



Review

Mitochondrial DNA involvement in human longevity

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Abstract

The main message of this review can be summarized as follows: aging and longevity, as complex traits having a significant genetic component, likely depend on a number of nuclear gene variants interacting with mtDNA variability both inherited and somatic. We reviewed the data available in the literature with particular attention to human longevity, and argued that what we hypothesize for aging and longevity could have a more general relevance and be extended to other age-related complex traits such as Alzheimer's and Parkinson's diseases. The genetics which emerges for complex traits, including aging and longevity, is thus even more complicated than previously thought, as epistatic interactions between nuclear gene polymorphisms and mtDNA variability (both somatic and inherited) as well as between mtDNA somatic mutations (tissue specific) and mtDNA inherited variants (haplogroups and sub-haplogroups) must be considered as additional players capable of explaining a part of the aging and longevity phenotype. To test this hypothesis is one of the main challenge in the genetics of aging and longevity in the next future.

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1. Introduction

Mitochondrial loss of function is considered a common feature in aging. Much less is known about the role of mitochondria in longevity, that is the capability of an organism to reach the extreme limits of the species-specific lifespan. Mitochondria are deeply involved in ATP synthesis, heat production, radical oxygen species (ROS) generation, fatty acid and steroid metabolism and apoptosis, among others. Thus, it is quite likely that dysfunction in mitochondrial metabolism

can, to some extent, be a cause of aging and, consequently, that mitochondrial determinants of aging can in turn be detrimental for longevity. Nevertheless, besides mitochondrial metabolism, a number of other hypotheses have been presented that link mitochondria and longevity through different mechanisms. In particular, a special attention is presently devoted to mitochondrial DNA (mtDNA), on the basis of several lines of evidence. First, mtDNA is maternally inherited, and heritability estimates for lifespan based on offspring–mother regressions are higher than those based on offspring–father regressions [1]. Second, many inherited variants of mtDNA do exist, that are geographically distributed, and some of them have been described to be associated with common complex traits [2] including longevity [3–6]. Third, from bioinformatics analysis it appears that number and length of direct repeat pairs in the mtDNA molecule constrain lifespan in long-living mammalian species [7]. Last

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but not least, mtDNA is the only repository of genetic information outside the nucleus and it is much more exposed to mutagenic events than the nuclear DNA (nDNA). According to the mitochondrial theory of aging, progressive accumulation of somatic mutations leads to a decline in mitochondrial function; therefore, the cell capability to counteract mtDNA somatic mutations may play a positive role in the age-related mitochondrial decline and thus in longevity [8].

In this review, we will focus our attention on data and hypotheses linking mtDNA and longevity in humans, and we will critically discuss the experimental models actually exploited to get insight in this subject, as well as their pros and cons. Finally, we will introduce an ongoing research project that should, in part, allow us to overcome such problems and to answer some questions on this topic. This project is named GEHA, as an acronym of “Genetics of Healthy Aging”, and it is supported by the FP6 of the European Commission (FP6-503270).

2. mtDNA abundance

Human mtDNA is a 16,569 bp double-stranded, circular genome. In each cell several hundreds to thousands mtDNA copies are present [9,10], and it is estimated that mtDNA accounts for 0.15 to 2.2% of the mass of the cellular diploid genome [11,12]. MtDNA is packaged into protein–DNA complexes, called mitochondrial nucleoids (mt nucleoids) that look like globular foci in human cells [13]. Among the proteins discovered to interact with mtDNA in mt nucleoids, there are a number of proteins involved in mtDNA replication and transcription, among which TFAM, Twinkle, mtSSB, polymerase gamma, BRCA1, PRSS15 (LON) are included (for a review, see [14]). Contrasting data are reported about the age-related changes in the copy number of mtDNA in both rodents and humans. In some cases mtDNA copies have been found to be increased during aging [15,16], while in other cases this number has been found to be decreased [17–21] or unaltered [10,12]. Such contrasting data may be due to the different methods used to quantify the amount of mtDNA (Real-Time PCR, Slot Blot) and, more importantly, to a marked tissue specificity. On the whole, no general consensus has been reached on the age-related changes of mtDNA content. Accordingly, several conceptualizations have been proposed to interpret these conflicting data: a lack of changes in mtDNA amount may reflect the incessant need of oxidative metabolism in tissues such as myocardium, while an increase in mtDNA copy number may be accounted for by a compensatory mechanism to counteract the age-related accumulation of mutated/deleted form of mtDNA [10,12,15,16]. For instance, it has been reported that deleted mtDNA replicates much faster than intact mtDNA [22]. On the other side, a decrease in mtDNA amount might mirror the decreased oxidative capacities of the aged tissues, such as liver and some skeletal muscles [17,18,20]. This contradictory scenario is even more complicated when considering the ratio between mtDNA copy number and the relative abundance of mt nucleoid proteins, such as TFAM, that has important roles not only in mtDNA packaging but also

