Survey of plasma proteins in children with progeria pre-therapy and on-therapy with lonafarnib

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BACKGROUND: Hutchinson–Gilford progeria syndrome (HGPS) is an ultra-rare, fatal, segmental premature aging syndrome caused by the aberrant lamin A protein, progerin. The protein farnesyltransferase inhibitor, lonafarnib, ameliorates some aspects of cardiovascular and bone disease.

METHODS: We performed a prospective longitudinal survey of plasma proteins in 24 children with HGPS (an estimated 10% of the world's population at the time) at baseline and on lonafarnib therapy, compared with age- and gender-matched controls using a multi-analyte, microsphere-based immunofluorescent assay.

RESULTS: The mean levels for 23/66 (34.8%) proteins were significantly lower and 7/66 (10.6%) were significantly higher in HGPS samples compared with those in controls ($P \leq 0.05$). Six proteins whose concentrations were initially lower normalized with lonafarnib therapy: interleukins 1α, 7, and 13, beta-2 microglobulin, C-reactive protein, and myoglobin. Alpha-2 macroglobulin, a protease inhibitor associated with stroke, was elevated at baseline and subsequently normalized with lonafarnib therapy.

CONCLUSION: This is the first study to employ a multi-analyte array platform in HGPS. Novel potential biomarkers identified in this study should be further validated by correlations with clinical disease status, especially proteins associated with cardiovascular disease and those that normalized with lonafarnib therapy.

Hutchinson–Gilford Progeria Syndrome (HGPS) is, in most cases, a sporadic, autosomal dominant, "premature aging" disease in which children die primarily of heart attacks at an average age of 14.6 years (range 1–26 years) (1). Incidence is estimated at 1 in 8 million live births (2), and prevalence is 1 in 20 million living individuals (3). Children experience normal fetal and early postnatal development. Between several months and 1 year of age, abnormalities in growth and body composition become readily apparent (4). Severe failure to thrive ensues, heralding generalized lipoatrophy, with apparent wasting of limbs, circumoral cyanosis, and prominent veins (5). Children reach a final height of ~1 m and weight of ~14 kg. Bone dysplasia includes clavicular resorption, coxa valga, distal phalangeal resorption, facial disproportion (a prominent, slim nose and receding mandible), and short stature. Dentition is severely delayed (6). Tooth eruption may be delayed for many months, and primary teeth may persist for the duration of life. Secondary teeth are present, but may or may not erupt. Skin looks thin with sclerodermatous areas and almost complete hair loss (7). Some skin findings are variable in severity and include areas of discoloration, stippled pigmentation, tightened areas that can restrict movement, and areas of the dorsal trunk where small (1–2 cm) soft bulging skin is present. Joint contractures, due to ligamentous and skin tightening, limit range of motion. Intellectual development is normal in HGPS. Transient ischemic attacks and strokes may ensue as early as 4 years of age, but more often they occur in the later years (8). Death results primarily from sequelae of widespread atherosclerosis. In a comprehensive retrospective study, causes of death in HGPS were cardiovascular failure (80%), head injury or trauma (10%), stroke (4%), respiratory infection superimposed on cardiovascular disease (4%), and complications from anesthesia during surgery (2%) (1).

We previously conducted a prospective single-arm clinical trial of lonafarnib for children with HGPS (NCT00425607) (9). Lonafarnib was well tolerated. The primary outcome measure (improved rate of weight gain) was achieved; cardiovascular distensibility, as assessed via carotid–femoral pulse wave velocity and carotid artery echodensity, was improved; radial bone structural rigidity and sensorineural hearing increased. There was preliminary evidence of decreased headache, transient ischemic attack, and stroke rates (10). To date, both the primary and secondary outcomes have been evaluated after 2 years of therapy.

The aim of this study was to initiate the development of shorter-term clinically meaningful biomarkers, so that

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evaluation of disease status and improvement with treatment can be assessed earlier than the current clinical outcomes. In recent years, large-scale multiplex assays have been developed and have succeeded in expanding the capacity to explore potential disease biomarkers for a wide variety of pediatric and adult conditions (https://myriadrbm.com/wp-content/themes/howes-child-rbm/includes/pdf.bibliography.php). Examples include identification of protein combinations that can discriminate bacterial from viral or misdiagnosed malaria in children presenting with a respiratory syndrome (11), identification of a multibiomarker disease activity score that can predict radiographic damage progression in rheumatoid arthritis (12), and integration of multiplex immunohistochemistry assays with genome-wide association studies and mass cytometry as tools to understand the basic biology and predict treatment benefit for cancers (13). To begin with the identification of potential biomarkers warranting further study in HGPS, we performed a prospective study of plasma proteins after only 1 year of lonafarnib therapy using a commercial multiplex, microsphere-based immunofluorescent assay.

METHODS

Study Approvals

This study was approved by the Institutional Review Boards of Hasbro Children's Hospital, Providence, RI, and Boston Children's Hospital, Boston, MA. Blood samples from children with HGPS were obtained as part of exploratory outcomes during a single-arm clinical trial administering the protein farnesyltransferase inhibitor lonafarnib, registered with Clinicaltrials.gov (NCT00916747) (9), entitled "Phase II Trial of Lonafarnib (a Farnesyltransferase Inhibitor) for Progeria".

Study Groups

Patients were 3 years of age and older with clinically and genetically confirmed c.1824 C>T, p. Gly608Gly classic HGPS, and adequate organ and marrow function. Consents were translated into the parent(s)' primary language and discussions were performed with interpreters. Assent was obtained from children old enough to comprehend. Twenty-four children with HGPS, an estimated 10% of the world's population at the time, donated blood at Boston Children's Hospital. Thirty-four healthy pediatric control children donated blood at Hasbro Children's Hospital after they and/or their parents gave informed consent. Healthy children who matched at least one HGPS child by age (±6 months) and gender were chosen for the controls; after matching was completed, for every HGPS child there was at least one child of the same age (±6 months) and gender in the control group.

