INTRODUCTION

HGPS is a rare disease that causes accelerated aging in children. Disease symptoms appear soon after birth and include skin abnormalities, alopecia, osteoporosis, and osteolysis with bone resorption at clavicles, phalanges and mandible, generalized lipodystrophy, and cardiovascular disorders leading to early death (Filgueiras-Rama et al., 2018; Gonzalo et al., 2017; Hamczyk et al., 2018). Searching therapeutic strategies for HGPS is still a challenge. Although an ongoing clinical trial exploiting a farnesyltransferase inhibitor elicited several positive results (Gordon et al., 2018), significant increase in life span and slowdown of organismal aging were not achieved.

Abstract

Hutchinson–Gilford progeria syndrome (HGPS) causes premature aging in children, with adipose tissue, skin and bone deterioration, and cardiovascular impairment. In HGPS cells and mouse models, high levels of interleukin-6, an inflammatory cytokine linked to aging processes, have been detected. Here, we show that inhibition of interleukin-6 activity by tocilizumab, a neutralizing antibody raised against interleukin-6 receptors, counteracts progeroid features in both HGPS fibroblasts and Lmna<sup>G609G/G609G</sup> progeroid mice. Tocilizumab treatment limits the accumulation of progerin, the toxic protein produced in HGPS cells, rescues nuclear envelope and chromatin abnormalities, and attenuates the hyperactivated DNA damage response. In vivo administration of tocilizumab reduces aortic lesions and adipose tissue dystrophy, delays the onset of lipodystrophy and kyphosis, avoids motor impairment, and preserves a good quality of life in progeroid mice. This work identifies tocilizumab as a valuable tool in HGPS therapy and, speculatively, in the treatment of a variety of aging-related disorders.

KEYWORDS
accelerated aging, ageing, anti-aging, cellular senescence, cytokines, inflammation, laminopathies, nuclear lamina
Moreover, poor quality of life characterizes HGPS due to very early onset and progressive worsening of osteoporosis, lipodystrophy, articular impairment, and cardiovascular disorders, which are barely improved by current treatments (Gordon et al., 2018).

The molecular defect causing HGPS is an heterozygous mutation in the LMNA gene, which encodes five A type lamins by alternative splicing, including lamins A and C (Eriksson et al., 2003). Most HGPS cases are linked to the c.1824C>T;p.G608G silent mutation in LMNA gene, which activates an aberrant splicing (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). The aberrantly spliced gene product is translated as progerin, a truncated prelamin A form that undergoes farnesylation at its C-terminus as wild-type prelamin A, but cannot be fully processed. Thus, progerin is maintained in HGPS cells as a permanently farnesylated protein precursor (Eriksson et al., 2003) and causes chromatin disorganization, aberrant nuclear lamina interaction with transcription factors and chromatin-binding proteins, upregulation of p21 and geroconversion of cells (Kreienkamp et al., 2016, 2018; Mattioli et al., 2018). Further, altered nucleo-cytoplasmic interplay involving tubulins and defective ion channel expression or activity have been shown in progeroid cells (Larrieu et al., 2018; Zironi et al., 2018). The outcome of such an altered scenario seems to be an unscheduled activation of stress response, marked by persistence of DNA damage markers as phosphorylated histone H2AX (γ-H2AX) and small telomeric DNA damage response RNAs (Aguado et al., 2019).

Progerin effects on the secretome have been observed in several preclinical models of HGPS (Gonzalo & Coll-Bonfill, 2019; Kreienkamp et al., 2018; Osmanagic-Myers et al., 2019; Osorio et al., 2012). In mice, it has been demonstrated that selective expression of progerin in endothelial cells causes dysregulation of circulating molecules and a condition leading to paracrine and profibrotic effects (Osmanagic-Myers et al., 2019; Sun et al., 2020). Activation of the senescence-associated secretory phenotype (SASP) by endothelium-targeted progerin affected most mouse tissues and induced premature aging in the whole organism (Sun et al., 2020). Systemic effects linked to aberrant NF-kB signaling and interleukin 6 (IL6) increase have been observed in Lmna \(^{G609G/G609G}\) and Zmpste24\(^{-/-}\) progeroid mice featuring progerin or prelamin A accumulation, while anti-inflammatory drugs have been shown to extend life span (Osorio et al., 2011, 2012). Moreover, recent studies showed that aberrant activation of JAK-STAT signaling occurs in HGPS cells and animal models and triggers SASP with increase of IL6 and IL8 (Griveau et al., 2020; Liu et al., 2019). Intriguingly, prelamin A-dependent SASP activation, including IL6 hypersecretion, has been also observed in human vascular smooth muscle cells undergoing calcification (Liu et al., 2013), a condition that is found in HGPS and contributes to disease severity (Gordon et al., 2016).

IL6 behaves as a pro-inflammatory cytokine, with features of anti-inflammatory molecule under certain conditions. In particular, canonical IL6 signaling, which relies on membrane-bound receptor alpha (IL6Ra) and on the ubiquitous receptor GP130 (Baran et al., 2018), triggers anti-inflammatory and pro-regenerative pathways, and it is restricted to cells harboring the receptor on their membrane, mainly hepatocytes and macrophages (Baran et al., 2018). Conversely, through the soluble IL6 receptor (sIL6R), IL6 stimulates pro-inflammatory response and pro-fibrotic processes in several cell types and target tissues (Baran et al., 2018). In this context, IL6 propagates inflammation signaling from cells that produce the cytokine to neighboring cells causing DNA damage in a sort of self-fueling circle activated by various stress conditions (Fang et al., 2014; Rodier et al., 2009; Storci et al., 2019).

