Ana Sofia Marques Leal

Strategies to reverse cellular senescence and enhance progerin clearance in Hutchinson-Gilford Progeria Syndrome

Dissertação para a obtenção do grau de Mestre em Investigação Biomédica sob orientação científica da Doutora Célia Aveleira e co-orientação do Doutor Henrique Girão

apresentada à Faculdade de Medicina da Universidade de Coimbra

Junho 2016

UNIVERSIDADE DE COIMBRA

Front cover: Accumulation of progerin (green) within the nuclei of dermal fibroblasts in culture, obtained from a Hutchinson-Gilford Progeria Syndrome patient. Image obtained by fluorescence microscopy.

Support

Progeria Research Foundation (USA) – PRF 2015-60

UID/NEU/04539/2013; SFRH/BPD/111710/2015; SFRH/BD/51965/2012

QREN - Projeto Mais Centro "Aging, Stress And Chronic Diseases: From Mechanisms To Therapeutics" (CENTRO-07-ST24-FEDER-002006)

Acknowledgments/Agradecimentos

Em primeiro lugar gostaria de agradecer ao Centro de Neurociências e Biologia Molecular da Universidade de Coimbra por me ter proporcionado todas as condições para a realização deste trabalho.

Agradeço igualmente à Professora Doutora Cláudia Cavadas pela orientação cíentifica e por me ter aceite no seu grupo de trabalho, proporcionando um ano de experiências enriquecedoras.

Agradeço à Doutora Célia Aveleira por me ter aceite neste projecto e por me ter acolhido no laboratório de forma tão calorosa. Quero agradecer de forma shakesperiana toda a ajuda, atenção, conselhos, preocupação e dedicação que recebi ao longo deste ano e agradecer sobretudo todas as lições que tirei para a vida dos teus ensinamentos e experiências partilhadas!

Pela boa disposição, pelo ambiente de entreajuda e de cooperação, pelas horas partilhadas queria agradecer a todos os elementos do grupo por terem feito deste ano, um ano mais agradável e simples!

Quero agradecer especialmente aos meus amigos MIBs, a Vanessa, a Adriana, o Diogo, a Filipa e à minha caríssima amiga Sofia Morais por terem estado sempre presentes em todos os bons e maus momentos! Obrigada por todos os almoços, jantares, lanches, saídas, jogos, bingos!, queimas, cinemas, telefonemas, lamurias, peripécias, receitas, compras ao pingo doce!, conselhos, concertos!, viagens… Obrigada por me ouvirem e por tornarem este caminho mais fácil, não teria sido um ano tão bom sem a vossa presença.

Um especial obrigada às minhas meninas emigrantes, à Rita e à Mariana. Mesmo longe sempre senti o vosso apoio, a vossa honestidade e a vossa alegria e entusiasmo! Que continuemos aqui sempre da mesma forma e independentemente daquilo que o futuro nos reserva sei que posso contar com a vossa amizade. Obrigada por fazerem parte da minha vida, tenho a maior sorte por ter amigas assim.

Ao Ricardo agradeço todo o apoio, toda a motivação, toda a compreensão, preocupação e dedicação. Obrigada por todos os pequenos e grandes gestos, por todas as palavras e por me motivares a ser mais e melhor! És um exemplo de tudo o que ainda aspiro poder ser, obrigada por me teres mostrado um outro lado de viver.

Aos meus pais, quero agradecer tudo aquilo que têm feito por mim. Por me fazerem acreditar que tudo é possível, por terem uma força incrível, por serem tão compreensivos e atenciosos, por serem um exemplo de integridade, por demonstrarem compaixão e dedicação e por serem uma fonte de inspiração constante… Nada mais posso fazer senão agradecer. Tudo o que sou, tudo o que tenho é porque o recebi de vocês por isso um enorme obrigada.

(À cidade de Coimbra, obrigada por seres perfeita e por teres trazido de volta a minha vida.)

Index

List of abbreviations

AgRP	Agouti gene-related proteins
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate (AMP)-activated protein kinase
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM Rad3 related
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cDNA	Complementary DNA
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
Ct	Cycle determination
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DSBs	DNA double-strand breaks
DTT	Dithiothreitol
ECF	Enhanced chemifluorescence
FOXO	Forkhead box protein O1
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
GHS	Growth hormone secretagogues
GHS-R	Growth hormone secretagogue receptor
GOAT	Ghrelin Octanoyl-acyltransferase
H2AX	Histone H2A variant H2AX
H3K27me3	Heterochromatin mark trimethylation of histone H3 lysine 27
HFD	High fat diet
HGPS	Hutchinson-Gilford progeria syndrome
hMSCs	Human mesenchymal stem cells
IGF-1	Insulin-like growth factor 1
LC3B	microtubule-associated protein light chain-3B
mRNA	RNA mensseger
MTOR	Mechanistic target of rapamycin
NAD	Nicotinamide adenine dinucleotide
NaF	Sodium fluoride
NPY	Neuropepetide Y

NRT	No reverse transcriptase control
NTC	No template control
OD	Optical density
ORTO	Sodium orthovanadate
PARP1	Poly [ADP-ribose] polymerase 1
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonylfluoride
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real time polymerase chain reaction
Rb	Retinoblastoma
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sulphate-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline and Tween 20

Abstract

Hutchinson-Gilford progeria syndrome (HGPS), a lethal genetic disorder, is characterized by premature accelerated aging. HGPS children have short stature, low body weight, lipodystrophy, alopecia, skin and bone abnormalities, and atherosclerosis. The cardiovascular system is severely affected and, in most of the cases, children succumb to myocardial infarction or stroke in their early teens, at an average age of 14.6. HGPS is most commonly caused by a *de novo* point mutation (G608G) within the lamin A/C gene (LMNA) that partially activates a cryptic splice donor site in exon 11, producing an abnormal lamin A protein termed progerin. This truncated abnormal Lamin A does not undergo the normal proteolytic processing, becoming permanently farnesylated, which alters lamins dynamics and their association with the inner nuclear membrane. Accumulation of progerin causes nuclear abnormalities, mitotic abnormalities and cell cycle arrest, ultimately leading to cellular senescence, and therefore, is one of the mechanisms underlying the progression of HGPS. Several therapeutic strategies have been developed in the last years for HGPS targetting progerin processing or its turnover. In fact, a recent study showed that treatment with rapamycin stimulated autophagy in HGPS cells, resulting in enhanced progerin clearance, reduced nuclear blebbing and postponed cellular senescence. Rapamycin, a macrolide antibiotic, is a well-established autophagy inducer, by inhibiting MTOR activity, the canonic molecular switch for autophagy induction. In fact, rapamycin is known to extend longevity in several organisms, including mammals, and this effect is at least in part dependent of autophagy activation. Although rapamycin treatment has been considered as a therapeutic strategy for HGPS, the substantial set of rapamycin-associated adverse effects, as well as the lack of aging-specific human data, should caution the routine use of rapamycin as an anti-aging agent. The identification of safer stimulators of autophagy, with other beneficial effects, for chronic treatment of HGPS patients is of utmost importance.

Ghrelin is 28 amino acid acylated peptide initially identified in the rat stomach as the endogenous ligand for the growth hormone secretagogue-receptor (GHS-R1). Ghrelin is a circulating hormone with multiple functions that has been shown to be beneficial in organs/cells affected by HGPS. It is envolved in growth hormone secretion, regulation of energetic and metabolic balance, regulation of cardiovascular functions (increase of cardiac output, decrease blood flow, protection against cardiac damage, antiapoptotic effects), bone remodeling (increase osteoblast differentiation and bone mineral density), inflammation (suppressing the production of cytokines), glucose metabolism, protection from ischemia/reperfusion injury as well as improving the prognosis of myocardial infarction and heart failure. Of importance, ghrelin and ghrelin analogues have been assessed in several clinical trials for the treatment of other diseases such as cachexia in chronic heart failure, anorexia nervosa and growth hormone deficiency-related disorders. Being an endogenous and ubiquitously expressed hormone it has been

proposed that ghrelin and its receptor can act positively in HGPS pathology and treatments with exogenous ghrelin promises a strategy to delay or block the premature aging of HGPS and potentially to increase lifespan. In line with this, the main goal of this study was to investigate the role of ghrelin in rescuing the aging phenotype of HGPS-patients derived cells.

In this study, primary human dermal fibroblasts cell cultures from HGPS patients were used. HGPS fibroblasts were treated with human ghrelin (1 nM) for 1 week and the effect of ghrelin on progerin accumulation and several hallmarks of cellular aging, such as loss of proteostasis, nuclear abnormalities, DNA damage and cellular senescence were evaluated. In HGPS cells, ghrelin decreased by 50 % progerin protein levels. As we observed no alterations on progerin mRNA levels, we next evaluated whether ghrelin decreased progerin levels through protein degradation. In fact, we observed that ghrelin increased autophagic flux in HGPS cells, supporting that ghrelin enhanced progerin degradation in HGPS cells. Concomitant with progerin clearance, ghrelin decreased the number of dysmorphic nuclei, a hallmark of HGPS cells, increased nuclear circularity and decreased nuclear area, rescuing nuclear morphology in HGPS cells. Ghrelin also decreased DNA damage in HGPS cells, as shown by a reduction in the number of γH2AX foci, a marker of DNA damage. In addition, ghrelin increased HGPS cells proliferative capacity, as determined by an increase in the number of Ki-67-positive cells, a marker of cell proliferation. Moreover, ghrelin decreased senescence-associated-beta-galactosidase (SA-beta-Gal) activity. Fewer SA-beta-Gal–positive cells were observed in ghrelin-treated cells, indicating that ghrelin slowed down the progression of cellular senescence in HGPS cells.

In conclusion, our study shows that ghrelin treatment i) decreases progerin levels, probably through autophagy stimulation, ii) rescues nuclear abnormalities, iii) decreases DNA damage, iv) increases cell proliferation, and v) delays cellular senescence in HGPS cells. Altogether, these results show that ghrelin rescues the senescent phenotype of HGPS cells and strongly support that ghrelin can be considered a promising strategy to delay or block the premature aging of HGPS.

Resumo

A Progeria, também conhecida como sindrome de Hutchinson-Gilford (HGPS) é uma doença genética extremamente rara e fatal, estimando-se que afecte 1 em cada 4 a 8 milhões de nascimentos, em todo o mundo. Esta doença afecta ambos os sexos e todas as raças de igual forma. Esta doença caracteriza-se por um envelhecimento acelerado e prematuro. As crianças com esta síndrome nascem aparentemente saudáveis e só começam a evidenciar sintomas de envelhecimento prematuro entre os 18 e os 24 meses, apresentando uma taxa de crescimento reduzida, baixa estatura, baixo peso corporal, lipodistrofia, alopécia, anomalias a nível da pele e do osso e aterosclerose. O sistema cardiovascular é severamente afectado e, na maior parte dos casos, as crianças morrem devido a enfarte do miocárdio ou acidente vascular cerebral com uma idade média de 14.6 anos. A HGPS é uma doença genética autossómca dominante causada, principalmente, por uma mutação pontual *de novo* (G608G) no exão 11 do gene que codifica a lamin A/C (LMNA) activando parcialmente um local de *splicing* alternativo, levando à síntese de uma proteína Lamina A truncada designada por progerina. Esta proteína truncada não é processada normalmente tornando-se permanentemente farnesilada, alterando a organização das laminas no envelope nuclear e a sua associação à membrana nuclear interna. A acumulação de progerina causa alterações na morfologia nuclear e divisão celular levando, em última instância à senescência celular, um dos mecanismos subjacentes à progressão da HGPS.

Várias estratégias terapêuticas têm vindo a ser desenvolvidas nos últimos anos para o tratamento da HGPS, focadas na inibição da farnesilação da progerina ou na sua degradação. Um estudo recente mostrou que o tratamento com rapamicina aumenta a autofagia em células HGPS, com a consequente degradação da progerina, melhoria da morfologia nuclear e retardamento da senescência celular. A rapamicina, um antibiótico macrólido, é um indutor da autofagia, através da inibição da actividade do MTOR, o interruptor molecular da via canónica para a indução autofagia. De facto, a rapamicina aumenta a longevidade em vários organismos, incluindo mamíferos, e este efeito é, pelo menos em parte, dependente da activação da autofagia. Embora o tratamento com rapamicina esteja a ser considerado como uma estratégia terapêutica para HGPS, os efeitos adversos associados ao tratamento com rapamicina, bem como a falta de dados em humanos no contexto do envelhecimento, sugerem cautela na adminstração crónica de rapamicina como um tratamento para retardar ou bloquear o envelhecimento. A identificação de fármacos indutores de autofagia, sem efeitos adversos, para o tratamento crónico de HGPS é por esta mesma razão, de extrema importância.

A grelina é um péptido com 28 aminoácidos e foi descrita inicialmente no estômago do rato sendo o ligando endógeneo para o recetor dos secretagogos da hormona de crescimento (GHS-R1a). A grelina é uma hormona periférica com múltiplas funções que têm vindo a ser demonstradas como benéficas em orgãos/células afetados pela HGPS. A grelina está envolvida

na secreção da hormona de crescimento, no equílibrio energético e metabolismo, na regulação das funções cardiovasculares (no aumento do débito cardíaco, na diminuição do fluxo sanguíneo, na proteção contra danos cardíacos, com efeitos antiapoptóticos), na remodelação óssea (no aumento da diferenciação de osteoblastos e na densidade óssea mineral), e na inflamação (suprimindo a produção de citocinas), no metabolismo da glucose, na proteção isquémica, melhorando o prognóstico de enfarte do miocárdio e da insuficiência cardíaca. É de salientar que a grelina e os seus analógos têm vindo a ser testados em ensaios clínicos para o tratamento de outras doenças humanas como caquexia, anorexia nervosa e doenças relacionadas com deficiências na hormona de crescimento. Sendo uma hormona endógenea expressa de forma ubíqua e tendo efeitos positivos em doenças associadas à HGPS, a grelina e o seu receptor podem ser considerados como alvos promissores para o desenvolvimento de estratégias terapêuticas para atrasar ou bloquear o envelhecimento prematuro que caracteriza a HGPS, aumentando a qualidade de vida e a esperança média de vida dos doentes com HGPS. Assim, o principal objetivo deste estudo foi investigar o potencial terapêutico da grelina em retardar o envelhecimento prematuro que caracteriza a HGPS.

Neste estudo foram usadas culturas primárias de fibroblastos da derme de doentes com HGPS, um modelo experimental frequentemente utilizado em estudos sobre esta patologia. As células foram tratadas com grelina (1 nM) durante uma semana e foi avaliado o efeito desta na acumulação de progerina e em diversas outras caracteristicas associadas ao envelhecimento celular como alteração da morfologia nuclear, danos no ácido desoxirribonucleico ADN, perda de proteostase e senescência celular.

Este estudo mostra que o tratamento com grelina diminui em cerca de 50% os níveis de progerina nos fibroblastos de doentes com HGPS. Como não foram observadas alterações nos níveis de mRNA da progerina, avaliou-se de seguida se a dimnuição nos níveis proteicos de progerina se deveriam a um aumento da degradação proteica, através da indução de autofagia. De facto, a grelina induz autofagia e aumenta o fluxo autofágico nos fibroblastos de doentes com HGPS, o que sugere que a grelina índuz a degradação de progerina nestas células. Concomitante com a degradação de progerina, a grelina diminuiu o número de núcleos dismórifcos, uma característica proeminente das células HGPS, o que se relaciona com um aumento na circularidade nuclear e diminuição da área nuclear, havendo uma melhoria na morfologia nuclear das células HGPS. A grelina também diminuiu os dano no ADN, demonstrado por uma redução no número de foci de γH2AX, um marcador de danos no ADN. Para além disso, a grelina aumentou a capacidade proliferativa das células e diminuiu a actividade da beta galatosidade associada à senescência demonstrando que a grelina reduz a progressão da senescência celular dos fibroblastos de HGPS.

