Role of Reticulin & Masson's Trichrome Stains in Evaluation of Extent of Fibrosis in Bone Marrow Biopsies: A Histochemical Study on 36 Cases of Primary Myelofibrosis

*Afroz S,[1] Dey BP,[2] Yasmin R,[3] Kabir E,[4] Shiraj-Um-Mahmuda S,[5] Islam T,[6] Shabnam US,[7] Kabir A[8]

Abstract

Background: Primary myelofibrosis (PMF) is a rare hematological malignancy with higher mortality and morbidity due to progression of bone marrow fibrosis (BMF). The amount of bone marrow reticulin and collagen fibrosis is detected by reticulin and Masson's trichrome (MT) stains, respectively. The study aimed to explore the pattern and degree of BMF in PMF cases with above mentioned special stains along with conventional Hematoxylin& Eosin (H&E) stain.

Methods: Thirty-six histopathologically suggested or diagnosed cases of PMF were studied at the Department of Pathology, BSMMU. Paraffin blocks of trephine biopsy specimens of the selected cases were collected. H&E, reticulin and MT stains were applied on the re-cut sections. Extent of fibrosis was assessed by examining the sections with each special stain using a proposed four scaled semiquantitative bone marrow fibrosis (MF) grading systems. The cases were divided into two groups. Patients having grade MF-0 to MF-1 were considered absent pathological BMF. Those cases with MF-2 to MF-3 were encountered with present pathological BMF.

Results: In this study, 19 (52.8%) PMF cases had pathological BMF diagnosed by only H&E. Moreover, thirty four (94.4%) patients had pathological BMF corresponding to MF-2 to MF-3 detected by special stains.

Conclusion: Significantly higher ($p<0.05$) detection of pathological BMF cases with use of additional special stains recommends that, both reticulin and MT stains can be used routinely to assess BMF properly according to special grading system.

[Journal of Histopathology and Cytopathology, 2023 Jul; 7 (2):103-111]

Keywords: Bone marrow fibrosis, Primary myelofibrosis, Reticulin fibrosis, Collagen fibrosis, Reticulin stain, Masson's trichrome stain, Hematoxylin & Eosin, Bone marrow fibrosis (MF) grade.

1. **Dr. Sadia Afroz, Medical Officer, Department of Histopathology, National Institute of Cancer Research & Hospital (NICRH), Mohakhali, Dhaka-1212, Bangladesh; sadiatoma2813@gmail.com*
2. *Dr. Bishnu Pada Dey, Associate Professor, Department of Pathology, BSMMU.*
3. *Dr. Rumana Yasmin, Assistant Professor, Department of Pathology, Dhaka Central International Medical College, Dhaka, Bangladesh.*
4. *Dr. Evana Kabir, Specialist, Laboratory, United Hospital, Gulshan-2, Dhaka, Bangladesh*
5. *Dr. Syeeda Shiraj-Um-Mahmuda, Lecturer, Department of Pathology, Dhaka Medical College & Hospital, Dhaka.*
6. *Dr. Tasmia Islam, Specialist (Pathology), Square Hospital Limited, Dhaka-1205, Bangladesh.*
7. *Dr. Ummey Salma Shabnam, , Assistant Professor, Department of Histopathology, National Institute of Cancer Research & Hospital (NICRH), Mohakhali, Dhaka-1212, Bangladesh.*
8. *Dr. A.K.M. Nurul Kabir, Professor, Department of Pathology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Shahbagh, Dhaka-1000, Bangladesh.*