Lonafarnib Dosage and Administration

Lonafarnib (Merck & Co., Kenilworth, NJ, USA) dosing was initiated at 115 mg/m^2 before increasing to 150 mg/m^2 after a minimum of 4 months' adjustment period. Patients experiencing drug-related grade 3 or 4 toxicity and also not responding to supportive care measures were dose-reduced back to 115 mg/m^2. Once reduced, patients were permitted to increase the dose of lonafarnib. Patients received oral lonafarnib either by oral capsule or by liquid suspension dispersed in Ora-Blend SF or Ora-Plus (Perrigan Company, Allegan, MI, USA) for every 12 ± 2 h. Patients were monitored for liver, kidney, and hematological toxicity each month for the first 3 months, and every 4 months for the duration of the study. Compliance with lonafarnib therapy was tracked using a daily patient log than included time and dose throughout the trial period. Patient logs were collected and verified at each patient visit to the trial site. Lonafarnib was generally well tolerated (9).

Sample Collection and Analysis

Lidocaine/prilocaine cream (2.5% EMLA (Astra Pharmaceuticals, LP, Wayne, Pa)) was applied to skin overlying a vein in the antecubital fossa for 60 min in all children with HGPS. Venipunctures were performed within 5 min of EMLA removal. Blood was collected into sodium heparin tubes and centrifuged at 4 °C for 15 min at 1,500*g*; plasma was removed and stored at − 80 °C before analysis. All blood samples were drawn in the morning and while patients were in a fasting state. For patients on lonafarnib therapy, blood was drawn just before morning therapy dosing, when lonafarnib was at a trough level.

Plasma samples were sent to Myriad Rules-Based Medicine (RBM, Austin, TX) for analysis of 90 analytes using the Human Multi-Analyte Profile (MAP), version 1.6 antigens. The RBM HumanMAP is a commercial validated platform, which measures a battery of proteins, including markers of autoimmunity, infection, cancer-related, hormones, cytokines, cardiovascular risk, acute phase reactants, and others. Samples were stored at − 80 °C until tested. They were thawed at room temperature, vortexed, spun at 13,000*g* for 5 min for clarification and 60 µl was removed for MAP analysis into a master microtiter plate. The rest of the process was fully automated per RBM procedures. Using automated pipetting, an aliquot of each sample was introduced into one of the fluorescently labeled capture microsphere multiplexes of the MAP, conjugated to antibodies encoded with unique fluorescent signatures. The beads were incubated with the sample at room temperature for 1 hour and antigens of interest allowed to bind to their targets. Multiplexed cocktails of biotinylated detection reagents for each multiplex were added to the sample followed by the addition of fluorescent reporter molecules, and incubated for an additional hour. Multiplexes were developed using an excess of streptavidin–phycoerythrin solution that was thoroughly mixed into each multiplex and incubated for 1 hour at room temperature. The volume of each multiplexed reaction was reduced by vacuum filtration and the volume was increased by dilution into matrix buffer for analysis. RBM determined analyte concentrations using The Luminex 100 instrument and the resulting data stream was interpreted using proprietary data analysis software developed at Rules-Based Medicine. For each multiplex, both calibrators and controls were included on each microtiter plate. RBM used a unique set of controls with known quantities of the protein of interest to create two sets of eight-point calibrators, which were run in the first and last column of each plate and three-level controls were included in duplicate. Testing results were determined first for the high, medium, and low controls for each multiplex to ensure proper assay performance. Concentrations were determined by fitting to standardized concentration curves using RBM curve-fitting routines. This provided a dynamic concentration range from fg/ml to mg/ml and intra-assay coefficient of variation and accuracy of <10%. Data were reported back as concentrations (average of two independent measures) and lower

Table 1. Study Population Demographics

	Control ($N = 34$)	HGPS pre-treatment ($N = 24$)	HGPS post-treatment[a] ($N = 23$)
Gender			
Male (%)	15 (44.1)	10 (41.7)	10 (43.5)
Female (%)	19 (55.9)	14 (58.3)	13 (56.5)
Age (years)			
Range	2.8–17.0	3.1–16.2	4.1–17.2
Mean ± SD	8.9 ± 3.7	7.42 ± 3.2	8.4 ± 3.2

HGPS, Hutchinson–Gilford Progeria Syndrome.
[a]Testing occurred after 1 year of lonafarnib therapy.

assay limits (lowest level of quantification, LLOQ). The plasma range was determined based on the testing of ~100 apparently healthy individuals. No assumption was made about the samples having a normal distribution. The range comprises the middle 95%, with the highest and lowest 2.5% of the samples excluded for each given assay. RBM MAP technology meets Clinical Laboratory Standard Institute standards.

Statistical Analysis

Descriptive statistics included the mean and SD for continuous variables, and counts and percentages for categorical variables. Assessment of the significance of linear trends in protein across age was assessed using simple linear regression separately for (i) healthy controls; (ii) lonafarnib-treated HGPS patients at baseline (before receiving lonafarnib); and (iii) lonafarnib-treated HGPS patients after 1 year of treatment with lonafarnib. The significance of the difference between healthy controls' mean protein and pretreatment lonafarnib patients' mean protein, and the difference between healthy controls' mean protein and post-treatment lonafarnib patients' mean protein were assessed using analysis of covariance adjusting for age. Group-by-age interaction was also assessed using analysis of covariance. *P* values presented are two-sided and are considered significant at the 0.05 level for direct measures and at the 0.1 level for interactions. There is no adjustment for multiple comparisons, given the exploratory nature of this analysis. All

Table 2. Plasma protein concentrations significantly lower in children with HGPS compared with healthy controls[a,b]

