Hutchinson-Gilford progeria syndrome (HGPS) is an extremely rare sporadic genetic disorder that is characterized by accelerated cardiovascular disease and premature aging. Most HGPS patients (>90%) carry a noninherited autosomal dominant de novo heterozygous mutation at codon 608 of the *LMNA* gene (c.1824C>T: GGC>GGT; p.G608G). This mutation activates a cryptic splice donor site, which causes the synthesis of progerin. Expression of this lamin A mutant disrupts the nuclear membrane architecture and causes multiple cellular alterations, including impaired DNA repair capacity and abnormal higher-order chromatin organization, gene transcription, and signal transduction. HGPS patients exhibit accelerated atherosclerosis and die at a mean age of 13.4 years (range, 8–21 years) predominantly of myocardial infarction or stroke. Additional clinical HGPS manifestations include dermal and bone abnormalities, alopecia, and joint contractures. Moreover, the aortas and aortic valves of HGPS patients are excessively calcified, and arteries from old transgenic mice carrying a human bacterial artificial chromosome that harbors the common HGPS mutation accumulate calcium deposits that are absent in age-matched controls. However, the mechanisms underlying excessive vascular calcification in HGPS remain unexplored.

**Background**—Progerin is a mutant form of lamin A responsible for Hutchinson-Gilford progeria syndrome (HGPS), a premature aging disorder characterized by excessive atherosclerosis and vascular calcification that leads to premature death, predominantly of myocardial infarction or stroke. The goal of this study was to investigate mechanisms that cause excessive vascular calcification in HGPS.

**Methods and Results**—We performed expression and functional studies in wild-type mice and knock-in *Lmna* G609G/+ mice expressing progerin, which mimic the main clinical manifestations of HGPS. *Lmna* G609G/+ mice showed excessive aortic calcification, and primary aortic vascular smooth muscle cells from these progeroid animals had an impaired capacity to inhibit vascular calcification. This defect in progerin-expressing vascular smooth muscle cells is associated with increased expression and activity of tissue-nonspecific alkaline phosphatase and mitochondrial dysfunction, which leads to reduced ATP synthesis. Accordingly, *Lmna* G609G/+ vascular smooth muscle cells are defective for the production and extracellular accumulation of pyrophosphate, a major inhibitor of vascular calcification. We also found increased alkaline phosphatase activity and reduced ATP and pyrophosphate levels in plasma of *Lmna* G609G/+ mice without changes in phosphorus and calcium. Treatment with pyrophosphate inhibited vascular calcification in progeroid mice.

**Conclusions**—Excessive vascular calcification in *Lmna* G609G mice is caused by reduced extracellular accumulation of pyrophosphate that results from increased tissue-nonspecific alkaline phosphatase activity and diminished ATP availability caused by mitochondrial dysfunction in vascular smooth muscle cells. Excessive calcification is ameliorated on pyrophosphate treatment. These findings reveal a previously undefined pathogenic process in HGPS that may also contribute to vascular calcification in normal aging, because progerin progressively accumulates in the vascular tissue of individuals without HGPS. (Circulation. 2013;127:2442-2451.)

**Key Words:** ATP ■ Hutchinson-Gilford progeria syndrome ■ muscle, smooth ■ progerin ■ pyrophosphate ■ tissue-non specific alkaline phosphatase ■ vascular calcification
Defective Extracellular Pyrophosphate in HGPS

Vascular calcification typically manifests as calcium-phosphate deposition (CPD) in distinct layers of the aortic wall.\textsuperscript{18,19} Calcium-phosphate deposits are composed of several members of the calcium orthophosphate family, including hydroxyapatite, octocalcium phosphate, and amorphous calcium phosphate.\textsuperscript{20} Medial calcification occurs within the elastin region of arteries and is almost exclusively associated with vascular smooth muscle cells (VSMCs). Elevated serum phosphorus (in the form of inorganic phosphate [Pi]) is a major risk factor for vascular calcification.\textsuperscript{21,22} In vitro and in vivo studies have shown that elevated Pi concentration in VSMCs triggers a transition to a bone-forming phenotype.\textsuperscript{18,19} This transition results in overexpression of osteochondrogenic markers, such as the transcription factor Runx2 (also named Cbfα1), which induces the expression of major bone matrix components.\textsuperscript{18,19}

