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Mitochondrial DNA and disease

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Abstract
The small circle of mitochondrial DNA (mtDNA) present in all human cells has proven to be a veritable Pandora's box of pathogenic mutations and rearrangements. In this review, we summarize the distinctive rules of mitochondrial genetics (maternal inheritance, mitotic segregation, heteroplasmy and threshold effect), stress the relatively high prevalence of mtDNA-related diseases, and consider recent additions to the already long list of pathogenic mutations (especially mutations affecting protein-coding genes). We then discuss more controversial issues, including the functional or pathological role of mtDNA haplotypes, the pathogenicity of homoplasmic mutations and the still largely obscure pathophysiology of mtDNA mutations.

Key words: *Haplotypes, heteroplasmy, homoplasmy, maternal inheritance, mitochondrial DNA, mtDNA*

Introduction

Mitochondrial DNA (mtDNA) is a fossil molecule proving that endosymbiosis did occur, when – about 1.5 billion years ago – protobacteria populated primordial eukaryotic cells and took permanent residence in the new environment (1). Unlike a fossil, however, mtDNA has lost its independence but not its life and it keeps functioning (sometimes malfunctioning, which is the reason for this article) under the overarching control of the nuclear genome.

Human mtDNA (Figure 1) is a 16,569-kb circular, double-stranded molecule, which contains 37 genes: 2 rRNA genes, 22 tRNA genes, and 13 structural genes encoding subunits of the mitochondrial respiratory chain, which is the 'business end' of oxidative metabolism, where ATP is generated (2). Reducing equivalents produced in the Krebs cycle and in the ß-oxidation spirals are passed along a series of protein complexes embedded in the inner mitochondrial membrane (the electron transport chain), which consists of four multimeric complexes (I to IV) plus two small electron carriers, coenzyme Q (or ubiquinone) and cytochrome c (Figure 2). The energy generated by the reactions of the electron transport chain is used to pump protons from the mitochondrial matrix into the space between the inner and outer mitochondrial membranes. This creates an electrochemical proton gradient, which is utilized by complex V (or ATP synthase), a tiny rotary machine that generates ATP as protons flow back into the matrix through its membrane-embedded F_0 portion, the rotor of the turbine (3).

Starting in 1988, when mutations in mtDNA were first associated with human disease (4,5), the circle of mtDNA has become crowded with pathogenic mutations, and three principles of mitochondrial genetics should, therefore, be familiar to the practicing physician.

1. Heteroplasmy and threshold effect. Each cell contains hundreds or thousands of mtDNA copies, which, at cell division, distribute randomly among daughter cells. In normal tissues, all mtDNA molecules are identical (homoplasmy). Deleterious mutations of mtDNA usually affect some but not all mtDNAs within a cell, a tissue, an individual (heteroplasmy), and the clinical expression of a pathogenic mtDNA mutation is largely determined by the relative proportion of normal and mutant genomes in different tissues. A minimum critical number of mutant mtDNAs is required to cause mitochondrial dysfunction in a particular organ or tissue and mitochondrial disease in an individual (threshold effect).

2. Mitotic segregation. At cell division, the proportion of mutant mtDNAs in daughter cells may shift and the phenotype may change accordingly. This

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Abbreviations

ATP	adenosine triphosphate
CK	creatine kinase
COX	cytochrome *c* oxidase
cyt *b*	cytochrome *b*
KSS	Kearns-Sayre syndrome
LHON	Leber hereditary optic neuropathy
LS	Leigh syndrome
MELAS	mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes
MERRF	myoclonus epilepsy and ragged-red fibers
MILS	maternally inherited Leigh syndrome
MRS	nuclear magnetic resonance spectroscopy
MtDNA	mitochondrial DNA
NARP	neuropathy, ataxia, retinitis pigmentosa
ND	NADH dehydrogenase
PCR	polymerase chain reaction
PEO	progressive external ophthalmoplegia
PS	Pearson syndrome
rRNA	ribosomal RNA
RRF	ragged-red fibers
tRNA	transfer RNA
SDH	succinate dehydrogenase

Key messages

- Mitochondrial DNA (mtDNA) is a Pandora's box of pathogenic mutations potentially affecting every organ of the body. mtDNA-related diseases are usually transmitted by non-Mendelian, maternal inheritance.
- Pathogenic mutations of mtDNA can be divided into two main groups, those affecting mitochondrial protein synthesis *in toto*, and those affecting individual proteins of the respiratory chain.
- The functional and possible pathogenic significance of mtDNA haplotypes is under intense investigation. Homoplasmy does not rule out pathogenicity.
- Although we have learnt a lot about mtDNA mutations, the pathophysiology of mtDNA-related diseases remains largely unknown.

phenomenon, called mitotic segregation, explains how certain patients with mtDNA-related disorders may actually shift from one clinical phenotype to a different one as they grow older.

