

## REVIEW-THEMED ISSUE

# Potential therapeutic effects of the mTOR inhibitors for preventing ageing and progeria-related disorders

**Correspondence** Dr Giovanna Lattanzi, CNR Institute for Molecular Genetics, Unit of Bologna, Bologna, Italy. Tel.: +39 05 1636 6857; Fax: +39 0 5158 3593; E-mail: lattanzi@area.bo.cnr.it

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Camilla Evangelisti<sup>1,2\*</sup>, Vittoria Cenni<sup>1,2\*</sup> and Giovanna Lattanzi<sup>1,2</sup>

<sup>1</sup>CNR Institute for Molecular Genetics, Unit of Bologna, Bologna, Italy, and <sup>2</sup>Rizzoli Orthopedic Institute, Laboratory of Musculoskeletal Cell Biology, Bologna, Italy

\*Contributed equally to this article.

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The mammalian target of rapamycin (mTOR) pathway is an highly conserved signal transduction axis involved in many cellular processes, such as cell growth, survival, transcription, translation, apoptosis, metabolism, motility and autophagy. Recently, this signalling pathway has come to the attention of the scientific community owing to the unexpected finding that inhibition of mTOR by rapamycin, an antibiotic with immunosuppressant and chemotherapeutic properties, extends lifespan in diverse animal models. Moreover, rapamycin has been reported to rescue the cellular phenotype in a progeroid syndrome [Hutchinson–Gilford Progeria syndrome (HGPS)] that recapitulates most of the traits of physiological ageing. The promising perspectives raised by these results warrant a better understanding of mTOR signalling and the potential applications of mTOR inhibitors to counteract ageing-associated diseases and increase longevity. This review is focused on these issues.

### Ageing and progeria-related diseases

Ageing is a physiological process aimed at the homeostasis of tissues through removal of cells bearing damaged genome sections or nonfunctional organelles. The advantage of ageing is that the neoplastic transformation of tissues and organs is avoided. The side effects of ageing include a loss of stem cells and a reduction in extracellular components, which are also associated with inflammatory processes contributing to tissue degeneration. The ageing traits are recapitulated in the majority of the so-called premature ageing (progeroid) syndromes, which are genetic diseases of variable severity, with onset in childhood or in the teens, and are described briefly in this section. Of note, all of the known progeroid syndromes are caused by defects in proteins that regulate chromatin dynamics and genome stability, from lamins to RepQ helicases up to proteins directly involved in DNA damage repair. The role of mammalian target of rapamycin

(mTOR) and mTOR inhibitors in the affected pathways is discussed in the section on mTOR molecular pathways in ageing.

### LMNA-linked progeroid syndromes

Progeric laminopathies are syndromic diseases associated with defects of the nuclear lamina protein lamin A/C. They represent a paradigm of age-related diseases as they encompass most of the disorders linked to normal ageing. Hutchinson–Gilford Progeria syndrome (HGPS) is caused by heterozygous mutations of the *LMNA* gene encoding lamin A and lamin C. Patients are healthy at birth but rapidly develop a senescent phenotype, starting at a few months of age, and are typically affected by ageing disorders, including hair loss, articular defects, skin atrophy and rigidity, atherosclerosis and coronary heart disease leading to premature death, in the first or second decade. The recurrent mutation

in HGPS patients is the silent G608G *LMNA* mutation, which activates a cryptic splice site, triggering the production of a truncated and farnesylated precursor of lamin A called progerin. Progerin is toxic to cells and causes nuclear dysmorphism and a severe loss of heterochromatin [1], mislocalization or loss of chromatin-associated proteins such as the DNA-bridging factor barrier-to-autointegration factor (BAF) and the DNA damage repair proteins poly(ADP-ribose) polymerase 1 (PARP1) and p53 binding protein 1 (53BP1) [2–4], and accumulation of irreparable DNA damage [5]. The same effects are observed in other progeroid syndromes featuring accumulation of farnesylated prelamin A, such as mandibuloacral dysplasia type A and B (MADA and MADB, respectively) [6–9] and atypical Werner syndrome (A-WS) [10, 11]. MADA is a rare disease characterized by growth retardation, bone resorption at specific sites (including the clavicles, phalanges and mandible), mottled cutaneous pigmentation, skin rigidity, partial lipodystrophy and insulin resistance. Patients develop premature ageing traits in the first or second decade. Similar clinical signs are also observed in A-WS and atypical progeria syndrome (APS), owing to mutations in the lamin A/C rod domain; these conditions do not necessarily feature accumulation of prelamin A and their pathogenetic pathways are still unclear [12]. In all premature ageing syndromes caused by mutations in the *LMNA* gene, the central nervous system is spared, owing to physiological downregulation by microRNA-9 controlling lamin A expression and its splicing isoform progerin in brain tissues [13, 14].

Cells from progeroid laminopathies are not only the best experimental model in which to test potential therapeutic approaches to these diseases, but also represent a powerful model for the study of the senescent phenotype associated with age-related disorders. However, the involvement of lamin in mechanisms that favour longevity has been determined by studying cells and tissues from very old healthy subjects. In a recent study [15], it was demonstrated that the lamin A precursor (prelamin A) plays a key role in healthy ageing, as a master regulator of the recruitment of nuclear factors implicated in genome stability. It has also been demonstrated that the nuclear envelope acts as a sensor of stress conditions and drives chromatin dynamics (heterochromatin decondensation, recruitment of 53BP1, rapid repair of damaged DNA) aimed at cell survival and genome maintenance [15]. Exacerbation of lamina remodelling as it occurs in progeroid laminopathies elicits the opposite, and deleterious, effects, mostly because of the accumulation of toxic prelamin A [4, 8, 16]. Thus, comparative analysis of lamin A and prelamin A role in normal and pathological ageing processes may give new and relevant insights into the understanding of ageing pathways, including those involving mTOR signalling and autophagy, as detailed below.

### Nucleotide excision repair (NER)-linked progeroid syndromes

Other diseases featuring premature ageing traits are associated with defects in the DNA repair machinery caused by mutations in genes encoding NER proteins. NER is a multistep mechanism able to identify and restore nucleotidic changes due to ultraviolet (UV) rays or chemical compounds, modifying DNA structure [17]. Mutations occurring to the proteins

involved in this machinery are responsible for the onset of genetic disorders, and all feature in the development of cancers and increased sensitivity to light.

Xeroderma pigmentosum (XP) is an autosomal recessive genetic disorder involving eight gene variants, all featuring an impaired ability to repair cellular DNA damaged by UV light exposure from the sun. The signs of XP usually appear in infancy or early childhood, when affected children develop severe sunburn after spending a few minutes in the sun. People with XP have a greatly increased risk of developing multiple basal cell carcinomas (basaliomas) and other skin malignancies on the face, lips and eyelids during their lifetime. Death occurs early, owing to metastatic malignant melanoma and squamous cell carcinoma [18].

XP mutations can occur in the following genes: damage-specific DNA binding protein 2 (*DDB2*), DNA excision repair crosscomplementation group 2, -3, -4, -5 (*ERCC2*, *ERCC3*, *ERCC4*, *ERCC5*), *XPA* and *XP5* [19]. The products of these genes are all involved in the DNA damage repair pathway, based on the NER mechanism.

