Supplemental Material

Plasma progerin in patients with Hutchinson-Gilford progeria syndrome: immunoassay development and clinical evaluation

Short Title: Progeria Lifespan Predicted by Plasma Progerin

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Contents

1 Supplemental Methods

1.1 SMC™ Progerin Immunoassay

Studies were performed with multiple reagent lots, 2 operators and 2 Erennas®. Progerin was measured by use of a single molecule counting (SMC) Immunoassay (EMD Millipore) as previously described[37], except that magnetic microparticles (MP) coated with capture antibodies and detection reagents were specific to the progerin assay (Fig. S1). Briefly, the progerin capture antibody (mouse Mab anti-Lamin A+C, clone: 131C3 Abcam, epitope between amino acid residues 319-566, common to Lamins A, C and progerin) was coated onto MP at 25 µg antibody/mg MP. Samples were exposed to the MP-capture antibody, to specifically bind analyte, followed by one plate wash to remove unbound material. Next, fluorescently labeled progerin-specific detection antibody (Mouse Mab, clone: 13A4, from Millipore, epitope between amino acids 604 and 611 of progerin, which cross the deletion region of lamin A), pre-diluted to 2,000 ng/mL in assay buffer and then filtered through a 0.2 µm filter (MilliporeSigma SLGP033RS), was added to wells. Plates were then washed 4 times to remove any unbound detection antibody. All MPs were then transferred from the initial 96-well assay plate to a new plate to avoid eluting any non-specific plate-bound detection antibody. Progerin-bound detection antibody was then eluted by pH shift using a proprietary glycine buffer, transferred to a new plate and detected by SMC using the Erenna Immunoassay System software (SgxLink™). The raw signal data and the interpolated value data obtained from SgxLink were transferred to Excel where precision was then calculated for the replicate wells. The acceptance specification for raw signal and interpolated values was precision and recovery bias ≤ 20%.

A 12-point standard curve of recombinant progerin (Abcam 93918) diluted in EMD standard diluent, a synthetic serum-based solution containing a proprietary mixture of Tris buffer and carrier protein (sigmaaldrich.com), was run on each plate in triplicate. The standard curve was used to determine progerin concentration in unknown samples. Samples were diluted in standard diluent so that anticipated progerin fell within the dynamic range of the standard curve. All reported progerin and SD are dilution-corrected. All samples were tested in duplicate, unless triplicate is specified. Signal at or below the background plus two standard deviations was reported as not detected (ND). The lower limit of quantification (LLoQ) was prospectively defined as the lowest point on the standard curve, from the top down, which was recovered within 20% of expected and had a % coefficient of variation (CV) of ≤20%.

To assure similar progerin quantitation between assays at high, mid and low values, the same set of endogenous plasma inter-assay quality controls (QC) were assayed on each plate in duplicate. Endogenous plasma progerin was derived from a homozygous transgenic mouse model of HGPS that expresses the human *LMNA* gene harboring the classic c.1824 C>T, p.G608G mutation[10]. This mouse plasma contains high levels of progerin and allowed QC generation without utilizing precious human HGPS plasma stores. Aliquots were diluted in normal human plasma for storage at -80ºC. QC plate acceptability parameters were locked in as mean ± 20% of the first 24 QC runs during assay development. The high and mid-QCs mean progerin was 188,206±10,580 pg/mL (%CV=5.6) and 19,513±1,235 pg/mL (%CV=6.3), respectively. The low QC was always below the LLoQ. To mirror the 1:25 dilution protocol for clinical

trial samples, QCs were filtered and diluted 25-fold on the same day as the assay was performed. Average in assay progerin for high- and mid-QCs in clinical trial sample plates (N=39) were 7,303±609 pg/mL (%CV=3.8) and 755±90 pg/mL (%CV=5.7), respectively. Thus, high- and mid-QC always fell within the analytical measurement range.

A valid run required the lower limit of quantitation calculated from the standard curve to be ≤ 59 pg/mL, and the controls to be within 20% of the established high and medium QCs.

Once final assay conditions were established, samples with interpolated concentrations that were above the upper limit of quantitation (ULoQ), or those with %CV > 20% were re-tested using appropriate dilution to assure progerin levels fell within the limits of quantitation of the assay. In addition, any plate in which the standard curve LLoQ was not met (59 pg/mL) or QCs (high and medium, with low control falling below LLoQ) fell outside of 20% of the mean and SD was repeated.