Extracellular pyrophosphate (ePPi) directly blocks CPD in vitro and in vivo and is therefore a major endogenous inhibitor of vascular calcification.\textsuperscript{23–26} Degradation of ePPi is catalyzed by tissue-nonspecific alkaline phosphatase (TNAP), which hydrolyzes inorganic pyrophosphate (Pi) to Pi. Importantly, calcification in ex vivo cultured rat aorta is induced by alkaline phosphatase and is prevented by TNAP inhibitors.\textsuperscript{26,27} TNAP is moreover upregulated in the aortas of uremic rats, which results in increased hydrolysis of ePPi and vascular calcification.\textsuperscript{28} The main enzyme involved in ePPi synthesis, both in aorta and in cultured VSMCs, is the ectoэнzyme nucleotide pyrophosphatase/phosphodiesterase-1 (eNPP1).\textsuperscript{26,29} Lack of eNPP1 leads to extensive and fatal arterial calcification in children and mice.\textsuperscript{30,31} The substrate for eNPP1 is ATP, which accumulates in the extracellular matrix via the action of several transporters,\textsuperscript{32} such as the multiple-pass transmembrane protein ANK.\textsuperscript{33}

To define the molecular mechanisms that lead to vascular calcification in HGPS, we analyzed Lmna\textsuperscript{G609G/G609G} knock-in mice, which express progerin as a consequence of aberrant splicing equivalent to the human mutation c.1824C>T (p.G608G).\textsuperscript{34} This mouse model of progeria mimics the main clinical manifestations of human HGPS, including cardiovascular aberrations and premature death.\textsuperscript{34} Our studies reveal profound alterations in ePPi homeostasis caused by progerin expression that exacerbate vascular calcification, which is inhibited by treatment with pyrophosphate.

Methods

Mice

Male Lmna\textsuperscript{G609G/G609G}, Lmna\textsuperscript{G609G/G609G}, and wild-type littermates were used.\textsuperscript{35} Animal studies were approved by the local ethics committee and conformed to directive 2010/63/EU and recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

Cell Culture

VSMCs were prepared from thoracic aortas of 30- to 32-week-old mice as described previously.\textsuperscript{36} Cells were grown in minimal essential medium that contained 1 mmol/L L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum at 37°C in a humidified atmosphere at 5% CO₂. VSMCs were used at passages 5 to 10. Cells were grown to confluence and used after an overnight quiescence step (0.1% fetal bovine serum). Calcification assays were performed on cells incubated for 7 days in minimal essential medium supplemented with 0.1% fetal bovine serum and 2 mmol/L Pi as described previously.\textsuperscript{37,38} For phosphate activity studies and ATP and Pi measurements, VSMCs were incubated in medium without Phenol Red.

Quantification of Calcium Deposition

Aortic arch and thoracic aorta from wild-type and Lmna\textsuperscript{G609G/G609G} mice (n=5 per genotype) were included in optimal cutting temperature compound (OCT; Sakura, Alphen aan den Rijn, Netherlands), and 4-μm cross sections were cut with a cryostat (Leica CM1950; Barcelona, Spain). Calcification in aortic tissue was revealed by Alizarin red staining as described previously.\textsuperscript{35,36} To quantify calcium deposits, 2 to 4 aortic cross sections of each mouse were analyzed with ImageJ (http://imagej.nih.gov/ij/; public domain). To quantify calcification content in VSMCs, cells were treated with 0.6 mol/L HCl overnight at 4°C and analyzed with a colorimetric QuantiChrom calcium assay kit (BioAssay Systems, Hayward, CA).