Cockayne syndrome (CS) is a rare autosomal recessive neurodegenerative progeroid pathology featuring clear premature ageing and severe light sensitivity [18]. It is characterized by growth failure, impaired development of the nervous system, photosensitivity, eye disorders and premature ageing. It is often associated with a group of disorders called leukodystrophies, which are conditions characterized by the degradation of neurological white matter. Two types of CS have been described and occur as a result of mutations of different genes. Type I CS occurs following mutations in the DNA excision repair crosscomplementation group gene 8 (*ERCC8*), encoding the CS-A protein. These mutations cause alternative splicing of the corresponding pre-mRNA, leading to a nonfunctional protein [20]. Type II CS is caused by mutations in the *ERCC6* gene, encoding the CS-B protein [21].

Trichothiodystrophy (TTD) is a rare autosomal recessive inherited disorder mostly characterized by brittle hair, due to a general sulfur deficiency, and by intellectual impairment [18]. Mild cases of TTD may involve only the hair. However, more severe cases may cause delayed development, significant intellectual disability and recurrent infections; severely affected individuals may survive only up to infancy or early childhood. Approximately half of all patients with TTD have photosensitivity, which divides the classification of the disease into syndromes with or without photosensitivity [22, 23]. In contrast to XP, patients affected by TTD do not show a higher risk of developing skin cancer.

### RecQ-associated progeria-related syndromes

The family of RecQ proteins groups enzymes with ATP-dependent helicase activity, required during the repair of damaged DNA to unwind the mutated double strand. Their actions prevent deleterious recombination and genomic instability [24]; therefore, mutations in the corresponding genes are associated with an increased predisposition to cancer and ageing phenotypes.

Bloom syndrome (BS) is a rare autosomal recessive progeroid syndrome characterized by a striking genomic instability associated with a high risk of cancer. Its most prominent features include short stature and a rash on all areas of the skin that are

exposed to the sun, developed in early age. Other clinical features include a high-pitched voice; distinct facial features, including a long, narrow face, micrognathia, and prominent nose and ears; and changes to skin pigmentation, including hypopigmented and hyperpigmented areas [25]. The genetic cause of BS is mutations in the RecQ2 gene, also known as the BLM gene, encoding the BS protein, which is one of the five RecQ DNA helicases identified so far. Two other RecQ-linked genetic diseases are caused by defects in WRN and RecQ4 genes where WRN encodes for a protein responsible, when mutated for a progeroid syndrome: Werner's syndrome [26].

Werner's syndrome (WS) is a genetic disorder due to mutations in the WRN gene, which encodes a RecQ helicase called WRN protein. The clinical phenotype is almost identical to that observed in A-WS syndrome linked to lamin A mutations [27]. Affected individuals can exhibit growth retardation, short stature and signs of premature ageing, such as premature greying and loss of hair, wrinkles, a beaked nose, skin atrophy, loss of fat tissues and atherosclerosis [28]. Interestingly, mutation of the WRN gene may cause a decrease in the stability of the corresponding mRNA. Mutations may also lead to the loss of the nuclear localization signal of the protein, leading to an aberrant accumulation of the WRN protein in the cytoplasm.

RecQ4 mutations cause Rothmund-Thomson syndrome (RTS). RTS patients exhibit some premature ageing symptoms similar to those of WS, including skin hyperpigmentation and widened blood capillaries, known as poikiloderma. Moreover, similar to progeric laminopathies, RTS patients present abnormal bone turnover but, unlike those affected by these syndromes, are predisposed to osteosarcoma.

### DNA polymerase-linked progeroid syndromes

The variant form of XP, known as XP-V, is caused by mutations in the POLH (DNA polymerase eta) gene, which encodes a DNA polymerase; its mutation leads to an overall increase in UV-dependent DNA damage, which ultimately causes the symptoms of XP [19].

Finally, mutations in the DNA polymerase delta p125 catalytic subunit (POLD1) have been recently linked to a form of mandibuloacral dysplasia, the mandibular hypoplasia, deafness, progeroid features and lipodystrophy syndrome (MDPL), which, from the clinical point of view, is identical to that caused by LMNA mutations but also features sensorineural deafness [29, 30]. Human POLD1 modulates cell cycle progression and DNA damage repair [31]. POLH, also known as DNA polymerase  $\eta$ , is involved in translesion DNA synthesis in the UV-induced DNA damage response and acts downstream of the WRN protein [32].

## mTOR signalling

### mTOR pathway

The mTOR pathway is a highly conserved signal transduction axis involved in many cellular processes, such as cell growth, survival, transcription, translation, apoptosis, metabolism, motility, autophagy and ageing [33]. This signalling pathway may be activated by various cellular stimuli, such as growth factors and cytokines, nutrients (e.g. glucose, amino acids) and inflammatory stimuli. Thus, considering the crucial role

played by mTOR signalling in several physiological processes, it is evident that dysregulation of this cascade could have dramatic consequences. Indeed, it is well established that alterations in its activity are linked to the pathogenesis of a variety of human diseases, such as metabolic disorders (obesity and type 2 diabetes), cardiovascular and neurodegenerative diseases, age-related disorders and cancer.

mTOR is a serine/threonine (Ser/Thr) kinase which belongs to the phosphoinositide kinase-related family of protein kinases (PIKK) [34]. In response to these growth signals, mTOR regulates cell growth and proliferation positively by promoting many anabolic processes and limiting catabolic processes such as autophagy [35] (Figure 1). mTOR collects input from several signal transduction networks, such as the phosphatidylinositol (PI) 3-kinase (PI3K)/Akt and the Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK). Moreover, recent studies have highlighted that mTOR also responds to inputs via the WNT and liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK) signalling pathways [36]. AMPK functions as a key sensor of intracellular energy balance: under metabolic stress conditions, LKB1 activates AMPK, which in turn directly inhibits mTOR [37].