3. Maternal inheritance. At fertilization, all mtDNA derives from the ovum. Therefore, the mode of transmission of mtDNA and of mtDNA point mutations (single deletions of mtDNA are usually sporadic events) differs from Mendelian inheritance. A mother carrying a mtDNA point mutation will pass it on to all her children (males and females), but only her daughters will transmit it to their progeny. A disease expressed in both sexes but with no evidence of paternal transmission is strongly suggestive of a mtDNA point mutation.

Over 150 point mutations and innumerable large-scale rearrangements have been associated with a bewildering variety of diseases (Figure 1). This is not surprising when one considers that mitochondria are ubiquitous organelles and all human tissues, in isolation or in various combinations, can be affected by mtDNA mutations (6). This concept is illustrated in Table I, a compilation of symptoms and signs described in mitochondrial encephalomyopathies due to single rearrangements (KSS, PS, and PEO), to point mutations in genes affecting protein synthesis *in toto* (MELAS; MERRF), and to a protein-coding gene (NARP and MILS). This table highlights the clinical features of the most common syndromes, which are difficult to miss in typical patients. It is also a reminder that any combination of these symptoms and signs ought to alert the astute clinician to the possibility of a mtDNA-related disorder. A third function of the table is to serve as a concise descriptor of the six most common mtDNA-related syndromes, which will not be described here in any more detail. If more details are needed, the reader is referred to textbook reviews (6).

As shown in Table II, mtDNA-related disorders fall into two major groups: 1) those due to mutations in genes involved in mitochondrial protein synthesis, and 2) those due to mutations in genes encoding individual proteins of the respiratory chain. Clinical features are not terribly useful in differentiating the two groups, but laboratory results, muscle biopsy, and muscle biochemistry are better discriminators and offer useful clues for a targeted molecular analysis. In defects of protein synthesis, lactic acidosis is seen more consistently and tends to be higher, and muscle biopsies almost invariably show RRF, which are characteristically COX-negative (except in typical MELAS, where RRF stain with the COX reaction, though not intensely so). Not

