Evaluation of thrombotic complications in essential thrombocythaemia and the most important mutations

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Ph.D. dissertation

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List of publications related to the thesis

I. Posfai E, Marton I, Szoke A, Borbenyi Z, Vecsei L, Csomor A, Sas K: **Stroke in essential thrombocythemia**. J Neurol Sci 2014;336(1-2): 260-262. **IF 2.243**

II. Posfai E, Marton I, Kiss-Laszlo Z, Kotosz B, Szell M, Borbenyi Z: **Thrombosis and risk factors in female patients with a rare acquired thrombophilia: chronic myeloproliferative disorder - polycythaemia vera and essential thrombocythaemia.** Eur Rev Med Pharmacol Sci 2014;18(24): 3810-3818. **IF 0.988**

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V. Pósfai E, Marton I, Nemes A, Borbényi Z: **Thromboticus események és az IPSET thrombosis rizikóbecslő pontrendszer jelentősége essentialis thrombocytaemiában.** Orv. Hetil 2015;156(14): 558–563. DOI: 10.1556/OH.2015.30117

List of abbreviations

BMI	body mass index
CALR	calreticulin
ET	essential thrombocythaemia
HR	hazard ratio
IPSET	International Prognostic Score of Thrombosis for Essential Thrombocythaemia
JAK	Janus kinase
MI	myocardial infarct
MPL	myeloproliferative leukaemia
MPN	myeloproliferative neoplasm
STAT	signal transducer and activator of transcription
NSTEMI	non-ST segment elevation myocardial infarction
STEMI	ST segment elevation myocardial infarction
TIA	transient ischemic attack
TPO	thrombopoietin
WHO	World Health Organization

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1. INTRODUCTION

Essential thrombocythaemia (ET), characterized by the excessive proliferation of megakaryocytes in the bone-marrow and the overproduction of circulating platelets in the peripherial blood, which was was first recognized as a distinct clinical syndrome by Emil Epstein and Alfred Godelin in 1934, and in 1951 was listed as one of the classical myeloproliferative neoplasms (MPNs) in the classification by William Dameshek [1-3].

MPNs are clonal haematopoietic stem cell malignancies characterized by an abnormal proliferation of one or more myeloid lineages (granulocytic, erythroid, megakaryocytic and mast cells). The revised World Health Organization (WHO) 2008 classification of myeloid neoplasms lists eight entities: *BCR-ABL1*-positive chronic myelogenous leukaemia, and seven *BCR-ABL1*-negative entities: chronic neutrophilic leukaemia; polycythaemia vera; primary myelofibrosis; ET; chronic eosinophilic leukaemia, not otherwise specified; mastocytosis and MPNs, unclassifiable [3]. In the group of *BCR-ABL1*-negative MPNs besides polycythaemia vera, myelofibrosis and ET are the most frequently diagnosed MPN. The data of regional centres indicate an annual incidence of ET in the range 0.21-2.27 per 100,000 inhabitants, with an estimated prevalence of 11 per 100,000 inhabitants [4-7]. The reported median age at the presentation of ET is 60-67 years, while 10% to 25% of the patients are younger than 40 years [6, 8, 9]. In females, the incidence of ET is approximately twice that in males [6, 10].

As ET is characterized by proliferation of the megakaryocytic lineage without significant expansion of the granulocytic and erythroid lineages, the bone-marrow biopsy result frequently reveals a hypercellularity picture with an increased number of enlarged hyperlobated, mature megakaryocytes, without notably increased marrow fibrosis (Fig.1/A) [11, 12]. The peripheral blood smear in many cases demonstrates enhanced numbers of platelets and megakaryocyte fragments (Fig.1/B) [11-13].

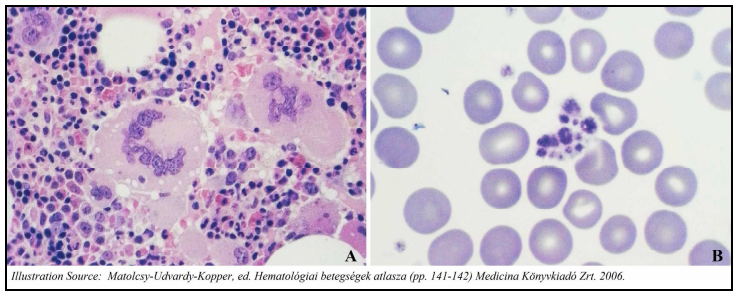

Figure 1.

Bone marrow in ET, hematoxylin-eosin stained section (Fig. 1/A). Peripheral blood smear in ET, May-Grunwald Giemsa staining (Fig.1/B) [12]

In the initial phase of the disease, many patients are symptomless and the diagnosis is commonly established only fortuitously via a routine blood count. An elevated peripheral platelet count is indicative of the diagnosis of ET if the sustained value is ≥ 450 giga/L and other evidence of reactive thrombocytosis, such as infection, inflammation and other chronic myeloid neoplasms or lymphoproliferative disorders, is excluded [13]. There are cases in which the presence of thrombohaemorrhagic events draw attention to ET as the underlying disease.

It has been reported that the most important complications that can exert major effects on the morbidity and mortality of ET patients are thrombohaemorrhagic events, the risk of which ranges around between 11-25%, although ET has a propensity to transform into myelofibrosis and acute leukaemia (the 10-year risk of leukaemic and fibrotic transformations has been stated to be <1% and 1%, respectively) [14-16]. The risk of microvascular (e.g. headaches, dizziness, visual disturbances, distal paraesthesia, acrocyanosis, and erythromelalgia) and major thrombotic complications (e.g. arterial events: myocardial infarction (MI), ischaemic stroke or a transient ischaemic attack (TIA); venous events: deep venous thrombosis) is higher than the risk of haemorrhagic complications [8, 14]. As concerns the major thrombotic events, arterial occur more often than venous thrombotic complications [14]. Detailed clinical characteristics of these thrombotic complications are mainly reported only in case reports or case series [17-27].

The ET-related haemostatic abnormalities and the pathogenesis of the major thrombotic complications or microvascular disturbances seen in ET still pose many questions, and in recent years this topic has been actively investigated [14, 28]. It is currently suggested that not merely the elevated platelet count in the periphery itself, but this together with the consequent qualitative abnormalities of the platelets and other possible additional thrombotic risk factors (e.g. cardiovascular risk factors, leukocytosis and the Janus kinase 2 *(JAK) V617F* mutation may influence the thrombotic complications seen in ET [14, 29-36].

Since the discovery of the JAK-STAT signalling pathway and the *JAK2* (9p24) mutation in 2005, their role in the pathogenesis of ET has been proven (Fig. 2) [8, 14, 37-40].

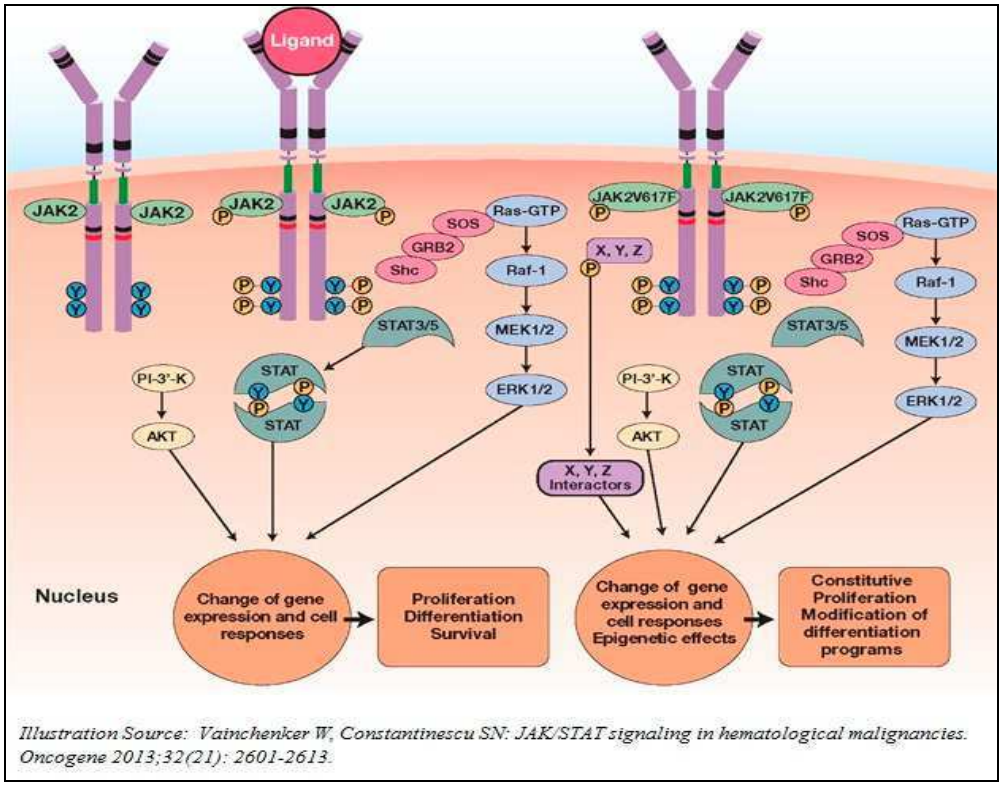

Figure 2.

Schematic illustration of the JAK/STAT signaling in hematological malignancies [41]

The *JAK2 V617F* mutation itself is an acquired point mutation, the result of a valine-to-phenylalanine substitution at codon 617 on the JAK-2 gene (a cytoplasmic tyrosine kinase with an important role in intracellular signal transduction), which leads to constitutive tyrosine phosphorylation activity [42]. In 2008, the testing for *JAK2 V617F* was recommended as a major diagnostic criterion for all suspected MPNs, among which ET patients are positive for the *JAK2 V617F* mutation in approximately 50-60% of the cases [8, 14, 37-39]. *JAK2 V617F* positive (*JAK2 V617F*(+)) ET patients have been reported to be older, and to display higher haemoglobin levels and leukocyte counts than *JAK2 V617F* negative (*JAK2 V617F*(-)) patients [43-45].

In ET patients who do not carry the *JAK2 V617F* mutation, besides the JAK-STAT pathway, the TPO-c-MPL system, which plays an important part in megakaryopoiesis and thrombopoiesis, is presumed to have a role in the clinical course of the disease (Fig. 3) [8, 46].

