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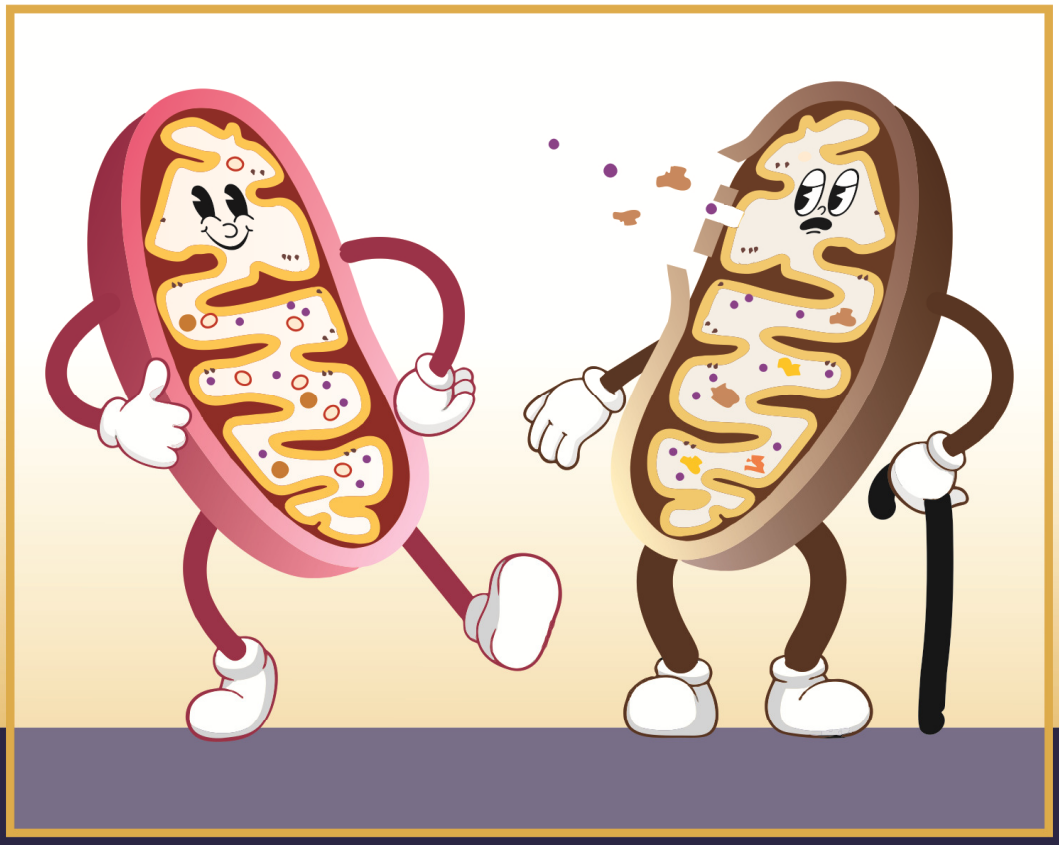

Oana ABUREL Danina MUNTEAN

MITOCHONDRIA and AGEING

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Piaţa Eftimie Murgu 2, cam. 316, 300041 Timişoara

Tel./Fax 0256 495 210

e-mail: evb@umft.ro

www.evb.umft.ro/ro/organizare-evb/

Director general: Prof. univ. dr. Sorin Ursoniu

Colecţia: ACADEMICA

Coordonatori colecţie: Prof. univ. dr. Andrei Anghel

Prof. univ. dr. Victor Dumitrașcu

Referent ştiinţific: Prof. univ. dr. Claudia Borza

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**"No amount of experimentation can ever prove me right;
a single experiment can prove me wrong."**

Albert Einstein

"Inside every old person is a young person wondering what happened."

Terry Pratchett

We started writing this book almost 10 years ago and, by a time, we considered ageing a great research topic (we still do) not a reality that we could face one day. The first motto belongs to one of the most famous scientists, while the second motto was postulated by one of the most imaginative yet realistic writers as the credo of all ageing people (including us). Both are right.

ABBREVIATIONS LIST:

ADP, D	adenosine diphosphate
Ama	antimycin A
ANOVA	analysis of variance
ATP	adenosine triphosphate
Atr*L*	atractylosyde *Leak*
C	cytochrome c
C I	complex I
C II	complex II
CF	coronary flow
CHD	coronary heart disease
CoQ	ubiquinone
COX	cytochrome c oxidase
CRC	calcium retention capacity
Cryo-EM	cryoelectron microscopy
D*P*	ADP induced oxidative phosphorylation
***E*, ETS**	electron transport system capacity
ETC	electron transport chain
F, FCCP	carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone
FMN	flavin mononucleotide
G	Glutamate
GM*L*	glutamate malate *Leak*
IMM	Inner mitochondrial membrane
L	LEAK respiration
M	Malate
MIA	Mitochondrial import and assembly machinery
MIM	Mitochondrial import

mtDNA	Mitochondrial DNA
mPTP	Mitochondrial permeability transition pore
OMM	Outer mitochondrial membrane
Omy	Oligomycin
OXPHOS	oxidative phopsphorylation
P	OXPHOS capacity
PAM	Presequence translocase-associated motor
PKC	protein kinase C
RCR	respiratory control ratio
ROS	radical oxygen species
ROX	Residual Oxygen consumption
Rot	Rotenone
S	Succinate
SAM	Sorting and assembly machinery
S(Rot)*L*	succinate (rotenone) *Leak*
S.E.M	standard error of mean
SOD	superoxide dismutase
SR	sarcoplasmatic reticulum
SUIT	substrate-uncoupler-inhibitor titration
SUMO	Small Ubiquitin-like Modifier
TIM	translocase of the inner membrane
TOM	translocase of the outer membrane
TPx	thioredoxin peroxidase
TTFA	thenoyltrifluoroacetone
VDAC	voltage-dependent anion channel
XO	xanthine oxidase

I. AGEING: AN OVERVIEW

Ageing of the population refers to the increased numbers of its elderly citizens and represents the successful story of mankind in increasing longevity and improving life expectancy (1).

Ageing at individual level is commonly defined as the inevitable, progressive decline in physiological organ functions required for survival and the persistent loss in fitness, being an intrinsic feature in all living beings.

Ageing in humans is a dynamic, multifaceted process that involves accumulation over time of biological, psychological, and social changes (2). As such, the science of gerontology (the comprehensive study of all aspects related to ageing and older adults) has constantly evolved with the increase in longevity; currently, biogerontology addresses the physical aspects of ageing whereas social gerontology studies its psychological and social parts.

Ageing in humans is the single greatest driving factor of multimorbidity (defined as the simultaneous presence of ≥ 3 chronic diseases). Accordingly, ageing increases the propensity of cardiometabolic and neurodegenerative diseases, cancer, geriatric syndromes, including the reduced ability to recover from illnesses (cognitive impairment, sarcopenia, fatigue, frailty, incontinence, etc.), ultimately, leading to death (3).

Biogerontology focuses on understanding the cellular and molecular mechanisms that underlie the age-related changes in order to prevent the onset of age-related diseases (4).

One of the challenging biomedical problems of the 21st century is to understand the basis of human ageing in order to increase lifespan, and more important, to maintain the physical or cognitive performances of the elderly, thus promoting resilience to disabling conditions and chronic diseases (5).

To this aim the era of *geroscience* has been launched, as an interdisciplinary science that studies the biological mechanisms of ageing underlying the chronic diseases (6) and develops novel therapeutic approaches, which are translatable from bench to bedside (7). These geroprotective interventions are not only aimed at delaying ageing, but also, to increase the "health span" defined as the period of life free of diseases and disabilities (i.e., the duration of health and independence) (3), thus promoting healthy longevity (8).

The hallmarks of ageing were defined more than one decade ago by the group of Guido Kroemer. In their landmark paper (cited more than 18 000 times) these authors formalized the aging phenotype into nine distinct, yet interconnected cellular and molecular processes: cellular senescence, DNA instability, mitochondrial dysfunction, loss of proteostasis (protein homeostasis), telomere attrition, epigenetic alterations, deregulated nutrient sensing, stem cell exhaustion, and altered intercellular communication (9), and was expanded it in 2023 with other 3 hallmarks: chronic inflammation, disabled macroautophagy, and dysbiosis (10). These hallmarks, further classified as primary, integrative and antagonistic are interdependent and highly interconnected with the major biochemical age-related processes, namely glycation, methylation and oxidation. This central framework that focuses on cellular and molecular damage has been substantiated in the past decades by a plethora of experimental and clinical research tackling the age-related diseases (11). Moreover, it has been crucial for advancing contemporary pharmaceutical strategies, such as metabolic modifiers, NAD+ precursors, mTOR inhibitors, senolytics, senomorphics, and clinical treatment approaches (such as stem cell therapy, depletion of senescent cells, etc.) and other pathomechanism-driven gerotherapeutics (12-14).

II. MITOCHONDRIA – GENERALITIES

Mitochondria are among the cell's most influential organelles. In eukaryotic organisms that do not depend on photosynthesis, they serve as the main source of adenosine triphosphate (ATP)—the energy currency that sustains essential cellular work, from mechanical activity and biosynthesis to protein turnover and the maintenance of membrane potential. In adult humans, the daily flux of ATP is enormous: mitochondrial ATP synthase continuously regenerates ATP from ADP and inorganic phosphate, with total turnover commonly estimated to reach tens of kilograms per day. Yet mitochondria are far more than ATP-producing "powerhouses." Alongside oxidative phosphorylation, they participate in core metabolic and signaling processes, including the production of reducing equivalents and high-energy intermediates through the citric acid cycle (e.g., NADH and GTP), the synthesis of amino acids and phospholipids needed for membrane biogenesis, intracellular calcium handling and signaling (15), coordinated responses to cellular stress (16), and the regulation of programmed cell death, with broad implications for ageing and age-related decline (17). Taken together, these roles help explain why mitochondrial integrity is so tightly linked to human health.

Mitochondria retain their own genetic system. They house a distinct genome and a translation machinery that includes ribosomes, tRNAs, and associated protein factors. Although mitochondrial DNA ultimately traces back to bacterial ancestors, its genetic code and organization have diverged over time from both those progenitors and the nuclear genome of the eukaryotic host (18). Advances in single-particle electron cryomicroscopy (cryo-EM) have

recently made it possible to resolve mitochondrial ribosomes in unprecedented detail, revealing features that parallel bacterial ribosomes as well as structural adaptations unique to the organelle (19). Over evolutionary time, however, mitochondria have transferred the overwhelming majority of their genes to the nucleus—often estimated at up to ~99% (20). As a result, most mitochondrial proteins are synthesized on cytosolic ribosomes and subsequently imported into the organelle through specialized protein translocases embedded in the mitochondrial membranes (21). Our understanding of mitochondrial architecture has progressed in step with imaging technology. In the 1990s, key structural insights came from electron tomography performed on thin plastic sections (22). Today, electron cryo-tomography (cryo-ET) allows three-dimensional visualization of mitochondria in a near-native state at sub-nanometer resolution, enabling the analysis of macromolecular assemblies and membrane organization with continually increasing clarity (22).

II.1. Mitochondrial structure

Because mitochondria are ubiquitous, semi-autonomous organelles, they are physically compartmentalized from the surrounding cytoplasm by a double-membrane system: an outer mitochondrial membrane and an inner mitochondrial membrane (Fig. 1) (23).

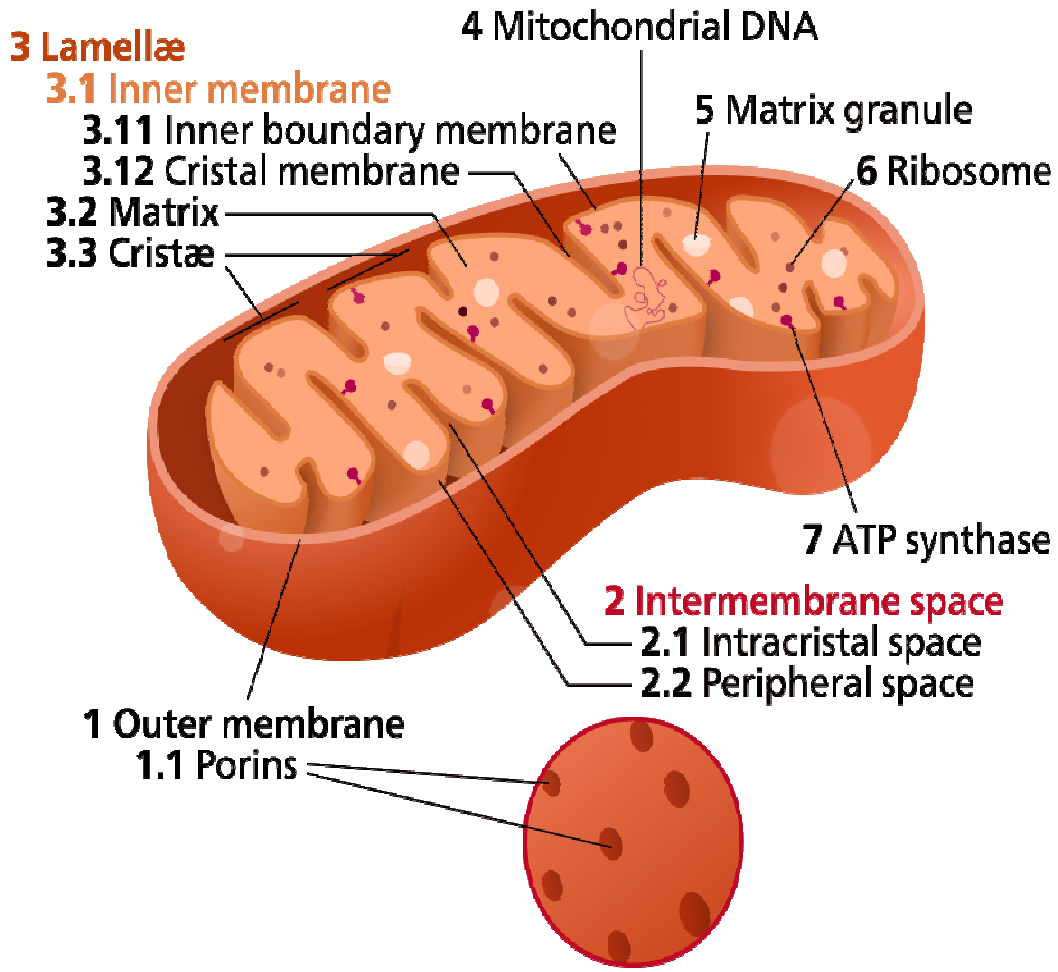

Figure 1. Structural organization of the mitochondrion.
Schematic representation illustrating the main structural compartments of the mitochondrion, including the outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM), intermembrane space, mitochondrial matrix, and cristae formed by invaginations of the inner membrane. This organization underlies the functional compartmentalization required for mitochondrial bioenergetics, metabolic integration, and regulation of cell survival pathways. *Adapted from* **"Mitochondrion mini.svg"**, licensed under the **Creative Commons Attribution–ShareAlike 4.0 International (CC BY-SA 4.0)** license, via *Wikimedia Commons* (https://commons.wikimedia.org/wiki/File:Mitochondrion_mini.svg).

The outer mitochondrial membrane (OMM) is relatively permeable. Small, uncharged molecules and many ions can pass through pore-forming proteins (porins), most notably the voltage-dependent anion channel (VDAC). In contrast, larger macromolecules—especially proteins destined for the matrix or inner compartments—require dedicated translocase systems to cross the mitochondrial membranes. Because the OMM is so porous, it does not sustain a meaningful transmembrane potential (24).

The inner mitochondrial membrane (IMM), by design, is a far stricter diffusion barrier. Ions and metabolites cannot cross it freely; exchange is mediated by highly specific transporters, each selective for particular solutes (25). This tight ionic control allows a steep electrochemical gradient to be established across the IMM, typically on the order of ~180 mV (25). As a consequence, the inner membrane is the functional hub of oxidative phosphorylation: respiratory-chain complexes generate the proton-motive force, and ATP synthase harnesses that gradient to produce ATP (25) .

Together, the outer and inner mitochondrial membranes partition the organelle into three major compartments, each defined by a characteristic biochemical environment and a distinct set of resident proteins.

Enclosed by the inner mitochondrial membrane, the mitochondrial matrix forms the innermost compartment (Fig. 1). Its pH is relatively alkaline—typically around 7.9–8.0, comparable to the chloroplast stroma (26). This alkalinity is not simply a chemical curiosity: it contributes directly to the proton-motive force across the inner membrane, a key component of the electrochemical gradient that powers ATP synthesis. The matrix is also the primary site for mitochondrial DNA (mtDNA) replication and transcription, mitochondrial protein synthesis, and a wide spectrum of enzymatic pathways. Within the matrix, mtDNA is packaged by TFAM (mitochondrial transcription factor A) into supramolecular structures known as nucleoids—commonly on

the order of ~1000 per cell—with each nucleoid typically containing a single mtDNA molecule (27). Mitochondrial ribosomes are closely associated with the inner membrane, consistent with their role in producing highly hydrophobic membrane-protein subunits that can be inserted into the membrane as they are translated (24). Among the matrix-centered pathways, the citric acid cycle is particularly central. Reflecting the density of enzymatic machinery required for these reactions, the matrix is remarkably protein-rich, reaching concentrations reported to be as high as ~500 mg/mL—approaching those found in protein crystals (24).

A second compartment, the intermembrane space, lies between the outer membrane and the outer surface of the inner membrane and is relatively narrow (approximately 20 nm) (Fig. 1). Because most nuclear-encoded mitochondrial proteins are synthesized in the cytosol and must reach the matrix or inner compartments, their import pathway necessarily brings them through this intermembrane region. Import is coordinated by translocase assemblies in the outer and inner membranes, which can align into functional supercomplexes spanning the intermembrane space; transiently moving polypeptide chains help stabilize this conduit during transport (28). In parallel, the inner membrane is densely populated by carrier proteins — such as the ~33 kDa ADP/ATP carrier (29) — that mediate the exchange of ATP, ADP, ions, and metabolites between the cytosol and the matrix, thereby coupling mitochondrial metabolism to cellular demands (28).

The inner mitochondrial membrane is not a smooth boundary. Instead, it forms extensive inward folds—known as cristae—that project deep into the matrix (Fig. 1). These invaginations create the third major mitochondrial subcompartment, the crista lumen.

The crista membranes are enriched in the molecular machinery of oxidative phosphorylation, including the electron transport chain (ETC) (30) and ATP synthase. Within this system, respiratory complexes I–IV transfer electrons derived from NADH and $FADH_2$ to molecular oxygen. As electrons flow through the chain, protons are actively moved from the mitochondrial matrix toward the intermembrane side of the inner membrane, building the proton-motive force that ultimately drives ATP production; at the terminal step, oxygen is reduced to water (Fig. 2).

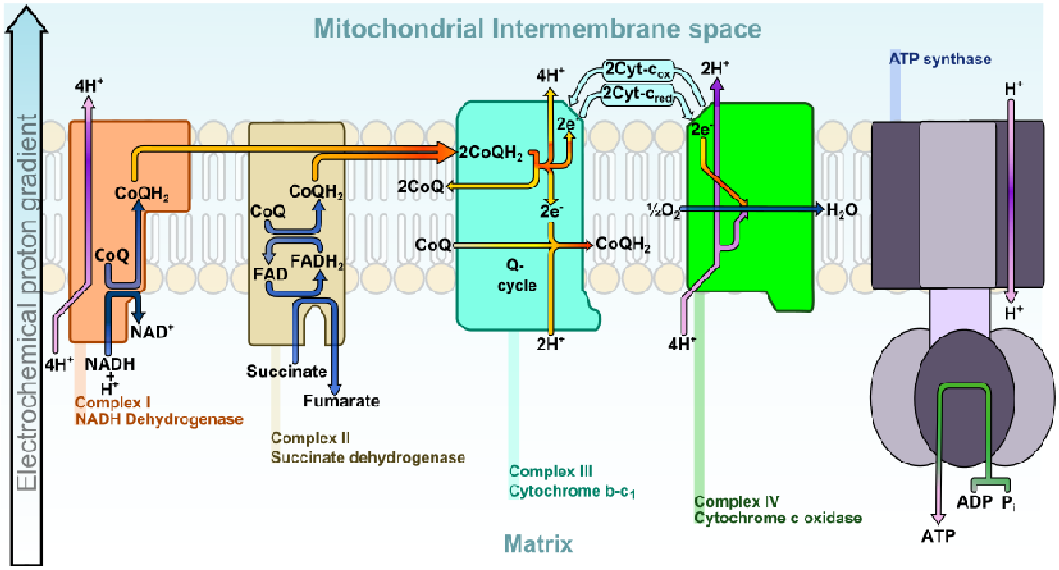

Figure 2. Mitochondrial electron transport chain.
Public domain image from Wikimedia Commons: "Electron transport chain" (CC0 1.0). Source: https://commons.wikimedia.org/wiki/File:Electron_transport_chain.svg

Cristae—disk-like lamellae, tubules, or sac-like extensions of the inner mitochondrial membrane—were first described in detail by electron microscopy (31, 32). They remain continuous with the inner membrane through narrow openings termed crista junctions. At these junctions, a dedicated architectural apparatus has been identified: the mitochondrial

contact site and cristae organizing system (MICOS), a complex composed of one soluble component and multiple membrane proteins. MICOS helps position and stabilize crista junctions and contributes to the physical linkage between cristae and the outer mitochondrial membrane (33). Functionally, MICOS is thought to act as a diffusion barrier at crista junctions, shaping how proteins and lipids move within the inner membrane. This organization may underlie a lateral segregation of membrane protein complexes between the cristae and the inner boundary membrane—an arrangement supported by observations from cryo-electron tomography (cryo-ET) (34).

