ORIGINAL RESEARCH ARTICLE

Vascular Smooth Muscle–Specific Progerin Expression Accelerates Atherosclerosis and Death in a Mouse Model of Hutchinson-Gilford Progeria Syndrome

BACKGROUND: Progerin, an aberrant protein that accumulates with age, causes the rare genetic disease Hutchinson-Gilford progeria syndrome (HGPS). Patients who have HGPS exhibit ubiquitous progerin expression, accelerated aging and atherosclerosis, and die in their early teens, mainly of myocardial infarction or stroke. The mechanisms underlying progerin-induced atherosclerosis remain unexplored, in part, because of the lack of appropriate animal models.

METHODS: We generated an atherosclerosis-prone model of HGPS by crossing apolipoprotein E–deficient (Apoe−/−) mice with LmnaG609G/G609G mice ubiquitously expressing progerin. To induce progerin expression specifically in macrophages or vascular smooth muscle cells (VSMCs), we crossed Apoe−/−LmnaLCS/LCS mice with LysMCre and SM22αCre mice, respectively. Progerin expression was evaluated by polymerase chain reaction and immunofluorescence. Cardiovascular alterations were determined by immunofluorescence and histology in male mice fed normal chow or a high-fat diet. In vivo low-density lipoprotein retention was assessed by intravenously injection of fluorescently labeled human low-density lipoprotein. Cardiac electric defects were evaluated by electrocardiography.

RESULTS: Apoe−/−LmnaG609G/G609G mice with ubiquitous progerin expression exhibited a premature aging phenotype that included failure to thrive and shortened survival. In addition, high-fat diet–fed Apoe−/−LmnaG609G/G609G mice developed a severe vascular pathology, including medial VSMC loss and lipid retention, adventitial fibrosis, and accelerated atherosclerosis, thus resembling most aspects of cardiovascular disease observed in patients with HGPS. The same vascular alterations were also observed in Apoe−/−LmnaLCS/LCS SM22αCre mice expressing progerin specifically in VSMCs, but not in Apoe−/−LmnaLCS/LCS LysMCre mice with macrophage-specific progerin expression. Moreover, Apoe−/−LmnaLCS/LCS SM22αCre mice had a shortened lifespan despite the lack of any overt aging phenotype. Aortas of ubiquitously and VSMC-specific progerin-expressing mice exhibited increased retention of fluorescently labeled human low-density lipoprotein, and atheromata in both models showed vulnerable plaque features. Immunohistopathological examination indicated that Apoe−/−LmnaLCS/LCS SM22αCre mice, unlike Apoe−/−LmnaG609G/G609G mice, die of atherosclerosis-related causes.

CONCLUSIONS: We have generated the first mouse model of progerin-induced atherosclerosis acceleration, and demonstrate that restricting progerin expression to VSMCs is sufficient to accelerate atherosclerosis, trigger plaque vulnerability, and reduce lifespan. Our results identify progerin-induced VSMC death as a major factor triggering atherosclerosis and premature death in HGPS.

Key Words: aging • atherosclerosis • cardiovascular diseases • models, animal • muscle, smooth, vascular • progeria

Sources of Funding, see page 281

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Clinical Perspective

What Is New?

- We have generated the first mouse model of progerin-induced acceleration of atherosclerosis, a major medical problem in patients with Hutchinson-Gilford progeria syndrome.
- We provide the first direct evidence that progerin expression restricted to vascular smooth muscle cells (VSMCs), but not to macrophages, is sufficient to induce premature atherosclerosis and death.
- We demonstrate that progerin-induced loss of VSMCs causes atherosclerotic plaque destabilization that may lead to myocardial infarction.
- We show that ubiquitous and VSMC-specific progerin expression increases low-density lipoprotein retention in the aortic media, likely accelerating atherosclerosis.

What Are the Clinical Implications?

- Apoe–/–LmnaG609G/G609G mice ubiquitously expressing progerin constitute the first preclinical animal model for the study of accelerated atherosclerosis and premature aging.
- Because atherosclerosis is the main disease sympto leading to death in patients with Hutchinson-Gilford progeria syndrome, our studies point to VSMCs as a potential target for future therapies for this devastating disease.

Atherosclerosis in Progeria

Cardiovascular disease (CVD) is the leading cause of death worldwide, despite major recent progress in its prevention, diagnosis, and treatment.1,2 Atherosclerosis, defined as the narrowing of the arterial lumen because of atheroma plaque buildup, underlies most manifestations of CVD and can lead to myocardial infarction or stroke.

Accelerated atherosclerosis is a predominant feature of the rare genetic disorder Hutchinson-Gilford progeria syndrome (HGPS). Classical HGPS is caused by a point mutation (c.1824C>T, p.G608G) in the LMNA gene resulting in ubiquitous expression of progerin, a mutant form of the nuclear envelope protein lamin A.3,4 Children with HGPS typically appear normal at birth, but within the first 2 years of life start to display the first signs of the disease, such as growth impairment and skin abnormalities. As childhood progresses, patients develop other symptoms, including alopecia, loss of body fat, joint stiffness, and osteoporosis, resembling many features of physiological aging.5,6 The most clinically relevant feature of HGPS is generalized atherosclerosis that leads to premature death from myocardial infarction or stroke at an average age of 14.6 years.8,7 Atherosclerosis in HGPS is accompanied by pathological changes in the aortic wall involving severe vascular smooth muscle cell (VSMC) depletion in the media, extracellular matrix deposition, calcification, and adventitial thickening.8–10

