Endothelin-1, vascular endothelial growth factor and systolic pulmonary artery pressure in patients with Chuvash polycythemia

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Background and Objectives. Endothelin-1 has been associated with development of hypoxia-related pulmonary hypertension and vascular endothelial growth factor (VEGF) with protection from this complication. In Chuvash polycythemia, homozygous germline von Hippel-Lindau (*VHL*) 598C→T leads to up-regulation during normoxia of hypoxia inducible factor-1α and several hypoxia-controlled genes including erythropoietin and VEGF. We postulated that endothelin-1 and pulmonary artery pressure may be elevated in Chuvash polycythemia.

Design and Methods. Systolic pulmonary artery blood pressure was estimated by Doppler echocardiography and plasma concentrations of endothelin-1, VEGF and erythropoietin were determined in 14 patients with Chuvash polycythemia and 14 controls.

Results. Plasma endothelin-1 (p=0.010), VEGF (p=0.022) and erythropoietin (p<0.0005) concentrations and Doppler-estimated systolic pulmonary artery pressures (p<0.0005) were higher in the patients while systolic systemic blood pressures were lower (p=0.001). Five (36%) patients and no controls had mild pulmonary hypertension defined as systolic pulmonary artery pressure ≥35 mmHg. Among the patients with Chuvash polycythemia, the trends of association of estimated pulmonary artery pressure with plasma concentrations of endothelin-1 (R = +0.236), VEGF (R = -0.389) and erythropoietin (R = +0.220) were not statistically significant.

Interpretations and Conclusions. Estimated systolic pulmonary artery pressure and plasma concentrations of endothelin-1 and VEGF are increased in patients with Chuvash polycythemia patients. The lack of significant associations of estimated systolic pulmonary artery pressure with plasma endothelin-1 and VEGF levels could conceivably be due to the small sample size. Further studies are indicated, especially in view of the reported efficacy of endothelin-1 receptor blockers in treating hypoxia-associated pulmonary hypertension.

Key words: Chuvash polycythemia, endothelin-1, vascular endothelial growth factor, VEGF, pulmonary embolism.

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Chuvash polycythemia, the only known congenital defect of augmented expression of hypoxia-controlled genes, is common in the Chuvash Republic of the Russian Federation[1] where approximately 200 cases are recognized among a population of about 1.5 million people. The condition also occurs in other parts of the world.[2,3] This form of polycythemia is caused by a homozygous mutation in the von Hippel-Lindau gene (*VHL* 598C→T) and is characterized by upregulation of hypoxia inducible factor 1α (HIF-1) under normoxic conditions.[4] HIF-1 is the principal transcriptional regulator of the response to hypoxia in mammalian cells, and a number of its target genes have been shown to be upregulated in Chuvash polycythemia, including erythropoietin (*EPO*), facilitated glucose transporter (*GLUT1*), plasminogen activator inhibitor 1 (*PAI-1*), transferrin (*TF*), transferrin receptor (*TFR*) and vascular endothelial growth factor (*VEGF*).[4,5] The expression of other HIF-1-regulated genes, such as endothelin-1[6] and endothelial nitric oxide synthase,[7] is likely to be increased in patients with Chuvash polycythemia, but this has not yet been studied. We have previously reported significantly decreased longevity and increased thrombotic complications in homozygotes for the Chuvash polycythemia mutation; however these complications did not correlate with the absolute level of elevated hematocrit, and were also seen in patients whose polycythemia was controlled by chronic phlebotomy.[5] Humans exposed to hypoxia have a tendency to develop pulmonary hypertension[8-10] in association with increased plasma levels of endothelin-1.[8,9] Similarly, experimental rats and mice exposed to chronic hypoxia develop elevated pulmonary artery pressures in association with increased expression of HIF1α[11-15] and endothelin-1.[13,14] Mice with partial deficiency of HIF1α or HIF2α are protected from hypoxia-induced pulmonary hypertension[11,12,14] and do not have increased expression of endothelin-1.[14] The finding that both selective inhibitors of the endothelin type A receptor and non-selective inhibitors of type A and B receptors block

the development of hypoxic pulmonary hypertension in experimental animals[16-18] supports the concept that increased expression of endothelin-1 is involved in the pathogenesis of this complication. Some but not all studies indicate that plasma VEGF concentrations are increased in humans with pulmonary hypertension or exposed to hypoxia.[19-24] The expression of VEGF is increased in the plexiform lesions of patients with pulmonary hypertension[25] and the lungs of rats with hypoxia-induced pulmonary hypertension,[26] but increased VEGF activity may not be causative. Rather, overexpression of VEGF-A and VEGF-B in the lungs protects from[27,28] and deficiency of VEGF-B[29] or blockade of VEGF receptor 2[30] contributes to the development of hypoxic pulmonary hypertension in rats. Because of the upregulation of the hypoxic response in Chuvash polycythemia[4] and despite the increased expression of VEGF,[5] we postulated that patients with this condition have elevated plasma endothelin-1 concentrations and a tendency to develop pulmonary hypertension even in the absence of hypoxia.

