Olive et al. Supplement material

Supplemental Methods:

The study was approved by the institutional review boards of Rhode Island Hospital and Brown University. Informed consent was obtained from the parents of HG001 and HG120.

Clinical Information: Medical information for HG001 and HG120 was obtained from The Progeria Research Foundation (PRF) Medical and Research Database (www.progeriaresearch.org/medical_database.html) at the Brown University Center for Gerontology and Health Care Research (Providence, RI).

Autopsy Specimens: Autopsy tissue from HG001 and HG120 were obtained from the PRF Cell and Tissue Bank (www.progeriaresearch.org/cell_tissue_bank.html) at Rhode Island Hospital (Providence, RI). Non-HGPS tissues were obtained from the CVPath Institute, Inc (Gaithersburg, MD).

Mutation Analysis: Mutational Analysis for HG001 and HG120 was performed via the PRF Diagnostics Program (www.progeriaresearch.org/diagnostic_testing.html). For HG001, fibroblasts (cell bank reference: HGADFN001) were cultured and DNA was isolated. Amplification and sequencing of the *LMNA* exon 11 was performed by PreventionGenetics (Marshfield, WI). For patient HG120, DNA was isolated from paraffin sections of the liver, submitted to two rounds of PCR followed by dideoxy sequencing. Amplification and sequencing of the LMNA exon 11 was performed by the Laboratory for Molecular Medicine (LMM) Cambridge, MA.

Histochemistry: Tissues were fixed in 2% paraformaldehyde and embedded in paraffin or frozen in OCT medium. Cross-sections (6 μm) were stained with hematoxylin/eosin and Movat

pentachrome. Images were captured using a Nikon Eclipse 6600 Microscope equipped with QImaging Retiga 1300 digital camera.

Immunohistochemistry (IHC): Lamin staining was previously described in detail.[1] Briefly, antibodies used in this study were: mouse monoclonal anti-lamin A/C non-diluted (MAB3211; Chemicon, pure); monoclonal anti-smooth muscle • -actin FITC-conjugated (1:100; clone 1A4; Sigma-Aldrich); Alexa Fluor 594 or 555-conjugated were used as secondary antibodies (1:500; Molecular Probes). Lamin A/C expression was explored using the MAB3211 antibody in 9 control individuals (3 individuals/age group: 0 to 20-years old, 80-years- old and above.)

Progerin antibody is a rabbit polyclonal 972 and used at 1/500 dilution.[2] Anti-progerin antibody stains progerin specifically, and does not cross-react with lamin.[3] Sections of non-HGPS individuals were subjected to a 2 min EDTA antigen retrieval treatment in a pressure cooker and further stained with the anti-progerin antibody. Slides were mounted in DAPI-containing medium (Vector Laboratories). Fluorescence emission images were obtained with a confocal microscope system (LMS 510; Zeiss) using 40x or 65x oil lenses. Progerin-positive cells and progerin negative cells were quantified on sections of LAD of non-HGPS individuals and a negative binomial generalized estimating equation used to model percent progerin staining as a function of age, allowing for within-subject correlation. Rate was calculated by modeling progerin-stained cell counts, offset by the logarithm of the total cell count (proc genmod, SAS version 9.2, SAS Institute, Cary, NC). There were no differences in inferences after Holm adjustment for multiplicity and so unadjusted p-values were reported.

Histological Evaluation of Extra Cellular Matrix: Decorin, biglycan and versican were detected with a one hour pre-incubation with chondroitinase ABC (Sigma)[4] followed by either rabbit polyclonal antiserum for decorin (LF-122 diluted 1:500, from Larry Fisher, National

Institute of Dental Research, Bethesda, MD), biglycan (LF-51 diluted 1:2000) or a mouse monoclonal antibody against human versican (2B1 (Calbiochem) diluted 1:1000). Macrophages were detected by heating sections in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 30 minutes followed by a monoclonal mouse anti-human CD68 (KP1 (Dako) diluted 1:100). Hyaluronan was detected with a biotinylated hyaluronan binding protein preparation (b-HABP, 3 μg/ml) made as described.[5] CD44 was detected with a mouse monoclonal anti-human CD44 (A3D8 (Abcam) diluted 1:50). Biotin-conjugated secondary antibodies were used and detected with Vectastain ABC kit (Vector) followed by peroxidase substrates NovaRed (Vector) or DAB (Vector). IgG isotype controls were carried out for each antibody (data not shown). Collagen was visualized with Picrosirius Red and viewed under polarized light. Lipid was detected in frozen sections of HG001 using Oil Red O. All images of ECM were obtained using a Leica (Deerfield, IL) DM2500 scope equipped with a Diagnostic Instruments (Sterling Heights, MI) Insight 4 megapixel color CCD camera with SPOT software.

