Universidade de Aveiro Departamento de Biologia
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MARIA CAROLINA COSTA E SANTOS BAPTISTA RODRIGUES

MODULATION OF MITOCHONDRIAL STRESS RESPONSE BY SESTRIN 2

MODULAÇÃO DA RESPOSTA MITOCONDRIAL AO STRESS PELA SESTRINA 2

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Professora Doutora Anabela Rolo do Departamento de Ciências da Vida da Universidade de Coimbra, do Professor Doutor João Serôdio do Departamento de Biologia da Universidade de Aveiro e co-orientação do Doutor Filipe Valente Duarte do Centro de Neurociências e Biologia Celular/Departamento de Ciências da Vida da Universidade de Coimbra.

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palavras-chave C2C12, stress, Sestrina 2, mitocôndria, disfunção mitocondrial, homeostase mitocondrial, autofagia, mitofagia, SIRT1, envelhecimento, diabetes, obesidade

Resumo

As mitocôndrias são organelos altamente dinâmicos com um papel crucial na homeostase celular. Uma rede de mitocôndrias funcionais é mantida por processos de biogénese e mitofagia, regulando desta forma o conteúdo e o metabolismo mitocondriais. Espécies reactivas de oxigénio (ROS) são formadas como consequência do processo normal de fosforilação oxidativa mitocondrial, desempenhando um papel importante na sinalização redox e regulação da função celular. Um aumento ligeiro na formação de ROS mitocondrial desencadeia o fenómeno de hormese mitocondrial, uma resposta adaptativa ao estado metabólico celular, ao stress e outros sinais intracelulares ou ambientais. Este mecanismo induz maior resistência a um stress posterior, tendo por isso efeitos benéficos para a saúde. Compostos que são tóxicos em doses maiores são conhecidos por induzir adaptações mitocondriais em doses mais baixas.

O excesso de equivalentes redutores fornecidos à cadeia transportadora de electrões (ETC) em condições de sobrenutrição/inactividade física ou danos acumulados/defesas antioxidantes mais baixas associadas com o envelhecimento, causam um aumento nas taxas de produção de ROS, provocando stresse oxidativo e danos irreversíveis em proteínas, lípidios e DNA. A disfunção mitocondrial resultante é permanente e compromete o estado energético de todo o organismo, aumentando a susceptibilidade a lesões, provocando a aceleração do envelhecimento e desenvolvimento de doenças metabólicas, tais como a resistência à insulina e fígado gordo. Um dos principais reguladores do sistema de defesa antioxidante celular é a Sestrina 2 (SESN2), induzida em condições de stress. A diminuição da actividade da SESN2 está associada a um aumento de danos oxidativos, disfunção mitocondrial, degeneração muscular e acumulação de gordura, resultando num envelhecimento mais rápido dos tecido. No entanto, os mecanismos pelos quais a SESN2 afecta as funções mitocondriais não estão definidos. A compreensão dos mecanismos moleculares e de como a SESN2 afecta a mitocôndria, pode fornecer novas pistas para alvos terapêuticos, a fim de atenuar e prevenir o envelhecimento e as patologias relacionadas com a obesidade.

Tendo em conta isto, este trabalho teve como objectivo avaliar se a SESN2 medeia uma resposta mitocondrial adaptativa protectora, desencadeada pela exposição de células C2C12 a menadiona, um estimulador da formação de aniões superóxido. Adicionalmente, e tendo em conta que a Sirtuina 1 (SIRT1) é um conhecido sensor metabólico e regulador da

função mitocondrial, este estudo avaliou de que forma a modulação de SIRT1 afecta a SESN2 no contexto de fígado gordo induzido por uma dieta rica em gordura.

Os resultados obtidos mostram um efeito da menadiona, dependente da dose, na viabilidade celular e a função mitocondrial. O tratamento com 10 µM de menadiona durante 1 h não alterou a formação de ROS, redução do MTT e o potencial de membrana mitocondrial, como avaliado 24 e 48 horas após a remoção de menadiona. No entanto, a exposição de células C2C12 a 30 µM menadiona durante 1 h, resultou no aumento da formação de ROS, diminuiu a redução do MTT e o potencial de membrana mitocondrial. Um aumento no conteúdo de SESN2 foi observado após 10 h de exposição a 10 µM de menadiona durante 1 h, enquanto 30 µM de menadiona resultou na diminuição do conteúdo em SESN2. Estes resultados sugerem que a indução de SESN2 por stress moderado, induzido pela menadiona, pode activar uma resposta mitocondrial protetora que preserva a viabilidade celular.

O silenciamento da SESN2 com siRNA resultou num aumento da morte celular, bem como numa diminuição no potencial de membrana mitocondrial induzida por ambas as concentrações de menadiona, sendo mais drásticas as alterações induzidas por 30 µM. Na presença de SESN2, a exposição a menadiona causou um aumento no padrão pontuado de distribuição de LC3, indicando a indução de autofagia. Contrariamente, a depleção de SESN2 com siRNA resultou numa diminuição da pontuação de LC3, quer em condições controlo quer após a exposição a menadiona. Colectivamente estes resultados sugerem que o stress moderado provocado pela menadiona induz a SESN2 e activa autofagia/mitofagia como uma estratégia de sobrevivência celular. A ausência de SESN2 resultou na acumulação de dano mitocondrial induzido por ROS e consequente diminuição da viabilidade celular. Em relação ao impacto da modulação da SIRT1 na SESN2, os resultados obtidos mostram que a expressão hepática do factor de transcrição c/EBPα (proteína alfa potenciadora de ligação CCAAT) foi estimulada pela dieta rica em gordura (HFD) e reduzida pelo tratamento com resveratrol, um activador da SIRT1. Em ratinhos sem SIRT1 (SIRT1 - KO) a expressão c/EBPα estava diminuída comparativamente ao controlo. A expressão hepática de SESN2 apresentou-se reduzida em animais HFD e SIRT1 - KO. O tratamento com resveratrol, em animais controlo, preveniu a diminuição da SESN2 induzida por HFD. A expressão de KEAP1 (proteína kelch 1 associada a ECH) também se verificou dependente de SIRT1, sendo que, em ratinhos SIRT1-KO, o tratamento com resveratrol não induziu nenhuma alteração em KEAP1. A degradação de KEAP1 é promovida pela SESN2, permitindo a translocação de Nrf2 (factor nuclear derivado de eritróide 2) para o núcleo e, consequentemente, a indução de genes antioxidantes. A expressão hepática de Nrf2 não foi afetada pela modulação de SIRT1. O envelhecimento diminuiu a expressão de todos os genes estudados.

Em conclusão, este trabalho demonstrou que a indução de SESN2 por stress ou compostos promotores de homeostase mitocondrial, como o resveratrol, aumenta a tolerância mitocondrial ao dano, através da modulação da autofagia/mitofagia. A estimulação da eliminação de mitocôndrias lesadas pela SESN2, pode ser uma via para evitar a acumulação de danos e, portanto, resultar num aumento da tolerância à sobrenutrição e ao envelhecimento.

Keywords C2C12, stress, Sestrin 2, mitochondria, mitochondrial dysfunction, mitochondrial homeostasis, autophagy, mitophagy, SIRT1, aging, diabetes, obesity

Abstract

Mitochondria are highly dynamic organelles with a crucial role in cellular homeostasis, with processes of biogenesis and mitophagy regulating mitochondrial content and metabolism and maintaining functional mitochondrial networks. Reactive oxygen species (ROS) are formed as a consequence of normal mitochondrial oxidative phosphorylation and are involved in redox signalling and regulation of cellular function. A mild increase in mitochondrial ROS triggers mitochondrial hormesis, an adaptive retrograde response to cellular metabolic state, stress and other intracellular or environmental signals that culminate in subsequently increased stress resistance with health promoting effects. Compounds that are toxic at higher doses are known to induce mitochondrial adaptations at lower doses.

Overflow of reducing equivalents to the electron transport chain (ETC) under conditions of overnutrition/physical inactivity or accumulated damage/lower antioxidant defenses associated with aging, causes higher rates of ROS formation, resulting in oxidative stress and irreversible damage to proteins, lipids, and DNA. As a result, permanent mitochondrial dysfunction compromises whole-body energetic status and increases susceptibility to injuries, resulting in accelerated aging and development of metabolic diseases such as insulin resistance and fatty liver.

One of the main regulators of the cellular antioxidant defense system is Sestrin 2 (SESN2), which is induced by several stress conditions. Decreased SESN2 activity is associated with increased oxidative damage, mitochondrial dysfunction, muscle degeneration and fat accumulation. However, the mechanisms by which SESN2 affects mitochondrial functions are not defined. Understanding the molecular mechanisms and how SESN2 affects mitochondria may provide new insights for novel therapeutic targets for attenuation and prevention of aging and obesity-related pathologies. In view of this, this work aimed to evaluate if SESN2 mediates an adaptive protective mitochondrial response in C2C12 cells triggered by menadione, a stimulator of superoxide anion formation. Additionally, and since Sirtuin 1 (SIRT1) is a known metabolic sensor and regulator of mitochondrial function, this work evaluated how modulation of SIRT1 affects SESN2 in the context of fatty liver induced by a high-fat diet.

Results showed a dose-dependent effect of menadione on cellular viability and mitochondrial function. Treatment with 10 µM menadione for 1 h did not alter ROS formation, MTT reduction and mitochondrial membrane potential, as evaluated 24 and 48 h after menadione removal. However, exposure of C2C12 cells to 30 µM menadione for 1 h

resulted in increased ROS generation, reduced MTT reduction and mitochondrial membrane potential. An increase in SESN2 content was observed 10 h after exposure to 10 µM menadione for 1 h, while 30 µM menadione resulted in SESN2 depletion. These results suggest that induction of SESN2 by mild stress induced by menadione may be involved in a mitochondrial protective response that preserves cell viability.

SESN2 silencing with siRNA resulted in increased cellular death as well as a decrease in mitochondrial membrane potential induced by both concentrations of menadione, being more potent the alterations induced by 30 µM menadione. In presence of SESN2, exposure to menadione caused an increase in the punctuated pattern of LC3 (microtubule-associated protein 1A/1B-light chain 3 - PE phosphatidylethanolamine) distribution, showing induction of autophagy. However, depletion of SESN2 with siRNA resulted in a decrease in LC3 punctuation, both in control and menadione conditions.

Altogether these results suggest that mild stress induced by menadione induces SESN2 and activates autophagy/mitophagy as a cell survival strategy. Absence of SESN2 results in accumulation of mitochondrial damage induced by ROS and consequent decrease in cell viability.

Regarding the impact of SIRT1 modulation on SESN2, results showed that hepatic expression of transcription factor c/EBPα (CCAAT-enhancer-binding protein - α) was up-regulated by high-fat diet (HFD) and down-regulated by resveratrol treatment, a SIRT1 activator. In SIRT1-knock-out (SIRT1 - KO) mice c/EBPα expression was decreased when compared to control. SESN2 expression was reduced by HFD and SIRT1-KO. Resveratrol treatment in wild-type animals prevented the decrease in SESN2 induced by HFD. KEAP1 (Kelch-like ECH-associated protein 1) expression was also dependent on SIRT1 and resveratrol treatment showed no effect on KEAP1 in SiRT1-KO mice. KEAP1 degradation is promoted by SESN2 and when degraded, induces Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) translocation to the nucleus and consequently induction of antioxidant genes. Hepatic Nrf2 expression was not affected by SIRT1 modulation. Aging decreased the expression of all of the evaluated genes.

The current work shows that SESN2 induction by mild stress or promotors of mitochondrial homeostasis, such as resveratrol acting on SIRT1, increase mitochondrial tolerance to damage, through modulation of autophagy/mitophagy. Elimination of damaged mitochondria is stimulated by SESN2 and may be the pathway to prevent accumulation of damage and thus result in increased tolerance to overnutrition and extend lifespan.

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List of Abbreviations

αKDH - α-ketoglutarate dehydrogenase

ABCA1 - ATP-binding cassette transporter

ACC - Acetyl-CoA carboxylase

Acetyl-CoA - Acetyl-coenzyme A

ADP - Adenosine diphosphate

AKT – Protein kinase B

AMPK - AMP-activated protein kinase

ANT - Adenine nucleotide translocator

ANOVA – Analysis of Variance

ATG – Autophagy-related genes

ATP – Adenosine Triphosphate

ARE - Antioxidant-response element

BAX - Bcl-2-like protein 4

BBC3 - Bcl-2-binding component 3

BCA - Bicinchoninic acid

BCL-2 - B-cell lymphoma 2

BMI – Body Mass Index

BNIP – BCL-2/adenovirus E1B 19 kd-interacting protein

Ca^{2+} - Calcium

cAMP - Cyclic adenosine monophosphate

CAT - Catalase

cDNA - Complementary DNA

CDK1 - Cyclin-dependent kinase 1

c/EBP - CCAAT/enhancer binding protein

CHOL - Cholesterol

CHX - Ciclohexamide

CMA – Chaperone mediated autophagy

CNS - Central nervous system

CR – Caloric restriction

CREB - Response element-binding protein

CYT C – Cytochrome c

CoQ/$CoQH_2$ - Coenzyme Q / ubiquinol

CO_2 – Carbon dioxide

CuSOD - Cooper-dependent superoxide dismutase

DBC1 - Deleted in breast cancer 1

DCF - 2,7- dichlorofluorescein

DJ-1 - Protein deglycase

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DRP1 - Dynamin related protein 1

ER – Endoplasmic reticulum

ETC – Electron transport chain

ETHD-1 - Ethidium homodimer-1

e^- - Electron

$FADH_2$ - Flavin adenine dinucleotide

FAS - Fatty acid synthase

FBS - Fetal bovine serum

FCCP - Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone

FH – Fumarate hydratases

FMN – Flavin mononucleotide

FOXO - Forkhead box transcription factor, subgroup O

FXR - Farnesoid x receptor

FOX01 - Forkhead box01

F-TFKO - Mouse model with disruption of TFAM specifically in fat

GCN5 - Histone acetyltransferase

GPx – Glutathione peroxidase

GSH/GSSG – Glutathione / glutathione dissulfide

HDAC – Histone deacetylase

HAT - Histone acetyltransferase

HEK293 - Human embryonic kidney 293 cell line

HFD – High fat diet

HNF1α - Hepatocyte nuclear factor 1 alpha

HI95 - Hypoxia-inducible gene 95

HIF-1 - Hypoxia-inducible factor 1

HS – Horse serum

HSC70 - Cytosolic chaperone

H_2DCF-DA - 2',7'-dichlorodihydrofluorescein diacetate

H_2O_2 – Hydrogen peroxide

H^+ - Proton

IDH – Isocitrate dehydrogenase

IMM – Inner mitochondrial membrane

IRS – Insulin receptor substrates

KEAP1 - Kelch-like ECH-associated protein 1

KO – Knock-out

LC3 - Microtubule-associated protein 1A/1B-light chain 3 - PE phosphatidylethanolamine

LKB1 - Serine/threonine kinase 11 or STK11

LNCaP - Androgen sensitive human prostate adenocarcinoma cells

LXR - Liver X receptor

L6 - Rat myoblast cell line

MCF7 - Breast cancer cells

MDH – Malate dehydrogenase

MEFs - Mouse embryo fibrobasts

MnSOD - Manganese-dependent superoxide dismutase

MPC - Mitochondrial pyruvate carrier

mRNA - Messenger RNA

mtDNA – Mitochondrial DNA

mTOR - Mammalian target of rapamycin

mTORC 1, 2 - Mammalian target of rapamycin complex 1, 2

MTT - 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

NAD^+/NADH - Nicotinamide adenine dinucleotide, oxidized and reduced

$NADP^+$/NADPH - Nicotinamide adenine dinucleotide phosphate, oxidized and reduced

NAFLD - Nonalcoholic fatty liver disease

NF-kB - Nuclear factor kappa B

NRF-1 - Nuclear respiratory factors

Nrf2 - Nuclear factor (erythroid-derived 2)-like 2

OXPHOS - Oxidative phosphorylation

O_2 – Molecular oxygen

$O_2^{\bullet -}$ - Superoxide anion

•OH - Hydroxyl radical

PAI-1 - Plasminogen activator inhibitor-1

PA26 - p53-activated gene 26

PBS - Phosphate-buffered saline

PCR - Polymerase chain reaction

PDC - Pyruvate dehydrogenase complex

PDH/PDHP - Pyruvate dehydrogenase/ pyruvate dehydrogenase phosphatase

PDK - Pyruvate dehydrogenase kinase

Pi – Inorganic phosphate

PIG3 - p53-inducible gene 3

PI3K - Phosphatidylinositol 3-phosphate

PINK1 - PTEN-induced putative kinase 1

PGC-1A - PPAR (peroxisome proliferator-activated receptor) coactivator-1α

POLɣ - Mitochondrial polymerase gamma

PPAPRα - Peroxisome proliferator-activated receptor alpha

PRX - Peroxiredoxin

PRX3- Peroxiredoxins 3

p38 - Mitogen-activated protein kinase

p53 - Tumor suppressor

p62 - Sequestossome 1

RESV - Resveratrol

RHEB - Ras homologues enriched in brain

RIPA – Radio-immunoprecipitation assay Buffer

RKO - Poorly differentiated colon carcinoma cell line

RNA - Ribonucleic acid

rRNA - Ribosomal RNA

ROS – Reactive Oxygen Species

RT – Reverse transcriptase

SCoA-S - Succinyl-CoA synthase

SD – Standard diet

SDH - Succinate dehydrogenase

SEM - Standard error of the mean

SESN1, 2, 3 – Sestrin1,2,3

SH – Cysteine thiol

siRNA - Small interfering RNA

SIRT1-7 – Sirtuin 1-7

SOD – Superoxide dismutase

SRB – Sulforodamine B

SREBP1c - Sterol regulatory element-binding protein-1c

SREBP - Factor sterol response element binding protein

SRX - Sulfiredoxins

S6K - S6 kinase

TCA – Tricarboxylic acid

TFAM - Mitochondrial transcription factor A

TIM/TOM - Mitochondrial inner/outer membrane transporter

TMRM - Tetramethylrhodamine methyl ester

TNF-α - Tumor necrosis factor-alpha

tRNA - Transfer RNA

TSC 1, 2 - Tuberous sclerosis 1 and 2

T2DM – Type 2 diabetes mellitus

ULK - Unc-51 like kinase

UCP2 - Uncoupling protein 2

VDAC - Voltage-dependent anion channel

WAT – White adipose tissue

WHO - World Health Organization

WT – Wild type

YY1 - Yin-Yang 1 transcription factor

ΔΨ – Mitochondrial membrane potential

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Table 1 - List of antibodies used for Western Blot

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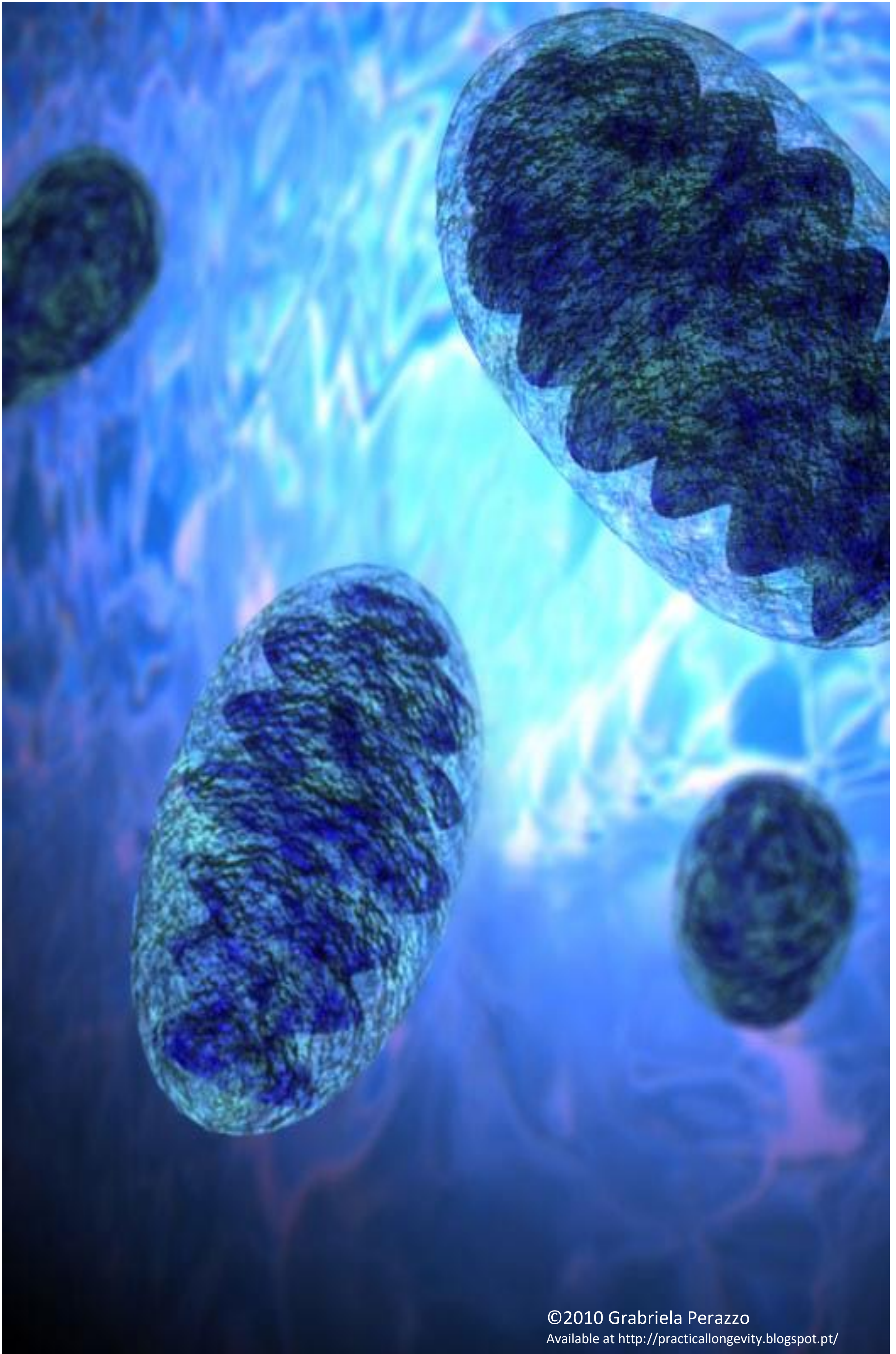
Introduction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1.1. Mitochondria

In the past years, recognition of a dynamic mitochondrial function and behavior in response to cellular signals supported the essential role of mitochondria for cellular homeostasis, as more than lonely participants working tirelessly to provide energy. This has prompted intense research regarding the modulation of mitochondrial function as a target for the prevention and treatment of pathologies such as aging and obesity-associated dysfunctions like cancer, neurodegenerative diseases and diabetes.

Mitochondria are indeed the powerhouses of the cells as they transfer intermediates derived from nutrients into energy in the presence of oxygen, in a process named oxidative phosphorylation. But several other metabolic pathways as well as the cellular redox status, besides other aspects of cell biology such as calcium signaling and programmed cell death are mitochondrial-related (Schatz et al., 1995). As such, disruption in mitochondrial homeostasis is closely associated with disease states, particularly evident in tissues with higher metabolic demands that have larger mitochondrial mass (Fig 1) (Johannsen et al., 2009).

The dynamic nature of mitochondria is highlighted by constant structural changes supported by fusion and fission processes, which are critical for a constant cycle of elimination and regeneration (Jheng et al., 2011; Palikaras and Tavernarakis, 2014). New mitochondria are formed in a process entitled mitochondrial biogenesis, which depends on the balanced execution of four processes (Scarpulla, 2006): replication of the mitochondrial DNA (mtDNA) and translational machinery (Wallace et al 2007), formation of double membrane boundary from phospholipids (Horibata and Sugimoto, 2010; Potting et al., 2010), import of mitochondrial nuclear-proteins and synthesis of the fundamental mtDNA-encoded proteins (Calvo and Mootha, 2010). Additionally, mitochondrial homeostasis is also dependent on the degradation of internal components, as well as the eventual autophagic digestion of the entire organelle by mitophagy (Barbour et al., 2014). Mitochondrial turnover minimizes the deleterious effects of reactive oxygen species (ROS) and other reactive molecules on proteins, lipids and mtDNA, since ROS are generated by mitochondria simultaneously to energy production by oxidative phosphorylation.

Therefore, cellular homeostasis relies on appropriate quality control of mitochondria, namely the selective degradation of damaged and less efficient organelles (Gottlieb et al., 2010). Such equilibrium is accomplished through autophagy based on casual identification of mitochondria that are surrounded by autophagosomes, preventing the accumulation of abnormal organelles.

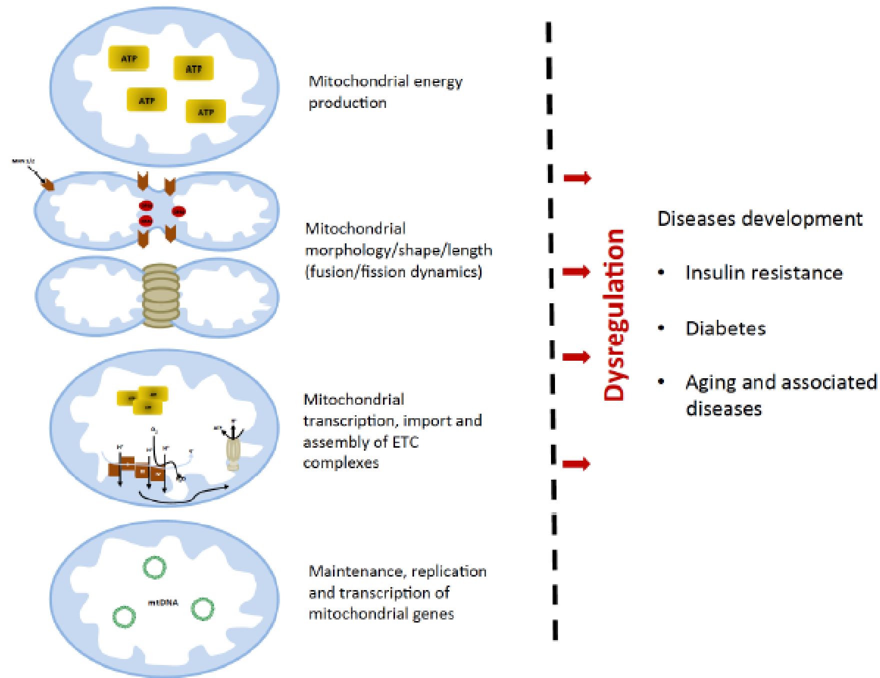


Fig 1 – Role of mitochondria in cellular homeostasis. Mitochondria are the main suppliers of cellular energy by generating ATP through oxidative phosphorylation and key regulators of cellular function. Mitochondrial fusion/fission dynamics, transcription, import and assembly of electron transport chain complexes together with mitochondrial biogenesis are essential for correct mitochondrial function. Therefore, mitochondrial dysregulation is associated with metabolic pathologies like diabetes, insulin resistance as well as aging (Duarte et al., 2015).

1.1.1 Mitochondrial structure

Mitochondria are thought to have evolved from bacteria that developed a symbiotic relationship of endosymbiosis with larger cells. They have about 0.5–1µm diameter, up to 7µm long and may appear as spheres, rods or filamentous bodies depending on the type and necessities of cell. But the general architecture is the same (Fig 2) (Lea and Hollenberg, 1989; Krauss, 2001). Mitochondria are double-membrane bound organelles, with the two membranes separated by an intramembrane space and composed by a phospholipid bilayer but still quite distinct in appearance and in physicochemical properties.

The outer membrane separates the mitochondria from the cytosol defining the outer perimeter and is about 50:50 of a protein-to-lipid ratio (Krauss, 2001), which allows to be widely permeable to ions and molecules smaller than 1000 daltons (Da). Channels formed by porins promote free diffusion of these molecules across the membrane. The inner mitochondrial membrane (IMM) encloses the mitochondrial matrix. Palade in 1952 and Daems and Wisse in 1966, described the presence of infoldings of the inner membrane that were attached to the IMM, named as cristae (Lea and Hollenberg, 1989). These cristae leads to an increase of the surface of the inner membrane, where is present the main enzymatic machinery for oxidative phosphorylation. IMM is impermeable to most small molecules and ions, including protons, and the only species that can cross it need specific transporters. Therefore, a separation of the matrix from the cytosolic environment is possible and essential for the conversion of energy, derived from oxidizable substrates by the electron transport chain (ETC), in adenosine triphosphate (ATP) by the ATPsynthase. This makes the inner mitochondrial membrane an electrical insulator and chemical barrier only crossed due to sophisticated ion transporters (Krauss, 2001). In the mitochondrial matrix, which is enclosed by the inner membrane, where the mitochondrial genetic system can be found along with the enzymes involved in central metabolic reactions. Mitochondrial DNA (mtDNA) are circular DNA molecules, present in n copies *per* organelle, encode only 13 proteins essential for the oxidative phosphorylation system, 2rRNA (ribosomal RNA) and 22tRNAs (transfer RNA). However, the vast majority of mitochondrial proteins required for oxidative phosphorylation and other metabolic functions are encoded by the nucleus and imported to mitochondria (Calvo and Mootha, 2010; Palikaras and Tavernarakis, 2014).