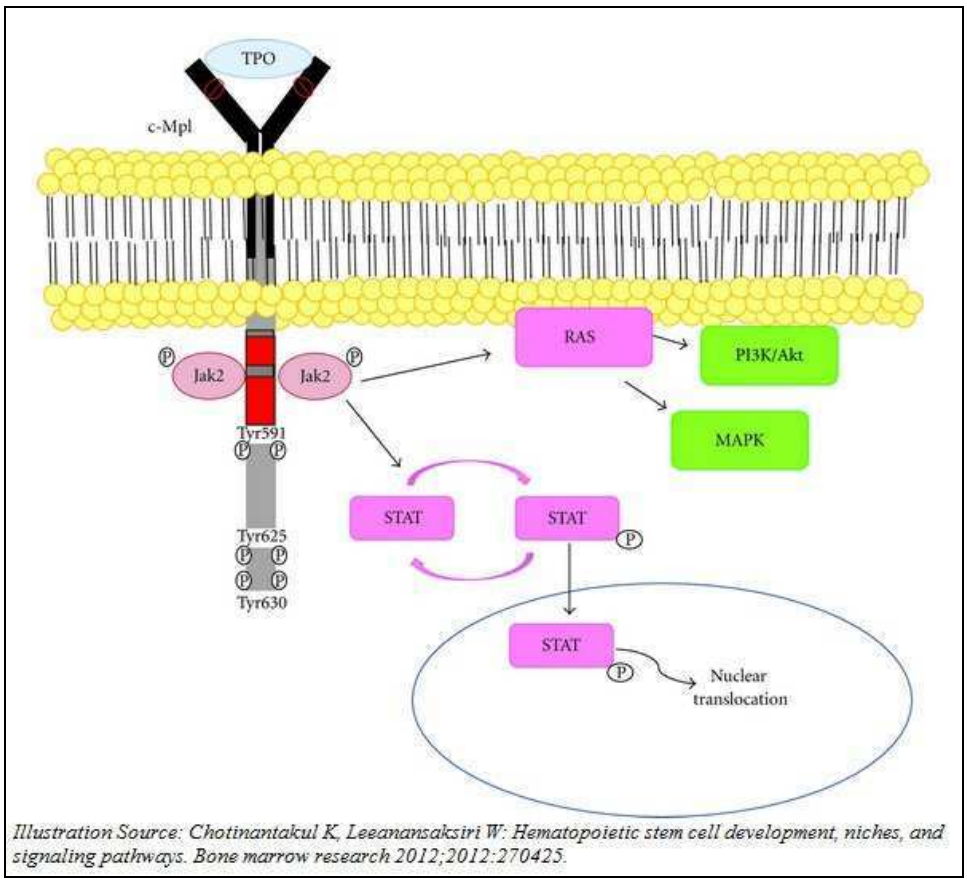

Figure 3.

Schematic illustration of the c-Mpl/TPO signaling pathway [47]

Myeloproliferative leukaemia virus oncogene (*MPL*) (1p34) mutations (*MPL W515L, MPL W515K, MPL W515R, MPL W515A*, and *MPL S505N*) are situated on the MPL gene (coding the thrombopoietin receptor) [8, 46, 48-51]. The presence of these gain-of-function mutations may lead to the overproduction of thrombopoietin and the activation of JAK-STAT signalling [8, 46, 48-51]. The most commonly reported detected *MPL* point mutation in ET is the *W515L* mutation (tryptophan-to-leucine substitution), with a frequency of around 1-5% [50, 52, 53]. *MPL* mutations have been reported to be mainly associated with an older age, a higher platelet count, a lower haemoglobin level and the female gender [49, 54]. However, the potential effect of this mutation on the clinical course of ET has not yet been fully established [50, 52, 53].

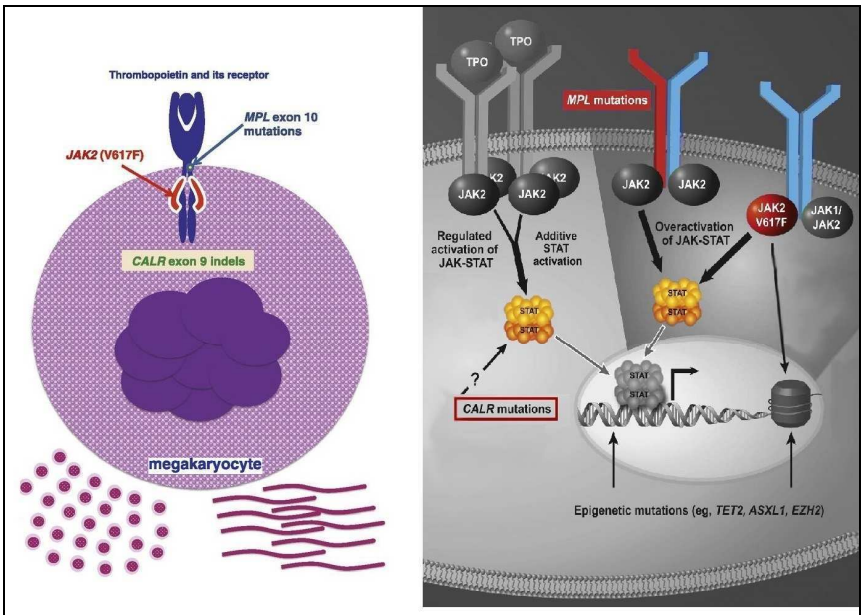

Illustration Source: Cazzola M, Kralovics R: From Janus kinase 2 to calreticulin: the clinically relevant genomic landscape of myeloproliferative neoplasms. Blood 2014;123(24):3714-3719. and Savona MR: Are we altering the natural history of primary myelofibrosis? Leuk Res 2014;38(9):1004-1012.

Figure 4.

Schematic illustration of the mutations in the thrombopoietin receptor gene MPL, in JAK2 (e.g., JAK2V617F) and the result in the constitutive activation of the JAK-STAT pathway and the suggested role of CALR mutations [55, 56]

In *JAK2 V617F* and *MPL* - unmutated ET, somatic calreticulin (*CALR*) (19p13.2) mutations were recently identified (Fig. 4). *CALR* has an important role in endoplasmic reticulum–retention signal, which is absent in mutant *CALR*, and leads to an impared Ca^{2+}-binding function [57]. These are a result of frameshift mutations caused by exon 9 deletions or insertions: e.g., type-1 (c.1092_1143del) and type-2 (c.1154_1155insTTGTC). The estimated frequencies are between 15% and 32% [35, 57-60]. According to the relevant literature, patients with the *CALR* mutation exhibit a more indolent clinical course with a lower incidence of thrombotic events and are characterized by the male sex, a younger age, a higher platelet count and a lower leukocyte count and haemoglobin level than in patients with the *JAK2 V617F* mutation [35, 57, 60-62].

The current haematological management strategy in ET is based on thrombosis risk-oriented recommendations: patients classified as at low risk (age <60 years, without prior thrombotic event) receive anti-platelet therapy (e.g.:aspirin) if necessary, while high-risk

patients (age >60 years and/or with a prior thrombotic event) receive cytoreductive drugs (e.g.:hydroxyurea) alone or in combination with anti-platelet therapy (Table 1) [63, 64].

Table 1. *Risk stratification in ET*

Risk categories	Risk factors
Low-risk	age <60 years and no thrombosis history
High-risk	age >60 years and/or presence of thrombosis history

The roles of the *JAK2 V617F* mutation and other suggested thrombosis risk factors (e.g. cardiovascular risk factors) have not yet been integrated into this currently used risk stratification. However, for more accurate thrombosis risk-guided management, other thrombotic risk factors too should be taken into account. In 2012, Barbui et al. published the idea of a new score system, IPSET (International Prognostic Score of Thrombosis for ET) [32]. The IPSET score includes a consideration of the predictive potential of the *JAK2 V617F* mutation and the cardiovascular risk factors (i.e. high blood pressure, diabetes and active tobacco use) (Table 2). Furthermore, instead of merely the classical high- and low-risk group stratification, it introduces a three-group (IPSET low-risk, IPSET intermediate-risk and IPSET high-risk) stratification model (Table 3) [32].

Table 2. *IPSET risk factors and related score values based on the results of multivariable analysis-derived hazard ratios [32]*

IPSET risk factors	Hazard ratios	Score
Age >60 years	*1.50*	1
Cardiovascular risk factors	*1.56*	1
History of thrombosis	*1.93*	2
JAK2 V617F mutation	*2.04*	2

Table 3. *IPSET risk categories [32]*

IPSET risk categories	Sum of score values
Low-risk	0-1
Intermedier-risk	2
High-risk	> 2

Although this score system appears more promising than the conventional two-categorical risk assessment, and may promote a better prediction of major thrombotic complications in ET, it requires confirmation through investigations at more clinical centres.

2. AIMS

The aim of the current Ph.D work was to create a retrospective study cohort of patients diagnosed with ET at the 2nd Department of Internal Medicine, University of Szeged, between 1999 and 2014, and to evaluate and analyse the following aspects:

1. the occurrence of thrombotic events previously and in the follow-up period;

2. the role of the leukocyte count at the haematological diagnosis as a suggested, but still controversial additional risk factor in the subsequent thrombotic complications;

3. the presence of *JAK2 V617F, MPL* mutations (*W515L, W515K, W515R, W515A, S505N*) and *CALR* (*type-1* and *type-2*) mutations and their clinicohaematological role;

4. the contributions of the main cardiovascular risk factors present at the time of the haematological diagnosis of ET as suggested additional risk factors in the subsequent thrombotic complications;

5. the main neurological and cardiological thrombotic complications from detailed clinical aspects as the most important major thrombotic complications of ET, and whether there are any special characteristics in the everyday clinical practice which could predict the development of these complications or whether any typical neurological or cardiological lesion may be identified which might be specific or characteristic of ET;

6. the clinical value of the recently introduced IPSET model as regards the thrombosis-free survival of the patients.

3. METHODS

3.1. PATIENTS AND DATA COLLECTION

Retrospectively, we established an MPN database for scientific research, including the cases of ET diagnosed at the 2nd Department of Internal Medicine, University of Szeged. In 2011, a basic database ("*database 99-11*") was established on patients diagnosed between 1999 and 2011. In 2014, a new enlarged database was created ("*database 99-14*"), in which patients diagnosed between January 1999 and July 2014 were enrolled. The main demographic and clinicohaematological characteristics of the study populations are presented in Table 4.

Table 4. *Main demographic and clinicohaematological characteristics of the study populations*

Main characteristics of the cohorts	Database 99-11	Database 99-14
Males [N, (%)]	29 (28.7)	49 (31.6)
Females [N, (%)]	72 (71.3)	106 (68.4)
Age at diagnosis, median (years) (range)	61 (20-95)	61 (20-96)
Median follow-up (months) (range)	30.4 (0.26-155.4)	27.9 (0.0-181.3)
Median leukocyte count at diagnosis (range) (giga/L)	9.4 (4.5-34)	9.3 (3.7-34)
Median platelet count at diagnosis (range) (giga/L)	664 (78-2240)	658 (78-2240)
JAK2 V617F (+) cases [N, (%)]	61 (60.4)	108 (69.7)
Conventional risk factors in ET		
Age >60 [N, (%)]	52 (51.5)	74 (47.7)
Prior thrombotic events	38	55

Database 99-11 was used to evaluate the contributions of the main cardiovascular risk factors and the leukocyte count at the time of the haematological diagnosis of ET in the subsequent thrombotic complications, and for a detailed neurological analysis, laboratory testing and the evaluation of clinicohaematological role of the *JAK2 V617F*, *MPL* and *CALR* mutations. *Database 99-14* served as the basis of detailed cardiological analyses and an evaluation of the clinical utility of the IPSET model as concerns the thrombosis-free survival of the patients.