transcription [23]. Indeed, the amount of TFAM associated with mtDNA may vary depending on cell type (reviewed by Chen and Butow, [14]), while during aging, TFAM mRNA and protein have been reported to be either increased [24] or unaltered [18]. Furthermore, a lack of correlation between the amount of mtDNA and mtRNA and mitochondrial polypeptides has been reported [9,17,25,26]. A deeper discussion of the complex relationship between mtDNA, packaging/transcription proteins and translated polypeptides is outside the scope of this review, but, whatever the meaning of age-related changes in mtDNA abundance can be, it is conceivable that a preserved capability to maintain a number of intact (non mutated) mtDNA copies can impinge upon aging and longevity. Data on accepted models of human longevity, such as centenarians, are still lacking. However, it has to be stressed that the above mentioned tissue-specific diversity in mtDNA abundance is likely a crucial point to be considered when addressing these studies. In particular, a different degree of age-related mosaicism has to be expected in different tissues from old people, likely depending on the need of energy supply, and thus comprehensive studies performed on a number of representative tissues have to be envisaged to draw meaningful results.

3. MtDNA somatic mutations

Because of its vicinity with ROS source (electron transport chain) and the presence of an incomplete repair system, mtDNA shows a high mutation rate. Mutated and wild-type mtDNA molecules can coexist within the same cell, tissue or organ, and this condition is called heteroplasmy. Cells containing only one type of mtDNA (mutated or wild-type) are indicated as homoplasmic [27]. However, the concept of homoplasmy is more apparent than real: indeed, because of clonal expansion, new mutations arising from random errors in mtDNA replication may occur at a variable level within cells and tissues, thus remaining undetected in tissue homogenates and blood buffy coats. In other words, heteroplasmy is detectable only if it occurs above a threshold whose level depends on tissue type, ROS production and stochastic factors. Although heteroplasmy is usually observed in post-mitotic tissues characterized by high energy demand, such as brain and skeletal muscle (for a review, see [28]), mtDNA mutations accumulate also in dividing cells, including stem cells [29,30].

The available literature suggests that a variety of deletions and mutations accumulate with age in mtDNA (see [28] Chomyn and Attardi, 2003). However, several important questions are still unanswered. The most important one regards the level of deletions/mutations attained in different cell types, tissues and organs during aging. Strictly correlated to this question is their functional importance, as it is believed that a critical threshold of deletions/accumulations must be attained in order to have functional consequences. The problem is further complicated by the fact that the different organs and tissues of the human body present a large difference regarding proliferative activity, apoptotic rate and cell renewal, abundance of stem cells, ROS production and mtDNA repair capability, among others. All these differences can likely have a strong

impact on mtDNA physiology and pathology (amount, replication rate, accumulation of mutations) and thus the overall scenario is extremely complex and best characterized by a mosaic model where the rate of aging, mtDNA mutation accumulation and dysfunction display a wide range of variability. Assuming, as we will argue later, that mtDNA mutations interact with nDNA genes and polymorphisms, as well as with inherited mtDNA variants (haplogroups), and taking into account the individual variability of both nuclear and mitochondrial genomes, we can have an idea of the enormous complexity posed by the involvement of mtDNA into a complex trait such as longevity. In any case, the correlation between level of mtDNA heteroplasmy, aging and longevity in the different cells, tissues and organs of the body is still debated. Recently, the fact that the accumulation of mtDNA mutations is a cause rather than an effect of aging has been addressed by experiments on a murine model, in which animals expressing a proofreading-deficient version of the mtDNA polymerase gamma accumulate mtDNA mutations and display features of accelerated aging [31,32]. The mechanism by which such an accumulation of mutations in mtDNA induces an aged phenotype is still controversial, but it appears that unexpectedly no increased oxidative stress occurs in such mtDNA mutator mice [32,33]. Different hypotheses have been proposed to explain the observed phenotype, from progressive defects in respiratory function to increase in apoptosis, and lately to depletion of stem cells [34]. However, a major pitfall of this experimental model is that it does not really mimic natural aging, but it is rather to be considered as a sort of genetic “disease” with progeroid features. In this specific case an exhaustion of stem cells could be the end result of the mtDNA mutator phenotype. This is suggested by the fact that the Authors found that the tissues mostly affected in such mouse model are those which actively proliferate (testis, thymus, intestine, blood), but not the post-mitotic ones (brain), as it is expected for normal aging. Again, the rate of mtDNA mutations reached in these mutator mice is much higher than that found in normally aged mice, and the observed effects are much larger and precocious than expected. On the whole, although the mouse model of accelerated aging provides strong evidence that mtDNA mutations can contribute to aging, further experimental studies are needed to confirm that such mutations are causative to aging and involved in longevity. This argument can be generalized, and in particular many gerontologists, including ourselves, think that models of *increased* longevity are to be preferred to models in which shorter lifespan is produced by genetic manipulations in a variety of organisms, from nematodes to mammals. These models are more complicated and more demanding but they represent the most interesting challenge in this field. The best examples of genes causally involved in aging and longevity are provided by non-mitochondrial genes such as *klotho* and *lin-4* which cause accelerated aging when mutated and which extend lifespan when over-expressed in mouse and *C. elegans*, respectively [35–37]. We are far from this type of situation in humans, and particularly in mtDNA, where studies on this topic are still in their infancy. However, it can be assumed that the capability to