Cristae architecture also varies with tissue metabolic demand. In high-energy tissues such as skeletal muscle and myocardium, mitochondria commonly display densely packed, lamellar cristae that occupy much of the organelle's volume. By contrast, in comparatively lower-demand tissues such as liver and kidney, cristae tend to be less tightly arranged, leaving proportionally more matrix space. Despite these differences, a consistent theme across tissues is that cristae account for a large fraction of inner membrane surface area, underscoring their central importance for mitochondrial—and cellular—physiology (34).

Complex V (F_1-Fo-ATP synthase) converts the proton-motive force generated by the respiratory chain into chemical energy by allowing protons to flow back into the matrix and coupling that return flux to ATP formation from ADP and inorganic phosphate (Fig. 2). For this reason, ATP synthase is one of the most prominent protein assemblies concentrated within crista membranes (25). Proton movement through the membrane-embedded Fo sector drives rotation of a ring built from c-subunits; the stoichiometry of this ring varies by species—commonly eight c-subunits in mammals (35) and around ten in yeast (36). The resulting torque is transmitted via a central stalk to the catalytic F_1 head, where a series of conformational transitions powers

ATP synthesis. A separate peripheral stalk stabilizes the complex and prevents the catalytic head from rotating relative to Fo, ensuring that rotational energy is productively converted into chemical work.

For many years, ATP synthase—and other oxidative phosphorylation complexes—was assumed to be distributed rather uniformly throughout the inner mitochondrial membrane. This view began to shift with electron microscopy studies that first visualized striking double rows of ATP synthase along cristae in *Paramecium* (37). Two decades later, cryo-electron tomography (cryo-ET) confirmed and generalized this organization, revealing that ATP synthase commonly assembles into dimers and that these dimers align into ordered rows across species (38, 39). These linear arrays tend to localize along crista ridges or wrap around narrow tubular cristae. Dynamic cryo-ET analyses further suggested that dimer formation promotes local bending of the lipid bilayer and that the geometry of the dimers favors the emergence of extended rows—implying that membrane elasticity can be a major driver of higher-order organization, potentially even in the absence of extensive protein–protein interactions (38). The linear arrays of ATP synthase dimers are located along the Structurally, each dimer includes elements of the a-subunit and long α-helical segments positioned near the Fo rotor, along with aqueous half-channels that provide proton access from either side of the membrane to the translocation pathway (38). This is an important observation which actually suggests that row formation requires only the energy driven from the elastic membrane deformation, and not protein interactions (38).

Collectively, these observations support the idea that ATP synthase dimers—and especially their assembly into rows—are not merely a byproduct of cristae architecture but an active determinant of it. By shaping membrane curvature and creating microenvironments where proton flow can be locally

concentrated, cristae invaginations may help limit proton dissipation and favor efficient capture of the proton gradient for ATP production.

In this sense, crista morphology and ATP synthase dimer rows can be viewed as structural mechanisms that optimize oxidative phosphorylation (40). Comparative work across species has also led to the broader hypothesis that cristae and ATP synthase oligomerization represent key evolutionary adaptations, enabling eukaryotic cells to meet higher energetic demands through increased membrane surface area and improved bioenergetic efficiency (41).

More recently, ATP synthase dimers have been proposed to contribute to the mitochondrial permeability transition pore (mPTP). While this connection is intriguing, the precise structural role—if any—remains incompletely resolved and continues to be actively investigated (42).

II.2. Mitochondrial energy production

A transmembrane proton gradient is established across the crista membranes primarily by three proton-pumping complexes of the respiratory chain—complex I (NADH-ubiquinone oxidoreductase), complex III (cytochrome *c* reductase), and complex IV (cytochrome *c* oxidase) (Fig. 2) (43). By coupling electron flow to vectorial proton movement, these assemblies generate the electrochemical driving force that ultimately powers ATP synthesis.

Complex I is among the largest macromolecular machines in the cell, with a mass of roughly ~1 MDa (44). It serves as the entry point for electrons donated by NADH, a soluble carrier, and passes them to ubiquinone (coenzyme Q; CoQ), which diffuses within the lipid bilayer of the inner membrane (43). This reaction produces ubiquinol that remains in the

membrane and regenerates NAD^+, which is recycled for continued substrate oxidation in the matrix. Despite major advances, the precise molecular mechanism that couples electron transfer within complex I to proton translocation is still not fully resolved (45). Structurally, complex I contains flavin mononucleotide (FMN) and multiple non-heme iron centers (Fig. 2), and it is classically inhibited by rotenone, a natural product historically derived from *Derris* roots and used as an insecticide. The free energy released during electron transfer is harnessed to pump four protons from the matrix toward the crista lumen/intermembrane side of the inner membrane (43).

Complex III accepts electrons from reduced ubiquinol and transfers them to cytochrome *c* (Fig. 2), a small, soluble electron carrier located in the intermembrane space (43). Proton translocation is coupled to this step through the Q-cycle, contributing to the proton-motive force. Complex III can be inhibited by the antibiotic antimycin A and contains several redox-active cofactors, including non-heme iron, cytochrome *b* hemes, and cytochrome c_1 (43). Beyond bioenergetics, cytochrome *c* has a pivotal signaling role: its release into the cytosol is a well-established trigger of apoptosis. This is one reason why maintaining outer membrane integrity—and tightly regulating membrane remodeling during mitochondrial fission and fusion—is essential to prevent inappropriate cytochrome *c* leakage (46).

Complex IV completes the chain by transferring electrons from cytochrome *c* to molecular oxygen, reducing it to water on the matrix side (Fig. 2). In doing so, electrons effectively traverse the inner membrane again. The catalytic core contains heme and copper centers capable of accumulating multiple electrons, enabling the full four-electron reduction of O_2 to H_2O in a controlled manner. This “clean” chemistry is crucial: incomplete reduction of oxygen yields reactive intermediates such as superoxide and hydrogen peroxide, which can damage cellular components if not tightly constrained.

In parallel, ***complex II*** (succinate dehydrogenase) feeds electrons into the chain by transferring them from succinate directly to ubiquinone (Fig. 2) but—unlike complexes I, III, and IV—it does **not** pump protons and therefore does not directly contribute to building the proton gradient. Complex II is unique in that it is simultaneously a Krebs cycle enzyme and a membrane-associated component of the electron transport chain. Its products include fumarate released into the matrix and ubiquinol retained within the membrane. The complex contains FAD and multiple non-heme iron centers and is inhibited by thenoyltrifluoroacetone (TTFA).

Additional dehydrogenases also funnel electrons into the ubiquinone pool. Major flavoproteins, such as acyl-CoA dehydrogenases involved in fatty acyl-CoA oxidation, and other entry routes, including glycerol-3-phosphate oxidation, can contribute directly or indirectly to CoQ reduction, linking diverse metabolic pathways to respiratory flux.

Notably, whereas ATP synthase tends to assemble into dimer rows along cristae, the proton-pumping respiratory complexes often organize into higher-order supercomplexes, frequently referred to as **respirasomes**.

These assemblies were first described in yeast and in bovine heart mitochondria (47). Subsequent studies—particularly in bovine heart—supported a canonical architecture consisting of one copy of complex I associated with a complex III dimer and one complex IV monomer (48, 49). Although multiple lines of genetic and biochemical evidence support the existence of respirasomes *in vivo* (48, 49), their physiological relevance was long debated, in part because early observations depended on detergent solubilization. As it became clear that oxidative phosphorylation components are not randomly dispersed within the inner membrane, attention shifted toward what these supramolecular arrangements might achieve functionally.

Proposed roles include more efficient electron transfer to and from ubiquinone through favorable spatial alignment of complexes I and III, reduced risk of nonspecific or deleterious protein–protein interactions within the crowded inner membrane, control of the stoichiometric balance among respiratory complexes, and enhanced long-term stability of respiratory-chain assemblies (50).

II.3. Mitochondrial translation

Until 2009 it was not known if in mammals are present specific translation activators like in yeast mitochondrial gene products. Shoubridge and Weraarpachai were the first who have identified two mammalian translation activators: TACO1, required for the translation of COX1 (a subunit of cytochrome *c* oxidase), and Pro 1853 required in Complex I assembly, as demonstrated by reduced level of CI subunits in case of its depletion (51).

Mitochondrial translation takes place in mitoribosomes (i.e., mitochondrial ribosomes) which are bound to IMM and it seems that Oxa1 translocase is responsible for the insertion of this end-by product into the membrane. In this view, it was reported an association between Oxa1 and Mrp20 & Mrpl40 within the mitoribosome, which facilitates the insertion of Oxa1 near the nascent chains for co-translational IMM insertion (52).

In yeast mitochondria it was also reported that Cox1 translation was altered by mutant ATP synthase. Another report showed that translation of ATP synthase subunits, Atp6 and Atp8, depends on the assembly of the α/β hexamer of the F1 complex of ATP synthase (52).

Finally, F1 directly activates the translation of *ATP8*/*ATP6* mRNA, which is responsible for an adequate mitochondria and nuclear gene expression required for an efficient ATP synthase (52).

Another unsolved problem is related to mitochondrial translation elongation and termination in human mitochondria. In this view, there were reports of 2 translation termination factors in humans: ICT1 which induces codon-independent peptidyl tRNA hydrolase activity that apparently hamper the mitoribosomes, and a mutant C12ORF65 factor which was demonstrated to induce in 2 patients a pathogenic mitochondrial translation defect (52).

II.4. Mitochondrial proteome

About 60% of mitochondrial proteins are imported from the cytoplasm with a cleavable presequence (53). Accordingly, there are five pathways of protein translocation identified so far:

The classical pathway named the presequence pathway: most proteins from matrix and numerous proteins form the IMM are synthesized with N-terminal presequences which actually function as targeting signals (53-55); these presequence-carrying preproteins are imported by the translocase of the outer membrane (TOM) and the presequence translocase of the inner membrane (TIM23) (56-58). The presequence translocase-associated motor imports proteins into the matrix (59, 60), where the presequences are cleaved by the mitochondrial processing peptidase (61, 62).

In all the other 4 pathways involved in protein import, the preproteins do not possess the cleavable presequences, but specific internal targeting signals. Still, the TOM complex works as the pivotal mitochondrial gate access for both types of preproteins: cleavable and the majority of noncleavable ones.

The second mitochondrial import pathway is the carrier pathway, involved in the import of hydrophobic carrier preproteins of the IMM via TOM, the small TIM chaperones of the inter-membrane space, and the carrier translocase of the inner membrane (TIM22) (63-65).

The third mitochondrial import pathway is the β-barrel pathway which import the precursors of β-barrel proteins of the OMM by the help of the TOM complex and small TIM chaperones; the insertion into the OMM is mediated by the sorting and assembly machinery (SAM) (66), also named TOB (topogenesis of outer-membrane β-barrel) (67).

The fourth mitochondrial import pathway is involved in the import of inter-membrane space proteins containing cysteine motifs, via TOM and the mitochondrial import and assembly (MIA) machinery of the inter-membrane space (68, 69).

The fifth mitochondrial import pathway is involved in the import of OMM proteins equipped with α-helical transmembrane segments, via the mitochondrial import (MIM) complex (70, 71).

Since for numerous preproteins, there are not yet identified the import pathways, the subject of mitochondrial protein import is still opened for further research and elucidations, despite the fact that till now we do not have high-resolution characterizations of protein channels. Moreover, the roles of lipids in the protein import and membrane insertion is still under intensive research (72-76).

Overall, it is clear that in order to have a comprehensive view of mitochondrial biogenesis, we need to completely understand the mechanisms of protein import regulation in different stress conditions and by thus its role over the cellular quality control (77).

II.5. Mitochondrial fusion and fission

Mitochondrial dynamics is tied to cell cycle regulation and quality control, and also to energy balance. Mitochondrial fusion is the process of two adjacent mitochondria coupling, while mitochondria fission is the process of splitting one mitochondria in two parts (Fig. 3) (78). These two dynamic events are in perpetual counterbalance: if one process is inactivated, the other one is not challenged anymore, and this imbalance actually is regulating the mitochondrial structure (79).

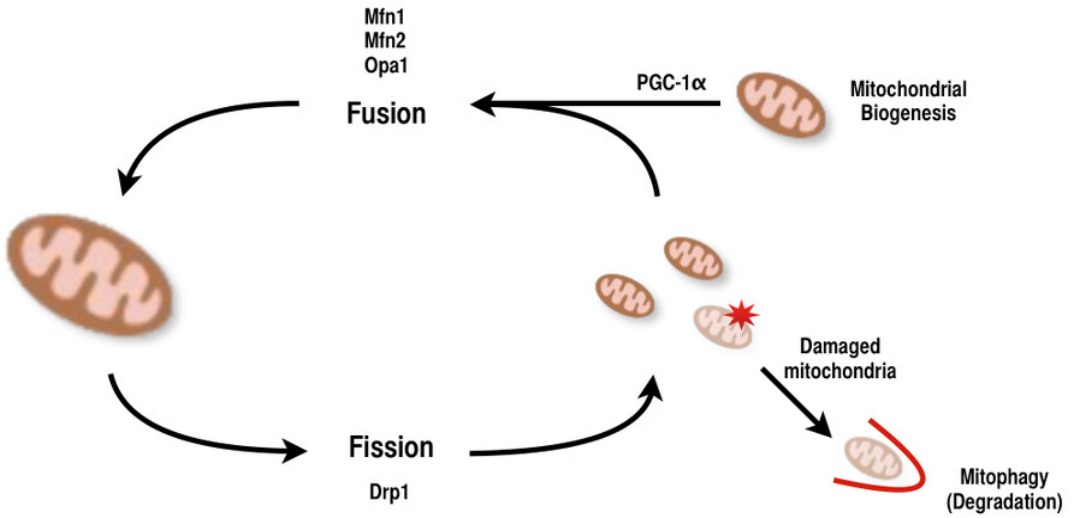

Figure 3. Mitochondrial Fission and Fusion [Internet].
Wikimedia Commons; 2017 [cited 2025 Dec 21]. Available from:
https://commons.wikimedia.org/wiki/File:Mitochondrial_Fission_and_Fusion_.png

In living cells, mitochondria fission and fusion is controlled by GTPases which are actually responsible for the appearance of either discrete tubules or interconnected networks (52).

It is known that DNM1 in yeast mitochondria and DRP1 in mammalian mitochondria (i.e., dynamin-related GTPase) are involved in a process of spirals assembly responsible for the constriction of OMM before fission (52).

In vitro experiments shown that Dnm1 is binding to the β-propeller domain of Mdv1 (i.e., mitochondrial division 1 prot in the cytoplasm (80). *In vivo*, this action induces Dnm1 spirals formation leading to mitochondrial membrane constriction. Recently, mammalian studies involving DRP1 changes, including phosphorylation and SUMOylation (i.e., a reversible post-translational modification induced by small ubiquitin-like modifier (SUMO) proteins) have been widely investigated and it was demonstrated that DRP1, as well as other mitochondrial proteins, are reversibly SUMOylated by the E3 ligase MAPL and the SUMO protease (81-84). Moreover, it was shown that during the G2/M phase of cellular cycle, SenP5 passes from the nucleolus to the mitochondrial surface, where actually takes place the SUMOylation activity leading to DRP1 construction and by thus to mitochondrial fission (83).

Another dynamin-related GTPase regulating the IMM fusion in yeast and mammals' mitochondria is Mgm1/OPA1. Mgm1/OPA1 presents two isoforms, expressed equally *in vivo*: the long isoform, l-Mgm1, which is attached to IMM, directed to the inter-membrane space, and the short isoform, s-Mgm1, which needs the IMM anchor. It was shown that these isoforms are distributed differently within mitochondria: while l-Mgm1 is located in cristae, s-Mgm1 is located at the inner boundary membrane (85).

Another interesting aspect is the fact that only s-Mgm1 needs a functional GTPase domain, which is suggestive for the hypothesis that these isoforms are differently involved during mitochondrial fusion. Zick et al. consider that the l-Mgm1 interactions actually tie down the opposing IMM which favors the later membrane fusion mediated by the s-Mgm1 isoform (85).

II.6. Mitophagy

Mitophagy represents an organelle specific process, which is an organelle specific process responsible for eliminating dysfunctional mitochondria by an autophagic response, in order to block the mitochondrial damaging oxidative stress, which actually will save the affected cell (Fig. 3).

Mitochondrial biogenesis involves multiple, connected pathways, which explain why an error occurred in one of these pathways leads to dysfunctional mitochondria that might activate cell death pathways. The intrinsic protective mechanism is well developed in mitochondria and is represented by mitochondrial proteases and the mitophagy process (86). If these protective mechanisms collapse, mitochondrial functioning will fall down, and this leads eventually to cell death.

The last years of intensive research were involved in the identification and characterization of mitochondrial proteases implicated in the removal of mislead mitochondrial, as well as in the identification of several molecules implicated in mitophagy (Fig. 4).

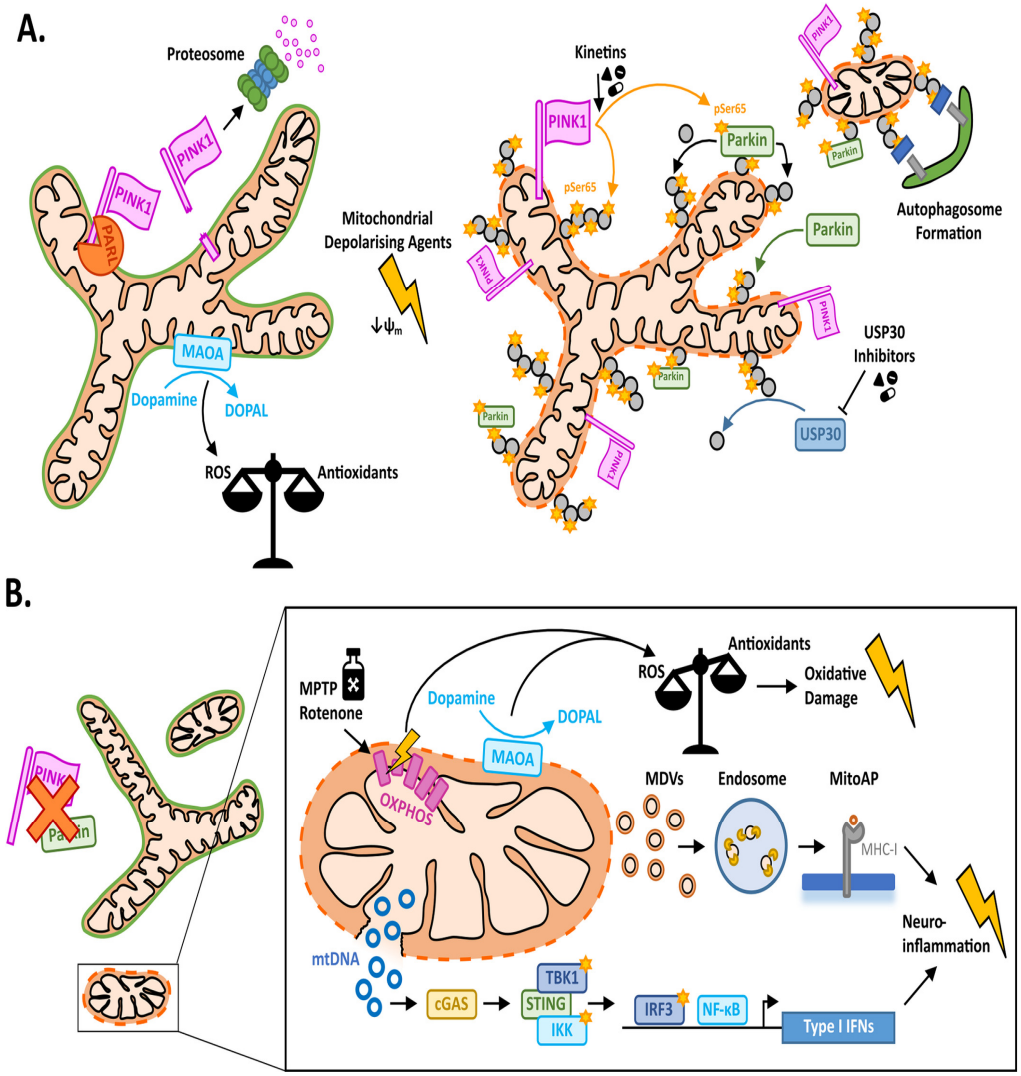

Figure 4. PINK1–Parkin-dependent mitophagy triggered by mitochondrial depolarization. In healthy mitochondria, PINK1 is imported and proteolytically processed (including cleavage by PARL), which prevents its accumulation and favors degradation, thereby keeping the pathway inactive. When mitochondria lose membrane potential, PINK1 import is impeded and PINK1 becomes stabilized at the OMM. PINK1 phosphorylates ubiquitin (Ser65), generating a high-affinity docking signal for Parkin recruitment, and phosphorylates Parkin itself, switching Parkin into an active E3 ligase conformation. Activated Parkin ubiquitinates numerous OMM substrates, expanding the ubiquitin "coat." PINK1 phosphorylates newly deposited ubiquitin chains, reinforcing a feed-forward amplification loop and ensuring selective labeling of damaged mitochondria. Ubiquitin/phospho-ubiquitin tags recruit autophagy receptors that couple mitochondria to LC3-decorated membranes, driving phagophore growth, autophagosome formation, and subsequent degradation of the organelle via the autophagy–lysosome pathway.
Abbreviations: OMM, outer mitochondrial membrane; ψm/ΔΨm, mitochondrial membrane potential; LC3, microtubule-associated protein 1 light chain 3.
Reproduced from O'Callaghan B, Hardy J, Plun-Favreau H. *PLOS Biology.* 2023;21(6):e3002196, **Figure 1, Creative Commons Attribution (CC BY).**

It is known that mitochondrial dysfunction is associated with neurodegenerative disorder, such as Parkinson disease. And several mutations, including Parkin, an E3 ligase, and Pink1 (87), a mitochondrial membrane-anchored kinase, were demonstrated to be responsible for several autosomal recessive forms of Parkinson disease.