This current scientific research was conducted with the approval of the Regional and Institutional Human Medical Biological Research Ethics Committee and adhered to the Declaration of Helsinki. Informed consent was not required. Patient DNA for genetic analyses

of the *JAK2 V617F*, *MPL* and *CALR* mutations was selected from the DNA bank at the Institute of Medical Genetics, University of Szeged.

Through use of the clinical centre data files (MedSol system), all the haematological results on these patients were reviewed. The thrombotic events before and after the clinical diagnosis of ET were retrospectively compiled for each patient, with special focus on cardiovascular (MI), cerebrovascular (stroke or a TIA) and venous thrombotic events (deep venous thrombosis or pulmonary embolism).

The retrospective analysis of the thrombotic events in the cohort in *database 99-11* revealed 38 prior vascular events in 33 (32.67%) patients: 11 cerebrovascular events (stroke/TIA), 16 cardiovascular events (MI) and 11 venous thrombotic events. During the haematological follow-up after the diagnosis of ET, 16 events were observed in 15 (14.85%) patients: 10 cerebrovascular events (stroke/TIA), 3 cardiovascular events (MI) and 3 venous thrombotic events. The enlarged *database 99-14* finally revealed 55 prior vascular events in 47 (30.32%) patients: 22 cerebrovascular events (stroke/TIA), 17 cardiovascular events (MI) and 16 venous thrombotic events. During the haematological follow-up after the diagnosis of ET, 25 events were observed in 23 (14.83%) patients: 13 cerebrovascular events (stroke/TIA), 7 cardiovascular events (MI) and 5 venous thrombotic events (Table 5).

Table 5. *History of vascular events before the clinical diagnosis of ET and follow-up vascular complications*

Vascular events [N]	Database 99-11	Database 99-14
Prior major vascular events	38	55
Cerebrovascular events (stroke/TIA)	11	22
Cardiovascular events (MI)	16	17
Venous thrombotic events	11	16
Follow-up major vascular complications	16	25
Cerebrovascular events (stroke/TIA)	10	13
Cardiovascular events (MI)	3	7
Venous thrombotic events	3	5

Data on cardiovascular risk factors present at the time of the clinical diagnosis of ET, i.e. hypertension (>140/80 mmHg), tobacco use, diabetes mellitus, hyperlipidaemia (hypercholesterolaemia or hypertriglyceridaemia or both), and obesity (body mass index (BMI) >30 kg/m^2) were collected (Table 6).

Table 6. *Distribution of the cardiovascular risk factors at the time of the clinical diagnosis of ET*

Distribution of cardiovascular risk factors [N, (%)]	Database 99-11	Database 99-14
High blood pressure	47 (46.5)	74 (47.7)
Hyperlipidaemia	13 (12.9)	28 (18.1)
Diabetes mellitus	7 (6.9)	10 (6.5)
Tobacco use	15 (14.9)	17 (11.0)
Obesity (BMI >30 kg/m^2)	16 (15.8)	19 (12.2)

Information relating to an inherited thrombophilic state was reported in only one case when lipoprotein A was diagnosed. In one patient ticlopidine- induced thrombotic thrombocytopenic purpura had developed (with a 78 giga/L platelet count at diagnosis), which drew attention to the underlying ET. In general, the haematological management strategy was based on risk-oriented recommendations: low-risk patients received anti-platelet therapy (e.g. aspirin) if necessary, while high-risk patients were given cytoreductive drugs (e.g. hydroxyurea) alone or in combination with anti-platelet therapy (Table 7). [15, 63, 64].

Table 7. *Haematological treatment of the patients*

Haematological treatment [N, (%)]	Database 99-11	Database 99-14
No treatment	16 (15.8)	19 (12.2)
Antiplatelets	43 (42.6)	79 (51.0)
Hydroxyurea (alone or in combination with antiplatelets)	42 (41.6)	57 (36.8)

3.2. STATISTICAL ANALYSES

Clinical and data for genetic analyses were compiled with Microsoft Office Excel, and subjected to statistical analysis with Statsoft Statistica v 9.1 (Statsoft) and SPSS 20 software (IBM).

Evaluation of the contribution of the leukocyte count

To analyse the possible contribution of the leukocyte count (measured at the time of ET haematological diagnosis) in the prediction of subsequent thrombotic complications, a current patient population-related cut-off value based on bivariate binary logistic regression was first calculated. Optimum regression was used to find the cut-off value of the leukocyte count which had the most balanced predictive value.

Mann–Whitney and multivariate binary logistic regression tests were performed in the cases of the presence or absence of thrombotic complications with the following variables: (a) the calculated leukocyte count cut-off related to the current population, and (b) leukocytosis with a median leukocyte count of at least 11.1 giga/L. For a detailed prudent analysis, the presence of prior thrombotic events, an age over 60, the *JAK2 V617F* mutation, the presence of at least one cardiovascular risk factor and the applied therapy (hydroxyurea or aspirin) as suggested important variables were incorporated in the multivariate binary logistic regression analyses and their effects were considered in its result.

To evaluate the probability of the thrombosis-free survival of patients at different leukocyte counts (as introduced above), the Kaplan–Meier method was used, followed by the log-rank test (Mantel–Cox) [65]. Statistical significance was set at 5% or 10%.

Evaluation of the clinicohaematological role of the JAK2 V617F mutation

The Mann–Whitney test was performed in the case of the *JAK2 V617F* mutation-positive and negative patients in order to compare the overall effects of series of variables: (a) all thrombotic events after the clinical diagnosis of ET, and their subtypes: cardiovascular (MI), cerebrovascular (TIA or stroke) or venous thrombotic events (deep venous thrombosis or pulmonary embolism); (b) age; and (c) the main clinical characteristics: median white blood cell count; median platelet count; median haemoglobin count; median red blood cell count; hepatomegaly; splenomegaly and hepatosplenomegaly. Multivariate binary logistic regression analyses were performed to estimate the probability of thrombotic events in the

presence of the *JAK2 V617F* mutation, and in this context, the conventional risk factors were also involved in the statistical model.

By means of the Kaplan–Meier method, followed by the log-rank test (Mantel–Cox), the probability of thrombosis-free survival was analysed and compared in four subgroups: (a) the *JAK2 V617F*(+), low-risk patients, (b) the *JAK2 V617F*(+) high-risk patients, (c) the *JAK2 V617F*(-) low-risk patients, and (d) the *JAK2 V617F*(-) high-risk patients [65]. The low-risk patients were those aged < 60 years without any prior thrombotic event, while the patients in the high-risk category were those aged > 60 years and/or with a prior thrombotic event [63, 64]. Statistical significance was set at 5% or 10%.

Evaluation of the contributions of cardiovascular risk factors

To evaluate and compare the overall and partial effects of cardiovascular risk factors present at the time of the haematological diagnosis, Mann–Whitney and multivariate binary logistic regression tests were performed in the cases of the presence or absence of thrombotic complications: cerebrovascular (ischaemic stroke or a TIA), cardiovascular (MI) and venous thrombotic events (deep vein thrombosis or a pulmonary embolism). In this context, besides the investigated cardiovascular risk factors (hypertension (> 140/80 mmHg), hyperlipidaemia (hypercholesterolaemia or hypertriglyceridaemia or both), tobacco use, diabetes mellitus and obesity (BMI > 30 kg/m^2)), the conventional risk factors (an age over 60 and prior thrombosis), the *JAK2 V617F* mutation and the therapy of the patients (hydroxyurea and aspirin) were additionally involved and compared. Statistical significance was set at 5% and, in view of the number of enrolled patients, statistical significance was also considered at 10%.

To evaluate and compare the probability of the thrombosis-free survival of (a) ET patients without cardiovascular risk factors and patients with at least one cardiovascular risk factor, and (b) ET patients with at most one cardiovascular risk factor and patients with two or more cardiovascular risk factors, the Kaplan–Meier method was used followed by the log-rank test (Mantel–Cox) [65].

Evaluation of the IPSET model on the thrombosis-free survival of the patients

The patients were subgrouped and the main clinical characteristics were compared on the basis of a 3-tiered prognostic model, IPSET [32]. In this model, patients were stratified into IPSET low-risk, IPSET intermediate-risk and IPSET high-risk groups based on the results of multivariable analysis-derived hazard ratios (HRs): patients age > 60 years (HR =

1.5; 1 point), a history of thrombosis (HR = 1.9; 2 points), cardiovascular risk factors (HR = 1.6; 1 point), and *JAK2 V617F* (HR = 2.0; 2 points) [32]. According to these results, patients with < 2 points could be classified in the IPSET low-risk group. In this model, an IPSET intermediate-risk group is introduced for patients with 2 points. The IPSET high-risk group comprising patients with > 2 points [32]. For the clinical utility of IPSET from the aspect of the thrombosis-free survival of the patients, the IPSET groups were compared by the Kaplan–Meier method followed by the log-rank test (Mantel–Cox) [65].

Limitations

One limitation of the current study is its retrospective design. From a statistical aspect, the current study population included a relatively low number of ET patients, and for appropriate conclusions in special cases, statistical significance was set not only at 5%, but also at 10%.

3.3. LABORATORY METHODS

Samples for genetic analyses were collected from the DNA bank at the Institute of Medical Genetics, University of Szeged, based on the information on the ET patients diagnosed at the 2nd Department of Internal Medicine, University of Szeged, between 1999 and 2014. From EDTA-stabilized peripheral blood samples, DNA was isolated and screened for the *JAK2 V617F* mutation as the part of diagnostic protocol with an allele-specific PCR method [38]. In the cases of patients whose haematological diagnosis was established before the *JAK2 V617F* mutation became an officially recommended part of the diagnostic protocol, their samples were collected retrospectively and also analysed.

The samples of the *JAK2 V617F*(-) patients were suitable for *MPL* mutation (*W515L*, *W515K*, *W515R*, *W515A* and *S505N*) analyses by allele-specific PCR reactions and subsequent agarose gel electrophoresis.

A forward primer *(5'-TGGGCCGAAGTCTGACCCTTT-3') (F)* and a reverse primer *(5'-GAA GTGGCGAAGCCGTAGGT-3') (R),* and an allele-specific forward primer *5'-GGGCCTGCTGCTGCTGAGGCT-3' (FW515L)* were used for *MPL W515L* and 5'-GGGCCTGCTGCTGCTGAGGCA -3' for *MPL W515K* analysis. PCR reactions were performed and the *MPL W515L* and *W515K* mutations were assessed as described by Bergamaschi et al. (Table 8) [66].

