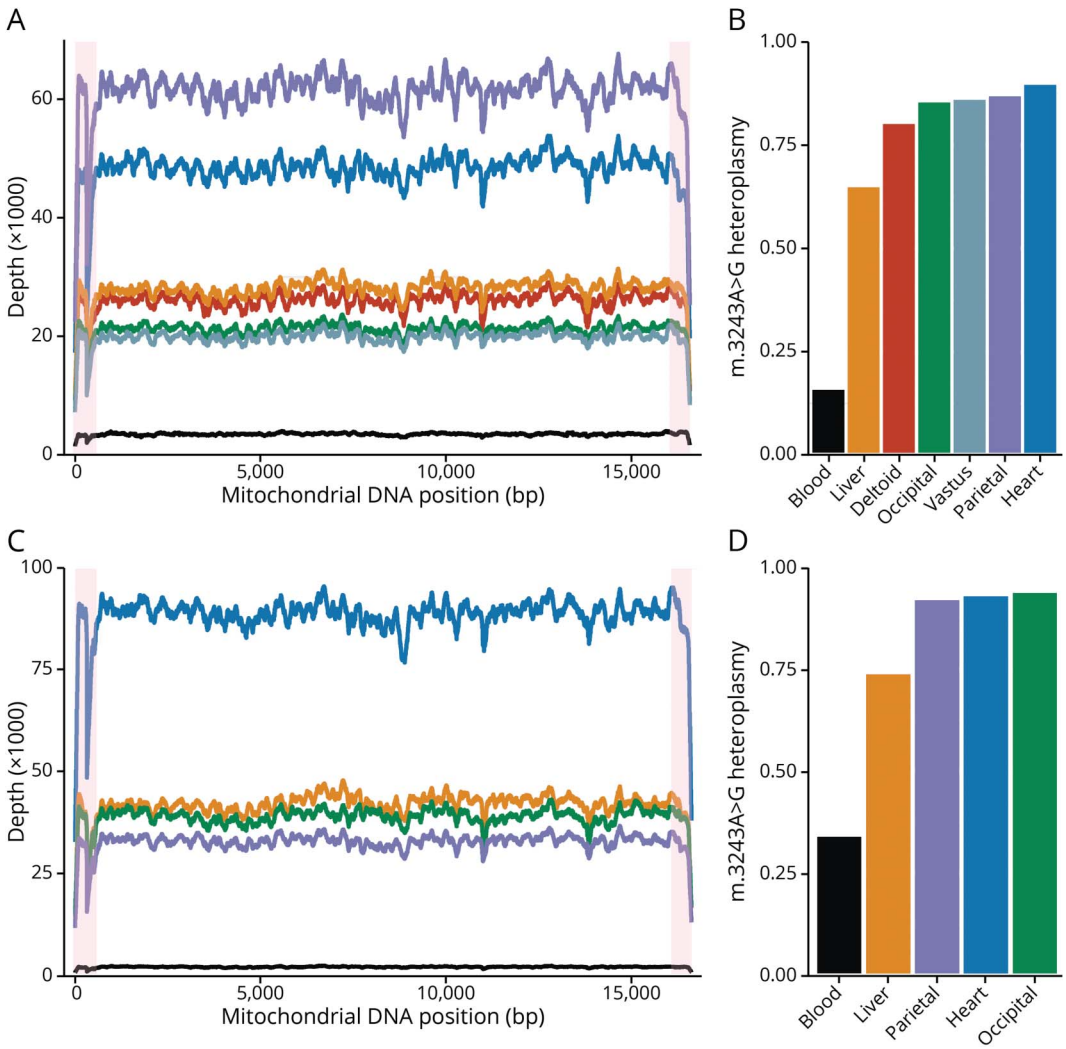
To analyze SNVs and insertion/deletion (indel) variants in mtDNA, we developed an analytical pipeline named "*mity*," which runs FreeBayes in an ultra-sensitive mode[24] and calculates variant quality accurately even for very low heteroplasmic variants. *mity* was developed using 13 replicates of the NA12878 control line, 2,570 healthy controls,[32] and 1 patient from this study with 2 independent genomic sequences. We optimized the analytical parameters as follows: Reads with mapping quality <30 were removed to minimize false-positive variants and spurious signals from nuclear mtDNA, only bases with base quality ≥24 were used for variant calling, and we required a variant to have at least 10 supporting reads or a variant allele frequency (VAF; i.e., the proportion of reads carrying the variant vs all reads) >1%.[24] We used the VAF as a direct measure of variant heteroplasmy. For variant interpretation, all mitochondrial variants were ordered by decreasing VAF, prioritizing known pathogenic variants and those linked to phenotypes in MITOMAP[33] and the literature.

To determine the capability of *ClinSV*[29] analysis of WGS to identify and quantitate mtDNA deletions, we studied DNA extracted from muscle taken at autopsy from a patient with KSS (sample 42, eTable 1, links.lww.com/WNL/C92).

Pyrosequencing

To evaluate the capability of *mity* in determining mtDNA variant heteroplasmy from WGS, we compared heteroplasmy determined by *mity* and quantitative pyrosequencing from 60

Figure 2 Different Tissues Provide High Depth of Coverage of the Mitochondrial Genome and High Levels of Heteroplasmy



(A and C) Depth of sequencing coverage across the mitochondrial genome and (B and D) m.3243A>G heteroplasmy varied between autopsy tissues from 2 patients, being considerably higher in solid tissues compared with blood. Colored lines in A and C are consistent with colored bars in B and D. Colors in A–D are consistent with those in Figure 1C for the different tissues.

samples (50 blood samples from individual patients and 10 autopsy tissue samples from 2 patients) known to have variable levels of the m.3243A>G variant. A custom m.3243A>G pyrosequencing assay was performed by the Australian Genome Research Facility (Perth, Australia). A standard curve was created using wild type or mutant gBlocks gene fragments of a 500 bp region around m.3243 (Integrated DNA Technologies, Singapore).

