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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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<sup>G609G/+</sup> mice expressing progerin, which mimic the main clinical manifestations of HGPS. Lmna<sup>G609G/+</sup> 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<sup>G609G/+</sup> 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<sup>G609G/+</sup> mice without changes in phosphorus and calcium. Treatment with pyrophosphate inhibited vascular calcification in progeroid mice.

Conclusions—Excessive vascular calcification in Lmna<sup>G609G</sup> 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-nonspecific alkaline phosphatase • vascular calcification

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. HP GS 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.
Vascular calcification typically manifests as calcium-phosphate deposition (CPD) in distinct layers of the aortic wall.18,19 Calcium-phosphate deposits are composed of several members of the calcium orthophosphate family, including hydroxyapatite, octacalcium phosphate, and amorphous calcium phosphate.20 Medial calcification occurs within the elastin region of arteries and is almost exclusively associated with vascular smooth muscle cells (VSMCs). Elevated serum phosphates (in the form of inorganic phosphate [Pi]) is a major risk factor for vascular calcification.21,22 In vitro and in vivo studies have shown that elevated Pi concentration in VSMCs triggers a transition to a bone-forming phenotype.18,19 This transition results in overexpression of osteochondrogenic markers, such as the transcription factor Runx2 (also named Cbfa1), which induces the expression of major bone matrix components.18,19,21

Extracellular pyrophosphate (ePPi) directly blocks CPD in vitro and in vivo and is therefore a major endogenous inhibitor of vascular calcification.23–26 Degradation of ePPi is catalyzed by tissue-nonspecific alkaline phosphatase (TNAP), which hydrolyzes inorganic pyrophosphate (PPi) to Pi. Importantly, calcification in ex vivo cultured rat aorta is induced by alkaline phosphatase and is prevented by TNAP inhibitors.26,27 TNAP is moreover upregulated in the aortas of uremic rats, which results in increased hydrolysis of ePPi and vascular calcification.28 The main enzyme involved in ePPi synthesis, both in aorta and in cultured VSMCs, is the ectoenzyme nucleotide pyrophosphatase/phosphodiesterase-1 (eNPP1).26,29 Lack of eNPP1 leads to extensive and fatal arterial calcification in children and mice.30,31 The substrate for eNPP1 is ATP, which accumulates in the extracellular matrix via the action of several transporters,32 such as the multiple-pass transmembrane protein ANK.33 To define the molecular mechanisms that lead to vascular calcification in HGPS, we analyzed LmnaG609G knock-in mice, which express progerin as a consequence of aberrant splicing that results from the LMNA c.1827C>T (p.G609G) mutation, which express progerin as a consequence of aberrant splicing that results from the LMNA c.1827C>T (p.G609G) mutation; this model mimics the main clinical transcriptome of human HGPS, including cardiovascular abnormalities and premature death.34 Our studies reveal profound cellular alterations in eNPP1 hemeostasis caused by progerin expression that exacerbate vascular calcification, which is inhibited by treatment with pyrophosphate.

