Review

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mtDNA copy number: molecular diagnostics and mitochondrial-nuclear crosstalk in frailty and ageing

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Keywords: ageing, mtDNA, copy-number, fusion-fission, OXPHOS, ROS, Sirtuins, Klotho.

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Quantitative assessment of mitochondrial DNA copy number is an emerging field of study in the diagnosis and evaluation of age-related pathology and frailty in the elderly, along with the assessment of other acute and chronic diseases that are often necessary within this patient cohort.

Aim: to analyze current advances in the quantitative assessment of mitochondrial DNA copy number as a tool for diagnosing and evaluating age-related pathology, frailty, and comorbid conditions in the elderly.

Material and methods. The author independently conducted a thorough review of literature available from the NIH, PubMed database, providing a detailed narrative of updates in the field. As clinical trials seek to further develop practical techniques, the author then undertook a search of relevant trials at ClinicalTrials.gov, including this as a table in the body text.

Results. The review provides a thorough examination of the theoretical foundation of mitochondrial biogenesis, fusion-fission processes, and control and repair of mitochondrial genetic material, and how advances in these topics may be applied to better understand the processes being measured in quantitative mitochondrial DNA analysis.

Conclusions. Detailed examination of the crosstalk between mitochondrial control proteins and nuclear factors, and the fundamental role of energy homeostasis apparatus within ageing processes underpins the advances in translational aspects of mitochondrial medicine and allows more effective exploitation of this emerging field.

Ключові слова: старіння, мтДНК, кількість копій, злиття – поділ, OXPHOS, ROS, сиртуїни, Klotho.

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Кількість копій мтДНК: молекулярна діагностика та мітохондріально-ядерні перехресні зв'язки при крихкості та старінні

Ч. Дж. Вард

Кількісне визначення кількості копій мітохондріальної ДНК – нова галузь досліджень у діагностиці та оцінюванні вікової патології та крихкості в осіб похилого віку, поряд з вивченням інших гострих і хронічних захворювань, що часто є доцільними для таких пацієнтів.

Мета роботи – проаналізувати сучасні досягнення щодо кількісного визначення копій мітохондріальної ДНК як інструмента діагностики та оцінювання вікової патології, крихкості та супутніх захворювань в осіб похилого віку.

Матеріали і методи. Здійснено ретельний огляд фахової літератури, що індексується в базі даних NIH, PubMed, з детальним описом найновіших досягнень у цій галузі. Оскільки під час клінічних випробувань прагнуть до розвитку практичних методів, на наступному етапі здійснено пошук даних щодо відповідних випробувань на ClinicalTrials.gov.

Результати. Здійснено дослідження теоретичних основ мітохондріального біогенезу, процесів злиття та поділу, а також контролю та репарації мітохондріального генетичного матеріалу. Описано, як досягнення у дослідженні з цих тем можуть бути застосовані для кращого розуміння процесів, що вимірюються під час кількісного аналізу мітохондріальної ДНК.

Висновки. Детальне дослідження взаємодії між мітохондріальними контрольними білками та ядерними факторами, а також фундаментальної ролі апарату енергетичного гомеостазу в процесах старіння є підґрунтям для розвитку трансляційних аспектів мітохондріальної медицини та забезпечує більш ефективне використання цього нового напряму.

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The author independently conducted a thorough review of literature available from the NIH, PubMed database, providing a detailed narrative of updates in the field. As clinical trials seek to further develop practical techniques, the author then undertook a search of relevant trials at ClinicalTrials.gov , including this as a table in the body text.

This review explores the molecular crosstalk between the nuclear proteins and the mitochondrial compartment and its role within ageing, with specific focus on the potential use of mitochondrial DNA (mtDNA) copy number in assessing age-related pathology, frailty syndrome and the diagnosis of acute and chronic disease. The usefulness

and validity of quantitative mtDNA analysis in clinical practice rests upon the foundational science of molecular ageing, and it is vital that this mechanistic process is fully explored before such advances can be translated into tools that might be useful at the bedside.

The mitochondrial theory of ageing, concepts of frailty and frailty syndrome and the investigation of molecular biomarkers related to ageing occupy the opening sections of this review. This is followed by a discussion on the role of quantitative mtDNA analysis and a detailed examination of the molecular nature of ageing in both nuclear and mitochondrial contexts, and the degree to which both processes rely on each other for progression. The final sections are an examination of past and current clinical trials, a critique of the validity of mtDNA copy number analysis, and a short discussion on the ethical considerations implicit in age-related and transgenic research before concluding remarks on the application of theoretical science to translational medicine and the future direction of travel in this exciting subject.

Results

The mitochondrial theory of ageing. Mitochondria have long been central to the understanding of the of ageing. Via the generation of ATP, reactive oxygen species (ROS) and age-related degenerative decline, ageing cells release components required for oxidative phosphorylation (OXPHOS), resulting in decreased ATP production [1]. A true breakthrough in molecular mitochondrial gerontology came with a 2004 landmark study generating mice expressing defective mtDNA polymerase γ1 (Pol γ1). During the earliest part of their lives the mice were phenotypically normal, yet they quickly displayed signs of ageing including alopecia, kyphosis, osteoporosis, anaemia, heart enlargement and greatly reduced fertility. During the animals' lifespan the number of mutations in mtDNA increased exponentially and the study determined a direct and causal link between increased mtDNA mutation, mitochondrial dysfunction in oxidative phosphorylation and the ETC and age-associated phenotypes [2].

Age-related pathology in the clinic and laboratory. Age-related pathology results in adverse outcomes in hospitalisation, quality of life and mortality. It is diagnosed clinically via assessments such as the mini-mental state exam (MMSE) [3]. Fried L. P. et al. defined a "phenotype of frailty" that could be used to identify aged patients at risk of morbidity, defining frailty syndrome where three or more of the following criteria are present: "shrinking" – unintentional weight loss ≥10 lbs in the past year or ≥5 % of body weight at annual follow-up; weakness as measured by grip strength; self-reported exhaustion, poor endurance and energy; slow walking speed; low physical activity [4].