Protein	Control (mean ± SD; *N* = 34)	HGPS untreated (mean ± SD; *N* = 24)	Age-adjusted *P* value	HGPS treated (mean ± SD; *N* = 23)	Age-adjusted *P* value
Proteins that did not normalize with treatment					
[c]Adiponectin (μg/ml) (16)	5.54 ± 1.8	2.56 ± 1.68	<0.001	2.72 ± 1.69	<0.001
Epithelial-derived neutrophil-activating protein 78 (ng/ml)	0.36 ± 0.18	0.20 ± 0.17	<0.001	0.16 ± 0.13	<0.001
Fibrinogen (mg/ml)	2.81 ± 0.55	1.86 ± 0.65	<0.001	1.93 ± 0.75	<0.001
Stem cell factor (pg/ml)	279.59 ± 68.30	219.15 ± 67.74	<0.001	218.64 ± 66.36	0.001
Vascular cell adhesion molecule-1 (ng/ml)	679.29 ± 135.34	495.75 ± 128.17	<0.001	489.82 ± 112.64	<0.001
von Willebrand Factor (μg/ml)	15.69 ± 6.83	7.80 ± 4.99	<0.001	9.69 ± 6.48	0.002
CD40 (ng/ml)	0.67 ± 0.15	0.56 ± 0.14	0.001	0.55 ± 0.12	0.002
[c]Leptin (ng/ml) (9,18)	5.54 ± 6.72	0.49 ± 0.32	0.001	0.33 ± 0.23	0.001
Apolipoprotein A1 (mg/ml)	0.27 ± 0.10	0.19 ± 0.06	0.002	0.18 ± 0.06	0.001
T-cell-specific protein RANTES (ng/ml)	2.26 ± 1.34	1.34 ± 1.41	0.002	1.04 ± 0.88	<0.001
Macrophage-derived chemokine (pg/ml)	376.6 ± 120.6	330.7 ± 98.5	0.002	271.65 ± 90.0	<0.001[c]
Tumor necrosis factor-RII (ng/ml)	4.00 ± 1.52	2.92 ± 1.04	0.003	2.87 ± 1.14	0.004
Brain-derived neurotrophic factor (ng/ml)	0.60 ± 0.29	0.37 ± 0.44	0.006	0.28 ± 0.26	<0.001
Interleukin-16 (pg/ml)	405.56 ± 190.61	301.86 ± 90.40	0.007	256.83 ± 71.38	<0.001
Extracellular receptor for advanced glycation end products (ng/ml)	14.87 ± 12.58	7.41 ± 2.93	0.010	9.73 ± 6.00	0.082
Interleukin-3 (ng/ml)	0.31 ± 0.11	0.24 ± 0.08	0.015	0.26 ± 0.07	0.049
Myeloperoxidase (ng/ml)	291.80 ± 295.04	121.05 ± 56.90	0.014	110.32 ± 58.69	0.007
Proteins that normalized with treatment					
Beta-2 microglobulin (μg/ml)	1.33 ± 0.28	1.07 ± 0.31	0.002	1.17 ± 0.44	0.113
Interleukin-13 (pg/ml)	177.46 ± 38.63	146.75 ± 55.65	0.009	155.54 ± 67.8	0.123
Interleukin-7 (pg/ml)	139.28 ± 57.22	102.73 ± 50.62	0.015	114.64 ± 61.63	0.138
[c]C-reactive protein (μg/ml) (5,16)	1.39 ± 2.77	0.22 ± 0.34	0.034	1.28 ± 2.82	0.788
Myoglobin (ng/ml)	7.28 ± 3.76	5.78 ± 2.42	0.035	6.32 ± 2.25	0.235
Interleukin-1 alpha (ng/ml)	0.01 ± 0.003	0.01 ± 0.004	0.043	0.01 ± 0.004	0.199

HGPS, Hutchinson–Gilford Progeria Syndrome.
[a]Sections are presented in order of ascending *P* values for control vs. HGPS untreated.
[b]Table does not include interleukin-1ra due to a significant group-by-age interaction (i.e., magnitude and direction of the difference in means between groups depended on patient age). Table does include adiponectin, which also shows a significant group-by-age interaction, the direction of the difference in means between groups was consistent across patient age (**Tables 4** and **5**).
[c]Previously measured in untreated HGPS patients using a clinical assay.

statistical analyses were carried out using SAS version 9.3. Pearson correlation values were between the proteins and patient age.

RESULTS

Table 1 summarizes demographics. Plasma samples from 24 lonafarnib-treated children with HGPS, age range of 3.1–16.2 years, were analyzed at pre-therapy baseline and again after 1 year of daily oral lonafarnib therapy, as described previously (9). One patient died after 4 months of therapy and was not included in the on-therapy sample group ($N = 23$). Healthy untreated non-HGPS control children were similarly analyzed ($N = 34$).

Supplementary Table S1 (online) lists the 90 proteins present in the assay, along with the least detectible dose for each protein. Of these, 23 proteins yielded values below the least detectible dose for at least 80% of the samples in each of the three patient sample categories, and one (interleukin (IL)-15) did so for at least 70% of the samples; these proteins are not included in the analyses presented here. Without treatment, the mean values for 23/66 (34.8%) proteins were significantly lower in HGPS compared with those in controls (**Table 2**), and 7/66 (10.6%) were significantly higher (**Table 3**; age-adjusted $P \leq 0.05$). Of these, six proteins whose levels were initially abnormally low in the untreated HGPS group normalized with lonafarnib therapy: ILs 1α, 7, and 13 (IL-1α, IL-7, IL-13), beta-2 microglobulin, C-reactive protein, and myoglobin (**Table 2**). Of the seven proteins whose means were significantly larger in untreated HGPS vs. controls, only alpha-2 macroglobulin normalized with lonafarnib therapy (**Table 3**).

There were 36 proteins that significantly increased or decreased with patient age (**Table 4**) for at least one of the three groups (healthy controls, HGPS patients before treatment, and/or HGPS patients after lonafarnib treatment). Correlation values between 0.2 and 0.6 were considered moderate and those at or above 0.6 were considered strong.