maintain mtDNA integrity and to avoid the accumulation of mtDNA mutations is likely a feature of longevity. To critically evaluate the informative capability of mouse models of accelerated aging as far as the role of the accumulation of mtDNA mutations, a comparison between polymerase gamma mutator mice, normal aged mice and long living humans is here proposed as far as their hematopoietic capability. The mutator mice are profoundly anemic [32], likely as a result of stem cells exhaustion, while normal aged mice are not anemic [38], and thus it is possible that in normal aged mice mtDNA mutations do not accumulate to such an extent capable of inducing stem cell depletion and consequent anemia. As far as humans are concerned, prevalence of anemia in persons 85 years and older is 26% in males and 20% in females (one third of the cases being due to nutritional deficiencies) and is associated with frailty and increased morbidity, disability and mortality [39,40]. Thus, the majority of older persons do not suffer of anemia, which still remains a major problem in the elderly. Our studies on centenarians also suggest that they are not anemic, thus indicating that their hematopoietic capability is quite preserved [41,42]. This in turn would support the hypothesis that in long-living people, hematopoietic progenitor cells, and likely their mtDNA integrity, is well preserved. Indeed, in healthy centenarians classified according to [43] we found a well preserved capability of circulating CD34+ cells to respond to hematopoietic cytokines and to form erythroid, granulocyte-macrophagic and mixed colonies in a way indistinguishable (number, size and morphology) from that of young subjects, despite their reduced absolute number [42]. These results have been recently replicated [44]. At present, no direct data regarding mtDNA mutations in long-living people and centenarians are available, and this topic deserves a specific attention in the next future. It is clear that such mice models are exaggeration of normal aging phenotypes, and that any extrapolation to the situation of non genetically manipulated animals, and particularly of humans, is premature.

4. Deleterious or beneficial? The special case of C150T mtDNA mutation

All the arguments discussed above are characterized by the implicit assumption that all mtDNA mutations are deleterious by definition *and fully explain the phenotype by themselves*. We challenge this tenet on the basis of recent data regarding the accumulation of a specific mtDNA mutation we obtained in centenarians. At least in theory, there is no *a priori* reason to consider *any* mtDNA mutation as detrimental. A mtDNA mutation might increase survival only at later ages, in the post-reproductive period, or increase the survival of a subject in earlier periods of life. This may be the case of a specific mtDNA mutation we described quite recently [45]. This mutation is a C to T transition found at position 150 of mtDNA control region (CR) and it is found to be over-represented in centenarians with respect to young and old people in Italian population [45]. This mutation likely changes the origin of replication of the mtDNA heavy strand at position 149, substituting for that at position 151. Age-related mutations in mtDNA CR usually show a tissue

specificity, as in the case of A189G, T414G and T408A. The C150T mutation was found in three different cell types, i.e., peripheral blood lymphocytes, granulocytes and fibroblasts. An intriguing characteristic of these mtDNA CR mutations is that in some cases they appear to be inherited mtDNA polymorphisms. The available data indicate that, on some occasions, the C150T mutation is undoubtedly the result of a somatic event, as suggested by its absence in fibroblasts derived from biopsy of adult subjects and its presence from a second biopsy taken 10 years later from the same subjects. On the other side, the possibility that C150T can also be an inherited polymorphism is indicated by the fact that this mutation has been found to be present at high frequency in both members of monozygotic and dizygotic twin pairs, also suggesting that it likely confers a selective advantage earlier in life (possibly during pregnancy) [45]. It is interesting to note that the C150T mutation has been found to be associated with longevity in three additional populations, such as Irish, Finnish and Japanese [6,46]. Thus, C150T might be advantageous throughout life as an inherited polymorphism or contributing to longevity as somatic mutation arising later in life. The above mentioned circumstantial evidence indicate that C150T mutation/polymorphism likely confers an advantage for survival, but the mechanism is still unclear. The following non-mutual hypotheses can be put forward: first of all, as originally suggested in our paper [45], the C150T mutation could confer a replicative advantage to the mtDNA, thus accounting for the homoplasmic or nearly homoplasmic level mostly found in cells from centenarians. Second, the C150T mutation might be related to the cellular origin of the mtDNA used in our study, i.e., lymphocytes and granulocytes [45] and to immunosenescence [47]. Indeed, the accumulation of megacloones of memory T lymphocytes, and their filling of the immunological space, concomitant with the exhaustion of naïve T cells, is one of the most important marker of immunosenescence and is correlated with morbidity and mortality in the elderly [48,49]. Third, the C150T mutation could confer a resistance to oxidative stress and apoptosis, and this topic is under scrutiny in our laboratories with the use of appropriate cybrids.