Molecular investigations in age-related pathologies are a long-running priority in gerontology and a significant literature exists regarding the molecular aspects of the ageing endocrine system. Insulin-like growth factor 1 (IGF-1) [5,6], SIRT1, growth differentiation factor 15 (GDF-15) [7], sex hormones such as testosterone and their precursors such as dehydroepiandrosterone (DHEA) and DHEA sulphate (DHEAS) [8] are the main candidates under investigation. Low serum IGF-1 has been associated with decreased cognitive and physical function in aged persons and provides the most well explored biomarker of age-related infirmity [6,9]. The causal linkage between decreased endocrine activity and frailty has yet to be established and, while well explored, it remains to be seen as to whether the age related decreased of these markers is causal of biological ageing or as a subsequent response to another process [10].

The main immunological markers associated with ageing are interleukin 6 (IL-6), CRP and TNF-α, along with alterations in the function of heat shock proteins (Hsps) [11] while alterations in small Hsps may be linked with reductions in muscle function [12]. Paradoxically, reduced Hsp function in rat models conferred resistance to myocardial ischaemia [13]. C-X-C motif chemokine ligand 10 (CXCL10) is a small protein chemokine secreted by neutrophils, eosinophils and lymphocytes in response to viral infection [14]. While strongly implicated in viral response [15], chemokines have also been implicated in autoimmune disease [16], as a novel mode of immunotherapy in cancers [17] and in age-related pathology. CXCL10 is induced in response to interferon-γ (IFN-γ) or viral infection and decreases mitochondrial activity, induces apoptosis and reduces cellular proliferation. Serum CXCL10 is increased in ageing and CXCL10 knockout animals have decreased response to interferon mediated immune responses [7]. Genetic material and nuclear proteins are also affected by ageing. Older cells have reduced histone synthesis, altered chromatin structure, reduced epigenetic histone methylation or acylation modification and overall loss of heterochromatin across the organism [18].

While clinical trials are progressing, the study of mtDNA is an underutilised and poorly explored aspect of molecular pathology of ageing.

The utility of mtDNA copy number in quantitative mtDNA assessment. mtDNA copy number is a measurement of the amount of mitochondrial genetic material in cells, assessed by quantitative real-time PCR (qPCR) and providing a quantitative assessment of the mitochondrial compartment of the cell [19]. Mitochondria are highly plastic organelles that adapt in form and quantity to cellular energy requirements, and it is anticipated that quantitative analysis of the mitochondrial compartment could provide an easy, cheap and effective understanding of the OXPHOS system of the cell from readily available peripheral blood samples [20].

The cellular mitochondrial compartment is highly heterogeneous and carries much variation across tissues with different energy requirements and across the organism in different physiologic states. In mice, primary oocytes have around 100,000 copies of mtDNA while sperm have around 100 [21]. Oocyte mtDNA copy number decreases with age and results in reduced reproductive success in both mice [22] and humans, also providing an avenue for fertility studies [21,23]. Similar changes in mtDNA copy number are true of other energy-rich tissues such as skeletal muscle, where mtDNA copy number can be used to predict the severity of sarcopenia in the aged [24] and nervous tissue, where reduced mtDNA

correlates with incipient Alzheimer's disease, Parkinson disease and other neurodegenerative conditions [25,26].

Attempting to determine association between mitochondrial dysfunction, oxidative stress, ageing and mortality, Ashar Moes et al. assessed alterations in mtDNA copy number against all-cause mortality and prevalent frailty. Using qPCR to determine mtDNA copy number in 13,444 patient samples from participants in the 1989–2006 Cardiovascular Health Study (CHS) and the 1987–2013 Atherosclerosis Risk in Communities (ARIC) study. Across the sample they determined a global reduction in mtDNA copy number against increasing indicators of frailty even while individual parameters of frailty did little to change the mtDNA copy number [27].

In 2010 J.-W. Lee et al. used qPCR to measure mtDNA copy number in peripheral leukocytes of 107 women aged over 60 in South Korea, finding that women who scored highly on tests for cognitive decline had significantly reduced mtDNA copy number when compared with those who were apparently healthy and performed significantly worse in mini-mental state examination, 6-minute walking test and chair-stand testing [28]. Similarly, muscle biopsies from 15 octogenarian world-class track and field master athletes revealed significantly higher mtDNA copy number among octogenarian master athletes when compared to control group non-athletes, along with greater preservation in muscle mass, increased abundance of mitochondrial proteins (specifically those involved in OXPHOS and the electron transport chain) and greatly increased abundance of mitochondrial ribosomes [29]. Mengel-From J. et al. used qPCR to measure the mtDNA copy number of 1,067 participants aged between 18 and 93 years. They noted age related decline in mtDNA copy number from 48 years old onwards with consistent association between higher mtDNA copy number and better physical and mental health outcomes, both in terms of physical performance and by self-rated questionnaire. Higher mtDNA copy number in aged participants was also associated with higher scores in cognitive testing [30].

Chronic disease and mtDNA copy number. Patients with chronic kidney disease (CKD) have sarcopenia, muscle weakness and limited exercise tolerance. Muscle atrophy and weakness correlates with impaired mitochondrial respiratory function and reduced mtDNA copy number [31]. Data from the ARIC study revealed that patients with CKD had a lower circulating mtDNA copy number and that a high mtDNA copy number reduced the incidence of CKD [32].

In advancing age the substantia nigra pars compacta (the primary site for dopamine production in the brain) has a particularly high level of mtDNA deletions when compared to other areas of the brain and immunohistochemistry reveals a higher rate of cytochrome c oxidase (Complex IV) depletion as age progresses [33,34]. A number of studies report that ageing brains acquire mutations in mtDNA rendering respiratory chain proteins defective and potentially providing for the pathogenesis of neurodegenerative diseases most commonly seen in the aged such as Parkinsonism [33,34]. This area could in future provide a rich seam of novel therapeutic endeavours for future research.