Em conclusão, o nosso estudo mostra que o tratamento com a grelina i) diminui os níveis de progerina, provavelmente através da indução de autofagia, ii) melhora a morfologia nuclear, iii) diminui os danos no ADN, iv) aumenta a proliferação celular e v) atrasa a senescência celular demonstrando que a grelina resgasta o fenótipo senescente dos fibroblastos de HGPS. Tudo o que foi concluído corrobora fortemente com o facto de que a grelina poderá ser uma estratégia promissora para atrasar ou bloquear o envelhecimento prematuro que caracteriza a HGPS.

Chapter 1
Introduction

1.1 Aging

Aging is essentially defined by an overall decline in the functional capacity of various organs to maintain tissue homeostasis and to respond effectively to physiological needs under stress, those being caused by lifelong accumulation of molecular and cellular damage (Kirkwood 2005). Aging is unquestionably correlated with the risk of developing chronical medical conditions such as cardiovascular diseases, arthropathies, pulmonary diseases, diabetes, strokes, dementia and cancer (Kirkwood 2008). However, it is possible to slow the rate at which these diseases appear and so increase human longevity. The aging process is regulated by the same signaling pathways and transcription factors as many other biological processes. The genetic pathways and biochemical processes involved in regulating age are conserved in evolution. These are: the insulin/IGF-1 pathway, TOR signaling, AMP kinase, sirtuins, inhibition of respiration, signals from reproductive system and telomeres. Through modulation of these pathways and transcription factors it may be possible not only to increase lifespan but to postpone age-related diseases (Kenyon 2010). Nevertheless, the process of aging has not been slowed; human senescence has only been delayed in the past few years due to improvements in lifestyle, medical and public health (Vaupel 2010).

1.1.1 Hallmarks of Aging

Improvements in lifestyle conditions and health care have led to a significant increase in lifespan over the last century, and consequently aging population, and its associated socio-economic implications. Therefore, one of the main goals of biomedical research is to find strategies to delay or reduce the deleterious effects of aging and provide a better quality of life. Aging is characterized by the gradual and overall loss of various physiological functions.

The hallmarks of aging such as genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication that underlie the aging phenotype generally contribute to the aging progression in later stages in life. These cellular aging mechanisms are common denominators of aging in differents species, particulary in mammals (Lopez-Otin, Blasco, Partridge, Serrano, & Kroemer, 2013).

1.1.1.1 Genomic instability

Within each cell all proteins, lipids and nucleic acids are susceptible to be damaged by endogenous and exogenous genotoxic agents and spontaneous reactions. For this reason throughout evolution there was an investment in cellular maintenance, mainly genomic maintenance. However, the DNA maintenance apparatus cannot cope with all the insults inflicted on the genome and when the DNA is damaged, depending on the type and severity of

DNA injury there is a DNA damage repair machinery responsible for cell survival, cellular senescence or cell death (Garinis *et al.* 2008).

This machinery acts on different levels including post-translational modifications such as phosphorylation, acetylation, ubiquitination, sumoylation and histone modification (Christmann & Kaina 2013) and by activating DNA damage response proteins such as ataxia telangiectasia mutated (ATM), ATM Rad3 related (ATR) and poly [ADP-ribose] polymerase 1 (PARP1) to protect the cell against genotoxic stress. Upon the activation of ATM and ATR there will be a phosphorylation cascade that is going to stimulate multiple proteins involved in cell cycle control, apoptosis, DNA repair and autophagy. These processes guarantee that genetic information remains intact during the entire lifetime of an organism but ultimately, DNA damage and mutations progressively accumulate being the leading cause of senescence and possibly of premature aging.

Besides DNA damage, loss of functionality of telomeres, failure to maintain mitochondrial DNA and defects in the nuclear architecture can cause genomic instability. The latter usually is responsible for premature aging syndromes such as Hutchinson-Gilford progeria syndrome (HGPS). This syndrome is characterized by abnormal nuclear morphology due the presence of progerin, a mutant isoform of lamin A protein, and it is a perfect example of the dramatic consequences of deficiencies in long-term maintenance of the nuclear architecture. However, cells from normal population also experience significant nuclear disintegration upon cellular stress stimuli; there is evidence that progerin accumulates over time leading to nuclear changes, being this one of the many causes for normal human aging. Therefore, there is a clear correlation between aging, DNA damage response, alterations in chromatin structure, genomic integrity and the nuclear architecture in which the lamin A protein has a evident conserved role. (Oberdoerffer & Sinclair 2007).

1.1.1.2 Telomeres attrition

Telomeres are specialized strutures in the ends of human chromosomes and are essential to protect and stabilize the integrity of the DNA (Shampay *et al.* 1984). Without these nucleoprotein caps there would be chromosomal fusion and, consequently, deleterious genomic instability (Levis 1989). To maintain the telomeres the enzyme telomerase is of major importance because it is the only DNA polymerase known with the ability to replicate the terminal ends of linear DNA molecules (Blackburn 2001). Most mammalian somatic cells do not express telomerase, and this results in progressive telomere attrition. Telomere exhaustion explains the limited proliferative capacity – or cellular senescence - of in-vitro cultured cells after a finite number of cell divisions, also called Hayflick limit (Hayflick & Moorhead 1961). Therefore, telomere shortening had been proposed to be the trigger that causes senescence. To confirm

this, enforced expression of telomerase in in-vitro cultured cells stabilized telomere length and confer unlimmited replicative potential to cells without malignent transformation showing that, there is causal correlation between senescence and telomeres (Bodnar *et al.* 1998). Besides telomere attrition, damaged telomeres also trigger senescence by inducing persistent DNA damage signals and sustained p53 activation (Sahin & Depinho 2010). Notably, telomere shortening is observed in normal aging (Blasco 2007). Mice with longer telomeres exhibit increased lifespan (Tomas-Loba *et al.*, 2008) and aging can be reverted by telomerase activation (Jaskelioff *et al.* 2011). In humans it was shown that there is a correlation between shorter telomeres and mortality risk (Boonekamp *et al.* 2013).

1.1.1.3 Epigenetic alterations

A variety of epigenetic alterations accumulate during our lifetime and influence gene expression. In recent studies on aging, it has been proposed that the number of alterations in the epigenetic patterns increases and these changes are fundamentally influenced by environmental factors and not familial factors (Talens *et al.* 2012). These variations in the epigenetic patterns are age-associated and include histone modifications, DNA methylation, chromatin remodeling and transcriptional alterations which affect multiple nuclear processes such as gene transcription and silencing, cell cycle progression, DNA replication/repair and telomere structure/function. Consequently, it affects genomic stability and the maintenance of heterochromatic domains. It has been proposed that epigenetic alterations correlate with age-related diseases, particularly cancer, since some of the epigenetic modifications in cancer and in aging are similar. This suggest that accumulation of these defects with age may lead to higher susceptibility to cellular malignant transformation and to develop cancer. Clearly, epigenetic changes are both responsive to and effectors of the aging process (Gonzalo *et al.* 2010).

1.1.1.4 Loss of Proteostasis

Continuous turnover between protein synthesis and protein degradation is a highly dynamic cellular process that prevents the accumulation of harmful substrates or components that lose functionality in the intracellular environment. This process is indispensable to keep the cellular machinery free from damaged proteins that could be toxic and could interfere with the normal cellular function (Kirschner 1999; Squier 2001; Kourie & Henry 2001).

In all eukaryotic organisms there are two major pathways to degrade intracellular targets (proteins or organelles): the ubiquitin-proteasome system and the lysosomal/autophagic system. Protein degradation by the ubiquitin–proteasome system is an indispensable cellular mechanism. This pathway is involved in several biological processes such as regulation of cell cycle and immune response and more recently it has been implicated in the process of aging (Löw 2011).

Ubiquitin is a highly conserved protein through evolution and ubiquitination of endogenous proteins is one of the key regulatory steps of protein degradation followed by regulation of proteasome activity, whose action decreases during the aging process (Chitra *et al.* 2012; Grillari *et al.* 2006). The ubiquitin molecule is crucial for the the ubiquitin-proteasome pathway, and only polyubiquitinated-proteins are degraded by this system. The process begins when the protein requiring degradation is tagged by the covalent attachment of ubiquitin to a lysine residue via an isopeptide bond (Pickart 2004). This reaction is catalyzed by three different types of enzymes: E1 - ubiquitin-conjugating enzyme (activates ubiquitin), E2 - ubiquitin-conjugating enzyme (promotes conjugation) and E3 - ubiquitin-protein ligase (promotes ligation) (Pickart 2004). Multiple ubiquitin molecules can be reversibly added to proteins that are targeted for proteolysis and elimination (Ciechanover and Gonen, 1990; Yewdell, 2001). The length of the ubiquitin chain and the lysine residue to which it is attached define the fate, stability and subcellular localization of the tagged protein (Christianson 2014). The ubiquitin-proteasome pathway plays a key role in degradating short-lived and misfolded soluble proteins present both in the cytoplasm and nucleus (Nath 2009).

Autophagy relies on a machinery highly conserved from yeast to mammals and this lysosomal degradation pathway is essential for survival, diferentiation, development and homeostasis (Reggiori & Klionsky 2002). It functions at a basal level to clear the cell from abnormal and useless intracellular material and in response to stress conditions, being the most-well known inducer of autophagy nutrient depletion. To guarantee the cell survival during starvation, the primary cellular response is to breakdown macromolecules into its essential components in the lysosoma to fuel the cell. Autophagy also functions to prevent neurodegeneration, as an anti-aging mechanisms, surpress tumors and regulate both innate and acquired immunity (Levine & Kroemer 2008; Mizushima *et al.* 2008; Mizushima & Klionsky 2007; Rubinsztein *et al.* 2007; Yang & Klionsky 2009). There are three different types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy and they differ in their mechanism, regulation and selectivity (Mizushima 2004; Cuervo 2004). For the purpose of this work, it will only be mentioned macroautophagy, hereafter referred to as autophagy. It is a process that involves the sequestration of cytoplasmic components (which can be entire organelles, lipid vesicles, or protein aggregates) within a double-membraned vesicle, the autophagosome. Autophagosomes fuse with lysosomes to generate autolysosomes, in which the autophagic cargo is degraded by acidic hydrolases (Seglen *et al.* 1996; Mortimore *et al.* 1996) (Figure 1.1). As previously said, autophagy is stimulated during nutrient depletion. In response to this stressful situation the protein MTOR the central regulator of cell growth and metabolism, is inhibited and autophagy is induced.

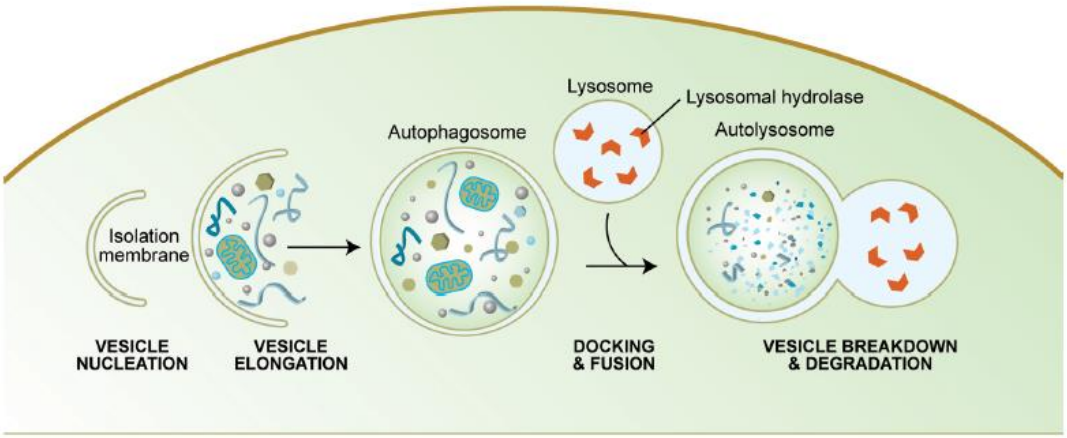

Figure 1.1. Schematic diagram of autophagy. Autophagy begins with the formation of an isolation membrane (vesicle nucleation step). The concerted action of the autophagy core machinery proteins leads to the expansion of the phagophore into an autophagosome (vesicle elongation). The autophagosome can engulf entire organelles, lipid vesicles or protein aggregates. When the outer membrane of the autophagosome fuses directly with a lysosome (docking and fusion steps), it forms an autophagolysosome. Finally, the autophagic cargo is degraded inside the autophagolyosome by the hydrolases (vesicle breakdown and degradation) and recycled (adapted from Melendez *et al.* 2009).

The MTOR pathway is present in a plethora of organisms and plays an evolutionarily conserved role in aging (Kaeberlein & Kennedy 2008). When MTOR activity is decreased, either blocked pharmacologically by rapamycin (Schmelzle & Hall 2000) or by limited nutrient pool, autophagy is stimulated and it is perceptible an increase in lifespan.

In aged individuals, lower levels of autophagy are responsible for different aspects of the aging phenotype such as: accumulation of damaged proteins and toxic aggregates, changes in regulatory plasma hormones and nutrients, insulin resistance, loss in immune functions, increased morbidity and mortality during infections, declining antibody response and higher incidence of cancer (Cuervo *et al.* 2005).

1.1.1.5 Deregulated nutrient sensing mechanisms

The IGF-1 pathway is one of the most conserved aging-controlling pathways in evolution. It is actived when there is nutrient abundance and in response to circulating levels of growth hormone (GH), release from the anterior pituitary, targetting, also the conserved, Forkhead box protein O1 (FOXO) family of transcriptional factors and the MTOR complexes, known to be involved in aging. Mutations and polymorphisms in any components of this pathway have been implicated in increasing human lifespan, demonstrating the importance of trophic and bioenergetic pathways on longevity.

The MTOR kinase belongs to two multiprotein complexes mTORC1 and mTORC2 that influences all aspects of anabolic metabolism, sensing high amido acids concentrations. When mTORC1 is inhibited genetically, longevity is extended in yeast, worms and flies. In mice when there is deficiencies in the S6K1 protein, the main mTORC1 replacement, but mTORC2 is active

longevity is also achieved. Thus, interferences in the mTORC1/S6k1 axis appears to be a fundamental pathway to increase lifespan. Although supression of MTOR activity appears to have a beneficial effect during aging, it also have less wanted effects such as insulin resistance, cataracts, impaired wound healing and testicular degeneration in mice. Therefore, it is extremely relevant to further investigate the beneficial and damaging effects of MTOR inhibition.

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) and sirtuins have an antagonist effect in comparison with mTOR and the IGF-1 pathway since AMPK signals low-energy states by detecting high AMP levels and sirtuins by detecting high nicotinamide adenine dinucleotide (NAD)+, regulating in this way, catabolism. AMPK when upregulated inhibits mTORC1 and, consequently, mediates lifespan increase in worms and mice and sirtuin when upregulated enhances antioxidant defenses, improves fatty acid oxidation and promotes mitochondriogenesis. In view of that, their upregulation favors healthy aging (Lopez-Otin *et al.* 2013).