1.2 Dilutional Linearity

Linearity of dilution was calculated by dividing the dilution corrected progerin value in the dilution series by its preceding dilution's progerin value and expressed as a percentage:

$DL = [obs]_B/[obs]_A * 100$, where B is the dilution corrected value of the higher dilution and A is the dilution corrected value at the previous dilution level.

1.3 Freeze-Thaw Specimen Stability

Plasma samples frozen at -80°C were thawed at room temperature (RT) for 15 minutes, mixed well, and aliquoted to create 3 additional aliquots for this study. All aliquots were then refrozen at -80°C prior to being subjected to additional freeze-thaw testing. On the day of testing, assigned aliquots were thawed to room temperature for 15 minutes and refrozen at -80°C for up to 4 freeze-thaw cycles. The final thawed samples were then diluted and assayed in triplicate. All samples were tested on the same day. The freeze-thaw sample stability was acceptable if the % difference at freeze-thaw cycle 2 and 5 was ±20% (industry standard).

The percent difference (% Diff) from Cycle 2 of each sample at freeze-thaw cycles 3, 4, and 5 was calculated using the following formula:

$$\%\ \text{Diff} = \frac{(\text{Mean C cycle \#2-4(pg/mL)} - \text{Mean C cycle \#1(pg/mL)})}{\text{Mean C cycle \#1 (pg/mL)}} \times 100$$

1.4 Detection of Native Progerin vs. Recombinant Progerin

Healthy nonHGPS plasma samples (commercially purchased) were diluted 50-fold in standard diluent and then spiked with either a 10% by volume standard diluent, recombinant progerin yielding a 1,000 pg/mL, or HGPS plasma designed to deliver 1,000 pg/mL progerin. The actual calculated progerin spike was determined from the buffer spiked sample HGPl110 control value and was 1,019 pg/mL.

Spike recovery (%)=(Spiked sample progerin (pg/mL)–endogenous progerin (pg/mL))/expected progerin spike (pg/mL).

1.5 Lonafarnib Interference Assay

Lonafarnib API (Batch #MK-6336-000R025) was obtained from The PRF Cell and Tissue Bank. HGPS plasma was spiked with 2 µL of either dimethyl sulfoxide (DMSO) or lonafarnib dissolved in DMSO 7.92 µg/mL.

Percent (%) interference = [(mean concentration of spiked sample – mean concentration of control)/mean concentration of control] x 100.

1.6 Serum Isolation

For serum isolation, blood was allowed to clot at room temperature for 60 minutes (± 10 minutes) and then centrifuged at RT for 10 minutes at 1100 x g.

2 Supplemental Results

2.1 Development of Quantitative Assay for Plasma Progerin Detection

Standard Curve and Reporting Range: To develop an SMC progerin immunoassay standard curve, assay sensitivity and the reliable reporting range were established. Recombinant progerin was spiked into buffer to achieve 30 ng/mL and serially diluted in steps of two-fold to generate a standard curve ranging from 30,000 to 29 pg/mL (Figure S2A,B). Signal was interpolated by SgxLink software. The dynamic detection range was 59 pg/mL (LLoQ) – 30,000 pg/mL (ULoQ). The mean value of the (measured concentration)/(expected concentration) was 100% (range 95–106%), and a linear response (R^2=0.9987) was observed from 59 pg/mL to 30,000 pg/mL.

Consequently, all samples were diluted in standard diluent to accommodate this range. Specifically, nonHGPS samples were assessed at 1:1 or 1:2; HGPS samples were below the ULoQ when diluted 1:25.

Dilutional Linearity: To assess the assay's ability to consistently measure plasma progerin with varying sample dilutions in HGPS, human HGPS plasma samples (N=3) were quantified in triplicate at 5, 25, and 125-fold dilution in assay buffer (Table S2). All patient samples were quantifiable at all the dilutions tested and ranged in final progerin from 15,835 pg/mL to 50,610 pg/mL. Mean linearity of dilution was 98.2% (range 92-110%). NonHGPS plasma (N=4) was assessed for linearity of 1:2 versus neat, to stay above the LLoQ.