Nevertheless, in conducting quantitative mtDNA analysis it is necessary to understand the mechanistic process by which mtDNA is generated, degraded and controlled throughout the organism. The following section provides an undertaking of this aspect of subcellular and molecular ageing.

Failure of the mitochondrial fusion-fission cycle in the pathophysiology of ageing. Wakabayashi T. defined structural change of mitochondria as a function of simple swelling. Free radicals and ROS produce change in the inner and outer mitochondrial membrane allowing accumulation of structural deficits and failure of normal fusion and fission, resulting in the formation of so called "megamitochondria". When these organelles become so dysfunctional that their membrane potential decreases sufficiently, cytochrome c is released causing caspase activation and induction of apoptosis [35]. He determined that mitochondrial swelling and the formation of pathological mitochondrial structures could be linked to free radical and ROS related degradation [36,37].

Mitochondria exist within a fluid continuum of fission-fusion events [38,39]. Large mitochondria spit into smaller more manageable units while smaller, more motile fragments coalesce. Taken as a whole, the mitochondrial mass of the cell can be considered a "compartment" responsible for energy production and with diverse functions controlling the innate immune system. Failure of the mitochondrial fusion-fission cycle has been linked to diseases as diverse as hepatocellular carcinoma, pancreatic adenocarcinoma, "triple-negative" breast cancers, neurological conditions such as sporadic type Parkinson disease and certain variants of Charcot-Marie-Tooth disease [39], along with hereditary inborn errors of the OXPHOS system including certain variants of subacute necrotising encephalomyelopathy ("Leigh syndrome") [40,41].

This cycle is tightly regulated by a series of inner mitochondrial, outer mitochondrial and cytosolic proteins. Inner membrane optic atrophy-1 protein (Opa1) controls fusion of the inner membrane. *Drosophilia* flies with mutated non-functional Opa1 soon display mitochondrial fragmentation and neural degeneration [31]. In the outer membrane, mitofusins -1 & -2 (Mfn-1 & Mfn-2) are highly conserved dynamin-related GTPases with diverse functions in the cell. While the two proteins are highly homologous, they are not functionally identical. Mfn-1 has the highest GTP affinity and is largely responsible for mitochondrial fusion [42]. Mfn-2 is best known as the defective protein in Charcot-Marie-Tooth (type IIa) sensory neuropathy. It is essential for the proper function of mitochondria as it tethers mitochondria to the endoplasmic reticulum (ER) and regulates ER shape and aspects of ER-mitochondrion calcium transfer [43].

During chronic disuse of skeletal muscles Mfn-2 and Opa1 are downregulated, leading to reduced fusion of mitochondria, increased organelle fragmentation and reduced mitochondrial mass within myofibrils [38]. In the innate immune system both Mfn-1 [44] and Mfn-2 activate mitochondrial antiviral signalling protein (Mavs) which, via activation of nuclear factor κB (NF-κB) upregulates production of interferons type I & III when activated by viral states or the release of mitochondrial RNA (mtRNA) [45]. Mfn-1 & -2 deficiency results in significant reduction in

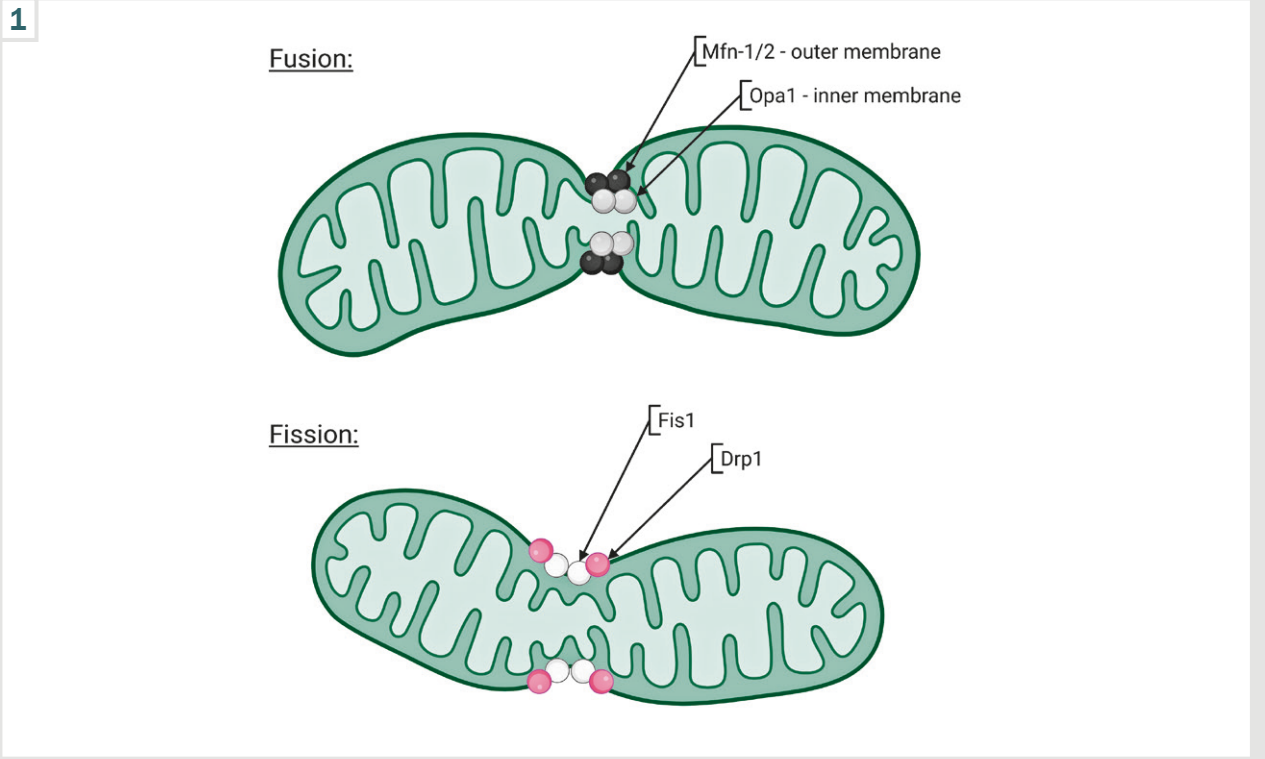

Fig. 1. Mitochondrial dynamics, showing fusion-fission apparatus of mitochondria (created in Biorender.com).

mitochondrial encoded electron transport chain complex proteins, yet no change in expression of nuclear encoded proteins of the TOM complex. Similarly, gross deficiency of Tfam expression is seen even while levels of Tfam-encoding mRNA remain constant, suggesting that mitofusins play a key role in post-transcription expression of Tfam. Other mitochondrial gene expression proteins such as Pol-γ and Twinkle remain constant, indicating alternative methods of modulating mtDNA replication take precedence [42].

