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Review

20 years of human mtDNA pathologic point mutations: Carefully reading the pathogenicity criteria

Julio Montoya [a,b,c,*], Ester López-Gallardo [a,b,c], Carmen Díez-Sánchez [a,b], Manuel J. López-Pérez [a,b,c], Eduardo Ruiz-Pesini [a,b,c,d]

[a] Departamento de Bioquímica, Biología Molecular y Celular, Universidad de Zaragoza, Spain
[b] CIBER de Enfermedades Raras (CIBERER), ISCIII, Spain
[c] Instituto Aragonés de Ciencias de la Salud, Spain
[d] Fundación ARAID, Miguel Servet 177, 50013-Zaragoza, Spain

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ABSTRACT

Despite the strong purifying selection that occurs during embryonic development, the particular location and features of mitochondrial DNA make it especially susceptible to accumulating point mutations, giving rise to a large number of mitochondrial DNA variants. Many of these will have moderate or no phenotypic effects but others will be the cause of very dramatic diseases, usually known as mitochondriopathies. Because of the abundance of different mitochondrial DNA variants, it is not easy to determine whether a new mutation is pathogenic. To facilitate this task, different criteria have been proposed, but they are often either too severely or too loosely applied. Citing examples from the literature, in this paper we discuss some critical aspects of these criteria.

1. Introduction

The first discoveries that open a new scientific field are usually the most obvious and they determine the bases of a new area of research. However, as the knowledge of the new field of research advances, the new findings defy the previous conceptions. Thus, very useful criteria have been proposed in the field of mitochondrial DNA (mtDNA) diseases [1–3] (Table 1), to decide whether a newly found point mutation in this genome is pathogenic and, therefore, if it is the cause of a disease. Nevertheless, there are many exceptions to the rule, and this makes it essential that we should be extremely careful in the application of these criteria and maintains an open mind in defining the pathogenicity of a new mutation, in order to progress in this field. In this paper we hope to create a general overview of these criteria and to discuss several examples found in the literature that will yield some clues on how to apply these criteria.

2. Mitochondria are unpopulated suburbs

Eukaryotic cells derive from an event of endosymbiosis. One of the organisms that participated in this endosymbiosis, the one that gave rise to mitochondria, was similar to an alpha-proteobacterium. Some of the genes contained in these protomitochondria, the redundant ones, were removed, and many others migrated to the nucleus. Thus, the DNA that remained in the mitochondria is much smaller than the bacterial genome [4]. There are several reasons for this reduction in size. The migration of the mitochondrial genes to the nucleus could represent an important evolutionary advantage due to the possibility of recombination. However, this does not explain why, in the mtDNA, there are almost no non-coding nucleotides between the different genes, and no introns within the genes, or why some of the genes overlap and several stop codons are not encoded in the mtDNA and have to be originated after polyadenylation of their corresponding mRNAs. Moreover, mtDNA-encoded genes are smaller than those encoded in the nuclear DNA (nDNA) or in bacteria [4]. A very extreme situation is found in the mitochondrial ribosomes. Bacterial and cytosolic ribosomes have a 2/1 ratio in the rRNA/protein proportion but this ratio is reversed—1/2—for the mitochondrial ribosomes. Very long segments of the MT-RNR genes have disappeared and their functions have been taken over by new proteins encoded in the nuclear chromosomes [5]. Thus, we might conclude that most of the neighbors in the suburbs died or moved downtown and that this is still happening, giving rise to many mitochondrial pseudogenes or numts [6]. The remaining genes decreased their size and removed every unessential nucleotide, providing an example of genetic economy. A possible explanation for the permanence of a genetic system within the mitochondria is that these genes are required for the correct functioning of the organelle, offering the quickest, finest and most local regulation [7], or because this allows the co-evolution of genes involved in the same function [8].

* Corresponding author. Departamento de Bioquímica, Biología Molecular y Celular, Miguel Servet 177, 50013-Zaragoza, Spain. Tel.: +34 976761640; fax: +34 976761612.
E-mail address: jmontoya@unizar.es (J. Montoya).

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Table 1
Pathogenicity criteria for mtDNA point mutations

* The mutation must be present in patients and absent in controls.
* The mutation must be found in different mitochondrial genetic backgrounds.
* The mutation must be the best mtDNA candidate variant to be pathologic.
* The percentage of the mutation must correlate with the phenotype.
* The mutation must affect highly evolutionary conserved nucleotides.
* The mutation must affect functionally important domains.
* The transference of the mutated mtDNA to another cell line must be accompanied by the transference of the cell or molecular defect.