Table 5 displays the proteins for which a significant group-by-age interaction existed (i.e., for which the differences between HGPS, treated or untreated, vs. controls differed as age increased). Of the 36 proteins in **Table 4**, 17 are also in **Table 5**. Four proteins are of particular interest, despite the significant interaction of groupings with age: their means in treated and untreated HGPS significantly differed from controls across the age range tested (**Tables 2** and **3**); they also decrease (adiponectin) or increase (creatine kinase MB, eotaxin, insulin) with increasing patient age in HGPS, but not in controls (**Table 5**). For adiponectin, eotaxin, and insulin, the differences between HGPS and controls became more pronounced with increasing patient age. Creatine kinase MB is detectable in only 12% of controls and 35.7% of HGPS samples, but those samples with detectable levels fall within the plasma normal range for this assay, at or below 1.1 ng/ml, indicating no increased cardiac or skeletal muscle damage (14). Although carcinoembryonic antigen, IL-1ra, and macrophage-derived chemokine levels are significantly different between HGPS patients (treated and untreated) vs. controls on average, their regression lines vs. age intersect with controls indicating the difference vs. control is not consistent across the age range; also results need to be interpreted with caution for this protein, given that many patients were below the least detectible dose. For 10 additional proteins listed in **Table 5**, patients with HGPS (treated or untreated) do not appear different from controls on average even in the presence of significant group-by-age interactions.

There were five proteins (CD40 ligand, ferritin, thyroxine-binding globulin, IL-8, and tumor necrosis factor alpha (TNF-alpha)) whose plasma levels for untreated patients with HGPS were similar to controls, but subsequently became abnormal with treatment when compared with controls

Table 3. Plasma protein concentrations significantly greater in children with HGPS compared with healthy controls[a]

Protein	Control (mean ± SD; $N = 34$)	HGPS untreated (mean ± SD; $N = 24$)	Age-adjusted P value	HGPS treated (mean ± SD; $N = 23$)	Age-adjusted P value
Proteins that did not normalize with treatment					
Complement 3 (mg/ml)	0.87 ± 0.13	1.00 ± 0.16	< 0.001	0.96 ± 0.15	0.017
[a]Insulin (μIU/ml)(5,18)	2.56 ± 1.29	9.03 ± 15.85	0.005 [c]	5.04 ± 6.60	0.014[c]
Eotaxin (pg/ml)	367.01 ± 145.34	477.29 ± 186.50	0.011 [c]	570.52 ± 193.72	< 0.001 [c]
Cancer antigen 19-9 (U/ml)	9.74 ± 13.09	19.29 ± 16.27	0.027	17.92 ± 13.97	0.034
Carcinoembryonic antigen (ng/ml)	1.11 ± 0.64	1.87 ± 1.54	0.038	1.77 ± 1.39	0.024 [c]
Creatine kinase MB (ng/ml)	0.44 ± 0.05	0.53 ± 0.21	0.047 [c]	0.55 ± 0.21	0.005
Proteins that normalized with treatment					
Alpha-2 macroglobulin (mg/ml)	0.81 ± 0.08	0.85 ± 0.98	0.050	0.84 ± 0.12	0.194

HGPS, Hutchinson–Gilford Progeria Syndrome.
[a]Sections are presented in order of ascending P values for control vs. HGPS untreated.
[b]Previously validated in untreated HGPS patients using a clinical assay.
[c]Age interaction was significant, $P \leq 0.1$.

Table 4. Correlations vs. age for proteins where significant trend with age was detected

Protein	Healthy control slope (*P* value)	Untreated HGPS slope (*P* value)	Lonafarnib-treated HGPS slope (*P* value)
Decrease with age in HGPS untreated[a] and treated, but not in controls			
[b]Adiponectin(16)	0.13 (0.5)	−0.73 (<0.001)	−0.56 (0.005)
[b]Leptin(9,18)	0.18 (0.32)	−0.56 (0.004)	−0.39 (0.07)
Interleukin-10	−0.10 (0.58)	−0.42 (0.04)	−0.37 (0.09)
Interleukin-16	−0.19 (0.28)	−0.40 (0.05)	−0.49 (0.02)
Decrease with age in HGPS untreated [a], but not in controls or HGPS treated			
Epithelial-derived neutrophil-activating protein 78	−0.22 (0.22)	−0.47 (0.02)	−0.12 (0.58)
Macrophage inflammatory protein-1 beta	−0.09 (0.613)	−0.42 (0.04)	0.04 (0.85)
Matrix metalloproteinase-3	0.27 (0.12)	−0.41 (0.05)	−0.27 (0.21)
Decrease with age in HGPS treated[a], but not in controls or HGPS untreated			
Prostatic acid phosphatase	0.07 (0.70)	−0.01 (0.97)	−0.50 (0.02)
Thyroid-stimulating hormone	−0.25 (0.15)	−0.31 (0.14)	−0.39 (0.06)
Increase with age in HGPS untreated[a] and treated, but not in controls			
Apolipoprotein CIII	0.24 (0.16)	0.69 (<0.001)	0.67 (<0.001)
Factor VII	−0.27 (0.12)	0.53 (0.008)	0.50 (0.02)
Plasminogen activator inhibitor-1	0.10 (0.57)	0.42 (0.04)	0.54 (0.007)
Eotaxin	−0.08 (0.64)	0.36 (0.08)	0.40 (0.06)
Increase with age in HGPS untreated[a], but not in controls or HGPS treated			
Serum amyloid P	0.19 (0.27)	0.52 (0.009)	−0.13 (0.54)
Haptoglobin	0.16 (0.38)	0.42 (0.04)	0.03 (0.88)
Immunoglobulin E	0.00 (0.99)	0.41 (0.05)	0.17 (0.44)
Complement 3	0.00 (0.99)	0.38 (0.06)	0.04 (0.85)
Decrease with age in controls[a], but not in HGPS untreated or treated			
Interleukin-18	−0.58 (<0.001)	−0.27 (0.20)	−0.18 (0.41)
Intercellular adhesion molecule-1	−0.50 (0.003)	0.14 (0.52)	−0.16 (0.45)
Tumor necrosis factor alpha	−0.32 (0.07)	−0.25 (0.24)	−0.28 (0.20)
Alpha fetoprotein	−0.30 (0.08)	−0.05 (0.80)	−0.26 (0.23)
Growth hormone	−0.30 (0.09)	−0.02 (0.92)	0.03 (0.90)
Interleukin-1ra	−0.30 (0.08)	0.18 (0.39)	0.03 (0.90)
Decrease with age in controls[a], HGPS untreated, and HGPS treated			
Macrophage-derived chemokine	−0.73 (<0.001)	−0.43 (0.03)	−0.37 (0.08)
Sex hormone-binding globulin	−0.48 (0.004)	−0.76 (<0.001)	−0.71 (<0.001)