Mitochondrial fission is controlled by dynamin-related protein (Drp1) and fission protein 1 (Fis-1). Drp1 is a GTPase which translocates to the outer mitochondrial membrane and develops active fission sites when recruited by Fis-1 [46]. Proinflammatory cytokine IL-6, highly expressed in chronic low-level inflammatory states in ageing tissue, stimulates production of Drp-1 and results in mitochondrial fission throughout skeletal muscle, which suggests a link between chronic inflammation of ageing and sarcopenia seen in advanced frailty syndrome [47].

Mitochondrial fusion and fission is a tightly controlled process regulating the size and surface area of organelles. Fusion is controlled by mitofusins 1 and 2 (Mfn-1 and Mfn-2) in the outer membrane and optic atrophy protein (Opa1) in the inner membrane. Fission is controlled by outer membrane proteins dynamin related protein 1 (Drp1), a GTPase which is recruited to the outer mitochondrial membrane by fission protein 1 *(Fig. 1)*.

Expression of mtDNA and mtDNA copy number is responsive to Mfn-1 Mfn-2 activity and both proteins are required to maintain proper mitochondrial structure. In mouse model studies, animals deficient in Mfn-1 alone had disorganised and fragmented mitochondria yet nevertheless maintained normal mtDNA copy number, maintaining glucose homeostasis in pancreatic β cells despite this fragmentation. Animals deficient in both Mfn-1 and Mfn-2 had fragmented mitochondria with reduced mtDNA copy number and were unable to maintain glucose homeostasis [42]. Traditional models of glucose tolerance dysregulation in ageing have focused on alterations in insulin receptor expression, function and response of second messenger systems [48,49,50,51]. The finding that mitochondrial aberrations can produce similar clinical results provides a fascinating alternate explanation and complements the hypothesis that correct mitochondrial homeostasis and morphological dynamics are essential to the proper functioning of metabolism.

Mitochondrial nucleoids house genetic information. Mitochondrial genetic material is housed in nucleoid DNA-protein ("nucleoprotein") configurations associated with the inner mitochondrial membrane (IMM) [52]. mtDNA polymerase (Pol γ1) is encoded in autosomal DNA and has both replicative and repair functions [53,54], showing a high degree of similarity to yeast and bacterial DNA polymerases [55]. Twinkle protein is a nuclear encoded mtDNA helicase with intramitochondrial nucleoid localisation, responsible for priming and unwinding mtDNA in replication and repair [53].

Of the many hundreds of proteins present in the mitochondrion, only 13 are encoded by mtDNA and all of those are subunits or components of subunits in the respiratory chain [56]. This complex interplay of nuclear and mitochondrial elements requires constant communication between nucleus and mitochondria, and this is accomplished via

nuclear respiratory factors 1 and 2 (NRF-1, -2). In addition to upregulating production of mitochondrial proteins such as cytochrome c oxidase, NRF-1 and -2 are the key activators of mitochondrial transcription factor A (Tfam). Tfam was one of the earliest proteins to be localised in both the nucleus and mitochondrion, and it holds a central role co-ordinating nuclear command and control of mitochondrial energy generation [52]. Tfam links NRF-1 & -2 to mitochondrial transcription and replication apparatus [57]. NRF-1 or -2 binding to Tfam causes activation of mitochondrial transcription, production of mtDNA and mitochondrial rRNAs and tRNAs. Tfam negative mice develop respiratory chain deficiencies, various cardiac abnormalities including arrhythmia and cardiomyopathy and have greatly reduced longevity [58]. On translation, nuclear-encoded proteins are conveyed via dense reticular networks to mitochondria, where they enter through translocator of outer membrane (TOM) complexes. The TOM complex is the primary means of transport for more than 90 % of mitochondrial proteins into the organelle and allows delivery of pre-protein components to mitochondrial sorting systems [59].

Repair and maintenance of the mitochondrial genome. Deletions and point mutations in mtDNA and mitochondrial tRNA cause diseases with typically heterogenous presentation and variable penetrance, the most common of which is subacute necrotising encephalomyelopathy ("Leigh syndrome") [40,41]. While mitochondria are the major cellular origin of oxidative stress, they do not appear to have a unique pathway of genetic repair and much of the nuclear DNA repair apparatus is "shared" with mitochondria. Excision repair apparatus is shared almost entirely with nucleic genetic material whereas mismatch repair is more reliant on mitochondrial proteins.

Oxidative damage is largely repaired by base excision repair, either by short-patch (SP) or long-patch (LP) pathways. SP repair is used when DNA polymerase-γ1 (Pol γ1) can repair a single nucleotide gap between a free 3' hydroxyl group and a 5' phosphate, while LP repair is used when there is a 5' end that cannot be ligated by a single nucleotide and a short single-stranded DNA (ssDNA) "patch" must be generated to fill the gap [54]. The LP repair system was first discovered in mitochondria in 1998 when it was demonstrated that oligonucleotides were utilised by mtDNA ligase and Pol γ1 to repair abasic sites [60].

