Thrombotic risk in congenital erythrocytosis due to up-regulated hypoxia sensing is not associated with elevated hematocrit

Thrombosis is a common complication in Chuvash erythrocytosis, the first genetic disorder of up-regulated hypoxia sensing to be discovered. We present an update supporting the concept that the occurrence of thrombosis in Chuvash erythrocytosis is independent of hematocrit and that the thrombotic risk seems to be increased by phlebotomy. We also present a six-generation pedigree with HIF-2α-mutated dominant erythrocytosis in which there is a high rate of thrombosis despite strict control of the hematocrit by phlebotomy. These results are consistent with the concept that inherited causes of erythrocytosis due to up-regulated hypoxia sensing are accompanied by physiological changes that could directly affect thrombotic risk, irrespective of the elevated hematocrit.

Increased erythropoietin (EPO) is the most common cause of secondary erythrocytosis. The transcription of the *EPO* gene is regulated by hypoxia *via* hypoxia inducible factors (HIF). Of these, HIF-2 is the principal regulator of *EPO* transcription. Congenital secondary erythrocytosis can be caused by relative tissue hypoxia (e.g. from high oxygen affinity hemoglobin variants, inherited low 2,3-BPG, or congenital methemoglobinemia) or by mutations that lead to inappropriate augmentation of hypoxia sensing. These hypoxia sensing pathway mutations include recessive loss-of-function mutations of *VHL* (encoding von Hippel Lindau protein, VHL) or *EGLN1* encoding prolyl hydroxylase 2 (PHD2), and dominant gain-of-function mutations of *EPAS1* encoding HIF-2α.[1] VHL and PHD2 are the principal negative regulators of HIF-1 and HIF-2, and these *VHL* and *EGLN1* mutations, unlike *EPAS1* mutations, lead to increase of both HIF-1 and HIF-2. Loss-of-function germ-line mutations of *VHL* and gain-of-function mutations of *EPAS1* can cause either erythrocytosis or a tumor predisposition syndrome. The molecular basis for these differences is not clear in *VHL* mutations,[1] but are better elucidated in HIF-2α-driven disease.[2]

Table 1. Predictors of new thrombosis during 11 years of follow up in a registry of 155 Chuvash erythrocytosis (CE) subjects and 154 matched controls.

	No new thrombosis	New thrombosis	Hazards ratio (univariate)	*P*	Hazards ratio (multivariate)	*P*
Combined analysis of CE subjects and controls						
N	272	37*				
Age (years)	33 (±16)	45 (±15)	1.04 (1.02-1.06)	<0.0001	1.06 (1.03-1.09)	<0.0001
Male sex	125 (46%)	21 (57%)	1.5 (0.8-2.9)	0.202	2.9 (1.3-6.4)	0.0095
Past history of thrombosis at enrollment:			3.0 (2.3-4.0)	<0.0001	2.0 (1.4-2.7)	<0.0001
0 events	256 (94%)	22 (59%)				
1 event	14 (5%)	7 (19%)				
2 events	1 (0.4%)	4 (11%)				
3 events	0 (0%)	4 (11%)				
History of smoking	52 (19%)	13 (35%)	2.2 (1.1-4.3)	0.022	1.5 (0.7-3.1)	0.34
Hematocrit (%)	46.5 (±9.9)	51.2 (±7.8)	1.04 (1.01-1.07)	0.008	0.95 (0.92-0.99)	0.026
VHLR200W homozygote	122 (45%)	33 (89%)	8.9 (3.2-25.2)	<0.0001	13.8 (4.2-45.7)	<0.0001
Analysis restricted to CE subjects						
N	122	33				
Age (years)	32 (±16)	43 (±14)	1.04 (1.02-1.06)	0.0004	1.04 (1.00-1.07)	0.027
Male sex	55 (45%)	18 (55%)	1.4 (0.7-2.8)	0.32	1.6 (0.7-3.8)	0.29
Past history of thrombosis at enrollment:			2.4 (1.8-3.2)	<0.0001	2.2 (1.6-3.2)	<0.001
0 events	110 (90.%)	18 (55%)				
1 event	11 (9%)	7 (21%)				
2 events	1 (0.8%)	4 (12%)				
3 events	0 (0%)	4 (12%)				
N. of cigarettes smoked per day past year	2.7 (±5.9)	5.1 (±7.6)	1.04 (1.00-1.09)	0.057	1.04 (0.99-1.10)	0.15
Past history of phlebotomy at enrollment:			2.0 (1.2-3.2)	0.004	1.9 (1.1-3.5)	0.028
0- none	46 (38%)	2 (6%)				
1- >1 year ago	25 (21%)	11 (33%)				
2- in past year	51 (42%)	20 (60%)				
Treatment with aspirin	44 (37%)	15 (46%)	1.3 (0.6-2.5)	0.52	0.9 (0.4-2.0	0.87
Hematocrit (%)	54.6 (±8.8)	52.5 (±7.1)	0.98 (0.94-1.02)	0.22	0.96 (0.92-1.00)	0.052

