Oxidative Stress Levels, JAK2V617F Mutational Status and Thrombotic Complications in Patients with Essential Thrombocythemia

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Essential thrombocythemia (ET) is a BCR-ABL1-negative myeloproliferative neoplasm associated with thrombotic and haemorrhagic complications. Reactive oxygen species (ROS) overexpression induces a growth advantage to JAK2V617F-positive clones and, in association with a higher number of immature platelets, leukocytosis, and additional cardiovascular risk factors, leads to an increased risk for thrombotic events. We evaluated oxidative stress by measuring ROS levels and the total antioxidant capacity (TAC) in 62 ET patients and investigated the relationship between oxidative stress, JAK2V617F mutational status and the development of thrombotic events. We found higher oxidative stress levels in JAK2V617F-positive vs. JAK2V617F-negative ET cases with no significant differences between homoygous and heterozygous genotypes. Increased ROS levels and thrombotic events were more frequent in ET patients with old age at diagnosis, higher haematocrit levels or leukocytosis.

Keywords: essential thrombocythemia, reactive oxygen species, total antioxidant capacity, JAK2V617F, thrombosis

Essential thrombocythemia (ET) is a BCR-ABL1-negative myeloproliferative neoplasm characterized by altered proliferation and differentiation of hematopoietic stem cells, persistent thrombopoietin levels > 450.000/mmc independent of thrombopoietin levels, excessive proliferation of bone marrow megakaryocytes with large, mature morphology, hyperlobulated nuclei and minor reticulin fibrosis. ET is also associated with thrombotic and haemorrhagic complications. Approximately a half of the patients have an acquired somatic mutation in the pseudo-kinase domain of the JAK2 gene as a result of a G-C to T-A transversion at nucleotide 1849 of exon 14, leading to the substitution of the amino acid valine by phenylalanine. Janus kinases mediate signal transduction from cytokine receptors and activate JAK-STAT (Signal Transducer and Activator of Transcription) signalling pathway which is essential for cytokine-mediated proliferation, survival and apoptosis. The JAK2V617F clone can escape regulation by the suppressor of cytokine signaling 3 and p27/kip1 and give rise to lineage-specific cells that are hypersensitive to cytokine stimulation [1-4].

Other mutations can be discovered in JAK2V617F-negative patients: JAK2 exon 12 mutations, mutations of the calreticulin gene (CALR 19p13.2) associated with altered activation of the JAK-STAT5 signalling pathway, or mutations in the exon 10 of the c-MPL gene (which codes for the thrombopoietin receptor) that lead to a permanent activation of the receptor and JAK-STAT pathway [5-9]. Other JAK2 gene mutations (V625F, F556V) and MPL gene mutations (T119L, S204F, F230G, Y591D) might be found in the rest of patients and only a very small number are indeed triple-negative [10-13]. Recent advances have elucidated the molecular basis of familial essential thrombocythemia as well: a novel germline JAK2 mutation, JAKR5640 [4].

Several studies have revealed that oxidative stress, via increased levels of reactive oxygen species (ROS), is associated with chronic inflammation and is involved in atherogenesis, obesity, neurodegenerative disorders, carcinogenesis and disease progression in myeloproliferative neoplasms through the activation of the NF-kB and NF-E2 pro-inflammatory pathways [14-20]. Moreover, in myeloproliferative neoplasms, the levels of catalase, an antioxidant enzyme, are decreased in the stem cell niche. Decreased antioxidant levels induce thus oxidative stress, oxidative DNA alterations, genomic instability and disease progression to myelofibrosis or acute leukaemia [21]. Some studies have shown that ROS overexpression induces a growth advantage to JAK2V617F-positive clones and, in association with a higher number of immature platelets, leukocytosis and additional cardiovascular risk factors, favoured thrombosis [22-26]. In ET, as well as in the other BCR-ABL1-negative neoplasms (polycythaemia vera and primary myelofibrosis), thrombotic and haemorrhagic events may be identified at the time of diagnosis or may occur during the evolution of the disease [27-29].

The aim of our study was to evaluate oxidative stress levels in patients with ET and to evaluate whether significant differences existed between JAK2V617F-positive vs. JAK2V617F-negative cases and between ET patients who had developed thrombosis vs. patients with no history of thrombosis.