Intra-assay and Inter-assay Variability: The precision of the SMC Progerin Immunoassay to reliably quantitate plasma samples within run (intra-assay) and day to day (inter-assay), with multiple replicates on multiple days was determined. Intra-assay variability (Table S3) demonstrated average CV of 7% (range 1-16%), while inter-assay variability (Table S4) demonstrated average CV of 12% (range 7-18%). In addition, excellent intra-assay precision was demonstrated with from high- and mid-QCs, which were run on each clinical trial sample plate (N=39) in duplicate. Average dilution-corrected high-QC was 182,593±15,229 pg/mL progerin with 3.8% CV; and mid-QC was 18,880±2,260 pg/mL progerin with 5.8% CV.

Freeze-thaw Stability: Samples presented in this study have been frozen prior to analysis. It is anticipated that only pre-frozen samples will be assessed routinely in clinical trials due to off-site assay execution. A freeze-thaw analysis of 4 freeze-thaw cycles demonstrated no differences between progerin (all p>0.05; Table S5).

2.2 Progerin Assay Does Not Cross-React with Lamin A

To assess assay specificity, potential cross-reactivity between progerin and lamin A detection was measured. Two, twelve-point sample curves, one containing human recombinant lamin A and another containing human recombinant progerin were run in triplicate in the SMC Progerin Immunoassay starting at 10,000 pg/mL and 2-fold serially diluting to 9.8 pg/mL with a zero pg/mL buffer-only anchor point on each curve. The assay had no detectable cross reactivity to lamin A (Fig. S3). Progerin recovery range was 97-111% of expected at the different concentrations tested, with R^2=0.9991. Thus, no signal was generated by the presence of lamin A that competed or interfered with the quantification of progerin.

2.3 Spike Recovery and Detection of Native Progerin vs. Recombinant Progerin

To evaluate the assay's ability to measure recombinant progerin in comparison to native progerin, either was spiked into 3 different healthy nonHGPS donor plasma samples that were diluted 50-fold with standard diluent before receiving a spike. Both native and recombinant progerin yielded similar progerin recovery in the expected range, with average recoveries of 96±6 and 120±15%, respectively (Table S6).

2.4 Exogenous Lonafarnib Does Not Interfere with the Progerin Assay

To assess whether changes in plasma progerin would accurately reflect the influence of lonafarnib therapy on plasma progerin levels, an assessment of whether lonafarnib interferes with progerin assay readings was performed. Because the Cmax determined from a pharmacokinetics study in children with HGPS treated with lonafarnib during a clinical trial was 2.64 µg/mL[18], plasma samples were spiked with 7.92 µg/mL lonafarnib, which is 3xCmax. Mean progerin was unaffected by spiking with lonafarnib in DMSO as compared with the control conditions (p=0.20; Table S7).

2.5 Similarity in Progerin Quantitation Between NaHeparin-isolated Plasma, K_2EDTA-isolated Plasma, and Serum

To assess whether the assay was suitable for progerin detection across different blood collection tube types, a comparative analysis of blood collected into tubes containing sodium heparin (plasma), K_2EDTA (plasma), and no anticoagulant (serum), from the same patient in the same blood draw (N=3 patients) was performed in triplicate. There were no significant differences between these 3 sample types; NaHeparin plasma vs. EDTA plasma p=0.98; NaHeparin plasma vs serum p=1.00; and EDTA vs serum p=0.97. Serum was used for two patient samples (1 nonHGPS and 1 HGPS ProLon1 trial visit 6) where plasma was not available.

2.6 Classic vs Nonclassic Genotypes Yield Similar Results

Both classic and nonclassic forms of HGPS are progerin-producing; classic due to optimization of an internal splice site and nonclassic due to de-optimization of the canonical splice site. Data from children with the classic genotype (c.1824 C>T, p.G608G,; N=74) were compared with nonclassic HGPS patient samples (LMNA c.1822G>A, p.G608S x 2, c.1968+1 G>A, c.1968+2 T>A, c.1968+5 G>C) (N=5; mean ±SD = 28,402±11,601 pg/mL). When comparing baseline off-therapy progerin to classic patient samples (N=74; mean ±SD =33,361±12,346 pg/mL), all nonclassic patient progerin levels fell within the range of classic patient levels, and there was no overall

difference detected (p=0.39). Similarly, baseline to end-of-study decreases in progerin with therapy (40.3-57.5%) were within the range of classic patients, and end-of-study values were not different from classic patients (p=0.85).

