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Departamento de Ciências Médicas

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular realizada sob a orientação científica da Doutora Sandra Maria Tavares da Costa Rebelo, Professora Auxiliar Convidada do Departamento de Ciências Médicas da Universidade de Aveiro e coorientação da Doutora Filipa de Sá Martins, Investigadora de pós-doutoramento do Departamento de Ciências Médicas da Universidade de Aveiro

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palavras-chave

Envelhecimento, senescência, funções nucleares, proteínas do envelope nuclear, interações proteína-proteína, ratinhos envelhecidos, fibroblastos humanos

resumo

O envelhecimento é caracterizado por um decréscimo generalizado da condição física durante o curso de vida. Assim, com a idade, aumenta o risco de desenvolver doenças neurodegenerativas, diabetes, cancro e doenças cardiovasculares. Vários estudos foram realizados no sentido de determinar as alterações moleculares e celulares adjacentes ao envelhecimento, contudo, os mecanismos moleculares exatos que regulam o processo de envelhecimento continuam pouco conhecidos. Ainda assim, a regulação de proteínas na periferia do envelope nuclear, nomeadamente da lamina e de proteínas nucleares associadas à lamina têm sido sugeridas como intervenientes no processo de envelhecimento. De facto, em modelos de envelhecimento prematuro, algumas alterações em proteínas do envelope nuclear foram reportadas. Neste trabalho, foi realizada uma análise bioinformática que nos permitiu elaborar um estudo amplo do processo do envelhecimento. Com recurso a duas bases de dados públicas, identificamos uma lista de genes relacionados com o envelhecimento. A análise dos dados evidenciou que mais de metade dos genes relacionados com o envelhecimento correspondem a proteínas nucleares, o que sugere um papel para a função nuclear no processo de envelhecimento. Ainda, a construção de redes de interação proteína-proteína levou-nos à identificação de clusters funcionais relacionados com regulação transcricional, remodelação da cromatina, processos metabólicos e reparação do DNA. Adicionalmente, investigamos as alterações dos níveis e de localização de proteínas do envelope nuclear durante o envelhecimento, em tecidos de ratinho e fibroblastos humanos. Os nossos resultados indicam claramente que os níveis da proteína lamina A/C e da proteína SUN1 aumentam com a idade tanto em modelos humanos como em ratinhos. Os níveis da nesprina-2, da emerina, da LAP1 e da lamina B1 aumentam com a idade em ratinhos, enquanto que os níveis da NUP133 e da NUP93 diminuem com a idade em fibroblastos humanos. Foi ainda evidenciada uma perda da integridade nuclear em fibroblastos humanos, assim como uma diminuição da fração nucleocitoplásmica da lamina A/C. Também observámos um aumento de *speckles* nucleares de emerina com a idade em fibroblastos humanos. Para concluir, a arquitetura nuclear e as proteínas do envelope nuclear parecem sofrer alterações durante o processo do envelhecimento. Assim, estudos futuros deveriam explorar e elucidar a sua contribuição para o processo de envelhecimento.

Keywords Ageing, senescence, nuclear functions, nuclear envelope proteins, protein-protein interaction, aged mice, human fibroblasts

Abstract

Ageing is characterized by an overall organismal fitness decrease across lifespan. Hence, the risk of developing complex diseases as neuropathologic disorders, diabetes, cancer and cardiovascular diseases increases with age. Despite a huge effort to determine the underlying molecular and cellular changes, the precise molecular mechanisms that regulate the ageing process remain elusive. Although, in the last few years, the protein regulation at the nuclear periphery, particularly of the nuclear lamina and lamina-associated proteins, has been pointed out as a major contributor to the ageing process. In fact, in premature ageing models, some nuclear envelope proteins have been mentioned to be altered. In this work, we performed a bioinformatic analysis that allowed us to develop an intricate study of the ageing process. Briefly, we assembled a list of age-related genes by combining the data of two public databases. Remarkably, the analysis of the data places the nucleus in the spotlight of the ageing process, given that almost half of the age-related genes are associated to nuclear functions. Moreover, protein-protein interaction network construction led us to the identification of prominent age-related functional clusters related with transcription regulation and chromatin remodelling, and DNA metabolic process and DNA repair. Further, in order to unravel the nuclear envelope proteins alterations during ageing, we performed immunoblotting and immunocytochemistry analysis in mice tissues and human fibroblasts. Our results clearly indicate that lamin A/C and SUN1 protein levels increases during ageing in both human and mice models. Moreover, nesprin-2, emerin, LAP1, and lamin B1 protein levels increase with age in mice models, whereas lamin B1, NUP133 and NUP93 protein levels decreases with age in human fibroblasts. Moreover, in aged human fibroblasts it is observable a loss of the nuclear integrity, combined with a decrease of the nucleocytoplasmic fraction of lamin A/C. Also, an increase of emerin nuclear speckles is noticeable in aged fibroblasts. To conclude, the nuclear architecture and the nuclear envelope proteins seems to be affected during the ageing process and further studies should be performed in order to elucidate their relative contribution to ageing.

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List of Abbreviations

A.U. – Arbitrary Units

AGEID – Aging Genes/Interventions Database

ANOVA – Analysis of variance

APC – Allophycocyanin

APS – Amonium persulfide

A-T – Ataxia Telangiectasia

ATP – adenosine triphosphate

BAF – barrier to autointegration factor

BCA – bicinchoninic acid

BMP – bone morphogenic protein

BSA – bovine serum albumin

Btf – death promoting transcriptional repressor

CEDCM-BEA – Animal welfare body of the Department of Medical Sciences of the University of Aveiro

DAPI – 4',6-diamidino-2-phenolyde

DAVID – The Database for Annotation, Visualization and Integrated Discovery

dH20 – distilled water

DMEM – Dulbecco's Modified Eagle Medium

DNA – deoxyribonucleic acid

DTT – Dithiothreitol

ECM – extracellular matrix

EDMD – Emery-Dreifuss muscular dystrophy

EDTA – Ethylenediamine tetraacetic acid

EGTA – Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid

ELB – extraction lysis buffer

ER – endoplasmic reticulum

FBS – Fetal Bovine Serum

FG – phenylalanine-glycine

g – times gravity

GCL – germ cell less

GO – Gene Ontology

h – hours

H3K27 – Histone H3 lysine 27

H3K4 – Histone H3 lysine 4

H3K9 – Histone H3 lysine 9

H4K20 – Histone H4 lysine 20

HEPES – N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid

HGPS – Hutchinson-Gilford progeria syndrome

HRP – Horseradish peroxidase

iBiMED – Institute for Biomedicine of University of Aveiro

ICC – immunocytochemistry

ICMT – isoprenylcysteinecarboxylmethyltransferase

INM – inner nuclear membrane

iPSCs – induced pluripotent stem cells

KASH – Klarsicht/ANC-1/SYNE homology

LAP1 – Lamina-associated polypeptide 1

LAP2 – Lamina-associated polypeptide 2

LAP2 – lamina-associated polypeptide 2

LB – loading buffer

LBR – Lamin B receptor

LINC – Linker of Nucleoskeleton and Cytoskeleton

mAb – monoclonal antibody

MAN1 – Inner nuclear membrane protein Man1

MAPK – mitogen activated protein kinases

min – minutes

Na3VO4 – Sodium orthovanadate

NaCl – sodium chloride

NAD – nicotinamide adenine dinucleotide

NE – Nuclear envelope

nm – nanometre

NPC – nuclear pore complexes

Nrf2 – nuclear factor erytroid-2-related factor 2

NUP – nucleoporin

ºC – degrees Celsius

ONM – outer nuclear membrane

PANTHER – Protein ANalysis THrough Evolutionary Relationships

PBS – phosphate buffered saline

PCNA – proliferating cell nuclear antigen

PI – propidium iodide

PN – passage number

PNS – perinuclear space

PP1 – protein phosphatase 1

PPI – protein-protein interaction

PTMs – Post-translational modifications

Rb – retinoblastoma

RNA – ribonucleic acid

ROS – reactive oxygen species

rpm – rotations per minute

RT – room temperature

SDS – sodium dodecyl sulfate

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel elextrophoresis

SEM – standard error

siRNA – small interfering RNA

SIRT – sirtuin

SUN – Sad1/UNC-84

TBS – Tris-buffered saline

TBS-T – Tris-buffered saline with Tween 20

TEMED - N,N,N′,N′-Tetramethylethylenediamine

TERT – telomerase reverse transcriptase

TGFβ – transforming growth factor beta

TOR1AIP1 – Torsin-1A-interacting protein 1

TPR – Nucleoporin TPR

TRF – telomere restriction fragment

VSMC – vascular smooth muscle cells

WB – western blotting

WT – wild type

YO – years old

ZMPSTE24 - Zinc Metallopeptidase STE24

CHAPTER I

General introduction and aims

1. Introduction

1.1.The Nuclear Envelope

The nuclear envelope (NE) is a very specialized double membrane that encloses the eukaryotic nuclear content (D'Angelo and Hetzer, 2006). The NE is crucial to keep the individuality of the nucleus and the cytoplasm as it represents the only barrier between these two compartments (Postberg *et al*., 2010). The NE is composed by the nuclear pore complexes (NPCs), the nuclear lamina, and two nuclear membranes, each of which is a lipid bilayer, namely the outer nuclear membrane (ONM) and the inner nuclear membrane (INM) (Watson, 1955; Yang *et al*., 2017). These membranes are separated by the perinuclear space (PNS) which corresponds to a 30 to 50nm gap (Stewart *et al*., 2007) (figure I.1).

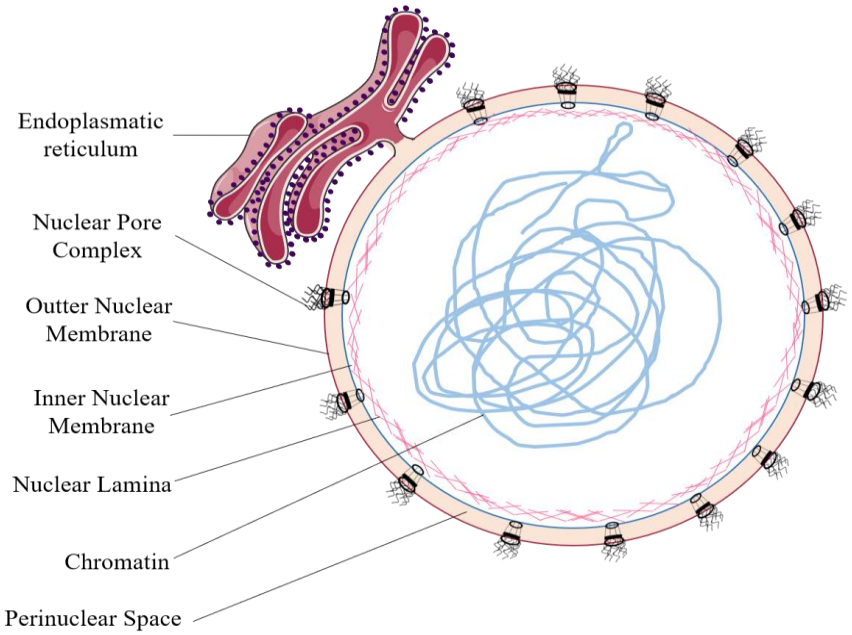

Figure I.1 – Schematic representation of the nuclear envelope. The nuclear envelope encloses the nucleus and is composed by a double nuclear membrane (the inner and the outer nuclear membrane). The perinuclear space is enclosed between the INM and the ONM. The nuclear lamina is localized close to the inner side of the nuclear membrane. The ONM is contiguous with the endoplasmic reticulum. The nuclear pore complexes go through the nuclear membranes.

The ONM is continuous with the rough endoplasmic reticulum (ER), which explains the presence of ribosomes on its cytoplasmic surface (D'Angelo and Hetzer, 2006; Newport

and Forbes, 1987) which are involved in protein synthesis (Alberts *et al*., 2008). The ONM also offers attachment sites for structural elements of the cytoplasm (Burke and Ellenberg, 2002). The link between the cytoskeleton and the ONM is vital to the NE integrity and to the location of the nucleus (Fernández-Álvarez and Cooper, 2017).

The INM is particular and encompasses many integral membrane-associated proteins (D'Angelo and Hetzer, 2006; Katta *et al*., 2014; Stewart *et al*., 2007). The INM contains many proteins that are involved in processes like genome regulation, inheritance and protection, counting peripheral and integral proteins (Ungricht and Kutay, 2017). The INM nucleoplasmic layer is closely associated with the nuclear lamina, which is a network of fibrous intermediate filaments that guarantees the integrity and supports the nuclear structure (Senior and Gerace, 1988; Alberts *et al*., 2008; Yang *et al*., 2017). Furthermore, the nuclear lamina is a thin matrix of ~10-20nm with multiple proteins constituted by A- and B-type lamins that are associated with proteins from the INM, establishing connections between the cytoskeleton, the nucleoskeleton, and the genome (Lee and Burke, 2017; Brady *et al*., 2018). It contacts directly with chromatin and might play a role in gene expression, DNA replication, DNA repair, chromosome organization, transcriptional control and heterochromatin maintenance (D'Angelo and Hetzer, 2006; Katta *et al.*, 2014; Fernández-Álvarez and Cooper, 2017).

The NPCs are aqueous multiprotein channels that penetrate both the inner and the outer nuclear membranes at many sites (Liu *et al*., 2012; Rothballer and Kutay, 2013). According to Hetzer (2010), these channels have an outer diameter of ~100 nm and a central transport channel measuring 40 nm in diameter (Hetzer, 2010). The main structure of NPCs can be represented by three rings, namely the nucleoplasmic ring at the top, the cytoplasmic ring at the bottom and in the middle: the inner ring. Some cytoplasmic filaments spread from the cytoplasmic ring. Below the nucleoplasmic ring, there is the nuclear basket which is connected with it (figure I.2) (De Magistris and Antonin, 2018).

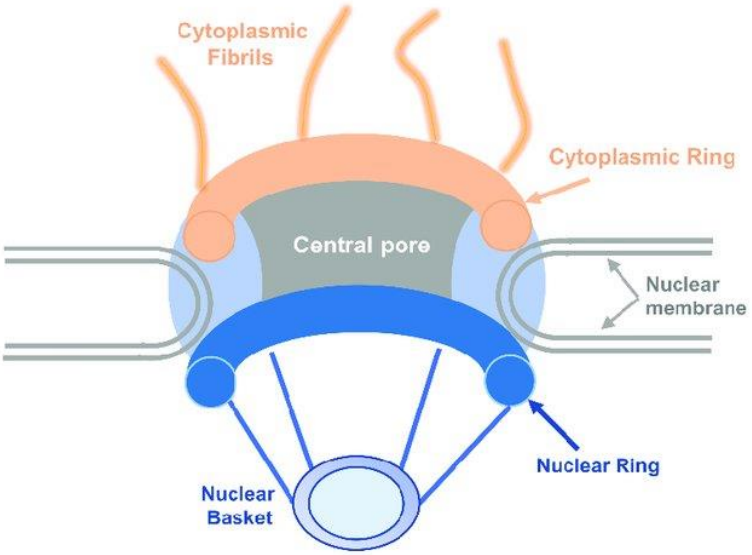

Figure I.2 – The Nuclear Pore Complex structure and composition. The nuclear pore complex is formed by cytoplasmic fibrils, the cytoplasmic ring, the central pore, the nuclear ring and the nuclear basket. Taken from Fu *et al.*, (2018).

The NPCs are formed by several complexes composed of nuclearporins. The main function of the NPCs is to enable the nucleocytoplasmic transport of small molecules (<40kDa and <5nm) (Patel *et al.*, 2007) and some larger protein complexes containing important signalling sequences (Robijns *et al.*, 2018). The NPC transport is arbitrated by a transient interaction between the NPC and karyopherins. Karyopherins are a transport receptors superfamily that links to their cargoes by recognizing precise nuclear localization or export signals. This link is controlled by the Ras-related GTPase Ran. Protein regulators of the GTPase cycle and subcellular localization supports Ran in this regulation (Pemberton and Paschal, 2005). Several proteins have been associated with this nucleocytoplasmic shuttling process, namely transcription factors, translation initiation factors, cell proliferation regulators, RNA binding proteins, and hormone receptors (Gama-Carvalho and Carmo-Fonseca, 2001; Hetzer, 2010; Liu *et al.*, 2012; Fu *et al.*, 2018).

1.1.1. The Nuclear Envelope Proteins

The NE proteins work together to perform individual structural, functional, and regulatory roles between the genome and the cytoskeleton, allowing the metazoan cells to answer various mechanical, chemical and inflammatory stimuli in a proper and timely manner (Brady *et al.*, 2018). The main NE proteins are represented in figure I.3.

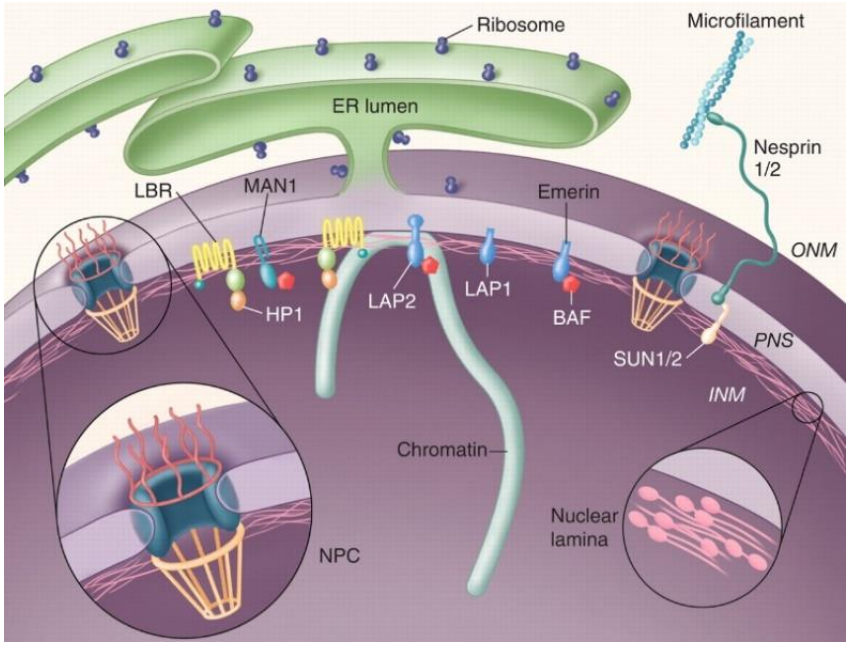

Figure I.3 – Structure of the nuclear envelope and associated proteins. LAP1, LAP2, BAF, Emerin, SUN1/2, Nesprin 1/2, LBR and MAN1 are the most abundant proteins of the nuclear envelope. Image taken from Stewart *et al*. (2007). INM, inner nuclear membrane; PNS, perinuclear space; ONM, outer nuclear membrane; NPC, nuclear pore complex.

- **Lamins**

The nuclear lamina is composed by members of the family of the intermediate filaments called lamins, which are a complex of proteins located close to the INM in a clear majority of the metazoan cells playing a fundamental role in the nuclear architecture (Reddy and Comai, 2016).The main elements of the nuclear lamina are two types of lamins: the A- and the B-type lamins. A-type lamins can be divided in lamin A, lamin C, lamin C2 and lamin C3, whereas B-type lamins are lamin B1, lamin B2 and lamin B3. Furthermore, the A-type lamins are encoded by a unique gene, the *LMNA*, by alternative splicing. B-type lamins are derived from two different genes: *LMNB1* that encodes lamin B1 and *LMNB2* that encodes lamins B2 and B3 (Schreiber and Kennedy, 2013). Lamins A/C are expressed only in differentiated cells, but C2 is uniquely expressed in germ cells. Recent studies reported the presence of low levels of lamin A/C in mouse embryonic stem cells (Eckersley-Maslin et al., 2013). Lamin B3 is specific of the testis tissue (Schütz *et al.*, 2005).

Lamins comprise a globular N-terminal head domain, a coiled-coil central rod domain, and a C -terminal tail domain. Lamins present singularities at the tail domain like a nuclear localization signal, an immunoglobulin fold domain, and a CaaX motif (C=Cysteine;

A= Alyphatic residue; X= any residue) (de Leeuw *et al.*, 2018). These proteins go through a long process of post-translational modifications (PTMs; figure I.4). A-, B1- and B2-lamin types are synthesised as prelamins containing a CaaX motif at their carboxy-terminal tails (Dittmer and Misteli, 2011).

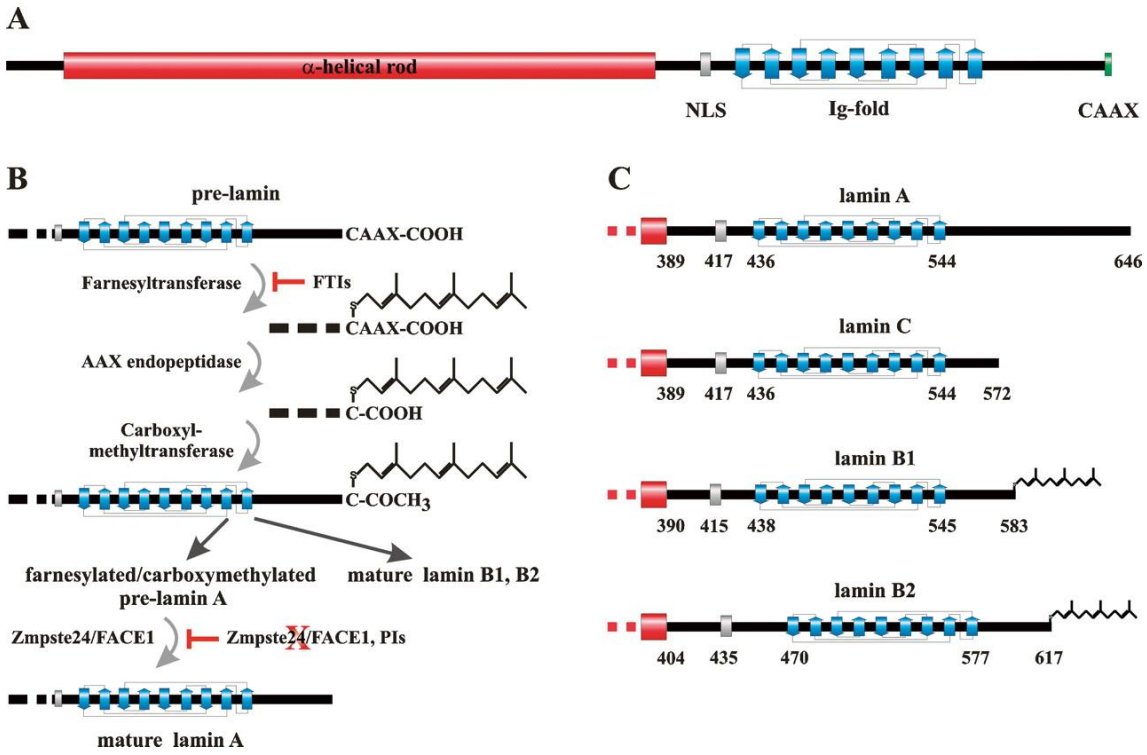

Figure I.4 – Schematic representation of the prelamin maturation process. Prelamin, the precursor to mature lamin A, lamin B1, and lamin B2 comprise a carboxyl-terminal *CaaX* motif that activates farnesylation and methylation of a carboxyl-terminal cysteine. Prelamin A undertakes one extra processing step: the last 15 amino acids of the protein, including the farnesylcysteine methyl ester, are removed, releasing the mature lamin A. Taken from Dechat *et al*. (2008).

The complex processing of the CaaX motif starts with an incorporation of the hydrophobic farnesyl group to the cysteine residue by farnesyltransferase and it is followed by the cleavage of the final three amino acids (-AAX) by an AAX endopeptidase like Zmpste24 or FACE1 metallopeptidases. An isoprenylcysteinecarboxylmethyltransferase (ICMT) adds a methyl group to the carboxylic acid group (-COOH) of the C-terminal cysteine (Dechat *et al*., 2008). In order to produce a mature Lamin A, another cleavage of 15 amino acids upstream of the farnesylated and methylated cysteine by Zmpste24 is needed (Carrero *et al.*, 2016).

Defects or failures in the removal of the farnesylated and methylated cysteine result in serious conditions and diseases like Hutchinson-Gilford progeria syndrome (HGPS) which is a very rare genetic disorder that presents clinical traits that mimic physiological ageing (Carrero *et al.*, 2016; de Leeuw *et al.*, 2018). This disorder occurs due to an accumulation of a permanently farnesylated and carboxy methylated prelamin A variant called progerin that results from the deletion of the second cleavage site (Reddy and Comai, 2016). The most frequent HGPS mutation occurs at codon 608 (G608G) and results in a cryptic splice site in the exon 11, excluding a proteolytic cleavage site in the expressed mutant lamin A (Pollex and Hegele, 2004). Other genetic disorders can be caused by mutations in the nuclear lamins or other NE proteins. These disorders are known as laminopathies and display various phenotypes and symptoms that can vary from muscular dystrophy to neuropathies to premature ageing syndromes (Schreiber and Kennedy, 2013).

- **Emerin**

Emerin is a nuclear protein encoded by the *EMD* gene and is attached to the inner nuclear membrane through its hydrophobic C-terminal domain (Samson *et al.*, 2017). Emerin is expressed in various tissues but it seems to play a prominent role in maintaining the regular function in skeletal and cardiac muscle (Wilson *et al.*, 2008). Emerin interacts with lamin A/C and mutations in any of these proteins can cause Emery-Dreifuss muscular dystrophy (EDMD) (Wilkinson *et al.*, 2003). EDMD is a disorder that influences skeletal and cardiac muscle, resulting in contractures, movement restriction, muscle weakness and wasting with propensity to get worse over time, heart conditions and a higher risk of unexpected death (Fairley *et al.*, 1999). Emerin is a member of the LEM-domain protein family. The LEM domain is found in LAP2β, emerin, and MAN1 (Lin *et al.*, 2000) proteins, is a 40-residue LEM-domain and also a conserved chromatin domain that connects with the BAF (barrier to autointegration factor)(Koch and Holaska, 2014) (figure I.5). Moreover, emerin binds to transcriptional repressors like GCL (germ cell less), Btf (death promoting transcriptional repressor) and the RNA-associated splicing factor YT521-B. Emerin also interacts with many other NE proteins, such as nesprin-1 and -2, MAN1, nuclear actin, nuclear myosin I, nuclear aII spectrin and lamins A, B and C (Bengtsson and Wilson, 2004). Collectively, these proteins are important in processes like the regulation of some genes,

nuclear assembly during cell division, supervising chemical signalling, aid chromatin tethering and sustaining the nuclear structure and stability (Berk *et al*., 2013).

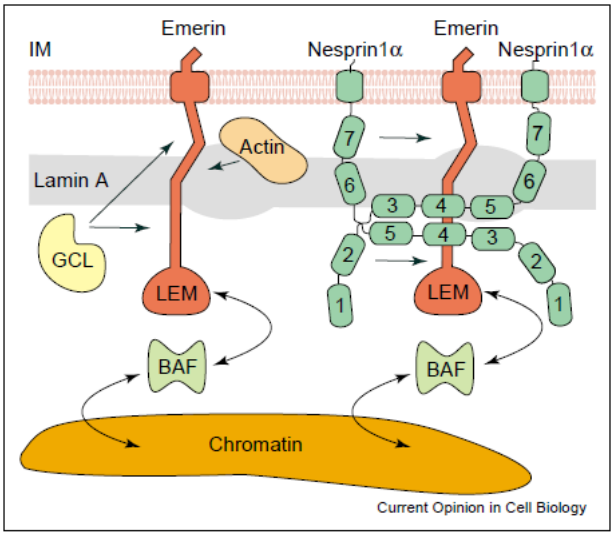

Figure I.5 – Schematic representation of emerin. Emerin is attached at the inner nuclear membrane, interacts with lamin A, GCL (germ cell less), BAF (barrier to autointegration factor), actin and a nesprin dimer. IM, inner nuclear membrane. Taken from Bengtsson and Wilson (2004).

- **LAP1 and LAP2**

Lamina-associated polypeptide 1 (LAP1) is a type II transmembrane protein of the INM and it is encoded by the *TOR1AIP1* gene (Senior and Gerace, 1988). LAP1 was one of first proteins to be associated with lamins (Foisner and Gerace, 1993). Historically, LAP1 was first identified in rat liver nuclei using a monoclonal antibody against lamina-enriched fractions (Senior and Gerace, 1988). Studies with null LAP1 fibroblasts show that it is essential for normal localization of emerin and A-type lamins in the INM (Shin *et al*., 2014). The association between LAP1 and lamins seems to be a key for lamins attachment to the NE during the interphase, playing a role in the preservation of the NE architecture (Foisner and Gerace, 1993; Rebelo *et al*., 2015; Santos *et al.*, 2015).

LAP1 has different species-specific isoforms described so far. There are three different LAP1 isoforms described in rats namely LAP1A, LAP1B and LAP1C with molecular weights of 75, 68, and 55kDa, respectively (Senior and Gerace, 1988). These isoforms only differ in the nucleoplasmic region. On the other hand, only two human LAP1 isoforms have been identified: LAP1B with a molecular weight of 68kDa and the more

recently reported LAP1C, a N-terminally truncated isoform of LAP1 with 55kDa (Santos *et al*., 2014). Three mouse LAP1 isoforms have been described: LAP1A with 64.7kDa, LAP1B with 58.3kDa and LAP1C with 43.9kDa (Shin *et al*., 2014).

The precise functions of LAP1 are not fully known yet. So far, some interactors of LAP1 have been uncovered like torsinA, emerin and protein phosphatase 1 (PP1) leading to the attribution of some functions to LAP1, such as nuclear architecture preservation and chromatin regulation (from the LAP1-lamins interaction) (Schirmer and Foisner, 2007), management of the torsinA ATPase activity, NE localization of torsinA and NE dynamics (from the LAP1-torsinA interaction) (Brown *et al*., 2014; Sosa *et al*., 2014) and the NE integrity and skeletal muscle maintenance (from the LAP1-emerin interaction) (Shin *et al*., 2013). The interaction between LAP1 and PP1 is reported to be involved in the control of the cell cycle progression (Santos *et al*., 2015). LAP1 has also been recently appointed as an important factor in the production of healthy sperm cells (Serrano *et al*., 2017).

Lamina-associated polypeptide 2 (LAP2) is a family of six proteins obtained by alternative splicing. Four of these proteins (LAP2 β, γ, δ, and ε) are type II membrane proteins. LAP2β binds to lamin B (Foisner, 2003). LAP2α is distributed in the nucleoplasm and interacts with lamin A/C, forming a LAP2α-lamin A/C complex that display a negative effect in cell proliferation and might be involved in cell differentiation and in chromatin organization and stabilization in the nucleus (Gesson *et al*., 2014). Regardless of lamins A/C, LAP2α is able to control extracellular matrix (ECM) components (Gesson *et al*., 2014; Vidak *et al*., 2015).

- **SUN1 and Nesprin-2**

SUN proteins are type II membrane proteins, normally encountered in the INM. They are conserved virtually in the entire eukaryotes' group, especially in mammalian somatic cells. They are composed of an N-terminal variable nucleoplasmic region that interacts with A- and B-type lamins and a transmembrane helix linked with a predicted coiled-coil C-terminal segment placed in the perinuclear space (Razafsky and Hodzic, 2009; Sosa *et al*., 2013).

SUN proteins are part of the Linker of Nucleoskeleton and Cytoskeleton (LINC) Complex (figure I.6). LINC complexes are composed by the Sad1/UNC-84 (SUN) INM

proteins and Klarsicht/ANC-1/SYNE homology (KASH) proteins (also known as nesprins, nuclear envelope spectrin repeat proteins) located in the ONM (Lee and Burke, 2017). These proteins interact with each other through the short KASH peptide and the SUN domain in the luminal space (Zhu *et al.*, 2017).

Similarly to the SUN proteins, KASH proteins are type II membrane proteins that have several spectrin-repeat-containing N-termini anchored to the ONM interacting with the cytoskeleton and other cytoplasmic proteins (Jahed *et al.*, 2018) followed by an highly conserved region of 35 aa rooted in the PNS (Razafsky and Hodzic, 2009). This short peptide connects with KASH binding sites on single SUN protein trimers (Lee and Burke, 2017).