In the clinical practice, an anti-IL6 receptor antibody (tocilizumab) is widely used to treat the abnormal inflammatory response that occurs in autoimmune diseases as rheumatoid arthritis and it is well tolerated by patients, even at very young age (Emery et al., 2019; Mallalieu et al., 2019; Mihara et al., 2011). The positive effect of the antibody relies on neutralization of IL6 activity through competition with soluble and membrane-bound IL6 receptors (Mihara et al., 2011). Efficacy of antibody treatment has been demonstrated particularly in the osteoarticular system in both murine experimental models (Kamiya et al., 2019) and patients (Safy-Khan et al., 2020). We reasoned that tocilizumab treatment could be beneficial in HGPS by reducing IL6-related progeroid features and tested this hypothesis in cultured HGPS fibroblasts, Lmna \(^{G609G/G609G}\) mouse cells, and in vivo in Lmna \(^{G609G/G609G}\) mice (Osorio et al., 2011, 2012; Zaghini et al., 2020). We show here that tocilizumab counteracts aberrant differentiation of adipocytes and osteoblasts from Lmna \(^{G609G/G609G}\) progeroid mice, improves adipose tissue dystrophy, aorta histological lesions, and skeletal deterioration, and positively impacts the overall condition of progeroid mice, while unexpectedly reducing progerin accumulation and its deleterious effects in mouse and human progeria cells.

2 | RESULTS

2.1 | IL6 secretion is increased in HGPS cells

Consistent with previous studies (Bidault et al., 2020; Liu et al., 2019), we observed that IL6 secretion is increased in HGPS fibroblasts carrying the classical G608G LMNA mutation and also in fibroblasts from Mandibuloacral Dysplasia, another LMNA-linked progeroid syndrome (Cenni et al., 2018; Filesi et al., 2005) (Figure 1a and Figure S1a). Further, the LMNA delta 50 mutation causing progerin expression, when transiently expressed in human HEK293 cells, induced hypersecretion of IL6 (Figure 1b). Although overexpression of wild-type LMNA elicited some increase in IL6 levels, much more significant enhancement of IL6 secretion was induced by progerin (Figure 1b) as well as by Mandibuloacral Dysplasia-linked R527H-mutated lamin A (Figure S1B). These results suggested that lamin A molecular defect was the primary cause of IL6 upregulation, which was linked to overexpression of IL6 gene (Figure 1c) and activation of IL6 promoter (Figure 1d). To confirm the latter finding, we used an IL6 luciferase mutant, which did not elicit any promoter activity signal neither in controls nor in HGPS fibroblasts (Figure 1D). Moreover, NF-kB promoter was
hyperactivated in HGPS cells (Figure 1e). We further observed that activated STAT3, a main effector of IL6 signaling, was accumulated to a significantly higher extent in the nucleus of HGPS fibroblasts relative to control fibroblast nuclei (Figure 1f). In fact, both Tyrosine 705 and Serine 727 STAT3 phosphorylation were increased in HGPS nuclei (Figure 1f).

### 2.2 Tocilizumab counteracts IL6 activity and bystander effects and progerin accumulation

In this context, we tested the effects of the neutralizing anti-IL6 receptor antibody tocilizumab in HGPS cells. STAT3 phosphorylation was significantly inhibited in HGPS fibroblasts subjected to

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**Figure 1** IL6 levels and activity are increased in HGPS cells. (a) IL6 secretion in culture media of normal human dermal fibroblasts (Control) or HGPS fibroblasts (HGPS) after 48 h in cell culture. IL6 levels were measured by ELISA. (b) IL6 secretion in culture media of HEK293 non-transfected (NT) or transiently transfected with plasmids carrying WT-LMNA (LA-WT) or Δ50-LMNA (LA-Δ50) and kept in culture for 48 h after transfection. IL6 levels were measured by ELISA. (c) qRT-PCR analysis of IL6 expression in control (Control) and HGPS fibroblasts (HGPS). (d) Activity of IL6 promoter in control (Control) and HGPS fibroblasts (HGPS) measured by a luciferase reporter assay. An IL6 luciferase mutant (IL6 Luc-mut) was also expressed in normal and HGPS fibroblasts as a negative control. (e) Activity of NF-kB promoter in control (Control) and HGPS fibroblasts (HGPS) measured by a luciferase reporter assay. (f) Immunofluorescence labeling of phosphorylated STAT3 in control (Control) and HGPS fibroblasts (HGPS). Mean fluorescence intensity values (MFI) are reported in the graph as arbitrary units (a.u.). Scale bar, 10 µm. Three biological replicates were used in all analyses (panels a, b, d, e, f), except in qRT-PCR (panel c, six biological replicates). Data are reported as means ± SEM. Statistically significant differences are indicated (*p < 0.05, **p < 0.01, ***p < 0.001).
Senescence-Associated beta-Galactosidase (SA-β-Gal) is upregulated in HGPS (Mattioli et al., 2018). These results suggested downregulation of the senescence marker p21 (Figure 2e), which consistently, in HGPS fibroblasts, tocilizumab treatment elicited was significantly increased, while tocilizumab prevented SA-β-Gal amount. Importantly, in HGPS-conditioned normal dermal fibroblasts, the efficacy of the proposed treatment on the bystander effect of HGPS cellular secretome and in particular of secreted IL6. Importantly, in HGPS-conditioned normal dermal fibroblasts, Senescence-Associated beta-Galactosidase (SA-β-Gal) staining was significantly increased, while tocilizumab prevented SA-β-Gal increase (Figure 2d). The latter result showed the inhibitory effect of tocilizumab on secretome-induced cellular senescence. Consistently, in HGPS fibroblasts, tocilizumab treatment elicited downregulation of the senescence marker p21 (Figure 2e), which is upregulated in HGPS (Mattioli et al., 2018). These results suggested inhibition of cellular senescence and a general improvement of HGPS cellular phenotype upon tocilizumab treatment. In agreement with this observation, significant improvement in nuclear shape, measured by the contour ratio algorithm, was observed in HGPS fibroblasts treated with tocilizumab, while lamin A/C immunofluorescence signal was not significantly affected (Figure 2f). Moreover, chromatin organization was improved in tocilizumab-treated cells, as also detectable from recovery of facultative heterochromatin areas (Figure 2f, arrowheads) (Lattanzi et al., 2007). Since nuclear dysmorphism has been directly correlated with the amount of progerin in the nuclear lamina (Columbaro et al., 2005), we hypothesized that inhibition of IL6 could reduce γ-H2AX amount. Importantly, in HGPS fibroblasts treated with tocilizumab, progerin amount in HGPS fibroblasts subjected to tocilizumab treatment (Figure 2h). Although reduced lamin A and C amount was also detected in antibody-treated cells, tocilizumab determined a significant increase in lamin A to progerin ratio (Figure 2h), which is relevant to the improvement of HGPS cellular phenotype (Pellegrini et al., 2015).