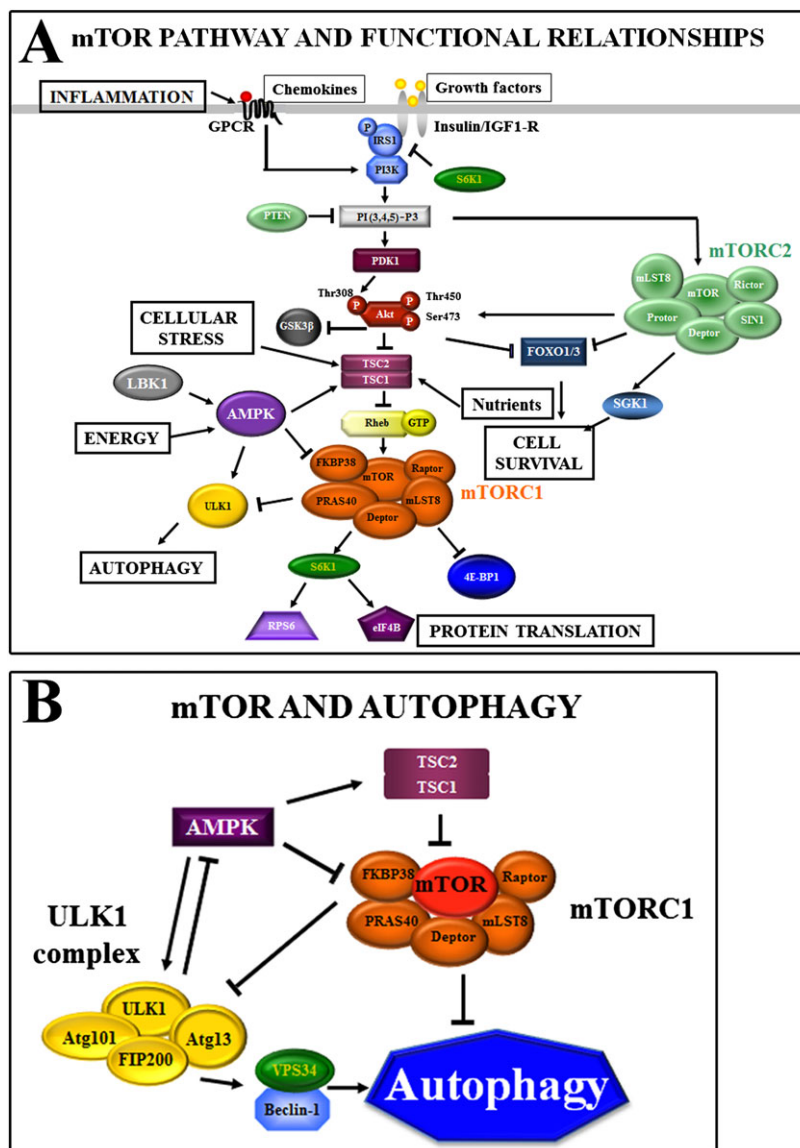
mTOR is the catalytic subunit of two functionally and structurally distinct multiprotein complexes: mTOR complex (mTORC) 1 and mTORC2, both of which are characterized by their different partner proteins and their substrate specificity [34].

mTORC1 is composed of mTOR, the regulatory associated protein of mTOR (Raptor), mammalian lethal with sec-13 protein 8 (mLST8), proline-rich Akt substrate of 40-kDa (PRAS40), FK-506-binding protein (FKBP) 38, and dep domain containing mTOR-interacting protein (Deptor) [34]. The best known function of mTORC1 is the regulation of translation. It can phosphorylate components of the protein synthesis machinery, including p70 ribosomal S6 kinase 1 (S6K1) and the translation inhibitor, eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) [38], leading to active translation of mRNAs involved in ribosome biogenesis [39].

mTORC2 comprises rapamycin-insensitive companion of mTOR (Rictor), mLST8, stress-activated protein kinase-interacting protein 1 (SIN1), protein observed with Rictor (Protor) and Deptor, and is generally described as being insensitive to rapamycin/rapalogs. It is mainly activated by growth factors through PI3K/Akt, and controls several downstream AGC kinases such as Akt itself (phosphorylation on Ser473), serum- and glucocorticoid stimulated kinase (SGK) and protein kinase  $\alpha$  (PKC $\alpha$ ). It regulates cell proliferation but is also involved in the spatial control of cell growth via cytoskeleton regulation [40]. Recently, it has been demonstrated that mTORC1, via activation of ribosome biogenesis and inhibition of autophagy-mediated ribosome turnover, indirectly controls mTORC2 [41] (Figure 1).

### mTOR and autophagy

Through the phosphorylation of other targets, mTORC1 triggers metabolic changes such as mitochondrial biogenesis, the promotion of glycolysis, lipid biogenesis and the suppression of autophagy [33]. Indeed, mTORC1 is a negative regulator of autophagy, a process required for the recycling of damaged



**Figure 1**

Cellular signalling upstream and downstream of mammalian target of rapamycin (mTOR) complexes. (A) mTOR pathways and functional relationships. mTOR forms two multiprotein complexes – mTOR complex (mTORC) 1 and mTORC2. The classical mTORC1-positive inputs are growth factors, chemokines, nutrients and cell energy balance. Growth factors stimulate mTORC1 through the phosphatidylinositol (PI) 3-kinase (PI3K)/Akt signalling pathway. Akt phosphorylates tuberous sclerosis complex 2 (TSC2). TSC2 is a GTPase-activating protein that functions in association with TSC1 to inactivate the small G protein Rheb, which, in turn, upregulates mTORC1 activity. mTORC1 activity is required for the phosphorylation and subsequent activation of ribosomal S6 kinase 1 (S6K1), which regulates the elongation step of protein translation, ribosomal protein S6 (RPS6) and eukaryotic translation initiation factor 4B (eIF4B), ultimately promoting the initiation of translation and elongation. mTORC1 also phosphorylates and inactivates the translation inhibitor eIF4E-binding protein 1 (4E-BP1), which inhibits cap-dependent translation. The mTOR pathway may be also regulated by the LKB1/AMP-activated protein kinase (AMPK) pathway. AMPK phosphorylates TSC2, activating the TSC1/TSC2 complex and thus repressing mTORC1 activity. Moreover, AMPK phosphorylates Raptor, inducing Raptor and mTORC1 disassembly/inhibition. mTORC2 regulates cell survival through serum- and glucocorticoid-activated kinase 1 (SGK1) and Akt. mTORC2 phosphorylates Akt at Ser473, priming Akt for further phosphorylation by phosphoinositide-dependent kinase 1 (PDK1) at the Thr308 residue. Loss of phosphorylation at the Ser473 site, however, affects only some Akt substrates, such as Forkhead Homeobox type O (FOXO) transcription factors, but not TSC2, in response to growth factor signalling. mTORC2 also associates with actively translating ribosomes to phosphorylate cotranslationally Akt (at Thr450), which prevents the ubiquitination and degradation of Akt. mTORC2 is involved in the spatial control of cell growth via cytoskeletal regulation. (B) mTOR and autophagy. Under nutrient-rich conditions, mTOR associates with and inhibits the UNC51-like kinase (ULK) complex by phosphorylating autophagy-related (ATG) 13 and ULK1. By contrast, stress signals or AMPK activation lead to mTORC1 dissociation from ULK1 and to the activation of the ULK complex, thereby triggering the autophagy machinery. Arrows indicate activating events, whereas perpendicular lines indicate inhibitory events. FIP200, focal adhesion kinase family-interacting protein of 200 kD; FKBP38, FK-506-binding protein; GPCR, G protein-coupled receptor; IGF1-R, insulin-like growth factor 1 receptor; GSK3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; LKB1, liver kinase B1; mLST8, mammalian lethal with sec-13 protein 8; PI(3,4,5)-P3, phosphatidylinositol (3,4,5)-trisphosphate; PRAS40, proline-rich Akt substrate of 40-kDa; PTEN, phosphatase and tensin homologue; SIN1, stress-activated protein kinase-interacting protein 1; VPS, vacuolar protein sorting



organelles and for cellular adaptation to nutrient starvation, growth factor withdrawal and oxidative stress [35]. Once activated, the autophagic machinery sequesters cytoplasmic components and, fusing with lysosomes, leads to the degradation of cell components and recycling of cellular building blocks. mTORC1 transduces anti-autophagic signals by binding to the UNC51-like kinase (ULK) multiprotein complex, which is essential for the initial steps of autophagy. mTORC1 directly phosphorylates ULK1 (which may also be a target for Akt following insulin stimulation), which in turn may regulate autophagy independently from mTORC1 function [42]. Conversely, during nutrient deprivation or pharmacological mTORC1 inhibition, mTORC1 dissociates from the ULK1 complex, leading to active forms of ULK1.

