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Study of Some Apoptotic and Fibrotic Markers in Myeloproliferative Neoplasms

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ABSTRACT

Background: Chronic myeloproliferative neoplasms are clonal hematopoietic stem cell disorders characterized by proliferation, in the bone marrow, of one or more of the myeloid lineages. One of the important bone marrow findings that overlap the various CMPN entities is fibrosis.

Objectives: To evaluate the role of NF-k ß in patients with Chronic Myeloproliferative Neoplasms (MPB) in relation to some apoptotic (cathepsin, TGF ß) and fibrotic markers (b-FGF, BM reticulin score).

Patients and Methods: The study included 20 patients with de novo CML (group A) who received imatinib 400mg/day for 3 months; group B included 20 patients with other MPN [Essential thrombocytosis (ET), polythycemia vera (PV) and idiopathic myelofibrosis (IMF) who received hydroxy-urea orally in a dose of 0.5-2g/day for 3 months. Ten age and sex matched healthy individuals served as a control group. All patients were subjected to thorough history taking, full physical examination and laboratory investigations including CBC, BM biopsy and reticulin stain. NF-k ß, cathepsin B, TGF-ß and b-FGF were measured using ELISA technique.

Results: The mean values of NF-k ß in CML patients (group A) and non CML-CMPN (group B) were 165.20± 26.96 and 200.79±92.00pg/ml respectively while after treatment the values were significantly decreased to 104± 25.36 and 134.2±96.89pg/ml respectively (p=0.013, p= 0.001). Cathepsin B increased significantly in group A after 3 months of therapy (From 71.90±39.85 to 126.80± 60.42pg/ml in CML, while in group 2 it decreased significantly from 123.47±50.67 to 67.50±42.50pg/ml (p=0.001 and 0.001 respectively). The level of TGF- ß decreased in CML from 101.20±38.16 to 52.20±22.06 while in group 2 it decreased from 128.53±63.54 to 87.45±48.87pg/ml (p=0.001) b-FGF level also decreased after treatment; in CML it decreased from 14.68±4.33 to 11.03±3.22 (p=0.042), while in group 2 it decreased from 35.65±9.85 to 15.52±3.2pg/ml (p=0.001). Reticulin stain score of BM biopsy showed significant decrease of BM fibrosis in group A and B (p=0.036 and 0.0001 respectively).

Conclusion: NF-κ ß, TGF-ß and Cathepsin B are beneficial in monitoring the disease progression and response to treatment in myeloproliferative neoplasms. These data support further use of these markers in the prognosis of CMPN.

Key Words: *CMPN = Chronic Myeloproliferative Neoplasms – NFK-b = Nuclear Kappa B cell – b-FGF = basic Fibroblast Gowth Factor – TGF-B = Transforming – Growth Factor Beta.*

INTRODUCTION

Chronic myeloproliferative neoplasms (CMPN) are clonal hematopoietic stem cell disorders characterized by proliferation, in the bone marrow, of one or more of the myeloid lineages **[1]**. One of the important bone marrow findings that overlap the various CMPN entities is fibrosis which is most likely caused by abnormal production of platelet derived growth factor (PDGF) and transforming growth factor-ß (TGF- ß) by Megakaryocytes **[2]**.

The caspases are a family of proteins that are one of the main executors of the apoptotic process **[3,4]**. Nuclear factor-kappa ß (NF-kß) is a protein that acts as a switch to turn inflammation on and off in the body. NF-kß acts in each of the main phases of cancer development **[5]**. NF-kß activation in inflammatory cells results in increased production of cytokines and other growth factors that support the growth, replication and invasion of cancerous cells. Such activated inflammatory cells provide new blood vessel formation **[5-7]**.

b-FGF is an endothelial cells growth factor. It is an important inducer of stromal cell activation; activated stromal cells, in turn, may

produce other inducers of angiogenesis such as b-FGF, IL-6 and IL-8 **[8]**.