Clinical Information:

Case history HG001

Early Course and Diagnosis: HG001 was a white female who died at age 9.9 years, after a clinical course typical for HGPS. She was born at 39 weeks gestation. Birth weight was 3-5%ile (2.5 kg) and hovered between 5-10%ile until age 5.7 months when she dropped below the 3%ile indefinitely. Birth height was 25%ile (47.6 cm) and gradually decreased to below the 3% ile at age 18 months. Despite nutritional intervention, she reached a maximum weight and standing height of 11.8 kg and 102 cm, respectively. Developmental milestones and intellect were normal throughout life. At age 2.5 months, the skin on the lower trunk and legs became mottled and

sclerotic. Over the next year, she developed typical signs of HGPS including prominent veins, receding mandible, high-arched palate, and delayed and crowded dentition; alopecia began at age 1.6 years. Skeletal findings included hypoplastic clavicles, coxa valga, and eventual bilateral avascular necrosis of the hips, without history of fracture. She was diagnosed clinically with HGPS at age 2.9 years. Karyotype analysis at age 6 months was normal 46XX. Genetic diagnosis was accomplished post-mortem (see Methods).

Family History: Parents were in good health and without history of hypertension or hyperlipidemia. Maternal grandparents developed hypertension and hypercholesterolemia in their sixth decade. Paternal grandparents had history of cancer as adults, with paternal grandfather deceased at 36 (pneumonia, cancer involving low back). There was no family history of lipodystrophy.

Blood Lipids: Lipid profiles were obtained at ages 7 and 22 months (Supplemental Table 2), with normal findings at 7 months and slightly elevated serum triglycerides at age 22 months. No lipid profiles were available at older ages.

Medications: The only medication taken routinely was low dose aspirin, which was taken daily between ages 4 and 7 years, and then resumed at 9.8 years of age. Ibuprofen and acetaminophen were taken intermittently for pain (hip pain, headache).

Glucose Tolerance: Glucose Tolerance: Fasting insulin and glucose levels were not measured.

Neurovascular: At age 7.5 years, HG001 began experiencing recurrent morning headaches. Over the next two years, the headaches increased in frequency, and were accompanied by nausea. Between ages 8 and 9.5 years, prolonged headaches preceded three transient ischemic attacks. At age 9.8 yrs, two weeks prior to death, she had a cerebrovascular accident characterized by syncope and fatigue, as well as severe headache with vomiting and seizure-like activities – body

stiffening followed by whole body limpness, incontinence of urine, and right-sided weakness. EEG demonstrated posterior slowing; MRI/MRA showed bilateral occipital-parietal edema, left greater than right.

Cardiac: Blood pressures were within normal limits for age throughout life; detailed blood pressure history is shown in Supplementary Table I. Annual electrocardiograms (EKG) and cardiac echocardiograms were normal from age 3 to 8.8 years, with a systolic vibratory Still's murmur noted. At age 8.8 yrs, she developed symptoms of occasional chest discomfort, a new murmur, and an echocardiogram demonstrating mild mitral stenosis, reserved ventricular function, and normal-appearing proximal coronary arteries. Chest pain frequency and duration increased over the next year, without additional findings on exam or EKG, until two weeks prior to death when she exhibited cardiomegaly on chest X-ray (CXR). EKG revealed LVH, and a new diastolic murmur was heard. On the day of death the patient presented with substernal chest pain, vomiting, fever, sinus tachycardia, a gallop rhythm, ST segment changes on EKG, and interstitial edema on CXR. Troponin-1 values were 4.0 and 6.0 (normal 0-0.8), consistent with acute myocardial infarct. Despite oxygen, morphine, nitroglycerine drip and propranolol, she developed bradycardia, followed by ventricular tachycardia, ventricular fibrillation, and asystole.

