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Low Mean Cell Haemoglobin is a Valuable Parameter of Thrombotic Risk Stratification in Patients with Polycythemia Vera

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Abstract

Objectives: Thrombosis is a leading cause of morbidity and mortality in patients with Philadelphia negative chronic myeloproliferative neoplasms (MPNs). There are many thrombosis risk stratifications used in this patient group taking into consideration the age, thrombosis history and cardiovascular factors (hypertension, hypercholesterinaemia, hyper-trigliceridaemia, thrombocytosis, smoking and diabetes mellitus). In this work we evaluated the possible role of iron deficiency in thrombotic events (TE) of the polycythaemia vera (PV) patients. Methods: We considered the low mean cell haemoglobin (MCH <28 pg) value as a parameter to assess the iron deficiency in the multicentre database (15 Hungarian haematology centres) of our HUMYPRON GROUP (Hungarian MPN Working Group). The MCH values, recorded at the time of diagnosis of 296 patients with polycythemia vera, were retrospectively analysed.

Results: The low MCH, at the diagnosis, was found to be a risk factor for thrombotic events occurring after diagnosis (OR: 1.966). It was also shown as an additive and independent parameter in the Tefferi high-risk patient groups, and combining it with Tefferi risk stratification an extremely high thrombotic risk group could be determined (Nagelkerke R square: 0.084). We have supposed that low MCH in PV reveals a disease form featured with a high proliferation activity. Our hypothesis was confirmed with a sub-study (n=52) showing that the high $JAK2^{V617F}$ allele burden was significantly correlated with the low MCH (p=0.005) and the high white blood cell count (WBC) (p<0.001).

Conclusions: Iron deficiency, existing at the time of diagnosis of PV, was proven to be a risk factor for imminent thrombotic events. The low MCH was found to be a strong additive factor when it was combined with the known thrombotic risk stratification systems. The low MCH showed significant correlation with the high $JAK2^{V617F}$ allele burden.

Keywords: Iron deficiency; Mean cell haemoglobin; Myeloproliferative neoplasms; Polycythemia vera; Thrombotic risk

Introduction

Philadelphia negative chronic myeloproliferative neoplasms (polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF)) are associated with higher thrombotic risk leading to excessive cardiovascular mortality [1-4].

A comprehensive epidemiological study involving PV patients, the European collaboration study on low dose aspirin in polycythemia (ECLAP), revealed that 41% of all mortality was of cardiovascular origin (1.5 deaths per 100 person per year). Coronary heart disease was responsible for 15% of all deaths, whereas congestive heart failure, non-haemorrhagic stroke and pulmonary embolism accounted for 8-8% each. The cumulative incidence of nonfatal thrombosis was 3.8 events per 100 people per year, without a difference between arterial and venous thrombosis [5].

The thrombophilic behaviour observed in these conditions manifests in microcirculatory disturbances, arterial and venous thrombosis. The possible mechanism resulting in thrombophilia in MPN patients has been widely investigated over the last decades, nevertheless there is no definite answer that could explain this phenomenon. Data from earlier studies are often conflicting or difficult to interpret. For instance, both the iron deficiency and the excess have been associated with increased thrombotic risk [6].

Epidemiological studies support the hypothesis of Sullivan [7], that elevated level of stored iron associates with higher incidence of cardiovascular morbidity [8-10]. However, iron deficiency was recognised as a risk factor of cerebral sinus thrombosis [11], carotid artery thrombosis [12], central retinal vein occlusion [13,14] and superior sagittal sinus thrombosis [15].

Donovan et al. [16] found that PV patients treated with phlebotomy alone suffered significantly more thrombotic events than those who received myelosuppressive regimens [17]. Iron deficiency is commonly detected in patients with PV. The phlebotomy can contribute to the further worsening of iron deficiency [18].

There is different risk stratifications used to assess the thrombotic risk at the time of diagnosis in clinical practice of Philadelphia negative MPNs. The risk stratification proposed by Tefferi estimates the

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possibility of a recurrent thrombosis in PV patients. There are two risk categories, the high (age >60 years or prior thrombotic event) and the low (age ≤ 60 years and no thrombosis history) [19]. The risk analysis by Landolfi, beside these variables, also takes general vascular factors into consideration [20].

The transferrin saturation, serum ferritin level, reticulocyte haemoglobin content and bone marrow iron content are the most used and suitable parameters to measure the body's iron status nowadays. On the other hand, more reports raised attention that red blood cell hypochromia and decreased red blood cell haemoglobin content indicate the presence of iron deficiency [21-26]. The low mean cell haemoglobin content (MCH) is an appropriate parameter which notes iron deficiency [21], and it is more available in retrospective databases in comparison to other iron status parameters.