Supplemental Tables

Table S1. Clinical Trial Details

Clinical Trial Acronym	Drug(s) Administered	Drug Dose; Frequency	Route of Administration	Time Between Trial Site Visits (months), Total Number of Sample Collection Visits	**Associated publications
ProLon1	lonafarnib	115 mg/m^2 for first 4 months, then 150 mg/m^2 thereafter; every 12±2 hours	Oral capsule or liquid	4,7	1, 2, 6, 7, 8, 10, 11, 12, 13, 14, 16, 17, 18, 19, 20
Triple Therapy	lonafarnib	150 mg/m^2; every 12±2 hours	Oral capsule or liquid	6,5	2, 3, 6, 7, 12, 14, 16, 18, 20
	pravastatin	*5 mg or 10 mg; once daily	Oral tablet		
	zoledronic acid	0.0125 mg/kg body weight initial infusion, then 0.05 mg/kg; every 6 months	Intravenous		
ProLon2	lonafarnib	150 mg/m^2; every 12±2 hours	Oral capsule or liquid	24,2	4, 5, 9, 10, 11, 12, 14, 15, 17, 18

*Children weighing less than 10 kg were given 5 mg pravastatin; children weighing greater than 10 kg were given 10 mg pravastatin.

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Table S2. Dilutional Linearity*						
Sample ID	**Dilution**	**Uncorrected progerin pg/mL**	**SD**	**%CV**	**Dilution corrected progerin pg/mL**	**% of lowest dilution****
HGPI154	1:5	3,300	302.97	9	16,499	NA
	1:25	637	21.62	3	15,933	97
	1:125	121	5.38	4	15,069	95
HGPI145	1:5	10,684	496.82	5	53,419	NA
	1:25	2,055	29.25	1	51,376	96
	1:125	376	34.63	9	47,035	92
HGPI110	1:5	7,593	362.48	5	37,963	NA
	1:25	1,500	44.53	3	37,489	99
	1:125	328	22.40	7	41,059	110
NPI1	1:1	244	2.47	1	244	NA
	1:2	101	5.78	6	201	83
NPI2	1:1	264	12.04	2	264	NA
	1:2	134	13.19	10	268	102
NPI3	1:1	115	0.77	1	115	NA
	1:2	50	5.30	11	100	87
NPI4	1:1	282	5.14	2	282	NA
	1:2	95	7.24	8	189	67

*HGPS (HGPI) and nonHGPS (NP) samples were assayed at different dilutions to assess linearity of dilution. Results are presented as a percentage of the progerin concentration of the lowest dilution for each sample tested.
**NA=not applicable

Table S3: Plasma Progerin Intra-assay Variability*						
Sample ID	**Replicate 1 progerin pg/mL**	**Replicate 2 progerin pg/mL**	**Replicate 3 progerin pg/mL**	**Mean progerin pg/mL**	**SD**	**%CV**
HGPI095	24,619	19,870	21,194	21,894	2,450	11
HGPI092	39,850	37,152	37,440	38,130	1,495	4
HGPI039	44,958	46,445	44,615	45,339	975	2
HGPI110	29,967	30,053	30,302	30,107	175	1
HGPI132	76,216	85,654	84,322	82,063	5,108	6
HGPI145	36,658	30,422	26,912	31,330	4,935	16
HGPI150	45,305	40,159	45,431	43,631	3,009	7
HGPI154	16,682	13,868	14,289	14,946	1,520	10

*Eight different HGPS plasma samples were assayed in triplicate wells on the same assay plate to assess inter-assay variability. Dilution factor for all samples was 1:50.

Table S4: Plasma Progerin Inter-assay Variability**									
Sample ID	**Plate 1 pg/mL**	**Plate 2 pg/mL**	**Plate 3 pg/mL**	**Plate 4 pg/mL**	**Plate 5 pg/mL**	**Plate 6 pg/mL**	**Mean pg/mL**	**SD**	**%CV**
HGPl095	21,894	23,143	21,395	20,537	28,427	28,817	24,036	3,653	15
* HGPl092	38,130	30,751	33,000	32,209	36,823	32,598	33,919	2,888	9
HGPl039	45,339	44,898	32,246	30,339	41,586	41,019	39,238	6,419	16
HGPl110	30,107	30,803	21,607	20,721	32,104	28,071	27,236	4,888	18
* HGPl132	82,064	59,555	73,036	71,949	74,650	69,186	71,740	7,373	10
HGPl145	31,330	31,932	28,455	26,866	33,499	33,522	30,934	2,724	9
* HGPl150	43,632	36,368	39,106	40,285	43,424	41,094	40,651	2,744	7
HGPl154	14,946	16,556	16,058	15,830	19,103	20,066	17,093	2,022	12

*These samples were repeated due to initial technical error during first series of plates.
**Dilution factor for all samples was 1:50; each sample run in triplicate in each of 6 assays. The average of each triplicate on a given plate is listed, followed by mean, SD and %CV for each sample ID.