Atherosclerotic disease in HGPS is particularly intriguing because patients lack the classical CVD risk factors, such as increased low-density lipoprotein (LDL) and C-reactive protein levels in serum.11 HGPS therefore offers a unique opportunity to study CVD in isolation from typical risk factors. Because progerin also accumulates during normal aging,10,12,13 studies of HGPS may enhance our knowledge about physiological aging and CVD.14

As of October 1, 2017, there were 111 identified children living with HGPS worldwide, all of them carrying a progerin-producing mutation in the LMNA gene.15 This extremely low number presents major difficulties for research into disease mechanisms and for clinical trials testing new therapeutic strategies. Several mouse models of HGPS have been generated, including LmnaG608G, LmnaG609G, and Zmpste24.16–22 Although these mice recapitulate many aspects of the disease, none have been reported to develop atherosclerosis, the life-threatening symptom of HGPS. Thus, the aim of this study was to generate a mouse model exhibiting progerin-induced atherosclerosis acceleration and to study the relative contribution to progerin-driven atherosclerosis of macrophages and VSMCs, the key players in atherogenesis.

METHODS

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure (available at the authors’ laboratories).

Study Approval

All experimental and other scientific procedures with animals conformed to EU Directive 2010/63/EU and Recommendation 2007/526/EC, enforced in Spanish law under Real Decreto 53/2013. Animal protocols were approved by the local ethics committees and the Animal Protection Area of the Comunidad Autónoma de Madrid (PROEX76/14, PROEX78/14, PROEX167/16). Blood for LDL isolation was obtained from healthy volunteers after informed consent in accordance with local ethics committee guidelines (Comité de Ética de la Investigación del Instituto de Salud Carlos III: CEI PI 12_2016-v2).

Mice

All mice used in this study were males on the C57BL/6J genetic background carrying the following genetic manipulations: Apoe–/– (The Jackson Laboratory, stock no: 002052),
Apoe–/–LmnaG609G/G609G expression (corresponding controls expressing normal lamin A/C (Apoe–/–Lmna+/+LCSSM22G609G), macrophage (myeloid)-specific progerin expression (Apoe–/–LmnaG609G/G609G), and atherosclerosis-resistant (Apoe+/+) backgrounds, we bred LmnaCSCLCS22αCre, LmnaCSCLCS, LmnaG609G/G609G, and Lmna+/+ mice.

Longevity Studies
Starting at 4 weeks of age, animals were weighed and inspected for health and survival at least once a week (checks were more frequent for Apoe–/–LmnaG609G/G609G mice). Diseased animals were examined by a specialized veterinarian blinded to genotype. Animals that met humane end point criteria were euthanized and the deaths recorded. Animals euthanized because of hydrocephalus, malocclusion, intermale aggression, or other reasons unconnected to phenotype were excluded from the analysis (normally at a very early stage of the study).

High-Fat Diet Experiments
For diet-induced atherosclerosis experiments, animals were maintained for 8 weeks on a high-fat diet (HFD) (10.7% total fat, 0.75% cholesterol, S9167-E010, Ssniff) starting at 8 weeks of age. Mice were euthanized at 16 weeks of age after overnight fasting.

Hematology and Serum Biochemical Analysis
Animals were fasted overnight for all blood analyses, which were performed by specialized staff from the Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC) Animal Facility. For hematology, blood samples were collected in Microvette 100 EDTA tubes (Sarstedt) and analyzed with a PENTRA 80 hematology analyzer (Horiba). For biochemical analysis, blood samples were collected in plastic tubes, incubated at room temperature for 2 to 3 hours to allow clotting, and centrifuged at 2000g for 5 minutes. Serum was stored at −80°C until samples from all experiments were collected. Because of volume limitations, serum samples were pooled from 2 to 4 animals of the same experimental group. Specimens with overt hemolysis were excluded from testing. Biochemical variables were analyzed using a Dimension RxL Max Integrated Chemistry System (Siemens Healthineers).

Quantification of Atherosclerosis Burden
Mouse aortas were fixed in 4% formaldehyde/phosphate-buffered saline (PBS), cleaned of fatty tissue, and stained with 0.2% Oil Red O (ORO, O0625, Sigma). The thoracic aorta and aortic arch were then cut open longitudinally and pinned out flat, intimal side up, for computer-assisted planimetric analysis. Images were taken with a digital camera (Olympus UC30) mounted on a stereo microscope (Olympus SZX3). The ORO-stained lesion was quantified as a percentage of total area with SigmaScan Pro 5 (Systat Software Inc) by an observer blinded to genotype.

Histology and Immunofluorescence
For all mice with an Apoe–/– background, formaldehyde-fixed aortic arches were cleaned of fatty tissue, incubated in 30% sucrose in PBS overnight at 4°C, and included in Tissue-Tek OCT compound (Sakura) for cryostat sectioning. Serial 8-µm sections were stained with ORO, hematoxylin and eosin, and Masson trichrome. For immunofluorescence, sections were blocked and permeabilized for 1 hour at room temperature (RT) in PBS containing 0.3% Triton X-100 (9002-93-1, Sigma), 5% bovine serum albumin (A7906, Sigma), and 5% normal goat serum (005-000-001, Jackson ImmunoResearch). Sections were then incubated for 2 hours at RT with anti-smooth muscle α-actin-Cy3 (SMA-Cy3, C6198, Sigma, 1:200) antibody and overnight at 4°C with the following antibodies: anti-CD68 (MCA1957, Serotec, 1:200), and anti-progerin/lamin A (sc-20680, Santa Cruz, 1:100). Next, samples were incubated with corresponding secondary antibodies (anti-rat Alexa Fluor-568, A-11077 and anti-rabbit Alexa Fluor-647, A-21245, Invitrogen) and Hoechst 33342 nucleic acid stain (B2261, Sigma) for 2 hours at RT and mounted in Fluoromount-G imaging medium (4958-02, Affymetrix ebioscience).

