Crosstalk between Oxidative Stress, Chronic Inflammation and Disease Progression in Essential Thrombocythemia

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Essential thrombocythemia (ET) is a Philadelphia-negative chronic myeloproliferative neoplasm characterized by acquired somatic mutations: JAK2, CALR or MPL. It is associated with low-grade chronic inflammation, oxidative stress, overproduction of reactive oxygen species (ROS) and antioxidant deficiency. In ET, chronic inflammation and oxidative stress contribute to the genomic instability, the clonal evolution to myelofibrosis and the leukemic transformation. We evaluated ROS levels and the total antioxidant capacity (TAC) in 62 ET patients and investigated the relationship between ROS, TAC, chronic inflammation, leukocytosis, JAK2V617F mutation, and disease progression to myelofibrosis or leukemic transformation. We observed increased levels of ROS and inflammation markers and a decreased TAC in ET patients vs. controls. The acute myeloid leukaemia transformation associated increased levels of oxidative stress and inflammation markers and increased leukocyte counts, while myelofibrosis progression associated an increase in ROS and serum ferritin.

Keywords: essential thrombocythemia, reactive oxygen species, inflammation, JAK2V617F

Essential thrombocythemia (ET) is a Philadelphia-negative chronic myeloproliferative neoplasm (MPN) characterized by JAK2, CALR or MPL acquired somatic mutations, persistently elevated platelet count over 450.000/mmc with morphologically and functionally modified thrombocytes, bone marrow modified megakaryocyte proliferation and inhibition of apoptosis [1-5].

Recent studies have shown that Philadelphia-negative MPNs (essential thrombocythemia, polycythaemia vera and primary myelofibrosis) are associated with low-grade chronic inflammation, oxidative stress, overproduction of reactive oxygen species (ROS) and antioxidant defence deficiency. Low-grade chronic inflammation is a result of constitutively activated JAK-STAT signalling pathways leading to uncontrolled secretion of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-11). IL-1β enhances cytokine release, NF-E2 expression with ROS overexpression and a relative deficiency of Nrf2 with subsequent downregulation of antioxidant genes [6-8]. Low-grade chronic inflammation increases genomic instability and clonal evolution to myelofibrosis and leukemic transformation [7-8]. On the other hand, overproduction of ROS enhances myeloid proliferation with leukocytosis, thrombocytosis, inflammation-mediated activation of leukocytes and platelets, continuous release of inflammatory products and a self-perpetuating vicious circle involving excessive ROS, growth advantaged JAK2V617F-positive clones, proliferation of megakaryocytes, genomic instability and disease progression [9-15].

The aim of the study was to evaluate oxidative stress levels in patients with ET and to investigate the relationship between ROS, TAC, chronic inflammation parameters, leukocytosis, JAK2V617F mutation and progression of disease to myelofibrosis or leukemic transformation.

Experimental part

Sixty-two patients diagnosed with ET based on the 2016 revised World Health Organization diagnostic criteria and 20 healthy volunteers with similar demographic characteristics were included in the study [1]. Informed consent was obtained from all subjects involved, the study protocol had the approval of the Ethics Committee of the University of Medicine and Pharmacy of Craiova (approval no. 79/23.02.2017) and was carried out in accordance with the standards imposed by the Declaration of Helsinki and the European Union 679/2016 regulation.

