Title

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Journal

American Journal of Hematology, 98(10)

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Publication Date

2023-10-01

DOI

10.1002/ajh.27021

Peer reviewed

HHS Public Access

Author manuscript

Am J Hematol. Author manuscript; available in PMC 2024 October 01.

Published in final edited form as:

Am J Hematol. 2023 October ; 98(10): 1532–1539. doi:10.1002/ajh.27021.

Increased transferrin protects from thrombosis in Chuvash erythrocytosis

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Abstract

Von Hippel-Lindau protein (VHL) is essential to hypoxic regulation of cellular processes. VHL promotes proteolytic clearance of hypoxia-inducible transcription factors (HIFs) that have been modified by oxygen-dependent HIF-prolyl hydroxylases. A homozygous loss-of-function VHL^{R200W} mutation causes Chuvash erythrocytosis, a congenital disorder caused by augmented hypoxia-sensing. Homozygous VHL^{R200W} results in accumulation of HIFs that increase transcription of the erythropoietin gene and raise hematocrit. Phlebotomies reduce hematocrit and hyperviscosity symptoms. However, the major cause of morbidity and mortality in Chuvash erythrocytosis is thrombosis. Phlebotomies cause iron-deficiency, which may further elevate HIF activity and transferrin, the HIF-regulated plasma iron transporter recently implicated in thrombogenesis. We hypothesized that transferrin is elevated in Chuvash erythrocytosis, and that iron-deficiency contributes to its elevation and to thrombosis. We studied 155 patients and 154 matched controls at steady-state and followed them for development of thrombosis. Baseline transferrin was elevated, and ferritin reduced in patients. VHL^{R200W} homozygosity and lower ferritin correlated with higher erythropoietin and transferrin. During 11 years of follow-up, risk of thrombosis increased 8.9-fold in patients versus controls. Erythropoietin elevation, but not hematocrit or ferritin, correlated with thrombosis risk. Unexpectedly, transferrin elevation associated with reduced rather than increased thrombosis risk. The A allele of the promoter *EPO*

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Authorship

Contribution: B.N.S. curated the biological samples and performed laboratory experiments; X.Z. performed genetic analyses; A.I.S. and G.Y.M. enrolled and followed up the research participants; A.I.S., G.Y.M., T.G., J.T.P. and V.R.G. designed the research; B.N.S., X.Z., T.G., J.T.P. and V.R.G. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Ethics approval. This work was approved by the Institutional Review Boards of the participating institutions.

Patient consent. Written informed consent was obtained according to the Declaration of Helsinki.

SNP, rs1617640, associated with elevated erythropoietin and increased thrombosis risk, whereas the A allele of the intronic *TF* SNP, rs3811647, associated with higher transferrin and protection from thrombosis in patients. Our findings suggest an unexpected causal relationship between increased transferrin and protection from thrombosis in Chuvash erythrocytosis.

Keywords

Chuvash erythrocytosis; thrombosis; erythropoietin; transferrin; ferritin; hypoxia-sensing

Introduction

Hypoxia-inducible factors (HIFs) are heterodimeric transcription factors composed of a constitutively expressed HIF-β subunit and one of several HIF-α subunits that are regulated post-translationally, principally by oxygen concentration.[1] Von Hippel Lindau (VHL) protein and prolyl hydroxylase domain proteins are critical to oxygen-related regulation of cellular HIF-α levels, which determine HIF levels and activity. VHL protein mediates rapid proteasomal degradation of HIF-1α and HIF-2α under normoxic conditions.[2] Prolyl hydroxylases are iron-dependent oxygen-requiring enzymes that hydroxylate HIF-α subunits on specific proline residues; this proline hydroxylation is required for HIF-α interaction with VHL[1,3] and degradation in normoxia. Degradation of HIF-α is decreased in response to hypoxia and iron-deficiency, and this leads to increased HIF heterodimers and increased transcription of hypoxia-inducible genes.[1,4]