5. MtDNA repair systems and longevity

The preservation of mtDNA integrity requires efficient detoxifying and repair systems. As far as detoxifying systems, two studies have been performed in Italian centenarians that investigated a possible association between superoxide-dismutase 2 (SOD2) gene variability and longevity. The first study, which considered only genetic data (allele and genotype frequencies), was not able to reveal any significant association [50]. However, when the same gene dataset was implemented by demographic data, a significant gene-environment interaction was observed by SOD2 allele T in modulating lifespan [51]. This type of study also suggests that sophisticated algorithms which integrate genetic and demographic data are needed to explore the subtle interactions between gene variability and lifespan [52]. Therefore, it cannot be excluded that other genes involved in ROS scavenging and antioxidant

systems can be associated, alone or in combination, to longevity. A recent paper has shed some light on this complex topic. In particular, the role of ROS in mammalian longevity has been addressed by the generation of transgenic mice over-expressing human catalase localized to the peroxisomes, the nucleus or the mitochondria [53]. Median and maximum life span were maximally increased of about 5 months in mice where catalase was targeted to mitochondria. It is interesting to note that in these mice a mild reduction of oxidative stress and damage was observed in tissues such as skeletal muscle and heart, as well as the reduction of mtDNA deletions. However, this last finding was evident in mice at 18–22 months of age and only in skeletal muscle, and the difference between wild type and transgenic mice was no more evident in older mice (33 months of age) in both skeletal muscle and heart. Thus, the possible causative role of the accumulation of mtDNA deletions in this model of lifespan extension is still unclear. Moreover, a different sensitivity of skeletal muscle and heart is also evident in this model, and skeletal muscle appears to be more sensitive to the beneficial effects of catalase over-expression. The Authors also comment their data regarding a possible diluting effect on catalase over-expression in mitochondria due to changes in the overall genetic background in successive generations of the transgenic mice. While rendering unclear some of the above mentioned results, this comment stresses once again the importance of the genetic context represented by the nuclear genome on mitochondrial function and mtDNA physiopathology. On the whole, these data further support the importance of mitochondria as a source of ROS and as a limiting factor in determining mammalian longevity.

As far as mtDNA repair systems, it has been recently reported that the base-excision repair (BER) activity is decreased in brain areas of aged mice [54]. It is possible that allelic variants of genes involved in BER system can confer different efficiency in mtDNA repair activity, and thus are putative longevity genes. Thus, such allelic variants are expected to be over-represented in long-living people and centenarians. We are currently testing this hypothesis in genetic association case-control studies in an Italian population (centenarians and young subjects). It has been reported that also *p53*, one of the pivotal players of nDNA repair can participate in mtDNA BER [55], likely through a physical interaction with polymerase gamma [56]. This interaction would increase polymerase gamma activity. It is known that *p53* carries a common polymorphism at codon 72, that yields an Arginine-to-Proline aminoacidic substitution. The two resulting isoforms of *p53* have, among other biological differences, a different capability to localize at the mitochondrial level [57]. It would be interesting to investigate whether subjects with different genotype of *p53* are characterized by a different level of polymerase gamma activity and of mtDNA instability. This would be of great interest, since *p53* codon 72 genotype has also been observed to modulate mortality by cancer and longevity in a study of Dutch nonagenarians [58]. It is remarkable that these results are apparently different from those obtained in other populations, thus suggesting a complex interaction between *p53* polymorphisms and longevity [59].

6. MtDNA inherited variants and longevity

Besides somatic mutations, mtDNA is characterized also by a series of inherited sequence point mutations that define a variety of haplotypes firstly described by the group of Wallace [60]. Phylogenetically related haplotypes are grouped together to form haplogroups that display a region-specific distribution [61–63].

The classification of mtDNA haplogroups is based on information gained from RFLP analysis of the coding region [64]. Haplogroups are coded with capital letters and subclusters with a running number. Three African (L₁, L₂, L₃), seven Asian (C, D, G, E, A, B, F) [65], and nine European (H, T, U, V, W, X, J, I, K) [64] main mtDNA haplogroups were identified. This research field is rapidly growing, and new sub-haplogroups are continuously emerging [66]. For instance, a recent paper reported that haplogroup H, the most common in Europe, can be subdivided into at least 15 sub-haplogroups [67], and formerly haplogroup K has been lately recognized as a sub-haplogroup of haplogroup U. To provide a comprehensive description of these haplogroups and subhaplogroups is outside the scope of this review. What it is interesting to us is that these variants are likely non neutral, and in particular a series of experimental evidence have been published suggesting that some mtDNA haplogroups are associated with longevity [3–5,46], as well as with mitochondrial diseases [68,69], and complex diseases [2]. In particular, in Caucasians such as northern Italians, haplogroup J is over-represented in long-living people and centenarians [4] thus suggesting a role for this mtDNA variant in longevity. In such study, both male and female centenarians were analyzed, but haplogroup J was over-represented only in male centenarians. This gender difference is quite common in studies on the genetics of longevity, where different polymorphisms of nuclear genes have been found to be associated with longevity only in males or females. Most of the positive correlations between nuclear gene polymorphisms and longevity we described regard male centenarians. The reason for such a gender difference is not clear, but it can be related to the fact that male centenarians are more selected, being less represented in the general population and having a lower probability to reach a hundred years of age in comparison to females. Accordingly, it can be speculated that males rely more upon genetics, either nuclear or mitochondrial, to attain longevity. In any case, such a male-specific connection between mtDNA haplogroups and longevity fits with the hypothesis that mtDNA variations have a higher impact on male than on female longevity [70]. This gender difference could be one of the consequences of the maternal inheritance of mtDNA, which in turn implies a higher relaxation and less purging of male-specific mitochondrial phenotype, being males evolutionary dead ends for mtDNA.

