Forecasting stroke-like episodes and outcomes in mitochondrial disease
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Abstract
In this retrospective, multicentre, observational cohort study, we sought to determine the clinical, radiological, EEG, genetics and neuropathological characteristics of mitochondrial stroke-like episodes and to identify associated risk predictors. Between January 1998 and June 2018, we identified 111 patients with genetically-determined mitochondrial disease who developed stroke-like episodes. Post-mortem cases of mitochondrial disease (n=26) were identified from Newcastle Brain Tissue Resource. The primary outcome was to interrogate the clinic-radio-pathological correlates and prognostic indicators of stroke-like episode in patients with mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes syndrome. The secondary objective was to develop a multivariable prediction model to forecast stroke-like episode risk. The most common genetic cause of stroke-like episodes was the m.3243A>G variant in MT-TL1 (n=66), followed by recessive pathogenic POLG variants (n= 22), and 11 other rarer pathogenic mitochondrial DNA (mtDNA) variants (n=23). The age of first stroke-like episode was available for 105 patients (mean [SD] age: 31.8 [16.1]); a total of 35 patients (32%) presented with their first stroke-like episode ≥40 years of age. The median interval (interquartile range) between first and second stroke-like episodes was 1.33 (2.86) years; 43% of patients developed recurrent stroke-like episodes within 12 months. Clinico-radiological, electrophysiological and neuropathological findings of stroke-like episodes were consistent with the hallmarks of medically refractory epilepsy. Patients with POLG-related stroke-like episodes demonstrated more fulminant disease trajectories than cases of m.3243A>G and other mtDNA pathogenic variants, in terms of the frequency of refractory status epilepticus, rapidity of progression and overall mortality. In multivariate analysis, baseline factors of body mass index, age-adjusted blood m.3243A>G heteroplasmy,
sensorineural hearing loss and serum lactate were significantly associated with risk of stroke-like episodes in patients with the m.3243A>G variant. These factors informed the development of a prediction model to assess the risk of developing stroke-like episodes that demonstrated good overall discrimination (area under the curve = 0.87, 95% CI 0.82-0.93; c-statistic = 0.89). Significant radiological and pathological features of neurodegeneration was more evident in patients harbouring pathogenic mtDNA variants compared with POLG: brain atrophy on cranial MRI (90% vs 44%, p<0.001) and reduced mean brain weight [SD] (1044g [148] vs 1304g [142], p=0.005). Our findings highlight the often idiosyncratic clinical, radiological and EEG characteristics of mitochondrial stroke-like episodes. Early recognition of seizures and aggressive instigation of treatment may help circumvent or slow neuronal loss and abate increasing disease burden. The risk-prediction model for the m.3243A>G variant can help inform more tailored genetic counselling and prognostication in routine clinical practice.

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Introduction

Stroke remains the second leading cause of death world-wide and the principal cause of serious long-term disability, with the prevalence of self-reported stroke in the general population, estimated at 2.5%. Speed of accurate diagnosis and rapid delivery of appropriate therapies, are central to modern stroke care models. Up to 30% of patients with suspected stroke have stroke ‘mimics’ representing a significant proportion of all acute hospital
admissions. A discrete group of individuals with mitochondrial disease experience a stroke ‘mimic’, termed stroke-like episode, as part of mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome. Stroke-like episodes among people with mitochondrial disease are often a devastating paroxysmal clinical event and a characteristic feature of MELAS syndrome.

The first description of a fatal neurodegenerative syndrome including stroke-like episodes, ragged red fibers and lactic acidemia was initially reported in 1975; with the acronym MELAS coined a decade later to encapsulate a distinct mitochondrial disease syndrome.

The m.3243A>G pathogenic variant in the mitochondrial DNA encoded MT-TL1 gene has been identified in 80% of MELAS syndrome patients. Identification of other pathogenic mtDNA variants and recessively-inherited POLG variants, have also emerged as rarer causes of MELAS. Predicting the risk of stroke-like episodes in mtDNA-related MELAS syndrome is further complicated by the peculiarities of mitochondrial genetics, including mtDNA heteroplasmy, threshold effect for biochemical and clinical manifestation, mtDNA copy number and mitochondrial genetic bottleneck. However, the extraordinary variability of phenotype and genotype in patients with mitochondrial disease, including those with MELAS syndrome, is recognized as a significant barrier to both the study and management of this condition, often delaying diagnosis and impacting negatively on patient care. Four decades since its first description of MELAS syndrome; the mechanistic basis and clinical diagnostic criteria of stroke-like episodes remain controversial.

Here we present the results of a multicentre, observational study evaluating the clinico-radiological, electrophysiological, laboratory, molecular and brain histopathological correlates to devise a new risk prediction model for stroke-like episodes in patients with genetically defined mitochondrial disease.

**Materials and methods**

**Study Population**

Three centres (Newcastle upon Tyne, London and Oxford) constituting the UK National Health Service (NHS) Highly Specialised Service for Rare Mitochondrial Disorders of Adults and Children retrospectively collected clinical, demographic and neuropathological data on all cases of stroke-like episodes that occurred in children and adults (age range 1 – 72 years)
with genetically confirmed mitochondrial disease between January 1998 to June 2018 inclusive. Data for patients without MELAS syndrome were retrieved from the UK Mitochondrial Disease Patient Cohort (REC reference number: 13/NE/0326, approved by the NRES Committee North East–Newcastle and North Tyneside 2) to permit survival analysis and construction of a risk prediction model.

**Study Design**

A stroke-like episode was defined as a discrete clinical event manifesting as focal neurological dysfunction, bilateral convulsion, altered consciousness, or any combination of these features. These paroxysmal events occurred in association with anatomically relevant acute (or subacute) cortical and subcortical neuroimaging abnormalities (Figure 1 and examples of CT head changes are provided in Supplemental Figure 1) and/or pathological EEG correlates. Participants with previous brain lesions or epileptogenic comorbidities considered unrelated to their primary mitochondrial disorder were excluded. The study was approved by the Newcastle and North Tyneside Local Research Ethics Committee (LREC 2005/202) and was conducted in accordance with the provisions of the Declaration of Helsinki and adhered to the Guidelines for Good Clinical Practice.