Case History HG120

HG120 was a white male who died at age 14.0 years, after a clinical course typical for HGPS. Early Course and Diagnosis: HG0120 was a white male born at 36 weeks gestation, weighing 2.98 kg (10%ile). Despite nutritional intervention, his weight was below the 3rd %ile at 11 months and remained so for his lifetime. Developmental milestones and intellect were normal throughout life. By one year of age, external features (alopecia, micrognathia, dystrophic nails,

absence of subcutaneous fat, and dental crowding) suggested HGPS. Clinical diagnosis of HGPS was made at age 3; karyotype analysis performed at age 7.5 was normal.

Family History: Parents and sibling reported good health, with no history hypertension or hyperlipidemia. Paternal extended family members reported to have late onset diabetes; paternal grandmother developed hypertension and myocardial infarction in later years. There was no family history of lipodystrophy.

Blood Lipids: Lipid profiles were obtained at ages 4.2, 7.0, 12.9 and 13.9 years (Supplemental Table II). The only abnormal findings were at age 12.9 years, when cholesterol and triglycerides were slightly elevated.

Medications: Aspirin was started at 12 years at dose of 240 mg twice per day, (reportedly for myalgias). At age 13, he was prescribed Theophyllin 100 mg TID. At age 13.6, he began daily Digoxin and diuretics (spironolactone and chlorothiazide), using nitroglycerin for chest pain. Age 13.8 years, after presenting with pulmonary edema he was treated acutely with furosemide and continued on above regimen. At age 14 years, daily oral furosemide was added, along with home oxygen and bed elevation for symptoms of dyspnea at rest.

Glucose Tolerance: There was mild insulin resistance by age 7, without frank diabetes. Insulin sensitivity was tested twice via intravenous stimulation tests. First, at age 4.2 - Tolbutamide and glucagon stimulation tests were normal, indicating normal insulin sensitivity. Second, at age 7.0, an intravenous carbohydrate tolerance test (insulin and glucagon stimulation test), revealed normal fasting blood sugar with normal response to glucagon, although blood sugar response to insulin was muted, suggesting mild insulin resistance.

Cardiac: HG120 was normotensive, and without clinical evidence of transient ischemic attacks or strokes throughout his life; detailed blood pressure history is shown in Supplementary Table I.

Cardiac exam, EKGs, CXR, and echocardiograms were initially within normal limits; at age 8, a grade I/VI systolic ejection murmur was detected, and at age 9, there was intermittent left lower chest pain. At age 12, he developed increased interstitial markings on CXR without overt pulmonary infection, and within the next 8 months experienced increasing episodes of angina, two-flight dyspnea on exertion, grade II/VI systolic murmur, prominent interstitial markings, peribronchial thickening, and small areas of atelectasis on CXR. At age 12.9, there was a grade III/VI systolic murmur and bilateral carotid bruits, as well as anginal episodes at rest 1-2 times each week. EKG showed LVH, and echocardiogram showed normal ejection fraction, but with septal wall motion abnormalities. Pulmonary function testing revealed obstructive lung disease with diffuse increased interstitial markings. Over the subsequent year, he developed increasing episodes of angina and dyspnea; physical exam revealed a prominent S3, hyperkinetic carotids, pulsatile jugular venous distension to 4 cm in the upright position, and the EKG showed inferior and lateral ischemia. Despite digoxin, diuretics, nitrates, and salicylate, he developed progressive congestive heart failure and angina; he died of cardiac arrest at home.

Autopsy Findings: Both the original review at the institutions where autopsies were performed and re-review by the study group are concordant.

HG001:

The heart displayed normally sized RV and RV wall thickness, and no abnormalities noted grossly or microscopically in the pulmonary circulation. There was gross and histologic evidence if myocardial infarction (MI) involving the posterior intraventricular (IV) septum as well as smaller infarctions involving the circumference of the left ventricle and papillary muscles. The MIs were of a variety of ages, ranging from remote (months) to subacute (2-3

weeks), with multifocal acute (2-3 day old) infarction. The coronary arteries all showed severe multifocal stenoses microscopically, with the right coronary focally exhibiting 95% chronic occlusion, the left circumflex artery up to 90% chronic occlusion distally, and the left coronary artery up to 50% chronic stenosis. There was no acute plaque hemorrhage, rupture, or thrombosis identified in any of the sampled coronary artery segments; additional wet tissue was not available for analysis. The findings point to both acute and chronic MI in the setting of severe three-vessel coronary artery disease, likely with a terminal arrhythmia as the cause of death.