PINK1 is processed by mitochondrial membrane proteases which lead to proteasomal degradation (Fig. 4). Accordingly it was shown that cytosolic Parkin migrates to dysfunctional mitochondria before its elimination from cells via mitophagy process (88).

Damaged mitochondria are characterized by a decrease of the mitochondrial membrane potential, which induces accumulation and activation of PINK1 in OMM. Further, the activated PINK 1 enrolls the cytosolic Parkin which stimulates the activity of Parkin E3, and by thus inducing mitochondrial elimination via mitophagy; Parkin 3 activity is also activated via the phosphorylation of Ser65 in the Ub1 domain Parkin, by PINK1 (Fig. 4) and (87). PINK1 also phosphorylates the cytosolic monomeric ubiquitin at Sr65 and the transitory interaction with the phosphorylated ubiquitin induces a Parkin conformational change and the activation of Parkin E3 (87).

Yet, we do not know which are the mechanisms involved in the formation of the primary ubiquitin chains within mitochondria, therefore the subject of research remains to be hopefully answered in the future.

II.7. Mitochondrial induced apoptosis

Apoptosis or programmed cell death is essential in both cellular developments, but also in cancer, aging and numerous neurological disorders. Apoptosis is activated by 2 pathways: the intrinsic pathway involving mitochondria, and the extrinsic pathway involving the activation of death

receptors (89). Ultimately, both pathways converge by activating the caspases which target the cell death, even though it was also reported an independent caspase pathway of apoptosis (90).

The mitochondrial pathway acts by the oligomerization of Bax and/or Bak in the OMM which induces the OMM permeabilization with subsequent release in the inter-membrane space of the proapoptotic cytochrome *c* (Fig. 5).

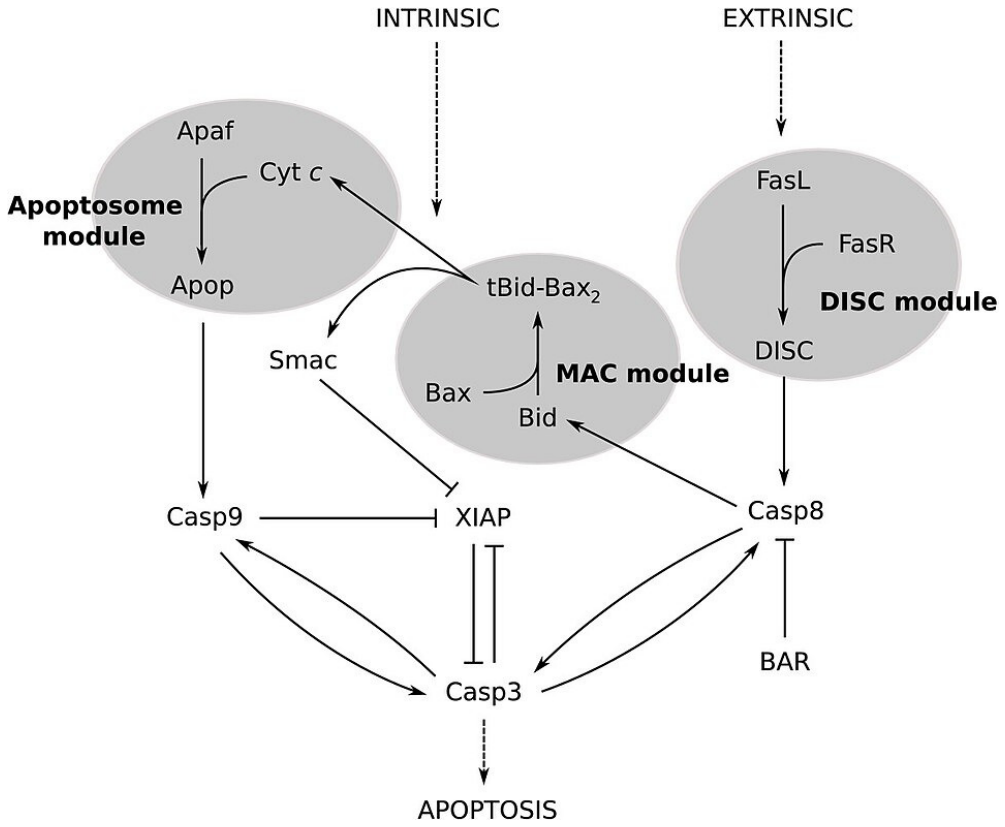

Figure 5. Mitochondria-mediated (intrinsic) apoptosis leading to caspase activation. Cellular stress activates pro-apoptotic BCL-2 family signaling and promotes mitochondrial outer membrane permeabilization (MOMP) (schematized here as the mitochondrial apoptosis-induced channel/module). This event enables the release of cytochrome c (Cyt c) and SMAC/DIABLO (Smac) into the cytosol. Cytochrome c binds Apaf-1 to assemble the apoptosome, which recruits and activates procaspase-9 (Casp9). Activated caspase-9 then cleaves and activates executioner caspases, particularly caspase-3 (Casp3), initiating the proteolytic cascade that produces the morphological and biochemical hallmarks of apoptosis. XIAP inhibits caspases, whereas SMAC antagonizes XIAP, facilitating executioner caspase activation. Reproduced from Harrington HA, Ho KL, Ghosh S, Tung KC. "Construction and analysis of a modular model of caspase activation in apoptosis." Theor Biol Med Model (2008). Image via Wikimedia Commons. Licensed under CC BY 2.5.

Cytochrome *c* combines with apoptosis protease activating factor (APAF-1) and pro-caspase 9 forming a complex called 'apoptosome' (91). The apoptosome induces the caspase 9 activation, which will further activate the effector caspases responsible for apoptosis end-point. DNA fragmentation and chromosomal condensation occurring during apoptosis are induced by AIF (92) and endonuclease G (93). Due to OMM permeabilization, besides cytochrome c are also released numerous proteins, such as Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP-associated binding protein with low pI) and Omi/HtrA2 (high temperature requirement A) which antagonize IAPs and by thus enhancing the caspase activation (94, 95).

Intensive research studies were focused on a possible connection between DRP1 GTPase-mediated mitochondrial fission and the permeabilization of OMM. Accordingly, it was demonstrated in several studies that OMM can be permeabilized without a connection to fission process. While other research studies have shown that alteration of DRP1 activity by different mutations altering mitochondrial functions or RNAi is delaying the fission process, and therefore the release of cytochrome c, and ultimately the apoptosis (52).

Recently, in a study that used a DRP1 assembly inhibitor showed that actually the GTPase plays a distinct role in OMM permeabilization which is not related to mitochondrial fission, but in the same time, the study did not explain the DRP1 effect on its apoptotic targets (96).

A more recent study demonstrated that DRP1 stimulates the tBID-induced oligomerization of Bax which actually does not depend on the GTPase activity of DRP1, but is sustained by hydrolysable and nonhydrolyzable adenine nucleotides (97). In this view it is mandatory to identify the mechanisms by which the nucleotides used in this process are involved in OMM permeabilization.

III. THEORIES OF AGEING

Over the past centuries, the *median* human lifespan has increased dramatically—often described as being several-fold higher than in the distant past—whereas the *maximum* observed lifespan appears far more stable, frequently cited around ~120 years. This contrast has helped shape the agenda of modern gerontology: not only to extend years lived, but to understand whether there are realistic ways to shift the upper boundary of human longevity. In its most ambitious form, the field has even asked whether life could be prolonged indefinitely. Yet, based on current knowledge, such an outcome seems highly unlikely. One major obstacle is that we still lack a definitive answer to a deceptively simple question: what is the primary driver of progressive cellular deterioration over time? (98).

Ageing itself is commonly framed as a complex, degenerative process in which function gradually declines and vulnerability to disease and death increases (99). Despite decades of investigation, there is still no universal agreement on the core pathomechanisms that govern this trajectory. In fact, an extraordinary number of conceptual models have been proposed—hundreds of "theories of ageing"—with many later revised, merged, or abandoned as evidence accumulated (98). To bring order to this diversity, ageing theories have been often grouped into two broad categories. The first, sometimes termed "classical," views ageing as a cumulative functional decline driven by multiple damaging influences acting over time. The second one, the "genetic" or "programmed" view, proposes that ageing is largely predetermined by genetic mechanisms (100). The programmed perspective remains controversial, in part because clear evidence for a dedicated genetic ageing program is lacking and because such a program would have to be reconciled with evolutionary pressures and natural selection (98).

Among the many proposals, two concepts have proven particularly durable because they continue to be supported by repeated experimental observations: the **free radical theory**—arguably the most widely discussed of all—and the **protein error** (or proteostasis failure) theory. Alongside these, the **cellular senescence/telomere** framework has also gained attention, offering a compelling angle on how replicative limits and senescence-associated changes could contribute to organismal ageing.

Other explanatory models have also been advanced, including **immunological** and **neuroendocrine** theories, although neither has achieved broad consensus as a singular, unifying explanation.

It is undisputed that ageing is accompanied by changes across essentially all physiological systems, including immune competence, yet the precise causes and dominant mechanisms of immune senescence remain debated (101). Age-related immune remodeling can distort inflammatory control and is thought to contribute to chronic, inflammation-linked disorders—an idea captured in the concept of **"inflammageing."** Several studies have associated longevity with specific immune alterations, but mechanistic clarity is still incomplete, and longitudinal studies are needed to better validate and refine immune-based models of ageing (101).

The **neuroendocrine theory** was articulated in influential form by Dilman and Dean in 1992 (102). In this view, ageing involves a progressive loss of precision in the hypothalamic–pituitary axis, the central regulatory network that helps maintain homeostasis, accompanied by reduced hormone receptor sensitivity (102). The downstream consequence is a decline in hormone secretion and a weakening of peripheral hormonal actions with advancing age (103).

More recently, aspects of this model have been revisited in light of evidence from *C. elegans*, where insulin/IGF-related signaling pathways

link stress responses to lifespan; specific mutations can increase resistance to environmental stressors and nutrient deprivation and, in turn, extend longevity (104).

From a clinical and functional standpoint, ageing trajectories are often described using three broad patterns (105). One is **ageing with disease**, where functional decline is strongly shaped by pathology. A second is **"usual ageing,"** characterized by the absence of major disease but with measurable functional loss. The third, often termed **"successful ageing,"** describes individuals who avoid significant pathology and experience only minor—or in some domains, negligible—physiological impairment (106).

Within this conceptual framework, mechanisms that support successful ageing are frequently discussed in relation to neuroendocrine and immune regulation (107) and are translated into practical strategies such as: (1) preserving normal function and plasticity; (2) restoring function through compensatory interventions (e.g., exercise, appropriate nutrition, and health education); (3) applying replacement approaches where diminished function can be safely supported; (4) modifying individual risk profiles to improve health outcomes (108); *(5) emphasizing disease prevention; and (6) strengthening social engagement and supportive networks* (109).

However, while the two major groups of theories, the error-based and the program-based have been continuously revisited, a critical view is that in ageing research a definite theoretical framework is still lacking (110). More recently, the pluralistic theoretical framework, placing the organisms in their ecological context and taking in account the life history, has been proposed as a possibility to accommodate the classic and emerging ageing theories (110)

III.1. The free radical theory of ageing

The free radical theory of ageing was introduced in 1956 by Harman, who proposed that reactive free radicals generated during aerobic metabolism gradually accumulate oxidative damage in cells, ultimately contributing to ageing and death in both humans and animals (111, 112).

In subsequent refinements of the concept, Harman emphasized the central role of mitochondria in the ageing process via the reactive oxygen species (ROS) generated during respiration (113). This pioneering hypothesis was further expanded by Miquel et al. who proposed that age-related elevations in mitochondrial ROS facilitate mutations and deletions in mitochondrial DNA (mtDNA), resulting in respiratory chain impairment and a self-perpetuating rise in mitochondrial ROS generation. In this framework, mitochondria become both the generator and the victim of oxidative stress, creating a self-reinforcing "vicious cycle" that amplifies cellular injury and promotes cell dysfunction and death (114). This mitochondria-centered extension of the theory was widely adopted and elaborated by later investigators (115-120). Later one, a question have been raised whether mtDNA mutations are the cause or the consequence of the ageing process, being suggested that mitochondrial point mutations are merely a consequence, while deletions play a causal role (121)

As summarized schematically in Figure 6, the model proposes that ageing is accompanied by a shift in which mitochondria emerge as a dominant source of ROS—and simultaneously a primary target of oxidative damage. Mitochondrial ROS can injure mtDNA, which encodes essential components of the oxidative phosphorylation (OXPHOS) machinery. Damage to these gene products compromises electron transport chain (ETC) performance, increasing the likelihood of electron leak and, in turn, further ROS generation. The resulting escalation of oxidative burden can drive additional mtDNA lesions and broader intracellular damage (30). Damaged mitochondrial respiratory chain

will further generate more ROS leading to ulterior mtDNA mutations (122, 123) and intracellular damage. As a result, mitochondrial membrane potential (ΔΨm), mitochondrial respiration, as well as regulation of Ca^{2+} homeostasis, will gradually decrease, contributing to ageing (124).

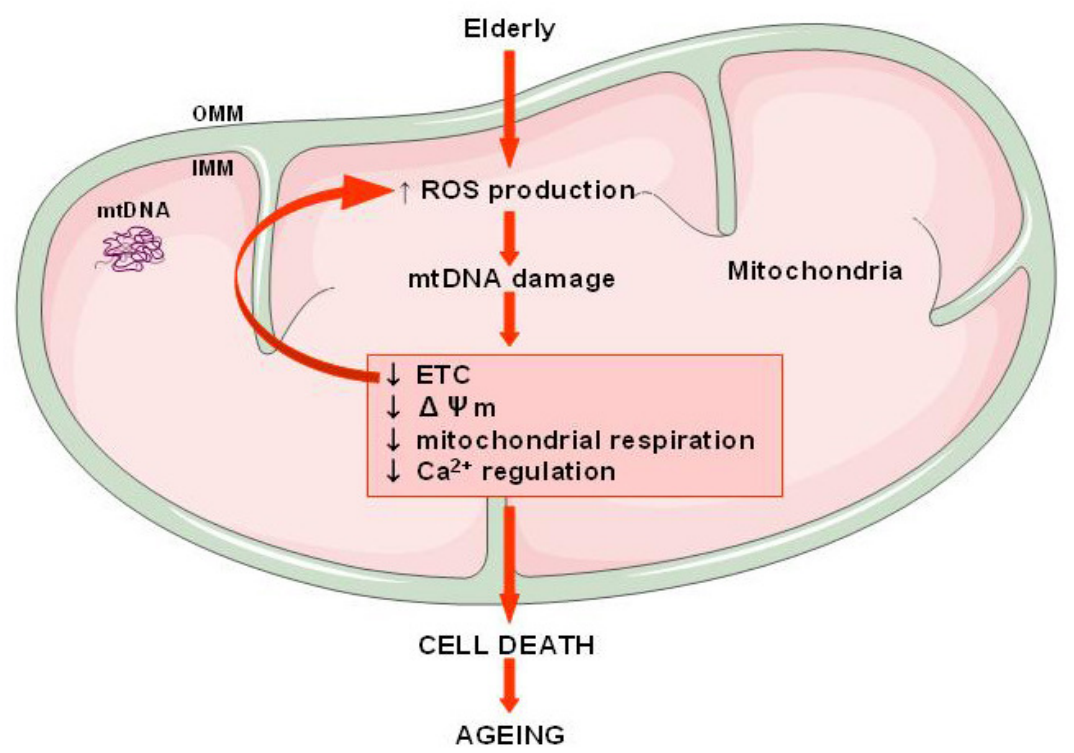

Figure 6. Schematic overview of the free radical theory of ageing (modified after (124)). With advancing age, mitochondrial production of reactive oxygen species (ROS) increases. These ROS damage mitochondrial DNA (mtDNA), compromising electron transport chain (ETC) function within the inner mitochondrial membrane (IMM). An impaired ETC, in turn, promotes further ROS generation, establishing a self-amplifying vicious cycle of progressive mitochondrial dysfunction. The cumulative outcome includes loss of mitochondrial membrane potential (ΔΨm), declining respiratory capacity, disturbed Ca^{2+} homeostasis, and, ultimately, cell death and ageing. *(IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane). Figure created using Servier Medical Art.*

Building on the implications of the free radical framework, Linnane and colleagues proposed back to 1989 that the burden of somatic mtDNA mutations in post-mitotic tissues increases with age and contributes substantially to human ageing and degenerative disease (125).

The group of Wei has extensively studied the occurrence of mtDNA mutations in various human tissues harvested from old persons and proposed that progressive, lifelong accumulation of somatic mtDNA deletions (and other mutations) gradually undermines mitochondrial function and thereby acts as a major contributor to human ageing (123).

Although the logic of the model is straightforward—oxidative stress injures mtDNA, impaired respiration increases ROS, and damage accelerates—the theory did not gain broad traction immediately. Interest in free radicals as biologically meaningful mediators was strengthened by the discovery of superoxide dismutase (SOD) by McCord and Fridovich in 1969, an enzyme that detoxifies superoxide radicals in living cells (126). Identifying an endogenous antioxidant defense system helped shift the field from speculative chemistry toward mechanistic biology and revived attention to how reactive species might shape ageing and disease (127). From this perspective, oxidative stress and mitochondrial oxidative injury were increasingly viewed as central elements of the ageing process (128).

While the mitochondrial theory of ageing stood the test of time for quite a while (129, 130), a more nuanced role of ROS in cellular regulation of ageing has progressively emerged (131). Indeed, a plethora of experimental studies unveiled that low amounts of mitochondrial ROS act as specific signaling molecules that regulate basic cellular processes and are part of stress responses, rather than being ageing toxins (132, 133). As such, the concepts evolved from ROS generation being the central piece of a unified theory of ageing (134) to the role of ROS as essential molecules to promote longevity in line with the concept of mitohormesis (135) - as mentioned in an excellent critical (and philosophical) paper providing pros-and cons of the theory (136). Nowadays, there is also unequivocal evidence that mitochondria can be variably affected by ROS based on their subcellular location and exhibit distinct age-dependent decline in their functions (137).

A substantial body of experimental work appeared to support the fact that in several model organisms, interventions aimed at limiting mitochondrial oxidative stress—particularly mitochondria-targeted antioxidants—were reported to extend lifespan (138-143).

Other studies suggested that mild mitochondrial uncoupling might also promote longevity in yeast and mice, potentially by reducing electron leak and ROS generation under certain metabolic conditions (144-147). These observations led some authors to argue that *mitochondrial ROS production itself* may be a more direct constraint on lifespan than the cumulative burden of oxidative damage measured at the whole-cell level (148).

In parallel, however, a major point of contention emerged when antioxidant strategies were evaluated in clinical contexts. A recurring criticism is that antioxidant supplementation has not reliably delivered the expected protective effects in human disease—or in slowing ageing. Indeed, a number of epidemiological studies delivered a significant challenge to the free radical theory of ageing by demonstrating that antioxidant supplementation did not reduce the incidence of numerous age-related disorders and, in certain instances, even increased the mortality risk (136).

Reflecting this skepticism, Howes argued that antioxidant use "has failed to quell the current pandemic of cancer, diabetes, and cardiovascular disease or (even) to stop or reverse the ageing process" (149), a view echoed by other investigators unable to reproduce consistent benefits with antioxidant approaches (30, 150-152).

One explanation proposed for these disappointing translations is that ageing is accompanied by a dual shift: antioxidant defenses tend to decline while oxidative load increases, pushing the oxidant–antioxidant balance toward a more pro-oxidant state. Jahangir and colleagues (2007) emphasized that this imbalance may heighten the vulnerability of cellular and subcellular

structures, effectively overwhelming the protective—or even signaling—roles that low, regulated ROS levels can play under physiological conditions (153). In other words, ROS are not purely detrimental; they are also messengers, and indiscriminate scavenging may disrupt adaptive pathways, a theory which was also embraced by numerous groups (154-158).

