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ORIGINAL ARTICLE

Thiol/Disulfide Balance in Older Patients with BCR-ABL Negative Myeloproliferative Neoplasms

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SUMMARY

Background: **The trio Essential Thrombocytosis (ET), Polycythemia Vera (PV), and Primary Myelofibrosis (PM) are BCR-ABL negative myeloproliferative neoplasms. All three diseases have the risk of transforming into acute leukemia. Oxidative stress and some genetic mutations increase the risk of leukemic transformation. The median age in patients with ET, PV, and MF is around 64 years, and it is expected to exceed 65 in the coming years. Since oxidative stress increases with age, we aimed to evaluate the oxidative stress parameters in older patients with myeloproliferative neoplasms.**

Methods: **The study included a total of 160 patients (57 patients with Essential Thrombocytosis, 52 patients with Primary Myelofibrosis, and 51 patients with Polycythemia Vera) and 56 healthy controls, aged 65 and over. Ischemia Modified Albumin (IMA) and thiol parameters (native thiol, total thiol, and disulfide) were studied from serum samples taken at the time of diagnosis.**

Results: **The median age of the patients was 69 (65 - 85) years. Patients had higher levels of IMA and lower levels of thiol compared to the control group ($p < 0.001$). When evaluated according to disease subgroups, it was observed that the highest IMA levels and the lowest thiol levels were in patients with PM ($p < 0.001$). Higher IMA levels and lower native thiol levels were found in patients with the ASXL1 mutation ($p < 0.001$).**

Conclusions: **Serum IMA and thiol levels are also significantly changed in older patients with BCR-ABL negative myeloproliferative neoplasia. Changes in these markers are independent of age. Disease-associated mutations such as ASXL1 can also affect the serum levels of these markers.**

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INTRODUCTION

There are four classic myeloproliferative disorders: Polycythemia Vera (PV), Essential Thrombocytosis (ET), Primary Myelofibrosis (PM), and Chronic Myeloid Leukemia (CML). In these disorders the BCR-ABL fusion gene is only found in CML. Among the remain-

ing three conditions, there are common mutations involved in their pathogenesis. JAK2 mutation is one of them. It is present in almost all cases with PV and 60 - 65% of those with PM and ET [1]. While the CALR mutation is seen in 20 - 25% of the patients with ET and PM; the MPL mutation is seen in 3% and 7% of the patients with ET and PM, respectively [2]. JAK2, CALR, and MPL mutations are known as driver mutations in myeloproliferative neoplasms as they start the proliferation of myeloid cells in the bone marrow by activating the JAK/STAT pathway [3]. ET and PV are diseases that may transform into both PM and acute leukemia. Among the myeloproliferative neoplasms, leukemic transformation is more common in patients with PM. In PM, the most common cause of death is leukemic transformation [4]. Therefore, predicting the clinical course and transformation into acute leukemia in patients with PM is important. The role of high molecular risk mutations such as the ASXL1 mutation has been gaining more importance in recent years. ASXL1 mutation is present in 20 - 35% of patients with PM, 7% of patients with PV, and 4% of patients with ET [5]. Tefferi et al. found that while median overall survival was 2.3 years in ASXL1 (+) and CALR (-) patients, it was 10.4 years in ASXL1 (-) and CALR (+) patients [6]. Once the prognostic significance of ASXL1 mutation is understood, it will be included in the new prognostic scores [7].

Many studies show that oxidative stress may also play a role in myeloproliferative neoplasm's pathogenesis [8-10]. Even in some studies, it has been suggested that oxidative stress could be predictive of transforming these patients into PM and acute leukemia [11].