Patients were classified based on age, sex, presence of JAK2V617F mutation and inflammation status. Haematological parameters (haemoglobin value, haematocrit levels, MCV, MCH, MCHC, RDWR, RDWA, leukocyte count, leukocyte formula, platelet count, mean platelet volume, PCT, PDW, LPCR, bone marrow aspiration or biopsy), lactate dehydrogenase, fibrinogen, C-reactive protein (CRP), serum ferritin level and neutrophil/lymphocyte ratio were measured. JAK2V617F detection was performed in a specialised molecular biology laboratory. Haematological parameters were analysed using a SYSMEX XN-450 analyser and biochemical parameters were determined using a KONELAB601 analyser by spectrophotometry. Serum ferritin level was estimated using the Enzyme-Linked Immunosorbent Assay (ELISA) method as per the manufacturer's guidelines. Oxidative stress was evaluated by measuring the level of reactive oxygen species (ROS) using a CyFlow Space Sysmex flow-cytometer (reagents from Abcam). Negative control was analysed immediately, patient samples were analysed after incubation at 37^0 Celsius for 30 minutes and the positive control was analysed after incubation at 37^0 Celsius for four hours. The total antioxidant capacity (TAC) was analysed using a FLUOstar Omega multi-detection microplate reader (reagents from Sigma-Aldrich) according to manufacturer's instructions. We compared

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inflammation markers (fibrinogen, CRP, serum ferritin, NLR, leukocytosis) and oxidative status parameters (ROS and TAC) in: 1) ET patients vs. the control group; 2) ET JAK2V617F-positive cases vs. JAK2V617F-negative cases; 3) ET patients vs. patients with disease progression (myelofibrosis or leukemic transformation). Statistical analysis was performed and a p-value < 0.05 was considered significant.

Results and discussions

The study group had a median age of 59.50 years and consisted in 25 males (40.30%) and 37 females (59.70%), revealing a female predominance. The JAK2V617F mutation was positive in 36 ET patients (58.06%) (heterozygous genotype in 30 patients and homozygous genotype in six patients) and negative in 26 ET patients (41.94%), according with literature data. ROS levels were increased (with high levels in JAK2V617F-positive ET patients vs. JAK2V617F-negative cases; mean ROS value = 2.73 mM/L vs. 2.60 mM/L; $p<0.05$) and TAC levels were decreased in patients with ET vs. the control group (with low levels in JAK2V617F-positive patients vs. JAK2V617F-negative patients, mean TAC value = 0.46 mM/L *vs.* 0.51 mM/L; $p<0.05$) [12, 15].

Fibrinogen levels were increased in ET patients *vs.* controls: 405.52 ± 73.60 mg/dL *vs.* 267.97 ± 37.27 mg/dL in controls, p-value<0.0001. CRP levels were increased in ET patients *vs.* controls: 5.75 ± 1.10 mg/dL *vs.* 3.24 ± 0.59 mg/dL in controls, p-value<0.0001. Serum ferritin levels were increased in ET patients vs. controls 411.70 ± 113.55 ng/mL *vs.* 158.60 ± 60.10 ng/mL in controls, p-value<0.0001 (fig. 1). The leukocyte count was increased in ET patients *vs.* controls: 11405.81 ± 5552.58 leukocytes/mmc *vs.* 6290.00 ± 134584 leukocytes/mmc in controls, p-value<0.0001. NLR was increased in ET patients vs. controls: 3.55 ± 2.44 (arbitrary units) *vs.* 2.30 ± 0.26 (arbitrary units) in controls, p-value=0.0067. We found no significant differences (p-value>0.05) between JAK2V617F-positive and JAK2V617F-negative cases.

We found positive weak correlations between ROS – fibrinogen r_s = 0.09424, p (2-tailed) = 0.46627, ROS – CRP r_s = 0.09688, p (2-tailed) = 0.45384, ROS – serum ferritin r_s = 0.16646, p (2-tailed) = 0.19598, and a negative weak correlation ROS - NLR r_s = -0.07457, p (2-tailed) = 0.56462, but there was no statistical significance (p-value>0.05). However, we found a moderate positive correlation ROS – leukocyte count: r_s = 0.28601, p (2-tailed) = 0.02423 (Figure 2).