Allele-specific primers *5'-TGGGCCTGCTGCTGCTGAGTC-3'* for *MPL W515R*, *5'-GGGCCTGCTGCTGCTGAGGGC-3'* for *MPL W515A* and 5'-*GCATCTAGTGCTGGGCCTCCA-3'* for *MPL S505N* were designed to detect futher mutations of exon 10 of the *MPL* gene and PCR reactions and subsequent agarose gel electrophoresis were performed as described by the Hungarian National Blood Transfusion Service Laboratory, Budapest, Hungary (Table 8). This method was used with their agreement.

Table 8. *Sequences of the used primers for the MPL mutation analysis*

Sequences of primers	
forward:	5'- TGGGCCGAAGTCTGACCCTTT -3'
reverse:	5'- GAAGTGGCGAAGCCGTAGGT-3'
MPL W515L:	5'- GGGCCTGCTGCTGCTGAGGCT -3'
MPL W515K:	5'- GGGCCTGCTGCTGCTGAGGCA -3'
MPL W515R:	5'- TGGGCCTGCTGCTGCTGAGTC -3'
MPL W515A:	5'- GGGCCTGCTGCTGCTGAGGGC -3'
MPL S505N:	5'- GCATCTAGTGCTGGGCCTCCA -3'

As a control experiment, samples were sequenced by an automated single capillary genetic analyser (ABI PRISM 310, Applied Biosystems, Life Technologies) (Fig. 5).

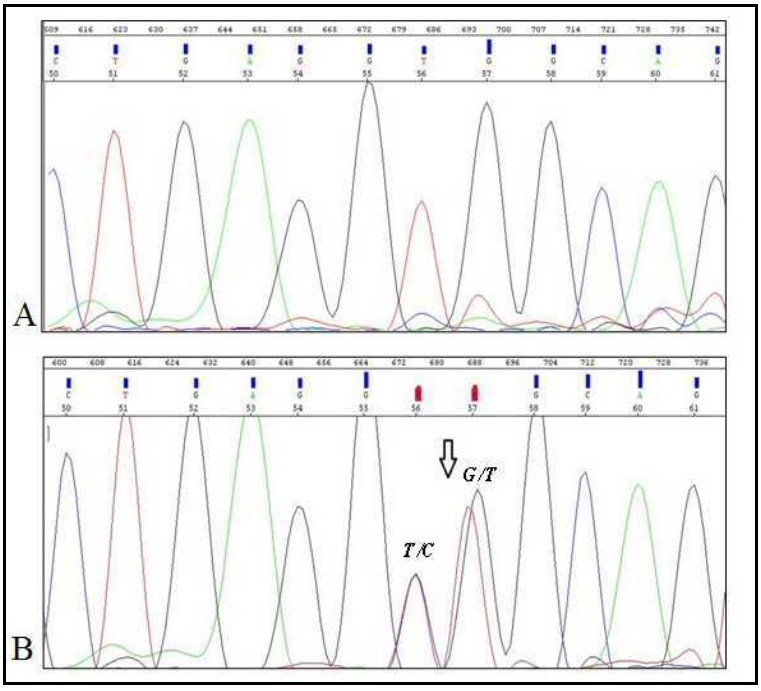

Figure 5.

Sequenogram of a negative (Fig. 5/A) and a positive sample (Fig. 5/B) carrying the MPL W515L mutation. The T->T/C and G ->G/T somatic mutations lead to a heterozygote status resulting in the W515L amino acid change

To assess the *CALR* mutation status, a fragment analysis with FAM-labelled primers (forward: 5'-*AGTTTGGCAACGAGACGTG*-3', reverse: 5'-*GAGTCTCACAGAGACATTATTTGG*-3') were performed on the samples of patients who had *JAK V617F*(-) essential thrombocythaemia. To characterize the types of *CALR* mutations, bidirectional Sanger sequencing was performed with the BigDye 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems). *CALR* mutation analyses were possible in a close cooperation with the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary (Table 9).

Table 9. *Sequences of the used primers for CALR mutation analysis*

Sequences of primers	
forward:	5'-AGTTTGGCAACGAGACGTG-3'
reverse:	5'-GAGTCTCACAGAGACATTATTTGG-3'

4. RESULTS

4.1. THE CONTRIBUTION OF THE LEUKOCYTE COUNT ON SUBSEQUENT THROMBOTIC COMPLICATIONS

On the basis of *database 99-11,* the median leukocyte count measured at the haematological diagnosis was 9.4 giga/L (range: 4.5-34.0 giga/L). Separately, in the cases when no thrombotic complications were observed during the follow-up period, the median leukocyte count was 9.4 giga/L (range: 4.5-28.6 giga/L), while in the cases when subsequent thrombotic complications were observed in the follow-up period, the median leukocyte count was 9.1 giga/L (range: 5.7-34.0 giga/L). In detaily, the patients who had cerebrovascular complications in the follow-up period (stroke or TIA) had a median leukocyte count of 7.5 giga/L (range: 5.7-13.3 giga/L) at the time of ET diagnosis. In the cases of cardiovascular complications (MI), the patients had a median leukocyte count of 10.4 giga/L (range: 9.3-16.7 giga/L), while the median leukocyte count in the cases of the patients who had subsequent venous thrombotic events was 16.7 giga/L (range: 10.1-34.0 giga/L).

As concerns the possible contribution of the leukocyte count at the time of ET diagnosis in the prediction of subsequent thrombotic complications, the optimal regression gave 9.15 giga/L as the cut-off value of the leukocyte count which had statistically the most balanced predictive value.

In the comparison of the two subgroups which differed in the presence or absence of major thrombotic complications in order to explore the overall and partial effects of the leukocyte count cut-off value 9.15 giga/L , Mann–Whitney univariate tests and multivariate binary logistic regression analyses revealed only (univariate: p=0.813; multivariate: relative risk: 0.528, 95% CI 0.156-1.785; p=0.304) non-significant tendencies. The analysis of the contribution of leukocytosis (at a median leukocyte count of at least 11.1 giga/L) also led to non-significant results (univariate: p=0.525; multivariate: relative risk: 0.325, 95% CI 0.071-1.487; p=0.147).

Similarly, a significant difference was not observed in connection with the Kaplan–Meier curves followed by the log-rank test (Mantel–Cox) in the comparison of the thrombosis-free survival of the patients who suffered and did not suffer thrombotic complications in the follow-up period with a leukocyte count cut-off of 9.15 giga/L (p=0.728) or with a median leukocyte count of 11.1 giga/L (p=0.478).

4.2. THE PRESENCE OF THE JAK2 V617F, MPL AND CALR MUTATIONS AND THEIR CLINICOHAEMATOLOGICAL ROLES

The JAK2 V617F mutation

From *database 99-11, JAK2 V617F*(+) cases were detected in 61 (60.39%) patients. The comparison of the *JAK2 V617F*(+) and *JAK2 V617F*(-) patients (Table 10) by means of univariate analysis revealed no statistically significant association with the thrombotic complications (p=0.651) or with the separately analysed cardiovascular events (p=0.849), cerebrovascular events (p=0.558) or venous thrombotic events (p=0.849). An age > 60 years (p=0.060) and the median platelet count (p=0.042), the haemoglobin level (p=0.000), the red blood cell count (p=0.000) and the haematocrit (p=0.000) were significantly different in the *JAK2 V617F*(+) and *JAK2 V617F*(-) groups. The median white blood cell count was not significantly higher in the *JAK2 V617F*(+) group than in the *JAK2 V617F*(-) group (p=0.401). At the haematological diagnosis of ET, a significantly higher number of hepatomegaly cases were observed in the *JAK2 V617F*(+) group than in the *JAK2 V617F*(-) group (p=0.045). However, the numbers of splenomegaly cases (in the *JAK2 V617F*(+) group vs. in the *JAK2 V617F*(-) group (p=0.973)), and hepatosplenomegaly cases (in the *JAK2 V617F*(+) group vs. in the *JAK2 V617F*(-) group (p=0.383)) did not show a significant difference (Table 10) [67].

Table 10. *Results of the Mann–Whitney tests for the comparison of the patients in the presence or the absence of the JAK2 V617F mutation*

Comparison of JAK2 V617F(+) patients and JAK2 V617F(-) patients		
VARIABLES		**Mann –Whitney p-value**
Thrombotic events after diagnosis	All	0.651
	Cardiovascular events	0.849
	Cerebrovascular events	0.558
	Venous thrombotic events	0.849
Age		**0.060***
Clinical characteristics and laboratory findings at ET diagnosis	Leukocyte count	0.401
	Platelet count	**0.042****
	Haemoglobin count	**0.000****
	Red blood cell count	**0.000****
	Haematocrit level	**0.000****
	Hepatomegaly	**0.045****
	Splenomegaly	0.973
	Hepatosplenomegaly	0.383

*Significant differences at 10% are marked by * and at 5% by ***

Multivariate binary logistic regression analysis on the subsequent thrombotic events after the diagnosis of ET revealed a significant partial effect of the prior thrombotic events (relative risk: 2.876, 95% CI 0.847-9.774; p=0.090), but a significant association was not observed between the *JAK2 V617F* mutation status (relative risk: 1.297, 95% CI 0.395-4.258; p=0.668), an age over 60 years (relative risk: 0.981, 95% CI 0.316-3.048; p=0.974) and the probability of subsequent thrombotic complications [67].

To estimate the contribution of the presence of the *JAK2 V617F* mutation on the probability of thrombosis-free survival during the follow-up period, the *JAK2 V617F*(+), low-risk patients, the *JAK2 V617F* (+) high-risk patients, the *JAK2 V617F* (-) low-risk patients and the *JAK2 V617F*(-) high-risk patients were compared via the Kaplan–Meier curves and the log-rank test (Mantel–Cox), which revealed only non-significant differences, p=0.548 (Fig. 6) [67].

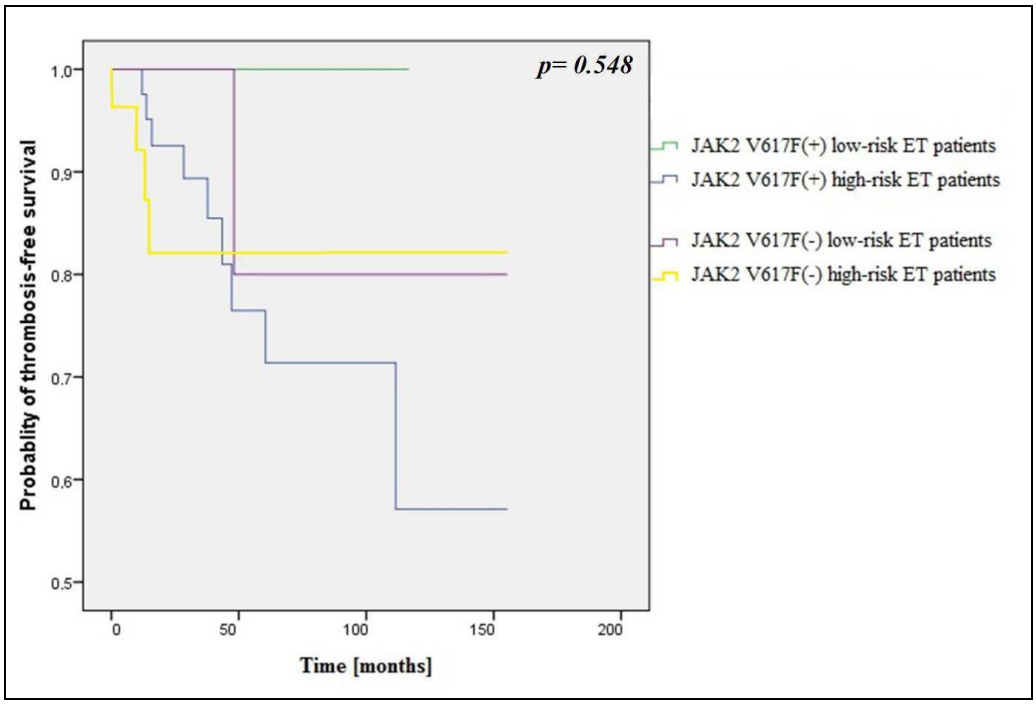

Figure 6.