Oxidative stress occurs when the balance between oxidant and antioxidant substances is disturbed. Many molecules in the literature can be used as an oxidative stress marker. Malondialdehyde, total homocysteine, glutathione peroxidase 1, and superoxide dismutase 2 are just a few of them. The most crucial problem of oxidative stress biomarkers is that they are affected by variables such as diet, lifestyle, age, and laboratory work methods. Therefore, an oxidative biomarker used in routine practice has not been found yet [12]. Thiol compounds are one of these oxidative stress markers. Free radicals oxidize the thiol groups and form reversible disulfide bonds. When disulfide bonds meet with antioxidant substances, they degrade into thiol groups, and thus hemostasis is maintained [13]. Oxidative stress creates an ischemia-like environment, especially in tumor cells. In ischemic conditions, albumin modified by free radicals is called ischemia modified albumin (IMA) [14]. Previous studies suggested that IMA could be used as an indicator for increased oxidative stress in many disorders such as acute coronary syndrome and diabetes mellitus [15-17]. There is no study showing the relationship between IMA levels and myeloproliferative disorders.

One of the features that limit oxidative stress markers in clinical practice is that the measurement methods are time-consuming and relatively expensive. Recently, Erel et al. developed a new, inexpensive, and simple automated spectrophotometric method to measure thiol compounds [18]. Clinical studies have confirmed that this method has significant results in many diseases [19-23].

This study aims to assess the change of thiol hemostasis measured with this new method in older patients with MPN to reduce the possibility of change with age. The secondary purpose is to examine the associations with disease subtypes and genetic features.

MATERIALS AND METHODS

The patients included in the study applied to the Mersin University Hematology outpatient clinic, which is the only tertiary hematology center in the Mersin region, between January 2019 and December 2020. The study included 57 patients with essential thrombocytosis, 52 patients with primary myelofibrosis, 51 patients with polycythemia vera, and 56 healthy controls, aged 65 and over. The diagnoses of the patients were confirmed according to the 2016 WHO diagnostic criteria. All of the patients were newly diagnosed patients. Patients under 65 years of age and those using antioxidant drugs (statins, N-acetylcysteine, NSAIDs) were excluded. Healthy controls were individuals without known chronic diseases and who did not use regular medication. After confirming the diagnoses, a blood sample was taken from each patient before starting treatment. After the samples were centrifuged, the separated serums were stored at -80°C. Disease-related genetic mutations (JAK2, CALR, MPL, ASXL1) of the patients were recorded.

IMA measurements were made by the ELISA method, and the results are given in ng/mL. Disulfide and thiol tests were studied with a novel technique described by Erel et al. [18]. Total thiol, native thiol, disulfide levels were given in mmol/L. Native thiol/total thiol, disulfide/total thiol, disulfide/native thiol ratios were calculated and shown as percentages.

This study was approved by the local ethics committee (2020/709) and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all healthy controls and patients.

Statistical method

Data distribution characteristics were evaluated with the Kolmogorov-Smirnov test, skewness and kurtosis assessment, coefficients of variations, probability plots, and histograms. The data showing normal distribution were reported as mean and standard deviation, and the data without normal distribution were reported as median (minimum-maximum). Student's *t*-test was used to compare continuous variables for independent samples. The Fisher's exact tests and chi-squared test were used to compare groups with categorical variables between groups. The Mann-Whitney U test was performed to compare groups that did not show normal distribution

Table 1. Comparison of ischemia modified albumin and thiol parameters with the control group and total patient group.

	Control			Patient			p-value
	Median	Min	Max	Median	Min	Max	
IMA (ng/mL)	0.67	0.32	0.83	0.75	0.49	1.49	p < 0.001
Native thiol (mmol/L)	431.38	415.15	871.40	299.90	111.70	565.80	p < 0.001
Total thiol (mmol/L)	466.42	428.80	921.10	331.00	135.40	624.70	p < 0.001
Disulfide (mmol/L)	18.25	3.04	27.65	14.77	4.26	32.40	p = 0.258
Disulfide/native thiol (%)	4.20	0.71	6.46	4.92	1.70	19.39	p < 0.001
Disulfide/total thiol (%)	3.87	0.70	5.72	4.50	1.70	13.97	p < 0.001
Native/total thiol (%)	92.26	88.55	98.60	91.04	72.06	96.60	p < 0.001

Abbreviations: IMA - ischemia modified albumin.

Table 2. The change of ischemia modified albumin and thiol parameters according to the disease subtypes.