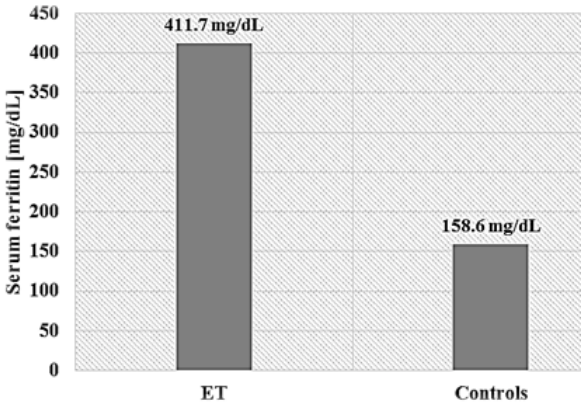

Fig. 1. Serum ferritin levels are increased in ET patients *vs.* controls

We found positive weak correlations between TAC – fibrinogen r_s = 0.07084, p (2-tailed) = 0.58428, TAC -CRP r_s = 0.02664, p (2-tailed) = 0.83716, TAC – serum ferritin r_s = 0.05653, p (2-tailed) = 0.66253, and moderate correlations between TAC -leukocyte count r_s = 0.22312, p (2-tailed) = 0.08131, and TAC – NLR r_s = 0.23242, p (2-tailed) = 0.06909. However, there was no statistical significance between the values.

In the ET patient who underwent transformation to acute myeloid leukaemia (AML), ROS levels increased (AML: 2.57 mM/L vs. ET: 1.97 mM/L) and TAC decreased (AML: 0.32 mM/L vs. ET: 0.48 mM/L). Also, following transformation to AML, we registered elevated numbers of leukocytes (29.300 leukocytes/mmc vs. 16390 leukocytes/mmc) and increased fibrinogen (686 mg/dL vs. 461 mg/dL), CRP (7.2 mg/dL vs. 6.2 mg/dL) and serum ferritin (1224 ng/mL vs. 402 ng/mL) values.

In the four ET patients who progressed to myelofibrosis (MF), ROS levels increased (MF: 3.14 ± 0.31 mM/L vs. ET: 2.27 ± 0.28 mM/L, p=0.0108) and TAC decreased (MF: 0.37 ± 0.02 mM/L vs. ET: 0.45 ± 0.04 mM/L, p=0.06), but only the ROS elevation had statistical significance. We recorded an increased leukocyte count (15427.50 ± 4922.37 leukocytes/mmc vs. 15900.00 ± 2081.67 leukocytes/mmc, p=0.8865) and elevated levels of fibrinogen (449.50 ± 54.29 mg/dL vs. 506.00 ± 21.97 mg/dL, p=0.1633), CRP (5.80 ± 1.35 mg/dL vs. 6.80 ± 0.14 mg/dL, p=0.2565) and serum ferritin (494.50 ± 137.42 ng/mL vs. 924.25 ± 108.24 ng/mL, p=0.0361) after transformation to MF, but only the rise in serum ferritin was statistically significant.