Table S5: Freeze-Thaw Specimen Stability						
F/T Cycle	**Mean progerin pg/mL**	**SD**	**%CV**	**Dilution Corrected progerin pg/mL**	**% Difference from F/T cycle 2**	**p value vs Cycle 2**
2	179	8.58	5	4,473		
3	201	24.57	12	5,015	12.13	0.26
4	202	14.01	7	5059	13.12	0.07
5	193	39.19	20	4,832	8.03	0.59
2	610	28.21	5	15,258		
3	599	28.62	5	14,976	-1.85	0.65
4	618	11.04	2	15,456	1.30	0.69
5	592	7.98	1	14,794	-3.04	0.37

All samples run in triplicate
*HGPS plasma samples were pre-diluted with normal human plasma in order to test F/T stability within the two desired concentration ranges. Samples were then diluted 1:25 in standard diluent prior to assay run. Paired sample t-tests were used to compare the values obtained from each freeze-thaw cycle (F/T 3-5) with F/T 2.

Table S6: Detection of native progerin vs. recombinant progerin		
	nonHGPS Plasma + HGPS Plasma pg/mL (N=3)	**NonHGPS Plasma + Recombinant Progerin pg/mL (N=3)**
Spiked concentration	1,019	1,000
Expected concentration = healthy plasma* + spike	1,051	1,032
Measured concentration	1,014±64	1,243±153
Average % recovery	96±6	120±15
* NonHGPS plasma + buffer concentration (N=3) = 32.4±33.3 pg/mL		

Table S7: Test of Exogenous Lonafarnib Interference with Plasma Progerin Detection							
Plasma ID	**Rep 1 progerin pg/mL**	**Rep 2 progerin pg/mL**	**Rep 3 progerin pg/mL**	**Mean progerin pg/mL**	**SD**	**%CV**	**Testing condition**
HGPI204	25,841	25,702	27,676	26,407	1,102	4	DMSO
HGPI204	26,681	25,854	26,406	26,314	421	2	lonafarnib in DMSO
HGPI201	48,868	46,242	51,008	48,706	2,387	5	DMSO
HGPI201	47,302	48,300	43,628	46,410	2,461	5	lonafarnib in DMSO
HGPI194	25,218	25,463	30,136	26,939	2,771	10	DMSO
HGPI194	22,562	24,115	22,378	23,018	954	4	lonafarnib in DMSO

Table S8: Trial Sample Collection Frequency and Number		
Trial Visit	**Time on Treatment (years)**	**Patients with Plasma Collected (N)**
ProLon1		
1	0	26
2	0.3	*25
3	0.6	*25
4	1.0	*25
5	1.3	24
6	1.6	22
7	2.2	25
**Triple Therapy Trial		
1	0	13
2	0.5	13
3	1.0	13
4	1.5	13
5	3.5	12
***6	5.0	10
***7	7.6	10
ProLon2		
1	0	0
2	2.4	26

*Not all the same patients at each of these time points
**Only includes patients who initiated trial naïve-to-therapy
***Lonafarnib monotherapy extension of Triple Therapy Trial

Table S9: Estimated Effect of Change in Plasma Progerin on Patient Risk of Mortality in Classic HGPS*				
Minimum Patient Inclusion Criteria	**Statistical Analysis**	**Decreased Risk of Mortality % (CI)**		**P value**
		1,000 pg/mL Progerin Decrease	**10,000 pg/mL Progerin Decrease**	
Naïve progerin (N=74) (9 patients with only naïve levels; 65 patients with naïve baseline plus on-therapy levels)	Cox Time-Dependent	4.8% (2.0,7.5)	39.0% (18.5,54.3)	0.0008
	Joint model	6.6% (5.0,8.1)	49.3% (40.0,57.2)	<0.0001
Naïve baseline and on-therapy progerin (N=65)	Cox Time-Dependent	4.1% (0.3,7.8)	34.2% (2.5,55.6)	0.0370
	Joint model	5.1% (2.0,8.2)	40.8% (17.9,57.3)	0.0017
*Effect of change in progerin on survival was calculated using the hazard ratios estimated from the Cox and joint models, relating measured plasma progerin to mortality.				