Aortic arches of LmnaCSCLCS22αCre and LmnaCSCLCS mice were fixed in 4% formaldehyde in PBS, cleaned of fatty tissue, dehydrated to xylene, and embedded in paraffin. Serial 4-µm sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin and Masson trichrome.

Mouse hearts were fixed in 4% formaldehyde in PBS, divided into 2 parts (the upper portion containing the aortic root and aortic valve was separated for atheroma plaque analysis), dehydrated to xylene, and embedded in paraffin. Serial sections were prepared at least at 6 levels. The aortic root was sectioned throughout the aortic valve. Serial 4-µm sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin, Masson trichrome, von Kossa, and Perl Prussian blue. For immunofluorescence, antigen retrieval was performed using 10 mmol/L sodium citrate buffer (pH 6). Then samples were blocked and permeabilized for 1 hour at RT in PBS containing 0.3% Triton X-100, 5% bovine serum albumin, and 5% normal goat serum, and incubated overnight at 4°C with anti-CD31 (ab28364, Abcam, 1:100) antibody, and for 2 hours at RT with anti-SMA-Cy3 antibody (C6198, Sigma, 1:200). Next, samples were incubated with anti-rabbit Alexa Fluor-647 secondary antibody (111-607-008, Jackson ImmunoResearch) and Hoechst 33342 nucleic acid stain (B2261, Sigma) for 2 hours at RT and mounted in Fluoromount-G imaging medium.

ORO, hematoxylin and eosin, Masson trichrome, von Kossa, and Perl Prussian blue–stained sections were scanned with a NanoZoomer-RS scanner (Hamamatsu), and images were exported using NDP.view2. Immunofluorescence images were acquired with a Zeiss LSM700 confocal microscope. Images were analyzed using ImageJ Fiji software by...
an observer blinded to genotype. Aortic media and adventitia thickness and medial lipid and VSMC content were analyzed in ≈3 sections of aortic arch per animal, and mean values were used for statistical analysis. Atherosclerotic plaque area, aortic perimeter affected by atherosclerosis, necrotic core size, collagen content, VSMC content, and plaque calcification were quantified in 3 different zones of the aortic root per animal (at the beginning, in the middle, and at the end of the aortic valve), and mean values were used for statistical analysis. Cardiac alterations were assessed near the aortic valve and at least at 6 different levels of the ventricles by a specialized pathologist blinded to genotype.

**mRNA Isolation and Reverse Transcription for PCR Detection of Lamin A and Progerin**

Liver, kidney, spleen, and heart were homogenized using a TissueLyser (Qiagen), and total RNA was extracted with QiAzo1 reagent (Qiagen). The RNA pellet was dissolved in RNase-free water, and concentration was measured in a NanoDrop spectrophotometer (Wilmington). RNA (2 µg) was transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

cDNA (200 ng) was amplified by PCR using DNA polymerase (Biotools). Lamin A and progerin mRNAs were detected according to a protocol adapted from Yang et al.24 PCR products were separated on a 2% agarose gel containing ethidium bromide. Images were taken with a Molecular Image Gel Doc XR+ System (BioRad) and analyzed with Image Laboratory (BioRad).

**LDL Retention in Vivo**

Fluorescently labeled human LDL was prepared as described.25 In brief, human blood from healthy donors was collected in K3EDTA-containing tubes (455036, Vacuette). After centrifugation, plasma was collected, mixed with KBr, and layered on a KBr density gradient column. The column was centrifuged at 256 000g for 18 hours in an Optima L-100 ultracentrifuge (Beckman). The 1.063 g/mL density layer containing LDL was collected, purified on a PD10 column (17085101, GE Healthcare), and conjugated to the fluorochrome Atto 565 N-hydroxysuccinimide ester (72464, Sigma-Aldrich). Atto565-LDL was purified on a PD10 column, and purity was tested at the CNIC Proteomics Unit by liquid chromatography-mass spectrometry (High Resolution Orbitrap-Fusion, Thermo Scientific) in an 8% to 27% acetonitrile gradient over 2 hours.

Mice received intravenous injections of Atto565-LDL (15 µg/g body weight). Approximately 20 hours postinjection, animals were anesthetized with pentobarbital (Dolethal, Vetoquinol) and lidocaine (Lidocaine 20 mg/mL, B. Braun Medical S.A.) solution in saline (pentobarbital 1:5, lidocaine 1:50) and perfused with 4% formaldehyde in PBS. Prefixed aortas were extracted, cleaned of fatty tissue, opened longitudinally, and pinned flat on a silicone-covered plate. After overnight fixation in 4% formaldehyde/PBS, aortas were cut into 3 pieces and mounted in glycerol on a microscope slide. Fluorescence images were acquired with a Zeiss LSM780 confocal microscope. Autofluorescence (background) was determined in 2 mice (of each genotype) not injected with Atto565-LDL. ImageJ Fiji software was used to quantify the percentage of the aortic area positive for red fluorescence. Videos were prepared using Imaris software (Bitplane).

**Electrocardiography**

Mice were anesthetized with 1.5% to 2% isoflurane, and 4 electrocardiography (ECG) electrodes were inserted subcutaneously into the limbs. ECG was performed in the morning using the MP36R system (Biopac Systems). ECG data were analyzed with AcqKnowledge software (Biopac Systems) by specialized staff blinded to genotype.

