Delineating MT-ATP6-associated disease
From isolated neuropathy to early onset neurodegeneration

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Abstract

Objective
To delineate the phenotypic and genotypic spectrum in carriers of mitochondrial MT-ATP6 mutations in a large international cohort.

Methods
We analyzed in detail the clinical, genetical, and neuroimaging data from 132 mutation carriers from national registries and local databases from Europe, USA, Japan, and China.

Results
We identified 113 clinically affected and 19 asymptomatic individuals with a known pathogenic MT-ATP6 mutation. The most frequent mutations were m.8993 T > G (53/132, 40%), m.8993 T > C (30/132, 23%), m.9176 T > C (30/132, 23%), and m.9185 T > C (12/132, 9%). The degree of heteroplasmy was high both in affected (mean 95%, range 20%–100%) and unaffected individuals (mean 73%, range 20%–100%). Age at onset ranged from prenatal to the age of 75 years, but almost half of the patients (49/103, 48%) became symptomatic before their first birthday. In 28 deceased patients, the median age of death was 14 months. The most frequent symptoms were ataxia (81%), cognitive dysfunction (49%), neuropathy (48%), seizures (37%), and retinopathy (14%). A diagnosis of Leigh syndrome was made in 55% of patients, whereas the classic syndrome of neuropathy, ataxia, and retinitis pigmentosa (NARP) was rare (8%).

Conclusions
In this currently largest series of patients with mitochondrial MT-ATP6 mutations, the phenotypic spectrum ranged from asymptomatic to early onset multisystemic neurodegeneration. The degree of mutation heteroplasmy did not reliably predict disease severity. Leigh syndrome was found in more than half of the patients, whereas classic NARP syndrome was rare. Oligosymptomatic presentations were rather frequent in adult-onset patients, indicating the need to include MT-ATP6 mutations in the differential diagnosis of both ataxias and neuropathies.

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ATP6 Study Group co-investigators are listed in the appendix 2 at the end of the article.

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Glossary

LS = Leigh syndrome; mtDNA = mitochondrial DNA; NARP = neuropathy, ataxia, and retinitis pigmentosa; NGS = next-generation sequencing; SARA = scale for assessment and rating of ataxia.

The MT-ATP6 gene of the mitochondrial DNA (mtDNA) (further named ATP6) encodes a subunit of the F1F0ATP-synthase complex, a key enzyme of mitochondrial energy metabolism.

Variants in ATP6 have long been recognized as a cause of mitochondrial disease.1 Since the first description of a pathogenic m.8993 T > G variant in 1992,1 more than 500 cases of ATP6-associated disease have been reported.

The initially described phenotype is today referred to as the syndrome of neuropathy, ataxia, and retinitis pigmentosa (NARP). A more severe form of ATP6-associated disorders, traditionally associated with high degrees of mutation heteroplasmy,2,3 is maternally inherited Leigh syndrome (LS), with early onset psychomotor developmental delay and symmetrical lesions in basal ganglia and brainstem, often leading to premature death.4 With the advent of next-generation sequencing (NGS), less typical manifestations have been observed, e.g., nonsyndromic sensorimotor neuropathy;5 any combinations of ataxia, neuropathy, diabetes mellitus, and hypergonadotropic hypogonadism;6 and mitochondrial myopathy, lactic acidosis, and sideroblastic anemia.7 Hence, the phenotypic spectrum of ATP6-associated mitochondrial disorders seems much broader than previously assumed. So far, no systematic study on the clinicogenetic spectrum of an extended cohort has been performed.

We report a cohort of 132 ATP6 mutation carriers from 11 countries, delineate phenotype and genotype, and evaluate the role of heteroplasmy as a disease modifier.

Methods

Probands and design

This is a multicenter, retrospective, cross-sectional cohort study. Probands with known pathogenic ATP6 variants were identified from the registry of the German network for mitochondrial disorders (mitoNET, n = 22) and 33 further diagnostic and research centers from Europe (n = 55), the USA (n = 6), China (n = 37) and Japan (n = 18).