Pyrophosphate Treatment In Vivo

PPi (Sigma-Aldrich product No. S6422; St Louis, MO) was dissolved in sterile saline (B. Braun Medical SA, Barcelona, Spain). Ten-week-old Lmna\textsuperscript{G609G/G609G} mice were treated with PPi for 9 weeks (daily intraperitoneal injection, 100 mg·kg⁻¹·d⁻¹), and the control group received saline (n=5 mice in each group). Quantification of aortic calcification was performed as indicated above.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from mouse aorta or VSMCs with QIAzol lysis reagent (Qiagen, Madrid, Spain). After DNase treatment, 2 μg of RNA was reverse transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Madrid, Spain). Quantitative real-time polymerase chain reaction was performed with Power SYBR Green in 384-well clear optical reaction plates on an ABI PRISM 7900HT sequence detection system according to the manufacturer’s instructions for calibrator normalization (Applied Biosystems). All reactions were performed in triplicate. The primers used for amplification were as follows: (1) BMP-2 (NM_007553): 5′-CAGCGTGCGACAGTCTCA-3′ (forward), 5′-CCGGCCGGTTTTCCTACTCA-3′ (reverse); (2) Runx2 (NM_001146038.1): 5′-CAATCCCAGGCACAGCAGTCTC-3′ (forward), 5′-ACAGCAGCGTTGGAGATG-3′ (reverse); (3) eNPP1 (NM_008813): 5′-GGATTGCCAATAAGGCT-3′ (forward), 5′-CAAGAAGCTTGTCCGTCGAG-3′ (reverse); (4) TNAP (NM_007431): 5′-CTATGTCCTGGAACCGCCTAGA-3′ (forward), 5′-AGCCTTTAGGATTTCGCTGTA-3′ (reverse); (5) Pit1 (NM_015747): 5′-CTGTCCTACAGGTGTTAGG-3′ (forward), 5′-TGTAAAATCTAGGCACGGAAAC-3′ (reverse); (6) Pit2 (NM_011394): 5′-CTTCTAAACGAGACCGTTGGA-3′ (forward), 5′-CATGAGCCGCAAAGAAGTT-3′ (reverse); (7) ANK (NM_020332): 5′-ACAGCGTGCGACAGTCTCA-3′ (forward), 5′-AATGCCAACACCTTT-3′ (reverse); (8) eNTPD1 (NM_009248): 5′-AGCTTCACACAGATGACCTT-3′ (forward), 5′-GCCACACTGAAACCCCTGTA-3′ (reverse); (9) fetoA (NM_013465): 5′-ATCGACAAAGTCAAGGTGTGG-3′ (forward), 5′-ACAAGAAATCCGAGGTTGAATG-3′ (reverse); (10) matrix Gla protein (MGP; NM_008597): 5′-AGGAGAACATGCAACCTCTT-3′ (forward), 5′-AGCAGAATCCGAGGTTGAATG-3′ (reverse). Expression was quantified by the comparative C_{T} method, with correction for the expression of the endogenous control gene acidic ribosomal phosphoprotein P0 (RPLP0, accession number NM_007475).
Immunoblots and Immunohistochemistry

VSMC lysates were prepared in lysis buffer (0.1% SDS, 25 mMol/L Tris-HCl pH 7.4, and protease inhibitors) and 30 to 50 µg of protein was separated by SDS-PAGE and blotted to polyvinylidene difluoride membrane as described previously.15 The following primary antibodies were used: Rabbit monoclonal anti-eNTPD1 (1/5000, ab108248, Abcam, Cambridge, United Kingdom), rabbit polyclonal anti-TNAP (1µg/mL, ab65834, Abcam), rabbit polyclonal anti-GFP (1/2500, ab6566, Abcam), and mouse monoclonal anti-tubulin (1/5000, sc-8035, Santa Cruz Biotechnology, Dallas, TX). After incubation with appropriate secondary antibodies, blots were revealed with the ECL system (Millipore, Billerica, MA). Mouse aortas were immunohistochemically stained with a rabbit polyclonal antibody to Runx2 (1/500, ab102711, Abcam).

Phosphatase Activity and ATP Hydrolysis Assays

Phosphatase activity in VSMCs or plasma was measured with the pNPF phosphatase assay kit and the QuantiChrom alkaline phosphatase assay kit (BioAssay Systems, respectively). ATP hydrolysis assays in VSMCs cultures were performed as described previously.20 Briefly, cells were seeded in 12-well plates and incubated for the indicated times with 1 µmol/L ATP and 1 µC/m (131I)ATP radiotracer (Perkin Elmer, Madrid, Spain). ATP, Pi, and PiP in the culture medium were then separated by chromatography on PEI-cellulose plates and were developed with 650 µmol/L KH2PO4 pH 3. After autoradiography, spots were excised and counted by liquid scintillation.