**For correspondence*

Introduction

Primary myelofibrosis (PMF) is a rare hematological malignancy encountered under the myeloproliferative neoplasms (MPNs) category. It is a clonal myeloproliferative disease characterized by the proliferation of mainly megakaryocytic and granulocytic components in the bone marrow, associated with reactive deposition of bone marrow connective tissue and extramedullary hematopoiesis.[1] Due to frequent association with poor cellularity of bone marrow aspirates in PMF, trephine biopsy of the marrow is recommended for evaluation of the marrow cellularity, topography, stromal changes and maturation pattern of the hematopoietic cells.[2] There is a stepwise evolution from an initial pre fibrotic to an overt fibrotic stage with marked reticulin or collagen fibrosis in the bone marrow, often with osteosclerosis. The marrow is hypercellular for age in the early or pre-fibrotic stage. There is an increase in abnormal megakaryocytes and granulocytes with reticulin fibrosis corresponding to normal bone marrow or not exceeding grade 1 (figure-1 a-b). Most cases of PMF are diagnosed initially in the overt fibrotic stage. The bone marrow is more often normocellular or hypocellular, with patches of active hematopoiesis alternating with regions of loose connective tissue and/or fat. The bone marrow shows increased reticulin or collagen fibrosis (grade 2 or 3) in this stage (figure-2 a-c). Very late in the course, the fibrotic marrow space may be converted into bone, a change called "osteosclerosis".[3-5]The revised criteria of "WHO classification of myeloid neoplasms, 2016" outlined the diagnosis of PMF into pre-PMF and overt PMF, which included modified grading of reticulin and collagen bone marrow fibrosis.[6]

Bone marrow stromal fibrosis is a reactive process associated with abnormalities in the number and/or function of megakaryocytes and platelets. PDGF, TGF-β, epidermal growth factor, endothelial cell growth factor, TNF-α, IL-1, IL-6 etc. can be produced by the cellular components of clonal proliferation. These act on fibroblasts to produce extracellular matrix leading to an increase in reticulinfibres (reticulin fibrosis) and/or collagen fibres (collagen fibrosis). These cytokines are also involved in the expression of proteases which inhibit enzymes responsible for the extracellular matrix degradation. Reticulinfibres are few and exist as a fine network of scattered fibres without intersection throughout the normal bone marrow. Bundles of reticulinfibres constitute collagen fibres. The latter usually is absent in the bone marrow interstitium. Connective tissue components, especially reticulin fibers, are poorly visualized in H&E-stained sections. Reticulin stain by silver impregnation techniques, such as Gordon & Sweet's and Gomori's stains, are used to observe reticulin fibrosis. Masson's trichrome (MT)stain identifies collagen fibrosis.[7-9]

The present study was designed to evaluate the degree of bone marrow fibrosis (BMF)according to the histological pattern of reticulin and collagen fibres deposition in the trephine biopsies of 36 PMF cases with reticulin and MT stains

Methods

This was a cross-sectional study performed on a total of 36 consecutive samples of formalin-fixed paraffin embedded trephine biopsies of patients received at the Department of Pathology, BSMMU in March 2020 to February 2022. Those included cases were histopathologically suggested or diagnosed with primary myelofibrosis. Myelofibrosis secondary to other neoplastic and non-neoplastic conditions, trephine biopsies with extensive crush artifact or inadequate sampling (e.g. < 1 cm long), and patients unwilling to give informed consent were excluded from the study. Re-cut sections were

obtained from paraffin blocks at about 5 µm thickness for staining with H&E, reticulin (Gordon & Sweet's method) and MT stains using a standard protocol.

Re-evaluation of bone marrow sections

1. *Using Haematoxylin & Eosin stain (H&E)*

Marrow architectural details were studied. Any focal or diffuse fibroblastic proliferation was considered as the presence of BMF in H&E-stained sections. The pattern of osteosclerosis was observed according to Kvasnicka et al.[10] The maximum pattern occupied in at least 30% of the marrow area ~~was~~ determined ~~as~~ the dominant osteosclerosis grade in heterogeneous cases.

2. *Using special stains*

BMF was evaluated withreticulin and MT stains for each case according to table I. The degree of myelofibrosis was determined according to a semiquantitative grading system as followed by Kvasnicka et al.[10] based on "European consensus on bone marrow fibrosis (MF) grading, 2005" within a range of MF-0 to MF-3. In cases with heterogeneous pattern of reticulin and/or collagen fibrosis, final 'MF' score was determined by the highest grade present in at least 30% of the marrow area.