**Statistical Analysis**

Experimental data are presented as the mean (error bars indicate SEM) for normally distributed data or median with interquartile range (error bars indicate minimum and maximum) for nonnormally distributed data. Based on all experiments, distribution of a variable (lesion size, nucleus count, etc) was assessed using the Kolmogorov-Smirnov and D’Agostino-Pearson normality tests. If the distribution was normal in most of the experiments, a 2-tailed t test was used. If the distribution of a variable was skewed, the 2-tailed Mann-Whitney test was used. To compare multiple groups, a 1-way ANOVA with the Tukey post hoc test was used for normally distributed data, and a Kruskal-Wallis test with the Dunn post hoc test was used for nonnormally distributed data. A log-rank (Mantel-Cox) test was used to compare Kaplan-Meier survival curves. Differences were considered significant at P<0.05. Statistical analysis was performed with GraphPad Prism 5.

**RESULTS**

**Ubiquitous Progerin Expression in Apoe−/− Mice Accelerates Atherosclerosis**

*Lmna*<sup>G609G/G609G</sup> knock-in mice ubiquitously expressing progerin recapitulate most clinical manifestations of HGPS, including postnatal growth impairment, lipodystrophy, vascular calcification, and reduced survival (average lifespan: ≈15 weeks).18,26 However, we observed no atherosclerosis in aortas of *Lmna*<sup>G609G/G609G</sup> mice even when animals were challenged with a HFD for 8 weeks starting at 8 weeks of age (Figure I in the online-only Data Supplement). This finding is consistent with the observation that mice, unlike humans, are extremely resistant to atherosclerosis development, in part, because of differences in cholesterol and lipoprotein metabolism.27 To circumvent this limitation, we generated an atherosclerosis-prone mouse model of HGPS by crossing *Lmna*<sup>G609G/+</sup> mice with apolipoprotein E-deficient (Apoe<sup>−/−</sup>) mice, a widely used atherosclerosis model.27 As expected, Apoe<sup>−/−Lmna*<sup>G609G/G609G</sup> mice showed reduced body weight and a shortened lifespan (median survival, 18.15 weeks) in comparison with Apoe<sup>−/−Lmna*<sup>G609G/+</sup> littermates with an intact *Lmna* gene (Figure 1A through 1C).
To study the influence of progerin on atherosclerosis, 8-week-old mice were challenged for 8 weeks with a HFD and euthanized at 16 weeks of age. Pre-HFD serum lipid levels, including total cholesterol, free cholesterol, LDL, and high-density lipoprotein were indistinguishable between experimental groups (Figure 1D). However, post-HFD levels were significantly lower in 16-week-old Apoe<sup>–/–</sup>Lmna<sup>G609G/G609G</sup> mice than in age-matched...
Apoe<sup>−/−</sup>Lmna<sup>+/+</sup> controls (Figure 1E), probably because of reduced food intake associated with the advanced disease progression in progeroid mice. HFD-fed Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice nonetheless had a 1.9-fold higher atherosclerosis burden in the aortic arch and a 2.8-fold higher burden in the thoracic aorta (Figure 1F) as assessed by ORO staining. Although both groups had focal aortic lesions, the aortic surface of progeroid Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice was largely covered with lipid deposits that were not seen in control Apoe<sup>−/−</sup>Lmna<sup>+/+</sup> mice (Figure 1F, high magnification in bottom images). Histological examination revealed that this abnormality was attributable to excessive lipid accumulation in the aortic tunica media in atheroma-free zones of Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> aorta (Figure 2A). It is remarkable that Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice developed other pathologies present in patients with HGPS, including adventitial thickening (Figure 2A) and massive loss of VSMCs within the media (Figure 2B). Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice also showed significant hematologic alterations, including lower pre- and post-HFD lymphocyte and monocyte counts (Figure II in the online-only Data Supplement).

**VSMC-Specific, But Not Macrophage-Specific, Progerin Expression in Apoe<sup>−/−</sup> Mice Aggravates Atherosclerosis and Shortens Lifespan**

Atherosclerosis is a multifactorial process involving many cell types, including VSMCs and macrophages. Immunohistopathological characterization of atherosclerotic plaques in patients with HGPS suggests that both cell types contribute to progerin-driven atherogenesis. Because Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice showed medial VSMC loss and reduced numbers of circulating monocytes (which differentiate to macrophages in ath-

![Diagram](https://example.com/diagram.png)