Chuvash erythrocytosis, a disorder prevalent in the Chuvash Republic of the Russian Federation,[5] is caused by a homozygous R200W substitution in *VHL*.[6] This mutation impairs binding of VHL to HIF-α subunits and therefore decreases ubiquitin-mediated degradation.[3,6] The resulting accumulation of HIF-1 and HIF-2 causes increased transcription of hypoxic response genes such as erythropoietin despite normoxia.[6] Erythropoietin binds to erythropoietin receptor on colony forming unit erythroid progenitors in the bone marrow leading to increased proliferation, maturation, and terminal differentiation,[7] and to erythrocytosis in Chuvash erythrocytosis. An inverse relationship exists between serum erythropoietin concentration and hemoglobin concentration in Chuvash erythrocytosis with a slope identical to this relationship in matched controls,[8] confirming that tissue hypoxia contributes to erythropoietin elevation in addition to VHL^{R200W}.

Homozygotes for VHL^{R200W} have increased incidence of stroke and other thromboembolic events,[8] but the cause for increased thrombosis is not clear. Many potentially thrombogenic HIF-regulated transcripts, including tissue factor, are differentially upregulated in peripheral blood mononuclear cells, granulocytes and reticulocytes of patients,[9–12] potentially causing a pro-thrombotic milieu.[11–13]

Iron chelators inhibit the activity of prolyl hydroxylases that would hydroxylate HIF-α-subunits and would direct them to proteasomal degradation; the increased activity of HIFs after iron chelator treatment confirms the iron-dependence of HIF degradation.[14,15] Iron deficiency itself has been implicated as a risk factor for thromboembolism[16] that may be

further exacerbated by associated thrombocytosis.[17] Patients with Chuvash erythrocytosis are sometimes treated with repeated phlebotomies, which lead to iron deficiency. This further increases HIF levels by reducing iron-dependent prolyl hydroxylase activity and it increases transcription of prothrombotic genes.[18] Transferrin, the hepatocyte-produced plasma iron carrier protein, is induced by both hypoxia and iron deficiency via HIF-1[19] and is upregulated in Chuvash erythrocytosis.[6] Increased transferrin has been reported to exert prothrombotic effects in the setting of atherosclerosis, possibly by binding to thrombin, factor XII, fibrinogen, and antithrombin-3, by promoting the enzymatic activity of thrombin and factor XIIa, and by blocking anti-thrombin activity.[20,21]

We previously reported a matched cohort study of 155 patients with Chuvash erythrocytosis and 154 age and sex-matched Chuvash controls in which there was a history of thrombosis in 27 Chuvash erythrocytosis patients and three controls at study entry.[22] Over a prospective follow-up of 11 years, 33 patients experienced 37 new thrombotic events, nine of them fatal, and four controls experienced five new events, two of them fatal. VHL^{R200W} homozygosity but not hematocrit was a strong independent predictor of thrombosis in this study.[22] We were unable to assess the relationships of erythropoietin and iron status to thrombotic risk because circulating erythropoietin and ferritin measurements were not available for many participants and transferrin measurements were not performed. We now report circulating erythropoietin and ferritin concentrations for nearly all subjects in the Chuvash matched cohort and for the first-time also report transferrin concentrations.

Here we tested the hypothesis that circulating erythropoietin and transferrin concentrations in Chuvash erythrocytosis have independent relationships to the VHL^{R200W} mutation, to hypoxia as reflected in the hematocrit, and to iron deficiency as reflected by the serum ferritin concentration. Therapy with recombinant erythropoietin and other erythropoiesis-stimulating agents has been associated with thrombotic complications, especially venous thromboembolism.[23] It has been reported that transferrin is sequestered by binding to fibrinogen at a molar ratio of 4:1 in normal conditions and that abnormally upregulated transferrin induces hypercoagulability by potentiating thrombin/factor XIIa and inhibiting antithrombin.[20,21] Elevated transferrin has been implicated in thrombosis associated with iron deficiency and thrombocytosis,[24,25] atherosclerosis,[21] high altitude[26] and Covid-19 infection,[27] but other evidence points to possible protection from thrombosis.[28,29] Therefore, we also tested the hypothesis that the severity of erythropoietin and transferrin increase may be independent markers for risk of thrombosis in Chuvash erythrocytosis. Genetic variation affects serum concentrations of erythropoietin and transferrin.[30,31] Thus, we evaluated whether erythropoietin gene (*EPO*) and transferrin gene (*TF*) single nucleotide polymorphisms (SNPs), identified in previous GWAS to influence gene expression,[30,31] affect erythropoietin and transferrin levels and risk of thrombosis in this study.