The degree of myelofibrosis (MF) was determined with examination of reticulin stained sections alone. The pattern of reticulin fibrosis was observed.Presence of admixed collagen fibres was estimated as bundles of thick fibres as reticulin stain with Gordon & Sweet's method can not discriminate between reticulin and collagen fibres. MF grade was calculated with the reticulin stain.

Assessment of above mentioned 'MF' scoring was done for each MT stained slide with observation of areas with collagen deposition as indicated by blue colour, irrespective of it'sreticulin stain finding. Focal MT positive area constituted MF-2 collagen fibrosis. The MT-positive marrow area ≥30% was regarded as MF-3. All the MT negative cases were MF-0. Mean (±SD) value of MF grade was calculated with the MT stained sections alone. Degree of collagen fibrosis was also assessed following another semiquantitative grading of collagen deposition within proposed four-grade systemwith consideration of the highest pattern present in at least 30% of the bone marrow area as the final score in heterogeneous cases.[10]

The final 'MF' grade was determined based on agreement among three observers (SA, BPD, AKMNK) according to examination of the staining pattern of same slides with both special stains under a multi-headed microscope. In the case of the same patient showing different findings in 'MF' grade in reticulin and MT-stained slides due to controversy in evaluating collagenized areas, the final grading was decided based on MT positivity as conclusive for collagen fibrosis. Ultimately, MF-2 and MF-3 cases were considered as having pathological BMF corresponding to overt fibrotic stage of PMF. Those cases with degree of fibrosis MF-0 to MF-1 were identified as pre-fibrotic patients with absent pathological BMF. Final mean (±SD) value of bone marrow fibrosis (MF) grade was calculated

Table I: Use of special stains[10,11]

NO.	Stain	Purpose	Colour	Internal control
1.	Reticulin	For demonstration of the presence of reticulin fibers, collagen type III.	Black (Gordon & Sweet's method) Yellowish or, brownish (Gomori's method)	Perivascular area
2.	Masson's trichrome	For demonstration of the presence of collagen fibers type I.	Blue	Perivascular area

Semiquantitative bone marrow fibrosis (MF) grading system based on "European consensus-2005" (Kvasnicka et al.[10])

- MF-0:Scattered linear reticulin with no intersections corresponding to normal bone marrow.
- MF-1:Loose network of reticulin with many intersections, especially in perivascular areas.
- MF-2: Diffuse and dense increase in reticulin with extensive intersections, occasionally with only focal bundles of thick fibres mostly consistent with collagen and /or focal osteosclerosis.
- MF-3:Diffuse and dense increase in reticulin with extensive intersections with coarse bundles of thick fibres mostly consistent with collagen usually associated with significant osteosclerosis.

Semiquantitative grading of collagen deposition (Kvasnicka et al.[04])

- Grade 0: Perivascular collagen only (normal).
- Grade 1**:** Focal paratrabecular or/and central collagen deposition without connecting meshwork.
- Grade 2**:** Paratrabecular or/and central collagen deposition with focally connecting meshwork or generalized paratrabecular apposition of collagen.
- Grade 3**:** Diffuse (complete) connecting meshwork of collagen.

Semiquantitative grading of osteosclerosis (Kvasnicka et al.[10])

- Grade 0: Regular bone trabeculae (distinct paratrabecular borders).
- Grade 1: Focal budding, hooks, spikes or paratrabecular apposition of new bone.
- Grade 2: Diffuse paratrabecular new boone formation with thickening of trabeculae, occasionally with focal interconnection.
- Grade 3: Extensive interconnecting meshwork of new bone with overall effacement of marrow space.

Results

Among 36 PMF cases, 27 (75%) were male and 9 (25%) were female with mean age 51.4±15.5 years (Table II). In this study, 19 (52.8%) patients had detectable BMF stained slides. Besides, thirty-four (94.4%) patients had detectable pathological BMF (MF-2 to MF-3) in reticulin and Masson's trichrome stained slides. Presence of detectable pathological BMF is significantly higher ($p<0.05$) with additional use of special stains when compared with the use of H&E stain alone (Table III). Thirty-two (88.9%) patients revealed pathological BMF in reticulin stained slides alone, whereas 29 (80.6%) patients showed pathological BMF in MT stained slides alone (Table IV).