Design and Methods

Participants

This research was approved by the Institutional Review Board of Howard University and all participants provided written informed consent. Fourteen patients with a diagnosis of Chuvash polycythemia and 14 age- and sex-matched control Chuvash individuals without polycythemia, all from the Chuvash Republic of the Russian Federation, were studied.

Polymerase chain reaction (PCR) analysis for the VHL 598C→T mutation

Genomic DNA was isolated from peripheral blood collected in EDTA and PCR reactions were performed in 50 μL volumes containing 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM dNTP, 300 nM primers, and 2.5 U/reaction Taq DNA polymerase (Life Technologies, Grand Island, NY, USA). The following primers were used for amplification of *VHL* exon 3: *VHL*3F 5'-CCTTGTACTGAGACCCTAG, *VHL*3R 5'-GCTGAGATGAAACAGTGTA. Ten microliters of PCR product were incubated with 5 U of *Fnu*4HI (New England Biolabs Inc, Beverly, MA, USA) for 2 hours to detect the mutation. The *VHL598C→T* mutation abolishes restriction sites for *Fnu4*HI resulting in an uncut 296 base pair band detected on 1.2% agarose gel.

Laboratory tests

The complete blood count was performed on EDTA-anticoagulated blood by an automated method. Plasma and serum samples were stored at -80°C until use. Plasma concentrations of endothelin-1, erythropoietin and VEGF were measured in duplicate by enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN, USA). The median (range) coefficient of variation was 3.5% (0% to 10.8%) for the endothelin-1 assay and 2.2% (0%-5.2%) for the VEGF assay.

Echocardiography

Cardiac measurements were performed according to the guidelines of the American Society of Echocardiography.[31] Transmitral flow, Doppler determinations of the severity of valvular regurgitation and left ventricular stroke volume were assessed and graded. Tricuspid regurgitation was assessed in the parasternal right ventricular inflow, parasternal short-axis, and apical four-chamber views, and a minimum of five sequential complexes were recorded. Continuous-wave Doppler sampling of the peak regurgitant jet velocity was used to estimate the right-ventricular-to-right-atrial systolic pressure gradient with the use of the modified Bernoulli equation (4 x [tricuspid regurgitant jet velocity]^2).[32] Pulmonary-artery systolic pressure was calculated by adding the Bernoulli-derived pressure gradient to an assumed right atrial pressure of 10 mmHg. Video tapes of the echodardiography procedures were independently reviewed by a cardiologist (PRG) at Howard University who was blinded to the diagnosis of Chuvash polycythemia versus control and the findings reported here were confirmed.

Statistical analysis

Continuous variables were compared between cases and controls with the paired t-test and dichotomous variables with Fisher's exact test. The relationship of estimated systolic pulmonary artery pressure and of systolic systemic blood pressure with other variables was examined with linear regression models. Calculations were made using Systat software.

Results

Characteristics of patients and controls. All 14 patients were homozygotes for the *VHL* 598C→T mutation and none of the control subjects had the mutation. The patients and controls were not acutely ill at the time of evaluation, did not reside at high altitude and were not suffering from chronic obstructive pulmonary disease, pulmonary embolism, sleep apnea or other conditions marked by hypoxia. Nine of the Chuvash polycythemia patients were undergoing phlebotomy therapy and six had hemoglobin concentrations within the normal range at the time of the study. The patients with Chuvash polycythemia had significantly higher concentrations of hemoglobin ($p<0.0005$) and plasma erythropoietin ($p<0.0005$) than controls and significantly lower systemic blood pressures ($p=0.001$) and platelet counts ($p=0.044$) (Table 1). Plasma concentrations of endothelin-1 ($p=0.010$) and VEGF ($p=0.022$) were significantly higher in the patients with Chuvash polycythemia than in the controls (Figure 1).