TGF- ß regulates cellular proliferation in a cell specific manner. In most endothelial and hematopoietic cells, TGF- ß is a potent inhibitor of cell proliferation. In cancer cells, mutations in the TGF- ß pathway have been described as it allows uncontrolled proliferation of the cells **[6-10]**. In response to increased production of TGF- ß by the tumor cells, they become more invasive and metastasize to distant organs **[9,11,12]**.

TGF-ß increases the biosynthesis of type I, III, IV collagens, fibronectin and proteoglycans. It blocks matrix degradation and acts as a potent angiogenic factor **[13]**.

Cathepsins are proteases found in many types of cells. Cathepsin B can participate in tumor invasion by degradation of extracellular matrix components. Cathepsin B enhances apoptotic pathways **[14-17]**.

In this study, we aimed to evaluate the role of NF-kß in patients with chronic myeloproliferative meoplasms (CMPN) in relation to some apoptotic (cathepsin, TGFB) and fibrotic markers (b-FGF, BM reticulin stain score).

PATIENTS AND METHODS

Patients: The study included 40 CMPN patients admitted to the Hematology Department, Main Alexandria University Hospital. They were divided into two groups: Group A comprised 20 (50%) de novo CML patients; they received imatinib 400mg/day for 3 months and group B comprised 20 patients with other MPN-ET 8 (20%), PV 5 (12.5%) and IMF7 (17.5%); they received 0.5gr-2gr/day oral hydroxyurea for 3 months. Three patients did not return back for follow-up in group B (missed). Ten healthy subjects with matched age and sex were a control group. The study was approved by the Institutional Review Board and a written informed consent was obtained from all subjects before enrollment.

Methods: All patients were subjected to complete blood count **[18]**, Renal and Liver profile **[19]**, BM biopsy using hematoxylin and eosin and reticulin stain **[20]**, PCR for BCR-ABL **[21]** and JAK2 V617F **[22]**, measurement of NF-Kb (NF-κB Transcription Factor Assay Kit, Rockland Immunochemicals, USA), Cathepsin B (Abcam's Human Cathepsin B in vitro ELISA, USA), TGF-ß (Human TGF-beta 2 Quantikine ELISA Kit R&D Systems), b-FGF (Human FGF basic Quantikine HS ELISA Kit-R&D system) **[23]**.

RESULTS

Demographic data: The age range of group A was 22-67 with a mean of 44.85±14.78 and a median of 50 years; for group B the range was 20-75 with a mean of 48.45±11.13 and a median of 51 years; and for the control, the range was 26-62 with a mean of 45.70±14.77 and a median of 42 years. The patients included 16 males (40%) and 24 (60%) females; the control included 4 males (40%) and 6 (60%) females. The patients included 20 (50%) cases with de Novo CML in chronic phase (Group A). Group B included 8 (20%) patients with essential thrombocythemia, 7 (17.5%) with idiopathic myelofibrosis and 5 (12.5%) with polycythemia vera.

Peripheral blood counts (Table 1): There was significant decrease in platelet counts as well as in WBCs in group A and B after treatment (p=0.001). In group A Hb level increased after therapy but not significantly.

Bone marrow cellularity: Regarding group A, BM was hypercellular in all 20 (100%) patients. The hypercellularity was secondary to myeloid metaplasia before therapy; after therapy with imatinib, all patients exhibited a decrease in cellularity. The nadir cellularity in 12 patients was normocellular to moderately hypocellular. The reduction in cellularity corresponded to prominent decrease in myeloid proliferation in all patients with the most severe reduction in cellularity characterized by decrease in both myeloid and erythroid production. The number of megakaryocytes remained normal in all except the 8 patients with marked hypocellularity, in whom the number was decreased.

Group B: Before therapy there were 4 patients (20%) with normocellular, 3 (15%) with hypocellular, and 13 (65%) with hypercellular BM; after treatment BM cellularity returned to normocellular in 9 patients (52.9%), 1 (5.9%)

remained hypocellular, 7 (41.2%) were hypercellular while 3 patients refused to repeat the trephine. There was significant improvement in BM cellularity after therapy (p=0.0001). Erythroid hyperplasia was prominent in PV and moderately to markedly reduced in IMF. Granulopoiesis showed a relevant increase in PV, but normal granulopoiesis in ET. In PV there was increase in clustered enlarged megakaryocytes with hyperploid nuclei.