1.1.1.6 Mitochondrial dysfunction

Mitochondrial function is vital for the cell since modifications in it are extensively linked to multiple degenerative and acute pathologies. It plays a fundamental role providing energy for the cell through a process called respiration. Respiration yields adenosine triphosphate (ATP) and as a side product it can produce reactive oxygen species (ROS). As we age, mitochondria ages too and it becomes less efficient and actually toxic to the cell. Age-related mitochondrial dysfunction may arise from different mechanisms including reduced biogenesis of mitochondria, alterations in mitochondrial dynamics resulting from mitochondria fission/fusion imbalance, accumulation of mutations and deletions in mtDNA, oxidation of mitochondrial proteins, destabilization of the respiratory chain complexes and defective quality control by mitophagy, an organelle-specific form of macroautophagy that targets deficient mitochondria for proteolytic degradation (Wang & Klionsky 2011). The combination of increased damage and reduced turnover in mitochondria, due to lower biogenesis and reduced clearance, may contribute to the aging process. Excessive ROS and mitochondria deficiencies are seemingly the driving forces in aging because they decrease cellular homeostasis, inflict mutations to the nuclear genome and possibly perpetrate damage to other organelles. As a result, mitochondria dysfunction can accelerate aging and promote disease (Green *et al.* 2011).

1.1.1.7 Cellular senescence

Cellular senescence is mostly trigger by telomere shortening, as mentioned above. However the primary role for senescence is to prevent the multiplication of damaged cells and to clear them by targetting the immune system. This mechanism conceivably is a beneficial compensatory response that helps the system dispose of hypothetically cancer cells and keep the tissues safe from harmful subtrates (Figure 1.2). The number of senescent cells increases with aging due to inefficient immune response. In aged organisms, the turnover system is less capable of cell replacement and the diminish mobilization of stem cells with regenerative capacity may result in the accumulation of cells phenotipically senescent, contributing in this manner to the aging process (Lopez-Otin *et al.* 2013).

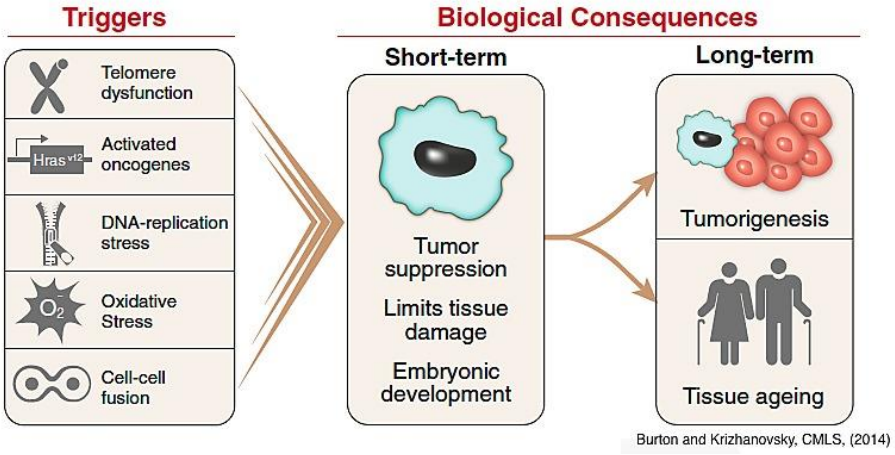

Figure 1.2. Schematic diagram of mechanisms that trigger cellular senescence and its biological consequences. Cellular senescence can be trigger by different mechanisms such as cell-cell fusion, oxidative stress, DNA-replication, activated oncogenes but mostly it can be trigger by telomere dysfunction. Differents outcomes could result from these depending on the time scale. In short-terms these outcomes are beneficial for the cell preventing tissue damage, supressing tumors and participating in embryonic development. In long-terms, these outcomes are harmful and can provoke tumorigenesis and ultimately, tissue aging (adapted from Burton and Krizhanosky, 2014).

1.1.1.8 Stem cell exhaustion

During aging, there is a decline in the cell replicative potential. This is particulary relevant for adult stem cells since the decrease in the regenerative potential of tissues is one of the most obvious characteristic of aging. This limited regenerative potential is linked to aging-associated damages like telomere shortening, accumulation of DNA damage, decrease in cell-cycle activity, deregulated nutrient sensing and cellular senescence demonstrating that stem cell exhaustion urges as the integrative response to numerous different types of damage. To overcome the process of aging, many studies had proposed rejuvenating interventions targetting stem cells

niches. Thus, there is a link between the genetic and molecular pathways of stem cell quiescence and selfrenewal and longevity (Lopez-Otin *et al.* 2013; Rando & Chang 2012) (Figure 1.3).

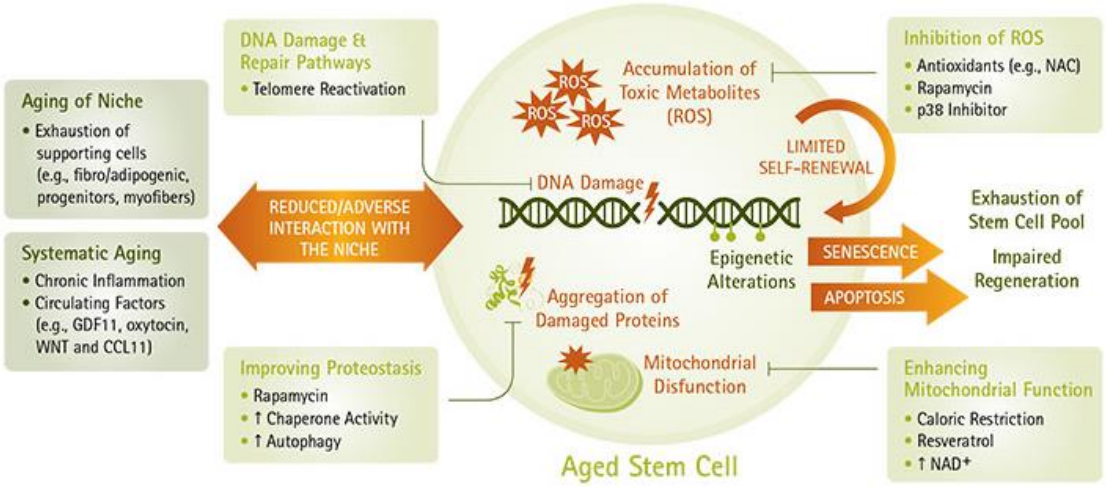

Figure 1.3. Schematic diagram of different interactions in a stem cell that leads to aging. The multiple pathways that contribute to stem cell loss and dysfunction in the aging process. Common aging phenotypes are represented centrally and strategies to overcome and reverse these are represented in lighter boxes (adapted from Oh *et al.* 2014).

1.1.1.9 Altered intercellular communication

With aging the alterations in intercellular communication become more and more significant. The augmented inflammatory response that accompanies aging is called inflammaging and it is the most prominent aging-associated intercellular alteration. It arises from multiple causes such as the accumulation of proinflammatory tissues damage, the decline of immunosurveillance against precancer cells and pathogens, the changes in the peri- and extracellular environment and the defective autophagy. An exacerbated proinflammatory reaction has been proposed to be causal with the aging phenotype because the molecular and genetic pathways activated in response to inflammatory and stress induce numerous aging-related changes. To restore deficiencies in intercellular communication that are present with aging, there are different approches that could be applied including pharmacological, nutritional and genetic therapies (Lopez-Otin *et al.* 2013).

There is an obvious correlation between all hallmarks and it is possible to categorize them into three different classes: the ones that cause damage (primary), the ones which try to repair the damage (antagonistic) and the ones responsible for the aging phenotype (integrative). The primary hallmarks are: genomic instability, telomere attrition and epigenetic alterations. These bring negative consequences in a ubiquitous manner for the cell. The antagonistic hallmarks are: loss of proteostasis, deregulated nutrient sensing and mitochondrial dysfunction. Depending on the intensity of their action, they can have both a beneficial and harmful effect. When overexpressed, these will certainly promote aging. The integrative hallmarks are: cellular senescence, stem cell exhaustion and altered intercellular communication. These affect mostly tissue homeostasis and function.

In summary, these hallmarks co-exist and co-occur during aging and understanding the exact connection and hierarchy between them is fundamental for future interventions to ameliorate human health span and longevity (Lopez-Otin *et al.* 2013) (Figure 1.4).

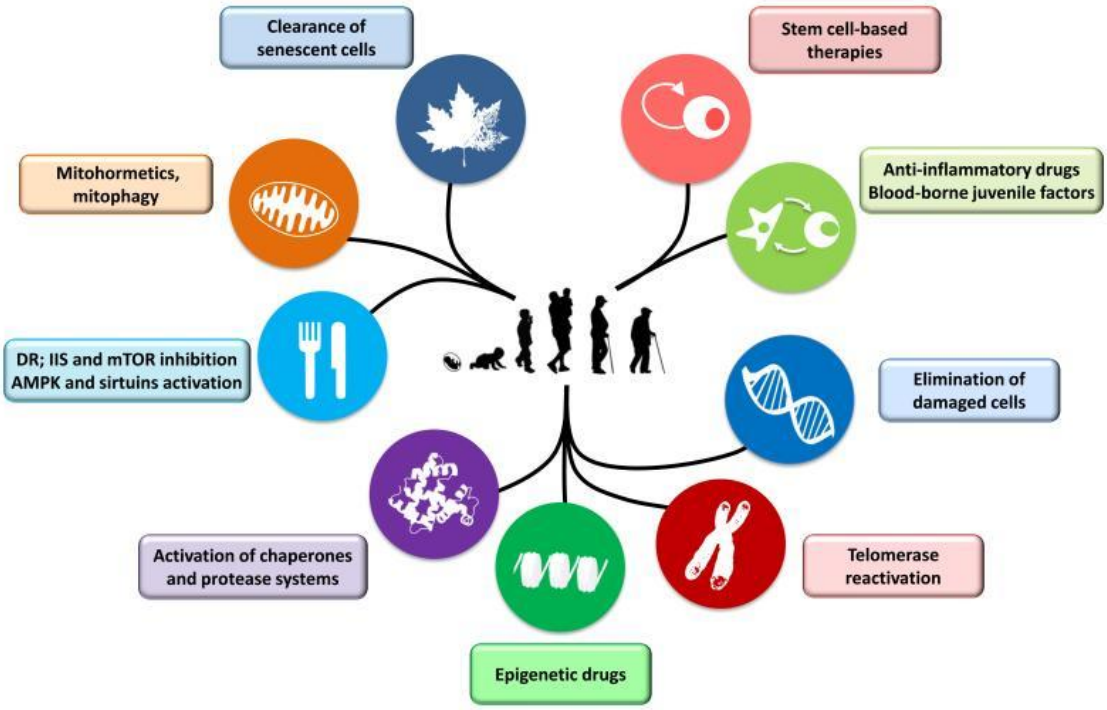

Figure 1.4. Interventions that might extend human healthspan. Although not yet recognized for humans the nine hallmarks of aging are shown together with adequate therapeutic strategies (adapted from Lopez-Otin *et al.* 2013).

1.2 Hutchinson-Gilford Progeria Syndrome (HGPS)

Laminopathies are diseases defined by mutations in lamin A (*LMNA)* gene and usually affect striated muscles, adipose tissue and peripheral nerves. Although its effect could be tissue-specific there are some situations in which the effect is ubiquitous causing premature aging syndromes such as Hutchinson-Gilford Progeria Syndrome (Parnaik *et al.* 2011).

For the first time in 1886, the general practitioner Jonathan Hutchinson described the Progeria Syndrome in a 3 year old boy. In 1895 a second case was mentioned briefly by Hutchinson but Hastings Gilford was the one who described it in great detail. Later, Gilford crossed the information between the two patients and designated the disease as a premature aging syndrome based on the overall resemblance of patients to aged individuals (Hennekam 2006). For more than a century, the characterization of this syndrome was extremely difficult due to the fact that it is one of the rarest diseases in the world, affecting scarcely 1 per 4-8 million children (Gordon L.B, 2013).

1.2.1 HGPS clinical features

Signs and some symptoms can oscillate depending on the degree of severity and age of onset but clinical features of HGPS children are usually very similar. These patients born looking healthy but within the first year of life, children begin to display many characteristics of HGPS such as growth impairment, alterations in the skin and craniofacial struture followed by alopecia, lypodystrophy, joint contractures, bone dysplasia and retarded primary tooth eruption. In the years following these children begin to suffer from conductive hear loss, dental crowding, keratitis, insulin resistance although without developing diabetes mellitus and lack pubertal development or secondary sex characteristics. Motor and intellectually these children are considered normal and as for the liver, kidney, gastrointestinal and immune functions, they are considered normal too. However there are vascular changes such as accelerated vascular stiffening, atherosclerotic plaque formation, vessel occlusive disease and, in later years, valvular and cardiac insufficiency. Consequently, these alterations in the vascular system leads to cardiovascular and cerebrovascular disease that ultimately, constitute the primary cause of morbidity and mortality in these patients. Deaths occurs by myocardial infarction or stroke between the ages of 6 to 20 years, with an average lifespan of 14.6 years (Merideth *et al.* 2008; Gordon *et al.* 2014, Hennekam 2006) (Figure 1.5).

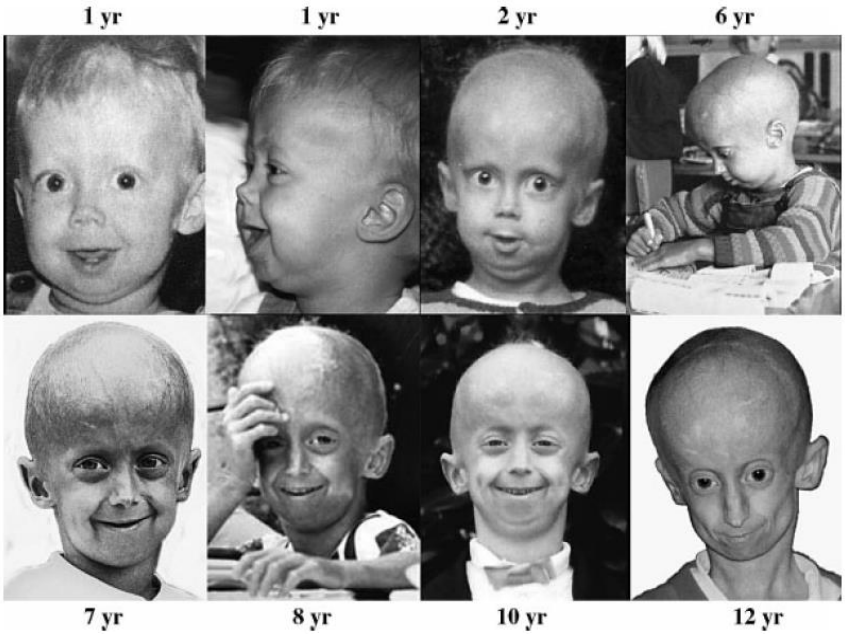

Figure 1.5. HGPS progression in a dutch patient at several different ages (adapted from Hennekam 2006).

1.2.2 HGPS pathophisiology

In the beggining of the 21st century, advances in medicine and genetics allowed the discovery of what causes HGPS. It is now clear that a point mutation in the *LMNA* gene , more specifically in codon 608 within exon 11 (G608G – change from glycine GGC to glycine GGT), is the reason why we observe this sporadic, autossomal dominant pathology where there is an accelerated ageing in children (Eriksson *et al.* 2003; Sandre-giovannoli *et al.* 2003) (Figure 1.6).