By contrast, AMPK is an important positive regulator of the autophagic machinery. Under metabolic stress conditions, LKB1 activates AMPK, which directly phosphorylates ULK1 at multiple sites, thus upregulating ULK1 activity, and activates tuberous sclerosis complex 2 (TSC2), an indirect inhibitor of mTORC1 activity [43].

Following mTORC1 inhibition, derepressed ULK1, by binding to ATG14L (ATG -Autophagy related-14 Like), is recruited to a molecular platform composed of vacuolar protein sorting 34 (Vps34) and beclin-1. This leads to the phosphorylation of beclin-1 on Ser 14, and activation of Vps34. VPS34 is the only class III PI3K member and plays an important role in regulating membrane trafficking and autophagy, releasing PI 3-phosphate at the nascent autophagosome, the phagophore [44]. In this condition, microtubule-associated protein 1 light chain 3 (LC3), a structural protein found in the cytoplasm in its precursor form (LC3I) is cleaved, lipidated (i.e. coupled to

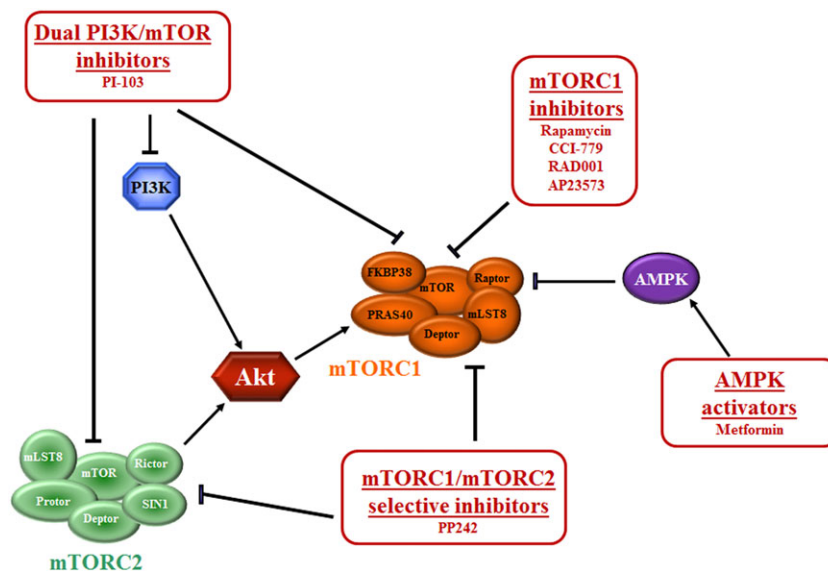
phosphatidylethanolamine) and converted into its active autophagosomal, membrane-bound form, LC3II [45].

## Potential therapeutic effects of mTOR inhibitors

Considering the numerous functions played by mTORC1 and mTORC2 in several physiological processes, it is evident that dysregulation of this axis could have important negative effects. Indeed, upregulation of mTOR signalling has been found to be involved in the onset and progression of many human diseases, and mTOR is therefore regarded as a key target for innovative therapeutic strategies. In fact, several mTOR inhibitors have been released by pharmaceutical companies. Three classes of mTOR inhibitors have been developed: rapamycin/rapalogs that are allosteric mTORC1 inhibitors; ATP-competitive, 'active-site' mTORC1/mTORC2 inhibitors that target the catalytic site of mTOR; and dual PI3K/mTOR inhibitors that target both PI3K and mTORC1/mTORC2 (Figure 2).

### Rapamycin

Rapamycin (also known as sirolimus) is a natural compound produced by the bacterium *Streptomyces hygroscopicus* that acts as an allosteric mTORC1 inhibitor [46]. It forms a gain-of-function complex with 12-kDa FKBP12, which binds to the FKBP12/rapamycin-binding (FRB) domain of mTOR only when mTOR is associated with other components of mTORC1. This complex leads to the dissociation of Raptor and loss of contact between mTORC1 and its substrates [47].



**Figure 2**

Mammalian target of rapamycin (mTOR) inhibitors. mTORC1 inhibitors are allosteric inhibitors that lead to the dissociation of Raptor from mTOR complex (mTORC) 1 and loss of contact between mTORC1 and its substrates. Dual phosphatidylinositol 3-kinase PI3K/mTOR inhibitors target both PI3K and mTORC1/mTORC2, acting on the catalytic sites of PI3K and mTOR. mTORC1/mTORC2-selective inhibitors target the catalytic site of the enzyme, thus acting on both mTORC1 and mTORC2. AMP-activated protein kinase (AMPK) activator has a negative effect on mTORC1. Arrows indicate activating events; perpendicular lines indicate inhibitory events. FKBP38, FK-506-binding protein; mLST8, mammalian lethal with sec-13 protein 8; PRAS40, proline-rich Akt substrate of 40-kDa

Therefore, rapamycin does not directly target the mTOR catalytic site and does not affect mTORC2 activity, although it has been demonstrated that long-term exposure can also affect mTORC2 assembly [48, 49].

The discovery of rapamycin immediately raised great interest in the scientific community for its numerous properties, e.g. as a powerful antibiotic, antiproliferative and immunosuppressant [50]. In 1991, rapamycin was approved by the US Food and Drug Administration (FDA) for the prophylaxis of renal transplant rejection [51] and as a chemotherapeutic agent against renal carcinoma. Moreover, rapamycin has been used to inhibit restenosis after the implantation of stents during coronary angioplasty [52, 53], and it entered numerous clinical trials for conditions such as lymphangioleiomyomatosis [54] and tuberous sclerosis.

### Rapalogs

The limitations in the solubility and pharmacokinetic properties of rapamycin led to the commercial development of new mTOR inhibitors, such as semi-synthetic rapamycin analogues, named rapalogs or active site inhibitors. Rapalogs have an improved bioavailability when compared with rapamycin, and include CCI-779 (temsirolimus or torisel), RAD001 (everolimus) and AP23573 (ridaforolimus), which have been, and continue to be, tested in a wide range of clinical trials for numerous chronic disease indications.

CCI-779 is a rapamycin ester derivative that was approved by the FDA for the treatment of advanced-stage renal cell carcinoma in 2007. In Europe, it is also approved for the treatment of mantle cell lymphoma [55]. Unfortunately, in patients affected by advanced renal cell carcinoma, CCI-779 has strong side effects, including hyperglycaemia, hyperlipidaemia, anaemia, peripheral oedema and dermatological adverse events [56].

RAD001 is another rapamycin analogue that has been approved by the FDA for patients affected by various malignancies, including renal cell carcinoma [57], subependymal giant cell astrocytoma [58] and progressive neuroendocrine tumours of pancreatic origin [59]. The orally available RAD001 is more efficacious than rapamycin as it has a higher affinity for FKBP12 [60].

More recently, two other classes of mTOR inhibitors, which target the catalytic site of mTOR, have been developed to overcome the side effects of rapalog 'active-site' mTORC1/mTORC2 inhibitors.

PI3K and mTOR belong to the PIKK family of kinases, and share high sequence homology in their catalytic domains. Dual PI3K/mTOR inhibitors target the active sites of both the PI3K and mTOR, inhibiting the PI3K/Akt/mTOR pathway both upstream and downstream of Akt, thus avoiding the Akt activation following abolition of the mTORC1/S6K1/insulin receptor substrate 1 negative feedback loop, which is known to occur with rapalogs [61]. The first compound of this class to be produced was the morpholinoquinazoline derivative PI-103 [62].