Long-Range PCR

To confirm mtDNA deletions were present in urine sedimentary cell DNA and absent in blood DNA, we amplified mtDNA as one full length fragment using overlapping primers and TaKaRa LA Taq, as previously described.[34]

Data Availability

Patients consented to genomic testing in a clinical setting and did not consent for the release of raw or processed genomic data. *mity* is available under an open source MIT license, from github.com/KCCG/mity.

Results

Patient Cohort

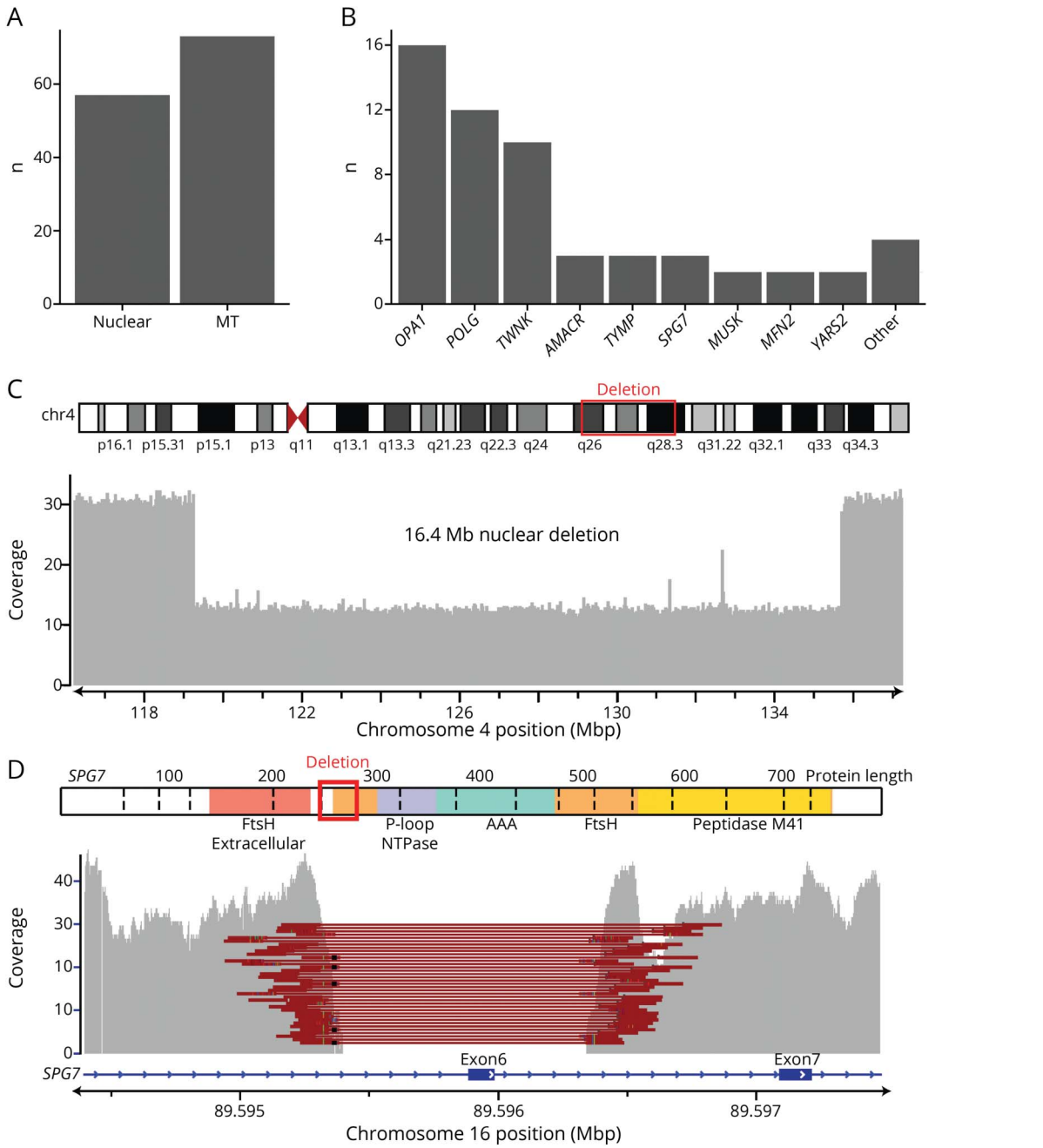
We recruited 242 patients (149 female patients and 93 male patients; eFigure 1, links.lww.com/WNL/C92) with a mean age at DNA sampling of 49.5 ± 16.8 years. According to the Nijmegen MD criteria,[17] 62 participants were classified as definite, 108 as probable, and 72 as possible.