Probability of thrombosis-free survival in the haematological follow-up period in the JAK2 V617F(+) low-risk, JAK2 V617F(+) high-risk, JAK2 V617F(-) low-risk and JAK2 V617F(-) high-risk ET patients

The MPL and CALR mutations

The *MPL W515L/K/R/A*, and *S505N* mutations were analysed on the basis of *database 99-11*. In the cases of 4 *JAK2 V617F*(-) ET patients, DNA was not available for further analyses, and thus samples from 36 *JAK2 V617F*(-) ET patients could be used for *MPL* analyses. The allele-specific PCR and the subsequent sequence analyses revealed *MPL W515L* mutation positivity in 4 samples, while for the *MPL W515/K/R/A* and *S505N* mutations the DNA tests of the 36 *JAK2 V617F*(-) patients were negative [67].

Table 11. *Demographic and clinicohaematological characteristics of JAK2 V617F(-),MPL W515L(-); JAK2 V617F(-), MPL W515L(+) and JAK2 V617F(-), MPL W515L(-), CALR type-2(+) patients*

CHARACTERISTICS	DATA		
	JAK2 V617F(-) MPL W515L(-) patients	**JAK2 V617F(-) MPL W515L(+) patients**	**JAK2 V617F(-) MPL W515L(-) CALR type-2(+) patients**
Male (N)	10	1	1
Female (N)	22	3	2
Median age at diagnosis (years)	56.5	70	49
Main clinical characteristics at ET diagnosis			
Median leukocyte count (giga/L)	8.7	9.9	7.4
Median platelet count (giga/L)	585	845.5	951
Median haemoglobin count (g/L)	128.5	125	120
Median red blood cell count (T/L)	4.3	4.5	3.8
Hepatomegaly cases	1	none	none
Splenomegaly cases	4	1	1
Hepatosplenomegaly cases	1	none	none

As regards a comparison of the *JAK2 V617F*(-) patients with or without the *MPL W515L* mutation (Table 11), the difference in the numbers of *MPL W515L*(+) and *MPL W515L*(-) patients did not allow a meaningful statistical analysis, but the *MPL W515L* mutation was predominantly observed in female patients, and in older patients (median age: 70 years). The *MPL W515L*(+) patients exhibited a higher median platelet count at the initiation of the haematological observation (845.5 giga/L) than that of the *JAK2 V617F*(-), *MPL W515L*(-) patients (585 giga/L). The comparison of the numbers of prior and follow-up thrombotic events interestingly revealed a lower number of thrombotic complications in the *JAK2 V617F*(-), *MPL W515L*(+) patients than in the *JAK2 V617F*(-), *MPL W515L*(-) patients (Table 12) [67].

Table 12. *History of vascular events before the clinical diagnosis of ET and follow-up vascular complications in the cases of JAK2 V617F(-), MPL W515L(-); JAK2 V617F(-), MPL W515L(+) and JAK2 V617F(-), MPL W515L(-), CALR type-2(+) patients*

CHARACTERISTICS	DATA		
	JAK2 V617F(-) MPL W515L(-) patients	**JAK2 V617F(-) MPL W515L(+) patients**	**JAK2 V617F(-) MPL W515L(-) CALR type-2(+) patients**
Prior vascular events			
Cerebrovascular events (stroke/TIA)	3	1	1
Cardiovascular events (MI)	8	no events	no events
Venous thrombotic events	3	2	no events
Major vascular events in follow-up			
Cerebrovascular events (stroke/TIA)	4	1	no events
Cardiovascular events (MI)	1	no events	no events
Venous thrombotic events	1	no events	no events

CALR type-1 and *type-2* mutation analyses were possible in the cases of 22 *JAK2 V617F*(-) and *MPL W515L*(-) ET patients where sufficient DNA was available for further genetic investigations. The allele-specific PCR and the subsequent sequence analyses revealed positivity only in the case of the *CALR* mutation type-2 (c.1154_1155insTTGTC), which was detected in 3 samples. These patients were relatively young (median age: 49 years), with a higher median platelet count (951 giga/L) and with a lower number of thrombotic complications in the follow-up period as compared with the *JAK2 V617F*(-), *MPL W515L*(-) or the *JAK2 V617F*(-), *MPL W515L*(+) patients (Table 11,12) [67].

4.3. THE CONTRIBUTIONS OF THE CARDIOVASCULAR RISK FACTORS TO SUBSEQUENT THROMBOTIC COMPLICATIONS

The univariate and multivariate statistical analyses based on *database 99-11* revealed a significant overall association between the thrombotic complications and a high blood pressure (univariate p=0.092; multivariate: relative risk: 2.174, 95% CI 0.531-8.899; p=0.280) and partial and overall effects in the case of hyperlipidaemia (univariate: p=0.011; multivariate: relative risk: 3.511, 95% CI 0.797-15.470; p=0.097). Tobacco use (univariate: p=0.545; multivariate: relative risk: 0.971 95% CI 0.193-4.890; p=0.971), diabetes mellitus (univariate: p=0.965; multivariate: relative risk: 0.735 95% CI 0.063-8.555; p=0.806) and obesity (univariate: p=0.634; multivariate: relative risk: 0.835, 95% CI 0.175-3.990; p=0.821)

were not associated with a risk of subsequent thrombosis. The presence of one cardiovascular risk factor (univariate: p=0.096) or two or more cardiovascular risk factors (univariate: p=0.025; multivariate: relative risk: 2.862, 95% CI 0.852-9.614; p=0.089) significantly increased the risk of thrombotic complications (Table 13) [68].

Table 13. *Mann–Whitney test and multivariate binary logistic regression results in the comparison of ET patient subgroups who did or who did not suffer thrombotic events in*

Comparison of ET patients who did or who did not suffer thrombotic events in the follow-up period				
VARIABLES	**MANN–WHITNEY UNIVARIATE ANALYSIS**	**MULTIVARIATE BINARY LOGISTIC REGRESSION ANALYSIS**		
	p	p	Odds ratio	95% CI
NEWLY SUGGETED RISK FACTORS IN ET				
Cardiovascular risk factors				
High blood pressure	**0.092***	0.280	2.174	0.531- 8.899
Hyperlipidaemia	**0.011****	**0.097***	3.511	0.797-15.470
Tobacco use	0.545	0.971	0.971	0.193- 4.890
Diabetes mellitus	0.965	0.806	0.735	0.063- 8.555
Obesity	0.634	0.821	0.835	0.175- 3.990
The presence of only one cardiovascular risk factor	**0.096***	*ND*		
The presence of two or more cardiovascular risk factors	**0.025****	**0.089***	2.862	0.852- 9.614
JAK2 V617F mutation	0.651	0.903	1.083	0.301- 3.891
CONVENTIONAL RISK FACTORS				
Prior thrombotic events	**0.066***	0.170	2.406	0.686- 8.437
Age >60 years	0.877	0.763	0.815	0.217- 3.067
THERAPY				
Antiplatelet	0.730	*ND*		
Hydroxyurea	0.319	*ND*		

*Significant differences at 10% are marked by * and at 5% by **.Abbreviation: ND, not determined*

In this context, the *JAK2 V617F* mutation, and the well-known conventional risk factors were also investigated. Univariate and multivariate statistical analyses revealed non-significant tendencies, from the aspects of the presence of the *JAK2 V617F* mutation (univariate: p=0.651; multivariate: relative risk: 1.083, 95% CI 0.301-3.891; p=0.903) and an age over 60 years (univariate: p=0.877; relative risk: 0.815, 95% CI 0.217-3.067; p=0.763). However the partial effect of the reported previous vascular events in the possible occurrence

of further thrombosis was significant (univariate: p=0.066; multivariate: relative risk: 2.406, 95% CI 0.686-8.437; p=0.170) (Table 13). The administered therapy was incorporated in the analysis in order to consider its potential influence on the thrombotic events in the follow-up period. Although differences could be observed between the patients treated with antiplatelet (univariate: p=0.730) or cytoreductive therapy (univariate: p=0.319) in the two subgroups, depending on the presence or absence of major thrombotic events, these differences were not significant (Table 13).

To compare the thrombosis-free survival of the patients in the presence or absence of the investigated cardiovascular risk factors, Kaplan–Meier curves and log-rank tests (Mantel–Cox) were utilized, which resulted in a significant difference between the thrombosis-free survival of the ET patients without cardiovascular risk factors (n=47) and those with at least one cardiovascular risk factor (n= 54) (p=0.011) (Fig.7) [68].

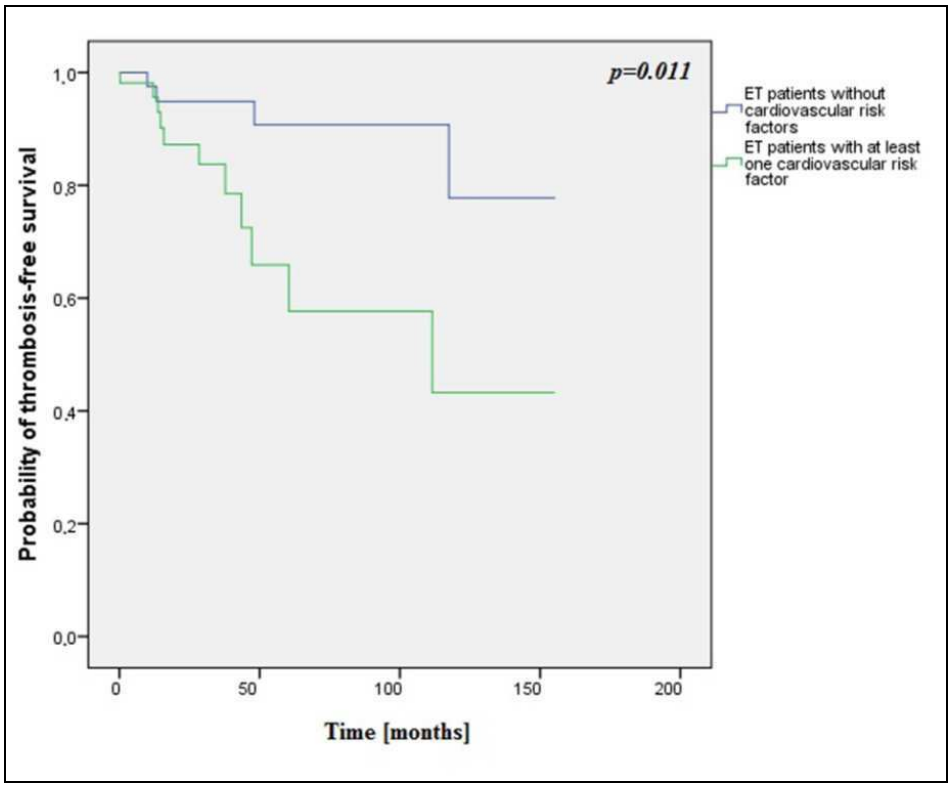

Figure 7.