The over-representation of haplogroup J in nonagenarians and centenarians has been replicated in Irish and Finnish long-living people [5,46], but not in southern Italians [71]. Indeed, when a large population (883 subjects with age from 18 to 108 years) from southern Italy was analyzed for the association of haplogroup J with longevity, no positive signal was found,

suggesting that in such a population haplogroup J *per se* does not contribute to longevity. On the other hand, in Finnish population, mtDNA mutations characteristic of J2 subhaplogroup (489C and 10398G) modified the association between the 150T mutation and longevity [6]. On the whole, these results support the idea that the effect of mtDNA inherited variants on longevity is geographically/population dependent.

7. MtDNA inherited variants and age-related diseases

MtDNA variants have been studied in a variety of diseases, including neuromuscular and neurodegenerative diseases, diabetes, cancer and sepsis (see for example [72–76]). Here we will discuss only the data available on two major neurodegenerative diseases associated with aging, such as Parkinson's and Alzheimer's Diseases, where several studies have been reported. For more a comprehensive review of other diseases, the reader is referred to a recent review by Wallace [2].

As far as Parkinson's Disease (PD), haplogroup J and subhaplogroup K have been found to be under-represented in patients with PD [77]. These results are in line with those obtained in an Italian population [78], and in an English population [79]. Other studies suggested an increased risk of PD with haplogroups clusters such as JT [80] and JTIWX [81].

Contrasting data are reported as far as Alzheimer's Disease (AD) is concerned. In particular, it has been reported that haplogroup T is under-represented in AD patients, while haplogroup J seems to be over-represented in AD patients [82]. Haplogroup U has been reported to be under-represented in females and over-represented in males Caucasian AD patients [83]. On the other side, it is to note that several studies did not find any association between mtDNA haplogroups and AD by studying different Caucasian populations [79,84–86]. On the whole, no general consensus has been reached as far as the correlation between mtDNA haplogroups and AD. These results are summarized in Table 1.

8. LHON as a paradigm of the role of mtDNA in complex traits

LHON is one of the best studied mitochondrial diseases, and most cases are associated with one of three mtDNA point mutations (G11778A, G3460A, and T14484C) affecting genes encoding complex I (NADH:ubiquinone oxidoreductase) subunits, and currently regarded as pathogenic. Despite the fact that all these mutations are homoplasmic in the large majority of families, the severity of the disease can be quite variable and gender-biased [87], suggesting that additional genetic components must be involved in order to explain the different penetrance of the mtDNA mutations. A key observation has been reported by Torroni et al. who found that *LHON is associated with mtDNA haplogroup J* [68], and this observation has been confirmed by other Authors [69,88]. Recently, it has been argued that the clinical heterogeneity of LHON can be also explained by assuming the critical role of an interaction between the mitochondrial mutations and a *second nuclear gene*, both necessary to explain the pathological effect of mtDNA mutations

Table 1
Studies on mtDNA haplogroups and complex traits (longevity, Parkinson's disease and Alzheimer's disease)

Complex trait	No. of cases vs. controls	Results	Ethnicity	Reference
Longevity	37 vs. 252	Over-represented: D	Asiatics	[3]
	109 vs. 125	Over-represented: J	Northern Italian (males)	[4]
	129 vs. 100	Over-represented: J	Northern Irish	[46]
	225 vs. 657	Over-represented: J and U, Under-represented: H	Finnish	[5]
	155 vs. 728	No association	Southern Italian	[71]
Parkinson's disease	609 vs. 340	Under-represented: J and K	Caucasians	[77]
	90 vs. 129	Over-represented: J+T	Northern Irish	[80]
	238 vs. 183	Over-represented: J+T+H+W+X	Finnish	[81]
	620 vs. 1995	Under-represented: K	Italians	[78]
	455 vs. 447	Under-represented: U+K+J+T	English	[79]
Alzheimer's disease	69 vs. 83	Under-represented: T	French-Canadian	[82]
	185 vs. 179	No association	English	[84]
	644 vs. 180	Under-represented: U	Caucasian females	[83]
	345 vs. 148	Over-represented: U	Caucasian males	[83]
	190 vs. 340	No association	Old Order Amish	[85]
	185 vs. 447	No association	English	[79]
	75 vs. 64	No association	US Americans;	[86]
70 vs. 64	No association	English		

MtDNA haplogroups and/or clusters having higher frequency in cases than controls (over-represented) are considered to have a positive association with the trait, while those under-represented are considered to have a negative association with the trait. D, H, I, J, K, T, U, W, X refer to haplogroup nomenclature as reported in MITOMAP (<http://www.mitomap.org>).