Echocardiographic findings in patients and controls

Transthoracic echocardiography was performed by a cardiologist (VIB) at the Republic Cardiac Center in Cheboksary, Chuvashia, Russia with an Acuson Computed Sonography 128 XP/10 system (Maintain View, CA, USA). Left atrial systolic diameter, left ventricular end diastolic diameter, and left ventricular ejection fraction did not differ significantly between

Table 1. **Characteristics of patients with Chuvash polycythemia and controls*.**

	Chuvash polycythemia (VHL 598C→T homozygotes) N=14	*Controls N=14*	*p†*
Demographic			
Age (years)	34±17	35±18	---
Female sex (no. and %)	8 (57)	8 (57)	---
Medical history			
History of smoking (no. and %)	4 (29)	5 (36)	1.0
History of alcohol use (no. and %)	2 (14)	1 (7)	1.0
History of thrombosis (no. and %)	2 (14)	1 (7)	1.0
Shortness of breath at rest	2 (14)	0 (0)	0.48
Dyspnea on exertion	5 (36)	0 (0)	0.041
Physical examination			
Body mass index (kg/m^2)	20.4±3.7	21.2±4.3	0.43
Systolic blood pressure (mmHg)	102±16	114±13	0.001
Diastolic blood pressure (mmHg)	73±12	77±12	0.35
Laboratory tests			
Hemoglobin (g/dL; mean ± SD)	16.8±2.5	13.3±1.2	0.0005
White blood cells (10^3/mm^3)	6.1±1.6	6.6±1.7	0.23
Platelets (10^3/mm^3)	193±53	236±57	0.044
Echocardiography			
Tricuspid rugurgitant jet velocity (m/sec)	2.2±0.6	1.5±0.5	0.001
Left atrial systolic diameter (mm)	33±5	33±6	0.81
Left ventricular end diastolic volume (mL)	99±23	99±24	0.79
Left ventricular ejection fraction	0.70±0.04	0.70±0.02	0.62
Stroke volume (mL)	69±6	68±16	0.89

**Plus-minus values are means ±SD. The body-mass index is the weight in kilograms divided by the square of the height in meters. †Two-sided p values for continuous variables were calculated with the independent sample t-test and p values for categorical variables were calculated with the Fisher exact test.*