For data collection, patients’ physicians were asked to complete a questionnaire which includes signs, symptoms, and laboratory parameters. Detailed clinical histories of individual patients are provided in table e-1, links.lww.com/NXG/A213. Examinations and diagnostic procedures were performed and evaluated by experienced medical professionals (neurologists, pediatricians, child neurologists, pathologists, radiologists, and clinical geneticists).

Review of the literature

A systematic review of the literature was conducted by searching PubMed for all articles published from 1990 with the terms “ATP6” and “ATPase6.”

Variants classified as confirmed disease-associated and reported mutations in MITOMAP (mitomap.org) were included in the analysis, for a total of 502. We reviewed the reported variants for the range of heteroplasmy in affected individuals and unaffected carriers, age at onset and phenotypic association (table e-2, links.lww.com/NXG/A214).

Molecular genetics

Individuals were screened for ATP6 mutations by using different approaches depending on the local facilities and year of analysis, including targeted single-gene sequencing, high-throughput panel sequencing, or whole-exome sequencing.

Levels of heteroplasmy in patients were assessed in leukocytes (n = 114), skeletal muscle (n = 20), urine (n = 23), and fibroblasts (n = 5).

Statistical analysis

Datasets were collected and organized in Microsoft Excel and LibreOffice Calc and subjected to a basic descriptive analysis. Deeper descriptive and explorative data analysis as well as statistical testing was then performed in R (version 3.5.1) and SPSS (version number 25). A group analysis of parametric data was performed by one-way analysis of variance (ANOVA) for comparison of means or by Welch test when Levene test for homogeneity of variances failed. A post hoc group-wise analysis was performed with Tukey Honestly Significant Difference test. Although ANOVA is considered comparatively robust to the violation of the assumption of normality, we additionally performed Kruskal-Wallis test with a post hoc group-wise analysis with Wilcoxon rank sum test as a nonparametric alternative when Shapiro-Wilk test for normal distribution of residues failed. Analysis of nominal and ordinal data was performed with χ² test where applicable, and Fishers exact test was used where the sample size did not permit for evaluation with χ² test. The significance level was corrected with the Bonferroni method for multiple comparisons with p = 0.00094.

Standard protocol approvals, registrations, and patients consents

All biological materials (tissue samples including blood, urine, skin, muscle, and nerve and derivatives including DNA extracted from tissues and cell lines), brain MRI scans, and medical and neurophysiologic reports were obtained with written informed consent of the probands, their parents, or legal guardians. The local institutional review boards approved the study.
Results

Our cohort of 132 mutation carriers consisted of 113 patients and 19 asymptomatic relatives. Twenty-three cases have been able on request by a qualified investigator. Requests should be made to Thomas Klopstock.

Data availability

Anonymized data presented in this study will be made available on request by a qualified investigator. Requests should be made to Thomas Klopstock.

Phenotypic spectrum

Clinical findings in our cohort are summarized in figure 3 and table e-1, links.lww.com/NXG/A213.

LS was the most frequent phenotype (62/112; 55%). In this subgroup, nearly half of the patients (30/62; 48%) harbored the m.8993 T > G variant, followed by m.9176 T > C (14/62; 23%), m.8993 T > C (12/62; 19%), and m.9185 T > C (5/62; 8%) and 1 patient with the rare variant m.8969 G > A. Most of the patients with LS became symptomatic in infancy and early childhood (55/60; 92%); oldest age at onset was 16 years (patient 29-1). In addition, 20 children showed clinical symptoms compatible with the diagnosis of LS, but brain MRI was either not available (11/20; 55%) or MRI findings were distinct from classic LS, showing no basal ganglia or brainstem lesions but delayed myelination (3/20; 15%), cerebellar atrophy/microcephaly (3/20; 15%), or no pathology at all (3/20; 15%). Only 9 patients in our cohort exhibited a NARP phenotype (8%), 6 of those with additional signs (e.g., cognitive dysfunction, global developmental delay, and seizures). In 6/9 patients (67%), the degree of heteroplasmy was 100%, whereas it was lower in the other 3 (90%, 80%, and 59%, respectively).

