# The heterozygote advantage of the Chuvash polycythemia *VHL*$^{R200W}$ mutation may be protection against anemia

Galina Y. Miasnikova,[1] Adelina I. Sergueeva,[2] Mehdi Nouraie,[3] Xiaomei Niu,[3] Daniel J. Okhotin,[4] Lydia A. Polyakova,[1] Tomas Ganz,[5] Josef T. Prchal,[6] and Victor R. Gordeuk[3]

[1]Chuvash Republic Clinical Hospital No. 1, Cheboksary Russia; [2]Cheboksary Children's Hospital, Cheboksary Russia; [3]Center for Sickle Cell Disease and Department of Medicine, Howard University, Washington, DC; [4]Russian Research Services, Camas, WA USA; [5]Dept. of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, USA; and [6]Division of Hematology, University of Utah and VAH, Salt Lake City, UT, USA

## ABSTRACT

The germ-line loss-of-function *VHL*$^{R200W}$ mutation is common in Chuvashia, Russia and occurs in other parts of the world. *VHL*$^{R200W}$ homozygotes have elevated hypoxia inducible factor (HIF)-1 and HIF-2 levels, increased hemoglobin concentration, propensity to thrombosis and early mortality. Because the mutation persists from an ancient origin, we hypothesized that there is a heterozygote advantage. Thirty-four *VHL*$^{R200W}$ heterozygotes and 44 controls over 35 years of age from Chuvashia, Russia were studied. Anemia was defined as hemoglobin less than 130 g/L in men and less than 120 g/L in women. Mild anemia was present in 15% of *VHL*$^{R200W}$ heterozygotes and 34% of controls without a mutated *VHL* allele. By multivariate logistic regression, the odds of anemia were reduced an estimated 5.6-fold in the *VHL*$^{R200W}$ heterozygotes compared to controls (95% confidence interval 1.4-22.7; $P$=0.017). In conclusion, heterozygosity for *VHL*$^{R200W}$ may provide protection from anemia; such protection could explain the persistence of this mutation.

Key words: heterozygosity, *VHL*$^{R200W}$, mutation, protection, anemia.

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## Introduction

The R200W mutation of the von Hippel Lindau gene (*VHL*) is present on the same haplotype in almost all persons of heterogeneous racial and ethnic backgrounds, indicating that the mutation may have originated in a founder prior to divergence of human races.[1] There is only one reported exception to this genetic linkage.[2] Homozygosity for *VHL*$^{R200W}$ is responsible for Chuvash polycythemia, the first recognized congenital disorder of augmented hypoxia sensing.[3] Chuvash polycythemia is common in the Chuvash Republic of the Russian Federation[4] and on the Italian island of Ischia;[5] the condition also occurs in other parts of the world.[2,6] Chuvash polycythemia is characterized by increased levels of HIF-1α and HIF-2α under ambient oxygen conditions[3,7] and upregulation of a number of the target genes of HIF.[3,8,9] Clinical manifestations include lower systemic blood pressure, higher pulmonary artery pressure and other changes in pulmonary vascular physiology, varicose veins, vertebral and hepatic hemangiomas, lower white blood cell and platelet counts, increased serum concentrations of inflammatory cytokines, changes in plasma thiol concentrations, arterial and venous thrombosis, major bleeding episodes, cerebral vascular events and premature mortality. In contrast to von Hippel Lindau cancer predisposition disease, malignant tumors typical of this syndrome have not been found, and no increased risk of cancer has been demonstrated.[8-13]

Given a negative selection for *VHL*$^{R200W}$ homozygotes, the mutation should be associated with some type of heterozygote advantage, albeit slight. However, the effect of heterozygosity for *VHL*$^{R200W}$ is not known. Only rare cases of polycythemia associated with *VHL*$^{R200W}$ heterozygosity have been reported.[2,6,14] In one study that included 9 Chuvash *VHL*$^{R200W}$ heterozygotes and 77 Chuvash participants with normal VHL alleles, the *VHL*$^{R200W}$ heterozygotes had significantly lower systemic blood pressures and higher serum PAI-1 concentrations. In addition, although no *VHL*$^{R200W}$ heterozygote was polycythemic, the mean hemoglobin concentration was higher by 4 g/L but this difference was not statistically significant.[8]

The present study was conducted to prospectively determine if heterozygotes for *VHL*$^{R200W}$ have discernable physiological and clinical differences from individuals without a mutated VHL allele, and if such differences exist, to consider whether they may represent a heterozygote advantage. We previously searched for a possible protection of *VHL*$^{R200W}$ heterozygotes from eclampsia, a major cause of maternal mor-