[89]. The nuclear gene likely does not induce any pathology *per se*, but it contributes to the pathogenic effect of the mitochondrial mutations [89].

This observation on LHON suggests that, even when the mtDNA mutations characteristic of the disease are homoplasmic, they are insufficient *per se* to fully explain the phenotype, and suggests that similar interactions within the mtDNA itself (mtDNA mutations plus mtDNA haplogroups and subhaplogroups) and between the mtDNA and nDNA (nuclear–mitochondrial cross-talk) might occur in other conditions where mtDNA is involved, including aging and longevity.

9. MtDNA variants and mitochondrial functions: an open field of research

On the whole, a series of circumstantial evidence suggest that the different mtDNA lineages are qualitatively different from each other, bearing mutations that can modulate the

mitochondrial function, and consequently influence longevity as well as diseases. It has been proposed that the observed geographic distribution of mtDNA lineages resulted from selection mainly driven by adaptation to climate and nutrition (the type of available food) [67,90]. However, the mechanism by which these haplogroups can impinge upon longevity and diseases is far from being clear. Few papers report that haplogroups confer a different biochemical activity to mitochondria. For example, it has been reported that haplogroups H and T display a significant difference in the activity of OXPHOS complexes I and IV [91]. These results can be accounted for by the possibility that non-synonymous sequence variations present in haplogroups and sub-haplogroups that can modulate mitochondrial activity do exist. In fact, it has been observed that some haplogroups that are more frequent in temperate and arctic areas contain very well conserved aminoacid variants that lie in coding genes, such as *ND2*, *ND4*, and *Cyt b* (see [90]), and it has been suggested that these aminoacid changes can modify the efficiency of electron transport chain, thus shifting mitochondrial activity towards an increased production of heat. Similarly, it could be hypothesized that such mtDNA-based modifications of mitochondrial activity can also impinge upon longevity by resulting in a reduction in OXPHOS efficiency, which in turn would reduce mitochondrial ROS stress and decrease apoptosis [44]. This hypothesis fits with some of the above mentioned mouse models of increased longevity attained by over-expression of catalase in mitochondria and with the major role of apoptosis in provoking the accelerated aging phenotype in mitochondrial polymerase gamma mutator mice. However, it is likely that in most cases the functional difference between mtDNA haplogroups is more subtle and relies upon more complex relationships between nuclear and mitochondrial genomes.

10. Nuclear–mitochondrial interactions

The nucleus and the mitochondria interact in a variety of physiological functions that are crucial for cell activity and survival. Different levels of interactions and cross-talk between nucleus and mitochondria can be envisaged:

- (i) the first is a major topic of proteomics and it is based on the fact that, during evolution, most of the original information encoded in mitochondrial genome translocated to the nucleus and thus the majority of structural mitochondrial proteins are encoded by nuclear genes. mtDNA encodes for only 13 of the more than 80 proteins involved in the respiratory chain, while nDNA encodes for all the remaining proteins. Moreover, the complete mitochondrial proteome is estimated to about 1000–2000 different proteins [92], which are all nuclear-encoded in order to perform the different mitochondrial functions, resulting in a coordinated regulation of the two genetic systems of the cell, crucial for cell physiology [93].
- (ii) Another group of nuclear-encoded proteins localize to mitochondria during important phenomena such as, for example, apoptosis [94]. Indeed, the nucleus not only

contains the great majority of genetic information for building up mitochondria (as reported above) but also strongly regulates their activity. This is suggested by the fact that an increasing amount of regulatory and adaptor proteins is reported to have a secondary mitochondrial localization, as already mentioned for *p53* (see previous paragraph and [55]) and *p66^{shc}* [95].

- (iii) Experimental data have been collected suggesting that a third level of communication occurs regarding nuclear and mitochondrial genomes. This finely tuned, nucleus-mediated, regulation of mitochondrial activity requires that the nucleus is constantly informed about the functional status of mitochondria, in a classical feedback loop fashion. Thus, a crucial component of cell signaling travels from mitochondria to the nucleus. This mitochondria-to-nucleus signaling, called “retrograde response” in yeast [96,97], provides information on the state of mitochondria and can serve to adjust carbohydrate and nitrogen metabolism [97]. Studies on model organisms have revealed a connection between retrograde response and modulators of aging such as ROS production, ATP levels, altered nutrient levels, metabolic shift resulting from changes in insulin and IGF-1 signaling pathways [98,99].

We surmised that similar retrograde response does exist in humans and impinges upon aging and longevity [100]. In particular, we found genetic evidence of interactions between specific mtDNA haplogroups and genes involved in human longevity. A non-random association between mtDNA haplogroups and Tyrosine hydroxylase polymorphisms has been found in aged people and centenarians but not in young people [50]. Furthermore, we reported that frequency of the a3 allele of *HRAS1* 3' variable number tandem repeat (*HRAS1* 3'VNTR) decreases in centenarians in respect to young people, and we estimated that during aging a3 carriers have an increased mortality risk and that the haplogroups K and U were significantly over-represented in a3 allele carrier centenarians. No interaction between *HRAS1* 3'VNTR a3 allele and mtDNA haplogroups was found in young individuals [101].