*33 Chuvash erythrocytosis subjects and four controls experienced at least one new thrombosis during follow up. Baseline characteristics according to the development of new thrombosis during follow up and hazards ratio (95% confidence interval) for first new thrombosis. Results for baseline characteristics in mean±Standard Deviation (SD) or number (N/n) (%) for baseline characteristics in mean (±SD) or n (%).

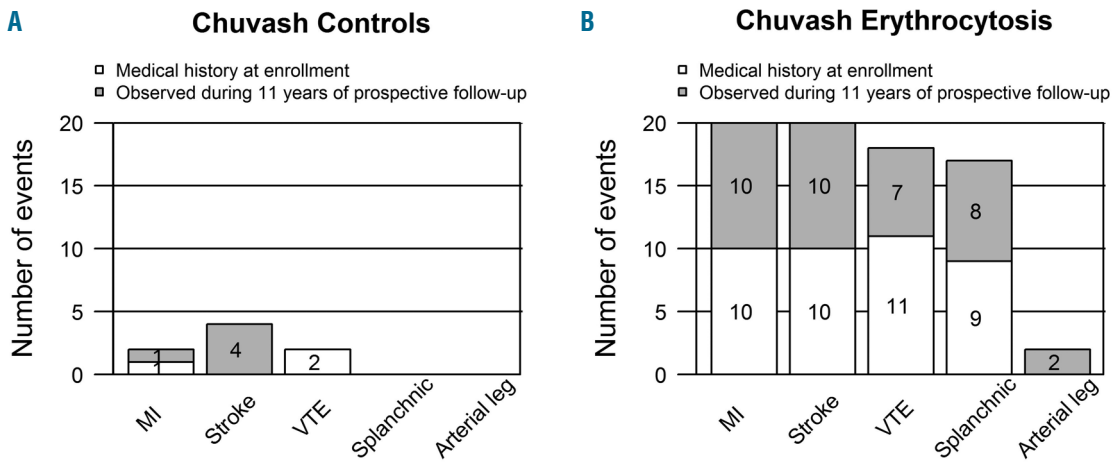

Figure 1. Thrombosis in Chuvash erythrocytosis (CE) and Chuvash control subjects by history at time of enrollment and prospectively during 11 years of observation. (A) Three of 154 controls had a history of thrombosis at study entry. Four controls developed five new thromboses during the observation period. (B) Twenty-seven of 155 CE subjects had a history of 40 thromboses at the time of enrollment. Thirty-three patients developed 37 new thromboses during the eleven-year observation period. MI: myocardial infarction; VTE: venous thromboembolism.