Among 36 cases, only 2 (5.6%) patients showed reticulin fibrosis grade MF1. Thirteen (36.1%) patients had bone marrow fibrosis MF2; five (13.9%) of them showed dense reticulin fibrosis without MT detected collagen fibrosis. Twenty-one (58.3%) patients had bone marrow fibrosis MF-3 with significant collagen fibrosis and osteosclerosis. All study subjects were divided into pre-fibrotic and overt fibrotic groups after evaluation of fibrosis comprising 2 (5.6%) and 34 (94.4%) patients respectively (Table V). Mean MF grade values of 36 cases using reticulin and MT alone were found 2.25±0.7 and 2.19±1.2 respectively; whereas using both of these special stains revealed 2.53±0.6. Mean MF grade was significantly higher (p-0.01) with using reticulin alone than MT alone (Table VI).Collagen fibrosis grade was assessed with findings of Masson's trichrome stain (Table VII). Only 5 (13.9%) overt fibrotic patients had absent collagen fibrosis (grade-0). Among 34 overt fibrotic patients, 33 (97.1%) had presence of osteoscleosis (Table VII).

Table II: Distribution of cases according to age (n=36)

Age range (yrs.)	Frequency	Percentage
≤40	10	27.8
41-50	6	16.7
51-60	8	22.2
≥61	12	33.3
Total	36	100.0

Table III: Comparison of H&E stain with combined use of reticulin and MT for detection of BMF

Stain	BMF present		BMF absent		*p*-value
	Frequency	Percentage	Frequency	Percentage	
H&E	19	52.8	17	47.2	<0.05
Special stains	34	94.4	2	5.6	

S- Significant, level of significance measured by Chi-square test

Table IV: Comparison of BMF detection reticulinand MT alone with combination of the two

BMF	Reticulin n (%)	MT n (%)	Both reticulin and MT n (%)	*p*-value
Present Absent	32 (88.9) 4 (11.1)	29 (80.6) 7 (19.4)		0.33 (NS)
Present Absent	32 (88.9) 4 (11.1)		34 (94.4) 2 (5.6)	0.39 (NS)
Present Absent		29 (80.6) 7 (19.4)	34 (94.4) 2 (5.6)	0.07 (NS)

NS- Not significant, level of significance measured by Chi-square test

Table V: Distribution of study subjects according to bone marrow fibrosis (MF) grades in 36 cases

Bone marrow fibrosis grade (MF)	n (%)	Pre-fibrotic(MT negative)	
0		0 (0.0)	
1		2 (5.6)	
	Overt fibrotic n (%)		
	MT negative	MT positive without osteosclerosis	MT positive with osteosclerosis
2	5 (13.9)	1 (2.8)	7 (19.4)
3	0	0	21 (58.3)
Total	2 (5.6) (MF-1) + 13 (36.1) (MF-2) + 21 (58.3) (MF-3) = 2 (5.6) (pre-fibrotic) + 34 (94.4) (overt fibrotic) = 36 (100)		

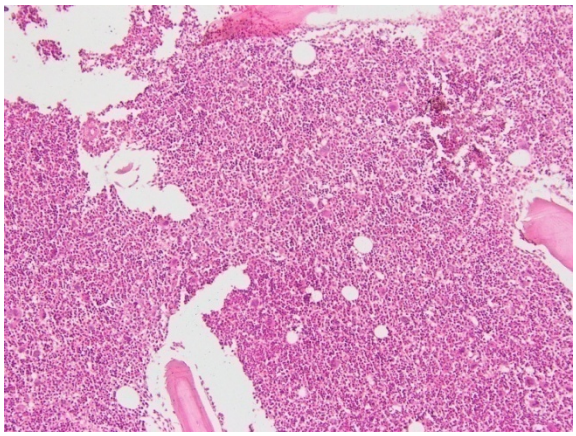
Table VI: Comparison of mean MF grade values with using reticulin and MT alone and in combination of the two (n=36)