Experimental part

We enrolled 62 ET patients and 20 healthy volunteers. Patients were diagnosed with essential thrombocythemia according to the 2016 revised diagnostic criteria of the World Health Organization (WHO) [30]. Informed consent was obtained from all patients and the study protocol was carried out in accordance with the standards imposed by the Declaration of Helsinki and with the approval of the Ethics Committee of the University of Medicine and Pharmacy of Craiova (approval no. 79/23.02.2017).
Patients were classified based on age, sex, JAK2V617F mutational status, history of vascular events, additional cardiovascular risk factors and comorbidities. Haemoglobin value, haematocrit levels, leukocyte count, leukocyte formula, platelet count, mean platelet volume, lactate dehydrogenase, acute phase proteins and other biochemical parameters were measured. Bone marrow aspiration or biopsy were performed and analysed. Electrocardiogram, echocardiography, abdominal ultrasound and computed tomography were employed when necessary. JAK2V617F detection was performed in a specialised molecular biology laboratory. Genomic DNA was isolated from peripheral blood leukocytes. The sequence of interest was amplified by ARMS-PCR and visualized on agarose gel electrophoresis.

Reactive oxygen species (ROS) and the total antioxidant capacity (TAC) levels were measured to evaluate oxidative stress status. Peripheral venous blood was drawn from the control group and from the patients at time of diagnosis in 6 mL tubes containing trisodium citrate. Blood samples were centrifuged at 3000 rpm for 15 minutes and 1.5 mL of plasma was collected to measure TAC. TAC was evaluated using a FLUOstar Omega multi-detection microplate reader (reagents from Sigma-Aldrich). Remaining plasma was transferred in another tube and centrifuged for 5 minutes. Concentrated plasma was used to prepare samples for the evaluation of ROS levels. ROS levels were measured by flow-cytometry using a CyFlow Space Sysmex flow-cytometer (reagents from Abcam). Negative controls were analysed immediately, samples from ET patients were measured following 30 minutes of incubation at 37°C of incubation; positive controls were evaluated after 4 hours of incubation at 37°C. Exclusion criteria were represented by smoking, alcohol consumption, iron deficiency and exogenous administration of antioxidants.

We compared oxidative status parameters in: 1) ET patients vs. the control group; 2) JAK2V617F-positive ET cases vs. JAK2V617F-negative cases; 3) ET patients with thrombotic events vs. ET patients without thrombotic events. Oxidative stress levels were compared according to the JAK2 status and history of thrombotic events using the Student’s t-test to detect significant differences between the two groups. Differences were considered significant at a p-value<0.05.

Results and discussions

The study group (mean age 59.50 years, range 22-82 years) consisted in 37 females (59.70%) and 25 males (40.30%). ROS levels were increased and TAC levels were decreased in patients with ET vs. healthy controls. We detected lower ROS levels and higher TAC values in the female group vs. the male group as previously reported elsewhere [31]. The JAK2V617F mutation was detected in 36 ET patients (58.06%), whereas 26 ET patients (41.94%) were JAK2V617F-negative. The percentages are similar to those found in other publications that estimate that 23-57% of ET cases are JAK2V617F-positive [1]. JAK2V617F homozygosity was detected in 6 ET patients (9.68% of the ET group) and JAK2V617F heterozygosity in 30 ET patients (48.39% of the ET group). In the ET group, JAK2V617F homozygous genotype was rare, similarly to other scientific articles in which JAK2V617F homozygosity was uncommon and found only in 3-7% of ET cases. However, it is frequent in polycythemia vera [3, 32].

Oxidative stress levels were higher in JAK2V617F-positive ET cases vs. JAK2V617F-negative cases. ROS levels were higher in JAK2V617F-positive cases vs. JAK2V617F-negative cases (mean ROS value = 2.73 mM/L vs. 2.60 mM/L; p<0.05). TAC evolved oppositely, as its value was lower in JAK2V617F-positive cases vs. JAK2V617F-negative cases (mean TAC value = 0.46 mM/L vs. 0.51 mM/L; p<0.05). No significant differences were found between the homozygous/heterozygous genotype and between JAK2V617F-positive and JAK2V617F-negative cases with regards to age, sex, lactate dehydrogenase levels or degree of splenomegaly. Old age at diagnosis, higher haematocrit levels and leukocytosis were more frequently encountered in the ET patients who developed thrombotic events, as highlighted by other publications as well [30, 33]. In ET, the JAK2V617F mutation has been associated with a significantly higher number of immature platelets and a higher rate of thrombotic events independently of thrombocytosis [25, 34-35]. Figure 1 depicts the fluorescence evaluation in ET vs. positive and negative controls.