Table 4 Continued

Protein	Healthy control slope (*P* value)	Untreated HGPS slope (*P* value)	Lonafarnib-treated HGPS slope (*P* value)
Decrease with age in controls[a], HGPS treated but not HGPS untreated			
Carcinoembryonic antigen	−0.36 (0.04)	−0.33, $P>0.1$ (0.11)	−0.44 (0.04)
Decrease with age in controls[a], HGPS untreated, but not HGPS treated			
Stem cell factor	−0.42 (0.01)	−0.50 (0.01)	−0.11 (0.62)
T-cell-specific protein RANTES	−0.39 (0.02)	−0.36 (0.08)	−0.09 (0.69)
CD40 ligand	−0.34 (0.05)	−0.41 (0.05)	−0.07 (0.74)
Creatine kinase MB	−0.33 (0.06)	−0.54 (0.006)	−0.32 (0.14)
Interleukin-1alpha	−0.30 (0.08)	−0.57 (0.004)	−0.24 (0.27)
CD40	−0.29 (0.09)	−0.36 (0.080)	0.11 (0.62)
Increase with age in both controls, HGPS untreated[a], and HGPS treated			
Insulin-like growth factor-1(5)	0.52 (0.002)	0.76 (<0.001)	0.62 (0.002)
Immunoglobulin A(5)	0.37 (0.03)	0.66 (<0.001)	0.62 (0.002)
Insulin(5,18)	0.42 (0.01)	0.47 (0.02)	0.56 (0.006)
Increase with age in both controls and HGPS untreated, but not HGPS treated			
Ferritin	0.31 (0.07)	0.40 (0.05)	0.09 (0.70)

HGPS, Hutchinson–Gilford Progeria Syndrome.
[a]Presented in order of decreasing correlation strength as per this patient group, regardless of positive or negative value.
[b]Previously demonstrated in untreated HGPS patients using a clinical assay.

(**Supplementary Table S2**. We speculate that lonafarnib affects these proteins regardless of disease state, although there were no lonafarnib-treated non-HGPS samples available. There were 30 detectable proteins whose means for patients with HGPS were similar between untreated and treated HGPS samples when compared with controls (**Table 6**).

Several proteins are of particular interest because their means are significantly lower in HGPS (treated and untreated groups) than controls regardless of age (no significant group-by-age interaction: apolipoprotein A1 (Apo-A1), brain derived neurotrophic factor (BDNF), CD40, epithelial-derived neutrophil-activating protein 78 (ENA-78), extracellular receptor for advanced glycation end products (EN-RAGE), fibrinogen, IL-3, IL-16, leptin, myeloperoxidase (MPO), tumor necrosis factor (TNF) receptor 2 (TNF-RII), C–C motif chemokine ligand 5 (RANTES), stem cell factor, von Willebrand Factor (vWF), and vascular cell adhesion molecule-1 (VCAM-1)) or greater than controls (cancer antigen 19-9 and complement 3), regardless of age (**Figure 1** and **Supplementary Figure S1**.

DISCUSSION

There is a paucity of validated disease-relevant biomarkers that can be utilized as indicators of disease status and treatment response for children with HGPS. This is the first multi-analyte study of plasma proteins for this disease. It seeks to open an avenue for biomarker discovery. Similar exploratory biomarker studies on this assay platform have been performed for diseases such as facioscapulohumeral muscular dystrophy (15) and chronic obstructive pulmonary disease (16). Within the 90 proteins surveyed, there was a subset of ILs and several other proteins that were lower than controls at baseline, and normalized with treatment: IL-1α, IL-7, IL-13, as well as beta-2 microglobulin, C-reactive protein, and myoglobin. Alpha-2 microglobulin was elevated and normalized with treatment. There were 15 proteins whose levels were lower than controls at baseline and did not change with treatment, but have not previously been identified as abnormal in HGPS: Apo-A1, BDNF, CD40, ENA-78, EN-RAGE, fibrinogen, IL-3, IL-16, MDC, MPO, RANTES, stem cell factor, TNF-RII, VCAM- 1, and vWF. Conversely, there were five proteins that were elevated over controls at baseline and did not change with treatment, but have not previously been identified as being abnormal in HGPS: cancer antigen 19-9, creatine kinase MB, carcinoembryonic antigen, complement 3, and eotaxin.

For a number of proteins whose means significantly differed from controls, age also influenced plasma levels. Eotaxin and insulin increased, and adiponectin decreased with increasing patient age in HGPS, but not in controls. The fact that these proteins had trend lines for HGPS that did not intersect with

Table 5. Proteins with disparate protein–age relationships, where characteristic changes in protein concentration with age differ between children with HGPS and control children ($P \leq 0.1$)[a]