Human mtDNA is exclusively inherited through the maternal lineage. Mitochondria from spermatozoa penetrate to the ovum but they are selectively marked with ubiquitin and apparently removed [9]. It has been recently found that even before the elimination of the spermatozoa mitochondria, the mtDNA is degraded [10]. Spermatozoa are germinal cells but the behavior of their mitochondria is similar to that of somatic cells. They are very active [11] and produce many mutagenic reactive oxygen species (ROS). Thus, by removing the paternal mtDNA, the possibility of transmitting mtDNA mutations decreases enormously. In fact, the only known human paternal contribution to the next generation is associated with a pathologic mutation in the mtDNA [12].

All these observations suggest that staying in the mitochondria, for most genes, was not evolutionarily advantageous.

3. Why are these suburbs so hostile?

mtDNA is located in the mitochondrial matrix close to the inner membrane where the electron transport chain (ETC) is embedded. The ETC is the main source of ROS in the cell and these compounds promote mutations in the mtDNA. In recognizing the importance of ROS for the cell, we must also note that some antioxidant enzymes necessary for the elimination of ROS, such as superoxide dismutase and catalase, are catalytically perfect, meaning their limiting factor is not the velocity of the enzyme but the chance to find the substrate [13]. The mutational process induced by ROS is facilitated because this genome is not covered with histones, although there are other proteins such as mtTFA organizing mitochondrial nucleoids. Moreover, mtDNA can replicate more than once in the cell cycle and, during replication, long segments of mtDNA may remain as single strands, allowing the formation of secondary structures that may hamper the progress of the DNA polymerase and promote the appearance of new mtDNA mutations. Conversely, the repair systems for mtDNA are not as abundant as those for nDNA [14]. For all these reasons, mtDNA may accumulate mutations at a higher rate than nDNA. Interspecific comparisons suggest that mtDNA may fix mutations 5 to 10 times faster than nDNA genes [15] but intraspecific comparisons have found even higher mutational rates. Furthermore, pedigree analysis has found rates 10 times higher than in the phylogenetic studies [16].

Since 92.7% of the mtDNA sequence encodes genes, most of the mutations in this genome will affect an mtDNA gene. Further, the non-coding segments contain regulatory sequences for replication and transcription and, very likely, mutations in these regions will also have phenotypic effects [17].

4. Frequent mtDNA pathologic point mutations

The frequency of mtDNA diseases is high. It has been estimated that one out of approximately 8000 individuals harbors a pathogenic mtDNA mutation [18]. However, only a few mtDNA pathologic point mutations account for the majority of cases (Table 2). Thus, the m.3243A>G and m.8344A>G mutations are at least twice as prevalent as all the other mtDNA point mutations known to cause disorders involving the central nervous system [19]. Moreover, only around half of the MT-ND and MT-ATP mutations have been reported in more than one pedigree, and practically all the MT-CYB and MT-CO mutations are unique to one patient or one family [20].

Why are these seven mutations of Table 2 so frequently found in comparison with other pathologic mutations [21]. One possibility is an evaluation bias since these mutations were among the first discovered and it is quite possible that they have been more frequently checked for. However, many other pathologic mutations reported during those years have rarely been found again. For example, the m.14459G>A LHON mutation was reported in 1994 [22]. Since then, we have checked this transition in 373 patients, negative for the most common mutations and suspected of suffering LHON, and none of them had this mutation. Another possibility is that scientific journals are not interested anymore in already-reported mtDNA mutations. However, the first pathologic point mutation to be described [23] and many other common mtDNA mutations continue being reported nowadays. An interesting common feature of these mutations is that they are usually associated with very well-defined phenotypes, although some particular mtDNA mutations are associated with very different phenotypes. In contrast, there are other pathologic mutations always reported in association with the same phenotype, such as the deafness mutation m.1494C>T, and they are not so frequent in the patient population. Another possible explanation for the high frequency of the common mutations is that they could be located in mtDNA regions with special sequence features. However, apparently only two of these six mutations (m.1555A>G, m.3243A>G) are located in genes with important secondary structures that could affect the DNA polymerase activity and increase the mutational rate. Moreover, there are no particular characteristic sequences around these six nucleotide positions, and single nucleotide polymorphisms (SNPs) are not frequently found in the 20 nucleotides around these pathologic positions. Another possible reason is that these mutations are not very pathologic and they survive more frequently the negative selection during embryonic development. Some of them present incomplete penetrance and they require the action of other factors to cause disease. For example, for some of the LHON mutations, the mitochondrial haplogroup (group of phylogenetically related mtDNA genotypes) strongly influences the chances of developing the disease [24]. In other cases, such as the deafness m.1555A>G mutation, environmental factors like antibiotics increase pathogenicity [25]. Still others, such as the MILS mutation, need to be in a high percentage of heteroplasmy to produce the disorder [26]. Mutations would be expected to occur randomly in mtDNA and many of them should be very pathologic. In fact, mutations provoking stop codons have been found very frequently in colonic crypt stem cells [27]. Thus, it is probable that mutations in other nucleotides suffer a strong negative selection, as has been recently shown [28–30]. However, other apparently less dramatic mutations, such as the previous mentioned m.1494C>T, are not very prevalent. Therefore, a combination of these and other possible reasons will probably be responsible for the relatively high frequency of the most common mtDNA pathologic point mutations.