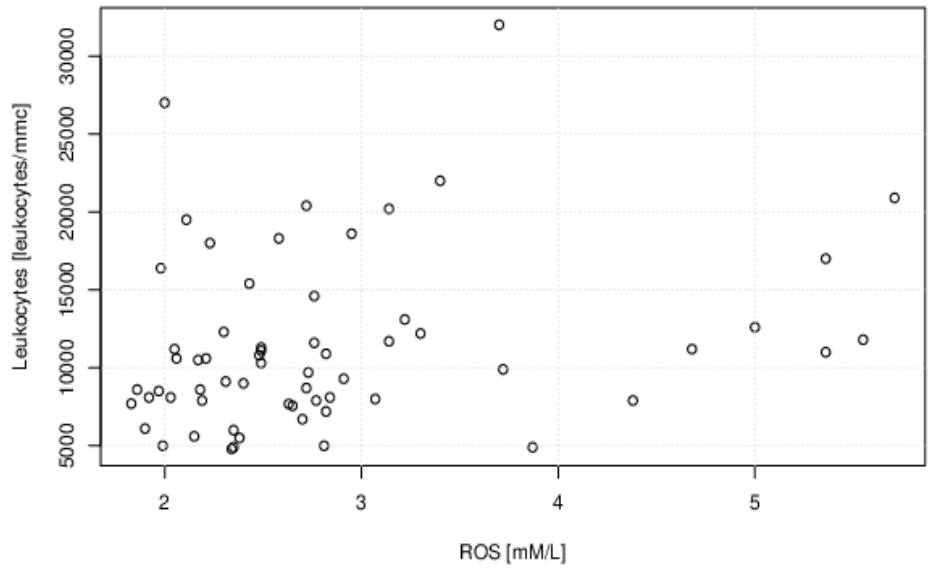

Fig. 2. ROS and leukocyte count correlation in ET patients

In the last years, experimental and clinical studies have shown that chronic inflammation, immune stimulation/ deregulation and oxidative stress are involved in the pathogenesis of MPNs. In MPNs, chronic inflammation might both initiate and drive the evolution of malignant clones, and may also be an active player in the promotion of atherosclerosis, cardiovascular and thromboembolic complications [6-8, 16-17]. MPNs are now considered a *Human Inflammation Model* and listed as *Oxyradical Overload Disorders* due to the chronic inflammation - transcription factor NF-kB activation - inflammatory cytokines release -ROS generation -oxidative stress - genomic instability -clonal evolution link [17].

It has been shown that the acquisition of the JAK2V617F mutation induces constitutive activation of JAK-STAT, NF-kB and PI3K/AKT signalling pathways and hypersensitivity of myeloid progenitors to hematopoietic cytokines [16]. STAT3 is responsible for neutrophil activation and STAT5 for cell proliferation [16]. STAT3 activates the release of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, IL-11, TNF-α, growth factors: vascular endothelial growth factor VEGF, fibroblast growth factor FGF) and metalloproteinases. IL-1β sustains induction of cytokines and inhibits the activation of transcription factor Nrf2 (nuclear erythroid-related factor 2) with subsequent downregulation of antioxidants genes. AKT is responsible for the negative regulation of the Forkhead O transcription factor family (FoxO) with downregulation of several antioxidant enzymes: glutathione peroxidase, catalase and superoxide dismutase [17-18]. Uncontrolled cytokine secretion induces a pro-inflammatory status in the bone marrow microenvironment and in the blood. Chronic inflammation in the bone marrow activates immune cells, increases NF-kB activity in hematopoietic and stromal cells. NF-kB is a redox-sensitive transcription factor with a major role in inflammation, innate immunity and carcinogenesis. The association between chronic inflammation, sustained proliferation of the myeloid lineage and the activation of cell signaling pathways ultimately damages the DNA of hematopoietic cells *via* ROS accumulation, tissue destruction, remodeling and fibrosis [16]. Leukocytosis and thrombocytosis reflect clonal myeloproliferation and the effect of chronic inflammation *per se* on malignant cells which are inherently hypersensitive to the stimuli of growth factors and cytokines [16].

It is known that inflammation is associated with increased ROS levels. H_2O_2, the most potent ROS, activates pro-inflammatory pathways (NF-kB) which in turn generate more ROS. The excessive ROS production in MPNs induces a proliferative advantage to JAK2-positive clones [17-18, 27]. On the other hand, the JAK2V617F mutation *per se* is responsible for an accumulation of ROS in the hematopoietic stem cell compartment, the excessive ROS production acting as a mediator of genomic instability, JAK2V617F-induced oxidative stress and DNA strand breaks and mutations [19]. Sevin *et al.* (2015) revealed that heat shock proteins (HSP) have a major role in inflammation in MPNs through their chaperone activity. HSP90 stabilizes many oncogenes, including JAK2, and, in association with HSP70, regulates the NF-5ØßB signaling pathway [31].