**Data Collection**

Clinical information including sex, age at disease onset and first stroke-like episode, duration between recurrent stroke-like episodes, survival status, clinical outcome, and disease burden as assessed by the Newcastle Mitochondrial Disease Adult Scale (NMDAS), were collected. Clinical manifestations, laboratory data, neuroimaging findings, EEG studies, and genetic test results were also collated.

**EEG data acquisition, analysis and interpretation**

All EEG data were acquired by qualified EEG physiologists at the Department of Clinical Neurophysiology, Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS Foundation Trust, UK. Reusable (autoclavable) gold cup electrodes were applied to the scalp in accordance with the international 10-20 system. A minimum of 8 electrodes was applied (in neonates, adults with challenging behaviours and in situations where large areas of scalp
were unavailable for recording e.g. extensive craniotomy, by necessity, the number of electrodes had to be reduced. Approximately 20 minutes of EEG was acquired with the eyes closed throughout most of the recording but with brief epochs of eye opening and eye closure. Routine activation procedures included hyperventilation and photic stimulation.

EEG changes observed during episodes of encephalopathy, particularly metabolic encephalopathies, are generally non-specific in terms of aetiology and can range from an EEG with a normal alpha rhythm with intermixed theta and delta activity to generalized high amplitude delta activity. The qualitative classification of EEG alterations was analysed based on the methods described elsewhere. There are a number of recognized EEG signatures of seizure activity but for the purposes of this study we limited our analysis to the presence or absence of lateralised periodic discharges (LPDs), as previously defined.

**Neuropathological Analysis**

Neuropathological reports were reviewed from 26 patients (16 with documented ante-mortem stroke-like episodes). We compared brain weights of mitochondrial diseases cases to those of every control case donated to Newcastle Brain Tissue Resource (NUTR) between January 2010 and July 2019. Formalin-fixed paraffin-embedded (FFPE) brain tissues from 10 patients with clinically and genetically defined mitochondrial disease and 10 cognitively normal, aged-matched controls were acquired from the Newcastle Brain Tissue Resource (Supplemental Table 1). Tissue processing and general neurohistopathological staining was performed as previously described.

**Immunofluorescence and confocal microscopy for identification of OXPHOS subunits in microvessels and neurons**

A quadruple immunofluorescence assay was developed to label OXPHOS subunits NDUFB8 and COX1 and mitochondrial mass marker porin in conjunction with \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) or GLUT-1 for smooth muscle (arterioles) and endothelial (capillaries) cells, respectively. Immunofluorescence was performed on 5um FFPE sections of cerebellum, temporal, and occipital cortices as previously described. The primary and secondary antibodies used are given in Supplemental Table 2.
Twenty cortical arterioles and capillaries per brain region were randomly selected on the basis of their immunoreactivity for α-SMA or GLUT-1 respectively and imaged using a confocal microscope (Nikon A1R) as previously described. Imaging was performed at x180 magnification (x60 objective with x3 electronic zoom) and z stacking (0.175µm increments). Laser settings were constant throughout imaging.

Quantification of OXPHOS subunit expression and deficiency
Velocity imaging software (v.6.1.1., Perkin Elmer) was used to identify arterioles and capillaries as 'objects' based on α-SMA or GLUT-1 immunoreactivity. Microvascular mitochondria were detected based on their porin immunoreactivity localised within either α-SMA or GLUT-1. The mean optical densities for porin, NDUFB8 and COX1 were then derived and used to determine z scores as previously described. Percentage levels of OXPHOS deficiency could be determined from z scores lower than -3.

Statistical Analyses
Statistical analyses were performed with the use of IBM SPSS Statistics software (version 23) and R programming language (version 3.5). Continuous data were presented as mean with standard deviation (SD); 95% confidence intervals (CI) were calculated where appropriate. Statistical significance was defined as p ≤ 0.05; where appropriate, p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method. Pearson chi-squared test was performed to examine relationships between categorical variables including clinical features associated with stroke-like episodes, neuroimaging, EEG and neuropathological findings between mtDNA and POLG variants. Minimal prevalence of MELAS syndrome was calculated for North East England using data from UK Mitochondrial Disease Patient Cohort and population estimates from the 2011 UK Census.

Developing a risk prediction model for the m.3243A>G variant
Survival curves were calculated and presented using the survival package within R. Putative predictors of stroke-like episodes were identified from the literature and clinical experience. Univariate analysis with Cox proportional hazards regression was then used to suggest significant predictors as candidates for a multivariate model. After confirming the assumptions of proportional hazards using Schoenfeld residuals, a multivariable Cox proportional hazards model using a backward stepwise elimination approach (with change in
Akaike Information Criterion for successive models as a threshold) was used to identify the variables that contribute to the final scoring system. Following the methodology described by Galvovic and co-workers, we derived a three-tier risk model of developing stroke-like episodes for carriers of the m.3243A>G variant, and applied the receiver-operating characteristics (ROC) curve techniques to evaluate the usefulness of our risk prediction model.

**Anderson-Gill Model**

An Anderson-Gill model was used to analyse the time gaps between stroke-like-episodes. As we have no entry point for individuals, we took the first stroke-like-episode as a starting point, and analysed the time to subsequent stroke-like-episodes. The model assumes that all events are ordered and equivalent.

**Data availability**

The data that support the findings of this study are available from the corresponding author (GSG) and first author (NZL), upon reasonable request.

**Results**

**Genetic Analysis**

Between Jan 1998 through to June 2018, a total of 111 patients with genetically-confirmed mitochondrial disease and stroke-like episodes were identified. The most common causative pathogenic defect was the m.3243A>G (MT-TL1) variant (n=72; 66%), followed by recessive POLG variants (n=22; 20%), and 11 other rarer pathogenic mtDNA variants (n=17; 14%). The other mtDNA variants were m.3764C>G (MT-ND1) (n=1), m.5543T>C (MT-TW) (n=1), m.7541T>C (MT-TD) (n=1), m.8344A>G (MT-TK) (n=1), m.1001T>C (MT-TG) (n=1), m.10158T>C (MT-ND3) (n=1), m.12147G>A (MT-TH) (n=1), m.12770A>G (MT-ND5) (n=1), m.13094T>C (MT-ND5) (n=3), m.13513G>A (MT-ND5) (n=4) and m.14430A>G (MT-ND6) (n=1).