WGS Capabilities

Coverage of nDNA and mtDNA in Blood vs Other Tissues

WGS provided a high depth of coverage of both nuclear and mitochondrial genomes from blood DNA. A mean nuclear

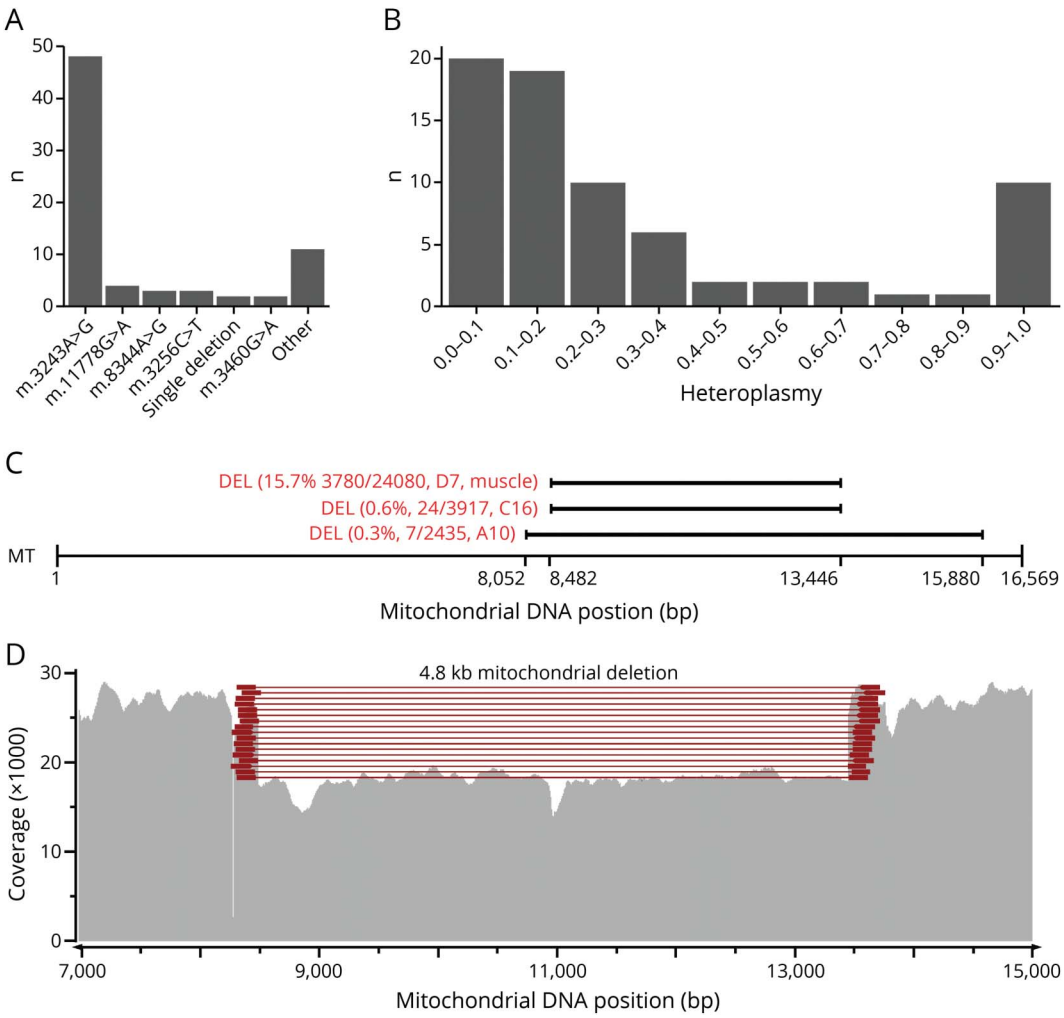
Figure 3 The Diagnostic Performance of WGS for Nuclear DNA–Based Mitochondrial Diseases



(A) The number of diagnoses made by WGS in the nuclear or mitochondrial genomes, from n = 242 patients with suspected mitochondrial disease. (B) The frequency of diagnoses made from mitochondrial disease genes in the nuclear genome. (C) WGS identified a hemizygous 16.4 megabase pair (Mbp) deletion on chromosome 4 in a patient with seizures, mild ophthalmoparesis, optic atrophy, cerebellar ataxia, myopathy, diabetes, recurrent pseudo-obstruction of the bowel, and liver dysfunction. (D) WGS identified a homozygous 946 base pair deletion in *SPG7* in a patient with hereditary spastic paraplegia complicated by cerebellar ataxia, ophthalmoplegia, and sensory neuropathy. MT = mitochondrial; WGS = whole-genome sequencing.

genome coverage of 30–40× was achieved (Figure 1A), with 80% of the genome covered to ≥10×. WGS simultaneously provided 3,000–4,000× mean coverage of the mitochondrial genome (Figure 1B), with >90% of the genome covered to >2,000×. Using our analytical pipeline with *mity*,[24] we were able to detect very low levels (<1%) of heteroplasmic mtDNA variants. Levels of m.3243A>G heteroplasmy quantified by *mity* analysis of WGS strongly correlated with pyrosequencing (n = 50, R^2 = 0.994, Figure 1C), with detection of the pathologic variants down to a heteroplasmic load of 0.35% (patient E53, eTable 4, links.lww.com/WNL/C92), well below the reliable limit of detection for pyrosequencing (~5%).[35] Although these ultra-low levels of heteroplasmy may be difficult to interpret clinically in a de novo situation, the fact they can be detected in blood to this level shows the sensitivity of WGS variant detection in blood but would require

Figure 4 The Diagnostic Performance of WGS for Mitochondrial DNA–Based Mitochondrial Diseases



(A) The number of causative mtDNA variants detected by WGS and (B) their corresponding variant heteroplasmy. (C) Two mitochondrial DNA single deletions were identified in blood, both at heteroplasmy below 1%. One mitochondrial deletion was found in autopsy muscle at a much higher heteroplasmy of 15.7%, shown in more detail in (D), which highlights the sequencing coverage and reads spanning the deletion. mtDNA = mitochondrial DNA; WGS = whole-genome sequencing.

validation of the variant in another tissue to confirm the genetic diagnosis.