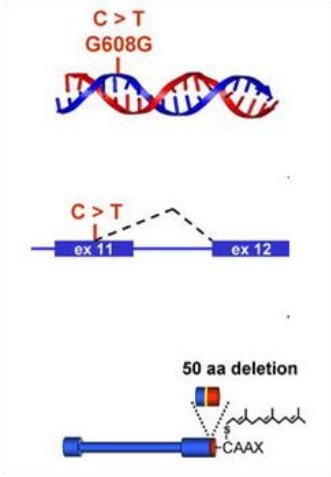

Figure 1.6. HGPS pathophisiology. Mutation in lamin A gene exon 11 (G608G – change from glycine GGC to glycine GGT) leads to HGPS phenotype. (adapted from Scaffidi *et al.* 2005)

1.2.3 Lamins

The lamins are type V intermediate filaments from a multiprotein-family and have globular tail domain, an α-helical rod domain responsible for dimerization and a short N-terminal head domain. They are located within the nucleus of eukaryotes and have multiples functions such as mechanical strengh and integrity, architectural determinants of the nuclear envelope, intermediate essential cell's processes like DNA replication and mRNA transcription (Goldman *et al.* 2002) and participate in signaling and gene regulation (Zastrow *et al.* 2004). There are three lamin genes: the *LMNA* gene which encodes A type-lamins, *LMNB1* gene which encodes lamin B1

protein and *LMNB2* gene which encodes LMNB2 lamins. For the purpose of this work it will be only mentioned the lamin A gene and protein.

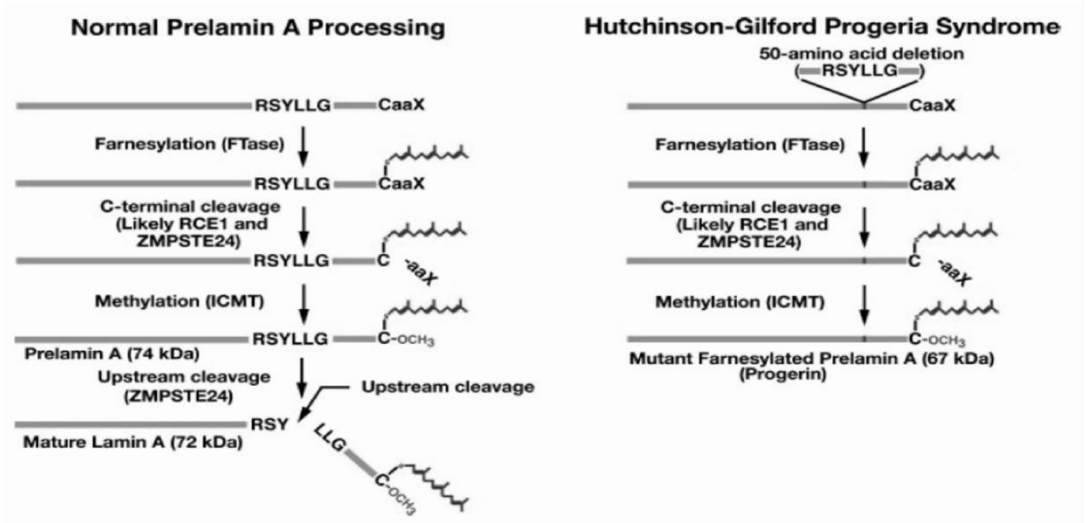

Figure 1.7. Lamin A (in the left) and progerin (in the right) processing. In HGPS, a 50-amino acid deletion in prelamin A eradicates the location for the second endoproteolytic cleavage resulting in a buildup of farnesyl-prelamin A. As a result, no mature lamin A is formed and a farnesylated mutant prelamin A (progerin) accumulates in cells (adapted from Coutinho et al 2009).

1.2.4 Lamin A synthesis and metabolization

Lamin A, as previously said, is a product of the *LMNA* gene. In the first instance it is produced via a prelamin A intermediate whose C-terminal tail ends with a CAAX group (cysteine – aliphatic aminoacid – apliphatic aminoacid – other). Then, such tail, goes through a series of post-translational modifications: first, farnesylation of the terminal cysteine; second, cleavage of –aaX group from C-terminal end by ZMPSTE24 endoprotease; third, carboxymethylation of the terminal cysteine and ultimately enzymatic cleavage of the terminal 15 amino-acids (including the farnesylated and carboxymethylated cysteine) by the ZMPSTE24 endoprotease again. The farnesylation is necessary for the correct localization of the mature lamin A protein in the nuclear envelope (Lutz *et al.* 1992; Glynn & Glover 2005).

In HGPS the point mutation (c.1824C>T; p.Gly608Gly) in the exon 11 causes an abnormal splicing event which removes about 150 nucleotides from that exon. As a result the ZMPSTE24 cleavage site is missing and the last step in the posttranslational processing of prelamin A (the removal of the last 15 amino-acids) does not occur. Hence, the presence of that last 15 amino-acids with farnesyl group leads to the accumulation of a mutant prelamin A isoform called progerin (Figure 1.7).

1.2.5 Progerin and Hallmarks of Aging

Considerable progress has been made in understanding the connection between HGPS and normal aging. In 2006, Scaffidi *et al.* first showed that the normal *LMNA* sequence could produce progerin at a low level in normal individuals, potentially implicating progerin in normal physiological aging (Scaffidi 2006). In addition, progerin-expressing cells from normal individuals mimic some aspects of HGPS cells, further supporting the idea that the premature aging disease HGPS and normal aging may share a common cellular and molecular basis (Cao 2007). Furthermore, a recent study has shown that progerin mRNA levels increase in late-passage cells *in vitro* (Rodriguez 2009), and progerin protein accumulates with age *in vivo* in normal individuals (McClintock 2007). However, the cause-and-effect relationship between progerin production and normal aging has not been clear.

1.2.5.1 Genomic instability

Progerin accumulates in the nuclear envelope and interacts with transcriptional factors and nuclear proteins leading to genomic instability. It can induce nuclear fragility and subsequently nuclear blebbing (Goldman *et al.* 2004), aggregate with the nuclear pores, disorganize and disrupt interactions between DNA and RNA leading to misregulation of gene expression (Coutinho *et al.* 2009) and heterodimerize with wildtype lamin A (Delbarre *et al.* 2006). Consequently there is loss of peripheral heterochromatin, reduce recruitment of DNA damage response and slow repair kinetics, disruption in DNA-lamin A/C association and changes in the spatial chromatin organization which, ultimately, leads to a disruption of the normal nuclear envelope scaffold leading to an irregular nuclei shape (Musich & Zou 2009; Mccord *et al.* 2013). However, irregular nuclei shape such as lobulation or distorted nuclear outlines are present in a noteworthy proportion in senescent fibroblasts from normal humans indicating that nuclear dysmorphology is a common characteristic of progeroid and senescent cells that contributes to the aging process of the cell (Ohshima 2008) (Figure 1.8).

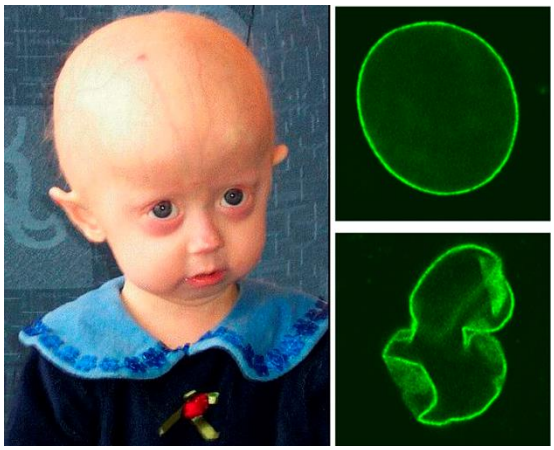

Figure 1.8. Hutchinson-Gilford Progeria Syndrome. HGPS is caused by mutations in the lamin A protein. In HGPS patients the cell nucleus has aberrant morphology (bottom, right) rather than the uniform shape typically found in healthy individuals (top, right) (adapted from Scaffidi *et al.* 2005).

1.2.5.2 Epigenetic alterations

In HGPS cells, A-type lamins are major components of epigenetic regulation. Progerin induces defects in repressive histones marks which are, usually, characteristic of physiological aging. Alterations in the facultative heterochromatin mark trimethylation of histone H3 lysine 27 (H3K27me3) and the decline of retinoblastoma family (Rb) members are the most plausible responsible mechanisms for epigenetic changes in HGPS cells (Gonzalo *et al.* 2010).

1.2.5.3 Telomere attrition

Progerin also appears to modulate telomeric function by activating DNA-damage signaling at the telomeres. In the presence of progerin, interactions between lamins and the ends of cromosomes are possibly disrupted, leading to inefficient mantainance or lack of repair in the telomeres. Likewise, progerin incidence was correlated with telomeric deformations such as telemore fusions establishing that, progerin-induced telomere dysfunction is in charge for the premature cellular senescence in the HGPS condition (Benson *et al.* 2010).

1.2.5.4 DNA damage

DNA damage accumulation not only in telomeres but also in all chromatin is considerate a hallmark of aging and undeniably causes genomic instability leading to cellular senescence. Alongside with these, DNA damage evokes a checkpoint response which influences cell cycle progression or arrest for repair of the damage. Firstly, the process begins with the recognition of what kind of damage was inflicted and then there is initiation of damage responses like cell cycle checkpoints and the phosphorylation of H2AX, a well-know marker of DNA double-strand breaks (DSBs). The ATM and ATR proteins are actived, being a fundamental part in the DNA damage checkpoints, and target checkpoint kinase 1 (Chk1) and Chk2 proteins for phosphorylation leading to a cascade of further down-stream activating signals like phosphorylation of p53. In HGPS cells it was demonstrated that there is accumulation of DSBs with continued passage in culture probably progerin-dependent and, consequently, there is higher levels of γ-H2AX foci, the phosphoralytaded form of H2AX, and higher levels of Chk1, Chk2 and p53 due to persistent ATM and ATR activation by damaged DNA in these cells. The mechanisms of DNA repair are not sufficient to overcome all damaged inflicted and a decrease in the cell cycle is observed alongside with a decline in the proliferative capacity of these cells and an intensification of nuclei dysmorphology (Musich & Zou 2009).

1.2.5.5 Cellular senescence

Cellular senescence is associated with a decrease in the growth rate and a terminal arrest of the cell cycle. In the cellular senescence of normal fibroblasts severals pathways have been implicated in DNA damage, including damage leading to telomere dysfunction such as the p53 and Rb tumor-suppressor pathway. In HGPS it has been reported that p53 pathway might be chronically activated inducing stress signaling pathways and in this way contributing to a decrease in the lifespan of HGPS patients. Recent studies demonstrate that the same effector pathways are involved in HGPS premature senescence as well as normal senescence suggesting that there is a checkpoint response actived by nuclear deficiencies derived from progerin build up in the nuclear envelope and backs up the concept that exarcebated activation of the tumour supressor p53 possibly will arise accelerated aging (Benson *et al.*, 2010; Varela *et al.*, 2005).

1.2.5.6 Mitochondrial dysfunction and loss of proteostasis

In recent studies, it was possible to correlated progerin expression with mitochondrial dysfunction and ROS overproduction. These effects cause an increase in protein misfolding and autophagic proteolysis leading to a generalized loss of proteostasis that undeniably take part in the premature aging process of HGPS (Mateos *et al.* 2015).

1.2.5.7 Stem cell exhaustion

Accelerated aging in HGPS patients is also linked to dysfunctions in adult stem cells and deterioration of tissues functions. Progerin presence in HGPS cells triggers several downstream components of the Notch signalling pathway, a major regulator of cell fate and stem-cell differentiation (Scaffidi & Misteli 2008). As the majority of tissues affected in HGPS are from mesenchymal origin, abnormalities in mesenchymal tissues derive, mostly, from progerin interference with the differentiation pathway of human mesenchymal stem cells (hMSCs). This contributes to reduce the differentiation potencial since progerin changes the molecular and cellular identity of hMSCs. In this manner, cell fate becomes compromise and there is a decline in the tissue homeostasis being these characteristics usually linked to the aging process (Scaffidi & Misteli 2008).

Taken together, the relationship between progerin and the hallmarks of aging is obvious suggesting a possible mechanism by which progerin causes premature aging symptoms in HGPS patients.

1.2.6 HGPS treatment

There is a large spetrum of potencial strategies to treat HGPS, with or without more specificity, since the DNA level until the tissue level. Studies from all over the world suggest two prominent treatments: one to prevent the accumulation of the mutant lamin A protein through farnesyltransferase inhibition (Glynn & Glover 2005; Gordon *et al.* 2012) and another to extend longevity through a combination of an aminobisphosphonate and a statin (Varela *et al.* 2008). In view of these, three independent clinical trials have been proposed to treat the patients with HGPS: one with lonafarnib (the farnesyltransferase inhibitor), another with pravastatin and zeledronate (the aminobisphophonate and statin) and ultimately a combination of both (ClinicalTrials.gov identifier NCT00425607; ClinicalTrials.gov identifier NCT00731016; ClinicalTrials.gov identifier NCT00916747). Treatments with lonafarnib, a farnesyltransferase inhibitor, intented to block the post-translational farnesylation of prelamin A and other proteins that are targets for farnesylation. Farnesylation is essential for the function of both mutant and non-mutant lamin A proteins, including progerin. Although lonafarnib is not curative some of HGPS features were improved by this treatment such as rate of weight gain, increased cardiovascular stiffness, increased bone struture and incresead audiological status. Therefore it offers a putative treatment to improve quality of life and extend longevity for HGPS patients (Gordon *et al.* 2012; Gordon, Massaro, *et al.* 2014). Treatments with pravastatin and zeledronate blocked prelamin A prenylation, had immunomodulatory and antiosteoporotic properties, targeted the proteasome degradation pathway, inhibited the synthesis of cholesterol and blocked angiogenesis (Varela *et al.* 2008). A phase II trial of lonafarnib in HGPS is currently ongoing in USA resorting the ultimately combination between lonafarnib, pravastatin and zoledronic acid. These therapies have been shown successful and could, in fact, ameliorate the HGPS condition (both in celular level and physiological level) (Figure 1.9).

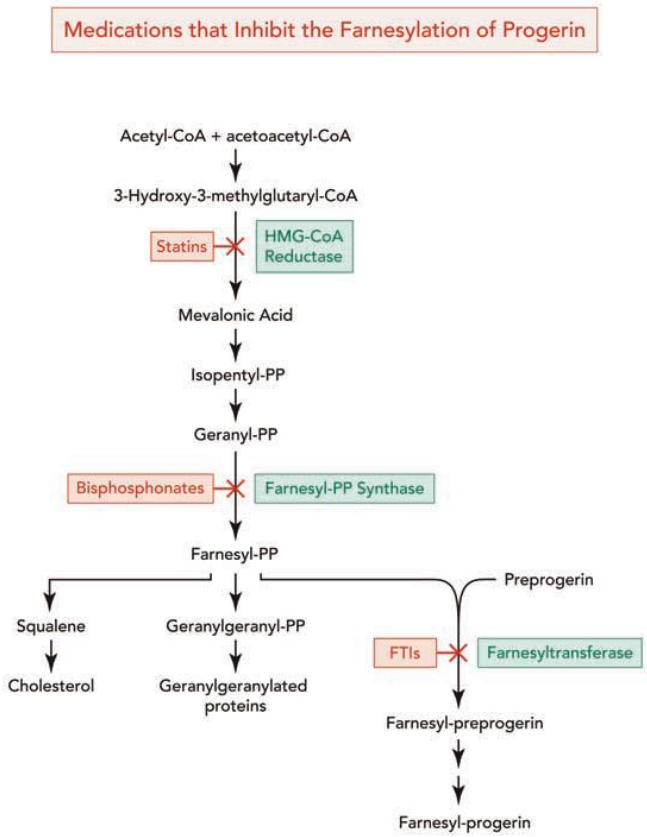

Figure 1.9. Mechanisms by which drugs work in Progeria. Pravastatin, Zoledronic Acid, and Lonafarnib act by blocking (inhibiting) the production or the attachment of the farnesyl group onto progerin (adapted from https://www.progeriaresearch.org).