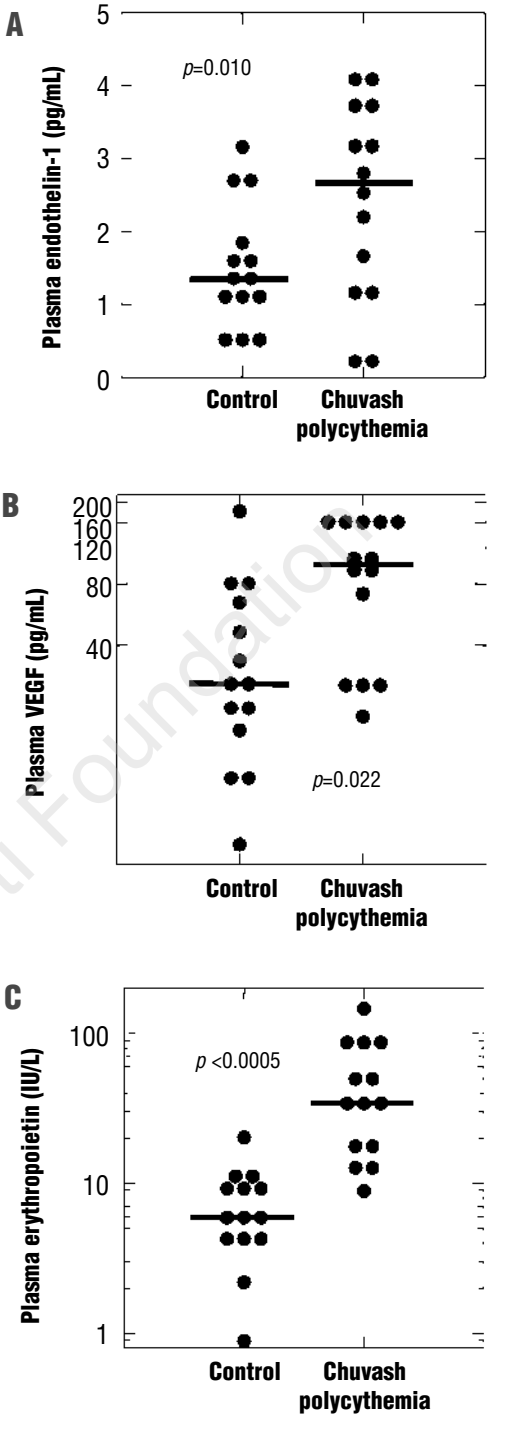

Figure 1. A. **Plasma endothelin-1 concentrations in 14 patients with Chuvash polycythemia (median 2.7 pg/mL; interquartile range of 1.3 to 3.6) and 14 controls (median 1.3 pg/mL; interquartile range of 1.0 to 1.8).** B. **Plasma VEGF concentrations in patients with Chuvash polycythemia (median 100 pg/mL; interquartile range of 28 to 152) and controls (median 28 pg/mL; interquartile range of 16 to 60).** C. **Plasma erythropoietin concentrations in patients with Chuvash polycythemia (median 33.7 IU/L; interquartile range of 16.2 to 79.6) and controls (median 6.0 IU/L; interquartile range of 4.6 to 9.2).**

patients with Chuvash polycythemia and controls ($p \leq 0.62$), but the tricuspid regurgitant jet velocity, which correlates with systolic pulmonary artery pressure, was significantly greater in the patients with Chuvash polycythemia ($p=0.001$) (Table 1). As defined by a tricuspid regurgitant jet velocity of at least 2.5 m/s or an estimated pulmonary artery systolic pressure of at least 35 mmHg, five (36%) of the patients with Chuvash polycythemia and none of the controls had elevated systolic pulmonary-artery pressures. Thus, systolic pulmonary blood pressures (as estimated by the modified Bernoulli equation and an assumed right atrial pressure of 10 mmHg) were significantly higher in the patients with Chuvash polycythemia than in controls while systolic systemic blood pressures were significantly lower (Figure 2). A preliminary partial report of these echocardiogram and systemic blood pressure findings has been published in abstract form.[33]

Relationship of hemoglobin, endothelin-1 and VEGF concentrations with systolic pulmonary and systemic blood pressures in univariate models

Among the patients with Chuvash polycythemia, univariate analyses revealed trends of positive association of estimated pulmonary artery pressure with plasma concentrations of endothelin-1 (R=0.236) and $\log_{10}$ erythropoietin (R=0.350) and trends of negative association with hemoglobin concentration (R=-0.425) and $\log_{10}$ plasma VEGF concentration (R=-0.389) but these were not statistically significant. Systolic systemic blood pressure had a trend of a positive association with hemoglobin concen-

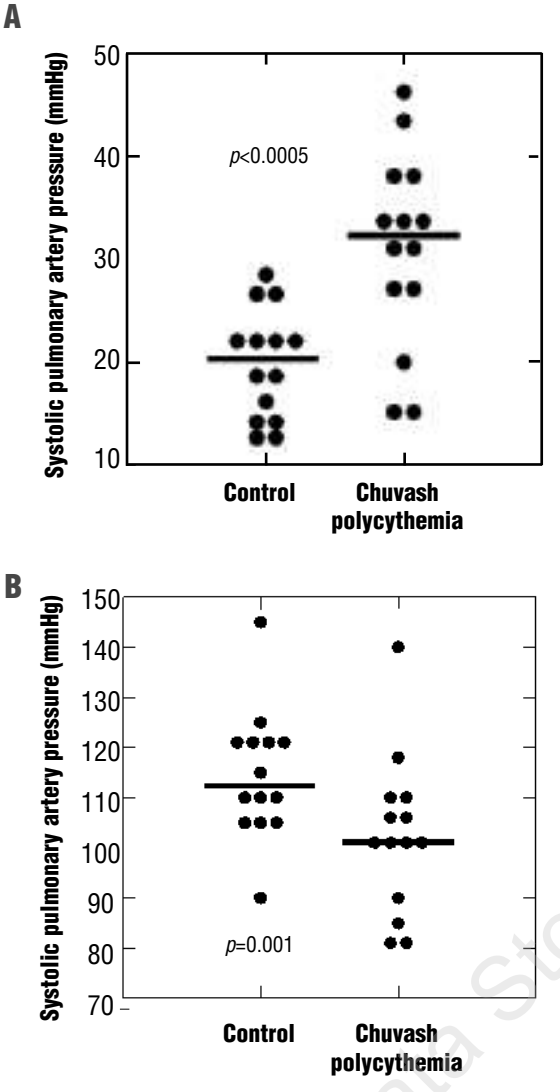

Figure 2. A. Estimated pulmonary artery systolic pressures in 14 patients with Chuvash polycythemia (median 32 mmHg, interquartile range of 26 to 37) and 14 control individuals (median 20 mm Hg, interquartile range of 17 to 22). B. Systemic systolic blood pressures in the same participants (median 102 mmHg, interquartile range of 90 to 110 in Chuvash polycythemia patients versus a median of 115 mmHg, interquartile range of 106 to 120) in controls.