Analysis of blood and postmortem tissues (n = 12) from 2 patients who died with m.3243A>G showed that the sequencing depth of mtDNA ranged from ~3,000× in blood to between ~20,000–90,000× in solid tissues with variable but high levels of heteroplasmy (Figure 2, A–D).

Diagnostic Yield

Using WGS and applying *mity*, we identified 57 patients with disease-causing nDNA variants and 73 patients with disease-causing mtDNA variants (Figure 3A, eFigure 1, links.lww.com/WNL/C92), obtaining an overall diagnostic yield of 53.7% (130 of 242; 95% CI 47.2%–60.1%). Data on the clinical features, family history, Nijmegen MD criteria classification, and variants identified are summarized in eTables 3 and 4.

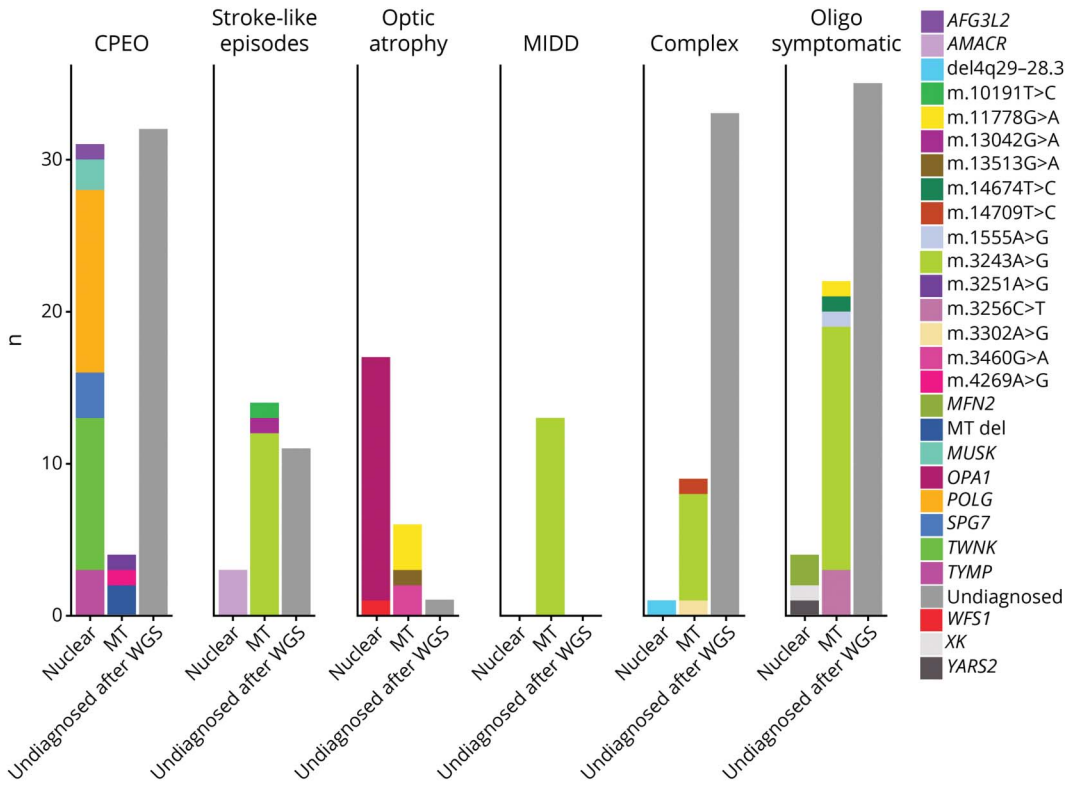
Detection and Impact of Variant Calling of nDNA-Encoded MDs

Fifty-seven patients were found to have causative nuclear gene variants, with pathogenic or likely pathogenic variants located in *AFG3L2*, *AMACR*, *MFN2*, *OPA1*, *POLG*, *SPG7*, *TWNK*, *TYMP*, *WFS1*, and *YARS2* (Figure 3B, eFigure 1, eTable 3, links.lww.com/WNL/C92). In addition, 11 patients had VUS identified, requiring further investigation of pathogenicity, but occurring in known MD-associated genes (eTable 3).

Nuclear Genome CNVs

Using *ClinSV*,[29] we were also able to detect CNVs and SVs, including a novel heterozygous 16.4 Mb de novo deletion of chromosome 4q26–q28.3 (Figure 3C), in a proband presenting with seizures, ophthalmoparesis, optic atrophy, ataxia, myopathy, diabetes, and recurrent pseudo-obstruction of the bowel (patient B3, eTable 3, links.lww.com/WNL/C92). The deletion encompassed numerous genes including *PRSS12*

Figure 5 Molecular and Diagnostic Heterogeneity Within Different MD Phenotypes



Patients were grouped into 6 clinical phenotypes (CPEO, stroke-like episodes, optic atrophy, MIDD, complex, and oligosymptomatic) to highlight the frequency of pathogenic nuclear gene and mitochondrial DNA variants identified by WGS. Precise molecular diagnoses are shown, and the number of undiagnosed cases in each subgroup is shown in gray. CPEO = chronic progressive external ophthalmoplegia; MD = mitochondrial disease; MIDD = maternally inherited deafness and diabetes; WGS = whole-genome sequencing.

(intellectual developmental disorder, autosomal recessive 1, Mendelian inheritance in man [MIM] 249500), *MRT29* (intellectual developmental disorder, autosomal recessive 29, MIM 614333), and *SPATA5* (epilepsy, hearing loss, and neurodevelopmental disorder, MIM 616577), without a detectable causative variant on the alternate allele. This finding was confirmed using comparative genomic hybridization array and was absent in the proband's parents. We also detected a homozygous exon 6 deletion in *SPG7* (Figure 3D) in a proband with spastic paraplegia complicated by cerebellar ataxia, ophthalmoplegia, and sensory neuropathy (patient E75, eTable 3).