Methods

Study design

We conducted a study of persons with the Chuvash erythrocytosis and controls matched by age, sex, residency and ethnicity in the Chuvash Republic of the Russian Federation (elevation <230 m).[22] Written informed consent was obtained according to the Declaration

of Helsinki. This work was approved by the Institutional Review Boards of the participating institutions.

Study participants

Subjects were enrolled between 2005 and 2009 and followed for 0.2–12.3 years for thrombotic events (median 10.9 years). Subjects were not hospitalized, were at steady-state, and at least four weeks had elapsed since any previous phlebotomy. Complete blood counts were performed by an automated analyzer (Sysmex XT 2000i; Sysmex Corporation). Serum samples and plasma from EDTA tubes were collected and stored at −80°C.

Erythropoietin

Erythropoietin levels were measured in 217 serum and 23 plasma samples using the U-Plex Human EPO Assay (Meso Scale Diagnostics, LLC, KV151VXK). We previously reported serum erythropoietin levels for 118 subjects as measured with enzyme immunoassay (R&D Systems, Minneapolis, MN, USA) in Chuvashia.[32] The values obtained with the new methodology correlated linearly with the previously reported values (Pearson R=0.87, P<0.00001). Results for erythropoietin measured in three serum and plasma samples collected at the same time also corelated (Pearson R = 0.99). Fifty of the results from Chuvashia were combined with the 240 results from our lab to give a total of 290 erythropoietin results (Table 1).

Ferritin

Ferritin was quantified in duplicate in 217 serum and 24 plasma samples using Roche Elecsys ferritin reagent (Roche Diagnostics 03737551) on a Meso Scale SQ120MM platform (Meso Scale Diagnostics, LLC). We previously reported serum ferritin levels for 118 of these subjects as measured with enzyme immunoassay (Ramco Laboratories Inc., Stafford, TX, USA) in Chuvashia.[32] The values obtained from the new methodology closely related to the previously reported values (R=0.98, P<0.00001). Results for ferritin measured in four serum and plasma samples collected at the same time also corelated (Pearson R = 0.97). For the present analysis, we included values for 49 samples measured in Chuvashia.[32]

Transferrin

Transferrin was measured in duplicate in 217 serum and 47 plasma samples using a Tina-quant Transferrin immunoturbidimetry assay (Roche Diagnostics, 03015050) on a Spectramax iD3 (Molecular Devices, LLC). Three controls were run with each plate: Lyphochek Anemia Control (Bio-Rad, 500X), Liquichek Immunology Control, Level 2 (Bio-rad, 592) and PreciControl ClinChem Multi (Roche Diagnostics, 05947626 160). The inter-assay CV (n=9 plates) was 7%. Transferrin can routinely be measured in either serum or EDTA plasma, but to document the consistency of determinations in plasma versus serum in our study we measured transferrin in plasma and serum samples collected from five research subjects at the same time: the mean values were 384 mg/dL in serum and 386 mg/dL in plasma and the Pearson correlation was 0.94.