HG120:

The right ventricle showed papillary muscle atrophy and RV dilation without hypertrophy. The main pulmonary artery was normal in size as were its branches. There is no mention of pulmonary artery atherosclerotic plaque in the original autopsy report (as a surrogate to assess for chronic pulmonary hypertension), nor was it visualized histologically. There was gross and histologic evidence of MI in the interventricular septum, extending into the anterior and posterior left ventricle in a focally transmural fashion. The MIs were of a variety of ages, ranging from remote (months) to subacute (2-3 weeks), with multifocal acute (2-3 day old) infarction. Microscopically, the coronary arteries all showed severe multifocal chronic stenoses (>95%) many of which were calcified. There was recent plaque hemorrhage in the left main coronary artery, but no areas of acute vessel thrombosis were sampled at the time of autopsy; additional wet tissue was not available for analysis. The findings point to both acute and chronic MI in the setting of severe three-vessel coronary artery disease, likely with a terminal arrhythmia as the cause of death.

Supplemental Tables and Figure Legends

Supplemental Table 1

HG001 Blood Pressures by Age			HG120 Blood Pressures by Age		
Age (yrs)	Systolic BP	Diastolic BP	Age (yrs)	Systolic BP	Diastolic BP
1.84	56	46	6.98	100	70
3.04	92	60	6.98	90	70
3.13	70	46	7.9	100	80
4.02	76	50	8.15*	100	66
4.15	100	50	9.48	90	30
5.15	83	53	10.05	80	50
5.15	100	60	12.7*	92	54
6.47	101	81	12.89*	110	80
6.47	100	70	13.57**	100	70
7.69	90	40	13.88*	80	40
7.99	80	52	13.88	90	40
7.99	80	52	13.89	95	30
8.79	90	50			
9.88*	101	52			
9.92	107	70			

*When a series of pressures was obtained over several days, one representative set is shown

**Age after which medications that could affect blood pressure were routinely administered

Supplemental Table II

HG001 Fasting Blood Lipids			HG120 Fasting Blood Lipids		
Test (units)	Result	Normal Range	Test (units)	Result	Normal Range
Age 7 months			Age 4.2 years		
Cholesterol (mg/dl)	138	64-170	Total Lipids (mg%)	670	400-800
Triglycerides (mg/dl)	89	35-135	Phospholipids (mg%)	100	100-250
Age 22 months			Age 7.0 years		
Cholesterol (mg/dl)	146	64-170	Total Lipids (mg%)	670	400-800
Triglycerides (mg/dl)	155*	35-135	Phospholipids (mg%)	100	100-250
HDL (mg/dl)	30	30-70	Cholesterol (mg%)	203	<205 mg%
LDL (mg/dl)	91	50-125	Age 12.9 years		

VLDL (mg/dl)	25	8-25	Cholesterol (mg%)	229*	<205 mg%
LDL/HDL ratio	3.04	1.00-3.22	Triglycerides (mg%)	176 *	<140 mg%
CHOL/HDL ratio	4.87*	1-4.44	Age 13.9 years		
			Cholesterol (mg%)	168	<205
			Triglycerides (mg%)	118	<140
*Abnormal Value					

Suppl. Figure I: A, Artery of the salivary gland surrounded by fibrosis (arrow). B, View of a hepatic vein with increased fibrosis (arrow) and C, dense fibrotic matrix is present in the hepatic triad (arrow) in HG120 (H&E stain). Perivascular fibrosis is also present in epicardial vein of D, patient HG001 (Movat stain) and E, patient HG120 and in F, the hilar lymph node vein (H&E stain). (Scale bars: 50 µm).

Suppl. Figure II: RCA of case HG001 was analyzed for the presence of lipid (Oil Red O), macrophages (CD68), the hyaluronan receptor CD44, and hyaluronan (HABP). Positive staining indicated in red. Sections were counterstained with hematoxylin. (Scale bars: 100 µm).

Suppl. Figure III: Endothelial Cells in HGPS patient have low levels of progerin. Small capillaries of the coronary arteries of HG001 are stained with progerin (red), CD31 (green) and DAPI (blue). (Scale bars: 10 µm).

Suppl. Figure IV: Lamin A/C in aging arteries

Lamin A/C is present in the normal coronary arteries of a 3-years-old control and in a typical coronary artery with complex plaque from a 84 years-old individual. Media, intima and adventitia were imaged. Lamin A/C (red), SMA (green) and DAP (blue). (Scale bars: 10 µm).