Fig. 1. Fluorescence evaluation in a 74-year-old patient with JAK2V617F-positive ET
Thrombotic events were found in 14 ET patients (22.58%). Vascular events were frequently caused by arterial thrombosis and less by venous thrombosis. Platelet-mediated microvascular disturbances (erythromelalgia) were discovered in 3 patients (4.84%) and 11 ET patients (17.74%) had major thromboembolic events: 5 ET patients (8.06%) had complications related to cerebrovascular disease (ischemic stroke: 3 cases, 4.84%; transient ischemic attack: 2 cases, 3.23%), 3 ET patients (4.84%) had coronary heart disease-related complications (acute myocardial infarction: 2 cases, 3.23%; unstable angina pectoris: 1 case, 1.61%) and 3 ET patients (4.84%) had venous thromboembolism-related events (deep vein thrombosis: 2 cases, 3.23%; pulmonary embolism: 1 case, 1.61%). ET patients who developed thrombosis had a significantly elevated platelet count (thrombocytes > 1.500 µL) associated with moderate leukocytosis, age at diagnosis > 60 years and traditional cardiovascular risk factors, such as arterial hypertension, diabetes mellitus, obesity or history of thrombosis. Oxidative stress levels were higher and thrombotic events vs. ET patients who did not develop thrombosis. We detected higher oxidative levels in JAK2V617F-positive ET cases vs. JAK2V617F-negative cases. However, we did not find any significant differences between ET patients with a JAK2V617F homozygous genotype vs. ET patients with a JAK2V617F heterozygous genotype. Oxidative stress levels were higher (elevated ROS levels and reduced TAC values) in ET patients who developed thrombosis (mean ROS value = 2.49 mM/L; mean TAC value = 0.46 mM/L) vs. ET patients who did not develop thrombosis (mean ROS value = 2.30 mM/L; mean TAC value = 0.51 mM/L) (p<0.05). In table 1, ROS, TAC, and JAK2V617F mutational status of ET patients were compared to ET patients with a JAK2V617F homozygous genotype vs. ET patients with a JAK2V617F heterozygous genotype. Oxidative stress levels were higher (elevated ROS levels and reduced TAC values) in ET patients who developed thrombosis vs. ET patients who did not develop thrombosis.

### Table 1

| No. | Thrombotic events     | JAK2V617F | Genotype      | ROS  | TAC  |
|-----|-----------------------|-----------|---------------|------|------|
| 1   | Ischemic stroke       | positive  | homozygous    | 2.37 | 0.48 |
| 2   | Transient ischemic attack | positive | heterozygous | 2.25 | 0.41 |
| 3   | Ischemic stroke       | positive  | heterozygous  | 2.66 | 0.51 |
| 4   | Ischemic stroke       | positive  | heterozygous  | 2.81 | 0.53 |
| 5   | Transient ischemic attack | negative | -             | 2.38 | 0.49 |
| 6   | Myocardial infarction | positive  | heterozygous  | 3.11 | 0.30 |
| 7   | Myocardial infarction | positive  | heterozygous  | 2.73 | 0.39 |
| 8   | Unstable angina       | positive  | heterozygous  | 2.61 | 0.35 |
| 9   | Deep vein thrombosis  | negative  | -             | 1.92 | 0.33 |
| 10  | Pulmonary embolism    | positive  | heterozygous  | 3.18 | 0.36 |
| 11  | Deep vein thrombosis  | positive  | heterozygous  | 2.90 | 0.52 |
| 12  | Erythromelalgia       | positive  | heterozygous  | 1.94 | 0.49 |
| 13  | Erythromelalgia       | positive  | heterozygous  | 1.81 | 0.50 |
| 14  | Erythromelalgia       | positive  | heterozygous  | 1.92 | 0.48 |

Conclusions

We detected higher oxidative levels in JAK2V617F-positive ET cases vs. JAK2V617F-negative cases. However, we did not find any significant differences between ET patients with a JAK2V617F homozygous genotype vs. ET patients with a JAK2V617F heterozygous genotype. Oxidative stress levels were more frequent in ET patients who had an old age at diagnosis, higher haematocrit levels or leukocytosis. Because the mechanism might apply also to ET. In another study on 43 patients with ET, Durmus et al. found a higher level of total oxidative status in patients with vascular complications compared to those without vascular events [37-38].

Bjorn and Hasselbalch observed that ROS overexpression in myeloproliferative neoplasms induces myeloid expansion with leukocytosis and excessive thrombocytosis. It also activates pro-inflammatory pathways (NF-kB, NF-E2) and leads to low-grade chronic inflammation. Chronic inflammation is associated with activation of leukocytes and platelets, promotes a sustained release of inflammation-related substances and increases ROS, contributing to a vicious cycle [16].

Marty et al. revealed that increased oxidative stress levels in hematopoietic stem cells from myeloproliferative neoplasms and an inflammatory bone marrow microenvironment can lead to ineffective DNA repair and DNA damage, genomic instability and disease progression to myelofibrosis or transformation to acute leukaemia [21].

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