EPO and *TF* Genotypes

Genomic DNA isolated from peripheral blood mononuclear cells of 144 Chuvash erythrocytosis patients was profiled on Infinium Global Screening Array-24 v1.0 (Illumina). Summarized feature intensities for each allele and each strand of the interrogated single nucleotide polymorphisms (SNPs) were parsed using R package “illuminaio”. Log feature intensity distribution was assessed per strand, based on which three samples were removed from further analysis due to inferior signal to noise ratio.[33] Log probe intensities were quantile normalized. Genotype calling used CRLMM algorithms.[33] Briefly, log intensity ratios of the two alleles were fitted for each sample to a mixture model to correct for SNP-specific hybridization effects and obtain initial genotype calls. SNP-specific genotype regions were estimated and updated through a two-level hierarchical model across samples. Final genotypes were called based on likelihood-based distance, with a minimum log likelihood ratio of 5 for homozygous calls and 1 for heterozygous calls. All 141 samples had <5% call missingness. SNPs with call rate <95% or minor allele frequency <0.01 or Hardy-Weinberg disequilibrium (P<0.0001) were removed. Autosomal SNPs for 141 patients were then examined for identity-by-descent using PLINK 1.9[34] and genetic relationships using GCTA.[35] One population outlier and three duplicated samples were further removed. SNPs of 137 samples were then phased using Shapeit 2[36] and imputed using Impute 2[37] to 1000 genomes phase 3 data with European population samples as references.

Statistical Analysis

Results for categorical variables are presented as number (%) and comparisons were made with Fisher exact test. Results for linear variables are presented as median and interquartile range, and comparisons were made with the Kruskal-Wallis test. Multiple linear regression was used to determine independent predictors of erythopoietin and transferrin concentrations. Cox proportional hazards analysis was used to determine the independent relationships of erythropoietin, ferritin, and transferrin to thrombosis in the context of homozygosity for the VHL^{R200W} mutation. Genetic associations for erythropoietin in Chuvash erythrocytosis patients used a linear model adjusted for gender and hemoglobin, and for *EPO* SNP association with follow-up thrombosis a Cox model adjusted for age, gender, and transferrin. Genetic associations for transferrin in patients used a linear model adjusted for log ferritin, and for *TF* SNP association with follow-up thrombosis a Cox model adjusted for age, gender, and log erythropoietin.

Results

Chuvash erythrocytosis patients and control subjects at study entry (Table 1)

Fifty-three percent of participants were females. Median ages were 34 years for patients and 35 years for controls. More patients had a history of smoking than controls (27% versus 15%) and 68% of patients had a history of phlebotomy therapy. As previously reported, compared to matched controls, Chuvash erythrocytosis patients had lower blood pressure (median mean arterial pressures of 88 vs. 93 mm Hg; p=0.001), random glucose concentrations (medians of 57 vs. 76 mg/dL; p=0.0004), hemoglobin A1C% (medians of 3.3 vs 4.1%; p=0.0004), neutrophil counts (medians of 2.9 vs 3.5 x 10^3/μL; p=0.00002) and platelet counts (medians of 224 vs. 257 x 10^3/μL; p=0.00004) and higher hematocrits

(medians of 53.5 vs 40.1 %; p<0.000001). The erythropoietin concentration was higher in patients compared to controls despite their having much higher hematocrits (medians of 42.3 vs 8.5 IU/L, p<0.000001), while circulating ferritin concentrations were lower (medians of 11 vs. 43 μg/L, p<0.000001). Consistent with its known induction by hypoxia via HIF-1[19] and our previous report,[6] transferrin levels were higher in the participants with Chuvash erythrocytosis (medians of 390 vs 296 mg/dL, p<0.000001). Fibrinogen levels were marginally lower in patients (medians of 285 vs. 330 mg/dl, p=0.019), which contrasts with a recent report that people living at high altitude have higher fibrinogen levels than low altitude dwellers.[26]

Independent predictors of erythropoietin and transferrin levels

In multivariate linear regression analysis (Online Supplementary Table 1), VHL^{R200W} homozygosity had the strongest correlation with erythropoietin concentration, but lower ferritin and hematocrit were also related in addition to older age, female gender, and smoking history. In a similar analysis, VHL^{R200W} homozygosity had the strongest relationship to transferrin concentration, but lower ferritin concentration and younger age were also independent predictors.