Probability of thrombosis-free survival in the haematological follow-up period in the subgroups of ET patients without cardiovascular risk factors, and ET patients with at least one cardiovascular risk factor

A significant difference was also observed between the ET patients with at most one cardiovascular risk factor (n=77) and those with two or more cardiovascular risk factors (n=24) (p=0.002) (Fig. 8).

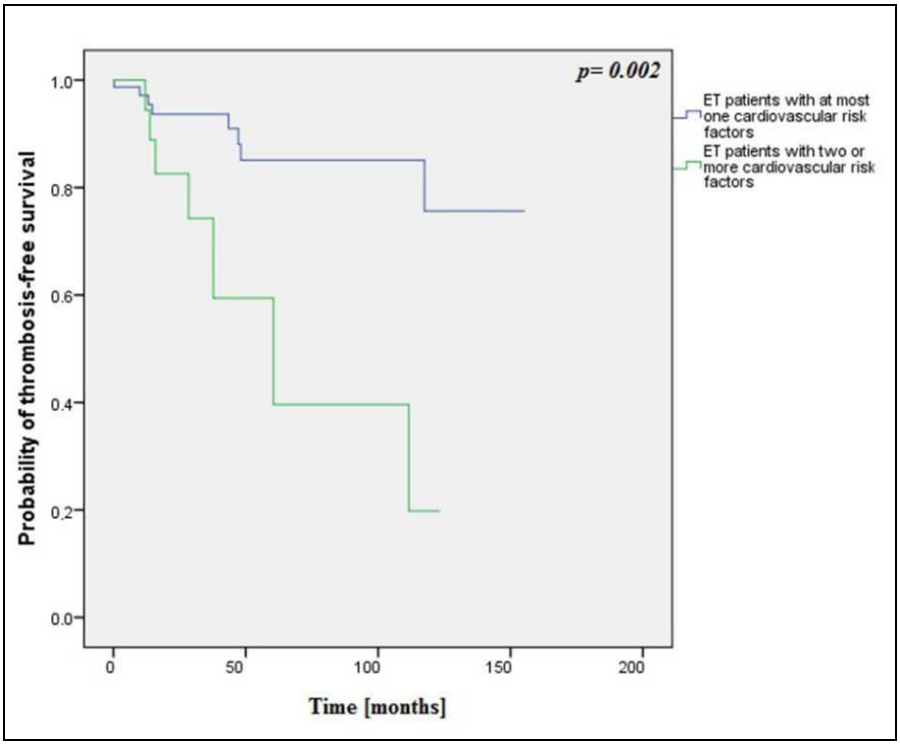

Figure 8.

Probability of thrombosis-free survival in the haematological follow-up period in the subgroups of ET patients with at most one cardiovascular risk factor, and ET patients with two or more cardiovascular risk factors

4.4. ANALYSES OF THE NEUROLOGICAL AND CARDIOLOGICAL CHARACTERISTICS OF THE CEREBROVASCULAR AND CARDIOVASCULAR COMPLICATIONS, AS THE MOST SEVERE THROMBOTIC COMPLICATIONS OF ET PATIENTS

Cerebrovascular complications

In 2012, *database 99-11* revealed cerebrovascular complications (stroke/TIA/ vertebrobasilar insufficiency) in 4 males and 7 females with a median age of 67 years [range: 45-82 years]). The median platelet count at hematological diagnosis was 658 giga/L [range: 514-1157 giga/L], while at the time of the cerebrovascular events it was 450 giga/L [range: 320-885 giga/L]. All of the 11 analysed cases were *JAK2 V617F*(+).

Mostly TIAs and/or minor strokes were noted. In most cases (8/11; 73%), the brain imaging modalities (skull CT and/or MRI) revealed periventricular and/or subcortical and/or basal ganglia lacunes or infarcts or confluent chronic white matter ischaemic lesions in all cerebral arterial regions. There were hardly any infratentorial lesions. Atrophy with diverse degrees of severity was frequently seen (7/11; 64%). Mainly large infarcts were detected in the middle and posterior arterial regions in 4 patients, 3 of whom exhibited posterior watershed-type infarcts. In one, patient with a 665 giga/L platelet count, a haemorrhagic transformation of a large parieto-occipital infarct was noted, without clinical deterioration. In 2 cases, the stroke complication itself drew attention to the presence of the underlying ET. Recurrent stroke or a vertebrobasilar insufficiency were seen despite the ongoing antiplatelet and cytoreductive therapy.

No correlation was found between the platelet count and the stroke type or occurrence of stroke, although supratentorial lacunar infarcts and chronic white matter lesions predominated. It is important to note that most patients (7/11; 64%) displayed at least two or more serious conventional vascular risk factors. It could be suggested that these could have influenced both the clinical course and the morphological alterations seen on brain imaging [69].

The cases are detailed individually in Table 14.

Table 14. *The main characteristics of ET patients with cerebrovascular complications*

CASE NO. AGE/GENDER/ DATE OF ET DIAGNOSIS	CARDIOVASCULAR RISK FACTORS	NEUROLOGICAL COMPLICATIONS		*TREATMENT AT THE TIME OF THE FIRST STROKE*	*TREATMENT AFTER THE FIRST STROKE*
		NEUROLOGICAL PRESENTATION	CT/MRI FINDINGS		
CASE 1 **66/M/2010**	hypertension hyperlipidaemia	**2011:** right MCA TIA **2012:** dementia syndrome	MRI: lacunes in basal ganglia in white matter on both sides, mild periventricular chronic white matter lesions CT: as above	aspirin + hydroxyurea	clopidogrel + hydroxyurea
CASE 2 **74/M/2009**	hypertension	**2010**: right MCA+PCA stroke **2010:** serious clinical condition,VBI	CT: right parieto-occipital infarct (diameter: 80 x 30 mm) CT: as above + circumscribed parieto-occipital atrophy, mild periventricular ischaemic white matter lesions	aspirin	clopidogrel
CASE 3 **79/F/2009**	hypertension hyperlipidaemia	**2010:** right MCA minor stroke **2011:** right MCA TIA	CT: some lacunes in frontal and occipital lobes on both sides, mild periventricular chronic ischaemic lesions CT: mild atrophy, periventricular and subcortical ischemic white matter lesions	aspirin + hydroxyurea	clopidogrel + hydroxyurea
CASE 4 **55/M/2008**	hypertension hyperlipidaemia cigarette smoking	**2008:** left MCA minor stroke ***(this stroke drew attention to the ET)*** **2011:** worsening of symptoms of prior left MCA stroke **2012:** recurrent left MCA stroke	CT: acute left parietal infarct (diameter: 15 x 20 mm) CT: mild atrophy, chronic left parietal and posterior watershed infarct (diameter: 60 x 40 mm) and some lacunes in basal ganglia on both sides CT: cerebral atrophy med. gr., left chronic parietal and watershed infarct (60 x 40 mm), some lacunes in basal ganglia on both sides	aspirin + clopidogrel	aspirin + clopidogrel + hydroxyurea
CASE 5 **67/F/2003**	hypertension	**2006:** left MCA minor stroke **2007:** recurrent VBI **2010:** recurrent VBI	CT: left MCA subcortical lacunar infarct CT: left MCA small chronic subcortical infarct (diameter: 15 x 20 mm); no new lesion CT: as above + periventricular chronic ischaemic lesions	clopidogrel + hyroxyurea	clopidogrel + hyroxyurea

CASE 6 **71/F/2001**	hypertension hyperlipidaemia	**2010:** left MCA stroke	CT: mild atrophy, widespread periventricular ischaemic lesions, multiple basal ganglia lacunes on both sides	aspirin	clopidogrel
CASE 7 **74/F/2007**	hypertension	**2011:** VBI	MRI: mild atrophy, widespread periventricular and subcortical chronic ischaemic lesions	nothing	clopidogrel
CASE 8 **60/F/2006**	hypertension hyperlipidaemia obesity	**2009:** left MCA minor stroke	CT, MRI: basal ganglia lacunes on both sides, periventricular, subcortical confluent chronic ischaemic lesions	aspirin + hydroxyurea	clopidogrel + hydroxyurea
CASE 9 **61/F/2001**	hypertension	**2006:** right MCA+PCA stroke	CT: cerebral atrophy med. gr., right posterior watershed infarct (diameter: 60 x 30 x 30 mm) with slight haemorrhagic transformation	clopidogrel	clopidogrel
CASE 10 **45/M/2006**	hypertension hyperlipidaemia cigarette smoking	**2006:** left MCA TIA ***(this stroke drew attention to the ET)*** **2011:** VBI	CT: negative CT: negative	aspirin	clopidogrel
CASE 11 **82/F/2003**	hypertension diabetes mellitus	**2005:** left MCA minor stroke	CT: mild atrophy; no further information since then	hydroxyurea	aspirin + hydroxyurea

Abbreviations: ET, essential thrombocythaemia; F, female; M, male; MCA, middle cerebral artery; PCA, posterior cerebral artery; TIA, transient ischaemic attack; VBI, vertebrobasilar insufficiency

Cardiovascular complications

Detailed analyses on the cardiovascular complications based on *database 99-14* revealed 7 ET patients (3 males and 4 females with a median age of 61 years [range: 38-76 years]) who suffered MI during the haematological follow-up period. With the exception of one case, the ET haematological diagnosis and the presence of the MI occurred within 12 months.

The median platelet count at hematological diagnosis was 647 giga/L [range: 562-732 giga/L], while at the onset of MI it was 630 giga/L [range: 346-1190 giga/L]. Four (57%) of the analysed 7 cases were *JAK2 V617F*(+). Most of the patients (6/7; 86%) displayed at least one serious conventional vascular risk factor.

The coronary angiography findings revealed ST segment elevation MI (STEMI) in 4 cases (1 subacute STEMI, 2 anterior STEMI and 1 inferior STEMI), while non-STEMI (NSTEMI) was observed in 3 patients. Stent implantation had been performed in most of the patients. In 5 cases, significant stenosis of the coronary arteries required percutaneous coronary intervention with a stent implantation. One patient had undergone a coronary artery bypass graft operation. Recanalization proved unsuccessful in one case. Reccurrent MI events were not observed in the follow-up period (Table 15).

