Mutations in the von Hippel-Lindau (*VHL*) tumor suppressor gene and *VHL*-haplotype analysis in patients with presumable congenital erythrocytosis

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Background and Objectives. Congenital erythrocytoses or polycythemias are rare and heterogeneous. A homozygous mutation (*VHLC598T*→Arg200Trp) in the von Hippel-Lindau (*VHL*) gene was originally identified as the cause of the endemic Chuvash polycythemia. Subsequently this and other mutations in the *VHL* gene were also detected in several patients of different ethnic origin. Haplotype analyses of the *VHL* gene suggested a common origin for the Chuvash-type mutation.

Design and Methods. Thirty-four patients with presumable congenital erythrocytosis due to an unknown underlying disorder were examined for *VHL* gene mutations and *VHL* region haplotypes.

Results. Four patients were homozygous and one patient heterozygous for the Chuvash-type mutation. One additional patient presented a previously not described heterozygous mutation *VHLG311→T* in exon 1. The haplotype analyses were in agreement with recently published data for three of the four patients with homozygous mutations as well as for the patient with a heterozygous Chuvash-type mutation. One patient of Turkish origin with homozygous Chuvash-type mutation had a haplotype not previously found in individuals with Chuvash-type mutation.

Interpretation and Conclusions. These results confirm that mutations in the *VHL* gene are responsible for a substantial proportion of patients with congenital erythrocytoses. Erythrocytoses due to a C598→T mutation of the *VHL* gene are not geographically restricted. The majority of patients with Chuvash polycythemia share a common *VHL* gene haplotype. The different haplotype in one of the patients with Chuvash-type mutation indicates that this mutation was not spread only from a single founder but developed independently in other individuals.

Key words: polycythemia, congenital erythrocytosis, von-Hippel-Lindau gene.

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Congenital erythrocytoses are rare and heterogeneous clinical entities. Two classes of absolute erythrocytosis — defined by an increase of the total red cell mass — must be distinguished. In primary erythrocytosis, the erythropoietic compartment is enlarged independently of extrinsic influences or by responding inadequately to them.[1] In contrast, secondary erythrocytosis is driven mostly by hormonal factors [predominantly erythropoietin (Epo)] extrinsic to the erythroid compartment. The erythroid progenitors respond normally to circulating cytokines. The increased Epo secretion may represent either a physiologic response to tissue hypoxia, an abnormal autonomous Epo production or a dysregulation of the oxygen-dependent Epo synthesis. The first defined congenital form of a secondary erythrocytosis — the abnormal hemoglobin Chesapeake — was reported in 1966.[2] Since then, more than 200 hemoglobin variants with an increased oxygen affinity have been described.[3] A similar pathophysiologic mechanism is the underlying cause of erythrocytosis in the rare congenital 2,3-bisphosphoglycerate (2,3-BPG) deficiency.[4,5]

It has been discussed for several years that a significant proportion of congenital erythrocytoses, e.g. Chuvash polycythemia, might be caused by disorders of the oxygen-dependent regulation of Epo synthesis. Chuvash polycythemia, the currently only known endemic form of an erythrocytosis is characterized by the presence of features of both primary (hypersensitivity of erythroid precursors to Epo) and secondary erythrocytosis (increased Epo levels).[6] It was discovered in the central Russian region of Chuvashia

among people of Asian Tatar-related ancestry.[7] In 2002, the underlying genetic defect, a homozygous mutation (*VHLC598T→*Arg200Trp) in the von Hippel-Lindau (*VHL*) gene, a known tumor suppressor gene was identified in patients with Chuvash polycythemia.[8,9] Together with other proteins (elongin B, C, Rbx1, Cul2), the VHL protein constitutes an E3-ubiquitin ligase complex which binds to the αchain of the transcription factor HIF-1 (hypoxia inducible factor-1) and leads to its polyubiquitination followed by its proteasomal degradation.[10,11] HIF plays a key role in the regulation of the oxygen-dependent expression of hypoxia inducible genes such as *EPO*.[12] Recently the *VHL598C→T* mutation was also detected in eleven individuals with sporadic or familial congenital erythrocytosis who do not belong to the Chuvash population, but to diverse ethnic groups.[6,13,14] Analyzing the *VHL* gene haplotype, two studies concluded that the Chuvash-type mutation in these patients was spread from a single founder.[14,15] It was also shown that the *VHL* gene haplotype in these patients not belonging to the Chuvash population was identical to that in polycythemic patients from Chuvashia.[15]