A correlation was also reported between mtDNA haplogroups and *APOE4* allele in relatively young AD patients, where a significant protective effect of haplogroups U and K in patients carrying the *APOE4* allele was found [102]. At variance, other Authors found that the effect of mtDNA haplogroups on AD risk is independent of *APOE* status, and a significant association of haplogroup U was only evident when data were stratified by sex [83]. Probably these differences can be explained by the geographic origin of the samples used in the two studies. Preliminary results from our lab in AD patients, while confirming the association between mtDNA haplogroups and AD, do not suggest an interaction between *APOE4* genotypes and mtDNA haplogroups (Santoro et al., unpublished data). Interestingly, the mean age of this sample of AD patients was 16 years higher than that of the samples analyzed by Carrieri and colleagues [102], and comparable with those of van der Walt and colleagues [83].

The lack of interaction between mtDNA haplogroups and *APOE* gene variants could be due to a decreased risk effect of *APOE4* allele in advanced age. More investigations are needed to clarify this topic.

On the whole, the few available data in humans are compatible with the hypothesis that mtDNA haplogroups could interact with nuclear gene variants and modulate their penetrance. As far as we know a totally unexplored area in humans is the predicted stronger interaction between mtDNA polymorphisms and genes present in X chromosomes on the basis of their maternal co-transmission. Indeed mtDNA and X chromosomes are co-transmitted through females two-thirds of the times, with a higher probability to maintain joint mitochondrial polymorphisms [70]. It is important to note the opposite case of Y chromosome which is never co-transmitted with mtDNA.

11. Nuclear–mitochondrial interactions: cybrids and new in vivo models

In vitro and in vivo models have been proposed to demonstrate that mtDNA inherited variants modulate biological functions. The best known in vitro model is represented by cytoplasmic hybrids. This technique allows the analysis of mtDNA mutations or inherited variants by minimizing the effect of the nuclear genome that is kept constant **Box 1**. Cybrid cell lines are relatively easy to obtain and easy to maintain (cells can be frozen and stored for years). This model is largely used to test the effect of specific mtDNA mutations. However, it has to be taken into account that it presents several possible limitations due to the technique used to obtain cybrid cells, that is, for example, culture in ethidium bromide, treatment with polyethylene glycol, selective media, etc. (see **Box 2**).

As far as in vivo models, an elegant approach has been developed by Roubertoux and coworkers [103], and this model appears to be particularly suitable to study the effect of mtDNA inherited variants on mammalian complex traits. In this model, the total, reciprocal substitution of mtDNA was achieved by 20 repeated backcrosses in two mice strains which differed for their nDNA as well as for their mtDNA (intraspecific mtDNA crosses). The Authors demonstrated that the substitution of mtDNA in such mice strains modified learning, exploration, sensory development and the anatomy of the brain with respect to parental strains, this effect becoming particularly evident in aged mice. This model, while providing additional evidence that mtDNA polymorphisms are not neutral, gives evidence that they can modulate biological functions and parameters, and suggests that interactions between the two genomes (the mitochondrial and the nuclear one), are particularly important for cognitive functions at advanced age. This in vivo model is particularly suitable for studies aimed to disentangle the role of and the interactions between mtDNA inherited variability and nuclear genes on mammalian longevity. The effect observed in short-living mammals may be even more important in long-living mammals such as humans whose life spans over decades. A

Box 1

Technical problems of studies on mtDNA mutations and variants

As for nuclear genes, when studying mtDNA variants both in case-control analyses and in other studies, stringent guidelines have to be followed in order to avoid analysis bias (see table below). Indeed, the inconsistency of the results could often be due to methodological complexities or limitations, and to mistakes in samples recruitment and selection. On this last point, it must be mentioned the deCODE study on Icelanders that shows the presence of a notable regional subdivision in the Icelandic gene pool [105]. This study strongly supports the crucial importance of a correct sampling to avoid false-positive results in association studies. Recommendations for the analysis of mtDNA variability:

1. To use an appropriate sample size, a task becoming more and more demanding as the resolution of the mtDNA variability increases.
2. To use a homogeneous population of cases and controls, matched for sex, ethnicity and geography.
3. To take into account the multifactorial nature of aging and age related diseases, and the possible interaction with nuclear genes [106].
4. To avoid technical problems related to:
 - non-accurate technique (necessity to check for the amplification of nuclear pseudogene by using an appropriate controls, *e.g.* ρ^0 cells).
 - possible sample contamination
 - quantification of mtDNA heteroplasmy by appropriate technique, *e.g.* DHPLC [107]
 - to ascertain whether DNA is directly extracted from cells/tissues or it derives from WGA (whole genome amplification)