	ET (n = 57)	PM (n = 52)	PV (n = 51)	p-value [a]
	Median (min - max)	Median (min - max)	Median (min - max)	
IMA (ng/mL)	0.71 (0.49 - 1.49)	1.03 (0.75 - 1.29)	0.71 (0.49 - 0.93)	p < 0.001 [b]
Native thiol (mmol/L)	307.15 (281.60 - 323.50)	270.25 (111.70 - 565.80)	304.50 (270.90 - 315.80)	p < 0.001 [b]
Total thiol (mmol/L)	341.40 (306.90 - 372.50)	299.55 (135.40 - 624.70)	336.40 (283.00 - 369.28)	p < 0.001 [b]
Disulfide (mmol/L)	15.63 (6.33 - 29.63)	14.73 (4.26 - 32.40)	14.66 (6.07 - 29.63)	p > 0.05
Disulfide/native thiol (%)	5.24 (2.03 - 9.80)	5.10 (1.70 - 19.39)	4.69 (2.03 - 9.80)	p > 0.05
Disulfide/total thiol (%)	4.75 (1.95 - 8.20)	4.65 (1.70 - 13.97)	4.29 (1.95 - 8.20)	p > 0.05
Native/total thiol (%)	90.51 (83.61 - 96.10)	90.75 (72.06 - 96.60)	91.42 (83.61 - 96.10)	p > 0.05

Abbreviations: IMA - ischemia modified albumin, ET - essential thrombocytosis, PM - primary myelofibrosis, PV - polycythemia vera.
[a] The p-values belong to the comparisons between three groups made with the Kruskal-Wallis test. The Mann-Whitney U test was performed to test the significance of pairwise differences using the Bonferroni correction to adjust for multiple comparisons.
[b] The p-value of the pairwise comparisons between PM vs. ET and PM vs. PV. It means that the main difference is due to patients with PM.

Table 3. Distribution of mutations within the total patient group.

	n	%
JAK2 mutation	105	65.6
ASXL1 mutation	27	16.9
CALR mutation	15	9.4
MPL mutation	7	4.4
Cases with no mutation detected	6	3.8

and analyze the significance of pairwise differences in disease subgroups. The Kruskal-Wallis test was used to compare parameters that did not show normal distribution with three or more groups.

RESULTS

A total of 160 patients with a myeloproliferative disorder and 56 healthy controls were included in this study. While the patient group's median age was 69 (65 - 85) years, it was 71 (66 - 78) years in the control group.

Table 4. The change of ischemia modified albumin and thiol parameters in patients with the ASXL1 mutation and ASXL1 naive patients.

	ASXL1 (-) (n = 133)			ASXL1 (+) (n = 27)			p-value
	Median	Minimum	Maximum	Median	Minimum	Maximum	
IMA (ng/mL)	0.73	0.49	1.49	0.98	0.69	1.29	p < 0.001
Native thiol (mmol/L)	302.40	111.70	427.00	282.20	158.10	565.80	p < 0.001
Total thiol (mmol/L)	332.80	135.40	466.20	312.10	166.60	624.70	p = 0.046
Disulfide (mmol/L)	13.71	5.72	29.63	16.91	4.26	29.50	p = 0.226
Disulfit/native thiol (%)	4.66	2.03	19.39	5.83	2.69	9.80	p = 0.128
Disulift/total thiol (%)	4.26	1.95	13.97	5.22	2.55	8.20	p = 0.130
Native/total thiol (%)	91.48	72.06	96.10	89.56	83.61	94.89	p = 0.130