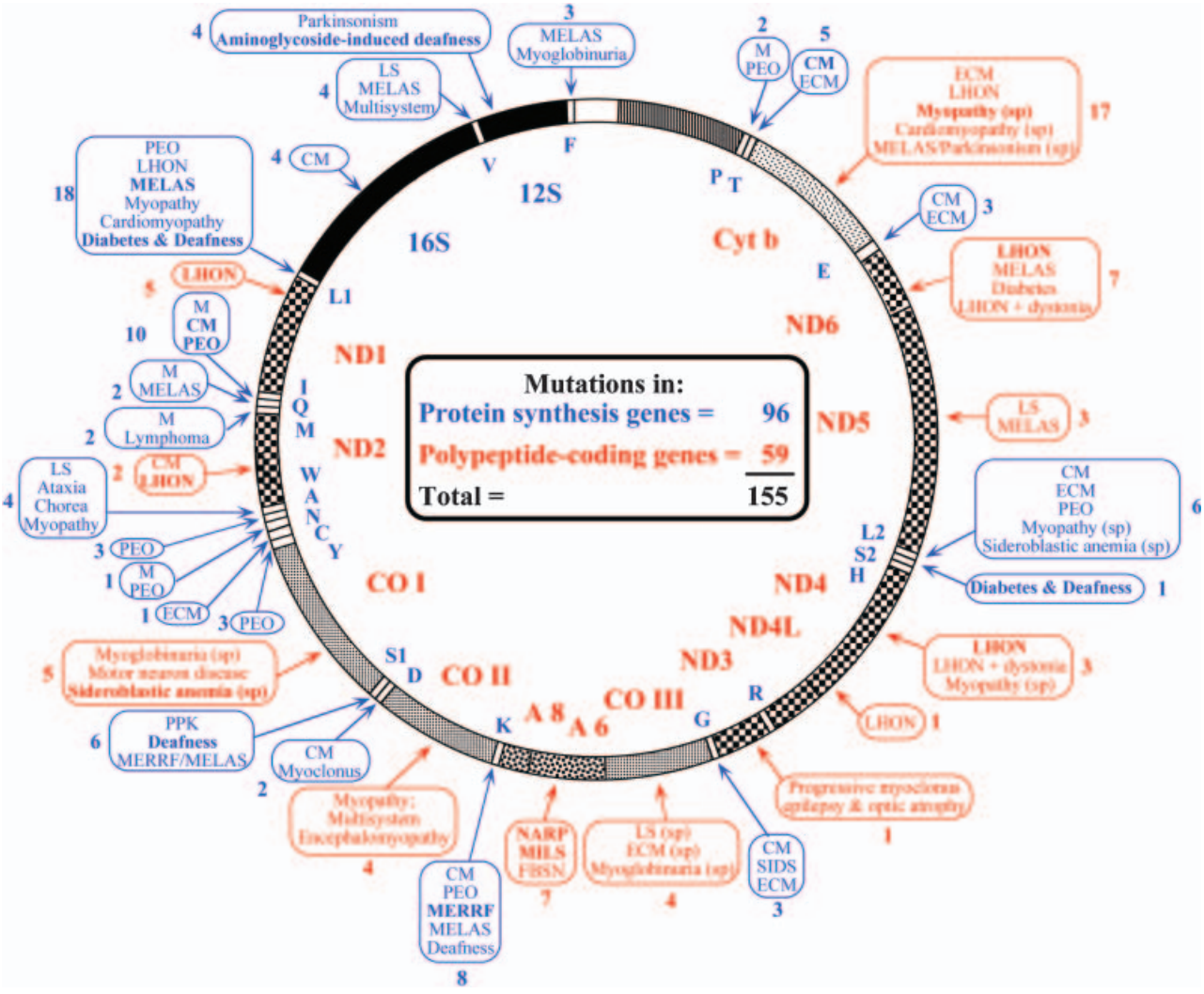

Figure 1. Morbidity map of the human mitochondrial genome. The map of the 16,569 bp mtDNA shows differently colored areas representing the protein-coding genes for the seven subunits of complex I (ND), the three subunits of cytochrome *c* oxidase (CO), cytochrome *b* (cyt b), and the two subunits of ATP synthase (A6 and A8), the 12S and 16S rRNAs (12S, 16S), and the 22 tRNAs identified by one-letter codes for the corresponding amino acids. See list of abbreviations. FBSN=familial bilateral striatal necrosis.