Supplemental Figures

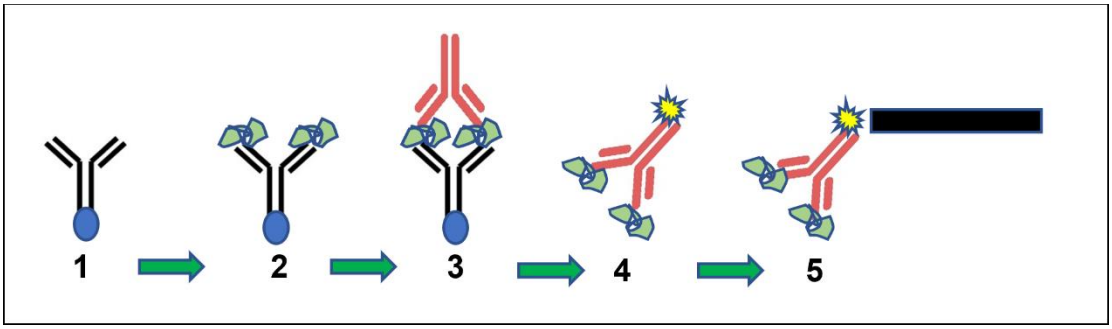

Fig. S1. SMC Immunoassay. 1. Lamin A/C capture antibody (black) is bound to magnetic microparticles (blue circle) at the heavy chain nonspecific region 2. Progerin-containing solution is added to wells and progerin (green) binds to Lamin A/C antibody capture antibody at antibody binding sites 3. Fluorescently labeled progerin-specific detection antibody (red) is added and binds to progerin 4. Elution – chemical separation of progerin + Fl-progerin-specific antibody from magnetic microparticles 5. Single molecule counting using laser detection of fluorescent label (black rectangle)

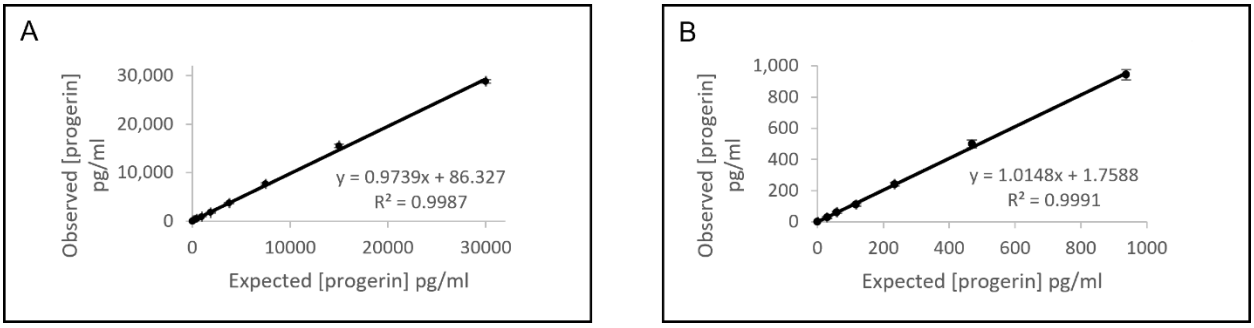

Fig. S2. Analytical performance of the progerin assay. Goodness of curve fit was generated using back interpolation of progerin calibration curves generated over 10 consecutive assay runs. A. full range of quantification. B. low-end range of quantification.