Twelve pathogenic POLG variants were identified in 23 individuals: p.Leu304Arg (n=2), p.His360Asp (n=1), p.Leu411Pro (n=1), p.Ala467Thr (n=27), p.Arg574Gln (n=1), p.Pro587Leu/p.Pro589Thr (in cis) (n=1), p.Arg597Trp (n=1), p.Thr606Alafs*64 (n=1), p.Arg574Gln (n=1), p.Pro587Leu/p.Pro589Thr (in cis) (n=1), p.Arg597Trp (n=1), p.Thr606Alafs*64 (n=1),
p.Arg627Gln (n=1), p.Trp748Ser (n=7), p.Gly848Ser (n=1) and p.Thr914Pro. Homozygosity for the p.Ala467Thr variant was present in nine individuals (39%).

**Clinical Characteristics of Stroke-Like Episodes**

Description on 229 stroke-like episodes were available (median [range], 2 [1-5]) with 63% of available data demonstrating radiological correlates (Table 1). There were no statistical differences in clinical, radiological and EEG data between cases of m.3243A>G and other mtDNA pathogenic variants (p>0.05), and therefore they were analysed collectively as a single “mtDNA” category. A total of 60 patients were women; mean (SD) age at last follow-up was 38.3 (16.2) years. There was a higher proportion of women in the POLG category compared to mtDNA (18/23 [78%] vs 42/88 [48%], p=0.009). The age of first stroke-like episode was available for 105 patients (mean [SD] age; range, 31.8 [16.1]; 0.6 - 71). Patient’s sex had no impact on age of first stroke-like episode nor age at last follow up. A total of 35 patients (32%) presented with their first stroke ≥40 years of age.

The majority of mtDNA-related MELAS cases (69/75; 91%) had exhibited antecedent symptoms suggestive of a mitochondrial disorder, whereas 45% (9/20) of POLG patients were considered asymptomatic, before presenting with their first stroke-like episode (p=0.005). Sensorineural hearing loss (SNHL) (45/74 [61%] vs 0%, p=0.005) preceding a stroke-like episode was more commonly associated with patients harbouring a pathogenic mtDNA variant while preceding ataxia was more frequently identified in patients with POLG variants (4/74 [30%] vs 6/20 [5%], p=0.007) (Table 2).

The most common symptoms associated with stroke-like episodes were confusion/drowsiness (69/86; 80%) followed by headache (60/80; 75%) and motor seizures (87/123; 71%) (Table 1 and Supplemental Figure 2). Visual symptoms most often manifested as either positive (44/84; 57%) or negative (52/91; 57%) visual phenomena (ranging from elementary visual hallucinations to visual loss; Supplemental Table 3). Focal onset status epilepticus was identified in almost all stroke-like episodes in patients with POLG variants compared to ~1/5th of mtDNA-related MELAS syndrome (27/28 [96%] vs 19/87 [22%], p=0.007). Dysphasia was significantly more common in mtDNA-related mitochondrial disease compared to POLG–related disease (28/55 [51%] vs 1/11 [9%], p=0.048) (Table 1). The mean age of last follow up was significantly younger and the overall disease burden
measured by NMDAS was significantly higher in patients with stroke-like episodes compared to those without stroke-like episodes irrespective of the genetic defects (Supplemental Table 4 and 5).

**Neuroimaging Findings**
Cranial MRI scans (n=144) performed during stroke-like episodes were available for analysis. Peri-ictal abnormalities in the temporal lobe (90/113 [80%] vs 5/32 [16%], p=0.004) and insular cortex (24/113 [21%] vs 1/32 [3%], p=0.042) were significantly more common in the mtDNA group compared to POLG cases; whereas thalamic involvement was less commonly observed in mtDNA group (7/113 [6%] vs 11/32 [34%], p=0.004) (Figure 1; Table 1). None of the subcortical white matter changes occurred in isolation. Cross-cerebellar diaschisis was identified in 11% of all scans; with no statistical difference between mtDNA and POLG cases (13/113 [12%] vs 8/32 [25%], p=0.139). Peri-ictal MRI changes were identified on all MRI head scans of mtDNA mutation cases whilst up to 16% of POLG scans did not identify any acute peri-ictal signal abnormality. MRI thalamic (12/46 [26%] vs 3/69 [4%], p=0.004) and cross-cerebellar diaschisis (12/46 [26%] vs 6/69 [9%], p=0.028) lesions were statistically more common in patients manifesting with status epilepticus compared to those not presenting in status epilepticus during a stroke-like episode (Supplemental Table 6). Imaging appearances of cerebral and cerebellar atrophy were significantly more evident in mtDNA compared to POLG cases (95/105 [90%] vs 12/27 [44%]; p<0.001 and 95/103 [92%] vs 16/28 [57%]; p<0.001), respectively; Supplemental Figure 3).

**EEG Findings**
EEG data (n=260) were available for analysis in 50 patients (m.3243A>G, n=30; POLG, n=13; others, n=7). Eighty percent of EEGs (80%; n=208) were performed during acute hospital admissions. Encephalopathic changes were more commonly identified in mtDNA than POLG-related MELAS syndrome (114/118 [97%] vs 65/89 [78%], p=0.036). Epileptic discharges were more frequently captured in POLG than mtDNA variants (67/89 [75%] vs 66/120 [55%], p=0.002) (Table 1) and more commonly identified in the posterior region in POLG compared to mtDNA cases (38/89 [43%] vs 23/120 [19%], p<0.001) (Supplemental Table 7). Periodic lateralising epileptic discharges were identified in 34% of all acute EEGs (28/91) irrespective of genotype and presence of clinical status epilepticus. Six episodes of
epilepsia partialis continua were documented clinically without EEG correlates in POLG cases.