In addition to the patients with a Chuvash-type mutation, patients with six additional mutations of the *VHL* gene were identified. These patients were either homozygotes or compound-heterozygotes in combination with a Chuvash-type mutation and presented with congenital erythrocytosis.[6,13]

We examined the *VHL* gene in patients who are currently living in Germany and who were diagnosed with presumable congenital erythrocytosis. The aim of this study was to analyze whether *VHL* mutations are responsible for erythrocytosis in these patients and — if this were the case — whether these mutations are Chuvash-type mutations or affect other parts of the *VHL* gene. When the *VHL 598C→T* mutation was found within this patient group, haplotype single nucleotide polymorphism (SNP) analysis of the *VHL* region was performed and the results compared with previously reported haplotypes.

Design and Methods

Patients

Thirty-four patients with presumed congenital erythrocytosis with a median age of 19 years (range 3-70 years) were included in the study. Patients were either referred to our laboratory because of a so far unexplored erythrocytosis or recruited by a recently introduced registry on congenital erythrocytosis and polycythemia vera in childhood and adolescence (PV-ERYKA 03). The group of patients was formed of one pair of siblings (male/female, 14/16 years) and a mother (29 years) with two (male/female, 3/5 years) of her three children. The mother's erythrocytosis was diagnosed at the age of 6 years, other relatives are not affected. All other patients with presumable congenital erythrocytosis do not have known relatives affected by an erythrocytosis. The presence of a congenital form of an absolute erythrocytosis was assumed if patients had been presenting with continuously elevated hemoglobin levels (greater than 18 g/dL in males, greater than 16.5 g/dL in females or greater than 2 SD above the median of the age-specific normal range in children) for a long time. The absence of splenomegaly or other characteristics of a myeloproliferative disorder was confirmed. Cardiac, pulmonary, renal, or neoplastic causes of secondary erythrocytosis were excluded. Hemoglobin oxygen affinity was normal in all patients. Some patients underwent either sporadic or regular phlebotomy treatment, explaining lower hemoglobin levels at the time of this study than those leading to the diagnosis. The patients' hematologic parameters are reported in Table 1. All patients gave their written informed consent to the molecular genetic analysis, ata analysis and publication. The study was approved by the local University ethics committee and performed in accordance with the World Medical Association Declaration of Helsinki of 1975, as revised in 2000.

Polymerase chain reaction (PCR) and sequencing of VHL gene DNA

Genomic DNA was isolated from peripheral blood using a silica matrix column (QIAGEN, Hilden, Germany). Polymerase chain reactions (PCR) were performed in a 60 µL volume containing 67mM Tris-HCL pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 5% glycerol, 0.8 mM dNTP, 330 nM each of forward and reverse primers, 2 U/reaction of either Taq DNA polymerase or Hot-Star Taq DNA polymerase (QIAGEN), and 300 ng of genomic DNA. The following sets of primers were used: VHL1F1 5'-AGCGCGTTCCATCCTCTAC-3', VHL1R1 5'-GCTTCAGACCGTGCTATCGT-3', VHL2F1 5'-GAGGTTTCACCACGTTAGCC-3', VHL2R1 5'-AGCCCAAAGTGCTTTTGAGA-3', VHL3F1 5'-CAGAGGCATGAACACCATGA-3', and VHL3R1 5'-AAGGAAGGAACCAGTCCTGT-3'. Amplification was initially performed for 15 minutes at 95°C, followed by 35 cycles of 1 minute at 95°C, 1 minute at 52°C (exons 1 and 2) or 56°C (exon 3), and 1 minute at 72°C in a GeneAmp® PCR System 9700 (Applied Biosystems, Darmstadt, Germany). PCR products were purified using the QIA Quick PCR Purification Kit (QIAGEN). Four microliters of the eluate were used as the tem-

Table 1. Hematologic parameters in patients with presumed congenital erythrocytosis.