The important fact is that, although the discovery of the same mutation in more than one pedigree and always associated to a

Table 2
Mitochondrial DNA mutations frequently associated to specific phenotypes

Phenotype	Mutation	Gene
LHON	m.3460G>A	MT-ND1
	m.11778G>A	MT-ND4
	m.14484T>C	MT-ND6
Leigh (MILS)/NARP	m.8993T>G	MT-ATP6
MELAS/diabetes	m.3243A>G	MT-TL1
MERRF	m.8344A>G	MT-TK
Non-syndromic deafness	m.1555A>G	MT-RNR1

MITOMAP: A Human Mitochondrial Genome Database, 2005. http://www.mitomap.org.

defined mitochondrial pathology is a good indicator of its pathogenicity, as we have previously mentioned, it is really extremely rare to find the same new pathologic mutation in more than one pedigree.

5. New mtDNA pathologic point mutations

Where should we look for new mutations when a frequent mtDNA pathologic point mutation is not responsible for mitochondrial disease? It is not unusual in mitochondrial pathology that the same phenotype is associated with very different mutations. For example, many genetic variants have been associated with Leigh Syndrome (LS), such as mutations in MT-ATP6 or in other mtDNA-encoded proteins or tRNA genes (www.mitomap.org). Nuclear mutations in structural proteins from several ETC complexes, mutations in OXPHOS complex assembly proteins, and even mutations in proteins not directly related with the OXPHOS system have also been associated with LS [31]. Thus, clinical data, except for very particular phenotypes, are not very useful to pinpoint a specific mtDNA gene as the cause of a disease. The subsarcolemmal accumulation of mitochondria and the presence of red ragged fibers (RRF) are frequently found associated with mtDNA depletion [32] and deletions, or mt-tRNA mutations [33], and they are occasionally found in other mitochondrial diseases caused by pathologic point mutations in mtDNA-encoded protein genes. Thus, histochemical data are not very useful in helping to select an mtDNA gene to examine for pathologic mutations. Usually, the respiratory complex activities can give some clues about the location of the pathologic mutation. Mutations in mt-tRNAs will produce multi-enzymatic defects, and mutations in mtDNA-encoded protein genes should cause isolated enzymatic defects. However, some mutations in mtDNA-encoded protein genes cause multi-enzymatic defects [34], probably because they increase ROS production and cause a secondary effect on other complexes or because they are necessary to stabilize the supercomplexes [35]. Thus, biochemical activities are not sufficient indicators to target an mtDNA gene. Probably, then, the only way to find a pathologic mutation is to sequence the whole mtDNA.

New mutations in coding genes may be good candidates to be pathologic, but we cannot rule out a new mutation in a non-coding nucleotide as the cause of the disease without a second thought [36]. It is curious that, as previously mentioned, large portions of the mt-rRNAs have disappeared during mtDNA evolution yet some small non-coding segments are still part of the mtDNA. Some of these non-coding mutations might affect RNA processing [37]. Mutations in segments of the control region without a defined function might also be important. There are sequences in the control region that are better conserved than sequences in some protein genes [38]. Moreover, other apparently non-pathologic mutations, such as synonymous changes affecting the stability of the mRNAs [39] or mitochondrial HREs, could also have phenotypic effects by altering the binding of transcription factors, as already shown for nuclear HREs [40]. Therefore, we need to be more cautious when ruling out these changes as possibly pathologic mutations [41].