To reconcile these apparent contradictions, Lane (2002) proposed renaming the free radical theory as a **"double-agent" theory**: oxidative stress contributes meaningfully to ageing and age-related pathology, yet antioxidant supplementation—particularly in individuals on balanced diets—may not slow ageing in practice (159). This framing preserves the central role of ROS while acknowledging that therapeutic manipulation is not as simple as "more antioxidants equal longer life."

Finally, the explanatory reach of the free radical framework extends beyond ageing itself. Elevated free-radical burden and dysregulated redox signaling have been implicated in the pathogenesis of many age-associated disorders, including diabetes, cardiovascular disease, cancer, and neurodegenerative conditions (160-166).

III.1.1. Ageing-associated mtDNA mutations

Human mitochondrial DNA (mtDNA) is a double-stranded spiral molecule that encodes 13 protein respiratory chain subunits and 24 RNA units (22 tRNAs and two RNAs) required for mitochondrial protein synthesis; ~ 1500 different mitochondrial proteins are encoded by nuclear DNA (DNA), which is translated into the cytoplasm and transported to the mitochondria (167). Hence, the mitochondrial respiratory chain biogenesis "*is a strongly dependent yet poorly understood crosstalk between mitochondrial and nuclear DNA*" (168).

Over the last years, certain human studies have shown that mtDNA damages and mutations are increasing with ageing, as reviewed in (169).

The underlying mechanism by which the accumulation of mtDNA mutations is responsible for the damage to respiratory function in the aging process is not yet clear, especially considering that the mtDNA mutations associated with ageing represent less than 1% of mtDNA (170). From this perspective, Lightlours et al. believed that such low levels of mtDNA mutations are not capable of causing significant dysfunction of mitochondria in aged tissues (171).

Johnson et al. suggested that mtDNA deletion may be due to downstream of other events (172), whereas Wei believed that the mtDNA mutations reported so far are only "*the tip of the iceberg of all the damages and mutations in mtDNA*" (123).

Piko reported for the first time in 1988 the involvement of mtDNA damage in old aged animals: he demonstrated by the use of electron microscopy the abundance of structural defects consistent with mtDNA deletions in older rats (173).

Multiple studies reported that mutation and oxidative modification of mtDNA increase with age in different animal tissues (174-177), but also in human tissues (178-184).

The accumulation of mtDNA mutations has been shown to be well correlated with the level of oxidative stress in mtDNA, thereby there are numerous studies that strongly support hypothesis that oxidative stress contributes significantly to mtDNA mutations (184-186).

Another important factor is the rate of mtDNA mutations which occurs more rapidly in short-lived animals than in long-term ones. This observation is actually consistent with the differences observed in mitochondrial ROS rate production (127). Therefore, it was speculated that the rate of mitochondrial

ROS production in each animal species determines the growth of mtDNA mutations with ageing (187).

In a recent study, Yao et al. hypothesized that mtDNA mutations in aged living tissues is regulated by the nuclear genome (188). This theory was sustained by another study which has shown that the transfer of HeLa cell nuclei (free of mtDNA) into the cells of old donors abolished the mitochondrial dysfunction (189).

III.2. The protein error theory of ageing

The protein-error theory of ageing aroused from various scientific data demonstrating that ageing declines the protein turnover rate as reviewed in (190).

Even from 1996, Rattan postulated the consequences of the age-dependent decline of the protein rate production: "*the implications and consequences of slower rates of protein synthesis are manifold, including a decrease in the availability of enzymes for the maintenance, repair and normal metabolic functioning of the cell, an inefficient removal of inactive, abnormal and damaged macromolecules in the cell, the inefficiency of the intracellular and intercellular signaling pathways, and a decrease in the production and secretion of hormones, antibodies, neurotransmitters and the components of the extracellular matrix*" (191).

In 1969, Young found out that the inclusion rate of aminoacids into a polypeptide chain is relaying on the intracellular level of carbohydrates, thereby he was the first researcher who prefigured the connection between the ATP production and the protein synthesis rate (192).

And since mitochondria represent the major site for ATP production, is rational to conclude that the respiratory chain alterations will lead to

decreased ATP synthesis, which eventually will alter the entire intracellular mechanisms depending on ATP, such as ion pumps, skeletal muscle contraction, or protein synthesis (193). In mammals, protein synthesis requires 20% of ATP synthesis, being more sensitive to various changes of intracellular ADP/ATP or GDP/GTP ratios, rather than on absolute ATP value (194). Thereby, a decreased ATP synthesis in aged mitochondria due to ETC alteration might affect the protein amount within the senescent cell.

Another important aspect is that protein damage is irreversible induced by oxidative stress (195). Eukaryotic cells are provided with an important proteolytic system, the proteasome, able to remove the oxidized proteins and to prevent its aggregation (196). Accordingly, it was demonstrated that ageing is affecting the proteasome efficiency, which leads to the accumulation of oxidized proteins, aggresome and lipofuscin, also named the “age pigment”. Experiments on cellular models that increased the proteasome expression and activity proved an increase by 15–20% in cellular longevity (197).

Actually, protein aggregation was identified as the common feature of neurodegenerative diseases in aged patients, such as Parkinson's and Alzheimer's disorders (198). It is known now that cells have the capacity to counteract the protein aggregation during an early stage of life by increasing the control of protein degradation, reducing protein synthesis and stimulating protein processing (199); still the underlying mechanisms of these process is far of being elucidated (200).

The proteolytic degradation system acts by preventing the accumulation of altered proteins. Accordingly, when the altered proteins are not recognized and thereby no longer degraded by the proteosomal activity, protein oxidation can take place, or covalent crosslink reactions to other proteins (201, 202), resulting the formation of protein aggregates, called the “aggresomes” (203). The proteasomal activity is low in aged cells and more

interestingly, if this activity is blocked in young cells, the accumulation of protein aggregates increases (204). In light with this, it was considered that an artificially enhanced proteosomal system activity would be an efficient anti-ageing approach (205). Yet, such strategies are not achievable so far, since we have little knowledge regarding the proteasome regulation, or the transcriptional regulation of the proteasome activation pathways (197).

III.3. The cellular senescence/telomere theory of ageing

This theory originates from 1965 when ageing was formulated as a limiting process of the cell divisions (206) after a certain amount of cell divisions and ends with physiological altered cells in the terminal stages (105).

Senescent cells can also be induced by numerous molecular events; accordingly, there are two types of cellular senescence: the replicative senescence – induced by cell replication and the stress-induced senescence – induced by a multitude of other causes (105).

The replicative senescence is specifically induced by the telomeres (i.e., specialized structures localized at the end parts of a chromosome, comprising composed a repeating DNA sequence loss (see Ref. (207)). After each cellular division, a minor part of DNA is lost at each chromosome end which leads to shorter telomeres with altered structure, and eventually to replicative senescence (207). This process can be prevented by the activation of telomerase enzyme able to regenerate telomeres (208).

SIS is induced by multiple stressors able to damage DNA, to alter the structure of heterochromatin, or to induce mitogenic signals as a result of oncogene expression (105). Because immortal cells (e.g., germ cells, T lymphocytes, and stem cells) have telomerase, they might keep the intact

length of the telomere or they can postpone the decrease of telomere length (209, 210). Moreover, the replicative senescence is avoided in malignant cells because they are able to activate the telomerase or another pathway of telomere extension (211, 212).

The first experiments with culture cells demonstrated that cells from older donors do not keep the same capacity for subsequent cell division. Also, senescence is more rapidly produced in short life span organisms as compared to those with longer life span. Nevertheless, these data are in contradiction with recent results, which urge a farther clarification about these discrepancies (207, 213, 214).

In numerous tissues it was observed an accumulation of aged cells presenting stress-induced markers (215, 216). In light with this, some recent data showed that atherosclerosis is actually induced by senescent modifications of the endothelial cells (217-219). The progeroid Werner's syndrome is characterized by a normal maturation evolution up to puberty, continued by multiple changes characteristic for senescence, such as a premature atherosclerosis (220). Also, cells cultured from patients with Werner's syndrome and a similar mouse model of this disease presented a sped up senescence (221, 222). Another hypothesis is that the alteration of physiological functions within the senescent cells might be responsible for ageing or malignant disorders via secondary effects on the contiguous cells (216). A prove for this is that senescent endothelial cells stimulate inflammation and mitogenesis by an upregulation of the proinflammatory cytokine IL-1α and EGF-like growth factors and by thus are responsible for the alteration of physiological functions or the occurrence of malignancy (223, 224).

P53, a tumor suppressor protein, has multiple functions such as the activation of transient cell cycle imprisonment and apoptosis, but is also

involved in replicative senescence and SIS induced by telomere loss and DNA damage (225). The cell response mediated by p53 depends on the type of the studied cell or the prototype of stress the cell is exposed to. Mutated p53 mice presented an increased cancer incidence (226), while ~80% of human cancers are associated with an alteration of p53 signaling (225), results that are a proof for the involvement of p53 in tumor suppression.

A possible way to combat malignancy is actually the replicative senescence and/or SIS via the limitation of the replicative capacity of any type of cell. Yet, if this indeed is true, we cannot explain why both cellular senescence and cancer are increasing with age (105). This discrepancy actually can be explained by the evolutionary hypothesis which claims that cellular senescence is aimed to suppress cancer in our first stage of life, but in the same time, in an aleatory way, will induce organ dysfunction and cancer in older subjects (216).

Since telomerase was proved to play a major role in human cellular immortality and since it was demonstrated the aged-associated shorten of telomeres it was hypothesized that the length of telomeres is controlling the in vivo replicative life span of the cells (105). This assumption was demonstrated so far only in rodents' gene targeting experiments which demonstrated that mice with telomerase-deficient phenotype do not deteriorate fast (227); this observation is a sufficient proof that telomere shortening do not justify normal ageing. As mentioned above, p53 is involved in ageing; in light with this, it was demonstrated that deficit of p53 inhibits the premature senescence in mice with telomerase-deficient phenotype (228); these data were further sustained by another study which found out that a mutant mouse with a p53 hyperactivity deteriorates fast and in plus presents a decreased incidence of malignancy (229). Still, recent scientific data indicate that p53 is actually needed for the cell senescence support: therapeutic approaches aimed to

inactivate p53 in aged cells induced the activation of replication cycle and cellular proliferation (230, 231), albeit human aged cells with p16 overexpression did not age due to p53 inhibition (230, 231).

So indeed, shorten telomeres are not responsible for the senescence process in mice, as previously argued, yet there is scientific data proving that telomeres might be involved in normal ageing in humans. The X-linked disorder, Dyskeratosis congenita (DKC) is considered to be induced by the alteration of stem cells in skin and bone marrow (232) and the DKC-induced mutant gene alters an enzyme that participates in the metabolic pathway of hTR (i.e., a telomerase RNA subunit) (233). Moreover, the dominant autosomal form of DKC is induced directly by a mutant hTR gene (234), which enhance the supposition that telomerase dysfunction is indeed responsible for DKC. Moreover, patients with the dominant DKC form are prone to much severe dysfunctions in later generations (234), similar to the delayed phenotype identified in mice with deficit of telomerase. The onset of DKC is correlated with the proportion of the telomerase deficit which depends on the severity of disease, X-linked or autosomal (e.g., the level of telomerase might decrease from two- to fivefold (105)). Accordingly, the most decrease level of telomerase is associated with the occurrence of specific pathologies at an early stage of life. Moreover, it was found an increased incidence of malignancies in some patients with DKC patients, an observation that is suggestive for the supposition that short telomeres might be involved in cancer occurrence (105).

In conclusion, the replicative senescence theory can be indeed considered a cause of ageing as a direct consequence of the number of cell divisions induced by the telomere length, while SIS is stress induced, especially by genome breakdown and DNA damage. In light with this, we should practically consider SIS as a general response to molecular changes

that occur with age and which enhance or precipitate the process of senescence. But we also have to admit, based on the scientific data, that these two theories are basically interconnected with the free radical and the protein error theories of ageing (105).

Instead of a final remark about the mechanism of ageing, which is far of being elucidated, we must acknowledge Trubitsyn who appreciated that: "*bioenergetics decrease and the lifespan depend not on the calendar time of an organism's existence, but from the number of past divisions in its critical tissues, i.e., the amount of the past divisions is a biological clock*" (98).

IV. MITOCHONDRIAL DYSFUNCTION AND AGEING

Even though age-induced mitochondrial physiology alteration is widely accepted, there are still multiple debates regarding the published data due to different methodologies used and to multiple species and tissues used in these experiments so far.

Another controversy is related to the degree of mitochondrial dysfunction related to senescence. Consistent with this issue, Pulliam et al. suggested that "*changes in mitochondrial function can be significantly diminished without noticeable physiological effects, yet small changes near the threshold (of mitochondrial function beyond which physiologic dysfunction becomes evident) can have dramatic effects*" (128).

Most commonly, the parameters assessed in order to characterize the age-dependent mitochondrial dysfunction and which will be further presented are the following: respiratory function, mitochondrial membrane potential, ROS generation, and the sensitivity of mitochondrial permeability transition pore (mPTP) to calcium overload.

Also, characterization of mitochondrial membrane in aged mitochondria is another important subject of analysis. The majority of scientific data generally suggest an age-dependent dysfunction of these parameters, but the results are still debatable, due to abundant literature data inconsistencies.

IV.1. Mitochondrial membrane rearrangement and ageing

Ageing is affecting all eukaryotic cells with a possible deterioration of mitochondrial membranes. In this regard, Cryo-ET technique demonstrated age-dependent changes of the IMM in a short-lived (~18 days) *Podospora*

anserina (235). As discussed in the first chapter, in normal mitochondria the cristae are profoundly projected into the matrix and cristae synthesis depends on the rows of ATP synthase dimers (38) and the MICOS complex within the crista junctions (236). In aged *P. Anserina* mitochondria, the cristae remain in the IMM which leads to the enlargement of the inter-membrane space (235). Further, the matrix is broken in vesicles within the OMM, while ATP synthase dimer rows dissipate which leads to the dissociation of dimers into monomers (235).

Another change concerns the sharp local curvature at the dimer rows which is inverted, and thus the ATP synthase monomers will be encompassed by a concave membrane and not by the convex arch at the crista ridges (235). Ultimately, the OMM disrupts with age with the release of cytochrome *c* into the cytoplasm (235); cytochrome *c* activates the cascade of proteolytic caspases which will eventually lead to the degradation of cellular proteins, apoptosis and cellular death (46). All these morphological changes in mitochondrial membranes of *P. anserina* might be induced by oxidative damage which is the key promoter of cellular senescence (237).

It's clear that aged mitochondria that are deficient in cristae and ATP synthase dimers are not capable to release ATP and thereby the cellular function will be altered. Oxidative stress is normally neutralized by oxygen radical scavenging enzymes (i.e., superoxide dismutase, or catalase) and by mitochondrial fission and fusion. In aged mitochondria, fission overwhelms fusion which induces mitochondrial network fragmentation and by thus is prevented the complementation of damaged mitochondria by fusion, deteriorating even more the already altered mitochondria (238).

IV.2. Mitochondrial respiratory function and ageing

An enormous scientific research was performed in the last two decades, on different animal models and human studies able to reveal a decline with age of oxidative phosphorylation (OXPHOS) and the electron transport activities of respiratory enzyme in highly energetic tissues, such as the skeletal muscle, heart, brain, and liver (170, 239-243). Accordingly, studies on old *vs.* adult mouse skeletal muscle mitochondria have generally reported in aged animals the decline of oxidative phosphorylation and respiratory complex ratio (RCR = the ratio of oxygen consumption in the presence versus the absence of ADP), as the indicator of the efficacy of oxidative ATP production.

On contrary, human skeletal muscle mitochondria isolated from elderly subjects did not show a significant decline of mitochondrial OXPHOS (244, 245). Moreover, Brierley et al. concluded that the respiratory decline of skeletal muscle in old individuals is rather induced by physical inactivity, than to the ageing process (246).

Numerous studies on aged mitochondria were also performed in rodent hearts from different species such as Fischer 344 or Wistar which demonstrated a decline of OXPHOS and/or of the RCR in old *vs*. adult animals (247-251).

Consistent with these results, our group of research demonstrated a statistically significant decrease in all bioenergetic parameters in old (20-24 months) *vs.* adult (4-6 months) Sprague Dawley rat heart mitochondria (RHM) respiring on glutamate+malate (252)): LEAK (i.e., State 2) 41.6±4.9 *vs.* 48.3±1.9 pmol.s-1.ml-1 (p<0.05), OXPHOS 480.17±85.2 *vs.* 673.38±32.5 pmol.s-1.ml-1 (p<0.0001), ETS 470.43±63.77 *vs.* 547.95±24.02 pmol.s-1.ml-1 (p<0.05) and RCR 11.58±1.9 *vs.* 13.62±1.1 (p<0.05) (252).

Nevertheless, there are some other studies on rat heart mitochondria that found no changes of OXPHOS and/or RCR (253, 254).

These dissimilarities of the respiratory chain activity in old rat heart mitochondria could be explained mostly by the chosen methodological approach of different labs.

Indeed, the majorities of the experimental studies have evaluated the entire population of heart mitochondria, a procedure that might probably disguise the potential changes that might occur with ageing in mitochondrial subpopulations, i.e. the subsarcolemmal (SSM) and interfibrillar mitochondria (IFM) (255). In light with this, Hoppel et al. demonstrated an age dependent decrease of OXPHOS only within the IFM cardiac mitochondria, but not in the SSM mitochondria (256). Similar results were acknowledged by Lesnefsky et al. who found a decrease in OXPHOS, in complex IV activity and in the oxidation rate of fatty acids in IFM (but not in SSM) mitochondria isolated from aged vs. adult Fischer 344 rat hearts (257).

Another problem which can affect the scientific results is the presence of myocardial fibrosis, which varies with ageing, and more importantly it might affect the accuracy of the isolation procedures.

Accordingly, in the above mentioned study we measured the heart/body weight ratio which represents an indirect indicator of cardiac hypertrophy; despite the fact that both animal and heart weights in the old group were significantly increased as compared to the adult one, the heart/body ratio was unchanged, suggesting that myocardial fibrosis was not installed in the old group (252).

Mitochondrial purity is another factor that might affect the experimental results, thereby normalization procedures must become a priority of all laboratories handling mitochondrial experiments (255). In this view, we measured the specific activity of citrate synthase which is commonly used as a quantitative marker enzyme for the content of intact mitochondria

(258). Our results showed no significant differences of CS activity between the analyzed groups: in adult group, it averaged 3185 ± 108 mU/mg proteins, whereas in old group it was 2794 ± 364.3 mU/mg proteins (p = NS) (252); in this respect, the normalization of respiratory rates and CRC per units of CS activity, did not affect the significance between groups (252).

Finally, it is worth mentioning that the majority of the studies evaluate the mitochondrial respiratory function in isolated mitochondria. And this is an important concern regarding the accuracy of the above mentioned results, since in these approaches the isolated mitochondria are no longer in their native state which obviously will alter mitochondrial interactions; moreover, the multiple isolation steps might affect also the properties of mitochondria (259). Because of these inconveniences, a better approach for mitochondrial function evaluation is the utilization of permeabilized cardiac or skeletal fibers, which are physiologically more relevant than the isolated mitochondria procedure (259).

In light with this, two studies using permeabilized heart fibers from Fischer 344 rats and permeabilized muscle fibers from C57Bl6 mice have also demonstrated an age-dependent decline of OXPHOS (250, 260).

Yet, in another study on gastrocnemius muscle from young adult *vs.* senescent rats, it was indeed identified a significant effect of ageing on mitochondrial respiration, oxidative stress, and sensitivity of permeability transition pore to Ca^{2+} when the experiments were performed on isolated mitochondria, effects that were no longer repeated in the case of permeabilized fibers (261).

The same group also reported in a study of permeabilized cardiomyocytes isolated from the myocardium of young adults (8 age-months old) and senescent (36 age-months old) Fisher 344/Brown Norway F1 hybrid (F344BN) rats, that senescent cardiomyocytes have relatively preserved

mitochondrial function and more modest differences as compared to previous studies performed on isolated mitochondria (262).

Another tissue which was intensively studied was the brain with also contradictory results related to mitochondrial function and ageing. Accordingly, in numerous studies it was demonstrated that mitochondria isolated from mouse and Fischer 344 rat brain present also a decrease of OXPHOS and CRC in aged animals (253, 263, 264). A complex analysis on 24 months aged rats was performed by Petrosillo et al. who demonstrated a reduced OXPHOS, together with a decline of mitochondrial membrane potential, but also a dysfunctional mitochondrial complex I induced by ROS-induced cardiolipin oxidation (265, 266).

However, a recent study of Gilmer et al. on synaptic and extra synaptic mitochondria isolated from the cortex of one hemisphere of young (3-5 months), adult (12-14 months), and aged (22-24 months) naïve Fischer 344 rats demonstrated no significant changes of mitochondrial respiration in aged rats (267). In light with this, Sanz et al. demonstrated no significant ageing-induced alteration of brain mitochondrial OXPHOS in Wistar rats (268).

Experiments on liver mitochondria also present contradictory data varying from no change up to a significant age-related decline of OXPHOS and/or the RCR (248, 269-271) Garcia-Fernandez et al., 2011).