**Figure 2.** Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice with ubiquitous progerin expression exhibit severe vascular pathology, including lipid retention and vascular smooth muscle cell (VSMC) loss in the media, and adventitial thickening. Mice were fed a high-fat diet for 8 weeks starting at 8 weeks of age. **A**, Representative staining of aorta sections with Oil Red O (ORO) and hematoxylin and eosin (H&E). Graphs show quantification of lipid content in atheroma-free zones of the media (as % of the ORO-positive area) and adventitia-to-media thickness ratio; n=6 to 8. Scale bar: 50 µm. **B**, Representative immunofluorescence images of aortas stained with anti–smooth muscle actin (SMA) antibody (red) and Hoechst 33342 (blue). Graphs show quantification of VSMC content in the media as either nucleus count or % of SMA-positive area; n=5 to 8. Scale bar: 50 µm. Box and whisker plots in **A** show medians, interquartile range, and minima and maxima; data in **B** are mean±SEM. Statistical analysis was performed by 2-tailed Mann-Whitney test in **A**, and 2-tailed t test in **B**. ***P<0.001. a indicates adventitia; and m, media.
eromata), we generated mice with specific progerin expression in VSMCs or macrophages to study the relative contribution of these cell types to the vascular pathology induced by progerin. We used knock-in Lmna<sup>−/−</sup>Lmna<sup>LCS/LCS</sup> mice expressing only lamin C, which produce progerin on crossing with transgenic mice expressing Cre recombinase. Apoe<sup>−/−</sup>Lmna<sup>LCS/LCS</sup> mice are apparently normal, but are slightly heavier and longer-lived than wild-type mice. Apoe<sup>−/−</sup>Lmna<sup>LCS/LCS</sup> SM22αCre mice (n=64) and Apoe<sup>−/−</sup>Lmna<sup>LCS/LCS</sup>LysMCre mice (n=36). Studies in A, B, and C were performed with mice fed normal chow, and those in D, E, and F were conducted with mice fed the HFD for 2 months starting at 8 weeks of age. Data in A, D, and E are mean±SEM. Box and whisker plots in F show medians, interquartile range, and minima and maxima. Statistical analysis was performed by log-rank test in B, by 1-way ANOVA with the Tukey post hoc test in A, D and E, and by the Kruskal-Wallis test with the Dunn post hoc test in F. ***P<0.001.
type controls. We generated Apoe−/−LmnaLCS/LCS mice and analyzed atherosclerosis in 16-week-old mice that received a HFD during the last 8 weeks. In comparison with control Apoe−/−Lmna−/− littermates, Apoe−/−LmnaLCS/LCS mice showed no significant differences in atherosclerosis burden, serum lipid levels, or hematologic parameters (Figure III in the online-only Data Supplement).

These results demonstrated that Apoe−/−LmnaLCS/LCS mice could serve as controls for the macrophase-specific Apoe−/−LmnaLCS/LCS LysMCre and VSMC-specific Apoe−/−LmnaLCS/LCS SM22αCre models. Progerin expression was examined by immunofluorescence in atheroma-containing aorta and by PCR in other organs. Apoe−/−LmnaLCS/LCS mice did not express progerin in any organ tested (Figures IV and V in the online-only Data Supplement). Apoe−/−LmnaLCS/LCS SM22αCre mice abundantly expressed progerin in VSMCs of the media, to a lesser extent in the adventitia (Figure IV in the online-only Data Supplement), and also in heart (Figure V in the online-only Data Supplement). Progerin expression in Apoe−/−LmnaLCS/LCS LysMCre mice was detected in intimal macrophages (Figure IV in the online-only Data Supplement), but was either absent or negligible in other organs (Figure V in the online-only Data Supplement).

Apoe−/−LmnaLCS/LCS LysMCre mice with macrophase-specific progerin expression showed no significant differences in organ size (Figure VI in the online-only Data Supplement), body weight, or longevity compared with controls (Figure 3A through 3C). Although Apoe−/−LmnaLCS/LCS SM22αCre mice with VSMC-specific progerin expression appeared normal early in life (Figure 3C and Figure VI in the online-only Data Supplement), they stopped gaining weight from ≈20 weeks of age (Figure 3A), and died prematurely at a median age of 34.3 weeks (Figure 3B). Atherosclerosis was studied with the same protocol used for the ubiquitously progerin-expressing Apoe−/−LmnaG609G/G609G mice. The 3 experimental groups showed no intergroup differences in serum lipid levels (Figure 3D and 3E) or hematologic parameters (Figure VI in the online-only Data Supplement), either pre- or post-HFD. Atherosclerosis burden in the thoracic aorta was similar in Apoe−/−LmnaLCS/LCS LysMCre and Apoe−/−LmnaLCS/LCS mice; in contrast, the lesion burden in Apoe−/−LmnaLCS/LCS SM22αCre mice was 4.4-fold higher (Figure 3F). Moreover, histological analysis in Apoe−/−LmnaLCS/LCS SM22αCre mice revealed the same marked aortic phenotype observed in the ubiquitous progeroid model, including massive VSMC loss, lipid accumulation in the media, and adventitial thickening (Figure 4).

Progerin Expression Accelerates Atherosclerosis in Apoe−/− Mice Fed Normal Chow

To gain insight into the progress of progerin-induced atherogenesis and to assess the role of cholesterol in this process, we performed experiments in 8- and 16-week-old mice fed normal chow. Histological examination of aortas from 8-week-old mice revealed no differences between the progeroid models and their controls, with tissue almost free of atherosclerosis and no obvious structural alterations (Figures VIIIA, VIIIC, and VIIIE in the online-only data supplement). In contrast, the thoracic aortas of 16-week-old Apoe−/−LmnaG609G/G609G and Apoe−/−LmnaLCS/LCS SM22αCre mice presented increased lesion formation than their controls (Figure VIIIB and VIIID in the online-only Data Supplement). Furthermore, aortas of both mutant models contained regions with VSMC loss (Figure VIIIE in the online-only Data Supplement). This vascular phenotype was slightly more pronounced in Apoe−/−LmnaLCS/LCS SM22αCre mice than in Apoe−/−LmnaG609G/G609G mice, with lipid retention in the media and adventital thickening (data not shown), probably because of normal food intake and a consequent cholesterol level similar to that of control mice.