tration (R=0.307) but only weak correlations (absolute R≤0.10) with plasma concentrations of endothelin-1, VEGF and erythropoietin among the patients. Among the controls, univariate analyses revealed trends of negative associations of estimated pulmonary artery pressure with hemoglobin concentration (R=-0.235), $\log_{10}$ plasma VEGF concentration (Rv-0.512) and plasma endothelin-1 concentration (R=-0.469), but these relationships were not statistically significant. There was no correlation with $\log_{10}$ plasma erythropoietin concentration (R=-0.006). Systolic systemic blood pressure had a trend of a positive association with $\log_{10}$ plasma erythropoietin concentration (R=0.287) but only weak correlations (absolute R<0.20) with concentrations of hemoglobin, $\log_{10}$ VEGF and endothelin-1 among the controls.

Table 2. Multivariate analyses of systolic pulmonary and systemic blood pressure in all participants.

Change in explanatory variable	*Estimated change in systolic pulmonary or systemic bood pressure (mmHg)*	*95 % confidence interval (mmHg)*	*p*
Model of Estimated Systolic Pulmonary Artery Pressure			
Presence of *VHL 598C→T* homozygosity	20	6 to 33	0.006
Increase by 1 pg/mL in plasma endothelin-1 concentration	1	-2 to 3	0.57
Increase by 1 log in plasma VEGF concentration	-7	-13 to 0	0.036
Increase by 1 log in plasma erythropoietin concentration	0	-10 to 10	0.95
Increase by 1 g/dL in hemoglobin concentration	-2	-4 to 0	0.068
Model of Systolic Systemic Blood Pressure			
Presence of *VHL 598C→T* homozygosity	-32	-2 to -61	0.035
Increase by 1 pg/mL in plasma endothelin-1 concentration	0	-6 to 5	0.90
Increase by 1 log in plasma VEGF concentration	2	-11 to 16	0.70
Increase by 1 log in plasma erythropoietin concentration	11	-10 to 32	0.28
Increase by 1 g/dL in hemoglobin concentration	3	-1 to 7	0.16

Multivariate analyses of systolic pulmonary and systemic blood pressures

In a multivariate logistic regression model of pulmonary pressure including all participants (both patients and controls), homozygosity for *VHL* 598C→T had a significant positive relationship with estimated systolic pulmonary artery pressure (p=0.006) while concentrations of VEGF (p=0.036) and hemoglobin (p=0.068) had negative relationships of borderline significance. In contrast, neither endothelin-1 nor erythropoietin concentrations had a significant relationship (p> 0.5) (Table 2). In a similar multivariate logistic regression model of systemic blood pressure, only homozygosity for *VHL* 598C→T had a significant relationship with systolic blood pressure, and this was a negative relationship (p=0.035) (Table 2).

Discussion

In this study, patients with Chuvash polycythemia had significantly higher plasma concentrations of endothelin-1 and VEGF compared to Chuvash controls and significantly higher systolic pulmonary artery pressures as estimated by Doppler echocardiography. Several of the patients had mild pulmonary hypertension based on estimated systolic pulmonary artery pressures of 35 mmHg or greater. Chuvash polycythemia is a condition of augmented hypoxia sensing marked by increased expression of HIF-1α in normoxia.[4] The present findings along with the observations that upregulation of HIF-1 is associated with pulmonary hypertension in animal models[11-14] provide a link between Chuvash polycythemia and conditions of

chronic hypoxia or high altitude, and suggest that upregulation of HIF-1 can lead to the development of pulmonary hypertension even in the absence of hypoxia. This is the first study showing increased pulmonary artery pressures and increased plasma concentrations of endothelin-1 in Chuvash polycythemia (Figure 1). Endothelin-1 thus joins the list of six other genes with a HIF-1 binding site in the promoter region that appear to be functionally up-regulated in Chuvash polycythemia.[4,5] A large number of studies have supported a role for endothelin-1 in the genesis of hypoxic pulmonary hypertension, including the findings of increased expression of endothelin-1 in hypoxic pulmonary hypertension and prevention or reversal by reducing endothelin-1 expression or by administering endothelin receptor inhibitors.[8–14,16–18,34] In the present study, plasma endothelin-1 concentration did not have a significant association with estimated systolic pulmonary artery pressure in either univariate analysis among the 14 patients with Chuvash polycythemia or in multivariate analysis among all 28 research participants (Table 2). However, systemic measurements of endothelin-1 may not precisely reflect expression in the lungs, since hypoxia in experimental animals leads to a preferential upregulation of endothelin-1 in the lungs.[35,36]