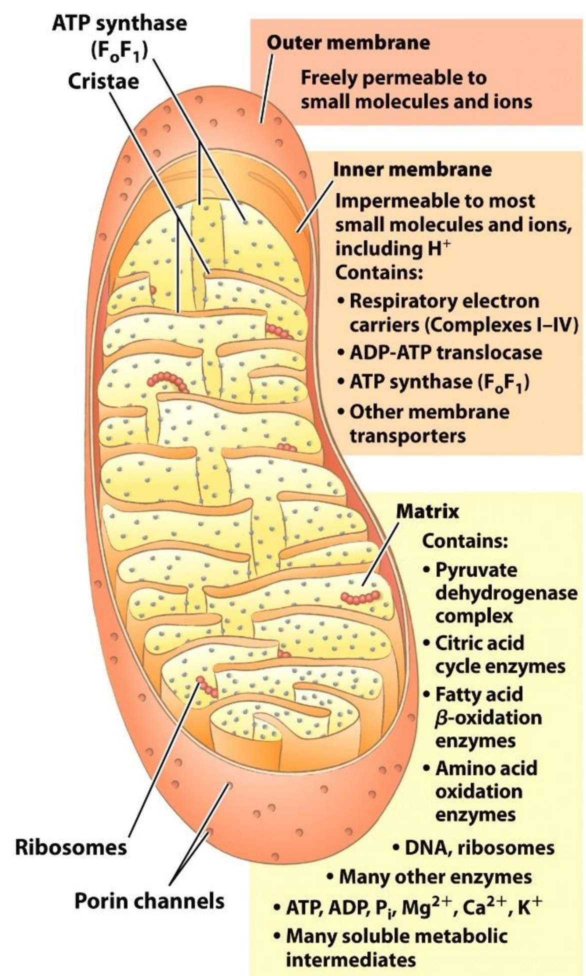


Fig 2- Schematic representation of the mitochondrial structure. Nelson, David L.; COX, Michael M.Principles of Biochemistry, 2008.Ffifth edition, W.H.Freeman and Company.

1.1.2 Mitochondrial respiratory chain and ATP production through oxidative phosphorylation

Mitochondria are responsible for the synthesis of approximately 95% of ATP needed by the cell (Erecinska and Wilson, 1982), as oxidative phosphorylation is the most efficient process to generate ATP (Fig 3). In the mitochondria, the energy contained in glucose, amino acids and fatty acids is used to form acetyl-coA that is metabolized in the tricarboxylic acid (TCA) cycle, generating redutive equivalents NADH (nicotinamide adenine dinucleotide) and $FADH_2$ (flavin adenine dinucleotide) . NADH and $FADH_2$ are then used in the oxidative phosphorylation process, occurring at the IMM, to produce up to 38 molecules of ATP.

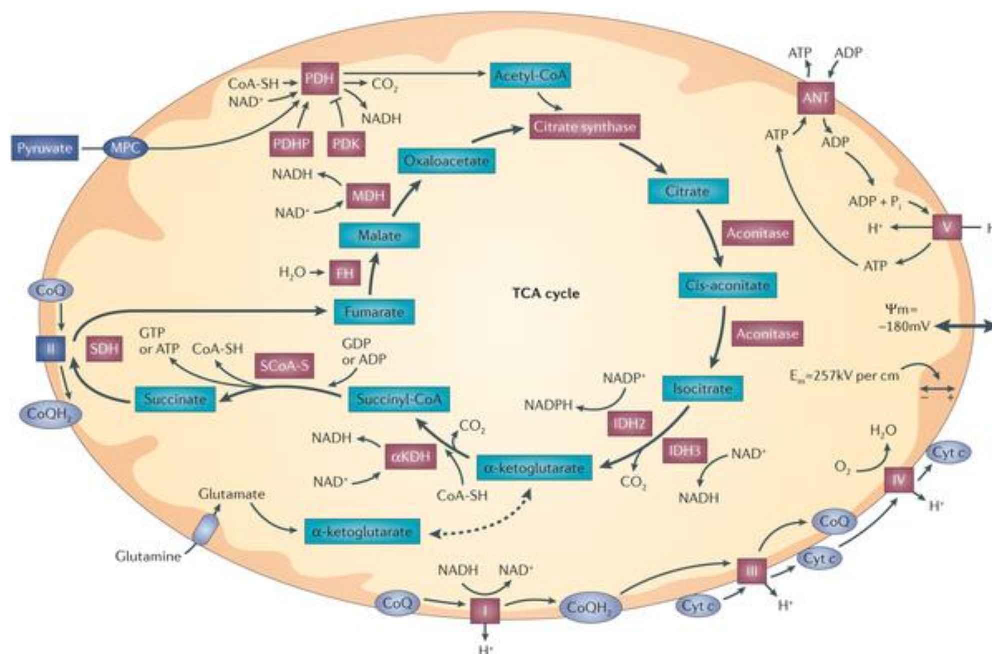


Fig 3 – Central role of mitochondria in cellular metabolism: ATP synthase generates ATP upon oxidation of reducing equivalents (NADH and $FADH_2$) by the electron transport chain. β-oxidation of fatty acids in peroxisomes and mitochondria provides Acetyl-CoA that enters the TCA cycle via its condensation with oxaloacetate to form citrate. Pyruvate formed upon oxidation of glucose (glycolysis) is delivered to the mitochondria by MPC (mitochondrial pyruvate carrier) where the pyruvate dehydrogenase complex (PDH) converts pyruvate to Acetyl-CoA. Amino acid metabolism also involves its conversion into metabolic intermediates that can be converted into glucose or oxidized in the TCA. Electrons (from NADH and $FADH_2$) are then funneled in the ETC to molecular oxygen down a redox-potential gradient. This electron flux is coupled to proton extrusion, creating the proton-motive force and the negative mitochondrial membrane potential (ΔΨ) of mitochondria, powering the ATP synthase (complex V). ATP is exchanged for cytosolic ADP by the inner membrane adenine nucleotide translocator (ANT). αKDH, α-ketoglutarate dehydrogenase; CYT C, cytochrome c; CoQ, coenzyme Q; $CoQH_2$, ubiquinol; FH, fumarate hydratase; IDH, isocitrate

dehydrogenase; MDH, malate dehydrogenase; PDH, pyruvate dehydrogenase; PDHP, pyruvate dehydrogenase phosphatase; PDK, pyruvate dehydrogenase kinase; P_i, inorganic phosphate; SCoA-S, succinyl-CoA synthase; SDH, succinate dehydrogenase; SH, cysteine thiol; I, complex I from ETC; II, complex II from ETC; III, complex III from ETC; IV, complex IV from ETC (Sabharwal and Schumacker, 2014).

Oxidative phosphorylation relies on a series of multimeric protein complexes, the ETC, as represented in Fig 4 (Krauss, 2001), where redox reactions take place, allowing the transfer of electrons from the reductive equivalents (NADH and $FADH_2$) to the final electron acceptor (molecular oxygen). The ETC is composed of several polypeptidic subunits grouped into four complexes, within the IMM: Complex I or NADH: Ubiquinone Oxiredutase, Complex II or Succinate Dehydrogenase, Complex III or Ubiquinol: Cytochrome c Oxiredutase and Complex IV or Cytochrome c Oxidase. The ETC also has two mobile components, an extrinsic small protein - Cytochrome c and a hydrophobic quinone - Coenzyme Q (coQ).

Complex I, also called NADH:ubiquinone oxidoreductase or NADH dehydrogenase, is a large enzyme that includes an FMN (flavin mononucleotide) -containing flavoprotein and six iron-sulfur centers. Here, two simultaneous and coupled processes occur by transferring two electrons from NADH to ubiquinone with a proton translocation across the membrane, releasing energy. The two coenzymes of complex I, FMN and CoQ are able to accommodate up to two electrons each in stable conformations and at the same time donate one or two electrons to the cytochromes of complex III. It is thought that four protons are pumped per pair of electrons (Krauss, 2001). Complex II is anchored in the membrane, facing the mitochondrial matrix and is responsible for electron transfer from succinate to ubiquinone, using FAD as coenzyme, three iron–sulfur clusters and cytochrome b_{560} (Krauss, 2001). Although smaller than complex I, complex II contains five prosthetic groups and four different protein subunits containing a heme group, a binding site for ubiquinone, final acceptor in this reaction, three 2Fe-2S centers and a binding site for succinate. The third complex, also called cytochrome bc1 complex or ubiquinone:cytochrome c oxydoreductase, is responsible for the transfer of electrons from cytochrome c, by performing to the intermembrane space two protons *per* pair of electrons. In complex III the redox groups comprise a 2Fe/2S center, located on Rieske protein, two b-type hemes located on a single polypeptide and the heme of CYT C (cytochrome c). At the end of the mitochondrial ETC is complex IV, where electrons are transferred from cytochrome c to O_2 reducing it to H_2O, the final step of respiratory chain. Mitochondrial subunit II of the complex IV, contains two Cu ions complexed with SH groups of two cysteine residues in a binuclear center, that look like the 2Fe-2S centers of iron-sulfur proteins. Subunit I

contains two heme groups designated a and a3 and other copper ion that together form a second binuclear center.

The coupling of electron flow to ATP synthesis as described by the Chemiosmotic Theory, proposed by Peter Mitchell (1961, Fig 4) is a great example of the relationship between mitochondrial function and structure and it postulates that the electrochemical energy formed by differences in proton concentration and separation of charge across the inner mitochondrial membrane, drives the synthesis of ATP in a proton flow back into the matrix. Since the inner membrane is impermeable to most ions and small molecules, the energy from oxidation of NADH and $FADH_2$ is converted to potential energy and stored in an electrochemical gradient that can be used to import proteins and Ca^{2+}, to generate heat by uncoupling proteins and to synthesize ATP. The flow of protons through the ATP synthase (complex V) back into the matrix releases energy that is used to convert ADP + Pi to ATP, which is then exchanged for cytosolic adenine diphosphate (ADP) by the inner membrane adenine nucleotide translocator (ANT) (Bernardi, 1999; Mitchel, 1966). A series of feedback and regulatory steps enables the rate of mitochondrial oxidative phosphorylation to match cellular ATP demands.

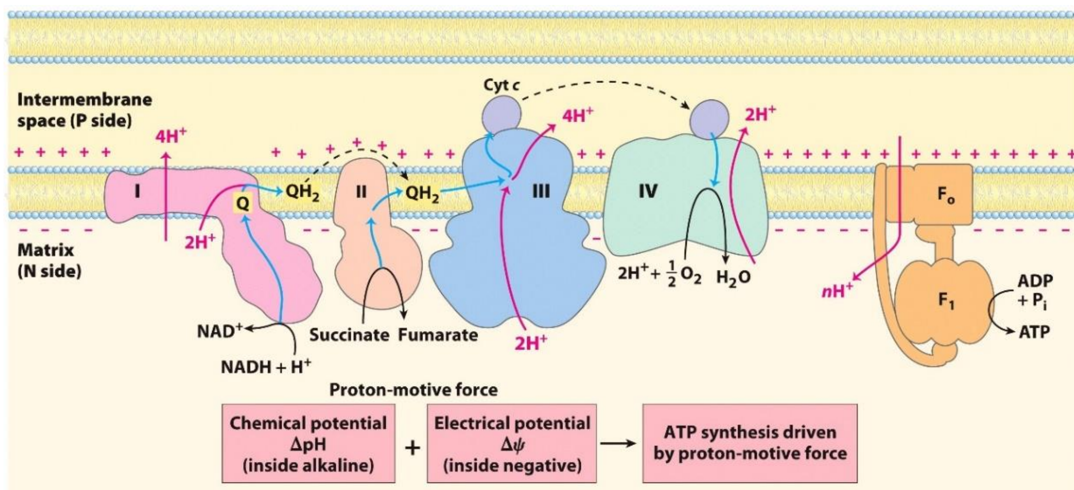


Fig 4 - Chemiosmotic Theory. Electron from NADH and other oxidizable substrates pass through a chain of carriers arranged asymmetrically in the inner membrane. Electron flow is accompanied by proton transfer across the membrane producing both a chemical gradient and an electrical gradient. The proton-motive force that drives protons back into the matrix provides the energy for ATP synthesis. - Nelson, David L.; COX, Michael M. Principles of Biochemistry, 2008.Fifth edition, W.H.Freeman and Company.

1.1.3 Mitochondrial generation of reactive oxygen species

Reactive oxygen species (ROS) is the collective term that generally describes a variety of molecules and free radicals derived from nitrogen and molecular oxygen including superoxide anions (O_2•-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH). These are products of normal metabolism with an important role in the organism, due to their action as redox messengers in intracellular signaling, growth arrest, apoptosis, cellular differentiation and defense against external microorganisms (Trachootham et al., 2008; Roberts and Sindhu,2009; Lee et al.,2012a). The redox state of the respiratory chain is the primary factor driving mitochondrial ROS generation (Skulachev, 1996; Lambert and Brand, 2004). The amplitude of the electrochemical proton gradient, which is known as respiratory control, regulates the overall rate of electron transport in the respiratory chain. When the electrochemical potential difference generated by the proton gradient is high (such as in high-fat or high-glucose states), or in conditions of inhibition of the ETC complexes, the life of superoxide generating electron transport intermediates, such as ubisemiquinone, is prolonged (Skulachev, 1996). This occurs because the transmembrane proton gradient and the membrane potential inherently govern the activity of the respiratory chain complexes as proton pumps. When sufficiently high, pH and potential inhibit the proton pumps.

ROS are inactivated by a natural antioxidant system that involves antioxidant enzymes and several non-enzimatic compounds. The primary antioxidant enzymes are superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). SOD is responsible for the dismutation of superoxide, the primary ROS produced in aerobic organisms, to oxygen and hydrogen peroxide. Three isoforms of SOD can be found in mammals: mitochondrial MnSOD (manganese-dependent superoxide dismutase), extracellular SOD and cytosolic Cu/ZnSOD (copper/zinc-dependent superoxide dismutase) (Roberts and Sindhu,2009; James et al., 2012). Hydrogen peroxide diffuses across the membrane and is degraded by CAT, GPx and thiorredoxin to water and hydroxyl radical. However, in the presence of reduced transition metals such as Cu or Fe, H_2O_2 is converted to hydroxyl radical by Fenton or Haber-Weiss reactions (Roberts and Sindhu, 2009). Glutathione peroxidase, that uses glutathione as hydrogen donor, reduces hydrogen peroxide, organic hydroperoxides and lipoperoxides into its hydroxylated compounds (Roberts and Sindhu, 2009).

The non-enzymatic antioxidant defenses include vitamin C or ascorbic acid, reduced glutathione, vitamin E and phenolic compounds (Roberts and Sindhu,2009), all of them with anticancer properties. While vitamin C needs to be incorporated on the diet through citric, fruits and vegetables, glutathione can be biosynthesized from amino acids like cysteine, glycine and glutamic acid. Vitamin E, one of the main biological defenders, is mainly found in peanuts and seed

oils as olive or sunflower oil. It can detoxify lipid peroxides and singlet oxygen directly. If the generation of ROS exceeds the antioxidant system, the intracellular environment turns extremely oxidized and reactive, causing DNA damage, lipid and protein oxidation, resulting in a decrease of cell function.

1.1.4. ROS generation induced by menadione

Menadione (2-methyl-1,4-naphtoquinone) has been widely used *in vivo* and *in vitro* to study the effects of increased intracellular formation of superoxide anion and hydrogen peroxide (Sun et al., 1999; Criddle et al., 2006). The lipophilic nature of menadione allows this compound to concentrate on lipid bilayers (McCormick et al., 2000). Reductive enzymes such as mitochondrial complex I and microsomal NADPH-cytochrome P450 reductase are known to mediate menadione one-electron reduction, forming an unstable semiquinone that enters into a redox cycling in the presence of oxygen, which will progressively damage mitochondria and impair several cellular processes (Fig 5) (McCormick et al., 2000; Basoah et al., 2005; Criddle et al., 2006,).

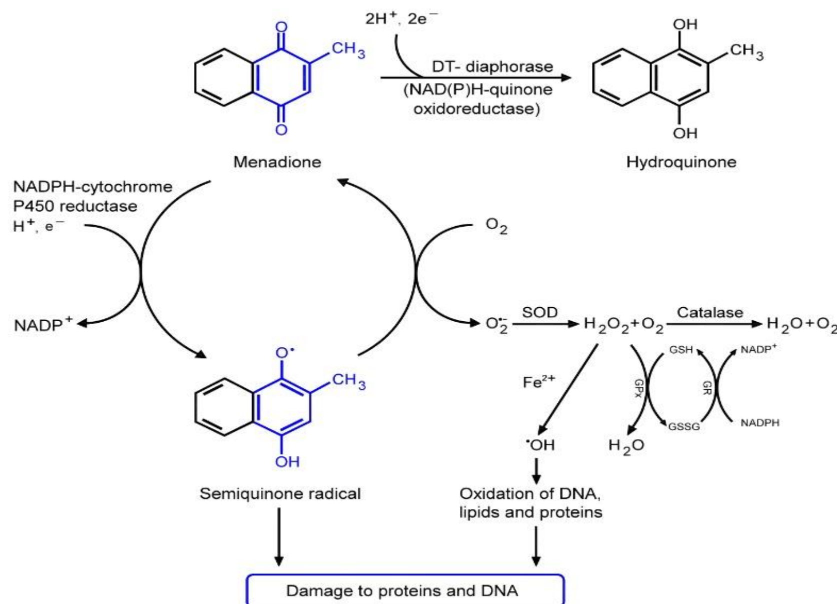


Fig 5 – Generation of reactive oxygen species induced by menadione. Menadione scavenge the electrons from ubiquinone directly into molecular oxygen and undergo cell-mediated one – electron reduction, forming a semiquinones. This reacts with molecular oxygen generating superoxide anions (O2•-) that are converted in H_2O_2 in a reaction catalyzed by SOD. Catalase and GPx (glutathione peroxidase) are other ROS metabolizing enzymes. Depletion of antioxidant defenses results in oxidative stress with lipid peroxidation and oxidative damage to proteins and DNA, resulting in cellular death. GSH, glutathione; GSSG, glutathione dissulfide; GR, glutathione reductase.

Several studies have been conducted using menadione as an inducer of ROS. In C2C12 myoblasts, exposure to menadione has been shown to induce mitochondrial oxidative stress that leads to increased degradation of newly synthetized mitochondrial proteins (Basoah et al., 2005). Other studies showed that L6 muscular cells in different states of differentiation, have differential responses to the same concentration of menadione, which can be explained by the activation by oxidative stress of signaling pathways, in different stages of differentiation, including the induction of pro-apoptotic factors (Lim et al., 2008). In hepatocytes, menadione has been reported to induce apoptosis, with increased superoxide leading to of caspases-6 and -9 activation (Conde de La Rosa et al., 2006). Accordingly, menadione has been also shown to induce apoptosis in pancreatic acinar cells by inducing permeability transition pore due to increased oxidation of intracellular glutathione and sulfydryl groups (Criddle et al., 2006).

1.1.5. Mitochondrial biogenesis

Mitochondrial biogenesis is defined as the growth and division of pre-existing mitochondria. This process is influenced by several environmental stress conditions such as low temperature, oxidative stress, caloric restriction (CR), division, renewal and differentiation of the cells (Jornayvaz and Shulman, 2010). Several studies also established the relationship between stimulation of mitochondrial biogenesis and physical activity in order to maximize energy formation in skeletal muscle. (Hoppeler et al., 1973).

This is a complex process and involves mtDNA transcription and translation, translation of nuclear transcripts, recruitment of newly synthesized proteins and lipids, as well as import and assembly of mitochondrial and nuclear products (Palikaras et al., 2015) to form mitochondria with a balanced function. The mRNAS translated in the cytosol have a pre-sequence that directs the unfolded polypeptidic chain to mitochondrial receptors that belong to TOM (translocase of the outer membrane) complex. Proteins are then conducted to TIM (translocase of the inner membrane) complex and in the matrix, protease cleaves the pre-sequence and proteins are folded by chaperones.

Mitochondrial biogenesis is regulated by several proteins linking environmental stimuli with metabolic responses (Palikaras et al, 2015). PPAR (peroxisome proliferator-activated receptor) coactivator-1α (PGC-1α), one of the major regulators of mitochondrial biogenesis, is a co-transcriptional regulation factor responsible for mitochondrial biogenesis by activation of other transcriptions factors (Jornayvaz and Shulman, 2010). It was described that PGC-1α induces mitochondrial biogenesis and respiration through the induction of uncoupling protein 2 (UCP2), Yin-

Yang 1 transcription factor (YY1), nuclear respiratory factors NRF-1 and NRF-2 and mitochondrial transcription factor A (TFAM) in order to induce transcription and mtDNA replication (Wu et al., 1999; Jornayvaz and Shulman, 2010; Wei et al., 2015). PGC-1α also induces nuclear factor- erythroid 2-related factor 2 (Nrf2) that belongs to a transcriptional complex network responsible for induction of an antioxidant response (Palikaras et al., 2015). Multiple extracellular and intracellular stimuli such as levels of calcium and ROS, hypoxia and lack of nutrients, all induce PGC-1α in order to modulate mitochondrial function and to guarantee proper cell function (Palikaras et al., 2015; Wei et al., 2015).

Through the years, several signaling events have been shown to be associated with the induction of mitochondrial biogenesis and prevention of disease development associated with mitochondrial dysfunction (Banks et al., 2008, Pearson et al., 2008; Fiorino et al., 2014). For example, glucose restriction as a therapeutic strategy, is associated with activation of PGC-1α via deacetylation by Sirtuin1 (SIRT1). AMP-activated protein kinase (AMPK) functions as a metabolic sensor that regulates energetic status since its activation by high AMP content triggers a response related with induction of mitochondrial biogenesis (Hardie, 2007). As demonstrated, AMPK activation increases NRF-1-binding activity and mitochondrial content as the result of induction of mitochondrial biogenesis by PGC-1α and NRFS (Bergeron et al., 2001). Conversely, aging-related decrease in AMPK is associated with mitochondrial dysfunction and dysregulated intracellular lipid metabolism (Jornayvaz and Shulman, 2010).

Mammalian target of rapamycin (mTOR) has been proposed to be a central regulator of lifespan (Blagosklonny, 2008, 2010) and being a sensor of nutrient status, mTOR affects mitochondrial homeostasis by modulation of PGC-1α transcriptional control of mitochondrial biogenesis (Cunningham et al., 2007; Palikaras and Tavernarakis, 2014; Wei et al., 2015). Mammalian cell growth is regulated by mTOR that forms two distinct complexes, mammalian target of rapamycin complex 1 (mTORC1) responsible for cell growth and protein synthesis and target of rapamycin complex 2 (mTORC2) responsible for cell spreading and control of the actin cytoskeleton (Budanov and Karin, 2008). Previous studies on this subject show that mTORC1 is present in mitochondria and its inhibition affects mitochondrial proteome, leading to a decrease in mtDNA copy number in skeletal muscle (Cunningham et al., 2007; Schieke and Finkel, 2006). Cunningham et al. identified the transcription factor YY1 as a common target of mTOR and PGC-1α. The proposed mechanism promotes the idea that decreased mTOR activity would inhibit YY1-PGC-1α axis, leading to a decrease in the expression of mitochondrial genes (Cunningham et al., 2007).

1.2 Autophagy and its relevance for metabolic homeostasis

Autophagy is a complex self-degradative process mediating the turnover of macromolecules and organelles within lysosomes/vacuoles. This process is highly regulated and conserved from yeast to mammals and arises as a response to alterations in cellular homeostasis induced by stimuli such as hypoxia, nutrient deprivation and alterations in redox status (Gelino and Hansen, 2012; Feng et al., 2014). First described by Christian de Duve in 1963 as the physiological process responsible for the clearance of defective organelles and misfolded or aggregated proteins, autophagy is also an adaptive mechanism essential for cell survival, since it promotes energy efficiency by acting as a recycling factory (Singh and Cuervo, 2011; Lee et al., 2012b; Gelino and Hansen, 2012). A decrease in ATP content induces autophagy through AMPK-inhibition of mTOR activity (Singh and Cuervo, 2011).

Autophagy can be divided in autophagosome formation, maturation, and lysosomal fusion, a complex process controlled and coordinated by more than 30 autophagy-related genes (ATG) (Singh and Cuervo, 2011; Lee et al., 2012b). Stimulation of the autophagic process leads to activation of the initiation complex ATG1/ULK (autophagy-related gene1/Unc-51 like kinase) and nucleation of the phagophore, the initial sequestering compartment consisting of an isolation membrane likely derived from the endoplasmic reticulum (ER) and/or the trans-Golgi and endosomes. Then, through PI3P (phosphatidylinositol 3-phosphate)-binding complex, it expands into a double-membrane structure that recruits the ATG5/ATG12 (autophagy-related gene 5/autophagy-related gene 12) conjugation system, which then associates with ATG16 (autophagy-related gene 16) and ATG8/LC3 (autophagy-related gene 8/microtubule-associated protein 1A/1B-light chain 3 - PE phosphatidylethanolamine) conjugation system in mammalian autophagy, thus forming the autophagosome. After finishing vesicle expansion, the mature autophagosome is ready for fusion with lysosomes, becoming an autolysosome whose contents are degraded by acidic hydrolases (Gelino and Hansen 2012; Singh and Cuervo, 2011). Amino acids and other products of degradation are exported to the cytoplasm by lysosomal permeases and transporters, becoming available for metabolism or building molecules.

Since LC3 is associated with the autophagossomal membrane, LC3 is the most used autophagy marker (Fig 6). LC3 is synthesized as pro-LC3 and two forms, LC3-I and –II, can be detected; LC3-I is cytosolic whereas LC3-II is membrane bound. Pro-LC3 is processed into a cytosolic, inactive form (LC3-I) by ATG4 (autophagy-related gene 4); LC3-I is then covalently linked to PE by E2-like enzymes such as ATG3 (autophagy-related gene 3). These proteins are particularly

sensitive to oxidative modifications due to the thiol groups on cysteine catalytic sites necessary for ubiquitin transfer (Cooper et al., 2002). The lipidation of LC3-I occurs during autophagosome formation and it converts LC3-I into the active, membrane-bound and cargo-recruiting LC3-II. Changes in LC3 localization and the level of conversion of LC3-I to LC3-II provides an indicator of autophagic activity (Lee et al., 2012b).

There are three types of autophagy regulated by signaling pathways: macroautophagy, microautophagy and chaperone-mediated autophagy (Lee et al., 2012b).

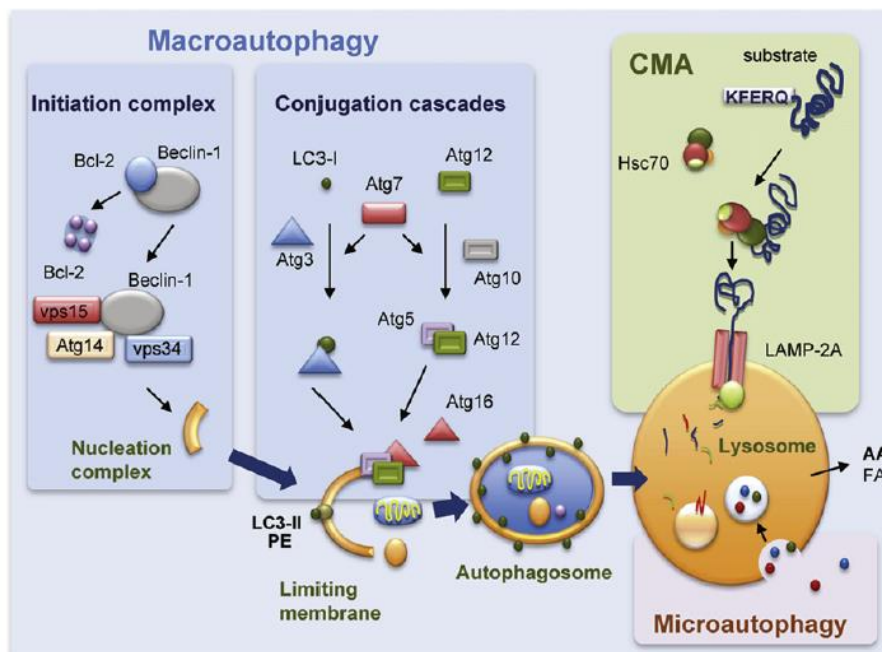


Fig 6 – Mammalian autophagic pathways: Macroautophagy begins with activation of initiation complex that together with PI3P-binding complex recruits ATGs and LC3 inducing the formation of a limiting membrane that engulf cytosolic components. The cargo is degraded by fusion between autophagosome and lysosome. Microautophagy is induced by invaginations in lysosomal membrane and sequestration of cytosolic components that are degraded by lysosomal hydrolases. Chaperone-mediated autophagy (CMA) occurs through recognition of targeting motifs like KFERQ in proteins by HSC70 (cytosolic chaperone) and delivery in lysosomal membrane where are internalized and degraded. (Singh and Cuervo, 2011).