**Survival Analysis**

A minimum prevalence of 0.42 (95% CI 0.38–0.46) per 100,000 was calculated for patients experiencing stroke-like episodes in the general population of North East England. POLG patients in this study, had a significantly higher risk of early death (mean age [SD], 20.4 [10.6]; HR 3.9, 95% CI 2.2-7.0, \( p < 0.001 \)) than individuals affected by m.3243A>G (mean age [SD], 45.8 [14.9]) or other pathogenic mtDNA variants (mean age [SD], 48 [11.9]). Moreover, stroke-like episodes were a significant predictor of early death in both m.3243A>G (HR 12.3, 95% CI 5.8 – 26.5, \( p<0.001 \)) (Supplemental Table 8) and POLG (HR 11.6, 95% CI 4.7-28.2, \( p<0.001 \)) patients (Figure 2).

**Risk Prediction of Stroke-Like Episodes associated with m.3243A>G**

Univariate analysis found significant associations with eleven putative predictors of stroke-like episodes (Supplemental Table 9). After simplification and model assessment using Akaike Information Criterion (AIC) a multivariate Cox proportional hazard analysis identified body mass index (BMI) z score (HR 6.6, 95% CI 2.0 – 22.1, \( p=0.003 \)), corrected blood m.3243A>G heteroplasmy \( \geq 70\% \) (HR 7.2, 95% CI 0.9 – 55.5, \( p=0.06 \)), serum lactate > 2mmol/L (HR 3.7, 95% CI 1.4 – 9.9, \( p=0.01 \)) and NMDAS hearing subscore \( \geq 3 \) (indicative of SNHL severity (HR 2.0, 95% CI 0.8 – 4.74, \( p = 0.1 \)) as four risk predictors of stroke-like episodes among m.3243A>G carriers (Figure 3A; Supplemental Figures 4 and 5; Supplemental Table 9). To account for sample size and enable clinical application (\( n=170 \)), a three-level hierarchical construct was defined which identified those patients at low (\( n=89; 52\% \)), intermediate (\( n=60; 35\% \)) and high risk (\( n=21; 12\% \)) of stroke-like episodes throughout their lifetime (Figure 3B). This has allowed us to devise a 6-point prediction model for the absolute risk of developing a stroke-like episode in m.3243A>G carriers (high risk: 6 points; intermediate risk: 3-5 points; low risk: <2 points) with good overall discrimination (area under the curve: 0.87, 95% CI 0.82-0.93; c statistics: 0.89 (Supplemental Figure 6).

The time interval between two consecutive stroke-like episodes was recorded in 65 patients, median 1.33 year (Q1 = 0.34, Q3 = 3.2, IQR = 2.86); there was no statistically significant
difference in median interval time between events among mitochondrial DNA and POLG variants. An Anderson-Gill model of ordered multiple events demonstrated no significant differences in the baseline cumulative hazard for subsequent stroke-like episodes, following the occurrence of a first event, suggesting that while stroke-like episodes are paroxysmal clinical events, the reoccurrence of an episode is independent of the initial event (Supplemental Figures 7 and 8).

Neuropathological Findings

Reduced brain weights and focal cortical necrosis

Neuropathological findings of 26 patients (16 with documented ante-mortem stroke-like episodes) were summarised in Supplemental Table 10. Brain weights of patients affected by primary mtDNA disease were significantly decreased compared to those with recessive POLG variants and controls ($p=0.005$; Figure 4A). Macroscopic necrotic cortical lesions were evident upon brain dissection (Figure 4B. i. and ii.) with neuronal cell loss, ranging from selective neuronal dropout (Figure 4C. i.) to laminar necrosis (Figure 4C. ii.), was a common neuropathological finding identified across all genotypes. This was associated with myelin deposits localised within necrotic foci (Figure 4D. i.) which were immune-positive for phagocytosing macrophages (Figure 4D. ii.) while underlying white matter remained myelinated. Astrogliosis is increased within necrotic foci (Figure 4D. iii). The brain regions most affected by laminar necrosis included cerebellum (23/25; 92%), brainstem (16/24; 67%), temporal (14/25; 56%) and occipital lobes (13/25; 52%). Frontal and parietal lobes appeared less affected (7/24; 29%) (Supplemental Table 11).

Respiratory chain deficiencies affecting the microvasculature

Immunofluorescent analysis demonstrated arterioles and capillaries have a low density of mitochondria (as judged by porin; Figure 5A and 5B). Quadruple immunofluorescence showed reduced expression of OXPHOS subunits, complexes I and IV, relative to mitochondrial mass in microvessels from patients with primary mtDNA disease (Figure 5A and 5B). In smooth muscle, mitochondrial mass was increased and demonstrated an atypical clumped appearance in patients who harboured pathogenic mtDNA variants (including m.3243A>G variant), suggestive of mitochondrial aggregation that was not observed in control tissues (Figure 5A. i.). Quantification confirmed the endothelium harboured higher levels of respiratory chain deficiency than smooth muscle (Figure 5A. ii. and B. ii.).
Furthermore, the severity of respiratory chain dysfunction differed across different brain regions within the same patient, although this was particularly apparent in patients harbouring the m.3243A>G variant. Amongst this patient group, a hierarchy of regional dysfunction was observed with occipital cortex more severely affected than cerebellum, which was more severely affected than temporal cortex. However, the severity of respiratory chain defect did not correlate with the number of necrotic lesions (Supplement Table 12).

Upregulation of SDHA protein and very mild reduction of COXI protein in microvessels
Triple immunofluorescence confirmed that SDHA protein expression was upregulated in patient vessels compared to porin and COX1 protein expression levels (Supplementary figure 9A. i.). Quantification confirmed that z scores were either within normal range or extremely high in patients’ vessels (Supplementary Figure 9A. ii).