2.3 Effects of tocilizumab in muscle, tendons, and bone of Lmna<sup>G609G/G609G</sup> mice

Based on these results, we treated progeroid mice with tocilizumab starting at weaning (4 weeks of age) and evaluated antibody effects in various tissues known to be affected in this mouse model (Zaghini et al., 2020). Figure 3 shows the outcome of tocilizumab treatment in the musculoskeletal system of Lmna<sup>G609G/G609G</sup> mice. Muscle fibers did not show irregular shape in progeroid mice (Figure 3a). However, in skeletal muscle of vehicle-treated Lmna<sup>G609G/G609G</sup> mice at 100 days of age, altered nuclear shape was observed by lamin A/C labeling of muscle cryosections (Figure 3a). Nuclear shape was significantly improved in muscle from age-matched tocilizumab-treated Lmna<sup>G609G/G609G</sup> mice, as measured according to the contour ratio algorithm in myonuclei observed by electron microscopy (Figure 3b,c). In fact, ultrastructural analysis showed severe nuclear morphological abnormalities in Lmna<sup>G609G/G609G</sup> mouse muscle consisting of nuclear envelope folding and loss of peripheral heterochromatin, which were recovered in muscle from tocilizumab-treated progeroid mice (Figure 3c). On the other hand, nuclear positioning and sarcomere ultrastructural organization were not altered in progeroid muscle.
mice (Figure 3a,c). Moreover, as observed in cultured HGPS cells, in vivo tocilizumab administration reduced progerin accumulation in Lmna<sup>G609G/G609G</sup> muscle tissue (Figure 3d). Noteworthy, in progeroid mouse muscle the lamin-binding partner emerin was reduced, while tocilizumab restored emerin amount (Figure 3d). We further investigated the muscle-specific oxidative stress responsive protein Ankrd2 (Cenni et al., 2019). In Lmna<sup>G609G/G609G</sup> mouse muscle, Ankrd2 amount was significantly reduced, while tocilizumab treatment increased Ankrd2 levels to a condition comparable to wild-type mouse tissue (Figure 3d).

Motor function in progeria might be also affected by defects in tendons. By ultrastructural analysis, misshapen nuclei were also
detected in tendons of Lmna<sup>G609G/G609G</sup> progeroid mice (Figure 3e). In particular, enlargement of perinuclear space with formation of vesicles was consistently observed in the vast majority of examined tendon nuclei (Figure 3e). These nuclear defects were abolished in mice subjected to tocilizumab (Figure 3e).

Then, we analyzed bone phenotype in Lmna<sup>G609G/G609G</sup> mice by microCT scan analysis and observed an altered structure of femur condyles (Figure 3f). In age-matched tocilizumab-treated progeroid mice, bone trabecular organization was improved (mean BV/VT 0.82 in tocilizumab-treated vs 0.42 in untreated progeroid mice, Figure 3f). Moreover, femur biomechanical length, which was significantly reduced in Lmna<sup>G609G/G609G</sup> mice, increased in tocilizumab-treated animals relative to untreated littermates (Figure 3g). However, trabecular thickness (Figure 3h) and other bone parameters were not significantly improved by tocilizumab treatment.

In cultured cells derived from Lmna<sup>G609G/G609G</sup> mouse muscle or bone, tocilizumab treatment also elicited positive effects. In
TABLE S1). Higher magnification of nuclei labeled by lamin A/C antibody is shown in the insets. Scale bars, 10 µm. (b) Contour ratio of muscle nuclei in tissue from three different Lmna+/+ or LmnaG609G/G609G mice (mean age 100 days), left untreated (NT) or treated (T) with tocilizumab. Muscle fibers are delineated by perinuclear staining (red). Higher magnification of nuclei labeled by lamin A/C antibody is shown in the insets. Scale bars, 10 µm. (c) Transmission electron microscopy analysis of tendon nuclei from LmnaG609G/G609G mice (T). Cell nuclei of LmnaG609G/G609G mouse tendons show vesicles in the perinuclear space (NT, round vesicles), which are not observed in tocilizumab-treated mice. Heterochromatin areas appear also disorganized in progeroid mouse tendon nuclei, while recovery of heterochromatin at the nuclear periphery is observed in tocilizumab-treated LmnaG609G/G609G mouse tissue. The analysis was performed in 50 nuclei per sample. Scale bars, 1 µm. (f) MicroCT scans of femur from LmnaG609G/G609G (NT) or tocilizumab-treated LmnaG609G/G609G mice. Mean values of relative bone volumes (bone volume/tissue volume (BV/TV)) of the corresponding samples are indicated under each picture. (h) Mean femur biomechanical length (length) and (i) trabecular thickness values (Tb.th) measured in groups of three Lmna+/+, untreated LmnaG609G/G609G mice (NT) or tocilizumab-treated LmnaG609G/G609G mice (T). Three biological replicates were used in each experiment. Data are reported as means ± SEM. Statistically significant differences are indicated (*p < 0.05, **p < 0.01, ***p < 0.001). Mean age of mice (all males), 100 ± 6 days.