Suppl. Figure V: Stenosis and VSMC loss in HGPS aorta

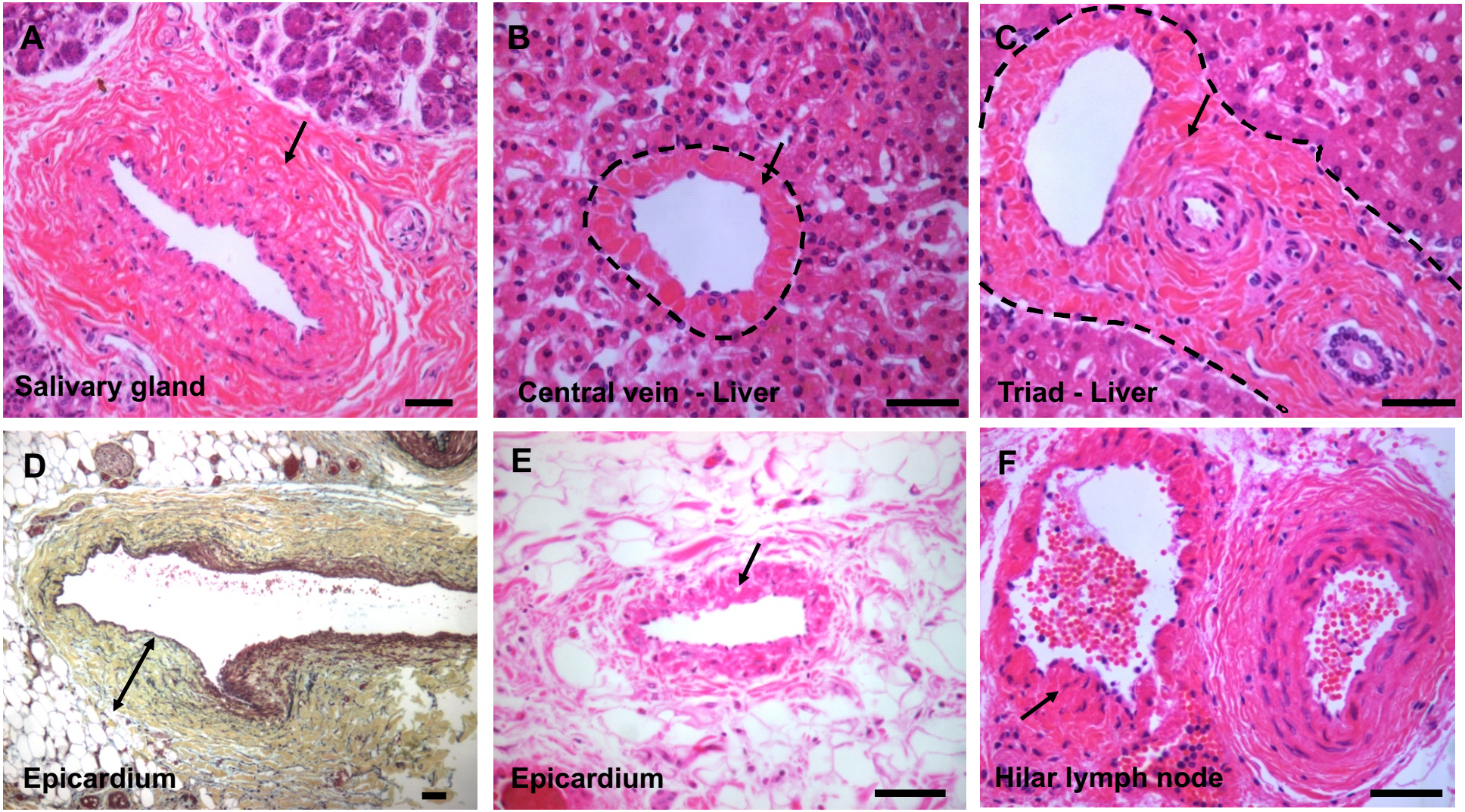
Comparison of a 16-year-old non-HGPS (left) and HGPS (right) aorta. A, Normal individual (16-year-old) and B, HGPS proximal aorta (H&E stain). HGPS proximal aorta has an enlarged intima (i) compared to the control. C, Control aorta, D, Dense and thickened adventitia of the distal aorta in HGPS (Movat stain). E, F, Higher magnification of the pictures of the media in C and D. White arrow indicates VSMC death. G, Control aorta stained with Lamin A/C (red) and SMA (green). H, Anti-progerin (red) and SMA antibodies (green) show VSMC loss in HGPS. I, Control aorta was stained with Lamin A/C (red) and SMA (green). J, Fibroblasts and small arterioles are progerin-positive in HGPS adventitia. K, Adventitia of a 16-years-old control aorta with less condensed collagen (yellow green) compared to L, highly condensed collagen fibers (red) present in HGPS adventitia (Picrosirius red stain). (Scale bars: A-D 500 µm, E-F 50 µm, G-J 10 µm, K-L 25 µm).