Neurons versus astrocytes versus microvessels
Immunofluorescent labelling of NDUFB8 and porin within neurons revealed high mitochondrial mass (as judged by porin; Figure 5C. i.) but variable levels of complex I subunit NDUFB8. Similar immunofluorescent labelling of astrocytes showed reduced levels of NDUFB8 and COXI in patients compared with controls (Figure 5C. ii.). To determine the extent of OXPHOS deficiency in neurons, astrocytes and microvessels, we used previously published data showing OXPHOS subunit expression in GAD65-67-positive interneurons and GFAP-positive astrocytes in the same cohort of patients. This analysis showed that neurons harbour greater percentage levels of OXPHOS deficiency relative to capillaries and arterioles (Figure 5C. ii.)

Discussion
Using a national cohort study and following well-established recommendations, we have developed a novel prognostic instrument to predict the risk of stroke-like episodes in individuals harbouring the pathogenic m.3243A>G variant. The multivariate model incorporates four items (BMI, blood lactate, age-corrected blood mtDNA heteroplasmy and sensorineural hearing loss severity) routinely available and easily applicable in clinical practice. Previous studies identified that patients with m.3243A>G-related MELAS have short stature and lower BMI, higher mtDNA heteroplasmy, and serum lactate levels.
in addition to more severe hearing loss compared to m.3243A>G carriers not manifesting with stroke-like episodes (non-MELAS). However, these studies only reported the differences in various parameters between MELAS and non-MELAS patients as singular entities, as opposed to the quantification of risk of their cumulative impact as we have attempted here. The availability of a simple tool to predict and stratify future risk of MELAS syndrome, will not only identify high-risk individuals and potentially enhance preventative strategies, but better inform patient counselling regarding their likely prognosis. Several stroke prediction models have been developed in disparate populations, but our prediction model demonstrates good overall discrimination and may pragmatically identify individuals with m.3243A>G-related mitochondrial disease at high risk of developing stroke-like episodes. These results, however, are only applicable in m.3243A>G-related mitochondrial disease, with other validated models widely recognised for other mechanistic forms of stroke.

Since the initial 2012 report, our study suggests the estimated minimum prevalence of MELAS syndrome is higher at approximately 0.42 per 100,000. Study design, genetic background, and population structure may, in part, explain this variability in prevalence. However, it is most likely due to improved awareness and case ascertainment. Almost one third of all m.3243A>G cases had their first stroke-like episode after the age of 40 years; this is in contrast with the original diagnostic criteria for this syndrome. Individuals harbouring POLG variants sustained their first stroke-like episode earlier in life (2nd decade versus 4th decade) had a more aggressive disease trajectory with a higher risk of death from status epilepticus, compared to patients with mtDNA-related MELAS syndrome who manifest with a more chronic disease course and died from predominantly non-CNS complications as reported elsewhere. Our cohort has a greater number of women harbouring pathogenic POLG recessive variants presenting with stroke-like episodes in their teens or early 20s, consistent with previous observations. It is intriguing that POLG disease comprises a continuum of clinical features including neonatal/infantile myocerebrohepatopathy spectrum, childhood/teenage-onset Alpers syndrome, teenage/young adult-onset ataxia neuropathy spectrum, myoclonic epilepsy, myopathy and sensory ataxia, adult-onset chronic progressive external ophthalmoplegia, with the phenotypic manifestations clustering with age. Indeed, our clinical experience (unpublished) would corroborate previous findings that patients with late-onset disease (>40 years) predominantly
present with chronic progressive external ophthalmoplegia, neuronopathy and cerebellar ataxia, whilst refractory epilepsy and stroke-like episodes are conspicuously rare.\textsuperscript{16, 50}

Approximately half of all \textit{POLG} cases had an explosive onset of overt, clinical seizures, and were otherwise asymptomatic, outside of CNS features, prior to first MELAS syndrome presentation. This was in contrast to m.3243A\textgreater{}G patients in whom preceding systemic features were almost universal. These manifestations should serve as ‘red flag’ symptoms for the potential development of stroke-like episodes in m.3243A\textgreater{}G-related mitochondrial disease. Ictal epileptic headaches (IEHs), recorded in three-quarters of individuals, often heralded the inception of a stroke-like episode with 75\% also exhibiting overt clinical seizure activity.\textsuperscript{54} IEHs were often accompanied by a crescendo of elementary visual hallucinations and blindness, consistent with occipital lobe predilection, irrespective of genotype.\textsuperscript{55, 56} Visual symptoms were almost invariably overlooked until more obvious clinical seizure activity, such as focal and/or generalised seizures, drowsiness and apathy of encephalopathy, ensued. This is likely to reflect the phenomenon of ictal spread\textsuperscript{57}, where subsequent epileptiform involvement of adjacent brain regions leads to clinical overshadowing of the original ictal focus. Extra-occipital manifestations, including temporal lobe seizure phenomena (encompassing autonomic and psychic peculiarities) and receptive or expressive speech deficits also arose. Limb focal motor deficits and limb apraxia, were incessantly subtle and often transient in nature. Detailed interrogation of seizure semiology often helped direct interpretation of clinical tests, including localization of the epileptogenic zone (particularly in cases of MRI negative focal epilepsy), predicted disease trajectory\textsuperscript{58}, and in rare cases advocated the use of novel interventions, as reported elsewhere.\textsuperscript{59}

MRI abnormalities related to stroke-like episodes involved both cortical and subcortical areas and demonstrated variable lesion reversibility, characteristic of seizure-induced signal changes on MRI.\textsuperscript{60-63} Radiological evidence of cross cerebellar diaschisis, the presence of the pulvinar sign\textsuperscript{64} and brain atrophy\textsuperscript{65} were indicative of prolonged seizure activity and favoured a particular pattern of seizure spread, including involvement of cortico-ponto-cerebellar pathways.\textsuperscript{66}

Our findings support core descriptions of relapsing and remitting stroke-like episodes, both clinically and radiologically, and for the first time, we have systematically investigated the interval between stroke-like episodes. We have demonstrated that the median time interval of
a second stroke-like episode was 1.33 year, and 25% of patients only experienced their second episode 3.2 years after the initial event. These findings highlight that the trajectory of stroke-like episodes is heterogeneous with important clinical implications. Clinicians, patients and their caregivers should be made readily aware of the nature of ictal phenomenology and risk of recurrent stroke-like episodes to ensure instigation of appropriate anticonvulsants. Moreover, the follow-up duration of any future trial of disease-modifying treatment for stroke-like episodes cannot be limited to ≤ 12 months based on the observation that half of the patients with MELAS syndrome only experience their second stroke-like episode 15 months after the first presentation.