2.4 Improvement of cardiovascular phenotype in tocilizumab-treated LmnaG609G/G609G mice

Aorta is a main target of HGPS pathology (Hamczyk & Andres, 2019; Hamczyk, del Campo, et al., 2018; Hamczyk, Villa-Bellosa, et al., 2018). Histological analysis of LmnaG609G/G609G mouse aorta sections showed a severe phenotype with loss of cellularity and myxoid degeneration (Figure 4a). In LmnaG609G/G609G mice, tocilizumab treatment reduced aorta degeneration (Figure 4a and Table S1).

As previously reported (Del Campo et al., 2019; Villa-Bellosa et al., 2013), the number of smooth muscle cells was dramatically reduced in LmnaG609G/G609G mouse aorta (Figure 4b). However, tocilizumab administration led to a significant increase in smooth muscle cell number (Figure 4b). As observed in skeletal muscle, in vivo tocilizumab administration elicited reduction of progerin levels in myocardium, while lamin C and emerin amount were not changed (Figure 4c). In progeroid mice, we observed cardiomyocyte hypertrophy (Figure 4d). In fact, mean cross-sectional diameter of cardiomyocytes from LmnaG609G/G609G mice aged 3 months was comparable to that measured in wild-type littermates aged 13 months (Figure 4d), despite wild-type mice weight reached 24 g, while LmnaG609G/G609G mice maximum weight was below 18 grams. In myocardium from tocilizumab-treated LmnaG609G/G609G animals, cardiomyocyte mean diameter was significantly reduced, suggesting a less severe heart disorder (Figure 4d,e).

2.5 Improvement of adipose tissue phenotype in tocilizumab-treated progeroid mice

Fat loss is rapid in LmnaG609G/G609G mice, and almost all white adipose tissue is quickly lost around 5 weeks (Zaghini et al., 2020), so that evaluation of chronic treatment starting at weaning is not possible. Thus, to test the effect of tocilizumab on adipose tissue, we analyzed subcutaneous fat from control and tocilizumab-treated LmnaG609G+/+ mice, which show slower progression of lipodystrophy, detectable at week 16 of age and progressing to complete atrophy of subcutaneous fat at week 43 (Zaghini et al., 2020). A dystrophic phenotype with high adipocyte diameter variability was observed in white fat from untreated progeroid mice aged 200 days (Figure 5a). In adipose tissue from tocilizumab-treated LmnaG609G+/+ mice, a more homogeneous adipocyte size and an overall increase in adipocyte mean diameter were observed (Figure 5a). Impaired terminal differentiation of adipocytes was observed by ultrastructural analysis in tissue from untreated LmnaG609G+/+ mice (Figure 5b), while fusion of lipid droplets appeared to be increased in tocilizumab-treated mouse adipose tissue (Figure 5c). As quantitative analysis confirmed the increase of
**FIGURE 4** Tocilizumab reduces aorta lesions and cardiomyocyte hypertrophy in progeroid mice. (a) Aorta medium layer histochemical analysis in *Lmna+/* and *LmnaG609G/G609G* mice left untreated (NT) or treated with tocilizumab (T). Hematoxylin–eosin (left panels) and Alcian Blue staining (right panels) show loss of cellularity and myxoid degeneration (accumulation of acidic mucopolysaccharides) in aorta from vehicle-treated *LmnaG609G/G609G* mice (NT) and rescue with tocilizumab (T). (b) Mean number of smooth muscle cells detected in aorta sections is reported in the graph. (c) Western blot analysis of Progerin, Lamin C and Emerin in myocardium lysates from untreated (NT) or tocilizumab-treated *LmnaG609G/G609G* mice (T). GAPDH bands are shown as loading controls. Densitometry of immunoblotted protein bands is plotted in the graphs in arbitrary units (a.u.). (d) Myocardium sections from 13 months old *Lmna+/* or 3 months old *LmnaG609G/G609G* mice vehicle-treated (NT) or treated with tocilizumab (T). Extracellular matrix surrounding cardiomyocytes was stained using an anti-collagen VI antibody. Bar, 10 µm. (e) Graphs reporting diameter of cardiomyocytes in samples shown in (d). Three biological replicates were used. Data are reported as means ± SEM. Statistically significant differences are indicated (*p < 0.05, **p < 0.01)
adipocyte dimensions in white fat from tocilizumab-treated mice (Figure 5d), we decided to test the effect of IL6 neutralization on adipocyte precursors. To this end, we established pre-adipocyte cultures from Lmna<sup>G609G</sup> white adipose tissue and induced adipogenic differentiation (Pellegrini et al., 2019). Differentiation of Lmna<sup>G609G</sup> pre-adipocytes was reduced, relative to Lmna<sup>-/-</sup> pre-adipocytes, and significantly improved when tocilizumab was added to differentiation medium (Figure 5e), as demonstrated by enhanced lipid vesicle size (Figure 5f) and increased percentage of differentiating cells (Figure 5g).

### 2.6 | Phenotype improvement in tocilizumab-treated progeroid mice

By visual inspection, the phenotype of tocilizumab-treated Lmna<sup>G609G/G609G</sup> mice appeared greatly improved with respect to their untreated littermates (Figure 6a). Alopecia was reduced, and the quality of the fur, which was typically altered in this mouse model (Zaghloul et al., 2020), was better preserved (Figure 6a). Motor activity was also significantly improved by antibody treatment, as demonstrated by open field tests (Figure 6b). Even in very old mice, motor activity was preserved (Figure 6b and Movie S1). Moreover, the onset of kyphosis was delayed in tocilizumab-treated mice (Figure 6c). Slowdown of weight loss was also determined by tocilizumab effect on progerin levels could depend on interconnected cellular pathways involving JAK/STAT signaling (Liu et al., 2019). On the other hand, STAT1-mediated inflammatory response, triggered by replication fork stalling and damaged DNA accumulation in the cytoplasm, has been demonstrated in HGPS cells and a synergistic effect with STAT3-related IL6 signaling on progeroid phenotype appears likely (Kreienkamp et al., 2018). Interestingly, any drug able to counteract progerin accumulation has been reported to attenuate STAT1 activity (Kreienkamp et al., 2018), reinforcing the view of a direct link between progerin levels and inflammatory response.