ATP and Pyrophosphate Quantification

ePi was measured with an enzyme-linked bioluminescence assay in which PPi reacts with adenosine 5'-phosphosulfate (A5508, Sigma-Aldrich) in the presence of ATP-sulfurylase (A8957, Sigma-Aldrich). After incubation with appropriate secondary antibodies, blots were revealed with the ECL system (Millipore, Billerica, MA). ATP, Pi, and PiP in the culture medium were then separated by chromatography on PEI-cellulose plates and were developed with 650 µmol/L KH2PO4 pH 3. After autoradiography, spots were excised and counted by liquid scintillation.

Generation of Retroviral Vectors and Stable Cell Lines

Wild-type prelamin A and progerin cDNAs were polymerase chain reaction amplified using as a template a previously published construct or Adgene plasmid #17663, respectively. Amplicons were reaction amplified using as a template a previously published construct or Adgene plasmid #17663, respectively. Amplicons were inserted into pECFP vector (Clontech, Mountain View, CA). Cyan fluorescent protein (CFP)-prelamin A and progerin-CFP were then subcloned into pMSCVpuro retrovector vector (Clontech) after digestion with XhoI/EcoRI to respectively generate pMSCVpuro/CFP-prelamin A (which is posttranslationally modified to generate mature lamin A) and pMSCVpuro/progerin-CFP. Correct cloning of both plasmids was verified by DNA sequencing. The precise primer sequences and cloning strategies are available on request.

Mitochondrial Assays

Mitochondrial ATP synthesis in digitonin-permeabilized VSMCs (2x10^6 cells) was measured by kinetic luminescence assay.
apyrase1/eNTPD1, and the phosphate transporter Pit2 in LmnaG609G/+ than in wild-type VSMCs, whereas eNPP1, ANK, and Pit1 were unaffected (Figure 2B). Western blot analysis confirmed the increase in the ectoenzymes TNAP and eNTPD1 in Lmna G609G/+ VSMCs (Figure 2C). Moreover, phosphatase activity was 2-fold higher in Lmna G609G/+ VSMCs (Figure 2D).

Given these findings, we next investigated the capacity of LmnaG609G/+ VSMCs to synthesize PPi using ATP as substrate. Cells were incubated for different times with 1 µmol/L ATP and [γ-32P]-ATP radiotracer, and radioactive ATP, PPi, and Pi were quantified in the culture medium after separation by thin-layer chromatography (Figure 2E). Wild-type and LmnaG609G/+ VSMCs showed indistinguishable rates of ATP hydrolysis, with ≈98% of ATP being hydrolyzed after 80 minutes (Figure 2E). However, the amount of ePPi produced was only 28% in LmnaG609G/+ VSMCs compared with 47% in wild-type cells (Figure 2E). Accordingly, the Pi:PPi ratio increased from ≈1:1 in wild-type VSMCs to ≈2.4:1 in LmnaG609G/+ VSMCs.

VSMCs Expressing Progerin Have Impaired ATP Synthesis and Mitochondrial Function

Extracellular ATP hydrolysis is the major source of ePPi in aorta and in VSMCs. We therefore analyzed intracellular ATP levels and extracellular ATP accumulation in wild-type and LmnaG609G/+ VSMCs. LmnaG609G/+ VSMCs showed significantly lower levels of extracellular ATP (Figure 3A) and intracellular ATP (Figure 3B). LmnaG609G/+ VSMCs also exhibited lower mitochondrial ATP production (Figure 3C). This was associated with a significantly lower COX:CS ratio (Figure 3D), which indicates impaired activity of COX, an essential component of the mitochondrial electron transport chain.