The discovery of a good candidate pathologic mutation in the mtDNA does not preclude the need to resequence the whole mtDNA. In 1997, a pathologic mutation associated with acute rhabdomyolysis was reported in the mtDNA MT-TF gene encoding $tRNA^{Phe}$ [42]. However, the complete sequencing of mtDNA some years later showed that the true pathogenic mutation was a novel mutation in the cytochrome oxidase subunit II gene [43]. These two studies by the same group, one of the teams with more experience in the mitochondrial pathology field, demonstrate the importance of whole mitochondrial genome sequencing. But does finding a better candidate mutation rule out the pathogenicity of the previous one? The m.606A>G/MT-TF mutation has been described four times, two of them in different haplogroups of control populations [44,45], and the other two times associated with patients suspected of suffering a mitochondrial disease [43]. The nucleotide position is not well conserved along the evolution, but a Watson–Crick (WC) base pair at this position is present in 103 out of 106 mammal species and the three species without a WC base pair at this position have developed new WC base pairs in the anticodon stem [46]. Moreover, only three mutations have been reported at nucleotides that interact with positions where pathologic mutations in the mt-tRNAs have been described [47]. These changes are likely pathologic mutations and the m.606A>G/MT-TF mutation is one of them, interacting with the m.618T>C/MT-TF associated with mitochondrial myopathy [48].

Despite the high rate of mutation [49], an mtDNA change is a rare event. However, it is possible that environmental, nuclear or mitochondrial genetic factors could increase the mutational rate and give rise to more than one pathologic mutation in the same mtDNA. Thus, a high level of natural radiation, such as that produced in Kerala (India) [50], or allelic variants in nuclear genes involved in mtDNA replication, could affect the mutational rate. In fact, patients with thymidine phosphorylase deficiency [51] or with defects either in the DNA helicase (Twinkle) or in the DNA polymerase γ (POLG) show enhanced accumulation of mtDNA mutations [52]. Finally, some mtDNA pathologic point mutations are associated with an increase in ROS production, and, as previously mentioned, they are mutagenic.

Then, is it possible to find two or more pathologic mutations in mtDNA? In fact this has already been observed on several occasions. The mutations at positions m.11778G>A and m.14484T>C have been found associated in LHON patients [53,54], the mutations at nucleotide positions m.14484T>C and m.1555A>G have been found in deafness-associated pedigree [55] and the m.5835G>A and m.4315delA mutations in a CPEO patient [56]. Therefore, to find the best candidate for pathological mutation and to detect more than one pathologic mutation, the entire mtDNA should be sequenced.

The complete sequence of the mtDNA will also provide knowledge about other mtDNA private variants that can act as modifiers of the main mutation. A cybrid cell line containing more than 99% of the m.3243A>G mutation in the MT-TL1 had a wild-type phenotype. These cells were heteroplasmic for another mtDNA mutation, m.12300G>A in the MT-TL2, which is apparently able to decode the UUR leucine codons [57]. Ancient variants can also modify the phenotypic effect of pathologic mutations, as frequently seen in the association between mtDNA haplogroup J and the m.11778G>A and m.14484T>C LHON mutations [24].

6. Absence of the candidate pathologic mutation in control individuals

One of the first criteria in considering the pathogenicity of a new mutation is its presence in patients and absence in controls. A pathologic mutation should not be found in normal individuals. But how many controls must we check? For example, a new mutation m.6489C>A was reported in the MT-CO1 gene in a patient suffering from epilepsia partialis continua. This mutation was not checked in controls, but it was not present at www.mitomap.org. The patient also harbored a polymorphism at position m.7028C>T and a 9 bp deletion m.8281-8289delCCCCCTCTA [58]. The same year 560 new mtDNA sequences were reported and this mutation, along with the other two mutations present in the patient, were found to define a small cluster in the mitochondrial haplogroup T (sequences 72, 103, 148, 435 and 537 from [59,60]). Therefore, it is very likely that the patient belongs to this cluster and the candidate pathologic mutation is probably a population polymorphism [20]. There are nowadays more than 3500 published mtDNA coding region sequences [60] and it takes no more than a few minutes, at no cost, to check all of them. Thus, we think that this is a good number of sequences to start to rule out the absence of a new mutation in

control individuals. For example, very recently a new mutation in the COI subunit, the m.6955G>A, was noted in publication, and the authors of the paper showed that it was not present in more than 4020 MT-CO1 sequences from patients and controls from all around the world, or MITOMAP (www.mitomap.org) [61].