Hasselbalch *et al.* (2014) reported that, in MPNs, there is a deregulation of oxidative stress and anti-oxidative stress genes. Their study revealed that ATOX1, DEFB122, GPX8, PRDX2, PRDX6, PTGS1, and SEPP1 genes were progressively upregulated, while AKR1B1, CYBA, SIRT2, TTN, UCP2, SOD2, CAT and Nrf2 genes were progressively downregulated [8]. Bjorn and Hasselbalch (2015) revealed that the hematopoietic stem cell niche in MPNs downregulates catalase and superoxide dismutase activity [7]. UCP2 and SIRT2 induce constitutively increased NF-kB activity and, when downregulated, production of pro-inflammatory cytokines [17]. An important role in the promotion of DNA repair, protection of telomere stability and differentiation of stem cells is played by sirtuins [24]. In MPNs, a downregulation of the Nrf2 gene, which encodes the transcription factor nuclear factor erythroid 2-related factor 2, occurs. Since Nrf2 modulates the migration and retention in the stem cell niche of hematopoietic stem cells, Nrf2 depletion results in the expansion of the compartment of hematopoietic stem and progenitor cells [20]. Inactivation of the tumor suppressor gene p53 and of FoxO3 has been shown to increase ROS levels and was linked to a loss of function of the hematopoietic stem cells, excessive oxidation of DNA, acquired mutations, genomic instability and disease progression to myelofibrosis or leukemic transformation [17, 21-22].

In MPNs, chronic inflammation has been suggested as a major player in disease progression, clonal evolution and myelofibrotic and leukemic transformation *via* TNF-α as a tumor promoter and inducer of clonal expansion of JAK2V617-positive cells, since the JAK2V617F mutation *per se* has been demonstrated to induce ROS overproduction, oxidative stress, DNA damage and genomic instability [6-7, 16, 25-27]. Oxidative stress activates the redox-sensitive transcription factor NF-kB, leading to an excessive release of pro-inflammatory cytokines which, in turn, can cause oxidative stress in hematopoietic cells. Thus, a self-perpetuating vicious circle develops: chronic inflammation -NF-kB activation -pro-inflammatory cytokines release -ROS generation -oxidative stress -acquired mutations -genomic instability -clonal evolution to myelofibrosis or leukemic transformation [6-8]. The sustained release of inflammatory cytokines and chronic oxidative stress cause a high-risk bone marrow microenvironment for induction of oxidative DNA damage in hematopoietic cells, mutations and epigenetic changes, such as DNA methylation and histone deacetylation, which contribute to tumor initiation, tumor progression and leukemogenesis [28-29]. Tefferi *et al.* (2016) and Hermouet & Vilaine (2011) reported that the JAK2 46/1 haplotype predisposes to additional mutations in the JAK2 gene and genomic instability, and associated with cytokine-mediated expansion of malignant clones, determines an inferior survival in primary myelofibrosis and increases the risk of myelofibrotic transformation in ET and polycythemia vera patients [1, 30]. According to recent studies, sustained JAK2 overexpression, as well as the presence of the JAK2V617F mutation, may induce genomic instability and disease progression from an early disease stage to the advanced myelofibrosis stage [6, 30].

Conclusions

In our study, we found high levels of inflammation markers in ET patients vs. controls, but without statistical significance between JAK2V617F-positive and JAK2V617F-negative cases. We found positive weak correlations between oxidative stress markers and fibrinogen, CRP, serum ferritin and a moderate positive correlation between oxidative stress -leukocyte count and NLR, respectively. Disease progression to AML associated increased levels of oxidative stress, elevated values of inflammation markers and increased leukocyte counts, while in patients who progressed to myelofibrosis, ROS and serum ferritin increased after vs. before transformation. Thus, we may hypothesize that chronic inflammation and oxidative stress

are involved both in ET initiation and also in disease progression to myelofibrosis or leukemic transformation. In this respect, anti-inflammatory and oxidative stress-modulating therapy, in addition to JAK2 inhibitors and IFN-α2, might be of aid in ET to slow down disease progression to myelofibrosis or leukemic transformation.

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