Aortas of Apoe−/−LmnaG609G/G609G and Apoe−/−LmnaLCS/LCS SM22αCre Mice Show Increased LDL Retention

Lipid accumulation in the aortic medial layer is a prominent characteristic of the vascular pathology in both the ubiquitous and the VSMC-specific progeroid mouse models. Because LDL retention in the aortic wall is one of the key processes triggering atherosclerosis initiation and progression, we performed in vivo LDL retention experiments by injecting normal chow–fed 16-week-old mice with human LDL labeled with the fluorochrome Atto565 (Figure 5A). Aortic Atto565-LDL content was quantified 20 hours postinjection, at which time exogenous human LDL is cleared from the circulation. In comparison with their respective control littermates, both Apoe−/−LmnaG609G/G609G and Apoe−/−LmnaLCS/LCS SM22αCre mice showed increased aortic LDL retention (Figure 5B and 5C and Figure IXA and IXB in the online-only Data Supplement). Although Atto565-LDL was localized in the intimal lesions in mice of all genotypes, its excessive retention in the medial layer was exclusive to the ubiquitous and VSMC-specific progeria mouse models (Figure 5D and 5E, Figure IXC and IXD in the online-only Data Supplement and Movies I through XVI in the online-only Data Supplement).

Progerin Triggers Plaque Vulnerability

Previous studies have shown that VSMC loss in the tunica media can promote atherosclerotic plaque instability; we therefore characterized atherosclerotic lesions in the aortic root. Consistent with our results...
in the aorta (Figures 1F and 3F), analysis of atheromatous lesions in the aortic root revealed a larger lesion area in HFD-fed Apoe−/−LmnaαCS/LCS and Apoe−/−LmnaCS/LCSαCre mice than in their respective controls, and this was accompanied by fibrosis and inflammation in the adjacent cardiac tissue (Figure 6A and 6B). Moreover, atherosclerosis affected a higher percentage of the aortic surface in both models (Figure 6A and 6B). Analysis of Apoe−/−LmnaαCS/LCSG609G/G609G and Apoe−/−LmnaαCS/LCSαCre mice revealed the presence in atheromata of erythrocytes, indicating healed plaque fissures (Figure 6C and 6D, top). We also found hemorrhage in the aortic valve itself (Figure 6C, bottom), and evidence of thrombus formation (Figure 6D, bottom).
Apoe−/− LmnaG609G/G609G Mice Develop Cardiac Electric Defects

Previous studies reported cardiac electric defects in progeroid LmnaG609G/G609G and Zmpste24-deficient (Zmpste24−/−) mice similar to those found in patients with HGPS.6,18,32 We therefore performed ECG in the ubiqui-
Figure 6. Enhanced aortic root atherosclerosis in Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> and Apoe<sup>−/−</sup>Lmna<sup>LCS/LCS</sup>SM22αCre mice is accompanied by inflammation of adjacent cardiac tissue and plaque destabilization. Mice were fed a high-fat diet for 8 weeks starting at 8 weeks of age. Serial sections of the aortic root were made and 3 different regions were stained with Masson trichrome. A and B, Representative photographs of atheromata in the aortic root of Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice (A) and Apoe<sup>−/−</sup>Lmna<sup>LCS/LCS</sup>SM22αCre mice (B), and their corresponding controls. Graphs show quantification of plaque area, with each point representing the mean for 3 aortic root regions; n=5. Scale bar: 200 µm. Dashed lines indicate medial perimeter (the last layer of elastin) and dotted lines indicate the luminal surface of the atheroma plaque. C, An example of intraplaque (Top) and intravalve (Bottom) hemorrhage in Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice. Scale bar: 100 µm. D, An example of intraplaque hemorrhage (Top) and thrombus formation (Bottom) in Apoe<sup>−/−</sup>Lmna<sup>LCS/LCS</sup>SM22αCre mice. Scale bar: 100 µm. Data are shown as mean±SEM. Statistical analysis was performed by 2-tailed t test. **P<0.01. ***P<0.001.
tous and VSMC-specific progeria models. As expected, 16-week-old Apoe<sup>+/−</sup>Lmna<sup>G609G/G609G</sup> mice exhibited severe bradycardia and prolonged QRS, QT, and QTc intervals (Figure XA in the online-only Data Supplement). Moreover, 2 of 5 mice had arrhythmias (Figure XB in the online-only Data Supplement). In contrast, 16-week-old Apoe<sup>+/−</sup>Lmna<sup>CSCS/CM22αCre</sup> mice presented no evident ECG alterations (Figure XIA in the online-only Data Supplement). Nevertheless, follow-up revealed a low-voltage QRS complex at 26 weeks of age (Figure IXB in the online-only Data Supplement), consistent with loss of viable myocardial tissue attributable to myocardial infarction. Likewise, many of the 26-week-old Apoe<sup>−/−</sup>Lmna<sup>CSCS/CM22αCre</sup> mice exhibited T-wave fattening or inversion (Figure IXB in the online-only Data Supplement and data not shown), further supporting the notion that these animals develop age-associated cardiac alterations probably caused by progressive atherosclerosis.

**Apoe<sup>−/−</sup>Lmna<sup>LCSC/LCSM22αCre</sup> Mice Die of Atherosclerosis-Related Causes**

Despite an almost identical vascular phenotype, ubiquitous and VSMC-specific atherosclerosis-prone progeria models differed in their cardiac phenotype and median survival (18.15 weeks versus 34.3 weeks, respectively). Apoe<sup>−/−</sup>Lmna<sup>LCSC/CM22αCre</sup> mice, which showed no overt aging phenotype, died suddenly between 26 and 66 weeks of age. It is interesting to note that Lmna<sup>LCSC/LCSM22αCre</sup> mice with an intact Apoe gene also presented VSMC loss and aortic adventitial thickening, but did not develop atherosclerosis and had a normal lifespan (Figure XII in the online-only Data Supplement). These findings point to progerin-driven atherosclerosis as the main cause of death in Apoe<sup>−/−</sup>Lmna<sup>LCSC/CM22αCre</sup> mice. In contrast, Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice progressively lost fat tissue, developed bone problems and muscle weakness, and died between 15 and 24 weeks of age, a median survival very similar to that of atherosclerosis-free Lmna<sup>G609G/G609G</sup> mice.<sup>18</sup> We therefore conclude that the cause of death is likely different in the ubiquitous and VSMC-specific progeria models, at least in the majority of the cases.