We found no correlation between the platelet counts and the MI type or the degree of stenosis.

Table 15. *Main characteristics of ET patients with myocardial infarction*

CASE NO. AGE/GENDER/ DATE OF DIAGNOSIS	TIME BETWEEN CARDIOLOGICAL EVENT AND ET DIAGNOSIS	CARDIOVASCULAR RISK FACTORS PRESENT AT ET DIAGNOSIS	JAK2 V617F MUTATION	CARDIOLOGICAL COMPLICATIONS		*HAEMATOLOGICAL TREATMENT AFTER ET DIAGNOSIS*
				CARDIOLOGICAL PRESENTATION	CORONARY ANGIOGRAPHY FINDINGS	
CASE 1 **67/M/2011**	4 months	hyperlipidaemia	negative	anterior STEMI	LAD: proximal critical and mid 40% stenosis (PCI-stent implantation) LCX: ostial 30% stenosis RC: normal	aspirin + clopidogrel
CASE 2 **54/F/2011**	3 months	hypertension, smoking	negative	anterior STEMI	LAD: mid occlusion (PCI-stent implantation) LCX: proximal borderline stenosis RC: chronic total occlusion (PCI-stent implantation)	clopidogrel + hydroxyurea
CASE 3 **38/F/2009**	1 months	smoking	*positive*	inferior STEMI	LAD: diagonal borderline stenosis LCX: normal RC: thrombotic subtotal occlusion (PCI-stent implantation)	aspirin + hydroxyurea
CASE 4 **61/F/2011**	9 months	hypertension, obesity	negative	subacute inferior STEMI	LAD: normal LCX: normal RC: occluded (unsuccessful recanalization)	aspirin + hydroxyurea
CASE 5 **55/M/1999**	139 months	none	*positive*	NSTEMI	LAD: 20% stenosis in LM (due to LM dissection PCI-stent implantation) LCX: I. OM branch ostial critical stenosis RC: proximal significant stenosis	aspirin + hydroxyurea
CASE 6 ***73/F/2013***	9 months	hypertension	*positive*	NSTEMI	LAD: significant stenosis in ostium of I. diagonal branch LCX: normal RC: proximal 80% stenosis (PCI-stent implantation)	aspirin
CASE 7 ***76/M/2012***	7 months	hypertension	*positive*	NSTEMI	LAD: stent in LM, ostial significant LAD stenosis, LIMA-LAD, SVG-diagonal LCX: proximal stent RC: SVG-CD (CABG)	aspirin + hydroxyurea

Abbreviations: CABG, coronary artery bypass grafting; ET, essential thrombocythaemia; F, female; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; LIMA, left internal mammarian artery; LM, left main artery; M, male; NSTEMI, non-ST segment elevation myocardial infarction; OM, obtuse marginal artery; STEMI, ST segment elevation myocardial infarction; PCI, percutaneous coronary interventions; RC, right coronary artery, SVG, saphenous vein graft

4.5. THE IPSET MODEL AND THE THROMBOSIS-FREE SURVIVAL OF THE PATIENTS

Patients (based on *database 99-14*) were subgrouped and the main clinical characteristics were compared (Table 16) on the basis of the IPSET score [32].

Table 16. *Main demographic and clinicohaematological characteristics of the patients according to the IPSET low-risk, IPSET intermediate-risk and IPSET high-risk groups*

CHARACTERISTICS OF THE COHORT	IPSET low-risk	IPSET intermediate-risk	IPSET high-risk
Patients [N, (%)]	26 (16.8)	36 (23.2)	93 (60.0)
Males (N)	8	16	25
Median age at diagnosis (years)	48.5	51.8	67.5
Median follow-up (years)	3.6	3.3	3.1
JAK2 V617F(+) cases [N, (%)]	0 (0.0%)	24 (66.7%)	84 (90.3%)
Blood counts at the time of the haematological diagnosis			
Mean platelet count (± standard deviation) (giga/L)	707.2 ± 336.8	696.3 ± 325.7	723.4 ± 321.4
Mean leukocyte count (± standard deviation) (giga/L)	8.6 ± 2.5	10.0 ± 5.8	11.1 ± 4.6
Mean red blood cell count (± standard deviation) (T/L)	4.3 ± 0.4	4.6 ± 1.0	4.8 ± 1.1
Mean haemoglobin value (± standard deviation) (g/L)	133.2 ± 14.1	138.8 ± 22.0	139.2 ± 23.5
Mean haematocrit value (± standard deviation) (%)	39.5 ± 4.7	40.6 ± 6.9	41.3 ± 7.2
Organomegaly at the time of the haematological diagnosis			
Hepatomegaly (N)	0	4	12
Splenomegaly (N)	5	4	14
Hepatosplenomegaly(N)	0	1	7
Major vascular events			
Prior vascular events Number of patients (%) Number of events	 0 (0.0%) 0	 4 (11.1%) 5	 43 (46.2%) 50
Follow-up period vascular complications Number of patients (%) Number of events	 1 (3.8%) 1	 5 (13.9%) 6	 17 (18.3%) 18
Distribution of cardiovascular risk factors [N, (%)]			
High blood preassure	4 (15.4)	7 (19.4)	63 (67.7)
Hyperlipidaemia	4 (15.4)	1 (2.8)	23 (24.7)
Diabetes mellitus	1 (3.8)	0 (0.0)	9 (9.7)
Tobacco use	2 (7.7)	0 (0.0)	15 (16.1)
Obesity	1 (3.8)	2 (5.6)	16 (17.2)
Distribution of patients according to the conventional two-categorical risk assessment [N, (%)]			
Low-risk patients	18 (69.2)	22 (61.1)	13 (14.0)
High-risk patients	8 (30.8)	14 (38.9)	80 (86.0)

From the data on the current cohort, the clinical characteristics of an intermediate-risk group of patients could be clearly differentiated from those of the low- and high-risk groups [70].

To compare the thrombosis-free survival of the patients categorized in the IPSET low-risk, IPSET intermediate-risk and IPSET high-risk groups, the Kaplan–Meier method was applied, followed by the log-rank test (Mantel–Cox), which resulted in significant differences between the different IPSET groups (p=0.002) (Fig. 9) [70].

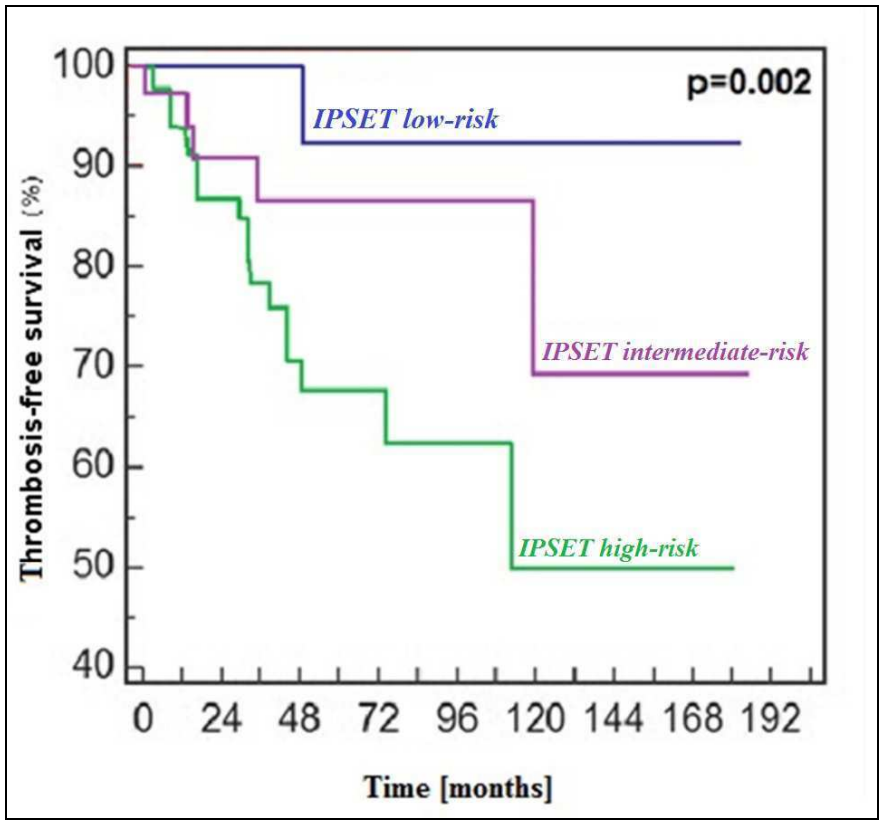

Figure 9.

Probability of thrombosis-free survival in the haematological follow-up period in IPSET low-risk, IPSET intermediate-risk and IPSET high-risk categories

5. DISCUSSION

ET is a chronic Philadelphia chromosome-negative MPN characterized by the overproduction of circulating platelets in the peripherial blood due to the excessive proliferation of megakaryocytes in the bone-marrow [71-73]. Epidemiological data on chronic Philadelphia chromosome-negative MPNs to estimate the incidence or the prevalance of ET are rare in the literature; only date from a few regional centres are available. The reported annual incidence of ET is in the range 0.21 to 2.27 per 100,000 inhabitants [4-7]. In the 2nd Department of Internal Medicine at the University of Szeged, which caters for approximately 1,297,735 inhabitants in the south-eastern Hungarian region, data on 155 ET patients were available in the period 1999-2014 [74].

Although ET is not a common disease in the general population and the patients have a relatively normal life expectancy, there is a possible risk that it will progress to myelofibrosis or/and acute myeloid leukaemia, and that thrombohaemorrhagic complications will occur, which are most commonly responsible for the morbidity and mortality. The reported incidence of thrombohaemorrhagic events in ET patients lies in the interval 11-25% [14-16]. Thrombotic events appear more commonly than haemorrhagic ones and among the thrombotic complications arterial thrombosis is more often observed than venous thrombosis [14]. In the current cohort in the period 1999-2014, major vascular complications were observed on 25 occasions in 23 patients (14.8%), arterial complications (80%) predominating over venous ones (20%).

The current goal of ET therapy is to prevent thrombohaemorrhagic complications without increasing the bleeding risk [40]. The haematological management strategy is based on risk-oriented recommendations, according to which the patients are stratified into low- and high-risk subgroups by the absence or presence of either an age > 60 years or a history of thrombosis [15, 63, 64]. However, recent publications suggest that this two-categorical classical risk stratification may not be sufficiently sensitive. The impact of other additional risk factors on thrombosis, such as classic cardiovascular risk factors, leukocytosis and the *JAK2 V617F* mutation, has recently been under active investigation [14, 29-34, 75]. Cardiovascular risk factors and the *JAK2 V617F* mutation are believed to be the most important additional risk factors, while the role of the leukocyte count measured at the time of ET diagnosis is still controversial in the thrombosis risk stratification [14, 16, 29-34, 76, 77].