In any case, 3500 mtDNA sequences are only 0.000006% of the whole human population and this first test does not preclude the need to check the mutation in the population from which the patient comes, to rule out a geographically restricted population polymorphism. Even though there is some homogeneity in geographic frequencies and distribution, studies with large sample sizes and better resolved mtDNA trees have shown that clades of mtDNA exhibit gradients [62] and these are more severe when more recent clusters are considered. Thus, by having a haplogrouped control population the number of required controls to be analyzed will decrease considerably, because it will only be necessary to check the control individuals that belong to the same mitochondrial cluster as the patient to rule out a geographically restricted polymorphism.

Another important question to take into account is the quality of the published sequences. As has often been noted [63], many reported sequences have errors, and this opens the possibility of finding a potentially pathologic mutation as part of one of those low quality sequences. It is also very important to know whether the sequences come from real control individuals or rather from the general population that includes some patients. In general, the disease status of the individuals contributing to these large data bases is often unknown [43]. For example, the sequences mentioned above [59], analyzed for a different purpose, were obtained from a general population including normal controls and patients with type 2 diabetes or with neurodegenerative disorders (Alzheimer and Parkinson diseases). Since some mutations in mtDNA are strongly associated with syndromes in which diabetes is a prominent clinical symptom [64], it is possible that some of the mutations found in these 'control' individuals were in fact pathologic.

Moreover, many mtDNA pathologic mutations have incomplete penetrance and they require other factors to be expressed. For example, the m.1494C>T mutation in the MT-RNR1 gene was found in one individual (wh6980) from a population study [65]. However, soon after this study, this mutation was defined as causing mitochondrial deafness [66]. Also, the mutations m.11778G>A and m.14484T>C are frequently found in homoplasmy in normal females of LHON pedigrees. If these individuals had been selected for population studies, these mutations would now be considered population polymorphisms. In fact, an individual carrying the m.14484T>C but who had normal vision was identified during a population screening [67].

When a new mtDNA mutation appears, a new branch is established in the mitochondrial phylogenetic tree. Thus, the newest mutations will be in the tips of the branches of the tree and those surviving evolution will pass to deeper branches. However, pathologic mutations will be removed by negative selection and they will not remain in populations for a long time [28,30]. Therefore, a thorough analysis of a large phylogeny, such as the one reported at www.mitomap.org [60], can yield other clues, because it is not expected to find a very pathologic mutation in an ancient branch of the phylogenetic tree. However, we need to be very careful in applying this and other pathogenicity criteria since the m.11778G>A and m.14484T>C LHON mutations and the m.1494C>T and m.1555A>G deafness mutations have been reported at internal branches of the phylogenetic tree [65,68–71].

The presence of a compensatory mutation would allow for the discovery of another pathologic mutation in a normal population. For example, a small cluster in the mitochondrial haplogroup A is defined by the pathologic mutation m.1494C>T [72]. This mutation gives rise to a new base pair with the nucleotide at position 1555 and makes the mitochondrial ribosome more susceptible to aminoglycosides and deafness. Thus, a pathologic mutation at m.1555A>G in this cluster would obviate the base pair and the phenotypic effect.

7. Homoplasmy and heteroplasmy

All mtDNAs from an individual are usually identical; this condition is called homoplasmy. When a new mutation appears, there will be two mtDNA types, the wild-type and the new mutant; this condition is called heteroplasmy. Heteroplasmy is taken as a good criterion for assigning pathogenicity. Germinal cell lines go through a tight bottleneck, thus reducing the number of mtDNAs that repopulate a new individual [73]. Therefore, a new mutation in the germinal cell line will reach homoplasmy in a few generations, or even in one [74]. The discovery of a heteroplasmic mutation, then, means that the mutation is recent and probably will not be found as an ancient polymorphism in the population. It is also commonly assumed that a heteroplasmic mutation cannot achieve homoplasmy because in this state it would be incompatible with life. However, heteroplasmy could be a normal condition for any kind of mutation until it attains the homoplasmic state.