Development of a new thrombosis over a median of eleven years of prospective follow-up

Twenty-seven (17.4%) of Chuvash erythrocytosis patients but only three controls had a history of thrombosis at enrollment. Patients with past thrombosis were older and had a higher BMI. Over 11 years of follow-up, new thrombosis occurred in 33 of 155 patients and 4 of 154 matched controls (P<0.000001). The new thrombotic events included cerebral vascular accident, deep vein thrombosis in extremties, mesenteric thrombosis, myocardial infarction, portal vein thrombosis, stroke, lung and pulmonary embolism.[22] Patients who developed a follow-up thrombosis were older (medians of 42 vs 30 years, p=0.0003), more often had been treated with phlebotomy (medians of 94% vs. 62%, p=0.002) and more often had a history of thrombosis (medians of 46 vs. 10%, p=0.00001) (Table 2). Baseline erythropoietin concentrations were higher (medians of 83 vs. 39 IU/L, p=0.0008) and baseline transferrin concentrations lower (medians of 357 vs. 402 mg/dL, p=0.0002) in those who developed a thrombosis with no difference in baseline hematocrits or ferritins. Fibrinogen levels did not differ in patients who developed a new thrombosis and the molar ratio of transferrin to fibrinogen trended lower rather than higher in those who developed a thrombosis. Multivariate Cox proportional hazards analysis confirmed that baseline elevation of erythropoietin was an independent predictor of developing new thrombosis (hazards ratio 1.59, 95% confidence interval 1.11-2.28) while baseline elevation of transferrin was an independent predictor of protection from new thrombosis (hazards ratio 0.29, 95% confidence interval 0.19-0.71) (Table 3).

Genetic variation of *EPO* and *TF* associates with circulating concentrations of these proteins and with risk of thrombosis (Table 4).

SNP rs1617640, located in the promoter region of *EPO*, was recently reported to be associated with circulating erythropoietin level in a meta-analysis of four cohorts with 6127 individuals.[30] The A allele of rs1617640 also associated with elevated level of erythropoietin in Chuvash erythrocytosis patients in the present study (P=0.0006) and with increased risk

of follow-up thrombosis (P=0.023). The increase per unit log EPO (IU/L) by the presence of rs1617640 was associated with a hazard ratio of 6.3 (95% CE 1.2-34) of follow-up thrombosis (P=0.032), estimated by the two-stage least squares method.[38]

Two SNPs located within the *TF* gene, rs3811647 and rs1830084, were previously identified in a genome-wide association study of serum transferrin in Australians.[31] The A allele of the lead SNP (rs3811647), an intronic SNP of *TF,* associates with increased serum transferrin concentration.[31] These two SNPs were linked in Chuvash erythrocytosis patients (r^2=0.95). The A allele of rs3811647 also associated with elevated circulating transferrin concentration in Chuvash erythrocytosis patients (P=0.059) and with reduced risk of developing a new thrombosis during the 11-year follow-up period (P=0.040). The increase by the presence of rs3811647 per mg/dL serum transferrin was associated with a hazard ratio of 0.95 (95% CI 0.90-1.0) of follow-up thrombosis (P=0.065).

Discussion

In addition to arterial and venous thrombosis, *VHL*^{R200W} homozygosity is associated with lower systemic blood pressure, higher pulmonary artery pressure, varicose veins, hemangiomas, lower leukocyte and platelet counts, increased inflammatory cytokines, lower glucose and Hb A1C, changes in plasma thiol concentrations, major bleeding episodes, and premature mortality. Malignant tumors typical of classical VHL tumor predisposition syndrome and increased risk of other cancers have not been described,.[8,32,39,40] Potentially thrombogenic HIF-regulated transcripts are differentially upregulated in Chuvash erythrocytosis peripheral blood mononuclear cells, including interleukin 1-beta, thrombospondin-1, NLR family pyrin domain containing 3, plasminogen activator inhibitor-1, and tissue factor.[9,11,12] Differential gene expression also exists in granulocytes and reticulocytes. Thus, increased HIFs likely cause a pro-thrombotic milieu.[13]