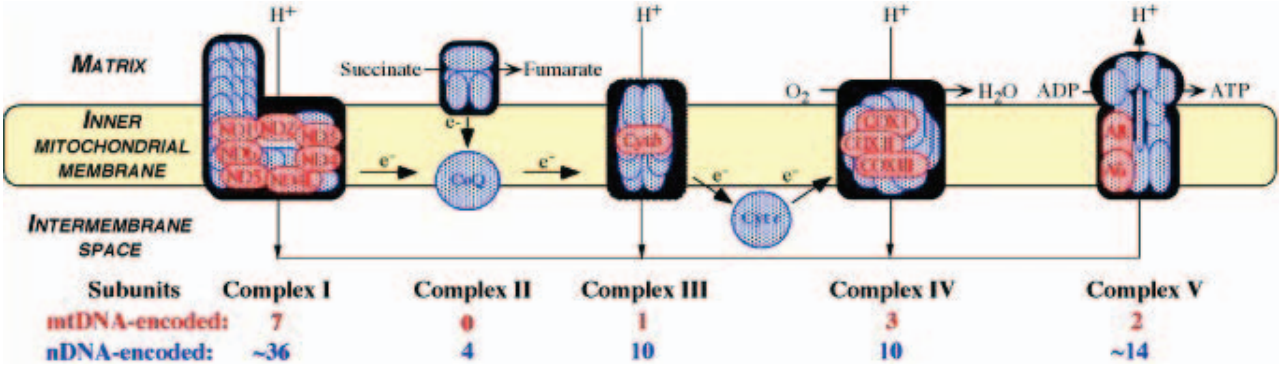

Figure 2. Schematic representation of the respiratory chain. Subunits encoded by mtDNA are in blue and subunits encoded by nuclear DNA are in red. Electrons (e-) flow along the electron transport chain, and protons (H+) are pumped from the matrix to the intermembrane space through complexes I, III, and IV, then back into the matrix through complex V, producing ATP. Coenzyme Q (CoQ) and cytochrome *c* are electron carriers.

Table I. Clinical features in mitochondrial diseases associated with mtDNA mutations. Clinical features of mtDNA-related diseases. Boxes highlight typical features of specific syndromes (except Leigh syndrome, which is defined by the neuroradiological or neuropathological alterations). CNS=central nervous system; PNS=peripheral nervous system; GI=gastrointestinal system; ENT=ear-nose-throat. Δ-mtDNA denotes deleted mtDNA. Other abbreviations are explained in the List of Abbreviations.

		Δ-mtDNA		tRNA		ATPase	
Tissue	**Symptom/sign**	**KSS**	**Pearson *n***	**MERRF**	**MELAS**	**NARP**	**MILS**
CNS	Seizures	−	−	+	+	−	+
	Ataxia	+	−	+	+	+	±
	Myoclonus	−	−	+	±	−	−
	Psychomotor retardation	−	−	−	−	−	+
	Psychomotor regression	+	−	±	+	−	−
	Hemiparesis/hemianopia	−	−	−	+	−	−
	Cortical blindness	−	−	−	+	−	−
	Migraine-like headaches	−	−	−	+	−	−
	Dystonia	−	−	−	+	−	+
PNS	Peripheral neuropathy	±	−	±	±	+	−
Muscle	Weakness	+	−	+	+	+	+
	Ophthalmoplegia	+	±	−	−	−	−
	Ptosis	+	−	−	−	−	−
Eye	Pigmentary retinopathy	+	−	−	−	+	±
	Optic atrophy	−	−	−	−	±	±
	Cataracts	−	−	−	−	−	−
Blood	Sideroblastic anemia	±	+	−	−	−	−
Endocrine	Diabetes mellitus	±	−	−	±	−	−
	Short stature	+	−	+	+	−	−
	Hypoparathyroidism	±	−	−	−	−	−
Heart	Conduction block	+	−	−	±	−	−
	Cardiomyopathy	±	−	−	±	−	±
GI	Exocrine pancreatic dysfunction	±	+	−	−	−	−
	Intestinal pseudo-obstruction	−	−	−	−	−	−
ENT	Sensorineural hearing loss	−	−	+	+	±	−
Kidney	Fanconi's syndrome	±	±	−	±	−	−
Laboratory	Lactic acidosis	+	+	+	+	−	±
	Muscle biopsy: RRF	+	±	+	+	−	−
Inheritance	Maternal	−	−	+	+	+	+
	Sporadic	+	+	−	−	−	−

Table II. Clinical, morphological, and biochemical features of mtDNA-related disorders.

Type	**Mutation**	**CLINICAL**	**LA**	**RRF**	**BIOCHEMISTRY**
Mutations affecting mitochondrial protein synthesis *in toto*	Single deletions	KSS	+	+(COX-)	⇓ I, III, IV
		PEO	+	+(COX-)	⇓ I, III, IV
		PS	−	−	
	tRNA mutations	MELAS.	+	+(COX)+	⇓ I, ↓ III, IV
		MERRF & other multisystemic	+	+(COX-)	⇓ I, III, IV
		myopathy	+	+(COX-)	⇓ I, III, IV
		LHON	−	−	↓ I (+/−)
	ND genes	MELAS, LS	+	+/−(COX+)	⇓ I
		myopathy	+/−	+(COX+)	⇓ I
Mutations in protein-coding genes	Cyt b	multisystemic	+/−	+(COX+)	⇓ III
		myopathy	+	+(COX+)	⇓ III
	COX genes	multisystemic	+/−	+/− (COX-)	⇓ IV
		myopathy	+	+(COX-)	⇓ IV
	ATPase 6 gene	NARP/MILS	+/−	−	⇓ V