Depending on the selective combination of ATG proteins there are two subsets of autophagy, with each process involving a core set of selective machinery that recognizes specifically cargo components (Feng et al., 2014). In non-selective autophagy, cargo are random cytoplasm components whereas selective autophagy targets organelles such as mitochondria and peroxisomes or microbes (Feng et al., 2014). When mitochondria are the specific target, the process is called mitophagy (Fig 7). Mitochondria are selected for mitophagy when they exhibit severe

damage and a sharp decrease in mitochondrial membrane potential and according to inducing conditions, different proteins can act as receptors (Feng et al., 2014). This process is also important to prevent apoptotic cell death since elimination of damaged mitochondria can prevent the release of pro-apoptotic factors (Lee et al., 2012b).

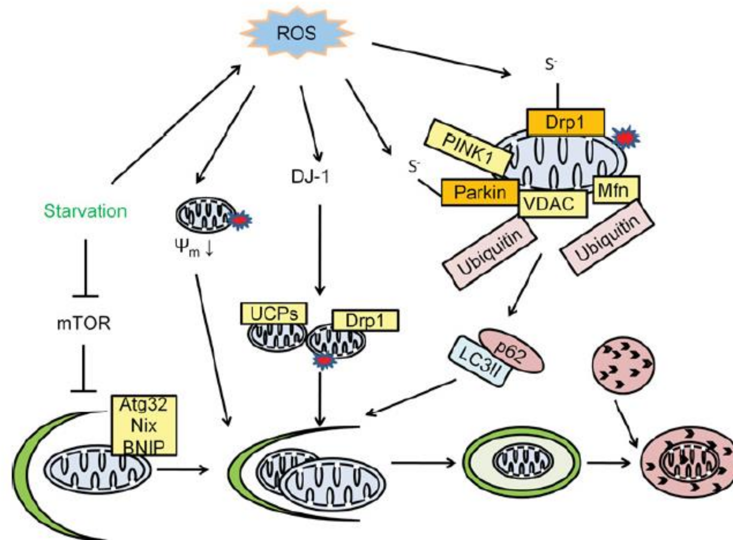


Fig 7 – Elimination of damaged mitochondria by mitophagy. Mitophagy is the degradation of mitochondria by autophagy and can be induced by several stimuli. In starvation, mTOR is inhibited and ATG32 (autophagy-related gene 32), NIX and BNIP targets mitochondria for degradation. Accordingly, Parkinson's disease genes can also induce mitophagy by encoding parkin α-synuclein, PINK1 (PTEN-induced putative kinase 1) and DJ-1 (Protein deglycase). PINK1 promotes parkin targeting and ubiquitinates VDAC (Voltage-dependent anion channel) that is recognized by p62 (sequestossome 1) and LC3, inducing autophagy. DJ-1 senses ROS. Fission events are also associated with mitophagy (Lee et al., 2012b).

Due to its complexity, autophagy is tightly regulated in order to be efficient and safe. mTOR is the best studied modulator of autophagy due to its function as a nutrient sensor, active in the presence of growth factors and abundant cellular nutrients (Singh and Cuervo, 2011; Lee et al., 201b2). Thus during starvation, mTOR remains inhibited allowing the activation of phosphatases and partial dephosphorylation of ATG13 (autophagy-related gene 13) which induces the initiation complex and leads to the formation of the autophagosome (Singh and Cuervo, 2011; Lee et al., 2012b). Another nutrient sensor that regulates autophagy is AMPK activated by conditions of energy depletion. This sensor has been shown to regulate the initiation complex in starvation, by reducing ULK1 phosphorylation promoting its release from mTOR and by inhibition of mTOR activity

through phosphorylation of TSC2 (tuberous sclerosis complex 2) (Singh and Cuervo, 2011; Lee et al., 2012b; Gelino and Hansen, 2012).

Years of research have started to show how important regulators of the autophagic process, including mTOR and AMPK, modulate the aging process. Although the mechanisms by which mTOR inhibition extends lifespan remains unclear, it is possible that mTOR affects lifespan through mitophagy mitochondrial hormesis, and apoptosis (Wei et al., 2015). Modulation of autophagy by redox signaling, including removal of defective proteins and/or the entire mitochondria by mitophagy, is a strategy to prevent perpetuation of mitochondrial dysfunction under conditions of increased ROS formation (Lee et al., 2012b). Superoxide anion and hydrogen peroxide play important roles in the regulation of autophagy as a response to nutrient starvation and rapamycin (Kissova et al., 2006; Scherz-Shouval et al., 2007; Chen et al., 2009). Although the exact mechanism remains to be established, it is known that superoxide induces iron release and promotion of lipid peroxidation while hydrogen peroxide is responsible for thiol modification of Cys^{18} residue of ATG4, (Scherz-Shouval et al., 2007; Chen et al., 2009). Autophagy is also regulated by the redox signaling Nrf2 (nuclear factor-erythroid 2-related factor 2)/KEAP1 (Kelch like ECH associated protein 1) pathway (Itoh et al., 2015). Oxidative conditions are responsible for the modification of a cysteine residue on KEAP1, resulting in Nrf2 release and subsequent translocation into the nucleus. Nrf2 binds to the antioxidant-response element (ARE), inducing transcription of antioxidant genes as well as autophagy-related, such as p62, resulting in ROS scavenging and removal of damaged proteins (Friling et al., 1990; Rushmore and Pickett, 1990; Wasserman and Fahl, 1997; Itoh et al., 1999; Malhotra et al., 2010).

Mitochondrial hormesis is an adaptive and protective response activated by a short and mild stress that culminates in subsequently increased long-term resistance (Ristow and Zarse, 2010). Conditions of mTOR inhibition are associated with only a moderate increase in ROS that helps to promote lifespan, as defended by mitohormesis (Wei et al., 2015). mTOR inhibition also induces mitophagy through ROS-regulating formation of the autophagosome. However, mTOR inhibition may also induce apoptosis caused by chronic and high oxidative insult. The activation of these three pathways is dependent on the level of ROS generation: low, mild levels induce a hormetic response, but ROS increase to a level that induces mitochondrial dysfunction, activates mitophagy. Oxidative stress conditions activates apoptosis (Fig 8) (Wei et al., 2015).

By allowing cells to recover from stress-induced damage, autophagy has been suggested as promoter of longevity (Singh and Cuervo, 2011; Gelino and Hansen, 2012). A number of pathologies

exhibit alterations in the process of autophagy and defective autophagy has been associated accumulation of damage and impairment of energetic status. Thus, reestablishment of normal autophagic activity may be an attractive therapeutic strategy (Singh and Cuervo, 2011).

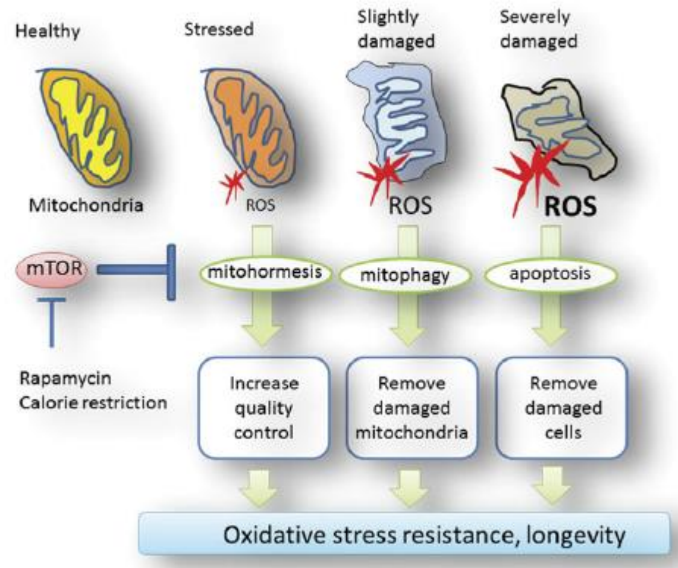


Fig 8 – Regulation of lifespan through mTOR inhibition and mitochondria. ROS act as inducers of multiple mitochondrial stress responses as mitophagy, mitohormesis or apoptosis, depending on ROS levels (Wei et al., 2015).

1.3 Obesity, aging and metabolic dysfunction

The prevalence of obesity, defined by the World Health Organization (WHO) as a body mass index (BMI) ≥ 30kg/m^2 (World Health Organization. (2015). Obesity and overweight Fact Sheet N°311. WHO Media Centre. Geneva, Switzerland), is largely increasing due to chronic overnutrition and physical inactivity (James et al., 2012). The associated dyslipidemia is linked to lipid accumulation not only in adipose tissue but also ectopically in non-adipose tissues including the liver, pancreas, heart and skeletal muscle, with adverse effects on health and decreased life expectancy (Unger et al., 2010). In fact, obesity amplifies the risk of developing various age-related diseases such as type 2 diabetes, cardiovascular and musculoskeletal disorders and certain types of cancer (Lavie et al., 2009; Samuel et al., 2010). The parallel increase in elderly subjects further aggravates detrimental health consequences with obesity accelerating age-related pathologies and aging causing a reduction in lean mass and a progressive increase and abnormal distribution of fat mass (Baumgartner et al., 1995; Gallagher et al., 1997). Moreover, aging is associated to metabolic disorders typical of obesity due to decreased basal metabolic rate and physical activity (Ahima, 2009; Hall et al., 2013).

Lipid overload in the skeletal muscle is associated with an impairment of insulin sensitivity, as well as in muscle maintenance and regeneration (Batsis and Buscemi, 2011), also maximal oxygen uptake decreases in skeletal muscle with age (Short et al., 2004; Proctor et al., 1998). Skeletal muscle is a major site of metabolic activity, accounting for about 40% of the total body mass, decreasing 3-10% per decade after the age of 25 (Johnson et al., 2013). Skeletal muscle fibers are an aggregate of multinucleated myotubes formed due to alignment, elongation, and fusion of mononucleated myoblasts in a process called muscle differentiation (Fig 9).

From all cell types and tissues, the skeletal muscle is the unique that possesses the capacity of increasing metabolic rate in transition from resting to maximal contractile activity, which combines with the mechanics of tendons and bones (Lanza et al., 2010). Contractile activity is dependent on ATP to regulate the maintenance of sarcolemmal membrane potential, calcium handling and interaction of contractile proteins (Lanza et al., 2010). Being mitochondria the main supplier of energy, mitochondrial density allows the maintenance of a steady-state of muscular work. In skeletal muscle, mitochondria are present as extensively branched reticula in two subpopulations. The subsarcolemmal mitochondria within plasma membrane and intermyofibrillar mitochondria distributed in contractile proteins (Lanza et al., 2010).

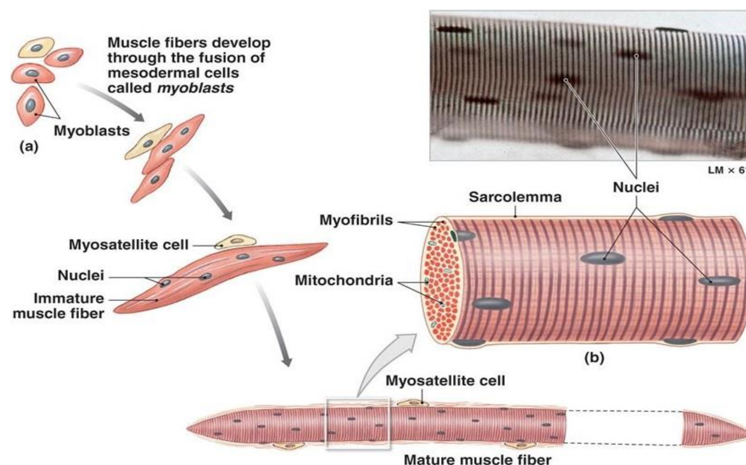


Fig 9 - Formation of skeletal muscle fibers. Precursor cells originate different populations of myoblasts (a) that fuse and form primary myotubes. These cells exhibit a central nucleus and are responsible for initiating myofibrils synthesis. Primary myotubes allow the formation of secondary myotubes derived from other populations of myoblasts. Separation of primary and secondary myotubes is necessary for differentiation of primary and secondary fibers, respectively. Some of the initial myoblasts remain as satellite cells that under some conditions will fuse and differentiate. Available in Basic Histology Lecture 3 by Marline Dorcinvil, accessed in August 20, 2015, https://quizlet.com/14872669/basic-histology-lecture-3-flash-cards/

Obesity and the development of skeletal muscle insulin resistance are closely related with decreased capacity of alternation between carbohydrate and fat as energy sources (Stump et al., 2006). The uptake of fat and carbohydrates is influenced by alterations in nutrition, hormonal signals like insulin as well as changes in oxidative metabolism (James et al., 2012). Promotion of insulin sensibility and oxidation of glucose and fatty acids are essential to stop age-related metabolic function (Hall et al., 2013). Several evidences promote the idea that obesity is linked to altered organ structure and function due to oxidative stress, genetic instability and disturbance of homeostatic pathways (Fig 10) (Russell and Kahn, 2007). Several studies demonstrate that incubation of human endothelial and smooth muscle cells with glucose induces oxidative stress (Ceriello et al., 1996; Du et al., 2000), which has been indicated as one of the main causes of obesity-associated pathologies. Reports show that oxidative stress may be an early event in the development of atherosclerosis, type II diabetes and hypertension (Roberts and Sindhu,2009).

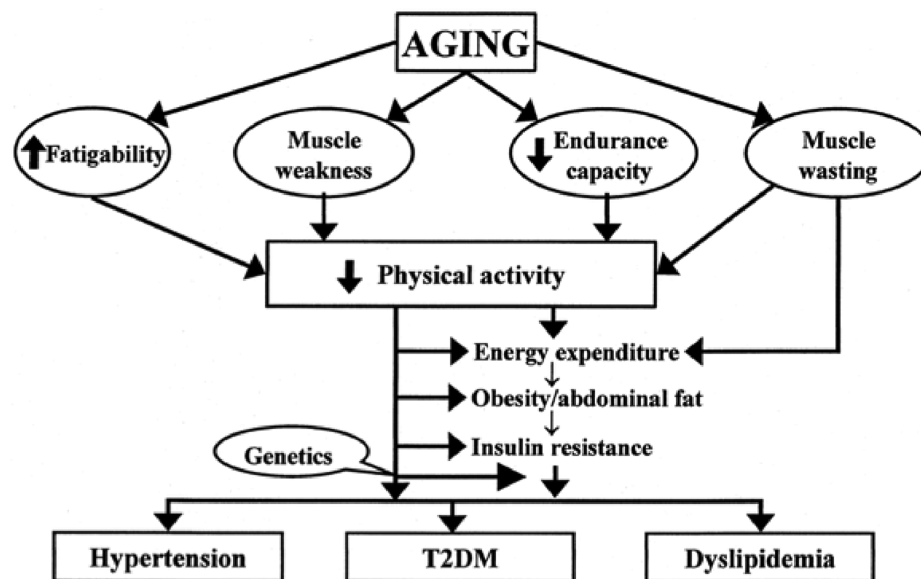


Fig 10 – Link between aging, physical activity and metabolic syndrome in skeletal muscle. In skeletal muscle, aging is related with a decrease in muscle mass and strength as well as in endurance and an increase of fatigability. Together these muscle conditions causes a reduction in physical activity. This reduction in energy expenditure potentiates obesity and abdominal fat accumulation, causing insulin resistance and contributing for development of hypertension, type 2 diabetes mellitus (T2DM) as dyslipidemia (Nair, 2005).

1.3.1 Mitochondrial alterations and its impact on obesity, aging and metabolic dysfunction

Mutations in mtDNA are associated with myopathies, neuropathies, diabetes, signs of premature aging and reduced lifespan (Vernochet and Kahn, 2012). Perturbation of mitochondrial function and its role in the development of metabolic disorders associated with aging, as well as insulin resistance and obesity-induced diabetes, has become a hot research topic.

Mitochondrial disorders mainly affects brain and muscles due to the high-energy requirements of these tissues. Decline in mitochondrial function and content are reported as the causes for the progressive decrease in skeletal muscle mass and strength that occurs with aging (Figueiredo et al., 2008), as shown by the decrease in oxygen consumption as well as the decline in maximal ATP production rate in aged tissues (Johnson et al., 2013). Indeed, not only do the number of mitochondria decrease in post mitotic tissues like heart, skeletal muscle and brain during aging (Samorajski et al., 1971; Tate and Herbener, 1976), but a number of age-associated structural changes of mitochondria have been reported as well (Tate and Herbener, 1976). Reductions in the expression of genes encoding mitochondrial proteins, as well as in proteins that regulate mitochondrial homeostasis, have been found in several organs of aged mice (Linford et al., 2007; Liu et al., 2004; Melov et al., 2007). Furthermore, accumulation of high levels of point mutations due to proofreading deficiency in mitochondrial polymerase gamma (POLɣ) causes premature aging phenotypes and a dramatic lifespan reduction in a mouse model (Kujoth et al., 2005; Trifunovic et al., 2005).

Data also shows that the decline in enzyme activities in aged skeletal muscle is more pronounced in slow-twitch fibers, which are enriched in mitochondria and responsible for sustaining ATP formation during endurance exercise (Johnson et al., 2013). This reduction in ATP production has been proposed as the basis for age-related reduction in muscle protein turnover (Nair, 2005).

Oxidative capacity in skeletal muscle relies on mitochondrial function and is directly related with insulin sensivity, decreased mitochondrial oxidative phosphorylation is correlated with insulin resistance (Bonnard et al., 2008). The fact that high fat-loads are correlated, in some circumstances, with reduction in mitochondrial mass in muscle, liver and adipose tissue suggests that insulin resistance may be triggered by a decrease in mitochondrial mass (Rolo et al., 2011). Moreover, the development of insulin resistance has also been associated with β-cell dysfunction caused by impairments in mitochondrial function (Wiederkehr and Wollheim, 2006), giving further strength to the key role of mitochondrial dysfunction in the development of diabetes.

Declines in mtDNA and in mitochondrial mass, reduced expression of genes related with

mitochondrial biogenesis, the TCA cycle and the ETC, have been shown to contribute to dysregulation of fuel metabolism creating an energy-deficient state in the muscle of pre-diabetic and diabetic patients as well as in mouse models of obesity (Kelley et al., 2002; Mootha et al., 2003; Lowell and Shulman, 2005; Crunkhorn et al., 2007; Koves et al., 2008; Toledo et al., 2013). Altered mitochondrial morphology as shown by swelling, fewer cristae and sometimes disruption of the inner and outer membranes (Bonnard et al., 2008) have been described in obesity and type 2 diabetes. However, mitochondrial plasticity is protected in the obese/insulin-resistant state since the overall oxidative capacity of mitochondria in skeletal muscles can improve just by modification in lifestyle, such as calorie restriction and intensive exercise training (Toledo et al., 2013). Indeed, caloric restriction (restriction of caloric intake by 30-40%) is the only known non-pharmacological intervention that extents lifespan in humans, and has been shown to induce mitochondrial biogenesis and prevent age-related mitochondrial decline (Gredilla et al., 2001; Sanz et al., 2005; Lopez-Lluch et al., 2006).

PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes (Patti et al., 2003; Rolo and Palmeira, 2006; Bonnard et al., 2008), being PGC-1α-related stimulation of mitochondrial biogenesis important for mitochondrial adaptations to aerobic exercise in rodents and humans, however not mandatory (Jonhson et al., 2013). AMPK-dependent modulation of mitochondrial biogenesis in response to nutrient or exercise stimuli, is also affected by aging. With age, AMPK signaling is attenuated leading to decreased mitochondrial protein synthesis and accumulation of damaged mitochondrial proteins (Johnson et al., 2013).

1.3.2 Mitochondrial oxidative stress, obesity and aging

ROS, formed as byproducts of normal mitochondrial oxidative phosphorylation, are necessary for redox signaling and regulation of mitochondrial and cellular metabolism. However, mitochondrial dysfunction results in increased ROS generation, with ROS causing oxidation of biomolecules and further impairing mitochondria (Dan Dunn et al., 2015). An increase in mitochondrial ROS generation has been correlated with the development of obesity and aging-related pathologies (James et al., 2012). For example, one of the main causes for neurodegenerative diseases as Parkinson's or Alzheimer's disease is aging, which is induced by inherited or age-accumulated mutations in mtDNA and increased net production of ROS, due to an imbalance between ROS removal and productive (Lin and Beal, 2006).

Mitochondria contain numerous enzymatic complexes involved in antioxidant defense and intermediary metabolism pathways, which play a role in nutrient adaptation. Within mitochondria,

superoxide anion produced by one-electron reduction of molecular oxygen is quickly decomposed to hydrogen peroxide in a reaction catalyzed by MnSOD. Previous studies revealed that mice lacking MnSOD suffer from metabolic disruption besides hypothermia, cardiac hypertrophy, growth retardation and accumulation of fat in skeletal muscle and liver (Li et al., 1995). Also MnSOD prevents the inactivation by superoxide anion of aconitase, an enzyme involved in TCA cycle (Li et al., 1995). In functional mitochondria, production of superoxide is low because electron carriers are oxidized and respiratory rate and proton motive force are decreased (Murphy, 2009). Overnutrition conditions result in increased formation of reducing equivalents due to higher availability of energy substrate in the form of glucose or free fatty acids. In these conditions, NADH and succinate generated in the tricarboxylic acid (TCA) cycle exceed the substrate oxidizing capacity of the ETC, resulting in mitochondrial hyperpolarization and higher transference of single electrons to molecular oxygen (Fig 11) (James et al., 2012; Teodoro et al., 2013). The increase in mitochondrial superoxide anion results in higher levels of hydrogen peroxide, which inhibits pyruvate dehydrogenase kinase 2 and in turn activates the pyruvate dehydrogenase complex (PDC), promoting the flow of acetyl-CoA-derived from glucose oxidation into the TCA cycle. Since this mechanism is coupled with inhibition of aconitase by superoxide, further oxidation of citrate is blocked, which is then deviated to the cytoplasm to be stored as fat, resulting in the decrease of circulating glucose, although the impairment of oxidative capacity (James et al., 2012). This response allows mitochondria to sense and respond accordingly to cellular conditions by slowing the TCA cycle and oxidation of fatty acids, while maintaining the oxidation of glucose to pyruvate (Fig 11).

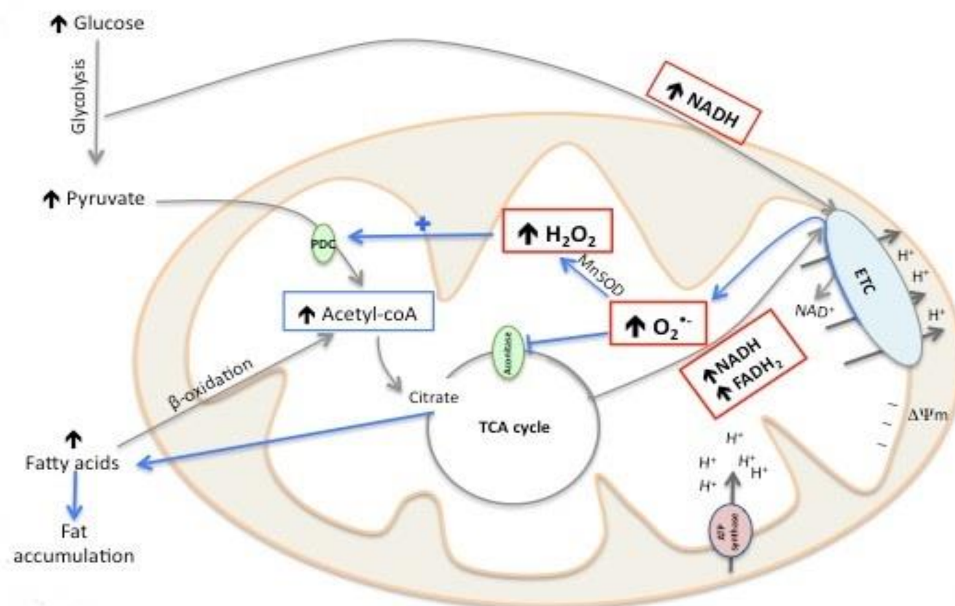


Fig 11 - Mitochondria as a sensor of cellular conditions. Normal mitochondrial bioenergetics is impaired by increased ROS generation under conditions of overnutrition and physical inactivity. Increased availability of glucose or free fatty acids results in increased delivery of electron donors to the ETC, exceeding its oxidizing capacity. This results in hyperpolarization of the mitochondrial membrane potential and a higher reduced state of the ETC complexes, favoring side reactions between semiquinones and O_2 that form superoxide anion (O_2•-). Under normal conditions, the mitochondrial MnSOD rapidly converts superoxide anion (formed at complexes I and III) to H_2O_2, but the sustained higher rate of NADH formation (due to overnutrition and physical inactivity) leads to an unbalance between ROS formation and antioxidant defenses, resulting in oxidative stress. A series of feedback steps are triggered in order to modulate metabolism, by slowing down the TCA cycle and decreasing the delivery of reducing equivalents to the ETC due to superoxide anion-dependent inhibition of aconitase. This is coupled to activation of the pyruvate dehydrogenase complex (induced by increased formation of H_2O_2) funneling acetyl-CoA derived from glucose into fatty acid synthetic pathway. This results in the decrease of glucose circulating levels and the accumulation of fatty acids (blue line).

It has been proposed that oxidative damage caused by hyperglycemia is the underlying cause for cumulative changes in long-lived macromolecules, which persist despite restoration of euglycemia (Rolo and Palmeira, 2006). Although the described attempt to decrease ROS by slowing down mitochondrial oxidative pathways and increasing acetyl-CoA funneling into fatty acid synthesis under overnutrition conditions, type II diabetes, whose development is linked to obesity

and insulin resistance, is associated with an increase in lipid, protein and DNA oxidation. Glycoxidation and lipoxidation products are increased in plasma and tissue from diabetic patients (Roberts and Sindhu,2009). Interestingly, hyperglycemia-induced overproduction of superoxide has been shown to inhibit glucose-6-phosphate dehydrogenase, the rate-limiting enzyme of the pentose phosphate pathway that provides reducing equivalents (NADPH) to the antioxidant cellular defense system (Nishikawa et al., 2000; Zhang et al., 2000).

States of insulin resistance or type II diabetes are associated with an alteration in free fatty acid metabolism as well as triglyceride accumulation. This promotes lipid peroxidation, linked to protein oxidation and mitochondrial dysfunction, as supported by studies in skeletal muscle of obese insulin resistant subjects (Russel et al., 2003; Roberts and Sindhu,2009). Visceral accumulation of triglycerides alters the panel of cytokine production by the white adipose tissue, which is linked to a state of systemic oxidative stress (Furukawa et al., 2004; Evans et al., 2005). Adipocytes produce a variety of biologically active molecules known as adipokines including leptin, TNF-α (tumor necrosis factor-alpha) and adiponectin, with pro- or anti-inflammatory action. The pathogenesis of obesity-associated metabolic syndrome is related with dysregulated production of these adipokines, with increased release of pro-inflammatory molecules such as TNF-α (Hotamisligil et al., 1993; Uysal et al., 1997). Conversely, plasma levels of adiponectin are inversely related with oxidative stress, with oxidative stress conditions causing downregulation of adiponectin expression (Furukawa et al., 2004). Studies with obese mice have shown an increase in hydrogen peroxide formation observed first in adipocytes, and only after in liver, aorta and skeletal muscle, suggesting that adipose tissue is the source of increased plasmatic ROS (Furukawa et al., 2004). It was proposed that upregulation of the expression of NADPH oxidase, observed in adipocytes from obese animals, combined with decreased antioxidant defenses, results in a vicious cycle of oxidative damage and increased release of pro-inflammatory adipokines, inducing systemic alterations (Fig 12) (Furukawa et al., 2004).