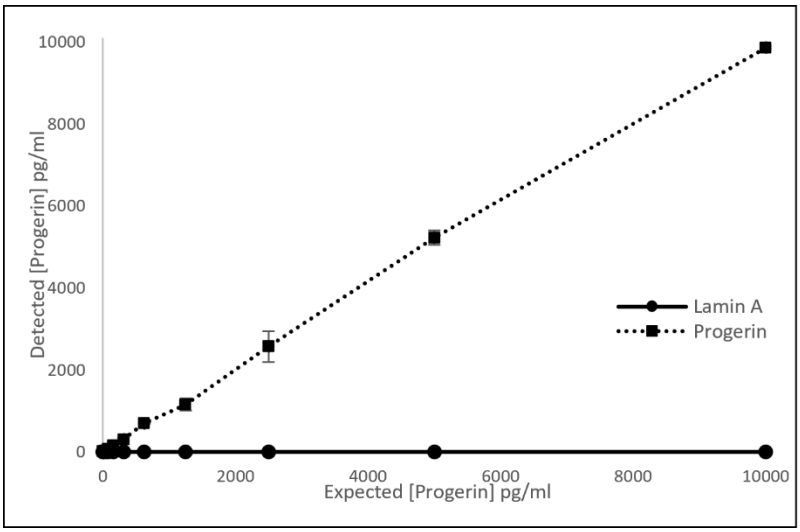

Fig S3. Lamin A Is Not Detected by the Progerin Assay. Solid line = detected progerin from lamin A samples. Dashed line = detected progerin from recombinant progerin samples. R^2=0.9991.