surprisingly, given the impaired synthesis of all 13 mtDNA-encoded subunits, biochemical analysis of muscle shows combined defects of all complexes containing such subunits, which are easily demonstrable for complexes I, III, and IV, but more difficult to document for complex V, which is not measured routinely. In contrast, the biochemical finding of a specific respiratory chain deficiency is a strong indicator that a mutation may be present in one of the mtDNA genes encoding subunits of that particular complex. Until a few years ago, it was believed that RRF were not seen in disorders due to mutations in protein-coding genes. This belief was based on our experience with LHON (due to mutations in three ND genes) and NARP/MILS (due to mutations in the ATPase 6 gene), but was proven erroneous by studies of patients with myopathy or multisystem disorders and mutations in ND, cyt b, or COX genes. In contrast to the COX-negative RRF typical of defects of protein synthesis, the RRF in these patients stain intensely both with the SDH stain and with the COX stain (7), except, of course, for patients with mutations in COX genes (8).

There have been some practical improvements in our diagnostic armamentarium, such as the observation that urinary sediment cells – obtainable more easily and less invasively than blood samples – are more sensitive than blood cells for the detection and quantitation of the A3243G mutation in oligosymptomatic MELAS patients (9,10). This is almost certainly true for other mtDNA mutations, although large series are not yet available. Another useful test in patients with MELAS and in their oligosymptomatic or asymptomatic relatives is proton magnetic resonance spectroscopy (MRS) of the brain, which allows the measurement of cerebrospinal fluid (CSF) lactate non-invasively. In a large cohort, there was a good correlation between cerebral lactic acidosis – estimated by ventricular MRS lactate levels – and severity of neurological and neuropsychological impairment (11). The rest of this review will be devoted to new and controversial issues regarding the pathology of mtDNA mutations.

Frequency of mtDNA-related diseases

A flurry of epidemiological studies in recent years (reviewed by Schaefer et al. (12)) has confirmed the notion that mitochondrial diseases – long considered of purely academic interest – are, in fact, among the most common genetic disorders and a major burden for society. When studies in children and adults are combined and both nuclear DNA and mtDNA mutations are considered, the minimum prevalence is at least 1 in 5,000 (12). To focus on patients with mtDNA mutations, a study of adult patients in northern England had shown an overall prevalence of 6.57/100,000 (13), with a particularly high prevalence of LHON (3.22/100,000) (14). An apparent discrepancy between the high prevalence of the MELAS-3243G-related symptoms in northern Finland (5.71/100,000) (15) and a much lower prevalence (0.95/100,000) in northern England (13), was apparently due to an underestimation of the affected English population (12). Two epidemiological studies of affected children have been conducted in antipodal countries, Sweden (16) and Australia (17). The results were remarkably similar, both in terms of overall prevalence of mitochondrial diseases (about 5/100,000) and in terms of prevalence of mtDNA-related disorders, which accounted for about 15% of the total.

Inheritance of single mtDNA deletions

By and large, single mtDNA deletions are neither inherited from the mother nor transmitted to the progeny and disorders due to single mtDNA deletions, KSS, PEO, and PS, are almost always sporadic. This phenomenon is attributed to a 'bottleneck' between the ovum (where the giant deletion probably occurs) and the embryo, such that only a small minority of maternal mtDNA populates the fetus (2). However, a few cases of maternal transmission have been reported (18–21), raising the question of how reassuring can we be in counseling carriers of single mtDNA deletions. A recent multicenter retrospective study of 226 families has confirmed that the risk of a carrier woman to have affected children is very small, but finite (1 in 24 births) and has disproved the idea that the risk increases with maternal age (22).