Detection and Impact of Pathogenic mtDNA-Encoded MDs

We identified 73 patients with disease-causing mtDNA variants (Figure 3A, eFigure 1, links.lww.com/WNL/C92). Using *mity*, we were able to confidently detect a broad range of mtDNA variants in blood DNA (Figure 4A, eTable 4), even if present at low levels of heteroplasmy (Figure 4B).

Using *ClinSV*[29] to identify and quantitate mtDNA deletions, we detected a 4.8 kb mtDNA deletion at 16% heteroplasmy (3,780/24,080 sequencing reads; Figure 4, C and D) in the control sample derived from the muscle tissue of a patient with KSS (sample 42, eTable 1, links.lww.com/WNL/C92). We were also able to detect single 8 and 5 kb mtDNA deletions in blood DNA of 2 additional patients at extremely low heteroplasmic loads of 0.29% (7/2,435 reads) and 0.61% (24/3,917 reads), respectively (Figure 4C; patients A10 and C16, eTable 4), and confirmed that they also had mtDNA deletions in other tissues, for example, muscle or urine (data not shown).

In 7 patients with clinical features of chronic progressive external ophthalmoplegia (CPEO), mtDNA deletions were detectable in muscle or urine using Southern blot or long-range PCR[34] (eTable 5, links.lww.com/WNL/C92). These deletions were not detected by WGS of blood DNA, presumably because they were not present in this tissue (eFigure 2). This is consistent with the known selection against mtDNA deletions in blood.[20]

Diagnostic Rates According to Clinical Phenotype and Age

We found that the diagnostic rate varied depending on the presenting clinical phenotype (Figure 5), rather than disease classification using the Nijmegen criteria (eFigure 3A, links.lww.com/WNL/C92). The highest diagnostic rates were achieved when patients with suspected MD presented with clear clinical phenotypes (Figure 5). For individuals with optic

Table 1 Case Studies of Mitochondrial Disease or Mitochondrial Mimics for Which a Diagnosis Led to a Change in Management

ID	Diagnosis	Variants identified by WGS	Clinical features	Change in management
E19	α-methylacyl-CoA racemase deficiency	Homozygous variants in *AMACR* (NM_014324.5:c.154T>C, NP_055139.4:p.Ser52Pro) (eTable 3, links.lww.com/WNL/C92).[e1] Variants in this gene are associated with α-methylacyl-CoA racemase deficiency (MIM 614307).	49-year-old woman who presented with seizures, encephalopathy, migrainous auras without headache (e.g., visual scotomas, right face and hand numbness, right hand weakness) and multiple focal neurologic deficits (e.g., transient visual loss, transient hearing loss, numbness of the upper limbs, left homonymous hemianopia) associated with multiple lesions on cerebral MRI (Figure 6A). Sister (sample B45, eTable 3) presented with seizures and a stroke-like episode (aphasia) associated with bilateral thalamic lesions of cerebral MRI (Figure 6B). Pristanic acid levels were markedly elevated for both participants (196–255 μM; normal <2.5 μM).	Commenced pristanic acid dietary restriction (Refsum diet[40]), which resulted in a reduction of seizures and improvements in immediate attention and working memory, information processing speed, letter and semantic fluency, as documented on formal neuropsychometric testing.
B43	Congenital myasthenia	Homozygous variant in *MUSK* (NM_005592.3:c.358+3G>T) (eTable 3). Variants in this gene are associated with congenital myasthenic syndrome 9 (MIM 616325).	77-year-old man with proximal myopathy, ophthalmoplegia, and respiratory difficulties since his 40s. Eldest of 11 siblings from a consanguineous marriage. Sister had a similar presentation. His muscle biopsy showed ragged-red fibers. The homozygous variant affected a donor splice site and affected on splicing, as indicated by transcript analysis from patient-derived fibroblasts (eFigure 4).	Genetic diagnosis ended a 20-y diagnostic odyssey, prompting a trial of salbutamol (albuterol)[41] and recommendations to avoid agents that block the neuromuscular junction.
B39	McLeod neuroacanthocytosis	Nonsense variant in the *XK* gene (NM_021083.2:c.268delT, NP_066569.1:p.Tyr90ThrfsX40) (eTable 3). Variants in this gene are associated with McLeod neuroacanthocytosis syndrome (MIM 300842).	A 58-year-old man with bilateral ptosis, proximal myopathy, seizures, tics, and COX-negative fibers on muscle biopsy. Blood film examination identified acanthocytes and confirmed the diagnosis of neuroacanthocytosis	Surveillance for cardiac abnormalities and seizures was instituted and recommendations to use Kx-negative blood or banked autologous blood for transfusions when required.

Abbreviations: COX = cytochrome *c* oxidase; ID = patient identification number for this study found in eTable 3 (links.lww.com/WNL/C92); WGS = whole-genome sequencing; MIM = Mendelian inheritance in man.