NF-k ß, Cathepsin B, TGF-ß, b-FGF and Reticulin stain score before and after treatment (Table 2):

In patients with CML (group A) and ET, PV and IMF (group B) the level of NF-k*ß* both before and after treatment was increased in relation to the control group (p=0.001), but there was significant decrease of NF-k*ß* after treatment in both groups (p=0.013, p=0.001 respectively). Cathepsin B increased significantly in group A and B after 3 months of therapy (p=0.001 and 0.001 respectively). Also the level of TGF-*ß* declined significantly in both groups (p=0.001and 0.001). Regarding b-FGF it decreased significantly in both groups (p=0.013, 0.001). Reticulin stain score of BM biopsy (Figs. 1, 2, 3 and 4) showed significant decrease in BM fibrosis after treatment in both groups (p=0.036 and 0.0001 respectively).

Correlation of NF-k ß, Cathepsin B, TGF-ß, b-FGF and Reticulin score with other parameters (Table 3):

There was significant positive correlation between NF-k *ß* and TGF- *ß*, b-FGF and Reticulin score, Hb, platelets and bone marrow Cellularity but no significant correlation with other parameters (RBC, erythopoiesis, Myelopoeisis , megakaryopoiesis and blast cells).

Table (1): Peripheral Blood parameters in 40 chronic myeloproliferaive patients before and after treatment.

Parameter	CML patients		Non-CML MPNs Patients		Control
	Before treatment	After treatment	Before treatment	After treatment	
WBCx10^9/L:					
Range	45.62-235.25	7.5-22.1	4.53-39.3	4.5-23	3.9-10.4
Mean±S.D.	182.11±65.11	15.89±4.90	16.68±10.36	10.39±5.17	7.51±2.27
p	0.0001*	0.0001*	0.001*	0.013*	
p_1	0.001*		0.001*		
RBCx10^{12}/L:					
Range	3.32-4.71	3.63-5.14	3.1-8.91	2.35-7.27	3.97-5.3
Mean±S.D.	3.99±0.23	4.22 ±1.01	5.13±1.90	5.20±1.13	4.85±0.47
p	0.233	0.365	0.412	0.365	
p_1	0.096		0.254		
Hb gm/dl:					
Range	8.8-10.20	7.1-11.3	6.9- 20	6.9-18	11.1-14.5
Mean±S.D.	9.18±2.05	10.16±2.43	11.95±3.91	12.65±2.62	12.68±1.62
p	0.022*	0.045*	0.265	0.652	
p_1	0.265		0.18		
HCT %:					
Range	27.9-46.1	22.9-42.7	23.8-57.4	22.6-53.0	35.1-42.2
Mean±S.D.	32.11±4.62	30.91±6.04	38.0±12.0	39.0±9.0	39.18±2.30
p	0.132	0.098	0.468	0.852	
p_1	0.236		0.336		
PLTx10^9/L:					
Range	198-550	216-445	110-1985	159-765	177-368
Mean±S.D.	365.11±76.28	327.11±81.94	766.45±588.7	356.20±155.65	274.2±77.76
p	0.016*	0.098	0.001*	0.089	
p_1	0.136		0.001*		

p : Comparison between control and other groups.

p_1 : Comparison between before and after treatment in the same group.

Table (2): NF-k ß, Cathepsin B, TGF- ß, b-FGF and Reticulin score before and after treatment in CML patients and Non-CML MPNs compared to control.