In vivo tocilizumab administration to Lmna<sup>G609G/G609G</sup> progeroid mice allowed us to show some tissue-specific effects. Tocilizumab elicited positive effects in skeletal muscle, including rescue of the muscle-specific stress responsive factor Ankrd2 (Cenni et al., 2019), which was decreased in progeroid muscle. This result is particularly relevant, as resolution of oxidative stress and inflammation could be fostered by Ankrd2-dependent NF-kB inhibition (Bean et al., 2014), with potential effects on IL6 levels and other NF-kB effectors of inflammatory signaling. Reduction of emerin in the skeletal muscle of progeroid mice is a novel finding, which could suggest defects in skeletal muscle regeneration associated with progerin expression (Capanni et al., 2009; Squarzoni et al., 2005). Emerin is the main lamin A/C binding partner, known to play a major role in striated muscle and linked to Emery–Dreifuss muscular dystrophy. We previously observed that emerin localization at the nuclear membrane is related to its interplay with prelamin A (Capanni et al., 2009), a mechanism that could be impaired in the presence of a defective prelamin A form as progerin. Of note, concomitant with progerin decrease, emerin levels are rescued in skeletal muscle from tocilizumab-treated Lmna<sup>G609G/G609G</sup> mice, while emerin levels are not altered in myocardium of progeroid mice nor they are affected by tocilizumab in heart tissue. Further, emerin levels are not altered in HGPS fibroblasts, again suggesting a tissue-specific deficiency. Given the role of emerin and emerin-prelamin A interplay in muscle physiology and pathology (Capanni...
et al., 2008, 2009; Squarzoni et al., 2005), deeper understanding of emerin fate in HGPS skeletal muscle might provide new insights not only into HGPS pathogenesis, but also on aging-associated muscle disorders as sarcopenia. Overall, altered nuclear structure and disorganized chromatin here observed both in muscle and tendons, as well as altered expression levels of emerin and Ankrd2 in skeletal muscle, indicate an insofar unrecognized condition that might contribute to motor function impairment in HGPS (Levy et al., 2018).

A limitation of our study is lack of electrophysiological evaluation of heart functionality. However, we focused on tissue morphology and were able to demonstrate improvement of aorta lesions, including smooth muscle cell loss, and amelioration of cardiomyocyte hypertrophy in myocardium from tocilizumab-treated mice. Hypertrophy of myocardium was previously observed in mouse models expressing endothelium-targeted progerin (Osmanagic-Myers et al., 2019; Sun et al., 2020). As SASP activation including IL6 hypersecretion was determined in one of those progeroid mouse strains, it is likely that cell

FIGURE 5 Tocilizumab improves adipose tissue phenotype in progeroid mice. (a) Light microscopy observation of semithin sections of subcutaneous adipose tissue from Lmna<sup>−/−</sup> or Lmna<sup>G609G/−</sup> vehicle-treated (NT) or tocilizumab-treated mice (T). Semithin sections were obtained from epon resin-embedded tissue prepared for electron microscopy analysis. (b, c) Electron microscopy analysis of adipose tissue samples shown in (a). Arrows indicate fusing lipid vesicle. (d) Quantitative analysis of adipocyte size in adipose tissue samples shown in (a–c). A trend toward wild-type size distribution in tocilizumab-treated Lmna<sup>G609G/−</sup> mice is observed. (e) Oil red O staining of white pre-adipocytes derived from Lmna<sup>−/−</sup> or Lmna<sup>G609G/−</sup> mouse subcutaneous fat. (f) Graph showing the distribution of lipid vesicle size. (g) Graph representing the percentage of differentiating pre-adipocytes in cell cultures. Data are in arbitrary units (a.u.). Three biological replicates were used. Scale bars: (a), 100 µm; (b) 5 µm; (c), 1 µm, (e), 10 µm. Statistically significant differences are indicated (***(p < 0.0001)
FIGURE 6  Tocilizumab improves progeroid phenotype and lifespan in progeroid mice. (a) Representative photographs of untreated (NT) and tocilizumab-treated (T) Lmna<sup>G609G/G609G</sup> mice at 80 days of age. (b) Motility test of untreated (NT, n = 3, all males) and tocilizumab-treated Lmna<sup>G609G/G609G</sup> mice (T, n = 3, all males). In the graph is reported the walking time (minutes) in a period of 30 min. Differences at 85 and 110 days are statistically significant (p < 0.01). (c) Age at kyphosis onset (days) of untreated (NT, n = 6, 3 females, 3 males) and tocilizumab-treated (T, n = 7, 5 females, 2 males) Lmna<sup>G609G/G609G</sup> mice. Statistically significant difference is indicated (*p < 0.05). (d) Body weight variation (%) of untreated and tocilizumab-treated Lmna<sup>G609G/G609G</sup> mice between 6 (6 w) and 12 weeks of age (12 w). Mean values measured in 5 mice per group (2 females, 3 males) are reported in the upper graph, weight referred to each animal (indicated as 1–10) is reported in the lower graphs (left bars, 6 weeks weight; right bars, 12 weeks weight). Statistically significant differences are indicated (**p < 0.01, ****p < 0.0001). (e) Kaplan–Meier survival plot showing the increase in life span of Lmna<sup>G609G/G609G</sup> mice treated with tocilizumab (n = 13, 7 females, 6 males) as compared with Lmna<sup>G609G/G609G</sup> untreated littermates (n = 18, 9 females, 9 males), p < 0.05, log-rank/Mantel-Cox test. (f) Kaplan–Meier survival plot showing the increase in life span of Lmna<sup>G609G</sup/+ mice treated with tocilizumab (n = 16, 9 females, 7 males) as compared with Lmna<sup>G609G</sup/+ untreated littermates (n = 20, 3 females, 17 males), p < 0.01, log-rank/Mantel-Cox test. The age at 50% survival (median survival) is indicated next to each graph (d, days).
extrinsic mechanisms contribute to hypertrophy of cardiac tissue (Sun et al., 2020), an hypothesis supported by our results.