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*Correspondence: Victor R. Gordeuk, M.D., Center for Sickle Cell Disease, Howard University, 2041 Georgia Ave. NW, Washington, DC 20060 USA. Phone: international +1.202.8651941. Fax: international +1.202.2326719. E-mail: vgordeuk@howard.edu*

bidity and mortality that is related to dysregulation of HIF-1-regulated VEGF, but this was not validated (Gordeuk *et al.*, unpublished data, 2011). In this study, we examined the effect of *VHL*$^{R200W}$ heterozygosity on anemia in an otherwise unselected Chuvash sample. We postulated that the identification of any *VHL*$^{R200W}$ heterozygote advantage could point to potential benefits of chronic augmentation of HIFs by pharmacological agents, such as inhibitors of PHDs.[15,16]

## Design and Methods

### Study objective

The study objective was to compare clinical and molecular characteristics of a group of *VHL*$^{R200W}$ heterozygotes over 35 years of age and a similar number of controls without a mutated *VHL* allele over 35 years of age in the Chuvash Republic of the Russian Federation. Budgetary restraints confined the study to less than 80 research participants.

### Research protocol

The Howard University Institutional Review Board approved the research and all participants provided written informed consent. The study was carried out in the Chuvash Autonomous Republic of the Russian Federation, which is located about 650 kilometers southeast of Moscow along the Volga River. *VHL*$^{R200W}$ heterozygotes over 35 years of age were identified by studying first-degree family members of patients with Chuvash polycythemia. In addition, unaffected, unrelated controls, also of Chuvash ethnicity, and of similar age and sex distribution were enrolled from the community in the same geographical area of Chuvashia without previous knowledge of their health status. The study participants, who were in their usual state of health, were characterized by medical history, physical examination including blood pressure and body weight, and laboratory tests of the peripheral blood.

### Laboratory procedures

Complete blood count was performed by an automated analyzer (Sysmex XT 2000i, Sysmex Corporation, Kobe, Hyogo, Japan). Serum ferritin concentration was determined by enzyme immunoassay (Ramco Laboratories Inc., Stafford, TX, USA). Plasma concentrations of VEGF and serum concentrations of erythropoietin and soluble transferrin receptor were determined by enzyme linked immunosorbent assay (ELISA) (R& D Systems, Minneapolis, MN, USA). Plasma PAI-1 concentration was also determined by ELISA (Innovative Research, Inc., Novi, MI, USA). Serum hepcidin concentration was measured by competitive ELISA as previously described.[17] Genotyping for *VHL*$^{R200W}$ was performed by PCR as previously described.[8]

### Statistics

The primary study comparison was between *VHL*$^{R200W}$ heterozygotes and genotypically normal subjects. Analysis of continuous variables was made by the Student's t-test (after normal transformation) or by analysis of variance with adjustment for significant covariates. Analysis of categorical variables was by Pearson's $\chi^2$ test or by logistic regression with adjustment for other significant variables. Skewed continuous variables were log-transformed to approximate a normal distribution. Analyses were performed with Stata 10.1 (StatCorp., College Station, TX, USA).

### Role of the funding source

Amgen helped in the design of the study but Dr. Gordeuk had full responsibility for implementing and conducting the study, collecting, managing and interpreting the data, and writing the manuscript.

## Results and Discussion

The clinical characteristics of the study participants are summarized in Table 1 according to *VHL* genotype. The mean value for MCV was lower among the *VHL*$^{R200W}$ heterozygotes ($P$=0.033) and the white blood cell counts were higher ($P$=0.036). The serum ferritin concentrations were similar in *VHL*$^{R200W}$ heterozygotes and controls. The mean (standard deviation [SD]) hemoglobin concentration was 134 (14) g/L in the *VHL*$^{R200W}$ heterozygotes and 128 (16) g/L in the controls without a mutated VHL allele ($P$=0.10). The study sample size of 78 has a power of 0.50 to detect the observed 6 g/L difference in hemoglobin concentration between *VHL*$^{R200W}$ heterozygotes and controls at a significance level of $P$<0.05. A sample size of 170 would have a power of 0.8 to detect such a difference and a sample size of 230 would have a power of 0.9.

Using the World Health Organization definition of hemoglobin (less than 130 g/L in males and less than 120 g/L in females),[18] 20 (26%) of the 77 study participants were anemic (Table 2) and the prevalence by gender was 15 (32%) of 47 females and 5 (17%) of 30 males. The anemia was mild and associated with decreases in the MCHC ($P$=0.002), ferritin concentration ($P$=0.007) and hepcidin concentration ($P$=0.009), and an increase in the erythropoietin concentration ($P$=0.06; Table 3), suggesting that the anemia may have been predominantly related to iron deficiency.