The aim of our retrospective multicentre study was to evaluate the impact of MCH, white blood cell count (WBC), platelet count (PLT), age and thrombotic events at the time of diagnosis and compare our findings to Tefferi and Landolfi thromboembolic risk stratifications in PV patients registered in the Hungarian Myeloproliferative Neoplasia Working Group (HUMYPRON GROUP) database [27].

Materials and Methods

After its establishment in 2012, the Hungarian MPN Working Group (HUMYPRON GROUP) introduced a simple, practical database for Philadelphia negative MPN patients with clinical and laboratory data collected from 15 Hungarian haematology centres [27].

Eligibility criteria

Eligibility criteria included the age, 18 years or older, and a previous confirmation of PV according to WHO 2008 criteria [28]. Clinical factors (age, gender, previous thrombotic history and risk stratifications by Tefferi and Landolfi and family history of thrombophilia) and laboratory parameters at diagnosis (white blood count: WBC, haemoglobin: Hb, haematocrit: Htc, mean cell haemoglobin: MCH, platelet: PLT and C-reactive protein: CRP) were collected. We used MCH value to assess the iron status at the time of MPN diagnosis. We excluded patients with thalassemia, myelofibrosis and end-stage kidney disease whose MCH alterations might have been associated with the original disease and not with the iron deficiency.

Diagnosis of thrombosis

Vascular thrombosis was defined according to Gisslinger et al. [29]. Major events included the following complications: peripheral vascular (peripheral arterial thrombosis, deep venous thrombosis and pulmonary thromboembolism), cardiovascular (myocardial infarction), central nervous system (stroke, retinal vessel thrombosis and sinus thrombosis) and intra-abdominal vascular events (splenic-portal vein thrombosis and Budd-Chiari syndrome). Minor events were angina pectoris, transient ischemic attack and superficial thrombophlebitis of the extremities, and they were taken into account only in patients who had no other major thrombotic events. Visual complaint, headache, dizziness, tinnitus or acroparesthesia were not considered as thrombotic events.

JAK2^{V617F} (c.1849G>T) activating mutation was screened by allele-specific polymerase chain reaction (PCR) [30]. In a subgroup of the JAK2^{V617F} mutant cases, real-time quantitative PCR was performed to determine the V617F allele burden [31].

Ethics and study management

The study was conducted according to the good clinical practice rules and the principles of the Helsinki Declaration. Written informed consent was obtained from the subjects for using their data anonymously after explaining them the purpose and nature of the study.

Statistical Analysis

Fisher's exact test was used to compare dichotomous variables, while Mann-Whitney test served to analyse continuous variables. Studying the development of thrombotic events, odd's ratios (OR) and 95% confidence intervals (95%CIs) were calculated by logistic regression in different subgroups according to the gender, age, diagnosis, previous thrombotic history, JAK2V617F, WBC, MCH, PLT, Landolfi and Tefferi risk groups. Only those factors which exerted significant impact on univariate analysis were involved in multivariable tests. As the previous thrombotic event, as a parameter, is involved in the Landolfi and Tefferi risk stratifications, it was not separately included in multivariable analyses. Statistical analyses were performed using IBM SPSS 24.0 and Social Science Statistics software package (www.socscistatistics.com). The cut-off value of significance was $p < 0.05$.

Results

Data of 296 PV patients were available from the date of diagnosis, allowing us to identify thromboembolic events throughout the following period of 61 months on average. Median age was 61.4 years and male dominance was observed. Only JAK2^{V617F} or exon 12 mutated patients were included. We adopted both the Landolfi and Tefferi risk analyses with the simplification of using only two categories (low and high risks) (Table 1).

Altogether 99 thromboembolic events were observed in 82 patients between date of diagnosis and date of study entry. Low MCH value

	PV (n=296)	
	No. of pts.	%
Sex		
Male	169	57.09%
Female	127	42.91%
Previous TE event		
No	224	75.68%
Yes	72	24.32%
MCH		
Low (<28 pg)	95	32.09%
Not low (≥ 28 pg)	201	67.91%
WBC		
Not high (≤ 10 × 10^9/l)	125	42.23%
High (>10 × 10^9/l)	171	57.77%
PLT		
Not high (≤ 450 × 10^9/l)	152	51.35%
High (>450 × 10^9/l)	144	48.65%
Landolfi risk		
Low (low+intermediate risk)	69	23.31%
High (high+very high risk)	227	76.69%
Tefferi risk		
Low	113	38.18%
High	183	61.82%
Median age at the diagnosis (years)		
Male	59.6	
Female	63.9	

Table 1: Sex, TE history, WBC, MCH, PLT, Landolfi- and Tefferi-risk, median age at the diagnosis in patients with PV (n=296).