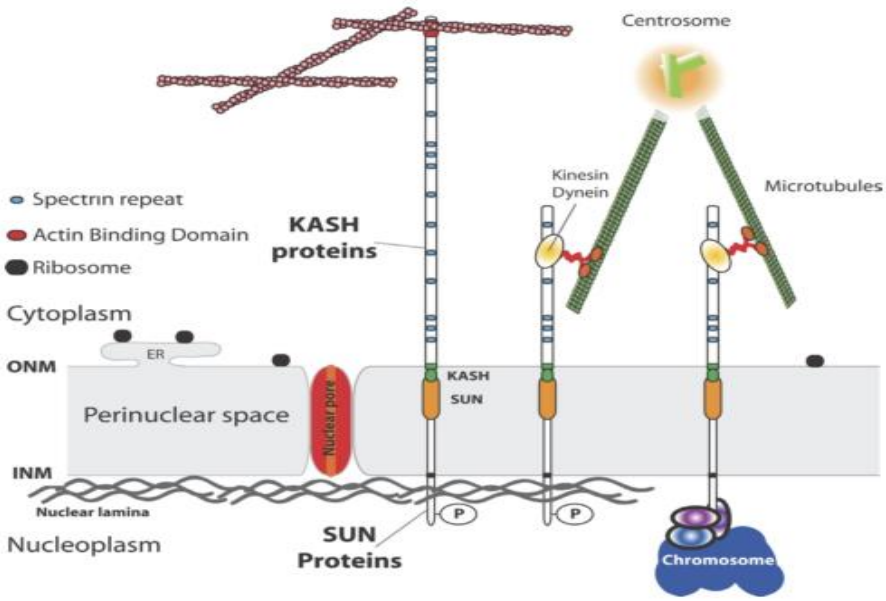

Figure I.6 – The LINC complex. LINC complexes are composed by the Sad1/UNC-84 (SUN) INM proteins and Klarsicht/ANC-1/SYNE homology (KASH) proteins located in the outer nuclear membrane. ONM, outer nuclear membrane; INM, inner nuclear membrane. Taken from Puckelwartz (2017) and Razafsky and Hodzic (2009).

The number of SUN proteins increases depending on the complexity of the organism. Simpler organisms, like unicellular ones, only have one SUN domain protein. However, more evolved organisms like mammalians display at least five different members of the SUN protein family (Sosa *et al.*, 2013; Horn, 2014).

Likewise, at least six genes corresponding to KASH proteins are encoded in mammals, namely nesprins 1-4, lymphoid-restricted membrane protein and KASH5. The members of these protein families are expressed differently in various tissues due to

alternative splicing (Horn, 2014; Lee and Burke, 2017; Jahed *et al*., 2018).

Nevertheless, the most commonly expressed SUN proteins in mammals are SUN1 and SUN2 while the most expressed KASH proteins in mammals are nesprins 1-4. These SUN and KASH proteins interact with each other. (Jahed *et al.*, 2018).

The LINC complex might play a role in the spread of signals from the cytoplasm to the nucleus and it is possibly involved in several functions like moving and shaping the nucleus, signal transduction, chromatin localization (Puckelwartz, 2017), and spermatogenesis (Horn, 2014). There is a certain redundancy of the ligation between SUN1 or SUN2 to KASH proteins, leading to interchangeable functions between them of coupling the nucleus of centrosomes in neuronal precursors in the developing brain, repairing DNA damage, nuclear positioning in the skeletal muscle, among other functions. SUN1 alone has been described as necessary for DNA double-strand break repairs, pairing meiotic chromosomes, taking part in RNA export as a messenger, nucleolar morphogenesis, etc. On the other side, SUN2 alone is responsible for the formation of actin cables that are able to move the nucleus (Jahed *et al*., 2018).

Nesprins have been associated with many diseases like myopathies, neurological disorders, cancer, hearing loss and arthrogryposis. Nesprin-1 and -2 are linked with EDMD and EDMD-like phenotypes. Lamin A/C mutations affect the LINC complex, altering nesprin-1, nesprin-2 and SUN2 localization. In progeroid syndromes regulated by lamin A/C, nesprin-2 giant is down-regulated, while SUN1 is significantly upregulated. In this case, nesprin-2 giant acts as a compensatory mechanism since its presence *in vitro* improves the deleterious progerin defects. Nevertheless, SUN1 tend to extend the lifespan of lamin A/C deficient and progeroid mice and nesprin-2 giant knock-out mice do not present an ageing phenotype (Cartwright and Karakesisoglou, 2014; Puckelwartz, 2017). Nesprin is also implicated in mRNA export, opening the possibility that nesprin helps anchoring SUN1 close to the NPC aiding and enabling it to accomplish its duty in mRNA export (Li *et al*., 2017).

- **Nucleoporins**

The NPCs are very large complexes with a molecular weight of approximately 120 MDa. Nevertheless, the NPCs are only constituted by ~30 nucleoporins (NUPs) highly

conserved during the evolution of the eukaryotes (DeGrasse *et al*., 2009; Kabachinski and Schwartz, 2015). Most of NUPs are named with a number that corresponds to their molecular weight. However, given that NUPs molecular weight changes between species there is no universal nomenclature for NUPs. NUPs can be divided according to their localization in the NPC. Commonly, they are divided in six principal categories: (1) Transmembrane NUPs; (2) Membrane-apposed coat NUPs; (3) Adapter NUPs; (4) Channel NUPs; (5) Nuclear Basket NUPs; and, (6) NPC cytoplasmic filaments (Figure I.7 and Table I.1) (Hoelz *et al.*, 2011; Nofrini *et al*., 2016).

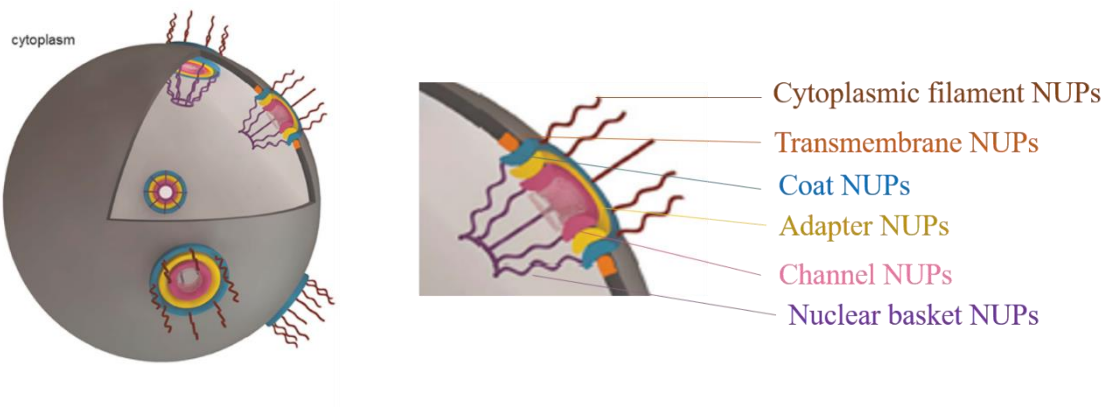

Figure I.7 – Schematic representation of the NPCs and the different nucleoporins categories (Adapted from Nofrini *et al.* (2016)).

Table I.1 – Classes of nucleoporins (Adapted from Nofrini *et al.* (2016)).

Transmembrane NUPs	Coat NUPs	Adapter NUPs	Channel NUPs	Nuclear basket NUPs	Cytoplasmic filament NUPs
NUP210	NUP160	NUP205	NUP62	NUP153	RANBP2
NDC1	NUP133	NUP188	NUPL1	TPR	NUP214
POM121	NUP107	NUP155	NUP45	NUP50	NUP98
	NUP96	NUP93	NUP54		NUP88
	NUP85	NUP35			ALADIN
	SEH1L				NUPL2
	NUP43				RAE1
	NUP37				
	SEC13				
	ELYS				

The transmembrane NUPs are integral membrane proteins, which have been associated with NPC assembly and anchorage to the NE (Hoelz *et al.*, 2011). The coat NUPs form a Nup107–160 complex, which comprehends the largest NPC subunit in size and complexity. This complex is necessary in the first stages of NPC assembly. The adapter NUPs form the NUP93 complex, the second largest NPC structural unit. NUP188 and NUP93 are part of the NUP93 complex and play a key role in delimiting the NE sub-compartments, creating a blockade that avoids the movement of membrane proteins from ONM to the INM. NUP155 also belongs to this complex and guarantee the right localization of the INM proteins. The channel NUPs are the ones in the deepest cylindrical layer of the NPC. NUP54 and NUP58 are part of these NUPs category and play a part in regulating nucleocytoplasmic traffic by establishing a ring in middle of the NPC that has the capacity of expanding reversibly (Solmaz *et al.*, 2011). The nuclear basket NUPs are constituted by NPC nucleoplasmic filaments, namely NUP153, NUP50 and TPR associated with nuclear import/export processes. NUP153 and NUP50 make a ring that links the TPR protein filaments, creating a basket structure that aids in nucleocytoplasmic traffic. NPC cytoplasmic filaments are made of seven proteins that works as fundamental interaction sites to the nucleocytoplasmic trafficking machinery. The mRNA export factor GLE1 and DEAD-box-

containing RNA helicase DDX19 are linked with cytoplasmic filaments and are described as part of the NPC. These two proteins are also involved in the mRNA transport (Hoelz *et al.*, 2011; Nofrini *et al.*, 2016).

Some nucleoporins have a domain with several phenylalanine-glycine (FG) motifs. FG NUPs act as binding sites for transport receptors, being fundamental for translocation within the NPC. Some FG NUPs are thought to associate and create the permeability barrier of the pore (Wente and Rout, 2010; Xu and Powers, 2013).

NPCs have been proposed to directly regulate transcriptional programs and to interact in the nuclear compartmentalization of the genome. Some interactions between the NPC and non-transcribing genomic regions and silencing machinery have also been reported. NUPs mutations have been linked to various human diseases that go from neurological disorders to cardiac diseases and cancer. NUPs differ according to the tissue we are looking at and have been described to alter tissue-specific embryonic development like differentiation of neurons and muscle (Pascual-Garcia and Capelson, 2014). Furthermore, NUPS and NPCs have been reported to play a diverse role in cellular functions like chromatin organization, regulation of gene expression and cell division (Hoelz *et al.*, 2011).

- **MAN1**

MAN1 is an INM that has been reported as an important factor in transforming growth factor beta/bone morphogenic protein (TGFβ/BMP) signalling. Three autosomal dominant diseases related with increased bone density have been associated with loss of MAN1 (Bengtsson and Wilson, 2004).

- **LBR**

Lamin B receptor (LBR) is an INM protein that is associated with the nuclear lamina. LBR is crucial for cholesterol synthesis and LBR mutations are implicated in congenital disorders such as Greenberg skeletal dysplasia or Pelger-Huët anomaly where it fails to rescue the cholesterol auxotrophy because of a loss-of-function mechanism (Tsai *et al.*,

2016). LBR contributes to the profile of the interphase nuclear architecture (Nikolakaki *et al.*, 2017).

1.2.The Ageing Process

All the organisms age. How cells and tissues age is still far from being fully understood and exploited. It is commonly accepted that there is no genetic program that determine the ageing process. Nevertheless, it is undeniable that a crossover between genetics and environmental factors has a tremendous impact on the degree and rate of ageing in same species-individuals and in the variances among species (Rando, 2013).

The lifespan seems to be affected by anatomical and physiological characteristics like size and metabolic rate possibly leading to primary mechanisms, but there are many crucial exceptions that teach us how difficult and complex is understanding the ageing process. Between species, there is a narrow variability of lifespan which points out to the strength of the physiological signalling networks behind the maintenance of cells and tissues. Whenever these signalling networks fail, ageing phenotypes arise. This lifespan variability within the same species can be explained by the environmental stress impact on cell, tissues and organism's responses changing the timing of the failure of homeostatic mechanisms that end up in ageing phenotypes. Caloric restriction and several genes and signalling pathways have been reported as successful options for extending the lifespan (Rando, 2013).

The ageing process is a high-risk factor for the most frequent diseases like cancer, diabetes, heart disorders and Alzheimer's disease. Over the years, lifespan expectancy has increased, but 100 years old seems to be the upper limit of the human ageing process. Understanding the underlying molecular signalling pathways might help us in our quest for increasing lifespan (Aunan *et al.*, 2016).

It is widely accepted that the general characteristic of ageing is an accumulation of genetic material damage (Gems and Partridge, 2013; Moskalev *et al.*, 2013). General accumulation of genetic damage happens in different species, namely in yeast, mice, and humans (Pitt and Kaeberlein, 2015). Cancer is a consequence of an abnormal gain of cellular fitness, and ageing is quite the opposite, being characterized by a loss of fitness. An accumulation of cellular damage is observed in many of pathologies linked with ageing involving unrestrained cellular overgrowth or hyperactivity like in atherosclerosis and inflammation (Blagosklonny, 2008). Similarly to cancer, in the last few years, ageing has

gained a new research interest with the publication of the nine postulated "Hallmarks of Aging" (López-Otín *et al*., 2013). The hallmarks of ageing were reported by López-Otín *et al.* (2013) as: genomic instability; telomere attrition; epigenetic alterations; loss of proteostasis; deregulated nutrient-sensing; mitochondrial dysfunction; cellular senescence; stem cell exhaustion and altered intercellular communication (figure I.8).

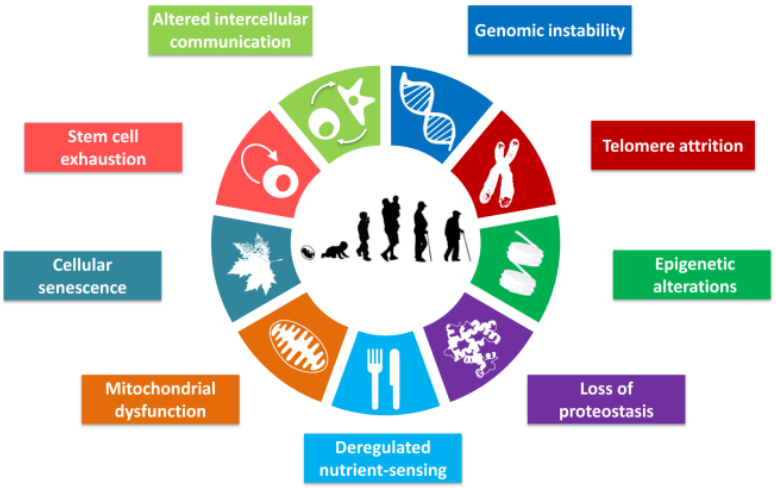

Figure I.8 – The hallmarks of ageing. Genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient,sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication. Taken from López-Otín *et al*. (2013).

The hallmarks can be organized into three main groups: the first group comprises the ones that are thought to be the real primary hallmarks, which diretly causes the damage; the second group corresponds to antagonistic halmarks, that try to respond to the damage; and the integrative ones, which are resultant of the ageing phenotype itself (figure I.9). Every one of the hallmarks of ageing should preferably achieve the subsequent principles: being present in normal ageing processes; resulting from deterioration of the ageing condition when it is intensified and when experimentally ameliorated it should slow down the normal ageing process and increase healthy lifespan. This last criteria is quite difficult to achieve, some of the hallmarks do not fulfil this requirement to the fullest (López-Otín *et al*., 2013).

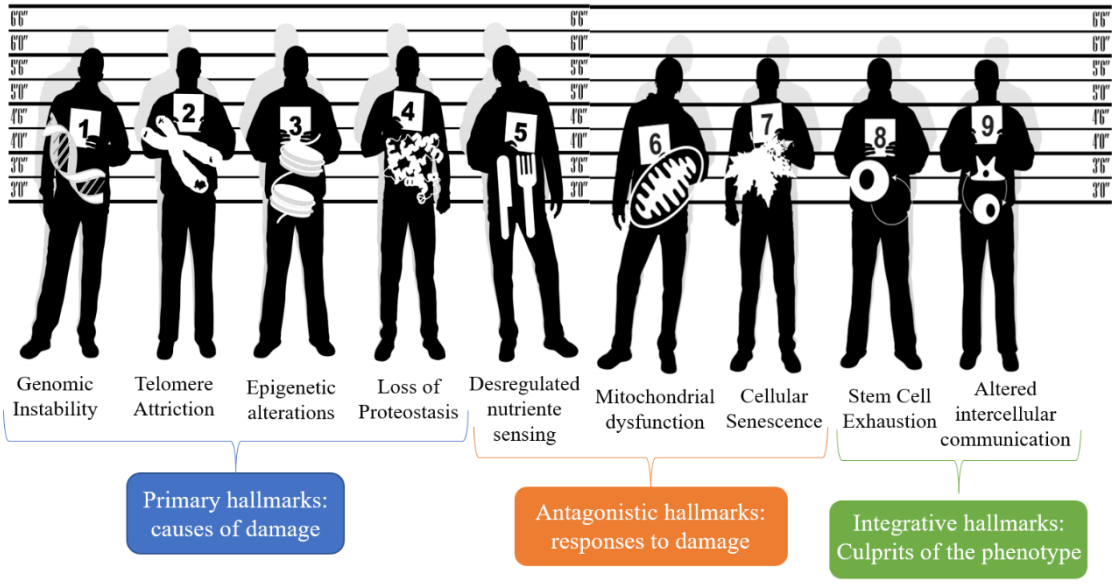

Figure I.9 – The three core groups of the hallmarks of ageing. The hallmarks of ageing can be grouped into three core groups: the primary hallmarks that are considered caused of damage; the antagonistic hallmarks, that are considered responses to damage; and the integrative hallmarks, that are considered the culprits of the phenotype. Adapted from López-Otín *et al*. (2013) and http://www.revgenetics.com/wp/hallmarks-aging-interventions.

Through lifetime, humans and other organisms are constantly exposed to external (like radiation, diet, etc.) and internal risk factors (like failure in replicating DNA, etc.) that causes damages at a molecular level, resulting in diseases and in a decline of the overall organ/tissue function. This accumulation of damage include hallmarks such as genomic instability, telomere attrition, epigenetic changes and loss of proteostasis (Aunan *et al.*, 2016).

- **Genomic instability**

During lifespan there are numerous opportunities for the cell to accumulate genetic damage. Possible mutagens include radiation, chemicals, free radicals, etc. The DNA repair machinery is highly effective way and may reverse many of these damages (Friedberg, 2003; Lord and Ashworth, 2012). Nevertheless, chromosomes get less stable during ageing, and mutations and damage accumulate through time (Moskalev *et al*., 2013). The older the cell the higher the possibility of having more genetic instability. The endogenous and environmental harmful effects can vary from small point mutations to more serious insults like retrotransposon movement and large chromosome rearrangements that play a bigger role in this process (Hoeijmakers, 2009). There is a variety of reports associating genomic and

epigenomic alterations to premature accelerated ageing, but it is not easy to relay and co-relate these alterations to the physiological ageing process once it only shows some features of normal ageing (Burtner and Kennedy, 2010).

Accumulated DNA damage, epigenetic alterations and microsatellite unsteadiness are more frequently found in older individuals. Genomic instability including DNA damage is currently linked with carcinogenesis and other age-related diseases like neurodegenerative disorders (Lombard *et al.*, 2005; van Heemst *et al.*, 2007; Kryston *et al.*, 2011; Ogino *et al.*, 2012). Compromised and decreased DNA repair systems is the main feature of genomic instability but there is no broad consensus whether this is the cause or a consequence of the ageing process. Nonetheless, increasing the DNA repair systems activity and reducing DNA damage could be positive and it could potentially be achieved through caloric and dietary restriction (Heydari *et al.*, 2007; Michan, 2014)

- **Telomere attrition**

One of the most known paradigm of cell division is that telomers get shorter in each cellular division. Ultimately, the telomerase is silenced and telomeres get too short for the division process to continue (Blackburn et al., 2015).Telomeres are prone to DNA damage and contrasting with the rest of the chromosome, this DNA damage is not fixed by DNA repair systems in order to avoid fused chromosome and genomic instability. Telomerase works in this particular process but it seems to be silent in several adult cells in an attempt to control extensive cell proliferation and carcinogenesis (Olovnikov, 1996).The shortening of the telomeres is associated with many age-related disorders such as atherosclerosis, atrial fibrillation, osteoarthritis and coronary heart disease (Hunt *et al.*, 2015; Kuszel *et al.*, 2015; Carlquist *et al.*, 2016).

- **Epigenetic alterations**

As stated in the genomic instability section, environmental factors contribute to alterations in epigenetic mechanisms in the cells. Epigenetic alterations are alterations in the gene expression, which result from nucleotide sequence alteration, such as DNA methylation. These kind of alterations accumulate with time and have been associated with ageing (Aunan *et al.*, 2016). Among other changes, decreased trimethylation of H3K9 and H3K27 and an increased trimethylation of H4K20 and H3K4 are associated with a reduction

in the quantity of heterochromatin and a higher state of chromosome fragility and transcriptional noise (D'Aquila *et al*., 2013).

- **Loss of proteostasis**

The variety of cellular systems and mechanisms responsible for keeping the homeostasis of the proteasome, the synthesis and turnover of human proteins are called proteostasis (Eisenstein, 2014). The accumulation and aggregation of misfolded proteins can be avoided by proteostasis. Therefore, the loss of this set of mechanisms results in failure of autophagy processes, unstable and misfolded proteins accumulation, which leads to a variety of age-associated diseases in human, such as Alzheimer's and Parkinson's disease and others diseases like cataracts (Clark *et al*., 2012; Labbadia and Morimoto, 2015). The reduced capacity of keeping homeostasis of the proteins with age along with protein aggregation are encountered in almost all tissues in older organisms (Koga *et al.*, 2011).

Inflammaging, the decrease of successful autophagy processes, and a worse removal of vesicles holding cellular waste by lysosomes result in a rise of intracellular ROS which lead to low levels of chronic inflammation generated by the inflammasome damage sensing mechanism, and ultimately result in additional accelerating ageing (Glick *et al*., 2010; Franceschi and Campisi, 2014). Upregulation of autophagy can lead to an extension of nematodes, flies and mice lifespan (Madeo *et al.*, 2015).

- **Deregulated nutrient sensing**

Human cells rely on their capacity to use nutrients and fluids in a sensitive manner in order to keep the body functioning and growing. However, through age, several of these sensors and molecular targets disappear or are downregulated (Barzilai and Ferrucci, 2012). The most frequent traits of human ageing embrace increase of visceral fat mass, diminishing lean mass, decreased sensitivity to insulin that results in an intolerance to glucose and muscle ragged red fibres (Pesce *et al.*, 2001).

Metabolic decline causes an accelerated ageing rate in cells. This decline can be translated into cellular damage caused by oxidative and ER stress, mitochondrial and calcium signalling dysfunction (Riera and Dillin, 2015). A dysfunctional nutrient sensing system might result for instance in a greater intake of food and calories than the ones that are needed. Therefore, obesity, diabetes and other metabolic disorders happen to be linked

to the ageing process. Furthermore, diabetes and obesity have an associated chronic inflammation that can additionally decontrol this nutrient sensor by itself. Up to now, the only effort towards expanding human lifespan that seems to work is the intermittent caloric restriction (also known as fasting) (Lee and Longo, 2011).

- **Mitochondrial dysfunction**

The constant exposure of the mitochondrial DNA to reactive oxygen species (ROS) makes the probability of oxidative damage and ageing happen very plausible and common. This is called the mitochondrial free radical theory of ageing (Harman, 2009). ROS can oxidize and insult proteins, lipids and nucleic acids. There is a reported increase of ROS linked with the loss of mitochondrial integrity since the mitochondria is the main manufacturer of ROS. An increase in the induced apoptosis has also been described to be associated with age. Below a certain level of ROS, they may function as an activator of cellular homeostasis signalling pathways, and it decelerates the ageing process. Nonetheless, above a certain limit, ROS are toxic and cause accelerating ageing or even cell death. Some molecules like metformin increase the overall fitness of the organism and might ameliorate the ageing phenotype (Hekimi *et al.*, 2011; Aunan *et al*., 2016).

- **Cellular senescence**

Cellular senescence can be defined as the moment that cells stop to divide and to growth permanently, suffering of chromatin and secretome alterations. Senescence can be caused by the lack or damage of essential components (Kuilman *et al.*, 2010).Young senescent cells are probably cleared by the immune system. On the contrary, older cells wonder around, staying and producing injurious proinflammatory signals such as interleukins that persist in damaging our organisms (Kuilman et al., 2010; Van Deursen, 2014).Other hallmarks that involve DNA damage, telomere shortening, and oxidative stress contribute to this senescence process (Collado *et al.*, 2007; Van Deursen, 2014).

- **Stem cell exhaustion**

As we get older, the cell stemness state capacity is lost, and the organism become incapable of substituting stem cells that migrated, differentiated or died, given that the stem cells are unable to divide. A decrease of the regeneration of stem cells play a fundamental

role in ageing. For instance, a decline in haematopoietic stem cell results in anaemia, mesenchymal stem cell exhaustion lead to osteoporosis and fractures, intestinal epithelial stem cell reduction results in perturbed intestinal function, and muscle stem cell loss is linked with ageing (López-Otín *et al*., 2013; Aunan *et al*., 2016).

- **Altered intercellular communication**

Age-related alterations that occur in a single tissue can affect and damage other tissues due to a complex system of intercellular communication of the ageing phenotype. Senescent cells seem to be able to induce senescence in proximal cells through gap-junction-mediated cell-cell contacts and processes concerning ROS. It is called the "bystander effect" (Nelson *et al*., 2012). Then, aiming a unique gene or protein can have an effect in other tissues (Durieux *et al*., 2011). Systems like renin-angiotensin or the insulin-to-insulin growth factor response can be deregulated by the changes in the intracellular communication. Immunosenescence (age related changes of the immune system) and inflammaging (chronic low-grade systemic inflammation) due to an increase secretion of cytokines by adipose tissue are in the origin of several age-related disorders like diabetes, arthritis, metabolic syndrome and cardiovascular diseases (Sergio, 2008; Capri *et al*., 2014; Szarc Vel Szic *et al*., 2015) leaving the organism more fragile and prone to morbidity and mortality in aged individuals (Aunan *et al*., 2016).

1.3.The NE dysfunction in the ageing process

There are some limitations in using animal ageing models once it does not completely mimic the human ageing phenotype in some aspects, as is the cases of telomeres that do not get as short in these animal models through ageing as in humans (Blackburn *et al.*, 2015). The long human lifespan and other limitations poses difficulties to study human normal ageing at a practical level. Ethical considerations are also a drawback (Mitchell *et al*., 2015).

The premature ageing disorders, also known as progerias, provide us with a unique opportunity to unravel the mechanisms inherent to physiological ageing (Kubben and Misteli, 2017). Nevertheless, it is important to remember that no premature ageing disorder is able to present all the physiological ageing phenotype and features, so they should be seen as segmental progerias (Van De Ven et al., 2006; Reddy and Comai, 2016).

Many premature ageing disorders have been associated with defects and alterations in the nuclear proteins, leaving the prospect of nuclear lamina and the INM as major regulators of the ageing phenotype (Serebryannyy and Misteli, 2018).Many indications that pose the nuclear periphery as a prospective controller of the ageing phenotype were obtained from studies in HGPS (Serebryannyy and Misteli, 2018). As previously described, this is a rare genetic disorder that causes systemic accelerated ageing in children due to a single mutation in the *LMNA* gene that leads to the synthesis of a truncated and toxic form of lamin A called progerin. Progerin assimilation into the nuclear lamina is supposed to alter negatively the lamin A and B grids, causing an overall structural and signalling defects (Delbarre *et al.*, 2006; Lee *et al.*, 2016). HGPS fibroblast cells present a poor and defective nuclear shape, DNA damage, down-regulation of various nuclear proteins and a different histone modification pattern (Scaffidi and Misteli, 2006a).

The nuclear integrity seems to be affected by ageing in human fibroblasts where telomere attrition and radiation damage induce progerin expression (Cao *et* al., 2011; Takeuchi and Rünger, 2013). In fact, an accumulation of prelamin A or progerin has been described in the past few years associated with the normal ageing phenotype (Scaffidi and Misteli, 2006a; Ragnauth *et al.*, 2010; Sieprath *et al.*, 2015).Mutations in *LMNA* gene are linked with older individuals (Conneely *et al*., 2012). The expression of altered isoforms of lamin A has been widely mentioned in the literature in physiological ageing models, such as fibroblast from skin biopsies (Scaffidi and Misteli, 2006a; McClintock *et al*., 2007; Rodriguez *et al.*, 2009), fibroblasts extensively passaged (Cao et al., 2011), fibroblasts from centenarians (Lattanzi *et al*., 2014), cardiovascular tissues (Olive *et al*., 2010) and normal and aged vascular smooth muscle cell (VSMC) (Ragnauth *et al.*, 2010).

According to Serebryannyy and Misteli (2018), the concept of sequestration of proteins at nuclear periphery, or loss thereof, may be responsible for prominent defects associated with premature and physiological ageing like diminished cell stemness, dysfunctional cytotoxicity, inhibition of proliferation, altered chromatin and telomere preservation (Serebryannyy and Misteli, 2018)

- **Cell stemness**

The capacity of the tissues regenerate (cell stemness) is compromised during ageing (López-Otín *et al.*, 2013). Notch activation controls proliferation, migration, and

differentiation, and it is vital to maintain stem cell homeostasis (Bray, 2016). The renovation of muscle stem cells is prevented by the loss of Notch signalling pathway which also plays a role in cardiovascular dysfunctions. On the other side, an upregulation of the Notch signalling is involved in carcinogenesis and inflammation processes through MAPK and TBFβ (Balistreri *et al*., 2016). Notch target genes are activated by progerin expression in human mesenchymal stem cells leads to aberrant stem cells and differentiation flaws (Scaffidi and Misteli, 2008). WNT seems to have a similar pathway that is implicated in stem cell proliferation and differentiation (Borggrefe *et al*., 2016).

- **Dysfunctional cytotoxicity**

Mitochondrial dysfunction is present in HGPS fibroblast cells as well as elevated ROS levels, meaning that the nuclear periphery can play a role in mitochondria homeostasis (Serebryannyy and Misteli, 2018). Nuclear factor erytroid-2-related factor 2 (Nrf2) is a transcriptional activator of resistance to ROS stress and other chemical insults (Ma, 2013). Noticeable, Nrf2 levels are altered through ageing and show a specific profile in different tissues and different models (Zhang et al., 2015a).

Nfr2 is inadequately sequestered to the nuclear periphery in HGPS fibroblasts by progerin, resulting in a decrease of its cytoprotective activity against ROS stress and a reduced concentration at the nucleus (Kubben *et al.*, 2016).

- **Proliferation**

Immunosenescence has been associated with loss of functions related to ageing. This can be related with a proliferation suppression of stem cells and an increase of senescent cells (Van Deursen, 2014).The cellular proliferation capacity can be controlled by a group of intrinsic checkpoints, signalling pathways in a close connection with the nuclear periphery according to results obtained in HGPS models (Varela *et al*., 2005).

A good example is PCNA, a crucial part of the DNA replication apparatus. It is associated with DNA damage repair systems, and telomere steadiness (Choe and Moldovan, 2017). With age, PCNA expression is reduced (Tanno *et al.*, 1996). PCNA localization is partly defined by A- and B-type lamins. Therefore, the loss of the nuclear lamina integrity due to lamin mutants or progerin leads to PCNA sequestration and aggregation at the nuclear periphery disrupting replication (Hilton *et al*., 2017). The contrary is also true: expression of

prelamin A or progerin conduct to the loss of PCNA correct functions, resulting in DNA damage (Cobb *et al.*, 2016). Having all that stated, it is not surprising that PCNA is being implicated in the ageing process.

- **Chromatin remodelling**

Modifications in DNA methylation, histone alteration, chromatin structure and epigenetic controlling systems are important ageing features (Zane *et al.*, 2014). Human sirtuin1 (SIRT1) is a NAD+ dependent protein deacetylase that play a part in many cellular processes, such as ageing, metabolism, cancer and stress responses. SIRT1 is connected and activated by lamin A. Resveratrol is an activator of SIRT1 by increasing SIRT1-Lamin A connection, consequently assisting in the localization of SIRT1 in the nuclear lamina. This connection can be jeopardized by prelamin A and progerin. In premature ageing mice and HGPS fibroblasts, resveratrol rescue adult stem cell due to the increased SIRT1-Lamin A binding. This ends up in an extended lifespan and an improvement in the progeroid traits. It can influence other characteristics such as chromatin remodelling, DNA damage combat and stem cell therapy (Ghosh *et al.*, 2013).

- **Telomere maintenance**

Telomeres shortening have been extensively associated with lifespan. There is a class of human telomers that are localized at the nuclear periphery posing the possibility that lamin organization modulate the telomere length and maintenance. Telomeres decline can result in progerin expression and uncontrolled DNA damage.