	All patients (n=34)		*Pts. with VHL mutation (n=6)*		*Pts. without VHL mutation (n=28)*	
	median	*range*	*median*	*range*	*median*	*range*
Hemoglobin (g/dL)	17.8	14.9-23.1	19.6	16.5-19.9	17.7	14.9-23.1
Hematocrit (%)	57	48-73	63	52-67	57	48-73
Leukocytes ($\times 10^3/\mu L$)	6.7	4.6-11.4	5.4	4.9-7.3	7.6	4.6-11.4
Thrombocytes ($\times 10^3/\mu L$)	261	106-385	176	141-222	264	106-385
Erythropoietin (mU/mL) (n.v.: 3.3-16.6 mU/mL)	16.9	5.6-64	19.6	7.9-33.2	12.6	5.6-64

plate for sequencing with a DNA Sequencing Kit (Big Dye™ Terminator Cycle Sequencing Ready Reaction, Applied Biosystems). The sequencing reaction was performed in the GeneAmp® PCR System 9700 (Applied Biosystems). The products were analyzed on an ABI 3100 DNA Genetic Analyzer (Applied Biosystems).

Haplotype analysis

Single nucleotide polymorphisms (rs776517, rs776517+68bp, rs374645, rs2600005, rs166538, rs458952) located in introns 1 and 2 of the *VHL* gene, 3kb 5', 3kb and 8kb 3' to the *VHL* gene, selected according to a previously reported study,[14] were used for haplotype analysis. Primer sets were used as described elsewhere.[12] Amplification was performed as described above with annealing temperatures of 54°C (rs776517, rs776517+68bp, and rs374645), 56°C (rs2600005 and rs458952), and 58°C (rs166538). PCR products were sequenced as described above. PCR amplification of a 1.9 kb spanning region including rs2600005 and *VHL* exon 3 was done in patients #1 and #8 using the primers rs2600005F1 and VHL3R1. PCR and sequencing conditions were the same as those used for analysis of *VHL* exon 3 apart from an elongation time of 2 minutes.

Reverse transcription polymerase chain reaction (RT-PCR)

RNA was isolated from 2×10^6 cells from an Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line using the QIA Rneasy Mini Kit (QIAGEN) including DNAse treatment. cDNA was synthesized using random hexamers and Superscript II RNase HT (Invitrogen, Karlsruhe, Germany) at 42°C for 50 minutes. PCR amplification of cDNA was done using the primers cVHL1-3F1 5'-CAGCTCCGCCCCGCGTCCGAC-3'(5'-untranslated region (UTR)) or cVHL2-3F1 5'-CTCTTCAGAGATGCAGGGACAC-3' (exon 2) and VHL3R1 (3'-UTR). PCR and sequencing were performed as reported for *VHL* exon 3 analysis.

Results

Sequencing of all three exons of the *VHL* gene revealed a homozygous C to T mutation at nucleotide 598 (Chuvash-type mutation) in four patients (Figure 1A), one heterozygous mutation at this nucleotide in another patient and one patient with a new heterozygous *VHL* 311G→T mutation in exon 1 (Figure 1B). Two of the homozygous patients with the Chuvash-type mutation are siblings. The parents are not consanguineous and — according to their knowledge — are of German origin. Neither a sister of these patients nor other family members are known to be affected by an erythrocytosis. The male sibling presented at the age of 14, the female sibling at the age of 16 years with hemoglobin levels of 19.8 and 16.5 g/dL, hematocrit values of 66 and 54%, and serum erythropoietin levels of 32 and 33 mU/mL, respectively. So far, they have not had any hyperviscosity symptoms or thromboembolic complications. The third patient with a homozygous *VHL 598C→T* mutation is the single daughter of consanguineous heterozygous parents. She presented at the age of 4 years with headache and had a hemoglobin level of 19.5 g/dL, a hematocrit of 64%, and a serum erythropoietin level of 11.5 mU/mL. Another homozygous patient is a 6-year old boy of Turkish origin who presented with erythrocytosis with a hemoglobin level of 16.1 g/dL already at the age of 12 months. His current hemoglobin level is 19.6 g/dL, hematocrit 62%, and serum erythropoietin level 17 mU/mL. At the age of 3 years the patient developed type-I diabetes mellitus. Additional clinical problems include a global developmental retardation, in particular of speech, and intermittent local bone pains (right knee and foot). The patient has two siblings; one of them is heterozygous for the *VHL 598C→T* mutation. The heterozygous parents deny consanguinity. The patient with a heterozygous *VHL 598C→T* mutation is a 45-year old female of German origin. Erythrocytosis was diagnosed at the age of 21 years. She