Lipodystrophy is a prominent phenotype in all progeroid laminopathies (Cenni et al., 2018). None of currently available drugs was able to counteract adipose tissue loss, either in less severe LMNA-linked lipodystrophies (Araujo-Vilar & Santini, 2019) or in HGPS (Gordon et al., 2018). On the other hand, in Lmna<sup>Δ609G/Δ609G</sup> mice subjected to high fat diet, an impressive life span extension was obtained (Kreienkamp et al., 2019; Kreienkamp & Gonzalez, 2020), suggesting a main role of adipose tissue loss in HGPS pathogenesis. Thus, amelioration of white adipose tissue condition by tocilizumab might contribute to the overall improvement of health status here observed in progeroid mice. An application of the antibody to other laminopathies featuring lipodystrophy warrants investigation.

Bone phenotype (Gargiulli et al., 2018) was also improved by tocilizumab treatment. In fact, increased femur biomechanical length and partial rescue of altered condyle trabecular structure along with delay in the onset of kyphosis were observed in antibody-treated animals. Our results obtained in cultured Lmna<sup>Δ609G/Δ609G</sup> osteoblasts confirmed the aberrantly increased differentiation rate of laminopathic osteoblasts previously demonstrated in bone progenitor cells carrying the human progeria G608G LMNA mutation (Scaffidi & Misteli, 2008) and Mandibuloacral dysplasia osteoblasts (Avnet et al., 2011). We cannot rule out the possibility, suggested in other studies, that osteoblast activity could be instead decreased in progeroid mice in different bone districts (Strandgren et al., 2015). However, here we show a differentiation rate comparable to wild-type cells in progeria osteoblasts subjected to tocilizumab, suggesting potential rescue of normal bone turnover. Since IL6 is an osteoclastogenic cytokine and tocilizumab has been shown to reduce the RANKL/OPG ratio, which regulates osteoclastogenesis (Kamiya et al., 2019), we predict that antibody administration to Lmna<sup>Δ609G/Δ609G</sup> mice might also reduce osteoclastogenesis by directly targeting the NF-κB/RANKL pathway. Along this line, a recent study using another IL6-neutralizing antibody showed amelioration of osteoporosis associated with hypersecretion of IL6 due to LMNA deficiency (Xiong et al., 2020).

A relevant outcome of tocilizumab treatment was the amelioration of motor activity in progeroid mice. Open field tests showed better performance of antibody-treated mice even at advanced age. Although skeletal abnormalities and motor impairment do not appear as life-threatening features of progeria, they severely impact on patient quality of life as children affected by progeria can only walk short distances due to articular impairment (Gordon et al., 2018). In this respect, it is worth to remind that tocilizumab is currently used to treat rheumatoid arthritis, where the antibody is expected to reduce IL6 inflammatory activity affecting articular cartilage (Mihara et al., 2011).

A main bias in this and almost all previously published studies conducted in progeroid mice is failure to identify an obvious cause of premature death (Hamczyk & Andres, 2019). The moderate increase in life span obtained by tocilizumab administration suggests that pathogenetic pathways specifically relevant to animal survival were not rescued. For instance, despite amelioration of adipose tissue turnover and attenuation of IL6 signaling, metabolic effects related to dysregulation of other cytokines (Bidault et al., 2020; Griveau et al., 2020; Liu et al., 2019) might suddenly establish a fatal condition. Optimization of tocilizumab dosage and combination with drugs or molecular approaches already explored for HGPS treatment may pave the way to effective therapeutic strategies (Cenni et al., 2020; Liu et al., 2019). For instance, tocilizumab could elicit a synergistic effect with lonafarnib, the farnesyltransferase inhibitor used in the ongoing HGPS clinical trial (https://www.clinicaltrials.gov/ct2/show/NCT00425607). Of note, lonafarnib has been reported to lower progerin levels in HGPS iPSCs-derived smooth muscle cells subjected to biomechanical strain (Ribas et al., 2017). Also, combination with statins, shown to improve HGPS cellular phenotype (Columbaro et al., 2005) and reduce IL6 levels (Ribas et al., 2017), warrants investigation.

Finally, our study indicates that tocilizumab could be explored in aging-associated disorders, including sarcopenia, cachexia, and motor impairment and more in general to mitigate the detrimental effects of age-related inflammation that impinge upon the onset of dysfunctions and disability at late ages and on the overall quality of life in the elderly.

4  |  MATERIALS AND METHODS

4.1  |  IL6 detection and neutralization

Tocilizumab, a monoclonal anti-IL6R neutralizing antibody (Mihara et al., 2011), was from Roche. IL6 levels in culture media of control and HGPS fibroblasts were measured by ELISA using kits from RD-Systems (Human IL-6 Quantikine HS ELISA Kit) following manufacturer’s instructions. A fluorescent plate reader (Infinite M200-6110; Tecan) was used to measure IL6 signal. For IL6 neutralization in HGPS fibroblast cultures, 100 µg/ml of neutralizing antibody was added to culture medium for 72 h. Tocilizumab was administered to Lmna<sup>Δ609G/Δ609G</sup> or Lmna<sup>G609G/G609G</sup> by intraperitoneal injection every three days starting at weaning (typically at post-natal week 4) and continued up to the humane endpoint, when the animals were euthanized. A dosage of 40 mg/kg (body weight) per week was estimated the most efficient. Saline solution was injected in parallel to animals to be used as controls.