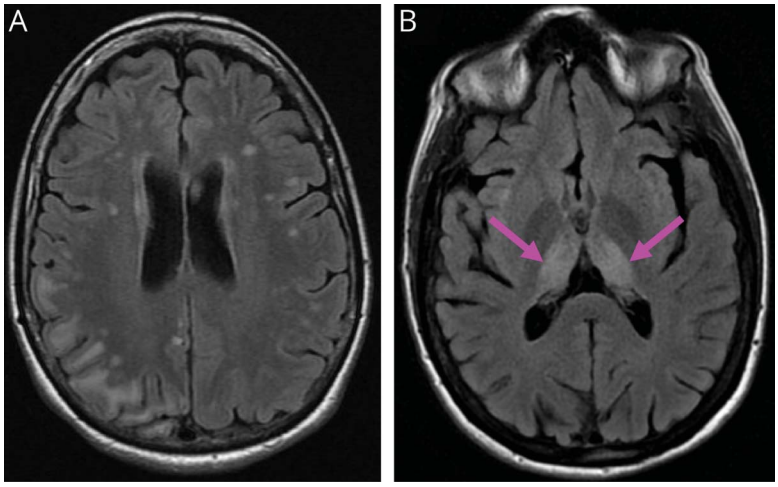
atrophy, 23 of 24 (95.8%; 95% CI 79.8%–99.3%) were diagnosed using WGS (n = 17 nDNA-encoded MDs; n = 6 mtDNA-encoded MDs). In patients presenting with stroke-like episodes, 17 of 28 (60.1%; 95% CI 42.4%–76.4%) were diagnosed (n = 3 nDNA-encoded MDs; n = 14 mtDNA-encoded MDs) and 35 of 67 patients with a CPEO phenotype (52.2%; 95% CI 40.5%–63.8%) also had a molecular cause identified (n = 31 nDNA-encoded MDs; n = 4 mtDNA-encoded MDs). Thirteen patients with m.3243A>G in our cohort had maternally inherited deafness and diabetes (MIDD). Diagnostic rates for nonsyndromic complex phenotypes (defined as >5 clinical features listed in the Nijmegen criteria) and oligosymptomatic phenotypes (defined as <5 clinical features listed in the Nijmegen criteria) were lower (10/43 complex = 23.3%; 95% CI 13.2%–37.8% and 26/61 oligosymptomatic = 42.6%; 95% CI 31.0%–55.1%) (Figure 5). The diagnostic rate using our WGS protocol was higher in patients younger than 50 years (odds ratio 2.29; 95% CI 1.36–3.84, $p < 0.002$; eFigure 3B).

Clinical Impact of Definitive Genetic Diagnoses Confirmed by WGS

Our approach led to genetic diagnoses that changed clinical management in patients (e.g., commencement of disease-specific clinical care, avoidance of disease-specific contraindicated care, and clarification of reproductive options) with both nuclear and mtDNA-encoded disorders. WGS identified patients with treatable MDs including mitochondrial neurogastrointestinal encephalopathy syndrome (n = 3; treatable by liver or allogeneic bone marrow transplantation[36]) and Leber hereditary optic neuropathy (n = 6; idebenone or potential gene therapy treatment), as well as identifying patients with causative variants in *POLG*, whereby recommendations to avoid contraindicated medications, such as valproic acid that can cause fulminant liver failure or life-threatening status epilepticus, are important for optimal management of the MD.[37,38] In addition, a 33-year-old woman (patient C44, eTable 3, links.lww.com/WNL/C92) with ptosis, optic atrophy, and proximal muscle weakness who was found to have compound heterozygous variants in *YARS2* causing MLASA2 became pregnant with the confidence from WGS findings that she would be highly unlikely to transmit MD to her child.[39] Thus, we were able to provide certainty for patients making reproductive decisions by obtaining definitive genetic diagnoses.

Regarding MD mimics, WGS was able to detect variants in a larger targeted gene list, thereby allowing diagnosis and

Figure 6 Cerebral MRI in α-Methylacyl-CoA Racemase Deficient Patients



(A) Representative axial T1 weighted images showing focal areas of abnormal signal intensity in the right parieto-occipital region after episodes of encephalopathy in patient E19. Note there are also small high signal intensities scattered throughout the subcortical white matter. (B) Magenta arrows indicate high signal areas in the thalami of patient B54, consistent with what is seen in adult-onset Leigh syndrome.

differentiation of patients with other treatable conditions, enabling appropriate care and treatment for their respective diseases, while ruling out MD. Three patients were diagnosed with α-methylacyl-CoA racemase (AMACR) deficiency,[e1] inclusive of 2 sisters (Table 1; patients B45 and E19, eTable 3, links.lww.com/WNL/C92) who had seizures, encephalopathy, and stroke-like episodes suggestive of MD. With the confirmed genetic diagnosis of AMACR deficiency, they were treated with dietary restriction of pristanic acid[40,41] that resulted in symptomatic improvement. Their cerebral MRIs showing T1 high signal intensities in the right parieto-occipital cortical ribbon or bilateral thalami before treatment were reported to be suggestive of the diagnosis of an MD (Figure 6). Furthermore, 2 siblings (Table 1; patients B42 and B43, eTable 3) who presented with progressive external ophthalmoplegia and proximal muscle weakness were found to have a novel homozygous splicing variant in the *MUSK* gene (c.358+3G>T; eFigure 4) and were subsequently treated with salbutamol.[42]

Discussion

WGS comprehensively and simultaneously sequenced both mitochondrial and nuclear genomes to a high depth of coverage from blood DNA, and in combination with *mity*, we were able to identify a range of SNVs, indels, and CNVs in both genomes to achieve precise genetic diagnoses for a broad spectrum of MDs and MD mimics. When applied as a first-line diagnostic blood test for MD, WGS achieved an overall diagnostic rate of 53.7%, a result that compares favorably with previous genetic disease cohorts sequenced using other next-generation sequencing methods, despite their enrichment with more stringent selection criteria.[43-46] Importantly, our findings demonstrate the simplicity of our comprehensive bigenomic sequencing diagnostic approach, that for most of the cases uses DNA from blood and alleviates the need for muscle biopsy or obtaining DNA from other tissues.