However, another therapy has been suggested to treat these patients: degradation of the mutant farnesyl lamin A protein using rapamycin. This approach has been strongly proposed because rapamycin can stimulate autophagy by targetting the MTOR pathway and, therefore, could promote progerin clearance by that mechanism (Cenni *et al.* 2011; Cao *et al.* 2011). In regard to that, there is already a clinical trial running to assess the combinatory effect of lonafarnib and everolimus (rapamycin) (ClinicalTrials.gov Identifier NCT02579044).

1.3 Ghrelin

Ghrelin is a natural endogenous ligand for the growth hormone secretagogue receptor (GHS-R), consisting of 28 amino-acids in which the serine 3 residue is *n*-octanoylated capable of stimulating growth hormone release from anterior pituitary gland (Kojima *et al.* 1999) (Figure 1.10).

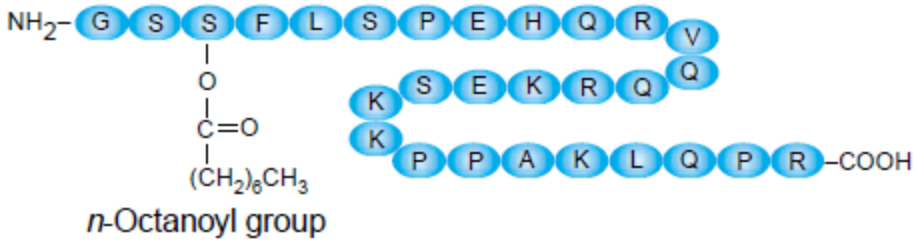

Figure 1.10 Structure of human ghrelin. Ghrelin is a 28-amino acid peptide, in which Ser3 is modified by a lipid, n-octanoic acid. This modification is vital for ghrelin's activity (adapted from Kojima *et al.* 2001).

1.3.1 Ghrelin Gene and ghrelin synthesis

Ghrelin gene is located on the cromossome 3p25-26 and has five exons (Wajnrajch *et al.* 2000; Nakai *et al.* 2004). The first exon is a short non coding region with only 20 bp, translated into a 5' flanking region which contains a TATA box, as well as putative binding sites for a variety of transcription factors. The exons 2 and 3 encode the 28 amino acids of the functional ghrelin peptide. There are two transcriptional initiation sites for the ghrelin gene and therefore two different mRNA transcripts (A and B) (Figure 1.11).

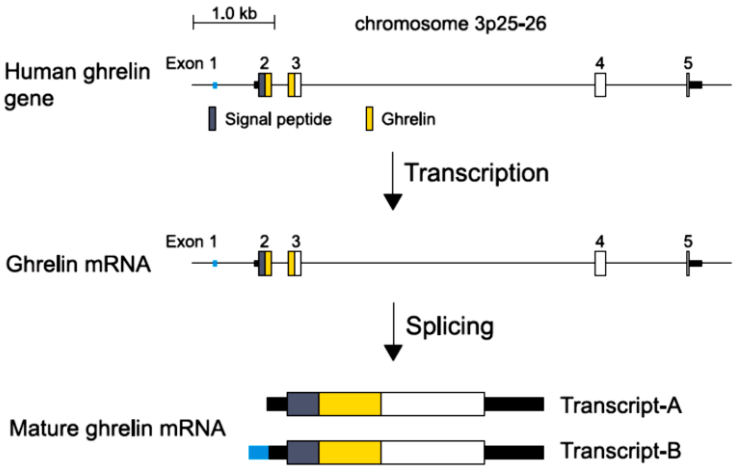

Figure 1.11 Ghrelin gene. The human ghrelin gene comprises five exons. The first exon encodes the 5-untranslated region and is very short. cDNA analyses of human ghrelin have revealed that transcript A, an alternative splicing product from exon 2 to exon 4, is the main form of human ghrelin mRNA *in vivo* (adapted from Kojima *et al.* 2005).

The transcript B is an alternative transcript due to a splicing event in the ghrelin's mRNA processing. That transcript lacks glutamine in N-terminal position 14 and it is called des-gln14 acylghrelin. Still, is fully active on the GHS-R 1a receptor (Hosoda *et al.* 2000). The transcript A is the main form of human ghrelin mRNA *in vivo* and it is translated into a 117 aminoacid preproghrelin (ghrelin percursor). The first 23 amino acids (signal peptide) of the 117 aminoacid preproghrelin are cleaved and a 94 amino acid proghrelin is created. Next, this proghrelin suffers protease cleveage by prohormone covertase 1/3 (Zhu *et al.* 2006) and acyl- modification by ghrelin octanoyl-acyltransferase (GOAT) (Yang *et al.* 2008) generating a mature 28 amino-acid acyl- ghrelin peptide (Kojima *et al.* 2005). It is important to refer that there is strutural conservation between amino acids sequences of mamalian ghrelins, particulary the first ten amino acids in the N-terminal region.

1.3.2 Acyl-ghrelin peptide

Ghrelin requirement to be modify by a fatty acid for its primary biological activity, is of central importance to the function of this peptide. Only acyl-ghrelin can activate its receptor and that is achieved by an intermediate enzyme named GOAT. The fundamental role of GOAT in activating ghrelin could be explained by the following: first, GOAT and des-acyl ghrelin are enough to restart the production of acyl-modified ghrelin in non-ghrelin, non-GOAT cells (Gutierrez *et al.* 2008; Yang *et al.* 2008); second, the tissue distribution of GOAT and ghrelin expressing cells is similar both in humans and mice (Gutierrez *et al.* 2008; Sakata *et al.* 2009; Lim *et al.* 2011); third, GOAT is highy conserved through evolution across vertebrates. From zebrafish, mice, rats to humans all display GOAT activity and sequences of aminoacids much alike to GOAT are present in other vertebrates, consistent with the existence of active octanoylated ghrelin across vertebrates (Gutierrez *et al.* 2008). For the correct ghrelin's metabolic effects, the length of the fatty acid used to acylate is of major importance. The outcome of changing the fatty acid length could result in differential activation of GHSR1a *in vitro* and could alter ghrelin's effect on food intake and adiposity *in vivo* (Heppner *et al.* 2012). Albeit preliminary, modulation of ghrelin's acylation might represent a potential therapy for future interventions.

1.3.3 Ghrelin receptor: Growth-hormone secretagogue-receptor 1

GH is a hormone with multiple essential functions such as regulation of body and cell growth, metabolism and water balance (Kellendonk, Tronche, Reichardt, & Schutz, 1999). It is secreted from the anterior pituitary and it is regulated by hypothalamic growth hormone-releasing hormone (GHRH), somatostanin (Jansson & Dickson 1999) and growth hormone secretagogues (GHS). The above-mentioned GHS are small, synthetic and exogenous molecules with the ability to stimulate and amplify the release of GH (Bowers 1998; Ghigo 1999; Bailey *et al.* 1999; Smith

et al. 1997). GHS chemically bind to the growth-hormone secretagogue receptor 1 (GHS-R1), a full length G-protein with seven transmembranar domains, predominantly expressed in anterior pituitary gland, hypothalamus, hippocampus (Guan *et al.* 1997), adrenal gland, myocardium, pancreas, spleen and thyroid gland (Gnanapavan *et al.* 2002).The *GHS-R1* gene is located on the cromossome 3 at position q26-27 (Smith *et al.* 1997) and has two distinct exons: the first exon encodes for the trasmembranar domain 1 to transmembranar domain 5 and the second exon encodes for the transmembranar domain 6 to transmembranar domain 7. Due to an aberrant splicing event this gene can conceal two transcripts: the GHS-R type 1a which encodes a fully functional receptor for its ligands and GHS-R type 1b which has only 5 transmembranar domains and therefore it is pharmacologically inactive (Davenport *et al.* 2005; Howard *et al.* 2006). The GHS-R is well conserved and widely distributed in all the vertebrate species analyzed indicating its biological relevance (Palyha *et al.* 2000). However, the existence of an endogenous ligand molecule for this receptor was not defined until the identification of ghrelin hormone in 1999 by Kojima and colleagues.

1.3.4 Biological functions of ghrelin

1.3.4.1 GH and food intake

Ghrelin was initially identified in the rat stomach and was correlated with the GH release from the pituitary, indicating that this molecule participates in this process by binding to GHS-R1a receptor. This implicated other regulating pathways besides hypothalamic GHRH for GH stimulation (Kojima *et al.* 1999). Thereafter, it was elucided that ghrelin circulates in blood through the gastric vagal afferent (Date *et al.* 2002), reachs the hypothalamus where via the secretion of the hypothalamic orexigenic hormones neuropepetide Y (NPY) and agouti gene-related proteins (AgRP) can control the food intake and stimulate appetite (Cummings 2001; Nakazato *et al.* 2001; Cowley *et al.* 2003; Toshinai *et al.* 2003; Wren *et al.* 2001). Ghrelin is affected by feeding pattern – fasting conditions induce higher levels of plasma ghrelin and these decrease after nutrient intake. It induces weight gain by promoting appetite and by reducing fat utilization (Cummings 2001; Nakazato *et al.* 2001; Tschop *et al.* 2001; Tschöp *et al.* 2000). NPY and AgRP peptides are essential for ghrelin's function. This was demonstrated in studies where ghrelin-treated NPY and AgRP KO mice did not increase the food intake (Wang *et al.* 2014).

1.3.4.2 Glucose metabolism

Beside regulating energy homeostasis, ghrelin has been discovered to be involved also in glucose metabolism. Ghrelin is expressed in multiple regions such as brain, stomach, intestine, pituary, heart, ovaries, lung, kidney and pancreatic islets (Gnanapavan *et al.* 2002). In the pancreatic islets, it has been elucidate that ghrelin administration influence insulin and glucose

levels by inhibiting insulin but stimulating glucose release (Tassone *et al.* 2003; Dezaki *et al.* 2004; Broglio *et al.* 2001). Hence, ghrelin and insulin are negatively correlated. The effects of ghrelin on insulin are specifically mediated by AgRP neurons in the hypothalamus. It was found that the reexpression of AgRP neuron-selective GHSR in GHSR-null mice totally restored the lowered blood glucose levels observed upon caloric restriction. These were associated with glucagon rises and hepatic gluconeogenesis induction (Wang *et al.* 2014).

1.3.4.3 Adiposity

During high fat diet (HFD) exposure, ghrelin deficient mice reveal that one of the physiological roles for ghrelin is to regulate adiposity and body weight, changing the metabolism and fat deposition. These ameliorations in body weight homeostasis probably lead to improvements in glucose homeostasis by enhancing glucose sensitivity and pancreatic beta cell function. In the same conditions mentioned above, GHSR deficient mice show improved glucose disposal and insulin sensitivity (Müller *et al.* 2015). During aging, deletion of the ghrelin receptor decreases adiposity and improves insulin sensitivity by regulating fat metabolism in white and brown adipose tissues (Lin *et al.* 2011). GH levels might be associated with the effects of ghrelin on glucose metabolism during aging because the circulating GH levels decreased with age and these are correlated with insulin resistance (Müller *et al.* 2015).

1.3.4.4 Cardiovascular system

Ghrelin also has a beneficial effect on cardiovascular diseases. Ghrelin stimulates GH release and its mediator IGF-1, both necessary for metabolic balance and myocardial growth. Since GH/IGF-1 can directly influence the cardiac struture and function, ghrelin is able to affect the cardiovascular system by inducing higher GH levels in blood. Likewise, the release of GH from the pituitary improves energy homeostasis and modulates the autonomic nervous system.

The administration of ghrelin has been validated to reduce cardiac afterload, decrease blood pressure, increase cardiac output without affecting the heart rate, dilate human artery, inhibit apoptosis in endothelial and cardiomyocytes through the activation of Akt serine kinases, supress the sympathetic nerve which is exceedingly activated in cardiac conditions and decrease of pro-inflammatory cytokines which by consequence leads to lesser inflammatory response during cardiac conditions. Together with the localization of GHS-R in the cardiac ventricules and blood vessels, these results suggest that ghrelin might have direct cardiovascular action and become one potential therapy in cardiac diseases such as heat failure, pulmmonary hypertension, myocardial infarction and fatal arrhythmias (Kishimoto *et al.* 2012; Du *et al.* 2014; Baldanzi *et al.* 2002; Okumura *et al.* 2002).

1.3.5 Ghrelin and aging

In addition to all physiological functions that have been mentioned aboved, growing body of evidence suggest that ghrelin also has an anti-aging action in bone metabolism (increase osteoblast differentiation and bone mineral density), inflammation (suppressing the production of cytokines), memory, learning and has a neuroprotective effect in neurodegenerative diseases and ischemic brain injury models (Ferrini 2009; Stengel 2012; Spencer 2013) enhancing cell survival. Ghrelin has also been shown to intermediates gastrointestinal signalling, stimulating gastrointetsinal motility (Strasser 2012) and improves GH/IGF-1 axis (Akamizu *et al.* 2012).

Moreover, a recent study showed that increasing ghrelin signaling ameliorated several age-related disorders and prolonged survival in several animal models of human aging, supporting endogenous ghrelin signaling as an important role in preventing aging-related diseases and premature death (Fujitsuka 2016).

Given the plethora of ghrelin actions, and the fact that its levels are decreased in elderly patients (Akamizu 2006; Nass 2014; Rigamonti 2002), ghrelin administration might be considered a promising strategy to block age-related deteriorations and to increase healthy lifespan.

1.4 Objectives

HGPS syndrome is characterized by premature aging. In HGPS, one of the most disconcerting conditions is accelerated premature cardiovascular disease that leads to fatal myocardial infarcton or stroke. It has been proposed that ghrelin can act positively in the cardiovascular system, in bone remodeling, in glucose metabolism and in inflammation. All of these beneficial effects of ghrelin could, in fact, ameliorate HGPS features, rescuing the aging phenotype and possibly increasing lifespan.

Thus, the aim of the present study is to investigate the potential of ghrelin to delay the premature aging phenotype of HGPS and also contribute to a better understanding of ghrelin's anti-aging role. In order to achieve these goals, primary cultures of human dermal fibroblasts from HGPS patients will be used. These cells will be expose to ghrelin treatment and different age-related cellular alterations will be assess such as progerin accumulation, aberrant nuclear morphology, DNA damage, impaired autophagy and cellular senescence.

Chapter II
Materials and Methods

2.1. HGPS cellular model

2.1.1. Primary cultures of human dermal fibroblasts from HGPS patients

In this study we used primary cultures of human dermal fibroblasts obtained from HGPS patients (HGPSDNF003 and HGPSDNF127) from Progeria Research Foundation, as an *in vitro* model of HGPS. Cells were cultured in high glucose (4.5 g/L D-glucose) Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 15% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Gibco) and 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) and maintained at 37°C and 5% CO_2/air.

2.2. Cell treatment

2.2.1. Ghrelin

To investigate the effect of ghrelin on the mechanisms of cellular senescence, primary cultures of human dermal fibroblasts from HGPS patients were exposed to 1 nM ghrelin during one week, unless otherwise stated, to mimic a chronic treatment. To investigate the effect of this hormone on autophagy, cells were exposed to chloroquine, a lysosomal protein degradation inhibitor (100 µM) 30 minutes before ghrelin treatment (6h).