TRF2 is a binding factor involved in maintaining telomere stability. TRF2 keep the telomere integrity at the nuclear periphery by interaction with the nuclear lamina, LAP2α and possibly LAP1 (Ludérus *et al.*, 1996; Chojnowski *et al.*, 2015; Serrano *et al.*, 2016). LAP2α levels decrease in case of progerin expression which might obstruct the TRF2-lamin A/C connection. HGPS cells without lamin A/C being expressed eventually stop having telomeres (Wood *et al.*, 2014). Human fibroblasts carrying a null TERT mutation show high levels of progerin. Fibroblasts expressing TRF2ΔBΔM result in uncapped telomeres and it is associated with higher progerin synthesis. The connection between aberrant telomere and progerin synthesis might induce cellular senescence (Cao *et al.*, 2011).

SUN1 seems to be upregulated in studies performed in centenaries fibroblasts, but not in an older group of fibroblasts from patients with 65-80 years old. Prelamin A is accumulated in fibroblasts from centenarians. Centenarian fibroblasts show an accumulation of prelamin A in cells that suffer stress and they do no present senescence traits (Lattanzi *et al*., 2014).

Some studies have reported a decrease in lamin B levels through ageing in *Drosophila* and human. In *Drosophila* the low levels of lamin B result in a general process of inflammaging. Lamin-B diminishing in a single tissue might disturb the role of other tissues through systemic inflammation (Chen *et al.*, 2015). Dysregulation of lamin B1 in human fibroblasts might cause cell senescence (Liu *et al.*, 2012). Studies point out to the fact that when lamin B1 is silenced, cellular ROS levels are decreased (Shimi and Goldman, 2014). Augmented levels of lamin B1 are connected with higher levels of ROS (Barascu et al., 2012a). Lamin B1 seems to be a signalling hub and its response is different according to what senescence pathway is activated (ROS-induced premature senescence, Replicative-induced senescence and Oncogene-induced senescence) (Hutchison, 2012).

There are some studies that mention a disruption in NPC associated with age in *C. elegans* post mitotic cells (D'Angelo *et al.*, 2009). Moreover, using aged VSMCs it was demonstrated that the lamin A precursor, prelamin A, accumulates and induces NE invaginations trapping the Nup153 and consequently compromising the Ran gradient and large cargo transportation to the nucleus (Cobb, et al., 2016). Progerin expression inhibits TPR import, and reduces trimethylation of H3K9 (Kelley, et al., 2011). A reduction of nuclear import rate is observed in Progeria. NPCs clustering mislocate NPC components such us Nup153 and TPR. Additionally, it was also shown that in aged leaky nuclei (where Nup93 was lost) there is also decrease signal of FG-nucleoporins, recognized by the mAb414 antibody that recognizes several nucleoporins including Nup62 (D'Angelo *et al*., 2009). These authors observed that Nup93 were lost in old and permeable nuclei (D'Angelo, et al., 2009).

Emerin has been reported to be increased and mislocated in aged-iPSCs when mutations in nesprins and lamin A occur (Zhang *et al*., 2007; Ho *et al.*, 2013; Petrini *et al.*, 2017). Petrini *et al*. (2017) results show that in young iPSCs (early passages) nesprin-2 is faintly detected. However, after passaging iPSCs for about one year (aged iPSCs) the nesprin-2 expression is uniformly increased through the nucleus.

Table I.2 summarizes the NE proteins already described to be involved in the ageing process.

Table I.2 - Nuclear envelope proteins involved in the ageing process. (INM – Inner Nuclear Membrane, VSMCs - vascular smooth muscle cells, HGPS - Hutchinson–Gilford progeria syndrome, NPC – nuclear pore complex).

NE protein	Alterations in ageing	Ageing model	References
Nuclear lamina			
Lamin A/C	WT lamin A, mutations in prelamin A and lamin A accumulate with age. Nucleoplasmic lamin A/C seems to be depleted	◆ Human VSMCs from old donors ◆ Human HGPS coronary arteries ◆ Human fibroblasts from old donors ◆ Human fibroblasts extensively passaged in culture	(Candelario et al., 2008; Cao et al., 2011; Lattanzi et al., 2014; McClintock et al., 2007; Olive et al., 2010; Ragnauth et al., 2010; Rodriguez et al., 2009; Scaffidi and Misteli, 2006a)
Lamin B1	Decrease or increase with age (depending on the senescence pathway being activated)	◆ Drosophila ◆ Human aged fibroblasts	(Chen *et al.*, 2015; Dreesen and Stewart, 2011; Freund *et al.*, 2012; Lattanzi *et al.*, 2014; Shimi and Goldman, 2014)
INM			
LAP2α	Decreases in case of progerin expression	◆ HGPS fibroblast cells	(Wood *et al.*, 2014)
SUN1	Upregulated with age and increased in progerias	◆ Human centenarian fibroblasts ◆ Human HGPS fibroblasts	(Meinke *et al.*, 2011; Chi *et al.*, 2012; Lattanzi *et al.*, 2014)
NPC (Nup93, Nup62, Nup153 and TPR)	Age-related deterioration of NPC	◆ *C. elegans*	(D'Angelo *et al.*, 2009)
Emerin	Increases and is mislocated with age	◆ Human aged-iPSCs	(Petrini *et al.*, 2017)
ONM			
Nesprin-2	Increases with age	◆ Human aged-iPSCs	(Petrini *et al.*, 2017)

1.4.Ageing models

To study the ageing process, it is important to define which model is adequate for the intended research. Studying human ageing is tough because of the long lifespan and ethical considerations. Over the years, ageing studies have been conducted in many type organisms including unicellular organisms like the *Saccharomyces cerevisiae*, other organisms such as *Caernohabditis elegans*, *Drosophila melanogaster*, zebrafish, rodents, and human derived cells were used (Mitchell *et al*., 2015).

S. cerevisiae is a yeast with a highly known and characterized genome and proteome that accepts well genetic manipulation. Its low doubling time (1.25 to 2h) and its 26 cell divisions average lifespan makes it an easier and cheap model. Several age-related genes have been identified using *S. cerevisiae* (Mitchell *et al*., 2015).

The extensive knowledge about *C. elegans* genome, developmental stages, and its short lifespan (2-3 weeks) makes it also an attractive model to study ageing. *C. elegans* has a translucid body which aids in microscopy studies without interfering or threatening its life course, and allows us to investigate the accumulation of heterochromatin at the nuclear periphery in specific tissues (González-Aguilera *et al*., 2014).

D. melanogaster conserve human disease genes that might be related to ageing disorders such as stem cell exhaustion, cardiovascular decline and Alzheimer's disease. It presents a short lifespan (up to 6 weeks) and a low maintenance cost (Brandt and Vilcinskas, 2012). Drosophila and *C. elegans* age differently than humans and they do not have several age-related diseases described in humans. It enlightens the very real possibility of most of the results obtained in these animals not being valid to humans (González-Aguilera *et al.*, 2014; Sinclair, 2002).

Zebrafish is starting to be considered an interesting ageing model since it has been used to study evolutionary and development origins of ageing. Genes linked to senescence phenotype uncovered in zebrafish have been linked with cellular senescence and chronological ageing, but more studies are required to clarify this (Kishi, 2014).

Rodents like mice and rat are considered to be the best ageing model available nowadays. They present a relative short lifespan (up to 3 years), can be genetically modified, and resemble most of the human ageing phenotypes. Moreover, they can be tested on the lab with therapeutic drugs to increase lifespan. There are many different strains available that can mimic many scenarios. So far, the mice model has given new

perspectives in several processes underlying ageing, such as caloric-restriction that ended up having a relevant interest in humans as well (Vanhooren and Libert, 2013).

Nevertheless, human cell cultures studies grow day by day since there are a strong resemble between them and what we aim to study: the human ageing. It is realist to think that at least some of the age-related processes that happen *in vitro* also happens *in vivo* (de Magalhães, 2004). Several ageing studies use fibroblasts obtained from young and old individuals biopsies (Scaffidi and Misteli, 2006; Cao *et al.*, 2011; Lattanzi *et al.*, 2014). Alterations related to ageing occurring in the human body due environmental stresses and intrinsic factors make fibroblasts a good candidate to study intrinsic and extrinsic ageing (Varani, 2010; Tigges *et al.*, 2014).

Aims

Ageing is a multifactorial process characterized by the loss of the overall organismal fitness. Bioinformatic approaches can provide us a unique opportunity to identify new putative players in the ageing phenotype and to uncover the main underlying biological functions and cellular components associated with this process. On the other hand, the nuclear envelope periphery has already been pointed as a potential regulator of the ageing phenotype. Nevertheless, there are still few studies regarding this topic and most of them focus on a single protein and/or are based on premature ageing disease model. Nevertheless, the significance of premature ageing disorders to physiological ageing is still uncertain since they do not fully recapitulate and mimic the physiological ageing.

Therefore, the main aim of this dissertation is to characterize the alterations of the nuclear envelope proteins during the physiological ageing process.

Thus, the specific aims of this dissertation are the following:

- Analyse and characterize age-related interaction networks using bioinformatic tools;
- Characterize the profile of nuclear envelope proteins during ageing using a mice model;
- Characterize the profile of nuclear envelope proteins during ageing using human fibroblasts;
- Evaluate the subcellular localization of nuclear envelope proteins during ageing using human fibroblasts.

CHAPTER II

Bioinformatic analysis of interaction networks in ageing

2.1. Introduction

The physiological ageing is defined as a functional decline at cellular, tissue and organismal level across the lifespan (López-Otín *et al.*, 2013). Ageing impacts many areas and increases the risk of developing several age-related diseases like cancer, diabetes, cardiovascular and neurodegenerative disorders. Despite all the efforts to determine the molecular and cellular changes associated with ageing, the underlying mechanisms remains elusive. Several studies have monitored aspects of the ageing in mammals, namely alterations in gene expression (de Magalhães *et al.*, 2009), transcription (Schumacher *et al.*, 2008) and proteome (Waldera-Lupa *et al.*, 2014; Walther and Mann, 2011). Although these studies have reported some age-specific changes, no substantial alterations were highlighted. In fact, when we look at a single gene or a single protein in a certain research field, whether we are looking into a specific disease or process, we are limiting our investigation. Genes and proteins work together. Biological systems are composed of many components that interact with one another at different levels. A single component of a complex network of interactions can affect the rest of them. Therefore, over the last few years, more integrative and comprehensive approaches have been used, bringing interactomes into the spotlight (Chautard *et al.*, 2010).

Consequently, it urges to look at the bigger picture and network approaches are essential for the understanding of the complex ageing process. This strategy provides important information since it allows for a systems approach of the protein complexes formed, rather than single interactions (Chautard *et al.*, 2010). Here, by building human age-related protein-protein interaction (PPI) networks we aimed to identify the underlying biological processes and the main signalling pathways of ageing.

2.2. Methodology

2.2.1. Collection of the human age-related proteins/genes

Ageing-associated genes already described in the literature were retrieved using the GenAge Human Database and the AGEID (Ageing Genes/Interventions Database) databases. GenAge is a curated database of genes associated to human ageing (de Magalhães and Toussaint, 2004; Tacutu *et al.*, 2018), whereas AGEID is a database of experimental results in any organism related to genes and interventions connected with ageing phenotypes (Kaeberlein *et al.*, 2002). Therefore, the list of genes retrieved in

AGEID from other organisms were extrapolated by homology to *Homo sapiens*. A total of 663 ageing-associated genes were retrieved from both databases (Table SII.1).

2.2.2. Functional enrichment analysis of Gene Ontology

Gene Ontology (GO) provides a system of terms to consistently describe and annotate gene products (Ashburner *et al.*, 2000; Mi *et al.*, 2017; The Gene Ontology Consortium, 2017). Functional annotation clustering analysis was performed online using the DAVID database (The Database for Annotation, Visualization and Integrated Discovery; available at https://david.ncifcrf.gov/tools.jsp; version 6.8) (Huang *et al.*, 2008, 2009) to enquire for GO terms that are over-represented using the annotation Cellular Component. Briefly, the 663 genes were submitted to analysis, and a classification stringency of 4 was used. The output retrieved was a table that lists and groups in clusters the significant shared GO terms (or parents of GO terms) used to describe the set of genes submitted to analysis, the fold enrichment (%) and the p-value. The statistical analysis included Bonferroni's correction for multiple testing and a term was considered as enriched if the adjusted p-value is below a significance threshold of 0.05.

2.2.3. Construction of an interaction network of ageing

The 308 proteins/genes assembled in the two first clusters of the cellular component enrichment analysis (Table II.1) were further used to construct a PPI network using the STRING database (Version 10.5) (Szklarczyk *et al.*, 2017), considering only physical contacts and co-expression as interaction sources. The network was visualized using the freely available Cytoscape software (version 3.7.0) and further analysed using the NetworkAnalyzer plugin version 3.3.1. Cluster analysis was carried out using the Cytoscape plugin clusterMaker (GLay), a specific tool which provides numerous algorithms for clustering nodes within a network. This network visualization approach allows the analysis of complex networks in a biological context. The PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system was used to perform a statistical overrepresentation test of the biological processes (Thomas *et al.*, 2006; Mi *et al.*, 2013, 2017) in order to analyse the genes retrieved from the first two clusters produced by clusterMaker.

A schematic representation of the followed methodology is presented in figure II.1.

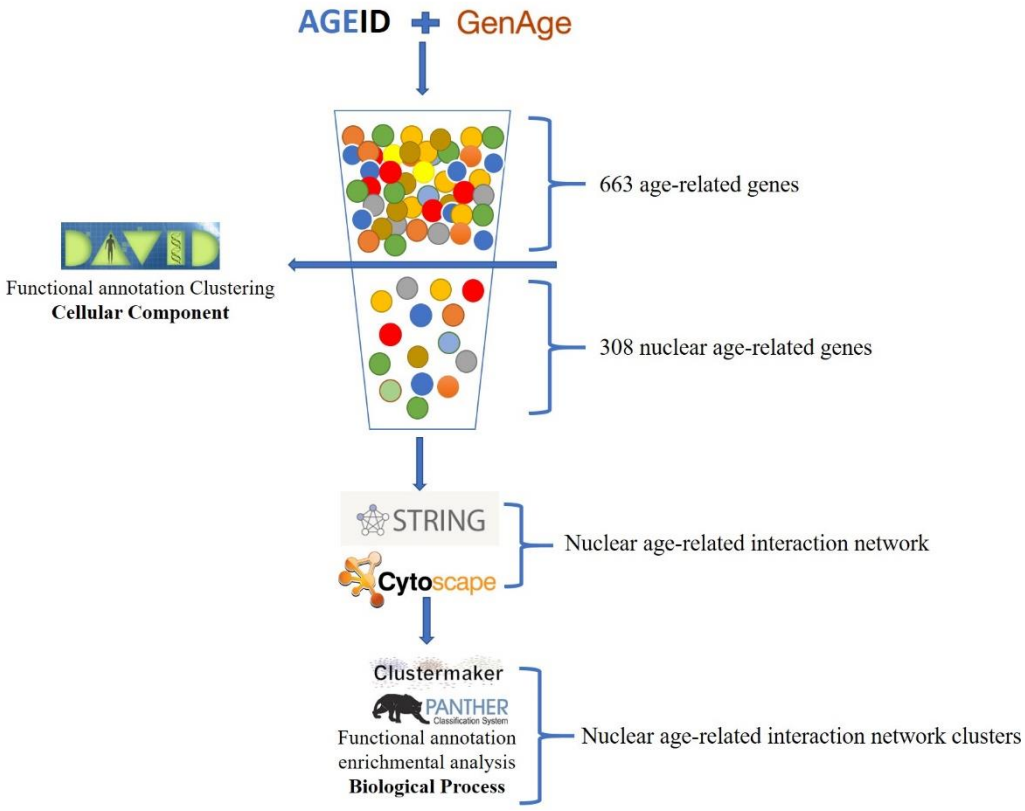

Figure II.1. – Methodological workflow overview. Schematic representation of the methodological workflow applied in the bioinformatic approach.

2.3. Results

The identification of genes that were associated to ageing, longevity or senescence was achieved using GenAge and AGEID databases. A total of 663 age-associated genes were identified as described in the methods section, several being human orthologs established in model organisms.

A functional annotation clustering analysis according to GO cellular component annotation using the DAVID online resource was performed. The resulting cellular component enrichment clusters are listed in Table II.1. The clusters with higher enrichment scores correspond to clusters 1 and 2 (enrichment scores of 28.28 and 27.42, respectively). Interestingly, the main cellular components were related to nucleus (chromosomal part, nuclear chromosome part, nuclear chromosome, intracellular

organelle lumen, nuclear part, nucleoplasm, and nuclear lumen). The remaining clusters have enrichment scores of 3.21 and lower (Table II.1).

The main cellular component categories of the first two clusters are shown in Figure II.2.

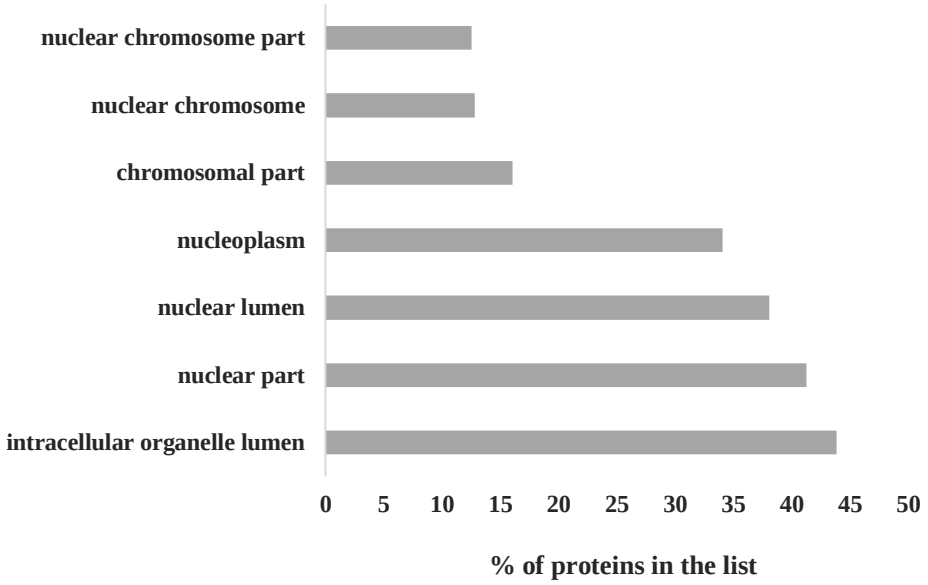

Figure II.2 – Functional annotation cellular component clustering analysis (clusters 1 and 2) of the age-related genes using DAVID online resource. The percentage of the age-related genes was calculated and plotted.

Table II.1 – Cellular component functional annotation clustering enrichment analysis of the age-related genes network using DAVID online resource. Enriched categories are those with Bonferroni adjusted p value <0.05.

	GO Term	Nr. Genes	% of genes	p-value
Cluster 1 Enrichment Score: 28.28	chromosomal part	105	16.06	1.53E-26
	nuclear chromosome part	82	12.54	1.95E-26
	nuclear chromosome	84	12.84	8.04E-26
Cluster 2 Enrichment Score: 27.42	intracellular organelle lumen	287	43.88	2.14E-26
	nuclear part	270	41.28	9.99E-26
	Nucleoplasm	223	34.10	3.58E-25
	nuclear lumen	249	38.07	2.39E-24
Cluster 3 Enrichment Score: 3.21	cytoplasmic vesicle	71	10.86	2.66E-01
	intracellular vesicle	71	10.86	2.78E-01
	cytoplasmic, membrane-bounded vesicle	66	10.09	3.23E-01
Cluster 4 Enrichment Score: 2.79	respiratory chain complex	11	1.68	3.63E-01
	mitochondrial respiratory chain	11	1.68	5.43E-01
	inner mitochondrial membrane protein complex	13	1.99	8.66E-01
Cluster 5 Enrichment Score: 2.66	RNA polymerase complex	14	2.14	4.69E-01
	nuclear DNA-directed RNA polymerase complex	13	1.99	8.11E-01
	DNA-directed RNA polymerase complex	13	1.99	8.11E-01
Cluster 6 Enrichment Score: 2.53	terminal bouton	10	1.53	2.24E-01
	neuron projection terminus	12	1.83	9.70E-01
	axon terminus	11	1.68	9.92E-01
Cluster 7 Enrichment Score: 1.72	muscle myosin complex	5	0.76	9.15E-01
	myosin filament	5	0.76	9.86E-01
	myosin II complex	5	0.76	1.00
	myosin complex	5	0.76	1.00
Cluster 8 Enrichment Score: 1.06	Z disc	9	1.38	1.00
	sarcomere	12	1.83	1.00
	I band	9	1.38	1.00
Cluster 9	contractile fiber part	13	1.99	1.00

Enrichment Score: 1.03	sarcomere	12	1.83	1.00
	contractile fiber	13	1.99	1.00
Cluster 10 **Enrichment Score: 0.94**	nuclear outer membrane-endoplasmic reticulum membrane network	49	7.49	1.00
	endoplasmic reticulum membrane	44	6.73	1.00
	endoplasmic reticulum part	50	7.65	1.00
Cluster 11 **Enrichment Score: 0.27**	respiratory chain complex I	3	0.46	1.00
	NADH dehydrogenase complex	3	0.46	1.00
	mitochondrial respiratory chain complex I	3	0.46	1.00
Cluster 12 **Enrichment Score: 0.21**	microbody lumen	3	0.46	1.00
	peroxisomal membrane	3	0.46	1.00
	microbody membrane	3	0.46	1.00
	peroxisomal part	4	0.61	1.00
	microbody part	4	0.61	1.00
Cluster 13 **Enrichment Score: 0.12**	synaptic vesicle membrane	3	0.46	1.00
	exocytic vesicle membrane	3	0.46	1.00
	transport vesicle membrane	3	0.46	1.00

Remarkably, 308 genes are associated with the categories of cluster 1 and 2, which correspond to almost half of the age-related genes identified. Hence, the protein-protein interactions of these 308 age-related genes were collected from the STRING database using physical contacts and co-expression only. The network was visualized using the Cytoscape program and is presented in Figure II.3. Further, the NetworkAnalyzer, a Cytoscape plugin, was used to analyse and calculate the following parameters: 1) the average number of neighbours, which indicates the average connectivity of a node in the network; 2) the mean clustering coefficient of the network, which characterizes how nearest neighbouring nodes are connected to each other.

The total number of proteins mapped in the nuclear age-related protein-protein interaction (PPI) network was 265 (nodes) and the total number of connections between them was 1443 (edges). Of note, only 43 of the 308 nuclear age-related genes are not connected to this PPI network. The average number of neighbours for a node in the network is 10.891, and the mean clustering coefficient of the network is 0.283. This value is 10 times larger than the clustering coefficient expected for a sparse random uncorrelated network (0.02) (Newman, 2006; Doncheva *et al*., 2012). Overall these results clearly indicate that the resulting nuclear age-related PPI network is strongly connected.

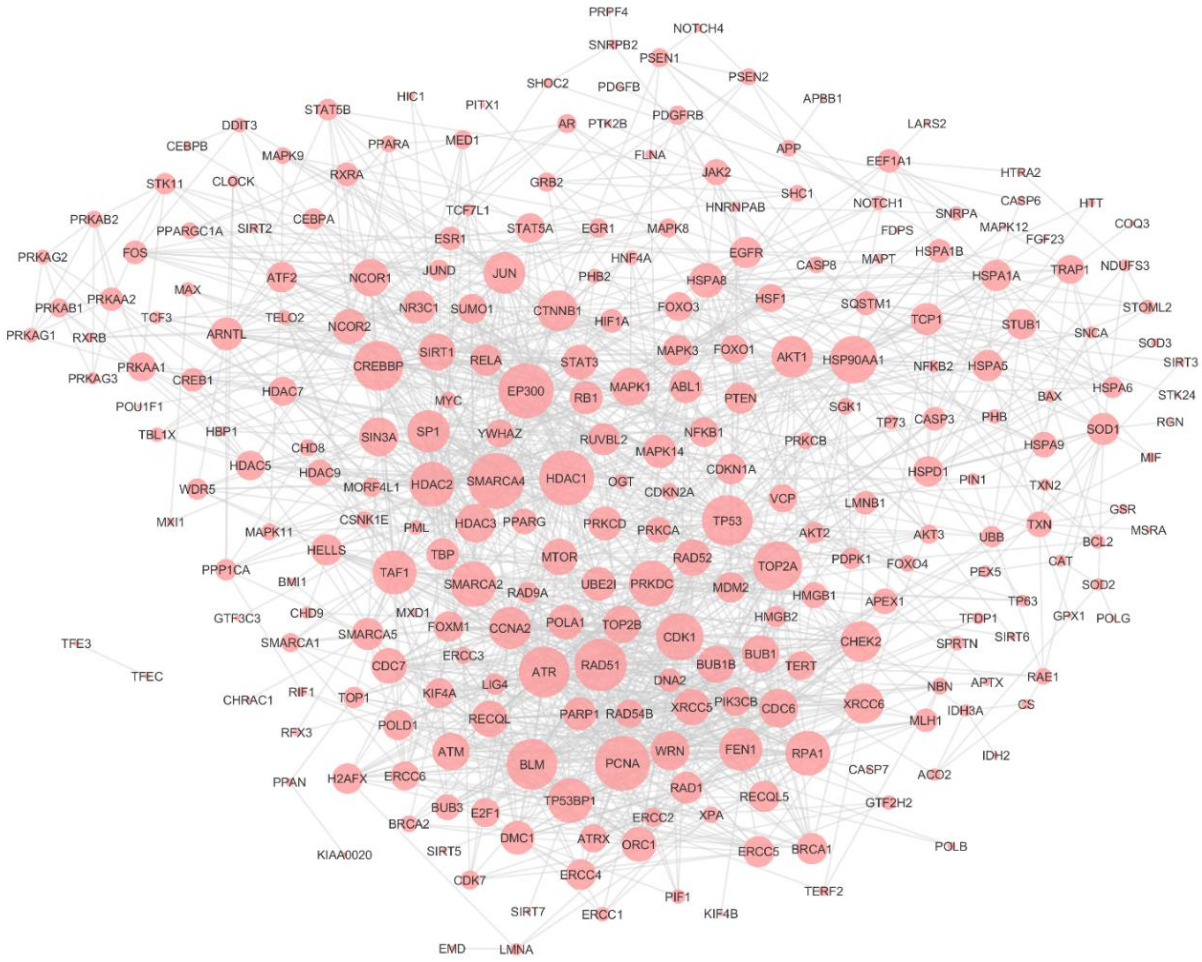

Figure II.3 – Protein-protein interaction (PPI) network of the nuclear age-related genes. Node size according to the degree (number of neighbours) in the network.

The nuclear age-related PPI network was further analysed using the Cytoscape plugin ClusterMaker (GLay) (Figure II.4) that allows the analysis of complex networks in a biological context and the identification of functional relationships. The analysis resulted in the identification of 9 clusters from which the composition of clusters 1 and 2 suggests the formation of enriched functional clusters (Figure II.4).

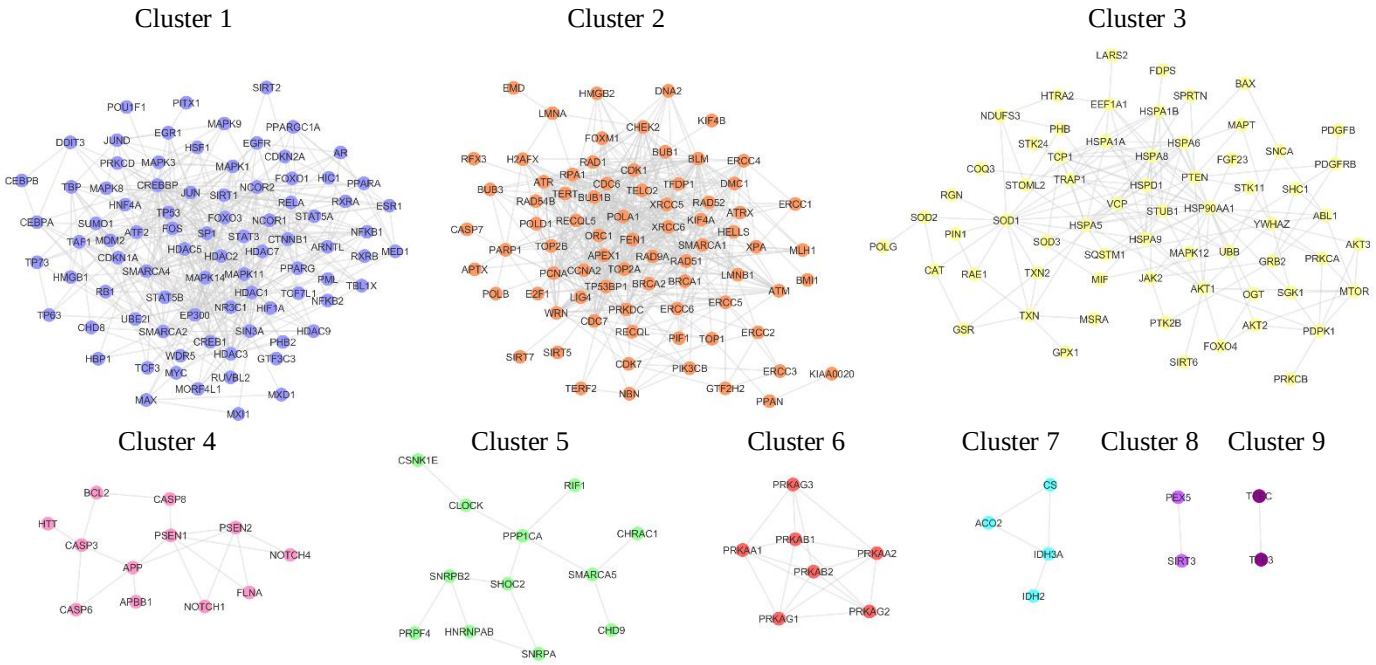

Figure II.4 – Nuclear age-related PPI network clusters produced using the Cytoscape plugin clusterMaker (GLay).

Therefore, we proceeded with further analysis of the two main clusters (Figure II.5). The total number of proteins mapped in the cluster 1 network was 85. The average number of neighbours for a node in the network is 10, and the mean clustering coefficient of the network is 0.3483. A total number of proteins mapped in the cluster 2 network was 77. The average number of neighbours for a node in the network is 11.992, and the mean clustering coefficient of the network is 0.423. Overall these results clearly indicate that our nuclear age-related PPI clusters are closely connected and therefore the proteins of these networks are closely functionally associated.

A. Cluster 1

B. Cluster 2

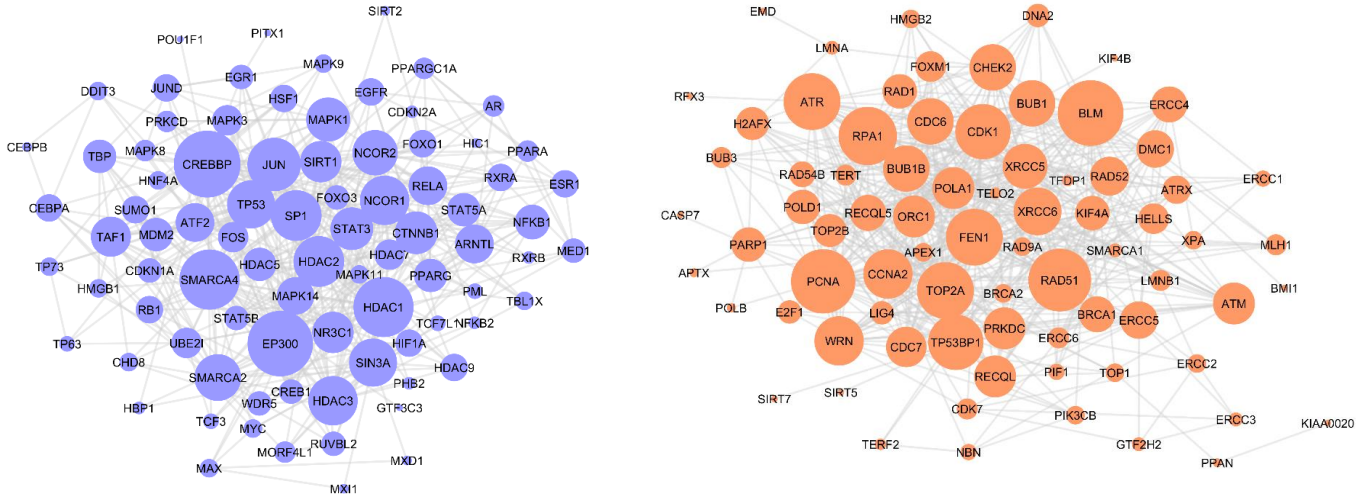

Figure II.5 – Nuclear age-related PPI network cluster 1 (A) and cluster 2 (B) produced using the Cytoscape plugin clusterMaker (GLay). Node size corresponding to node degree.

