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

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POSTERS AND PRESENTATIONS

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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].

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 [4]. The peripheral blood smear in many cases demonstrates enhanced numbers of platelets and megakaryocyte fragments [4, 5]. 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 $\geq$ 450 giga/L and other evidence of reactive thrombocytosis, such as infection, inflammation and other chronic myeloid neoplasms or lymphoproliferative disorders, is excluded [5]. 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 approximately ranges between 11-25% [6-8]. 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 [6, 9].

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 [6, 10]. 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 *JAK2 V617F, MPL* and *CALR* mutations may influence the thrombotic complications seen in ET [6, 11-18].

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 [19, 20]. 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) [14]. 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). Furthermore, instead of merely the classical high- and low-risk group stratification, it introduces a three-subgroup (IPSET low-risk, IPSET intermediate-risk and IPSET high-risk) stratification model [14]. 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:

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

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

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

IV. 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;

V. 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;

VI. 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 1.

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.

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. 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 [7, 19, 20].

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) [21]. Statistical significance was set at 5% and 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 [21]. 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 [19, 20]. Statistical significance was set at 5% and 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 venous 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 and set 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) [21]. Statistical significance was set at 5% and 10%.

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 [14]. In this model, patients were stratified into IPSET low, intermediate and high risk categories 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) [14]. According to these results, patients with < 2 points could be classified in the low-risk group. In this model, an intermediate-risk group is introduced for patients with 2 points. The IPSET high-risk risk group comprising patients with > 2 points [14]. For the clinical utility of IPSET from the aspect of the thrombosis-free survival of the patients, the IPSET subgroups were compared by the Kaplan–Meier method followed by the log-rank test (Mantel–Cox) [21].

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 [22]. 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.

PCR reactions were performed and the *MPL W515L* and *W515K* mutations were assessed as described by Bergamaschi et al. [23]. Allele-specific primers for *MPL W515R*, for *MPL W515A* and 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. This method was used with their agreement. As a control experiment, samples were sequenced by an automated single capillary genetic analyser (ABI PRISM 310, Applied Biosystems, Life Technologies). To assess the *CALR* mutation status, a fragment analysis with FAM-labelled primers were performed on the samples of patients who had *JAK2 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.

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 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 [24].

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 [24].

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 [24].

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 [24].

As regards a comparison of the *JAK2 V617F*(-) patients with or without the *MPL W515L* mutation, 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 [24].

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 [24].

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.

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).

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, but these differences were not significant.

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). 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) [25] .

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 [26].

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 (57.1%) of the analysed 7 cases were *JAK2 V617F*(+). Most of the patients (85.7%) 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. We found no correlation between the platelet counts and the MI type or the degree of stenosis.

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 on the basis of the IPSET score [14]. 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.

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) [27].

5. SUMMARY AND CONCLUSIONS

1. The reported incidence of thrombohaemorrhagic events in ET patients lies in the interval 11-25% [6-8]. Thrombotic events appear more commonly than haemorrhagic ones and among the thrombotic complications arterial thrombosis is more often observed than venous thrombosis [6]. In the current cohort in the period 1999-2014 retrospectively, the numbers of major thrombotic events that occurred previously were 30.32% and during the follow-up period 14.83% were evaluated. Arterial complications (80%) predominating over venous ones (20%).

2. The current goal of ET therapy is to prevent thrombohaemorrhagic complications [28]. 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 [7, 19, 20]. 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 leukocytosis, *JAK2 V617F* mutation and the classic cardiovascular risk factors has recently been under active investigation [6, 11-16, 29].

   a) 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 [30-35]. Our findings on the current study population likewise did not confirm this suggestion. 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 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.

   b) 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 In ET, approximately 50-60% of the patients possess the *JAK2 V617F*

mutation [6, 14]. In the current study population, 60-69.7% of the patients were found to harbour the mutation. It has been reported that *JAK2 V617F*(+) ET patients are older, have a higher rate of thrombohaemorrhagic complications, higher hemoglobin levels and a higher leukocyte counts than *JAK2 V617F*(-) patients [36-40]. 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. However, the presence of the mutation was not associated statistically significantly with an increased risk of thrombosis: only non-significant tendencies were observed. In the IPSET risk stratification model, the predictive potential of the *JAK2 V617F* mutation on subsequent thrombosis was incorporated, and promising results were reported [14]. 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 *JAK2 V617F* mutation in thrombotic complications [28].

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*) [9, 41-45]. In ET, the most commonly detected (1-5%) *MPL* mutations are the *W515L/K* mutations [28, 46-48]. 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 [43, 49]. 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*(-) patients. In the cases of *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% [17, 50, 51]. 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 [17, 50, 51]. 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.

c) 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. Based on these results the contribution of cardiovascular risk factors as newly suggested additional risk factors to the subsequent thrombotic complications of ET was confirmed in indicating the importance of the identification and consideration of cardiovascular risk factors in a more accurate thrombosis risk-guided management.

3. 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.

4. 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 [14]. In addition to the classical two-categorical risk assessment, it includes an intermediate-risk group of patients who were previously not clearly identified [14]. 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 subgroups, 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. In the relevant literature up to September 2014, only one external validation result is available on IPSET [52]. That analysis too demonstrated the significant differences in the cumulative thrombosis-free survival of ET patients classified by IPSET [52].

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