References

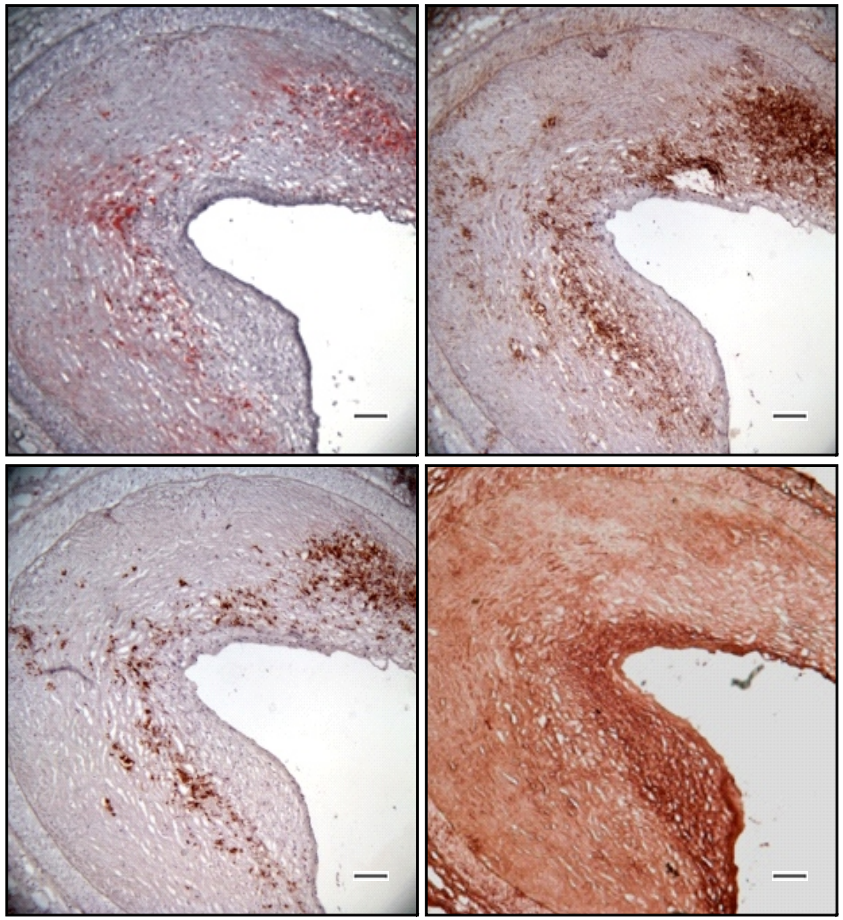
1. Varga R, Eriksson M, Erdos MR, Olive M, Harten I, Kolodgie F, Capell BC, Cheng J, Faddah D, Perkins S, Avallone H, San H, Qu X, Ganesh S, Gordon LB, Virmani R, Wight TN, Nabel EG, Collins FS. Progressive vascular smooth muscle cell defects in a mouse model of Hutchinson-Gilford progeria syndrome. *Proc Natl Acad Sci U S A.* 2006;103:3250-3255.
2. McClintock D, Ratner D, Lokuge M, Owens DM, Gordon LB, Collins FS, Djabali K. The mutant form of lamin A that causes Hutchinson-Gilford progeria is a biomarker of cellular aging in human skin. *PLoS One.* 2007;2:e1269.
3. McClintock D, Gordon LB, Djabali K. Hutchinson-Gilford progeria mutant lamin A primarily targets human vascular cells as detected by an anti-Lamin A G608G antibody. *Proc Natl Acad Sci U S A.* 2006;103:2154-2159.
4. Lark MW, Yeo TK, Mar H, Lara S, Hellstrom I, Hellstrom KE, Wight TN. Arterial chondroitin sulfate proteoglycan: localization with a monoclonal antibody. *J Histochem Cytochem.* 1988;36:1211-1221.