Our neuroanatomical study represents the largest cohort reporting clinical and pathological correlates of stroke-like episodes in patients with mitochondrial disease. Post-mortem brain weights demonstrated marked reductions, particularly in patients harbouring pathogenic mtDNA variants versus patient with POLG-related disease, corroborating the disparity in tissue diminution observed on neuroimaging in our patient cohort. It is tempting to speculate that a more marked loss of brain weight in patients with mtDNA variants reflects their typically insidious onset and protracted course of disease, while patients with POLG-related MELAS have an explosive onset and rapid progression. Surprisingly, macroscopic neuropathological analysis of focal necrotic lesions were relatively innocuous compared to the striking nature of their neuroimaging alterations. Microscopically, they feature selective neuronal dropout to morphological changes of neurodegeneration, pan-cortical necrosis, astrogliosis, presence of microglial cells, blood brain barrier breakdown, and secondary axonal loss, which are now recognised pathological hallmarks of various forms of medically refractory focal epilepsy were homologous across genotypes.

While some of our findings are confirmatory of previous studies that stroke-like episodes are not primarily driven by a vascular mechanism, we have shown for the first time that neurons harbour more severe mitochondrial respiratory chain deficits than vessels. Furthermore, our neuropathological data do not support a causal role of microvascular impairments driving the formation of focal necrotic cortical lesions. Mitochondrial respiratory chain deficiencies, involving complexes I and IV, were evident and detected at low levels within endothelial and smooth muscle cells of microvessels, particularly in patients harbouring pathogenic mtDNA variants. This contrasts to previous COX/SDH histochemistry data that demonstrated overt COX-deficiency within the microvasculature. Our data support increased SDHA protein
levels within vessels which is not matched by either COX1 or porin protein expression; the
relevance of a selective increase in SDH activity and expression in microvessels is not
understood but does explain the apparent high levels of COX-deficiency. To measure the
contribution of angiopathic changes to neuronal necrosis, we compared mitochondrial
respiratory chain deficiencies within microvessels to the levels detected in inhibitory
interneuron and astrocytic populations. This provided clear evidence that the neuronal deficit
is more pronounced, and given the preponderance of inhibitory interneuron involvement provides further evidential support that these paroxysmal stroke-like events are seizure-
induced, and are not driven by angiopathic changes. Whether these paroxysmal events result
from concomitant dysfunctional neuroinflammatory response to various triggers, involving
the interaction of neuronal, vascular and innate immune processes, as in other refractory
seizure disorders, that is ultimately predicated on mitochondrial dysfunction remains to be
determined.

There are several potential sources of bias identified including the retrospective nature of
study design and cohort, variable data acquisition, inherent assumptions made for the
development of the Cox model, limitation of surface EEG ability to detect deep lying seizure
foci, potential sampling errors with the measurement of serum lactate, and the lack of
external validation of our prediction model. While other tissue heteroplasmy levels (including
muscle) were available, numbers were insufficient and precluded their use in the model.
Another potential limitation of the m.3243A>G- prediction model relates to ‘age’ that may
represent yet another factor in prognostication. The age profile of first stroke-like episode is
significantly different between m.3243A>G and POLG-related cases. Among 72 patients
who had m.3243A>G-related stroke-like episodes in our study, only 4 patients developed
their first stroke-like episode before the age of 18 years. Our findings and clinical experience
would suggest that m.3243A>G-related stroke-like episodes are uncommon in the paediatric
population. The cumulative risk of developing stroke-like episodes for individuals harbouring
the m.3243A>G variant may evolve over time, depending on the number of risk factors (as
illustrated in Figure 3B). However the cumulative risk barely changes over time if an
individual scores two or less in our prediction model, suggesting age is not an independent
risk predictor.

Given no murine model has yet been developed that fully recapitulates the complex
heterogeneity and pathogenesis of MELAS syndrome, the chronological analysis of pervasive
stereotypical clinical features, neuroimaging alterations, EEG abnormalities and neuropathological findings to provide clarity around mechanisms underlying stroke-like episodes pathogenesis and physiology is imperative in our quest for curative therapies, and were strongly supportive of pharmacological refractory seizures driving the clinico-radio-pathological correlates of stroke-like episodes and neurodegeneration across genotypes. In addition, we have pioneered the development of an tool to predict those individuals with genetically defined m.3243A>G-related mitochondrial disease, who are at high risk of developing stroke-like episodes and who may benefit from appropriate early clinical intervention. Our findings support the urgent need to revise current patient care standards, prioritising instigation of anti-convulsant therapy early in the treatment strategy.7
CONTRIBUTORS

YSN lead the data collection, data interpretation, clinical and radiological analyses, statistical analysis, prepared the figures and prepared the first draft. NZ-L and DE undertook the data collection, neuropathological data analysis and interpretation, prepared the figures, and critically reviewed and approved the final Article. AP-B performed the statistical analyses, prepared the figures and critically reviewed and approved the final Article. MB, ML, RG-W, AG and AW collected and reported EEG data, and approved the final Article. TP and CM-M performed the neuropathological study and approved the final Article. RT, JH, CF, ME-F, CH, MR, CL, AC, KM, TB, PM, CD, JP, and MH contributed to the data collection and approved the final Article. VN, RDS-P and AM-S contributed to the data collection, data interpretation, and critically reviewed and approved the final Article. EB contributed to data collection, genetic analysis and approved the final Article. RW-T, DM-T and RM obtained funding, contributed to data collection and interpretation, critically reviewed and approved the final Article. GSG led the design, supervised the data collection and interpretation, and writing, critically reviewed and approved the final Article, and obtained funding.