Falanga et al. raised the possibility that the circulating leukocytes may be another prothrombotic factor in MPNs, as demonstrated for other conditions, such as sickle cell or peripheral arterial diseases [78]. It has been reported that leukocyte adhesion to the endothelium and platelets plays an important role in the activation of the coagulation cascade in which platelet–leukocyte interactions might be indicative of platelet activation, but to date there have been only a few reports on this issue [79-83]. Some studies on ET have evaluated the clinical role of the leukocytes, and suggested the predictive role of leukocytosis in subsequent thrombotic complications, although other reports have not verified this [81, 84-88]. As an example, Carobbio et al. concluded that a leukocyte count above the median cut-off level of 8.7 giga/L makes an evident contribution in the prediction of thrombotic complications, especially in untreated low-risk as compared with treated high-risk patients [89]. In contrast, Gangat et al. found that the leukocyte count at the time of haematological diagnosis defined by a cut-off level of either 9.4 or 15 giga/L did not appear to be predictive of thrombotic complications [86]. The leukocyte count cut-off value at the time of ET diagnosis relating to the current patient population (9.15 giga/L) was not associated with a higher risk of subsequent thrombotic complications either; likewise, the leukocytosis analysis did not indicate any potential association. The 2015 update on the diagnosis, risk stratification and management of ET states that, as the findings of different research groups are conflicting and inconclusive, leukocytosis is not considered to be a thrombosis risk factor; it is biologically plausible, but clinically still uncertain [40, 90, 91].

From the aspect of genetic mutations, the role of *JAK2 V617F* is regarded as the most important [32, 40]. In ET, approximately 50-60% of the patients possess the *JAK2 V617F* mutation [14, 32]. In the current study population, 60-69.7% of the patients were found to harbour the mutation. It has been reported that *JAK2V617F*(+) ET patients are older, have a higher rate of thrombohaemorrhagic complications, higher hemoglobin levels and a higher leukocyte counts than *JAK2V617F*(-) patients [39, 43-45, 92]. In the current study, the *JAK2 V617F*(-) cases differed significantly from the *JAK2 V617F*(+) cases from the aspects of the platelet, haemoglobin, red blood cell and haematocrit counts and hepatomegaly. Interestingly, all the patients with cerebrovascular thrombotic complications harboured the *JAK2V617F* mutation. However, the presence of the mutation was not associated statistically significantly with an increased risk of thrombosis: only non-significant tendencies were observed. These results concerning the risk of thrombosis are similar to those of the few published single-

centre reports likewise involving small patient populations [93, 94]. In the IPSET risk stratification model, the predictive potential of the *JAK V617F* mutation on subsequent thrombosis was incorporated, and promising results were reported [32]. However it is important to note that the 2015 update on the diagnosis, risk stratification and management of ET does not provide a clear-cut standpoint as concerns the predictive role of the *JAK V617F* mutation in thrombotic complications [40].

A noteworthy proportion (40-50%) of the ET cases did not harbour the *JAK2 V617F* mutation, though the thrombotic complications could also be observed in these patients during the course of the ET. In *JAK2 V617F*(-) cases, other mutations have been investigated from the aspect of their influence in thrombotic complications, e.g. the *MPL* mutations (*MPL W515L, MPL W515K, MPL W515R, MPL W515A,* and *MPL S505N*) [8, 46, 48, 49, 95, 96]. In ET, the most commonly detected (1-5%) *MPL* mutations are the *W515L/K* mutations [40, 50, 52, 53]. In current study population, genetic analysis of the *JAK2 V617F*(-) cohort revealed a 3.96% incidence of the *MPL W515L* mutation. It has been reported that *MPL* mutations may be associated with higher platelet counts, older age and a female predominance [49, 54]. In current study the patients who displayed the *MPL W515L* mutation were also older, were predominantly female and had higher platelet counts as compared with the *JAK2 V617F*, *MPL W515L* - negative patients. Beer et al. found that this mutation lacked prognostic significance from the viewpoint of thrombotic complications [49]. The number of our patients who gave laboratory analysis data did not allow meaningful statistical analysis. However, in the *JAK2 V617F*(-) and *MPL W515L*(+) patients, we could observed lower numbers of thrombotic complications as compared with the *JAK2 V617F*(-) and *MPL W515L*(-) patients.

In *JAK2 V617F*-unmutated ET, the presence of *CALR type-1 or type-2* somatic mutations has recently been identified with an estimated frequency of 15-32% [35, 57, 60]. Additionally, recent relevant literature data suggest that *CALR*-mutated ET may comprise a distinct MPN entity [95, 97]. Genetic analysis of our *JAK2 V617F*(-) cohort revealed *CALR type-2* mutation positivity in 3 cases. It has been reported that a more indolent clinical course, with a younger age and a lower leukocyte count, but a higher platelet count and a decreased risk of thrombosis, may be observed among patients with *CALR* mutations [35, 57, 60]. In the current study the patients who harboured the *CALR type-2* mutation were relatively young and

had a lower median leukocyte count and a higher median platelet count at diagnosis, and in these cases a lower number of thrombotic complications were observed.

During this single-centre analysis, we evaluated the roles of cardiovascular risk factors in the major thrombotic complications of ET. Our results demonstrated that the most important cardiovascular risk factors contributing to an enhanced thrombotic tendency were high blood pressure and hyperlipidaemia. The presence of one or two or more cardiovascular risk factors significantly increased the risk of thrombosis during the follow-up period of the ET patients. The thrombosis-free survival was also significantly different between patients without cardiovascular risk factors and those with at least one cardiovascular risk factor, and between patients with at most one cardiovascular risk factor and those with two or more cardiovascular risk factors. These results on a single-centre cohort involve a relatively low number of patients, but large multicentric studies have published similar findings [16, 29, 32, 98, 99]. The IPSET in 2012 incorporated the cardiovascular risk factors in its thrombosis-risk stratification [32]. In contrast, however, the 2015 annual clinical update on haematological malignancies mentions IPSET and the cardiovascular risk factors as additional risk factors, but still does not consider changes in the currently used classical two-categorical risk stratification model [40].

Vascular complications are mostly responsible for the morbidity and mortality of ET patients, and arterial complications occur more frequently than venous ones. In the current cohort, the cerebrovascular and cardiovascular thrombotic complications of the patients were therefore analysed in detail. The cardiovascular and cerebrovascular complications revealed differences. The cerebrovascular complications occurred within a wide range of time during the course of ET, whereas the cardiovascular events were predominantly observed within 12 months after the diagnosis of ET. During the haematological follow-up period, no recurrent cardiovascular events were observed, but despite the ongoing antiplatelet and cytoreductive therapy, recurrent stroke or a vertebrobasilar insufficiency was observed in the case of cerebrovascular complications. The *JAK V617F* mutation was positive in all patients who had cerebrovascular complications, but not in the case of cardiovascular complications. In these cases, almost all the patients displayed cardiovascular risk factors. We did not detect a correlation with the platelet count. Comparison of our observations is difficult in this context, as mostly only case reports and limited case series have been reported on these complications with clinical details [17-25]. However, our findings are in agreement with the concept that it

is not the increased platelet count itself, but rather the consequent qualitative abnormalities of the platelets, and other additional risk factors in ET (e.g. cardiovascular risk factors or genetic mutations) that are together responsible for the major thrombotic complications seen in ET [14, 32, 36, 100].

The new IPSET score system published by Barbui et al. in 2012 incorporates the predictive potential of the cardiovascular factors and the *JAK2 V617F* mutation [32]. In addition to the classical two-categorical risk assessment, it includes an intermediate-risk group of patients who were previously not clearly identified [32]. Consideration of the thrombosis-free survival of patients in the current cohort stratified into the low, intermediate and high-risk IPSET groups revealed significant differences. In the relevant literature up to September 2014, only one external validation result is available on IPSET [101]. That analysis too demonstrated the significant differences in the cumulative thrombosis-free survival of ET patients classified by IPSET [101]. At this point, this new score system appears promising and in the future may facilitate the better prediction of major thrombotic complications of ET, but this requires confirmation from further clinical centres.

6. SUMMARY

CONCLUSIONS AND CLINICAL IMPLICATIONS OF THE CURRENT WORK

- Epidemiological data on ET, a chronic Philadelphia chromosome-negative MPN, are rare in the literature. We have provided important new data.

- Retrospectively, the numbers of major thrombotic events that occurred previously (30.32%) or during the follow-up period (14.83%) were evaluated.

- Some literature studies suggest the contribution of the leukocyte count and leukocytosis at the diagnosis of ET to subsequent thrombotic events, but other reports did not verify this. Our findings on the current study population likewise did not confirm this suggestion. The leukocyte count measured at the time of the ET diagnosis, is probably not a sufficiently sensitive marker; its contribution is biologically plausible, but clinically uncertain.

- The presence of the *JAK2 V617F*, *MPL* and *CALR* mutations in the current patient population was analysed. The revealed occurrence of these mutations and their impact on the clinicohaematological findings were in line with the relevant literature.

- The contribution of cardiovascular risk factors as newly suggested additional risk factors to the subsequent thrombotic complications of ET was confirmed in our patient population, indicating the importance of the identification and consideration of cardiovascular risk factors in a more accurate thrombosis risk-guided management.

- As concerns the clinical characteristics of the neurological and cardiological compliations of ET, from the aspect of the cerebrovascular complications in the current cohort, mostly TIAs and/or minor strokes were observed within a wide range of time during the course of ET. Recurrent stroke or a vertebrobasilar insufficiency were seen despite the ongoing antiplatelet and cytoreductive therapy. We did not detect a correlation between the platelet count and the stroke type or the occurrence of

stroke. Most patients displayed at least two or more serious conventional vascular risk factors, which could be suggested to have influenced both the clinical course and the morphological alterations seen on brain imaging.

- As regards cardiovascular complications, both STEMI and NSTEMI were present. Interestingly, most of the MI complications occurred within 12 months after the haematological diagnosis of ET. No recurrent MI was subsequently observed during the follow-up period. No correlation with the platelet count was identified. Most patients displayed at least one conventional vascular risk factor. Our findings lead us to suppose that the early diagnosis and percutaneous coronary intervention and the personalized management of the patient's cardiovascular risk factors may greatly facilitate the prevention of these further vascular events. The importance of the close cooperation of the haematologist and other specialists in the field of vascular medicine is emphasized.

- The clinical value of the IPSET model, especially as concerns the thrombosis-free survival of the patients, was examined for the first time in a cohort of ET patients diagnosed in a single Hungarian haematological centre. Significant differences were observed in the thrombosis-free survival of patients stratified into low, intermediate and high-risk IPSET groups, suggesting that this score system provides more information than the conventional thrombosis risk assessment. However, further prospective investigations are required to establish the potential advantages of the IPSET score system in everyday practice, and to determine whether cytoreductive treatment is necessary in the IPSET intermedier-risk subgroup.

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9. APPENDIX