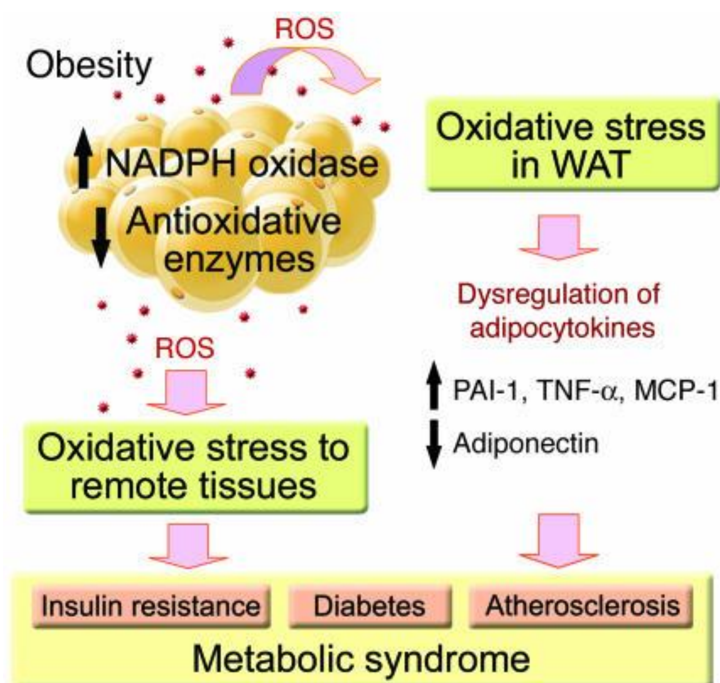


Fig 12 - Alterations of redox status due to increased NADPH oxidase in obesity are linked to the development of metabolic syndrome. Fat accumulation in white adipose tissue (WAT) induces NAPDH oxidase and a decrease in antioxidant defenses, creating a vicious and systemic cycle of oxidative damage that alters the panel of adipokines release by the WAT and damages other organs including liver and skeletal muscle (Furukawa et al., 2004).

An association between oxidative stress in skeletal muscle and mitochondrial dysfunction has been established in the context of obesity and aging (Lee et al., 2012b). Bonnard et al showed that, in skeletal muscle of diet-induced diabetic mice, intramyocellular lipid accumulation is linked to an increase in both mitochondrial and cytoplasmic ROS production, causing mitochondrial dysfunction. These effects were reversed by normalization of glycemia or antioxidant treatment. This work proposed that mitochondrial alterations do not precede the onset of insulin resistance but increased mitochondrial ROS induced by high glucose/fatty acids is the initial trigger promoting mitochondrial alterations, lipid accumulation, and insulin resistance (Nishikawa et al., 2000; Schrauwen and Hesselink, 2004; Evans et al., 2005; Bonnard et al., 2008). As discussed earlier, high glucose/fatty acids conditions causes the increase in electron leakage from the ETC, creating a vicious cycle that causes mitochondrial dysfunction (Bonnard et al., 2008; Johnson et al., 2013). Proteins, lipids and DNA damage impair mitochondrial functions and biogenesis, contributing to altered bioenergetics and accumulation of lipids in the muscle (Bonnard et al., 2008).

Although their physiological functions, ROS are involved in numerous cell- and tissue-alterations. In skeletal muscle ROS are decisively involved in the pathogenesis of fatigue, aging or exercise-induced muscle damage (Kerkweg et al., 2007). Studies showed that small increases in ROS induce hormetic signals in order to delay its accumulation to detrimental levels and consequently extending lifespan (Wei et al., 2015). Perturbation of mitochondrial function can accelerate or delay the aging process; by acting as messengers mitochondrial ROS are able to induce multiple mitochondrial stress responses like mitophagy and mitohormesis. Mitochondrial proteins with minor damage are degraded by proteases, while severe mitochondrial damage with drastic loss of membrane potential induces fission and degradation by mitophagy (Johnson et al., 2013). These mechanisms are disrupted in the skeletal muscle of older mice. In aged cells, an increase in ROS can cause mitochondrial dysfunction due to damage in cellular lipids, proteins and mtDNA (Johnson et al., 2013). Caloric restriction that is known to increase lifespan by delaying the aging process in several species, including flies and mice (Weindruch et al., 1997), has been shown to control cellular ROS production and damage on cellular macromolecules in various tissues. In mammals, CR reduces generation of ROS by mitochondria as well as improves energetic status in the whole body, by inducing mitochondrial proliferation related with AMPK activation (Bevilacqua et al., 2004).

Strategies preventing oxidative stress but promoting mild ROS-mediated protective response may also be beneficial in treating metabolic-related pathologies. A protective response mediated by ROS and involving mitochondria is supported by studies with a mouse model exhibiting disruption of the mitochondrial transcription factor A (TFAM) in the adipose tissue. These animals (F-TFKO) exhibit decreased mtDNA copy number, altered ETC protein content, decreased complex I activity but higher rates of uncoupled-oxygen consumption (Vernochet et al., 2012). Interestingly, exposure of these animals to stress conditions such as high-fat, shows that although the increase in oxidative stress markers in adipose tissue, its oxidative capacity is increased and a resistance to diet-induced obesity, insulin resistance and fatty liver is observed (Fig 13) (Vernochet et al., 2012).

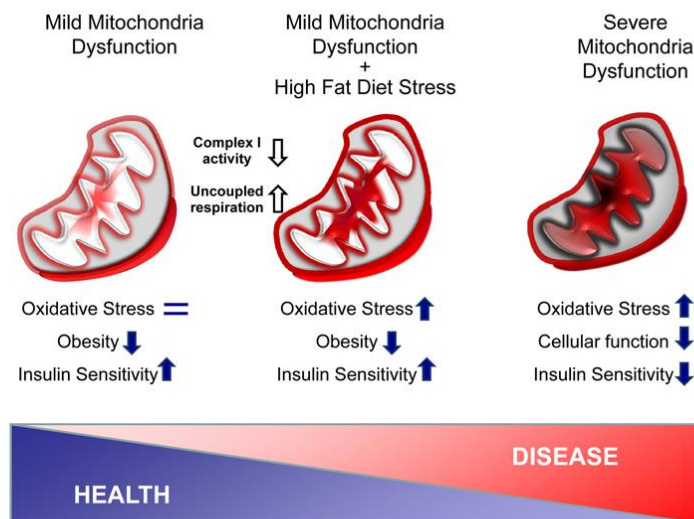


Fig 13 – Effects of different levels of ROS in mitochondria and health. A short and mild stress like small increases in ROS formation induce hormetic signals that trigger an adaptive and protective response, able to extend lifespan and to prevent the development of metabolic alterations associated with overnutrition. In contrary, severe mitochondrial dysfunction is linked to a vicious cycle of oxidative damage, impairing cellular homeostasis (Vernochet and Kahn, 2012).

1.4 Sestrins: a stress-inducible family essential for cell survival

In the 90's, the sestrin family was isolated and identified as a conserved family of proteins, ubiquitously expressed in all adult tissues, although at different levels (Budanov et al., 2010). Sestrins expression is up-regulated in response to stress like DNA damage, oxidative stress, hypoxia, aging, obesity, nutrient signaling, diabetes and cancer (Budanov et al., 2010; Lee et al., 2010a; Lee et al., 2010a). All members of sestrin family are induced by oxidative stress, although, induction mechanisms are different. Three isoforms encoded by three independent loci were described: p53-activated gene 26 (PA26) encodes for sestrin 1 or (SESN1) and hypoxia-inducible gene 95 (HI95) encodes to sestrin 2 (SESN2), and sestrin 3 (SESN3) was identified as a FOXO target (Velasco-Miguel et al., 1999; Budanov et al., 2002; Nogueira et al., 2008).

Several studies were performed in order to better understand the biochemical functions associated to sestrins and in 2004 Budanov and colleagues reported the antioxidant function of SESN1 and SESN2 as regenerators of peroxiredoxins (PRX) (Budanov et al., 2004). Later it was found that SESN2 also induces Nrf2-dependent antioxidant gene transcription, through stimulation of the autophagic degradation of KEAP1 (Bae et al., 2013). Another important function of SESN2 is the capacity to suppress mTORC1 through direct association with AMPK or through indirect transcriptional regulation by LKB1 (Serine/threonine kinase 11 or STK11) (Budanov and Karin 2008;

Chen et al., 2010). SESN2 can activate AMPK in response to alterations in the redox state of cells, leading to mTOR inhibition and autophagy activation (Budanov AV and Karin M, 2008; Hay N, 2008). In all cases, SESN2 activation extends lifespan: increased antioxidant capacity decreases ROS and inhibits apoptosis through regeneration of PRX while inhibition of mTORC1 promoting autophagy mechanisms is also responsible for elimination of dysfunctional mitochondria (Chang et al., 2004;.Lee et al., 2013; Li et al., 2013)

1.4.1 SESN2: regulation and role in antioxidant response

Genetic deficiency in the SESN2 gene has been shown to accelerate aging and obesity-related pathologies, precipitating the development of glucose intolerance, insulin resistance, fatty liver, muscle degeneration and mitochondrial dysfunction (Lee et al., 2012a; Lee et al., 2013). This work highlighting the essential role of SESN2 lead research into the investigation of the mechanisms involved in the regulation of SESN2 expression/activity under physiological and pathological situations.

SESN2 inhibits ROS accumulation, which is linked to regeneration of 2-Cys PRXs and consequent modulation of cellular hydrogen peroxide concentration (Sanchis-Gomar, 2013). Peroxiredoxins act as H_2O_2 scavengers which cause PRX to be in an inactive oxidized form until sestrins restore its catalytic activity (Sanchis - Gomar, 2013). The mammalian family of PRXs is composed by six members that encode proteins in different cellular compartments. PRX3 is the mitochondrial form responsible for scavenging mitochondrial H_2O_2. H_2O_2 oxidizes the redox – sensitive Cys residue of each PRX subunit to Cys-SOH, which reacts with another Cys-SH residue, creating a disulfide bridge (Chang et al., 2004). This disulfide bound is then indirectly reduced by SESN2 and sulfiredoxins (SRXs), through Nrf2 activation (Budanov et al., 2008; Budanov, 2011; Bae et al., 2013). It has been recently described that SESN2 is necessary for SRX activation in liver. It was demonstrated that SESN2 promotes the autophagic degradation of KEAP1 mediated by sequestossome 1 (p62), increasing Nrf2 translocation to the nucleus and activating SRX transcription (Rhee and Bae, 2015). Depletion of PRX3 leads to induction of the mitochondrial apoptotic pathway, associated with increased ROS, mitochondrial membrane permebilization and release of pro-apoptotic factors such as cytochrome c, initiating the apoptotic cascade which results in caspase activation (Chang et al., 2004).

Genotoxic conditions induce SESN2 expression mediated by p53 upregulation (Velasco-Miguel et al., 1999; Budanov et al., 2002). The tumor suppressor p53, the guardian of the genome, is activated by genotoxic damages, oxidative damage, nutrient deprivation and hypoxia (Budanov, 2011). Studies on the role of p53 in antioxidant defense came up with the discovery of SESN2 as a

target (Sablina et al., 2005), with oxidative environment activating p53 and inducing SESN2 expression. Concerning hypoxia, in cancer cell lines is known that hypoxic conditions up-regulate SESN2 in p53-independent manner (Budanov et al., 2002) and accordingly, studies in mouse epithelial tracheal cells have shown that transcriptional activation of SESN2 is hypoxia-inducible factor 1 (HIF-1) dependent (Olson et al., 2011). However, other studies have also shown HIF-1-independently hypoxic induction of SESN2, probably activated by pathways involved in energy deprivation due to prolonged hypoxia since several compounds that reduce ATP content, like glycolysis inhibitor (2-deoxyglucose) or inhibitor of mitochondrial respiration (metformin) induce SESN2 (Budanov et al., 2002; Ben-Sahra et al., 2013).

Interestingly, in 1997 Polyak and collaborators showed that p53 activation mediates a pro-oxidant response to stress, tightly related with apoptosis induction. This work showed that p53 stimulation is responsible for the activation of oxidorreductase genes that increase mitochondrial ROS formation and lead to caspase activation (Polyak et al., 1997). Years later, (Sablina et al., 2005) demonstrated that p53 plays a dual role depending on the stress level and has a pro-oxidant function that results in apoptosis induction, but it can also induce antioxidant defenses such as SESN2 activation (Fig 14). This protective antioxidant response dependent on SESN2 activation preserves cell viability, as shown by previous studies in which absence of SESN2 sensitizes cells to stress-induced apoptosis (Ben-Shara et al., 2013).

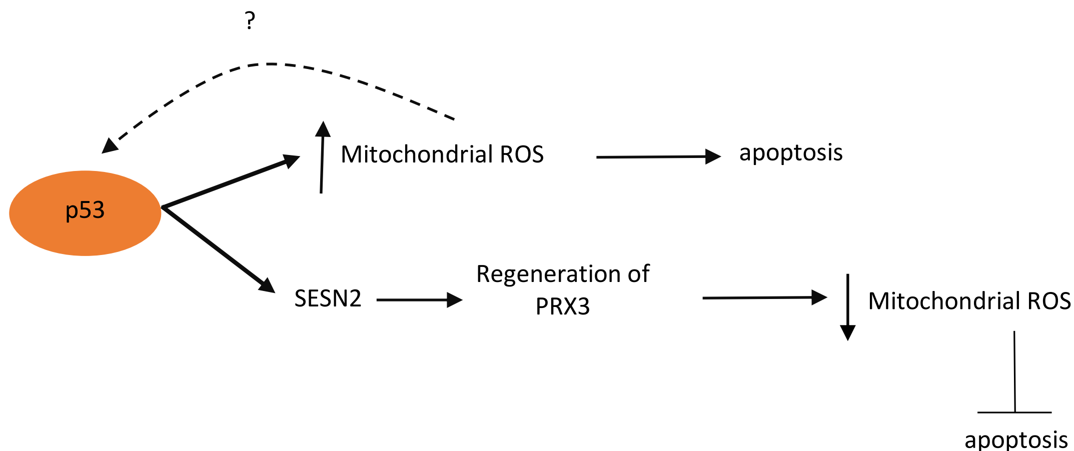


Fig 14– Model of p53 dual role. p53 activation results in increased ROS generation or induction of an antioxidant response depending on cell type and stimuli.

p53 has been pointed as a major regulator of mTORC1 with p53 activation impairing mTORC1 signaling (Hay, 2008). Studies have shown that SESN2 is essential for the inhibition of mTORC1 by p53, resulting in autophagy induction (Fig 15) (Budanov et al., 2010). Although the

precise mechanisms remains to be established, excessive intracellular ROS results in p53 activation and consequently increases SESN2 expression. SESN2 interacts with the α-catalytic subunits of AMPK, therefore promoting AMPK activation (Hay, 2008). This association between SESN2 and AMPK recruits and induces TSC1:TSC2 (tuberous sclerosis 1 and 2) complex activation by phosphorylation of TSC2. Activation of TSC1:TSC2 complex leads to inhibition of a small GTPases, Ras homologues enriched in brain (RHEB) which inhibits mTORC1 activity (Budanov et al., 2010). Inhibition of mTORC1 promotes dephosphorylation of ATG13, leading to autophagosome formation and activation of autophagy (Budanov et al., 2010).

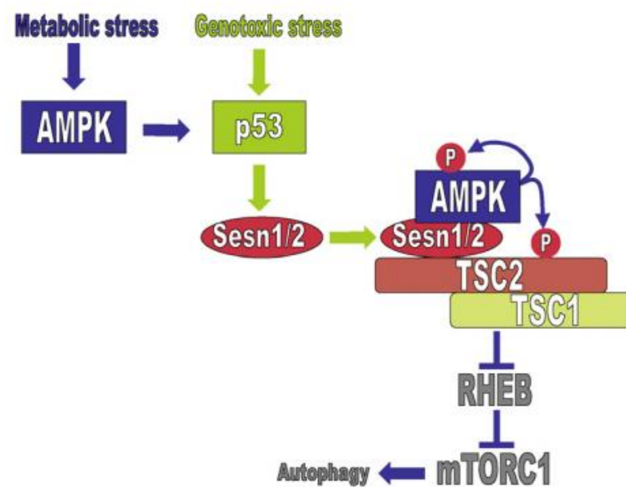


Fig 15 - Links between oxidative/genotoxic stress and Sestrin 2. Oxidative and genotoxic conditions induce both p53 and SESN2. Activation of SESN2 promotes AMPK phosphorylation and activation forming a complex that then binds to TSC2 and promotes its phosphorylation and consequent activation. This results in RHEB and mTORC1 inhibition and promotes autophagy. Metabolic stress can also activate p53 through activation of AMPK (adapted from Hay, 2008).

Therefore, SESN2 is an essential regulator of cellular antioxidant defense and critical for maintaining a basal level of autophagy. Under stress conditions, this mechanism is vital to cellular homeostasis due to possibly stimulating elimination of dysfunctional mitochondria and prevention of a vicious cycle of oxidative damage.

1.4.2 SESN2: role in metabolic homeostasis

SESN2 has been shown as the only sestrin activated by obesity and metabolic syndrome with beneficial effects related to mTORC1 modulation (Lee et al., 2013). Since strategies to extend

lifespan, such as caloric restriction, results in mTORC1 inhibition and induction of autophagy, and taking into account the connection SESN2-mTORC1 (Budanov et al., 2010), it was proposed that SESN2 induction has an anti-aging effect by preserving metabolic homeostasis.

The role of SESN2 in metabolism is also supported by its role in tumor progression, although still matter of discussion. Cancer is a pathology highly linked to genomic instability, metabolic dysregulation and oxidative stress. SESN2 induction as response to DNA damage, contributes to tumor suppressor functions of p53, suppressing cell growth plus cellular senescence by mTORC1 inhibition (Lee et al., 2010a). It is known that in cancer cells the mTORC1 pathway is frequently activated, while SESN2 was found downregulated in several cancer cell types, due to inactivation of p53 (Loayza-Puch et al., 2013). SESN2 downregulation promotes chronic activity of mTORC1 pathway and consequently inhibition of autophagy, thus favoring cancer development (Budanov et al., 2010). Therefore loss of SESN2 results in cells more susceptible to oncogenic transformation (Budanov and Karin, 2008). Recently, a mutation in SESN2 gene was shown associated with myeloproliferative neoplasm however, how the mutation affects SESN2 is not yet defined (Hou et al., 2012). Despite the clear impact of SESN2 in tumor suppression and genome protection, sestrins are still active in many cancers and moreover, are necessary for maintenance of cancer cells under certain conditions (Budanov et al., 2002; Lee et al., 2013). Up-regulation of sestrins expression is beneficial for cancer cells in order to fight high levels of ROS, chronic inflammation and prolonged growth factor signaling, that can be harmful for survival and propagation of tumor cells (Lee et al., 2013). Additionally, sestrins up-regulation and activation of autophagy can support tumor growth and metabolism under conditions of limited oxygen supply, as the ones found in the tumor microenvironment (Maiuri et al., 2009; Ishihara et al., 2013)

As already established, mTORC1 hyperactivity is related with the development of several metabolic pathologies such as obesity and type II diabetes. For example, inhibition of autophagy causes hepatic lipid accumulation (Budanov et al., 2010). Also mTORC1 can promote lipid synthesis due to an increase in activity of the lipogenic transcription factor sterol response element binding protein (SREBP) and in its targets, such as, fatty acid carboxylase, acetyl-CoA carboxylase (ACC), acetyl-CoA synthase and fatty acid synthase (Laplante and Sabatini, 2009). Through substrate S6K (S6 Kinase), mTORC1 activation induces an inhibitory phosphorylation of insulin receptor substrates (IRS), attenuating PI3K/AKT (Phosphatidylinositol 3-phosphate/protein kinase B) signaling with consequent development of insulin resistance (Um et al., 2004; Budanov et al., 2010). Sestrins up-regulate AKT signaling in an AMPK and mTORC2 dependent manner, which suggest that sestrins modulate signaling activity between mTORC1 and mTORC2 through AMPK (Lee et al., 2013) In

obesity conditions, SESN2 was shown to be essential for the maintenance of blood sugar homeostasis, insulin response in adipose tissue and suppression of insulin resistance in hepatocytes (Lee et al 2012 a; Lee et al., 2013). Chronic inhibition of autophagy in liver and endoplasmic reticulum (ER) stress both induced by activation of mTORC1, can attenuate insulin signaling and induce insulin resistance (Ozcan et al., 2008; Yang et al., 2010) Consequently, prolonged activation of mTORC1 in response to prolonged overnutrition can induce insulin resistance, type II diabetes and increase in blood glucose (Lee et al., 2013). Pharmacological inhibitors of mTOR are an inappropriate treatment for metabolic pathologies since mTORC2 inhibition stimulate insulin resistance. Thus, attenuation of mTORC1 by sestrins may constitute an alternative for prevention of diabetes, obesity and insulin resistance (Lamming et al., 2012).

1.5 Sirtuins, the deacetylase superfamily

Sirtuin family members were first identified in *Saccharomyces cerevisiae* as silence information regulators (SIRs) by Rine and Herskowitz, 1987. The founding sirtuin (SIRT) family member is pointed as the SIR2 in *S.cerevisiae*, but there are seven homologs in humans, SIRT1-7 divided in four classes after phylogenetic analysis. SIRT1 is the most studied mammalian sirtuin, SIRT2 is present primarily in the cytoplasm but in G2/M phase shuttles to the nucleus, SIRT3, SIRT4 and SIRT5 are found in mitochondria and are involved in responses to oxidative stress and in energy metabolism, SIRT6 and SIRT7 are predominantly found in nucleus and have been implicated in cell proliferation and genomic stability (North et al, 2003; Li et al., 2007;Gan et al., 2008; Fiorino et al., 2014).

Phylogenetically conserved from bacteria to humans, sirtuins regulate cell functions by deacetylating both nonhistone and histone targets. Acetylation of lysine residues of histone tails is one of the most studied post-translational modifications and results from the balance between histone deacetylase (HDAC) and histone acetyltransferase (HAT) activity (Fiorino et al., 2014). Eukaryotic HDACs belong to an ancient family of proteins constituted by two subfamilies that have different HDAC activity, sirtuins family and the classical HDAC family. Sirtuins activity is dependent on nicotinamide adenine dinucleotide (NAD^+) as a substrate for removal of acetyl group from proteins, that is transferred from the lysine side chain of a substrate to NAD^+, generating deacetylated proteins, nicotinamide and O-acetyl-ADP-ribose (Fiorino et al., 2014; Gan et al., 2008).

Mammalian sirtuins share a conserved core domain, including a large well-organized and conserved Rossman domain characteristic of NAD^+/NADH binding proteins, a more variable smaller zinc-binding domain and a NAD^+ binding site between these domains. Differing among sirtuins are

zinc domains, proved to be the cause for the different activities of these proteins (Fiorino et al., 2014). These proteins link transcriptional regulation directly to intracellular energetics, being involved in the coordination of several cellular functions, such as metabolism, cell cycle, DNA damage response, apoptosis and autophagy. N- and C-terminal flanking regions differ in length and sequence among sirtuins, also explaining differences in sirtuins activities with protein-specific regulatory roles (Fiorino E et al., 2014).

Depending on cell type and pathophysiological circumstances, activation of a given sirtuin can induce diverse outcomes. For example, in cells with DNA damage, activation of p53, FOXO and SIRT1 promotes cell-cycle arrest and leads to survival by inhibiting apoptosis. However, in tumor cells with damaged DNA but without p53 or FOXO, SIRT1 activation promotes tumorigenesis by allowing damaged cells to proliferate (Gan et al., 2008).

1.5.1 SIRT1: Regulation and role in metabolic homeostasis

SIRT1 is present in several organs such as liver, brain, pancreas, heart, muscle and adipose tissue and its subcellular localization is influenced by different factors (Fiorino et al., 2014). Although mainly localized in the nucleus, in specific conditions this protein can shuttle to the cytoplasm, allowing the control of nuclear and cytoplasmic proteins. SIRT1 is the HDAC with the best-characterized regulation that occurs at multiple levels. It can be regulated by transcription factor such as FOX01, peroxisome proliferator-activated receptors α (PPARα) and cAMP (cyclic adenosine monophosphate) response element-binding protein (CREB), under calorie restriction (Hayashida et al., 2010; Noriega et al., 2011). Additionally, its expression is reduced by poly (ADP-ribose) polymerase 2 (PARP2) that is involved in DNA repair and apoptosis (Fiorino et al, 2014). Regulation of SIRT1 also includes post-transcriptional modifications mediated by CyclinB/cyclin-dependent kinase 1 (CDK1) complex and c-Jun N-terminal kinase (JNK) in order to lead SIRT1 to specific targets and increase its activity (Sasaki et al., 2008; Nasrin et al., 2009). Additionally to regulation by NAD^{+}, SIRT1 is regulated by complex formation and usually complexes formed between sirtuins and other proteins negatively regulate SIRTs activity (Fiorino et al, 2014).

As previously discussed, maintenance of energy homeostasis requires a tight balance between energy intake, storage and expenditure, dependent on modulation of metabolism by metabolic sensors. In mammals, SIRT1 acts as a pleiotropic energy sensor activating an appropriate physiological response to changes in nutritional status through deacetylation of key factors in several organs including PGC-1α, PPARα and HIF-1, among others (Fig 16) (Haigis and Sinclair, 2010).

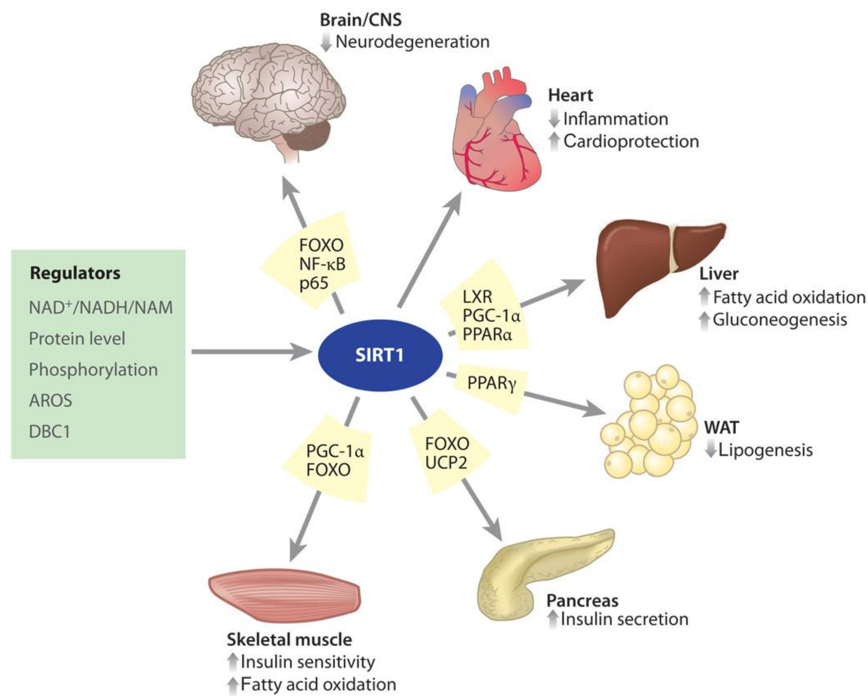


Fig 16 – Role of SIRT1 in metabolism. SIRT1 activation protects against metabolic diseases. SIRT1 is a pleiotropic energy sensor that activates an appropriate physiological response to changes in nutritional status through deacetylation of key factors in several organs. Sirtuins activity is dependent on nicotinamide adenine dinucleotide (NAD^+) as a substrate for removal of acetyl group from proteins. NAM – Nicotinamide; DBC1, deleted in breast cancer1; WAT, white adipose tissue; CNS , central nervous system; FOXO, forkhead box transcription factor, subgroup O; LXR - liver X receptor; NF-kB - nuclear factor kappa B; PGC-1α**,** peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PPARα**,** peroxisome proliferator activated receptor alpha; UCP2, uncoupling protein 2 (Haigis and Sinclair, 2010).