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COMPETING INTERESTS

The authors report no competing interests.
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Figure Legends

Figure 1. Stroke-like lesions identified on MRI head. Fluid-attenuated inversion recovery (FLAIR) sequence shows characteristic right occipital, temporal, parietal (A-i) and left parietal (A-ii) changes with restricted diffusion (A-iii&iv) and mixed ADC map changes (A-v&vi) in a 20 year old man with the m.3243A>G variant presenting with encephalopathy, occipital seizures, left hemianopia and apraxia. FLAIR sequence shows signal abnormalities involving left occipital lobe and pulvinar nucleus (B-i) and multiple, cortical signal abnormalities with restricted diffusion (B-ii&iii) in a 25 year old woman with recessive POLG variants presenting with right hemianopia and status epilepticus. (C) Diffusion weighted imaging (DWI) identifies bilateral signal abnormalities approximate to pre-central gyri in a 16 year old woman with recessive POLG variants presenting with refractory epilepsy partialis continua. FLAIR sequence demonstrates the phenomenon of cross-cerebellar diaschisis where signal abnormalities are present in right temporal and insular cortices (D-i) and contralateral cerebellar hemisphere (D-ii). Coronal view of FLAIR sequence demonstrates bilateral temporal lobe changes (E-i) at baseline, and the signal abnormalities in left mesial temporal lobe (hippocampus) evolved to involve the remaining temporal lobe on the interval scan a week later (E-ii) in a 34 year old man with the m.3243A>G variant presenting with headache, right hemianopia, global aphasia and focal motor seizures. Multiple, confluent signal abnormalities identified in both cerebral hemispheres (F) in a 24 year old woman with the m.3243A>G variant presenting with recurrent stroke-like episodes; only the signal abnormalities of left frontal lobe are associated with restricted diffusion (not shown) and there is evidence of generalised brain atrophy.

Figure 2. Kaplan-Meier Estimates of Survival. Panel (A) shows the number of patients affected by stroke-like episodes at risk of death in both mtDNA and POLG groups. Panel (B) shows the number of patients at risk of death in all individuals who harboured the m.3243A>G variant. Panel (C) shows the number of patients at risk of death in all individuals who harboured the recessive POLG variants.

Figure 3. Multivariate analyses of predictors for stroke-like episodes among individuals who harbouring the m.3243A>G variant. (A) Forest plot of the odds ratios (with 95% CI) for the four risk predictors of stroke-like episodes among m.3243A>G carriers. Corrected blood m.3243A>G heteroplasmy is derived from the methods detailed elsewhere.73 (B)
Cumulative incidence of stroke-like episodes among the carriers of m.3243A>G variant according to their risk: high risk indicates the presence of all four risk predictors (6 points), intermediate the presence of three predictors (3-5 points), with low risk indicating between zero and two risk predictors (0-2 points). There are much fewer patients aged>60 years in our study that may impact on the risk estimation.

**Figure 4. Reduced brain weight and focal cortical necrosis are prominent across all genotypes.** Post-mortem brain weights (g) were significantly lower in patients with primary mtDNA disease relative to those harbouring POLG variants and control individuals (p=0.005) particularly in those harbouring mtDNA variants (A). Macroscopic necrotic cortical lesions were evident in temporal lobe of patient 3 (B. i.; m.3243A>G), and frontal cortex (BA9) of patient 14 (B. ii.; POLG). Scale bars = 1cm. Stages of lesions in the temporal cortex range from selective laminar dehiscence (C. i.; cresyl fast violet stain, CFV) to total necrosis of the temporal cortex in patient 3 (C. ii.; CFV) relative to normal cortex in a control (C. iii.; CFV). Evidence of intact myelination under the cortex of laminar dehiscence with myelin deposits in the lesioned grey matter in patient 3 (D. i.; myelin basic protein), these deposits also label with macrophages which may be phagocytosing damaged myelin (D. ii.; CD-68). Astrogliosis is observed circumferential to necrotic lesions in patient 3 (D.iii.; GFAP). Scale bars = 100µm.

**Figure 5. Immunofluorescence showed mitochondrial OXPHOS deficiencies evident in microvessels, neurons and astrocytes in the occipital cortex.** Control arterioles (α-SMA; blue) demonstrated matched protein expression of OXPHOS subunits for complexes I (NDUFB8; magenta) and IV (COX1; green) relative to mitochondrial mass (porin; red; A. i.). Arterioles in patients with primary mtDNA disease featured atypical “clumping” of mitochondria (A. i.) and downregulation of NDUFB8 (magenta) and COX1 (green) proteins relative to porin (red) and therefore a higher percentage level deficiency (A. ii.), while no such alterations were observed in POLG patients. Control capillaries (GLUT-1; blue) have lower mitochondrial mass compared to the neuropil and do not show loss of OXPHOS subunit expression for complexes I (NDUFB8; magenta) and IV (COX1; green) relative to mitochondrial mass (porin; red; B. i.). Capillaries in patients demonstrated decreased NDUFB8 (magenta) and COX1 (green) expression relative to porin (red; B. ii.). Quantification showed a higher percentage of deficiency, particularly in patients with primary mtDNA disease (B. ii.) Scale bars = 14 μm. Immunofluorescent labelling of
NDUFB8 (green) and porin (red) proteins showed neurons contain high mitochondrial mass
with clear deficiencies of complex I (Patient 23 (POLG1; C. i.; complex I deficient neurons
shown by asterix). Scale bar = 100μm. Immunofluorescent labelling of astrocytes (C. ii.
GFAP = blue) and their mitochondria (magenta), NDUFB8 (red) and COXI (green) in patient
and controls show reduced OXPHOS expression. Scale bar = 10μm. Quantification of
NDUFB8 and COXI within inhibitory interneurons (C.iii.; GAD65-67-positive neurons; red)
and astrocytes (GFAP-positive astrocytes; yellow) reveals high percentage levels of
deficiency compared to microvessels (capillaries (GLUT1); green) and arterioles (α-SMA);
blue).
Figure 1