To assess whether the alterations observed in LmnaG609G/+ VSMCs are a direct consequence of progerin expression,
we performed retrovirus-mediated gain-of-function experiments. Western blot analysis demonstrated CFP-lamin A and progerin-CFP expression in primary mouse VSMCs infected with retrovirus encoding these proteins (Figure 4A). In agreement with many previous studies in different cell types, infected VSMCs expressed CFP-lamin A predominantly in the perinuclear rim, whereas progerin-CFP was found in both the perinuclear rim and in nucleoplasmic aggregates (Figure 4B). Compared with VSMCs expressing CFP-lamin A, forced progerin expression in VSMCs led to increased TNAP and apyrase1/eNTPD1 mRNA steady state levels without affecting the expression of other enzymes and transporters involved in vascular calcification. Representational immunoblotting and quantification of tissue-nonspecific alkaline phosphatase (TNAP) and ectonucleoside triphosphatase diphosphohydrolase 1 (eNTPD1) expression after normalization to α-tubulin (a.u. indicates arbitrary units). D. Alkaline phosphatase activity. Results in A through D are means±SEM of 3 independent experiments. E. Cells were incubated with 1 μmol/L ATP plus 1 μCi/mL [γ-32P]ATP. At time points indicated in the graphs, inorganic phosphate (Pi), PPi, and ATP in the culture medium were separated by thin-layer chromatography. A representative autoradiograph is shown on the left. Graphs show quantification of ATP, Pi, and PPi. Results are means±SEM of 6 wells. Similar results were obtained in 2 additional experiments. *P<0.05, **P<0.01, and #P<0.0001 vs wild-type. CPM indicates counts per minute.

Figure 2. Vascular smooth muscle cells (VSMCs) expressing progerin exhibit impaired ATP-dependent inorganic pyrophosphate (PPi) synthesis and extracellular Pi (ePi) accumulation. Primary VSMCs were obtained from wild-type and LmnaG609G/+ mice. A. Quantification of ePi accumulated over 2 days in culture. B. Quantitative polymerase chain reaction analysis of ectoenzymes and transporters involved in vascular calcification. C. Representative immunoblots and quantification of tissue-nonspecific alkaline phosphatase (TNAP) and ectonucleoside triphosphatase diphosphohydrolase 1 (eNTPD1) expression after normalization to α-tubulin (a.u. indicates arbitrary units). D. Alkaline phosphatase activity. Results in A through D are means±SEM of 3 independent experiments. E. Cells were incubated with 1 μmol/L ATP plus 1 μCi/mL [γ-32P]ATP. At time points indicated in the graphs, inorganic phosphate (Pi), PPi, and ATP in the culture medium were separated by thin-layer chromatography. A representative autoradiograph is shown on the left. Graphs show quantification of ATP, Pi, and PPi. Results are means±SEM of 6 wells. Similar results were obtained in 2 additional experiments. *P<0.05, **P<0.01, and #P<0.0001 vs wild-type. CPM indicates counts per minute.

Progeroid Mice Have Alterations in Plasma Parameters Involved in Vascular Calcification

Because hyperphosphatemia and hypercalcemia lead to CPD,19 we quantified Pi and calcium in plasma from wild-type and LmnaG609G/+ mice (Figure 5A). These parameters were similar in both genotypes (phosphorus: 8.2±0.2 in wild-type versus 7.7±0.3 mg/dL in LmnaG609G/+ mice; calcium: 14.4±0.3 in wild-type versus 14.8±0.3 mg/dL in LmnaG609G/+ mice; P>0.05). However, the plasma of LmnaG609G/+ mice exhibited higher alkaline phosphatase activity (29.5% higher) and lower concentrations of ATP (8-fold) and ePi (9-fold; Figure 5A).

Pyrophosphate Treatment Inhibits Aortic Calcification in Progeroid Mice

Recent studies showed that injection of exogenous PPi prevents uremic vascular calcification in rats and mice.24,39 We therefore investigated the effect of PPi injection on vascular calcification in progeroid mice. For these studies, we used homozygous LmnaG609G/+/G mice, which are more severely affected than heterozygous LmnaG609G/+ mice, with
Red–stained aortic cross sections (Figure 3A, B). Results are mean±SEM of 12 determinations in 2 to 3 independent experiments. #P<0.0001. COX indicates cytochrome c oxidase; and CS, citrate synthase.

Table 3. Vascular smooth muscle cells (VSMCs) from progeroid LmnaG609G/+ mice have low ATP levels and mitochondrial dysfunction. Wild-type and LmnaG609G/+ VSMCs were cultured to quantify the level of extracellular ATP (A), intracellular ATP (B), mitochondrial ATP synthesis (C), and the COX:CS activity ratio (D). Results are mean±SEM of 12 determinations in 2 to 3 independent experiments. #P<0.0001. COX indicates cytochrome c oxidase; and CS, citrate synthase.