To analyze cardiovascular alterations, which could reveal the cause of death, we collected aortas and hearts from chow-fed Apoe<sup>+/−</sup>Lmna<sup>G609G/G609G</sup> and Apoe<sup>−/−</sup>Lmna<sup>LCSC/CM22αCre</sup> mice at an age close to their maximum survival (21–23 weeks and 51 weeks, respectively). Atherosclerosis burden in the aortic arch and thoracic aorta was significantly higher in Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice than in Apoe<sup>+/−</sup>Lmna<sup>G609G/G609G</sup> controls (Figure 8A). In comparison with Apoe<sup>+/−</sup>Lmna<sup>LCSC/LCS</sup> controls, Apoe<sup>−/−</sup>Lmna<sup>LCSC/CM22αCre</sup> mice showed a higher lesion burden in the thoracic aorta but not in the aortic arch (Figure 8B), likely because this athero-prone aortic region is saturated with plaques in 51-week-old (=1 year) Apoe<sup>−/−</sup> mice. It is important to note that vascular pathology was much more severe in the aorta of 51-week-old Apoe<sup>−/−</sup>Lmna<sup>CSCS/CM22αCre</sup> mice than in 21- to 23-week-old Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice (Figure 8A through 8C).

Consistent with the findings in the aorta, pathological findings in the aortic root were more severe in Apoe<sup>−/−</sup>Lmna<sup>CSCS/CM22αCre</sup> mice than in Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice (Figures XIII through XVI in the online-only Data Supplement). Plaques in Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice were generally immature, frequently exhibiting intraplaque hemorrhages that were absent in age-matched controls (Figure XIIIIA in the online-only Data Supplement). Of the Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> animals, 3 of 9 showed signs of coronary atherosclerosis (Figure XVI, zone 1, in the online-only Data Supplement). In Apoe<sup>−/−</sup>Lmna<sup>LCSC/CM22αCre</sup> mice, atheromas were mature and severely calcified, and many of them had chondroid metaplasia (Figures XIII and XIV in the online-only Data Supplement). The noncalcified atheromas had thin fibrous caps and large necrotic cores (Figure XIIIIB in the online-only Data Supplement). Moreover, 4 of 9 of the analyzed Apoe<sup>−/−</sup>Lmna<sup>LCSC/CM22αCre</sup> mice developed coronary atherosclerosis (Figure XVI, zone 1, in the online-only Data Supplement). Even though plaques of both mutant models showed features of vulnerable plaques, such as lower collagen and VSMC content (Figure XIIIIB in the online-only Data Supplement), Apoe<sup>−/−</sup>Lmna<sup>LCSC/CM22αCre</sup> mice had more atherosclerosis, fibrosis, and inflammation in the aortic root (Figure XV in the online-only Data Supplement).

We also performed a histopathologic evaluation of the heart ventricles. Apoe<sup>+/−</sup>Lmna<sup>G609G/G609G</sup> hearts were characterized by cardiomyocyte vacuolization, which was absent in Apoe<sup>−/−</sup>Lmna<sup>−/−</sup>, Apoe<sup>−/−</sup>Lmna<sup>LCSC/CM22αCre</sup>, and Apoe<sup>−/−</sup>Lmna<sup>CSCS</sup> mice (Figure XVII in the online-only Data Supplement and data not shown). Furthermore, 3 of 9 Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> hearts had coronary atherosclerosis (Figure 8D and Figure XVI in the online-only Data Supplement) and 2 had a collagen scar in the septum, indicative of a nonfatal infarct (Figure XVIII in the online-only Data Supplement). Nevertheless, many of the animals analyzed showed no detectable alterations apart from the cardiomyocyte vacuolization (Figure 8D and 8E).

Most Apoe<sup>−/−</sup>Lmna<sup>LCSC/CM22αCre</sup> hearts presented perivascular and interstitial fibrosis, intimal hyperplasia (without cellular infiltrates) and coronary atherosclerosis (Figure 8D and 8E, Figures XV and XVI in the online-only Data Supplement). Some of the animals analyzed also showed evidence of coronary plaque calcification (Figure 8D). These pathological alterations in the heart suggest that Apoe<sup>−/−</sup>Lmna<sup>CSCS/CM22αCre</sup> animals are, like patients with HGPS, susceptible to microinfects.
during their lifetime (Figure 8E). Collectively, these histopathologic findings in the aorta and heart indicate that Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> animals die of atherosclerosis complications, whereas Apoe<sup>−/−</sup>Lmna<sup>CSLCS/G609G</sup> mice mostly die of atherosclerosis-independent processes.