	CML Patient No=20		Non-CML MPNs Patients: No=20		Control No=10
	Before treatment	After treatment	Before treatment	After treatment	
NF-k ß: pg/ml:					
Range	39-233	33-179	60-359	35-390	20-65
Mean±S.D.	165.20±26.96	104.0±25.36	200.79±92.0	134.2±96.89	45.2±16.57
p	0.001*	0.001*	0.001*	0.001*	
p_1	0.013*		0.001*		
Cathepsin B: (pg/ml):					
Range	28-137	17-198	11-200	10- 240	21-123
Mean±S.D.	71.9±39.85	126.80±60.42	123.47±50.6	67.50±42.50	69.4±41.41
p	0.236	0.001*	0.001*	0.685	
p_1	0.001*		0.001*		
TGF-ß (pg/ml):					
Range	30-174	18- 83	10-222	7-188	10-28
Mean±S.D.	101.2±38.16	52.20±22.06	128.53±63.54	87.45±48.87	17.9±7.17
p	0.001*	0.001*	0.001*	0.001*	
p_1	0.001*		0.001*		
bFGF (pg/ml):					
Range	6.5-18	7-14	18-68	9-20	8-15
Mean±S.D.	14.68 ±4.33	11.03±3.22	35.65±9.85	15.52 ±3.21	11.25±2.98
p	0.042*	0.526	0.001*	0.107	
p_1	0.013*		0.001*		
Reticulin Stain score:					
Min-Max.	0-2	0-1	2-4	1-3	
Mean±S.D.	1.42±0.31	0.561±0.12	3.05±0.76	2.00±0.79	
	p=0.036*		*p*=0.001*		

p = Comparison before and after treatment.
p_1 = Comparison between group A and group B.

Table (3): Correlations between different studied parameters in all patients.

		NF-k ß	Cathepsin	TGF- ß	b-FGF	Reticulin stain score
NF-kB (pg/ml)	*r*		0.477**	0.711**	0.404**	0.644**
	p		0.001	0.001	0.006	0.001
Cathepsin B (pg/ml)	*r*	0.509**		0.807**	0.622**	0.787**
	p	0.001		0.001	0.001	0.001
TGF-B (pg/ml)	*r*	0.699**	0.898**		0.455**	0.791**
	p	0.001	0.001		0.002	0.001
b-FGF (pg/ml)	*r*	.401**	.455**	.442**		.347*
	p	0.006	0.002	0.002		0.019
Reticulin stain score	*r*	.327*	.601**	.615**	.298*	
	p	0.028	0.001	0.001	0.047	
RBC	*r*	0.234	0.099	0.112	0.29	0.279
	p	0.122	0.52	0.465	0.053	0.063
HGB	*r*	.455**	0.064	.349*	0.182	0.208
	p	0.002	0.677	0.019	0.232	0.17
PLT	*r*	.381*	.475**	0.143	.412**	0.243
	p	0.011	0.001	0.348	0.005	0.108
BM Cellularity	*r*	.307*	.470**	.631**	0.205	.435**
	p	0.04	0.001	0.001	0.176	0.003
Erythropoiesis	*r*	0.283	0.132	0.178	0.174	0.254
	p	0.059	0.387	0.241	0.252	0.093
Myelopoeisis	*r*	0.122	0.276	0.262	0.164	0.247
	p	0.201	0.066	0.083	0.282	0.102
Megakaryopoiesis	*r*	0.174	.344*	0.234	0.099	0.112
	p	0.23	0.021	0.122	0.52	0.465
BLASTS	*r*	0.185	–0.087	–0.14	0.168	–0.099
	p	0.225	0.571	0.361	0.271	0.516