2.3. Gene expression analysis

2.3.1. Purification and quantification of total RNA

Total ribonucleic acid (RNA) was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

Brifely, cells were disrupted with lysis buffer (RLT) and homogenized by pipetting up and down to disrupt cell clumps. To ensure a complete disruption and homogenization of starting material, the lysate was passed through a QIAshredder homogenizer spin column and centrifuged for 2 min at 14,100 g. Next, 70 % ethanol was added to the homogenized lysate and this was transferred to an RNeasy spin column and centrifuged for 15 seconds at 8,000 g. The total RNA was retained in the RNeasy spin column silica matrix, washed with the recommended buffers and eluted with 30 µL of RNase-free water by centrifugation for 1 min at 8,000 g.

The quantification of total RNA in each sample was done by optical density (OD) measurements using a ND-1000 Nanodrop Spectrophotometer (Thermo Scientific), and the purity was assessed by measuring the ratio of OD at 260 and 280 nm. To avoid any contamination with genomic DNA, RNA samples were treated with RNase-free DNAse (Qiagen). RNA samples were kept at -80 °C until use.

2.3.2. Reverse transcription

Reverse transcription into cDNA was carried out using the iScript Select cDNA Synthesis Kit (Bio-Rad) following the supplier's intructions.

Briefly, 1 µg of total RNA from each sample was reverse transcribed into cDNA in a 20 µL reaction containing 50 units of reverse transcriptase, 1x reaction buffer and 1x random primers. Reverse transcription reactions were executed in a thermocycler at 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and 4 °C for 5 min. cDNA samples were then stored at -20 °C until use.

2.3.3. Quantative-real time Polymerase Chain Reaction (qRT-PCR)

HGPS cells mRNA expression was measured by qRT-PCR performed in the StepOnePLus™ Real-Time PCR System (Applied Biosystems) using 96-well optical plates (Thermo) and the SsoAdvanced™ Universal SYBR® Green Supermix (BioRad). For amplifying human progerin mRNA, the sequence of the forward PCR primer was 5′-CTCAGGAGCCCAGAGCC-3′ and for the reverse primer was 5′-GGCATGAGGTGAGGAGGAC-3′. GAPDH was used as housekeeping gene to normalize progerin expression; the sequence of the forward PCR primer was 5′-TGTTCGACAGTCAGCCGCATCTTC-3′ and for the reverse primer was 5′-CAGAGTTAAAAGCAGCCCTGGTGAC-3′.

A master mix was prepared for each primer set, containing the appropriate volume of 2× SsoAdvanced™ Universal SYBR® Green Supermix and 500 nM of each specific gene primer.
For each reaction, 7 µL of master mix were added to 3 µL of template cDNA (1:50). All reactions were performed in duplicates (two cDNA reactions per RNA sample). No template control (NTC) and no reverse transcriptase control (NRT) were used as negative controls. The reactions were performed according to the manufacturer's recommendations: 95 °C for 3 min, followed by 40 cycles at 95 °C for 5 sec and 59°C for 15 sec. The melting curve protocol started immediately after amplification. The amplification efficiency for each gene and the threshold values for threshold cycle determination (Ct) were determined automatically by the StepOnePLus Software (Applied Biosystems). Relative mRNA quantification was performed using the ΔCt method for genes with the same amplification efficiency. The results are expressed as the relative amount compared to control.

2.4. Protein expression analysis

2.4.1. Cell lysates

After cell treatments, the cell culture plates were rapidly placed on ice, the culture media was removed by aspiration and each well was rinsed twice with ice-cold PBS. The cells were lysed with radio-immunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5

mM EDTA; 1 % (v/v) Triton X-100; 0.5 % (w/v) deoxycholate 0.1 % (w/v) sodium dodecyl sulphate (SDS); 200 µM phenylmethylsulfonylfluoride (PMSF); 1 mM dithiothreitol (DTT); 1 mM sodium orthovanadate (ORTO); 10 mM sodium fluoride (NaF)], supplemented with complete mini protease inhibitor cocktail tablet (Roche). Cells were then collected by scrapping, using a rubber cell-scrapper. The cell lysates were kept on ice for 15 min, being vortexed every 5 min and, thereafter, frozen and preserved at -20 °C, until use. The bicinchoninic acid (BCA) protein assay was used to quantified the protein content in each sample. Before protein determination, samples were centrifuged at 16,100 g, for 10 min, at 4 °C, to remove cell debris, and each supernatant collected to a new tube. The bovine serum albumin (BSA) solution (2 mg.mL-1) was used as standart. After following the supplier´s instructions for protein quantification, the samples were denatured with 6x concentrated electrophoresis sample buffer (0.5 M Tris-HCl, pH 6.8, 30 % (v/v) glycerol, 10.4 % SDS (w/v), 0.6 M DTT, 0.012 % bromophenol blue (w/v)), boiled for 5 min at 95 °C and stored at -20 °C until use.

2.4.2. Western blot

Western Blotting technique was used in order to immunodetect the expression of progerin and LC3B, an autophagy marker. Equal amounts of protein were loaded per lane and proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using 10 %-12 % gels. The electrophoresis was run on a Tris-Bicine buffer (25 mM Tris; 25 mM Bicine; 1 % SDS (w/v); pH 8.3), first at 70 V, for 10 min, and then, at 120-140 V, for 90-120 min. The protein samples were then transferred electrophoretically from the gels to methanol-activated PVDF membranes, in CAPS transfer buffer (10 mM CAPS, pH 11.0; 10% (v/v) methanol), at a 750 mA – 1 A constant current, for 2.5 h, at 4 °C. Afterwards, the membranes were blocked for 1 h at room temperature (RT), in 5 % (w/v) non-fat milk in a Tris-buffered saline (TBS) (20 mM Tris; 137 mM NaCl; pH 7.6) containing 0.1 % (v/v) Tween 20 (TBS-T), and incubated overnight with the primary antibodies, in TBS-T with 5 % (w/v) BSA or non-fat milk, at 4 °C. The primary antibodies used (all at a dilution of 1:1,000) were: mouse monoclonal anti-laminA/C (Developmental Studies Hybridoma Bank; MANLAC1 (4A7)) and rabbit polyclonal anti- microtubule-associated protein light chain-3B (LC3B) (Cell Signaling). After the incubation, membranes were washed with TBS-T and incubated with an alkaline phosphatase-linked secondary antibody, specific to rabbit or mouse IgG, in a 1:10 000 dilution in TBS-T with 5 % (w/v) BSA or non-fat milk, for 1 h, at RT. The membranes were then rinsed in TBS-T and protein immunoreactive bands were visualized by chemifluorescence using the ECF substrate (GE Healthcare). Fluorescence was detected on Versa Doc Imaging System (Bio-Rad). The optical density of the bands was quantified with the Quantity One Software (Bio-Rad). Membranes were, then, reprobed overnight with mouse monoclonal anti β-tubulin I immunoreactivity (Sigma), in

a 1:1 000 dilution in TBS-T with 5 % (w/v) non-fat milk, at 4 °C, for protein loading control. After being washed in TBS-T, the membranes were incubated with an alkaline phosphatase-linked secondary antibody, specific to mouse IgG, in a 1:10 000 dilution in TBS-T with 5 % (w/v) non-fat milk, for 1 h, RT. The OD of the bands was quantified with the Quantity One Software (Bio-Rad). The results are normalized to β-tubulin and are expressed as the relative amount compared to control.

2.4.3. Immunocytochemistry

After the treatments, cells were washed twice with PBS (pH 7.4) at 37°C and then fixed in 1 mL of ice-cold 4% paraformaldehyde for 15 minutes. Next, cells were rinsed three times with ice-cold PBS and permeabilized with 0.1 % (v/v) TX-100/PBS for 10 minutes at room temperature. Once more, cells were washed twice with PBS and block with 500 µL of 3% BSA/10% goat serum/ PBS for 1h at room temperature. Then, cells were incubated with primary antibody overnight at 4°C. The primary antibodies used were mouse monoclonal anti-progerin (1:500; Sigma), mouse monoclonal anti-H2AX (1:500; Millipore) and mouse monoclonal anti-Ki67 (Dako). After incubation, cells were washed three times for 5 min with PBS and incubated with the secondary antibody (50 µL of blocking solution plus antibody/Hoechst) for 1h at room temperature. The secondary antibodies used were Alexa-Fluor 488- or Alexa Fluor 594-conjugated goat anti-mouse IgG. The nuclei were stained with Hoechst 33342 (2 µg.mL-1; Sigma-Aldrich) during secondary antibody incubation. Lastly, cells were rinsed three times with PBS and the coverslips were mounted on glass slides with Aqua-Polymount (Polysciences, Inc.) mounting medium. Cells were analyzed in a Axio Observer Z1 fluorescence microscope or LSM 710 confocal microscope (Carl Zeiss).

Quantification of Ki-67-positive cells and misshapen/blebbing nuclei was done by direct counting of positive cells in 40 randomly chosen, non-overlapping fields (x400 magnification), normalized to the total number of nuclei stained with Hoechst for each coverslip of each experimental condition. Results represent the mean ± SEM of four independents experiments (at least 400 cells analyzed for each experimental condition) and are expressed as percentage of HGPS. The analysis of nuclei morphology parameters was performed using FIJI (Fiji is Just ImageJ) software, through the use of a home-made macro. Briefly, images were thresholded and nuclei were automatically detected based on their size (100-infinite) and circularity (0.3-1). Then, all images were manually reanalysed to confirmed the automatic selection. Finally, the following parameters were measured for each selected nucleus: area, perimeter and circularity (4π*area/perimeter^2).

For H2AX foci quantification 20 randomnly choosen, non-overlapping z-stacking images were acquired on a Carl Zeiss LSM 710, using a Plan ApoChromat (63x/1,4 oil-objective, and laser

lines DPSS 561-10 for H2AX staining and Diode 405 for Hoechst staining. First, the nuclei in all images using the nuclear morphology parameters macro above described. Then, the FindFoci plugin was applied (Herbert *et al.* 2014) in order to identify the peak corresponding to each foci in each nuclei, and setting the minimum peak size above saddle on 5. The number of foci/nuclei, the number of foci/nuclei area and the percentage of area were calculated for each nuclei and condition.

2.4.4 Senescence associated β galactosidase assay (SA- β-Gal)

Medium from HGPS treated cells was removed and cells were washed twice with PBS at room temperature. Next, cells were fix with ice-cold 4% paraformaldehyde for 3 minutes. Cells were then rinsed twice with PBS at room temperature. Afterwards, cells were exposed to fresh SA-β-Gal staining solution (2 mL) composed of 1x citric acid/sodium phosphate buffer (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, and 1 mg/mL X-Gal in water and were incubated overnight in a non-CO_2 incubator, at 37°C. Staining solution was washed away, cells were rinsed twice with PBS and, lastly, the coverslips were mounted on glass slides with Aqua-Polymount mounting medium). Cells were analysed by brightfield microscopy on a Zeiss Axio Imager Z2 microscope (Carl Zeiss, Oberkochen, Germany). Quantification of SA-β-Gal-positive cells was performed by direct counting of positive cells in 30 randomly chosen, non-overlapping fields (x200 magnification), for each coverslip of each experimental condition.

2.5. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's post test, or Student's unpaired t test with two-tailed p value, as indicated in figure legends. A value of $p<0.05$ considered significant. Prism 6.0 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis.

Chapter 3
Results

3.1. Ghrelin decreases progerin accumulation in HGPS fibroblasts

HGPS is commonly caused by a *de novo* point mutation in exon 11 (residue 1824,C →T) of the *LMNA* gene that partially activates a cryptic splice donor site, producing an abnormal lamin A protein termed progerin (Cao *et al.* 2003; Eriksson et al. 2003; Sandre-giovannoli et al. 2003). Accumulation of progerin within the nucleus causes nuclear and mitotic abnormalities and cell cycle arrest, ultimately leading to cellular senescence (Cao *et al.* 2007; Dechat *et al.* 2007; Goldman *et al.* 2004; McClintock *et al.* 2006), and therefore, is one of the mechanisms underlying the progression of HGPS. To evaluate the hypothesis that ghrelin could ameliorate HGPS fibroblasts senescent phenoype, we first evaluated ghrelin effect on progerin levels. HGPS fibroblasts were incubated with ghrelin (1 nM) for one week, to mimic a chronic treatment, and progerin protein levels were evaluated by Western blotting and immunocytochemistry. As shown in Figure 3.1 A and B, ghrelin treatment induced a significant decrease in progerin protein levels (51±12% of HGPS) in HGPS cells. We also observed that progerin immunoreactivity decreased or is absent in HGPS fibroblasts (Figure 3.1 C). These observations suggest that ghrelin promotes progerin degradation in HGPS fibroblasts.

To investigate if progerin degradation was due to a decrease in progerin expression, total RNA was isolated from non-treated and ghrelin-treated HGPS fibroblasts and progerin transcript levels were analysed qRT-PCR. As shown in Figure 3.1 D, progerin mRNA levels were not altered upon ghrelin treatment (85.1±10.0% of HGPS), suggesting that the effect of ghrelin on progerin occurs most likely at the translational or post-translational level.

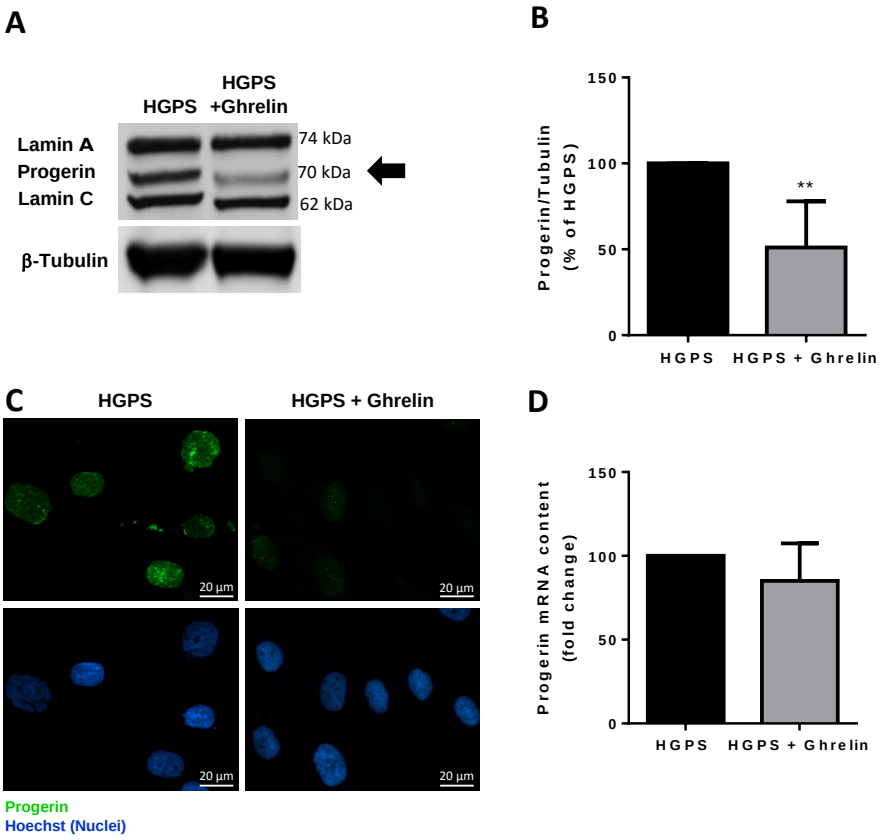

Figure 3.1 Ghrelin decreases progerin levels in HGPS fibroblasts.

Primary HGPS fibroblasts were exposed to ghrelin (1 nM) for 1 week (HGPS + Ghrelin). Untreated cells were used as control (HGPS). (A) Whole cell extracts were assayed for Lamin A/Progerin/Lamin C (A) and β-tubulin (loading control) immunoreactivity through Western blotting analysis. Representative Western blot of four independent experiments is shown. (B) Densitometric quantification of progerin levels in HGPS cells. (C) Ghrelin decreased progerin immunoreactivity. Cells were immunolabeled for progerin (green) and nuclei were stained with Hoechst (blue). Representative images of three independent experiments are shown. (D) qRT-PCR analysis of progerin mRNA levels in HGPS fibroblasts. The results represent the mean ± SEM of, at least, four independent experiments, and are expressed as percentage of HGPS. **p<0.01, significantly different compared to HGPS; as determined by Student's t test.