To reduce toxicity due to the use of these inhibitors, efforts were undertaken to design a new-generation mTOR inhibitor (mTORC1/mTORC2-selective inhibitor) that competes with ATP for binding to the kinase domain of mTOR, thus having a specific blocking effect on its kinase

activity. In the same manner as with dual PI3K/mTOR inhibitors, acting on both mTOR complexes, mTORC1/mTORC2-selective inhibitors display stronger effects than rapalogs in the treatment of a wide variety of cancers, and they might offer an efficient alternative to rapalogs. Their use also minimizes the reactivation feedback loops of Akt that are seen with rapalogs. The prototype of this class of drugs is PP242 [63].

Recently, novel compounds that target mTOR indirectly are attracting attention for their better safety profiles, such as resveratrol (3,4',5-trihydroxy-trans-stilbene) and metformin (N,N-dimethylbiguanide).

### Resveratrol

Resveratrol is a natural polyphenol that shows numerous beneficial effects, acting as an antioxidant, anti-inflammatory and anticancer drug. Moreover, it seems to have protective effects against a number of cardiovascular and neurodegenerative diseases [64]. It has been reported that resveratrol can inhibit mTORC1 by blocking the interaction between Deptor and mTOR [65].

### Metformin

Metformin belongs to the biguanide class of oral hypoglycaemic agents, which has been approved by the FDA for the treatment of type 2 diabetes since 1995. Intriguingly, metformin treatment has also been associated with a reduced risk of cancer [66] and of cardiovascular diseases [67]. There is a growing body of evidence that metformin inhibits mTORC1 through a different mechanism, by activating AMPK. AMPK, in turn, blocks mTORC1 [68] regulating it through Ras-related GTP binding (Rag) GTPases [69] and indirectly activating regulated in development and DNA damage responses 1 (REDD1), a mTOR inhibitor that promotes TSC2 activity [70].

## mTOR molecular pathways in ageing

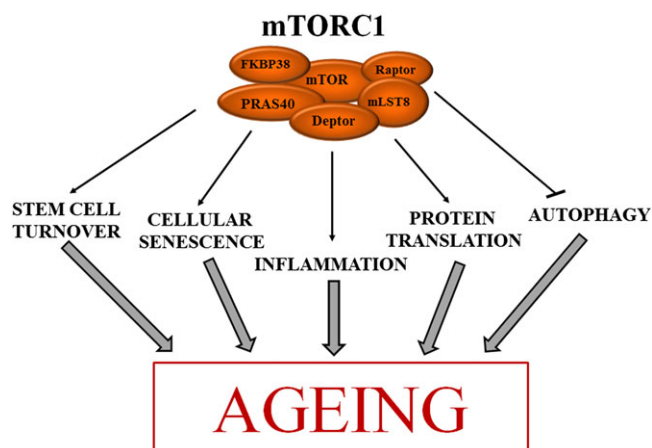
Several processes downstream of mTOR signalling contribute to ageing, including the regulation of protein translation, autophagy, stem cell pool homeostasis, inflammation and cellular senescence (Figure 3).

### Protein translation

The inhibition of mTORC1-dependent translation may extend lifespan by reducing the burden on the protein-folding machinery, leading to improved protein quality. Reduced translation via knockout of S6K1 extends longevity and blocks the onset of age-related pathologies [71]. Moreover, genetic depletion of ribosomal proteins, as well as inhibition of translation initiation factors, can similarly extend lifespan in yeast and worms [72, 73].

### Autophagy

Ageing is characterized by the accumulation of unprocessed material and by the decrease in autophagy. The anti-ageing effects of rapamycin are also dependent on the induction of this catabolic mechanism in yeast, worms and flies [74–76].



**Figure 3**

The mammalian target of rapamycin (mTOR) complex (mTORC1) signalling pathway contributes to ageing through various cellular processes. Active mTORC1 activates stem cell turnover, cellular senescence and protein translation (arrows), while it inhibits autophagy. These conditions contribute to the ageing of an organism. See text for details. FKBP38, FK-506-binding protein; mLST8, mammalian lethal with sec-13 protein 8; PRAS40, proline-rich Akt substrate of 40-kDa

For instance, in *Caenorhabditis elegans*, a loss-of-function mutation in the insulin-like growth factor (IGF) pathway causes autophagy, whereas inhibition of autophagy by mutation of essential Atg genes prevents the acquisition of longevity [77].

### Stem cell pool turnover

mTORC1 is a key regulator of growth signals that drives the exit of tissue stem cells from quiescence. Stem cell activation contributes to tissue turnover but affects the stem cell pool, progressively eliciting stem cell niche depletion. Thus, rapamycin represents a tool for preserving the stem cell pool and, as a consequence, the functionality of tissues and organs over time. In support of this, six weeks of treatment with rapamycin in elderly mice improved the regenerative capacity of haematopoietic stem cells [78].

### Regulation of the inflammatory response

Recent exciting advances in the study of the well-defined mechanism of inflammaging [79] have suggested that tissue damage or stress trigger a specific secretory phenotype, starting from interleukin (IL) 1 $\alpha$  production and nuclear factor- $\kappa$ B activation [80]. The environment elicited by cellular damage corresponds to a senescence-associated secretory phenotype (SASP) and, unexpectedly, favours tissue repair and cellular senescence as a side effect [80–82]. Cytokines produced during the inflammatory response signal through mTOR (Figure 3) either by activating PI3K, which targets mTORC2 complex, or by stimulating the p38/Erk pathway to activate mTORC1 [83]. Downstream of these biochemical pathways, cellular proinflammatory cytokines are mostly inhibited but ageing-related molecules such as IL6 are activated [83]. Thus, modulation of mTOR activity can affect inflammaging positively or negatively. It has been shown

that rapamycin inhibits the expression of tumour necrosis factor and IL-6, two main players in the ageing-associated inflammatory response [83].

### Cellular senescence

The mTOR pathway also drives cellular senescence [84], a cellular decline that follows development and maturation, characterized by loss of capacity to replicate without undergoing apoptosis and by a proinflammatory secretory phenotype (SASP) [85]. In proliferating cells, mTOR drives cellular proliferation through activation of protein translation and modulation of nutrient sensing and the cellular response. Under stress conditions, the cyclin-dependent kinase inhibitor p21 is upregulated to trigger cell cycle arrest and allow damage repair. In this context, mTOR contributes to converting irreversible cell cycle arrest to cellular senescence, an event referred to as geroconversion [86, 87]. Geroconversion is a form of futile growth that occurs during cell cycle arrest. It leads to hypersecretory and hypertrophic cellular phenotypes and the activation of a specific inflammatory response, as also detailed above (Figure 3). In both human and rat cells, geroconversion [84] is suppressed by rapamycin and other rapalogs, maintaining cellular quiescence [88, 89]. Recently, it has been demonstrated that low doses of dual mTORC1/mTORC2-selective inhibitors (PP242 and Torin1) can also constrain cellular senescence [87].

### mTOR inhibition to counteract ageing

There is an increasing interest in genetic and pharmacological mTOR inhibition aimed at extending longevity and improving the healthy lifespan.

The effects of mTOR inhibition in slowing down the ageing process have been demonstrated over the years in many animal models. Initial experimental data showed that reduced TOR activity in yeast, worms and flies may be sufficient to delay ageing [74, 90, 91].