Accordingly, our research group recently evaluated mitochondrial respiratory function in liver mitochondria from old (20-24 months) *vs.* adult (4-6 months) Sprague Dawley healthy rats and we found a significant decline in both State 2 and OXPHOS respiratory rates in mitochondria isolated from senescent rats with both NAD(+) (20.68 +/- 2.43 *vs*. 24.46 +/- 2.38, $p < 0.001$; 101.11 +/- 5.40 *vs.* 121.60 +/- 17.74, $p < 0.001$, respectively) and FAD(+) (33.55 +/- 3.28 *vs.* 42.75 +/- 1.83, $p < 0.0001$; 123.92 +/- 5.02 *vs.* 140.69 +/- 15.37, $p < 0.001$, respectively) linked substrates (272).

The effects of ageing on the activities of individual mitochondrial complexes of the respiratory chain also are presented as contradictory results (extensively reviewed by (273)). Marin-Garcia et al. investigated the levels of specific mitochondrial enzyme activities and content during cardiac growth and development from the early neonatal period (10-20 days) to adulthood (67 years), demonstrating that ageing is not associated with changes of the levels of cytochrome c oxidase (274) and complex V specific activity, mtDNA copy number and COX subunit II (275). Miro et al. also did not find any alterations of the respiratory chain complexes' activities in aged patients, although increased lipid peroxidation was found to be age-dependent in these patients (276). All these results were reinforced by a study performed on mitochondria isolated from quadriceps muscle of healthy adult (20+ y.o.a.) and aged (70+y.o.a.) individuals which did not find any age-related alteration of pyruvate dehydrogenase, tricarboxylic acid cycle, respiratory chain, and ATP synthesis; in light with these results the final conclusion of the study was that "*these results are incompatible with the mitochondrial theory of ageing*" (244).

Contradictory results are also noticed regarding the activity/expression of mitochondrial complex IV (cytochrome c oxidase). Müller-Höcker identified a COX age-dependent deficiency in numerous tissues, such as the heart, limbs, diaphragm, and extraocular muscle of healthy individuals, but also the fact that the number of COX-negative muscle fibers were higher in aged humans, but also in aged animals (277, 278). These data are in agreement with an old report of Abu-Erreish and Sanadi who were the first that identified an age-related decrease of COX activity in heart muscle and brain homogenates, and in purified heart mitochondria, but not in liver and kidney homogenates (279). On contrary, Ozawa et al. demonstrated an increased amount of cytochrome c +c1

and unmodified cytochrome oxidase in liver mitochondria isolated from 67 biopsy specimens of normal human liver obtained at operation (280).

Pulliam et al. substantially reviewed the age-dependent decrease of ATP production noticing that most of the studies demonstrated a decrease ATP induced by age in both human and animal mitochondria (128). Moreover, Short et al. demonstrated that ATP production in human skeletal mitochondria decreases by ~ 5% every 10 years starting with the second decade of life (281).

However, according to Pulliam et al. (2012) it is not feasible to affirm that ATP decline is indeed physiologically relevant since these studies did not take into account the glycolysis, the second pathway for ATP synthesis (128).

IV.3. Mitochondrial membrane potential and ageing

Mitochondrial membrane potential ($\Delta\psi$) is another parameter of which evaluation is essential in characterizing mitochondrial function.

The electrons passing through ETC mitochondrial protein complexes I-IV will induce a chemical free energy to drive protons against their concentration gradient across the IMM (i.e., out of the mitochondrial cytoplasm) which is used to generate a H^+ electrochemical potential gradient, expressed as the proton-motive force (Δp) (282). This leads to a net H^+ storage outside the membrane, which will further flow back into the mitochondria via the Vth mitochondrial complex, namely the F_1/F_0 ATP-synthase, producing ATP and closing the ETC. The force driving protons into the mitochondria (i.e., Δp) represents an association of $\Delta\psi_m$, as a charge or electrical gradient, and the mitochondrial pH gradient (ΔpH_m), as the H^+ concentration gradient. Generally, $\Delta\psi$ (i.e., the electric potential across the IMM) represents the predominant constituent of Δp, with a value of approximately 200 mV (282).

The role of Δ*p* is to drive ADP phosphorylation and to end the electron flow in the absence of ADP, in a controlled metabolic condition; the mitochondrial membrane potential also is supplying the driving force for K^+ and Ca^{2+} uptake by mitochondria (237). Thereby, any dysfunction of the ETC will lead to the impairment of mitochondrial membrane potential, since Δψ is essential for ATP synthesis.

The preservation of mitochondrial membrane potential is essential thus for the protein transportation and process within mitochondria, while its alteration might signal a subsequent selective degradation by mitophagy.

Numerous studies using mitochondria isolated from different rodent tissues (e.g., heart, skeletal muscle, or liver) tissues demonstrated an age-related decline of mitochondrial membrane potential (as extensively reviewed by (128). An important observation related to all of these scientific data, is the fact that age-dependent decrease of $\Delta\psi_m$ was substrate independent, since mitochondria were energized with both, complex I- and complex II-dependent substrates. Moreover, Murphy demonstrated a solid correlation between mitochondrial oxidative stress and Δψ, NADH/NAD+ ratio, CoQH2/CoQ ratio, and the local O_2 concentration (283).

Sastre et al. (1998) evaluated for the first time the effects of ageing on mitochondria in intact liver cells using flow cytometry: they found out that age is associated with a decrease in mitochondrial membrane potential (30%), an increase in mitochondrial size, and an increase in mitochondrial peroxide generation (23%), while intracellular peroxide levels were also increased (284).

Few years later, LaFrance et al. (2005) evaluated cortical and striatal mitochondria were isolated from Fischer 344 rats at 2, 5, 11, 24 and 33 months of age. They found out that $\Delta\psi_m$ from both brain regions was not modified up to 24 months, but slightly declined at 33 months. They also

showed that the ability of calcium to induce mitochondrial swelling and depolarization was also unchanged through 24 months of age and increased at advanced ages only for cortical, but not striatal, mitochondria, concluding that indeed mitochondrial functions decline with advanced age, but this decline depends on brain region (285).

Lin et al. (2007) also showed more depolarized (lower) mitochondrial membrane potential in astrocytes cultured from old (26-29 months) mice as compared to young (4-6 months) mice, but their study represent just an indirect evidence for supporting $\Delta\psi$ decrease, since they showed the impairment of the H^+ permeability of the inner membrane in old astrocytes (286). In line with this, Navaro and Boveris consider a simple speculation that the increased H^+ permeability of the IMM might be suggestive for a further failure in maintaining the H^+ electrochemical gradient in aged mitochondria (237).

Accordingly, in our study on senescent Sprague Dawley rats, simultaneously with the respiration measurements we evaluated the mitochondrial membrane potential in mitochondria energized with glutamate+malate (252), using an ion selective electrode (ISE) filled with the tetraphenylphosphonium (TPP+) solution. Our data showed a statistically significant decrease of $\Delta\psi$ ($p < 0.005$) in old *vs.* adult group, as follows: LEAK $\Delta\psi$ = 165.9±0.88 *vs.* 213.8 ±2.57 mV ($p < 0.005$); OXPHOS $\Delta\psi$ = 100.6±6.58 *vs.*190.1±3.14 mV ($p < 0.005$); $\Delta\psi$ after Omy addition = 145.2±1.727 *vs.* 193.2±3.773 mV ($p < 0.005$); ETS $\Delta\psi$ 100.8±2.662 *vs.* 160±2.9 mV ($p < 0.005$) (252).

IV.4. Mitochondrial ROS production and ageing

It is clear now and widely accepted that mitochondrial dysfunction is the key player in ageing (see the extensive review of (287)). Particularly, mitochondrial ROS synthesis, which is tissue-, species-, and substrate-dependent, is highlighted as the major contributor of age-dependent mitochondrial dysfunction, since mitochondrial ETC represents the major site for ROS generation (287).

As previously discussed, mitochondrial respiration takes place in the IMM as a result of the electrons flow through the ETC protein complexes (complex I, II, III, and IV), while the last one (complex V) plays the key role in ATP synthesis. Accordingly, the energy induced by the electron transfer is further used by ETC in protons translocation from the mitochondrial matrix to the inter membrane space. As a result, across the IMM is attained an electrochemical gradient, which be further utilized in ATP synthesis as the protons will re-enter the mitochondrial matrix via the F1-F0-ATP synthase (36). Accordingly, any alteration of the mitochondrial protein complexes will break the electron flow, leading ultimately to mitochondrial dysfunction.

Mitochondrial ROS production is a physiological event occurring during these stages of ATP synthesis: thus, during mitochondrial respiration electrons will evade from ETC and will interact with a highly and unstable oxygen molecule, generating superoxide anion ($O_2^{\bullet -}$) and other types of ROS (288), including hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), and hydroxyl ion. Firstly, oxygen is converted to a superoxide anion by xanthine oxidase (XO) or mitochondrial ETC complexes I and III (289). Complex III produces a superoxide anion in both matrix and the inter membrane space (290). The superoxide anion is further converted to hydrogen peroxide by superoxide dismutase (SOD); hydrogen peroxide can

either be detoxified to water and oxygen by glutathione peroxidase, catalase or thioredoxin peroxidase (TPx) (291, 292), or it can be converted to hydroxyl radical and hydroxyl ion *via* the Fenton reaction (293).

Therefore, the major sites for ROS production are represented primarily by complex I, and to a lesser extent, by the ubiquinone binding sites of complexes II and III. Accordingly, it was demonstrated that the inhibition of complexes I or III leads to an eloquent increase of ROS production, which is suggestive for the unanimously accepted conclusion that the ETC impairment is indeed harmful for cell survival (294).

It is thus reasonable to consider that aged-induced decline of mitochondrial ATP production and membrane potential (already demonstrated by a multitude of scientific data, as presented above) will induce an increased mitochondrial ROS production with age, as proposed by the free radical theory of ageing (111) and supported by the results from numerous studies. However, as ascertained by Pulliam et al. these changes are "*far from universal and absolute, and often, the degree of dysfunction is modest*" (128).

In this regard, the group of Garcia-Ruiz et al. demonstrated that oxidative stress, followed by electrons leakage out of ETC, is indeed involved in the alteration of mitochondrial function (295). Few years later, in an experiment conducted on skeletal and heart muscle of the MnSOD-deficient mice, it was shown that increased oxidative stress correlates with the decline of mitochondrial Complex I and Complex II activities (296), an observation which is an indubitable proof that mitochondrial respiratory function is affected by the oxidative stress.

Numerous ulterior studies also informed about higher levels of ROS in old *vs.* adult mitochondria energized with either complex I- or II-linked substrates (see the review of (128)).

In this view, we recently demonstrated that ROS production is indeed increased in aged rat heart mitochondria, regardless the substrate/substrate+inhibitor combination (252).

However, there are inconsistent research data regarding the age-dependent decline of ROS mitochondria: some studies demonstrated indeed this decline in oxygen consumption and/or alterations in ETC complex activities with increasing age, whereas others reported no significant age-related ROS changes (as reviewed in (128)).

The same contradictory results were identified in mitochondria isolated from human skeletal muscles regarding the mitochondrial ROS production in aged patients (245, 297, 298). The first study conducted on human skeletal muscle biopsy samples did not confirm the hypothesis of increased oxidative stress in aged muscle since the authors showed that the level of mitochondrial ROS and measurements of antioxidative defense (muscle content of glutathione, glutathione redox status, antioxidative enzymes activity) were not significantly different between old vs. adult subjects (245).

On contrary, Capel et al. showed that in human mitochondria isolated from vastus lateralis biopsies, muscle oxidative capacity was preserved with age but the ROS production was markedly increased in aged individuals. The study also demonstrated that rotenone (i.e., the inhibitor of ETC Complex I) abolished this increase, suggesting that the increase of ROS production occurs during the reverse electron transfer (i.e., from complex II towards complex I) (297).

Finally, Ghosh et al. performed a study on mitochondria isolated from vastus lateralis biopsies in old vs. adult subjects and they acknowledged an age-dependent decline of mitochondrial function as demonstrated by reduced ATP production, but their study did not identify an increased ROS production in aged mitochondria (298).

Another important aspect which must be emphasized is that several research data also reported the potential beneficial role of ROS as redox signaling molecules. Accordingly, it was shown that low doses of ROS exposure actually decreased the mortality rate and increased stress resistance, while higher doses induced the opposite effects (299).

In line with these results, it was reported that antioxidants actually impair the mitochondrial ROS signal altering thereby general health status and preventing the lifespan extension (135, 300). This concept was termed mitochondrial hormesis or mitohormesis (301) and it was reported that mitohormesis extended the lifespan in numerous model organisms, such as *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans* and mice (135, 302). The mitohormesis concept is actually more complex since it is based on the physiological effects of caloric, glucose, and macronutrients restriction, as well as physical exercises, which are essential in providing a healthy status and thereby longevity, while ROS represent vital signaling molecules involved in all these processes (303).

IV.5. Mitochondrial Ca^{2+} overload and ageing

Another concept unanimously accepted today is the central role of calcium deregulation in the induction of apoptosis and necrosis (304, 305). Alternatively to death, stress also may induce either senescence or autophagy (306). Accordingly, numerous studies (as reviewed in (307)) reported calcium overload involvement in senescence progression, despite the fact that we still do not have a clear depicted picture of this mechanistic insight.

In animal models of ischemia-reperfusion injury, it was reported that the aged heart was prone to a greater damage, both in aged animals and humans as reviewed in (308), presenting a reduced restoration of contractile function (309)

and a higher risk for further rhythm troubles incidence (310). Moreover, during ischemia, the ventricle functional recovery is inversely proportional to intracellular calcium loading, an aspect which is more evident in the aged heart (310).

Aged heart also present important alterations of sarcolemmal Na^+/Ca^{2+} exchanger, and of Ca^{2+}-ATPase and ryanodine receptor from the sarcoplasmatic reticulum (SR), these changes affecting the cytosolic calcium in the aged heart (308). Summarizing all these changes, is evident that an aged heart will present an increased intracellular calcium overload due to decreased Ca^{2+} release and Ca^{2+} blockage within the SR (309).

The increased calcium overload in the aged heart induces important cellular damage via at 2 major pathways: (1) a *direct pathway*, via the activation of calcium-dependent phospholipases, proteases, and nucleases, and (274) an *indirect pathway*, via the inhibition of OXPHOS, the increased ATP consumption as a result of calcium ATPases activation; thereby, the global effect of the indirect pathway is an important depletion of cellular ATP (309).

Over the time, numerous research data have come to another important conclusion: the increase in mitochondrial calcium overload in aged heart is induced by the opening of the mitochondrial permeability transition pore (mPTP), due to cardiolipin depletion in aged mitochondria, as reviewed by (311). Moreover, the research group of Padova assumed that age-induced mitochondrial alteration is linked to ROS accumulation and implies the mPTP (255).

Most of the studies reported an increased intracellular calcium accumulation during oncogene-, rotenone-induced, or replicative senescence (312-314), focusing on the role of mitochondrial calcium overload as the major cause for increased mitochondrial ROS production and mitochondrial dysfunction in aged cells.

Other researchers focused on the link between calcium and transcription factor p53 within the context of ageing, proposing that calcium homeostasis is actually affected by the activation of age-dependent p53 activation (315), although there is no evidence so far about the correlation between p53 and calcium.

As previously discussed, another important stress response associated with ageing is autophagy. It is not doubtful the involvement of intracellular calcium overload in autophagy regulation (316, 317), albeit, the exact mechanism is far of being elucidated. Yet it is hypothesized that Ca2+ signaling promotes the autophagic activity in response to stress (316).

IV.5.1. Mitochondrial permeability transition pore (mPTP)

Mitochondria are the key players in metabolic homeostasis by controlling the intracellular Ca^{2+} level and producing ATP which is essential for cell survival.

Under physiological conditions, the IMM is impermeable to almost all metabolites and ions. On contrary, different pathological conditions, such as mitochondrial Ca^{2+} overload associated with increased ROS production, high phosphate level, and low adenine nucleotides, is inducing the occurrence of mPTP (Fig. 7) within the IMM (318).

mPTP opening allows the passive diffusion for molecules of <1.5 kDa, disrupting thus the IMM permeability barrier leading to mitochondrial uncoupling and swelling which eventually induces mitochondrial energetic failure. Hence, due to marked ATP depletion the integrity of cell structure and functionality cannot be preserved anymore, thus ionic homeostasis occurs, which finally leads to cell death, predominantly via necrosis (318).

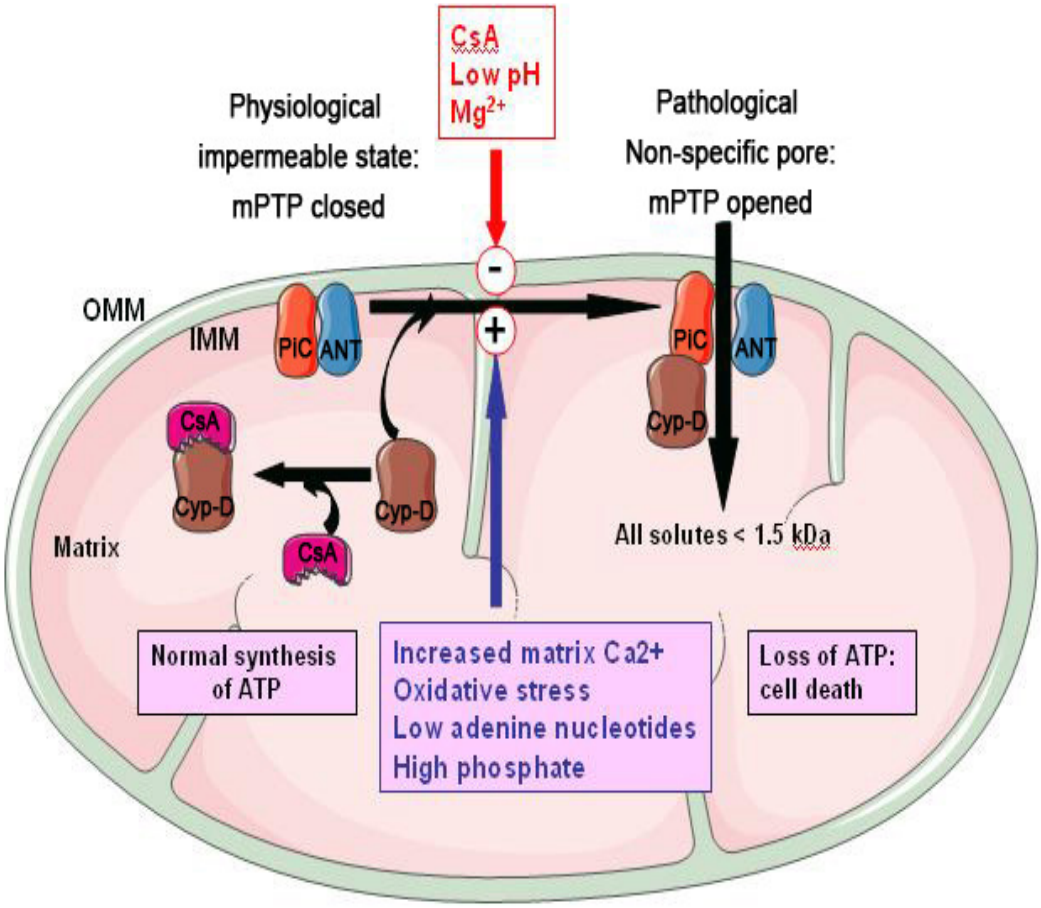

Figure 7. Schematic overview of the proposed mitochondrial permeability transition pore (mPTP) and its role in cell death. *(Modified after (318)).* The mPTP is generally described as a high-conductance channel that forms in the inner mitochondrial membrane (IMM). In the model illustrated here, pore formation involves IMM carriers—most notably the phosphate carrier (PiC) and the adenine nucleotide translocase (ANT)—together with cyclophilin D (CypD), a key regulatory component located in the mitochondrial matrix. Under physiological conditions, the pore remains closed, oxidative phosphorylation proceeds normally, and ATP production is preserved, thereby supporting cell survival. In pathological settings—such as mitochondrial Ca^{2+} overload, oxidative stress, depletion of adenine nucleotides, or accumulation of inorganic phosphate—the pore may open. When this occurs, the IMM becomes non-selectively permeable to solutes below ~1.5 kDa, promoting osmotic influx into the matrix, mitochondrial swelling, and impairment of ATP synthesis; if pore opening is sustained, these events can culminate in cell death. mPTP opening can be attenuated by cyclosporine A (CsA), acidic pH, or Mg^{2+}.
(*Figure created using Servier Medical Art*).

Despite more than 30 years of intensive research, the exact molecular structure of mPTP is still not identified. Nowadays, two molecules are considered as the key components of mPTP structure: the phosphate intracellular carrier (PiC), and adenine nucleotide translocase (ANT) - Fig. 7, both located within the IMM (318). Another component of the mPTP, cyclophilin D (Cyp-D) which is located in the mitochondrial matrix, is rather viewed as a modulatory factor of the mPTP (319).

Cyp-D is a nuclear encoded mitochondrial isoform from the cyclophilin family, which was reported firstly by Crompton et al. (320) as an important part of mPTP: they basically demonstrated the inhibitory effect of the immunosuppressant cyclosporine A (CsA) on pore opening, via the blockage of Cyp-D (see Fig. 7).