(<28 pg) was found in 36 patients. The male/female ratio, median age, median follow up, Tefferi- and Landolfi risk results were similar in the groups of low and normal/high MCH values (≥ 28 pg) (Table 2).

Univariate analysis found significant correlation between the thromboembolic events and the previous thrombosis history, high Tefferi and Landolfi risk groups, high WBC as well as low MCH value. No other parameter was found to correlate with thromboembolic events (Table 3).

	Low MCH (n=95)		Normal/high MCH (n=201)		
	No. of pts.	%	No. of pts.	%	p
Sex					
Male	49	51.58%	120	59.70%	0.209
Female	46	48.42%	81	40.30%	
Landolfi risk					
Low (low+intermediate risk)	20	21.05%	49	24,38%	0.559
High (high+very high risk)	75	78.95%	152	75.62%	
Tefferi risk					
Low	31	32.63%	82	40.80%	0.201
High	64	67.37%	119	59.20%	
Pts. having TE after diagnosis					
Yes	35	36.84%	46	22.89%	0.017
No	60	63.16%	155	77.11%	
Minor arterial events	25		24		
Major arterial events	1		5		
Minor venous events	20		22		
Major venous events	8		17		
Median age (years)	62.5		60.9		
Follow up (months)	60		62		

Table 2: Comparison of the TE events, Tefferi- and Landolfi risk, sex, median age, median follow up in low MCH (<28 pg) and normal/high MCH (≥ 28 pg) groups.

Univariate analysis (n=296)	TE events (n=81)	no TE events (n=215)	OR (CI 95%)	p
Sex			1.502 (0.887-2.545)	0.13
male	52 (64%)	117 (54%)		
female	29 (36%)	98 (46%)		
Age			1.265 (0.754-2.123)	0.374
>60 years	48 (59%)	115 (53%)		
≤ 60 years	33 (41%)	100 (47%)		
Previous TE			12.783 (6.870-23.782)	<0.001
yes	49 (60%)	23 (11%)		
no	32 (40%)	192 (89%)		
WBC			1.948 (1.133-3.350)	0.015
high	56 (69%)	115 (53%)		
not high	25 (31%)	100 (47%)		
MCH			1.966 (1.156-3.343)	0.012
low	35 (43%)	60 (28%)		
not low	46 (57%)	155 (72%)		
PLT			1.041 (0.625-1.736)	0.877
high	40 (49%)	104 (48%)		
not high	41 (51%)	111 (52%)		
Landolfi			3.614 (1.644-7.947)	<0.001
high	73 (90%)	154 (72%)		
not high	8 (10%)	61 (28%)		
Tefferi			2.771 (1.538-4.993)	<0.001
high	63 (78%)	120 (56%)		
not high	18 (22%)	95 (44%)		

Table 3: Univariate analysis of factors measured at the time of diagnosis affecting thrombotic risk (WBC high: >10 × 10^9/l; WBC not high: ≤10 × 10^9/l; MCH low: <28 pg; MCH not low: ≥ 28 pg; PLT high: >450 × 10^9/l; PLT not high: ≤ 450 × 10^9/l).

After diagnosis, most patients were treated with the combination of hydroxiurea and aspirin. No conclusion could have been drawn from the treatment effect on thrombotic risk, as the follow-up period was long and the patients usually received more than one therapies.

Multivariable analysis revealed that the low MCH is an independent risk factor for thrombotic events. An additive effect to Tefferi risk was observable regarding both WBC and MCH. Male gender, together with the other three factors, also has a significant effect (Table 4).

The low MCH level may be resulted by an interaction between the high proliferation activity and the iron demand. Supporting our hypothesis, we analysed a random subgroup (n=52) of 296 patients. The consistency of this subgroup did not differ in age, gender or any other parameters from the group which had been originally examined. The average age was 63.9 years. The distribution of female and male patients was 46% and 54%, respectively. Comparing JAK2V617F allele burden to MCH value and white blood cell count, we found that, the incidence of low MCH and high WBC was significantly higher in cases featured with the high JAK2V617F burden (Figures 1 and 2 and Table 5). In those patients whose JAK2V617F allele burden has exceeded 20% the low HCH occurred in a significantly higher proportion (p=0.005).

A similar rise, though a less remarkable, was found regarding WBC count (p<0.001).