Methods

Mice

Male LmnaG609G+/−, LmnaG609G/G609G, and wild-type littermates were used.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.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.37,38 For phosphatase activity studies and ATP and Pi measurements, VSMCs were incubated in medium without Phosphatase 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 LmnaG609G/G609G mice were treated with PPi for 9 weeks (daily intraperitoneal injection, 100 µg·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′-CACCCTGGGACAGCCTTCA-3′ (forward), 5′-CCGCGGCGTTCATCCCACTCA-3′ (reverse); (2) Runx2 (NM_001146038.1): 5′-CAATCCCGGAGCAGGACAGCTG-3′ (forward), 5′-ACACCCCGGTGATTGGAATG-3′ (reverse); (3) eNPP1 (NM_008813): 5′-GGATTGTGCCAATAAGGACT-3′ (forward), 5′-CAAGAACTGTTGCTGCTGGAG-3′ (reverse); (4) TNAP (NM_007431): 5′-CAGATCCCAGGCAGGCACAGTC-3′ (forward), 5′-CAAGGCGGTGTTGGAATG-3′ (reverse); (5) Pit1 (NM_015747): 5′-CTGCTTCAGATGGTATGAGG-3′ (forward), 5′-TGTGAACCTACGGCAGAAGG-3′ (reverse); (6) Pit2 (NM_011394): 5′-ACACCCCGGTGATTGGAATG-3′ (forward), 5′-ACAGCCCGGTGTTGGAATG-3′ (reverse); (7) ANK (NM_001146038.1): 5′-AGCCTTTGAGGGTTTTCGGTA-3′ (reverse); (8) TNAP (NM_007431): 5′-CTATGTCTGGAACCGCTACTGA-3′ (forward), 5′-AGCCTTTGAGGGTTTTCGGTA-3′ (reverse); (9) Pit1 (NM_015747): 5′-CTGCTTCAGATGGTATGAGG-3′ (forward), 5′-TGTGAACCTACGGCAGAAGG-3′ (reverse); (6) Pit2 (NM_011394): 5′-CTTCTACACGGGACATCACCTGA-3′ (forward), 5′-CATGAGCCGAAAGAAGTG-3′ (reverse); (7) ANK (NM_020332): 5′-CATCCGCCAATCTTCTCTGTA-3′ (forward), 5′-ACACCCCGGTGTTGGAATG-3′ (reverse); (8) eNTPD1 (NM_009848): 5′-AGCCTTCCACAGCATCTCCTT-3′ (forward), 5′-GCCACCACTTGAACACTCTGAT-3′ (reverse); (9) fetuin A (NM_013465): 5′-ATCGCAAGAATAGTCAAGGTTTG-3′ (forward), 5′-CAACCTACAGACGTCTCTTG-3′ (reverse); and (10) matrix Gla protein (MGP; NM_008597): 5′-AGGAGAATGGCCACACCTTT-3′ (forward), 5′-ACGAAAACTCACCACACACATATT-3′ (reverse). Expression was quantified by the comparative Ct method, with correction for the expression of the endogenous control gene acidic ribosomal phosphoprotein P0 (RPLP0, accession number NM_007475).
Immunobots 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.19 The following primary antibodies were used: Rabbit monoclonal anti-eNTPD1 (1/1500, ab108248, Abcam, Cambridge, United Kingdom), rabbit polyclonal anti-TNAP (1 µg/mL, ab65834, Abcam), rabbit polyclonal anti-GFP (1/2500, ab65356, 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 pNPP phosphatase assay kit and the QuantiChrom alkaline phosphatase assay kit (BioAssay Systems), respectively. ATP hydrolysis assays in VSMCs cultures were performed as described previously.19 Briefly, cells were seeded in 12-well plates and incubated for the indicated times with 1 µmol/L ATP and 1 µCimol/L [γ32P]ATP radiotracer (Perkin Elmer, Madrid, Spain). ATP, Pi, and PPI 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
ePPI was measured with an enzyme-linked bioluminescence assay in which Pi reacts with adenosine 5′-phosphosulfate (A5508, Sigma-Aldrich) in the presence of ATP-sulfurylase (A9857, Sigma-Aldrich) to generate ATP.29 For each sample, the blank (reaction without ATP-sulfurylase) was subtracted to obtain the true PiPPI amount. ATP was measured by a coupled luciferin/luciferase reaction with an ATP determination kit (Invitrogen, Paisley, United Kingdom).

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 construct5 or Addgene 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 cloninger 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 (2×106 cells) was measured by kinetic luminescence assay, Cytochrome c oxidase (COX) and citrate synthase (CS) enzymatic activities were measured in cell lysates (30–50 µg of protein) as described previously20 and expressed as international units (IU) per milligram of protein. COX activity was normalized to CS, a Krebs cycle enzyme used as an index of mitochondrial mass.

Statistical Analysis
Results are expressed as mean±SEM. For the studies shown in Figures 1, 2, 3, 4, and 5A, results were analyzed by unpaired Student t test with Welch correction for 2 samples with possibly unequal variance. The results in Figure 5B were analyzed by paired Student t test. In all cases, statistical significance was assigned at P<0.05.

Results
Vascular Smooth Muscle Calcification Is Increased in Progeroid LmnaG609G/+ Mice
HGPS patients carry the LMNA mutation in heterozygosis and express both lamin A and progerin.1,2 We therefore performed studies with heterozygous LmnaG609G/+ mice that also express lamin A and progerin and have a significantly shortened life span (average 34 weeks compared with >2 years for wild-type controls).14 We used 30- to 32-week-old LmnaG609G/+ mice. Gross examination of Alizarin Red–stained vessels revealed prominent medial calcification in aortic arch and thoracic aorta of LmnaG609G/+ compared with wild-type mice, and computer-assisted planimetric analysis showed statistically significant differences between genotypes (Figure 1A). Consistent with these findings, quantitative polymerase chain reaction revealed that calcified aortas from LmnaG609G/+ mice have high mRNA levels of BMP-2 and Runx2, 2 osteogenic markers expressed during vascular calcification, but no changes were observed in the anticalcification agents MGP and fetuin A (Figure 1B and 1C). Immunohistochemical analysis confirmed the increase in Runx2 expression in LmnaG609G/+ aortas compared with aortas from wild-type mice (Figure 1D).