The phenotypic effect will depend on the mutational load: a higher proportion of mutants will correlate with a more dramatic defect. However, the phenotypic effect will also depend on the mitochondrial energy needs of a particular tissue. This is known as the threshold effect. Those tissues that are more dependent on mitochondrial energy will have a lower threshold because a lower mutational load will produce some phenotypic effects. However, this relationship between the proportion of the mtDNA mutation and phenotypic effect will also depend on other factors, such as the mtDNA levels. For example, it has been shown that segments of human skeletal muscle fibers harboring a pathogenic mtDNA mutation retain normal cytochrome oxidase activity by maintaining a minimum amount of wild-type mtDNA. The segment of the multinucleated cell responds by non-selectively proliferating its entire mtDNA content, to restore wild-type mtDNA to its optimal level [75].

It is normally assumed that 10–30% of wild-type mtDNA is sufficient to compensate for a mutation in a mitochondrial tRNA. However, sometimes the presence of extremely low levels of a pathologic mutation is associated with a mitochondrial disease. For example, some patients with type II diabetes show very low levels of the m.3243A>G mutation [76]. These percentages were found in blood cells, and post-mitotic tissues usually have much higher levels of the mutant mtDNA. However, there was also a report about a patient with very low levels of the m.3243A>G mutation in blood and lower levels in muscle [77]. Thus, a dominant effect must be involved in the pathogenicity of this case. In this sense, the m.5545C>T transition in the MT-TW at low levels of heteroplasmy—below under 25%—caused a severe multisystem disorder and respiratory chain deficiency. The pathogenic threshold for the mutation in cybrids in this case was between 4% and 8%, implying a dominant mechanism. These findings pose new diagnostic challenges, because such mutations may easily go undetected in normal tests [78].

Single muscle fiber analysis correlates the mutational load with the staining pattern for the cytochrome oxidase activity of different single muscle fibers. Therefore, this test only can be performed for heteroplasmic mutations that affect complex IV activity. A higher proportion of the mutation will be associated with a lower COX activity, thus directly relating the genotype with the phenotype. However, the results are not always straightforward. For example, the ancient polymorphism previously mentioned, m.6489C>A, and therefore probably homoplasmic, was reported to be heteroplasmic and with differing proportions of the mutation in different muscle fibers [58]. Furthermore, another homoplasmic mutation [43] was also reported to be heteroplasmic and with different mutation loads depending on the particular muscle fiber in question [42]. In both

cases, malfunction of the restriction enzyme used for diagnosis could explain the heteroplasmy, but the difference in the proportion of the mutation was not explained. It is quite possible that mtDNA pseudogenes, integrated in the nuclear genome, are responsible for this problem. In fact, it was reported some years ago that patients with Alzheimer disease were heteroplasmic for several missense mutations in the mtDNA genes. These mutations were also present at low levels in normal individuals [79]. Immediately after these theoretically important findings, two different research groups demonstrated that the cause of this heteroplasmic state was amplification of pseudogene sequences, and it was very likely that the DNA extraction method differentially enriched the samples in nuclear DNA depending on the physiologic state of the cell [80–82]. There may be a similar explanation for the anomalous results and for the previously noted errors in individual muscle fiber analysis.

8. Conservation index and substitution type

The histidines 82, 96, 182 and 196 of the cytochrome b subunit of complex III are conserved in 1121, 1122, 1123 and 1118 out of 1124 species, respectively, from protists to mammals. These histidines bind the heme groups to fulfill the electron transfer reactions performed by this protein, and likely their absence in some species is due to sequencing errors. This example gives us the logic for one of the pathogenicity criteria commonly used in the mitochondrial disease field, namely, functionally very important amino acids will be highly conserved throughout evolution.