We performed an analysis of the Biological Processes involved in the age-related genes that comprise the clusters 1 and 2 using Panther Online Resource (Tables II.2 and II.3, respectively). The main biological processes associated with cluster 1 proteins are related to transcription (namely, TAF1, TCF3, TP53 and CREBBP) and transcription regulation by chromatin remodelling/organization (namely, EP300, HDAC1, HDAC 2, HDAC3, HDAC5, HDAC7, SIRT1 and SMARCA4). In cluster 2, the most prominent biological processes are DNA metabolic process and DNA repair (namely, APTX, PCNA, RAD51, BLM, ATM, H2AX and *TP53BP1*). Noticeably, the cluster 2 comprises the lamins (*LMNA*, *LMNB1*) genes which were also previously associated to DNA damage response and consequently to the ageing process (Mahen *et al*., 2013). In fact, both A- and B-type lamins are required for the recruitment of factors involved in DNA repair, namely 53BP1, BRCA1 and RAD51 (reviewed in Gonzalo, (2014).

Table II.2. – Biological processes functional annotation enrichment analysis of the cluster 1 using Panther online resource. Enriched categories are identified as those with Bonferroni adjusted p value <0.05.

GO Biological Process	Nr of genes	Fold enrichment	p-value
regulation of transcription from RNA polymerase II promoter	27	11.18	1.55E-18
transcription from RNA polymerase II promoter	29	9.28	5.56E-18
transcription, DNA-dependent	32	7.77	5.97E-18
RNA metabolic process	32	5.05	1.19E-12
metabolic process	58	2.44	3.90E-12
chromatin organization	14	12.70	1.99E-09
biosynthetic process	29	4.11	5.07E-09
negative regulation of apoptotic process	9	22.50	1.18E-07
nucleobase-containing compound metabolic process	34	3.01	2.28E-07
nitrogen compound metabolic process	29	2.84	2.32E-05
primary metabolic process	40	2.08	1.72E-04
response to abiotic stimulus	6	19.29	2.44E-04
intracellular signal transduction	16	3.70	1.39E-03
developmental process	19	3.13	1.75E-03
cellular process	54	1.62	2.25E-03
response to stimulus	25	2.31	1.14E-02
JAK-STAT cascade	3	43.69	1.60E-02
MAPK cascade	8	5.82	1.87E-02
organelle organization	15	3.06	2.40E-02
regulation of biological process	23	2.31	2.66E-02
biological regulation	26	2.16	2.75E-02
regulation of cell cycle	6	7.99	2.94E-02

Table II.3. – Biological processes functional annotation enrichment analysis of the cluster 2 using Panther online resource. Enriched categories are identified as those with Bonferroni adjusted p value <0.05.

GO Biological Process	Nr of genes	Fold enrichment	p-value
DNA metabolic process	40	27.60	1.22E-44
DNA repair	30	48.46	6.86E-39
response to stress	32	13.22	4.96E-25
nucleobase-containing compound metabolic process	51	4.92	1.03E-23
DNA recombination	13	67.44	2.74E-17
nitrogen compound metabolic process	42	4.49	1.53E-16
primary metabolic process	53	3.01	5.19E-15
metabolic process	58	2.66	6.70E-15
regulation of cell cycle	12	17.40	1.95E-09
response to stimulus	33	3.33	2.19E-08
cell cycle	18	6.72	3.66E-08
DNA replication	10	17.18	1.35E-07
organelle organization	21	4.67	5.45E-07
meiosis	7	30.46	1.50E-06
cellular process	56	1.83	1.77E-06
cellular component organization	24	3.30	2.48E-05
chromosome segregation	7	19.67	2.48E-05
phosphate-containing compound metabolic process	21	3.55	5.85E-05
cellular component organization or biogenesis	24	3.08	8.43E-05
reproduction	7	7.77	9.05E-03
biosynthetic process	18	2.78	1.38E-02

2.4. Discussion

Ageing is a multifactorial and complex process, so studying ageing should never be focused in a unique protein, gene or pathway. Network approaches provide a framework for understanding the complexity of biological processes and diseases, looking further than to a simple gene, protein or pathway. More than looking at a specific level, interaction networks analysis allows us to better understand the protein complexes formed, rather than single interactions, enabling us to identify new disease/trait/process players.

In this chapter, the integration of multiple bioinformatic tools allowed the development of an intricate study of the ageing process. Briefly, we assembled a list of age-related genes by combining the data of two public databases. Remarkably, the analysis of the data places the nucleus in the spotlight of the ageing process, given that almost half of the age-related genes correspond to nuclear proteins (308 proteins). Moreover, PPI network construction lead us to the identification of prominent age-related functional clusters related with transcription regulation and chromatin remodelling (Cluster 1), and DNA metabolic process and DNA repair (Cluster 2).

In fact, numerous of the described hallmarks of ageing are correlated with epigenetic alterations that regulate transcription (López-Otín *et al.*, 2013). Changes in the chromatin-transcriptional pathways during ageing have been reported. In physiological ageing models, alterations in transcription and aberrant RNA splicing have been described, which might be due to chromatin structure changes (Zahn and Kim, 2007; Harries *et al.*, 2011). At molecular level, in aged tissues was reported alterations in multiple histone modifications, as is the case of histone acetylation which is a major regulator of transcription by regulating the interactions between DNA and histones (Benayoun *et al*., 2015). Moreover, alterations in several histones and histone-modifying enzymes occur through lifespan (López-Otín et al., 2013; Zane *et al*., 2014). Among these are histone deacetylases (HDAC), as is the case of sirtuins (SIRT), that is well established to regulate longevity through their roles in genomic stability, metabolic regulation and chromatin remodelling (Ghosh *et al.*, 2013).

Moreover, using *in vitro* aged cells and aged tissues it was observed cumulative occurrence of DNA damage which might be due to a functional decline of DNA repair systems contributing for the genomic instability that increases with age (reviewed in Chen *et al*., (2007). Several studies suggest defective DNA repair pathways on the origin of

premature ageing disorders since several of this syndromes are related to defects in DNA repair or in the processing of DNA damage (Brosh and Bohr, 2007; Vijg, 2008; Campisi and Vijg, 2009; Martin, 2011). Although the HGPS is not directly related to mutations in genes involved in DNA damage repair, the mutant lamin A accumulate and sequester replication and repair factors, namely RAD50, RAD51 and 53BP1 resulting in delayed checkpoint response and defective DNA repair, culminating with an accumulation of DNA damage in HGPS cells (Liu *et al.*, 2005; Krishnan *et al.*, 2011). Of note, lamin A and lamin B1 in our PPI network analysis was found in the cluster 2, a cluster of proteins functionally enriched in DNA repair. Remarkably, both A- and B-type lamins are required for the recruitment of factors involved in DNA repair, namely 53BP1, BRCA1 and RAD51 (reviewed in Gonzalo, (2014)). So, it appears that expression of lamin A mutant proteins is strongly associated with an impairment in the capacity of cells adequately repair DNA damage and keep telomere homeostasis, resulting in genomic instability (Gonzalo, 2014).

All together, our data and previous studies indicate that the nuclear functions are very compromised during the ageing process. Remarkably, the nuclear periphery has been recently pointed out as potential regulator of the ageing phenotype (Serebryannyy and Misteli, 2018), and in particular the lamina together with inner nuclear membranes represents a scaffold platform for the genome integrity maintenance. In fact, several alterations in the nuclear transport, chromatin organization and telomere maintenance were already proposed to be involved in the ageing process (Lombard *et al.*, 2005; van Heemst *et al.*, 2007; Kryston *et al.*, 2011; Ogino *et al.*, 2012). However, defects in the NE architecture and in the nuclear envelope proteins in ageing are still poorly studied. It urges the need of studying them further and understand what kind of alterations they could present with ageing. Moreover, the identification of additional proteins involved in the ageing are crucial for the understanding the ageing phenotype.

The NE integrity and its dynamic remodelling are crucial for cellular homeostasis, and so, when perturbed, NE dysfunction occurs. Therefore, revealing the mechanisms underlying NE dysfunction will significantly contribute to our understanding of the precise mechanisms underlying the ageing process.

Supplementary Data

Table SII.1 – Age-related genes obtained from the online databases GenAGE and GENEID. Uniprot accession numbers, gene and protein names are listed.

Uniprot accession	Gene name	Protein name
P10912	GHR	Growth hormone receptor
P01286	SLIB	Somatoliberin
P29353	SHC1	SHC-transforming protein 1
P28069	PIT1	Pituitary-specific positive transcription factor 1
O75360	PROP1	Homeobox protein prophet of Pit-1
P04637	P53	Cellular tumor antigen p53
O14746	TERT	Telomerase reverse transcriptase
Q13315	ATM	Serine-protein kinase ATM
P00749	UROK	Urokinase-type plasminogen activator
P18074	ERCC2	General transcription and DNA repair factor IIH helicase subunit XPD
Q13216	ERCC8	DNA excision repair protein ERCC-8
Q14191	WRN	Werner syndrome ATP-dependent helicase
P02545	LMNA	Prelamin-A/C
P08069	IGF1R	Insulin-like growth factor 1 receptor
P10599	THIO	Thioredoxin
Q9UEF7	KLOT	Klotho
Q01094	E2F1	Transcription factor E2F1
Q06124	PTN11	Tyrosine-protein phosphatase non-receptor type 11
Q00653	NFKB2	Nuclear factor NF-kappa-B p100 subunit
P51692	STA5B	Signal transducer and activator of transcription 5B
P40763	STAT3	Signal transducer and activator of transcription 3
P42229	STA5A	Signal transducer and activator of transcription 5A
Q02297	NRG1	Pro-neuregulin-1, membrane-bound isoform
O15379	HDAC3	Histone deacetylase 3
P01241	SOMA	Somatotropin
P16871	IL7RA	Interleukin-7 receptor subunit alpha
P05019	IGF1	Insulin-like growth factor I
P01344	IGF2	Insulin-like growth factor II
P01308	INS	Insulin
P01138	NGF	Beta-nerve growth factor
P35568	IRS1	Insulin receptor substrate 1
P18031	PTN1	Tyrosine-protein phosphatase non-receptor type 1
Q9Y4H2	IRS2	Insulin receptor substrate 2
P31749	AKT1	RAC-alpha serine/threonine-protein kinase
P42338	PK3CB	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform
P08138	TNR16	Tumor necrosis factor receptor superfamily member 16
P01112	RASH	GTPase HRas
P01106	MYC	Myc proto-oncogene protein
P00533	EGFR	Epidermal growth factor receptor
P04626	ERBB2	Receptor tyrosine-protein kinase erbB-2

P06213	INSR	Insulin receptor
O75376	NCOR1	Nuclear receptor corepressor 1
O60934	NBN	Nibrin
P17535	JUND	Transcription factor jun-D
P60568	IL2	Interleukin-2
P01127	PDGFB	Platelet-derived growth factor subunit B
P01133	EGF	Pro-epidermal growth factor
P31785	IL2RG	Cytokine receptor common subunit gamma
P01100	FOS	Proto-oncogene c-Fos
P09619	PGFRB	Platelet-derived growth factor receptor beta
P19235	EPOR	Erythropoietin receptor
P61278	SMS	Somatostatin
Q05655	KPCD	Protein kinase C delta type
Q07869	PPARA	Peroxisome proliferator-activated receptor alpha
P07949	RET	Proto-oncogene tyrosine-protein kinase receptor Ret
P16885	PLCG2	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2
P50542	PEX5	Peroxisomal targeting signal 1 receptor
P15923	TFE2	Transcription factor E2-alpha
Q9HCS4	TF7L1	Transcription factor 7-like 1
P09874	PARP1	Poly [ADP-ribose] polymerase 1
P38398	BRCA1	Breast cancer type 1 susceptibility protein
Q13526	PIN1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1
P60484	PTEN	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN
Q92793	CBP	CREB-binding protein
Q16665	HIF1A	Hypoxia-inducible factor 1-alpha
P0CG47	UBB	Polyubiquitin-B
P27694	RFA1	Replication protein A 70 kDa DNA-binding subunit
P54132	BLM	Bloom syndrome protein
P10415	BCL2	Apoptosis regulator Bcl-2
P04271	S100B	Protein S100-B
P55072	TERA	Transitional endoplasmic reticulum ATPase
P54098	DPOG1	DNA polymerase subunit gamma-1
P17936	IBP3	Insulin-like growth factor-binding protein 3
P07900	HS90A	Heat shock protein HSP 90-alpha
P04150	GCR	Glucocorticoid receptor
P18146	EGR1	Early growth response protein 1
P15692	VEGFA	Vascular endothelial growth factor A
P00519	ABL1	Tyrosine-protein kinase ABL1
P51587	BRCA2	Breast cancer type 2 susceptibility protein
P11388	TOP2A	DNA topoisomerase 2-alpha
Q02880	TOP2B	DNA topoisomerase 2-beta
P19838	NFKB1	Nuclear factor NF-kappa-B p105 subunit
P11387	TOP1	DNA topoisomerase 1
Q06609	RAD51	DNA repair protein RAD51 homolog 1

P63279	UBC9	SUMO-conjugating enzyme UBC9
P01375	TNFA	Tumor necrosis factor
O15530	PDPK1	3-phosphoinositide-dependent protein kinase 1
P49715	CEBPA	CCAAT/enhancer-binding protein alpha
P17676	CEBPB	CCAAT/enhancer-binding protein beta
P50539	MXI1	Max-interacting protein 1
P01137	TGFB1	Transforming growth factor beta-1
Q03468	ERCC6	DNA excision repair protein ERCC-6
Q15831	STK11	Serine/threonine-protein kinase STK11
Q09472	EP300	Histone acetyltransferase p300
Q7Z2E3	APTX	Aprataxin
P29590	PML	Protein PML
P49841	GSK3B	Glycogen synthase kinase-3 beta
P31645	SC6A4	Sodium-dependent serotonin transporter
P42858	HD	Huntingtin
P17252	KPCA	Protein kinase C alpha type
P32745	SSR3	Somatostatin receptor type 3
Q9NRZ9	HELLS	Lymphoid-specific helicase
P02656	APOC3	Apolipoprotein C-III
P13639	EF2	Elongation factor 2
P19447	ERCC3	General transcription and DNA repair factor IIH helicase subunit XPB
P54274	TERF1	Telomeric repeat-binding factor 1
P78527	PRKDC	DNA-dependent protein kinase catalytic subunit
P04040	CATA	Catalase
Q6IB77	GLYAT	Glycine N-acyltransferase
P28715	ERCC5	DNA repair protein complementing XP-G cells
P10275	ANDR	Androgen receptor
Q13888	TF2H2	General transcription factor IIH subunit 2
P13010	XRCC5	X-ray repair cross-complementing protein 5
P12004	PCNA	Proliferating cell nuclear antigen
P39748	FEN1	Flap endonuclease 1
P25445	TNR6	Tumor necrosis factor receptor superfamily member 6
P49327	FAS	Fatty acid synthase
Q15554	TERF2	Telomeric repeat-binding factor 2
P12956	XRCC6	X-ray repair cross-complementing protein 6
P28340	DPOD1	DNA polymerase delta catalytic subunit
Q07812	BAX	Apoptosis regulator BAX
P06400	RB	Retinoblastoma-associated protein
P50402	EMD	Emerin
P62993	GRB2	Growth factor receptor-bound protein 2
O43524	FOXO3	Forkhead box protein O3
Q12778	FOXO1	Forkhead box protein O1
Q00613	HSF1	Heat shock factor protein 1
P23025	XPA	DNA repair protein complementing XP-A cells
Q9UJ68	MSRA	Mitochondrial peptide methionine sulfoxide reductase

O94761	RECQ4	ATP-dependent DNA helicase Q4
P04179	SODM	Superoxide dismutase [Mn], mitochondrial
P00441	SODC	Superoxide dismutase [Cu-Zn]
Q08050	FOXM1	Forkhead box protein M1
Q99807	COQ7	5-demethoxyubiquinone hydroxylase, mitochondrial
O00555	CAC1A	Voltage-dependent P/Q-type calcium channel subunit alpha-1A
P98164	LRP2	Low-density lipoprotein receptor-related protein 2
O95831	AIFM1	Apoptosis-inducing factor 1, mitochondrial
P09936	UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1
P05067	APP	Amyloid-beta A4 protein
P02649	APOE	Apolipoprotein E
P01023	A2MG	Alpha-2-macroglobulin
O76070	SYUG	Gamma-synuclein
Q06830	PRDX1	Peroxiredoxin-1
P27169	PON1	Serum paraoxonase/arylesterase 1
Q04206	TF65	Transcription factor p65
P05231	IL6	Interleukin-6
Q15493	RGN	Regucalcin
P48047	ATPO	ATP synthase subunit O, mitochondrial
P43351	RAD52	DNA repair protein RAD52 homolog
O95985	TOP3B	DNA topoisomerase 3-beta-1
P07992	ERCC1	DNA excision repair protein ERCC-1
Q96EB6	SIRT1	NAD-dependent protein deacetylase sirtuin-1
Q13547	HDAC1	Histone deacetylase 1
P38646	GRP75	Stress-70 protein, mitochondrial
P07203	GPX1	Glutathione peroxidase 1
P00390	GSHR	Glutathione reductase, mitochondrial
P48637	GSHB	Glutathione synthetase
O15217	GSTA4	Glutathione S-transferase A4
P09211	GSTP1	Glutathione S-transferase P
P00395	COX1	Cytochrome c oxidase subunit 1
P10809	CH60	60 kDa heat shock protein, mitochondrial
P0DMV8	HS71A	Heat shock 70 kDa protein 1A
P0DMV9	HS71B	Heat shock 70 kDa protein 1B
P22061	PIMT	Protein-L-isoaspartate
P45983	MK08	Mitogen-activated protein kinase 8
P63104	1433Z	14-3-3 protein zeta/delta
Q14289	FAK2	Protein-tyrosine kinase 2-beta
Q05397	FAK1	Focal adhesion kinase 1
P13232	IL7	Interleukin-7
Q16539	MK14	Mitogen-activated protein kinase 14
P11362	FGFR1	Fibroblast growth factor receptor 1
P08047	SP1	Transcription factor Sp1
Q8N907	DAND5	DAN domain family member 5
P17948	VGFR1	Vascular endothelial growth factor receptor 1

P05412	JUN	Transcription factor AP-1
O95243	MBD4	Methyl-CpG-binding domain protein 4
Q15648	MED1	Mediator of RNA polymerase II transcription subunit 1
P45984	MK09	Mitogen-activated protein kinase 9
P27361	MK03	Mitogen-activated protein kinase 3
P09429	HMGB1	High mobility group protein B1
P20248	CCNA2	Cyclin-A2
P26583	HMGB2	High mobility group protein B2
Q99683	M3K5	Mitogen-activated protein kinase kinase kinase 5
P21675	TAF1	Transcription initiation factor TFIID subunit 1
P20700	LMNB1	Lamin-B1
Q99643	C560	Succinate dehydrogenase cytochrome b560 subunit, mitochondrial
P98177	FOXO4	Forkhead box protein O4
Q9UBX0	HESX1	Homeobox expressed in ES cells 1
P27986	P85A	Phosphatidylinositol 3-kinase regulatory subunit alpha
Q96G97	BSCL2	Seipin
O15120	PLCB	1-acyl-sn-glycerol-3-phosphate acyltransferase beta
P35226	BMI1	Polycomb complex protein BMI-1
P68104	EF1A1	Elongation factor 1-alpha 1
P05549	AP2A	Transcription factor AP-2-alpha
P23560	BDNF	Brain-derived neurotrophic factor
P16220	CREB1	Cyclic AMP-responsive element-binding protein 1
P15336	ATF2	Cyclic AMP-dependent transcription factor ATF-2
P20226	TBP	TATA-box-binding protein
P27695	APEX1	DNA- (apurinic or apyrimidinic site) lyase
O60381	HBP1	HMG box-containing protein 1
Q8IV16	HDBP1	Glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1
O60566	BUB1B	Mitotic checkpoint serine/threonine-protein kinase BUB1 beta
P35354	PGH2	Prostaglandin G/H synthase 2
P11142	HSP7C	Heat shock cognate 71 kDa protein
Q96ST3	SIN3A	Paired amphipathic helix protein Sin3a
P06493	CDK1	Cyclin-dependent kinase 1
Q14186	TFDP1	Transcription factor Dp-1
P35638	DDIT3	DNA damage-inducible transcript 3 protein
P09884	DPOLA	DNA polymerase alpha catalytic subunit
P10636	TAU	Microtubule-associated protein tau
P29279	CTGF	Connective tissue growth factor
Q92769	HDAC2	Histone deacetylase 2
P61244	MAX	Protein max
Q05195	MAD1	Max dimerization protein 1
Q00987	MDM2	E3 ubiquitin-protein ligase Mdm2
P63165	SUMO1	Small ubiquitin-related modifier 1
P16104	H2AX	Histone H2AX
P09629	HXB7	Homeobox protein Hox-B7
P09017	HXC4	Homeobox protein Hox-C4

O60674	JAK2	Tyrosine-protein kinase JAK2
P03372	ESR1	Estrogen receptor
P41159	LEP	Leptin
O15243	OBRG	Leptin receptor gene-related protein
P48357	LEPR	Leptin receptor
P25963	IKBA	NF-kappa-B inhibitor alpha
P10909	CLUS	Clusterin
Q02643	GHRHR	Growth hormone-releasing hormone receptor
P35222	CTNB1	Catenin beta-1
P49768	PSN1	Presenilin-1
Q9NYJ7	DLL3	Delta-like protein 3
P42771	CDN2A	Cyclin-dependent kinase inhibitor 2A
Q8N726	ARF	Tumor suppressor ARF
P62136	PP1A	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit
Q16643	DREB	Drebrin
Q13253	NOGG	Noggin
P15502	ELN	Elastin
P20848	A1ATR	Putative alpha-1-antitrypsin-related protein
Q13535	ATR	Serine/threonine-protein kinase ATR
Q9H6X2	ANTR1	Anthrax toxin receptor 1
P55916	UCP3	Mitochondrial uncoupling protein 3
O75844	FACE1	CAAX prenyl protease 1 homolog
Q9H3D4	P63	Tumor protein 63
P55851	UCP2	Mitochondrial uncoupling protein 2
P06746	DPOLB	DNA polymerase beta
P48506	GSH1	Glutamate--cysteine ligase catalytic subunit
P48507	GSH0	Glutamate--cysteine ligase regulatory subunit
Q8N6T7	SIRT6	NAD-dependent protein deacetylase sirtuin-6
O43684	BUB3	Mitotic checkpoint protein BUB3
P78406	RAE1L	mRNA export factor
P20382	MCH	Pro-MCH [Cleaved into: Neuropeptide-glycine-glutamic acid
P40692	MLH1	DNA mismatch repair protein Mlh1
P49674	KC1E	Casein kinase I isoform epsilon
Q9UNE7	CHIP	E3 ubiquitin-protein ligase CHIP
O15297	PPM1D	Protein phosphatase 1D
O96017	CHK2	Serine/threonine-protein kinase Chk2
P35558	PCKGC	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]
Q07960	RHG01	Rho GTPase-activating protein 1
P60953	CDC42	Cell division control protein 42 homolog
O00327	BMAL1	Aryl hydrocarbon receptor nuclear translocator-like protein 1
O15516	CLOCK	Circadian locomoter output cycles protein kaput
Q14526	HIC1	Hypermethylated in cancer 1 protein
Q13219	PAPP1	Pappalysin-1
O95622	ADCY5	Adenylate cyclase type 5
Q9UBK2	PRGC1	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

P36969	GPX4	Phospholipid hydroperoxide glutathione peroxidase
P25874	UCP1	Mitochondrial brown fat uncoupling protein 1
Q9GZV9	FGF23	Fibroblast growth factor 23
Q12805	FBLN3	EGF-containing fibulin-like extracellular matrix protein 1
Q92889	XPF	DNA repair endonuclease XPF
P11597	CETP	Cholesteryl ester transfer protein
P37231	PPARG	Peroxisome proliferator-activated receptor gamma
P30556	AGTR1	Type-1 angiotensin II receptor
Q8N5K1	CISD2	CDGSH iron-sulfur domain-containing protein 2
O43324	MCA3	Eukaryotic translation elongation factor 1 epsilon-1
Q12929	EPS8	Epidermal growth factor receptor kinase substrate 8
P22001	KCNA3	Potassium voltage-gated channel subfamily A member 3
Q9NRC8	SIR7	NAD-dependent protein deacetylase sirtuin-7
Q9BZW2	S13A1	Solute carrier family 13 member 1
O14508	SOCS2	Suppressor of cytokine signaling 2
P29144	TPP2	Tripeptidyl-peptidase 2
Q12888	TP53B	TP53-binding protein 1
Q9NTG7	SIR3	NAD-dependent protein deacetylase sirtuin-3, mitochondrial
Q9Y618	NCOR2	Nuclear receptor corepressor 2
O94901	SUN1	SUN domain-containing protein 1
Q16611	BAK	Bcl-2 homologous antagonist/killer
P18065	IBP2	Insulin-like growth factor-binding protein 2
P32322	P5CR1	Pyrroline-5-carboxylate reductase 1, mitochondrial
O15350	P73	Tumor protein p73
P21554	CNR1	Cannabinoid receptor 1
Q16236	NF2L2	Nuclear factor erythroid 2-related factor 2
P38936	CDN1A	Cyclin-dependent kinase inhibitor 1
P16234	PGFRA	Platelet-derived growth factor receptor alpha
P42336	PK3CA	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform
P02745	C1QA	Complement C1q subcomponent subunit A
P42772	CDN2B	Cyclin-dependent kinase 4 inhibitor B
Q9GZV4	IF5A2	Eukaryotic translation initiation factor 5A-2
P03971	MIS	Muellerian-inhibiting factor
P14174	MIF	Macrophage migration inhibitory factor
O75907	DGAT1	Diacylglycerol O-acyltransferase 1
P04732	MT1E	Metallothionein-1E
Q9NSA1	FGF21	Fibroblast growth factor 21
O43464	HTRA2	Serine protease HTRA2, mitochondrial
P49840	GSK3A	Glycogen synthase kinase-3 alpha
P36639	8ODP	7,8-dihydro-8-oxoguanine triphosphatase
O14920	IKKB	Inhibitor of nuclear factor kappa-B kinase subunit beta
Q13501	SQSTM	Sequestosome-1
P50613	CDK7	Cyclin-dependent kinase 7
P28799	GRN	Granulins
P05121	PAI1	Plasminogen activator inhibitor 1

Q9H040	SPRTN	SprT-like domain-containing protein Spartan
Q6R327	RICTR	Rapamycin-insensitive companion of mTOR
Q16619	CTF1	Cardiotrophin-1
Q12931	TRAP1	Heat shock protein 75 kDa, mitochondrial
Q58FF3	ENPLL	Putative endoplasmin-like protein
Q8NER1	TRPV1	Transient receptor potential cation channel subfamily V member 1
Q14494	NF2L1	Endoplasmic reticulum membrane sensor NFE2L1
P01574	IFNB	Interferon beta
O95390	GDF11	Growth/differentiation factor 11
Q13131	AAPK1	5'-AMP-activated protein kinase catalytic subunit alpha-1
P00813	ADA	Adenosine deaminase
P48736	PK3CG	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform
O00443	P3C2A	Phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit alpha
Q96BI3	APH1A	Gamma-secretase subunit APH-1A
Q8WW43	APH1B	Gamma-secretase subunit APH-1B
P06576	ATPB	ATP synthase subunit beta, mitochondrial
Q9HD33	RM47	39S ribosomal protein L47, mitochondrial
P13861	KAP2	cAMP-dependent protein kinase type II-alpha regulatory subunit
P31323	KAP3	cAMP-dependent protein kinase type II-beta regulatory subunit
Q14457	BECN1	Beclin-1
O43683	BUB1	Mitotic checkpoint serine/threonine-protein kinase BUB1
P10606	COX5B	Cytochrome c oxidase subunit 5B, mitochondrial
Q9UK39	NOCT	Nocturnin
Q9UQ13	SHOC2	Leucine-rich repeat protein SHOC-2
P17987	TCPA	T-complex protein 1 subunit alpha
Q13972	RGRF1	Ras-specific guanine nucleotide-releasing factor 1
O14827	RGRF2	Ras-specific guanine nucleotide-releasing factor 2
Q07889	SOS1	Son of sevenless homolog 1
Q99741	CDC6	Cell division control protein 6 homolog
Q13415	ORC1	Origin recognition complex subunit 1
Q9Y619	ORNT1	Mitochondrial ornithine transporter 1
O00311	CDC7	Cell division cycle 7-related protein kinase
P42574	CASP3	Caspase-3
P55210	CASP7	Caspase-7
P55212	CASP6	Caspase-6
P42575	CASP2	Caspase-2
Q14790	CASP8	Caspase-8
P61964	WDR5	WD repeat-containing protein 5
O60907	TBL1X	F-box-like/WD repeat-containing protein TBL1X
Q8NCM8	DYHC2	Cytoplasmic dynein 2 heavy chain 1
P21333	FLNA	Filamin-A
O75390	CISY	Citrate synthase, mitochondrial
Q9Y4R8	TELO2	Telomere length regulation protein TEL2 homolog
Q9NZJ6	COQ3	Ubiquinone biosynthesis O-methyltransferase, mitochondrial
P20674	COX5A	Cytochrome c oxidase subunit 5A, mitochondrial

P08574	CY1	Cytochrome c1, heme protein, mitochondrial
O60266	ADCY3	Adenylate cyclase type 3
P17568	NDUB7	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7
Q9HBG6	IF122	Intraflagellar transport protein 122 homolog
P48380	RFX3	Transcription factor RFX3
Q33E94	RFX4	Transcription factor RFX4
P48382	RFX5	DNA-binding protein RFX5
Q13705	AVR2B	Activin receptor type-2B
P37173	TGFR2	TGF-beta receptor type-2
P36897	TGFR1	TGF-beta receptor type-1
Q13188	STK3	Serine/threonine-protein kinase 3
Q13043	STK4	Serine/threonine-protein kinase 4
P10632	CP2C8	Cytochrome P450 2C8
P08684	CP3A4	Cytochrome P450 3A4
P11712	CP2C9	Cytochrome P450 2C9
Q9NR63	CP26B	Cytochrome P450 26B1
P04798	CP1A1	Cytochrome P450 1A1
P11509	CP2A6	Cytochrome P450 2A6
O43174	CP26A	Cytochrome P450 26A1
Q9NQZ3	DAZ1	Deleted in azoospermia protein 1
P51530	DNA2	DNA replication ATP-dependent helicase/nuclease DNA2
P49917	DNLI4	DNA ligase 4
Q7Z6J0	SH3R1	E3 ubiquitin-protein ligase SH3RF1
Q15822	ACHA2	Neuronal acetylcholine receptor subunit alpha-2
P17787	ACHB2	Neuronal acetylcholine receptor subunit beta-2
P13637	AT1A3	Sodium/potassium-transporting ATPase subunit alpha-3
Q13976	KGP1	cGMP-dependent protein kinase 1
Q8N8Q8	COX18	Cytochrome c oxidase assembly protein COX18, mitochondrial
O00213	APBB1	Amyloid-beta A4 precursor protein-binding family B member 1
Q99729	ROAA	Heterogeneous nuclear ribonucleoprotein A/B
P50616	TOB1	Protein Tob1
P62324	BTG1	Protein BTG1
P14324	FPPS	Farnesyl pyrophosphate synthase
P42345	MTOR	Serine/threonine-protein kinase mTOR
Q9Y478	AAKB1	5'-AMP-activated protein kinase subunit beta-1
O43741	AAKB2	5'-AMP-activated protein kinase subunit beta-2
P20594	ANPRB	Atrial natriuretic peptide receptor 2
P16066	ANPRA	Atrial natriuretic peptide receptor 1
P51841	GUC2F	Retinal guanylyl cyclase 2
P05090	APOD	Apolipoprotein D
P46531	NOTC1	Neurogenic locus notch homolog protein 1
Q99466	NOTC4	Neurogenic locus notch homolog protein 4
P63096	GNAI1	Guanine nucleotide-binding protein G
P09471	GNAO	Guanine nucleotide-binding protein G
P19087	GNAT2	Guanine nucleotide-binding protein G