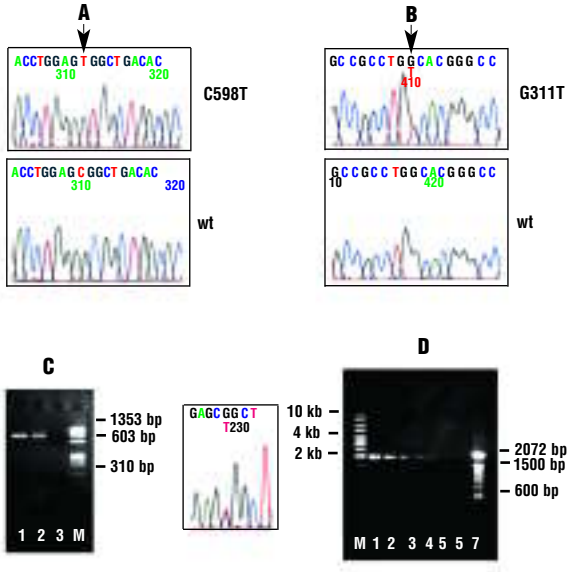

Figure 1. Detection of *VHL* gene mutations. A. Sequence analysis of exon 3 of the *VHL* gene detected a homozygous base change 598C→T in patients #2, 3, 15, and 46. B. Patient #9 presented with a heterozygous G to T mutation at nucleotide 311 in the first exon 1 of the *VHL* gene. C. Left: Agarose-gel (1.5%). The major product of RT-PCR (primer cVHL1-3F1 + VHL3R1) is a ~750 bp cDNA fragment comprising exons 1, 2, and 3 (transcript variant 1) of the *VHL* gene (lane 1: control; lane 2: patient #15; lane 3: dH_2O). Right: Sequence analysis of the product confirmed the presence of both a wild-type and a 598C→T mutated transcript in patient #15. D. Agarose-gel (0.8%). Amplification of a 1.9 kb spanning a DNA fragment containing both exon 3 and SNP rs 2600005 revealed a single PCR product in patients #1 (lanes 2-4, decreasing amounts of the PCR product) and #8 (lanes 5-7, decreasing amounts of the PCR product).

now has a hemoglobin level of 19.9 g/dL, a hematocrit of 67% and a serum erythropoietin level of 22.2 mU/mL. According to the knowledge of the patient, other family members were not affected by erythrocytosis, but an uncle possibly died from a cerebrovascular insult. Detailed data on other family members are not available.

The previously not described heterozygous *VHL 311G→T* mutation was detected in a 61-year old female with a history of long-standing erythrocytosis. She is the only family member with a clinical picture of erythrocytosis and presented with a hemoglobin level of 17.2 g/dL, a hematocrit of 52% and a normal serum Epo level (7.9 mU/mL). This patient initially consented to the *VHL* gene analysis reported here, but later refused further investigations.

Since individuals with a heterozygous *VHL 598C→T* mutation are usually only carriers but not affected by clinical erythrocytosis, the possibility of non-expression of the wild-type allele in the patient

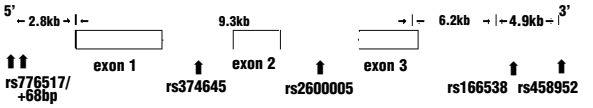

Figure 2. Schematic diagram of the *VHL* haplotype.

with a heterozygous Chuvash-type mutation and normal DNA sequence in all other *VHL* gene exons was considered. To test this hypothesis, cDNA of this patient was amplified and sequenced after RNA isolation and reverse transcription. RT-PCR using two primers in the 5'- and 3'-UTR of the gene revealed both full length transcripts (transcript variant 1) and a minor portion of transcript variant 2 (lacking the in-frame coding exon 2) of both alleles (Figure 1C). The presence of full length transcripts (variant 1) of both alleles was further confirmed by RT-PCR using the primers cVHL2-3F1 and VHL3R1. The results from sequencing analyses and RT-PCR together exclude the exclusive transcription of the mutated allele as well as the presence of an aberrant transcript due to mutations in either the 5'-UTR or one of the introns. The 3'-UTR was not analyzed.