The CsA effect is mediated via the inhibition of peptidyl-prolyl-cis trans isomerase (PPIase) activity of Cyp-D: CyP-D forms a complex with a target protein, which determines a conformational change that generates the occurrence of a channel on the IMM (318). It was also reported that the matrix volume overload will increase both CyP-D binding and mPTP opening in response to Ca^{2+} load (318). CsA binds to CyP-D preventing thus the Cyp-D to further attach to its target protein – Fig. 7. Studies using mitochondria isolated from Cyp-D knockout mice, have showed a lower sensitivity to Ca^{2+} and a delayed mPTP opening that was insensitive to CsA (321, 322).

Halestrap and Davidson were the first who reported ANT as a component of the mPTP molecular structure (323). Their finding was further confirmed by several studies which used the bongkrekic acid (Bka), the ANT inhibitor which showed the inhibition of the mPTP opening by decreasing its sensitivity to Ca^{2+}, while carboxyatactyloside and ANT depletion were able to trigger pore opening (324, 325).

Yet, other relevant study performed on knockout mice demonstrated that liver mitochondria of animals lacking ANT1 and ANT2 isoforms induced a Ca^{2+}-activated mPTP which was still sensitive to CsA (326). The same study showed that mPTP opening in ANT knockout mitochondria needed a 3 fold higher quantity Ca^{2+} load in order to trigger mPTP vs. the wild type mitochondria (326). In light with this, Halestrap considered that there are other isoforms of the ANT family which can replace ANT in mPTP constriction or at least are involved in its regulation (318).

The potential role of phosphate carrier (PiC) in mPTP construction was shown in a study which demonstrated that CyP-D binds to PiC in a CsA-sensitive manner; moreover this connection is enhanced by the oxidative stress that sensitizes the pore opening to Ca^{2+} (327). This potential role of the PiC was also confirmed in another study which demonstrated that phosphate is requested for the inhibition of mPTP opening via the blocking of Cyp-D (328).

For a certain period of time, VDAC (voltage dependent anion channel), a class of porin ion channel located on OMM, was also considered as a part of the mPTP structure, yet, recent data showed that the mPTP still functions in mitochondria from VDAC1-VDAC3 knockout mice and also in VDAC 1/3_/_ cells with silenced VDAC2 (329).

Therefore, VDAC is no longer validated as an essential component of mPTP molecular structure, nor as a regulatory molecule of mPTP. Yet, other proteins such as the peripheral benzodiazepine receptors, creatine kinase, hexokinase and Bcl-2 family members are still controversial as components of the mPTP structure, therefore further researches must elude their contribution to mPTP function (311). Also, mitochondrial F1FO (F)-ATP synthase dimers, monomers or c-subunit ring alone have been implicated as crucial components. While several models have been proposed for its molecular identity (330), despite being studied for more the half century the molecular structure of mPTP still remains elusive (331).

IV.5.2. The relationship between mPTP and ageing

The relationship between mPTP and ageing was extensively investigated, but there is still a numerous aspect which are far of being elucidated. Accordingly, alterations of calcium handling, ANT, voltage-dependent anion channel, or cyclophilin D have been all considered to be involved in the pathomechanism of ageing (as reviewed by (311)).

The age-dependent increased sensitivity to mPTP is widely accepted, since it was identified in lymphocytes, neurons, hepatocytes and cardiac myocytes (332-335). The first studies demonstrating the age-induced increased sensitivity to mPTP opening were carried out in mitochondria from lymphocytes (334) and murine liver (332), while the latter one demonstrated along to faster rates of mPTP in response to Ca^{2+} overload, a lower OXPHOS and a higher state 4 respiratory rate in aged hepatic mitochondria (335). Lastly, in a study conducted on brain and liver mitochondria isolated from 20-month-old mice, Mather and Rottenberg reported a reduced threshold for Ca^{2+}- induced mPTP activation (333). Moreover, in all these studies, the calcium retention capacity (CRC) and the mitochondrial swelling rate of aged animals were restored to control values after CsA addition, which suggest that the age-related alterations are assigned to mPTP (255).

Consistent with these first research data, more recent studies also demonstrated an age-dependent increased sensitivity to mPTP opening in heart mitochondria and permeabilized cardiomyocytes (262, 336, 337).

Accordingly, we also measured the amount of total mitochondrial Ca^{2+} retained before the mPTP opening mitochondria isolated from aged vs. adult rat hearts (252) and we evaluated the results in the presence vs. the absence of the classical pore desensitizer, cyclosporine A. Our measurements showed an increased sensitivity to Ca^{2+}-induced mPTP opening in old vs. adult groups (626.7±69.79 vs. 1045±114.8 nmol $CaCl_2$/mg prot, $p < 0.05$) (252).

Moreover, the cardioprotective effect of CsA was significantly reduced in old animals as compared to the adult ones (991.7±103.5 vs. 1787±90.43 nmol $CaCl_2$/mg prot, $p < 0.0001$).

Despite the fact that these data clearly demonstrated the increased susceptibility to mPTP opening in aged animals, this phenomenon has 2 major limitations: (1) there is no experiment performed so far in myocardium in vivo, and (274) its underlying mechanism(s) are still a matter of debate (255). In particular, three major hypothesis have been formulated as possible explanations for the age-dependent mPTP opening: (i) the pore opening triggered by calcium is the a primary event; (ii) the pore opening triggered by an increased ROS formation is a later event; (iii) the initial pore opening triggers a vicious cycle that, "albeit involving only a fraction of mitochondria, might result in mitochondrial dysfunction" (255).

Indeed mPTP opening might be induced by oxidative stress in aged mitochondria as demonstrated in numerous studies as extensively reviewed in (311), albeit the causal relationship between mPTP opening and ROS release is not scientifically explained. Increased ROS release during ageing triggers the structure and functional alterations of several lipid constituents of the IMM, especially cardiolipin (338). Accordingly, various studies proved that cardiolipin is involved in the regulation of several mitochondrial bioenergetic processes, via the optimization of the activity of key proteins involved in OXPHOS; thereby, abnormalities in the structure and/or content of cardiolipin might be responsible for mitochondrial dysfunction in ageing (311).

Due to their high content of unsaturated fatty acids and to their location in proximity of the site of ROS production, cardiolipin molecules are particularly exposed to the oxidative damage (311). It is thus possible that mitochondria become increasingly susceptible to Ca^{2+}-induced mPTP opening with age, because of oxidation/depletion of cardiolipin. Consistent with this,

Petrosillo et al. reported an increased susceptibility to Ca^{2+}-induced mPTP opening together with an oxidation/depletion of cardiolipin in aged heart mitochondria (337). Even more, treatment of aged rats with melatonin, a known antioxidant which is able to protect cardiolipin from ROS attack to its fatty acid constituents was actually able to prevent the above mentioned pathological processes (337).

Ageing is also inducing changes in the level and structure of several proteins associated with the mPTP: the ratio between Cyp-D and ANT was found to be increased in old male Fischer 344 and Brown Norway rats, and this increase was suggested to be responsible for higher susceptibility to mPTP opening (339).

ANT, as an important component of mPTP molecular structure, was found to be one of the mitochondrial proteins most susceptible to ROS attack during ageing (340). Accordingly, increased ROS production with age may be thus responsible for ANT deformation and destabilization. Age-dependent increased oxidative stress might transform ANT into a non-selective pore, allowing free access of small ions and metabolites across the inner mitochondrial membrane (311). An age-associated rise in mPTP activity specifically in interfibrillar cardiac mitochondria was observed with no apparent increase in either Cyp-D or ANT immunoreactivity (336). All these data provided significant evidence that in heart mitochondria, mPTP activity increases with ageing, suggesting that the sensitivity to inhibition by cyclosporine A may also decrease with age (255).

Finally, an increased probability of mPTP opening might be caused by alterations of the signaling pathways that characterize the ageing process in different tissues (reviewed in (311)). Accordingly, mPTP opening would convert the decreased response of protective pathways, such as Akt activation into bioenergetic failure and ultimately to cell death (341).

Currently it is not known if ageing-associated changes in mPTP sensitivity described in rodent mitochondria are also present in human cardiac mitochondria, and it is also unclear whether interventions able to prevent mitochondrial calcium overload or mPTP opening during experimental models of I/R injury are effective in protecting the senescent human heart (342).

Nowadays it is unequivocally recognized that the activity of this highly regulated, multi-component mega-channel, is increased in ageing and contributed to the ageing-related degenerative diseases via multiple mechanisms (343).

IV.6. Ageing and mitochondrial dynamics alterations

Currently it is known that mitochondria are dynamic organelles connected in a network, with alternate configurations that interact with the bioenergetic properties, that maintain the normal mitochondrial function and to participate in different fundamental processes. What is important to highlight is that even though they can function as united organelles, mitochondria can also function, and be morphologically independent in the cells.

To maintain the network and also the individual integrity, the mitochondria undergo several dynamic processes that include: fusion (joining of two mitochondria into one), fission (division of one mitochondrion into two), transport (along the cytoskeletal network), and mitophagy. Consecutively, studies have concentrated on elucidating the role of these dynamic behaviors and their consequences. Even though these processes appear to have a distinctive mechanism from the biochemical and metabolic processes that are taking place in mitochondria, an increasing number of studies have provided links between mitochondrial metabolism and dynamics. Moreover,

studies demonstrated the importance of the dynamic processes in both the normal and pathological state, by modulating mtDNA integrity, cellular redox status and apoptosis (344). By interfering in mitochondrial communication and exchange with cytosol, mitochondrial shape control and quality control, the dynamic processes also regulate mitochondrial function (345).

In ageing, all the biochemical and bioenergetic changes that occur in mitochondria (increased ROS production, mtDNA damage, impairment of Ca^{2+} homeostasis and respiratory chain efficiency) are also accompanied by alterations in mitochondrial dynamics, more precisely by a decreased mitochondrial biogenesis, perturbations in fusion/fission and turnover (mitophagy) and also by an increased mitochondria-mediated apoptosis (346). However, the complete mechanisms by which changes in mitochondrial dynamics are involved in ageing have not been completely elucidated yet.

Mitochondrial morphology and organization occurs as a result of various intracellular and extracellular signals (344). The morphology is tightly dictated by the balance between fusion and fission; hence any perturbation may result either in an excessive fragmentation, either in long interconnected mitochondria caused by a decreased fission. These processes are modulated by different mitochondria-associated proteins and by conformational transitions in the IMM. Studies have demonstrated that depending on mitochondrial morphology, cells present different energetic states. In human cells, elongated mitochondria occurs in conditions associated with increased ATP production, to efficiently increase energy production and distribution across significant distances within a cell (347, 348). Opposite, a fragmentation of mitochondrial network can ease the recruitment of mitochondria in compartments in need of ATP (349). Any disturbance in mitochondrial dynamics can produce mitochondrial defects in different sites like placenta, neurons, skeletal muscle and vascular smooth cells (350).

Mitochondrial fusion is highly important for the regulation of OXPHOS activity and also mtDNA integrity, by being able to facilitate the mixing of mitochondrial compartments. In ageing, fusion can act like a "double-edged sword", on one hand it can prevent the mutations of mtDNA that are produced by respiratory dysfunction and on the other hand, fusion can produce the accumulation of mutated mtDNA, otherwise removed by fission or mitophagy (351, 352). Mitochondrial fusion is also involved in an efficient energy transfer achieved by an "electrical coupling" between two fusing mitochondria that will transmit to each other their ΔΨm (353), thus suggesting that fusion is a mean of mitochondrial communication. Studies have also demonstrated that inhibition of mitochondrial fusion can promote cell death in response to apoptotic signals (354).

As a process, mitochondrial fusion consists in multiple steps that include: mitochondrial tethering and fusion of the OMM, docking and fusion of IMM and finally, intramitochondrial components mixture. In mammalian cells, fusion is under the control of two GTPases, mitofusin 1 and 2 (Mfn1/Mfn2), and optic atrophy (Opa1) protein. Mfn1 and 2 mediates the tethering and fusion of OMM, whereas Opa1 is responsible for IMM docking and fusion.

In order to investigate in more detail, the regulation of mitochondrial fusion and its implications, researchers have tested in isolated organelles the effect of respiratory chain substrates addition. It was found that the OMM fusion does not respond to changes in metabolic state, whereas IMM fusion is stimulated by substrates that increases OXPHOS activity via ATP-dependent metalloprotease Yme1L that proteolytically processes Opa1 (355). Proteolysis of Opa1 can be achieved via a membrane potential-dependent metalloprotease called Oma1. Scientist believe that the activation of Oma1 when ΔΨm is dissipated leads to a complete cleavage and inactivation of Opa1, that can contribute to mitochondrial fragmentation found in mitochondrial

dysfunction, while a transient depolarization may lead to Oma1 partial activation, thus to a pro-fusogenic action (356). Furthermore, it was demonstrated that conditions associated with an increased mitochondrial ATP function and with a tight coupling produced an increased fusion and in contrary, signals that lead to mitochondrial uncoupling produced fusion inhibition (357). Oxidative stress represents another metabolic mechanism that regulates mitochondrial fusion. It was demonstrated that high concentrations of oxidized glutathione increased the dimerization of mitofusin and stimulated mitochondrial tethering (358).

Various studies suggested a context dependent cell sensibility to decreased mitochondrial fusion. To support this theory, it was found that a partial defect in mitochondrial fusion, produced by a loss of either Mfn1 or 2, can be easily tolerated without bioenergetic alterations, in mice embryonic fibroblasts, whereas neurons cannot survive if Mfn2 is removed, due to loss of respiratory chain activity (359). Contrary, a genetic deletion of the genes involved in fusion produces a severe mitochondrial network fragmentation and suppression of intermitochondrial content exchange. More specific, removal of Mfn2 caused the death of cerebellar Purkinje neurons and removal of both Mfn or Opa1 caused a reduced growth and significant loss of mtDNA content, $\Delta\Psi m$ and respiratory chain function, in mice tissues and cultured cells (360). These consequences are probably caused by the ability of Mfn2 to maintain the levels of coenzyme Q and Opa1 to maintain the mitochondrial structure and respiratory chain assembly, as suggested by the group of Mourier et al. (361) and Cogliati et al (362). Moreover, studies demonstrated the important role fusion in embryonic development. The loss of Mfn1 and 2 was shown to cause an important decrease of mitochondrial fusion, responsible for midgestional lethality due to improper placenta development (363). A study demonstrated Mfn2 had a lower gene expression in skeletal

muscle of older persons and the mutation of Opa1 is responsible for a decreased OXPHOS and ATP production (364, 365).

Mitochondrial fission is a key player in mtDNA integrity and mitochondrial quality maintenance. Furthermore, it facilitates autophagy and transport, is involved in apoptosis and is also required in periods of high ATP demand (346). Indeed, as reviewed by Peterson et al. (346), mitochondria excised by fission are a target for autophagy because they often have a lower ΔΨm. Mitochondrial fission occurs early during apoptosis, just before caspase activation and membrane swelling and almost at once with cytochrome c release, thus is universally linked with apoptosis (366). Still, fission can appear independently from apoptosis, due to either exposure to uncoupling substances or viral infections (366). However, several components involved in mitochondrial fission (Drp1, Fis1, Endophilin B1) have been found involved in programmed cell death progression.

In mammalian cells, fission is under the control of dynamin-related protein 1 (Drp1), a large GTPase recruited to the surface of OMM by different receptor proteins (Mff, MiD49, and MiD50) and fission protein 1 (Fis1) that interacts with Drp1 and assembles into foci used as scission sites for fission events (367). There have been identified, on the surface of Drp1, multiple phosphorylation sites linked to various signaling pathways that can either activate or inhibit Drp1, depending on the site. As presented by the group of Seo (344), there are also several accessory proteins involved in fission, such as: endophilin B1, mitochondrial protein of 18 kDa (MTP18), death-associated protein 3 (DAP3) and ganglioside-induced differentiation-associated protein 1 (GDAP1). It was found that the inhibition of Drp1 transforms mitochondria into long and interconnected organelles, promotes ATP production and organelle structure and contrary, the overexpression of Drp1 can produce an incorrect fragmentation of mitochondria in dividing cells (344). Moreover, suppression

of either Drp1 or Fis1 was found to produce an increase in ROS production and mtDNA damage (368). The involvement of Drp1 in apoptosis was strengthened when several papers revealed that the expression of a dominant negative mutant (Drp1K38A) or the down-regulation by RNAi of Drp1 can delay caspase activation, cytochrome c release, mitochondrial fragmentation and apoptosis (369, 370). Also, it was found that mitochondrial fission and apoptosis can be induced by Fis1 overexpression (371).

During ageing, the balance between fusion and fission, required to maintain a normal mitochondrial function, is perturbed by the accumulation of dysfunctional, damaged mitochondria. Ageing is associated with a decrease of mtDNA integrity and functionality, ultrastructural abnormalities, highly interconnected networks, a reduced antioxidant defense, a reduced oxidative phosphorylation and ATP production, and more significant, an increase in ROS generation. Also, giant mitochondria were found in aged tissues and cells, suggesting that the alteration of mitochondrial dynamics can contribute to the mitochondrial deterioration with age. Thus, a proper function mitochondrial fission is very important in order to eliminate the damaged mitochondria and prevent its accumulation. In fact, it was demonstrated in human cells that impairment of mitochondrial fission leads to cell senescence, produced by high ROS production and low $\Delta\Psi m$ (368, 372). Studies suggested that a decrease of mitochondrial fission proteins represents an adaptive response, in order to increase the resistance against oxidative stress produced by mitochondrial dysfunction, or a defensive response in order to maintain a functional mitochondria by allowing the escape of mitochondrial from (373).

Furthermore, it was demonstrated an inverse connection between mitochondrial fusion and respiration rates of complexes I, III and IV, in both mammals and humans (374). Moreover, an increasing tendency of mitochondrial fusion during ageing can represent a mechanism used to

maintain mitochondrial activity. In cell models of ageing it was found that cultured cells entered a state of cellular senescence after a finite number of divisions. The cells displayed different biochemical and morphological changes corresponding to ageing. Particularly, they found an increased mitochondrial length in senescent cells and also that an experimental increasing of length by Fis1 depletion determined a senescence-like state that was accompanied with a decrease of ΔΨm, increased ROS production and mtDNA damage (368). The presented data indicate that dysfunction in the regulation of mitochondrial dynamics can be linked with the accumulation of damaged mitochondria during ageing.

The previously described dynamic processes are intertwined tightly with mitochondrial motility and any alteration of either can affect the signaling pathways and cell function. Mitochondrial motility holds a great importance for the transport of mitochondria to areas where there is an increased energy demand and also in the return of them to be degraded in areas where the lysosome concentration is higher. Moreover, mitochondrial movement results in the rearrange of ATP production Ca^{2+} buffering spatial pattern. Studies have shown that defects in mitochondrial motility and an altered distribution are involved in the pathogenesis of various neurological disorders (375). Mitochondria movement can be uniform over a long distance or it can be shorter and punctuated with a pause followed by a change in direction.

It was identified, in smooth muscle cells, that mitochondrial motility is decreased with age. Similarly, the same observation was made in neurons (376). In smooth muscle cells mitochondrial movement was described as a directed and Brownian-like motion that occurred in a higher percentage in cells from young animal models, as compared to cells from older animals. Intriguingly, the same study revealed that extent of mitochondrial movement was greater in organelles located near the periphery of cells (377). Moreover,

stem cells can spatially-segregate mitochondria by age, thus younger mitochondria are placed at the periphery and hence, motility can be associated with the age of each individual organelle (377).

When energy demand exceeds respiratory capacity, mitochondrial biogenesis is triggered. Particularly, it can be caused by ROS production, hypoxia and as a response to stress, temperature, different hormones and exercise. Mitochondrial biogenesis represents the increase of existing mitochondrial content, either by growth of mitochondrial network, which will result in an increase of mass and either by division, which will result in an increase of mitochondria number.

Mitochondrial biogenesis is induced by the co-transcriptional regulation factor PGC-1α (p eroxisome-proliferator-activated receptor γ co-activator-1α) that in turn activates several transcription factors, such as nuclear respiratory factor 1 and 2 (NRF-1 and NRF-2) and mitochondrial transcription factor A (mtTFA). In turn, the activity of PGC-1α is regulated by AMPK, a protein kinase that acts as an energetic sensor of cells and a major regulator of mitochondrial biogenesis. More important, AMPK represents one link between mitochondrial biogenesis and ageing, being demonstrated that its activity is decreased with ageing (378). Other major contributors to ageing are NRF-1 and NRF-2. They increase the transcription of several key mitochondrial enzymes and more important, they interact with mtTFA, which later activates the transcription and replication of mtDNA (379). There is some scientific proof that suggests that the binding of NRF-1 to mtTFA is increased with age (380), Other triggers of mitochondrial biogenesis are thought to involve alterations in Ca2+ flux and calcium/calmodulin-dependent kinases, p38 MAPK and protein kinase C (PKC) that will induce the expression of: key signaling molecules (SIRT1), transducers of regulated cAMP response element-binding protein- binding proteins (TORCs) (381, 382).