Chuvash erythrocytosis (CE) is a worldwide condition, with increased prevalence in Chuvashia in Russia and Ischia in Italy, that is caused by homozygosity for a C→T missense mutation in *VHL* (*VHL* c.598C>T; resulting in VHL p.R200W).[3-5] The mutation impairs interaction of VHL with the HIF-α subunits, thereby reducing the rate of ubiquitin-mediated HIF-α degradation by the proteasome. Consequently, the levels of HIF-1 and HIF-2 heterodimers increase, leading to increased expression of many target genes, including *EPO*.[4,6-8] CE patients have increased EPO levels mediated by increased HIF-2, a feature of secondary erythrocytosis.[4] CE erythroid progenitors also display hypersensitivity to EPO, a feature of primary erythrocytosis.[1,4] To explain this hypersensitivity, it has been proposed that the VHL protein with the p.R200W mutation hinders suppression of cytokine signaling (SOCS1)-mediated JAK2 degradation.[9] This does not explain the fact that another closely positioned VHL erythrocytosis mutation, p.H191D, is not associated with EPO hypersensitivity[10] while other, more upstream, mutations such as VHLP138L are associated with increased sensitivity of erythroid progenitors to EPO.[11]

Individuals with CE have a propensity for both arterial and venous thrombosis at sea level that is comparable to or even higher than that of polycythemia vera.[7,12,13] The p.R200W variant in VHL protects from anemia in heterozygotes[14] but causes augmented hypoxia sensing with elevated hematocrit in homozygotes.[4,7] It is not associated with tumors characteristic of the von Hippel-Lindau tumor predisposition syndrome. Thrombosis largely accounts for the morbidity and mortality in CE, although affected individuals have lower body mass index, systolic blood pressure, blood glucose, HbA1c, white blood cell count and platelet count compared to controls.[13,15] The high rate of thrombosis in CE begins in childhood[12] and increases with age.[13] Many HIF-regulated genes that are potentially thrombogenic are differentially up-regulated in CE peripheral blood mononuclear cells, including *IL1B*, encoding interleukin 1-beta, *TSP1*, encoding thrombospondin-1, *NLRP3*, encoding NLR family pyrin domain containing 3, *SERPINE1*, encoding plasminogen activator inhibitor-1 (PAI-1), and *F3* encoding tissue factor (TF).[6] There is also a differential gene expression in granulocytes and reticulocytes, and plasma TSP-1 concentrations are increased.[13] Thus, increased HIF may cause a prothrombotic milieu in CE.[16] Here we provide new data that question hematocrit as the primary risk factor for thrombosis in CE and in erythrocytosis secondary to HIF-2α gain of function mutations.

We analyzed 155 CE adult and pediatric patients and 154 matched controls followed for a median of 11 years. Previously, the findings in adults (n=13) and children (n=12) had been reported separately with a median follow up of nine years. The present report includes the same patients, increases the duration of follow up by more than 20%, and provides a combined analysis with more robust power to identify the predictors of thrombosis. There was a history of 40 thrombotic events in 27 CE subjects at enrollment, and 37 new events occurred in 33 subjects during the prospective 11-year observation (Figure 1), nine of which were fatal. There was a history of thrombosis in only three controls at enrollment, and five new events developed in four subjects during observation. Among the patients and controls, homozygosity for the c.598C>T *VHL* variant was a stronger predictor of new thrombosis over 11 years of follow up than baseline hematocrit in univariate Cox proportional hazards analysis (P=3.6×10^{-5} *vs.* P=0.008) (Table 1A). Age and history of past thrombosis or cigarette smoking were also predictors of new thrombosis in univariate analysis. In a multivariate Cox proportional hazards analysis that included all of these variables, homozygosity for the c.598C>T *VHL* variant was a strong independent predictor of new thrombosis (hazard ratio 13.8, P<0.0001). Higher hematocrit tended to be independently associated with a lower risk of thrombosis (hazard ratio 0.95, P=0.026) (Table 1A). In analyses restricted to CE subjects, age and past thrombosis were independent predictors of new thrombosis. Twelve subjects with a past history of thrombosis did not experience a repeat thrombosis during follow up and 18 subjects without a past history of thrombosis did experience a thrombosis during follow up. Fifteen subjects had both a past history of thrombosis and developed a new thrombosis during follow up. Higher hemat-