	Control vs. HGPS untreated (*P* value)	Control vs. HGPS treated (*P* value)	Interaction description	Trend lines intersect
Disparate age relationships in proteins whose means differ in HGPS compared with healthy controls				
Creatine kinase MB	0.002	$P > 0.1$ (0.15)	Mean HGPS greater than controls overall. HGPS decreased with age over time, but controls did not change with age; untreated HGPS levels approached normal levels by ~age 10 y	No
[b]Insulin(5,18)	0.005	0.002	HGPS increased with age over time, but control levels did not change with age. Mean HGPS greater than controls	No
[b]Adiponectin(16)	0.016	0.097	HGPS significantly decreased with age, both before and after treatment, but control levels did not change with age. Mean for HGPS smaller than that for controls	No
Eotaxin	0.06	0.04	HGPS increase with age over time, but control levels did not change with age. Mean HGPS greater than controls	No
Interleukin-1ra	0.09	$P > 0.1$ (0.23)	HGPS untreated increased with age. Controls decreased with age. Mean HGPS less than controls	Yes
Macrophage-derived chemokine	$P > 0.1$ (0.14)	0.051	Controls decreased with age; HGPS did not decrease with age. Mean untreated and treated HGPS less than controls	Yes
Carcinoembryonic antigen	$P > 0.1$ (0.3)	0.097	Mean HGPS greater than controls. HGPS-treated group levels decreased with age over time, but not controls; levels become lower than controls by ~age 13 y	Yes
Disparate age relationships in proteins whose means do not differ in children with HGPS (treated or untreated) compared with healthy controls				
Apolipoprotein CIII	< 0.001	0.001	HGPS untreated and treated increased with age	Yes
Factor VII	0.001	0.002	HGPS untreated and treated increased with age	Yes
Sex hormone-binding globulin	0.017	0.059	HGPS decreased with age	Yes
Intercellular adhesion molecule-1	0.033	$P > 0.1$ (0.30)	HGPS untreated increased with age	Yes
Matrix metalloproteinase-3	0.037	$P > 0.1$ (0.10)	HGPS untreated decreased with age	Yes
Plasminogen activator inhibitor-1	0.067	$P > 0.1$ (0.10)	HGPS untreated increased with age	Yes
Macrophage inflammatory protein-α	0.086	$P > 0.1$ (0.42)	HGPS untreated decreased with age	Yes
Immunoglobulin E	0.094	$P > 0.1$ (0.60)	HGPS untreated increased with age	Yes
Thyroid-stimulating hormone	$P > 0.1$ (0.33)	0.075	HGPS treated decreased with age	Yes
Prostatic acid phosphatase	$P > 0.1$ (0.79)	0.051	HGPS treated decreased with age	Yes

HGPS, Hutchinson–Gilford Progeria Syndrome.
[a]Sections are presented in order of ascending *P* values for control vs. HGPS untreated.
[b]Previously demonstrated using a clinical assay.

Table 6. Thirty proteins with similar levels in plasma of children with HGPS and healthy controls ($P > 0.05$)[a]

Protein	Control (mean ± SD; $N = 34$)	HGPS untreated (mean ± SD; $N = 24$)	Age-adjusted P value	HGPS-treated (mean ± SD; $N = 23$)	Age-adjusted P value
Haptoglobin (mg/ml)	0.90 ± 0.53	1.17 ± 0.76	0.053	1.22 ± 0.91	0.102
Granulocyte colony-stimulating factor (pg/ml)	18.47 ± 21.14	10.19 ± 8.87	0.073	15.25 ± 17.83	0.508
Interleukin-10 (pg/ml)	17.85 ± 5.62	16.0 ± 1.6	0.089	16.04 ± 1.14	0.122
Interleukin-18 (pg/ml)	240.09 ± 91.25	335.47 ± 226.99	0.089	301.37 ± 147.78	0.072
Cancer antigen 125 (U/ml)	11.38 ± 6.56	16.39 ± 12.93	0.090	13.39 ± 6.7	0.310
Interleukin-4 (pg/ml)	171.32 ± 55.43	151.88 ± 46.04	0.096	166.65 ± 59.57	0.716
Immunoglobulin E (ng/ml)	90.65 ± 248.23	190.69 ± 282.59	0.108 [b]	136.33 ± 189.37	0.445
Alpha-1 Antitrypsin (mg/ml)	2.44 ± 0.46	2.25 ± 0.40	0.119	2.33 ± 0.42	0.332
Lipoprotein A (µg/ml)	79.98 ± 80.84	48.27 ± 68.33	0.137	45.47 ± 76.37	0.116
Sex hormone-binding globulin (nmol/l)	70.17 ± 29.85	67.48 ± 38.35	0.148 [b]	65.17 ± 36.34	0.289 [b]
Prostatic acid phosphatase (ng/ml)	0.20 ± 0.08	0.23 ± 0.07	0.151	0.20 ± 0.07	0.908 [b]
Matrix metalloproteinase-3 (ng/ml)	0.86 ± 0.63	0.65 ± 0.32	0.208 [b]	0.63 ± 0.25	0.118
Immunoglobulin A(5)(mg/ml)	0.83 ± 0.45	0.62 ± 0.39	0.210	0.74 ± 0.43	0.557
Intercellular adhesion molecule-1 (ng/ml)	137.8 ± 27.82	151.54 ± 37.78	0.213 [b]	140.01 ± 29.58	0.930
Tissue inhibitor of metalloproteinases 1 (ng/ml)	54.67 ± 8.19	49.90 ± 14.81	0.232	48.68 ± 15.12	0.072
Vascular endothelial growth factor (pg/ml)	370.85 ± 102.43	337.87 ± 107.78	0.280	347.35 ± 115.09	0.449
Apolipoprotein CIII (µg/ml)	68.36 ± 20.95	71.23 ± 55.41	0.293 [b]	59.21 ± 42.71	0.367[c]
Serum glutamic oxaloacetic transaminase (µg/ml)	8.36 ± 2.96	7.66 ± 1.63	0.323	8.52 ± 2.54	0.848
[d]Thyroid-stimulating hormone(5) (µIU/ml)	2.13 ± 0.67	2.46 ± 1.24	0.381	2.19 ± 1.85	0.992[b]
Macrophage inflammatory protein-1beta (pg/ml)	129.04 ± 37.85	126.03 ± 32.16	0.533	135.29 ± 59.75	0.645
Monocyte chemoattractant protein-1 (pg/ml)	335.53 ± 126.58	361.88 ± 100.3	0.540	376.39 ± 130.63	0.274
Insulin-like growth factor-1 (ng/ml)	179.44 ± 143.31	130.63 ± 109.47	0.566	135.32 ± 127.62	0.302
Macrophage inflammatory protein-1 alpha (pg/ml)	47.84 ± 13.96	45.72 ± 10.36	0.613 [b]	41.4 ± 8.42	0.064
Plasminogen activator inhibitor-1 (ng/ml)	15.18 ± 8.98	15.18 ± 14.38	0.698 [b]	13.14 ± 8.12	0.462
Alpha fetoprotein (ng/ml)	1.86 ± 1.73	2.13 ± 1.99	0.808	1.78 ± 1.77	0.740
Apolipoprotein H (µg/ml)	182.49 ± 46.73	183.35 ± 42.72	0.867	184.71 ± 34.38	0.817
Serum amyloid P (µg/ml)	10.88 ± 3.81	10.31 ± 3.19	0.901	11.16 ± 5.08	0.793
Growth hormone (ng/ml)	2.77 ± 3.11	2.93 ± 3.68	0.917	5.28 ± 8.25	0.125
Factor VII (ng/ml)	526.74 ± 121.5	520.17 ± 189.15	0.992[b]	529.78 ± 173.78	0.904 [c]
Immunoglobulin M(5)(mg/ml)	0.99 ± 0.33	0.96 ± 0.33	0.994	0.85 ± 0.32	0.124