Due to the key metabolic role of mitochondria in bioenergetics, maintenance of mitochondrial homeostasis, including modulation of biogenesis, is an essential process for the prevention and treatment of metabolic disorders. SIRT1 regulates mitochondrial biogenesis by deacetylating and activating PGC-1α (Gerhart-Hines et al., 2007), increasing mitochondrial mass and function in several tissues (Lagouge et al., 2006). In neurodegenerative disease, more than 20% of the proteins acetylated on lysine residues are proteins with a role in longevity and metabolism. PGC-1α activation by SIRT1 has also been shown to exert protection against neuronal injury induced by hydrogen peroxide (Gan et al., 2008). As such, in the last years intense research has been evaluating SIRT1 activators as therapeutic approaches against chronic stress and aging-related pathologies (Hall et al., 2013).

Enhanced activity of PGC-1α due to deacetylation by SIRT1 increases the expression of transporters and catabolic enzymes essential for the uptake and oxidation of free fatty acids (Fig 17). This mechanism is accentuated during exercise in skeletal muscle (Hall et al., 2013). Thus, increased activity of SIRT1 protects from metabolic dysfunction associated with obesity by reducing blood glucose and plasma insulin levels and through maintenance of β-cell integrity and function (Hall et al., 2013). Attenuation of inflammatory responses by SIRT1 diminishes hepatic fat accumulation induced by overnutrition, ROS and adipose tissue inflammation (Hall et al., 2013). However, is important take in account the context and tissues specificity to apply SIRT1-targeted therapy (Hall et al., 2013).

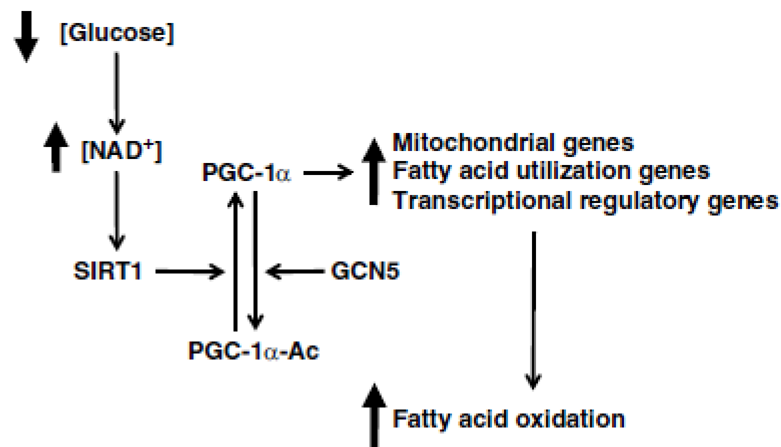


Fig 17 – Glucose control of fatty acid oxidation through SIRT1/PGC-1α. Decline in glucose blood glucose levels activate SIRT1 that deacetylates PGC-1α, inducing genes of mitochondrial function and fatty acid oxidation. This mechanism protects skeletal muscle cells from metabolic dysfunction (Gerhart-Hines et al., 2007). GCN5 - Histone acetyltransferase

1.5.1.1 SIRT1: Regulation of hepatic metabolism

Liver is the body's second largest organ and is involved in the regulation of several aspects of lipid metabolism, like fatty acid oxidation, lipoprotein uptake and secretion and lipogenesis. As such, dysregulation of lipid metabolic pathways leads to the development of non-alcoholic fatty liver and contributes to chronic hepatic inflammation, insulin resistance and liver damage (Sanyal, 2005). SIRT1 has been pointed as a regulator of hepatic fatty acid metabolism through several mechanisms, including inhibition of lipogenesis (Fig 18) (Haigis and Sinclair, 2010). Liver X receptors (LXRs) are members of the nuclear superfamily receptors of the ligand-activated transcription factors and act as lipid sensors that increase lipid synthesis in the liver, regulating insulin-stimulated lipogenesis (Jin et al., 2013). In animals with NAFLD (nonalcoholic fatty liver disease) the mRNA of

LXRα is elevated suggesting that LXRα induces NAFLD by regulation of lipogenic genes that promote fat accumulation (Jin et al., 2013).

As reported, SIRT1 is a LXR positive regulator. SIRT1 deacetylates LXRs at lysine K432, in an adjacent region to the ligand activation domain and, consequently, activates LXR target gene transcription of the encoding the ATP-binding cassette transporter (ABCA1), important in HDL synthesis and reverse cholesterol transport (Fiorino et al, 2014). Accordingly, Chen and colleagues 2008, demonstrated that SIRT1 LKO (lacking SIRT1 in the liver) mice avoid deacetylation and activation of LXR, decreasing hepatic expression of LXR targets like ABCA1, SREBP1C (sterol regulatory element-binding protein 1) and lipogenic enzyme fatty acid synthase (FAS). The polyphenol resveratrol was early described by Howitz et al 2003 as the most potent SIRT1 natural activator and, in the majority of studies in *S. cerevisae, C. elegans* and *D. melanogaster* it was reported to increase lifespan in a Sirtuin-dependent manner (Howitz et al., 2003). In obese mice, resveratrol induces a variety of health benefits, including improvement of vascular function, decreased fatty liver, greater endurance, decreased insulin resistance, increased mitochondrial function and prolonging survival in mice fed a high-fat diet. Both SIRT1 and AMPK have been described as important mediators of resveratrol's beneficial effects (Price et al, 2012; Gan et al., 2008). SIRT1 knockout mice revealed that SIRT1 is required for resveratrol to induce phosphorylation of AMPK, increasing mitochondrial function and biogenesis (Price et al., 2012). In contrast, other studies have shown that resveratrol's effects are independent of this signaling pathway.

Oxidative stress has been pointed as an important factor in NAFLD progression and it is known that antioxidant enzymes activities are decreased in NAFLD patients (Oliveira et al., 2002; Browning and Horton, 2004; Videla et al., 2004). Recently it was shown that treatment of HFD fed-mice with resveratrol increases hepatic SESN2 expression in hepatocytes, which is decreased by HFD (Jin et al., 2013). In addition, it was established that Nrf2 plays an important role in SESN2 induction and that resveratrol activates Nrf2 which is responsible for the inhibition of LXRα-DNA binding ability and LXRα-mediated hepatic steatosis (Shin et al., 2012; Kay et al., 2011; Jin et al., 2013). Accordingly with studies that demonstrate the inhibitory action of resveratrol on LXRα (Jin et al., 2013), the described mechanism was suggested as plausible molecular pathway for resveratrol inhibition of lipogenesis mediated by LXRα (Jin et al., 2013).

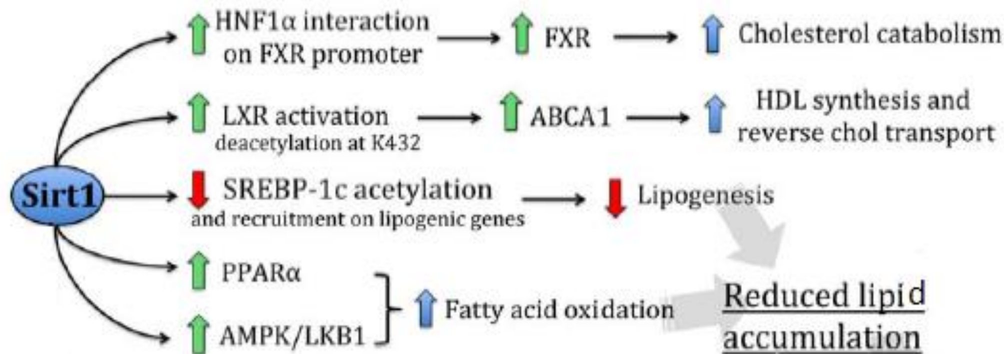


Fig 18 – Central role of SIRT1 in hepatic metabolism. SIRT1 promotes activation of AMPK/LKB1 signaling pathway and PPARα, leading to an increase in fatty acid oxidation and inhibition of lipogenic pathways. Under fasting conditions SIRT1 interacts with SREBP-1c decreasing lipogenesis. In cholesterol homeostasis, SIRT1 activates LXR and HNF1α (hepatocyte nuclear factor 1 alpha) with FXR (farnesoid x receptor) promoter (adapted from Fiorino et al., 2014). Chol- Cholesterol.

Another mechanism by which SIRT1 regulates glucose homeostasis and fat metabolism, involves two members of CCAAT/Enhancer Binding Protein (C/EBP) family, C/EBPα and C/EBPβ. Highly expressed in the liver, these binding proteins play critical roles in liver regulation as transcription factors that interact with several genes promoters. Ectopic SIRT1 expression may correct liver proliferation by reduction of C/EBPα protein but not C/EBPα mRNA (Jin et al., 2011).Taken together, these results suggest a relationship between SESN2 and SIRT1, involving Nrf2.

1.6 Objective

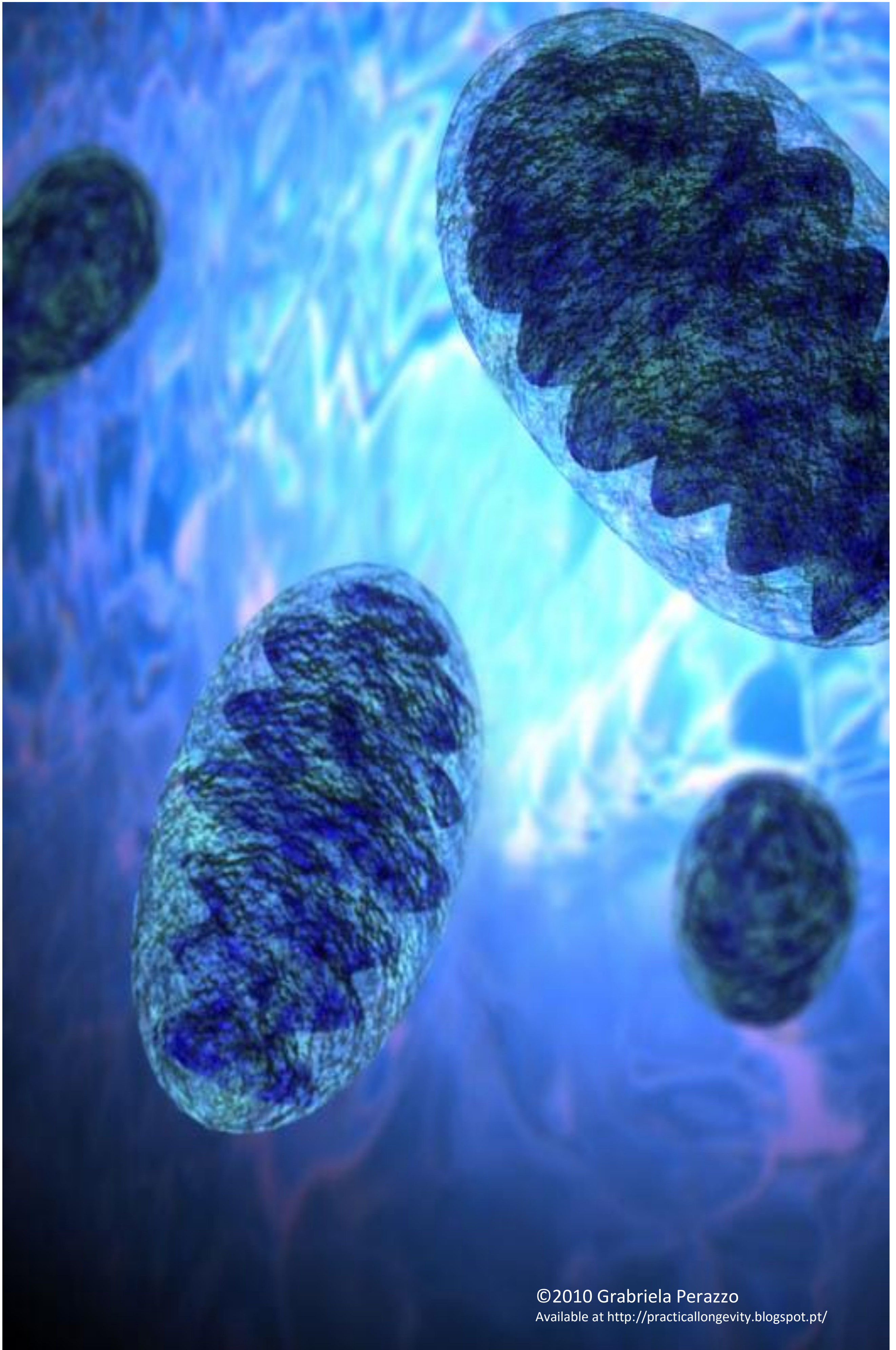
Through the years, mitochondria has been studied and recognized as highly dynamic organelles whose function is crucial for the maintenance of cellular homeostasis. Mitochondria are the main target and generator of ROS in the cell and its dysfunction is linked to metabolic-related pathologies such as obesity and aging. Sestrins are a family of antioxidant proteins and SESN2 is described as one of the main regulators of the cellular antioxidant defense system, in which mitochondria and mitochondrial proteins like peroxiredoxin 3 (PRX3) are involved, promoting metabolic homeostasis. Accordingly with several studies, inactivation of SESN2 results in increased oxidative damage, mitochondrial dysfunction, muscle degeneration and fat accumulation, resulting

in accelerated tissue aging. However, the mechanisms by which SESN2 affects mitochondrial functions are not well defined.

Given the potentially important role of SESN2 in mitochondrial stress response and its impact in preventing disease development, the present work aimed to evaluate if a mild increase in mitochondrial ROS generation, induced by menadione, triggers mitochondrial adaptations dependent on SESN2 activity. Understanding the molecular mechanisms and how SESN2 affects mitochondria may provide new insights for novel therapeutic targets for attenuation and prevention of aging and obesity-related pathologies.

Additionally, and since SIRT1 is a known metabolic sensor and regulator of mitochondrial function, this work evaluated how modulation of SIRT1 affects SESN2 in the context of fatty liver induced by a high-fat diet.

Methods


©2010 Grabriela Perazzo
Available at http://practicallongevity.blogspot.pt/

Except when noted, all compounds were purchased from Sigma-Aldrich (St. Louis, MO). All reagents and chemicals used in this work were of the highest grade of purity commercially available.

2.2 Studies with culture of C2C12 cells

2.2.1 Cell culture

Cells were obtained from American Type Culture Collection (ATCC). The C2C12 cell line is an adherent cell type, derived from mouse C3H muscle myoblast. It differentiates rapidly, forming contractile myotubes and producing characteristic muscle proteins. All cell care and maintenance was done according to the protocol recommended by ATCC. Cells were grown in 75-cm^2 culture flasks at 37 °C in a humidified atmosphere with 5% CO_2 and maintained in Dulbecco's Modified Eagle Medium (DMEM, GIBCO 31600-083), supplemented with 3,7 g/L $NaHCO_3$, antibiotic/antimycotic 1% (Thermo Fisher Scientific) and 10% of fetal bovine serum (FBS, Thermo Fisher Scientific). Cells were passaged or harvested for experiments when reaching 80% of confluence, by detachment with 0,05% trypsin and 0,5 mM EDTA (TrypLE Express, Thermo Fisher Scientific). 5 ml of trypsin TrypLE were added to a T75 and after 5 min in the incubator, 5ml of DMEM with 10% FBS was added to block the action of trypsin. Cells were collected in a 15 ml falcon tube and centrifuged at 200 x *g* for 3 min. Cellular pellet was ressuspended in 12 ml of DMEM with 10% FBS and 1 ml of this dilution was subcultured in case of experiments with differentiated cells, in DMEM with 10% FBS for 1 day and then the medium was replaced by DMEM with 2% Horse Serum (HS, Thermo Fisher Scientific) and were cultured for 6 days. For experiments with undifferentiated cells, cells were counted in a cell counter (TC10 Automated Cell Counter, Bio-Rad Laboratories, Hercules, CA) and subcultured (10000 cells for 6-well plates and 5000 cells for 12-well plates) for 2 days in DMEM with 10% FBS.

For treatments, menadione (prepared in DMSO) was added to culture media to a final concentration of 10 μM or 30 μM, while the control was treated just with DMSO. After 1 h incubation, culture medium was replaced with DMEM containing 2% HS for differentiated cells and with DMEM containing 10% FBS for undifferentiated cells. Cells were incubated for 10, 24 or 48 h of recovery after menadione removal. Cells were checked for morphological alterations prior to use in experiments.

2.2.2 Sulforhodamine B colorimetric assay

The method was originally developed by Shekan and colleagues in 1990. The assay allows the determination of cell density based on the cellular protein content in a simple and accurate way, producing reproducible results. Providing a colorimetric end point that is non-destructive, visible to naked eye and indefinitely stable, Sulforhodamine B (SRB) also affords a sensitive measure of drug-induced cytotoxicity. Cells were seeded in 12–well plates in a total medium volume of 1 ml per well and treated. After treatment with menadione, cells were gently fixed in 12-well plates with ¼ volume of cold 10% (wt/vol) acid trichloroacetic acid (TCA) and incubated for 1 h at 4 °C. Subsequently, the cells were washed 4 times with water and air dried. Afterwards, 300 µl SRB solution (0,5% in 1% acetic acid) was added to each well followed by 30 min incubation at room temperature. Cells were washed 4 times with 700 µl of 1% acetic acid to remove unbound excess dye and dried at room temperature. Finally, 1 volume of 10 mM Tris (pH10) was added to solubilize protein-bound dye for 5 min at room temperature in an orbital shaker. Finally absorbance was determined at 540 nm in Victor^3 plate reader (Perkin–Elmer).

2.2.3 Evaluation of ROS generation

ROS generation was fluorometrically determined using 2,7–dichlorofluorescein diacetate (H_2DCF-DA), as previously described (Varela et al., 2010). This probe easily penetrates cellular membrane and is hydrolyzed by cellular esterases to DCFH. The oxidation of DCFH by reactive species (ROS) yields 2,7- dichlorofluorescein (DCF). Consequently, the emitted fluorescence is proportional to the ROS levels in the cellular compartment. After incubation with menadione in 12–well plates, culture media was replaced by DMEM without HS or FBS and phenol red and 50 µM H_2DCF-DA prepared in DMSO. After 30 min incubation at 37°C in the dark, the medium with H_2DCF-DA was removed was replaced by culture medium without phenol red and measurements were done in a fluorescence plate reader Victor (Perkin–Elmer), at 37 °C, with an excitation wavelength of 485 nm and an emission wavelength of 538 nm (Zhou et al., 2001). Data was normalized taking into account protein count as determined in each well by the Sulforhodamine B method.

2.2.4 Evaluation of MTT reduction

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) colorimetric assay was used to assess cell metabolic activity, as a function of redox potential. Viable cells convert the water-soluble MTT to an insoluble purple formazan, that is then solubilized and its concentration determined by optical density.

After treatments with menadione in 12–well plates, 10% volume of MTT (from a working solution of 5 mg/ml in PBS) was added directly to 1 ml culture medium and incubated for 3 h allowing MTT reduction. After incubation, culture medium was discarded and remaining crystals were dissolved in 1ml of isopropanol and placed in the shaker during 30 min at 150 *rpm* at room temperature. After this period, cells were incubated at 37 °C for 5 min (Duarte et al., 2011). The absorbance of purple solution formed was read at 540 nm in Victor^3 plate reader.

2.2.5 Evaluation of Mitochondrial Membrane Potential

The electrical potential across the inner mitochondrial membrane (ΔΨ) was evaluated by a probe-based assay (Rolo et al., 2003). Tetramethylrhodamine methyl ester (TMRM) is a lipophilic cation that, due to its charge and solubility, electrophoretically accumulates in mitochondria in proportion to their ΔΨ. In depolarized mitochondria probe will be leaked to the cytoplasm, increasing fluorescence detection since high concentrations of TMRM inside of mitochondria prevents fluorescence detection.

After treatments with menadione in 12–well plates, the culture medium was aspirated and the cells were loaded with 6,6 µM TMRM in DMSO in 1 mL of DMEM without HS or FBS and phenol, and incubated at 37 °C, in the dark, for 15 min. After incubation, and replacement of the culture medium, fluorescence was measured using Victor^3 plate reader. Fluorescence was measured using excitation and emission wavelengths of 485 and 590 nm respectively, at 37 °C. After recording the baseline fluorescence for 15 min, mitochondrial depolarization was induced by adding 2 µM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP). FCCP is an ionophore that disrupts ATP synthesis by permeabilizing the mitochondrial membrane to proton transport. Data was normalized taking into account protein count as determined in each well by the Sulforhodamine B method.

Mitochondrial membrane potential was also evaluated with TMRM and without FCCP addition for non-transfected and transfected cells, visually by fluorescence microscopy using a fluorescence microscope Niko Eclipse TS100 with software NIS-Elements Imaging software (Nikon).

2.2.6 Protein extraction and BCA quantification

After treatments with menadione, cells were washed three times with PBS (pH 7.2) and lysed in cell lysis buffer (10 mM Tris–HCl, pH 7.5, 10 mM NaH_2PO_4, 130 mM NaCl, 1% Triton X-100). Lysates were then sonicated and centrifuged at 12 000 *rpm* for 10 min, at 4 °C. The supernatant was removed for protein assays and the pellet was discarded.

Protein content was determined by the BCA (bicinchoninic acid assay) method, described by Smith in 1985. BCA is a stable, water-soluble compound that in alkanine conditions forms purple complexes with copper ion (Cu^{+}) with colour increasing proportionally to protein content (Smith et al., 1985). For this assay, was necessary to create a standard curve with known amounts of BSA by successive dilutions, as indicated in Fig 19. Samples were diluted 1:20 in order to stay in concordance with standard curve. The assay was performed in 96-well plate and to each well (including samples and standard curve) 25 µl of sample and 200 µl of BCA solution were added to the plate was then incubated for 15 min at 60 °C and absorbance was measured at 540 nm in Victor[3] plate reader.

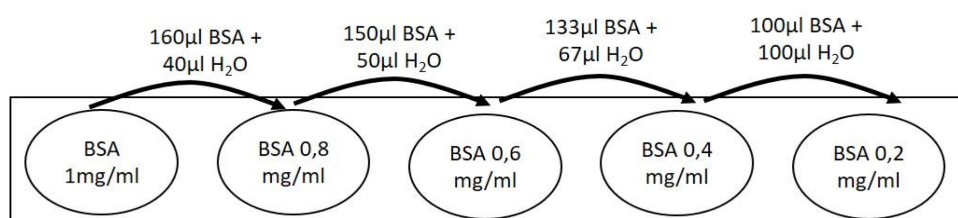


Fig 19 – Standard curve of BCA method.

2.2.7 Western Blot analysis

After protein extraction and quantification, samples were prepared with equal parts of Laemmli buffer 2x supplemented with β-Mercaptoethanol 5% (Bio-Rad Laboratories) and RIPA plus protein in order to load 50 µg of protein from each sample. Equal amounts of protein were loaded and electrophoresed on 8% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories). Membranes were blocked with 5% non-fat milk, at room temperature for 2 h, and incubated with primary antibody anti-SESN2 or anti-β-Actin overnight at 4 °C. After incubation, the membranes were washed 3 times for 15 min with TBS- 0,5% Tween solution and incubated with an anti-mouse secondary antibody (when incubated with anti-β-Actin, B2763) or anti-rabbit (when incubated with anti-SESN2, W10142) for 1 h at room temperature. Membranes were then washed 3 times for 10 min with TBS - 0,5% Tween and incubated with substrate Qdot 625 streptavidin conjugate (Thermo Fisher Scientific) for 15 min. Membranes were imaged using Bio-Rad Gel Doc™ EZ Imager equipment and with aid of Image Lab 4.1 Bio-Rad software. Antibodies utilized are summarized in Table 1.

Table 1 - List of antibodies used for Western Blot

Antibody	Dilution	Specie	Predicted molecular weight	Supplier	Catalog Number
SESN2	1:250	Rabbit	60-66 kDa	Proteintech	21346-1-AP
Actin	1:5000	Mouse	42 kDa	Sigma	A5441

2.2.8 Transfection of siRNA for SESN 2

For transfection, as described in Kang 2014, with minor modifications, cells were seeded in 6-well culture plates in 2 ml of DMEM with 10% FBS per well. SESN2 small interfering RNA (siRNA) (EMU050341) and control siRNA (SIC001) were purchased from Sigma. The siRNAs were transfected into C2C12 cells according to the manufacturer's instructions using Lipofectamine Transfection Reagent (Thermo Fisher Scientific). For each well, it was first prepared a mixture containing 5µl of Lipofectamine and 250 µl of OPTIMEM (Reduced Serum Medium, Thermo Fisher Scientific) and another one containing siRNA (100 ng) mixed with 250 µl of OPTIMEM. After mixing these two and waiting 20 min, 500 µl of the final mixture was added to a culture medium volume of 1,5 ml.

2.2.9 Determination of cell viability

Cell viability was determined by the LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Thermo Fisher Scientific) by fluorescence microscopy (Palmeira et al., 2007). This is a quick and easy two-color assay to determine of cells viability based on plasma membrane integrity. Viable cells are characterized by an intracellular esterase activity that can be determined by conversion of nonfluorescent cell-permeant calcein AM to the fluorescent calcein, generating a green fluorescent light. The fluorescent dye is retained in viable cells in way that viable cells are identified as green cells. This assay can also identify the damaged cells due to Ethidium homodimer-1 (ETHD-1); this dye enters damaged cells and binds to nucleic acids generating a red fluorescent light.

Cells were seeded in 6-well plates with each well containing a round coverslip. After treatments with menadione, 2 µM Calcein AM and 4 µM Ethidium homodimer-1 were added to each well in a total medium volume of 2 ml and incubated for 30 min at 37 °C. Following incubation period, medium was removed and PBS was added in order to wash and remove the excess of working solution. Coverslips were then inverted and mounted on microscope slide and cells were analyzed by fluorescence microscopy with a Zeiss Axioscop 2 Plus with AxioCam MRC (Zeiss) and software Axiovision 4 (Zeiss), with green, blue and red filters.

2.2.10 Immunocytochemistry

Previously cells were seeded in 6-well culture plates in a total medium volume of 2ml per well. After each treatment cells were fixed with a solution of paraformaldehyde 4%, permeabilized with PBS with 0,1% Triton and 2% BSA, and finally blocked with 3% BSA in PBS solution. After washing with PBS, cells were incubated with primary antibodies for SESN2 and for LC3 (antibodies described in table 2) overnight at 4 °C, protected from light and under agitation. The following day, cells were washed and incubated with 0,001 mg/ml Alexa Fluor 594 (Invitrogen) conjugated with anti-rabbit antibody for 2 h and after which Hoechst (1 mg/ml) was added for 10 min in order to stain the nuclei. Cells were imaged using a fluorescence microscope Niko Eclipse TS100 with software NIS-Elements Imaging software (Nikon).

Table 2 - List of antibodies used for immunocytochemistry

Antibody	Dilution	Specie	Supplier	Catalog Number
LC3	1:1000	Rabbit	Thermo Fisher Scientific	L10382
Alexa 594	1:2000	Rabbit	Thermo Fisher Scientific	A21207
SESN2	1:250	Rabbit	Proteintech	21346-1-AP

2.3. Animal experiments and treatments

In this section, hepatic tissue collected from adult-inducible SIRT1 knockout (SIRT1 - KO) mice and C57BL/6J mice of 6 or 30 months of age was used to determine SESN2 expression, as previously described in Price 2012. Wild-type and SIRT1-KO mice were maintained on experimental diets for 8 months. Different types of diets were administrated, an AIN-93G standard diet (SD), an AIN-93G modified to provide 60% of calories from fat (HFD), an HF diet with the addition of 0.04% resveratrol (HFD + RESV) as previously described (Baur and Sinclair, 2006).

2.3.1 RNA isolation and determination of genetic expression by semi-qPCR

RNA extraction was done with a PureLink® RNA Mini Kit from Thermo Fisher Scientific, accordingly to manufacturer’s instructions Hepatic tissue previously collected in liquid nitrogen, was pulverized and ressuspended in 200 µl RNA lysis buffer. After lysis, the solution was passed in a syringe and tissue was homogenized, one volume of 70% ethanol was mixed and 700 µl of the sample was transferred to the Spin Cartridge centrifuged at 12,000 x *g* for 15 s at room temperature. Then, flow-through was discarded and the Spin Cartridge was reinserted in the same

collection tube. These steps were repeated until all the sample was processed. Then 700 µl of wash buffer I was added and the sample was centrifuged at 12,000 x *g* for 15 s at room temperature. Flow-through was discarded and the Spin Cartridge was placed into a new collection tube. At this time, 500 µl of wash buffer II with ethanol was added and again the sample was centrifuged at 12,000 x *g* for 15 s at room temperature. In the end the flow was discarded, and this last step repeated. While in the recovery tube, the spin cartridge was centrifuged at 12,000 x *g* for 1 min at room temperature. Finally 30 µl of RNase-free water was added, incubated for 1 min and centrifuged for 2 min at 12,000 x *g* at room temperature. Purified RNA was stored at -80 °C.