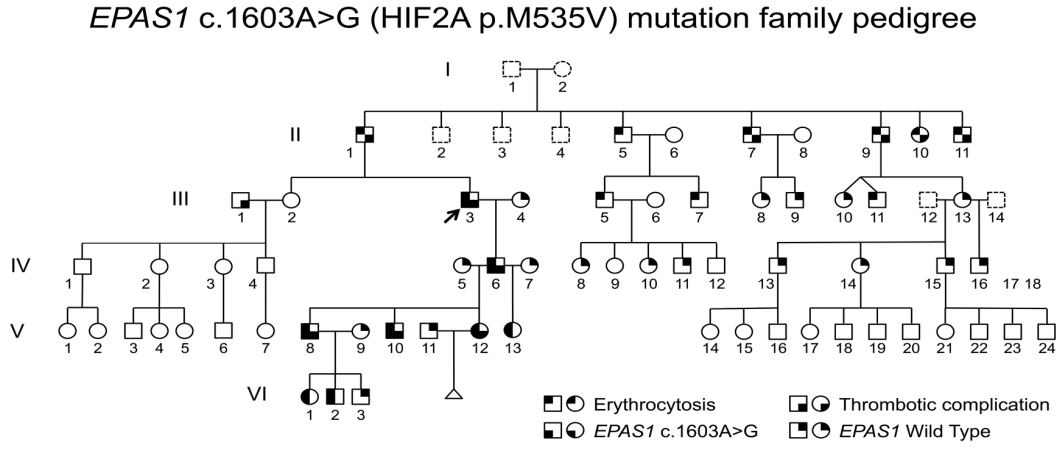

Figure 2. Thrombosis in patients with *EPAS1* mutation. A six-generation pedigree with erythrocytosis inherited in a dominant fashion due to a c.1603A>G variant in *EPAS1* (encoding p.M535V missense mutation in HIF2A). The *EPAS1* variant was present in all genotyped subjects with erythrocytosis and in no genotyped subjects without erythrocytosis. We encountered thrombotic complications in young individuals with the *EPAS1* mutation whose hematocrit was maintained below 45% by phlebotomy therapy. For each family member in the figure for whom a sample was able to be genotyped, the result is designated as heterozygous mutant (*EPAS1* c.1603A>G) by filling the lower left quadrant, or wild type, by filling the upper right quadrant. If neither the lower left nor upper right quadrants are filled then no genotypic information is available for that family member. The pedigree symbols drawn with a dashed line indicate no clinical information was available regarding erythrocytosis or thrombotic complications.

ocrit was not a predictor of increased thrombotic risk in either univariate analysis (hazard ratio 0.98, *P*=0.22) or multivariate analysis (hazard ratio 0.98, *P*=0.052). Furthermore, a history of therapeutic phlebotomy was associated with an increased risk of thrombosis in both univariate (hazard ratio 2.0, *P*=0.004) and multivariate (hazard ratio 1.9, *P*=0.028) analysis (Table 1B). Patients were not receiving therapeutic anticoagulation at the time of the second event. About half of the patients with a past history of thrombosis were receiving aspirin 75 mg/day at entry into the study, but this did not appear to have a protective effect (Table 1B).