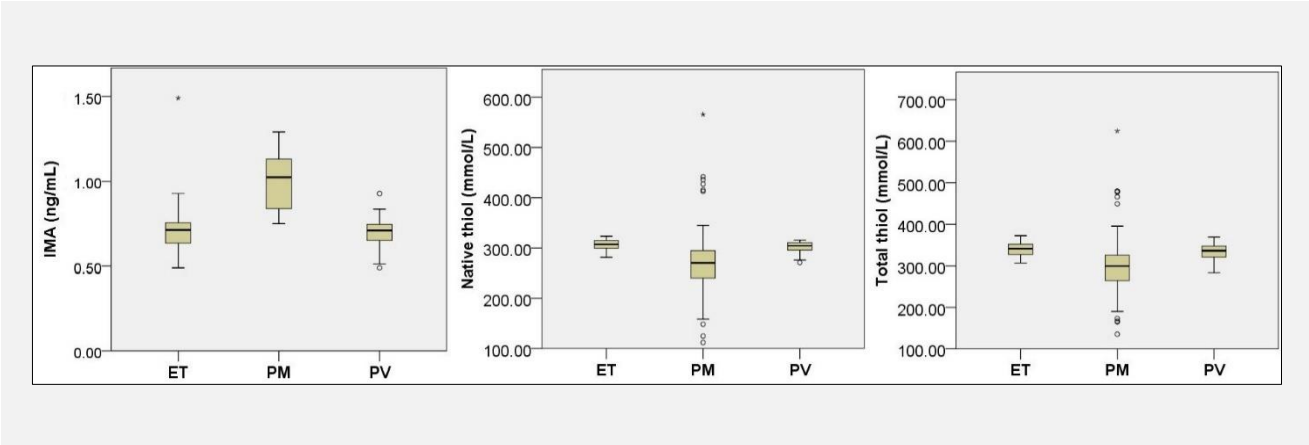

Figure 1. Box-plot graphs showing the change of IMA, native thiol, and total thiol levels in disease subtypes.

Abbreviations: IMA - ischemia modified albumin, ET - essential thrombocytosis, PM - primary myelofibrosis, PV - polycythemia vera. It is seen that the significant differences seen in Table 2 are caused by patients with PM.

Eighty-seven (54.4%) of the patients and 31 (55.4%) of the control group were female.

When the patient and control groups were compared in terms of the measured parameters, it was found that the parameters except disulfide showed a statistically significant difference between the patient and control groups (Table 1).

When oxidative stress parameters were compared according to disease types, statistically significant differences were found in IMA, native thiol, and total thiol levels (Table 2). In pairwise comparisons, it was realized that this significant difference was due to the results of patients with PM. The comparisons between PM and ET and between PM and PV were statistically significant ($p < 0.001$), and no significant difference was found in the comparisons between ET and PV ($p > 0.05$) in terms of IMA, native thiol, and total thiol levels (Table 2 and Figure 1).

The genetic mutations detected in the patients are shown in Table 3. When IMA and thiol parameters were compared according to genetic mutations in patients, it was found that there was a statistically significant difference in terms of IMA, native thiol, and total thiol in cases with the ASXL1 mutation (Table 4). In the presence of other mutations, no significant difference was found between the parameters studied.

DISCUSSION

DNA is one of the cellular structures affected by oxidative stress. It has been shown in many studies that the increase in free oxygen radicals causes structural DNA damage. Because of this feature, it has been suggested that oxidative stress may play a role in the etiopathogenesis of many cancer types [24]. Since the relationships of oxidative stress with aging and antioxidant drugs are known, we think it is necessary to minimize

the effects of the age factor and antioxidant drug usage on laboratory parameters in oxidative stress studies. To the best of our knowledge, this study is the first study in the BCR-ABL negative MPN patient group in which the case and control group were over 65 years old. Exclusion of patients using antioxidant drugs and inclusion of only newly diagnosed patients who have not yet started treatment increased the strength of the study. This study provides a significant contribution to the literature in terms of showing that oxidative stress is increased in older patients compared to the control older population. Moreover, it is the first study in the literature to show that patients with the ASXL1 mutation have significantly increased oxidative stress compared to ASXL1 naive patients.

Although various scoring systems that integrate clinical and genetic features are widely used to show the prognosis in myeloproliferative diseases, none of these scores can precisely predict the clinical course. Among myeloproliferative diseases, it is known that there is a direct relationship between BCR-ABL copy number in CML and leukemic transformation. CML is distinguished from other myeloproliferative disorders by this aspect. Therefore, other myeloproliferative diseases are referred to as bcr-abl negative myeloproliferative diseases. Our study aimed to evaluate BCR-ABL negative myeloproliferative conditions in which prognostic scores are not as straightforward as CML.