HGPS, Hutchinson–Gilford Progeria Syndrome; TSH, thyroid-stimulating hormone.
[a]Proteins are presented in order of ascending P values for control vs. HGPS-untreated.
[b]Age interaction was significant, $P < 0.1$.
[c]Age interaction was significant, $P < 0.001$.
[d]TSH previously published as being within normal clinical limits.

control trend lines supports their future investigation as meaningful disease biomarkers in HGPS. In addition, the differences between HGPS and controls became more pronounced with increasing patient age, implying that the abnormality worsens with disease progression. Although carcinoembryonic antigen, IL-1ra, and macrophage-derived

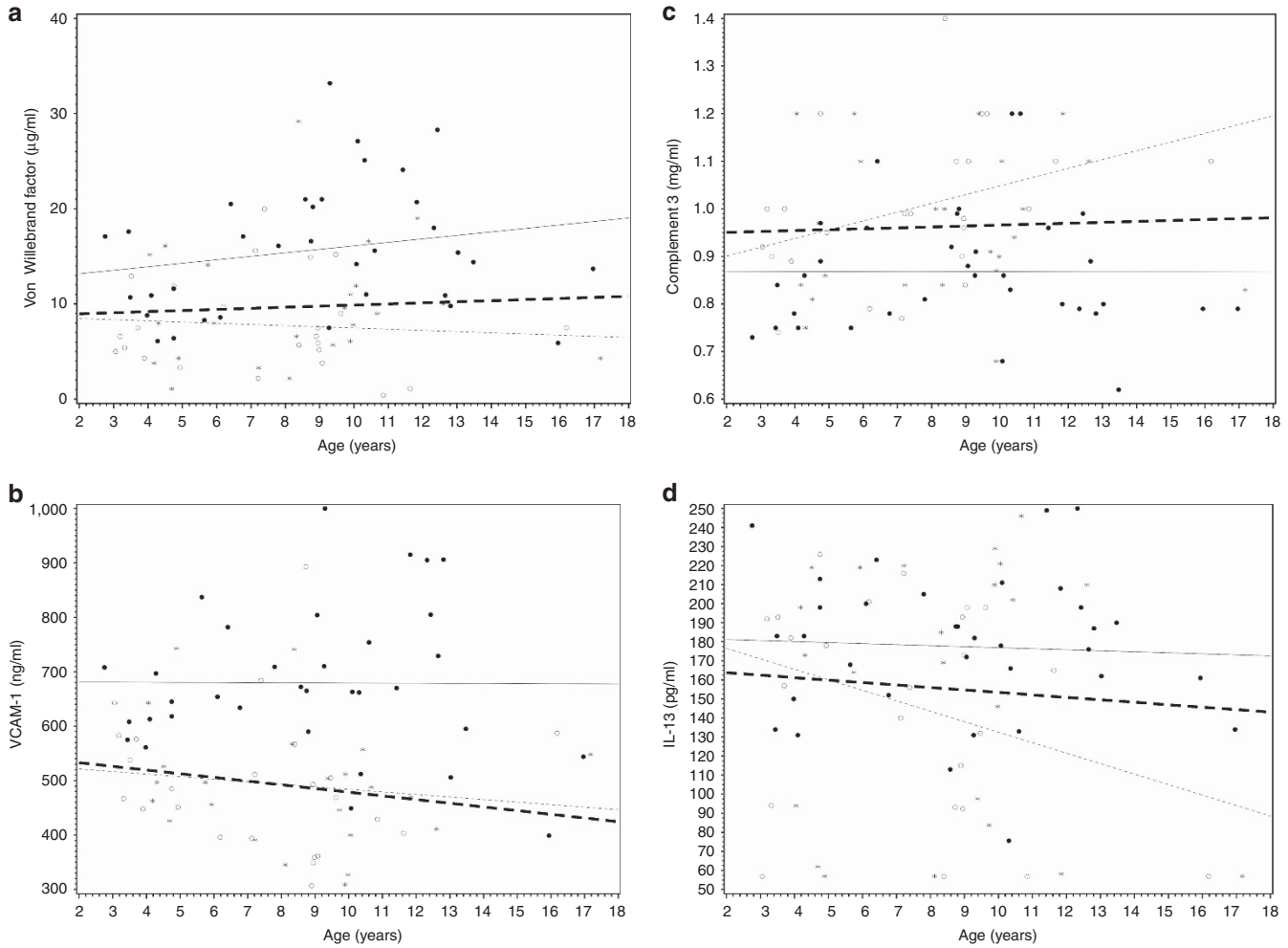

Figure 1. Plots representing various result categories. Symbols are individual patient levels and lines are trend lines. (**a**,**b**) Levels of von Willebrand Factor and VCAM-1 are significantly lower in HGPS compared with controls regardless of treatment or age. (**c**) Levels of complement 3 are increased over controls, and normalize with lonafarnib treatment. (**d**) Levels of interleukin-13 are decreased compared with controls, and normalize with lonafarnib treatment. Control: ●_____ ; HGPS Baseline: ○———— ; HGPS Lonafarnib-treated: *_ _ _ _. HGPS, Hutchinson–Gilford Progeria Syndrome.