Pathogenic mutations in mtDNA: Recent arrivals

Although the small circle of mtDNA is getting saturated with pathogenic mutations, it is true now – as it was 5 years ago (23) – that we are not yet scraping the bottom of the barrel. Novel mutations are still being reported, especially in protein-coding genes, albeit at a slower pace. A first flurry of new mutations in protein-coding genes was associated with the all-too-frequent but often elusive syndrome of exercise intolerance (with or without episodic myoglobinuria). In retrospect, it was surprising that these symptoms, long associated with defects in the

utilization of the two major 'muscle fuels', glycogen and fatty acids, had not been seen in defects of the respiratory chain, the quintessential energy-yielding pathway (24). Within a few years, numerous patients with exercise intolerance were found (or rediscovered) to have mutations in genes encoding subunits of complex I (25–27), complex IV (8,28,29) and especially in the one mtDNA gene for complex III, *cyt b* (7,30–36). One reason that delayed the identification of these patients is that these mutations are *de novo* events occurring in myoblasts or myoblast precursors after germ-layer differentiation: thus, contrary to the 'rules' of mitochondrial genetics, these patients are sporadic and the disease is confined to skeletal muscle (7). As an unfortunate clinical consequence, these patients are often misdiagnosed as having chronic fatigue syndrome, fibromyalgia rheumatica or conversion syndrome. As a matter of curiosity, one of these mutations, a microdeletion in the *ND2* gene (27), generated a furor in mitochondrial circles not in and by itself, but because it led to the discovery that most skeletal muscle mtDNA in this patient was of paternal origin. This revolutionary finding was shown to be the proverbial exception that confirms the rule (37–39) but the unique coexistence of paternal and maternal mtDNA in the same tissue made it possible to document that mtDNA molecules can recombine (40).

More recently, another protein-coding gene has been the object of much attention and has reached 'hotspot' status, *ND5*. Several mutations have been described, all associated with maternally inherited multisystemic disorders (Table III). One mutation (G13513A) seems to be particularly common and causes MELAS (41,42), LS (43–45) or MELAS/LHON overlap (46). Five other mutations in the same gene have been associated with MELAS (47,48), MELAS/LS (49), MELAS/MERRF (50) and even a three-way overlap syndrome, MELAS/LS/LHON (48). Muscle biopsy in these patients occasionally shows RRF or pre-RRF (fibers with subsarcolemmal rims of hyperintense SDH stain) that are invariably COX-positive (Table III).

Two recently reported mutations are of special interest because of their unusual clinical phenotypes. The first, G12147A in $tRNA^{His}$, was described in a previously normal 19-year-old man, who had an unusually dramatic presentation, with stroke following a seizure and complicated by severe brain edema leading to emergency temporal lobectomy (51). The stroke was preceded by rhabdomyolysis (serum CK 37, 880 IU/L) and further complicated by Reye-like liver failure. The take-home message here is that some mtDNA mutations can debut in a very stormy fashion, suggesting, as in this young man, venous infarction or encephalitis. The second mutation, G611A in $tRNA^{Phe}$, is unusual in that it presented in a young woman with the clinical phenotype of MERRF (52), which thus far has been associated only with mutations in the $tRNA^{Lys}$ gene (53).

The question of homoplasmy

Because mtDNA mutates spontaneously at a high rate and most changes are neutral polymorphisms, a situation exploited in forensic medicine (54,55), a set of canonical rules has been established to prove the pathogenicity of a novel mtDNA mutation. First, the mutation should not be present in normal individuals of the same ethnic group. Second, it should alter a site conserved in evolution and, therefore, functionally important. Third, it should cause single or multiple respiratory chain enzyme deficiencies in affected tissues or defects of mitochondrial protein synthesis and respiration demonstrable in cybrid cell lines. Fourth, there should be a correlation between degree of heteroplasmy and clinical severity as well as a correlation between

Table III. Clinical syndromes, abundance of RRF, and complex I deficiency in patients with mutations in the ND5 gene. B, brain; L, liver; M, muscle.

Mutation	Syndrome	RRF	Complex I	Reference
G13513A	MELAS	+(COX+)	46% (B); 52% (L)	(41)
	MELAS/LHON	5% (COX+)	'isolated defect' (M)	(46)
	MELAS	1% (COX+)	42% (M)	(42)
	LS	0	35% (M)	(43)
	LS	n.d.	partial defect'' (M)	(44)
	LS	0	25% (M)	(45)
A13514G	MELAS	0-several	40%–60% (M)	(47)
A13084T	MELAS/LS	0	85% (M)	(49)
A12770G	MELAS	0	normal	(48)
A13045C	MELAS/LHON/LS	0	'mildly reduced' (M)	
G13042A	MELAS/MERRF	0	15%–57% (M)	(50)

degree of heteroplasmy and cell pathology (best documented by single fiber PCR).