Figure 3. Vascular smooth muscle cells (VSMCs) from progeroid LmnaG609G/+ mice have low ATP levels and mitochondrial dysfunction. Wild-type and LmnaG609G/+ VSMCs were cultured to quantify the level of extracellular ATP (A), intracellular ATP (B), mitochondrial ATP synthesis (C), and the COX:CS activity ratio (D). Results are mean±SEM of 12 determinations in 2 to 3 independent experiments. #P<0.0001. COX indicates cytochrome c oxidase; and CS, citrate synthase.

Discussion

The aim of the present study was to identify molecular mechanisms that contribute to excessive vascular calcification in HGPS, a rare premature aging disorder caused by abnormal expression of progerin.12 Available evidence indicates that vascular calcification involves both passive and active processes.18,19,25 (Figure 6). CPD in the aortic wall and in vitro models is the main feature of vascular calcification. At physiological serum concentrations of calcium and phosphate, CPD is a passive process that does not require cellular activity.20,25,36,40 Active processes involved in vascular calcification include reduced capacity of VSMCs to synthesize and secrete calcification inhibitors, such as MGP, fetuin A, and ePPi, and the transition of VSMCs to a bone-forming phenotype, a process that is enhanced via CD-dependent overexpression of osteochondrogenic factors, including Runx2.18,19,36,41 Pi is essential for the synthesis of ATP, the main source of ePPi.26 It has thus been proposed that defective ATP production by VSMCs may impair the synthesis of CPD inhibitors.42 Previous studies revealed that old transgenic mice carrying the common HGPS mutation exhibit a vascular calcification not seen in age-matched controls17; however, the mechanisms by which progerin exacerbates vascular calcification remain unknown. Here, we have shown that progerin expression in LmnaG609G/+ mice causes excessive vascular calcification associated with VSMC abnormalities that lead to impaired mitochondrial function and ATP production and reduced PPI synthesis (Figure 6), without affecting expression of the anticalcification agents MGP and fetuin A. The causal relationship between progerin-induced low plasma ePPi and vascular calcification is reinforced by our observation that aortic calcification in progeroid mice is reduced by treatment with Pi. It is noteworthy that physiological aging of non-HGPS individuals is associated with mitochondrial dysfunction43,44 and with a progressive accumulation of progerin in several tissues.45–48 including vascular tissue.9 Thus, the present findings might be relevant to vascular calcification not only in HGPS patients but also in the elderly.

Because HGPS patients carry the Lmna mutation in heterozygosity,1,2 we studied heterozygous LmnaG609G/+ mice that express both lamin A and progerin.34 We found excessive aortic calcification in progeroid LmnaG609G/+ mice, which was associated with increased aortic expression of Runx2 and Bmp-2, 2 osteochondrogenic markers expressed in VSMCs during calcification in vivo and in vitro49 that are induced by CPD.36,41 Importantly, VSMCs from LmnaG609G/+ mice also exhibited an impaired capacity to inhibit CPD in vitro, which might explain the excessive medial calcification and the consequent overexpression of osteogenic markers in the aorta of LmnaG609G/+ mice. To investigate the molecular mechanisms through which progerin triggers vascular calcification, we focused on the enzymes and transporters involved in ePPi homeostasis, a complex process that involves a balance between synthesis and degradation. The present results provide evidence that excessive vascular calcification in LmnaG609G/+ mice is the consequence of alterations in VSMCs, including impaired synthesis of PPI, which leads to reduced accumulation of ePPi (Figure 6). Additional factors contributing to progerin-dependent reduction in ePPi levels include the upregulation of the ectoenzymes TNP (the main enzyme involved in ePPi hydrolysis) and apyrase1/eNTPD1 (an enzyme that hydrolyzes ATP to release P), as well as low synthesis of ATP (the major substrate for ePPi synthesis). Our studies suggest that impaired ATP synthesis in LmnaG609G/+ VSMCs is caused by mitochondrial dysfunction associated with reduced complex IV COX activity. Importantly, retrovirus-mediated overexpression of progerin in primary wild-type VSMCs recapitulates the alterations observed in VSMCs isolated from LmnaG609G/+ mice, including increased expression of TNP and apyrase1/eNTPD1, reduced extracellular and intracellular ATP, diminished mitochondrial COX:CS ratio and ATP synthesis, and lower ePPi accumulation. A-type laminas have been linked to the regulation of multiple cellular processes (eg, higher-order chromatin organization, DNA repair and replication, signal transduction, gene transcription, and cell proliferation, differentiation, and migration), many of which are altered on progerin expression.1,4 Therefore,
Additional studies are required to refine our understanding of the molecular mechanisms through which progerin expression alters VSMC behavior to promote calcification.