2.3.2. RNA quantification

Extracted RNA was quantified by using the Qubit® RNA assay kit (Thermo Fisher Scientific).Working solution was prepared by diluting Qubit®RNA Reagent 1:200 in Qubit® RNA Buffer and 200µL of Qubit® Working Solution were prepared to each standard and sample. Working solution, standard and user sample were mixed in the assay tubes (0,5 ml PCR tubes) according to manufacturer's instructions, and mixed by vortex for 3 s and incubated for 2 min at room temperature. In order to calibrate with standards and read the samples the option RNA assay was selected on the Qubit® 2.0 Fluorometer. Purified RNA was stored at -80 °C.

2.3.3. cDNA synthesis

In order to analyse the genetic expression, cDNA (complementary DNA) was synthesized using the reverse transcriptase (RT) method. RT is an enzyme used for synthetizing complementary DNA (cDNA) using as template RNA molecules in a process reverse transcription with random primers. With this purpose, the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories) was used and 5x iScript reaction mix, iScript reverse transcriptase, Nuclease-free water and 1 µg RNA template were mixed according to manufacturer's instructions. The mix was run in a PCR protocol of 5 min at 25 °C, 30 min at 42 °C and finally 5 min at 85 °C. cDNA was stored at 4 °C until was used for semi-quantitative real time-PCR.

2.3.4. Semi-quantitative real rime- PCR

Aiming to evaluate gene expression of c/EBPα, SESN2, KEAP1 and Nrf2, a semi-quantitative real-time PCR was conducted. This type of PCR allows to compare treated samples with control samples and understand if the treatment increased or decreased gene expression, relatively to control.

Primers (Table 3) were designed based on sequence information in GenBank. Semi-quantitative real-time PCR (semi-qPCR) reactions were prepared with IQ™SYBR ® Green Supermix Reagents and according to manufacturer´s instrutions (Bio-Rad Laboratories). For one reaction 300 nM of each primer (sense and anti-sense) was added, mixed with IQ™SYBR ® Green Supermix 1x and cDNA template 2 µl diluted 1:10 and nuclease free-water in order to complete the reaction volume of 20 µl. The sample were subjected to a PCR protocol of 90 °C during 3 min and after that 40 cycles of 90 °C for 5 s, 60 °C for 10 s and 72 °C for 20 s. Following the PCR, a melting curve was done from 55 °C to 95°C, increasing 0,5 °C every 5 s, in a In MiniOpticon Real-Time PCR System (Bio-Rad Laboratories). The 18S was the housekeeping gene used as a reference standard.

TABLE 3 – LIST OF PRIMERS USED FOR SEMI-QUANTITATIVE REAL TIME PCR

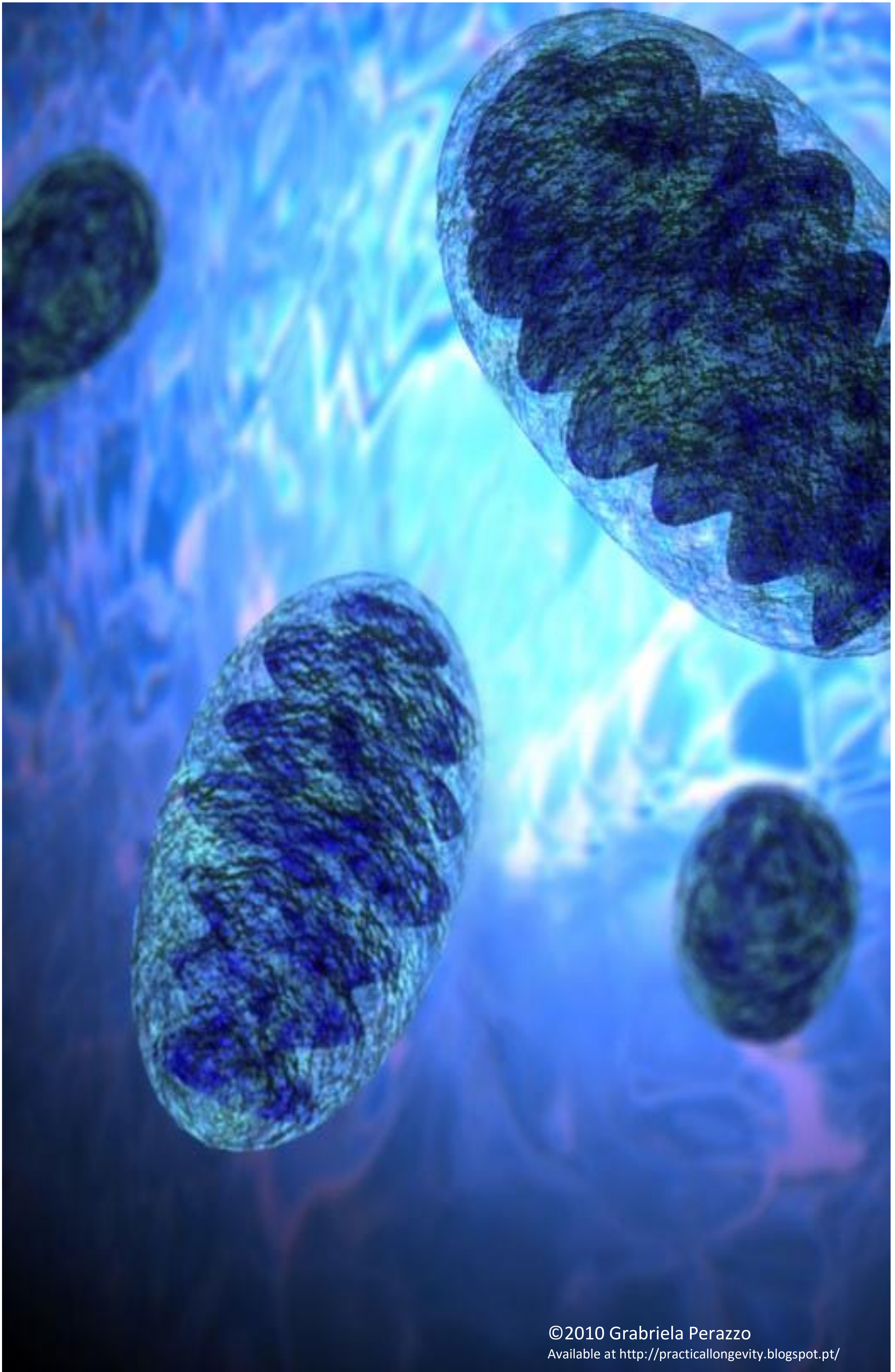
Gene	Specie	Sense	Anti-sense
c/EBPα	Mouse	TTACAACAGGCCAGGTTTCC	CTCTGGGATGGATCGATTGT
SESN2	Mouse	TAGCCTGCAGCCTCACCTAT	GATTTTGAGGTTCCGTTCCA
KEAP1	Mouse	ATGGCCACACTTTTCTGGAC	TCCTGTTGTCAGTGCTCAGG
NRF2	Mouse	CTCGCTGGAAAAAGAAGTGG	CCGTCCAGGAGTTCAGAGAG
18 S	Mouse	GCCCGAGCCGCCTGGATAC	CCGGCGGGTCATGGGAATAAC

A standard curve composed by cDNA of a mix of several control samples serially diluted as 1:10, 1:20, 1:40, 1:60 and 1:100 was prepared. Genetic expression was analysed by comparing samples to the standard curve.

2.3.5. Statistical Analysis

Data was presented as Means ± SEM. Statistical significance was evaluated by using one-way ANOVA (for three groups comparison) with Tukey. A p-value < 0.05 was considered statistically significant.

Results



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3.1. Mitochondrial dysfunction induced by menadione is aggravated by Sestrin 2 depletion

3.1.1 Exposure of C2C12 cells to menadione increases ROS generation in a dose-dependent manner

As previously referred, increased oxidative stress in obesity and aging is associated with impaired skeletal muscle metabolism and loss of tissue homeostasis (Johnson et al., 2013). Currently, the exact causes for this increased susceptibility to free radical-induced damage are still matter of discussion but it may involve a decrease in the expression of cytoprotective proteins and accumulation of dysfunctional mitochondria (Roberts and Sindhu, 2009).

Since menadione has been shown to trigger a dose-dependent effect, lower concentrations activate protective cascades while higher concentrations induce toxic oxidative stress and cell death (Basoah et al., 2005), differentiated C2C12 cells were exposed for 1 h to 10 µM and 30 µM menadione and ROS generation evaluated 24 or 48 h after the removal of menadione. Cells were treated for 1 h with menadione because less time showed no effects. After 1 h of exposure, menadione was removed and cells cultured for 24 or 48 h in order to allow the induction of long-term effects.

As shown in Fig 20, treatment with 30 µM menadione for 1 h induced a statistically significant increase in ROS generation, both detected 24 and 48 h after menadione removal. 10 µM menadione however did not affect cellular ROS, when compared to control (Fig 20).

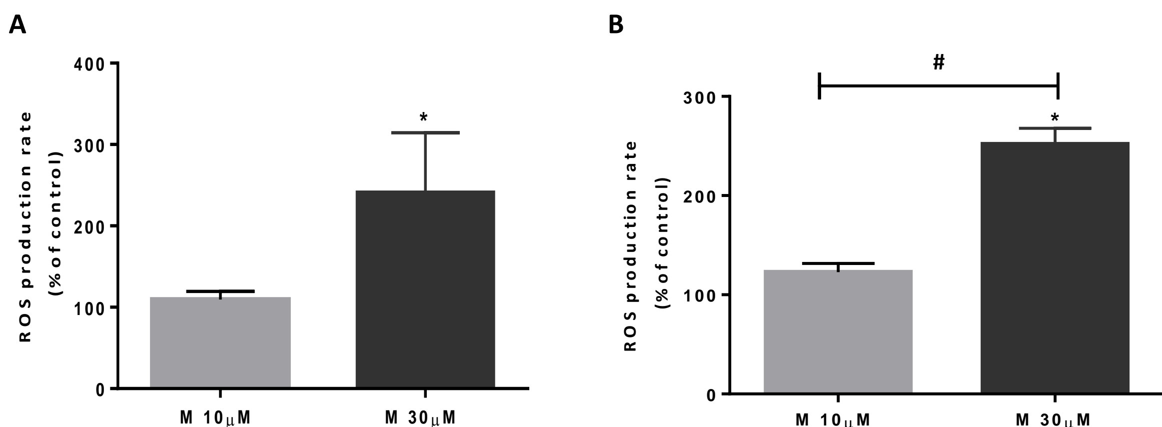


Fig 20 – ROS generation in differentiated C2C12 cells 24 h (A) and 48 h (B) after exposure for 1 h to 10 and 30 µM menadione (M). ROS generation was fluorometrically assayed using the probe H_2DCF-DA, as described in 2.2.3 of Methods section. Data are means ± SEM for cells treated with M 10 and M 30 µM for 1 h. * indicates statistically significant difference in M 30 µM versus control ($p < 0.05$) and # indicates statistically significant difference in M 10 µM versus M 30 µM ($p < 0.05$). This figure is representative of an n=5.

3.1.2 Exposure of C2C12 cells to menadione decreases MTT reduction in a dose-dependent manner

Given that 30 µM menadione increased ROS generation, the MTT assay was used to assess MTT reduction as an indicator of cell viability. 10 µM menadione had no effect on MTT reduction. Cells exposed to 30 µM showed a decrease in MTT reduction when compared either to control or to cells exposed to 10 µM menadione, and after 24 or 48 h of recovery (Fig 21).

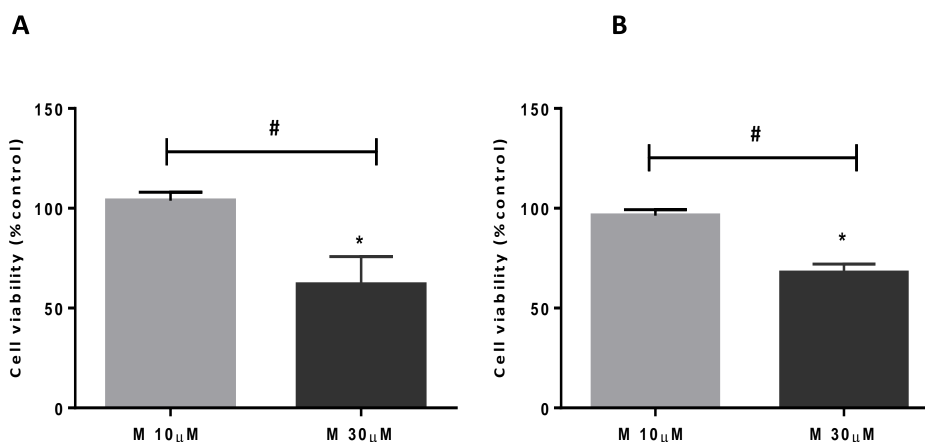


Fig 21 – MTT reduction, as an indicator of cell viability, in differentiated C2C12 cells 24 h (A) and 48 h(B) after exposure for 1 h to 10 and 30 µM menadione (M). Cellular viability was colorimetrically assayed using MTT, as described 2.2.4 of Methods section. Data are means ± SEM of cells treated with M 10 and M 30 µM for 1 h. * indicates statistically significant difference in M 30 µM versus control ($p < 0.05$) and # indicates statistically significant difference in M 10 µM versus M 30 µM ($p < 0.05$). This figure is representative of an n=5.

3.1.3 Menadione-induced increase in ROS generation is associated with impaired mitochondrial membrane potential

Mitochondrial membrane potential is closely related to cellular ATP production by oxidative phosphorylation. In order to determine if the tested concentrations of menadione were associated with altered mitochondrial bioenergetics, mitochondrial membrane potential was evaluated in C2C12 cells by using the fluorescent dye TMRM.

While 1 h of incubation with 30 µM menadione decreased mitochondrial membrane potential, an effect observed 24 and 48 h (Fig 22) after menadione removal, the result obtained with 10 µM menadione was similar to control cells. Therefore, data suggests that the increase in ROS generation caused by 30 µM menadione is associated with disruption of the electron transport chain.

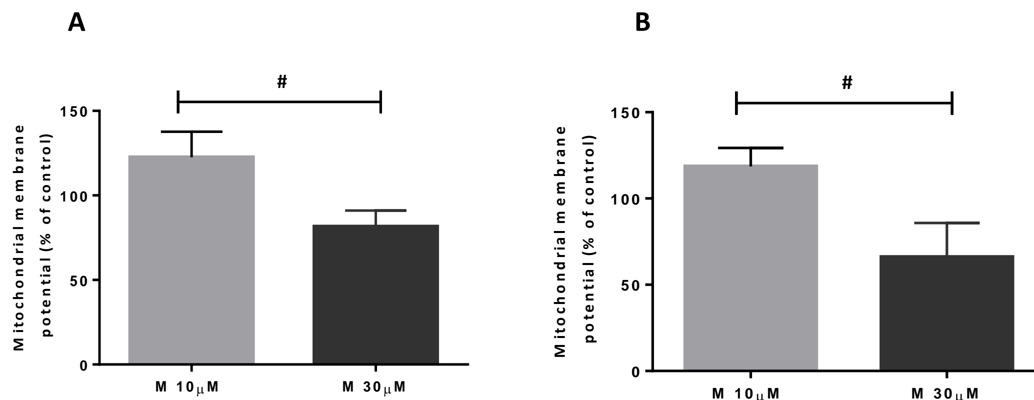


Fig 22 – Evaluation of mitochondrial membrane potential in differentiated C2C12 cells 24 h (A) and 48 h (B) after exposure for 1 h to 10 and 30 µM menadione (M). Mitochondrial membrane potential was fluorometrically assayed using TMRM, as described 2.2.5 of Methods section. Data are means ± SEM for cells treated with M 10 and M 30 µM for 1 h. # indicates statistically significant difference in M 10 µM versus M 30 µM ($p < 0.05$). This figure is representative of an n=5.

Mitochondrial membrane potential was also assessed visually by fluorescence microscopy. 24 h after menadione (30 µM) removal, a decrease in fluorescence was observed (Fig 23). Morphological alterations induced by alterations in differentiation state such as a decrease of in length of myotubes, were observed with 10 µM menadione, although this concentration did not statistically alter mitochondrial membrane potential.

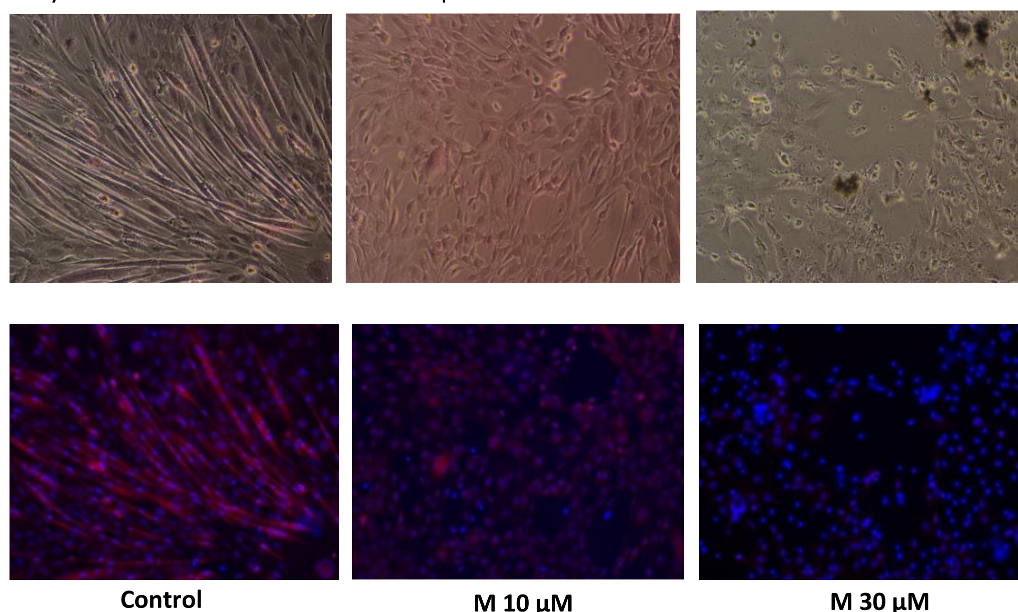


Fig 23 – Evaluation of mitochondrial membrane potential in differentiated C2C12 cells 24 h (after exposure for 1 h to 10 and 30 µM menadione (M). Mitochondrial membrane potential was assayed using TMRM in fluorescence microscopy, as described 2.2.5 of Methods section. Data are means ± SEM for cells treated with M 10 µM and M 30 µM for 1 h. This figure is representative of an n=5.

48 h after menadione removal, it was also observed a decrease in fluorescence with 30 µM menadione (Fig 24), and an increase in the number of dead cells. Morphological alterations were observed with 10 µM menadione exposure, although this concentration did not statistically alter mitochondrial membrane potential.

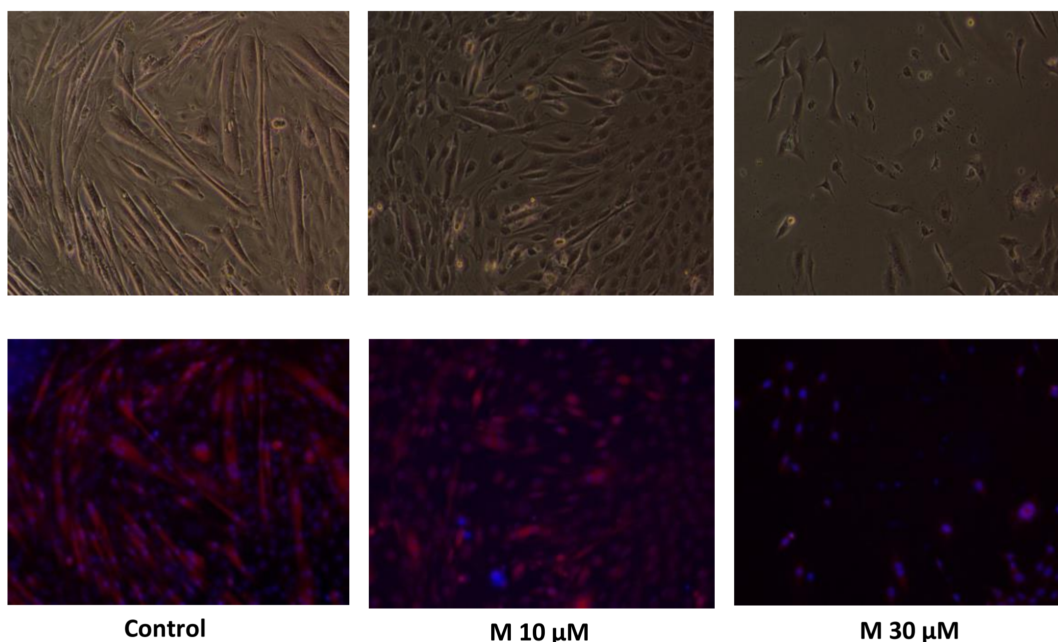


Fig 24 – Evaluation of mitochondrial membrane potential in differentiated C2C12 cells 48 h after exposure for 1 h to 10 and 30 µM menadione (M). Mitochondrial membrane potential was assayed using TMRM in fluorescence microscopy, as described 2.2.5 of Methods section. Data are means ± SEM for cells treated with M 10 µM and M 30 µM for 1 h. This figure is representative of an n=5.

3.1.4 Exposure of C2C12 cells to menadione is associated with an adaptive response mediated by Sestrin 2

Sestrin 2 regenerates mitochondrial PRX3 and its depletion was found to induce an increase of ROS generation, by increasing mitochondrial ROS and mitochondrial dysfunction (Rhee and Bae, 2015).

In order to elucidate the pathways underlying the dose-dependent effect of menadione, SESN2 content was evaluated 10, 24 and 48 h upon exposure of C2C12 cells for 1 h to menadione (Fig 25). Actin was used as loading control. 10 µM menadione induced an increase in SESN2 content, noticeable at 10 h, but not after 24 or 48 h, suggesting a possible protective early response induced by lower concentrations of menadione. In contrast, 30 µM menadione was associated with a decrease in SESN2 content at all time points, suggesting that this concentration induces a rate of ROS generation that unbalances the cellular antioxidant capacity and is cytotoxic.

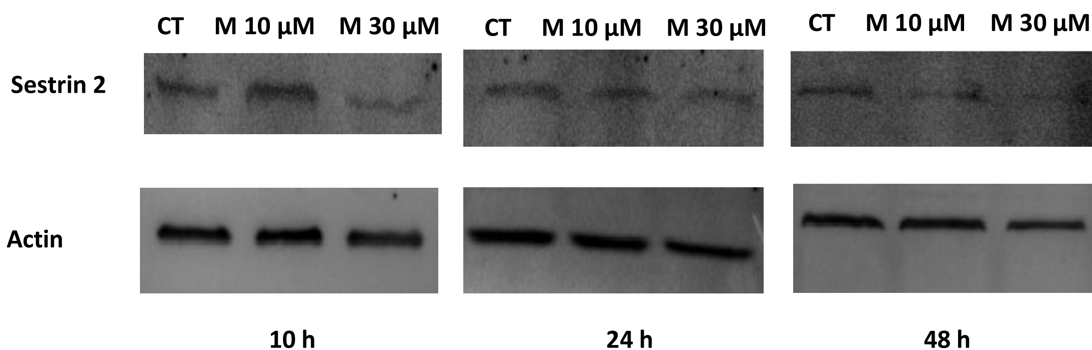


Fig 25 – Sestrin 2 content by Western Blot after treatment with menadione. Sestrin 2 content was evaluated by Western Blot as described in 2.2.7 of Methods section, in cells treated with menadione 10 and 30 µM for 1 h and incubated 10, 24 and 48 h after menadione removal. This figure is representative of an n=5.

To further establish the role of SESN2 in the preservation of mitochondrial function against ROS deleterious effects, C2C12 cells were transfected with siRNA for SESN2 after 1 h incubation with 10 or 30 µM menadione. 10 h after transfection, immunocytochemistry was performed in order to verify the effects of siRNA in SESN2 content (Fig 26). A decrease in SESN2 in cells transfected with siRNA was observed, demonstrating the effectiveness of transfection.

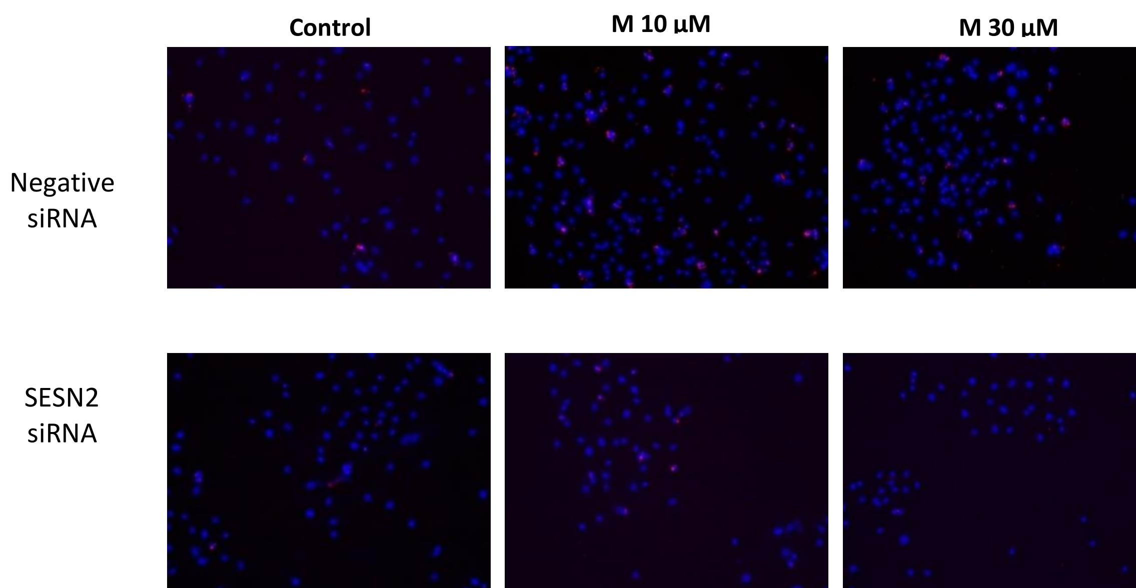


Fig 26 – Sestrin 2 content evaluated by immunocytochemistry in C2C12 cells transfected with siRNA for SESN2 or with negative siRNA. For control condition**,** cells were exposed to vehicle (DMSO) and the treatments were exposed to menadione 10 and 30 µM for 1 h, transfected with negative siRNA and SESN2 siRNA and incubated for a 10 h of recovery. In After recovery time, immunocytochemistry was done as described in 2.2.8 of Methods section. This figure is representative of an n=3.

Aiming to identify the effects of SESN2 in cell survival, cells under the conditions described above were tested with the Live/Dead assay. Live cells were stained green (calcein) and dead cells were stained red (ethidium). In Fig 27, it is visible that exposure to menadione increases cell death in a dose-dependent manner. In cells transfected with siRNA for SESN2, the damage caused by menadione was noticed in both concentrations, although yet dose-dependent. Control cells transfected with siRNA for SESN2 also exhibited an increase in cell death, but smaller than in the presence of menadione. These results demonstrate that SESN2 is an important protein in the maintenance of cell survival and in protection against oxidative insults such as menadione exposure.