As the last criterion implies, a corollary of these guidelines is that pathogenic mutations are usually heteroplasmic whereas neutral polymorphisms are homoplasmic. In general this is true, but there are many exceptions and an increasing awareness of the possible or documented pathogenicity of homoplasmic mutations. In fact, the first point mutation (G11778A in *ND4*) associated with a human disease, LHON, was homoplasmic (5), as are other mutations causing LHON (56). Similarly, most non-syndromic forms of deafness are due to homoplasmic mutations, including A15555G in the 12S rRNA gene (57), and two mutations in the $tRNA^{Ser(UCN)}$ gene, A7455G (58) and T7511C (59). A homoplasmic mutation (A4300G) in $tRNA^{Ile}$ caused maternally inherited hypertrophic cardiomyopathy in two families (60), and a homoplasmic mutation (C1624T) in $tRNA^{Val}$ caused multiple neonatal deaths and LS in the offspring of an also homoplasmic but mildly affected woman (61). The T14709C mutation in the $tRNA^{Glu}$ gene, typically associated with myopathy and diabetes (62), attained homoplasmy in several members of one large family, some of whom – strangely – were asymptomatic (63). The latest 'cause celèbre' of homoplasmy regards a large pedigree in which the 'metabolic syndrome' (syndrome X or dyslipidemic hypertension), which includes various combinations of central obesity, atherogenic dyslipidemia, hypertension, hypomagnesemia, and insulin resistance, was transmitted maternally and attributed to a homoplasmic mutation (T4291C) in the $tRNA^{Ile}$ (64). Because the metabolic syndrome afflicts about one fourth of the US population, this finding did not go unnoticed (65). The challenge with this, as with other homoplasmic mutations, is to go beyond the association and to document a deleterious functional effect. Nuclear magnetic resonance spectroscopy (MRS) and muscle biopsy were performed in a single 55-year-old member of the family with the metabolic syndrome. MRS showed decreased ATP production and a few pre-RRF were seen in the biopsy – rather meager evidence of mitochondrial dysfunction, especially considering the age of the patient studied.

Most of the canonical criteria for pathogenicity listed above do not apply to homoplasmic mutations, except for biochemical evidence of respiratory chain dysfunction or direct evidence that the expression of mutated genes is impaired. Defective ATP production due to distinct respiratory chain dysfunctions has been documented in cybrid cells harboring different LHON mutations (66,67), and high resolution Northern blots have shown decreased steady-state levels of $tRNA^{Ile}$ in the heart of patients with the A4300G mutation (60), and decreased levels of $tRNA^{Glu}$ in muscle of patients with the T14709C mutation (63). Similar studies are necessary to document a pathogenic role of the T4291C mutation in patients with the metabolic syndrome.

Another major question underlying the pathogenic mechanism of homoplasmic mtDNA mutations is why they are expressed in some family members but not in others and why they can result in different clinical phenotypes. At least four factors can influence phenotypic expression: environmental factors, mtDNA haplotype, nuclear DNA background, and tissue-specific expression of interacting genes. The importance of environmental factors is exemplified by the deleterious effect of aminoglycoside exposure in triggering deafness in carriers of the A1555G mutation (57,68). The influence of mtDNA haplotype was shown by the higher penetrance of the A7455G mutation in a family that also harbored three 'secondary' LHON mutations (69). The influence of nuclear background was illustrated by the different severity of complex I deficiency in two cybrid cell lines, both homoplasmic for the A3460G LHON mutation, but derived from different rho^0 cells (osteosarcoma and lung) (70).