Hyperphosphatemia causes vascular calcification, thus underscoring the importance of Pi homeostasis in CPD. However, in a study of 15 HGPS patients with a wide age range of 1.6 to 17.8 years, Merideth et al. reported a plasma phosphate concentration of 5.5±0.2 mg/dL, which is within the normal range in humans (2.7–5.5 mg/dL). We also found that Lmna<sup>G609G</sup>/+ mice have plasma phosphate concentrations in the normal range, undistinguishable from their wild-type littermates (≈8 mg/dL; normal range in mice, 5.7–9.2 mg/dL). Likewise, plasma calcium levels were normal in progeroid mice. These results indicate that hyperphosphatemia and hypercalcemia do not contribute to excessive vascular calcification in Lmna<sup>G609G</sup>/+ mice, thus emphasizing the importance of reduced synthesis of ePPi by VSMCs as a key mechanism triggering excessive vascular calcification in HGPS. Although plasma phosphate levels are normal in Lmna<sup>G609G</sup>/+ mice, increased accumulation of Pi in the tunica media may contribute to CPD in these animals, because the present results in primary VSMCs from Lmna<sup>G609G</sup>/+ mice show augmented Pi production from ATP hydrolysis accompanied by a high Pi:PPi ratio of ≈2.4:1, compared with ≈1:1 in wild-type cells (Figure 2E). The defect in ePPi production by progerin-expressing VSMCs is unlikely to be counterbalanced systemically, because Pi concentration in plasma from Lmna<sup>G609G/+</sup> mice was also significantly reduced compared with wild-type controls, probably as a result of lower ATP concentration and higher alkaline phosphatase activity in plasma. Our observation that Pi treatment inhibits aortic calcification in progeroid mice underscores the role of defective ePPi homeostasis as a key factor underlying excessive vascular calcification in HGPS. We did not observe changes in body weight or mortality on Pi treatment. However, these studies were performed in homozygous Lmna<sup>G609G/G609G</sup> mice that have an average life span of only 15 weeks, and treatment was started at 10 weeks of age, when the animals already exhibit severe symptoms. HGPS patients carry the LMNA mutation in heterozygosis, and therefore, future studies are warranted in very young heterozygous Lmna<sup>G609G/+</sup> mice (1–2 weeks old) to ascertain whether chronic treatment with Pi starting early in life, when symptoms are absent or very mild, can not only inhibit vascular calcification but also improve the general health and life span of progeroid mice.

Because imbalances in the degradation and synthesis of ePPi have been shown to lead to pathological calcification of articular cartilage, the systemic defect in circulating Pi...
associated with progerin expression might promote not only excessive vascular calcification but also the appearance of joint contractures, a characteristic of both HGPS patients and 

Lmna<sup>G609G/G609G</sup> mice. 34 Treatment with PPi might thus ameliorate both vascular calcification and joint contractures. It is noteworthy that combined treatment with statins and bisphosphonates (nonhydrolyzable pyrophosphate analogues) significantly extends the average life span of progeroid Zmpste24-null mice, another model of premature aging caused by abnormal accumulation of prelamin A. 50 On the basis of these findings,