P21695	GPDA	Glycerol-3-phosphate dehydrogenase [NAD
P46091	GPR1	G-protein coupled receptor 1
Q13952	NFYC	Nuclear transcription factor Y subunit gamma
Q9NRG0	CHRC1	Chromatin accessibility complex protein 1
O14733	MP2K7	Dual specificity mitogen-activated protein kinase kinase 7
P17066	HSP76	Heat shock 70 kDa protein 6
Q9Y230	RUVB2	RuvB-like 2
O75351	VPS4B	Vacuolar protein sorting-associated protein 4B
P04792	HSPB1	Heat shock protein beta-1
O14618	CCS	Copper chaperone for superoxide dismutase
P08294	SODE	Extracellular superoxide dismutase [Cu-Zn]
Q99757	THIOM	Thioredoxin, mitochondrial
O43396	TXNL1	Thioredoxin-like protein 1
P52789	HXK2	Hexokinase-2
P48735	IDHP	Isocitrate dehydrogenase [NADP], mitochondrial
P50213	IDH3A	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial
Q13183	S13A2	Solute carrier family 13 member 2
P14616	INSRR	Insulin receptor-related protein
P47985	UCRI	Cytochrome b-c1 complex subunit Rieske, mitochondrial
O43808	PM34	Peroxisomal membrane protein PMP34
Q9H2D1	MFTC	Mitochondrial folate transporter/carrier
P27544	CERS1	Ceramide synthase 1
P22749	GNLY	Granulysin
P15090	FABP4	Fatty acid-binding protein, adipocyte
P05413	FABPH	Fatty acid-binding protein, heart
P02689	MYP2	Myelin P2 protein
P01116	RASK	GTPase KRas
P01111	RASN	GTPase NRas
Q8IXJ6	SIR2	NAD-dependent protein deacetylase sirtuin-2
Q9Y6E7	SIR4	NAD-dependent protein lipoamidase sirtuin-4, mitochondrial
Q9NXA8	SIR5	NAD-dependent protein deacylase sirtuin-5, mitochondrial
Q15031	SYLM	Probable leucine--tRNA ligase, mitochondrial
P09012	SNRPA	U1 small nuclear ribonucleoprotein A
P08579	RU2B	U2 small nuclear ribonucleoprotein B"
Q6ZRY4	RBPS2	RNA-binding protein with multiple splicing 2
Q9Y6E0	STK24	Serine/threonine-protein kinase 24
Q9NQU5	PAK6	Serine/threonine-protein kinase PAK 6
Q99798	ACON	Aconitate hydratase, mitochondrial
Q9UBU8	MO4L1	Mortality factor 4-like protein 1
P11021	BIP	Endoplasmic reticulum chaperone BiP
Q8TB72	PUM2	Pumilio homolog 2
Q14671	PUM1	Pumilio homolog 1
O15440	MRP5	Multidrug resistance-associated protein 5
Q96J65	MRP9	Multidrug resistance-associated protein 9
Q96J66	ABCCB	ATP-binding cassette sub-family C member 11

P48960	CD97	CD97 antigen
P55157	MTP	Microsomal triglyceride transfer protein large subunit
P14314	GLU2B	Glucosidase 2 subunit beta
P19793	RXRA	Retinoic acid receptor RXR-alpha
P28702	RXRB	Retinoic acid receptor RXR-beta
P41235	HNF4A	Hepatocyte nuclear factor 4-alpha
P30419	NMT1	Glycylpeptide N-tetradecanoyltransferase 1
O60551	NMT2	Glycylpeptide N-tetradecanoyltransferase 2
P40261	NNMT	Nicotinamide N-methyltransferase
Q6XQN6	PNCB	Nicotinate phosphoribosyltransferase
P01303	NPY	Pro-neuropeptide Y
O00115	DNS2A	Deoxyribonuclease-2-alpha
Q8WZ79	DNS2B	Deoxyribonuclease-2-beta
O75489	NDUS3	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial
Q96LB3	IFT74	Intraflagellar transport protein 74 homolog
Q9Y496	KIF3A	Kinesin-like protein KIF3A
O15066	KIF3B	Kinesin-like protein KIF3B
O14782	KIF3C	Kinesin-like protein KIF3C
O95239	KIF4A	Chromosome-associated kinesin KIF4A
Q2VIQ3	KIF4B	Chromosome-associated kinesin KIF4B
Q12840	KIF5A	Kinesin heavy chain isoform 5A
O15294	OGT1	UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase 110 kDa subunit
Q9Y5Q9	TF3C3	General transcription factor 3C polypeptide 3
Q9Y366	IFT52	Intraflagellar transport protein 52 homolog
O14753	OVOL1	Putative transcription factor Ovo-like 1
Q9BRP0	OVOL2	Transcription factor Ovo-like 2
O00110	OVOL3	Putative transcription factor ovo-like protein 3
O60260	PRKN	E3 ubiquitin-protein ligase parkin
Q3L8U1	CHD9	Chromodomain-helicase-DNA-binding protein 9
Q9HCK8	CHD8	Chromodomain-helicase-DNA-binding protein 8
Q07343	PDE4B	cAMP-specific 3',5'-cyclic phosphodiesterase 4B
O76083	PDE9A	High affinity cGMP-specific 3',5'-cyclic phosphodiesterase 9A
Q08499	PDE4D	cAMP-specific 3',5'-cyclic phosphodiesterase 4D
Q9Y243	AKT3	RAC-gamma serine/threonine-protein kinase
Q02156	KPCE	Protein kinase C epsilon type
P31751	AKT2	RAC-beta serine/threonine-protein kinase
P05771	KPCB	Protein kinase C beta type
Q9NZ42	PEN2	Gamma-secretase subunit PEN-2
Q8N697	S15A4	Solute carrier family 15 member 4
P46059	S15A1	Solute carrier family 15 member 1
Q16348	S15A2	Solute carrier family 15 member 2
Q9H611	PIF1	ATP-dependent DNA helicase PIF1
P35232	PHB	Prohibitin
P27105	STOM	Erythrocyte band 7 integral membrane protein
Q99623	PHB2	Prohibitin-2

Q13393	PLD1	Phospholipase D1
Q8TE04	PANK1	Pantothenate kinase 1
Q9BZ23	PANK2	Pantothenate kinase 2, mitochondrial
P49810	PSN2	Presenilin-2
Q15397	PUM3	Pumilio homolog 3
Q15274	NADC	Nicotinate-nucleotide pyrophosphorylase
Q8WWV3	RT4I1	Reticulon-4-interacting protein 1, mitochondrial
O60671	RAD1	Cell cycle checkpoint protein RAD1
Q5T890	ER6L2	DNA excision repair protein ERCC-6-like 2
Q9Y620	RA54B	DNA repair and recombination protein RAD54B
P51532	SMCA4	Transcription activator BRG1
P51531	SMCA2	Probable global transcription activator SNF2L2
O60264	SMCA5	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5
P28370	SMCA1	Probable global transcription activator SNF2L1
Q14565	DMC1	Meiotic recombination protein DMC1/LIM15 homolog
Q8IV36	HID1	Protein HID1
Q9UKC9	FBXL2	F-box/LRR-repeat protein 2
Q99638	RAD9A	Cell cycle checkpoint control protein RAD9A
P46100	ATRX	Transcriptional regulator ATRX
P62070	RRAS2	Ras-related protein R-Ras2
Q96NR8	RDH12	Retinol dehydrogenase 12
Q8TC12	RDH11	Retinol dehydrogenase 11
Q92781	RDH1	11-cis retinol dehydrogenase
O75452	RDH16	Retinol dehydrogenase 16
Q8IZV5	RDH10	Retinol dehydrogenase 10
Q9NYR8	RDH8	Retinol dehydrogenase 8
Q969S3	ZN622	Zinc finger protein 622
Q5UIP0	RIF1	Telomere-associated protein RIF1
Q9Y581	INSL6	Insulin-like peptide INSL6
Q8WUI4	HDAC7	Histone deacetylase 7
Q9UKV0	HDAC9	Histone deacetylase 9
Q9UQL6	HDAC5	Histone deacetylase 5
P62899	RL31	60S ribosomal protein L31
Q02878	RL6	60S ribosomal protein L6
P62834	RAP1A	Ras-related protein Rap-1A
P61224	RAP1B	Ras-related protein Rap-1b
P61225	RAP2B	Ras-related protein Rap-2b
O14948	TFEC	Transcription factor EC
P19532	TFE3	Transcription factor E3
P54107	CRIS1	Cysteine-rich secretory protein 1
P16562	CRIS2	Cysteine-rich secretory protein 2
P54108	CRIS3	Cysteine-rich secretory protein 3
P48060	GLIP1	Glioma pathogenesis-related protein 1
Q01995	TAGL	Transgelin

P51911	CNN1	Calponin-1
P37802	TAGL2	Transgelin-2
Q9UI15	TAGL3	Transgelin-3
O00141	SGK1	Serine/threonine-protein kinase Sgk1
Q96BR1	SGK3	Serine/threonine-protein kinase Sgk3
Q9HBY8	SGK2	Serine/threonine-protein kinase Sgk2
P46063	RECQ1	ATP-dependent DNA helicase Q1
O94762	RECQ5	ATP-dependent DNA helicase Q5
Q9NPE6	SPAG4	Sperm-associated antigen 4 protein
P31948	STIP1	Stress-induced-phosphoprotein 1
O15264	MK13	Mitogen-activated protein kinase 13
P28482	MK01	Mitogen-activated protein kinase 1
P53778	MK12	Mitogen-activated protein kinase 12
Q15759	MK11	Mitogen-activated protein kinase 11
P37840	SYUA	Alpha-synuclein
P54646	AAPK2	5'-AMP-activated protein kinase catalytic subunit alpha-2
Q9UGJ0	AAKG2	5'-AMP-activated protein kinase subunit gamma-2
P54619	AAKG1	5'-AMP-activated protein kinase subunit gamma-1
Q9UGI9	AAKG3	5'-AMP-activated protein kinase subunit gamma-3
P50416	CPT1A	Carnitine O-palmitoyltransferase 1, liver isoform
Q92523	CPT1B	Carnitine O-palmitoyltransferase 1, muscle isoform
O95198	KLHL2	Kelch-like protein 2
Q9Y573	IPP	Actin-binding protein IPP
Q9BTW9	TBCD	Tubulin-specific chaperone D
P29992	GNA11	Guanine nucleotide-binding protein subunit alpha-11
P50148	GNAQ	Guanine nucleotide-binding protein G
P30679	GNA15	Guanine nucleotide-binding protein subunit alpha-15
O95467	GNAS3	Neuroendocrine secretory protein 55
P63092	GNAS2	Guanine nucleotide-binding protein G
P84996	ALEX	Protein ALEX
Q5JWF2	GNAS1	Guanine nucleotide-binding protein G
Q9NQ55	SSF1	Suppressor of SWI4 1 homolog
P53701	CCHL	Cytochrome c-type heme lyase
Q9NQW8	CNGB3	Cyclic nucleotide-gated cation channel beta-3
P29973	CNGA1	cGMP-gated cation channel alpha-1
Q14028	CNGB1	Cyclic nucleotide-gated cation channel beta-1
P07333	CSF1R	Macrophage colony-stimulating factor 1 receptor
Q04912	RON	Macrophage-stimulating protein receptor
P35916	VGFR3	Vascular endothelial growth factor receptor 3
P29320	EPHA3	Ephrin type-A receptor 3
P50607	TUB	Tubby protein homolog
Q9UBI4	STML1	Stomatin-like protein 1
Q9HCH5	SYTL2	Synaptotagmin-like protein 2
Q9UPW8	UN13A	Protein unc-13 homolog A
O14795	UN13B	Protein unc-13 homolog B

Q8NB66	UN13C	Protein unc-13 homolog C
Q70J99	UN13D	Protein unc-13 homolog D
O95267	GRP1	RAS guanyl-releasing protein 1
Q00975	CAC1B	Voltage-dependent N-type calcium channel subunit alpha-1B
Q13936	CAC1C	Voltage-dependent L-type calcium channel subunit alpha-1C
Q01668	CAC1D	Voltage-dependent L-type calcium channel subunit alpha-1D
Q15878	CAC1E	Voltage-dependent R-type calcium channel subunit alpha-1E
O60840	CAC1F	Voltage-dependent L-type calcium channel subunit alpha-1F
Q9UJZ1	STML2	Stomatin-like protein 2, mitochondrial
O14595	CTDS2	Carboxy-terminal domain RNA polymerase II polypeptide A small phosphatase 2
Q99259	DCE1	Glutamate decarboxylase 1
Q05329	DCE2	Glutamate decarboxylase 2
Q9Y600	CSAD	Cysteine sulfinic acid decarboxylase
P20711	DDC	Aromatic-L-amino-acid decarboxylase
P19113	DCHS	Histidine decarboxylase
Q9UBI9	HDC	Headcase protein homolog
O15056	SYNJ2	Synaptojanin-2
O43426	SYNJ1	Synaptojanin-1
P11230	ACHB	Acetylcholine receptor subunit beta
Q05901	ACHB3	Neuronal acetylcholine receptor subunit beta-3
P30926	ACHB4	Neuronal acetylcholine receptor subunit beta-4
P78337	PITX1	Pituitary homeobox 1
O75364	PITX3	Pituitary homeobox 3
Q99697	PITX2	Pituitary homeobox 2
P23759	PAX7	Paired box protein Pax-7
Q15699	ALX1	ALX homeobox protein 1
O14813	PHX2A	Paired mesoderm homeobox protein 2A
Q9ULU8	CAPS1	Calcium-dependent secretion activator 1
Q16623	STX1A	Syntaxin-1A
P61266	STX1B	Syntaxin-1B
Q13277	STX3	Syntaxin-3
P32856	STX2	Syntaxin-2
O75558	STX11	Syntaxin-11
O15400	STX7	Syntaxin-7
Q93050	VPP1	V-type proton ATPase 116 kDa subunit a isoform 1
Q13488	VPP3	V-type proton ATPase 116 kDa subunit a isoform 3
A6NJT0	UNC4	Homeobox protein unc-4 homolog
O60902	SHOX2	Short stature homeobox protein 2
O75569	PRKRA	Interferon-inducible double-stranded RNA-dependent protein kinase activator A
Q9Y2V3	RX	Retinal homeobox protein Rx
P40425	PBX2	Pre-B-cell leukemia transcription factor 2
Q86WB7	UN93A	Protein unc-93 homolog A
Q9H598	VIAAT	Vesicular inhibitory amino acid transporter
P18505	GBRB1	Gamma-aminobutyric acid receptor subunit beta-1
P47870	GBRB2	Gamma-aminobutyric acid receptor subunit beta-2

P28472	GBRB3	Gamma-aminobutyric acid receptor subunit beta-3
P24046	GBRR1	Gamma-aminobutyric acid receptor subunit rho-1
P23415	GLRA1	Glycine receptor subunit alpha-1
O00591	GBRP	Gamma-aminobutyric acid receptor subunit pi
Q9Y623	MYH4	Myosin-4
P11055	MYH3	Myosin-3
P12883	MYH7	Myosin-7
P13535	MYH8	Myosin-8
Q9UKX3	MYH13	Myosin-13
O00634	NET3	Netrin-3
Q9Y6N6	LAMC3	Laminin subunit gamma-3
P11047	LAMC1	Laminin subunit gamma-1
P55268	LAMB2	Laminin subunit beta-2
P25391	LAMA1	Laminin subunit alpha-1
O95631	NET1	Netrin-1
Q99689	FEZ1	Fasciculation and elongation protein zeta-1
Q9Y250	LZTS1	Leucine zipper putative tumor suppressor 1
Q9UHY8	FEZ2	Fasciculation and elongation protein zeta-2
O75083	WDR1	WD repeat-containing protein 1
O14727	APAF	Apoptotic protease-activating factor 1
O43172	PRP4	U4/U6 small nuclear ribonucleoprotein Prp4
Q9P2D8	UNC79	Protein unc-79 homolog
Q8N2C7	UNC80	Protein unc-80 homolog
P18283	GPX2	Glutathione peroxidase 2
P22352	GPX3	Glutathione peroxidase 3
Q96SL4	GPX7	Glutathione peroxidase 7
Q9UI12	VATH	V-type proton ATPase subunit H
Q8N8U9	BMPER	BMP-binding endothelial regulator protein
Q9Y6R7	FCGBP	IgGFc-binding protein
O75443	TECTA	Alpha-tectorin
Q96KJ9	COX42	Cytochrome c oxidase subunit 4 isoform 2, mitochondrial
P13073	COX41	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial
Q99643	C560	Succinate dehydrogenase cytochrome b560 subunit, mitochondrial
Q06830	PRDX1	Peroxiredoxin-1
P47985	UCRI	Cytochrome b-c1 complex subunit Rieske, mitochondrial
Q96RD7	PANX1	Pannexin-1
Q96H78	S2544	Solute carrier family 25 member 44
O14558	HSPB6	Heat shock protein beta-6
P02795	MT2	Metallothionein-2
P29353	SHC1	SHC-transforming protein 1

CHAPTER III

Characterization of the nuclear envelope protein alterations during ageing in mice models

3.1. Introduction

Ageing is a very complex process to study about. As previously stated, studying ageing in humans poses various difficulties like ethical problems, long lifespan, uncontrollable environment impacts, among others. Hence, diverse animal models have been used to study the ageing process and biology. One of the most used ageing animal model is mice. Mice are very similar genetically and physiologically to humans and over the years, they have been used in many research areas. Mice offers advantages like being possible to raise them until full term life in less than 3 years, controlling their growing environment, registering sudden changes (Vanhooren and Libert, 2013).

Mice life cycle is quite different from humans and mice maturate a lot faster than humans. Since they are born and until they complete 1 month old, their maturation rate is 150 times faster than in humans. Between their 1 to 6 months old, they maturate 45 times faster than a human. After they complete 6 months, they mature 25 times faster than a human being. In terms of translating their ages to "human age" equivalents, there has been reported that a 3 to 6 months old mouse is considered a mature adult mouse, corresponding to 20 to 30 years old human (Figure III.1). A 10 to 14 months old mouse is considered a middle-aged mouse, corresponding to a 38 to 47 years old human (Figure III.1). An 18 to 24 months old mouse is considered old, which corresponds to a 56 to 69 years old human. After the 24 months old, 50% of the mice die (Figure III.1). Their maximum lifespan goes up to 36 months (Figure III.1) (The Jackson Laboratories booklet, 2017).

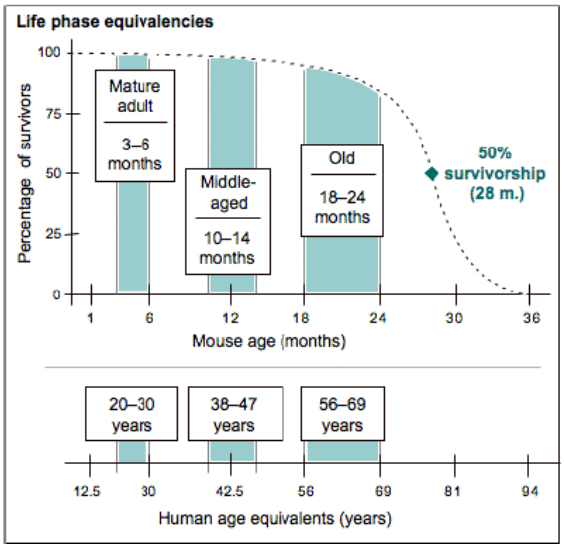

Figure III.1 - Life phase equivalencies between mice and human. Taken from (The Jackson Laboratory booklet, 2017).

Given all the ethical constrains to work with humans, the animal models represent a very powerful and attractive model for the study of human physiology and pathology. In our case it was chosen to study the ageing process, particularly to characterize the nuclear envelope proteins alterations during ageing.

3.2. Materials and Methodology

3.2.1. Animals

C57BL/6 female mice with 6, 18 and 24 months old were obtained from Charles River Laboratory and housed at iBiMED animal facility according the Directive 2010/63/EU recommendations. The procedures were approved and supervised by the Animal welfare body of the Department of Medical Sciences of the University of Aveiro (CEDCM-BEA). Upon arrival the animals were kept one week in quarantine and then were sacrificed by cervical stretching followed by decapitation, and their organs collected, immediately frozen in liquid nitrogen and stored at -80ºC. During all the procedure efforts were made to minimize the number of animals used and their suffering.

In this study, the tissues used were liver and kidney from 4 different mice with 3 specific ages (6,18 and 24 months). Image III.2 summarizes the tissues collected at the different ages.

Figure III.2 – Illustrative scheme of the tissues (liver and kidney) collected of four different mice at different ages of 6, 18 and 24 months old.

3.2.2. Protein extraction

The collected tissues were ground to powder using liquid nitrogen in a mortar and stored for later use at -80ºC. For protein extraction 10 volumes of ELB buffer (0.05M HEPES, 0.25M NaCl, 1mM DTT, 1mM NaF, 2mM EDTA, 1mM EGTA, 1mM Na3VO4 and 0.5% Triton X-100, containing protease inhibitors) was added. The tissues were sonicated twice, for about 15 seconds/pulses. Then, the lysates were centrifuged for 15 min at 4ºC and the supernatant was kept at -30ºC. The resulting lysates were then subjected to a cold acetone protein precipitation protocol. Briefly, it was added 9 parts of cold acetone (-20ºC) to each part of the sample. The samples were centrifuged at 14,000g for 2h at 4ºC. The supernatant was discarded, and the pellets were resuspended in boiling 1% SDS. The samples were boiled for 10 min at 95ºC, sonicated twice during 15 seconds/pulses and stored at -30ºC.

3.2.3. Protein Quantification

The *Thermo Scientific™ Pierce™ BCA Protein Assay Kit* was used to determine the protein concentration of the samples. This kit is a colorimetric assay based on a combination of the reduction of Cu^{2+} to Cu^{+} by protein in a basic medium with an extremely accurate colorimetric detection of the cuprous cation (Cu^{+}) by bicinchoninic acid (BCA). The first complex formed is a light blue one that is formed due to the chelation of copper with protein in a basic setting. Then, BCA reacts with the reduced cuprous cation formed previously. This is called the colour development reaction. The result of the chelation of two molecules of BCA with one cuprous ion is an intense purple-coloured reaction product. The complex formed (BCA/copper complex) is soluble in water and displays a linear absorbance at 562nm that increased with the increasing concentration of protein that is compared to a protein standard. The working range of this kit is 20-2000µg/ml.

Firstly, standards A, B, C, D, E, F, G, H and I were prepared in duplicate according to the manufacturer's instructions (see the appendix). The standards were prepared in autoclaved dH_20. Then, 10µl of each standard were pipetted in duplicated to a 96-well plate. After that, the samples were prepared in a microtube (1µl of the original sample plus 24µl of autoclaved dH_20). Then, 10µl of each sample was pipetted in duplicated to the 96-well plate. At that point, the working solution was prepared being the proportion

of the reagent A to B, 50A: 1B, and 100µl of the working solution were pipetted in each well (samples + standards). After that, the plate was incubated at 37ºC for 30 min. The absorbance at 562nm was read in a microplate reader (e.g. Infinite® 200 PRO from Tecan). The results were analysed having into account the dilution factor and by comparison with the standard curve.

3.2.4. SDS-Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a very used method for protein separation by electrophoresis. It uses a discontinuous polyacrylamide gel as a support medium and SDS to denature the proteins. When SDS is dissolved, it has a net negative charge as it is an anionic detergent. The complex structures presented by proteins are destroyed by the negative charges on SDS. The higher molecular weight of the protein, the higher the amount of SDS bonded to a polypeptide chain. The negative charges of SDS are very attracted to an anode in an electric field. Larger molecules cannot migrate as fast as the smaller ones as they are restrained by polyacrylamide gels (Caprette, 2012). Samples were separated in 5-20% gradient SDS-PAGE gels in order to obtain a wider separation range and distinguish better some protein isoforms with close molecular weights. The gradient resolving gels were prepared (see composition in the appendix) and mixed in the Hoefer® GS50 Gradient Maker and allowed to polymerize at room temperature (RT). Then, the 3.5% stacking gel was prepared and loaded on the top of gradient gels. The combs were inserted, and the gels were left to polymerize at RT. Prior to loading, the samples were prepared by the addition of 4x Loading buffer (LB), boiled for 10 min and spinned down. The combs were removed, and the samples were carefully loaded into the wells. Molecular weight protein markers (Precision Plus Protein™ Dual Colour Standards; Bio Rad) were also loaded in the gels. The gels were subjected to electrophoresis in a Hoefer® electrophoresis system at 90mA for approximately 3h.

3.2.5. Immunoblotting

When the electrophoresis was completed, proteins were transferred from the gel to a nitrocellulose membrane (0.2 µm pore size; Amersham™ Protran®) at 200mA for 18h in a Hoefer™ TE42 Transfer Tank Blotting Unit. After transfer, the membranes were hydrated with 1x TBS-T (see appendix for composition) for 5 min at RT with gentle

agitation. Ponceau S staining was used as a loading control and the membrane scanned in Molecular Imager GS-800 Calibrated Densitometer (Bio-Rad). Membranes were washed in 1x TBS-T until the bands coloured with Ponceau S staining disappeared.

In order to reduce the background and unspecific signals, the blocking solution (5% BSA in 1x TBS-T) was incubated for 22h (4h at RT and overnight at 4ºC with agitation). After that, the primary antibodies were incubated for 2-4h at RT and also overnight at 4ºC (both with gentle agitation). The antibodies used were: LAP1 Antibody RL13, lamin A/C Antibody (346), lamin B1 Antibody (B-10), emerin Antibody (H-7), Syne-2 Antibody (C-1), SUN1 antibody (see table III.1 for further details). All the primary antibodies were diluted in 3% BSA in 1x TBS-T.

Table III.1 – Primary antibodies used in order to detect the proteins analysed by immunoblotting.

Antibody	Antibody Company	Antibody type	Target	Dilution
Lamin A/C Antibody (346) sc-7293	Santa Cruz Biotechnology	Mouse, monoclonal	Lamin A/C	1:500
Lamin B1 Antibody (B-10) sc-374015	Santa Cruz Biotechnology	Mouse, monoclonal	Lamin B1	1:500
Emerin Antibody (H-7) sc-393247	Santa Cruz Biotechnology	Mouse, monoclonal	Emerin	1:500
LAP1 Antibody RL13 MA1-074	Thermo Fisher Scientific	Mouse, monoclonal	LAP1	1:1000
SUN1 antibody	Kindly provided by Ya-Hui Chi	Rabbit, polyclonal	SUN1	1:2000
Syne-2 Antibody (C-1) sc-365097	Santa Cruz Biotechnology	Mouse, monoclonal	Nesprin-2	1:500

Anti-mouse or anti-rabbit horseradish peroxidase (HRP)-linked secondary antibodies (GE Healthcare) were used and diluted in 3% milk in 1x TBS-T. The secondary antibodies were incubated for 2h at RT. Between the primary and the secondary antibodies, the membranes were washed 4 times for 5 min with agitation. The washes were also performed after removing the secondary antibodies. The membranes were incubated with Luminata Crescendo™ Western HRP Substrate (Millipore) for 5 min

before image acquisition in the ChemiDoc™ Touch Imaging System (Bio-Rad). Luminata Crescendo™ Western HRP Substrate (Millipore) is a chemiluminescent reagent used to detect immobilized specific antigens conjugated directly or indirectly with HRP-labelled antibodies.

3.2.6. Data Analysis and Statistics

Quantitative analyses of immunoblots was performed using Image Lab™ Software (Bio-Rad Laboratories; Version 6.00 Build 25). All data were corrected relative to loading control (Ponceau S) and were expressed as mean ± SEM (standard error). Statistical significance analysis was conducted using GraphPad Prism 6 software and data were analysed using one-way ANOVA followed by the Dunnett's multi-comparisons test, for comparison of data with control values.

3.3. Results

Several studies have pointed protein sequestration at the nuclear envelope periphery as a potential regulatory mechanism of the ageing phenotype. Here, the protein levels of six relevant nuclear envelope proteins were evaluated across mice lifespan in liver and kidney. Therefore, liver and kidney protein extracts from mice with 6, 18 and 24 months (4 animals for each time point) were obtained and further analysed by SDS-PAGE followed by immunoblotting using the specific antibodies against NE proteins. The NE proteins analysed were lamin A/C, lamin B1, emerin, LAP1, SUN1, and nesprin-2. The results are presented in Figure III.3A-E and Figure III.4A-F,referent to mice liver and mice kidney, respectively.

Regarding liver tissue, the first proteins analysed were the nuclear lamina proteins, particularly lamin A/C and lamin B1. The lamins protein levels are presented in figure III.3A and III.3B and we observe a significant increase in lamin A/C protein levels at 18 months old (p-value 0,001; 3.98 fold change) and a tendency to increase at 24 months old (2.35 fold change) in comparison with the 6 months old mice. Moreover, lamin B1 protein levels (Figure III.3B) also increases significantly at 18 months old (p-value 0.0164; 2.41 fold change) and also presents a tendency to increase at 24 months old (1.15 fold change) when compared with the 6 months old. The inner nuclear membrane proteins (INM) analysed were emerin and LAP1. The emerin protein levels are presented

in Figure III.3C, and revealed a significant increase comparing the 6 to 18 months old (p-value=0,0175; 4.72 fold change). The same pattern was observed when comparing the 6 months samples to the 24 months old ones (2.24 fold change). The levels of LAP1 total protein (Figure III.3D) increase significantly between 6 and 18 months old (p-value=0.0287; 2.70 fold change), and also between 6 and 24 months old animals (p-value= 0.0059; 2.91 fold change). Finally, the nesprin-2 protein levels are present in Figure III.3E, seems to increase with age, presenting a tendency to increase at 18 months (6.49 fold change) and being significantly increased in 24 months mice (p-value= 0,0145; 18.59 fold change) when compared with 6 months old mice.

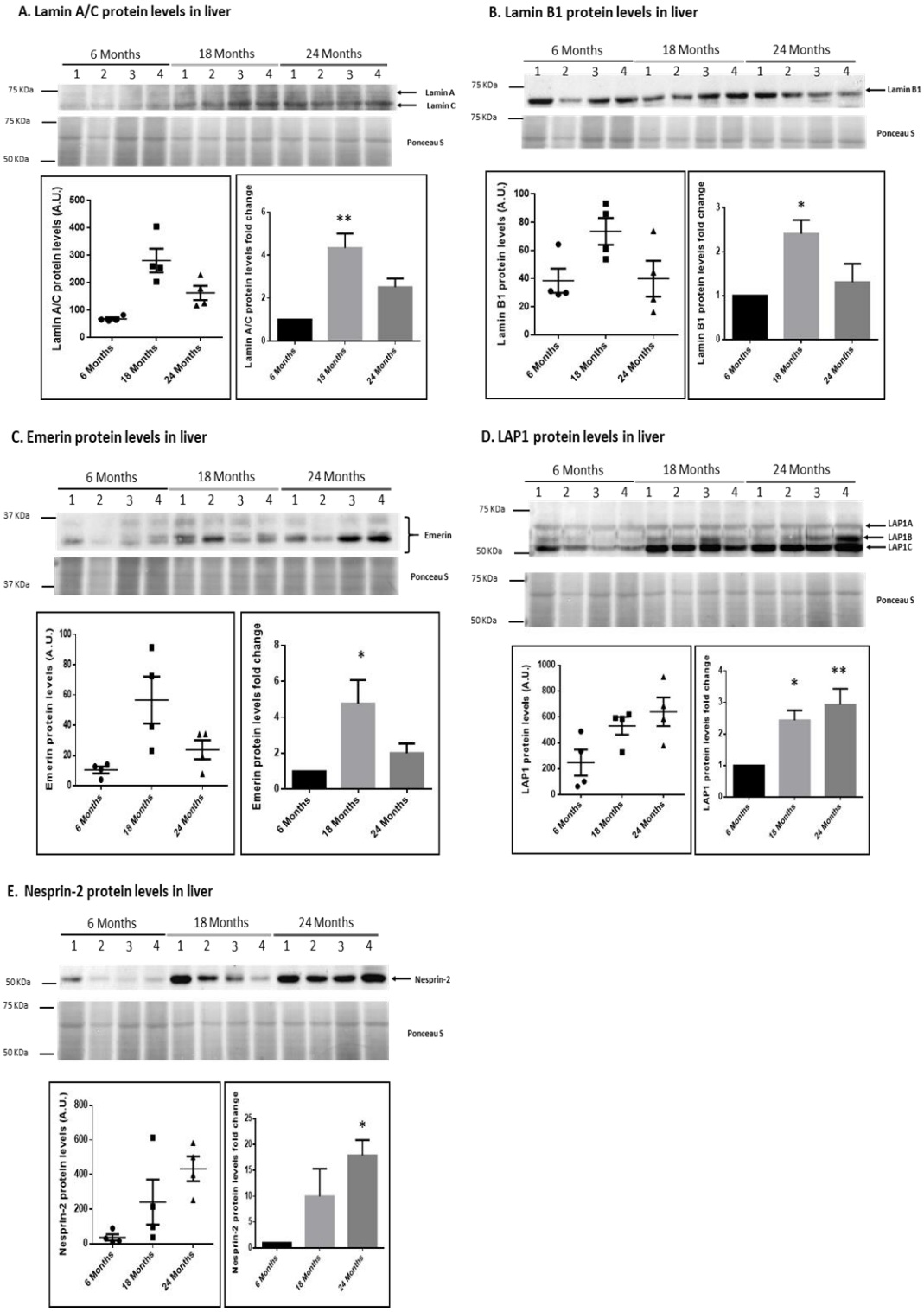

Figure III.3- Protein levels of specific nuclear envelope proteins during ageing in mice liver. Analysis of nuclear lamina protein levels [lamin A/C (A) and lamin B1 (B)], the INM protein levels [emerin (C), and LAP1 (D)] and the ONM protein levels [nesprin-2 (E)] by immunoblotting. Three different ages were analysed (6, 18 and 24 months old). Ponceau S staining was used as a loading control. The quantitative data are presented as mean ± SEM of the 4 independent animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Statistical symbols: (*) for comparisons to control (6 months) by using one-way ANOVA followed by the Dunnett's test. (A.U.=Arbitrary Units).