4.2  |  Mice

All animal studies were performed in accordance with EU regulations, the guidelines of the Italian Ministry of Health and the local committee for animal welfare. Experiments were also in compliance with ethical rules and the experimental protocol was approved by the Italian Ministry of Health (No. 653/2016-PR issued on 07/04/2016 and update No. AC750.13-1105). Homozygous or heterozygous mice (C57BL/6 strain) carrying the mouse Lmna G609G mutation, equivalent to LMNA G608G mutation of human HGPS,
were kindly provided by Prof. Carlos Lopez-Otin (Oviedo University, Spain) (Osorio et al., 2012).

Phenotype and molecular features of Lmna<sup>G609G/G609G</sup> and Lmna<sup>G609G+/+</sup> progeroid mice have been characterized and previously reported (Osorio et al., 2011; Zaghini et al., 2020). Co-managed wild-type littermates (Lmna<sup>+/+</sup>) were used as controls. Two to five mice were housed in each cage, at a constant temperature of 22 ± 1°C under a 12-hour light/12-hour dark cycle with free access to food and water. Animals were randomly assigned to each group. Analyses of phenotypes of mice were performed at different times throughout their lifespan, and at time of death. Procedures performed in mice include: weight monitoring, performance tests to assess locomotor activity (open field test), micro-computed tomography (microCT) analysis of femurs with a microCT scan and tissue collection. As a whole, 10 Lmna<sup>+/+</sup> male mice and 10 females, 22 Lmna<sup>G609G+/+</sup> males and 20 females and 13 Lmna<sup>G609G/G609G</sup> males and 7 females were used in this study.

To assess motor activity, a subset of mice (3 mice per genotype, all males) underwent the open field test at 85, 95, 105 and 110 days of age. A semi-transparent plastic white rectangular box (70 cm × 50 cm), was used as arena (Zaghini et al., 2020). At the beginning of the test, each mouse was set in the middle of the arena. Experienced operators during a 30 min period observed and recorded and evaluated: travelled quadrants, time spent moving, time spent in the central area, and vertical movements. The room was isolated from sound, and unintentional interruptions were avoided.

### 4.3 | Skeletal microCT

Mouse femurs immersed in saline solution within a plastic block were analyzed on a microCT model SkyScan 1072 (Bruker Corp., MicroCT unit). The scanning parameters were set at a voxel resolution of 10.78 µm, 50 kV, 200 µA, 1 mm aluminum filter, exposure time 5936 ms, image averaged on 2 frames, rotation 180° and a rotation step of 0.9°. Tomographic image reconstruction was based on NRecon software (Bruker Corp., MicroCT unit). A global threshold was applied to select bone tissue (gray threshold value 117/255). Mouse femur and trabecular bone were also qualitatively analyzed on 3D reconstructions thanks to CT-Vox software (Bruker Corp., MicroCT unit) to identify any possible alteration. Trabecular and cortical morphology of the femur were investigated by use of CT-Analyzer software (Bruker Corp., MicroCT unit). Trabecular bone was measured on the following parameters: bone volume fraction (bone volume/total volume or BV/TV, %), trabecular bone thickness (TbTh, mm), trabecular separation (TbSp, mm), and trabecular bone number (TbN, 1/mm). Volume of interest for trabecular tissue was selected with the anatomical reference of the distal growth plate, starting about 0.215 mm proximally from the growth plate level (offset of 50 image slices), and extending in the direction of the femoral head for about 1.72 mm (height of 450 image slices). This section of the diaphysis is defined in coincidence to the length investigated by mechanical testing. The femur biomechanical length was defined as the longitudinal distance between the cranial side of the intertrochanteric fossa and the intercondylar fossa.

### 4.4 | Cell cultures and transfection

All human cell cultures used in this study were from BioLaM biobank (Rizzoli Orthopedic Institute Ethical Committee approval no. 0018250–2016, in compliance with all local and EU ethical rules). Control and HGPS fibroblast cultures had been obtained from skin biopsies as previously described (Columbaro et al., 2005). Necropsy of Lmna<sup>+/+, G609G/G609G</sup> and Lmna<sup>G609G+/+</sup> progeroid mice was performed according to local and EU ethical rules. Cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (D5648; Sigma) supplemented with 20% fetal bovine serum (FBS) (10270-106; Thermo Fisher), 100 IU/ml penicillin and 100 µg/ml streptomycin (15140122; Thermo Fisher) (growth medium) in a 5% CO₂ humidified atmosphere at 37°C.

Adipocyte, osteoblast, tenocyte and myoblast cultures were established as previously described from mouse subcutaneous fat, rib bone and skeletal muscle (vastus lateralis), respectively, and differentiated according to established protocols (Antonie et al., 2020; Avet et al., 2011; Mattioli et al., 2011; Pellegrini et al., 2019).

Differentiation of cultured mouse pre-adipocytes was assessed by Oil Red O staining. Cells were washed twice with PBS and fixed with 10% Formalin Solution (HT501128, Fisher) in distilled water for 45 min. After a 5 min incubation with 60% isopropanol, Oil Red working solution was added to the fixed cells for 5 min at room temperature. Images were obtained by using a Zeiss Axio A1 inverted microscope equipped with a digital camera and ZEN software.

Mineralized matrix formation in osteoblast cultures was detected by alizarin red S (ARS) staining. Cells were washed twice with PBS and fixed with 10% Formalin Solution in distilled water for 5 min, washed twice with distilled water and stained with ARS working solution for 5 min. Samples were observed using a Zeiss Axio A1 inverted microscope equipped with a digital camera. Pictures were taken using ZEN software.