A high conservation index (CI) is a good clue for the functional importance of a particular amino acid, but the contrary is not necessarily true. The methionine at amino acid position 64 of the p.MT-ND6 subunit is only conserved in 89 (12.6%) out of 817 species from protists to mammals. However, the LHON m.14484T>C pathologic mutation affects this particular amino acid, p.MT-ND6:Met64Val. Thus, a low CI does not directly rule out the potential pathogenicity of a new mutation. Then why are some amino acids with a low CI important? The Met64 at p.MT-ND6 appeared at the top of the radiation of primates. The p.MT-ND6 subunit is part of respiratory complex I and is located in the subcomplex Iα [83]. This complex contains 45 other subunits, and it has recently been shown that some mtDNA and nDNA-encoded complex I structural subunits, such as NDUFA1, have evolved faster in primates [84]. NDUFA1 is located in the subcomplex Iα-λ, as p.MT-ND6 [83]. Therefore, parallel co-evolution may explain the low CI of Met64 at p.MT-ND6. In this sense, xenomitochondrial cybrids with human nucleus and chimpanzee mitochondria show a complex I deficiency [85], thus highlighting the importance of strong interaction between nDNA and mtDNA-encoded structural subunits. In fact, it has been shown that the mtDNA-encoded COX residues in close physical proximity to nDNA-encoded COX residues evolve more rapidly than the other mitochondrial-encoded COX residues, and thus display an optimizing interaction because the faster mtDNA mutation rate will allow sampling of more residues in the interacting region by accommodating mutations important for subunit interaction [86].

Compensatory mechanisms may also explain why population polymorphisms can affect highly conserved amino acids. We have already seen that the m.6489C>A, p.MT-CO1:Leu196Ile defines a cluster in the mitochondrial haplogroup T. Leu196 is conserved in 1234 (94.4%) of 1307 species. This is an interesting issue to consider since if a homoplasmic mutation is present in a particular pedigree, it is possible that only the index case would develop the disease if he/she also harbored a particular nuclear allele. It would also be possible that the transfer of these mitochondria to a rho0 cell line without the particular nuclear allele would not reproduce the pathologic phenotype. In these cases, the sequencing of the genes for those subunits that interact with the one presenting the mutation could produce interesting surprises.

The conservation index is a good criterion when it is correctly applied because the results will be dependent on the species analyzed. Which species and how many of them should be considered? There are now more than 1000 complete mtDNA sequences from different species (www.mitomap.com). Why restrict ourselves to a small number of species when we can select a large number of them, from protists to humans? Relevant to this point, a new pathologic substitution p.MT-CO1:Gly351Asp, m.6955G>A has recently been described; it was conserved in 1302 (99.6%) of 1307 species from protists to mammals [61]. Moreover, the CI of a candidate pathologic mutation can be compared with that of other pathologic mutations reported in the same mtDNA gene. Thus, the CI of the mutation previously described is higher than that of the only other described p.MT-CO1 pathologic missense mutations (m.6328C>T, p.MT-CO1:Ser142Phe) [87] associated with a classical mitochondriopathy, in 1208 (92.4%) of 1307 species.

Another criterion used to define pathogenicity is the type of amino acid substitution. In general, the existence of great differences in the chemical properties of two amino acids increases the probability of there being a pathologic substitution. However, every amino acid can be classified in many different groups depending on its chemical properties, although polarity and size are the most frequently used. Thus, practically every substitution can be defined as non-conservative, if a particular property is considered. Moreover, the phenotypic effect of a severe substitution will also depend on the protein domain affected. An analysis of a particular change in a particular protein in the human phylogeny or in different species may yield some clues as to the effect of this substitution. For example, a Gly to Asp replacement has never been described in the human p.MT-CO1 but it has been found several times in *Saccharomyces cerevisiae* and *Rhodobacter sphaeroides* COX subunit I, and all of them are respiration-deficient mutants [61].

9. The definitive evidence: the transmitochondrial cell line (cybrid)?

The previously mentioned criteria will give some evidence as to the pathogenicity of a particular point mutation. However, functional evidence is always needed if we want to confirm the pathogenicity of a new mutation. In this sense, the transmitochondrial cell line model is nowadays considered to provide the strongest evidence to define a candidate mutation as pathologic. The transfer of a particular phenotype by repopulating human cell lines emptied of their mtDNA (ρ0-cells) with another mitochondrial background is very good evidence of the pathogenicity of the particular mutated mtDNA [88,89]. This technique has been used to determine the pathogenicity of human mtDNA point mutations since 1991 [90], but the results are not always straightforward.