26 of the 34 patient with elevated WBC (>10x10^9/l) had low MCH (<28 pg), while only 8 of 18 patient with low WBC (≤10 ×10^9/l) had low MCH (p=0.021).

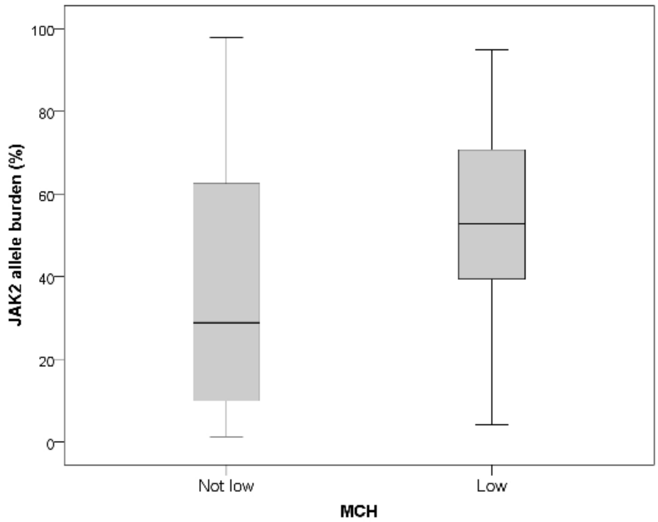
Discussion

According to our current knowledge, the thrombophilic state observed in myeloproliferative neoplasms is of multifactorial origin. The previous thrombotic event, elevated WBC, high JAK2^{V617F} allele burden, advanced age, high body mass index (BMI), hypertension, type II diabetes mellitus and hyperlipidaemia are acknowledged as contributors of the increased thrombotic risk [20].

Clinical observations indicate that the course of PV is individually different. The proliferation rate of erythroid precursors in PV is higher in some patients than in the others. The low MCH suggests that the erythropoesis in PV exceeds the amount of available iron thus the

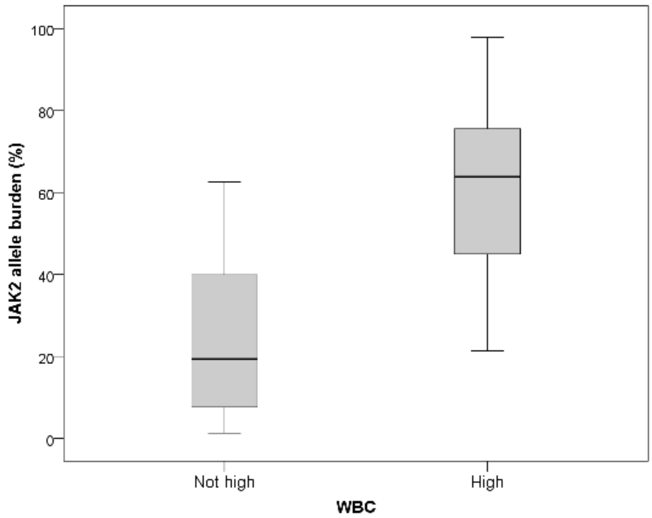
Tefferi risk
Tefferi risk: OR=2.771 (1.538-4.993) p=0.001
Overall classification rate: 72.6%
Nagelkerke R square: 0.061
Tefferi risk+MCH
Tefferi risk: OR=2.681 (1.481-4.853) p=0.001
MCH: OR=1.871 (1.088-3.217) p=0.024
Overall classification rate: 72.6%
Nagelkerke R square: 0.084
Tefferi risk+MCH+WBC
Tefferi risk: OR=2.881 (1.561-5.318) p=0.001
MCH: OR=1.915 (1.099-3.335) p=0.022
WBC: OR=1.863 (1.057-3.284) p=0.016
Male sex: OR=2.004 (1.142-3.521) p=0.016
Overall classification rate: 72,6%
Nagelkerke R square: 0.130
The effect of PLT is not significant (p=0.958), thus it is not present in model set by the forward LR method.

Table 4: The additional effect of low MCH and WBC to Tefferi risk stratifications in TE events (forward LR method). Nagelkerke R square means the proportion of the thrombotic event which is explained by the variables in the model.



Case Summaries						
	N	Mean	Std. Deviation	Median	Minimum	Maximum
.00	18	36.2262222	29.33774981	28.8580000	1.30000	97.92430
1.00	34	54.7471138	23.35507530	52.7100000	4.20437	94.45000
Total	52	48.3360360	26.81426579	48.6000000	1.30000	97.92430

Figure 1: JAK2^{V617F} allele burden correlation with MCH (Mann-Whitney test, p=0.021).