5. Underhill CB, Nguyen HA, Shizari M, Culty M. CD44 positive macrophages take up hyaluronan during lung development. *Dev Biol.* 1993;155:324-336.

Olive et al, Suppl. Fig. I



HG001 - RCA



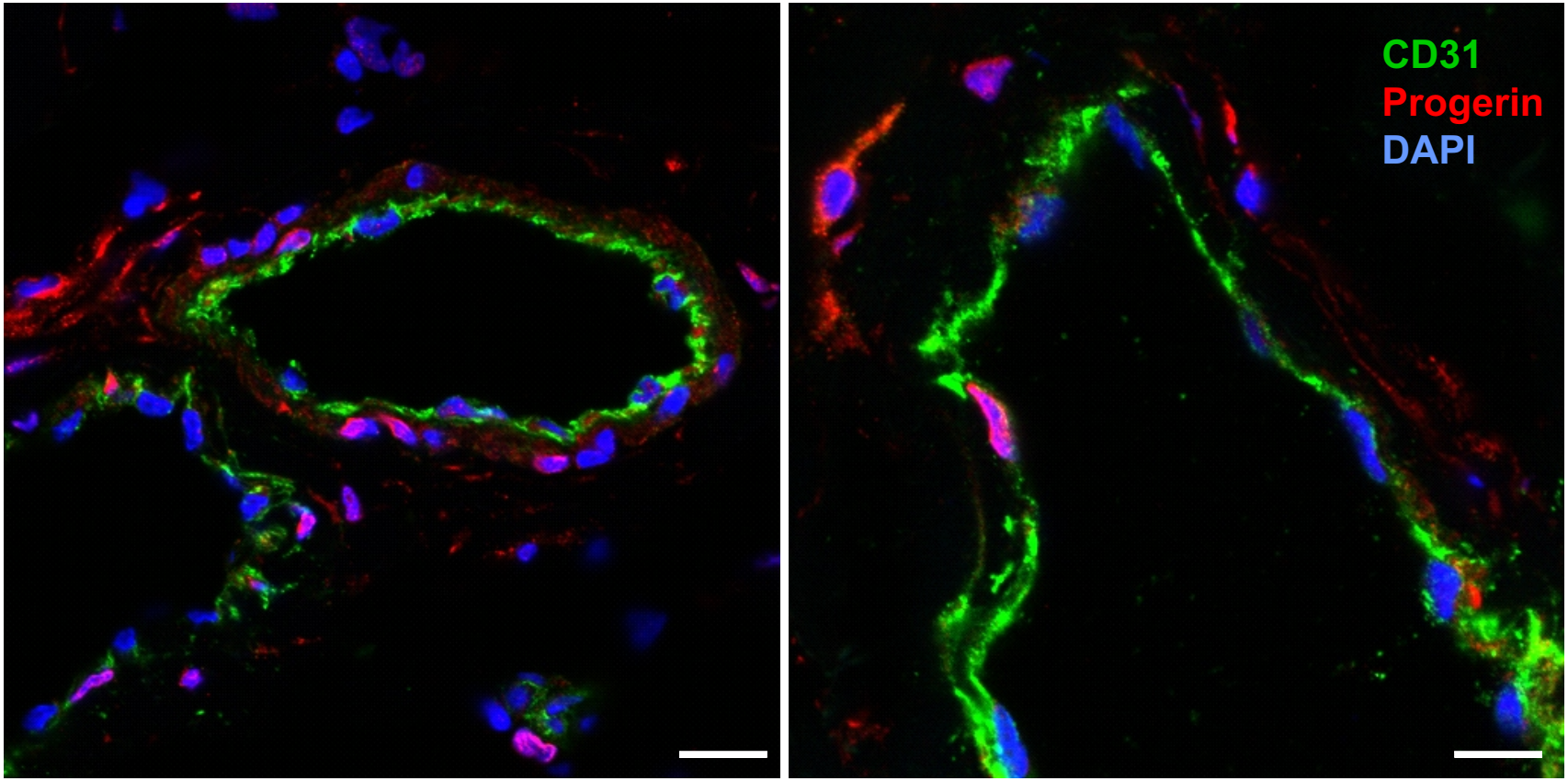