The median age in patients with ET, PV, and MF is around 64 years, and it is expected to exceed 65 in the coming years [25]. Moreover, it is known that overall survival for all three diseases decreases with advancing age [26]. Therefore, prognostic scores are needed to predict the prognosis of these patients, especially in the older patient group. Although the integration of oxidative stress markers into these scores seems quite logical in the light of the evidence so far, the effect of oxidative stress markers may decrease their reliability in this age group. This study was designed to include only older patients and controls to eliminate the effect of age on oxidative stress.

As seen in Table 1, when we compare all disease subtypes with the control group collectively, we noticed that all parameters except disulfide show that oxidative stress increased significantly in these patients. Then, when we compared the disease subtypes separately, we observed that oxidative stress was more dominant in myelofibrosis patients (Table 2). Among these three disease groups, the disease group with the lowest probability of survival is myelofibrosis. Since there is no chance of allogeneic bone marrow transplantation in high-risk myelofibrosis patients over 65 years of age, survival is even lower [26]. *In vitro* studies have shown that oxidative stress triggers hypoxia in bone marrow stem cells in myelofibrosis patients [27]. Hypoxia correlates with the hypoxia inducible factor-alpha (HIF-a) level in these patients and increases proteins involved in maintaining the healthy environment of hematopoietic stem cells such as FoxO3 [27-29]. The higher serum IMA levels in PM compared to ET and PV may indicate that the hypoxia environment is more severe in this patient group. This may be one of the reasons for the higher frequency of leukemic transformation in patients with PM.

Apart from the factors mentioned above, the genetic characteristics of the disease may also affect oxidative stress. An increase in oxygen free radical formation in myeloproliferative conditions with JAK2 positivity was previously shown [30]. CALR is a protein that drives cell death through oxidative stress. It has been demonstrated that free radical-associated cellular toxicity and oncogenic transformation are increased in the presence of mutant CALR [31-33]. The ASXL1 mutation is also known to decrease overall survival and increase leukemic transformation, especially in patients with myelofibrosis and MDS [34]. However, its relationship to oxidative stress is not clear yet. As seen in Table 4, we found significant changes in oxidative stress parameters and serum IMA levels in patients with the ASXL1 mutation when patients with ASXL1 mutation and non-patients were compared. We believe that this finding is one of the most important results of this study. Although we know the effects of ASXL1 on prognosis, it is unclear on what mechanism it causes this situation. Based on these findings, it can be said that *in vivo* and *in vitro* studies on oxidative stress are needed inpatients with the ASXL1 mutation.

Although there are many studies on antioxidant drugs in cancer treatment, the studies generally involve low patient numbers, and the results are conflicting [35]. Therefore, the role of oxidative stress in the pathogenesis of the disease should be fully revealed after limiting all the factors that may affect oxidative stress, such as age. Then studies should be planned on the effects of antioxidant treatments. Ruxolitinib is a JAK1/JAK2 tyrosine kinase inhibitor and is widely used in the management of PM and PV worldwide. It has been shown that ruxolitinib reduces the production of superoxide from monocytes, and it is thought that it may have antioxidant properties [12]. In light of all these, prospective studies comparing pre-treatment and post-treatment parameters can be designed to understand how ruxolitinib changes oxidative stress parameters in these patients. Our study had several limitations. First of all, the number of patients was relatively small. Studies with higher patient numbers may facilitate interpreting the results regarding the effects of relatively rare genetic factors on oxidative stress markers. Second, due to the short follow-up period, we could not provide data on overall survival and leukemic conversion of the patients. In order to clearly understand the effect of oxidative stress markers on factors such as survival and leukemic transformation, controlled prospective studies aiming at the long-term follow-up of these patients are needed.

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Declaration of Interest:
The authors declare that they have no conflict of interest.

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