269x172mm (300 x 300 DPI)
Figure 2

174x276mm (300 x 300 DPI)
Figure 3

190x258mm (300 x 300 DPI)
Figure 4

264x195mm (300 x 300 DPI)
Figure 5

299x237mm (200 x 200 DPI)
Table 1 Summary of the differences between mtDNA- and POLG-related stroke-like episodes

| Demographic data       | mtDNAa | POLG  | p value |
|------------------------|--------|-------|---------|
| N                      | 88     | 23    | -       |
| Male : Female          | 46:42  | 5:18  | 0.009   |
| No. of family pedigrees| 80     | 23    | -       |
| Mean age of 1st stroke-like episode (SD) (95% CI) | 35.2 [14.6] (32.0 – 38.4) | 18.3 [25.6] (11.2 – 25.4) | <0.001 |
| Mean age of last follow up (SD) (95% CI)       | 42.6 [13.3] (39.6 – 45.6) | 22.1 [14.7] (15.5 – 28.5) | <0.001 |
| Death                  | 34 (39%) | 17 (74%) | 0.017 |
| Mean age of death (SD) (95% CI)                | 46.5 [14.4] (41.2 – 51.8) | 23 [17.5] (12.9 – 33.1) | <0.001 |

| Features of stroke-like episodes                  | mtDNAa | POLG  | p value |
|---------------------------------------------------|--------|-------|---------|
| Headache                                          | 51/68  (75%) | 9/12  (75%) | 0.894   |
| Nausea/vomiting                                   | 15/29  (52%) | 5/7   (71%) | 0.500   |
| Positive visualb                                  | 37/67  (55%) | 7/17  (41%) | 0.329   |
| Negative visual                                   | 42/72  (58%) | 10/19 (53%) | 0.628   |
| Focal weakness                                    | 30/65  (46%) | 6/14  (43%) | 0.704   |
| Dysphasia                                         | 28/55  (51%) | 1/11  (9%)  | 0.048   |
| Sensory disturbance                               | 14/46  (30%) | 3/14  (21%) | 0.689   |
| Acute hearing loss                                | 8/46  (17%) | 0/10  | 0.286   |
| Confusion/drowsiness                              | 57/68  (84%) | 12/18 (67%) | 0.234   |
| Neuropsychiatricc                                 | 29/53  (55%) | 4/13  (31%) | 0.263   |
| Seizures                                          |        |       |         |
| Motor seizures                                    | 60/95  (63%) | 27/28 (96%) | 0.007   |
| Occipital seizures                                | 38/73  (52%) | 7/21  (33%) | 0.234   |
| Status epileptic                                  | 19/87  (22%) | 27/28 (96%) | 0.007   |

| Neuroimaging changes                              |          |       |         |
|---------------------------------------------------|----------|-------|---------|
| Frontal                                           | 26/113  (23%) | 13/32 (41%) | 0.051   |
| Insular                                           | 24/113  (21%) | 1/32  (3%)  | 0.042   |
| Parietal                                          | 80/113  (71%) | 16/32 (50%) | 0.053   |
| Temporal                                          | 90/113  (80%) | 5/32  (16%) | 0.004   |
| Occipital                                         | 86/113  (76%) | 20/32 (63%) | 0.167   |
| Thalamus                                          | 7/113   (6%)  | 11/32 (34%) | 0.004   |
| Cross-cerebellar                                  | 13/113  (12%) | 8/32  (25%) | 0.139   |

| EEG findings (acute)                              |          |       |         |
|---------------------------------------------------|----------|-------|---------|
| Encephalopathy                                    | 114/118 (97%) | 65/89 (78%) | 0.036   |
| Epileptic discharge\d                             | 66/120  (55%) | 67/89 (75%) | 0.002   |
| PLEDs                                             | 7/35   (20%) | 21/66 (38%) | 0.163   |

p values were adjusted where appropriate for multiple comparisons. ADC= apparent diffusion coefficient. BG= basal ganglia. CI= confidence interval. EEG= electroencephalogram. GTCS= generalised tonic-clonic seizures. N/A= not applicable. NCSE= non-convulsive status epilepticus. PLED= periodic lateralising epileptic discharge. SD= standard deviation. SLE= stroke-like episode.

\textsuperscript{a}The most common pathogenic mtDNA variant associated with stroke-like episodes is the m.3243A>G variant (72/88; 82%). There are no statistical differences in clinical, radiological and EEG data between cases of m.3243A>G and other mtDNA pathogenic variants, and therefore they are analysed collectively as one “mtDNA” category.

\textsuperscript{b}The details of positive visual symptoms are catalogued in Supplemental Table 3.

\textsuperscript{c}The range of neuropsychiatric symptoms included agitation and aggressiveness, severe anxiety, psychosis and behavioural changes reported by the family.

\textsuperscript{d}More details about the location of epileptic discharge are available in Supplemental Table 7.
Table 2 Clinical manifestations before the emergence of first stroke-like episode

|                | mtDNA* | POLG         | p value |
|----------------|--------|--------------|---------|
| No pre-existing symptom | 6/75 (9%) | 9/20 (45%) | 0.005   |
| Deafness       | 45/74 (61%) | 0/20        | 0.005   |
| Ataxia         | 4/74 (5%) | 6/20 (30%)  | 0.007   |
| Headache       | 16/73 (22%) | 0/20        | 0.053   |
| Diabetes mellitus | 20/75 (27%) | 1/20 (5%)  | 0.076   |
| Renal          | 6/74 (8%)  | 0/20        | 0.269   |
| Gut dysmotility | 10/73 (14%) | 1/20 (5%)  | 0.318   |
| Seizures       | 10/74 (14%) | 3/20 (15%) | 0.864   |
| Exercise intolerance/myopathy | 6/73 (8%)  | 0/20        | 0.269   |
| Cardiac        | 4/73 (6%)  | 0/20        | 0.318   |
| Renal          | 6/74 (8%)  | 0/20        | 0.269   |

p values were adjusted where appropriate for multiple comparisons.

*There are no statistical differences in clinical data between cases of m.3243A>G and other mtDNA pathogenic variants, and therefore they are analysed collectively as one "mtDNA" category.