Recent evidences suggested that mTOR plays the same important role in regulating lifespan in mammals. Indeed, Selman and colleagues demonstrated that deletion of S6K1 extends the median lifespan of mice and counteracts the onset of age-related pathologies, such as bone, immune and motor dysfunction, as well as loss of insulin sensitivity [71]. The effect on lifespan extension was also demonstrated in mice bearing heterozygous deletions of mTOR and mLST8, corroborating this hypothesis [49]. Moreover, Wu and colleagues recently showed that, in a mTOR hypomorphic mouse model, there was an increase in median lifespan, associated with a reduction in the age-dependent decline in tissue and organ functionality [92].

The specific effects of mTOR on lifespan have also been confirmed concurrently by pharmacological inhibitors. Rapamycin was the first mTOR inhibitor to be tested in mice, and was found to extend both mean and maximum lifespan [93].

In recent years, the National Institute of Ageing (NIA) Intervention Testing Program (ITP) embarked on a phase II study to test the possible clinical translational potential of rapamycin to delay ageing, and thus to extend the healthy



lifespan in mice. Indeed, mTOR inhibition seems to be an innovative strategy for the treatment of a wide range of age-associated conditions and diseases. The effects of rapamycin on lifespan extension have been reported when treatment is begun late in mouse life, at 20 months of age, in both genders [93]. Slightly larger effects have been observed when the drug is administered to young mice [94]. Rapamycin's efficacy in mice has been also confirmed starting at four months of age and continued throughout life [95]. It is interesting that rapamycin-treated mice live three to four months longer (the equivalent of ten human years), with the same quality of life at the time of death, than mice that do not receive the drug [96].

Importantly, rapamycin delayed different hallmarks of ageing in mice, including cardiac hypertrophy, liver degeneration, and a decline in adrenal gland and endometrial function and tendon elasticity [97].

Various independent groups have reported that rapamycin also affects cognitive performance. Neff *et al.* revealed that learning and memory were improved after rapamycin treatment [98], while other independent groups found that rapamycin enhanced cognitive function in young adult mice and blocked age-associated cognitive decline in older animals [99, 100]. In addition, in transgenic mouse models of Alzheimer's disease (AD), rapamycin was able to delay the onset of the neurodegenerative disease, restoring memory [101–103].

In spite of the initial encouraging results on the anti-ageing therapeutic potential of resveratrol in mice and primates [104, 105], recent studies have shown that this drug does not affect lifespan or longevity in male or female mice [94], or in a cohort of more than 800 people over the age of 65 years [106]. Moreover, the use of resveratrol in progeric mice carrying an *LMNA* mutation did not improve the senescence phenotype [107].

With regard to metformin, evidence has shown that it extends healthy lifespan in worms [104], rats [108] and mice [105, 109, 110]. In a promising NIA ITP study, long-term treatment with low-dose metformin in male mice, starting in middle age, showed a substantial extension of healthy lifespan and longevity [110].

Interestingly, a long-term study in human patients, including the UK Prospective Diabetes Study, found that the treatment of diabetic patients with metformin reduced mortality from all causes, including diabetes-related mortality, cancer and myocardial infarction [111, 112]. All of these results are hopeful, given that metformin has been already demonstrated to be safe and effective in humans, and, thus, may represent a novel strategy to delay ageing and counteract age-related diseases.

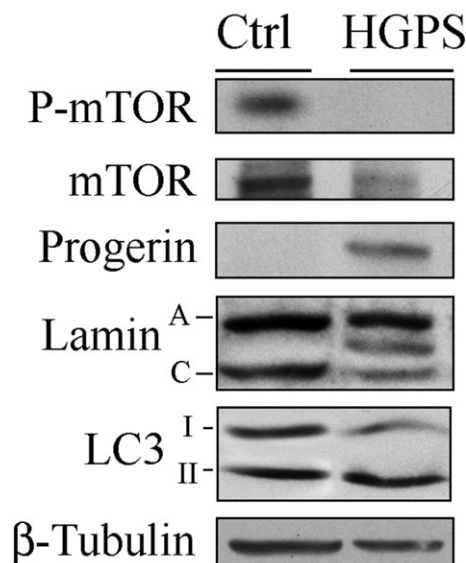
In mice, calorie restriction (CR) may counteract the onset of age-related diseases such as stroke, diabetes, cancers, AD and Parkinson's disease (PD) [113]. Clinical studies have also been carried out in humans, with promising results, showing that CR is linked to a decreased risk of cancer, diabetes and overall mortality [113]. The positive effects of CR are attained, at least in part, through down-regulation of the mTOR signalling pathway in yeast [114], worms and mice [115]. Thus, mTOR inhibition by rapamycin or its analogues may mimic CR and counteract ageing processes.

## mTOR inhibitors and progeria-related disorders

### Regulation of autophagy and protein accumulation

The importance of mTOR in ageing and age-related diseases is mostly related to its function as a master regulator of autophagy. Increased basal autophagic activity has been reported in progeroid laminopathies [8, 116] and WS cells [117]. Interestingly, as shown in Figure 4, increased autophagy observed in HGPS fibroblasts is coupled to a reduction in mTOR activity. Besides being related to alterations in mTOR signalling, the increased autophagy observed in progeria cells is also a response to the cellular accumulation of misfolded and/or aggregated proteins, typical of these cells [118]. Although it is not an aspect common to all the progeroid syndromes studied so far, the mutated proteins responsible for the onset of the pathology, may be themselves targets of autophagic degradation. This has been demonstrated in MADA, for example, in which autophagy contributes, in part, to prelamin A degradation, thus reducing protein toxicity [8]. However, progerin, the toxic prelamin A form that accumulates in HGPS, is minimally affected by the autophagic process under basal conditions [116, 119].

### Human fibroblasts



**Figure 4**

Undetectable mammalian target of rapamycin (mTOR) activity in Hutchinson–Gilford Progeria syndrome (HGPS) fibroblasts. HGPS fibroblasts at passage 20 were cultured and lysed, as described by Cenni *et al.* [116]. Immunoblotting was performed with the following antibodies: progerin (Enzo Life Sciences, Inc. Farmingdale, NY, USA.), phospho-mTOR [P-mTOR (Ser2448)], mTOR and microtubule-associated protein 1 light chain 3 (LC3), respectively [#2971, #2972 and #4108 (Cell Signaling Technology Inc. Danvers, MA, USA)]. Lamin A/C (#6215, Santa Cruz Biotechnology, Inc. Dallas, TX, USA),  $\beta$ -tubulin and flag (both from Sigma-Aldrich, Saint Louis, MO, USA). The immunoblotted band between lamin A and lamin C bands corresponds to progerin