Haplotype SNP analysis of the *VHL* gene was performed in 24 / 34 patients including all patients with a *VHL* gene mutation (Figure 2, Table 2). The SNP status of three of the four patients with homozygous and the patient with a heterozygous *VHL598C→T* mutation corresponded to a haplotype reported in one previous study including Pakistani, Bangladeshi and one English patient.[14] In contrast, the Turkish boy with polycythemia due to a Chuvash-type mutation, had a haplotype — as confirmed by sequence analyses of the *VHL* regions in his parents — which had previously only been found in unaffected individuals.

Except for two, all patients without a *VHL* gene mutation did display SNP sequences in accordance with the haplotype(s) previously described in normal individuals.[14] In the remaining two patients, sequence analyses of the *VHL* gene consistently revealed absence of the *VHL598C→T* mutation. To exclude a possible deletion of or within exon 3 including nucleotide 598 in these latter patients, a PCR of a DNA segment spanning 2 kb and including both the SNP rs2600005 and exon 3 was performed. However, a single PCR product was obtained again presenting the heterozygous SNP sequence (C/T) but homozygosity for nucleotide 598 (C) in exon 3 of the *VHL* gene (Figure 1D).

Table 2. VHL-haplotype analysis in patients with presumably congenital erythrocytosis.

Patients	*VHL-gene mutation*	*Haplotype*					
		rs776517	*rs776517 + 68bp*	*rs374645*	*rs2600005*	*rs166538*	*rs458952*
#2	598C→T/598→CT	G	C	T	A	C	A
#3	598C→T/598→CT	G	C	T	A	C	A
#15	598C→T/598→CT	G	C	T	A	C	A
#46	598C→T/598→CT	A	A	C	G	C	G
#20	598C→T/wt	G/A	C/A	T/C	A/G	C/T	A/G
#01	wt/wt	G/A	C/A	T/C	A/G	C	A/G
#08	wt/wt	G/A	C/A	C	A/G	C/T	A/G
#09	311G→T/wt	A	A	C	G	C/T	G
other pts. (n=16)	wt/wt	A	A	C	G (#26: A/G)	T (n=10) C/T (n=6)	G (#26: A/G)

Discussion

For the first time, mutations causing congenital erythrocytosis were identified in the von Hippel-Lindau gene in patients with Chuvash-polycythemia.[8,9] Chuvashia is an autonomous region in central Russia and the presence of an endemic form of congenital polycythemia in this region among people of Asian Tatar-related ancestry had already been reported several years ago.[7,16] In 2002, it was demonstrated that a homozygous C to T mutation at nucleotide 598 of the *VHL* gene is responsible for the development of erythrocytosis in these patients.[8,9] The *VHL* gene plays an essential role in the regulatory pathway of oxygen-dependent gene expression through the initiation of HIF1α degradation.[10] Although it was shown that the *VHL598C→T* mutation leads to only a partial loss of function with regard to HIF1α- binding and its degradation, cell culture experiments revealed a significant increase of the expression of usually hypoxia-induced genes such as *EPO* following transfection of the defective *VHL* gene.[9] After the initial description of the Chuvash-type mutation, this aberration was subsequently found in several other patients of Afro-American, Caucasian and Asian ethnicity living in the United States, the United Kingdom, Denmark, and Croatia.[6,13,14] As shown in this study, the Chuvash-type mutation of the *VHL* gene is also present in patients of German and Turkish origin. However, in contrast to other reports, in which up to 50% of the patients with apparently congenital erythrocytosis and elevated serum Epo appear to be affected by a *VHL* gene mutation,[13] only about 20% of the patients examined in this study display a mutation of at least one *VHL* allele. Although the Chuvash-type mutation of the *VHL* gene usually leads to erythrocytosis only when present in homozygosity (heterozygous carriers of the mutation have normal hemoglobin, hematocrit, and serum Epo levels), one of the patients reported here presented with severe erythrocytosis despite only a heterozygous *VHL598C→T* mutation. A similar case of a 34 year-old male of English ancestry who presented with heterozygous Chuvash-type mutation and erythrocytosis, has previously been described.[14] cDNA studies were not performed in this patient. In the patient reported here, RT-PCR of mRNA confirmed the expression of both the normal and the mutated allele. The presence of an aberrant transcript due to mutations in either the 5'-UTR or one of the introns could also be excluded. Although possible small quantitative gene expression differences due to changes within regulatory parts of the gene were not assessed with this assay, one could speculate whether an additional genetic aberration affecting other parts of the oxygen-sensing pathway may contribute to the erythrocytosis in the heterozygous patient reported here. All known VHL gene mutations in patients with congenital erythrocytosis affect either exon 3 — such as the Chuvash-type mutation and the *VHL 562C→G, VHL 571C→G,* and *VHL 574C→T* mutations — or exon 2 (*VHL 376C→T*, *VHL 388G→C*). Here we report the first mutation in the first exon of the *VHL* gene in a patient with idiopathic erythrocytosis. The heterozygous *VHL311G→T* mutation results in a Gly104Val amino acid exchange. According to the model of the three-dimensional structure of the VHL-HIF1α-elonginB-elonginC-complex, this amino acid is situated in immediate contact with the HIF1α-binding site in front of the lysine residue at position 574 of the HIF1α protein.[17,18] Therefore, the amino acid change in the *VHL* gene at this position might directly affect HIF1α-VHL binding. Assuming that the *VHL 311G→T* mutation does have such an influence, it