Telomeric ageing controls mitochondrial ageing. Telomerase reformulates eukaryotic chromosomal telomeres by the addition of nucleotide tandem repeats allowing continued replication of chromosomes [61], and subsequent discoveries have demonstrated that telomerase is highly conserved across eukaryotic kingdoms [62]. Chromosomal telomere dysfunction activates p53 to arrest growth, produce senescence and initiate apoptosis. While commonly described as a tumour-suppressor gene and the "guardian" of the genome, the role of p53 is varied, wide ranging and displays many characteristics of both a classical tumour suppressor gene and oncogene. Wild-type p53 acts as a negative regulator of cell growth, yet the atypical variants found in cancers express multiform gain of function mutations that drive tumour growth [63]. p53 is the most prolific mutation found in cancer and, most understandably, its role in oncogenesis has been by far its most studied aspect [64].

It is now clear that the role of p53 in governing mitochondria and energy balance across the cell is vital to its function as a key regulator of cell growth and differentiation. This relationship was first elucidated by the discovery that p53 activation suppressed "Warburg-type" glycolysis and increased cytochrome c production and aerobic mitochondrial respiration in severely nutrient depleted glioblastoma cells [65]. This is achieved through activation of proliferator-activated receptor gamma, coactivator 1 α and β (PGC-1α and PGC-1β) and PGC-1-related coactivator (PRC) which act as high-level regulators of mitochondrial function and are regulated by P53 (*Fig. 2*) [66]. PGC-1α was first discovered as a cold-induced coactivator of thermogenic-associated nuclear receptors increasing transcriptional activity of PPARγ and thyroid hormone receptors in thermogenic tissue. Activation of PGC-1α promotes expression of mitochondrial respiratory chain enzymes, thermogenic uncoupling protein 1 (UCP-1) and increases the expression of mtDNA across the cell [67]. PRC is not upregulated in thermogenic tissue and does not have the same cold-induction properties of PGC-1α/β and is instead expressed constitutively in skeletal and cardiac muscle [68].

Since this discovery, the pathway through which PGC-1α/β and PRC regulate high-level mitochondrial function and energy homeostasis has been further researched. Increasing cellular NAD+ (indicating low cellular energy balance) activates mitochondrial deacylase silent information regulator 1 (Sirt-1) which deacylates and activates PCG-1α and PCG-1β. In contrast, increased cellular acetyl-CoA (indicating high cellular energy state) activates general control non-repressed protein 5 (GCN5), an acyltransferase inhibiting PCG-1α [69].

Similarly, increased circulating IL-6 as seen in low level chronic inflammation causes suppression of PCG-1α gene expression via activation of extracellular regulated kinase 1/2 (ERK1/2), resulting in mitochondrial depletion and skeletal muscle catabolism [47]. Specifically, IL-6 causes inactivation of MEK [70] (the primary downstream target of second messenger Raf [71]), which downregulates a mitogen activated protein kinase (MAPK) and the MAPK signalling pathway, resulting in reduced translation and mRNA expression of PCG genes [70].

In mouse models, telomere dysfunction directly causes quantitative deficits in downstream PCG-1α and PCG-1β expression resulting in downregulation of genes for oxidative phosphorylation proteins, defence against ROS (such as glutathione peroxidase), gluconeogenesis, and the synthesis of fatty acids and cholesterol. Consequently, cellular mitochondrial mass and energy production are inversely proportional to the degree of telomere dysfunction. The is especially marked in tissues with a high basal rate of ATP production such as the heart and liver which, in ageing, show reduced mtDNA copy number, reduced Complex I & IV activity and reduced ATP production [72].

Potentially, neoplastic cells with inappropriately activated telomerase maintain telomere stability allowing pathological hyperplasia and hypertrophy. Aged cells with reduced telomere stability show a concomitant reduction in activation of PCG-1α/β and therefore decreased mitochondrial gene expression, function and mass within the cell.