chemokine levels were significantly different from controls on average, their trend lines with age intersected with controls, diminishing enthusiasm for further exploring them as potential biomarkers. Ten additional proteins listed in Table 5 were not different from controls on average, and also intersected with controls when age trends were plotted, even though there was a significant age interaction. This diminishes our enthusiasm for further exploring them as potential biomarkers.

Several cardiovascular and neurovascular disease-associated proteins not previously identified as abnormal in HGPS are of particular interest for further validation. Elevations in serum alpha-2-macroglobulin levels are associated with high-grade white matter lesions in the general population (17), and increased odds of stroke and deep vein thrombosis in children (18). The fact that this protein was elevated at baseline, but normalized with treatment, adds to its potential value. In addition, levels of vWF were about half of those measured in healthy age- and gender-matched controls, which may constitute a clinically significant finding. Low level or dysfunctional vWF protein results in von Willebrand disease, which typically manifests with mucocutaneous bleeding symptoms such as epistaxis and heavy menstrual bleeding. Although initially recognized for its role in hemostasis, vWF has also been demonstrated to influence angiogenesis. vWF is secreted by vascular endothelial cells and platelets. The potential etiology of low vWF is not clear from the pathogenesis of HGPS. However, the impact of vWF on both intracellular and extracellular pathways regulating angiogenesis could be influenced (19), as there is evidence for both abnormal extracellular matrix (20,21) and endothelial cell involvement in HGPS (22). Another protein of significant interest in the setting of HGPS is Apo-A1. Apo-A1 represents the primary apolipoprotein constituent of HDL particles, and HDL is reduced in HGPS (23). Decreased Apo-A1 is associated with increased coronary events in the general population and poor glycemic control (24).

There are a number of protein abnormalities identified in this study that are associated with cardiovascular disease, neurovascular disease, and dysfunctional glucose metabolism in the general population, including adiponectin, alpha-2 macroglobulin, Apo-A1, BDNF, leptin, and insulin (25,26). In HGPS, it is possible that these abnormalities contribute in a cumulative manner to the characteristic insulin resistance and premature atherosclerosis seen. As with other diseases (27–29), a panel of biomarkers that incorporates these proteins could be extremely valuable if it is predictive of cardiovascular status, stroke events, or survival.

Support for the validity of the results of this study comes from proteins in the assay that have been assessed in HGPS using clinically validated assays as decreased, increased, or similar to the healthy control population, and previously published. For example, we have previously reported that adiponectin was decreased in HGPS, and, unlike controls, decreased significantly with increasing patient age (23). Leptin was detected in controls, and was largely undetectable in HGPS, both untreated and treated groups (9). This correlates with the characteristic extreme paucity of subcutaneous fat in children with HGPS. Lack of subcutaneous fat is likely a major contributor to insulin resistance in HGPS, as reflected in the extremely low levels of proteins secreted mainly by subcutaneous fat cells, including leptin and adiponectin. It is not surprising that these proteins and overall insulin resistance were not improved by treatment with lonafarnib, as the fat compartment was not affected by lonafarnib as measured using dual X-ray absorptiometry (9). Insulin levels were elevated in HGPS and significantly improved with treatment, although they were still elevated over controls, correlating with the insulin resistance detected in HGPS (9). Growth hormone (5,9), IGF-1 (refs 5,9), IgA (5), IgM (5), and TSH (9) values, all published as being within normal limits for healthy individuals, were found to be similar to healthy control levels in this study. Although C-reactive protein has been assessed as normal in HGPS (23), this study found that it was lower in HGPS than in controls at baseline, but not with lonafarnib treatment.

There are several weaknesses that should be addressed in follow-up studies. As this study was exploratory, we did not fully account for multiple comparisons. Instead, we presented the proteins in order of ascending *P* values or descending correlation strength when comparing controls with untreated HGPS samples, to highlight which proteins may be of greatest interest. Future validation studies will likely be performed with individual proteins, and therefore abrogate the need for this statistical consideration. In addition, when validation studies are performed for individual proteins and protein subsets identified in this exploratory study, it will be necessary to analyze relationships to disease outcomes such as cardiovascular status, neurovascular status, or survival, in order to further evaluate the protein as a disease-relevant biomarker. Finally, control samples were not collected longitudinally, so that within-subject variability for control participants could not be evaluated.

CONCLUSIONS

To date, no validated FDA-approved circulatory biomarker exists for HGPS. We have identified a set of plasma proteins in children with HGPS, before and 1 year after initiating treatment with the farnesyltransferase inhibitor lonafarnib. This is an initial survey step in identifying serologic biomarkers that may provide insight into the natural history of disease, and/or be indicative of improvements with treatment. This is the first study of HGPS to employ a multi-analyte array platform to identify potential plasma biomarkers in HGPS.

The goal of this study was to make these data available worldwide to the research community studying this ultra-rare disease. This Rules-Based Medicine, HumanMAP panel was not specifically designed for this rare disease, but was utilized as proof-of-principle for this type of approach in biomarker discovery for HGPS. In this nonspecific protein panel, more than 40% of analyzed proteins differed from the age- and gender-matched control population. This implies that such an approach may have significant potential for identifying meaningful plasma protein biomarkers for children with HGPS. Future studies should focus on essential studies that will further evaluate plasma proteins identified here as having abnormal levels in HGPS, namely validation and clinical disease correlations.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

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