Case Summaries						
	N	Mean	Std. Deviation	Median	Minimum	Maximum
.00	18	24.6325594	19.91906075	19.3850000	1.30000	62.50000
1.00	34	60.8849353	20.93036257	63.8755000	21.36000	97.92430
Total	52	48.3360360	26.81426579	48.6000000	1.30000	97.92430

Figure 2: JAK2^{V617F} allele burden correlation with WBC (Mann-Whitney test, p<0.001).

produced erythroid mass becomes iron depleted. Our analysis has proved that the low MCH is a sign of the high proliferation rate in PV.

We consider that the iron deficiency exists in the background of the low MCH level [21-26]. The incidence of thromboembolic events was significantly higher in the low MCH group, supporting our hypothesis that low MCH is an independent risk factor for TE morbidity.

Possible pathogenic mechanisms which explain the role of iron deficiency in thromboembolic events include reactive thrombocytosis caused by iron deficiency [6], blood flow pattern alterations due to the reduced deformability and increased viscosity of microcytic red blood cells [32] and the metabolic stress increase because of the anaemic hypoxia [33]. Thus, alterations in platelet count and function may

n=52	JAK2^{V617F} allele burden		p
	<20%	≥ 20%	
MCH low (<28 pg)	2	32	0.005
MCH not low (≥ 28pg)	7	11	
WBC high (>10×10^9/l)	0	34	<0.001
WBC not high (≤ 10×10^9/l)	9	9	

Table 5: Correlation of JAK2^{V617F} allele burden with MCH and WBC (Fisher's exact test).

contribute to thrombus formation, especially in the presence of an underlying atherosclerotic disease [34].

In our former article, we have hypothesized that elevated lipocalin2 (LCN2) expression level in PV and ET may also play a crucial role in the development of arterial and venous thrombosis [35]. Examining LCN2 gene expression levels, we found that, the higher relative expression correlated with the occurrence of the thrombotic events. LCN2 is a small 25 kDa glycoprotein, which was first identified as a bacteriostatic agent produced by activated neutrophils, that acts by sequestering bacterial ferric siderophores and interfering with bacterial iron uptake [36].

LCN2 binds iron particles, the siderophores, and transports them into the cells producing increased cytoplasmic iron levels [37]. Experimentally induced iron deficient anaemia resulted a marked elevation of LCN 2 expression. After phlebotomy and alimentary iron depletion in the murine model [38-40], elevated LCN 2 levels were observed in blood, spleen and liver, and at the same time, a decrease in the overall iron levels was detected. Presumably, this mechanism may be responsible for the higher efficacy of cytoreduction compared to phlebotomy and therefore frequent phlebotomy is not recommended.

According to our hypothesis, the excessive cell turnover found in PV patients (erythropoeisis) consumes iron and decreases its level. This leads to overexpression of LCN2 in the neutrophil granulocytes transporting iron into endothelium. The resulted significant increase in endothelial cytoplasmic iron levels (labile iron) leads to oxidative stress induction in the endothelial cells and contributes to the development of thrombosis.

Conclusions

Earlier publications proved that PV patients had an iron deficiency because of elevated red blood cell mass, and this condition could be corrected with cytoreductive therapy while phlebotomy might worsen it [18]. However we did not find any data in the literature if iron deficiency enhanced TE risk in patients with PV. In the HUMYPRON database, we found low MCH in 95 cases out of 296 PV patients. After excluding patients with HD because of uraemia, thalassemia and myelofibrosis we considered the low MCH as a sign of iron deficiency [21]. Comparing TE events in the two groups, the patients with low MCH were found to have significantly more thromboembolic events. Both of the internationally accepted risk stratifications, Tefferi and Landolfi, showed significant correlation with TE events in our analysis. Using multivariable analysis, the low MCH was found to be independent of and additive to the Tefferi risk stratification. We have shown that low MCH among PV patients reveals a disease with high proliferation activity with an iron demand that cannot be maintained by a normal diet. We confirmed our hypothesis with a sub-study showing that the high JAK2^{V617F} allele burden was significantly correlating with the low MCH and the high white blood cell count.

Taking our findings in consideration we propose the phlebotomies to be performed with special attention to the iron level of those PV patients who had been diagnosed with low MCH (iron depletion).

Limitations and Essentials

The lack of a normal control group and its retrospective nature are the limitations of this publication. Polycythemia vera is often accompanied by thromboembolic events. We have investigated the potential role of the low mean cell haemoglobin content (MCH). Data of 296 patients was analysed retrospectively. Low MCH was found to be an independent risk factor for thrombosis.

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