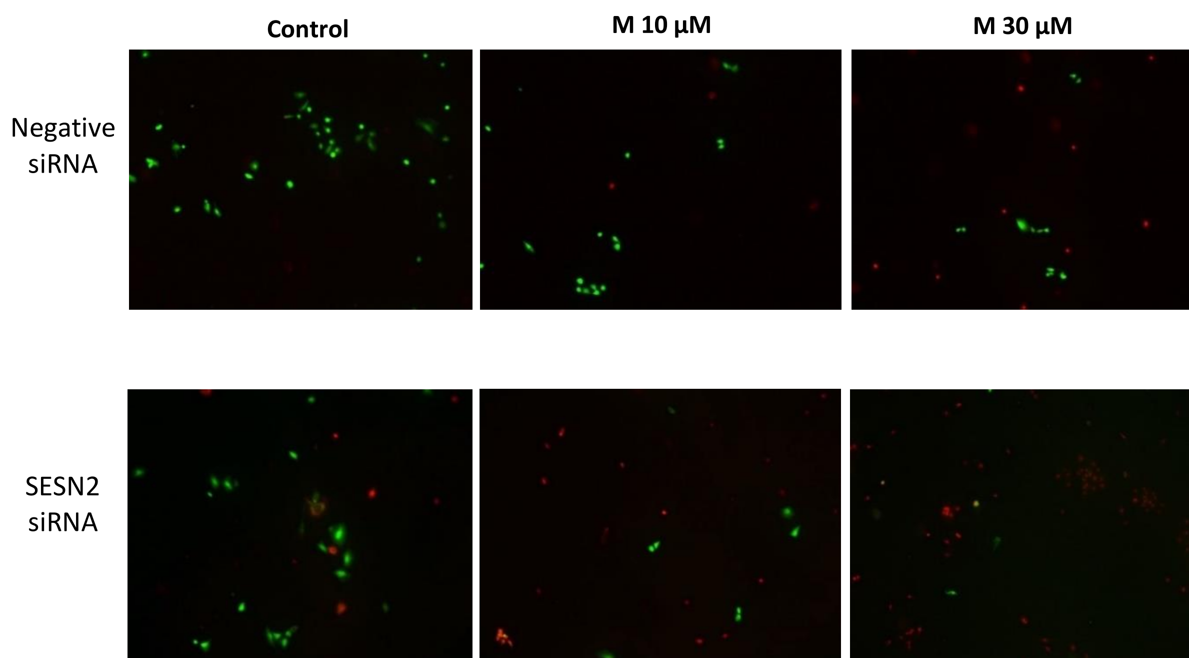


Fig 27 – Effects of Sestrin 2 in cell survival evaluated by Live/Dead in C2C12 cells transfected with siRNA for SESN2 or with negative siRNA. For control condition**,** cells were exposed to vehicle (DMSO) and the treatments were exposed to menadione 10 and 30 µM for 1 h, transfected with negative siRNA and SESN2 siRNA and incubated for a 10 h of recovery. After recovery time, Live/Dead kit was used as described in 2.2.9 of Methods section. This figure is representative of an n=3.

Mitochondrial membrane potential was also evaluated in conditions of SESN2 silencing and menadione exposure. As shown in Fig 28, SESN2 depletion aggravated the impairment of mitochondrial membrane potential induced by 30 µM menadione. Furthermore, transfection of cells with SESN2 siRNA also decreased TMRM fluorescence in both 10 µM and control cells. These results show that SESN2 is an important protein for adequate mitochondrial function, since its absence is associated with decreased mitochondrial membrane potential.

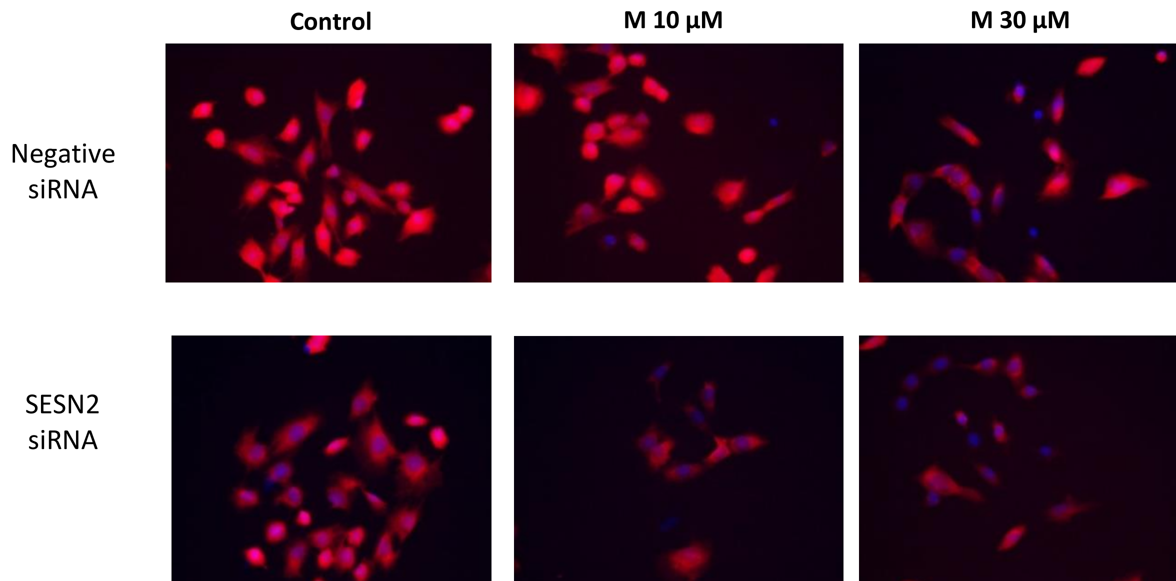


Fig 28 – Effects of Sestrin 2 knockdown in mitochondrial membrane potential in C2C12 cells transfected with siRNA for SESN2 or with negative siRNA. For control condition**,** cells were exposed to vehicle (DMSO) and the treatments were exposed to menadione 10 and 30 µM for 1 h, transfected with negative siRNA and SESN2 siRNA and incubated for a 10 h of recovery. In After recovery time, TMRM was performed as described in 2.2.5 of Methods section. This figure is representative of an n=3.

Taking into account that SESN2 is a key player in autophagy and that defective regulation of autophagy is associated with metabolic dysfunction, cells transfected with siRNA for SESN2 and exposed to menadione, were assayed for LC3 cellular pattern distribution (Fig 29).

In the presence of SESN2, exposure to menadione caused an increase in the punctuated pattern of distribution of the LC3 protein, in accordance with studies that describe induction of autophagy as one of the main functions of SESN2 (Hay, 2008; Budanov et al., 2010). However, when SESN2 content was reduced (by using siRNA), a decrease in LC3 punctuation was observed, both in control and menadione conditions. Together, these results demonstrate that SESN2 is essential for autophagy and the maintenance of cell homeostasis after a stress.

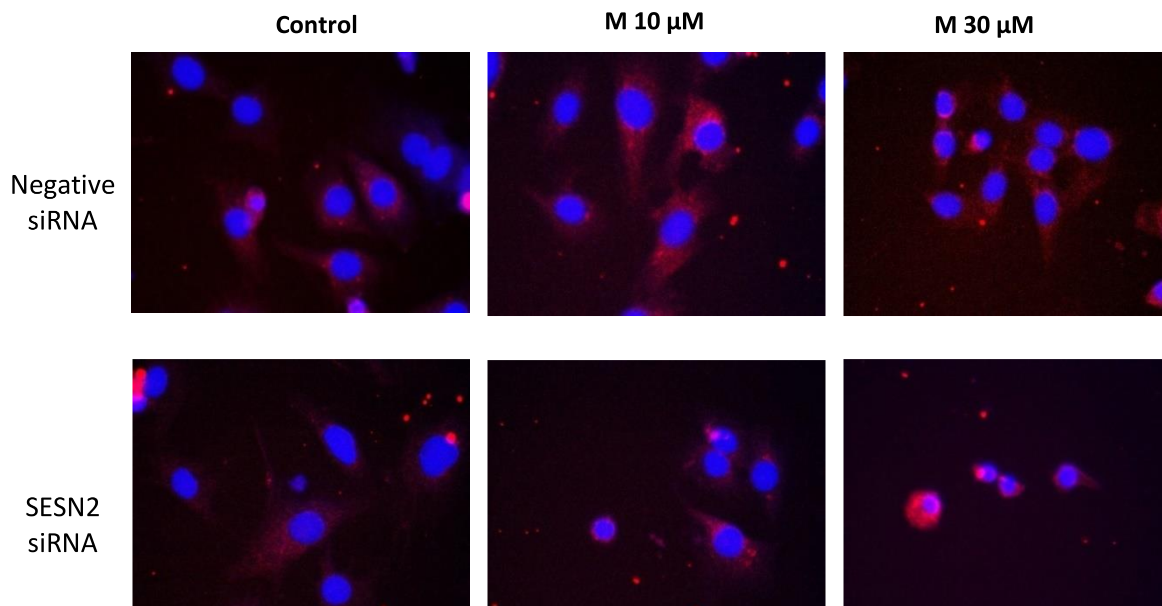


Fig 29 – LC3 content and distribution pattern in C2C12 cells transfected with siRNA for SESN2 or with negative siRNA. For control condition, cells were exposed to vehicle (DMSO) and for treatments were exposed to menadione 10 and 30 µM for 1 h, transfected with negative siRNA and SESN2 siRNA and incubated for 10 h of recovery. In After recovery time, immunocytochemistry was performed as described in 2.2.10 of Methods section. This figure is representative of an n=3.

3.2 Aging and metabolic dysfunction is associated with altered sestrin-2 expression

Sirtuin 1 is a key regulator of mitochondrial function, with aged tissue exhibiting loss of mitochondrial homeostasis and SIRT1 silencing resulting in an imbalance between nuclear and mitochondrial-encoded genes (Gomes et al., 2013). In order to explore how SIRT1 modulation impacts SESN2, hepatic expression of c/EBPα, SESN2, KEAP1 and Nrf2 was addressed in wild-type (WT) and SIRT1 knockout mice (SIRT1 - KO). These mice were fed with standard diet (SD), high-fat-diet (HFD) or high-fat diet treated with resveratrol (HFD + RESV). Mice with 6 or 30 months of age and fed a standard diet were also evaluated.

3.2.1 SIRT1 controls expression of c/EBPα

Wild-type (WT) mice fed with a high-fat diet and treated with resveratrol (HFD + RESV) showed decreased hepatic expression of c/EBPα, comparing either to WT mice exposed to standard (SD) or to high-fat (HFD) diet. In contrast, in knock-out (KO) mice, HFD + RESV treatment induced an increase in c/EBPα expression comparatively with HFD SIRT1-KO mice. In the absence of SIRT1, HFD induced a decrease in c/EBPα gene expression when compared with SIRT1-KO treated with SD. For 30 months old mice, a decrease of c/EBPα expression was visible, accordingly with previous

studies (Jin et al., 2011). These data indicate that gene expression of c/EBPα transcription factor is dependent on the expression of SIRT1 (Fig 30).

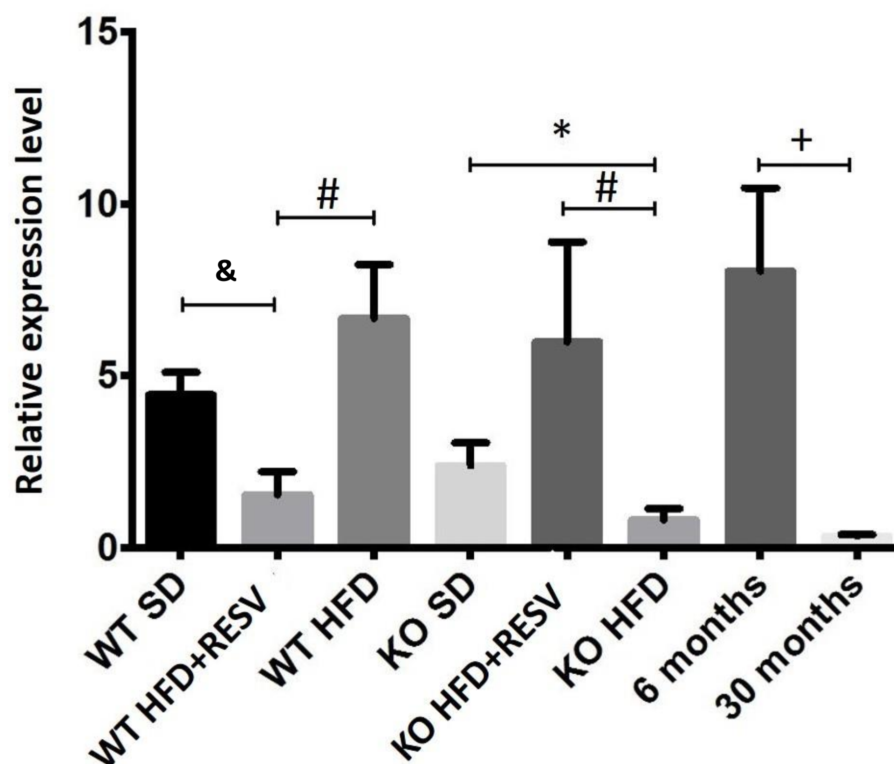


Fig 30 – Profile of hepatic c/EBPα expression in wild-type (WT) mice and SIRT1 knockout mice (KO) fed a standard diet (SD), high-fat (HFD) or high-fat diet treated supplemented with resveratrol (HFD + RESV). 6 or 30 months old mice were also evaluated. RNA extraction and quantification, cDNA synthesis and semi-quantitative real-time PCR were performed as described in 2.3 of Methods section. Data are means ± SEM for at least n=5. For control, 18S was used as an housekeeping gene. * indicates a statistically significant difference in SD versus HFD, # indicates a statistically significant difference in HFD + RESV versus HFD, & indicates a statistically significant difference SD versus HFD + RESV, + indicates a statistically significant difference in 6 months versus 30 months, $p<0.05$.

3.2.2. SIRT1 modulates SESN2 expression

Regarding SESN2, WT mice with HFD exhibited decreased expression when compared to SD. HFD + RESV treatment was able to prevent the decrease in SESN2 expression induced by HFD, suggesting that activation of SIRT1 by resveratrol modulates SESN2 expression. However, HFD + RESV treatment prevented the decrease in SESN2 induced by HFD in SIRT1-KO animals, suggesting induction of SESN2 expression by Resv is only partially SIRT1-dependent (Fig 31). Analysis of aged mice showed a decrease of SESN2 expression, as expected from literature.

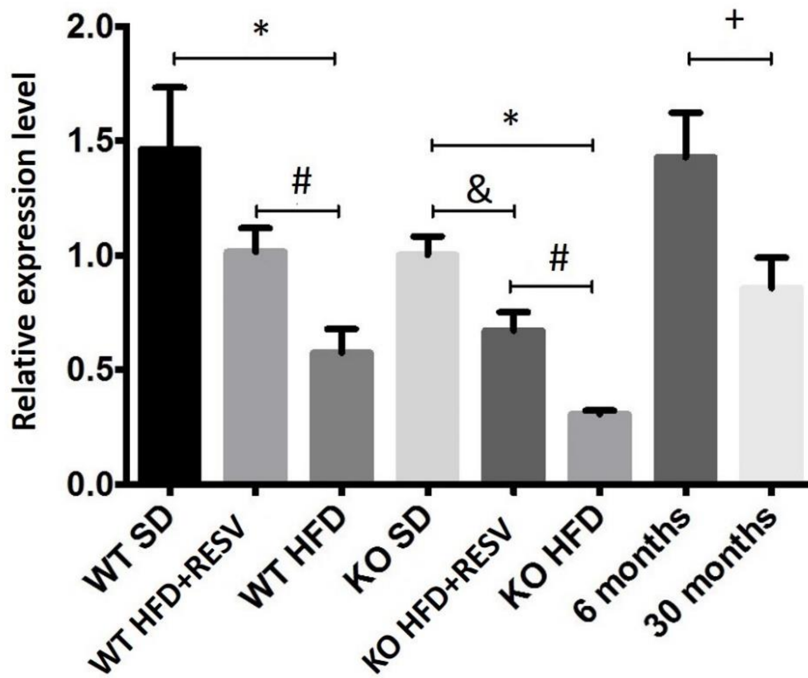


Fig 31 – Profile of Sestrin 2 expression in wild-type (WT) mice and SIRT1 knockout mice (KO) fed a standard diet (SD), high-fat (HFD) or high-fat diet treated supplemented with resveratrol (HFD + RESV). 6 or 30 months old mice were also evaluated. RNA extraction and quantification, cDNA synthesis and semi-quantitative real-time PCR were performed as described in 2.3 of Methods section. Data are means ± SEM for at least n=5. For control 18S was used as an housekeeping gene. * indicates a statistically significant difference in SD versus HFD, # indicates a statistically significant difference in HFD + RESV versus HFD, & indicates a statistically significant difference SD versus HFD + RESV, + indicates a statistically significant difference in 6 months versus 30 months, $p<0.05$.

3.2.3 SIRT1 triggers KEAP1 gene expression

Since it has been previously reported that KEAP1 is activated under stress conditions by a mechanism involving SESN2 (Rhee and Bae, 2015), hepatic expression of KEAP1 was evaluated. In WT mice, KEAP1 expression was decreased in HFD mice comparatively to a standard diet. HFD + RESV treatment prevented the effect of HFD in WT animals. However, in SIRT1-KO mice, HFD + RESV treatment was not able to prevent HFD-induced decrease in KEAP1, providing a strong evidence that KEAP1 is regulated by a SIRT1-dependent mechanism (Fig 32). Older WT mice showed a decrease in KEAP1 expression.

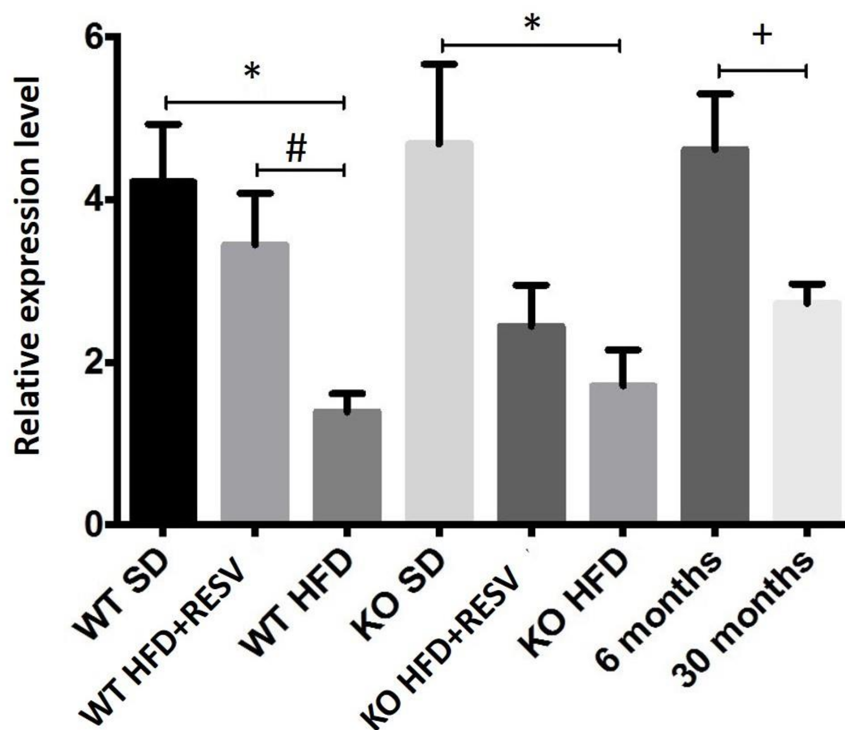


Fig 32 –Profile of KEAP1 expression in wild-type (WT) mice and SIRT1 knockout mice (KO) fed a standard diet (SD), high-fat (HFD) or high-fat diet treated supplemented with resveratrol (HFD + RESV). 6 or 30 months old mice were also evaluated. RNA extraction and quantification, cDNA synthesis and semi-quantitative real-time PCR were performed as described in 2.3 of Methods section. Data are means ± SEM for at least n=5. For control 18S was used as an housekeeping gene. * indicates statically significant difference in SD versus HFD, # indicates statically significant difference in HFD+RESV versus HFD, + indicates statically significant difference in 6 months versus 30 months, P<0.05.

3.2.4 Aging affects Nrf2 gene expression

Because Nrf2 is an important regulator of cellular stress response, regulated by KEAP1 and SESN2 (Rhee and Bae, 2015), its gene expression was also analyzed. Results showed that expression of Nrf2 was not affected by resveratrol or HFD treatment, presence or absence of SIRT1 (Fig33). Only for aged mice, a decrease of this gene expression was observed.

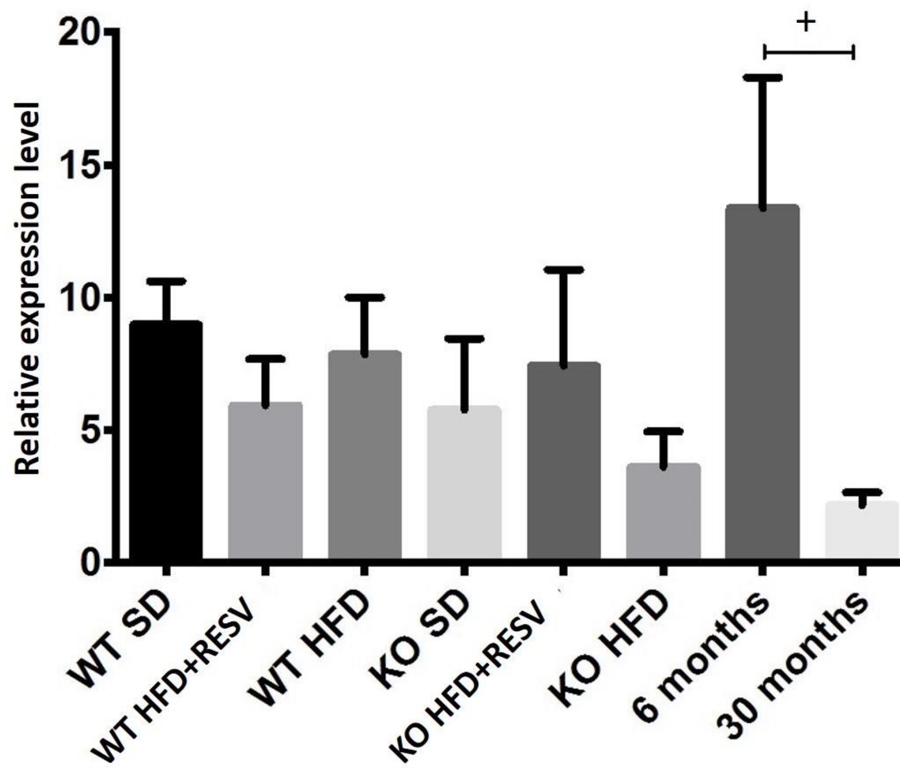
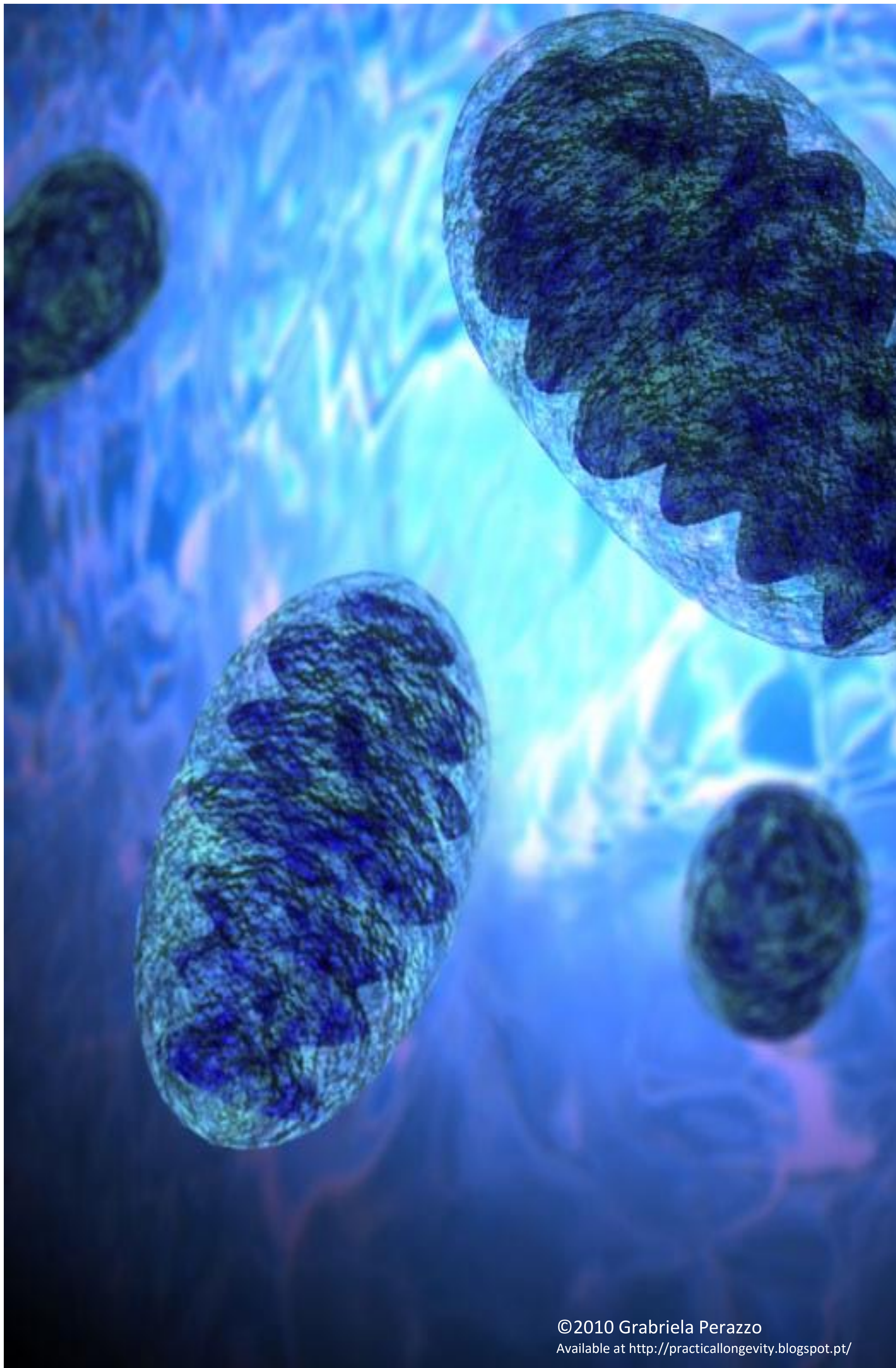


Fig 33 –Profile of Nrf2 expression in wild-type (WT) mice and SIRT1 knockout mice (KO) fed a standard diet (SD), high-fat (HFD) or high-fat diet treated supplemented with resveratrol (HFD + RESV). 6 or 30 months old were also evaluated. RNA extraction and quantification, cDNA synthesis and semi-quantitative real-time PCR were performed as described in 2.3 of Methods section. Data are means ± SEM for at least n=5. For control 18S was used as an housekeeping gene. + indicates statistically significant difference in 6 months versus 30 months, $p<0.05$.

Discussion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4.1 Induction of Sestrin 2 by lower concentrations of menadione preserves mitochondrial function

Sestrins are an emerging family of stress-inducible proteins that act as physiological regulators of cellular metabolism (Lee et al., 2013). Conditions of metabolic stress, such as aging, obesity and diabetes are associated with impairment in sestrin function. Several studies have shown that genetic deficiency in the SESN2 gene accelerates aging and diabetes-induced by obesity, precipitating the development of glucose intolerance, insulin resistance, fatty liver, muscle degeneration, mitochondrial dysfunction and formation of protein aggregates (Ro et al., 2014; Park et al., 2014; Lee et al., 2010a; Lee et al., 2010b; Lee et al., 2012a). In this context, research has been conducted in order to develop therapeutic strategies based in sestrin modulation (Eid et al., 2013; Tao et al., 2014).

Aging and obesity-related pathologies are accompanied by a disruption in energy balance and mitochondrial dysfunction, with consequent accumulation of oxidative damage (Lee et al., 2013; Duarte et al., 2015). Mitochondria are the major suppliers of cell energy but are also responsible for the vast majority of cellular ROS generation as a by-product of NADH and $FADH_2$ oxidation via oxidative phosphorylation. Under normal circumstances, an efficient antioxidant system prevents the development of oxidative stress and subsequent damage to lipids, proteins, DNA, leading to cell death. However, depletion of antioxidant defenses and/or unbalanced metabolism results in ROS overproduction leading to a vicious cycle in which increased ROS impair mitochondrial function, causing the formation of more oxidants and less ATP. Eukaryotes developed several mechanisms of quality control in order to preserve mitochondrial and cellular homeostasis (Palikaras and Tavernarakis, 2014). SESN2 regulates one of the most important antioxidant systems in the cell, making it essential for cell survival.

Sestrins accumulate in cells exposed to stress, being its expression controlled by the ROS-activated transcription factors p53 and FOXO (Budanov et al., 2002; Velasco-Miguel et al., 1999; Nogueira et al.,2008). The regulation of cellular homeostasis by sestrins depends not only on their capacity to suppress oxidative damage, but also by acting as antioxidants (in particular SESN2) that control the activity of peroxiredoxins, which scavenge ROS (Lee et al., 2010a; Budanov et al., 2004), but as well by acting as activators of AMPK, thus inhibiting mTORC1 signaling and leading to autophagy (Lee et al., 2010b; Budanov et al., 2004). mTORC1 inhibition by SESN2 has been pointed as critical for the prevention of mitochondrial and cellular aging (McCormick et al., 2011; Jonhson et al., 2013).