Most patients evaluated for cerebellar symptoms showed cerebellar ataxia (68/84; 81%), 26% of those in combination with a sensory ataxia (18/68; 26%). One patient showed signs of sensory ataxia without having additional cerebellar signs. Symptoms in 16% of adult patients (18/112) were rated using
Peripheral neuropathy was suspected in 36 of 40 (90%) patients assessed for clinical symptoms and signs of peripheral nerve dysfunction; in 19 patients (19/40, 48%), nerve conduction studies and EMG confirmed the diagnosis. One patient without clinically apparent neuropathy showed neurophysiologic findings of a peripheral neuropathy. Neuropathy was predominantly axonal and sensory in 10 patients, whereas in the other 9 patients, detailed electrophysiologic data were not available.

Any form of cognitive dysfunction was reported in 49% (43/87) of the patients, ranging from mild learning disabilities to severe mental retardation or secondary cognitive decline.

Epileptic seizures were reported in 37% (35/95) of patients, mostly in Leigh or Leigh-like syndrome (30/35, 86%) but also in NARP syndrome (4/9, 44%).

Overall, 14 of 70 patients (20%) with an ophthalmologic assessment showed (nonmuscular and noncerebellar) ocular symptoms. Ten patients (10/70, 14%) had a retinopathy, mostly (9/10, 90%) in the context of NARP syndrome. Other ocular symptoms included optic atrophy in 4/70 (6%) and cataract in 3/70 (4%) patients.

Cardiac symptoms were reported in 11/80 patients (14%). Three had cardiac arrhythmia, which manifested as Wolff-Parkinson-White syndrome in 2 (carrying the mutations m.9185 T > C and m.8993 T > C). Six patients had cardiomyopathy.
One patient (85-1) carrying the m.9176 T > C variant had a chronic progressive external ophthalmoplegia as a first sign, which is unusual in reported ATP6 patients.

We examined correlation of distinct genotypes with the occurrence of specific phenotypes or symptoms. A Leigh/Leigh-like syndrome (43/47; 91%) was more common in patients harboring the m.8993 T > G variant (22/39; 56%) than in patients carrying other variants. No other pathogenic variant appeared to be predictive of distinct symptoms or signs (table 1). There was, however, a tendency toward a higher proportion of seizures in patients with the m.8993 T > G variant (22/39 patients, 56%) as compared with patients with other variants (12/51 patients, 24%) (p = 0.0021) and a trend toward lower proportion of cognitive dysfunction in patients with the m.9176 T > C variant (4/21 patients, 19%) as compared with patients with other variants (34/59 patients, 58%) (p = 0.0044).

Twelve of our patients manifested in adulthood were monosymptomatic or oligosymptomatic and had a relatively mild disease course. One patient showed isolated sensorimotor neuropathy (patient 2-3) and another had pure cerebellar ataxia (patient 96-1). Six patients exhibited signs of neuropathy plus cerebellar ataxia, spasticity, or cognitive dysfunction. Two adult patients presented with a NARP phenotype (patients 25-1 and 28-1).

Neuroimaging data
Brain MRI findings in ATP6 patients are presented in figure 4, A–F. Brain MRI data were available for 85 patients, which showed abnormalities in 81 (95%) patients. Basal ganglia and brain stem lesions, usually found in classic LS, were reported in 65% (55/85) and 32% (27/85) of the patients, respectively, with 22% (19/85) patients showing both alterations. Cerebellar atrophy was present in 16% (14/85), and cortical/subcortical atrophy was identified in 13% (11/85). Other less frequent signs were white matter lesions in 5 patients, delayed myelination in 4 patients, and microcephaly in 2 patients. Brain MRI was reported normal in 4 patients.

In 20 cases, muscle biopsy specimens were studied histologically, and evaluation was either normal (9/20 cases) or disclosed unspecific changes (11/20 cases). Ragged red fibers were found only in 1 adult patient carrying a m.9035 T > C mutation who presented with ataxia in his late 50s and displayed no clinical signs of myopathy.