In many age-related diseases, protein aggregate formation is a hallmark of the mutated cellular phenotype. Aside from the afore-mentioned progeroid cells, neurodegenerative pathologies also show a clear accumulation of aggregates and highly insoluble structures both inside the cells and in the extracellular matrix, negatively affecting the regulation of the nervous connections [120]. In AD, these aggregates consist of intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein, and of extracellular plaques containing abnormally folded amyloid-beta ( $A\beta$ ) aggregates.  $A\beta$  is a short peptide obtained at the end of the pathological processing of the amyloid precursor protein, whose function is unclear but is thought to be involved in neuronal development.  $A\beta$  oligomers and plaques act by blocking proteasome function, inhibiting mitochondrial activity, altering intracellular calcium levels and stimulating inflammatory processes.  $A\beta$  also interacts with the signalling pathways that regulate the phosphorylation of the microtubule-associated protein tau, which, in normal conditions, regulates axonal transport. Hyperphosphorylation of tau disrupts its normal function and leads to the accumulation of neurofibrillary tangles and toxic species of soluble tau. In addition, the degradation of hyperphosphorylated tau by the proteasome is inhibited by the actions of  $A\beta$  [121]. In PD, the aggregates consist of  $\alpha$ -synuclein fibrils, which assemble to form lethal Lewy bodies in the nerve cells. In normal conditions,  $\alpha$ -synuclein is found mainly in the presynaptic terminals of neurones, where it regulates the neuronal Golgi apparatus and vesicle trafficking [122]. Finally, in Huntington's disease (HD), toxic aggregates of mutated huntingtin (mHtt) are formed (see below). Wild-type huntingtin is associated primarily with vesicles and microtubules, and is also involved in the upregulation of the expression of brain-derived neurotrophic factor [123, 124].

### *mTOR inhibitors and lamin A/C-dependent progeroid syndromes: HGPS and MADA*

Our group has proved that, by promoting autophagy, rapamycin has a powerful negative effect on the expression level of progerin and farnesylated forms of prelamin A in both HGPS [4, 116, 119] and MADA cells [8]. In 2011, we described that, by promoting the autophagic clearance of progerin and prelamin A, rapamycin treatment of HGPS fibroblasts rescues their chromatin phenotype, including histone methylation status and nuclear envelope protein distribution patterns [116]. Importantly, rapamycin treatment did not affect lamin C protein levels but increased the relative expression of ZMPSTE24 (Zinc Metalloproteinase 24), which is the endoprotease involved in prelamin A maturation, suggesting that not only prelamin A degradation, but also prelamin A processing may be enhanced by drug treatment [116]. Further, the results from Collins's laboratory showed that, by reducing progerin accumulation, the treatment of progeric cells with rapamycin reverses cellular phenotypes, such as nuclear blebbing, and delays the onset of cellular senescence [119].

Very recently, we demonstrated that all-trans retinoic acid is synergistic with low-dose rapamycin in reducing progerin accumulation in HGPS fibroblasts [4]. This effect is obtained through transcriptional downregulation of *LMNA* and by mutated protein degradation, and elicits an

improvement in heterochromatin organization and the DNA damage response, ultimately ameliorating cell cycle dynamics. These data are very promising, particularly because the low doses of the drugs required for this combined treatment avoid the potential side effects associated with chronic treatment.

Nuclear lamina turnover and protein modifications, e.g. occurring at cell division or during cellular differentiation, are mediated by several protein kinases that selectively lamin A/C and lamin B [125]. For example, lamin A phosphorylation sites have been identified and related to myogenic differentiation [126]. In addition, we have also reported that lamin A/C and nonfarnesylated prelamin A are phosphorylated by Akt1 at Ser404. This leads to lamin association to 14.3.3 proteins which is an event required for lamin A proteosomal degradation [127–129]. Intriguingly, these mechanisms also affect prelamin A stability, both under physiological conditions and in premature ageing syndromes. For instance, mutated prelamin A accumulates in MADA cells, and prelamin A levels increase *in vitro* with the number of passages in culture and *in vivo* with the age of the patients, contributing to a worsening of the disease [6]. Two forms of prelamin A accumulate in MADA cells [7]. However, while nonfarnesylated prelamin A is, in part, degraded through autophagy, farnesylated prelamin A accumulates to toxic levels. This is because nonfarnesylated prelamin A is highly phosphorylated at Ser404, while the farnesylated form of the lamin A precursor is not, suggesting the existence of an objective hindrance to its lysosomal clearance [8]. Nevertheless, in 2014, we demonstrated that the treatment of cultured MADA fibroblasts with rapamycin triggered the autophagic degradation of farnesylated prelamin A efficiently and selectively.

The treatment of progeroid cells with rapamycin elicited the recovery of several cellular parameters [8]. In particular, rapamycin treatment of MADA cells, which feature very low levels of the nicotinamide adenine dinucleotide-dependent sirtuin 1 (SIRT-1) in the nuclear matrix, reduced soluble SIRT-1 levels, to the benefit of the insoluble matrix-bound protein. Recovery of SIRT-1 binding to the nuclear matrix is expected to improve enzyme activity and to be relevant to metabolic processes governed by SIRT-1 [130]. Rapamycin treatment also led to the recovery of heterochromatin organization, as seen by the recovery of trimethylated Lysine (K) 9 on Histone 3, (usually indicated as trimethyl H3K9) distribution patterns and levels [8]. The latter is a key objective of laminopathy treatment as heterochromatin loss and disorganization are a major feature of laminopathic cells [1, 4, 6, 131].

Farnesylated prelamin A accumulation observed in MADA cells is also responsible for the recruitment of the stress-induced transcription factor Oct-1 at the nuclear lamina. As a consequence of its sequestration, Oct-1 activity is impaired. By reducing farnesylated prelamin accumulation, rapamycin administration was seen to elicit the release of Oct-1, restoring its transcriptional activity. Finally, rapamycin treatment of MADA fibroblasts partially rescued cell cycle dynamics. In fact, although the doubling time of MADA fibroblasts was not significantly changed by rapamycin, a significant proportion of cells re-entered the cell cycle following drug treatment, and the S-phase length was restored [8].

We recently reported on the efficacy of rapamycin treatment in rescuing cell type-specific defects associated with LMNA-linked progeroid syndromes. In osteoblast-like U2OS cells overexpressing a mutant form of lamin A found in MADA (LA-R527H), osteoclast differentiation markers were affected [9]. According to our evidence, this may have been due to the positive effect of the LA-R527H mutant on the secretion of transforming growth factor- $\beta$ 2, which, in turn, triggers osteoclastogenesis, an effect previously reported in primary MADA osteoblasts [132]. A reduction in mutated prelamin A levels by rapamycin treatment reduced aberrant osteoclastogenesis [9]. Thus, mTOR inhibitors may prove useful in the rescue of bone loss that affects MADA patients.

### *mTOR inhibitors and other progeria-related disorders*

As already stated, progeric laminopathies represent a paradigm of age-related diseases as they summarize most of the disorders linked to normal ageing. Nonetheless, a number of pathologies feature some aspects of physiological ageing. A list of progeria-related disorders for which mTOR inhibitor administration has been evaluated is presented below.

Recent findings have proven that the mTOR pathway is altered in cells from nevi and melanoma from XP patients with defective DNA repair. Masaki *et al.* [133] revealed that these cancerous cells have a very high frequency of UV-type mutations in the phosphatase and tensin homologue (PTEN) tumour suppressor gene. PTEN is a phosphatase that removes 3'-phosphate from PI(3,4,5)-trisphosphate to yield PI(4,5)-bisphosphate, thus switching off the PI3K/Akt/mTOR signalling pathway. XP-induced mutation in the PTEN gene results in a loss of the protein activity, leading to a constitutive PI3K/Akt/mTOR cascade activation. The administration of PTEN/mTOR inhibitors in patients with these lesions may therefore offer an opportunity to prevent the development of melanoma [133, 134]. It should be noted, however, that Populo and colleagues describe the therapeutic inhibition of mTOR to induce cell death and prevent the progression of melanomas, rather than to induce autophagy. trisphosphate [134].