The group of Dillon et al. (383) demonstrated in an animal model with mitochondrial muscle myopathy that an increased expression of PGC-1α can lead to an increased mitochondrial mass, respiration and ATP production. Moreover, overexpression of PGC-1α in skeletal muscle of ageing wild-type mice can protect from mitochondrial dysfunction and sarcopenia, which are associated with normal ageing, while both heart and muscles, it can confer protection by increasing the mitochondrial pool that in turn minimizes the partial defects associated with increased levels of mtDNA mutations (383). It was also found that with increasing age, the skeletal muscle suffers a significant decrease in mitochondrial density, which suggests a decrease in mitochondrial biogenesis. As overexpression of PGC-1α in skeletal muscle of old animals was shown to improve oxidative capacity, suppress muscle atrophy and mitochondrial degradation, consecutively, low levels of PGC-1α can be incriminated for the reduction of mitochondrial biogenesis (346). However, there are studies that report a reduced expression, while others found no change in gene expression in aged skeletal muscle (346). The group of Chabi et al. (384), reported a 30% reduction in mitochondrial content and lower PGC-1α protein concentration, in muscles of senescent rats, while another study found a decrease in the expression of other genes encoding some ETC subunits in aged human muscle (385).

Increased Mitochondria-Mediated Apoptosis

Mitochondria have emerged as cellular respiratory regulators, involved in different signaling pathways in ageing, pathways associated with apoptosis, necrosis and autophagy. Alteration of redox potential, electron transport chain, oxidative phosphorylation and ATP synthesis, release of cytochrome c that activates the caspase family proteases and mitoptosis, are several mechanism by which mitochondria is involved in apoptosis.

The pathway independent of caspase activation, called mitoptosis, can be regulated by either pro- (386) and anti-apoptotic (Bcl-2) proteins and by mPTP. What is interesting is that mitochondrial fragmentation occurs almost with the release of proapoptotic factors when mitochondrial fission in increased via Drp1 and parallel with the mitochondrial fusion blockade (366).

In human, it was demonstrated that the percent of cells that undergoes apoptosis is increasing with age. Moreover, mitochondrial dysfunction precedes and is needed for the induction of apoptosis both in age-related sarcopenia and disuse muscle atrophy (387). Other studies sustain that the caspase-independent pathway is the one up-regulated with age, while no change was recorded in caspase-3 or caspase 7 activity (346). Experiments on rats revealed that apoptotic susceptibility is age-specific, in older skeletal muscle apoptosis is more likely produced via increased EndoG levels, while skeletal muscle from younger rats is more likely produced via increased caspase-2 activity (346).

The role of mitoproteases in mitochondrial dynamics

Mitoproteases are a large and variate group of enzymes located in mitochondria or in the cytosolic compartments, forming the mitodegradome. They are of great importance for several processes involved in maintaining mitochondrial function and homeostasis. Based on their location, function, and structural and proteolytic characteristics, these enzymes are classified intro three major categories: intrinsic (function mainly into mitochondria), pseudo-mitoproteases (are catalytically deficient) and transient mitoproteases (translocate temporarily to mitochondria where they perform some proteolytic activities). Besides their degradative role, mitoproteases are involved in regulating different proteolytic reactions like protein synthesis, quality control, mitophagy, mitochondrial dynamics and biogenesis and cell

death, thus their important role in maintaining mitochondrial function and homeostasis. Thus, it was proposed that any defect in these enzymes may concur to the mitochondrial dysfunction observed in ageing (388). Indeed, an impaired or dysregulated function of mitoproteases was found to be associated with ageing and also with many pathological conditions, like: neurodegenerative disorders, metabolic syndrome and cancer (389).

Studies oriented to find the role and importance of mitoproteases in ageing has identified several mitoproteases involved in the modulation in ageing process. Studies have found that the upregulation of LONP can confer protection against stress, while levels and activity is decreasing with age (390). As reviewed by Quiros et al. (389), evidence supporting this study came when it was discovered that the deletion of the gene encoding this protease in yeast produces accelerating ageing.

Deletion of other genes that encode Agf312, Parl and Clpp mitoproteases produced a shortening of lifespan due to mitochondrial dysfunction (391, 392). Moreover, the inactivation of mouse Immp21 was found to increase oxidative stress that promoted an onset of age-associated disorders (393). Other studies realized on yeast revealed that the deletion of Icp55, the orthologue of mammalian gene encoding XPNPEP3, is able to increase the lifespan by increasing the resistance to oxidative stress (389). These studies suggest that mitoproteases are very important in regulation of ageing and the study of them can bring new understandings on this complex process.

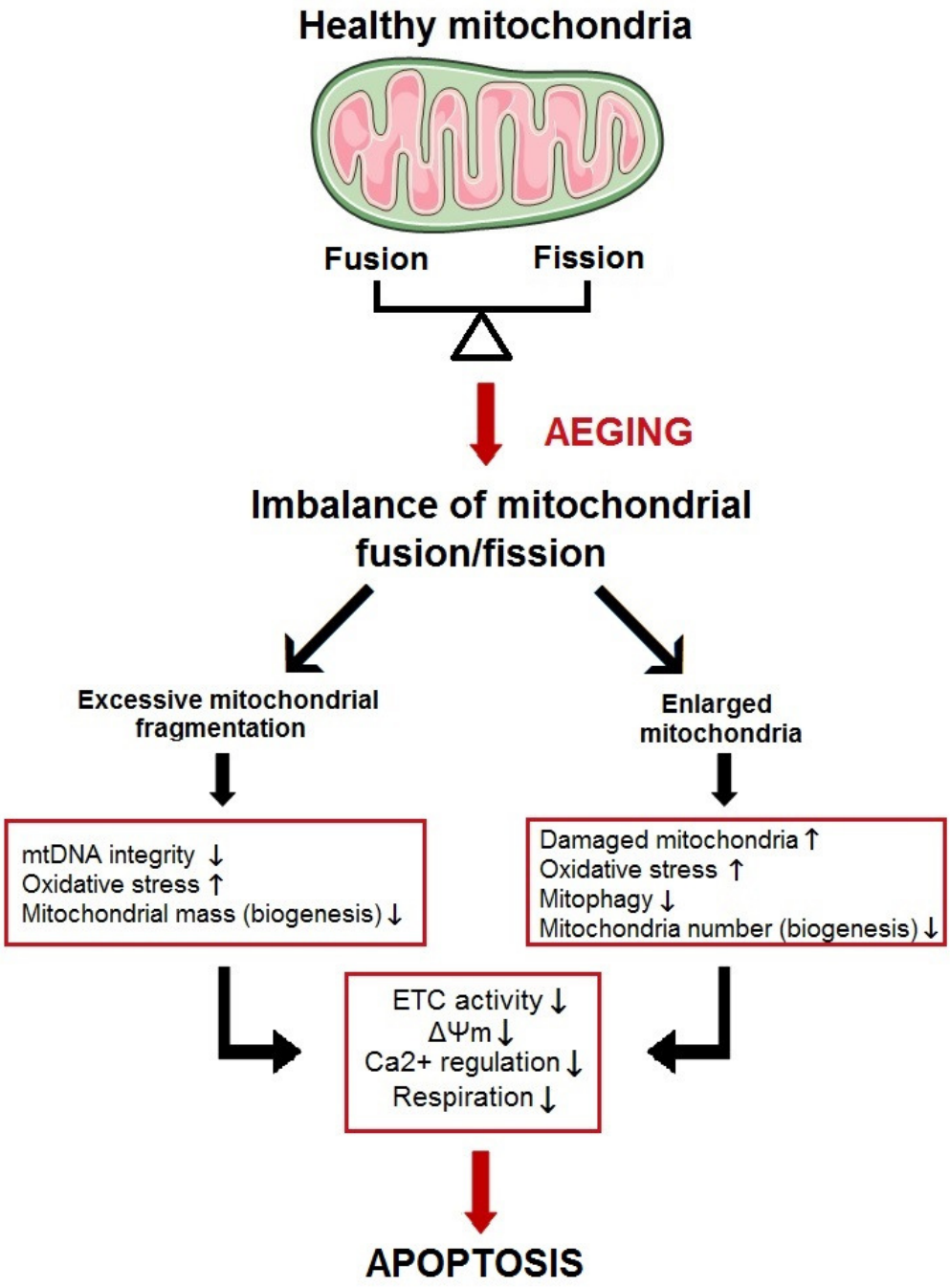

Figure 8. Schematic representation of mitochondrial dynamics changes in aging
(Illustration realized thanks to Servier Medical Art)

IV.6. Ageing and mitophagy

Even though there are many mitochondrial quality control mechanisms (proteasome-mediated degradation and endogenous proteases), the only mechanism that can produce the entire degradation of organelles is the autophagic mechanism. The term mitophagy refers to the selective autophagy of mitochondria. Mitophagy accomplishes a great number of distinct physiological needs, such as: adaptation to starvation (by mitochondrial degradation to gain nutrients), quality control (by removing the damaged macromolecules and toxins) and in the cellular renewal process. In this process, mitophagy is needed for the renewal of daughter cells in cell divisions of replicative aged mother cells, mechanism that also leads to deposition of damaged molecules in aged mother cells (394). Moreover, it can be viewed as a process by which cells regulate the size, number and quality of their mitochondrial network in order to adjust to energy demands. It was found that mitophagy can promote longevity when mitochondrial function is mildly attenuated or with dietary restriction. Besides this effect, mitophagy can offer protection against starvation, genotoxic and oxidative stress (395).

Mitophagy starts when there is an excessive mitochondrial number or in the presence of damaged mitochondria. The entire mitochondrion is then sequestered in vesicles with double-membranes, called autophagosomes, and delivered to lysosomes for further degradation. In both yeast and mammalian cells, mitochondrial fission occurs before mitophagy, in order to divide the elongated mitochondria into small pieces for encapsulation and for quality control segregation to realize a selective removal of damaged mitochondrial.

The molecular pathways involved in mitophagy have been best described in yeast. Although mammalian homologues of the principal genes and proteins involved in mitophagy have not been found, present studies are

trying to reveal the molecular mechanisms that are behind mitophagy regulation.

In yeast, mitophagy is regulated by a protein called Atg32 that labels the damaged mitochondria and recruits phagophores by interacting with Atg8. Researchers found that a critical decline in ΔΨm leads to the translocation of Atg32, observed in ageing (394). Intriguingly, Atg32 is required only for mitophagy that occurs as response to enforced respiration but not for non-selective (396). Another mitochondrial-targeted protein is Uth1, one of the four genes involved in "youth", which can prolong yeast life span during starvation (397). As stated above, the mammalian homologues have not been found.

In mammalian cells, mitophagy is regulated via more complex process, being identified several ways for damaged mitochondria recognition by phagophores. As reviewed by Saito et al. (398), these mechanisms involves ubiquitination of mitochondrial proteins followed by interaction with the adaptor proteins connecting ubiquitin with microtubule-associated protein 1 light chain 3 (LC3), ubiquitination of mitochondrial proteins by kinase PTEN-induced putative kinase protein 1 (PINK1)-Parkin pathway, phosphorylation of p62 at Ser 403 by CK2 and the use of NIP3-like protein X (Nix/Bnip3L), BCL2/adenovirus E1B 19kDa interacting protein 3 (Bnip3), FUN14 domain containing 1 (FUNDC1), and cardiolipin as receptors for LC3.

Mitophagy mediated via BNIP3L pathway is a programmed mitophagy found in red blood cells, while a selective mitophagy can be achieved in a PINK1-dependent or independent way. When ΔΨm decreases, PINK1 is anchored on the OMM, where it begins to phosphorylate and activate the ubiquitin-conjugating E3-type enzyme (399). Contrary, mitochondria that presents high ΔΨm are spared from mitophagy dependent on PINK1. Besides this, PINK-1 phosphorylates ubiquitin itself and Mfn-2, whereas Parkin directs

the ubiquitination of Mfn-1 and Mfn-2. The OMM proteins are then ubiquitylated. As PINK1 phosphorylates ubiquitin, the recruitment of adaptor proteins, such as: optineurin, nuclear dot protein 52 (NDP52) and p62 leads to the recruitment of UNC51‑like kinase 1 complex (ULK1), and finally to the formation of autophagosome (a double lipid membrane-bound vesicle) though LC3. The mitochondrion will then be swallowed by the autophagosome that will further fuse with one lysosome to disintegrate it.

An interesting crosstalk between mitophagy and apoptosis involves Sirtuin 1 (SIRT1), responsible for the activation of both. SIRT1 is a nuclear sirtuin that regulates various pathways and is involved in the delay of ageing progression through deacetylation of various substrates, including here the PGC-1α. Moreover, SIRT1 controls mitophagy by regulating the ΔΨm and therefore PINK1 integrity. The inactivation of SIRT1 leads to the acetylation of p53 and subsequent cellular death, as well as to an increase in mitochondrial ROS, the main factor incriminated in ageing (400, 401). All the presented data suggests the ability of SIRT1 to counteract ageing.

A defective mitophagy was found to be implicated in neurodegenerative disorders like Alzheimer and Parkinson disease and also in pathological ageing (402). Studies have shown that the decline in mitophagy observed with ageing is attributed to the increase of oxidative stress and apoptosis and also to the accumulation of damaged mitochondria (403). Current studies have shown that mtDNA mutations accumulates with age, process that leads to abnormal or even blockade of protein synthesis and mitochondrial dysfunction, thus mitophagy is very important for mutant mtDNA and dysfunctional mitochondria elimination. The accumulation of mtDNA with age can be a result of decreased recognition of signal for mitophagy by certain mutations (404).

An excessive accumulation of mitochondria was found to be a common feature of different cell types, in a number of pathological conditions, during ageing. Moreover, to sustain this theory, it was found that inhibition of mitophagy will increase mitochondrial mass that can in turn recapitulate the effects of ageing in young individuals (395).

In skin fibroblasts, mitophagy declines as the age of the donor increases. The same group showed that if the cells are forced to rely on mitochondria to produce energy, mitophagy in stimulated in order to remove the mutant mtDNA, thus suggesting this explain the beneficial effects of exercise and caloric restriction in slowing the ageing process

Evidence that supports the idea that genetically increasing autophagy delays ageing was found in flies, mice and worms. The importance of mitophagy in ageing and lifespan was validated by numerous studies. In Drosophila, the overexpression of Parkin was associated with a decreased protein aggregation and with an increased lifespan, whereas in the brain it produced the same effect on protein aggregation, in parallel with an increase in neuron longevity. Administration of lithium on worms, produced improved mitochondrial function and lifespan via stimulation of macroautophagy. To present day, more and more studies reveal that a dysfunction in mitophagy can contribute to the deterioration of mitochondrial function and quality that produce an ageing phenotype.

All these observations highlight the importance of mitophagy in mitochondria quality, function and integrity, as well as in the ageing process.

IV.7. Ageing and mitochondrial clonal (DNA) mutations

More than three decades ago, the first theory about the major role played by mitochondria in ageing was proposed (405). This theory implied that ageing occurs due to an increased mitochondrial ROS production, responsible for membrane, protein damage and DNA damage (406). Since many studies have oriented to the investigation of DNA damage in ageing, over years, another theory has emerged. This theory, mitochondrial theory of ageing, enunciate that accumulated somatic mutations of DNA produce alterations that are randomly transmitted during cellular and mitochondrial division, alterations that will promote a respiratory chain deficiency that leads further to an increased ROS production, further responsible for more mtDNA damage.

Mammalian cells have a high number of mitochondrial genome copies to ensure that one mutation of one copy will not produce an overall alteration in mitochondrial function. However, it does not stop the growth of the novo mutations developed in single mtDNA molecule (168). If mtDNA molecules are distributed randomly it can induce during cell division to increased amounts of mutated mtDNA molecules in one daughter cell. More precisely, even if a cell is carrying small amounts of mutated mtDNA, it can still rise a daughter cell with higher mutation levels.

A new unifying theory, the redox mechanism of ageing has emerged, suggesting the existence of a link between oxidative stress, ageing and apoptosis.

The mitochondrial genome is a double-stranded, circular molecule, composed of 16,569 bp that encodes 37 genes. It is maternally inherited and present in one to thousands copies in a cell, number that varies based on the bioenergetic needs of the cell. All genes found in mtDNA encodes the

complexes of ETC, except for complex II that is encoded by nuclear DNA. MtDNA replication occurs in a cell cycle-independent way, with the participation of a small number of enzymes known so far, such as mitochondrial RNA polymerase, transcription factors B2 and A (TFB2M, TFAM), Twinkle, mitochondrial single-stranded DNA-binding protein (mtSSB) and POLG-α.

Mutation rate of mtDNA is 10 up to 15-fold higher than the rate of nuclear DNA mutation. The increase of mtDNA mutations, over a critical threshold, produces adverse effects in mitochondria and leads to improper function or damage of respiratory chain complexes, thus these mutations alter oxidative phosphorylation producing mitochondrial dysfunction and increased ROS production. Therefore, the repair mechanisms of damaged mtDNA holds an important role in protecting the mitochondrial function. Among these some well characterized repair mechanisms include: the 3–5 proofreading activity of POLG and base excision repair (BER). Even though there are known many mtDNA repair proteins, with the exception of POLG, LIG3, APE1 and NEIL1, they somehow prove to be less efficient on mtDNA as compared to nuclear DNA (407, 408). However, considering the large number of mtDNA molecules, the degradation of these is understandable, as compared to nuclear DNA where disposal is not an option.

Even though there are up to several thousand copies of mtDNA per cell, studies have demonstrated that a decrease in mtDNA copy numbers is associated with ageing. Consecutively, studies realised suggested an age-associated decrease of mitochondrial biogenesis due to a reduced expression of the nuclear regulatory factors and repair proteins that are involved in the maintenance of mtDNA. More precisely, in a rodent model was found a decreased AMPK activation and a low response to stimuli, whereas in young animals these stimuli produced and increased of NFR-1, TFAM and PGC-1α

(409). Additionally, activation of PGC-1α in POLG-α mutator mouse produced an increase in heart and skeletal mutations and an increase in mitochondrial biogenesis (410). Another mechanism involved in mtDNA maintenance, BER, presented an age-dependent decrease in activity, which was also tissue-dependent (411).

The general idea of all studies regarding mtDNA is that expression of it decreases, whereas mutations accumulate with age. The relevancy of mtDNA mutations in ageing is however not a current one. The first study published in 1988 by the group of Piko et al. revealed a high prevalence of deletions in both senescent rats and mice (173). The deletion of 5 kilobase between nucleotides 8470 and 13447, associated with mitochondrial diseases, was found in the hearts, skeletal muscle and brains of ageing humans, especially at the level of substantia nigra and the caudate, regions that are known to be sensitive to respiratory chain dysfunction (411-413). Furthermore, a high number of studies revealed other deletions that involved different parts of mtDNA also increased with age (414, 415). The group of Michikawa et al. (416), found an increasing base substitution in the displacement loop (D-loop, a small noncoding of mtDNA necessary for transcription and replication) in the fibroblasts of aged individuals as compared to younger subjects. With other study that found the same increase in the striatum of aged individuals, the strengthened idea is that D-loop is the region more prone to single-nucleotide variants mutations (417).

Despite the remarkable progress made in the research of the ageing process, the exact mechanism is still controversial and under debate, thus different theories of ageing have emerged over the past decades.

The free radical theory of ageing, first published in 1954 (418), revealed the underlying mechanisms involved in ageing. This theory stated that ageing is a consequence of the reaction of active free radicals with the

cellular components. Indeed, in ageing tissues it was detected an increased amount of ROS produced by mitochondria and also an increased 7,8-dihydro-8-oxodeoxyguanosine(8-oxo-dG) content in mtDNA, thus revealing the role of mtDNA damage as a contributory factor (419). Consecutive studies realized on various animal models, such as fly, mice and worms demonstrated that a decrease in ROS production or an increase in antioxidant defense can increase lifespan (420). A great body of research further brought evidences that strengthened the central role of mitochondrial ROS production in the ageing process. However, some studies challenge the free radical theory of ageing. Thereby, one study revealed that mice models with decreased levels of MgSOD and increased oxidative damage presented a normal lifespan (421). When SOD1 or catalase (antioxidant enzymes) were overexpressed, it was found no increase in lifespan (422). Moreover, various studies reported no effect on lifespan when animal models were treated with antioxidants neither a correlation between ageing and level of oxidative damage (287). In Drosophila, mutations that produced a loss-of-function in SOD or in the enzyme Ogg1 (a DNA repair enzyme) did not influenced the somatic mtDNA mutation frequency (423).

ROS are by-products of aerobic respiration and various metabolic processes. Within a cell exists different sites were ROS are produced, but the main producer is mitochondrial electron transport chain (30), as a result of inefficient electron transport at ETC level. Moreover, ROS can be produced as a response to numerous environmental stimuli: pro-inflammatory cytokines, growth factor, radiation, UV light, chemotherapeutics, toxins and various oxidant. Other sources of ROS production include: NAPDH oxidase, monoamine oxidase, xanthine oxidase and nitric oxide synthase.

The cellular level of ROS is maintained in normal range by a series of endogenous antioxidant systems, such as SOD, glutathione peroxidase and

reductase, catalase, thioredoxin and glutaredoxin. Any imbalance between the antioxidant defense and ROS production, with the consecutive increase in ROS concentration, will lead to oxidative stress.

ROS produce a wide range of effects. Among the deleterious effects, ROS were shown to react and cause oxidative damage to proteins, lipids and even nucleic acids. By reacting with nucleic acids, ROS produces various DNA lesions including oxidation of DNA bases, DNA strands breakage and apurinic sites development, lesions that further produce genomic instability. The most important DNA lesion produced by ROS is oxidation of a guanine derivative, 8-oxo-dG, thus creating a highly mutagenic lesion that produces G: C to T: A transversion (424). However, G -T mutations, were found not to significantly increased with age (425).