Baseline erythropoietin was two-fold higher in patients who developed a new or recurrent thrombosis over 11 years of follow-up, and this represented a hazards ratio of 1.7 on multivariate analysis (Table 3; P=0.0008). Our finding that SNP rs1617640, located in in *the EPO* promoter region, associated with erythropoietin level and thrombotic risk, suggests that elevated erythropoietin may have a direct relationship with thrombosis in Chuvash erythrocytosis. Increased erythropoietin would also reflect augmentation of HIF levels that facilitate increased expression of prothrombotic genes with accompanied prothrombotic state as in polycythemia vera[11,12] and high altitude hypoxia.[41] Therapy with recombinant erythropoietin has been associated with venous thromboembolism.[23]

We examined the role of iron deficiency as measured by ferritin in upregulating the hypoxic response as reflected in erythropoietin and transferrin levels. Transferrin elevation is a classic feature of iron deficiency that may be induced by both hypoxia and iron deficiency via HIF-1.[19] Impaired degradation of HIF-α subunits due to homozygous *VHL*^{R200W} and impaired function of prolyl hydroxylase-2 due to iron deficiency have additive effects in increasing HIF signaling. In point of fact, iron chelation was found to increase erythropoietin levels prior to the discovery of HIFs.[15] Iron deficiency inhibits the function of prolyl hydroxylase-2 in the hydroxylation of HIF-α subunits that is necessary

for recognition and degradation by VHL[1,2] and may therefore enhance hypoxic responses. At the same time, iron deficiency may repress the translation of HIF-2α protein through the action of iron regulatory proteins[42] and thereby decrease HIF-2-mediated hypoxic responses. We found that in addition to the homozygous *VHL*^{R200W} mutation, lower iron stores as reflected in lower ferritin concentrations were highly predictive of elevated erythropoietin and elevated transferrin (Online Supplementary Table 1).

Transferrin was reported to have prothrombotic effects,[20,21] but there is also evidence that higher circulating transferrin may be protective from thromboembolism.[28] Here we examined how iron status relates to risk of thrombosis over 11 years of follow-up. Specifically, we asked whether low serum ferritin concentrations and/or elevated transferrin concentrations add to the thrombotic risk in Chuvash erythrocytosis. In multivariate analysis (Table 3), we found that circulating ferritin concentration did not correlate with thrombotic risk, but that higher circulating transferrin concentration was protective from thrombosis. Furthermore, an intronic *TF* SNP, rs3811647, previously reported to be associated with elevated serum transferrin in Australians,[31] was associated with higher transferrin levels in this study and with protection from the development of new thrombosis during the prospective follow-up period, implying a potential causal relation between increased transferrin and lower risk for thrombosis. These findings suggest that high transferrin, perhaps because of transferrin's role in binding and neutralizing potentially toxic forms of iron associated with non-transferrin carriers (non-transferrin bound iron, NTBI) may have an anti-thrombosis effect in Chuvash erythrocytosis. We previously reported that, at a given serum ferritin concentration, serum hepcidin concentrations are lower in *VHL*^{R200W} homozygotes than controls;[43] this might imply an increased tendency for enterocytes, macrophages and hepatocytes to release iron to plasma via ferroportin in Chuvash erythrocytosis. *VHL*^{R200W} homozygotes in that report had been frequently phlebotomized and the geometric mean serum ferritin (a measure of iron stores) was less than one-fourth the value in controls while the geometric mean values for serum iron and transferrin saturation (reflecting iron circulating in plasma) were more than half the value in controls.

It remains to be established if the antithrombotic effect of transferrin is present in other conditions. Thromboses are the major cause of morbidity and mortality in polycythemia vera.[11,12] wherein significant dysregulation of iron metabolism exist,[11,44] and they are important complications in therapy with prolyl hydroxylase inhibitors,[45] wherein the activity of HIFs is augmented. Treatment of polycythemia vera therapy by hepcidin analogues may also lead to augmentation of HIF activity.[46] The expression of Kruppel-like factor 2 (KLF2) is decreased in polycythemia vera neutrophils and platelets and this likely contributes to thrombosis.[47] KLF2 is a transcription factor downregulating expression of genes associated with inflammation and thrombosis. Knockdown of *KLF2* in endothelial cells increases prothrombotic gene expression including *F3,* encoding tissue factor, and *SERPINE1*, encoding plasminogen activator inhibitor 1; low neutrophil KLF2 activates neutrophils, induces tissue factor and leads to arterial and venous thromboses.[48–50] Whether, the KLF2 level is decreased in Chuvash erythrocytosis and also contributes to thromboses remains to be established by future studies.