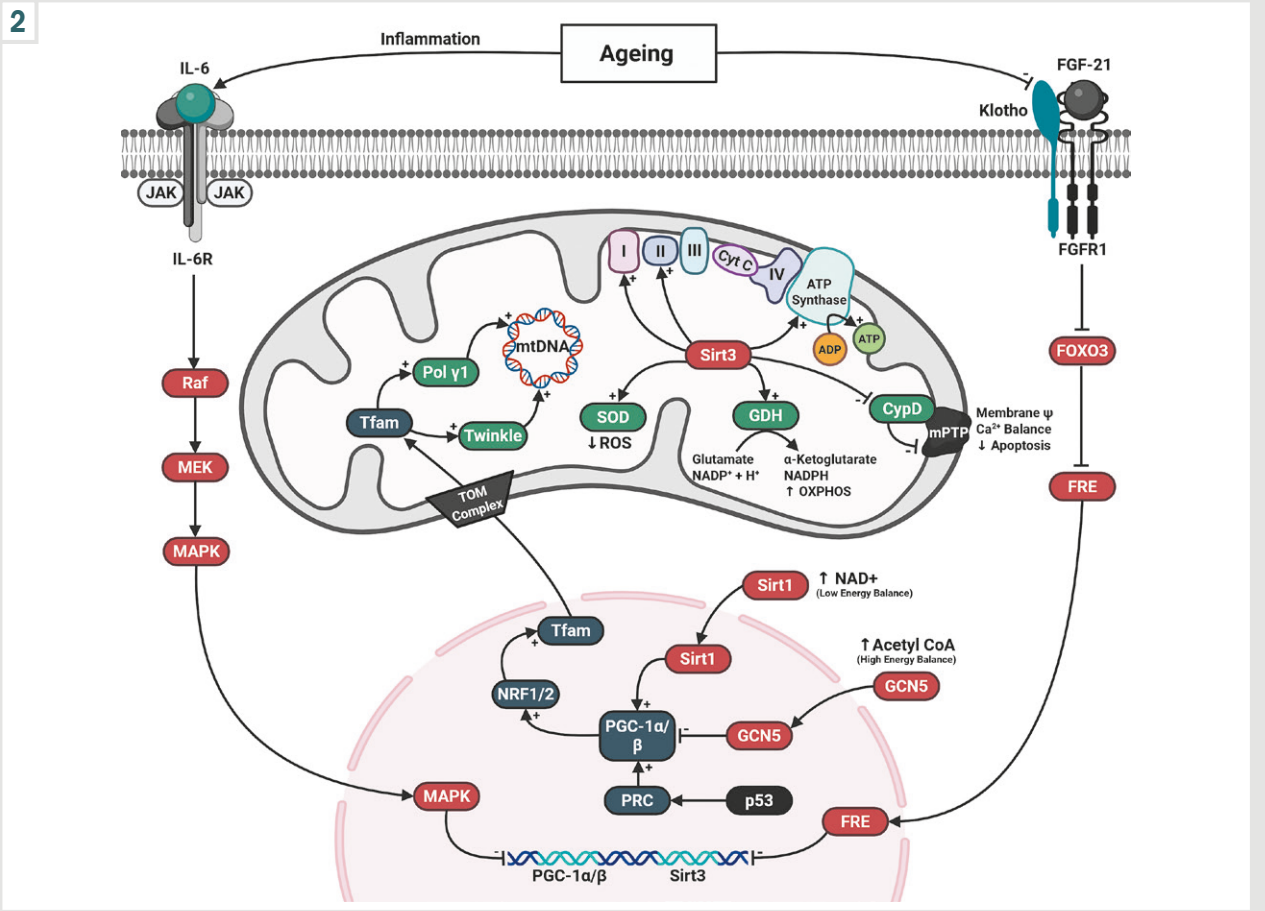

Fig. 2. The effect of ageing on nuclear regulation of mitochondrial energy homeostasis.

Sirtuins, klotho & fibroblast growth factors in mitochondrial ageing. Sirtuins are the protein product of silent information regulator (SIR) genes and act as NAD-dependent acylases / deacylases found throughout the cytoplasm, nucleus and mitochondria. Sirtuin protein complexes are highly expressed in the nucleolus and regulate the transcription of ribosomal subunits. In the simple yeast *Saccharomyces cerevisiae*, sirtuins 2, 3 and 4 are linked to longevity, and knockout cells with inactivated Sirt-2, -3 & -4 have significantly shortened lifespans [73].

Sirt-3 also functions as a major mitochondrial NAD-dependent deacylase responsible for epigenetic deacylation of lysine residues. In mitochondria, Sirt-3 deacylation of TCA cycle malate dehydrogenase and complexes II and V of the ETC produces increased ATP levels, while deacylation of superoxide dismutase (SOD) of the antioxidant system provides much needed protection against the ROS so readily produced in the matrix [74,75]. Sirt-3 also directly regulates complex I of the ETC. Mitochondria deficient in Sirt-3 have around 50 % lower resting ATP levels in comparison to controls, along with increased complex I activity on addition of exogenous Sirt-3 [76]. Glutamate dehydrogenase (GDH) is a significant target of Sirt-3 deacylation, inducing enzyme activation and increasing production of α-ketoglutarate, stimulating OXPHOS within the cell [74].

Sirt-3 has another intriguing role as the regulator of mitochondrial permeability via Sirt-3 cyclophilin D (cypD) interaction. CypD is a chaperone of the mitochondrial permeability transition pore (mPTP) which, when induced to bind to adenine nucleotide translocator, initiates the opening of the pore allowing transfer of material between the mitochondrial matrix and the cytosol, loss of membrane potential, disruption of calcium metabolism and the OXPHOS system and cell death [77,78].

Klotho, which has been linked to age-related pathology suppression, is a three-isoform protein co-receptor allowing fibroblast growth factor (FGF) binding to the receptor complex. All isoforms are transmembrane proteins acting in association with FGF receptor 1 (FGFR1) complexes, and there also exists a soluble form found in circulation [79,80]. Klotho proteins are essential for the binding of FGF-19, FGF-21 and FGF-23 to FGF receptor 1 (FGFR1) complexes. FGF-21 is a stress hormone produced in response to starvation and exercise [75], FGF-19 results from satiety [80] while FGF-23, in association with vitamin D and parathyroid hormone (PTH), regulates calcium and phosphorus balance [81].

FGF-21 and Klotho promote Sirt-3 expression and are essential control points in the maintenance of mitochondrial OXPHOS and antioxidant systems. FGF-21 binding FGFR1 induces a MAPK cascade converging on transcription factor forkhead box protein O3 (FOXO3)

which, via FOXO3 response elements (FRE) stimulates production of Sirt-3. This has been demonstrated in FOXO3 inhibited mouse models where FGF-21 is no longer able to induce production of Sirt-3 [75]. Immediate control of Sirt-3 is provided for via the SENP1-Sirt-3 axis, where small ubiquitin-like modifier (SUMO) proteins are covalently attached to Sirt-3 by SUMO-specific protease SENP-1 [82]. In addition to the action of FGF-21, Klotho acts as an anti-ageing protein by preventing senescence through suppression of RIG-1 mediated activation of NF-κB [83,84] and the decreased ability of ROS to activate the caspase-9 and -3 apoptotic pathway [85].

In tissues undergoing normal oxidative phosphorylation, PGC-1α/β sit at the centre of nuclear control of metabolism. P53, acting via PRC activates it, as does a reduced energy balance across the cell (signified by increased NAD+), which acts via Sirt1. When there is a high energy balance across the cell (signified by increased acetyl-CoA), PGC-1α/β is deactivated. Activation of PGC-1α/β causes translocation of Tfam to the mitochondrial matrix where, acting through Pol γ1 and Twinkle, mtDNA replication and the production of mitochondrial OXPHOS proteins is upregulated. Similarly, the activation of Sirt3 results in increased production of OXPHOS proteins, increased glutamate dehydrogenase (GDH) activity (resulting in increased α-ketoglutarate and increased tricarboxylic acid cycle flux) and increased superoxide dismutase activity, resulting in less ROS production. Acting via cyclophilin D, Sirt3 modulates the activity of the mitochondrial permeability transition pore (mPTP), resulting in membrane potential stability, maintenance of proper calcium balance and reduction in apoptosis *(Fig. 2)*.