3.2. Ghrelin improves nuclear morphology in HGPS fibroblasts

HGPS is mostly characterized by aberrant nuclear morphology due to accumulation of progerin protein in the nuclear envelope (Cao *et al.* 2007; Dechat *et al.* 2007; Goldman *et al.* 2004; McClintock *et al.* 2006). To investigate whether ghrelin could ameliorate nuclear morphology of HGPS fibroblasts, cells were exposed to 1 nM ghrelin during one week and immunocytochemistry with a nuclear dye was performed to assess the number of dysmorphic nuclei, nuclear circularity and area. As shown in Figure 3.2 A and B, ghrelin decreased the number of dysmorphic nuclei in HGPS fibroblasts. In addition, when analyzing the circularity index of these nuclei, we observed that ghrelin decreased by 2.5-fold the frequency of nuclei with a circularity index <0.6 (aberrant nuclear shape) and increased by ~2-fold the frequency of nuclei with a circularity >0.8 (normal nuclear shape) (Figure 3.2 C), suggesting that ghrelin ameliorated nuclear morphology in HGPS fibroblasts. The nucleus of a senescent cell is often irregular and lobed and its area is larger compared to the nucleus of a normal cycling cell, being therefore considered as a marker of cellular senescence (Cristofalo *et al.* 1993). Concomitant with a nuclear circularity improvement, ghrelin decreased the nucleus area of HGPS fibroblasts (Figure 3.2 D and E). In addition, we observed that the number of nuclei with a smaller area increased but the number of nuclei with a larger area decreased upon ghrlein treatment, suggesting an increase in cellular proliferation and a decrease in cellular senescence, as shown in Figure 3.2 F, suggesting that ghrelin improves nuclear morphology in the HGPS condition.

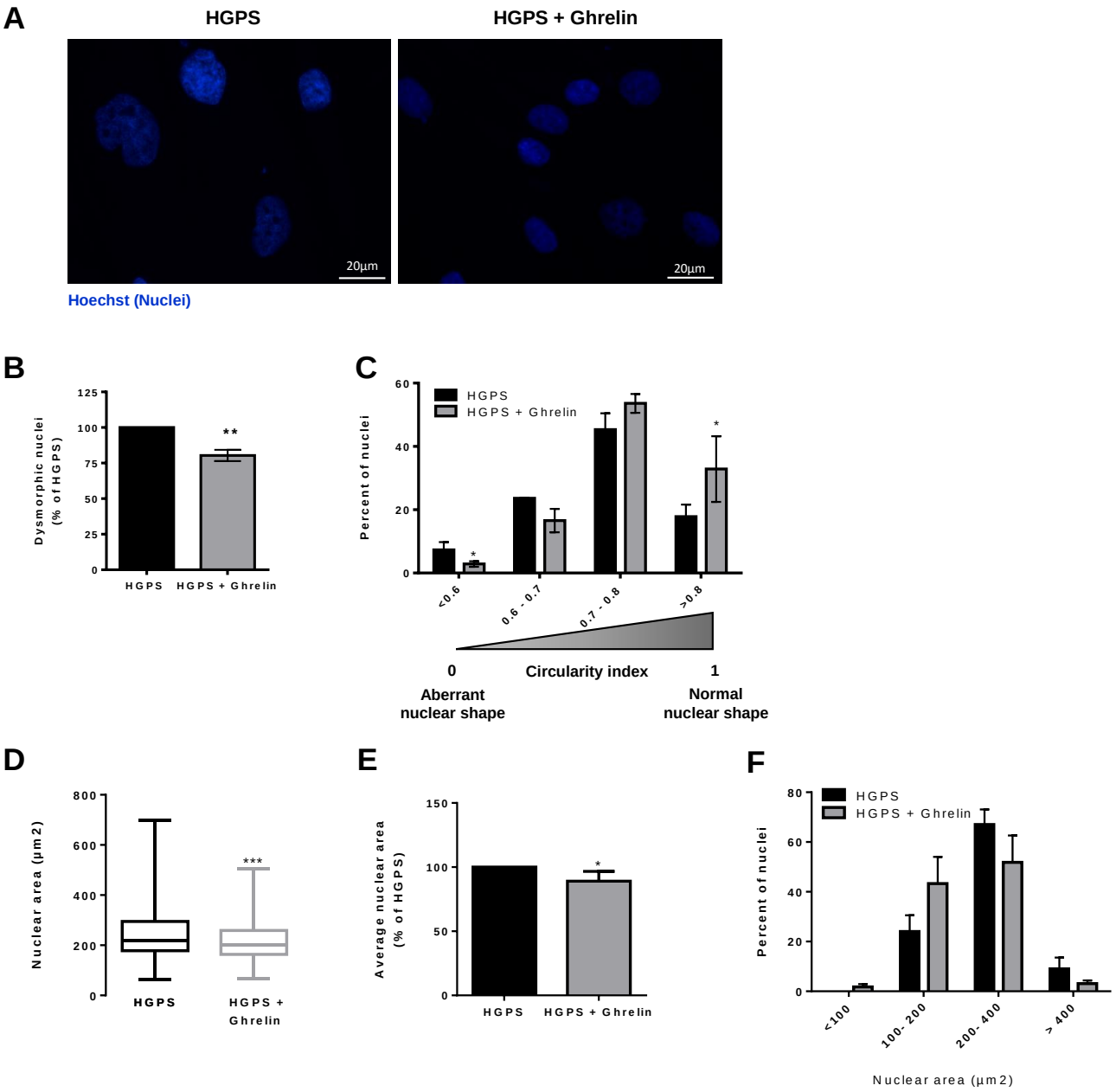

Figure 3.2. Ghrelin decreases the number of dysmorphic nuclei and improves nuclear morphology in HGPS fibroblasts.

HGPS fibroblasts were exposed to ghrelin (1 nM; HGPS + Ghrelin) for 1 week. Untreated cells were used as control (HGPS). (A) HGPS fibroblasts nuclei were stained with Hoechst (blue). Representative images of four independent experiments are shown. Quantification of the number of dysmorphic nuclei (B), nuclear circularity (C) and area (D and E) upon ghrelin treatment. For each condition, an equal number of nuclei (>400) were randomly analyzed. Circularity (defined as: 4* π *area/perimeter^2) and area (μm^2) was measured using ImageJ. A circularity value equal to 1 corresponds to perfect circular nuclei. (D) Boxplot of the nucleus area of all the cells analyzed in each experimental group. (E) Nuclear area average of all the cells analyzed in each experimental condition. (F) Distribution of nuclei per nuclear area ranges. The results represent the mean ± SEM of four independents experiments, and are expressed as percentage of HGPS. *p<0.05, **p<0.01 and ***p<0.001, significantly different from HGPS, as determined by Student's t test.

3.3. Ghrelin decreases DNA damage in HGPS cells

It is well accepted that cellular DNA damage accumulation is a hallmark step leading to premature aging (Lopez-Otin *et al.* 2013). A common feature of progeria syndromes is a premature aging phenotype accompanied by an accumulation of DNA damage arising from a compromised repair system (Benson *et al.* 2010; Musich & Zou 2009; Musich & Zou 2010; Liu *et al.* 2005; Liu *et al.* 2013; Liu *et al.* 2013a). Progerin accumulation causes chromatin alterations which result in the formation of DSBs and an abnormal DNA-damage response (DDR). The accumulation of these DSBs causes genome instability, eventually leading to cellular senescence (Benson et al. 2010; Musich & Zou 2009). As ghrelin decreased progerin accumulation in HGPS cells, we hypothesized that ghrelin treatment could also decrease DNA damage in these cells. To test this hypothesis, we evaluated the effect of ghrelin on DNA damage by evaluating γH2AX foci, a DSBs marker. HGPS cells were incubated with ghrelin (1 nM) for one week and then immunocytochemistry was performed against γH2AX. As shown in Figure 3.3 A, there was a decrease in γH2AX immunoreactivity alongside with a significant decrease in the number of γH2AX foci (Figure 3.3 B and C) in ghrelin-treated HGPS fibroblasts. These observations suggest that ghrelin decreases DNA damage in HGPS fibroblasts, probably by decreasing progerin accumulation.

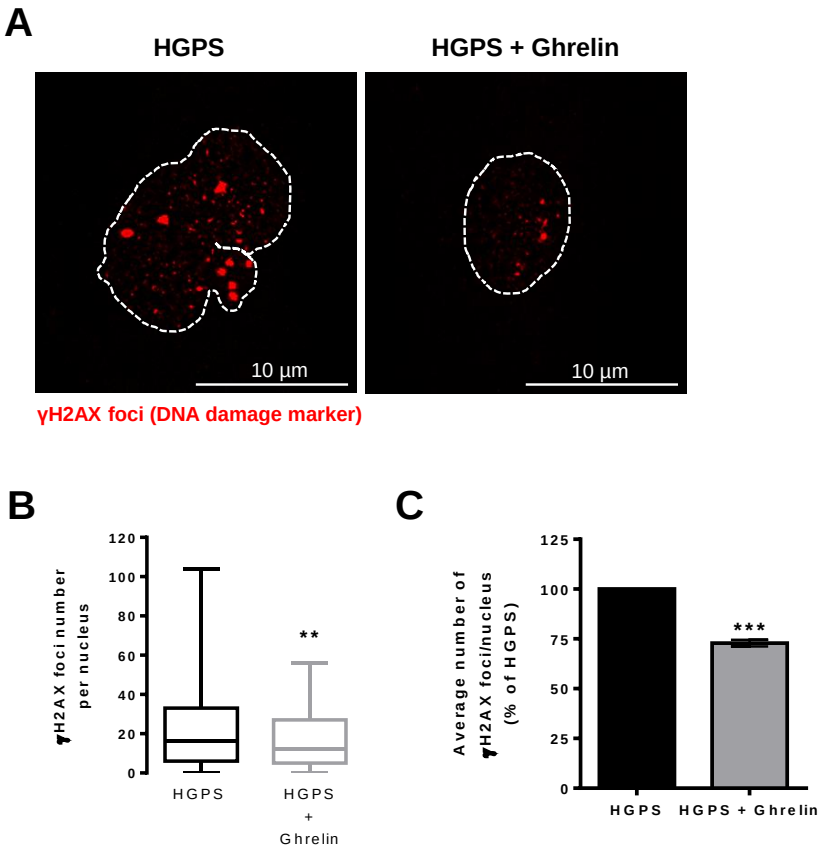

Figure 3.3 – Ghrelin decreases DNA damage in HGPS fibroblasts.

HGPS fibroblasts were exposed to ghrelin (1 nM; HGPS + Ghrelin) for 1 week. Untreated cells were used as control (HGPS). (A) Ghrelin decreased γH2AX immunoreactivity. Cells were immunolabeled for γH2AX (red) and nuclei were stained with Hoechst (blue). Representative images of three independent experiments are shown. (B-D) Quantification of γH2AX foci number using ImageJ analysis and customized macros of three independent experiments; >200 cells analysed (B) Boxplot of the number of γH2AX foci per nucleus in all the cells for each experimental group. (C) Average number of γH2AX foci per nucleus. Data are expressed as percentage of HGPS. **p<0.01; ***p<0.001, significantly different from HGPS, as determined by Student's t test.

3.4. Ghrelin enhances autophagy in HGPS cells

HGPS patients display features of premature aging due to accumulation of progerin within the nucleus. Impared autophagy and loss of proteostasis could be one of the leading causes for progerin build up (Cao et al. 2011). To analyze if ghrelin could improve this hallmark of aging, enhancing autophagic flux, HGPS fibroblasts were treated with ghrelin (1 nM) for 6 hours, in the presence and absence of chloroquine (Chloroq, 100 μM), a lysosomal degradation inhibitor. Then, the whole extracts were collected and the levels of LC3B, an autophagy marker was evaluated by Western blotting. Upon autophagy induction, the cytoplasmic form of LC3B is converted by cleavage and lipidation to a transient, autophagosomal membrane-bound form of LC3B (LC3B-II). LC3B-II is an essential component of autophagosomal membrane and a marker of all autophagic structures, immature and mature autophagosomes as well as autophagolysosomes. Thus LC3B-II levels are widely used as marker for monitoring the autophagic process (Klionsky *et al.* 2012).

As shown in Figure 3.4 A, ghrelin increased LC3B-II levels (128.7±4.8% of HGPS) in HGPS fibroblasts. To rule out the possibility that the increase of LC3B-II was due to an inhibited autophagosome degradation rather than autophagosome formation, we evaluated endogenous autophagic flux in the absence and presence of chloroquine, an inhibitor of autophagic degradation (Klionsky *et al.* 2012). In the presence of chloroquine, the increase in LC3B-II levels were significantly higher than in cells treated with ghrelin alone (Figure 3.4 A). In addition, when comparing the differences in the amount of LC3B-II between samples in the presence and absence of chloroquine (autophagic flux), we observed that ghrelin increased LC3B-II net flux (145.8±16.8% of HGPS), in HGPS fibroblasts (Figure 3.4 B). These results suggest that ghrelin enhances progerin clearance, probably through autophagy flux stimulation.

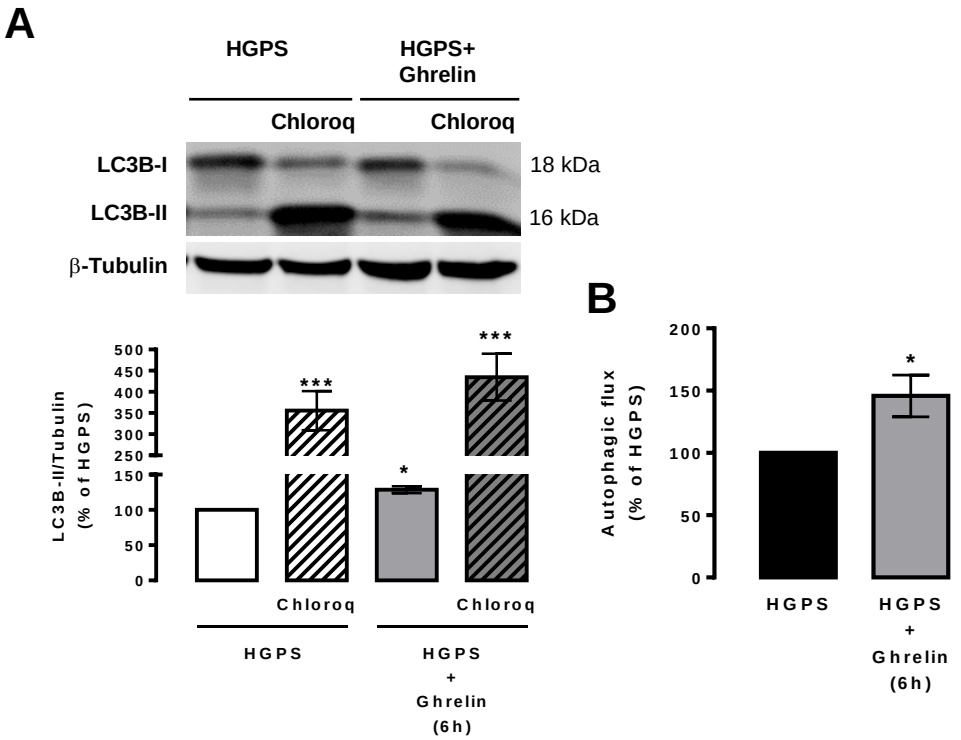

Figure 3.4. Ghrelin increases autophagic flux in HGPS fibroblasts.