Recent studies indicated that Cockayne's Syndrome group B protein, CSB, a protein involved in the DNA repair machinery that is mutated in CS, is enriched in the mitochondria. This suggests a role for CSB in mitochondrial DNA repair [135, 136] following oxidative stress. In CS cells, the lack of CSB leads to an increase in the release of reactive oxygen species, coupled with mitochondrial dysfunction and increased metabolism. In these cells, another effect of the impairment of autophagic processes, is a defective mitochondrial turnover. Scheibye-Knudsen *et al.* demonstrated that, by promoting autophagy, rapamycin was able to promote mitochondrial turnover, rescuing the bioenergetic profile [137].

Talaei and colleagues have that fibroblasts from WS patients show a strong activation of the mTOR pathway, in spite of their high level of basal autophagy [117]. This has been demonstrated by an increase in mTOR activity, as well as in S6K1 [117]. What is more, according to Talaei *et al.*, the simultaneous activation of these pathways in WS cells may result in a signal that overrides suppression of autophagy by mTOR [117]. Recent studies have found that WS

cells also exhibit low levels of intracellular thiol groups, which are known to be antioxidants. This is probably responsible for the highly vulnerability of WS cells to hydrogen peroxide treatment, UV radiation and cytostatic drugs [138]. Thiol groups, which are increased in sodium hydrosulfide treatment, were found to attenuate hydrogen peroxide-induced DNA fragmentation in WS cells, suggesting a beneficial effect at the DNA level. In addition, sodium hydrosulfide treatment was also found to be responsible for an intense repression of the mTOR pathway [117]. Although the molecular mechanism is still obscure, the inhibition of the mTOR pathway by thiol groups produces the same beneficial effects as rapamycin treatment – i.e. a decrease in the level of insoluble proteins and in the percentage of aggregates, a restored nuclear morphology and a normalized phenotype of WS cells [117].

Unfortunately, published data on possible alterations of the mTOR pathway, increased autophagy and the use of rapalogs as treatment for TTD or BS are still missing. As stated above, these progeroid syndromes are associated with deficiencies in DNA repair mechanisms that lead to highly damaged DNA, which is ultimately responsible for a high vulnerability to stress. At present, scientists believe that increasing the efficiency of DNA repair might be associated with an extended healthy lifespan [139, 140]. In this context, mTOR inhibitors may not be useful as it has been reported that, by inhibiting p21 activity, they suppress the repair mechanisms [86].

### *mTOR inhibitors and other ageing-related diseases*

Mouse models of AD show increased activity of mTOR and its downstream kinases. This leads to an abnormal hyperphosphorylation of tau, the accumulation of which is toxic to cells. Nevertheless, recent findings support a role for A $\beta$  aggregates as positive regulators of mTOR activity [101]. By activating autophagy, the treatment of AD mice with rapamycin has been reported to reduce A $\beta$  protein levels and reverse cognitive deficits [102, 141]. Thus, mTOR inhibitors may represent a promising therapy for AD by reducing the hyperphosphorylation of tau, decreasing A $\beta$  levels, and inducing autophagy [141].

Pathological protein deposits of  $\alpha$ -synuclein, A $\beta$  and tau proteins can also be found in cells from patients with PD. This degenerative disorder also features the death of specific populations of cells, such as dopaminergic cells in the substantia nigra. According to recent theories, oxidative stress plays an important role in the evolution of some forms of PD, as the increase in oxidative stress precedes the signs of neuronal degeneration. Toxin accumulation and oxidative stress in PD are likely to be responsible for the activation of RTP801, a stress response protein that drives neuronal cell death by leading to the inactivation of the survival kinase Akt. Published evidence obtained from cell cultures and *in vivo* PD models suggests that the treatment of PD patients with rapamycin regulates RTP801 expression negatively by reducing its stability, thereby protecting the brain against neuronal death [142].

Dopaminergic cell death in PD has been hypothesized also to be related to oxidative stress-induced accumulation

of nonfunctioning autophagosomes. By inducing autophagy, rapamycin was shown to restore lysosome levels and autophagosome clearance, attenuating dopaminergic cell death in both *in vitro* and *in vivo* models of PD [143]. Moreover, rapamycin treatment has also been shown to counteract the accumulation of  $\alpha$ -synuclein aggregates in a mouse model of PD [144].

In HD, nerve cells appear full of aggregates made up of portions of mHtt, which is the product of HTT gene [145]. The severity of this neurodegenerative disorder is related to the number of polyglutamine (polyQ) repeats generated by pathological HTT mutation. Accumulation of mHtt is highly toxic and is associated with neuronal death. In mouse models and in the brains of humans with HD, mTOR has been shown to be sequestered in polyQ aggregates. mTOR inhibitors can enhance autophagy and consequently reduce mutated-huntingtin (mHtt) accumulation and neuronal death in cellular and animal models of HD [146, 147]. Furthermore, it has been reported that rapamycin treatment can improve motor deficits in mouse models of HD [146].

On the whole, the administration of chemical compounds that act by inhibiting the mTOR pathway has led to excellent and promising results, as seen in primary cultures obtained from patients affected by progeria-related syndromes and in cellular models reproducing these pathologies. However, it should be noted that, probably due to its immunosuppressant properties, the long-term use of rapamycin is sometimes associated with many side effects (e.g. interstitial pneumonia, high levels of triglycerides, reduced wound healing and anaemia) which might also increase the risk of cancer and preclude its chronic use in age-related diseases [148].

As the use of rapamycin monotherapy in a broad spectrum of diseases, especially in treating cancers, is limited due to its poor efficacy, it would be reasonable for rapamycin treatment of age-related diseases to be combined with other compounds.

## Perspectives

Several mTOR-dependent processes are likely to contribute to the effects of mTORC1 inhibitors on ageing and diseases associated with premature ageing. Among those processes, activation of autophagy leading to the degradation of damaged organelles and misfolded or toxic proteins, as occurs in rapamycin-treated cells from progeroid laminopathies, plays a beneficial role in counteracting cellular senescence. In this context, mTOR inhibition mimics and overcomes CR and its role in extending the lifespan. Moreover, hyperactivation of mTOR may be associated with inflammation, and mTOR inhibitors have anti-inflammatory effects [81, 82]. Thus, reduction of age-associated inflammation [149] is another possible mechanism by which mTORC1 inhibition could slow ageing-related pathologies in humans. However, a key issue is avoiding uncontrolled effects due to improper dosage of rapamycin or its analogues. The proposed combination with other drugs, such as retinoic acid or oxidative stress inhibitors, acting in synergy with mTOR inhibitors to activate longevity pathways is a promising perspective.

## Competing Interest

All authors have completed the Unified Competing Interest form at [www.icmje.org/coi\\_disclosure.pdf](http://www.icmje.org/coi_disclosure.pdf) (available on request from the corresponding author) and declare: GL had support from Associazione Italiana Progeria Sammy Basso (A.I.Pro.Sa.B) and Fondazione Del Monte di Bologna e Ravenna for the submitted work; VC and GL had support from 'fondi 5 per mille 2012 Istituto Ortopedico Rizzoli'.

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