Discussion
Our study presenting the currently largest series of patients with mitochondrial disease has 2 major results: First, it broadens the clinical spectrum of ATP6-associated mitochondrial disorders...
and shows that the clinical spectrum is more heterogeneous than previously reported. Second, it shows that the degree of mutation heteroplasmy does not strictly correlate with disease severity, with homoplasmic mutations found not only in LS but also in milder cases and even asymptomatic probands.

The phenotypic spectrum in our cohort ranged from asymptomatic mutation carriers to fatal, early onset, and multisystemic neurodegenerative disease with severe disability. Most affected children presented with an overall homogeneous Leigh or Leigh-like syndrome phenotype (73%; 82/112) with developmental delay and hypotonia as first signs. One exception is patient 29-1 who showed first symptoms at the age of 16 years with acute brainstem dysfunction with dysarthria, dysphagia, tetraparesis, and ophthalmoplegia, followed by respiratory insufficiency and coma. Brain MRI revealed extensive lesions in the basal ganglia, brainstem, and cerebellum. Patient 85-1 showed isolated chronic progressive external ophthalmoplegia at the age of 10 years, followed by respiratory and cardiac failure. Brain MRI revealed Leigh-like abnormalities in the dorsal midbrain and hypothalamus. About half of the deceased patients in our cohort, mostly severely affected children, carried the mutation m.8993 T > G. Together with the higher proportion of seizures in the m.8993 T > G group, this implies that the m.8993 T > G variant might be associated with a more severe course of disease.14

Only 9 patients in our cohort (8%) presented with a NARP phenotype, and 6 of those showed severe additional signs and symptoms such as epilepsy, cognitive dysfunction, delayed myelination, and Leigh-like changes. This low frequency of the NARP phenotype was unexpected because NARP has not previously been considered typical for Leigh syndrome. Later onset showed a significant correlation with a larger delay until the molecular diagnosis was established. One obvious explanation for this finding is that there is a clear need for a fast and broad diagnostic workup if the disease starts early in life and takes a life-threatening course such as in children with LS, whereas staged diagnostic approaches for milder presentations such as neuropathy and ataxia rarely include ATP6 diagnostics. To avoid this unnecessary delay in diagnosis, ATP6-associated mitochondrial disorders should be considered in monosymptomatic or oligosymptomatic presentations that have not previously been considered typical for ATP6-associated disease, such as isolated neuropathy or ataxia. The ATP6 gene should be included in routine NGS panel diagnostics for the differential diagnosis of both ataxias and neuropathies and comprehensive genetic testing via whole-exome sequencing should cover mtDNA in addition to nuclear DNA in a clinical diagnostic setting.16

Clinical variability between patients with the same genotype was high in our cohort, not only interfamilial but also intrafamilial. Of note, the monozygotic twin sisters 3-1 and 3-2, both harboring the homoplasmic m.8993 T > C mutation show very different disease courses. Patient 3-1 showed a severe gait ataxia because of a combination of sensorimotor polyneuropathy and cerebellar ataxia starting at age 2 years and has been has been using a wheelchair from the age of 16 years (SARA score 18.5/40), whereas her sister was only slightly affected with mild gait ataxia from the age 7 of years. She was able to walk unaided at age 16 (SARA score 3/40).