Another possible source of confounding results is the occurrence of mtDNA recombination. Indeed, the dogma that mtDNA does not recombine has been recently broken. mtDNA recombination has been described to occur at a cell level both *in vivo* and *in vitro* [108,109]. Whether mtDNA recombination is a common event in humans remains to be determined. Indeed, there is only little evidence that it can occur at population level [110]. These findings have possible important implications for mtDNA-related diseases, the interpretation of human evolution and population genetics and forensic analyses based on mtDNA genotyping [109,110]. To better understand this problem it could be useful to perform studies on human pedigrees, which are probably the most informative way to investigate this topic.

recent study in *Drosophila* that used an experimental design similar to that of Roubertoux, but extended to interspecific crosses, showed important epistatic interactions between mtDNA and nDNA on longevity which were more evident in interspecific introgression lines in comparison to intraspecific lines [104]. Owing to these mitonuclear epistases the quantitative effect of mtDNA variability on longevity might be highly dependent on significant interactions with ubiquitous nuclear allelic variation.

12. Conclusions

All the data considered in this review support the hypothesis that mtDNA is involved in aging and longevity. A number of different possible mechanisms have been proposed to explain this involvement, from mtDNA abundance and accumulation of somatic mutations, to inherited variability and cross-talk with nuclear genome. The study of the precise role of mtDNA in aging and longevity has been addressed using a variety of experimental models, while the most part of studies involving human subjects are genetic association studies. These latter

Box 2

CYBRIDS: a method to study the interaction between mtDNA variants and the nuclear genome

Beside animal models, one of the most popular and commonly used model for mtDNA study is represented by cytoplasmic hybrids, or cybrids. Developed by King et al. in Attardi's laboratory [111], the technique relies on the creation of a mtDNA-null (ρ^0) cell from cancer cell lines by long exposure to 3.8-diamino-5-ethyl-6-phenylphenanthridiniumbromide (EtBr) [112]. This intercalating agent is toxic to the mitochondrial genome by inhibiting mtDNA replication, while it is supposed to have almost no effect on nDNA [113]. This specificity is due to the the intercalation of EtBr into mtDNA but not in nDNA, that is strictly packed as chromatin. Recently it has been observed that genetic instability occurs in cells upon mtDNA depletion [114] and thus particular attention should be devoted to possible chromosomal changes when addressing the study of nuclear-mitochondrial cross talk using cybrid cells. After creation of a ρ^0 cell line which is completely depleted of mtDNA, a fusion of enucleated cells with the ρ^0 cell is induced by means of polyethylene glycol 1500. This produces a new cell line in which the nDNA comes from the parental ρ^0 cells, and the mtDNA comes from the donor cells [115]. To select the cytoplasmic hybrids requires several weeks of culture in selective uridine-free medium containing pyruvate to sustain the true cybrids and to eliminate ρ^0 cells. Moreover, if only the faster growing colonies are picked up, a bias could be created.

suffer in general for a low statistical power, due on one side to the ever increasing complexity of inherited lineages (haplogroups and sub-haplogroups), and to the relative tissue-specific importance of mitochondria on the other side. To overcome these pitfalls, it is necessary: i. to study a large number of aged subjects; ii. to take into account technical tips; iii. to study the entire sequence of mtDNA in order to consider its whole complexity. To this end, in the framework of the European Project “Genetics of Healthy Aging” (GEHA), an unprecedented number of old subjects will be studied as far as mtDNA sequence variation is concerned. In particular, 2,650 couples of sibs both older than 90 years of age as well as 2,650 young controls will be collected all along 11 European Countries. We are confident that this study will be able to provide important data on a large number of young and old subjects belonging to different geographic origins, including countries from southern and northern Europe.

Another important aim of the GEHA Project is to perform a genome-wide analysis of the 2650 sib pairs in order to identify candidate chromosomal regions which harbor longevity genes and to perform more dense analysis in order to finally identify the gene variants involved in the trait. Moreover, all subjects within the GEHA Project will be studied for *APOE* variants ($\epsilon 2$, $\epsilon 3$, $\epsilon 4$), assumed as the gold standard gene, whose variants have been found associated with aging and longevity in all the studies. One of the characteristics of the GEHA Project is to combine the data which will emerge from the study of the nuclear genome with those emerging from the study of the mitochondrial genome.

In conclusion, the main message of this review can be summarized as follows: aging and longevity, as complex traits having a significant genetic component, likely depend on many nuclear gene variants interacting with mtDNA variability both inherited and somatic. We also surmise that what we hypothesize for aging and longevity could have a more general relevance and be extended to other complex traits, such as age-related diseases like cardiovascular diseases and diabetes, besides AD and PD, where nDNA–mtDNA interactions as well as mtDNA–mtDNA interactions can play a critical role. The genetics which emerges for complex traits, including aging and longevity, is thus even more complicated than previously thought as mtDNA variants (both somatic mutations and inherited variants) must be considered as additional players capable of interacting with nDNA variants, thus possibly explaining a part of the aging and longevity phenotype. To test this hypothesis is one of the main challenge in the genetics of aging and longevity in the next future.

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