We next studied primary cultures of VSMCs isolated from aortic tissue of wild-type and LmnaG609G/+ mice. Previous in vitro studies showed that CPD is more prominent with lysed VSMCs than with live cells, which suggests that VSMCs synthesize CPD inhibitors.23,35,36 We therefore compared the CPD-inhibitory capacity of wild-type and LmnaG609G/+ VSMCs. Cells were incubated for 7 days in calcifying medium, and the difference in CPD between lysed and live cells (∆Ca) was calculated. LmnaG609G/+ VSMCs showed a significantly lower capacity to inhibit calcium deposition than equivalent numbers of control cells, after normalization for protein concentration (Figure 1E).

LmnaG609G/+ VSMCs Have an Impaired Capacity to Synthesize Extracellular Pyrophosphate
Because excessive calcification in LmnaG609G/+ aorta was not associated with reduced expression of MGP and fetuin A (Figure 1B), we quantified in VSMC-conditioned culture medium the accumulation of ePPI, the major endogenous inhibitor of vascular calcification.21,23–26 After 2 days in culture, ePPI levels were 3.8-fold lower in LmnaG609G/+ VSMCs than in wild-type controls (Figure 2A). Reverse transcription–polymerase chain reaction analysis of enzymes and transporters involved in ePPI metabolism demonstrated significantly higher expression of TNAP,
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 [γ32P]-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 49% 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 (Figure 3A). 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 ePPi metabolism (Figure 4C).

Western blot analysis confirmed the increase in TNAP and eNTPD1 in Lmna<sup>G609G</sup>/+ VSMCs that overexpress progerin (Figure 4D). Consistent with the results in primary Lmna<sup>G609G</sup>/+ VSMCs, we also found significant reductions in extracellular and intracellular ATP concentration, mitochondrial ATP synthesis, COX:CS ratio, and ePPI levels in progerin-CFP–overexpressing VSMCs compared with CFP-lamin A–expressing controls (Figure 4E).

Progeroid Mice Have Alterations in Plasma Parameters Involved in Vascular Calcification

Because hyperphosphatemia and hypercalcemia lead to CPD, we quantified Pi and calcium in plasma from wild-type and Lmna<sup>G609G</sup>/+ 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 Lmna<sup>G609G</sup>/+ mice; P>0.05). However, the plasma of Lmna<sup>G609G</sup>/+ mice exhibited 29.5% higher alkaline phosphatase activity and 8-fold lower concentrations of ATP (8-fold) and ePPI (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. We therefore investigated the effect of PPI injection on vascular calcification in progeroid mice. For these studies, we used homozygous Lmna<sup>G609G/G609G</sup> mice, which are more severely affected than heterozygous Lmna<sup>G609G</sup>/+ mice, with
It has thus been proposed that defective ATP production by vascular smooth muscle cells (VSMCs) from progeroid *Lmna*^G609G/+^ mice have low ATP levels and mitochondrial dysfunction. Wild-type and *Lmna*^G609G/+^ 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 means±SEM of 12 determinations in 2 to 3 independent experiments. #P<0.0001. COX indicates cytochrome c oxidase; and CS, citrate synthase.

![Graphs](image)

Figure 3. Vascular smooth muscle cells (VSMCs) from progeroid *Lmna*^G609G/+^ mice have low ATP levels and mitochondrial dysfunction. Wild-type and *Lmna*^G609G/+^ 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 means±SEM of 12 determinations in 2 to 3 independent experiments. #P<0.0001. COX indicates cytochrome c oxidase; and CS, citrate synthase.

an average life span of only 103 days. 14 Ten-week-old homozygous *Lmna*^G609G/G609G^ males received daily intraperitoneal injections of saline (control group) or PPI over 9 weeks (100 mg·kg⁻¹·d⁻¹, n=5 mice in each group). We did not observe differences in body weight and mortality during follow-up, but postmortem analysis demonstrated a statistically significant reduction in aortic calcification in PPI-treated mice compared with controls, as revealed by planimetric analysis of Alizarin Red–stained aortic cross sections (Figure 5B).

![Graphs](image)