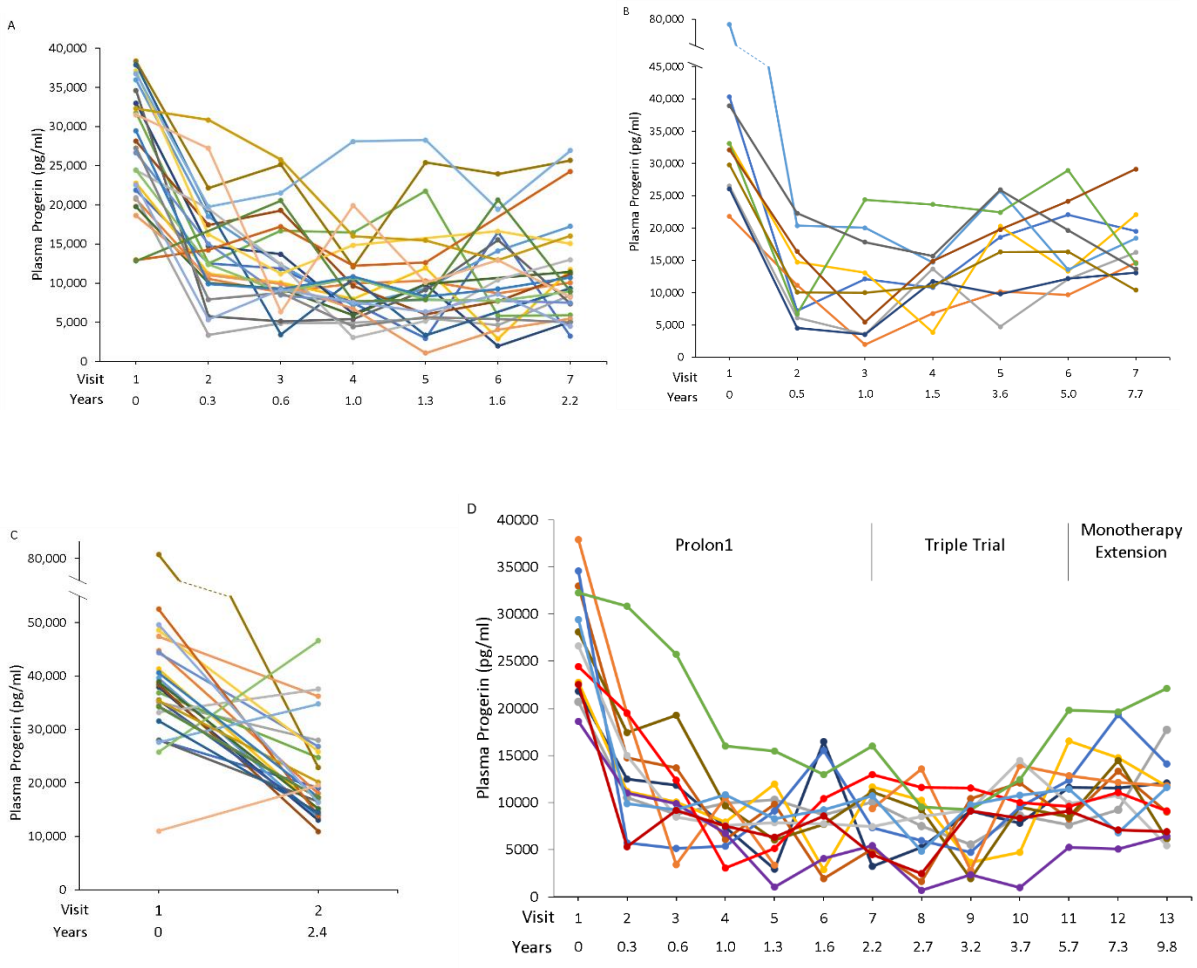

Fig. S4. Individual subject plots of plasma progerin for clinical trial patients. On-site trial center visit numbers and time on therapy vs. plasma progerin. Trial visits to BCH occurred at various times post-visit 1 for different trials. All visit 1 patients were naïve to therapy. A. ProLon1, treated with lonafarnib (N=25) B. Triple trial patients treated with triple therapy from baseline to visit 5 (N=13), then switched to lonafarnib monotherapy thereafter (visits 6,7; N=10). C. ProLon2 lonafarnib monotherapy (N=25) D. Long-term continuous therapy (N=13).

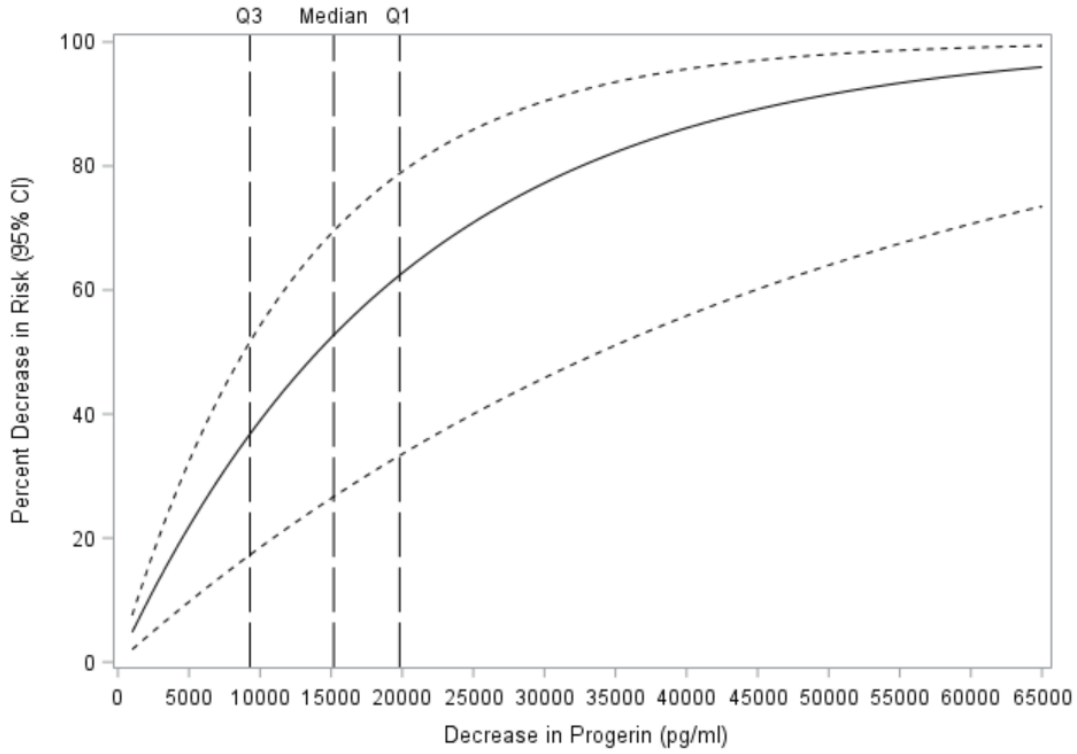

Fig. S5. Progerin Level Is Associated with Survival (p=0.0008). Decreased risk of death with decreased plasma progerin. Time-dependent Cox Model using progerin to predict survival, adjusting for age and sex. Change in mortality risk (y-axis) versus all values in range of observed progerin decrease (x-axis) from 0 to 65,000 pg/mL. Solid line is % decrease in risk if death; dashed lines = 95% CI. N=74 subjects (9 untreated with single samples and 65 treated with untreated baseline plus multiple on-therapy samples).