Lipid
Oil red O

Hyaluronan
Receptor
CD44

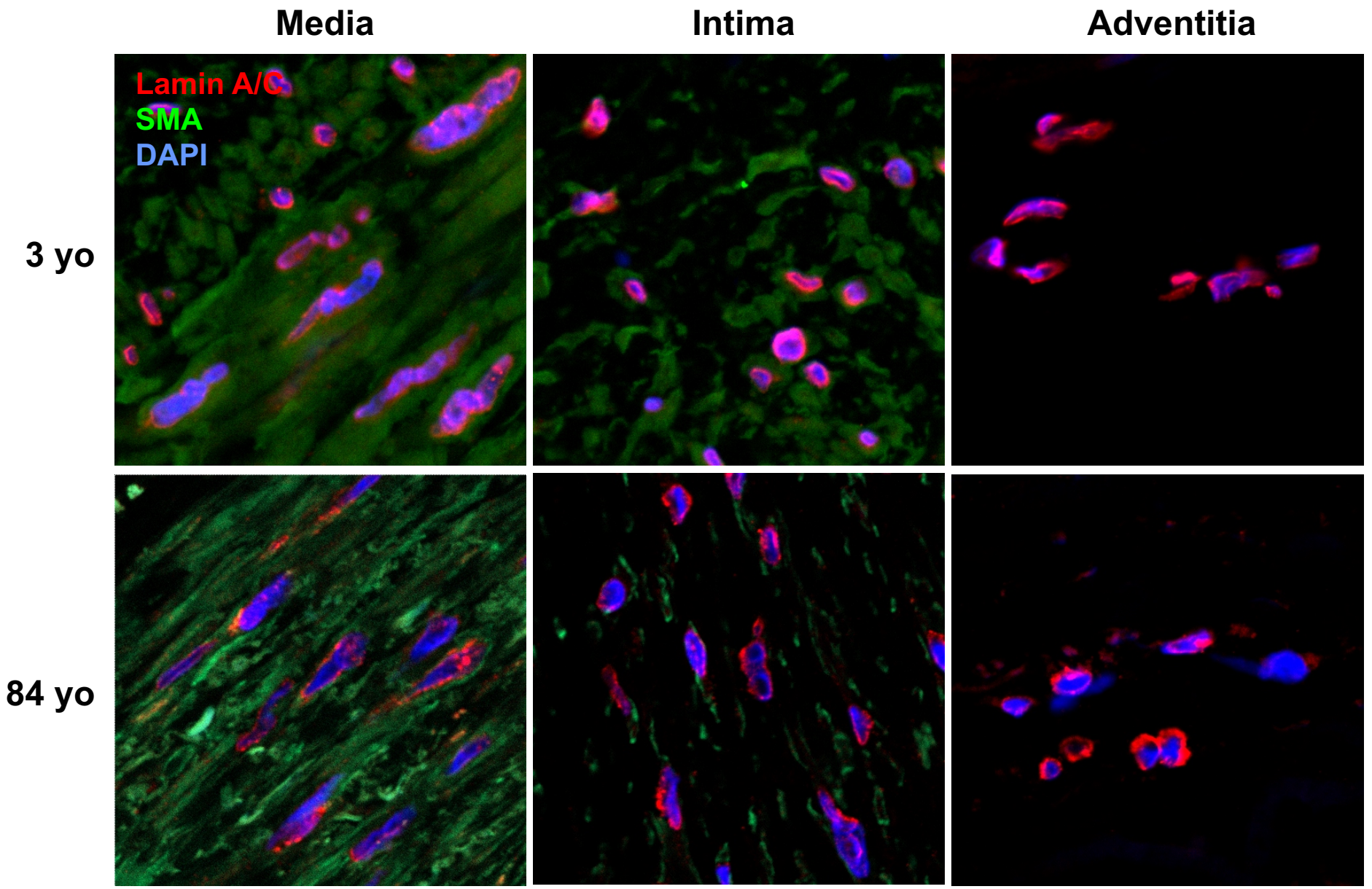
Macrophages
CD68

Hyaluronan
HABP

Olive et al, Suppl. Fig. III



Olive et al, Suppl. Fig. IV



Olive et al, Suppl. Fig. V