**DISCUSSION**

Several mouse models of HGPS have been generated<sup>16–22</sup>; however, only a few of these models presented cardiovascular alterations and none showed evidence of atherosclerosis, the pathological process that leads to myocardial infarction– or stroke-related death in most children with HGPS. Based on these models, various molecular mechanisms have been proposed to explain how progerin drives HGPS. Treatments targeting candidate pathways have significantly ameliorated disease progression and extended lifespan in mice; however, these treatments have only had modest therapeutic effects in patients with HGPS.<sup>33–35</sup> One possible explanation is that progeric mice, unlike humans, die of atherosclerosis-independent causes. It is important to note that mice are particularly resistant to atherosclerotic plaque formation, in part, because of the murine blood lipid profile, with high atheroprotective high-density lipoprotein and low atherogenic LDL.<sup>36</sup> Thus, to study atherosclerosis in mouse models, it is usually necessary to introduce a genetic mutation (such as in-
activation of the Apoe or Ldlr genes), to alter their lipid metabolism and make them more prone to developing atherosclerosis.37,38

In this study, we generated Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice, the first mouse model of progerin-induced atherosclerosis acceleration. In addition to enhanced atherosclerosis, these mice exhibited more severe cardiovascular phenotypes, including increased intimal hyperplasia, coronary atherosclerosis, perivascular fibrosis, interstitial fibrosis, and plaque calcification compared to control strains. The data were analyzed using 2-tailed Mann-Whitney test. *P<0.05, **P<0.01. LV indicates left ventricle; RV, right ventricle; and S, septum.
Plaque formation, these animals exhibit massive loss of medial VSMCs and prominent adventitial thickening and fibrosis, important hallmarks of vascular disease in HGPS. These features were previously observed in knock-in Lmna<sup>G609G/G609G</sup> and transgenic G608G BAC mouse models; however, in earlier models, these features did not manifest as early or as severely as described here and were not accompanied by atherosclerosis development. The Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> model is also the first to show lipid accumulation in atheroma-free zones of the aortic media, which may contribute to enhanced plaque formation. Furthermore, our studies with Apoe<sup>−/−</sup>Lmna<sup>CsclC5/SM22αCre</sup> mice (VSMC-specific progerin expression) and Apoe<sup>−/−</sup>Lmna<sup>G609G/</sup>LysM<sup>Cre</sup> mice (macrophage-specific progerin expression) provide the first direct evidence that restricting progerin expression to VSMCs, but not macrophages, is sufficient to accelerate atherosclerosis and reduce lifespan. Although limiting progerin expression to macrophages does not increase atherosclerosis burden or reduce survival, it remains to be determined whether it affects plaque composition. Further studies are also required to ascertain why progerin-expressing Apoe<sup>−/−</sup> mice have increased lipid retention in the media (eg, increased vascular permeability, changes in the extracellular matrix amount and composition).

Our findings in both the ubiquitous and the VSMC-specific progerin mouse models indicate that VSMCs are particularly sensitive to progerin accumulation. Progerin-induced VSMC death results in increased LDL retention in the media, adventitial thickening, and accelerated atherosclerosis. Similarly to Clarke et al., we found that VSMC death triggers features of plaque vulnerability, which may lead to myocardial infarction and premature death. Our results demonstrate the importance of VSMCs in progerin-driven atherosclerosis and identify this cell type as a key target for future treatments.

It is remarkable that we observed that Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice have a similar average lifespan to non-atherosclerotic Lmna<sup>G609G/G609G</sup> mice, suggesting that mice with ubiquitous progerin expression die of atherosclerosis-independent causes, unlike human patients with HGPS. In contrast, Apoe<sup>−/−</sup>Lmna<sup>CsclC5/SM22αCre</sup> mice appear to die of atherosclerosis-related problems, because Lmna<sup>CsclC5/SM22αCre</sup> mice, with VSMC-specific progerin expression but an intact ApoE gene, did not develop atherosclerosis and had a normal lifespan. This hypothesis is supported by histopathologic findings in mice at ages close to their maximum survival, which reveal much more severe aortic and coronary atherosclerosis in Apoe<sup>−/−</sup>Lmna<sup>CsclC5/SM22αCre</sup> mice than in Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice. It may be that plaque disruption leading to infarction and death occurs in Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice, but only in animals surviving to near 26 weeks of age. This is well beyond the median survival in this model (18.15 weeks), but is the age at which Apoe<sup>−/−</sup>Lmna<sup>CsclC5/SM22αCre</sup> mice begin to die. Although Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice may die of heart failure, the cause would likely be related to complications such as arrhythmia, rather than atherosclerosis, as supported by ECG studies in this model. Similarly, we previously reported that Lmna<sup>G609G/G609G</sup> mice have a prolonged QRS-complex duration, indicating defective cardiac conduction. Likewise, our studies in Zmpste24-null mice, another model of premature aging caused by defective prelamin A processing, revealed PQ and QRS prolongation and T-wave flattening, which may increase the risk of lethal arrhythmias in the setting of progeria. This result is also consistent with the observation that patients with HGPS show repolarization abnormalities resulting in an increased risk of arrhythmias and premature death.

In summary, atherosclerosis-prone Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> mice faithfully recapitulate most aspects of human HGPS, including growth retardation, shortened lifespan, and early CVD onset, and is therefore a valuable tool for investigation of the general mechanisms underlying HGPS. We have also demonstrated that VSMC-restricted progerin expression in Apoe<sup>−/−</sup>Lmna<sup>CsclC5/SM22αCre</sup> mice is sufficient to induce the vascular features of progeria independently of growth retardation and other disease symptoms, thus providing a unique preclinical model for the study of atherosclerosis and atherosclerosis-associated premature death in isolation from other signs of premature aging. Another advantage of these animals is the lack of overt growth defects, which might permit the testing of diagnostic techniques and interventional strategies that are unavailable in the undersized Apoe<sup>−/−</sup>Lmna<sup>G609G/G609G</sup> model.


REFERENCES