To measure nuclear circularity, the contour ratio algorithm was used and calculated by following the formula:

\[
\text{contour ratio} = 4\pi \times \frac{\text{nuclear area}}{\text{nuclear perimeter}^2}
\]

HEK293 cells were transiently transfected with plasmids expressing wild-type prelamin A (LA-WT), which undergoes normal maturation, progerin (LA-Δ50), which cannot be processed by ZMPSTE24 endoprotease, or R527H-mutated lamin A, associated with MADA (Lattanzi et al., 2007). Transfections were performed using lipofectamine-2000 (18324012; Invitrogen) according to the manufacturer’s instructions. After transfection, cells were incubated for 48 h, if not differently stated.
4.5 | Biochemical analysis

Cells were fixed in 4% paraformaldehyde, post-fixed using absolute methanol for 5 min, and stained according to previously published protocols (Mattioli et al., 2011). Primary antibodies were applied overnight at 4°C, and secondary antibodies were used for 1 hour at room temperature. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Sample observation and image acquisition were performed using a Nikon Eclipse Ni epifluorescence microscope equipped with a digital CCD camera and NIS-Elements 4.3 AR software. Photoshop CS and Photoshop 7 were used for image processing. Mean fluorescence intensity (MFI) was measured using NIS-Elements 4.3 AR.

For Western blot analysis, tissues and cells were lysed in buffer containing: 20 mM Tris-HCl (pH = 7.5), 1% SDS, 1 mM Na3VO4, 1 mM PMSF, 5% beta-mercaptoethanol, and protease inhibitors. Proteins were subjected to SDS gradient gel (5%-20%) electrophoresis and transferred to nitrocellulose membrane overnight at 4°C. After incubation with primary and secondary antibodies, immunoblotted bands were revealed by Invitrogen ECL detection system. Densitometry was performed by a Bio-Rad GS800 Densitometer equipped with Quantity One Software. Densitometric values were normalized to corresponding GAPDH bands if not differently stated.

4.6 | Histology

Skeletal muscle or myocardium fragments from Lmna+/- or LmnaG609G/G609G mice were frozen in melting isopentane and stored in liquid nitrogen. Unfixed cryosections were subjected to immunofluorescence staining as detailed above.

Samples of aortic arch promptly after necropsy were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. Semithin sections were stained with toluidine blue for preliminary optical microscopy sample observation. Ultrathin sections were treated for transmission electron microscopy observation as described (Fiesli et al., 2005). After post-fixation with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour, samples were dehydrated in an ethanol series, infiltrated with propylene oxide, and embedded in Epon812 epoxy resin following standard procedures. Ultrathin sections (60 nm thick) were stained with uranyl acetate and lead citrate and observed at a 0° tilt angle with a JEOL JEM-1011 transmission electron microscope operated at 100 kV.

4.7 | Antibodies

Antibodies employed in this study were as follows: anti-STAT3 phosphorylated on tyrosine 705 (P-STAT3 Y705), anti-STAT3 phosphorylated on Serine 727 (P-STAT3 S727), and anti-STAT3 from Thermo Fisher Scientific; anti-γ-H2AX, rabbit polyclonal (4937; Cell Signaling); anti-p21, rabbit polyclonal (MA5-14949; Invitrogen); anti-Emerin, mouse monoclonal (MONX10804; Monosan); anti-β-tubulin; anti-lamin A/C, goat polyclonal (SC-6215; Santa Cruz Biotechnology); anti-Progerin, mouse monoclonal (13A4; Enzo); anti-GAPDH, mouse monoclonal (MAB374; Millipore); anti-Ankrd2, mouse monoclonal, clone YAS11 (LS-Bio); and anti-collagen VI, monoclonal (Millipore).

4.8 | Transmission Electron Microscopy

Tissue fragments (tendon, skeletal muscle and adipose tissue) were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. Semithin sections were stained with toluidine blue for preliminary optical microscopy sample observation. Ultrathin sections were treated for transmission electron microscopy observation as described (Fiesli et al., 2005). After post-fixation with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour, samples were dehydrated in an ethanol series, infiltrated with propylene oxide, and embedded in Epon812 epoxy resin following standard procedures. Ultrathin sections (60 nm thick) were stained with uranyl acetate and lead citrate and observed at a 0° tilt angle with a JEOL JEM-1011 transmission electron microscope operated at 100 kV.

4.9 | Luciferase assays

One day before transfection, human control or HGPS fibroblasts were seeded on 6-well plates and transfected with 500 ng of luciferase reporters driven by the −2,161 to −41 bp IL6 promoter fragment (kindly provided by W. L. Farrar, NCI-Frederick Cancer Research and Development Center, USA, Papi et al., 2012). Firefly Luciferase was normalized by co-transfecting 10 ng of Thymidine Kinase Renilla Luciferase reporter (Promega Corporation). All luciferase assays were performed in triplicate following manufacturer’s instructions (Promega).

4.10 | Real-time PCR

Total RNA was extracted using the TRI Reagent Solution (Invitrogen) and treated with TURBO DNase (Invitrogen). cDNAs were produced using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer’s protocol. Gene expression was determined by qPCR and Power SYBR Green PCR master mix (Applied Biosystems). Expression analysis was performed using the Applied Biosystem 7900HT real-time PCR system. Fold change of expression levels was analyzed by the ΔΔCT method, and transcript levels were normalized by using the housekeeping reference gene GAPDH. The qPCR primer list is reported in Table S2.
4.11 | Statistical analysis

Statistical analysis was performed with GraphPad Prism version 7 (Palm). Data were expressed as means ± standard error of the mean (SEM), as indicated in the figure legends, and tested using one-way ANOVA (for multiple comparisons) or two-tailed Student’s t test (two groups). For the comparison of different groups in Kaplan–Meier survival plots, we used a log-rank (Mantel-Cox) test. P values of ≤0.05 are considered statistically significant. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; Non-significant (NS), p ≥ 0.05.

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CONFLICT OF INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

SS and GL involved in conceptualization. ES, PS, EM, CC, VC, MRDA, DA, CB, MS, VP, AF, and GS involved in investigation. GL contributed to manuscript writing. GS, FB, MB, SS, and AZ contributed to supervision. GL involved in funding acquisition. AZ and GL contributed to resources.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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