The prevalence of anemia was 15% in the *VHL*$^{R200W}$ heterozygotes and 34% in the controls ($P$=0.061 by Pearson's $\chi^2$ test). In a logistic regression analysis that adjusted for

**Table 1. Clinical characteristics of study participants according to VHL genotype.***

| | *VHL*$^{R200W}$ heterozygotes N = 34 | Controls with normal *VHL* alleles N = 44 | *P* |
|---|---|---|---|
| Age (years) | 53 (12) | 53 (12) | 0.9 |
| Female gender | 19 (56%) | 29 (66%) | 0.4 |
| History of smoking | 6 (18%)[1] | 5 (11%) | 0.4 |
| History of alcohol consumption | 8 (24%)[1] | 9 (20%) | 0.7 |
| History of bleeding in the past year | 3 (9%)[1] | 3 (7%) | 0.7 |
| History of thrombosis | 4 (12%)[1] | 5 (11%) | 0.9 |
| BMI (kg/m$^2$) | 26.1 (4.3)[2] | 25.7 (5.2) | 0.4 |
| Systolic blood pressure (mm Hg) | 136 (28)[1] | 134 (24) | 0.9 |
| Diastolic blood pressure (mm Hg) | 87 (14)[1] | 85 (11) | 0.8 |
| Mean blood pressure (mm Hg) | 103 (18)[1] | 101 (15) | 0.8 |
| Hemoglobin (g/L) | 134 (14) | 128 (16) | 0.10 |
| Mean corpuscular volume (fL) | 88 (4.5) | 90 (6.1) | 0.026 |
| White blood cells (×10$^9$/L ) | 7.6 (1.7) | 6.9 (1.5) | 0.036 |
| Platelets (×10$^9$/L) | 238 (69.5) | 258 (56.0) | 0.09 |
| Ferritin (μg/L)** | 62 (24-159) | 56 (21-147) | 0.6 |

*Results in mean (SD) or n. (%). Comparison by Student's t-test or Pearson's $\chi^2$ test. **Geometric mean and SD range. [1]n=33; [2]n=32

**Table 2.** Clinical characteristics of study participants according to the presence or absence of anemia (hemoglobin <130 g/dL men, <120 g/dL women).*

| | Anemia N = 20 | No anemia N = 57 | *P* |
|---|---|---|---|
| Age (years) | 53 (12) | 53 (12) | 0.8 |
| Female sex | 15 (75%) | 32 (56%) | 0.14 |
| *VHL*$^{R200W}$ heterozygote | 5 (25%) | 28 (49%) | 0.061 |
| History of alcohol consumption | 4 (20%) | 13 (23%) | 0.8 |
| History of bleeding in the past year | 3 (15%) | 3 (5%) | 0.16 |
| BMI (kg/m$^2$) | 24.2 (3.3) | 26.5 (5.1) | 0.040 |
| WBC (×10$^9$/L) | 6.7 (1.2) | 7.4 (1.8) | 0.07 |
| Hemoglobin (g/L) | 112 (10) | 138 (11) | --- |
| MCV (fL) | 89 (7.3) | 89 (4.9) | 0.4 |
| MCHC (g/dL) | 32.1 (1.3) | 33.2 (1.9) | 0.002 |
| RDW | 14.0 (3.1) | 13.8 (3.9) | 0.9 |
| Platelets (×10$^9$/L) | 270 (77) | 242 (56) | 0.18 |
| Ferritin (μg/L )** | 33 (12-92) | 71 (30-167)[1] | 0.007 |
| Transferrin receptor (mMol/L)** | 18.4 (12.3-2.0)[2] | 20.0 (15.8-25.4)[3] | 0.06 |
| Erythropoietin (IU/L)** | 12 (7-19) | 9 (6-15) | 0.06 |
| Hepcidin (ng/mL)** | 25 (9-78) | 58 (22-153) | 0.009 |

*Results in mean (SD) or n. (%). Comparison by the Student's *t*-test or Pearson's $\chi^2$ test. **Geometric mean and SD range. [1]n=56; [2]n=16; [3]n=49.

**Table 3.** Products of HIF-regulated genes in study participants according to VHL genotype.*

| | *VHL*$^{R200W}$ heterozygotes N = 34 | *VHL* wildtype controls N = 44 | *P* |
|---|---|---|---|
| Erythropoietin (IU/L)** | 11 (10-12) | 10 (9-10) | 0.18 |
| Hepcidin (ng/mL)*** | 46 (41-53) | 48 (42-55) | 0.8 |
| Plasminogen activator inhibitor-1 (ng/mL) | 3.1 (2 1-4.5)[1] | 2.0 (1.4-2.9)[1] | 0.41 |
| Transferrin receptor (nMol/L)**** | 20.2 (19.5-20.9)[1] | 18.1 (17.5-18.7)[1] | 0.026 |
| Vascular endothelial growth factor (ng/mL) | 17.5 (14.9-20 5)[1] | 12.3 (10.4-14.6)[1] | 0.14 |