WGS provides substantial advantages over targeted mitochondrial sequencing panels, which are less comprehensive, provide lower diagnostic rates,[47] and would require DNA from muscle or urine to achieve the same detection rates seen here. Although whole exome sequencing largely provides adequate coverage of nDNA protein coding exons, the average coverage of mtDNA is much lower (~50×) and thus less sensitive when compared with WGS.[48] Whole exome sequencing of nDNA and mtDNA is performed in parallel and is subject to incomplete coverage and target enrichment bias during library preparation, when compared with WGS. Of further benefit, analysis of WGS provides more capability when identifying CNVs and SVs, which can be challenging when using targeted sequencing panels or whole exome sequencing.[49] The superior detection sensitivity of WGS,[50] in combination with *mity*, is of particular importance for adults with MD because our study shows that most of the diagnosed patients (73 of 130; 56.2%) had pathogenic mtDNA variants, rather than nDNA variants (Figure 3A). However, when there is a strong maternal inheritance pattern and typical clinical phenotype indicative of a specific common mtDNA variant, alternatives, such as RFLP analysis for common mtDNA pathogenic variants (e.g., m.3243A>G or m.8344A>G) or full mtDNA sequencing, may be more cost-effective, although careful tissue selection to address the issues of low heteroplasmy needs to be considered. Once a variant has been identified by WGS, cascade testing in relatives can also be approached using diagnostic targeted sequencing, although these methods cannot always provide an estimate of the heteroplasmy of the pathogenic mitochondrial variant involved.

Diagnostic rates were further increased (up to 95%) when patients were stratified by deep clinical phenotyping, underpinning the critical value of clinical expertise and assessment in

combination with our WGS pipeline. We demonstrate that a high index of clinical suspicion and knowledge of specific clinical phenotypes, such as CPEO, optic atrophy, and stroke-like episodes (MELAS) that all demonstrate genetic heterogeneity across both genomes, justifies simultaneous dual genomic analysis with WGS (Figure 5, eTables 3 and 4, links.lww.com/WNL/C92). WGS diagnostic rates differed depending on the specific phenotype; patients presenting with optic atrophy, MELAS, CPEO, or MIDD had a higher diagnostic rate than those who presented with nonsyndromic, complex phenotypes, possibly indicating that patients with MD mimics may still satisfy the standard clinical diagnostic criteria (Figure 5).

An important limitation highlighted here when using WGS on DNA extracted from blood is that mtDNA deletions may not be present[20] and therefore are not able to be detected using this readily obtainable and often used tissue (Figure 5, eFigure 2, links.lww.com/WNL/C92). Thus, to increase detection of deletions in patients with CPEO or KSS phenotypes, where single or even multiple mtDNA deletions are suspected, WGS or long-range PCR of an alternative tissue, such as muscle, saliva, or urine, may be required if initial sequencing of blood fails to identify a causative variant or if only a low-level heteroplasmic mtDNA deletion is identified by WGS. Despite this caveat, our study still diagnosed 52.2% of patients with CPEO using WGS, showing the value of initial testing of DNA sourced from blood.

In our analysis, we also considered variants in neuromuscular disease–associated genes and identified patients presenting to our clinic who had disorders mimicking MD (e.g., congenital myasthenia and neuroacanthocytosis), although these patients had muscle biopsy abnormalities and clinical symptomology consistent with the diagnosis of an MD (Table 1). The provision of a molecular diagnosis and confirmation of an MD mimic led to changes in medical management (Table 1), as well as informing the risk of transmission of their disorder to their offspring. In addition, 3 patients with neurologic presentations (focal neurologic deficits, seizures associated with abnormalities on cerebral MRI) suggestive of MELAS or Leigh syndrome were identified as having AMACR deficiency[e1] (Figure 6, eTable 3, links.lww.com/WNL/C92), demonstrating that WGS was able to change clinical management by identifying disorders that are treatable by simple dietary restriction,[41] as well as providing a diagnosis for MD phenocopies that have treatment options (Table 1).

Identification of the precise genetic cause of MD is clinically important and clarifies reproductive options for affected patients and their families. For instance, patients with causative nDNA variants can undergo prenatal genetic diagnosis, whereas those with pathogenic mtDNA variants are now able to consider the novel in vitro fertilization option, mitochondrial donation.[21,50] This advantage of WGS is further underpinned by its ability to quantify mtDNA heteroplasmy in blood because this provides predictive information regarding disease transmission in mtDNA disorders.[21]

A limitation of this study was that we conservatively restricted our variant calling to known disease-causing variants and those that fulfilled the stringent pathogenicity classification criteria. Although the diagnostic rate was high, a number of patients still remain undiagnosed. These patients may have causative variants in novel disease genes that are yet to be discovered or associated with MD. Furthermore, subsequent analysis may reveal disease-causing variants in noncoding regions as atypical splice variants or as tissue-specific mtDNA variants. Other patients may have VUS in known disease genes that require confirmation of pathogenicity with functional studies (eTable 3, links.lww.com/WNL/C92). Moreover, mtDNA variants (particularly deletions) that typically disappear in blood with advancing age[19,20] may be more difficult to detect. Reanalysis with updated variant and gene lists and further functional genomic investigation of novel variants has the potential to identify additional diagnoses in the future. Where parents can be recruited, trio analysis of WGS may further increase the diagnostic yield through improved filtering of autosomal recessive or de novo nuclear disorders.[52] However, this is often challenging with adult patients with MD, and familial segregation studies may be the only option, although these can also be challenging for various reasons (disease penetrance, insurance considerations, and denial by family members).