Mean MF grade (± SD)	Reticulin	MT	Both reticulin & MT	*p*-value
	2.25±0.73	2.19±1.17		0.01 (S)
	2.25±0.73		2.53±0.61	0.07 (NS)
		2.19±1.17	2.53±0.61	0.07 (NS)

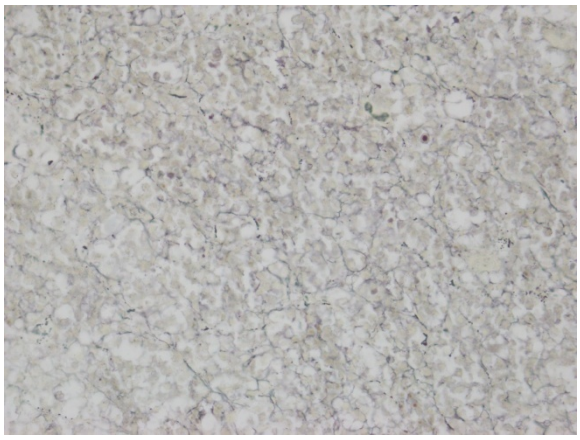
SD- Standard deviation NS- Not significant, S- Significant.
Level of significance measured by unpaired t-test

Table VII: Distribution of total cases according to collagen fibrosis and osteosclerosis grade (n=36)

Collagen fibrosis grade	Pre-fibrotic (n=2) n (%)	Overt fibrotic (n=34) n (%)
0	2 (5.6)	5 (13.9)
1	0	6 (16.7)
2	0	10 (27.8)
3	0	13 (36.1)
Osteosclerosis grade		
0	2 (5.6)	1 (2.8)
1	0	8 (22.2)
2	0	11 (30.6)
3	0	14 (38.9)