HGPS fibroblasts were exposed to ghrelin (1 nM) for 6 h (HGPS + Ghrelin) in the presence or absence of chloroquine (Chloroq, 100 μM), a lysosomal degradation inhibitor. Untreated cells were used as control (HGPS). Whole cell extracts were assayed for LC3B (A) and β-tubulin (loading control) immunoreactivity through Western blotting analysis. Representative Western blots for each protein are presented above each respective graph. Autophagic flux analysis in HGPS cells (B) is shown. Autophagic flux was determined in the presence of the lysosomal inhibitor chloroquine, and expressed as "Autophagic flux" calculated by subtracting the densitometric value of LC3B-II - Chloroq from those corresponding LC3B-II + Chloroq values. The results represent the mean ± SEM of, at least, four independents experiments, and are expressed as percentage of HGPS. *p<0.05, ***p<0.001, significantly different compared to HGPS; as determined by ANOVA, followed Bonferroni's post test, or Student's t test.

3.5. Ghrelin increases cell proliferation and delays cellular senescence in HGPS fibroblasts

Senescence, a hallmark of aged fibroblasts, is characterized by a loss of proliferation and an increase in SA-β-Gal activity (Dimri *et al.* 1995). To investigate the potential proliferative effect of ghrelin, HGPS fibroblasts were incubated with 1 nM ghrelin for one week and the immunoreactivity of Ki-67, a well known proliferation marker, was evaluated by immunocytochemistry. We observed that ghrelin increased HGPS fibroblasts proliferative capacity, as determined by an increase in the number of Ki-67-positive cells (150.1±6.4% of HGPS; Figure 3.5 A and B). To assess whether this increase in cell proliferation was correlated with a decrease in cellular senescence, we evaluated SA-β-Gal activity in HGPS fibroblasts. As shown in Figure 3.5 C and D, ghrelin decreased SA-β-Gal (53.1±5.9% of HGPS). Fewer SA-β-Gal–positive cells were observed in ghrelin-treated cells, indicating that ghrelin slowed down the progression of cellular senescence. These results suggest that ghrelin enhances cell proliferation and delays cellular senescence in HGPS fibroblasts.

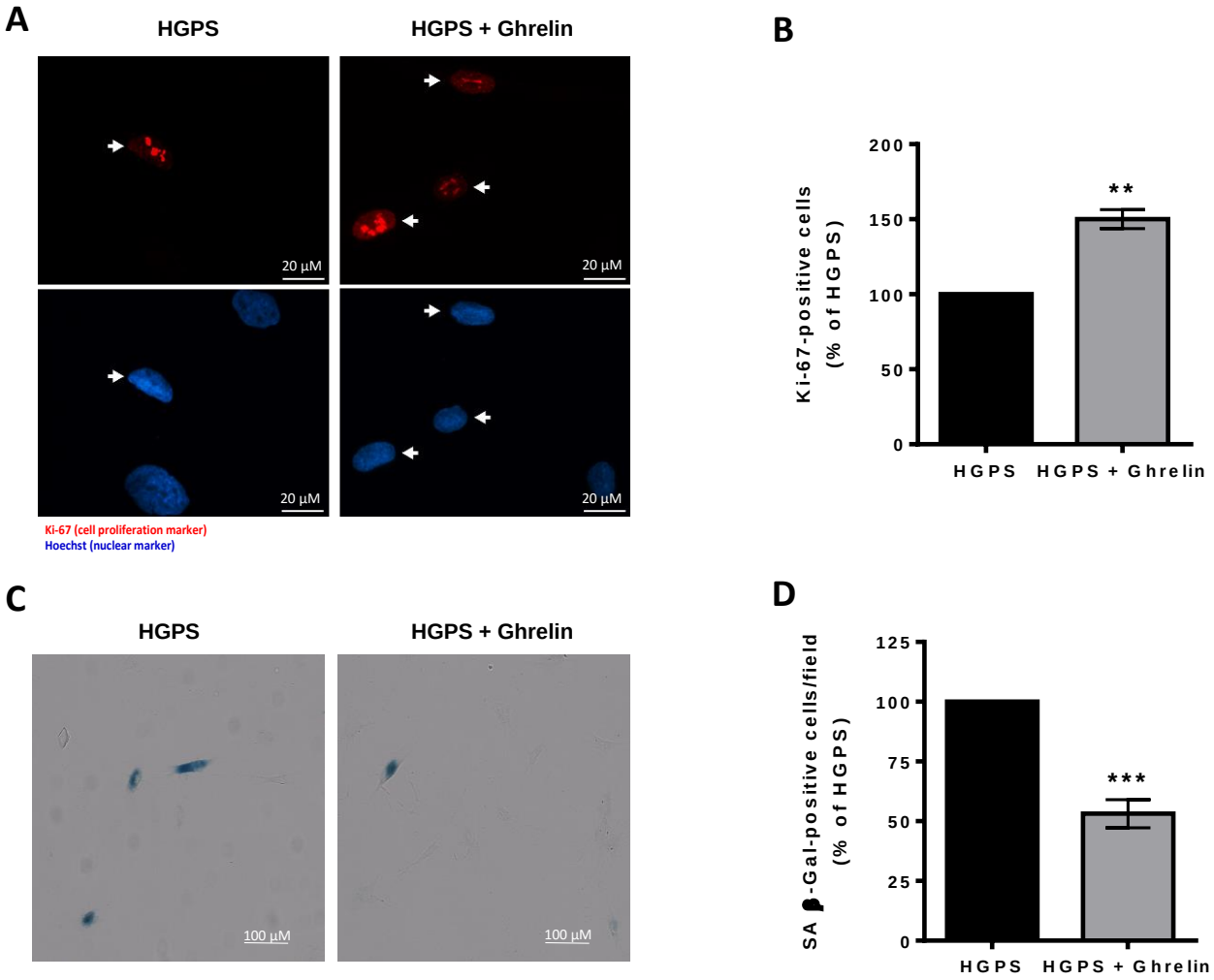

Figure 3.5. Ghrelin increases cell proliferation and decreases cellular senescence in HGPS fibroblasts.

HGPS fibroblasts were exposed to ghrelin (1 nM; HGPS + Ghrelin) for 1 week. Untreated cells were used as control (HGPS). (A) Ghrelin increases cell proliferation, as determined by Ki-67 immunoreactivity. Cells were immunolabeled for Ki-67 (red) and nuclei were stained with Hoechst (blue). Representative images of five independent experiments are shown. (B) Quantification of the number of Ki-67-positive cells in HGPS and ghrelin-treated HGPS cells. (C) Ghrelin decreases the number of senescent cells, as determined by SA-β-Gal activity. Representative images of four independent experiments are shown. (D) Quantification of SA-β-Gal-positive cells. All the results represent the mean ± SEM of, at least, four independents experiments, and are expressed as percentage of HGPS. **p<0.01, ***p<0.001, significantly different from HGPS, as determined by Student's t test.

Chapter 4
Discussion

HGPS is a rare, fatal genetic disorder affecting 1 per 4–8 million new borns that is characterized by a premature and accelerated aging. The most disturbing aspect of this disease is accelerated, premature cardiovascular disease that leads to fatal myocardial infarction or stroke by an average age of 14.6 years (Hennekam *et al.* 2006; Capell *et al*. 2006). Several evidences support that progerin accumulation, a mutant lamin A isoform, is responsible for the HGPS phenotype (Columbaro *et al*. 2005; Scaffidi et al. 2005; Shumaker *et al.* 2006). In line with this, several treatments have been proposed to improve the HGPS condition by acting on progerin. Two clinical trials were performed independently using pravastatin and zeledronate, a aminobisphophonate and statin, respectively, and lonafarnib a farnesyltransferase inhibitor. Both clinical trials shown successful results in ameliorating the HGPS condition such as rate of weight gain, increased cardiovascular stiffness, increased bone struture, incresead audiological status, blocked prelamin A prenylation, had immunomodulatory and antiosteoporotic properties, targeted the proteasome degradation pathway, inhibits the synthesis of cholesterol and block angiogenesis. A phase II trial of lonafarnib in HGPS is currently ongoing resorting the ultimately combination between lonafarnib, pravastatin and zoledronic acid. However different approaches beside these have been proposed, possibly the most promissing one , until moment, is from Cao and colleagues study which shows that treatment with rapamycin stimulated autophagy in HGPS fibroblasts, enhancing progerin clearance, improvements in nuclear blebbing, cellular proliferation, less epigenetic dysregulation and genomic instability (Cao *et al.* 2011). Rapamycin treatment is known to extend longevity in several organisms, including mammals (Harrison et al. 2009; Miller *et al*. 2014; Powers RW 2006), and has been considered as a therapeutic strategy for HGPS. In fact, a third clinical clinical trial it will assess a two-drug combination of lonafarnib plus everolimus (rapamycin) based in the fact that lonafarnib can block progerin from developing and that rapamycin can promote progerin clearance more rapidly targetting a different pathway than lonafarnib. However, the adverse effects associated with rapamycin, as well as the lack of aging-specific human data, should be taken in consideration before using it as an anti-aging agent.

Ghrelin is a circulating peptide hormone that, through GHS-R1a activation (Kojima *et al*. 1995), targets the hypothalamus and the pituitary to release GH and regulate the energetic and metabolic balance by increasing food increase, body weight and adiposity (Kojima *et al.* 1995; Howard *et al.* 1996; Nakazato *et al.* 2001; Tschop *et al.* 2000; Wren *et al.* 2001; Finger *et al.* 2011; Wren *et al.* 2001a; Wren *et al.* 2000). Besides its orexygenic actions, ghrelin is involved in the regulation of cardiovascular functions (shown to be cardioprotective after heart damage), bone remodeling and inflammation (Sato *et al.* 2012; Stengel *et al.* 2012; Nagaya *et al.* 2004; Nagaya *et al.* 2001). Moreover, ghrelin is already being used in several clinical trials as a therapeutic strategy for the treatment of cachexia in chronic heart failure, cancer, endstage-renal-disease or cystic

fibrosis, frailty in elderly, anorexia nervosa, growth hormone deficient patients and sleep-wake regulation (Akamizu *et al.* 2012; Strasser 2012).

In this study we explored the potential of ghrelin to delay the premature aging phenotype of HGPS. Using primary cultures of human dermal fibroblasts from HGPS patients as an HGPS experimental model, we observed that ghrelin decreased progerin accumulation, the major cause of HGPS. The exact mechanism by which progerin protein is decreased is not clear, although as we observed no alterations on progerin mRNA content upon ghrelin treatment, the results suggest that ghrelin acts on the translation or post-translational level. Moreover, the fact that ghrelin increases autophagic flux in a significant manner in HGPS fibroblasts, as rapamycin do (Cao *et al.* 2011), autophagy could provide the putative mechanism by which ghrelin enhances progerin clearance. To confirm the hypothesis that progerin is eliminated by autophagy it is relevant to assess the levels of this protein in the presence of chloroquine, an autophagy inhibitor degradation, and ghrelin.

Irregular nuclei shape is one of the most prominent features of HGPS due to the accumulation of progerin. Since progerin is cleared in HGPS fibroblasts treated with ghrelin, as stated before, we hypothesized that nuclear morphology could be improved in the absence of this mutant isoform. In fact, our results show that ghrelin improves nuclear circularity and decreases nuclear area, decreasing the number of dysmorphic nuclei, which can be explained by progerin's absence from the nuclear envelope. As follows, normal lamin A protein could line together along the nuclear envelope, devoided of progerin intereferences, and reconstitute the normal scafold of the nucleus confering, again, to these cells nuclear steadiness and integrity. Also, since changes in the nuclear morphology are related with age, ghrelin can improve the aging phenotype of HGPS fibroblasts.

Since ghrelin can ameliorate the HGPS condition by promoting progerin clearance and consequently rescuing nuclear morphology, other cellular senescence markers were analysed to investigate if ghrelin's actions could be beneficial. In recent studies several dysfunctions were related to the aging phenotype, being denominated hallmarks of aging (Lopez-Otin *et al.* 2013). In this study it was investigated if ghrelin could enhance DNA damage response, increase cellular proliferation and delay cellular senescence, HGPS cells treated with ghrelin show a significant decrease in the number of yH2AX foci in the nucleus. This may be because ghrelin promotes progerin clearence, as stated before, restablishing the normal connection between the lamins and the DNA. Consequently, normal communication between those cellular components leads to a decrease in the damage inflicted to the DNA, ultimately leading to improvements in genomic instability. We also observed that ghrelin increase cell proliferation and delay cellular senescence in HGPS fibroblasts rescuing the senescent phenotype of HGPS fibroblasts. In recent studies the p53 pathway has been urging as a common denominator between cellular senescence and DNA

damage (Benson *et al.* 2010). It is known that in HGPS patients p53 is chronically activated by permanent DNA damage (Liu *et al.* 2005; Liu *et al.* 2006). By clearing the progerin from the fibroblasts DNA damage decreases, because the checkpoint response to nuclear abnormalities is no longer activated, and the stress signalling pathways stimulated by p53 are arrested leading to probably an augmentation in the DNA damage response machinery and to re-activation of the cell cycle. (Wang *et al.* 2009; Benson *et al.* 2010; Varela *et al.* 2005). Further investigation will be necessary to assess the effect of ghrelin in cell cycle repressors pathways such as p53/p21. It is also necessary to evaluate the upstream regulators of p53/p21 such as Chk1, Chk2, ATM and ATR that are also engaged in DNA damage response. Moreover it would be interesting to see if ghrelin could induce changes in the IGF-1 axis, since this via could improve the cardiovascular system in HGPS patients by the stimulation of GH release and to investigate if there is a possible relationship between IGF-1 and MTOR kinase as one of the targets of IGF-1 is the MTOR complexes. Furthermore since cellular senescence is deeply related with telomere attrition it is of upmost importance to assess what occurs in the telomeres of HGPS patients. It will be relevant to assess if upon treatment with ghrelin there is any change in the telomere length. Besides this, it would be important to use DNA damage markers such as H2AX to measure the levels of DNA damage in the telomeres, by performng co-localization immunocytochemistry experiments with H2AX and the well known telomere marker TRF-1 or performing a CHIP assay to measure the levels of DNA damage at telomeres and to observe if treatment with ghrelin could be beneficial. Alongside with this, it is necessary to evalute all mechanisms of senescence that characterize the aging phenotype in the presence of ghrelin such as stem cells exhaustion, mitochondrial dysfunction, epigenetic alterations, altered intercellular communication and deregulated nutrient sensing. In the long run to confirm that all beneficial effects observed are indeed performed by ghrelin pharmacological blockage of ghrelin response using a specific antagonist for GHS-R1a and GHSR1a knockdown via shRNA are strategies to be applied. *In vivo* studies are also desirable to further confirm ghrelin's actions.

Given ghrelin actions related with HGPS and all the positive effects that it could exert on HGPS condition demonstrated here in this study, treatment with exogenous ghrelin could be considered as a promising strategy to delay or block the premature aging of HGPS and potentially to increase lifespan.

In conclusion, the results of the present show that ghrelin i) enhances progerin clearance, probably through autophagy stimulation, ii) rescues nuclear abnormalities, DNA damage, and iii) decreases cellular senescence of HGPS cells. Altogether, these results show that ghrelin reverses cellular hallmarks of premature aging of progeria fibroblasts and strongly support that ghrelin can be considered a promising strategy to delay or block the premature aging of HGPS as well as normal cellular aging.

Chapter 5
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