*Geometric mean (SE range) from ANOVA. **Adjusted for anemia. Two outliers excluded. ***Adjusted for ferritin and anemia. ****Adjusted for ferritin and anemia. Four outliers excluded. [1]n=33

age, gender, and an interaction between age and gender, the estimated odds of anemia were 5.6-fold lower in the *VHL*$^{R200W}$ heterozygotes than the controls (95% confidence interval 1.4-22.7; *P*=0.017). Among the 5 *VHL*$^{R200W}$ heterozygotes with anemia, one 39-year old woman had a serum ferritin concentration of 6 μg/L and hepcidin of 2.5 ng/mL, indicating iron deficiency, and 3 women and one man had unexplained anemia. Among the 15 controls with anemia, six women 53 years of age or under had serum ferritin concentration less than 20 μg/L and hepcidin less than 21 ng/mL indicating iron deficiency. Two men in the control group had changes consistent with alcohol effect (macrocytosis and a history of alcohol consumption), one man and 4 women under 60 years of age had unexplained anemia, and one man and 2 women over 65 years of age had otherwise unexplained anemia (commonly referred to as 'unexplained anemia of the elderly'). Thus, this study suggests that, in contrast to the deleterious effects of homozygosity for *VHL*$^{R200W}$ in the form of Chuvash polycythemia, heterozygosity for *VHL*$^{R200W}$ may lead to protection from anemia. Based on our data, this protective mechanism may apply to iron deficiency anemia and to anemia in general; whether it is pertinent to anemia of inflammation or anemia of the elderly in particular cannot be answered in this relatively small study. However, these questions could be addressed in a larger study in humans and in experiments utilizing a mouse model of Chuvash polycythemia.[7]

It seems possible that the observed lower risk for anemia in *VHL*$^{R200W}$ heterozygotes may be due to a mild increase in HIF activity in normoxia. HIF transcription factors are known to up-regulate erythropoietin, plasminogen activator inhibitor-1, transferrin receptor, and vascular endothelial growth factor and down-regulate hepcidin.[19,20] Concentrations of these products as adjusted for significant covariates are presented in Table 3. There were higher transferrin receptor concentrations (*P*=0.026), and a trend to higher vascular endothelial growth factor (*P*=0.14) and erythropoietin (*P*=0.18) concentrations among the *VHL*$^{R200W}$ heterozygotes. Hepcidin concentrations did not differ by VHL genotype. Although there were no significant differences in the circulating erythropoietin concentrations between *VHL*$^{R200W}$ heterozygotes and VHL wildtype controls in this study, there is evidence for direct stimulation of erythropoiesis by HIFs independent of erythropoietin concentration, as *VHL*$^{R200W}$ homozygote erythroid progenitors have a heightened response to erythropoietin.[3,21] Evidence from a mouse model suggests that, in addition to promoting erythropoietin and transferrin receptor expression and down-regulating hepcidin expression, HIF likely also stimulates erythropoiesis by an iron-dependent mechanism yet to be defined.[22]

A limitation to this study is the small sample size. To confirm the present findings and identify other possibly subtle benefits of the heterozygous state for an autosomal recessive disease, study of a larger cohort would be required. Another potential drawback is that the overall prevalence of anemia in this study seems high. Information on the hematologic status of adults in Russia in general or Chuvashia in particular is scarce. In our previously published study from Chuvashia,[8] the prevalence of anemia among adult controls from the community was similar to the present study (24% overall, 34% in women, 14% in men) (*V. Gordeuk, unpublished observations, 2011*).

In conclusion, a level of protection from anemia might explain a heterozygote advantage for the *VHL*$^{R200W}$ allele, despite the high mortality of the homozygotes. Furthermore, the data presented here suggest that mild increases of HIF-1 and HIF-2 signaling in normoxia do not compromise erythropoietic activity under normal physiological conditions to a degree that would noticeably change the hemoglobin concentration in healthy Chuvash heterozygotes. However, when a pathological insult increases demand on erythropoiesis, augmented signaling of HIFs in heterozygotes may increase erythropoiesis to a discernible degree, resulting in either prevention or improvement of anemia. Thus, the cumulative effect of *VHL*$^{R200W}$ heterozygosity may provide a degree of protec-

tion from anemia that might afford an advantage for maintaining or even increasing the frequency of this genetic polymorphism in humans. Furthermore, our findings suggest that raising levels of HIF-α by pharmacological inhibition of PHDs in humans might be a safe means to increase erythropoietin levels for the correction and prevention of anemia and/or to produce other potentially beneficial aspects of the hypoxic response.[15]

## Authorship and Disclosures

*The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.*

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