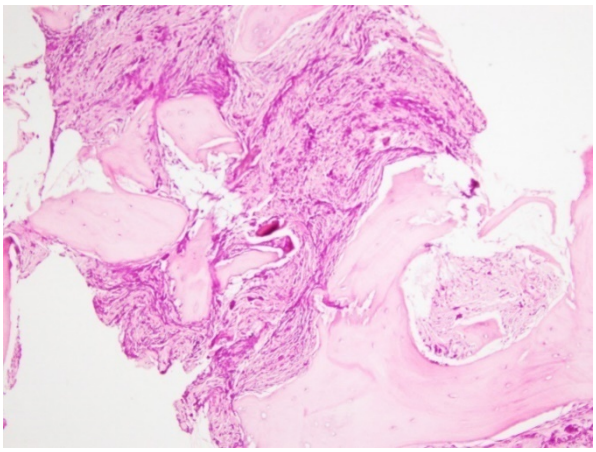


A

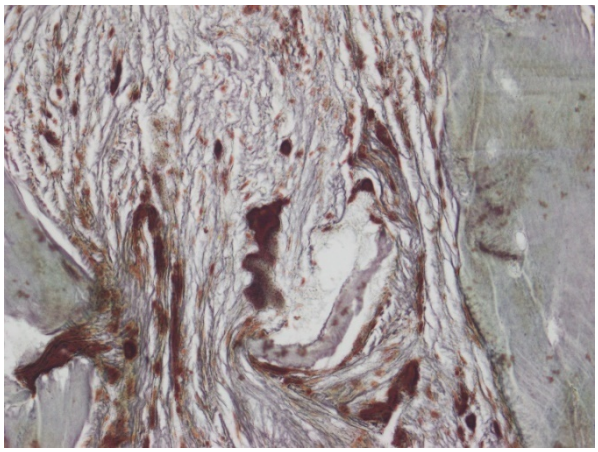


B

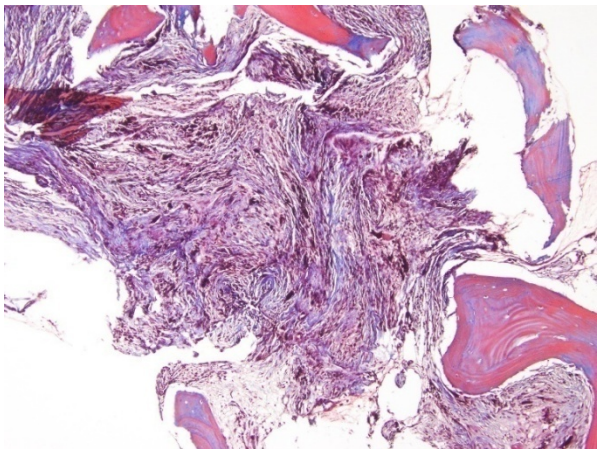
Figure 1. Photomicrograph showing a pre-fibrotic case of primary myelofibrosis, (a) H&E stain (100x) revealed, hypercellular marrow with predominance of granulocytic and megakaryocytic population, (b) MF-1 with reticulin stain (400x).



A



B



C

Figure 2. Photomicrograph showing an advanced fibrotic case of primary myelofibrosis, (a) H&E stain (100x) revealed, severely hypocellular marrow with distorted architecture and osteosclerosis, (b) MF-3 with reticulin stain (400x), (c) difuse meshwork of collagen fibrosis grade-3 with MT stain (100x).

Discussion

In the present study, 55.6% of the patients were over 50 years old and 44.4% were ≤50 years. Most of the participated patients were male. PMF is most commonly diagnosed in the 6^{th} – 7^{th} decades of life. Usually, men and women are nearly equally affected by PMF.[6] The significantly higher detection of pathological BMF cases with special stains and significant difference between reticulin and MT assessed mean MF grade can suggest to establish that, the routine use of both special stains is essential to evaluate the amount and nature of marrow fibrosis properly than performing either one of these stains or H&E alone. Those hypercellular fibrotic cases necessitate application of reticulin stain that resemble to prefibrotic stage of PMF with H&E stain.

In our study, five (13.9%) cases exhibited pathological BMF by reticulin stain. All of them were negative in MT stain. Their final fibrosis grade was determined as 'MF-2'. Three (8.3%) cases showed 'MF-3' fibrosis in reticulin stain. Ultimately, these cases revealed trichrome positive 'MF-2' for focal (<30%) collagenized areas. Oppositely, reticulin fibrosis can be underscored in inadequately performed reticulin stain due to inappropriate fixation, embedding, shrinkage of tissue, etc.[10]Two (5.6%) cases in the present study were estimated as pre-fibrotic in reticulin stain. All of them demonstrated pathological BMF in MT stain. Eleven (30.6%) cases were MF-2 in reticulin stain. They were confirmed for 'MF-3' in MT stain. Reticulin stain often fails to discriminate between reticulin and collagen fibres. Reticulin stain revealed coarse bundles of thick black fibres are mostly consistent with collagen fibres. Reticulinfibres can be diffusely increased without positive trichrome staining due to deposition of immature collagen.[10,12]Reticulin fibrosis, often reversible after successful treatment, may exhibit a correlation to disease prognosis. Collagen fibrosis signifies more severe disease conditions of the marrow, and less likely to be reversible with treatment.[7,11,13] Osteosclerosis is usually associated with areas of collagenization. Five (13.9%) patients in our study had demonstrable osteosclerosisinspite of being MT negative for collagen fibrosis. They were MF-2 for diffusely increased reticulinfibres. It possibly observed due to increased deposition of osteoid reflecting higher osteoblastic activity in PMF.[14]

Conclusion

Evaluation of BMF has the prognostic importance in myelofibrotic patients. H&E stain alone, or by using either reticulin or MT stain may not be sufficient to evaluate all type of fibrosis. So, combined use of these additional special stains can be preferred routinely in trephine biopsies to get a complete picture as well as extension of bone marrow stromal fibrosis.