### Table 1 Comparison of the frequency of phenotypes and symptoms among carriers of different MT-ATP6 mutations

| Variant                  | Leath/Leigh-like syndrome | LS   | Ataxia | Cognitive dysfunction | Neuropathy | Seizures | Retinitis pigmentosa | Cardiac symptoms |
|--------------------------|---------------------------|------|--------|-----------------------|------------|----------|---------------------|-----------------|
| m.8993T > G vs m.8993T > C, m.9176T > C, m.9185T > C | 0.0005a | 0.4274a | 0.7711a | 0.022a | 0.0019a | 0.0021b | 1.0b | 0.4732b |
| m.8993T > C vs m.8993T > G, m.9176T > C, m.9185T > C | 0.1126a | 0.3492a | 0.3329b | 0.7931a | 0.2742b | 0.8011a | 0.4375b | 0.6771b |
| m.9176T > C vs m.8993T > G, m.9185T > C | 0.1126a | 0.8146a | 0.1025a | 0.0044a | 0.6618b | 0.0191a | 0.1868b | 0.6771b |
| m.9185T > C vs m.8993T > G, m.9176T > C | 0.2593b | 0.7370b | 0.19212 | 1.0b | 0.0473b | 0.3084b | 0.6255b | 0.2878b |

Abbreviation: LS = Leigh syndrome.

p values of χ² Pearson or Fishers exact are shown. Significance level was defined as p < 0.0009 after Bonferroni correction for multiple testing.

a Chi square Pearson (2-sided p values).

b Fishers Exact (2-sided p values).
A generally accepted preconception about mitochondrial disease because of mtDNA variants is a direct correlation of symptom severity to degree of heteroplasmy. It is widely believed that a percentage of heteroplasmy >90% for the m.8993 T > C/G variant leads to LS, whereas a heteroplasmy load between 70% and 90% results in a NARP phenotype.17,18 This idea has recently been challenged.19 Our findings of a high grade of heteroplasmy in the asymptomatic carrier group (7/17 ≥ 90%, 4/17 homo-plasmic for known pathogenic mutations) and the observation of moderate degrees of heteroplasmy in several early onset symptomatic patients support the view that heteroplasmy load alone is insufficient to explain disease severity. For instance, patient 54-1 presenting with a LS showed onset in infancy and harbored the m.8993 T > C variant at moderate degrees of heteroplasmy (68% in leukocytes and 65% in urine cells). By contrast, the asymptomatic mother (proband 59-2) of a patient with LS harbored the same variant even at higher degrees of heteroplasmy (78% leukocytes and 88% urine cells). In 3 asymptomatic carriers harboring the m.8993 T > G (proband 49-1) and the m.9176 T > C (proband 69-3, 69-4) variants, the high degrees of heteroplasmy have to be interpreted with caution because they are children and might still develop symptoms later in life.

Degrees of heteroplasmy in our multicentric study were determined in different tissues, at different ages, and with different methods, warranting careful interpretation. However, age differences in measurements of heteroplasmy may have a negligible impact at least for the m.8993 variant because it has been shown that heteroplasmy of m.8993 T > G/C does not change significantly over time.20 Moreover, we found a very high correlation of degrees of heteroplasmy between different tissues in 21 probands (table e-1, links.lww.com/NXG/A213).

Further modifying factors determining disease manifestation may include the mtDNA background, which has been shown to play an important role in modulating the biochemical defects and clinical outcome.21 For example, the variants m.8741 T > G and m.8795 A > G have been reported to be
protective factors for the m.8993 T > G mutation causing a LS.\textsuperscript{22} mtDNA copy number may be another modifying factor of disease severity because it has recently been shown for the m.3243 A > G mutation,\textsuperscript{23} but so far, there are no data on this in ATP6-associated disorders. Furthermore, ATP6 variants may not be fully penetrant, even when homoplasmic.\textsuperscript{8}

In essence, our study demonstrates that ATP6 variants cause a continuous disease spectrum rather than a group of distinct clinical syndromes. Early onset was associated with a more severe course of disease, especially in patients carrying the m.8993 T > G variant. Onset after the age of 6 years leads to a considerable delay in diagnosis, most likely because of monosymptomatic and oligosymptomatic presentations, especially in the adult group. Including ATP6 mutation screening in routine diagnostics for neuropathy and ataxia will benefit patients regarding a prompter and correct diagnosis and genetic counseling.

Our observations strongly support the notion that the degree of heteroplasmy alone cannot reliably predict disease severity. Further studies are needed to identify factors that modulate prognosis.

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**Disclosure**
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## Appendix 1 (continued)

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## Appendix 1 (continued)

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Continued
### Appendix 2 (continued)

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