The importance of mtDNA haplotypes

In their migration out of Africa, human beings have accumulated distinctive variations from the mtDNA of our ancestral 'mitochondrial Eve', resulting in several haplotypes characteristic of different ethnic groups (71). It has been suggested that different mtDNA haplotypes may modulate oxidative phosphorylation, thus influencing the overall physiology of individuals and predisposing them to, or protecting them from, certain diseases (71). Among the functional characteristics reportedly influenced by mtDNA haplotypes are intelligence quotient (IQ) (72), spermatozoa swiftness (73) and adaptation to cold climates (74). Among the diseases, cardiomyopathy (75), Alzheimer disease and dementia with Lewy bodies (76), and multiple sclerosis (77) have been associated with specific mtDNA haplotypes. Also, patients with LHON and certain mtDNA haplogroups have a higher risk of developing blindness (78), whereas no link has been established between the variable phenotypic expression of MELAS-3243 and mtDNA haplotypes (79). In fact, a recent retrospective multicenter study of patients with MELAS and the A3243G mutation (80) has also failed to confirm a previously reported association between a polymorphic variant (A12308G) and

increased risk of stroke (81). Clearly, much work remains to be done to better define both the pathogenic role of homoplasmic mutations and the modulatory role of haplotypes in health and disease.

Pathogenesis

A recent review article by Neil Howell on mitochondrial diseases was aptly subtitled: 'answering questions and questioning answers' (78). Seventeen years after the discovery of pathogenic mutations in mtDNA, we have lots of answers, i.e. molecular causes, but precious little understanding of how the different molecular defects cause different syndromes. In fact, it is surprising that mtDNA mutations should cause different syndromes in the first place. If, as conventional wisdom dictates, mtDNA rearrangements and point mutations in rRNA or tRNA genes impair mitochondrial protein synthesis and ATP production, it would be logical to expect a clinical swamp of ill-defined and overlapping symptoms and signs, as was originally predicted by the 'lumpers' (82). Although clinical overlap does occur in mtDNA-related diseases (see above), it is fair to say that the 'splitters' won the day in that most mutations result in well defined and rather stereotypical syndromes.

One major obstacle to functional studies of mtDNA mutations is the lack of animal models due to the still unsurmountable problem of introducing mtDNA into the mitochondria of mammalian cells. An alternative approach has been to use cybrid cells, that is, established human cell lines first depleted of their own mtDNA then repopulated with various proportions of mutated genomes (83). This technique has largely confirmed the prediction that single deletions or mutations in tRNA genes impair respiration and protein synthesis and decrease ATP production (84). Elegant as it is, the *in vitro* cybrid system cannot replace animal models in understanding clinical expression. To be sure, two transmitochondrial mice or 'mito-mice' were obtained through clever, if circuitous, stratagems, one harboring mtDNA deletions (85) and the other harboring a point mutation for cloramphenicol resistance (86). Although these animal 'prototypes' are important proofs of principle (87), there has been no recent progress in this area.

In an attempt to explain the distinctive brain symptoms in patients with KSS, MERRF, and MELAS, the different mutations have been 'mapped' indirectly through immunohistochemical techniques. Consistent with clinical symptomatology and laboratory data, immunocytochemical evidence suggests that the 3243-MELAS mutation is abundant in the walls of cerebral arterioles (Tanji and Bonilla, unpublished), the 8344-MERRF mutation is abundant in the dentate nucleus of the cerebellum (88), and both the MELAS mutation and single deletions abound in the choroid plexus (89). However, these data do not explain what 'directs' each mutation to a particular area of the brain.

Finally, mutations in different tRNA genes may have different mechanisms of action, as suggested by the selective tissue vulnerability associated with mutations in certain tRNAs: for example, cardiopathy is often associated with mutations in $tRNA^{Ile}$; diabetes is a frequent manifestation of the T14709C mutation in $tRNA^{Glu}$ (62) and multiple lipomas have been reported only in patients with mutations in $tRNA^{Lys}$ (53). But, again, these are associations, not explanations. It is fair to conclude that the pathogenesis of mtDNA-related disorders is still largely *terra incognita*.

This review does not exhaust the subject of the title, 'mitochondrial DNA and disease'. We have only considered disorders whose primary causes are well-defined mtDNA mutations, but presumably secondary mtDNA alterations are also involved in aging and neurodegenerative disorders (90) – a vast chapter that deserves a separate review. Nor have we considered therapy of mtDNA-related disorders, which is still woefully inadequate and largely palliative. However, at least at an experimental level, interesting strategies are being developed, mostly aimed at shifting downward the percentage of mutant mtDNAs in affected tissues (91,92). Because the pathogenic threshold of most mtDNA mutations is both high and steep, a small shift in heteroplasmy may result in a disproportionate clinical benefit. There is a glimmer of light at the end of the present therapeutic tunnel.

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