A limitation to this study is that we measured only baseline circulating concentrations of erythropoietin and transferrin while the study follow-up continued for a median of almost 11 years. However, the promoter SNP of *EPO* and the intronic SNP of *TF* that contribute to the elevated circulating concentrations of erythropoietin and transferrin also significantly influenced the risk of thrombosis- increased in the case of erythropoietin and decreased in the case of transferrin. The effect of these SNPs would be constant throughout the observation period.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by grants from Amgen Corporation, Incyte Corporation (V.R.G.), and the National Institutes of Health (grants P01CA108671 and R01 HL137991) (J.T.P.) (grants 1P50HL118006 and R01HL079912} (V.R.G.)

Data availability:

Requests for data should be addressed to the corresponding author.

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Table 1.

Baseline clinical characteristics in Chuvash erythrocytosis patients and controls. Results in number (%) or median (interquartile range). Significance determined by the Fisher exact test for proportions and the Kruskal-Wallis test for continuous variables.

	Chuvash erythrocytosis		Chuvash controls		P
	N	Result	N	Result	
Female gender	155	82 (52.9%)	154	81 (52.6%)	1.0
Age (years)	155	34.1 (21.0-47.2)	154	35.5 (21.0-48.0)	0.71
Mean arterial pressure (mm Hg)	155	88 (81-97)	154	93 (87-100)	0.001*
Body mass index (kg/m^2)	155	21.0 (19.4-23.5)	154	22.6 (20.1-25.7)	0.001*
Smoking history, enrollment	155	42 (27.1%)	154	23 (14.9%)	0.012
History of thrombosis, enrollment	155	27 (17.4%)	154	3 (2.0%)	0.000005*
Phlebotomy therapy	155	106 (68.4%)	154	0 (0%)	---
Hematocrit (%)	141	53.5 (48.5-58.8)	153	40.1 (36.8-43.0)	<0.000001*
Mean corpuscular volume (fL)	136	82.3 (74.7-87.9)	153	86.7 (83.0-90.1)	<0.000001*
White blood cells (x 10^3/μL)	154	5.7 (4.6-7.1)	154	6.4 (5.4-7.6)	0.0001*
Neutrophils (x 10^3/μL)	119	2.9 (2.2-3.7)	148	3.5 (2.9-4.4)	0.00002*
Platelets (x 10^3/μL)	154	224 (173-281)	154	257 (218-322)	0.00004*
Random glucose (mg/dL)	60	57 (36-75)	44	76 (65-84)	0.0004
HbA1C (%)	50	3.3 (3.1-3.7)	72	4.1 (3.3-4.6)	0.0004*
Erythropoietin (IU/L)	143	42.3 (25.4-92.9)	147	8.5 (6.1-10.8)	<0.000001*
Transferrin (mg/dL)	140	390 (351-424)	124	296 (256-341)	<0.000001*
Ferritin (μg/L)	143	11 (8-21)	147	43 (22-73)	<0.000001*
Fibrinogen (mg/dL)	73	285 (238-340)	63	330 (251-391)	0.019
Transferrin to fibrinogen molar ratio	71	5.9 (4.8-7.2)	59	4.1 (3.2-5.2)	<0.00001

*Significant after Bonferroni correction

Table 2.

Baseline clinical characteristics in Chuvash erythrocytosis patients who developed a new thrombosis over 11 years of follow-up versus those who did not. Results in number (%) or median (interquartile range). Significance determined by the Fisher exact test for proportions and the Kruskal-Wallis test for continuous variables.