Concerning nuclear lamina protein levels in the kidney tissue, the results are presented in Figure III.4A. We observe a significant increase in lamin A/C protein levels in 18 months old (p-value =0.0187; 9.23 fold change) and a tendency to increase at 24 months old (7.63 fold change) in comparison with the 6 months old mice. Moreover, lamin B1 protein levels (Figure III.4B) also presents a significant increase at 18 (p-value 0.0003; 2.51 fold change). At 24 months old, a tendency to decrease is noticed (0.80 fold change) when compared with the 6 months old samples.

Concerning the INM proteins, the results of emerin protein levels are shown in Figure III.4C, and revealed a significant increase comparing the 6 to 18 months old mice (p-value=0.011; 63.41 fold change). The same significant pattern was observed when comparing the 6 month samples to the 24 months old (p-value 0.0056; 63.42 fold change). The LAP1 total protein levels (Figure III.4D) also increase significantly between 6 and 18 months old (p-value 0.0057; 5.36 fold change), and also presents a significant increase between 6 and 24 months old animals (p-value 0.0456; 3.26 fold change) although not so pronounced.

Regarding the LINC complex proteins, the SUN1 protein levels (Figure III.4E) present a tendency to increase between 6 and 18 months old (1.23 fold increase) and also when comparing the 6 months samples to the 24 months old ones (1.18 fold change). As indicated in Figure III.4F, nesprin-2 protein levels increase significantly between 6 and 18 months (p-value=0.0059; 37.44 fold change), and also when comparing 6 to 24 months mice (p-value= 0.003; 35.99 fold change).

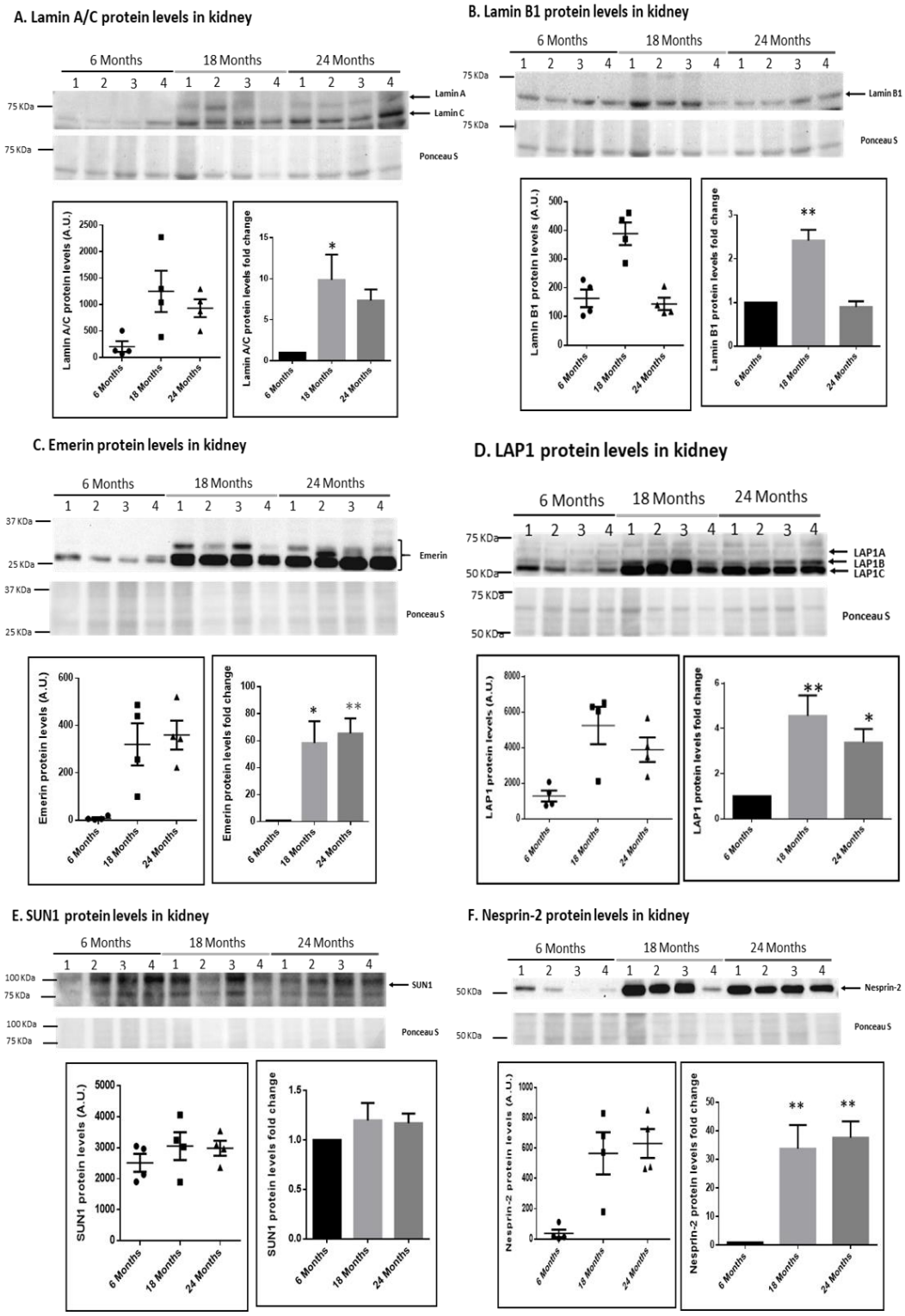

Figure III.4 - Protein levels of specific nuclear envelope proteins during ageing in mice kidney. Analysis of nuclear lamina protein levels [lamin A/C (A) and lamin B1 (B)], the INM protein levels [emerin (C), LAP1 (D), and SUN1 (E)] and the ONM protein levels [(nesprin-2 (F) by immunoblotting. Three different ages were analysed (6, 18 and 24 months old). Ponceau S staining was used as a loading control. The quantitative data are presented as mean ± SEM of the 4 independent animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Statistical symbols: (*) for comparisons to control (6 months) by using one-way ANOVA followed by the Dunnett's test. (A.U.=Arbitrary Units).

3.4. Discussion

Ageing is a highly complex process and can be described as a continued loss of physiological integrity that leads to altered function and a higher susceptibility to death. Many important diseases arise due to this impairment and deterioration of organism physiology, like cancer, diabetes, cardiovascular and neurodegenerative disorders (López-Otín et al., 2013). Nevertheless, there are some aspects that cannot be recapitulated in mice (Blackburn *et al.*, 2015). It is important to keep in mind that a crossover between genetics and environmental factors has a tremendous impact on the degree and rate of ageing in same species-individuals and in the variances among species (Rando, 2013).

Interestingly, defects or alterations in the nuclear proteins have been associated to various premature ageing disorders. It leads to the main idea that the nuclear lamina together with associated INM can effectively be central regulators of the ageing phenotype (Serebryannyy and Misteli, 2018). Mutations in *LMNA* outcome in disorders linked with skeletal balance, muscle, heart, vascular tissue, and premature ageing (Barascu et al., 2012b).

The results presented in this chapter indicated that lamin A/C, lamin B1, emerin, LAP1, and nesprin-2 protein levels increase in both tissues during ageing. Emerin levels increase in both tissues, but it increases more significantly in the kidney tissue. The same happens with nesprin-2. SUN1 seems to increase with age in kidney, but intriguingly it is not detected in liver. Both of these results might be attributed to a tissue-specific expression. Of note, the results presented are referent to four different mice that have individual-specific differences between them.

LAP1 protein is still poorly studied and there are no reports in the literature about its possible effect in the ageing process. Nevertheless, LAP1 was one of first proteins to be associated with lamins (Foisner and Gerace, 1993) and this association between LAP1 and lamins seems to be crucial for the preservation of the nuclear envelope architecture and to the lamins attachment to the nuclear envelope during the interphase (Foisner and Gerace, 1993; Rebelo *et al.*, 2015; Santos *et al*., 2015). Since LAP1 is closely associated with lamins, it is not surprising that after so many reports of lamin A/C being involved in the ageing process, that LAP1 is also important/involved in this process.

Previous results from Petrini *et al*. (2017) have shown that in young human iPSCs (early passages) nesprin-2 is faintly detected. However, after passaging iPSCs for about

one year (aged iPSCs) the nesprin-2 expression is uniformly increased through the nucleus which are in line with our results. Nesprins have been associated with many diseases like myopathies, neurological disorders, cancer, hearing loss and arthrogryposis. Nesprin-1 and -2 are linked with EDMD and EDMD-like phenotypes since it binds to emerin which is crucial for its proper localization. Lamin A/C mutations affect the LINC complex, consequently altering nesprin-2 localization (Cartwright and Karakesisoglou, 2014; Petrini *et al*., 2017).

Several studies indicated that an accumulation of prelamin A or progerin has been associated with the normal ageing phenotype (Scaffidi and Misteli, 2006; Ragnauth *et al.*, 2010; Sieprath *et al*., 2015). The expression of altered isoforms of lamin A has been widely mentioned in the literature in human physiological ageing models (Cao et al., 2011; Lattanzi et al., 2014; McClintock et al., 2007; Olive et al., 2010; Ragnauth et al., 2010; Rodriguez et al., 2009; Scaffidi and Misteli, 2006). Augmented levels of wild-type lamin A have been described to be associated with a shorter lifespan and nuclear aberrancies that result in apoptosis and senescence in human dermal fibroblasts, common features of HGPS phenotype. Over-expressing ZMPSTE24, a metalloproteinase in charge of removing the farnesylated carboxyl terminal region of lamin A, rescues the normal phenotype (Candelario *et al*., 2008).

The vast majority of the literature correlates an accentuated loss of lamin B with age (Freund *et al.*, 2012; Chen *et al.*, 2015; Lukášová *et al.*, 2017). However, this is not consensual. Interestingly, some studies indicate that overexpression of lamin B1 in wild-type human fibroblasts is enough to induce senescence without the induction of DNA damage. Different studies showed that if lamin B1 level is altered, a nuclear shape alteration is observed (Barascu et al., 2012b). Some studies in drosophila point to the evidence that an overexpression of lamin B1 leads to nuclear shape alterations (Padiath *et al*., 2007; Taimen *et al.*, 2009; Barascu *et al.*, 2012). These nuclear shape alterations possibly result in a change in lamina organization, driving to senescence (Barascu et al., 2012b). Cellular senescence is a hallmark of ageing (López-Otín *et al*., 2013) and can be defined as the moment the cells stop cell division and growth permanently suffering chromatin and secretome alterations (Kuilman *et al.*, 2010). Other hallmarks that involve DNA damage, telomere shortening, and oxidative stress contribute to this senescence process (Collado *et al.*, 2007; Van Deursen, 2014). Furthermore, when mutations in lamin A and nesprins occur, emerin can be mislocated. In human aged-iPSCs, emerin was found

overexpressed although it was mislocated (Zhang *et al*., 2007; Ho *et al*., 2013; Petrini *et al.*, 2017). It seems clear that emerin might play a role in the ageing phenotype.

SUN1 is part of the LINC complex. Lamin A/C interacts with the LINC complex proteins mediating the interaction between the actin cytoskeleton and the nucleus that results in movement in the cell (Meinke *et al*., 2011). SUN1 was found over-accumulated in human progeric laminophaties. Deleting SUN1 in *Lmna*-deficient mice proved to ameliorate cellular and organs disorders of premature ageing in mice and knocking-out SUN1 corrected the nuclear aberrancies and the senescence tendencies of HGPS skin fibroblasts. Ageing is a result of inappropriate location of accumulated proteins. Correcting the over-accumulated proteins like SUN1, should be considered when working on possible treatments (Chen *et al.*, 2012; Chi *et al*., 2012).

Over the years, some studies referenced the possibility of INM proteins accumulate with age as in the HGPS. NPC is clustered, emerin is mislocated and overexpressed, LAP2 is diminished and SUN1 and nesprin-2 are increased. What leads some of these proteins to be mislocated and overaccumulated is still not understood, but some speculate that INM proteins might occupy non-random territories in the INM and perhaps migrate to certain locations in the INM to play different functions. Lamin A might be controlling their movement (Chi *et al*., 2012).

The results presented and discussed in this chapter, places the nuclear envelope proteins in the spotlight in the ageing process. Most of them seem to accumulate with age. Most of what we know so far about the possible involvement of the nuclear envelope proteins in the ageing process comes from evidences observed in premature ageing disorders like progerias and other ageing models different from mice. The results presented here are particularly important because it evaluates the nuclear envelope proteins alterations in physiological ageing. In order to further confirm these results, more animals should be used, and more tissues should be analysed. Moreover, it would be worthy to investigate whether this also happens in humans and it would be interesting to look at the protein localization since various studies mention protein mislocalization. Therefore, in the next chapter of this thesis, we will address some of these issues.

Supplementary Data

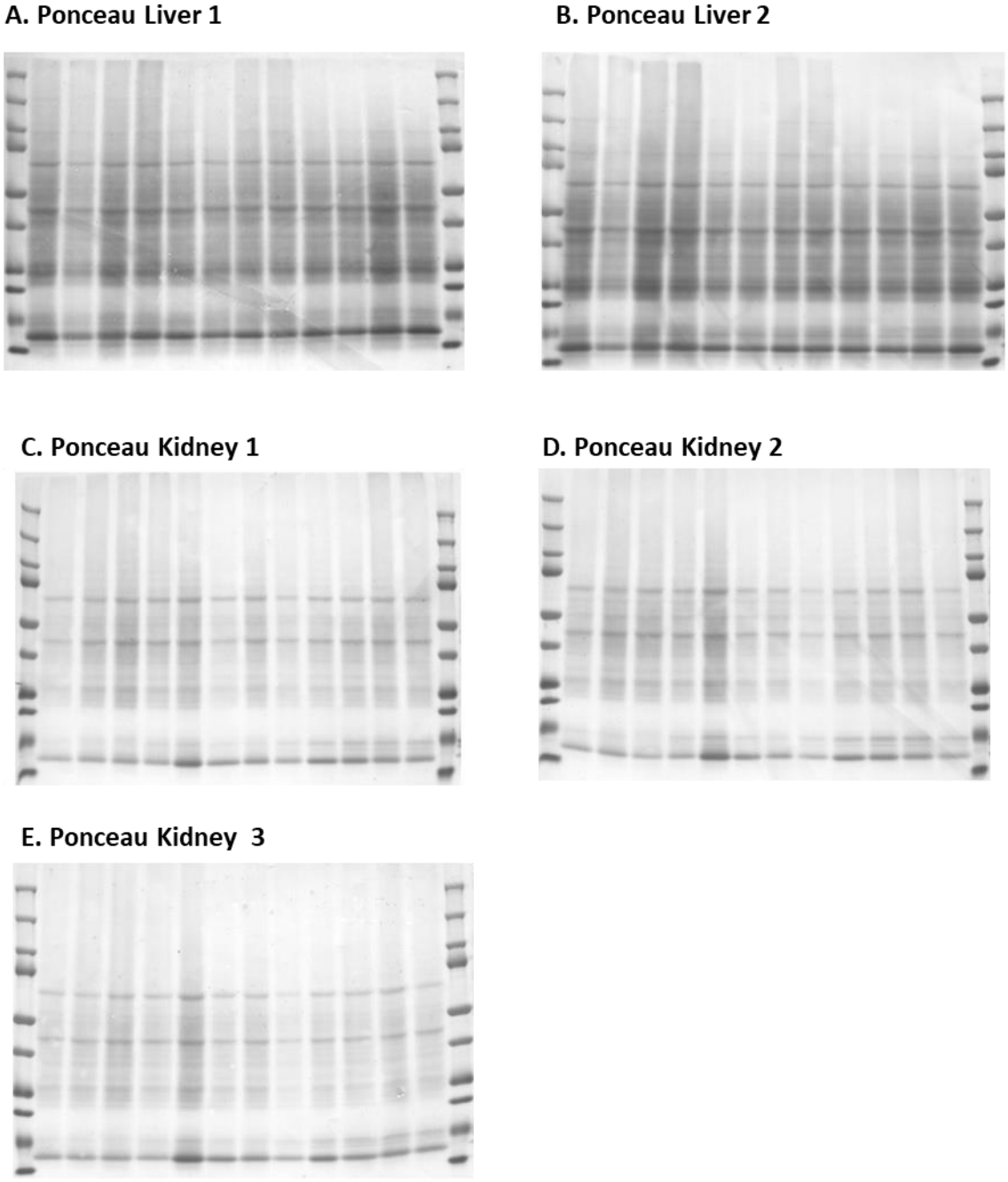

Figure SIII.1 – Ponceau S scans used to normalize the mice samples. Regarding the liver tissue, LAP1 and nesprin-2 were immunoblotted in membrane liver 1 (A) and emerin, lamin A/C and lamin B1 were immunoblotted in membrane liver 2 (B). Concerning the kidney tissue, emerin and SUN1 were immunoblotted in membrane kidney 1 (C), LAP1 and nesprin-2 were immunoblotted in membrane kidney 2 (D), and lamin A/C and lamin B1 were immunoblotted in membrane kidney 3 (E).

CHAPTER IV

Characterization of the nuclear envelope protein alterations during ageing in human models

4.1.Introduction

Physiological ageing is a multifaceted process that is impacted by genetic and environmental factors that lead to a decline in organismal fitness that can lead to numerous diseases, such as dementia, cancer, among others. Therefore, it urges to understand the underlying mechanisms of the ageing process (Ludovico *et al.*, 2012).

Human fibroblasts have been widely used as an excellent ageing model. They are long-lived cells that are impacted by various exogenous influences that can promote extrinsic ageing. It is believed that fibroblasts can provide an overview of the ageing process going on the organism. Fibroblasts aged *in vivo* and *in vitro* recapitulate most of the hallmarks of ageing postulated by López-Ótin *et al*. (2013). Aged fibroblasts *in vitro* present the following hallmarks of ageing: genomic instability, telomere attrition, epigenetic alterations, mitochondrial dysfunction, cellular senescence, altered intercellular communication, and loss of proteostasis (figure IV.1). The other hallmarks have either not been tested yet or are not observable. Not all of these hallmarks mentioned to be observed in fibroblasts aged *in vitro* are observable in fibroblasts normally aged *in vivo* (Tigges *et al*., 2014).

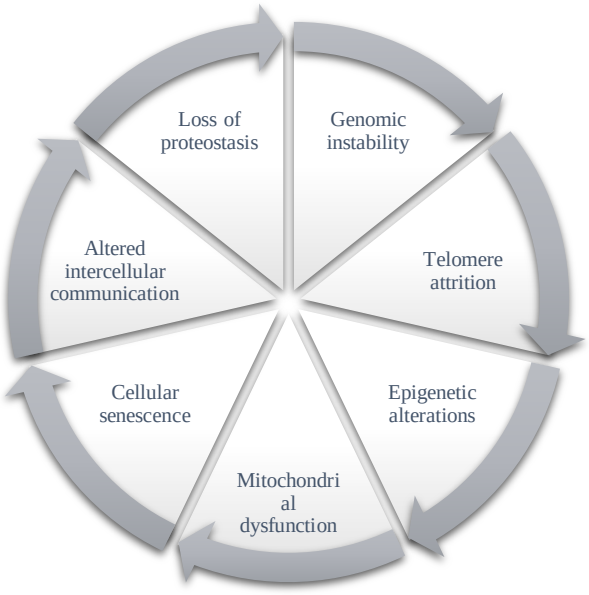

Figure IV.1 – Schematic representation of the ageing, confirmed *in vitro* by using aged fibroblasts as model.

4.2.Materials and Methods

4.2.1. Samples

Fibroblasts from biopsies from donors with a range of ages: 24 years old (AG09319, Coriell Institute), 25 years old AG09429, Coriell Institute), 46 years old (AG02258, Coriell Institute), 69 years old (3027122310VA, Axol Bioscience) and 80 years old (3027033110VA, Axol Bioscience) were obtained. All the donors were female, Caucasian, and apparently healthy (Figure IV.2).

Figure IV.2 – Categorization of the fibroblasts samples across lifespan. Biopsies were performed on healthy Caucasian women with ages between 24 yo (years old) to 80 yo. Image adapted from (http://www.longlonglife.org/en/transhumanism-longevity/aging/biological-causes-aging-lifespan-limitation/).

4.2.2. Cell Culture

Fibroblasts were cultured in DMEM (Dulbecco's Modified Eagle Medium, high glucose) (Gibco™) supplemented with 15% FBS (Fetal Bovine Serum, Origin South America) (Gibco™). All the washes performed were made using Dulbecco's 1X PBS (Phosphate bufferd Saline), without $Ca^{2+}$and Mg^{2+} (BioConcept Ltd. Amimed®). Fibroblasts were subcultured whenever 85-90% confluence was reached. It was incubated 0.05% Trypsin-EDTA (Gibco™) for 5 min to separate the cells from the cell flask whenever subculture was needed. Cell cultures were maintained at 37ºC under 5% CO_2.

Fibroblasts were maintained until passage number 10 (10-13) was reached. At passage 10, cells were harvested for western blotting and flow cytometry and prepared for immunocytochemistry analysis (see below).

4.2.3. Flow Cytometry

In order to evaluate the phenotype and robustness of our human ageing model, parameters like apoptosis and necrosis were assessed using flow cytometry.

4.2.3.1.Annexin V/Propidium Iodide Staining

The two main mechanisms of cell death are apoptosis and necrosis. Apoptosis happens almost naturally in order to eliminate aberrant cells and keep the homeostasis. Necrosis results from environmental perturbations with uncontrolled release of inflammatory cellular components (Fink and Cookson, 2005). The flipping of phosphatidylserine of the plasma membrane from the inside surface to the outside surface is characteristic of an early apoptosis. Annexin V bind specifically in presence of Ca^{2+} ions to negatively charged phospholipids like phosphotidylserine (Reutelingsperger and van Heerde, 1997). APC conjugated annexin V can be used as a fluorescence probe to label apoptotic cells. Propidium iodide (PI) can also be used in conjunction with annexin-V-APC detection to detect necrotic cells. Viable cells (with proper membrane integrity) are not permeable to PI. Overall, this flow cytometric method allows us to distinguish between viable, apoptotic and necrotic cells.

Briefly, fibroblasts of all ages were trypsinized and centrifuged at 1000rpm for 3 min. The supernatant was removed, and $0.5x10^6$cells were resuspended in 500µl of binding buffer (1x PBS with Ca^{2+}). Another centrifugation at 1000rpm for 3min was performed. The supernatant was discarded, and the cells were resuspended in 70µl of binding buffer. It was added 4µl of annexin V-APC (31490016, ImmunoTools®) to the cell suspension and incubated for 15 min at RT in the dark. The cell suspension was immediately analysed in BD Accuri™ C6 (BD Biosciences®). Further, it was added 10µl of PI (10µg/ml) to the cell suspension and the analysis was performed again. Proper negative and positive controls were also analysed. The negative control corresponds to cells without annexin V-APC or PI. The positive control was performed with cells that were incubated for 20 min at 50ºC and properly stained with annexin V-APC and PI. The data were analysed using BD Accuri™ C6 Software (BD Biosciences®).

4.2.4. Antibodies

The following primary antibodies were used: Trimethyl Histone H3, lamin A/C, lamin B1, emerin, LAP1, SUN1, Nup133, and Nup93 (see table IV.1 for further details).

Table IV.1 – Primary antibodies used in order to detect the multiple proteins. WB, Western Blotting; ICC, immunocytochemistry.

Antibody	Antibody company	Antibody type	Target	Dilution
Trimethyl Histone H3 (6F12-H4) sc-130356	Santa Cruz Biotechnology	Mouse, monoclonal	H3K9me3	WB: 1:500
Lamin A/C Antibody (E-1) sc-376248	Santa Cruz Biotechnology	Mouse, monoclonal	Lamin A/C	WB: 1:500 ICC: 1:250
Lamin B1 Antibody (H-90) sc-20682	Santa Cruz Biotechnology	Rabbit, polyclonal	Lamin B1	ICC: 1:50
Emerin Antibody (H-12) sc-25284	Santa Cruz Biotechnology	Mouse, monoclonal	Emerin	WB 1:3000 ICC: 1:750
LAP1 Antibody	Kindly provided by Dr. Dauer	Rabbit, polyclonal	LAP1	WB: 1:20000 ICC: 1:4000
SUN1 antibody	Kindly provided by Ya-Hui Chi	Rabbit, polyclonal	SUN1	WB: 1:2000 ICC: 1:500
Nup133 Antibody (E-6) sc-376763	Santa Cruz Biotechnology	Mouse, monoclonal	NUP133	WB 1:500
Nup93 Antibody (F-2) sc-374400	Santa Cruz Biotechnology	Mouse, monoclonal	NUP93	WB 1:500

The secondary antibodies used in Western Blotting (WB) were HRP-conjugated (GE Healthcare). The secondary antibodies used in immunocytochemistry were Invitrogen Alexa Fluor 488 dye from Thermo Fisher Scientific (mouse or rabbit, accordingly).

4.2.5. Western Blotting

4.2.5.1.Protein Extraction

Fibroblasts at PN10-PN13 were lysed using 1% SDS (300µl per confluent T75 cell flask), and the cell lysates boiled at 95ºC for 10 min and then sonicated for 15 seconds.

4.2.5.2.Preparation of Soluble and Insoluble Protein Fractions from Fibroblasts

Cells were collected in 300µl of extraction buffer (50mM Tris-HCl pH 7.5, 1%Triton X-100 and Protease Inhibitors – Roche protease inhibitor cocktail and PMSF). The cells were incubated in the buffer for 20 min on ice. Then, protein was quantified as described in 3.2.3. section. Homogenates with 150µg and a final volume of 100µL were prepared and centrifuged at 20000xg for 15 min at 4ºC. The supernatant was collected to another microtube and corresponds to the soluble fraction. To the supernatants was added 25µl of 4x Loading Buffer (LB) and then were boiled at 95ºC for 10min. The pellet is considered the insoluble fraction and was solubilized in 125µl 1x LB and boiled at 95ºC for 10 min. Total extracts with 75µg were also prepared.

4.2.5.3. Protein quantification

The *Thermo Scientific™ Pierce™ BCA Protein Assay Kit* was used to determine the protein concentration of the samples, following the same protocol mentioned in the previous chapter (see 3.2.3 section).

4.2.5.4.Sample preparation by SDS-PAGE

Prior to loading, samples with 75µg of total protein were prepared by the addition of ¼ volume of 4x LB, boiled at 95ºC for 10 min and spinned down.

4.2.5.5.Gradient SDS Polyacrylamide Gel Electrophoresis

Gradient SDS Polyacrylamide Gel Electrophoresis was performed as described in the previous chapter (see 3.2.4. section).

4.2.5.6.Immunoblotting

The transfer to membranes were performed as described in the materials and methods of 3.2.5 section. The overall protocol is very similar, apart from the fact that blocking time was 2-4h using 5% BSA in 1x TBS-T. The primary antibodies were incubated 2h at RT plus overnight at 4ºC (both with gentle agitation). The primary

antibodies used are the ones described in 4.2.4 section (also see table IV.1 for further details).

4.2.6. Immunocytochemistry

Fibroblasts of the donors at passage 10 (PN10-PN12) were grown in 6-well plates (Corning) with coverslips. Then, cells were washed once with 1x PBS and 4% paraformaldehyde (PFA) was added for 20 min in a ventilated hood to each well of the plate (cell fixation). Cells were washed three times with 1x PBS. Then the cells were permeabilized using 0.2% Triton-X in 1x PBS for 10 min. Cells were then washed with 1x PBS. After that, cells were incubated with a blocking solution (3% BSA in 1x PBS) for 1h to avoid unspecific binding, and the primary antibodies were incubated for 2h at RT. Further, cells were washed with 1x PBS and the adequate fluorescence secondary antibodies were incubated for 1h in the dark. A final wash was performed with 1x PBS. Coverslips were mounted on a microscope slide with VECTASHIELD® Mounting Media with 4',6-diamidino-2-phenolyde (DAPI) (Vector Laboratories) and observed in an epifluorescence microscopy Zeiss AxioImager Z1 (Zeiss) motorized microscope equipped with a Plan-ApoCHROMAT 63x/1.40 oil objective lens. Photographs were taken with a digital AxioCam HR3 (Soft Imaging System).

4.3.Results

4.3.1. Characterization of our human ageing model

Three well established ageing markers were used to validate our human cellular model. Essentially the histone 3 K9 trimethylation (H3K9me3) proteins levels were determined, as well as the percentage of apoptosis and necrosis using annexin V and PI staining.

4.3.1.1. Histone H3 K9 trimethylation (H3K9me3) protein levels

Fibroblasts of the donors at passage 10 (PN10-PN12) were subjected to SDS-PAGE and immunoblotting analysis and the H3K9me3 protein levels were evaluated (Figure IV.3). The results revealed a decrease of the H3K9me3 protein levels during ageing. In fact, when comparing the fibroblasts from the aged group (46, 69 and 80 yo)

to the young group (24 and 25 yo) it was possible to observe a decrease (0.66 fold change) in H3K9me3 (Figure IV.3).

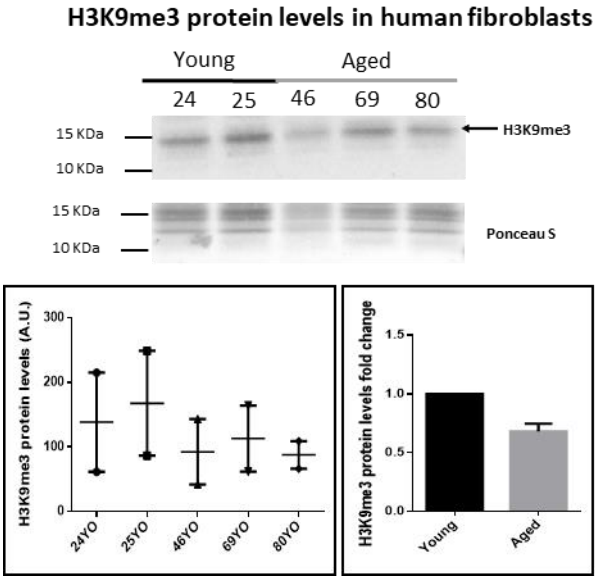

Figure IV.3. – H3K9me3 protein levels evaluated by immunoblotting in human fibroblasts from five different patients analysed separated or grouped into 2 groups: young (24, and 25yo) and aged (46, 69 and 89yo). Ponceau S staining was used to access gel loading. The quantitative data are presented as mean ± SEM of 2 independent experiments (A.U.=Arbitrary Units; YO = Years Old)

4.3.1.2.Annexin V/Propidium Iodide staining using flow cytometry

Fibroblasts of the donors at passage 10 (PN10-PN12) were collected, stained with annexin V-APC and PI, followed by flow cytometry analysis. The results are presented in figure IV.4. The percentage of cells annexin V positive/PI negative was of about 0.1% at 24 yo; 1.4% at 46 yo; 0.8% at 69 yo, and 2.1% at 80 yo. The percentage of cells Annexin V positive/PI positive is 4.2% at 24 yo; 8.2% at 46yo; 4.3% at 69 yo, and 7.0% at 80 yo. Briefly, the results revealed a tendency to increase both Annexin V positive/PI negative and Annexin V positive/PI positive cells during ageing.