In contrast to endothelin-1, a number of studies suggest that increased expression of VEGF may protect against the development of hypoxia-associated pulmonary hypertension.[27-30] Although there was not a significant relationship between plasma VEGF concentration and estimated systolic pulmonary artery pressure among the patients with Chuvash polycythemia in this study, the significant inverse association in the multivariate analysis including both patients and controls (Table 2) might be consistent with a pulmonary artery pressure lowering effect of VEGF. Erythropoietin can increase pulmonary endothelial nitric oxide synthase levels and exert anti-smooth muscle and anti-fibroblast proliferative effects in transgenic mice.[37] Despite the fact that such effects could modulate pulmonary vascular remodeling in Chuvash polycythemia, we found no significant relationship of plasma erythropoietin concentrations with estimated systolic pulmonary artery pressure in this study.

The divergence in the changes in estimated systolic pulmonary artery pressures and systolic systemic blood pressures in the patients with Chuvash polycythemia in this study (Figure 2) may reflect different responses of the pulmonary and systemic vasculature to the global up-regulation of hypoxia-responsive genes in this condition. Increased circulating endothelin-1 would tend to lead to higher systemic blood pressure[38-40] while increased circulating VEGF[41] and nitric oxide[38,39] to lower systemic blood pressure, although a significant association of these markers with systolic blood pressure was not observed in multivariate analysis in this study (Table 2). Exposure to high altitude hypoxia has been reported to lead to an increase in pulmonary artery pressure without an increase in systemic blood pressure in some[9,42] but not all[43] studies. Pulmonary hypertension has been described as a complication of chronic acquired clonal myeloproliferative disorders characterized by myelofibrosis and hypercoagulability, namely myelofibrosis, essential thrombocytosis and polycythemia vera.[44-46] While repeated pulmonary emboli and/or polycythemia may be implicated in the development of pulmonary hypertension in this setting, it is interesting that patients with primary pulmonary hypertension[47] as well as patients with myeloproliferative disorders[48] (*J.T. Prchal, unpublished data*) may have dysregulation of bone morphogenetic protein receptor 2. Chronic hypoxic conditions are often associated with polycythemia, and some data from experimental animals have suggested that polycythemia itself may lead to pulmonary hypertension because of increased blood viscosity[37] or through inactivation of endothelium-derived nitric oxide.[49] These considerations raise the possibility that the elevated pulmonary artery pressures in patients with Chuvash polycythemia are related to the polycythemia and hypercoagulability associated with this condition[5] rather than to the upregulation of HIF-1. However, all five patients with elevations in estimated systolic pulmonary artery pressure in the present study had a history of phlebotomy therapy and three of them had normal hemoglobin concentrations at the time of the echocardiographic evaluation. Furthermore, in multivariate analysis an inverse relationship between hemoglobin and estimated systolic pulmonary artery pressure was observed among all study participants. Major limitations of this study are the small sample size and the intrinsic limitations of the methodology used to estimate systolic pulmonary artery pressure, i.e. tricuspid regurgitant jet velocity. Nevertheless, our data suggest that pulmonary hypertension may be common among patients with polycythemia due to inherited constitutive augmentation of hypoxia sensing and that HIF-1 may have a central role in the pathogenesis. Further studies are needed to understand the exact molecular basis of this complication, to investigate the divergent changes in pulmonary and systemic blood pressure and to determine whether pulmonary hypertension contributes to the early mortality[5] associated with Chuvash polycythemia. While writing this manuscript, endemic polycythemia due to the identical mutation of Chuvash polycythemia was reported in the southern Italian island of Ischia,[3] providing the unique opportunity to collaboratively investigate the effects of this mutation on systemic and pulmonary blood pressures using two populations with different genetic backgrounds and environments.

VIB contributed to the study conception and design and data analysis. GYM, AIS and LAP, and DO contributed to the study design and data analysis; PRG contributed to data analysis; ZD and SN contributed to study conception and data analysis. OLC contributed to the study conception and revising the manuscript. JTP contributed to the study design and revising the manuscript. VRG led the study group, contributed to the study conception and design and data analysis and drafted the manuscript.

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