In this study, we did not compare the diagnostic utility of WGS from different tissues, such as muscle or urine, or to other diagnostic methods such as limited gene panels, WES, long-range PCR, and whole mtDNA genome sequencing. Rather, we applied the standard clinical situation to determine the capability of WGS when used as a single diagnostic test that could be standardized, scaled, and widely implemented. Although direct gene testing or whole mitochondrial genome sequencing may lead to confirmation of a genetic diagnosis in some patients who have typical clinical presentations, given the limited genotype-phenotype correlation of this disorder,[9] WGS could prove to be more cost-effective and may become preferential as costs decrease, especially when considering the high diagnostic rate observed in this study. At present, the cost of WGS is falling and will become more accessible, requiring evidence and analytical pipelines, as presented here, to support its routine clinical uptake for MD diagnosis in the future. Further evaluation of the cost effectiveness of WGS compared with conventional genetic testing methods (e.g., targeted mtDNA variant, single gene, or gene panel analysis) will be important because the benefits of a single diagnostic blood test to inform directed genetic testing in an extensive number of family members are considerable regarding time to diagnosis and the costs of testing.[53]

Comprehensive simultaneous sequencing of both mitochondrial and nuclear genomes by WGS from blood is an accurate and minimally-invasive test to diagnose patients with MDs, avoids tissue biopsies, and has the capability to transform the MD diagnostic pathway. Improvements in health outcomes from early genetic diagnosis, appropriate intervention and treatment, avoidance of adverse events, reduced costs of inappropriate therapy, and potential to prevent disease inheritance are all advantages that could be enabled by the introduction of our WGS analysis pipeline and emphasizes the benefits for integrating WGS into future clinical practice.

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Disclosure

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Appendix Authors

Name	Location	Contribution
Ryan L. Davis, PhD	Department of Neurogenetics, Kolling Institute, Faculty of Medicine and Health, University of Sydney and Royal North Shore Hospital, Northern Sydney Local Health District, St Leonards; Kinghorn Centre for Clinical Genomics, Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data
Kishore R. Kumar, MBBS, FRACP, PhD	Department of Neurogenetics, Kolling Institute, Faculty of Medicine and Health, University of Sydney and Royal North Shore Hospital, Northern Sydney Local Health District, St Leonards; Kinghorn Centre for Clinical Genomics, Garvan Institute of Medical Research, Darlinghurst; Department of Neurology, Royal North Shore Hospital, Northern Sydney Local Health District, St Leonards, New South Wales, Australia	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data
Clare Puttick, MStat	Kinghorn Centre for Clinical Genomics, Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data
Christina Liang, MBBS, FRACP, PhD	Department of Neurogenetics, Kolling Institute, Faculty of Medicine and Health, University of Sydney and Royal North Shore Hospital, Northern Sydney Local Health District; Department of Neurology, Royal North Shore Hospital, Northern Sydney Local Health District, St Leonards, New South Wales, Australia	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; analysis or interpretation of data
Kate E. Ahmad, MBBS, FRACP	Department of Neurogenetics, Kolling Institute, Faculty of Medicine and Health, University of Sydney and Royal North Shore Hospital, Northern Sydney Local Health District; Department of Neurology, Royal North Shore Hospital, Northern Sydney Local Health District, St Leonards, New South Wales, Australia	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data
Fabienne Edema-Hildebrand, RN	Department of Neurogenetics, Kolling Institute, Faculty of Medicine and Health, University of Sydney and Royal North Shore Hospital, Northern Sydney Local Health District; Department of Neurology, Royal North Shore Hospital, Northern Sydney Local Health District, St Leonards, New South Wales, Australia	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; analysis or interpretation of data
Jin-Sung Park, PhD	Department of Neurogenetics, Kolling Institute, Faculty of Medicine and Health, University of Sydney and Royal North Shore Hospital, Northern Sydney Local Health District, St Leonards, New South Wales, Australia	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data
Andre E. Minoche, PhD	Kinghorn Centre for Clinical Genomics, Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia	Major role in the acquisition of data; analysis or interpretation of data

Appendix *(continued)*

Name	Location	Contribution
Velimir Gayevskiy, PhD	Kinghorn Centre for Clinical Genomics, Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia	Major role in the acquisition of data; analysis or interpretation of data
Amali C. Mallawaarachchi, MBBS, FRACP, PhD	Kinghorn Centre for Clinical Genomics, Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia	Major role in the acquisition of data; analysis or interpretation of data
John Christodoulou, MBBS, FRACP, PhD	Brain and Mitochondrial Research Group, Murdoch Childrens Research Institute, Parkville; Department of Paediatrics, University of Melbourne, Victoria, Australia	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data
Deborah Schofield, PhD	GenIMPACT: Centre for Economic Impacts of Genomic Medicine, Macquarie University, Macquarie Park, New South Wales, Australia	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data
Marcel E. Dinger, PhD	Kinghorn Centre for Clinical Genomics, Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data
Mark J. Cowley, PhD	Kinghorn Centre for Clinical Genomics, Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data
Carolyn M. Sue, MBBS, FRACP, PhD	Department of Neurogenetics, Kolling Institute, Faculty of Medicine and Health, University of Sydney and Royal North Shore Hospital, Northern Sydney Local Health District, St Leonards; Kinghorn Centre for Clinical Genomics, Garvan Institute of Medical Research, Darlinghurst; Department of Neurology, Royal North Shore Hospital, Northern Sydney Local Health District, St Leonards, New South Wales, Australia	Drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data

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