EPAS1 (HIF2A) gain-of-function mutations are characterized by reduced binding efficiency to the HIF-α inhibitor PHD2, decreased ubiquitination, increased HIF-2α stability, and increased transcription of HIF-regulated genes in granulocytes.[17] Over the last two decades, we (JTP and FRL) studied a six-generation pedigree (Figure 2) with dominantly inherited erythrocytosis and elevated EPO. Initially, we did not find co-segregation of polymorphisms in the *EPO, EPOR, EGLN1, HIF1A*, or *EPAS1* genes with the erythrocytosis phenotype. However, we identified a variant in *EPAS1* (c.1603A>G resulting in the missense change p.M535V in HIF2A) through whole exome sequencing of two affected individuals. Further testing revealed that the c.1603A>G variant in *EPAS1* was present in eight genotyped subjects with erythrocytosis but not in 16 genotyped subjects not having erythrocytosis or one genotyped subject for whom knowledge of erythrocytosis phenotype is missing. The p.M535V variant in HIF2A has previously been reported to be a cause of erythrocytosis in a single patient[18] in whom thrombotic complications have not occurred[18] (*MF McMullin, personal communication, 2019*). We observed a high rate of thrombotic complications (stroke, myocardial infarction, deep vein thrombosis and Budd-Chiari syndrome) in the present pedigree in association with the p.M535V HIF2A allele. Thrombotic complications occurred in 5 of 8 subjects with the p.M535V HIF2A variant compared to none of 17 HIF2A wild-type individuals (*P*=0.001 by the Fisher exact test). Among 40 additional pedigree members for whom genotyping is not available, thrombosis occurred in 5 of 9 subjects with a history of erythrocytosis and in only one of 31 who were known not to have erythrocytosis (*P*=0.001 by the Fisher exact test). The proband is at position III,3 and indicated by the arrow on the pedigree in Figure 2. He, and later his affected son, were initially thought to have polycythemia vera, and both underwent a rigorous phlebotomy program to keep their hematocrit below 45%. Nevertheless, the proband died in his 60s of coronary artery disease and congestive heart failure and his son died at the age of 44 years after suffering strokes. All affected offspring of these two patients (see Figure 2 on the left portion of the pedigree as the fourth and fifth generations) have been on a similar rigorous phlebotomy program and their hematocrits have been maintained below 45%. Nevertheless, some still suffered thrombotic complications and none had hypertension, diabetes or dyslipidemia as risk factors. A son of the proband's son from a second marriage had a myocardial infarction at age 24 years, followed by two coronary artery stents; he later developed Bud-Chiari syndrome. His sister had a stroke at the age of 21 years during her pregnancy followed by a spontaneous abortion. All thrombotic events were up-dated as of March 2019. Since some of the thromboses occurred in the distant past, we have been unable to obtain verifiable data on the duration and type of therapeutic anticoagulation.

In aggregate, these data indicate that in CE due to a homozygous loss-of-function VHL mutation, and in erythrocytosis due to a HIF-2α gain-of-function mutation, the thrombotic risk may be independent of elevated hematocrit and viscosity and is instead related to the up-regulated hypoxic responses associated with these mutations. This hypothesis needs to be tested further; examination of vascular cells derived from induced pluripotent stem cells (iPSC) (prepared from their $CD34^+$ hematopoietic cells) is in progress. These conditions are characterized by diverse cellular and metabolic changes that could

be directly associated with thrombotic risk, irrespective of hematocrit level. The challenge in these conditions is to elucidate factors for the thrombotic risk independent of elevated hematocrit. The routine practice of phlebotomy for elevated hematocrit, with its inevitable iron deficiency (which leads to inhibition of PHD2, increased HIF, and increased EPO) and potential detrimental thrombotic effects should be re-evaluated. We provide evidence here that phlebotomy therapy may not be beneficial in reducing thrombotic risk in these two conditions. More studies are needed to define the specific molecular basis of thrombosis in erythrocytosis due to up-regulated hypoxia sensing and to develop targeted approaches for the prevention and therapy of thrombotic complications.

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doi:10.3324/haematol.2019.216267

Funding: this work was supported in part by grants from the National Institutes of Health [R01HL079912 (Gordeuk), R01HL137991 (Prchal), and institutional funds from the University of Illinois at Chicago.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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