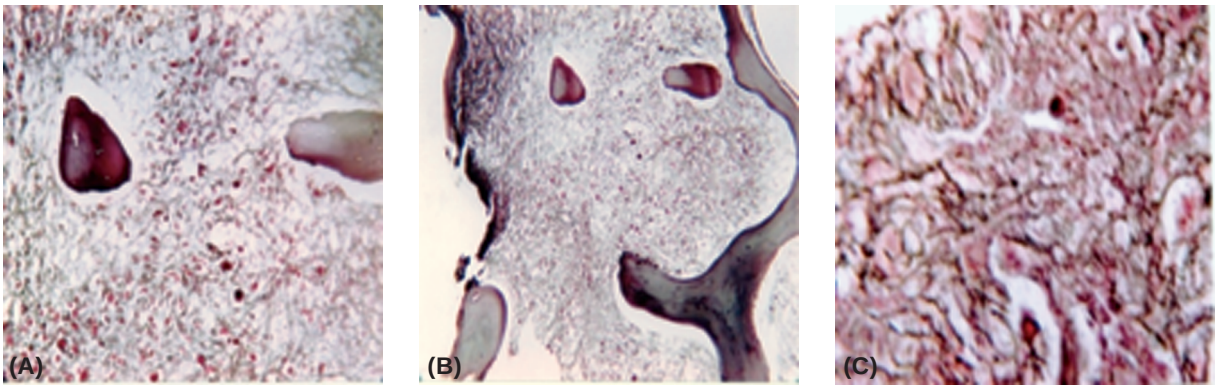

Fig. (1-A,B,C IMF): Grade 1 BM fibrosis: Reticulin stain showing normal scattered fine fibers (200x magnification).

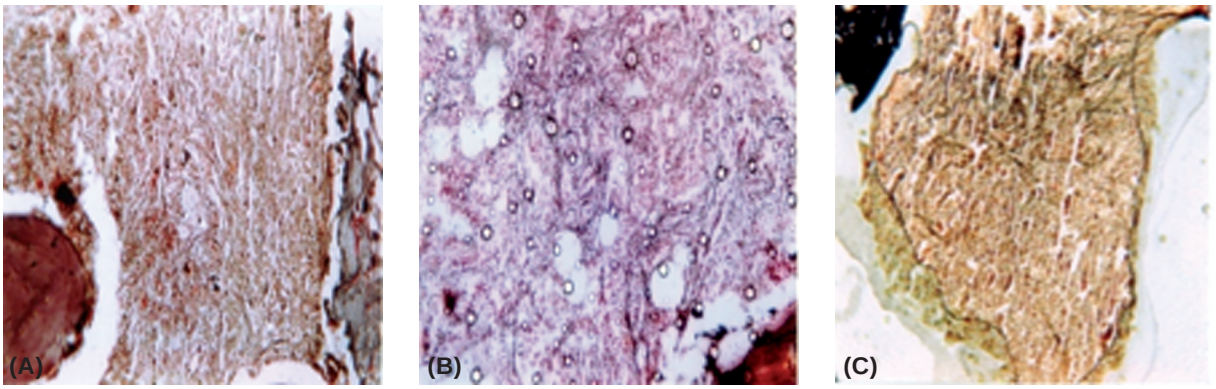

Fig. (2-A,B,C ET): Grade 2 BM fibrosis: Reticulin stain showing a fine network of fibers with no coarse fibers (200x magnification).

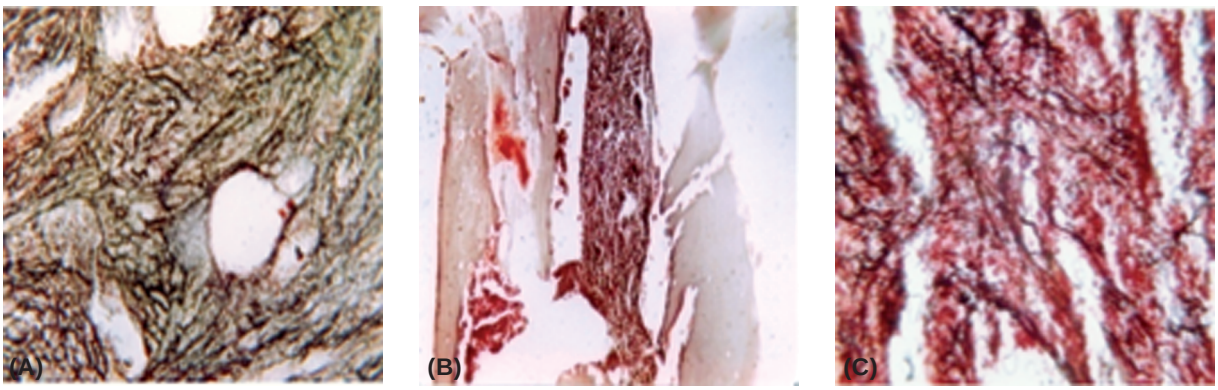

Fig. (3-A,B,C CML): Grade 3 BM fibrosis: Reticulin stain showing diffuse fine reticulin and scattered thick coarse fibers, with no mature collagen (200x magnification).