Senescence, is a natural process that defines the irreversible growth of normal somatic cells, after a well-defined number of cellular divisions. It is believed that senescence contributes to organismal ageing (426). Senescent cells present a high concentration of ROS and an extended oxidative damage, contrary to immortal cells that are more resistant cu oxidative damage. The major cause of cellular senescence is considered to be telomere shortening, which is directly associated with oxidative stress (427). The direct effects of ROS on telomere shortening were demonstrated in studies realized when cells were exposed to mild oxidative stress or when certain antioxidants enzymes were overexpressed. The results showed that under mild oxidative stress telomere shortening is increased, whereas SOD overexpression was able to decrease telomere shortening rate (428). By producing 8-oxo-dG, ROS interferes directly in telomere maintenance. 8-oxo-dG diminishes the formation of intramolecular G quadruplexes and decreases the affinity of telomeric DNA for telomerase (287). Indirectly, ROS interferes with telomeres maintenance by interacting with telomerase reverse transcriptase (TERT), the

catalytic subunit of telomerase. The decrease of TRET activity produced by ROS leads to an accelerated onset of cellular senescence (287). Moreover, by interacting with DNA, ROS indirectly produces the activation of p53, a major player in cellular senescence. p53 is further responsible for the transactivation of E3 ubiquitin ligase Siah1 that in turn mediates ubiquitination and degradation of TRF2 (a component that protects the integrity of telomeres) (287).

Finally, another study brought evidence to sustaining that the free radical theory of ageing is not a valid one. Experiments realized on mouse models with mutated POLG-α (mitochondrial DNA α polymerase) did not find an increased level of protein oxidative damage, neither an age-dependent increase of mtDNA oxidative damage (411). Moreover, the mouse in which POLG-α-deficient protein is expressed ubiquitously, developed a premature ageing phenotype, due to a significant increase of mtDNA deletions and point mutations level, but without any signs of increased oxidative damage to mitochondrial (411).

α

The new theory of mtDNA mutation involvement in ageing that emerged, states that in the cells were the mutation arises, occurs a clonal expansion that will lead to a threshold of mtDNA mutations with age, which if exceeded, pathological changes start developing, thus affecting the whole tissue in time. Studies reported different thresholds for inducement of respiratory chain dysfunction, depending on the type of heteroplasmic mtDNA mutation and also on the localization of mitochondria (168).

Intermyofibrillar mitochondria showed an age-dependent decrease of oxidative phosphorylation, whereas subsarcolemal mitochondria did not (411). Both inherited and de novo mutations can increase with age through clonal expansion. In order to explain the clonal expansion and mutated mtDNA

accumulation, two processes have been incriminated, such as an advantage in replication of the mutant mtDNA vs the wild one and also a defective autopagocytosis (411).

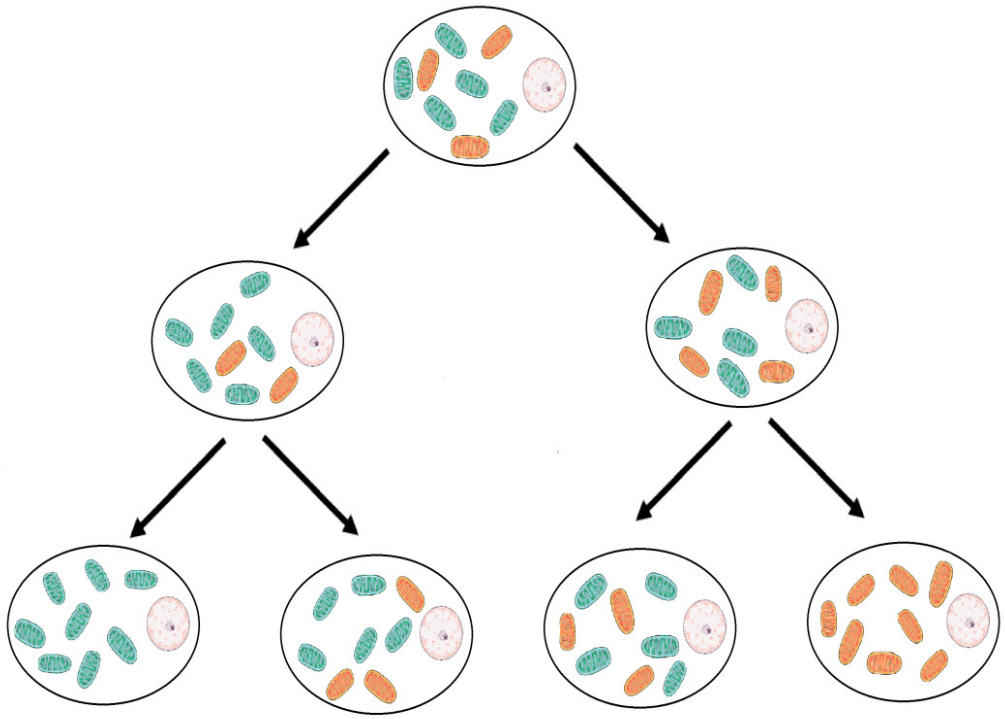

Figure 9. Possible changes of mtDNA heteroplasmy throughout the lifetime of an individual (Modified after Stewart et al., Nature Reviews Genetic, 2015; Illustration realized thanks to Servier Medical Art)

More important, the clonal expansion of mutated mtDNA will impair the respiratory chain until the whole cell is dysfunctional, whereas mtDNA mutations reported to the overall tissue remains just slightly affected. Even though some studies support the idea that the level of overall tissue level of mtDNA is too low to produce tissue dysfunction, other studies suggest an inter-/intracellular mosaic-like pattern in the distribution of mtDNA mutations that could cause age-related tissue dysfunction if critical cells are involved, even if the overall level of mutations is relatively low (411).

Extensive experimental research has demonstrated that human ageing process is liked with the occurrence of mtDNA mutations and clonal expansion of these, processes that leads to respiratory chain dysfunction (LARSSON). This mosaic-like respiratory chain deficiency has been found in numerous types of ageing tissues, such as heart, colon, skeletal muscle, dopaminergic and hippocampal neurons and choroid plexus (429).

MtDNA encodes 13 protein subunits and 24 RNA components (used for mitochondrial protein synthesis), thus the biogenesis of respiratory complexes is highly dependent on mtDNA integrity (TRIUMF 2008). Hence, any alteration in mtDNA will result in a deterioration of OXPHOS activity and ATP production. The decrease of respiratory chain function is responsible for a consecutive increased ROS production that in turn will decrease mtDNA integrity by enhancing damage and mutagenesis at this level. Thereby, a" vicious-cycle" is established until the cells suffer an extensive dysfunction, which culminates in cell death.

Several studies demonstrated the accumulation with age of mtDNA mutations and deletions in a large number of tissues including cardiac and skeletal muscle, brain, liver and colon (430, 431). As reviewed by Baines et al. (432), current studies found that respiratory chain defects in single COX-deficient cells are accumulating in various ageing tissues like the colon, small intestine, liver, stomach and pancreas, and these defects were directly caused by clonally expanded somatic point mutations of mtDNA. Moreover, it is known that in mitotic tissues, stem cells are the only long-lived cells hence, these mtDNA mutations must be occurring at this level.

In individual cells, the mtDNA defects expand clonally and when they exceed the threshold level, they trigger a biochemical defect. This effect was histochemical observed as cytochrome c oxidase (274) deficiency. The study of Muller-Hocker found that in myocytes and cardiomyocytes of old

individuals, there is a high number of single and randomly distributed cells that don't present COX activity (433).

Experiments realized on filamentous fungus (used frequently for the study of mitochondrial metabolism implication in longevity) revealed that inactivation of subunit 5 of COX (COX5) results in an increased longevity (5 years compared with normal 16 days) together with mitochondrial genome stabilization (434). A more interesting fact is that inhibition of COX5 resulted in the use of an alternative respiratory pathway and lead to an important decrease of ROS production.

It was discovered, in COX deficient muscle fibers of aged individuals, increased levels of clonally expanded point mutations, such as tRNALeu (274) A3243G, tRNALys A8344G mutations, tRNAMet T4460C and G4421A, mutations responsible for s significant impairment of mitochondrial function of an individual cells, even though the overall level of mtDNA mutations in muscle was low (435).

One of the irrefutable pieces of evidence that mtDNA mutations clonally expanded are the cause of age-related dysfunction, was offered by recent studies in human brain. An age-increasing COX-deficiency has been reported in hippocampal neurons and in choroid plexus epithelial cells (436).

In intestinal crypts of aged humans, Taylor et al. found a high incidence of COX-negative cells (437). Another study stated that the crypts that carrying mtDNA mutations are clonally expanded by fission (438).

Regarding the human ageing skin, many studies portray both somatic deletions and point mutations of mtDNA, however, it is not elucidated yet if these are or not clonally expanded. In this regard, a specific point mutation of T414G located in fibroblasts, specifically in the control region of mtDNA, was found to be significantly correlated with ageing (416). Moreover, this mutation

was found to be present in both muscle and skin of aged persons, especially if sun exposed, but absent in the brain (429).

A better understanding of COX deficiency and clonally expanded mutations of mtDNA mechanisms and consequences at cellular level in aged tissues, will lead definitely to a better knowledge of the role played by mitochondria in ageing.

The redox theory of ageing links both free radical theory and mitochondrial theory of ageing. Particularly, it suggests that ageing produced by the decline of the adaptive interface, plasticity of the functional genome and exposome (environment) that occurs as a consequence to cell and tissue differentiation and exposure memory systems (439). Contrary to its name, the theory is not limited only to redox processes, but holes a redox-dependent character, mainly due to the central role of electron transport in energy production. Moreover, in this theory are included and presented, as linked and interconnected processes, all the hallmarks of ageing, such as failure of oxidative or xenobiotic defenses, oxidative stress, compromised mitochondrial integrity, failure of proteostasis, DNA mutations, immune system dysfunction, barrier failure, cellular senescence, telomere shortening and regenerative dysfunction (9).

In his review, Jones (439) suggestively described that the differentiation and adaptive structures permits an individual genome to be influenced and shaped by the exposures throughout life, thus improving opportunity for success in reproduction. In order to gain survival advantage, the protective mechanisms, such defenses against infection, food obtaining and discrimination between good and bad food and danger response have evolved. Thus, the theory has emerged stating that this exposure memory system is using mechanisms in parallel with developmental programs, at a cost that is considered to play a central role in ageing. So, execution of

programs in response to exposure and of organogenesis programs will produce a decrease in the flexibility of genome to respond to other programs or to other exposures. The redox network's role is to maximize the flexibility and adaptability to the environment by controlling organismic defense, cellular energetics, molecular order and reproduction. All these networks are interconnected in function, a failure in one produces a compensation in the other that will ultimately fail. For example, the inter-conversion of metabolism that is redox-dependent and defense mechanisms means that a deficiency in metabolism will lead to a failure of defense against infection mechanisms.

From the ageing point of view, this theory suggests that all systems age and death is the collapse of the entire networks that are supporting the genome-exposome interaction.

IV.8. Unfolded protein response

An imbalance in proteostasis (balance between the number of unfolded proteins and total folding capacity) has been recorded in the ageing process, imbalance that occurs due to an increased protein oxidative damage associated with an impairment of the ubiquitin-proteasome system (440). As a result, misfolded proteins accumulates and mitochondria reacts by triggering a specific mitochondrial unfolded protein response (UPR^{mt}) that aims to restore the proteostasis equilibrium in the cells.

In order to protect the cells from damage and to maintain mitochondrial homeostasis, eukaryotic cells have evolved various quality control mechanisms. The mitochondrial homeostasis has to be strictly and continuously maintained by a balance between organelle biogenesis and quality control mechanisms. Currently, there are known more than 1000 proteins/peptides in mitochondrion, involved in different processes like

synthesis of amino acids, nucleotides, redox status regulation, calcium homeostasis, cell survival and death signaling. Mitochondrial proteins that are encoded in mtDNA are translated on mitochondrial ribosomes and then transported in the IMM. Parallel, mitochondrial proteins nuclear encoded are translated on cytosolic ribosomes and subsequently targeted by a mitochondrial amino-terminal targeting sequence (MTS) or by internal sequences. In order to cross the IMM through a translocase of inner membrane (441) complex, the MTS are cleaved at this level, whereas proteins folds into their functional conformation (442). Passage of proteins over IMM requires a functional respiratory chain (to produce a membrane potential) and mitochondrial matrix chaperones. It was demonstrated that in various pathological states the mitochondrial protein import is altered, process that has recently emerged as a major step in metabolism and stress response dysregulation (443).

Mitochondria contains chaperones and proteases responsible for quality control that participate in protein import, trafficking, folding and activation, thus they are central players in mitochondrial proteostasis. Any disruption in adequate protein import or in the protein folding process will lead to un- /mis-folded proteins, which in turn will accumulate and will consecutively lead to an altered proteostasis, mitochondrial damage and dysfunction and cell damage. Proteostasis is maintained and regulated by the UPR^{mt}, response that was studied intensely in *Caenorhabditis elegans.* These pioneering studies link UPR^{mt} with ageing, longevity and healthy lifespan (444-446).

Besides mitochondrial chaperones and several antioxidant systems (SOD2, GPX1, PRDX3 and 5) that maintain cell homeostasis and function, there are also involved quality control proteases that stabilize mitochondria, by promoting protein folding and also degradation of mis-/ or un-folded proteins

(447). Any process that causes mitochondrial dysfunction (morphological changes, mitochondrial dynamics alteration, mtDNA mutations, oxidative stress and aggregation/accumulation of mis-/ or un-folded proteins) leads to a "retrograde signaling", a mitochondria-nucleus communication that carries the information necessary for the cell to adapt to the mitochondrial defect or to resolve the mitochondrial stress. Since these changes of nuclear gene expression are not limited to mitochondrial proteins encoded in the nucleus, retrograde signaling changing also the global nuclear pattern of gene expression, these signaling responses gained increasing attention of scientific researchers in the last two decades (448) and particularly to UPR^{mt}. The UPR^{mt} represents a signaling cascade activated in stress conditions in order to maintain mitochondrial function and content homeostasis. Even if the regulatory mechanism of UPR^{mt} response in mammals is still extensively unknown, the involvement of some mitoproteases has been recently discovered. The signaling cascade of UPR^{mt} response consists of ClpP protease that detects the misfolding of proteins in mitochondria and generates a signal that activates different downstream genes like ubiquitin-like protein (UBL-5), that in turn activates the transcription factor DVE-1. Together, UBL-5 and DVE-1 produce the increase of mitochondrial chaperones (hsp-6 and hsp-60) expression that results in nuclear-encoded genes up-regulation (449, 450) that encodes mitochondrial stress proteins like Cpn60 and Cpn10. Another sensor of mitochondrial stress is ATFS-1 that accumulates in cytosol as a response to stress where it triggers UPR^{mt} response (451).

As presented herein, mitochondrial dysfunction is involved in the normal ageing process, however there is evidence that demonstrates that a mild mitochondrial perturbation can increase lifespan by almost 50% in worms (452) but also in mice, flies and yeast (453). Studies also suggest that an increase in longevity linked with mitochondrial dysfunction involves UPR^{mt}

activation (453) that produces pleiotropic physiological changes in the experimental animal model (445). UPR^{mt} activation was demonstrated to reduce the size and perturb the muscle structure, while the age-related decline of both muscle function and structure is diminished in worms and flies in which UPR^{mt} was chronically activated (454).

As a more compelling proof, major age-related neurodegenerative diseases, like Alzheimer's and Parkinson's disease, have been found to present an increased accumulation of misfolded proteins in mitochondria and also in endoplasmic reticulum (440).

Thus, future studies focused on the research of UPR^{mt} signaling can shed light on the molecular mechanisms involved in ageing and lifespan, which could be further exploited for therapeutic purposes.

V. FUTURE PERSPECTIVES: RESUSCITATING AGEING MITOCHONDRIA

More and more studies place mitochondrial dysfunction in the center of an increasing number of diseases. A better understanding of the mechanisms involved, and also a better knowledge of how to modulate mitochondrial behavior can lead to the development of new therapeutic approaches aiming mitochondria in this numerous disease states.

It is now established that mitochondrial dysfunction represents a crucial hallmark of ageing. However, the questions that still arise are many and the answers are partially or not even discovered yet. The progress in understanding the exact mechanism of the ageing process has been limited by different issues such as the absence of appropriate biomarkers that can properly quantifying the extent/'degree' of ageing at both molecular and cellular level, the diversity of phenotypic presentations and different time of onset of this process among population. More important, it is crucial to determine whether mitochondrial dysfunction is a direct cause or a consequence of ageing and ageing-associated diseases. Evidence from recent studies supports both theories, it was proven that deficits in mitochondrial bioenergetics have been demonstrated to compromise the cellular adaptation capacity to different physiological stresses and also that mitochondrial dysfunction caused by specific diseases can lead further to the depletion of compensatory biogenesis reserve, and enter a vicious cycle that ultimately will led to loss of function and tissue deterioration.

Alterations in mitochondrial function are accompanied by changes in mitochondrial architecture, and now it is known that many age-related

pathologies present an altered mitochondrial fusion and fission and hence mitochondrial morphology. Even if many progresses have been made in understanding the relationship between mitochondrial dynamics and mitochondrial function, it is still difficult to visualize directly the fusion and fission processes in *in vivo* experimental models of aged mammals due to the dynamic nature of mitochondria. In order to achieve a better understanding, new techniques and methods were applied, but still many questions remained unanswered. Currently it is not known what are the upstream molecule involved in the signaling pathway for mitochondrial fusion and fission proteins, how are the factors involved integrated in the ageing process and how mitochondrial dynamics interrelate to maintain and guarantee quality control. In the near future, when specific genetic tools that can modulate the levels of these proteins will be available, then it will be possible a full understanding of the complex role of mitochondrial morphology proteins and their use in preventing the ageing process and age-related diseases. To prevent cellular damage, the dysfunctional mitochondrial have to be rapidly repaired or degraded. Alterations in the quality control mechanisms, genome maintenance and mitochondrial dynamics and proteostasis lead to mitochondrial dysfunction that underlines the ageing process. The importance of mitoproteases and mitochondrial proteostasis has been highlighted by recent research that have discovered that modulation of these processes can trigger a mitochondrial stress response that can in turn influence longevity and the ageing process. One newly discovered stress response is UPRmt that seems to increase lifespan by three possible mechanisms that include: changes the mitochondrial metabolism, activation of Nrf2/SKN-1 transcription factor (involved in a longevity pathway) and improvements in the folding environment of mitochondrial proteins by increasing the ROS detoxification capacity, mitochondrial fission, mitophagy and concentration of chaperones

(455). Recent studies suggests that a low stress can induce a pro-longevity signal that is regulated by mitoproteases and directly impacts mitochondrial function. The elucidation of the function, regulation and interactions of these mitoproteases can further lead to the discovery of their roles in different pathological conditions associated with ageing and can open the pathway to the discovery of clinical treatments for these pathologies and also to improve human health span and lifespan.

A healthy ageing depends on the ”mitochondrial fuel” and hence by the maintenance and integrity of respiratory chain complexes that are involved in both ATP production and ROS generation. Particularly, more and more recent studies are oriented on the complex role positive and negative that ROS generation and modulation plays in both the physiological and respectively, pathological ageing process (456). Many experiments were focused on manipulating ROS-scavenging mechanisms in order to elucidate the role it plays, yet the inability of antioxidants to mitigate ageing in mice led to the progressive deterioration of the free radical theory of ageing.

Impairment of mitochondrial function due to alterations in mtDNA was also linked with ageing and numerous age-related pathologies. It was demonstrated that ageing humans present an increased level of somatic mtDNA mutations that go through a clonal expansion that further causes a mosaic respiratory chain deficiency that leads to increased apoptosis, in different tissues, like the heart, skeletal muscle, brain and gut (429). There is experimental evidence that incriminates ROS production in these alterations, however, the certain role has not been established yet; some lines of evidence suggesting an inherent polymerase γ error may be responsible for mtDNA mutations (429).

Therefore, additional experimental evidence from conditional knockout mice's and tissue-specific mutator alleles is needed in order to reveal the exact cause and the degree by which the focal respiratory chain dysfunction can cause overall tissue-organ dysfunction and age-related pathologies.

Taken together, all these observations may lead to the conclusion that mitochondrial (dys)function is intimately connected to numerous processes associated with ageing and senescence. Further studies my reveal that mitochondrial biology is the missing link in the complete understanding of physiological ageing, longevity and also age-related diseases. However, it is still to be clarified what are the exact perturbations of mitochondrial function that have the biggest and earliest impact on the ageing process.

Moreover, there is an unmet need for mitoprotective strategies and a recent position paper has provided consensus opinion from landmark basic scientists and clinicians regarding the mitochondrial impact of risk factors, comorbidities and comedications that have been responsible for the failure of clinical translation so far (457).

We expect in years to come, an increase in the number of studies focused on the elucidation of the mechanisms that link mitochondria with ageing, with fruitful results that will bring us one step closer to finding the elusive "Fountain of Youth".

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