### 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. 1,2 Available evidence indicates that vascular calcification involves both passive and active processes. 3,18,19,25 (Figure 6). CPD in the aortic wall and in vivo 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 CDP-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 controls 17; however, the mechanisms by which progerin exacerbates vascular calcification remain unknown. Here, we have shown that progerin expression in *Lmna*^G609G/+^ 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 PPI. It is noteworthy that physiological aging of non-HGPS individuals is associated with mitochondrial dysfunction 43,44 and with a progressive accumulation of progerin in several tissues, 45–48 including vascular tissue. 5 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 *Lmna*^G609G/+^ mice that express both lamin A and progerin. 34 We found excessive aortic calcification in progeroid *Lmna*^G609G/+^ 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 vitro that are induced by CPD. 36,41 Importantly, VSMCs from *Lmna*^G609G/+^ 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 *Lmna*^G609G/+^ 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 *Lmna*^G609G/+^ 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 TNAp (the main enzyme involved in ePPi hydrolysis) and apyrase1/eNTPD1 (an enzyme that hydrolyzes ATP to release Pi), as well as low synthesis of ATP (the major substrate for ePPi synthesis). Our studies suggest that impaired ATP synthesis in *Lmna*^G609G/+^ 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 *Lmna*^G609G/+^ mice, including increased expression of TNAp 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,
Forced progerin expression in vascular smooth muscle cells (VSMCs) induces alterations in extracellular inorganic pyrophosphate (Pi) metabolism. Primary VSMCs obtained from wild-type mice were infected with retroviral vectors encoding cyan fluorescent protein (CFP)-lamin A or progerin-CFP. A, Western blot analysis using anti-green fluorescent protein antibody. B, Confocal microscopy to visualize CFP-lamin A and progerin-CFP. C, Quantitative polymerase chain reaction of enzymes and transporters involved in extracellular Pi metabolism. Results are normalized to mRNA levels in CFP-lamin A controls. D, Representative immunoblots and quantification of TNAP and eNTPD1 expression after normalization to α-tubulin (a.u. indicates arbitrary units). E, Determination of extracellular and intracellular ATP, mitochondrial ATP synthesis, COX/CS ratio, and extracellular PPi. Results are mean±SEM of 3 independent experiments. *P<0.05, **P<0.01, ***P<0.0001 vs CFP-lamin A. COX indicates cytochrome c oxidase; CS, citrate synthase; eNTPD1, ectonucleoside triphosphatase diphosphohydrolase 1 (eNTPD1); and TNAP, tissue-nonspecific alkaline phosphatase.

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 phosphorus 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 LmnaG609G/+ 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 LmnaG609G/+ mice, thus emphasizing the importance of reduced synthesis of ePi by VSMCs as a key mechanism triggering excessive vascular calcification in HGPS. Although plasma phosphate levels are normal in LmnaG609G/+ mice, increased accumulation of Pi in the tunica media may contribute to CPD in these animals, because the present results in primary VSMCs from LmnaG609G/+ mice show augmented Pi production from ATP hydrolysis accompanied by a high Pi:Pi ratio of 2.4:1, compared with 1:1 in wild-type cells (Figure 2E). The defect in ePi production by progerin-expressing VSMCs is unlikely to be counterbalanced systemically, because Pi concentration in plasma from LmnaG609G/+ 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 ePi 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 LmnaG609G/G609G 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 LmnaG609G/+ 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 ePi 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\(^{G609G/G609G}\) mice. 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 5.** Inorganic pyrophosphate (PPi) homeostasis in mouse plasma and treatment with exogenous PPi. A, The indicated parameters were analyzed in plasma of 50- to 52-week-old male wild-type and Lmna\(^{G609G/G609G}\) mice. Results are means±SEM of 8 to 10 mice. B, Ten-week-old male Lmna\(^{G609G/G609G}\) mice received for 9 weeks a daily intraperitoneal injection of saline or exogenous PPi (n=5 mice in each group). Images show representative examples of Alizarin Red–stained aortic tissue (boxed areas are shown at higher magnification). Graph shows quantification of calcium deposition by planimetric analysis of stained cross sections. ***P<0.001 and #P<0.0001. ALP indicates alkaline phosphatase activity.

**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\(^{G609G/G609G}\) 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\(^{G609G}\) mice promote excessive aortic calcification that can be inhibited on treatment with PPi.
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.\textsuperscript{5,22} Moreover, bisphosphonates have been shown to induce the rupture of atherosclerotic plaques in apolipoprotein E–null mice.\textsuperscript{23} 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.\textsuperscript{24,39} Moreover, TNAP and PHOSPHO1 inhibitors can inhibit vascular calcification.\textsuperscript{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 FTI and statins is more beneficial than current strategies.

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**Disclosures**

None.

**References**


**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 understudied in this disease. Here, we have shown that progerin-expressing *LmnaG609G/+* mice, which mimic the main clinical manifestations of HGPS, develop excessive VC. Moreover, primary vascular smooth muscle cells from *LmnaG609G/+* mice exhibit increased tissue-nonspecific alkaline phosphatase and apyrase 1/eNTPD1 and reduced mitochondrial ATP synthesis and extracellular ATP accumulation. Accordingly, *LmnaG609G/+* 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 *LmnaG609G/+* mice. Pyrophosphate administration to severely ill progeroid *LmnaG609G/+* 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 *LmnaG609G/+* 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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