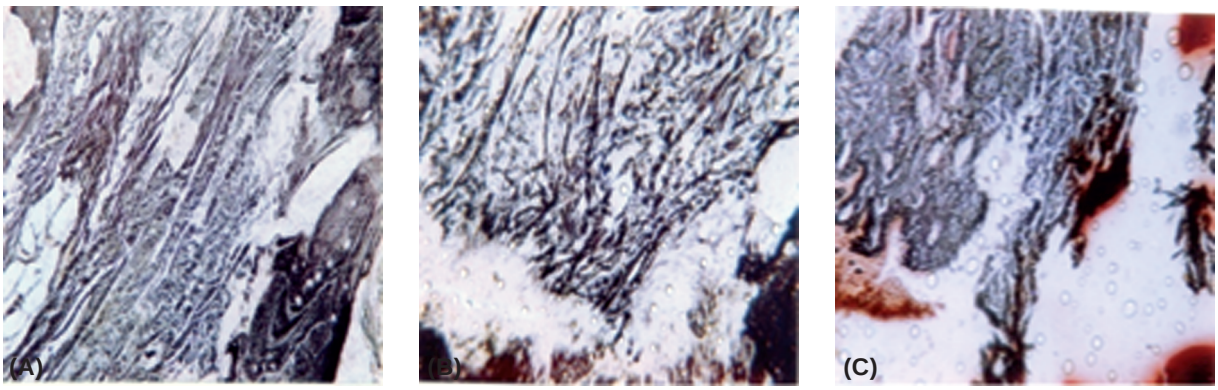

Fig. (4-A,B,C PV): Grade 4 BM fibrosis: Reticulin stain showing coarse reticular fiber network, with collagen deposition (200x magnification).

DISCUSSION

Little is known about the signal transduction pathways involved in the development of CMPNs. However, an activation of the JAK/STAT pathway is involved in myeloproliferative disorders and BCR-Abl tyrosine kinase in CML for which the molecular mechanisms have been characterized **[17]**. In the present study, we showed that other signal transduction pathways, NF-k*ß*, TGF-*ß*, cathepsin B, and b-FGF were also activated.

Kidney functions improved after treatment in both groups. The improvement was significant for creatinine in both patients group A & B, before treatment it was 1.89 and 1.11mg/dl while after treatment the mean value was 1.35 and 0.69mg/dl respectively. Comparing these values in the patient groups before and after treatment, a statistically significant differences were found (p=0.036, 0.01). Plomely et al., **[24]** reposted a case of PV which was found to have a high blood urea nitrogen and serum creatinine. The patient was initially subjected to phlebotomies and showed symptomatic improvement and serum creatinine decreased with the normalization of its level.

Regarding CML patients, bone marrow was hypercellular in all 20 patients (100%) before treatment, after 3 months therapy with imatinib, all patients exhibited a decrease in cellularity and decreased myelopoiesis. These observations are in agreement with Hasserijian et al., **[25]** who concluded that reduction in marrow cellularity in response to imatinib was independent of cytogenetic response. However John et al. found that longer follow-up of the histopathologic features of bone marrow biopsy specimens showed striking differences between patients who remained positive or negative for BCR-Abl **[26]**.

Reticulin stain score of BM biopsy showed significant decrease in fibrosis. A reduction in marrow fibrosis in CML cases treated with imatinib mesylate has been reported **[27]** even in cases in which the malignant clone is not eradicated.