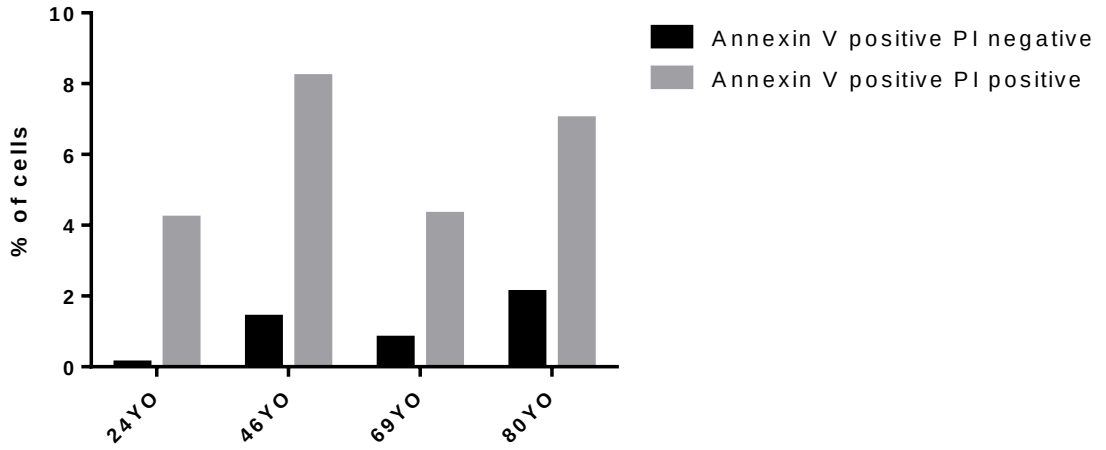

Figure IV.4. – Percentage of fibroblasts annexin V positive/PI negative stained (black bars) and annexin V positive/PI positive stained (grey bars) of four different donors (24, 46, 69 and 80 yo). Proper negative and positive controls were used. No statistical analysis was performed since only one experiment was performed (YO = Years Old; PI – Propidium Iodide).

4.3.2. Evaluation of Nuclear Envelope Proteins during ageing

Quite a few studies have pointed protein sequestration at the nuclear envelope periphery as a potential regulatory mechanism of the ageing phenotype. Here, the protein levels of six relevant nuclear envelope proteins were evaluated across human lifespan using samples of human fibroblasts. Therefore, lysates of human fibroblasts from donors with 24, 25, 46, 69, and 80 years old at passage number 10 were analysed by SDS-PAGE and immunoblotting.

The proteins analysed were lamin A/C, emerin, LAP1, SUN1, NUP133, and NUP93. The results are presented in Figures IV5-7.

Regarding nuclear lamina protein levels, in Figure IV.5A., we observe a small tendency of lamin A/C protein levels to increase in the aged group (1.04 fold change) when comparing to the young one. In Figure IV.5B it is also possible to observe a drastic reduction of the soluble lamin A/C protein levels (0.05 fold change). The insoluble fraction seems to increase with age (1.20 fold change) when comparing the aged to the young group.

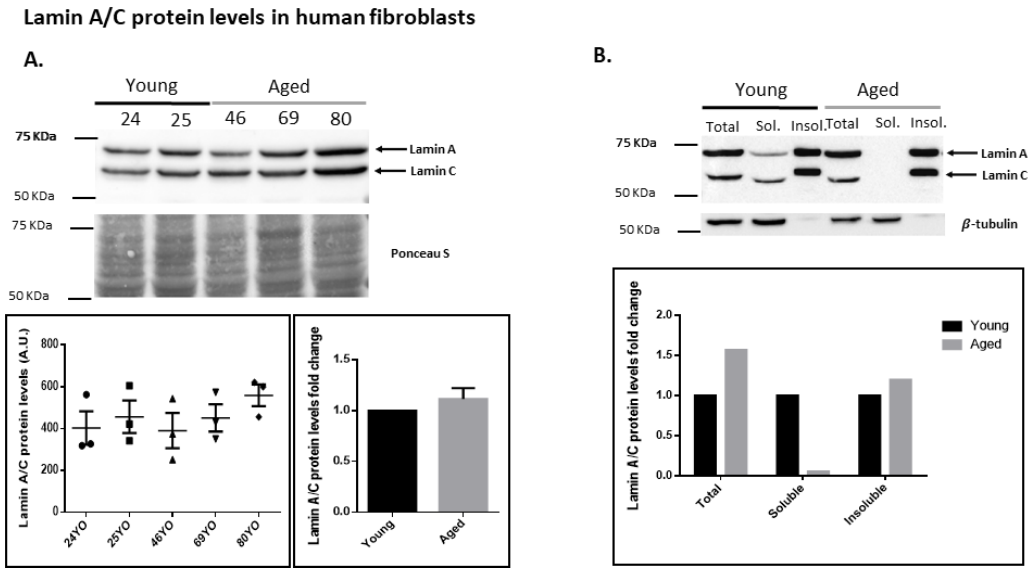

Figure IV.5 – Lamin A/C protein levels during ageing in human fibroblasts. A – Lamin A/C protein levels were evaluated in total extracts from donors with 24, 25, 46, 69, and 80 yo, analysed separated or grouped into two groups: young (24 and 25yo) and aged (46, 69, and 80yo). Ponceau S staining was used as loading control. The quantitative data are presented as mean ± SEM of 3 independent experiments by comparison between the young and aged group by using t-student's test. B - Lamin A/C protein levels were evaluated in detergent soluble and insoluble fractions from a young donor (24 yo) and an aged donor (69 yo). β-tubulin was used to access the total protein loading. No statistical analysis was performed as the data results from only one experiment (A.U.=Arbitrary Units; YO = Years Old).

Regarding the inner nuclear membrane proteins, emerin protein levels do not seem to change significantly with age (0.95 fold change) (Figure IV.6A). On the other hand, as presented in Figure IV.6B, the SUN1 protein levels present a significant increase between the young and aged group (p-value 0.0112; 1.26 fold change).The LAP1 protein levels (Figure IV.6C) do not seem to change significantly between the aged and the young group (0.99 fold change). Nevertheless, LAP1 isoforms levels were analysed separately and in fact it was possible to observe a significant increase in LAP1B protein levels with age (p-value 0.0189; 1.52 fold change).LAP1C seems to present a small decrease (0.89 fold change) when comparing the aged group to the young group (Figure IV.6C).

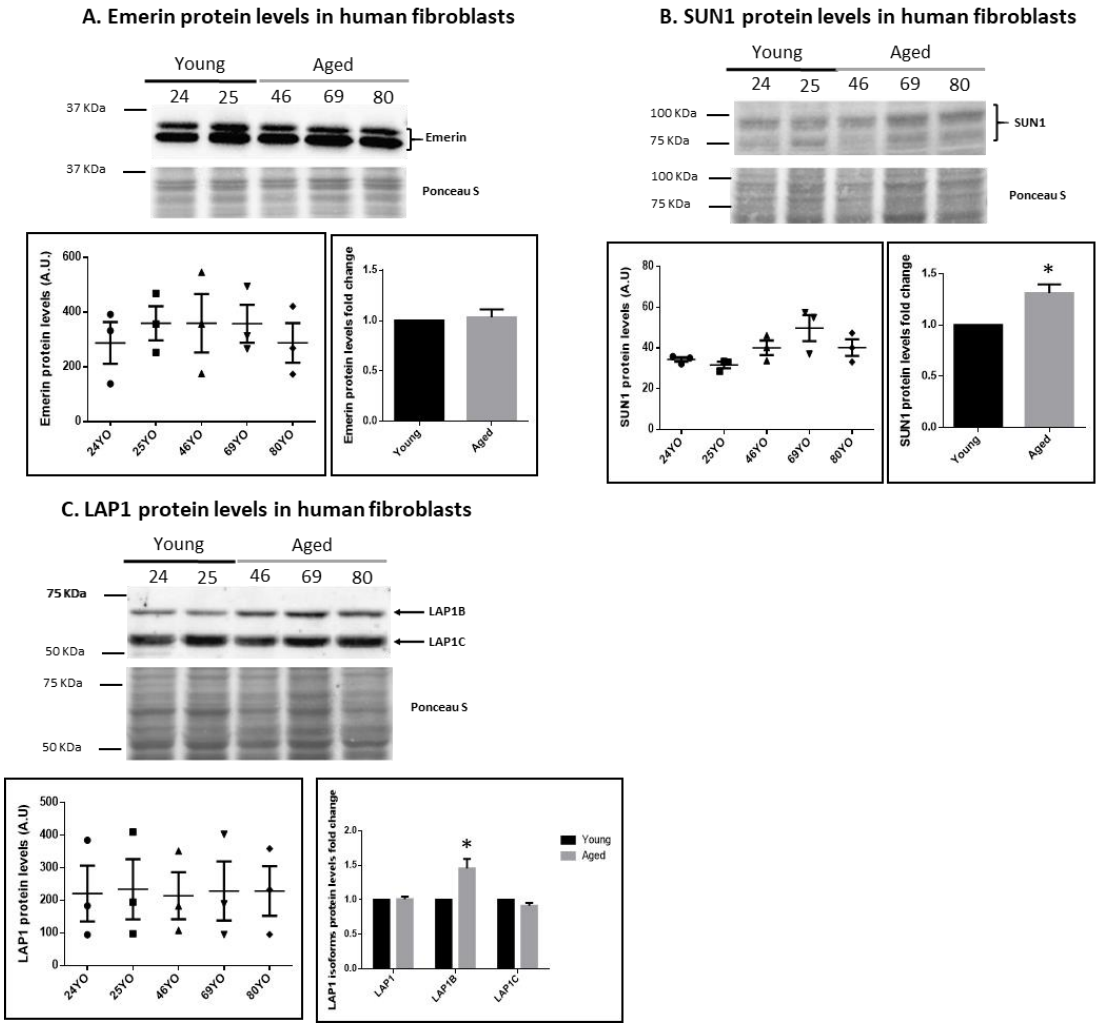

Figure IV.6 – The inner nuclear membrane proteins levels during ageing in human fibroblasts. Analysis of emerin (A), SUN1 (B), and LAP1 protein levels (C) by immunoblotting in human fibroblasts lysates from five different patients were analysed separated and grouped in 2 groups: young (24 and 25 yo) and aged (46, 69 and 80 yo). Ponceau S staining was used to access gel loading. The quantitative data are presented as mean ± SEM of 3 independent experiments by comparison of the young and aged group by using t-student's test. Statistical significance symbol represents * $p < 0.05$. (A.U.=Arbitrary Units; YO = Years Old)

Concerning, the nuclear pore complex proteins, as indicated in Figure IV.7A, the NUP133 protein levels decrease significantly when comparing the young to the aged group ($p<0.0001$; 0.65 fold change). NUP93 protein levels (Figure IV.7B) also decrease significantly when comparing the young to the aged group ($p<0.0001$; 0.63 fold change).

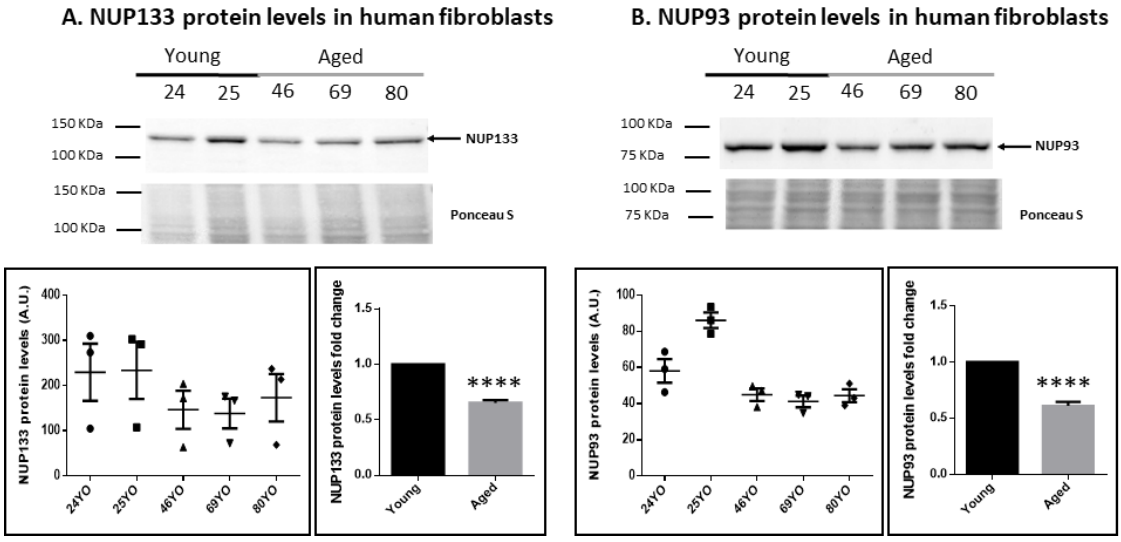

Figure IV.7 – Nuclear Pore Complex Proteins levels during ageing in human fibroblasts. Analysis of NUP133 (A) and NUP93 protein levels (B) by immunoblotting in human fibroblasts lysates from five different patients analysed separated or grouped into 2 groups: young (24 and 25 yo) and aged (46, 69 and 80 yo). Ponceau S staining was used to access gel loading. The quantitative data is presented as mean ± SEM of 3 independent experiments by comparison of the young and aged group by using t-student's test. Statistical significance symbol represent **** $p<0.0001$. (A.U.=Arbitrary Units; YO = Years Old)

4.2.3. Nuclear Envelope Proteins localization during ageing

In order to investigate the localization of the nuclear envelope proteins during ageing, immunocytochemistry was performed. Briefly, human fibroblasts from representative ages (24, 46, 69 and 80 years old) at passage number 10 were labelled with lamin A/C (Figure IV.8), lamin B1 (Figure IV.9), emerin (Figure IV.10), LAP1 (Figure IV.11), and SUN1 (Figure IV.12). Additionally, the lamin A/C protein localization of the 24 yo sample was also analysed at passage number 20 (Figure IV.13).

The overall signal of lamin A/C seems to be increasing with age, whether the nucleoplasmic signal is significantly depleted (reduced) with age (p-value 0.0072 comparing 24 to 46 yo; 0.0058 comparing 24 to 69 yo; and 0.0001 comparing 24 to 80 yo). Lamin A/C dependent-nuclear abnormalities are significantly increased with age (p-value <0,0001 comparing 24 to 46 yo; 0.0018 comparing 24 to 69 yo; and 0.0009 comparing 24 to 80yo) (Fig. IV.8).

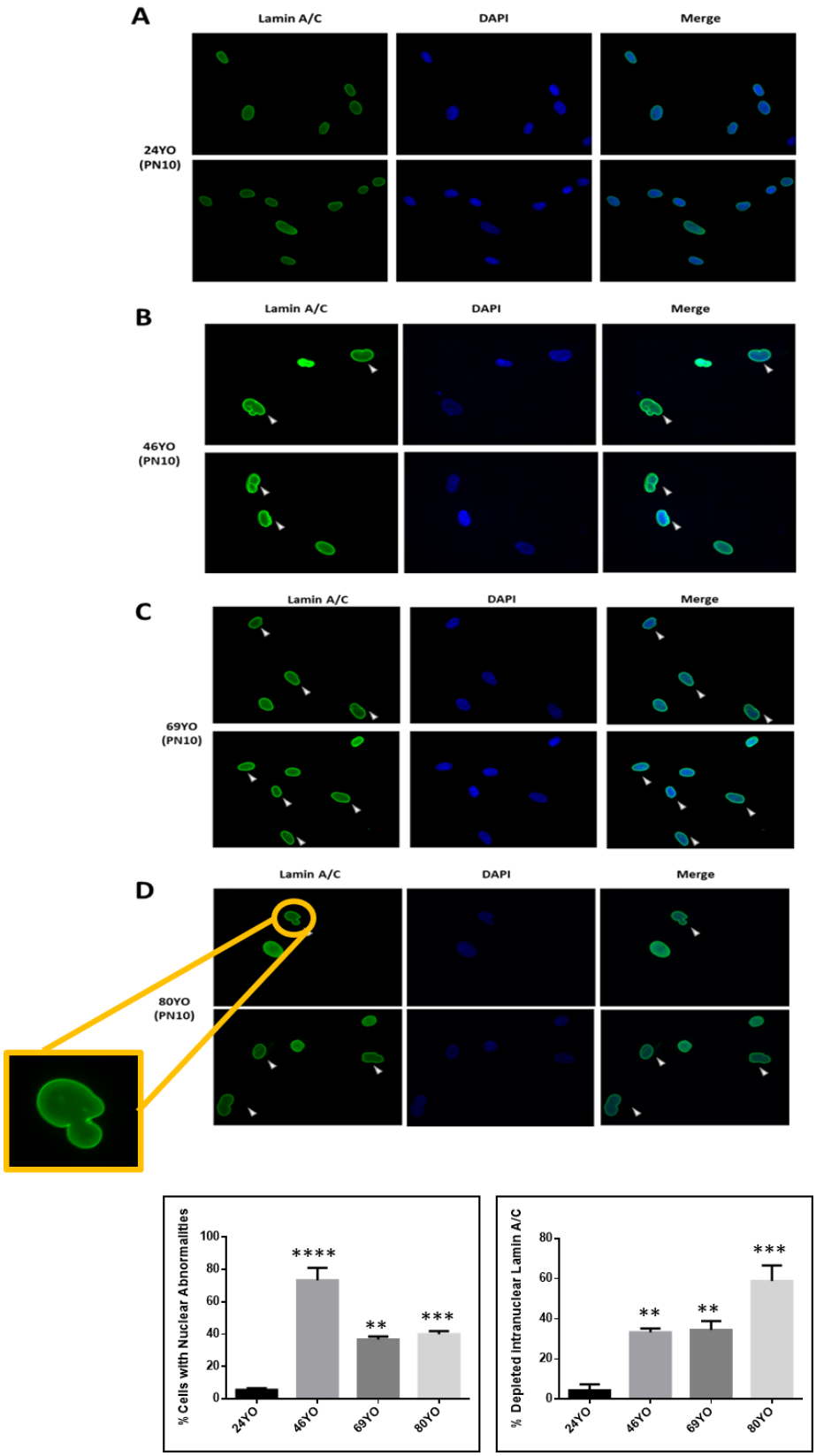

Figure IV.8. – Subcellular distribution of lamin A/C in fibroblasts from donors' samples with 24 (A), 46 (B), 69 (C) and 80 yo (D). Lamin A/C was detected using an anti-mouse Alexa-488 conjugated secondary antibody (green). Nucleic acids were stained using DAPI (blue). Lamin A/C dependent-nuclear abnormalities and depletions of nucleoplasmic lamin A/C are also

observed in subnuclear structures (arrows). The quantitative data are presented as mean ± SEM and was obtained by analysing at least 30 cells per condition from three independent experiments. Statistical significance symbols: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ for comparisons to control (24 yo) by using one-way ANOVA followed by the Dunnett's test. (A.U.=Arbitrary Units).

Remarkably, the depletion of lamin A/C at nucleoplasm is even more pronounced at PN20 fibroblasts when comparing with the observed at PN10 (p-value 0.0006). Moreover, lamin A/C dependent-nuclear abnormalities increases about 50% at PN20 (p-value 0.0002) when comparing with PN10 fibroblasts (Figure IV.9).

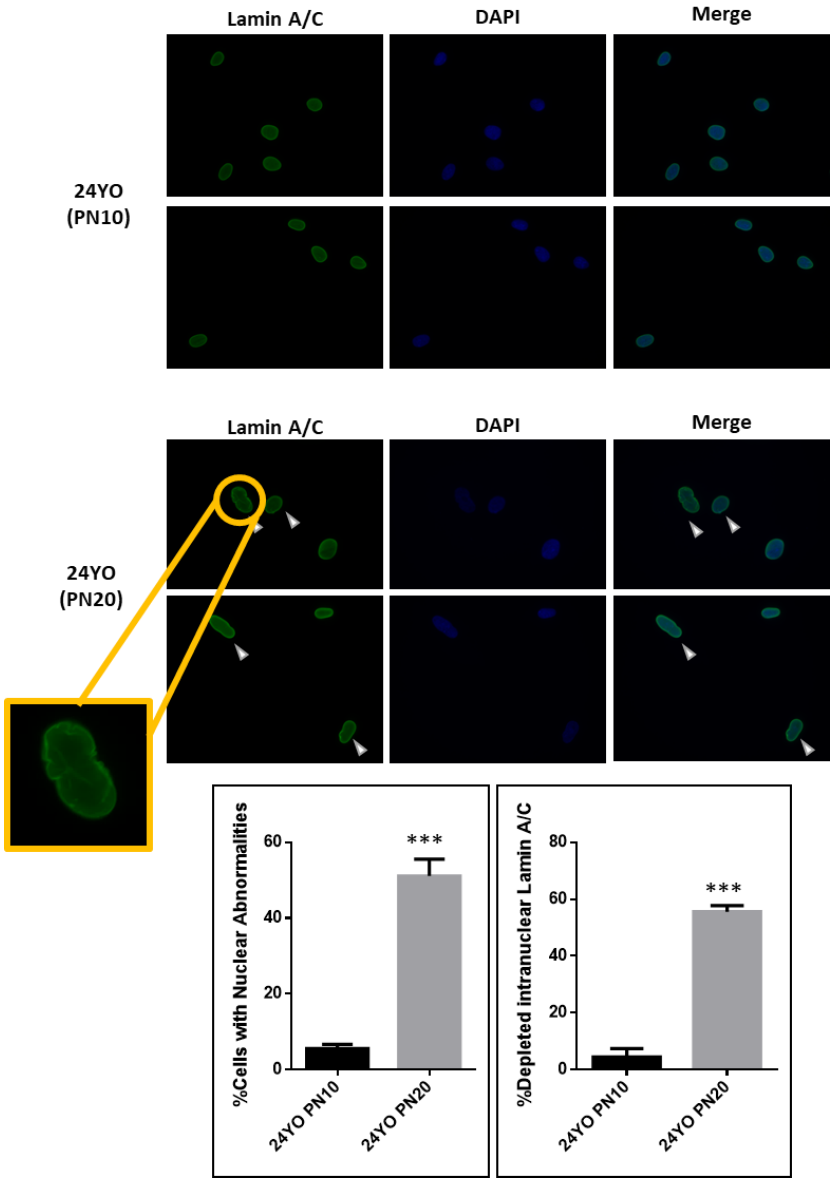

Figure IV.9. – Subcellular distribution of lamin A/C in fibroblasts from donors' samples with 24 yo at PN10 and at PN20. Lamin A/C was detected using an anti-mouse Alexa-488 conjugated secondary antibody (green). Nucleic acids were stained using DAPI (blue). Lamin A/C

abnormalities and depletions of nucleoplasmic lamin A/C are observed in subnuclear structures (arrows). The quantitative data are presented as mean ± SEM and was obtained by analysing at least 30 cells per condition from three independent experiments. Statistical significance symbols: *** $p < 0.001$ for comparisons to control (24YO PN10) by using t-student's test. (A.U.=Arbitrary Units; PN = Passage Number)

Subcellular localization of lamin B1 in human fibroblasts revealed, as expected, a localization at the inner surface of the nuclear envelope. Moreover, lamin B1 staining seems to decrease with age in human fibroblasts (Figure IV.10), although no fluorescence intensity quantification was performed.

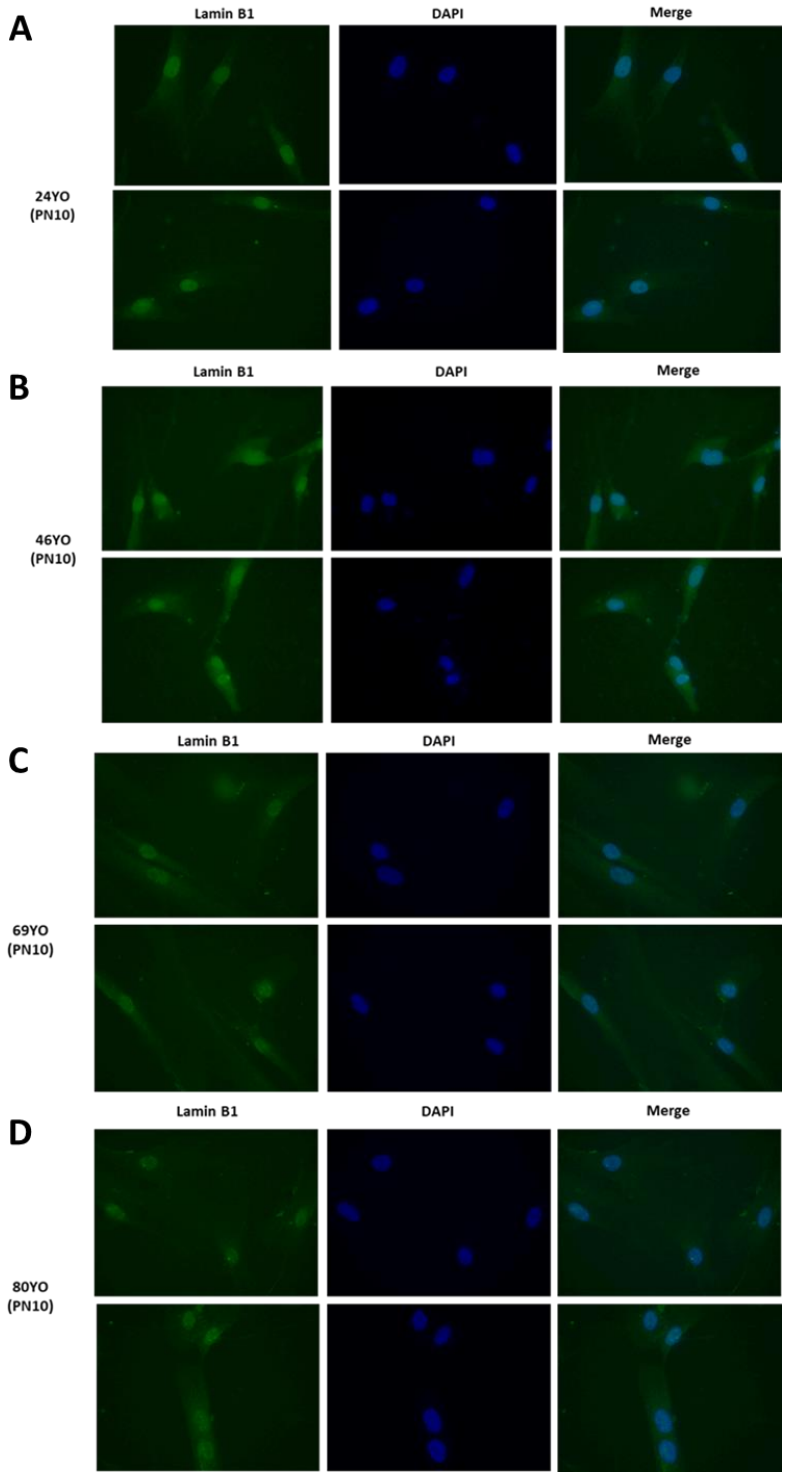

Figure IV.10. – Subcellular distribution of Lamin B1 in fibroblasts from donors' samples with 24 (A), 46 (B), 69 (C) and 80 yo (D). Lamin B1 was detected using an anti-rabbit Alexa-488 conjugated secondary antibody (green). Nucleic acids were stained using DAPI (blue).

Regarding emerin distribution in human fibroblasts, it was detected in the nuclear envelope at all ages analysed (Figure IV.11). In addition, it was possible to observe the

presence of emerin nuclear speckles, which are significantly increased in the aged human fibroblasts (p-value <0.0001; 1.39 fold change) (Fig. IV.10).

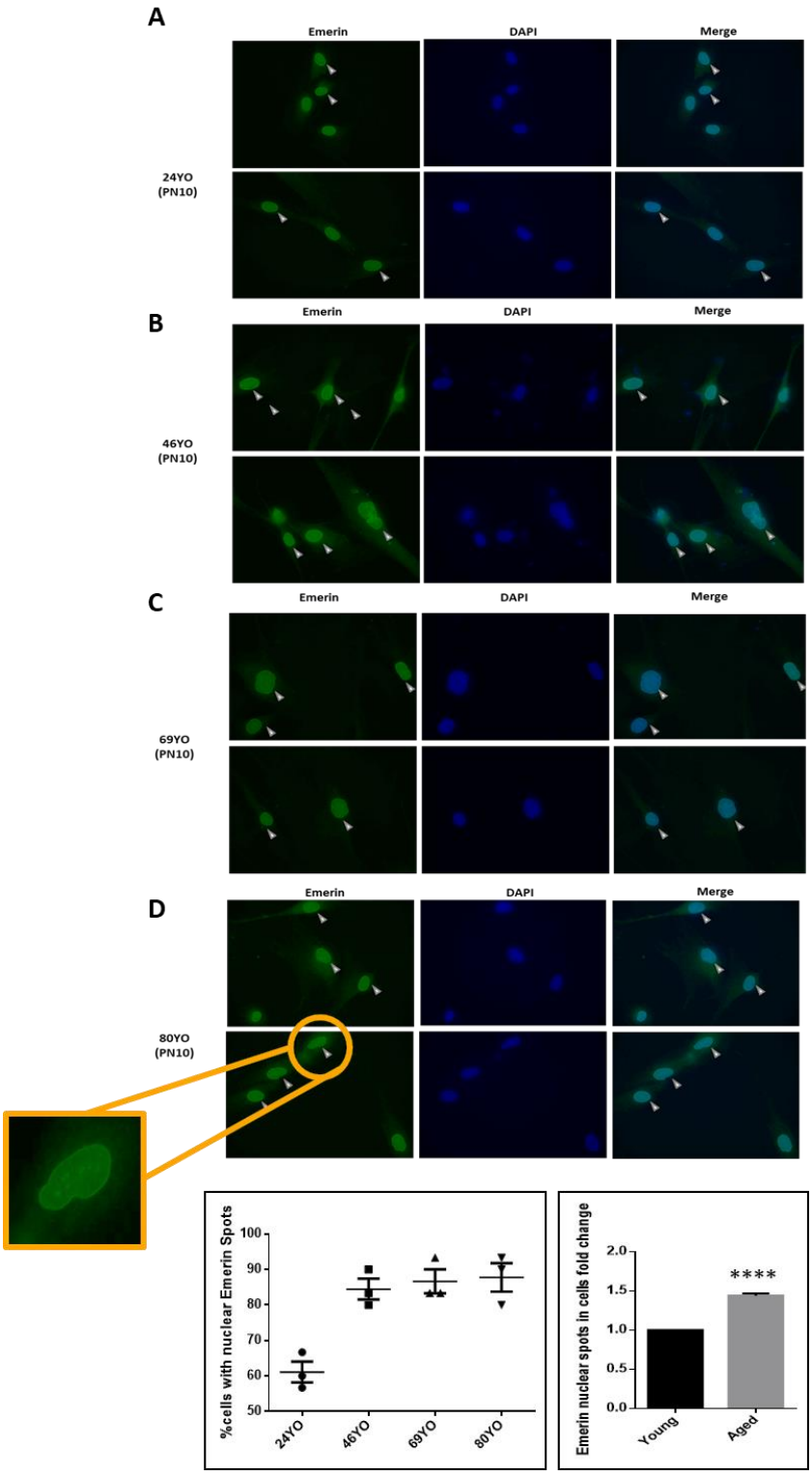

Figure IV.11 – Subcellular distribution of emerin in fibroblasts from donors' samples with 24 (A), 46 (B), 69 (C) and 80 yo (D). Emerin was detected using an anti-mouse Alexa-488 conjugated

secondary antibody (green). Nucleic acids were stained using DAPI (blue). Emerin nuclear speckles are observed (arrows). The quantitative data are presented as mean ± SEM and was obtained by analysing at least 30 cells per condition from three independent experiments. Statistical significance symbols: **** $p < 0.0001$ for comparisons of the young group (24 yo) to the aged group (46, 69 and 80 yo) by using t-student's test (A.U.=Arbitrary Units).

SUN1 also reveals a nuclear envelope staining in human fibroblasts at all ages (Figure IV.12). By performimg a qualitative analysis of the images acquired it was possible to observe an increase in the SUN1 protein staining with age (Figure IV.12) which in fact, are in accordance with the imunnoblotting data.