Acknowledgement

Authors would like to express their gratitude towards all the mentors of the Department of Pathology, BSMMU for the tremendous support in conducting the research work.

Conflict of interest

The authors declare no financial or personal conflict of interest.

References

1. Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, Advani R, Ghielmini M, Salles GA, Zelenetz AD, Jaffe ES. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. Blood, The Journal of the American Society of Hematology. 2016 May 19; 127(20):2375-90.
2. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A,

Harris NL, Le Beau MM, Hellström-Lindberg E, Tefferi A, Bloomfield CD. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood, The Journal of the American Society of Hematology. 2009 Jul 30; 114(5):937-51.

3. Tefferi A. Primary myelofibrosis: 2023 update on diagnosis, risk-stratification, and management. Am J Hematol. 2023 May; 98(5):801-821.
4. Thiele J, Kavasnicka HM, Orazi A, Gianelli U, Barbui T, Barosi G et al. Primary myelofibrosis. In: Swerdlow SH, Campo E, Nancy LH, Jafee ES, Pileri SA, Stein H et al. WHO classification of tumours of haematopoietic and lymphoid tissues. 2017 Revised 4th edition , pp. 44-49. Lyon: IARC.
5. Thiele J, Kvasnicka HM. Hematopathologic findings in chronic idiopathic myelofibrosis. In Seminars in oncology 2005 Aug 1 (Vol. 32, No. 4, pp. 380-394). WB Saunders.
6. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, Bloomfield CD, Cazzola M, Vardiman JW. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood, The Journal of the American Society of Hematology. 2016 May 19; 127(20):2391-405.
7. Kuter DJ, Bain B, Mufti G, Bagg A, Hasserjian RP. Bone marrow fibrosis: pathophysiology and clinical significance of increased bone marrow stromal fibres. British journal of haematology. 2007 Nov; 139(3):351-62.
8. AbouZahr A, Salama ME, Carreau N, Tremblay D, Verstovsek S, Mesa R, Hoffman R, Mascarenhas J. Bone marrow fibrosis in myelofibrosis: pathogenesis, prognosis and targeted strategies. Haematologica. 2016 Jun; 101(6):660.
9. Layton C and Bancroft JD: Connective and mesenchymal tissues with their stains. Bancroft's Theory and Practice of Histological Techniques, 11th Edition. Edited by Suvarna KS, Layton C, Bancroft JD. Elsevier Health Sciences; 2013, pp. 187-214
10. Kvasnicka HM, Beham□Schmid C, Bob R, Dirnhofer S, Hussein K, Kreipe H, Kremer M, Schmitt□Graeff A, Schwarz S, Thiele J, Werner M. Problems and pitfalls in grading of bone marrow fibrosis, collagen deposition and osteosclerosis–a consensus□based study. Histopathology. 2016 May; 68(6):905-15.
11. Al-Khafaji AK, Al-Shammari HH, Al-Obeidi SR. Bone Marrow Fibrosis in Chronic myeloid leukemia (CML) and other Myeloproliferative Disorders Evaluated by Using Special Histochemical Stains for Collagen. Journal of the Faculty of Medicine Baghdad. 2011 Oct 2; 53(3):296-300.
12. Nazha A, Khoury JD, Rampal RK, Daver N. Fibrogenesis in primary myelofibrosis: diagnostic, clinical, and therapeutic implications. The oncologist. 2015 Oct;20(10):1154-60.
13. Das A, Talukdar L, Biswas R, Deka MK, Bhuyan K. Reticulin Pattern in Adult Bone Marrow in Haematological Malignancies"-A Hospital Based Study.IOSR Journal of Dental and Medical Sciences. 2021 August; 20(8):38-44
14. Spampinato M, Giallongo C, Romano A, Longhitano L, La Spina E, Avola R, Scandura G, Dulcamare I, Bramanti V, Di Rosa M, Vicario N. Focus on osteosclerotic progression in primary myelofibrosis. Biomolecules. 2021 Jan 19;11(1):122.