In our cohort, 65% of non CML MPN (group B) had hypercellular BM before treatment, 15% hypocellular and 20% monocellular, while after treatment 41.2% had hypercellular and 59% normocellular BM. Our results are in agreement with Thiele et al., who reported significant decrease in erythroid precursors in CML compared to control, while this cell lineage was most prominent in PV and moderately to markedly reduced in IMF with clusters of small to giant sized megakaryocytes in PV. In IMF more than 80% of patients present with some degree of myelofibrosis-osteosclerosis at diagnosis, while the rest show an initial prefibrotic, hypercellular stage **[28]**.

In this study, the values of NF-k*ß* decreased significantly after treatment in both CML and non CML patients (p=0.013, p=0.001). Guicciardi et al., **[15]** reported that NF-k*ß* activates multiple target genes whose products can block the apoptotic program triggered by death receptors or the mitochondrial pathway; NF-k*ß* inducible anti-apoptotic factors include those that inhibit caspase function and those that inhibit NF-k*ß* signaling after TNF-R1 stimulation. Stimulation of TNF-R1 induces the breakdown of the lysosome and the induction of apoptosis by cathepsins released into the cytoplasm.

In the current study, cathepsin B level was significantly increased in all patients (p=0.001) after treatment. Foghogaard et al., **[14]** stated that several observations may lead to conclude that the induction of serine protease inhibitor (SPi2A) by NF-k*ß* protects cells from TNF-alpha by inhibiting the lysosomal pathway of apoptosis. Inhibition of cathepsin B protects NF-k*ß* from TNF-alpha induced apoptosis, confirming the observation that cathepsin B plays a direct role in apoptosis in other cell types. Scaffidi et al., **[29]** suggested that after ligation of TNF-R1, Spi2A inhibits cytoplasmic cathepsin B activity and so may prevent apoptosis by inhibiting the cleavage of Bid by cathepsin B and subsequent activation by proteins released from mitochondria.

The increase in cathepsin B after therapy in our study agrees with the previous studies as its increase denotes caspase activation **[30]**. Cathepsin B is a potent inducer of apoptosis and is released into the cytoplasm where it activates caspase dependent and caspase-independent pathways of cell death.

In this study, we reported that the level of TGF-*ß* was significantly decreased after treatment in both patient's groups (p=0.001). This

is in agreement with others who found that an abnormal release of growth factors such as TGF-ß could be responsible for the development of marrow fibrosis **[30]** and that activation of NF-k ß leads to increase of TGF- ß expression **[31,32].**

In non CML patients the mean value of reticulin stain score of BM biopsy had markedly decreased after treatment (p=0.001) raising the possibility that fibrosis has decreased due to the inhibitory effect of NF-k ß on TGF- ß.

In our patients the level of b-FGF was significantly higher in non CML in comparison with CML patients; it significantly decreased in both groups post treatment (p=0.001, 0.013). Increased plasma levels of b-FGF have also been reported by others in patients with IMF, ET and PRV **[33,34].**

There was significant positive correlation between NF-k ß and TGF- ß, b-FGF and Reticulin score, Hb, platelets and bone marrow Cellularity but no significant correlation with other parameters (RBCs, erythopoiesis, Myelopoeisis , megakaryopoiesis and blast cells). To the best of our knowledge, such correlations have not been previously addressed. Our results might indicate that the inhibition of NF-kß by treatment leads to the activation of the apoptotic pathway and that the release of cathepsin B is a sign of apoptosis progression. The NF-κß inhibits TGF-ß and b-FGF which are markers of fibrosis.

Conclusion:

The use of NF-κß, TGF-ß and Cathepsin B is beneficial in monitoring the disease progression and response to treatment in myeloproliferative neoplasm. These data support further use of these markers in monitoring the prognosis of CMPN. More prospective randomized controlled trials and large sample size are still required to precisely understand the role of NF-κß, TGF-ß and Cathepsin B in the pathology of CMPN. Implementation of the use of NF-κ ß, TGF-ß and Cathepsin B as prognostic markers of CMPN is strongly recommended.

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