![Figure 6. Proposed model of altered pyrophosphate homeostasis in Hutchinson-Gilford progeria syndrome. Extracellular inorganic pyrophosphate (PPi) is a major inhibitor of calcium phosphate deposition that is synthesized from ATP by the ectoenzyme nucleotide pyrophosphatase/phosphodiesterase-1 (eNPP1) and degraded to inorganic phosphate (Pi) by tissue-nonspecific alkaline phosphatase (TNAP). Pit1 and Pit2 mediate the cellular uptake of Pi, which is essential for ATP synthesis, mainly via mitochondrial oxidative phosphorylation (OX-phos). ATP is released to the extracellular matrix via the transporter ANK. Progerin expression in vascular smooth muscle cells (VSMCs) augments TNAP and apyrase/ectonucleoside triphosphatase diphosphohydrolase 1 (eNTPD1) and impairs mitochondrial function and ATP synthesis. These alterations lead to reduced extracellular concentrations of PPI and a high ratio of Pi to PPI ratio in the arterial wall. The plasma of Lmna<sup>G609G/G609G</sup> mice exhibits normal phosphorus and calcium levels, but higher alkaline phosphatase activity (ALP) and lower concentrations of ATP and extracellular PPI. These alterations in VSMCs and blood of progeroid Lmna<sup>G609G</sup> mice promote excessive aortic calcification that can be inhibited on treatment with PPi.](http://circ.ahajournals.org/issue/1/8/2449/fig6)
ongoing clinical trials are assessing the efficacy of statin plus bisphosphonate (pravastatin plus zoledronate) with or without farnesyl transferase inhibitor (http://www.progeriaresearch.org/clinical_trial.html). However, although treatment with bisphosphonates decreases aortic calcification, animal studies have identified adverse effects of these compounds in bone.55 Moreover, bisphosphonates have been shown to induce the rupture of atherosclerotic plaques in apolipoprotein E–null mice.68 In contrast, recent studies have shown that exogenous PPI injected daily into uremic rats and mice reduces the calcium content in calcified aortas without producing adverse effects on bone.24,39 Moreover, TNAP and PHOSPHO1 inhibitors can inhibit vascular calcification.27,54 Future studies in HGPS mouse models are thus warranted to investigate whether treatment with PPI in combination with TNAP or PHOSPHO1 inhibitors and FTY and statins is more beneficial than current strategies.

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Disclosures
None.

References


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**CLINICAL PERSPECTIVE**

Progerin is a mutant form of lamin A responsible for Hutchinson-Gilford progeria syndrome (HGPS), a rare premature aging disorder featuring excessive vascular calcification (VC). However, calcium dysfunction is understood in this disease. Here, we have shown that progerin-expressing *Lmna<sup>G609G</sup>* mice, which mimic the main clinical manifestations of HGPS, develop excessive VC. Moreover, primary vascular smooth muscle cells from *Lmna<sup>G609G</sup>* mice exhibit increased tissue-nonspecific alkaline phosphatase and apyrase 1/eNTPD1 and reduced mitochondrial ATP synthesis and extracellular ATP accumulation. Accordingly, *Lmna<sup>G609G</sup>* vascular smooth muscle cells show defective production and extracellular accumulation of pyrophosphate, a major inhibitor of VC. We also found increased alkaline phosphatase activity and reduced ATP and pyrophosphate levels in plasma of *Lmna<sup>G609G</sup>* mice. Pyrophosphate administration to severely ill progeroid *Lmna<sup>G609G</sup>* mice carrying the *LMNA* mutation in homozygosis inhibited VC but did not improve body weight or mortality. Because HGPS patients carry the *LMNA* mutation in heterozygosis, future studies are warranted in very young heterozygous *Lmna<sup>G609G</sup>* mice to ascertain whether chronic treatment with pyrophosphate starting early in life cannot only inhibit VC but also improve the general health and life span of progeroid mice. Moreover, it will be of interest to investigate whether treatment of progeroid mice with pyrophosphate in combination with tissue-nonspecific alkaline phosphatase or PHOSPHO1 inhibitors and farnesyl transferase inhibitors and statins, which are currently under evaluation in clinical trials of HGPS, is more beneficial than current strategies. Because progerin progressively accumulates in the vascular tissue of non-HGPS individuals, these studies should shed light into VC associated with both premature and physiological aging.
Defective Extracellular Pyrophosphate Metabolism Promotes Vascular Calcification in a Mouse Model of Hutchinson-Gilford Progeria Syndrome That Is Ameliorated on Pyrophosphate Treatment

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