Ageing organisms encounter persistent low-level inflammation and the depletion of necessary cofactors, such as Klotho, required for the binding of FGF-21. Via a series of intracellular cofactors such as MEK, MAP kinase (MAPK), forkhead box protein O3 (FOXO3) and FOXO3 response element (FRE), production of essential modulators of cellular energy metabolism PGC-1α/β and Sirt3 are downregulated. This causes downregulation of mitochondrial energy metabolism, mtDNA transcription, and mitochondrial protein synthesis, along with reduced mitochondrial stability, disruption of membrane potential and ultimately apoptosis.

Discussion

Relevant clinical trials. A number of clinical trials have been conducted or are currently taking place investigating the application of quantitative mtDNA analysis into the diagnosis or evaluation of risk or progression in disease and ageing. Several focus on the use of mtDNA copy number as a biomarker in disease states, frailty syndrome. Of particular interest is the use of mtDNA copy number to assess risk of neurocognitive impairment, sarcopenia and muscle function. A full list of past and current trials can be found in *Table 1*.

A critique of the validity of mtDNA copy number. In peripheral blood there are a wide variety of cells and cellular components that have a greatly varying mitochondrial complement. Red blood cells have a negligible mitochondrial fraction and contain only trace amounts of mtDNA, which is why peripheral blood mononuclear cells (PBMCs) are the tested fraction in whole blood [20]. This "buffy coat" includes a wide and uneven distribution of CD4+ Th cells, CD8+ Tc cells, B cells, NK cells, neutrophils, monocytes, eosinophils and basophils; all present in different samples in differing quantities, influenced by a wide variety of physiological and pathological factors and themselves having different energy requirements and mitochondrial composition [86]. Platelets do not have nuclear DNA yet do have mitochondria and mtDNA and frequently contaminate and "stick" to fractioned PBMCs, potentially altering the observed mtDNA copy number [87,86].

While it is often thought that decreased mtDNA copy number is indicative of disease states some studies have reported a paradoxical rise in mtDNA. In cell lines derived from an individual with chronic progressive external ophthalmoplegia, a disease-causing weakness of the eye muscles resulting from mtDNA deletions of components of the OXPHOS system or mtDNA maintenance apparatus, an increased in mtDNA copy number and mitochondrial mass within the cell was observed even while OXPHOS was absent [88]. Similarly, diseases exist which logically exclude the utility of quantitative mtDNA analysis. Leber hereditary optic neuropathy, for example, is an inherited mitochondrial disease resulting from mtDNA base pair mutations in ETC complex I. Patients often display increased mitochondrial mass and mtDNA copy number in comparison to controls owing to a hypothesised upregulation of mitochondrial genetic material and mitochondrial biogenesis in order to compensate for reduced OXPHOS capacity [89]. While researchers have produced techniques and proposed correction formulae to reduce introduced bias [90], there nevertheless remain questions about whether studies of mtDNA copy number are truly comparing like with like, and whether the techniques can overcome these challenges to gain validity at the bedside.

Ethical considerations. Advances in medical technology inevitably open new frontiers in medical ethics. Whether ageing should be considered as a disease (and should therefore be treated) or as a natural part of the pattern of life is as much a question of philosophy as medical science. The topic is discussed at length in both the scientific literature [91,92,93] and the popular press, eliciting strong and sometimes polarising reactions. Changes in life expectancy brought by life-extending medical treatments and declining patterns of fertility in Western nations also stimulates discussion regarding future demographic and economic challenges in countries with declining birth rates and ageing populations [94,95].

Meanwhile, emerging transgenic mitochondrial fertility treatments produce debate regarding the ethical considerations, with early concerns raised regarding the long-term downstream effect of "transmitochondrial babies", and arguments made for renewed public and legal scrutiny [96]. These topics should be discussed sensitively and in depth, with full regard to cultural expectations which may differ across communities [97]. A thorough examination of these topics remains beyond the scope of this article, yet it is nevertheless important that research into ageing, life-extending treatment and transgenic cellular biology is conducted within a robust ethical and philosophical framework.

Table 1. Clinical trials relevant to the quantitative study of mtDNA, the effects on ageing and mitochondrial diseases (from ClinicalTrials.gov, National Library of Medicine, Bethesda, MD USA)