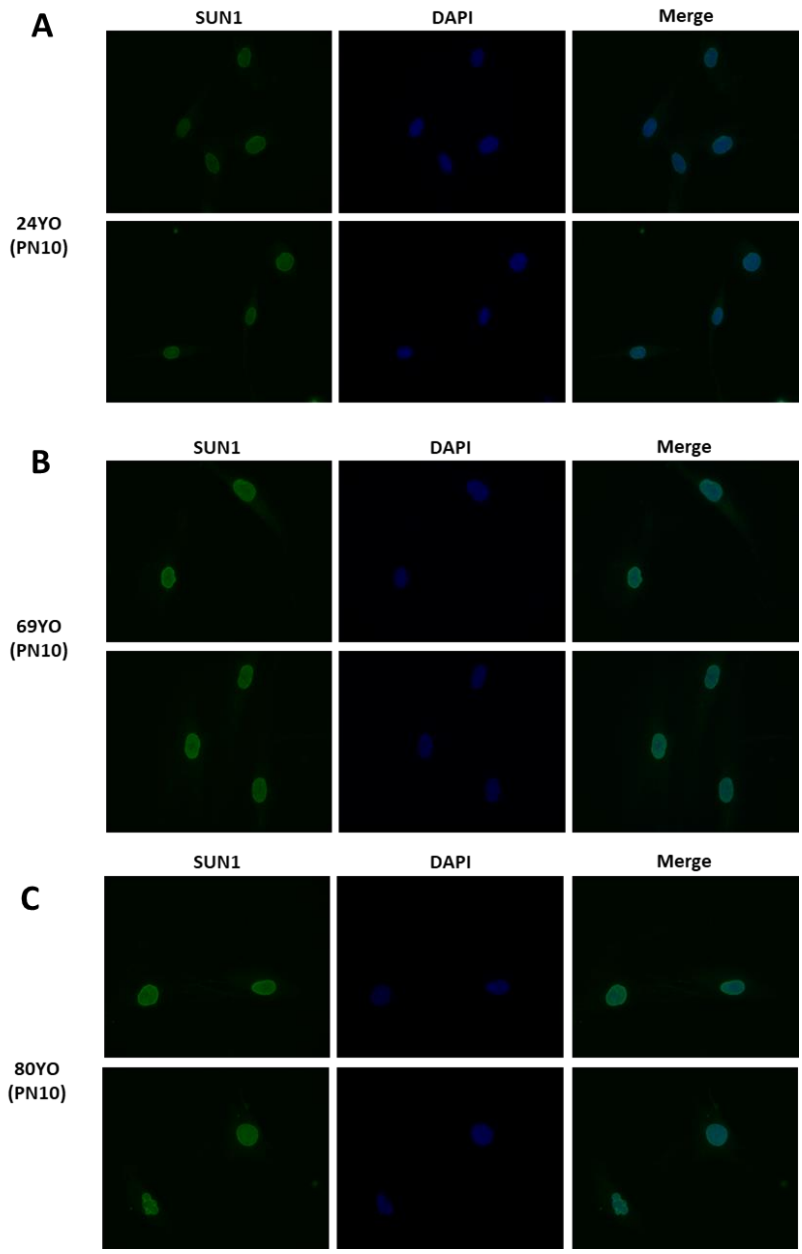

Figure IV.12 – Subcellular distribution of SUN1 in fibroblasts from donors' samples with 24 (A), 69 (B) and 80 yo (C). SUN1 was detected using an anti-rabbit Alexa-488 conjugated secondary antibody (green). Nucleic acids were stained using DAPI (blue).

Lastly, LAP1 subcellular localization was also investigated in human fibroblasts, and a nuclear envelope staining is observed (Figure IV.13). Noticeably, it seems that LAP1 is being recruited to the invaginations and abnormalities that occur during ageing (Figure IV.13 arrows) and further studies should be performed in order to pursue this feature.

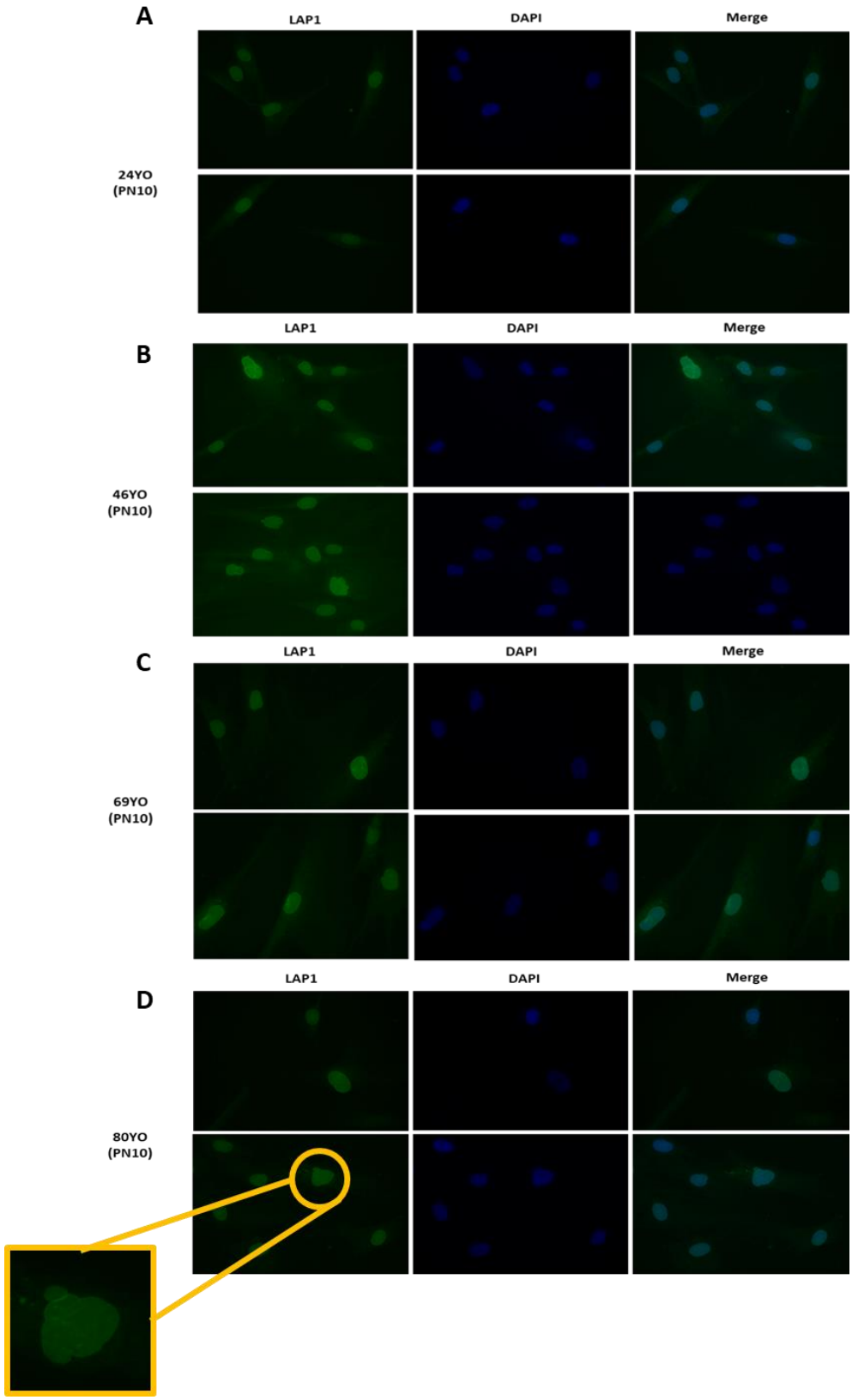

Figure IV.13 – Subcellular distribution of LAP1 in fibroblasts from donors' samples with 24 (A), 46 (B), 69 (C) and 80yo (D). LAP1 was detected using an anti-rabbit Alexa-488 conjugated

secondary antibody (green). Nucleic acids were stained using DAPI (blue). Arrows point to LAP1 staining in nuclear abnormalities.

4.4.Discussion

The underlying mechanisms of ageing have been studied in diverse cellular and animal models. Fibroblasts cultures derived from patients and healthy donors have been employed in studies from diverse research areas. Moreover, fibroblasts have been used to study ageing at a cellular level. Nevertheless, it is important to highlight that any results obtained in any model should be interpreted with caution when trying to translate to human ageing (Schneider, 1979; Ludovico *et al.*, 2012). Selecting samples of a normal human population to investigate ageing is a complex mission. Trying to find aged individuals without serious conditions and, whether we should even choose to do so, can be tricky. It is almost impossible to distinguish between the normal alterations from the pathological ones that come with ageing. Therefore, there is no ideal population to study ageing (Schneider, 1979).

Over the years, studies have pointed to the nuclear periphery as a possible intervenient in the ageing phenotype. In fact, lamins, nesprin-2, emerin and some nucleopore complex proteins have already been described to be altered mainly in premature ageing disorders (Scaffidi and Misteli, 2006, 2008; Barascu *et al.*, 2012; Lattanzi *et al.*, 2014; Lukášová *et al.*, 2017; Petrini *et al.*, 2017; Serebryannyy and Misteli, 2018).

In the work here presented, we started by performing a characterization of our ageing model. Our results evidenced a decrease of the H3K9me3 protein levels (Figure IV.3). In fact, levels of H3K9me3 has been well described as decreased with age in the literature (Villeponteau, 1997; Tsurumi and Li, 2012; Zhang *et al.*, 2015; Maleszewska *et al.*, 2016). H3K9me3 is able to recruit heterochromatin protein-1 (HP1), which is thought to be involved in heterochromatin formation. The model of heterochromatin loss for cell ageing is based on the assumption that over the years the heterochromatin domains are shortened. This shortening affects the gene expression at the periphery of the heterochromatin domains since the transcription factors become more accessible. This could lead to many small but important alterations in gene expression patterns with ageing (Villeponteau, 1997). Moreover, we analysed fibroblasts for the Annexin V/Propidium Iodide staining by flow cytometry. Annexin V is capable of distinguish early apoptotic cells from the viable ones. Propidium iodide only stains necrotic cells. Viable cells should

not be stained by any of these two compounds. Our results suggest that early apoptotic cells and necrotic cells are increasing with age (Figure IV.4). Whether apoptosis is increased with ageing is still debatable in the literature. Nevertheless, studies point to a higher level of apoptosis in diverse aged cell types and organs. In these cases, apoptosis is seen as a protective tool of the organism in order to fight the increase and spread of abnormal cells. Consequently, an increase of the apoptosis rate can be seen as marker of an overall decline in fitness of the cell, organ and organism (Muradian and Schachtschabel, 2001; Lu *et al.*, 2012). Higher rates of apoptosis and necrosis have also been reported in older donors fibroblasts samples compared with the young donors fibroblast samples (Dekker *et al.*, 2009). Altogether, our results strongly suggest that our human ageing model is adequate and accurate.

Of notice, the 46 yo fibroblasts sample is quite different from the rest ages, presenting a higher apoptotic and necrotic rate than the older samples, and in general comprises a different phenotype both in immunoblotting and immunocytochemistry. This fact might be explained by environmental influences and/or any other underlying problem not reported. This case enlightens the urge of increasing the number of samples in order to further characterize the observed alterations with more confidence.

A short recapitulation of the main results obtained for the protein levels and localization of the nuclear envelope proteins evaluated in this work are presented in Figure IV.14.

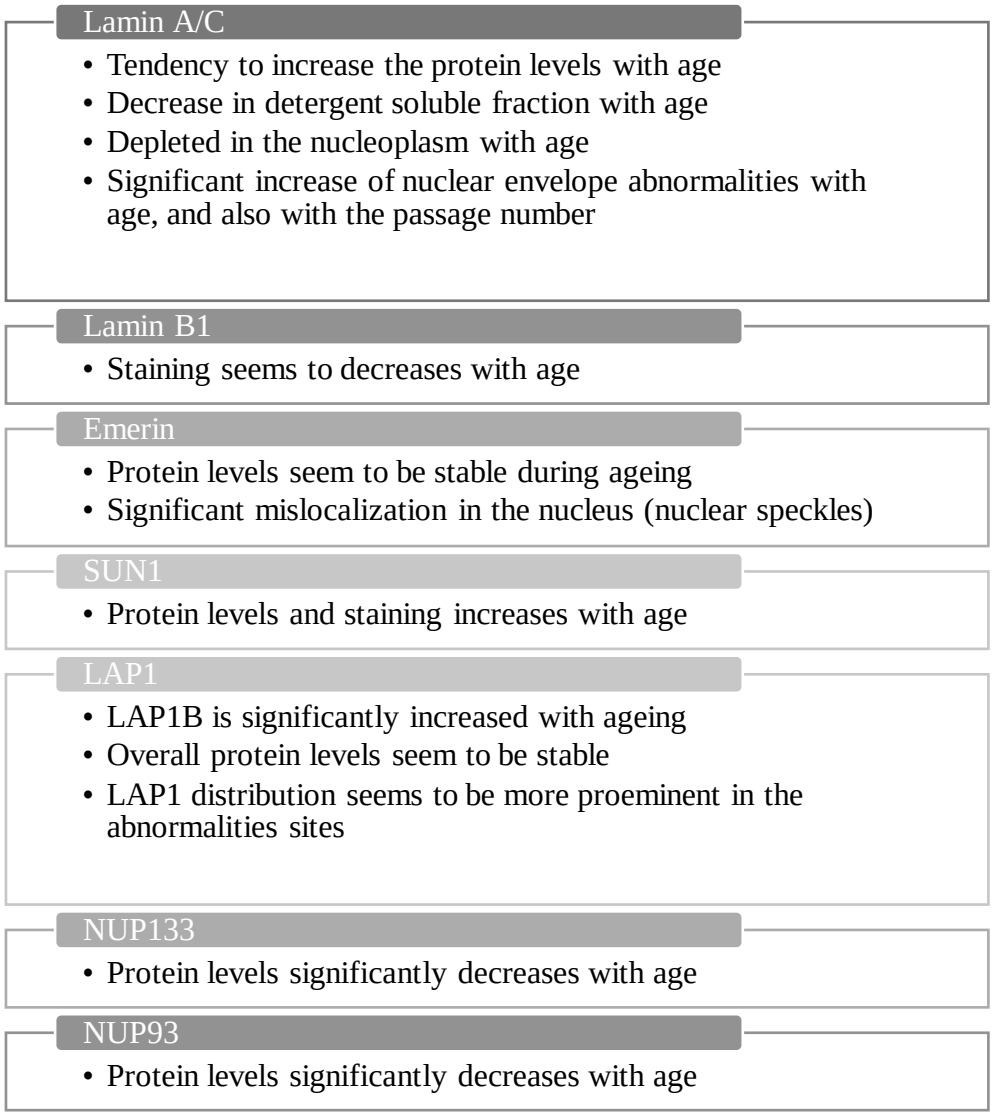

Figure IV.14 – Schematic summary of the main results obtained by immunoblotting and immunocytochemistry analysis of lamin A/C, lamin B1, emerin, SUN1, LAP1, NUP133, and NUP93 in human fibroblasts.

Our results reveal a small tendency of lamin A/C to accumulate with age both *in vivo* and *in vitro* (evaluated by increasing passage number; Figures IV.8 and Figure IV.9). Moreover, we could observe a number of lamin A/C-dependent nuclear abnormalities which increases with age. There is also a depletion of the nucleoplasmic lamin A/C, corroborating the strong diminution of the lamin A/C protein levels observed in the aged fibroblasts soluble fraction. Increased levels of wild-type lamin A have been previously reported to be associated with a shorter lifespan and nuclear aberrancies that result in apoptosis and senescence, common features of the HGPS phenotype. Over-expression of the ZMPSTE24, the metalloproteinase in charge of removing the farnesylated carboxyl terminal region of lamin A, rescues the normal phenotype (Candelario *et al.*, 2008).

Furthermore, high *LMNA* mRNA levels are expressed in aged iPSCs (induced pluripotent stem cells), showing a thicker nuclear lamina and nuclear abnormalities like blebs, doughnut-shaped nuclei and folded nuclear envelope (Petrini *et al*., 2017). Scaffidi and Misteli. (2006), describe a depletion on the nucleoplasmic lamin A/C and consequent accumulation at the nuclear rim, as well as irregularities of the NE in wild-type aged fibroblasts, which is in accordance with our results. The nucleoplasmic lamin A/C has been correlated with functions like chromatin organization and gene expression. Therefore, the depletion of nucleoplasmic lamin A/C can exacerbate the ageing phenotype (Serebryannyy and Misteli, 2018). An accumulation of mutant or normal lamin A can impact mitosis, producing DNA damage, premature senescence and an overall problematic nuclear architecture that can be attributed to the ageing process.

Moreover, immunolocalization studies suggest that lamin B1 is decreasing with age in human fibroblasts which is in line with the vast majority of the literature that correlate a loss of lamin B1 with increased cellular senescence. The decrease of lamin B1 have been reported in primary human and murine cell strains, WI-38 cells, E145K cells, HGPS patient derived fibroblasts, as well as fibroblasts and keratinocytes undergoing replicative senescence (Taimen *et al.*, 2009; Shimi *et al.*, 2011; Freund *et al.*, 2012; Dreesen *et al*., 2013a, 2013b).

Concerning emerin, the protein levels do not seem to increase with age in human fibroblasts. However, a significant increase of emerin nuclear speckles is noticed when comparing the young group to the aged one. Emerin has been reported to be increased and mislocated in aged-iPSCs when mutations in nesprins and lamin A occur (Zhang *et al*., 2007; Ho *et al*., 2013; Petrini *et al.*, 2017). Nuclear emerin with a granular brighter speckles appearance is reported to be seen in fibroblasts, differently to what happens in sections of skin or cardiac muscle where emerin is generally localized at the nuclear periphery (Morris et al., 2002). Whether or not this is important to the ageing process is still not clear and should be explored in the future.

SUN1 protein levels are significantly increased in human fibroblasts and it also seems to be increasing with age in our immunocytochemistry analysis, although no fluorescence intensity quantification was performed. As previously stated, SUN1 was found to be increased in progerias (Chen *et al*., 2012). In studies where SUN1 was deleted in *Lmna*-deficient mice, the overall cellular and organs premature disorders ameliorated, correcting nuclear aberrancies and diminishing their course of senescence in HGPS skin

fibroblasts (Chen *et al*., 2012). Taking all into account, there should be a crescent interest in targeting SUN1 to slow down the ageing phenotype.

Our results suggest that LAP1 protein levels do not change with age. However, LAP1B isoform was found to be significantly increased with age in human fibroblasts. Furthermore, LAP1 staining seems to be more intense in nuclear abnormalities sites. LAP1 is still poorly studied and not much is known about its precise physiological function. However, it is known that LAP1 binds to lamins and chromatin contributing to the maintenance of the nuclear architecture. Since lamin A/C-dependent abnormalities have been observed, and LAP1 binds to lamins, it is not surprising that LAP1 might be also localized in the abnormality's sites. Studies report that rat LAP1A and LAP1B bind directly to lamin A/C *in vitro* but not rat LAP1C (Foisner and Gerace, 1993). Human LAP1C is similar to rat LAP1C (Santos *et al*., 2014), and in fact, both LAP1C are easily solubilized from lamina in a Triton X-100 low salt concentrations buffer (Foisner and Gerace, 1993; Santos *et al*., 2014). In our work, total lamin A/C seems to increase with age and so does LAP1B. Therefore, we speculate that there might exist a functional relationship between lamin A/C and LAP1B during ageing, impacting nuclear architecture.

Regarding the nuclear pore complex protein, our results show that both NUP133 and NUP93 protein levels decreases significantly with age. NUP133 is part of membrane-apposed coat NUPs. The membrane-apposed coat NUPs form a Nup107–160 complex, which comprehends the largest NPC subunit in size and complexity, which is necessary in the first stages of NPC assembly (Nofrini *et al*., 2016). Specific NPC nuclear transport events impacts ageing (Lord *et al.*, 2015). A defective NPC will alter the balance and localization of the nuclear envelope proteins. The adapter NUPs form the NUP93 complex, the second largest NPC structural unit that play a key role in delimiting the NE sub-compartments, creating a blockade that avoids the movement of membrane proteins from ONM to the INM (Solmaz *et al.*, 2011). The loss of NE integrity leads to a gradual decrease in nucleocytoplasmic transport, to selective loss and degradation of NE components that culminates in rupture inducing aberrant molecules transport between the nucleus and cytoplasm (Robijns *et al.*, 2018). In fact, previous studies indicated that the long-lived NPC structure deteriorates with time, thereby increasing the nuclear permeability across lifespan. In particular, some studies have observed that Nup93 were lost in old and permeable nuclei (D'Angelo *et al.*, 2009). Interestingly, Nup93 has previously been functionally associated with NPC permeability barrier (Galy *et al.*, 2003)

which explains the correlation between the age-related loss of this nucleoporin and the increased nuclear permeability observed (D'Angelo et al., 2009).

Noticeably, some literature mention that some of the differences in the protein levels and/or mislocalization during ageing are only observed in extensively passaged fibroblasts (Cao et al., 2011), which is not the case of our fibroblasts that were at passage number 10. Therefore, we hypothesised that at passage number 20 (or later), a number of other or more prominent changes might be observed.

To sum up, the work here presented puts the nuclear envelope proteins in the spotlight in the ageing process. Some of our results are pioneering in physiological ageing, since most of the studies available out there are based on premature ageing models, and can be the foundation for future studies regarding the nuclear envelope dysfunction and its contribution to the ageing process

CHAPTER V

General discussion and concluding remarks

Ageing is a multi-factorial process that urge to be studied profoundly if we ever want to unpuzzle this complex process. The nuclear periphery has caught the attention of some researchers in the last few years, especially due to the fact of some progeroid/premature ageing syndromes being due to defects in nuclear envelope proteins. Remarkably, the PPI network analysis of the age-associated genes available in the public databases places nuclear proteins and their associated functions in the spotlight of the ageing process (Chapter 2). Moreover, our results using the mice as ageing model suggest that lamin A/C, lamin B1, emerin, LAP1, nesprin-2, and SUN1 protein levels increases during ageing (chapter 3). In human fibroblasts from healthy individuals passage-matched, lamin A/C, LA1PB and SUN1 protein levels seems to increase with age, whereas lamin B1, NUP133 and NUP93 protein levels seems decrease with age. In addition, concomitantly with increasing levels of lamin A/C, the significant increase of lamin A/C dependent abnormalities and depletion of nucleoplasmic lamin A/C were observed with age (chapter 4). A short recapitulation and comparison of the main results obtained from both ageing models (mice and humans) is presented in Figure V.1.

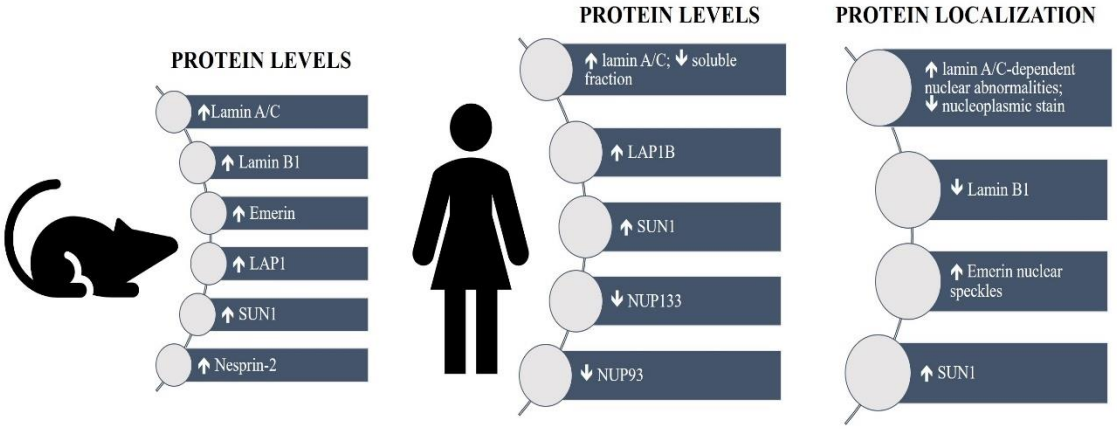

Figure V.1 – Schematic summary of the main results obtained with both ageing models studied in this work (mouse model left, and human fibroblast model right). ↑ indicates the increase and ↓ indicates decrease of each protein level. The subcellular localization of some proteins are also indicated.

Comparing the results obtained from the two models, it becomes clear that lamin A/C, emerin, LAP1 and SUN1 seem to be altered during the ageing process. Lamin A/C seems to increase with age in both models. Concomitantly, lamin A/C-dependent nuclear abnormalities increase with age and the nucleoplasmic fraction (soluble fraction) of lamin A/C decreases with age. These results are in agreement with previous studies that have shown an increase of lamin A with ageing (Candelario *et al.*, 2008) and an increase of depleted nucleoplasmic lamin A/C in human fibroblasts (Scaffidi and Misteli, 2006). In

our hands, emerin is mislocalized in human aged fibroblasts and seems to be increasing with age in mice. In a previous study performed in iPSCs aged *in vitro*, emerin seems to be increasing and it is mislocalized around the nuclei (Petrini *et al*., 2017). We do not observe this kind of mislocalization, but human fibroblasts had previously been reported to have a different emerin localization (Morris *et al*., 2002). LAP1 increases with ageing in mice and LAP1B increases in human aged fibroblasts. This is the first time to our knowledge that any efforts were made to characterize LAP1 alterations during ageing. SUN1 is also increased in both of our ageing models which is in line with what we already knew from mice studies (deletion of SUN1 in *Lmna*-deficient mice ameliorates their nuclear aberrancies and improves their overall fitness) and from HGPS fibroblasts (SUN1 is increased in progerias) (Chen *et al.*, 2012; Chi *et al.*, 2012). NUP133 is decreased with ageing in human fibroblasts. No previous studies related NUP133 with ageing, but other nucleoporins had already been linked with a leaky nuclear pore complex during the ageing process (D'Angelo et al., 2009) as it is the case of NUP93 which is also decreased with age in our human fibroblasts. Nesprin-2 is increased with age in our mice model. This result is corroborated by the study performed in aged human iPSCs that reported an increase of nesprin-2 protein levels with age (Petrini *et al.*, 2017).

Regarding the lamin B1, the results in mice depicted an increase of protein levels with age at 18 months and then decrease with 24 months, whereas in human fibroblasts cellular model it seems to decrease. Barascu *et al.*, (2012) reported that in premature ageing syndrome Ataxia Telangiectasia (A-T), higher levels of ROS synthesis and defective nuclear shape results in higher levels of lamin B1. Overexpression of lamin B1 results in nuclear architecture defects leading to a state of premature senescence in normal cells. The higher expression of lamin B1 seems to be due to ROS synthesis. Pro-oxidant stimulation in normal cells enhanced lamin B1 protein levels. Although, anti-oxidant treatment of A-T cells led to decreased lamin B1 protein levels. Therefore, it seems that ROS-dependent higher lamin B1 expression is connected with p38 MAP kinase that is associated with senescence, pointing to a new ATM-independent stress-sensing that results in a senescence. On the other hand, other studies performed in fibroblasts derived from an atypical progeria patient that quickly became senescence show a strong loss of lamin B1protein levels (Taimen et al., 2009). Moreover, other work reports a diminution of lamin B1 protein levels in either replicative or oncogene-induced senescence fibroblasts (Shimi and Goldman, 2014). Silencing lamin B1 with siRNA resulted in lower proliferation rates and enhanced cellular senescence. The silencing of lamin B1 was

linked with higher p53 expression and cells entered in a state of senescence dependent of the activity of p53 and Rb. Silencing lamin B1 was linked with a p53-dependent decrease in the mitochondrial ROS synthesis (Shimi and Goldman, 2014). This might seem conflicting results and conclusions, but there is a possibility of the both results being plausible. Lamin B1 seems to be a signalling hub and its response could be different according to what senescence pathway is activated (ROS-induced premature senescence, Replicative-induced senescence and Oncogene-induced senescence) (Hutchison, 2012).

The integrative results of this thesis are particularly important since are one of the only results available out there performed that explores the nuclear envelope proteins in physiological ageing. Nevertheless, more samples should be used.

To sum up, the nuclear envelope proteins are definitely affected by the ageing phenotype and further studies should be performed in order to uncover their role in this complex process. Perhaps in the future, the knowledge acquired about the nuclear envelope proteins role in the ageing process can be put into use to design therapeutics targeting the nuclear dysfunction.

Therefore, we propose the following future perspectives:

- Complement this study by increasing the number of mice samples and human fibroblasts;
- Evaluate the mRNA levels of the NE proteins during ageing, in mice tissues and human fibroblast samples;
- Characterize the nuclear envelope alterations reported in this study in extensively passaged fibroblasts.

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Appendix

ELB Lysis buffer

Samples

Stock ELB (Vf≈10 ml) → **Complete ELB (Vf≈5ml)**

Triton X-100 100% 50 µl

HEPES pH 7 1M 500 µl

NaCl 5M 500 µl

H_20 8,95 ml

ELB stock 4,645 ml

DTT 1M 5 µl

NaF 1M 5µl

EDTA 0.5M 20µl

EGTA 100.0mM 50µl

Na3VO4 100.0mM 50µl

When working with samples, distribute 955µl of complete ELB in each microtube and store it like that. After, at the time you will use it, complete it with 20µl of cOmplete™, EDTA-free Protease Inhibitor Cocktail Roche and 25µl of PMSF Protease Inhibitor.

SDS-PAGE (1D)

29:1 Acrylamide:Bis Acrylamide Ratio (3.3% cross-linker concentration)

- **Gradient gel**

Lower Gel (Gel with 1.5mm):

½ system		
	LG (5%)	**LG (20%)**
H_20	9.29ml	3.67ml
LGB	3.75ml	3.75ml
Acrylamide	1.875ml	7.5ml
APS	75µl	75µl
TEMED	7.5µl	7.5µl

1 system		
	LG (5%)	**LG (20%)**
H_20	18.59ml	7.34ml
LGB	7.5ml	7.5ml
Acrylamide	3.75ml	15ml
APS	150µl	150µl
TEMED	15µl	15µl

3.5% Upper Gel (gel with 1.5mm)		
	½ system	1 system
H_20	6.92ml	13.83ml
Acrylamide	0.88ml	1.75ml
UGB	2ml	4ml
SDS 10%	100µl	200µl
APS 10%	100µl	200µl
TEMED	10µl	20µl

10% APS (ammonium persulfate)

0.1g em 1ml de dH_20 aµtoclaved

Note: Always prepare it fresh before using it.

LGB (Lower Gel Buffer)

V=0.5L

Tris 90.825g

SDS 2g

Note: It is necessary to heat. Adjust pH 8.9. Keep at 4ºC.

UGB (Upper Gel Buffer)

Vf=0.5L

Tris 37.845g

Note: Adjust pH 8.9. Keep at 4ºC.

10% SDS (sodium dodecilsulfate)

Dissolve 10g of SDS in 100ml H20d.

Tris-Gly 10x Stock

30.3g TRIS (250mM)

144.1g Glycine (1.92M)

Make up to 1L with dH20

Do not adjust pH 8.3

LB 4X

LB 4x (Loading Buffer 4x)	**Vf=10ml**
Tris 1M	2,5ml
SDS	0,8g
Glycerol	4ml
2-Mercaptoethanol	2ml
Bromophenol blue	0.001g

Stock Tris 1M

Dissolve 30.3g Tris in 250ml of dH_20. Adjust pH 6.8. Store at 4ºC.

TBS 10X

	Vf=1L
Tris	12.11g
NaCl	87.66g

Adjust pH 8,0. Store at 4ºC.

TBST 10x

	Vf=1L
Tris	12.11g
NaCl	87.66g
Tween 20	5ml

Adjust pH 8,0. Store at 4ºC.

Running Buffer (1L)

800ml dH20

100ml Tris-Gly 10x Stock

10ml SDS 10%

Make up to 1L with dH20

Transfer Solution (1L)

100ml Tris-Gly 10x Stock

700ml dH20

200ml methanol

Stripping Solution

	Vf=500ml
Tris	3.76g
SDS	10g

Note: It is necessary to heat the solution. Adjust pH to 6.7. Store at RT. Immediately before usage, add 175µl of 2-Mercaptoethanol for each 25ml of solution.

Dilution scheme for Standard Teste Tube Protocol and Microplate Procedure (Working range 20-2,000µg/mL)

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 =Blank

Ponceau S

0.1% Pounceau in 5%acetic acid

(example Vf=50ml: add 0.05g of Ponceau S to 47.5ml of dH_2O. In the hood, add 2.5ml of acetic acid).

Store at 4ºC.