The current work focused on SESN2, highly expressed in skeletal muscle (Sanchis-Gomar, 2013) and well characterized in liver and adipose tissues (Lee et al., 2012a). Although the relation between mitochondria and SESN2 remains to be established, its existence seems clear since they are both involved in aging, cell survival, antioxidant defense and disease development. Previous studies showed that SESN2 knockdown decreases mitochondrial ATP content, highlighting that SESN2 is important for maintenance of the energetic homeostasis in muscular cells (Ben-Sahra et al., 2013). Furthermore, the fact that the deleterious effects of SESN2 ablation on metabolic homeostasis are reversed by AMPK activation (Lee et al., 2012a) and that AMPK activation is linked to both mitochondrial biogenesis and degradation of dysfunctional mitochondria (Rolo et al., 2011), this further suggests a role for SESN2 in mitochondrial homeostasis. Minimizing mitochondrial dysfunction is essential in the prevention/treatment of several diseases and it is dependent on a mitochondrial quality control system. This system includes recognition and degradation of dysfunctional mitochondria by autophagy and a response involving an increase in chaperones and mitochondrial proteases, aiming to improve the folding and preventing aggregation of proteins within mitochondria (Butow and Avadhani, 2004; Schieke and Finkel 2006).

ROS have a dual role in cellular fate: at low levels, ROS act as signaling molecules but at high levels, they damage organelles, particularly the mitochondria. Taking into account that a number of pathways are regulated by redox state (Finkel, 2003) and that one major sensor of redox signaling is at the switch of stress adaptation/cell death is autophagy (Lee et al., 2012b), this work proposes that activation of SESN2 by a mild increase in ROS, could trigger a pathway of adaptation to stress in which ROS are specific signaling molecules that potentially regulate mitochondrial homeostasis, via SESN2-dependent quality control. Since SESN2 activation promotes autophagic catabolism, it is proposed that SESN2-induced mitophagy is critical for maintaining proper cellular functions, upon oxidant exposure. Mitophagy regulates the number of mitochondria to match the metabolic or developmental demands and is also a part of a quality control system based on the removal of dysfunctional mitochondria (Palikaras et al., 2015). Mitophagy can be triggered by mild oxidative stress, in a process dependent on modulation of mitochondrial dynamics via DRP1-dependent induction of mitochondrial fission, thus preventing the accumulation of cytotoxic mediators and the initiation of a cycle of oxidative stress (Frank et al., 2012)

Exposure to oxidants such as menadione causes oxidation of cellular environment due to an increase in ROS formation like superoxide, hydrogen peroxide or hydroxyl radical. Menadione generates intracellular ROS at multiple cellular sites through futile redox cycling (McCormick et al., 2000; Lim et al., 2008), as demonstrated in muscle, liver and pancreatic cells (Lim et al., 2008;

Criddle et al., 2006; Conde de la Rosa et al., 2006). In mitochondria, menadione induces ROS generation by capturing electrons from ubiquinone and transporting them directly onto oxygen, producing superoxide (Basoah et al., 2005). Previous studies with L6 myoblasts and myotubes exposed to 5 µM to 75 µM menadione (Lim et al.,2008; Basoah et al., 2005) have shown that cell viability decreases only for concentrations above 20 µM in L6 myotubes but above 10 µM in L6 myoblasts (Lim et al.,2008). Furthermore, menadione has been shown to induce distinct dose-dependent cellular responses: lower concentrations trigger cytoprotection mediated by alterations in gene expression (Chuang et al., 2002; Heinzel et al.,2005) while higher concentrations induce cell death caused by sustained oxidative stress (Sakagami et al., 2000; Grishko et al., 2001; Loor et al., 2010).

The current work shows that exposure of differentiated C2C12 cells to 10 µM menadione for 1 h does not lead to alterations in ROS formation, MTT reduction or mitochondrial membrane potential, as evaluated 24 or 48 h after menadione exposure. Accordingly, previous work has shown that 10 µM menadione decreases viability only in undifferentiated cells (Lim et al., 2008), as myotubes are more resistant to apoptosis than myoblasts. This is due to gene reprogramming induced by differentiation, including a decrease in p53 and apoptotic protease activating factor 1 (APAF-1) (Smith et al., 2009; Fortini et al., 2012). The absence of cell death 24 or 48 h after exposure to 10 µM menadione for 1 h may be explained by the fact that superoxide and hydrogen peroxide formation caused by menadione (Lim et al., 2008) is not high enough to induce severe oxidative cellular damage. It can be proposed that mild ROS formation induced by 1 h exposure to 10 µM menadione induces SESN2, activating an antioxidant defense response and inducing autophagy due to mTORC1 inhibition by SESN2. Nevertheless, 10 µM menadione induced changes on morphological characteristics of differentiated C2C12 cells.

As expected, exposure to 30 µM menadione for 1 h resulted in sustained oxidative conditions since increased ROS generation was observed 24 or 48 h after menadione exposure (Fig 20). This may be due to the fact that superoxide formation caused by 30 µM menadione exceeded the cellular antioxidant capacity, creating a more oxidative intracellular environment and a subsequent cycle of mitochondrial and cellular damage. Mitochondria are highly susceptible to oxidative stress, due to the concentration in oxidizable lipids and abundant redox-proteins, which amplify oxidative damage (Lee et al., 2012b). Accordingly, MTT reduction was decreased by exposure to 30 µM menadione (Fig 21) as well as mitochondrial membrane potential (Fig 22).

Overexpression of MnSOD (manganese-dependent superoxide dismutase) in HeLa cells has been shown to decrease superoxide formation and autophagy, suggesting that mitochondrial-

dependent superoxide generation mediates an autophagic response (Lee et al., 2012b). Under normal conditions, SESN2 has a protective antioxidant function in order to prevent damage from physiological insults resulting from cellular metabolism, such as oxidative phosphorylation or DNA replication (Lee et al., 2010a). An increase in ROS generation caused by unbalanced metabolism or external insults are expected to induce SESN2 expression in order to maintain cell viability (Budanov and Karin, 2008; Lee et al., 2010b). Therefore, SESN2 content was evaluated upon menadione exposure. Differentiated C2C12 cells exposed to 10 µM menadione exhibited an increase in SESN2 content, as evaluated 10h after menadione exposure (Fig 25). However, 24 or 48 h upon menadione exposure, SESN2 content was similar to control, suggesting that mild oxidative conditions caused by 10 µM menadione induce SESN2 expression as an early protective response, that may include increased action of ROS detoxifying systems and autophagy induction. Opposingly, 30 µM menadione did not induce SESN2 expression (Fig 25).

In order to clarify if mild oxidative stress triggers a mitochondrial protective response involving SESN2, RNA silencing experiments were conducted in undifferentiated C2C12 cells. Data shows any concentration of menadione induces SESN2 expression (Fig 26) and that depletion of SESN2 increases cell death in both control and stress conditions (menadione 10 and 30 µM) (Fig 27), highlighting the critical role of SESN2 for cellular homeostasis.

SESN2 is an oxidoreductase, with a direct role on PRXs regeneration. PRXs act as ROS scavengers (Sanchis-Gomar, 2013) by cycling through oxidation of catalytic cysteine and formation of a disulfide bridge. To complete the enzymatic catalytic cycle, Cys-SH groups are regenerated, for example by the thioredoxin-thioredoxin reductase system (Fourquet et al., 2008) PRX3 is the mitochondrial isoform responsible for scavenging mitochondrial H_2O_2. As described by Budanov and colleagues, 2004, SESN2 silencing resulted in ROS accumulation as well as dysfunctional mitochondria and accumulation of protein aggregates in cultured RKO (poorly differentiated colon carcinoma cell line) and MCF7 (breast cancer cells) cells, and oxidative stress in *Drosophila* skeletal muscle (Budanov et al., 2004; Lee et al., 2013). The current work demonstrates that decreasing SESN2 content with SESN2 siRNA leads to a sharper decrease in mitochondrial membrane potential (Fig 28), indicating an important upstream role of SESN2 in preventing mitochondrial dysfunction caused by oxidative conditions. It has been reported in cancer cells that an increase in ROS, as induced in the current experiments by 30 µM menadione or by SESN2 silencing, may result in mitochondrial membrane permebilization and induction of mitochondrial transition pore, leading to the release of pro-apoptotic proteins (Li et al., 2013). The results obtained in the current work suggest that SESN2 is up-regulated in response to a mild increase in ROS (as induced by 1 h exposure

to 10 µM menadione) and that maintenance of SESN2 levels is critical in increasing mitochondrial resistance to stress conditions and thus preventing cell death. It can be proposed that conditions of mild increase in ROS induced by 10 µM menadione induces SESN2 expression that once active, regenerates PRX3 which reestablishes antioxidant defense, degrades mitochondrial ROS, maintaining mitochondrial redox balance and protecting the cell from a cycle of oxidative damage (Fig 34). PRX3 has been shown to protect cell from apoptosis induced by H_2O_2 originated from menadione exposure (Chang et al., 2004). In cervical cancer cells, down-regulation of PRX3 caused an increase in ROS and, consequently, an increase in apoptotic cells (Li et al., 2013). Thus, is proposed that in C2C12 cells, after the introduction of SESN2 siRNA, PRX3 regeneration is blocked, causing an increase in mitochondrial ROS, mitochondrial dysfunction and an increase in cell death.

Sestrin induction by tumor suppressor p53 mediates its antioxidant function related with prevention of cell transformation and aging-associated pathologies (Budanov, 2011). It is known that while sustained and severe stress conditions activate pro-apoptotic genes, normal and mild stress induces genes involved in antioxidant defense, DNA repair, cell signaling and metabolism (Vousden et al., 2007). SESN2 induction also occurs by a p53-independent mechanism such as under hypoxic conditions (Budanov et al., 2002; Velasco-Miguel et al., 1999).

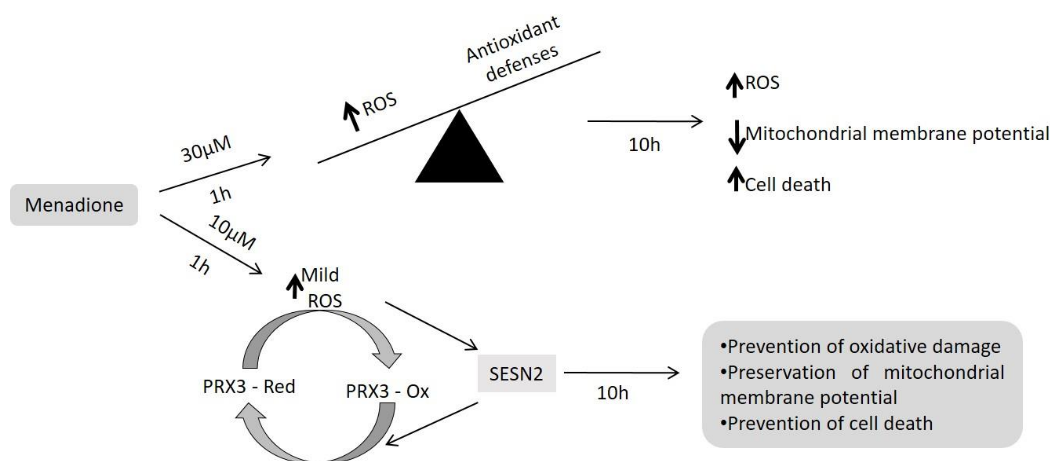

Fig 34 – Illustration of interaction between SESN2 and mitochondrial PRX3 and cell protection. A mild increase of ROS induced by menadione leads to inactivation of PRX3. However, this increase in ROS also activates SESN2 which reduces and regenerates PRX3 leading to cellular protection. Without SESN2, PRX3 remains inactive increasing oxidative damage induced by menadione and inducing cellular death. With a severe increase of ROS induced by 30 µM of menadione, the antioxidant systems is not effective and leads to increases in cellular oxidative damage. PRX3-Red – peroxiredoxin 3 reduced; PRX3-Ox - peroxiredoxin 3 oxidized.

The crosstalk between autophagy/mitophagy, redox signaling and mitochondrial dysfunction is still unclear, but dysregulated redox signaling and mitochondrial function has been shown to induce autophagy. It has also been shown that mitochondrial dysfunction leads to sustained oxidative stress, especially when the autophagic process is disrupted or in the absence of SESN2 (Lee et al., 2013).

It is known that SESN2 up-regulation, independently of its redox-regulating activity inhibits mTORC1 (Ro et al., 2014). Activation of AMPK by SESN2 is critical for the maintenance of metabolic homeostasis. SESN2 binds to AMPK and induces the activation of its catalytic subunit, resulting in mTORC1 inhibition and activation of autophagy (Hay, 2008). In mammalian cells, mTOR inhibition in response to stress is linked to ATG13 dephosphorylation, leading to autophagosome formation (Lee et al., 2012b). Pre-autophagosome assembly requires beclin-1-class III PI3K complex and LC3 insertion into the membrane (Lee et al., 2012b). LC3 is also essential in mitophagy, a type of selective autophagy linked to mitochondrial quality control that preserves a healthy population of mitochondria and prevents cell death (Lee et al.,2012b; Palikaras et al., 2015).

The increase in LC3 staining induced by menadione, in a dose-dependent manner, was blocked in C2C12 cells exposed to menadione with SESN2 silenced by siRNA (Fig 35). These results suggest that activation of autophagy by SESN2 is essential for the adaptive response induced by 10 µM menadione, since SESN2 silencing lead to increased cell death. Although not evaluated, it is proposed that stimulation of mitophagy by 10 µM menadione allows the elimination of dysfunctional mitochondria and prevents oxidative stress.

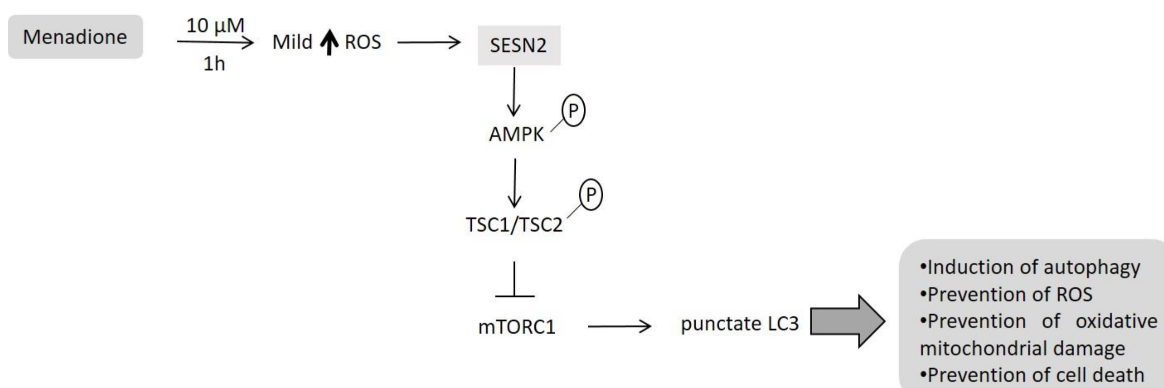

Fig 35 - Illustration of induction of autophagy by Sestrin 2. Stress induced by 10 µM menadione induces SESN2 expression and activates AMPK. AMPK phosphorylates TSC2 leading to activation of TSC1/TSC2 complex, inhibition of mTORC1 and activation of autophagy, showed by increased pattern of punctuated LC3. Activation of this mechanism prevents mitochondrial oxidative damage and preserves cell viability.

SESN2 has also been shown to modulate several pathways, probably depending on the nature of the stress. Several studies have shown that increased SESN2 expression inhibits cell growth as well as protects cells from low glucose, ischemia or H_2O_2 (Budanov and Karin, 2008; Budanov et al., 2002). In cancer cells, absence of PRX3, a SESN2 target, results in apoptosis induced by a combination of TNF-α and ciclohexamide (CHX) or by staurosporine (Chang et al., 2004), showing an anti-apoptotic role of SESN2 by regeneration of PRX3. Accordingly, studies performed by Ben-Sahra et al., 2013, reveal that in several cell lines, LNCaP (androgen sensitive human prostate adenocarcinoma cells) and MEFs (mouse embryo fibrobasts) cells, absence of SESN2 promotes apoptosis induced by energetic stress. However, other studies in HEK293 (human embryonic kidney 293 cell line) demonstrate that SESN2 is capable of negatively regulate cell growth, by inducing apoptosis (Budanov et al., 2002). Recent studies showed that polyphenolic compounds used in cancer treatment, induces apoptosis through SESN2/AMPK/p38(mitogen-activated protein kinase) pathway (Kim et al., 2014). The role of SENS2 as pro or anti-apoptotic protein remains controversial. It is known that by up-regulating antioxidant gene expression, p53 can induce a strong anti-apoptotic response (Sablina et al., 2005). However, p53 has a pro-oxidant response, by increasing mitochondrial ROS content, which induces caspases activation and promotes apoptosis (Polyak et al., 1997). Several proteins induced by p53, such as p53-inducible gene 3 (PIG3), bcl-2-like protein 4 (BAX), proline oxidase and Bcl-2-binding component 3 (BBC3) known as PUMA, are activated during apoptotic events and promote ROS formation (Vousden and Ryan, 2009). Recently, it was shown that premature aging observed in caspase-2 deficient mice involves increase oxidative damage and reduced activity of antioxidant enzymes and SESN2 (Shalini S et al 2012).

Taking into account that knockdown of SESN2 decreases the basal concentration of ATP (Ben-Sahra et al., 2013) and the results showing that SESN2 silencing by siRNA leads to a decrease in mitochondrial membrane potential, it is proposed that SESN2 depletion results in increased cell apoptotic cell death caused by an exponential increase in mitochondrial ROS and accumulation of damaged mitochondria. A decrease in SESN2 prevents PRX3 regeneration and blocks AMPK-mTORC1/2 axis signaling, conditions associated with increased apoptosis. SESN2 has been described as a regulator of AKT phosphorylation through AMPK-mTORC1/2 axis signaling in several cell types (Lee et al., 2012a). Accordingly, it has been demonstrated that oxidative stress caused by menadione induces PI3K/AKT signaling and apoptosis in several cells types including muscular cells (Criddle et al., 2006; Lim et al., 2008).

A previous study showed that exposure to 50µM menadione induced apoptotic cell death, involving superoxide formation and activation of caspases 9, 6 and 3 (Conde de La Rosa et al., 2006). It was also shown that low doses of menadione (10 µM) induces apoptosis shown by decreased BCL-2/BAX in L6 myoblasts. Expression of BCL-2 and BAX can be modulated by activation of AKT (Conde de La Rosa et al., 2006). Activation of PI3K/AKT by an increase in superoxide generation was previously reported by Kosmidou et al., 2001, suggesting that the cell death induced by menadione in the current model may involve apoptosis induction.

In summary and taking in consideration all the results obtained in this work, it is proposed that exposure of C2C12 cells to mild oxidative stress (induced by 10 µM menadione), activates antioxidant mechanisms in which SESN2 is a key regulator. When SESN2 is present, autophagy/mitophagy is induced probably by inhibition of mTORC1. Since the autophagic pathway is activated, the apoptotic pathway remains inhibited by SESN2. However, SESN2 depletion leads to an increase in mitochondrial ROS generation, decrease in mitochondrial membrane potential and inhibition of LC3 staining, but increased cell death, possibly through induction of apoptosis. Increased ROS generation by menadione inactivates PRX3 and promotes PI3K/AKT signaling, induction of pro-apoptotic mitochondrial proteins such as BAX, further impairing mitochondrial function.

4.2 Unravelling SESN2-SIRT1 as a protective axis against metabolic dysfunction in aging and obesity

SIRT1 acts as an energy sensor that modulates whole-body energy expenditure, being induced by low energy status. Mitochondrial decay associated with aging is characterized by decreased NAD^+ content and SIRT1 activity (Gomes et al., 2013). Previous studies have shown that SIRT1 improves insulin sensitivity by alleviating mitochondrial dysfunction and oxidative stress in skeletal muscle (Zhang et al., 2015). Also SIRT1 activation protects against high-fat diet-induced fatty liver, an effect related with stimulation of fatty acid oxidation (Lagouge et al., 2006; Feige et al.,2008) Accordingly to that, hepatic SIRT1 silencing leads to the development of NAFLD (Purushotham et al., 2009). Experiments with sirtuin activating compounds have shown that SIRT1 activation increases mitochondrial mass and stimulates oxidative metabolism, protecting from obesity-induced deleterious effects (Feige et al., 2008; Lagouge et al., 2006; Price et al., 2012). Resveratrol is a polyphenol that can be found in grapes and red wine and to which have been assigned beneficial effects in metabolic diseases and aging, due to activation of SIRT1 and AMPK (Seo et al., 2014,Baur and Sinclair, 2006; Price et al., 2012).

The maintenance of mitochondrial homeostasis in conditions of SIRT1 stimulation is probably dependent on balanced mitochondrial turnover, combining increased mitochondrial biogenesis with proper clearance of damaged organelles. The fact that a decrease in NAD^+ inhibits the autophagic flux (Hsu et al., 2009), while an increase in NAD^+/NADH ratio induces mitophagy (Jang et al., 2012) suggests a role for SIRT1-mediated autophagy in mitochondrial homeostasis.

Liver has a key role in glucose and fat metabolism. Members of CCAAT/Enhancer Binding Protein (c/EBPα and c/EBPβ) are highly expressed in the liver and important regulators of hepatic metabolism, playing an important role in the control of cell differentiation and proliferation (Jin et al., 2011). Previous studies revealed that ectopic expression of SIRT1 down-regulates c/EBPα, a transcription factor with hepatic growth inhibitory activity (Iakova et al., 2003; Jin et al., 2011). In this work, this relation was studied by modulating SIRT1 content with diet, aging and resveratrol. The results demonstrate that HFD induces an increase in c/EBPα expression. Treatment with resveratrol reverted the effects of HFD. It can be speculated that by preserving SIRT1 activity in HFD animals, resveratrol maintains normal hepatic metabolic function essential for hepatocyte function. This effect was shown to be SIRT1-dependent since in SIRT1-KO animals none of the previous results were observed.

A decrease in SESN2 expression was observed in HFD animals but resveratrol treatment reverted the HFD effects, leading to an increase in SESN2 expression. This is in accordance with previous data (Jin et al., 2013) showing induction of SESN2 and inhibition of lipogenesis by resveratrol in livers of mice fed a high-fat diet. SIRT1-KO led to a shaper decrease in SESN2 expression even in standard-diet conditions, suggesting that SESN2 is partially regulated by resveratrol and by SIRT1.

Recently it has been proposed that resveratrol induces SESN2 expression, which is linked to prevention of mitochondrial dysfunction and Nrf2 activation (Seo et al., 2014). Induction of SESN2 in hepatocytes is linked to Nrf2 activation as a preventive response against oxidative stress (Shin et al., 2012) since Nrf2 is an essential sensor in oxidative metabolism and is responsible for the induction of antioxidant pathways. As a natural anti-oxidant, resveratrol has the capacity to stimulate Nrf2 activity and consequently its targets expression (Seo et al., 2014). Recently, SIRT1 has been shown to increase Nrf2 expression with consequent activation of Nrf2-ARE anti-oxidative pathway (Huang et al., 2015).

SESN2 stimulates Nrf2 activity by stimulating p62-autophagic degradation of KEAP1 (Bae et al., 2013). We observed that KEAP1 expression is decreased in HFD animals, which may be due to increased ROS generation in the livers of these animals demanding the activation of transcription

factors associated with an antioxidant response, such as Nrf2. Resveratrol treatment increased KEAP1 expression. This seems to be a contradiction; however, this work only evaluated KEAP1 mRNA. We can´t exclude the degradation of KEAP1 at the protein level, allowing the activation of Nrf2. Additionally, the improvement of mitochondrial function by resveratrol treatment and associated decrease in oxidative stress may act as a stimuli for KEAP1 induction and consequent normalization of the antioxidant response. In SIRT1-KO animals, resveratrol had no effects on KEAP1 mRNA suggesting that SIRT1 is essential for resveratrol's effect on KEAP1. Studies with resveratrol precursors have shown to up-regulate SIRT1 levels in vitro and decrease KEAP1 cytoplasmic content while increasing KEAP1 in the nuclear fraction (Huang et al., 2015).

Aging is known to be responsible for a decrease in metabolic function in several tissues and a characteristic increase in ROS and accumulation of oxidized and modified proteins (Budanov et al., 2010). Accordingly this work shows that c/EBPα, SESN2, KEAP1 and Nrf2 expression decreases with age, highlighting possible therapeutic targets to delay aging.

With this work is demonstrated that mild oxidative stress is capable of preventing cell death by inducing a mitochondrial protective response, regulated by SESN2 in a dependent-manner. SIRT1 is also a regulator of SESN2 and antioxidant genes together with Resveratrol, promoting the increase of fatty acids accumulation. Together the results can provide new insights for treatment of Metabolic Syndrome.

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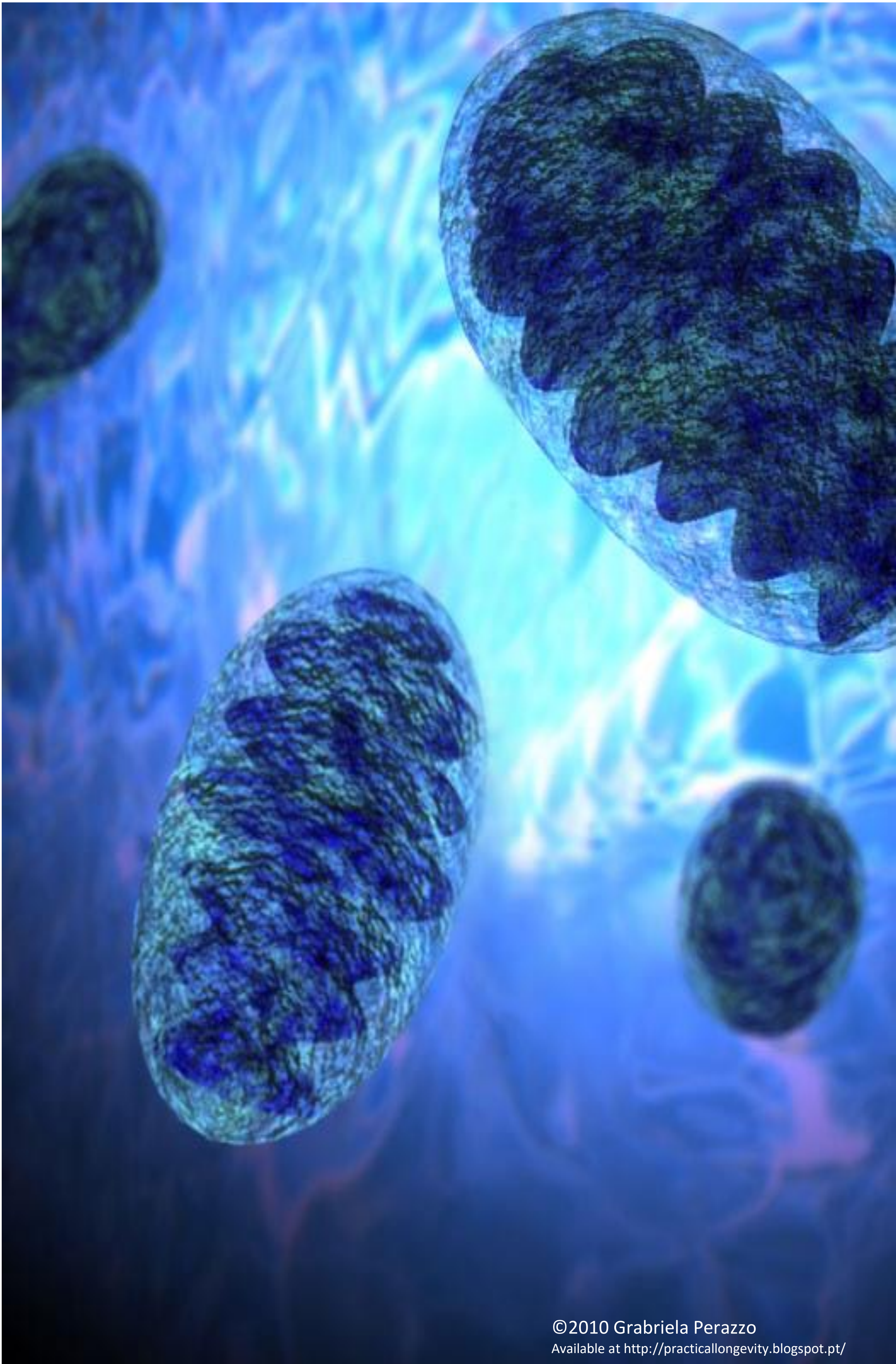
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