Trial ID	Start Date	Title	Sponsor	Location	Investigation	Status	Hyperlink
NCT00831948	01/12/2008	Identification of Large-Scale Mutations of POLG Gene by QMPSF in Patients With Mitochondrial DNA Instability	Centre Hospitalier Universitaire de Nice	Nice, France	To determine the role of POLG mutation in diseases of mtDNA instability	Unknown	https://clinicaltrials.gov/study/NCT00831948
NCT02132091	01/03/2011	Practicality of Intermittent Fasting and Its Effect on Markers of Aging and Oxidative Stress	University of Florida	Gainsville, Florida, USA	To determine the effect of intermittent fasting on markers of ageing (ROS, sirtuins, mitochondrial biogenesis factors) and oxidative stress	Completed	https://clinicaltrials.gov/study/NCT02132091
NCT01931540	01/06/2012	Developmental ORIgins of Healthy and Unhealthy AgeiNg: the Role of Maternal Obesity (DORIAN)	Turku University Hospital	Turku, Finland	To determine the effect of obesity on ageing using biomolecular markers, PET-CT imaging and clinical parameters	Completed	https://clinicaltrials.gov/study/NCT01931540
NCT01993082	01/01/2014	Fitness, Cellular Aging, and Caregiver Stress Study (FAST)	University of California, San Francisco	San Francisco, USA	Assessment of mitochondrial function and telomere length (among other parameters) and determination of whether aerobic training alters patterns of immune cell ageing	Completed	https://clinicaltrials.gov/study/NCT01993082
NCT02116166	01/05/2014	Skeletal Muscle Inflammation, Oxidative Stress and DNA Repair in Age-Related Sarcopenia	University of Florida	Gainsville, Florida, USA	Investigating the molecular basis of age-related sarcopenia with multi-omics techniques	Completed	https://clinicaltrials.gov/study/NCT02116166
NCT03290040	01/01/2015	dentification of Predictors for Early Cognitive Decline in Men	Rigshospitalet, Denmark	Glostrup, Denmark	Assessment of mitochondrial genetics (and other molecular and clinical features) to explore age-related brain pathology	Completed	https://clinicaltrials.gov/study/NCT03290040
NCT02500823	01/04/2015	Association Between Mitochondrial DNA Copy Number in Peripheral Blood Cells and Risk of Developing Coronary Heart Disease	Air Force Military Medical University, China	Xi'an, Shanxi, China	To determine the association between mtDNA copy number from peripheral blood and risk of coronary heart disease in 200 previously healthy individuals	Completed	https://clinicaltrials.gov/study/NCT02500823
NCT02472340	01/06/2016	Assessment and Reproducibility of Mitochondrial Function and Mitophagy Measurements in Human Muscle Tissue of Active and Pre Frail Elderly Males	Amazentis SA	Leiden, Netherlands	Assessment of decline in mitochondrial health associated with impairment in muscle function, along with levels of mitophagy and autophagy using mitochondrial biomarkers	Completed	https://clinicaltrials.gov/study/NCT02472340
NCT03077672	10/02/2017	Mitochondrial DNA as a Biomarker of Sepsis Severity	Weill Medical College of Cornell University	New York, USA	Determination of sepsis severity using mtDNA from peripheral blood	Completed	https://clinicaltrials.gov/study/NCT03077672
NCT03340571	12/03/2018	mtDNA Damage in Alzheimer's Disease (AD)	Duke University	Durham, North Carolina, USA	Analysis of blood biomarkers of mtDNA damage to determine diagnosis and disease progression in Alzheimer disease patients	Completed	https://clinicaltrials.gov/study/NCT03340571
NCT03929458	07/05/2019	Association of Uremic Sarcopenia and Mitochondrial Copy Number and Its Clinical Correlates	Tungs' Taichung Metroharbour Hospital	Taichung, Taiwan	To determine whether mtDNA copy number can be used to predict age-related sarcopenia	Completed	https://clinicaltrials.gov/study/NCT03929458
NCT04763291	01/09/2021	Cardiovascular and InflammAging Study	Green Beat	Graz, Austria	To determine whether ingestion of encapsulated juice powder and plant based omega fatty acid supplements affect biomarkers of cardiovascular health and biological ageing	Recruiting	https://clinicaltrials.gov/study/NCT04763291
NCT05600192	18/10/2021	Target Mitochondrial Fitness, Chronobiology and Metabolism	Fundación Pública Andaluza para la Investigación de Málaga en Biomedicina y Salud	Malaga, Spain	To determine the effect of aerobic and anaerobic exercise on mitochondrial function at different times of day	Unknown	https://clinicaltrials.gov/study/NCT05600192
NCT05123859	05/01/2022	Patterns of Natural Aging and the Role of Senescence Registry	University of North Carolina, Chapel Hill	Chapel Hill, North Carolina, USA	Collecting age-related biomarkers to model ageing across cohort lifespan	Completed	https://clinicaltrials.gov/study/NCT05123859
NCT03539497	06/12/2023	Prognostic Value of Plasma Mitochondrial DNA and Cytochrome C After Cardiac Arrest	University Medical Centre Ljubljana	Ljubljana, Slovenia	To determine whether plasma mtDNA copy number and cytochrome c holds prognostic valye after cardiac arrest	Active	https://clinicaltrials.gov/study/NCT03539497
NCT06334107	01/05/2024	Mitochondrial DNA Signatures of Poor Aerobic Exercise Trainability in Young Adults Born Preterm	Texas Tech University	Lubbock, Texas	To determine whether a specific mtDNA profile is associated with poor aerobic exercise tolerance in individuals born extremely preterm	Recruiting	https://clinicaltrials.gov/study/NCT06334107
NCT06433427	29/05/2024	Metabolic Dysregulation as Biomarker of Frailty: Role of the Mitochondrial Dysfunction (FRAMITO)	University Hospital of Ferrara	Ferarra, Italy	Assessment of the role of mitochondrial dysfunction in frailty through analysis of peripheral blood mononuclear cells	Not yet recruiting	https://clinicaltrials.gov/study/NCT06433427

Conclusions

1. Advanced techniques in molecular biology have allowed the detailed elucidation of the control systems that govern mitochondrial biogenesis, degradation and repair, and it is particularly fascinating that this energy metabolism apparatus directly participates in the molecular process of ageing. The high degree of influence by nuclear factors on these systems allows a mechanistic explanation of how ageing organelles and cells can produce clinical syndromes that are seen in the clinic. Detailed examination of the role of ROS in producing genetic mutation and the fascinating apparatus, highly conserved across millennia, that exists to repair this damage enables much greater understanding of ageing as a molecular process and potentially provides avenues for investigation and treatment.

2. Nevertheless, much work remains to be done to translate these advances into practical techniques that can be employed at the patient's bedside. Despite concerns around theoretical viability and practical employment, investigation of mtDNA copy number remains a field where significant developments may yet be made. As a general summary, high mtDNA copy number in PBMCs corresponds to states of health and positive activity or diseases of low penetrance and severity, while low mtDNA copy number is a poor prognostic marker and is linked to frailty and a wide range of disease states. Current and ongoing trials aim to further clarify this while also determining whether the techniques can prove to be widely applicable in day-to-day practice.

3. Whether this is causative has yet to be determined, and the answer will be key to producing treatments for the many diseases in which mitochondrial dysfunction is an aetiological agent. The elucidation of mitochondrial repair mechanisms and the further determination of the molecular basis of mitochondrial dysfunction open new pathways for the treatment and management of many currently incurable diseases. The prospect of a quick, simple, bedside method to quantitatively determine the risk of frailty and age-related pathology is tantalisingly close. While it is tempting to opine that all that remains is the refinement of the method and production of commercial standards, the basic methods employed need further development. Advances in understanding the fundamental background of mitochondrial biogenesis, control of energy homeostasis and the implicit role of these processes in ageing enable us to answer with greater clarity when, on drawing the peripheral blood sample for quantitative mtDNA analysis, we ask what, exactly, are we looking at?

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