seems possible that this mutation exerts a dominant effect either due to the lack of sufficient compensatory expression of wild-type *VHL* protein or by blocking the VHL binding site of HIF1α. However, the possibility that both *VHL*-alleles are differentially expressed due to an additional mutation in a regulatory domain cannot be excluded. Another two patients (siblings) with a heterozygous *VHL* gene mutation other than Chuvash-type were reported previously.[6] The G to T mutation in nucleotide 376 in the second exon of the *VHL* gene leads to an Asp126Tyr amino acid exchange. In those patients the presence of an inherited second null allele was excluded by real-time RT-PCR. Since the father of the patients showed the same *VHL* gene mutation but was not affected by erythrocytosis, the authors concluded that the *VHL376G→T* mutation might be an autosomal-dominant polycythemia-causing mutation with an incomplete penetrance. Unfortunately, in the patient reported here, it was impossible to examine other family members in order to test whether the *VHL311G→T* mutation was inherited in a similar manner as reported for the siblings with the *VHL376G→T* mutation.

Previous studies on *VHL* gene haplotypes concluded that a common founder was the origin for all currently living Chuvash-polycythemia patient.[14,15] The first study describing a common haplotype in affected individuals included eight patients not belonging to the Chuvash population and their heterozygous or normal relatives.[14] In the second study patients and controls from Chuvashia (61 patients), eleven non-Chuvash individuals or families, and several hundreds of unrelated healthy individuals were examined.[15] This study, based on different SNP distributed over a >300 kb segment spanning the *VHL* gene region in chromosome 3, revealed a common haplotype for Chuvash and non-Chuvash individuals with a *VHL 598C→T* mutation. It was calculated that the mutation arose in a single ancestor at least 14,000 to 62,000 years ago.[15] Haplotype SNP analyses in the patients reported here, using the SNP reported from the first study,[14] showed the common Chuvash haplotype in three of four patients with a homozygous Chuvash-type *VHL* gene mutation. However, from the presence of a different haplotype in a homozygous boy of Turkish origin and his family it can now be concluded that the Chuvash-type mutation developed independently from the suggested common founder in other affected individuals.

Mutations of the von Hippel-Lindau gene seem to be responsible for congenital erythrocytosis in a substantial number of patients with so far unexplored erythrocytosis worldwide. Nevertheless, in a significant number of patients with presumable congenital erythrocytosis the etiology remains unknown. Increasing knowledge about the oxygen-sensing pathway and the possibility that a congenital aberration that affects key players of this pathway may result in erythrocytosis, should lead to the identification of other potential etiological factors for congenital secondary erythrocytosis within the next few years.

HC was the principal investigator and wrote the manuscript. KS and EK were particularly involved in the design and conception of the study. In addition, KS contributed essentially to data analysis and interpretation and to the draft of the article. EK was also responsible for specific hematologic aspects of the study(e.g. hemoglobin analysis), contributed essentially to the recruitment of patients, and revised the manuscript. NJ, UK, PEP, DTS were responsible for the clinical management of patients and data acquisition. KMD promoted this trial and was involved in the conception of the study. In addition, NJ, UK, PEP, DTS and KMD reviewed and revised the manuscript critically. The authors declare that they have no potential conflicts of interest.

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