	New thrombosis		No new thrombosis		P*
	N	result	N	result	
Female gender	33	15 (45.5%)	122	67 (54.9%)	0.43
Age at baseline (years)	33	42 (34-53)	122	30 (19-44)	0.0003*
Mean arterial pressure (mm Hg)	33	91 (86-99)	122	87 (80-96)	0.036
BMI	33	22.4 (19.9-25.3)	122	20.8 (19.1-23.0)	0.025
Smoking history, enrollment	33	12 (36.4%)	122	30 (24.6%)	0.19
History of phlebotomy therapy	33	31 (93.9 %)	122	75 (61.5%)	0.002*
Past history of thrombosis	33	15 (45.5%)	122	12 (9.8%)	0.00001*
White blood cells ($x10^3/\mu L$)	32	5.0 (4.4-7.2)	122	5.9 (4.7-7.0)	0.28
Hematocrit (%)	32	52.2 (47.8-58.2)	122	55.2 (49.3-59.6)	0.23
Mean corpuscular volume (fL)	28	82.8 (74.2-90.1)	108	82.2 (74.7-87.5)	0.64
Neutrophils	22	2.7 (1.8-3.3)	97	3.0 (2.3-3.8)	0.28
Platelets	32	222 (161-289)	122	226 (179-280)	0.49
Glucose (mg/dL)	14	57.5 (47-75)	46	54.1 (36-75)	0.79
Hb A1C(%)	11	3.3 (2.9-3.8)	39	3.3 (3.1-3.7)	0.95
Erythropoietin (IU/L)	32	82.8 (39.1-115.4)	111	38.9 (20.9-74.5)	0.0008*
Ferritin (µg/L)	32	12 (9-36)	111	11 (7-20)	0.3
Transferrin (mg/dL)	31	357 (325-385)	109	402 (361-435)	0.0002*
Fibrinogen (mg/dL)	14	305 (222-400)	59	285 (240-335)	0.90
Transferrin to fibrinogen molar ratio	14	4.9 (4.1-6.2)	57	5.9 (4.9-7.3)	0.051

*Significant after Bonferroni correction

Table 3.

Hazards ratio (95% confidence interval) for new thrombosis in patients with Chuvash erythrocytosis.

Baseline values	Hazards ratio (univariate)	P	Hazards ratio (multivariate*)	P	Hazards ratio (multivariate)**	P
Erythropoietin (IU/L; natural log)	1.69 (1.23-2.33)	0.001	1.71 (1.25-2.34)	0.0008	1.59 (1.11-2.28)	0.011
Hematocrit (%)	0.97 (0.94-1.01)	0.21	0.99 (0.94-1.03)	0.51	0.98 (0.94-1.02)	0.41
Ferritin (µg/L; natural log)	1.37 (.925-2.03)	0.12	1.08 (0.73—1.61)	0.70	1.25 (0.83—1.88)	0.29
Transferrin (mg/ml)	0.28 (0.15-0.53)	0.0009	0.28 (0.12- 0.63)	0.002	0.29 (0.19- 0.71)	0.007

*Erythropoietin, hematocrit, ferritin, and transferrin are all included in the model.

**Additionally adjusted for age at baseline, past history of phlebotomy, and past history of thrombosis.

Table 4.

Genetic variation in *EPO* and *TF*

A. Relationship of genetic variation in *EPO* promoter to log (EPO) concentration and to thrombosis.								
SNP	**Reference allele**	**Effect allele**	**Effect allele frequency**	**Imputation r^2**	**Log (EPO)**		**Follow-up thrombosis**	
					β (95% CI)	**P**	**HR (95% CI)**	**P**
rs1617640	C	A	0.52	1.00	0.38 (0.17-0.59)	0.00062	1.8 (1.1-3.0)	0.023
B. Relationship of genetic variation in *TF* to transferrin concentration and to thrombosis.								
SNP	**Reference allele**	**Effect allele**	**Effect allele frequency**	**Imputation r^2**	**Transferrin**		**Follow-up thrombosis**	
					β (95% CI)	**P**	**HR (95% CI)**	**P**
rs3811647	G	A	0.40	1.00	15 (−0.42-30)	0.059	0.51 (0.27-0.97)	0.040
rs1830084	A	T	0.39	0.997	12 (−3.7-27)	0.14	0.55 (0.29-1.0)	0.067