As previously noted, m.14484T>C is an LHON-associated pathological mutation. However, cybrids harboring this mutation did not show a decrease in growth rate or in complex I activity [91], although more recently a big decrease in the ATP levels has been reported [92]. Thus, it is very important to analyze the correct parameters to demonstrate the pathogenicity of the mutation. Further, the m.5703G>A/MT-TN mutation has been associated with a severe mitochondrial protein synthesis defect and a reduction in steady-state levels of $tRNA^{Asn}$ but several galactose-resistant clones with restored OXPHOS function and higher levels of the mutant tRNA were found. These cells contained homoplasmic levels of the mutation and no other detectable alteration in the MT-TN gene. Apparently, nuclear-encoded factors can compensate for the pathogenic mutation [93]. Confirming this fact, the homoplasmic point mutation m.1624C>T/MT-TV caused a profound metabolic disorder that resulted in the neonatal deaths of many siblings [94]. In transmitochondrial cell lines established from the proband, the marked respiratory deficiency was lost, and by inducing the

Table 3
Considerations for the pathogenicity criteria of mtDNA point mutation

* The control population must be correctly defined. A high number of geographically and genetically matched controls are important.
* The mutation should be found in the tips of the mtDNA phylogenetic tree but potential compensatory changes must be considered for mutations at internal branches of the tree.
* The whole mtDNA should be sequenced to rule out another better candidate mutation or the existence of two pathologic mutations.
* Dominant effects for mtDNA mutations must be considered.
* It is important to considerer interaction between nucleotides or amino acids.
* The function of many mtDNA-encoded subunits or protein domains is still unknown.
* Cybrids are good models but they are not free of problems.

overexpression of human mitochondrial valyl-tRNA synthetase in cybrids, the steady-state levels of the mutated mt-tRNA^{Val} were partially restored [95].

MtDNA-encoded factors can also compensate for pathogenic mutations. Thus, it has been reported that cybrids with 99% of the m.3243A>G/MT-TL1 mutation were phenotypically wild-type, because they contained 10% of a suppressor mutation m.12300G>A/MT-TL2. This anticodon mutation generates a MT-TL2 able to decode UUR leucine codons. Very importantly, the proportion of the mutant sequence was barely detectable by direct sequencing of the PCR product [57].

Most mtDNA mutations are recessive and they have to reach a high percentage in a specific tissue or cell line before producing a phenotype. However, it has recently been reported that the pathogenic threshold for the m.5545C>T/MT-TW mutation in cybrids is between 4% and 8%, implying a dominant mechanism of action, again barely detectable by direct sequencing [78].

In all these examples, the cybrid model would have given negative results and only prior knowledge that the mutations were pathologic allowed the search for other parameters or compensatory mechanisms. However, the cybrid model requires some precautions, as wisely noted by Swerdlow [96]. A pathologic mutation m.13528A>G in the MT-ND5 gene in a MELAS patient has recently been reported. Measurements of oxygen consumption with complex I substrates in cybrids revealed a complex I deficiency [97]. However, the complex I activity on muscle homogenate was normal and the mutation had been previously found at internal branches of the mtDNA phylogenetic tree [60]. The number of culture passages or the mtDNA levels of these cybrids was not reported [97]. Therefore it is possible that these cells had not recovered, after the cybridization, the normal mtDNA levels for that particular growth conditions; as it is well known, mitochondrial function is very much dependent on the mtDNA levels in cultured cells [98].

10. Conclusion

Although pathogenicity criteria are very useful tools to demonstrate whether a newly found point mutation in mtDNA is pathogenic, as we have seen there are many examples in the literature that do not in fact fulfill all of them. Therefore, this is not a rare situation; the field of mitochondrial pathology is very complex and one cannot be too strict in the application of these criteria (Table 3).

In 2000, Salvatore DiMauro, talking about mtDNA pathologic mutations, wondered whether we were scraping the bottom of the barrel [99]. His answer was a resounding 'no'. Many of the latest findings lead us to reformulate the question: Are we still scraping the top of the barrel? It is likely that many new mtDNA mutations remain to be found, that some population polymorphisms will be associated with diseases in particular circumstances, and that some pathologic mutations will be found in normal individuals. The reason for this is that mtDNA-encoded proteins are only a part of the mitochondrial proteome and its interaction with the other nuclear-encoded proteins, will determine the pathological state of the individuals [100]. Furthermore, very little is known about the role of mtDNA mutations in age-linked diseases. These are considered multifactorial disorders, with genetic and environmental factors involved. The phenotypic effect of these variants will be more moderate and their need to interact with other factors to produce the pathological phenotype will render the classic cybrid model an extremely simplistic approach. Other more elaborate approaches will be required, for example by considering possible compensatory mechanisms [101].

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